Hematology/Hemostasis Reference Intervals

Unless otherwise noted, data for reference interval tables were compiled from multiple sources and may vary slightly from intervals listed within chapters. Each laboratory must establish its particular intervals based on its instrumentation, methodology and demographics of the population it serves.

### COMPLETE BLOOD COUNT REFERENCE INTERVALS (ADULT)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Units</th>
<th>Reference Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, male</td>
<td>× 10⁶/µL (× 10¹²/L)</td>
<td>4.20–6.00</td>
</tr>
<tr>
<td>RBC, female</td>
<td>× 10⁶/µL (× 10¹²/L)</td>
<td>3.80–5.20</td>
</tr>
<tr>
<td>HGB, male</td>
<td>g/dL (g/L)</td>
<td>13.5–18.0</td>
</tr>
<tr>
<td>HGB, female</td>
<td>g/dL (g/L)</td>
<td>12.0–15.0</td>
</tr>
<tr>
<td>HCT, male</td>
<td>% (L/L)</td>
<td>40–54</td>
</tr>
<tr>
<td>HCT, female</td>
<td>% (L/L)</td>
<td>35–49</td>
</tr>
<tr>
<td>MCV</td>
<td>fL</td>
<td>80–100</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>26–34</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>32–36</td>
</tr>
<tr>
<td>RDW</td>
<td>%</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>RETIC</td>
<td>× 10³/µL (× 10⁹/L)</td>
<td>20–115</td>
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### COMPLETE BLOOD COUNT REFERENCE INTERVALS (FEMALE)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Units</th>
<th>Reference Intervals</th>
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</thead>
<tbody>
<tr>
<td>RBC, female</td>
<td>× 10⁶/µL (× 10¹²/L)</td>
<td>3.80–5.20</td>
</tr>
<tr>
<td>HGB, female</td>
<td>g/dL (g/L)</td>
<td>12.0–15.0</td>
</tr>
<tr>
<td>HCT, female</td>
<td>% (L/L)</td>
<td>35–49</td>
</tr>
<tr>
<td>MCH</td>
<td>pg</td>
<td>26–34</td>
</tr>
<tr>
<td>MCHC</td>
<td>g/dL</td>
<td>32–36</td>
</tr>
<tr>
<td>RDW</td>
<td>%</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>RETIC</td>
<td>× 10³/µL (× 10⁹/L)</td>
<td>20–115</td>
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### HEMOGLOBIN FRACTION REFERENCE INTERVALS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Adult Reference Intervals (%)</th>
<th>Newborn Reference Intervals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>&gt; 95</td>
<td>10–40</td>
</tr>
<tr>
<td>Hb F</td>
<td>0–2.0</td>
<td>60–90</td>
</tr>
<tr>
<td>Hb A₂</td>
<td>0–3.5</td>
<td></td>
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</tbody>
</table>
### BONE MARROW ASPIRATE REFERENCE INTERVALS (ADULT)

<table>
<thead>
<tr>
<th>WBC Differential</th>
<th>Reference Intervals (%)</th>
<th>Erythrocyte Series</th>
<th>Reference Intervals (%)</th>
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</thead>
<tbody>
<tr>
<td>Blasts</td>
<td>0–3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>1–5</td>
<td>Pronormoblasts</td>
<td>0–1</td>
</tr>
<tr>
<td>N. myelocytes</td>
<td>6–17</td>
<td>Basophilic NB</td>
<td>1–4</td>
</tr>
<tr>
<td>N. metamyelocytes</td>
<td>3–20</td>
<td>Polychromatophilic NB</td>
<td>10–20</td>
</tr>
<tr>
<td>N. bands</td>
<td>9–32</td>
<td>Orthochoromic NB</td>
<td>6–10</td>
</tr>
<tr>
<td>N. segmented (polymorphonuclear)</td>
<td>7–30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0–3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophils</td>
<td>0–1</td>
<td>M:E ratio</td>
<td>1.5–3.3:1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>5–18</td>
<td>Megakaryocytes</td>
<td>2–10/lpf</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>0–1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>0–1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histiocytes (macrophages)</td>
<td>0–1</td>
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### COMPLETE BLOOD COUNT REFERENCE INTERVALS (PEDIATRIC)

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<thead>
<tr>
<th>Assay</th>
<th>Units</th>
<th>0–1 d</th>
<th>2–4 d</th>
<th>5–7 d</th>
<th>8–14 d</th>
<th>15–30 d</th>
<th>1–2 mo</th>
<th>3–5 mo</th>
<th>6–11 mo</th>
<th>1–3 y</th>
<th>4–7 y</th>
<th>8–13 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>× 10^12/L</td>
<td>4.10–6.10</td>
<td>4.36–5.96</td>
<td>4.20–5.80</td>
<td>4.00–5.60</td>
<td>3.20–5.00</td>
<td>3.40–5.00</td>
<td>3.65–5.05</td>
<td>3.60–5.20</td>
<td>3.40–5.20</td>
<td>4.00–5.20</td>
<td>4.00–5.40</td>
</tr>
<tr>
<td>HGB</td>
<td>g/dL</td>
<td>(165–208)</td>
<td>16.4–20.4</td>
<td>15.2–20.4</td>
<td>15.0–19.6</td>
<td>12.2–18.0</td>
<td>10.6–16.4</td>
<td>10.4–16.0</td>
<td>10.4–15.6</td>
<td>9.6–15.6</td>
<td>10.2–15.2</td>
<td>12.0–15.0</td>
</tr>
<tr>
<td>RDW</td>
<td>%</td>
<td>1.8–5.8</td>
<td>1.3–4.7</td>
<td>0.2–1.4</td>
<td>0–1.0</td>
<td>0.2–1.0</td>
<td>0.8–2.8</td>
<td>0.5–1.5</td>
<td>0.5–1.5</td>
<td>0.5–1.5</td>
<td>0.5–1.5</td>
<td>0.5–1.5</td>
</tr>
<tr>
<td>RETIC</td>
<td>%</td>
<td>1.5–4.3</td>
<td>1.5–4.3</td>
<td>1.5–4.3</td>
<td>1.5–4.3</td>
<td>1.5–4.3</td>
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<td>1.5–4.3</td>
<td>1.5–4.3</td>
<td>1.5–4.3</td>
</tr>
<tr>
<td>RETIC</td>
<td>× 10^11/L</td>
<td>73.8–353.8</td>
<td>56.7–290.1</td>
<td>8.4–81.2</td>
<td>0.0–56.0</td>
<td>6.4–50.0</td>
<td>27.2–140.0</td>
<td>18.3–75.8</td>
<td>18.0–78.0</td>
<td>17.0–78.0</td>
<td>20–78.0</td>
<td>20–124.2</td>
</tr>
<tr>
<td>WBC</td>
<td>× 10^12/L</td>
<td>(200–500)</td>
<td>2–24</td>
<td>5–9</td>
<td>0–1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4.5–13.5</td>
</tr>
<tr>
<td>NEUT</td>
<td>× 10^9/L</td>
<td>(9.0–25.0)</td>
<td>9.0–37.0</td>
<td>8.0–24.0</td>
<td>5.0–21.0</td>
<td>5.0–21.0</td>
<td>5.0–21.0</td>
<td>6.0–20.0</td>
<td>6.0–18.0</td>
<td>6.0–18.0</td>
<td>6.0–18.0</td>
<td>5.5–17.5</td>
</tr>
<tr>
<td>MONO</td>
<td>× 10^9/L</td>
<td>(3.7–30.0)</td>
<td>3.7–30.0</td>
<td>2.6–17.0</td>
<td>1.5–12.5</td>
<td>1.2–11.6</td>
<td>1.0–9.5</td>
<td>1.2–8.1</td>
<td>1.1–7.7</td>
<td>1.2–8.1</td>
<td>1.2–8.9</td>
<td>1.5–11.0</td>
</tr>
<tr>
<td>EO</td>
<td>× 10^10/L</td>
<td>(0.1–4.4)</td>
<td>0.1–4.4</td>
<td>0.2–3.4</td>
<td>0.2–3.6</td>
<td>0.2–3.6</td>
<td>0.1–3.2</td>
<td>0.2–2.5</td>
<td>0.1–2.0</td>
<td>0.1–2.0</td>
<td>0.1–1.9</td>
<td>0.1–1.9</td>
</tr>
<tr>
<td>BASO</td>
<td>× 10^9/L</td>
<td>(0.0–0.7)</td>
<td>0.0–0.7</td>
<td>0.0–0.4</td>
<td>0.0–0.4</td>
<td>0.0–0.4</td>
<td>0.0–0.4</td>
<td>0.0–0.4</td>
<td>0.0–0.4</td>
<td>0.0–0.3</td>
<td>0.0–0.3</td>
<td>0.0–0.3</td>
</tr>
</tbody>
</table>

*The RDW is markedly elevated in newborns, with a range of 14.2% to 19.9% in the first few days of life, gradually decreasing until it reaches adult levels by 6 months of age. Pediatric reference intervals are from Riley Hospital for Children, Indiana University Health, Indianapolis, IN.

Some reference intervals are listed in common units and in international system of units (SI units) in parenthesis.

ANC, absolute neutrophil count (includes segmented neutrophils and bands); BAND, neutrophil bands; BASO, basophilia; d, days; EO, eosinophils; ESR, erythrocyte sedimentation rate; HB, hemoglobin fraction; HCT, hematocrit; HGB, hemoglobin; if, low power field; LYM/C, lymphocytes; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; M:E, myeloid:erythroid; mo, month; MONO, monocytes; MPV, mean platelet volume; N, neutrophil; NB, normoblast; NEUT, neutrophils; NRBC, nucleated red blood cells; PLT, platelets; RBC, red blood cells; RDW, red blood cell distribution width; RETIC, reticulocytes; WBC, white blood cells; y, year.

Please see inside back cover for additional reference interval tables.
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REGISTER TODAY!
To my students for being great teachers, and to Camryn, Riley, Harper, Stella, Jackie, Alana, Ken, and Jake for reminding me about the important things in life.

EMK

To my wonderful mentors and students who have taught me so much about laboratory medicine.

LJS

To my teachers, both formal and informal, for all this fascinating knowledge in clinical laboratory sciences which made possible my interesting career.

JMW
Special Dedication

To Bernadette “Bunny” F. Rodak, with great admiration and gratitude for your vision, perseverance, and courage to first publish Hematology: Clinical Principles and Applications in 1995; for your over 20-year commitment to publish the highest quality text through five editions; for your mentorship and guidance of five co-editors and over 50 authors; and for sharing your great enthusiasm for hematology and hemostasis and lifelong learning that has inspired a generation of students and faculty in this country and around the world.

Special Acknowledgment

To George A. Fritsma, with our sincere gratitude for your dedication and reasoned approach that has kept Hematology: Clinical Principles and Applications at the leading edge as a comprehensive, state-of-the-art, yet practical textbook, guided by you as co-editor for two editions and through the multiple number of chapters that you have authored. We are indebted to you for sharing your vast knowledge in hematology and hemostasis and for your unwavering commitment to the profession of clinical laboratory science.
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Preface

The science of clinical laboratory hematology provides for the analysis of normal and pathologic peripheral blood cells, hematopoietic (blood-producing) tissues, and the cells in non-vascular body cavities such as cerebrospinal and serous fluids. Laboratory hematology also includes the analysis of cells and coagulation proteins essential to clinical hemostasis. Hematology laboratory assay results are critical for the diagnosis, prognosis, and monitoring treatment for primary and secondary hematologic disorders. Similarly, hematology results are used to establish safety in the perioperative period, monitor treatments during surgical procedures, and monitor transfusion needs in trauma patients.

Clinical laboratory hematology has been enhanced by profound changes as reflected in the numerous updates in the fifth edition of Rodak’s Hematology: Clinical Principles and Applications. Automation and digital data management have revolutionized the way blood specimens are transported and stored, how assays are ordered, and how results are validated, reported, and interpreted.

Molecular diagnosis has augmented and in many instances replaced long-indispensable laboratory assays. Hematologic disorders have been reclassified on the basis of phenotypic, cytogenetic, and molecular genetic analyses. Diagnoses that once depended on the analysis of cell morphology and cytochemical stains now rely on flow cytometry, cytogenetic testing, fluorescence in situ hybridization (FISH), end-point and real-time polymerase chain reaction assays, gene sequencing, and microarrays. Traditional chemotherapeutic monitoring of leukemias and lymphomas at the cellular level has shifted to the management of biologic response modifiers and detection of minimal residual disease at the molecular level. Hemostasis has grown to encompass expanded thrombophilia testing, methods that more reliably monitor available antplatelet and anticoagulant drugs, molecular analysis, and a shift from clot-based to functional and chromogenic assays.

Rodak’s Hematology: Clinical Principles and Applications systematically presents basic to advanced concepts to provide a solid foundation of normal and pathologic states upon which readers can build their skills in interpreting and correlating laboratory findings in anemias, leukocyte disorders, and hemorrhagic and thrombotic conditions. It provides key features for accurate identification of normal and pathologic cells in blood, bone marrow, and body fluids. The focus, level, and detail of hematology and hemostasis testing, along with the related clinical applications, interpretation, and testing algorithms, make this text a valuable resource for all healthcare professionals managing these disorders.

ORGANIZATION

Rodak’s Hematology: Clinical Principles and Applications fifth edition is reorganized into 7 parts and 45 chapters for enhanced pedagogy. Chapter highlights and new content are described as follows:

Part I: Introduction to Hematology
Chapters 1 to 5 preview the science of clinical laboratory hematology and include laboratory safety, blood specimen collection, microscopy, and quality assurance. The quality assurance chapter was significantly updated to include enhanced sections on statistical significance; assay validation with applications of the Student’s t test, ANOVA, linear regression, and Bland-Altman difference plots; and assessment of diagnostic efficacy.

Part II: Blood Cell Production, Structure, and Function
Chapters 6 and 7 use photomicrographs and figures to describe general cellular structure and function and the morphologic and molecular details of hematopoiesis. Chapters 8, 12, and 13 discuss erythropoiesis, leukopoiesis, and megakaryopoiesis using numerous photomicrographs demonstrating ultrastructure and microscopic morphology. Chapters 9 and 10 examine mature red blood cell metabolism, hemoglobin structure and function, and red blood cell senescence and destruction.Iron kinetics and laboratory assessment in Chapter 11 was substantially updated with new figures and updated coverage of systemic and cellular regulation of iron. Chapter 13 includes detailed descriptions of platelet adhesion, aggregation, and activation with updated figures.

Part III: Laboratory Evaluation of Blood Cells
Chapter 14 describes manual procedures such as microscopy-based cell counts, hemoglobin and hematocrit determinations, and point-of-care technology. Chapter 15 has been substantially updated to include descriptions and figures of the latest automated hematology analyzers. Chapter 16 describes peripheral blood film examination and the differential count correlation to the complete blood count. New figures correlate red blood cell and platelet histograms to their morphology. Chapter 17 follows up with bone marrow aspirate and biopsy collection, preparation, examination, and reporting. Chapter 18 describes methods for analyzing normal and pathologic cells of cerebrospinal fluid, joint fluid, transudates, and exudates, illustrated with many excellent photomicrographs.

Part IV: Erythrocyte Disorders
Chapter 19 provides an overview of anemia and describes cost-effective approaches that integrate patient history, physical examination, and symptoms with the hemoglobin, red blood cell indices, reticulocyte count, and abnormal red blood cell morphology. Chapters 20 to 22 describe disorders of iron and DNA metabolism and bone marrow failure. New algorithms help the reader to distinguish types of microcytic and macrocytic anemias. Chapters 23 to 26 discuss hemolytic anemias due to intrinsic or extrinsic defects. Chapter 23 is fully updated with new figures that detail extravascular and intravascular hemolysis and hemoglobin catabolism. Chapters 27 and 28 provide updates in
pathophysiology, diagnosis, and treatment of hemoglobinopathies (such as sickle cell disease) and the thalassemias.

Part V: Leukocyte Disorders
Chapter 29 is significantly updated with many excellent photomicrographs and summary boxes of nonmalignant systemic disorders manifested by the abnormal distribution or morphology of leukocytes. These include bacterial and viral infections, various systemic disorders, and benign lymphoproliferative disorders. Chapter 30 provides details on traditional cytogenetic procedures for detection of quantitative and qualitative chromosome abnormalities and more sensitive methods such as FISH and genomic hybridization arrays. Chapter 31 covers molecular diagnostics and was fully updated with new and enhanced figures on basic molecular biology, end-point and real-time polymerase chain reaction, microarrays, and DNA sequencing, including next generation sequencing. Chapter 32 describes flow cytometry and its diagnostic applications. It includes numerous scatterplots of normal and leukemic conditions. Chapters 33 to 36, with significant updating, provide the latest classifications and pathophysiologic models for myeloproliferative neoplasms, myelodysplastic syndromes, acute lymphoblastic and myeloid leukemias, chronic lymphocytic leukemia, and solid tumor lymphoid neoplasms, such as lymphoma and myeloma, with numerous full-color photomicrographs and illustrations.

Part VI: Hemostasis and Thrombosis
Chapter 37 provides the plasma-based and cell-based coagulation models and the interactions between primary and secondary hemostasis and fibrinolysis with updated illustrations. Chapter 38 details hemorrhagic disorders, including the management of the acute coagulopathy of trauma and shock. Chapter 39 updates the currently recognized risk factors of thrombosis and describes laboratory tests that identify venous and arterial thrombotic diseases, particularly for lupus anticoagulant and heparin-induced thrombocytopenia (HIT) testing. Chapters 40 and 41 detail the quantitative and qualitative platelet disorders using additional tables and figures, and Chapter 42 details laboratory assays of platelets and the coagulation mechanisms with helpful new figures and diagrams. Chapter 43 covers the mechanisms and monitoring methods of the traditional warfarin and heparin-derived antithrombotic drugs, as well as all thrombin and factor Xa inhibitor drugs. It also includes methods for monitoring the different classes of antiplatelet drugs, including aspirin. Chapter 44 reviews the latest coagulation analyzers and point of care instrumentation.

Part VII: Hematology and Hemostasis in Selected Populations
Chapter 45 provides valuable information on the hematology and hemostasis laboratory findings in the pediatric and geriatric populations correlated with information from previous chapters.

READERS

Roddak’s Hematology: Clinical Principles and Applications is designed for medical laboratory scientists, medical laboratory technicians, and the faculty of undergraduate and graduate educational programs in the clinical laboratory sciences. This text is also a helpful study guide for pathology and hematology-oncology residents and fellows and a valuable shelf reference for hematologists, pathologists, and hematology and hemostasis laboratory managers.

TEXTBOOK FEATURES

Elaine M. Keohane, PhD, MLS, Professor, Rutgers University, School of Health Related Professions, Department of Clinical Laboratory Sciences, co-editor in the fourth edition, and lead editor in the fifth edition, is joined by Larry J. Smith, PhD, Coagulation and Satellite Laboratory Director, Memorial Sloan Kettering Cancer Center, Adjunct Professor at Rutgers University, School of Health Related Professions and York College, CUNY, Department of Health Professions, and Jeanine M. Walenga, PhD, MT(ASCP), Professor, Loyola University Chicago, Stritch School of Medicine, Clinical Coagulation Laboratories Director, Loyola University Health System.

The outstanding value and quality of Rodak’s Hematology: Clinical Principles and Applications reflect the educational and clinical expertise of its current and previous authors and editors. The text is enhanced by nearly 700 full-color digital photomicrographs, figures, and line art. Detailed text boxes and tables clearly summarize important information. Reference intervals are provided on the inside front and back covers for quick lookup. Each chapter contains the following pedagogical features:

- **Learning objectives** at all taxonomy levels in the cognitive domain.
- One or two **case studies** with open-ended discussion questions at the beginning of the chapter that stimulate interest and provide opportunities for application of chapter content in real-life scenarios.
- A bulleted **summary** at the end of each chapter that provides a comprehensive review of essential material.
- **Review questions** at the end of each chapter that correlate to chapter objectives and are in the multiple-choice format used by certification examinations.
- **Answers** to case studies and review questions that are provided in the Appendix.

The Evolve website has multiple features for the instructor:
- An **ExamView test bank** contains multiple-choice questions with rationales and cognitive levels.
- **Instructor’s manuals** for every chapter contain key terms, objectives, outlines, and study questions.
- **Learning Objectives with taxonomy levels** are provided to supplement lesson plans.
- **Case studies** have been updated and feature discussion questions and photomicrographs when applicable.
- **PowerPoint presentations** for every chapter can be used “as is” or as a template to prepare lectures.
- The **Image Collection** provides electronic files of all the chapter figures that can be downloaded into PowerPoint presentations.

For the student, a Glossary is available as a quick reference to look up unfamiliar terms electronically.
The editors express their immense gratitude to Bernadette F. (Bunny) Rodak, who laid the foundation for this textbook with her expert writing, editing, detailed figures, and especially her contribution of over 200 outstanding digital photomicrographs over the past 2 decades. Now in its fifth edition, she has authored three chapters, provided invaluable contributions and assistance with additional photomicrographs and figures, and provided the opportunity for us to continue her work on this outstanding textbook. We sincerely thank George A. Fritsma for his significant contribution to this text as a previous coeditor and author, for sharing his immense expertise in hemostasis, for updating and authoring ten chapters in the fifth edition, and for his constant support and encouragement. We thank Kathryn Doig for her contributions as coeditor for the third edition; author in previous editions; and for her tenaciousness, creativity, and care in updating the five chapters authored in the fifth edition. The editors also thank the many authors who have made and continue to make significant contributions to this work. All of these outstanding professionals have generously shared their time and expertise to make Rodak’s Hematology: Clinical Principles and Applications into a worldwide educational resource and premier reference textbook for medical laboratory scientists and technicians, as well as pathology and hematology practitioners, residents, and fellows.

We also express our appreciation to Elsevier, especially Ellen Wurm-Cutter, Laurie Gower, Kellie White, Sara Alsup, Megan Knight, and Rebecca Corradetti, whose professional support and reminders kept the project on track, and to Debbie Prato for her editorial assistance.

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Elaine M. Keohane, PhD, MLS
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PART I  Introduction to Hematology

An Overview of Clinical Laboratory Hematology

George A. Fritsma

The average human possesses 5 liters of blood. Blood transports oxygen from lungs to tissues; clears tissues of carbon dioxide; transports glucose, proteins, and fats; and moves wastes to the liver and kidneys. The liquid portion is plasma, which, among many components, provides coagulation enzymes that protect vessels from trauma and maintain the circulation.

Plasma transports and nourishes blood cells. There are three categories of blood cells: red blood cells (RBCs), or erythrocytes; white blood cells (WBCs), or leukocytes; and platelets (PLTs), or thrombocytes. Hematology is the study of these blood cells. By expertly staining, counting, analyzing, and recording the appearance, phenotype, and genotype of all three types of cells, the medical laboratory professional (technician or scientist) is able to predict, detect, and diagnose blood diseases and many systemic diseases that affect blood cells. Physicians rely on hematology laboratory test results to select and monitor therapy for these disorders; consequently, a complete blood count (CBC) is ordered on nearly everyone who visits a physician or is admitted to a hospital.

HISTORY

The first scientists such as Athanasius Kircher in 1657 described “worms” in the blood, and Anton van Leeuwenhoek in 1674 gave an account of RBCs, but it was not until the late 1800s that Giulio Bizzozero described platelets as “petites plaques.” The development of Wright stain by James Homer Wright in 1902 opened a new world of visual blood film examination through the microscope. Although automated instruments now differentiate and enumerate blood cells, Wright’s Romanowsky-type stain (polychromatic, a mixture of acidic and basic dyes), and refinements thereof, remains the foundation of blood cell identification.

In the present-day hematology laboratory, RBC, WBC, and platelet appearance is analyzed through automation or visually using 500 to 1000 light microscopy examination of cells fixed to a glass microscope slide and stained with Wright or Wright-Giemsa stain (Chapters 15 and 16). The scientific term for cell appearance is morphology, which encompasses cell color, size, shape, cytoplasmic inclusions, and nuclear condensation.

RED BLOOD CELLS

RBCs are anucleate, biconcave, discoid cells filled with a reddish protein, hemoglobin (HGB), which transports oxygen and carbon dioxide (Chapter 10). RBCs appear pink to red and measure 6 to 8 μm in diameter with a zone of pallor that occupies one third of their center (Figure 1-1, A), reflecting their biconcavity (Chapters 8 and 9).

Since before 1900, physicians and medical laboratory professionals counted RBCs in measured volumes to detect anemia or polycythemia. Anemia means loss of oxygen-carrying capacity and is often reflected in a reduced RBC count or decreased RBC hemoglobin concentration (Chapter 19). Polycythemia means an increased RBC count reflecting increased circulating RBC mass, a condition that leads to hyperviscosity (Chapter 33). Historically, microscopists counted RBCs by carefully pipetting a tiny aliquot of whole blood and mixing it with 0.85% (normal) saline. Normal saline matches the osmolality of blood; consequently, the suspended RBCs retained their intrinsic morphology, neither swelling nor shrinking. A 1:200 dilution was typical for RBC counts, and a glass
pipette designed to provide this dilution, the Thoma pipette, was used routinely until the advent of automation. The diluted blood was transferred to a glass counting chamber called a hemacytometer (Figure 14-1). The microscopist observed and counted RBCs in selected areas of the hemacytometer, applied a mathematical formula based on the dilution and on the area of the hemacytometer counted (Chapter 14), and reported the RBC count in cells per microliter (µL, mL, or cc), or liter (L).

Figure 1-1 Normal cells in peripheral blood: A, Erythrocyte (red blood cell, RBC); B, Neutrophil (segmented neutrophil, NEUT, SEG, polymorphonuclear neutrophil, PMN); C, Band (band neutrophil, BAND); D, Eosinophil (EO); E, Basophil (BASO); F, Lymphocyte (LYMPH); G, Monocyte (MONO); H, Platelet (PLT).

Visual RBC counting was developed before 1900 and, although inaccurate, was the only way to count RBCs until 1958, when automated particle counters became available in the clinical laboratory. The first electronic counter, patented in 1953 by Joseph and Wallace Coulter of Chicago, Illinois, was used so widely that today automated cell counters are often called Coulter counters, although many high-quality competitors exist (Chapter 15). The Coulter principle of direct current impedance is still used to count RBCs in many automated hematology profiling instruments. Fortunately, the widespread availability of automated cell counters has replaced visual RBC counting, although visual counting skills remain useful where automated counters are unavailable.

Hemoglobin, Hematocrit, and Red Blood Cell Indices

RBCs also are assayed for hemoglobin concentration (HGB) and hematocrit (HCT) (Chapter 14). Hemoglobin measurement relies on a weak solution of potassium cyanide and potassium ferricyanide, called Drabkin reagent. An aliquot of whole blood is mixed with a measured volume of Drabkin reagent, hemoglobin is converted to stable cyanmethemoglobin (hemoglobin cyanide), and the absorbance or color intensity of the solution is measured in a spectrophotometer at 540 nm wavelength. The color intensity is compared with that of a known standard and is mathematically converted to hemoglobin concentration. Modifications of the cyanmethemoglobin method are used in most automated applications, although some automated hematology profiling instruments replace it with a formulation of the ionic surfactant (detergent) sodium lauryl sulfate to reduce environmental cyanide.

Hematocrit is the ratio of the volume of packed RBCs to the volume of whole blood and is manually determined by transferring blood to a graduated plastic tube with a uniform bore, centrifuging, measuring the column of RBCs, and dividing by the total length of the column of RBCs plus plasma. The normal ratio approaches 50% (refer to inside front cover for reference intervals). Hematocrit is also called packed cell volume (PCV), the packed cells referring to RBCs. Often one can see a light-colored layer between the RBCs and plasma. This is the buffy coat and contains WBCs and platelets, and it is excluded from the hematocrit determination. The medical laboratory professional may use the three numerical results—RBC count, HGB, and HCT—to compute the RBC indices mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) (Chapter 14). The MCV, although a volume measurement recorded in femtoliters (fL), reflects RBC diameter on a Wright-stained blood film. The MCHC, expressed in g/dL, reflects RBC staining intensity and amount of central pallor. The MCH in picograms (pg) expresses the mass of hemoglobin and parallels the MCHC. A fourth RBC index, RBC distribution width (RDW), expresses the degree of variation in RBC volume. Extreme RBC volume variability is visible on the Wright-stained blood film as variation in diameter and is called anisocytosis. The RDW is based on the standard deviation of RBC volume and is routinely reported by automated cell counters. In addition to aiding in diagnosis of anemia, the RBC indices provide stable measurements for internal quality control of counting instruments (Chapter 5).

Medical laboratory professionals routinely use light microscopy at 500× or 1000× magnification (Chapters 4 and 16) to visually review RBC morphology, commenting on RBC diameter, color or hemoglobinization, shape, and the presence of cytoplasmic inclusions (Chapters 16 and 19). All these parameters—RBC count, HGB, HCT, indices, and RBC morphology—are employed to detect, diagnose, assess the severity of, and monitor the treatment of anemia, polycythemia, and the numerous systemic conditions that affect RBCs. Automated hematology profiling instruments are used in nearly all laboratories to generate these data, although visual examination of the Wright-stained blood film is still essential to verify abnormal results.

Reticulocytes

In the Wright-stained blood film, 0.5% to 2% of RBCs exceed the 6- to 8-µm average diameter and stain slightly blue-gray. These are polychromatophilic (polychromatophilic) erythrocytes, newly released from the RBC production site: the bone marrow (Chapters 8 and 17). Polychromatophilic erythrocytes are closely observed because they indicate the ability of the bone marrow to increase RBC production in anemia due to blood loss or excessive RBC destruction (Chapters 23 to 26).
Methylene blue dyes, called nucleic acid stains or vital stains, are used to differentiate and count these young RBCs. Vital (or “supravitral”) stains are dyes absorbed by live cells. Young RBCs contain ribonucleic acid (RNA) and are called reticulocytes when the RNA is visualized using vital stains. Counting reticulocytes visually by microscopy was (and remains) a tedious and imprecise procedure until the development of automated reticulocyte counting by the TOA Corporation (presently Sysmex Corporation, Kobe, Japan) in 1990. Now all fully automated profiling instruments provide an absolute reticulocyte count and, in addition, an especially sensitive measure of RBC production, the immature reticulocyte count or immature reticulocyte fraction (Chapter 15). However, it is still necessary to confirm instrument counts visually from time to time, so medical laboratory professionals must retain this skill.

### WHITE BLOOD CELLS

WBCs, or leukocytes, are a loosely related category of cell types dedicated to protecting their host from infection and injury (Chapter 12). WBCs are transported in the blood from their source, usually bone marrow or lymphoid tissue, to their tissue or body cavity destination. WBCs are so named because they are nearly colorless in an unstained cell suspension.

WBCs may be counted visually using a microscope and hemacytometer. The technique is the same as RBC counting, but the typical dilution is 1:20, and the diluent is a dilute acid solution. The acid causes RBCs to lyse or rupture; without it, RBCs, which are 500 to 1000 times more numerous than WBCs, would obscure the WBCs. The WBC count ranges from 4500 to 11,500/µL. Visual WBC counting has been largely replaced by automated hematology profiling instruments, but it is accurate and useful in situations in which no automation is available. Medical laboratory professionals who analyze body fluids such as cerebrospinal fluid or pleural fluid may employ visual WBC counting.

A decreased WBC count (fewer than 4500/µL) is called leukopenia, and an increased WBC count (more than 11,500/µL) is called leukocytosis, but the WBC count alone has modest clinical value. The microscopist must differentiate the categories of WBCs in the blood by using a Wright-stained blood film and light microscopy (Chapter 16). The types of WBCs are as follows:

- **Neutrophils** (NEUTs; segmented neutrophils, SEGs, polymorphonuclear neutrophils, PMNs; Figure 1-1, B). Neutrophils are phagocytic cells whose major purpose is to engulf and destroy microorganisms and foreign material, either directly or after they have been labeled for destruction by the immune system. The term segmented refers to their multilobed nuclei. An increase in neutrophils is called neutrophilia and often signals bacterial infection. A decrease is called neutropenia and has many causes, but it is often caused by certain medications or viral infections.

- **Bands** (band neutrophils, BANDs; Figure 1-1, C). Bands are less differentiated or less mature neutrophils. An increase in bands also signals bacterial infection and is customarily called a left shift. The cytoplasm of neutrophils and bands contains submicroscopic, pink- or lavender-staining granules filled with bactericidal secretions.

- **Eosinophils** (EOS; Figure 1-1, D). Eosinophils are cells with bright orange-red, regular cytoplasmic granules filled with proteins involved in immune system regulation. An elevated eosinophil count is called eosinophilia and often signals a response to allergy or parasitic infection.

- **Basophils** (BASOs; Figure 1-1, E). Basophils are cells with dark purple, irregular cytoplasmic granules that obscure the nucleus. The basophil granules contain histamines and various other proteins. An elevated basophil count is called basophilia. Basophilia is rare and often signals a hematologic disease.

- **Lymphocytes** (LYMPHs; Figure 1-1, F). Lymphocytes comprise a complex system of cells that provide for host immunity. Lymphocytes recognize foreign antigens and mount humoral (antibodies) and cell-mediated antagonistic responses. On a Wright-stained blood film, most lymphocytes are nearly round, are slightly larger than RBCs, and have round featureless nuclei and a thin rim of nongranular cytoplasm. An increase in the lymphocyte count is called lymphocytosis and often is associated with viral infections. Accompanying lymphocytosis are often variant or reactive lymphocytes with characteristic morphology (Chapter 29). An abnormally low lymphocyte count is called lymphopenia or lymphocytopenia and is often associated with drug therapy or immunodeficiency. Lymphocytes are also implicated in leukemia; chronic lymphocytic leukemia is more prevalent in people older than 65 years, whereas acute lymphoblastic leukemia is the most common form of childhood leukemia (Chapters 35 and 36). Medical laboratory scientists and hematopathologists classify lymphocytic leukemias largely based on Wright-stained blood films, flow cytometric immunophenotyping, and molecular diagnostic techniques (Chapters 31 to 32).

- **Monocytes** (MONOs; Figure 1-1, G). The monocyte is an immature macrophage passing through the blood from its point of origin, usually the bone marrow, to a targeted tissue location. Macrophages are the most abundant cell type in the body, more abundant than RBCs or skin cells, although monocytes comprise a minor component of peripheral
blood WBCs. Macrophages occupy every body cavity; some are motile and some are immobilized. Their tasks are to identify and phagocytose (engulf and consume) foreign particles and assist the lymphocytes in mounting an immune response through the assembly and presentation of immunogenic epitopes. On a Wright-stained blood film, monocytes have a slightly larger diameter than other WBCs, blue-gray cytoplasm with fine azure granules, and a nucleus that is usually indented or folded. An increase in the number of monocytes is called monocytosis. Monocytosis may be found in certain infections, collagen-vascular diseases, or in acute and chronic leukemias (Chapters 29, 33, and 35). Medical laboratory professionals seldom document a decreased monocyte count, so the theoretical term monocytopenia is seldom used.

**PLATELETS**

Platelets, or thrombocytes, are true blood cells that maintain blood vessel integrity by initiating vessel wall repairs (Chapter 13). Platelets rapidly adhere to the surfaces of damaged blood vessels, form aggregates with neighboring platelets to plug the vessels, and secrete proteins and small molecules that trigger thrombosis, or clot formation. Platelets are the major cells that control hemostasis, a series of cellular and plasma-based mechanisms that seal wounds, repair vessel walls, and maintain vascular patency (unimpeded blood flow). Platelets are only 2 to 4 μm in diameter, round or oval, anucleate (for this reason some hematologists prefer to call platelets “cell fragments”), and slightly granular (Figure 1-1, H). Their small size makes them appear insignificant, but they are essential to life and are extensively studied for their complex physiology. Uncontrolled platelet and hemostatic activation is responsible for deep vein thrombosis, pulmonary emboli, acute myocardial infarctions (heart attacks), cerebrovascular accidents (strokes), peripheral artery disease, and repeated spontaneous abortions (miscarriages).

The microscopist counts platelets using the same technique used in counting WBCs on a hemacytometer, although a different counting area and dilution is usually used (Chapter 14). Owing to their small volume, platelets are hard to distinguish visually in a hemacytometer, and phase microscopy provides for easier identification (Chapter 4). Automated profiling instruments have largely replaced visual platelet counting and provide greater accuracy (see Chapter 15).

One advantage of automated profiling instruments is their ability to generate a mean platelet volume (MPV), which is unavailable through visual methods. The presence of predominantly larger platelets generates an elevated MPV value, which sometimes signals a regenerative bone marrow response to platelet consumption (Chapters 13 and 40).

Elevated platelet counts, called thrombocytosis, signal inflammation or trauma but convey modest intrinsic significance. Essential thrombocythemia is a rare malignant condition characterized by extremely high platelet counts and uncontrolled platelet production. Essential thrombocythemia is a life-threatening hematologic disorder (Chapter 33).

A low platelet count, called thrombocytopenia, is a common consequence of drug treatment and may be life-threatening. Because the platelet is responsible for normal blood vessel maintenance and repair, thrombocytopenia is usually accompanied by easy bruising and uncontrolled hemorrhage (Chapter 40). Thrombocytopenia accounts for many hemorrhage-related emergency department visits. Accurate platelet counting contributes to patient safety because it provides for the diagnosis of thrombocytopenia in many disorders or therapeutic regimens.

**COMPLETE BLOOD COUNT**

A complete blood count (CBC) is performed on automated hematology profiling instruments and includes the RBC, WBC, and platelet measurements indicated in Box 1-1. The medical laboratory professional may collect a blood specimen for the CBC, but often a phlebotomist, nurse, physician assistant, physician, or patient care technician may also collect the specimen (Chapters 3 and 42). No matter who collects, the medical laboratory professional is responsible for the integrity of the specimen and ensures that it is submitted in the appropriate anticoagulant and tube and is free of clots and hemolysis (red-tinted plasma indicating RBC damage). The specimen must be of sufficient volume, as “short draws” result in incorrect anticoagulant-to-specimen ratios. The specimen must be tested or prepared for storage within the appropriate time frame to ensure accurate analysis (Chapter 5) and must be accurately registered in the work list, a process known as specimen accession. Accession may be automated, relying on bar code or radio-frequency identification technology, thus reducing instances of identification error.

Although all laboratory scientists and technicians are equipped to perform visual RBC, WBC, and platelet counts
using dilution pipettes, hemacytometers, and microscopes, most laboratories employ automated profiling instruments to generate the CBC. Many profiling instruments also provide comments on RBC, WBC, and platelet morphology (Chapter 15). When one of the results from the profiling instrument is abnormal, the instrument provides an indication of this, sometimes called a flag. In this case, a “reflex” blood film examination is performed (Chapter 16).

The blood film examination (described next) is a specialized, demanding, and fundamental CBC activity. Nevertheless, if all profiling instrument results are normal, the blood film examination is usually omitted from the CBC. However, physicians may request a blood film examination on the basis of clinical suspicion even when the profiling instrument results fall within their respective reference intervals.

**BLOOD FILM EXAMINATION**

To accomplish a blood film examination, the microscopist prepares a “wedge-prep” blood film on a glass microscope slide, allows it to dry, and fixes and stains it using Wright or Wright-Giemsa stain (Chapter 16). The microscopist examines the RBCs and platelets by light microscopy for abnormalities of shape, diameter, color, or inclusions using the 50× or 100× oil immersion lens to generate 500× or 1000× magnification (Chapter 4). The microscopist then visually estimates the WBC count and platelet count for comparison with their respective instrument counts and investigates discrepancies. Next, the microscopist systematically reviews, identifies, and tabulates 100 (or more) WBCs to determine their percent distribution. This process is referred to as determining the WBC differential (“diff”). The WBC differential relies on the microscopist’s skill, visual acuity, and integrity, and it provides extensive diagnostic information. Medical laboratory professionals pride themselves on their technical and analytical skills in performing the blood film examination and differential count. Visual recognition systems such as the Cellavision® DM96 or the Bloodhound automate the RBC and platelet morphology and WBC differential processes, but the medical laboratory professional or the hematopathologist is the final arbiter for all cell identification. The results of the CBC, including all profiling and blood film examination parameters and interpretive comments, are provided in paper or digital formats for physician review with abnormal results highlighted.

**ENDOTHELIAL CELLS**

Because they are structural and do not flow in the bloodstream, endothelial cells—the endodermal cells that form the inner surface of the blood vessel—are seldom studied in the hematology laboratory. Nevertheless, endothelial cells are important in maintaining normal blood flow, in tethering (decelerating) platelets during times of injury, and in enabling WBCs to escape from the vessel to the surrounding tissue when needed. Increasingly refined laboratory methods are becoming available to assay and characterize the secretions (cytokines) of these important cells.

**COAGULATION**

Most hematology laboratories include a blood coagulation-testing department (Chapters 42 and 44). Platelets are a key component of hemostasis, as previously described; plasma coagulation is the second component. The coagulation system employs a complex sequence of plasma proteins, some enzymes, and some enzyme cofactors to produce clot formation after blood vessel injury. Another 6 to 8 enzymes exert control over the coagulation mechanism, and a third system of enzymes and cofactors digests clots to restore vessel patency, a process called fibrinolysis. Bleeding and clotting disorders are numerous and complex, and the coagulation section of the hematology laboratory provides a series of plasma-based laboratory assays that assess the interactions of hematologic cells with plasma proteins (Chapters 42 and 44).

The medical laboratory professional focuses especially on blood specimen integrity for the coagulation laboratory, because minor blood specimen defects, including clots, hemolysis, lipemia, plasma bilirubin, and short draws, render the specimen useless (Chapters 3 and 42). High-volume coagulation tests suited to the acute care facility include the platelet count and MPV as described earlier, prothrombin time and partial thromboplastin time (or activated partial thromboplastin time), thrombin time (or thrombin clotting time), fibrinogen assay, and D-dimer assay (Chapter 42). The prothrombin time and partial thromboplastin time are particularly high-volume assays used in screening profiles. These tests assess each portion of the coagulation pathway for deficiencies and are used to monitor anticoagulant therapy. Another 30 to 40 moderate-volume assays, mostly clot-based, are available in specialized or tertiary care facilities. The specialized or tertiary care coagulation laboratory with its interpretive complexities attracts advanced medical laboratory scientists with specialized knowledge and communication skills.

**ADVANCED HEMATOLOGY PROCEDURES**

Besides performing the CBC, the hematology laboratory provides bone marrow examinations, flow cytometry immunophenotyping, cytogenetic analysis, and molecular diagnosis assays. Performing these tests may require advanced preparation or particular dedication by medical laboratory scientists with a desire to specialize.

Medical laboratory scientists assist physicians with bedside bone marrow collection, then prepare, stain, and microscopically review bone marrow smears (Chapter 17). Bone marrow aspirates and biopsy specimens are collected and stained to analyze nucleated cells that are the immature precursors to blood cells. Cells of the erythroid series are precursors to RBCs (Chapter 8); myeloid series cells mature to form bands and neutrophils, eosinophils, and basophils (Chapter 12); and megakaryocytes produce platelets (Chapter 13). Medical laboratory scientists, clinical pathologists, and hematologists review Wright-stained aspirate smears for morphologic abnormalities, high or low bone marrow cell concentration, and inappropriate cell line distributions. For instance, an increase
in the erythroid cell line may indicate bone marrow compensation for excessive RBC destruction or blood loss (Chapter 19 and Chapters 23 to 26). The biopsy specimen, enhanced by hematoxylin and eosin (H&E) staining, may reveal abnormalities in bone marrow architecture indicating leukemia, bone marrow failure, or one of a host of additional hematologic disorders. Results of examination of bone marrow aspirates and biopsy specimens are compared with CBC results generated from the peripheral blood to correlate findings and develop pattern-based diagnoses.

In the bone marrow laboratory, cytochemical stains may occasionally be employed to differentiate abnormal myeloid, erythroid, and lymphoid cells. These stains include myeloperoxidase, Sudan black B, nonspecific and specific esterase, periodic acid–Schiff, tartrate-resistant acid phosphatase, and alkaline phosphatase. The cytochemical stains are time-honored stains that in most laboratories have been replaced by flow cytometry immunophenotyping, cytogenetics, and molecular diagnostic techniques (Chapters 30 to 32). Since 1980, however, immunostaining methods have enabled identification of cell lines by detecting lineage-specific antigens on the surface or in the cytoplasm of leukemia and lymphoma cells. An example of immunostaining is a visible dye that is bound to antibodies to CD42b, a membrane protein that is present in the megakaryocytic lineage and may be diagnostic for megakaryoblastic leukemia (Chapter 35).

Flow cytometers may be quantitative, such as clinical flow cytometers that have grown from the original Coulter principle, or qualitative, including laser-based instruments that have migrated from research applications to the clinical laboratory (Chapters 15 and 32). The former devices are automated clinical profiling instruments that generate the quantitative parameters of the CBC through application of electrical impedance and laser or light beam interruption. Qualitative laser-based flow cytometers are mechanically simpler but technically more demanding. Both qualitative and quantitative flow cytometers are employed to analyze cell populations by measuring the effects of individual cells on laser light, such as forward-angle fluorescent light scatter and right-angle fluorescent light scatter, and by immunophenotyping for cell membrane epitopes using monoclonal antibodies labeled with fluorescent dyes. The qualitative flow cytometry laboratory is indispensable to leukemia and lymphoma diagnosis.

Cytogenetics, a time-honored form of molecular technology, is employed in bone marrow aspirate examination to find gross genetic errors such as the Philadelphia chromosome, a reciprocal translocation between chromosomes 9 and 22 that is associated with chronic myelogenous leukemia, and t(15;17), a translocation between chromosomes 15 and 17 associated with acute promyelocytic leukemia (Chapter 30). Cytogenetic analysis remains essential to the diagnosis and treatment of leukemia.

Molecular diagnostic techniques, the fastest-growing area of laboratory medicine, enhance and even replace some of the advanced hematologic methods. Real-time polymerase chain reaction, microarray analysis, fluorescence in situ hybridization, and DNA sequencing systems are sensitive and specific methods that enable medical laboratory scientists to detect various chromosome translocations and gene mutations that confirm specific types of leukemia, establish their therapeutic profile and prognosis, and monitor the effectiveness of treatment (Chapter 31).

### ADDITIONAL HEMATOLOGY PROCEDURES

Medical laboratory professionals provide several time-honored manual whole-blood methods to support hematologic diagnosis. The osmotic fragility test uses graduated concentrations of saline solutions to detect spherocytes (RBCs with proportionally reduced surface membrane area) in hereditary spherocytosis or warm autoimmune hemolytic anemia (Chapters 24 and 26). Likewise, the glucose-6-phosphate dehydrogenase assay phenotypically detects an inherited RBC enzyme deficiency causing severe episodic hemolytic anemia (Chapter 24). The sickle cell solubility screening assay and its follow-up tests, hemoglobin electrophoresis and high performance liquid chromatography, are used to detect and diagnose sickle cell anemia and other inherited qualitative hemoglobin abnormalities and thalassemias (Chapters 27 and 28). One of the oldest hematology tests, the erythrocyte sedimentation rate, detects inflammation and roughly estimates its intensity (Chapter 14).

Finally, the medical laboratory professional reviews the cellular counts, distribution, and morphology in body fluids other than blood (Chapter 18). These include cerebrospinal fluid, synovial (joint) fluid, pericardial fluid, pleural fluid, and peritoneal fluid, in which RBCs and WBCs may be present in disease and in which additional malignant cells may be present that require specialized detection skills. Analysis of non-blood body fluids is always performed with a rapid turnaround, because cells in these hostile environments rapidly lose their integrity. The conditions leading to a need for body fluid analysis are invariably acute.

### HEMATOLOGY QUALITY ASSURANCE AND QUALITY CONTROL

Medical laboratory professionals employ particularly complex quality control systems in the hematology laboratory (Chapter 5). Owing to the unavailability of weighed standards, the measurement of cells and biological systems defies chemical standardization and requires elaborate calibration, validation, matrix effect examination, linearity, and reference interval determinations. An internal standard methodology known as the moving average also supports hematology laboratory applications. Medical laboratory professionals in all disciplines compare methods through clinical efficacy calculations that produce clinical sensitivity, specificity, and positive and negative predictive values for each assay. They must monitor specimen integrity and test ordering patterns and ensure the integrity and delivery of reports, including numerical and narrative statements and reference interval comparisons. As in most branches of laboratory science, the hematology laboratory places an enormous responsibility for accuracy, integrity, judgment, and timeliness on the medical laboratory professional.
REFERENCES

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Define standard precautions and list infectious materials included in standard precautions.
2. Describe the safe practices required in the Occupational Exposure to Bloodborne Pathogens Standard.
3. Identify occupational hazards that exist in the hematology laboratory.
4. Describe appropriate methods to decontaminate work surfaces after contamination with blood or other potentially infectious material.
5. Identify the regulatory requirements of the Occupational Exposure to Hazardous Chemicals in Laboratories standard.
6. Describe the principles of a fire prevention program, including details such as the frequency of testing equipment.
7. Name the most important practice to prevent the spread of infection.
8. Given a written laboratory scenario, assess for safety hazards and recommend corrective action for any deficiencies or unsafe practices identified.
9. Select the proper class of fire extinguisher for a given type of fire.
10. Explain the purpose of Safety Data Sheets (SDSs), list information contained on SDSs, and determine when SDSs would be used in a laboratory activity.
11. Name the specific practice during which most needle stick injuries occur.
12. Describe elements of a safety management program.

OUTLINE
Standard Precautions
   Applicable Safety Practices
      Required by the OSHA Standard
   Housekeeping
   Laundry
   Hepatitis B Virus Vaccination
   Training and Documentation
   Regulated Medical Waste Management
Occupational Hazards
   Fire Hazard
   Chemical Hazards
   Electrical Hazard
   Needle Puncture
Developing a Safety Management Program
   Planning Stage: Hazard Assessment and Regulatory Review
   Safety Program Elements

CASE STUDY
After studying the material in this chapter, the reader should be able to respond to the following case study:

Hematology Services, Inc., had a proactive safety program. Quarterly safety audits were conducted by members of the safety committee. The following statements were recorded in the safety audit report. Which statements describe good work practices, and which statements represent deficiencies? List the corrective actions required for identified unsafe practices.

1. A hematology laboratory scientist was observed removing gloves and immediately left the laboratory for a meeting. She did not remove her laboratory coat.
2. Food was found in the specimen refrigerator.
3. Hematology laboratory employees were seen in the lunchroom, wearing laboratory coats.
4. Fire extinguishers were found every 75 feet of the laboratory.
5. Fire extinguishers were inspected quarterly and maintained annually.
6. Unlabeled bottles were found at a workstation.
7. A 1:10 solution of bleach was found near an automated hematology analyzer. Further investigation revealed that the bleach solution was made 6 months ago.
8. Gloves were worn by the staff receiving specimens.
9. Safety data sheets were obtained by fax.
10. Chemicals were stored alphabetically.
Any conditions in the laboratory have the potential for causing injury to staff and damage to the building or to the community. Patients’ specimens, needles, chemicals, electrical equipment, reagents, and glassware all are potential causes of accidents or injury. Managers and employees must be knowledgeable about safe work practices and incorporate these practices into the operation of the hematology laboratory. The key to prevention of accidents and laboratory-acquired infections is a well-defined safety program.

Safety is a broad subject and cannot be covered in one chapter. This chapter simply highlights some of the key safe practices that should be followed in the hematology laboratory. Omission of a safe practice from this chapter does not imply that it is not important or that it should not be considered in the development of a safety curriculum or a safety program.

**STANDARD PRECAUTIONS**

One of the greatest risks associated with the hematology laboratory is the exposure to blood and body fluids. In December 1991, the Occupational Safety and Health Administration (OSHA) issued the final rule for the Occupational Exposure to Bloodborne Pathogens Standard. The rule that specifies standard precautions to protect laboratory workers and other health care professionals became effective on March 6, 1992. Universal precautions was the original term; OSHA’s current terminology is standard precautions. Throughout this text, the term standard precautions is used to remind the reader that all blood, body fluids, and unfixed tissues are to be handled as though they were potentially infectious.

Standard precautions must be adopted by the laboratory. Standard precautions apply to blood, semen, vaginal secretions, cerebrospinal fluid, synovial fluid, pleural fluid, any body fluid with visible blood, any unidentified body fluid, unfixed slides, microhematocrit clay, and saliva from dental procedures. Adopting standard precautions lessens the risk of health care worker exposures to blood and body fluids, decreasing the risk of injury and illness.

Bloodborne pathogens are pathogenic microorganisms that, when present in human blood, can cause disease. They include, but are not limited to, hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). This chapter does not cover the complete details of the standard; it discusses only the sections that apply directly to the hematology laboratory. Additional information can be found in the references at the end of this chapter.

**Applicable Safety Practices Required by the OSHA Standard**

The following standards are applicable in a hematology laboratory and must be enforced:

1. **Hand washing** is one of the most important safety practices. Hands must be washed with soap and water. If water is not readily available, alcohol hand gels (minimum 62% alcohol) may be used. Hands must be thoroughly dried. The proper technique for hand washing is as follows:
   a. Wet hands and wrists thoroughly under running water.
   b. Apply germicidal soap and rub hands vigorously for at least 15 seconds, including between the fingers and around and over the fingernails (Figure 2-1, A).
   c. Rinse hands thoroughly under running water in a downward flow from wrist to fingertips (Figure 2-1, B).
   d. Dry hands with a paper towel (Figure 2-1, C). Use the paper towel to turn off the faucet handles (Figure 2-1, D).
   e. After completion of work
   f. After gloves are removed and between glove changes
   g. Before leaving the laboratory
   h. Before and after eating and drinking, smoking, applying cosmetics or lip balm, changing a contact lens, and using the lavatory
   i. Before and after all other activities that entail hand contact with mucous membranes, eyes, or breaks in skin
2. Eating, drinking, smoking, and applying cosmetics or lip balm must be prohibited in the laboratory work area.
3. Hands, pens, and other fomites must be kept away from the mouth and all mucous membranes.
4. Food and drink, including oral medications and tolerance-testing beverages, must not be kept in the same refrigerator as laboratory specimens or reagents or where potentially infectious materials are stored or tested.
5. Mouth pipetting must be prohibited.
6. Needles and other sharp objects contaminated with blood and other potentially infectious materials should not be manipulated in any way. Such manipulation includes resheathing, bending, clipping, or removing the sharp object. Resheathing or recapping is permitted only when there are no other alternatives or when the recapping is required by specific medical procedures. Recapping is permitted by use of a method other than the traditional two-handed procedure. The one-handed method or a resheathing device is often used. Documentation in the exposure control plan should identify the specific procedure in which resheathing is permitted.
7. Contaminated sharps (including, but not limited to, needles, blades, pipettes, syringes with needles, and glass slides) must be placed in a puncture-resistant container that is appropriately labeled with the universal biohazard symbol (Figure 2-2) or a red container that adheres to the standard. The container must be leakproof (Figure 2-3).
8. Procedures such as removing caps when checking for clots, filling hemacytometer chambers, making slides, discarding specimens, making dilutions, and pouring specimens or fluids must be performed so that splashing, spraying, or production of droplets of the specimen being manipulated is prevented. These procedures may be performed behind a barrier, such as a plastic shield, or protective eyewear should be worn (Figure 2-4).
9. **Personal protective clothing** and equipment must be provided to the laboratory staff. The most common forms of personal protective equipment are described in the following section:

a. **Outer coverings**, including gowns, laboratory coats, and sleeve protectors, should be worn when there is a chance of splashing or spilling on work clothing. The outer covering must be made of fluid-resistant material, must be long-sleeved, and must remain buttoned at all times. If contamination occurs, the personal protective equipment should be removed immediately and treated as infectious material.

b. **Gloves** must be worn when the potential for contact with blood or body fluids exists (including when removing and handling bagged biohazardous material and when decontaminating bench tops) and when venipuncture or skin puncture is performed. One of the limitations of gloves is that they do not prevent needle sticks or other puncture wounds. Provision of gloves to laboratory staff must accommodate latex allergies. Alternative gloves must be readily accessible to any laboratory employee with a latex allergy.

Cloth laboratory coats may be worn if they are fluid resistant. If cloth coats are worn, the coats must be laundered inside the laboratory or hospital or by a contracted laundry service. Laboratory coats used in the laboratory while performing laboratory analysis are considered personal protective equipment and are not to be taken home.

All protective clothing should be removed before leaving the laboratory; it should not be worn into public areas. Public areas include, but are not limited to, break rooms, storage areas, bathrooms, cafeterias, offices, and meeting places outside the laboratory.

A second laboratory coat can be made available for use in public areas. A common practice is to have a different-colored laboratory coat that can be worn in public areas. This second laboratory coat could be laundered by the employee.

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**Figure 2-1** Proper hand washing technique. A, Wet hands thoroughly under running water, apply soap, and rub hands vigorously for at least 15 seconds. B, Rinse hands thoroughly under running water in a downward flow from wrist to fingertips. C, Dry hands with a paper towel. D, Turn off faucet with paper towel. (From Young AP, Proctor DB: Kinn’s the medical assistant, ed 11, St Louis, 2011, Saunders.)

**Figure 2-2** Biohazard symbol.
10. **Phlebotomy trays** should be appropriately labeled to indicate potentially infectious materials. Specimens should be placed into a secondary container, such as a resealable biohazard-labeled bag.

11. If a **pneumatic tube system** is used to transport specimens, the specimens should be transported in the appropriate tube (primary containment), and placed into a special self-sealing leakproof bag appropriately labeled with the biohazard symbol (secondary containment). Requisition forms should be placed outside of the secondary container to prevent contamination if the specimen leaks. Foam allergy. Gloves must be changed after each contact with a patient, when there is visible contamination, and when physical damage occurs. Gloves should not be worn when "clean" devices, such as a copy machine or a "clean" telephone, are used. Gloves must not be worn again or washed. After one glove is removed, the second glove can be removed by sliding the index finger of the ungloved hand between the glove and the hand and slipping the second glove off. This technique prevents contamination of the "clean" hand by the "dirty" second glove (Figure 2-5). Contaminated gloves should be disposed of according to applicable federal or state regulations.

c. **Eyewear**, including face shields, goggles, and masks, should be used when there is potential for aerosol mists, splashes, or sprays to mucous membranes (mouth, eyes, or nose). Removing caps from specimen tubes, working at an automated hematology analyzer, and centrifuging specimens are examples of tasks that could produce an aerosol mist.

Figure 2-3 Examples of sharps disposal systems. **A**, Molded foot pedal cart with hinged or slide top lid. **B**, In-room system wall enclosures. **C**, Multipurpose container with horizontal drop lid. **D**, Phlebotomy containers. (Courtesy Covidien, Mansfield, MA.)

Figure 2-4 Examples of safety shields. **A**, Face shield. **B**, Adjustable swing arm shield. (Courtesy Steve Kasper.)
inserts for the pneumatic tube system carrier prevent shifting of the sample during transport and also act as a shock absorber, thus decreasing the risk of breakage.

When specimens are received in the laboratory, they should be handled by an employee wearing gloves, a laboratory coat, and other protective clothing, in accordance with the type and condition of specimen. Contaminated containers or requisitions must be decontaminated or replaced before being sent to the work area.

12. When equipment used to process specimens becomes visibly contaminated or requires maintenance or service, it must be decontaminated, whether service is performed within the laboratory or by a manufacturer repair service. Decontamination of equipment consists of a minimum of flushing the lines and wiping the exterior and interior of the equipment. If it is difficult to decontaminate the equipment, it must be labeled with the biohazard symbol to indicate potentially infectious material. Routine cleaning should be performed on equipment that has the potential for receiving splashes or sprays, such as inside the lid of the microhematocrit centrifuge.

**Housekeeping**

Blood and other potentially infectious materials can contaminate work surfaces easily. Contamination can be caused by splashes, poor work practices, and droplets of blood on the work surface. To prevent contamination, all work surfaces should be cleaned when procedures are completed and whenever the bench area or floor becomes visibly contaminated. An appropriate disinfectant solution is household bleach, used in a 1:10 volume/volume dilution (10%), which can be made by adding 10 mL of bleach to 90 mL of water or 1½ cups of bleach to 1 gallon of water to achieve the recommended concentration of chlorine (5500 ppm). Because this solution is not stable, it must be made fresh daily. The container of 1:10 solution of bleach should be labeled properly with the name of the solution, the date and time prepared, the date and time of expiration (24 hours), and the initials of the preparer. Bleach is not recommended for aluminum surfaces. Other solutions used to decontaminate include, but are not limited to, a phenol-based disinfectant such as Amphyl®, tuberculocidal disinfectants, and 70% ethanol. All paper towels used in the decontamination process should be disposed of as biohazardous waste. Documentation of the disinfection of work areas and equipment after each shift is required.

**Laundry**

If nondisposable laboratory coats are used, they must be placed in appropriate containers for transport to the laundry at the facility or to a contract service and not taken to the employee’s home.

**Hepatitis B Virus Vaccination**

Laboratory employees should receive the HBV vaccination series at no cost before or within 10 days after beginning work
in the laboratory. An employee must sign a release form if he or she refuses the series. The employee can request and receive the hepatitis vaccination series at any time, however. If an exposure incident (needle puncture or exposure to skin, eye, face, or mucous membrane) occurs, postexposure evaluation and follow-up, including prophylaxis and medical consultation, should be made available at no cost to the employee. Employees should be encouraged to report all exposure incidents, and such reporting should be enforced as standard policy.

Training and Documentation
Hematology staff should be properly educated in epidemiology and symptoms of bloodborne diseases, modes of transmission of bloodborne diseases, use of protective equipment, work practices, ways to recognize tasks and other activities that may result in an exposure, and the location of the written exposure plan for the laboratory. Education should be documented and should occur when new methods, equipment, or procedures are introduced; at the time of initial assignment to the laboratory; and at least annually thereafter.

Regulated Medical Waste Management
Specimens from the hematology laboratory are identified as regulated waste. There are different categories of regulated medical waste, and state and local regulations for disposal of medical waste must be followed. OSHA regulates some aspects of regulated medical waste such as needle handling, occupational exposure, labeling of containers, employee training, and storing of the waste. The Occupational Exposure to Bloodborne Pathogens Standard provides information on the handling of regulated medical waste. Detailed disposal guidelines are specific to the state disposal standards. When two regulations conflict, the more stringent standard is followed.

OCCUPATIONAL HAZARDS

Four important occupational hazards in the laboratory are discussed in this chapter: fire hazard, chemical hazards, electrical hazard, and needle puncture. There are other hazards to be considered when a safety management program is developed, and the reader is referred to the Department of Labor section of the Code of Federal Regulations for detailed regulations.²

Fire Hazard
Because of the numerous flammable and combustible chemicals used in the laboratory, fire is a potential hazard. Complying with standards established by the National Fire Protection Association, OSHA, the Joint Commission, the College of American Pathologists, and other organizations can minimize the dangers. A good fire safety/prevention plan is necessary and should consist of the following:
1. Enforcement of a no-smoking policy.
2. Installation of appropriate fire extinguishers. Several types of extinguishers, most of which are multipurpose, are available for use for specific types of fires.

3. Placement of fire extinguishers every 75 feet. A distinct system for marking the locations of fire extinguishers enables quick access when they are needed. Fire extinguishers should be checked monthly and maintained annually. Not all fire extinguishers are alike. Each fire extinguisher is rated for the type of fire that it can suppress. It is important to use the correct fire extinguisher for the given class of fire. Hematology laboratory staff should be trained to recognize the class of extinguisher and use a fire extinguisher properly.

Table 2-1 summarizes the fire extinguisher classifications. The fire extinguishers used in the laboratory are portable extinguishers and are not designed to fight large fires. In the event of a fire in the laboratory, the local fire department must be contacted immediately.

4. Placement of adequate fire detection and suppression systems (alarms, smoke detectors, sprinklers), which should be tested every 3 months.
5. Placement of manual fire alarm boxes near the exit doors. Travel distance should not exceed 200 feet.
6. Written fire prevention and response procedures, commonly referred to as the fire response plan. All staff in the laboratory should be knowledgeable about the procedures. Employees should be given assignments for specific responsibilities in case of fire, including responsibilities for patient care, if applicable. Total count of employees in the laboratory should be known for any given day, and a buddy system should be developed in case evacuation is necessary. Equipment shutdown procedures should be addressed in the plan, as should responsibility for implementation of those procedures.
7. Fire drills, which should be conducted so that response to a fire situation is routine and not a panic response. Frequency of fire drills varies by type of occupancy of the building and by accrediting agency. Overall governance can be by the local or state fire marshall. All laboratory employees should participate in the fire drills. Proper documentation should be maintained to verify that all phases of the fire response plan were activated. If patients are in areas adjacent to the hematology laboratory, evacuation can be simulated, rather than evacuating actual patients. The entire evacuation route should be walked to verify the exit routes and clearance of the corridors. A summary of the laboratory’s fire response plan can be copied onto a quick reference card and attached to workers’ identification badges to be readily available in a fire situation.

<table>
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<tr>
<th>TABLE 2-1 Fire Extinguisher Classifications and Use</th>
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<tr>
<td>Class/Type of Extinguisher</td>
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8. Written storage requirements for any flammable or combustible chemicals stored in the laboratory. Chemicals should be arranged according to hazard class and not alphabetically. A master chemical inventory should be maintained and revised when new chemicals are added or deleted from the laboratory procedures.

9. A well-organized fire safety training program. This program should be completed by all employees. Activities that require walking evacuation routes and locating fire exit boxes in the laboratory area should be scheduled. Types of fires likely to occur and use of the fire extinguisher should be discussed. Local fire departments may request a tour of the laboratory or facility to become familiar with the potential fire hazards prior to an actual fire occurring in the laboratory.

Chemical Hazards
Some of the chemicals used in the hematology laboratory are considered hazardous and are governed by the Occupational Exposure to Hazardous Chemicals in Laboratories Standard. This regulation requires laboratories to develop a chemical hygiene plan that outlines safe work practices to minimize exposures to hazardous chemicals. The full text of this regulation can be found in 29 CFR (Code of Federal Regulations) 1910.1450.2

General principles that should be followed in working with chemicals are as follows:
1. Label all chemicals properly, including chemicals in secondary containers, with the name and concentration of the chemical, preparation or fill date, expiration date (time, if applicable), initials of preparer (if done in-house), and chemical hazards (e.g., poisonous, corrosive, flammable). Do not use a chemical that is not properly labeled as to the identity or content.
2. Follow all handling and storage requirements for the chemical.
3. Store alcohol and other flammable chemicals in approved safety cans or storage cabinets at least 5 feet away from a heat source (e.g., Bunsen burners, paraffin baths). Limit the quantity of flammable chemicals stored on the workbench to 2 working days’ supply. Do not store chemicals in a hood or in any area where they could react with other chemicals.
4. Use adequate ventilation, such as fume hoods, when working with hazardous chemicals.
5. Use personal protective equipment, such as gloves (e.g., nitrile, polyvinyl chloride, rubber—as appropriate for the chemical in use), rubber aprons, and face shields. Safety showers and eye wash stations should be available every 100 feet or within 10 seconds of travel distance from every work area where the hazardous chemicals are used.
6. Use bottle carriers for glass bottles containing more than 500 mL of hazardous chemical.
7. Use alcohol-based solvents, rather than xylene or other particularly hazardous substances, to clean microscope objectives.
8. The wearing of contact lenses should not be permitted when an employee is working with xylene, acetone, alcohols, formaldehyde, and other solvents. Many lenses are permeable to chemical fumes. Contact lenses can make it difficult to wash the eyes adequately in the event of a splash.
9. Spill response procedures should be included in the chemical safety procedures, and all employees must receive training in these procedures. Absorbent material should be available for spill response. Multiple spill response kits and absorbent material should be stored in various areas and rooms rather than only in the area where they are likely to be needed. This prevents the need to walk through the spilled chemical to obtain the kit.
10. Safety Data Sheets (SDS), formerly known as Material Safety Data Sheets (MSDS), are written by the manufacturers of chemicals to provide information on the chemicals that cannot be put on a label. In 2012, the Hazard Communication Standard (29 CFR 1910.1200(g)) was revised to align with the United Nations Globally Harmonized System (GHS) of Classification and Labeling of Chemicals. The significant revisions required the use of new labeling elements and a standardized format for Safety Data Sheets (SDS). The standardized information on the SDS uses a 16-section format, and the implementation date is June 1, 2015. The OSHA website on Hazard Communication Safety Data Sheets specifies the content for the 16 sections of the SDS as follows:3

- Section 1. Identification includes product identifier, manufacturer or distributor (name, address, emergency phone number), recommended use, and restrictions on use.
- Section 2. Hazard(s) identification includes all hazards of the chemical and required label information.
- Section 3. Composition/information on ingredients includes information on chemical ingredients and trade secret claims.
- Section 4. First-aid measures includes symptoms, acute and delayed effects, and required treatment.
- Section 5. Firefighting measures provides extinguishing techniques and equipment and chemical hazards from fire.
- Section 6. Accidental release measures lists emergency procedures, protective equipment, and methods of containment and cleanup.
- Section 7. Handling and storage lists precautions for safe handling and storage and incompatibilities with other chemicals.
- Section 8. Exposure controls and personal protection lists OSHA’s permissible exposure limits, threshold limit values, engineering controls, and personal protective equipment.
- Section 9. Physical and chemical properties includes properties such as boiling point, vapor pressure, evaporation rate, appearance, and odor.
- Section 10. Stability and reactivity lists chemical stability and the possibility of hazardous reactions.
- Section 11. Toxicological information lists the routes of exposure, related symptoms, acute and chronic effects, and measures of toxicity.
Section 12. Ecological information (nonmandatory) provides information to evaluate the environmental impact if chemical was released.

Section 13. Disposal consideration (nonmandatory) provides guidance on proper disposal practices and recycling or reclamation of the chemical.

Section 14. Transport information (nonmandatory) provides classification information for shipping and transporting the chemical.

Section 15. Regulatory information (nonmandatory) lists safety, health, and environmental regulations for the chemical that are not listed in the other sections.

Section 16. Other information includes the date of SDS preparation or last revision.

A SDS management system should be considered to track the incoming SDSs received in the laboratory. A notice should be posted to alert the hematology staff when new or revised SDSs have been received. SDSs may be obtained electronically by means of computer, fax, or Internet. If an electronic device is used in the laboratory to receive and store SDSs, each employee must be trained on the use of the device. The training must include emergency procedures in case of power outages or malfunctions of the device. The device must be reliable and readily accessible during the hours of operation. In the event of emergency, hard copies of the SDSs must be accessible to medical staff. SDSs are required to be kept for 30 years after employment of the last employee who used the chemicals in the work area, and they should be documented with the date when the chemical was no longer used in the laboratory.

Electrical Hazard

Electrical equipment and outlets are other sources of hazard. Faulty wiring may cause fires or serious injury. Guidelines include the following:

1. Equipment must be grounded or double insulated. (Grounded equipment has a three-pronged plug.)
2. Use of “cheater” adapters (adapters that allow three-pronged plugs to fit into a two-pronged outlet) should be prohibited.
3. Use of gang plugs (plugs that allow several cords to be plugged into one outlet) should be prohibited.
4. Use of extension cords should be avoided.
5. Equipment with loose plugs or frayed cords should not be used.
6. Stepping on cords, rolling heavy equipment over cords, and other abuse of cords should be prohibited.
7. When cords are unplugged, the plug, not the cord, should be pulled.
8. Equipment that causes shock or a tingling sensation should be turned off, the instrument unplugged and identified as defective, and the problem reported.
9. Before repair or adjustment of electrical equipment is attempted, the following should be done:
   a. Unplug the equipment.
   b. Make sure the hands are dry.
   c. Remove jewelry.

DEVELOPING A SAFETY MANAGEMENT PROGRAM

Every accredited laboratory is required to have a safety management program. A safety management program is one that identifies the guidelines necessary to provide a safe working environment free from recognizable hazards that can cause harm or injury. Many medical laboratory scientists assume positions as supervisors or laboratory safety officers. Responsibilities in these positions require knowledge of the safety principles and the development, implementation, and maintenance of a laboratory safety program. This section provides an overview of the elements that should be considered in developing a safety program.

Planning Stage: Hazard Assessment and Regulatory Review

Assessment of the hazards found in the laboratory and awareness of the standards and regulations that govern laboratories is a required step in the development of a safety program. Taking the time to become knowledgeable about the regulations and standards that relate to the procedures performed in the hematology laboratory is an essential first step. Examples of the regulatory agencies that have standards, requirements, and guidelines that are applicable to hematology laboratories are given in Box 2-1. Sorting through the regulatory maze can be challenging, but the government agencies and voluntary standards organizations are willing to assist employers in complying with their standards.

Safety Program Elements

A proactive program should include the following elements:

- Written safety plan. Written policies and procedures should be developed that explain the steps to be taken for all of the occupational and environmental hazards that exist in the hematology laboratory.

Needle Puncture

Needle puncture is a serious occupational hazard for laboratory personnel. Needle-handling procedures should be written and followed, with special attention to phlebotomy procedures and disposal of contaminated needles (Chapter 3). Other items that can cause a puncture similar to a needle puncture include sedimentation rate tubes, applicator sticks, capillary tubes, glass slides, and transfer pipettes.

Disposal procedures should be followed and enforced. The most frequent cause of a needle puncture or a puncture from other sharp objects is improper disposal. Failure to check sharps containers on a regular basis and to replace them when they are no more than three-quarters full encourages overstuffing, which sometimes leads to injury. Portable bedside containers are available for personnel when performing venipunctures or skin punctures. Wall-mounted needle disposal containers also are available and make disposal convenient. All needle punctures should be reported to the health services or proper authorities within the institution.
BOX 2-1 Government Regulatory Agencies Providing Laboratory Safety Standards

**Department of Labor: 29 Code of Federal Regulations Part 1910**
- Air Contaminants: Permissible Exposure Limits: 29 CFR 1910.1000
- Occupational Noise Level Standard: 29 CFR 1910.95
- Personal Protective Equipment: 29 CFR 1910.132
- Eye and Face Protection: 29 CFR 1910.133

**Medical Waste Standards Regulated by the State**
- State medical waste standards

**Department of the Interior, Environmental Protection Agency: 40 Code of Federal Regulations Parts 200-399**
- Resource Conservation and Recovery Act (RCRA)
  - Clean Air Act
  - Clean Water Act
  - Toxic Substances Control Act (TSCA)
  - Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA)
  - Superfund Amendments and Reauthorization Act (SARA)
  - SARA Title III: Community Right to Know Act

**Voluntary Agencies/Accrediting Agencies/Other Government Agencies**
- The Joint Commission
- College of American Pathologists (CAP)
- State public health departments
- Centers for Disease Control and Prevention (CDC)
- Clinical and Laboratory Standards Institute
- National Fire Protection Association (NFPA)
- Department of Transportation (DOT): Regulated Medical Waste Shipment Regulations: 49 CFR 100-185

**CFR, Code of Federal Regulations.**

- **Training programs.** Conducted annually for all employees. New employees should receive safety information on the first day that they are assigned to the hematology laboratory.
- **Job safety analysis.** Identifies all of the tasks performed in the hematology laboratory, the steps involved in performing the procedures, and the risk associated with the procedures.
- **Safety awareness program.** Promotes a team approach and encourages employees to take an active part in the safety program.
- **Risk assessment.** Proactive risk assessment (identification) of all the potential safety, occupational, or environmental hazards that exist in the laboratory should be conducted at least annually and when a new risk is added to the laboratory. After the risk assessment is conducted, goals, policies, and procedures should be developed to prevent the hazard from injuring a laboratory employee. Some common risks are exposure to bloodborne pathogens; exposure to chemicals; needle punctures; slips, trips, and falls; and ergonomics issues.
- **Safety audits and follow-up.** A safety checklist should be developed for the hematology laboratory for use during scheduled and unscheduled safety audits. Any unsafe practices should be corrected. Actions taken to correct the unsafe practice should be documented and monitored to verify the actions are effective in correcting the practice.
- **Reporting and investigating of all accidents, "near misses," or unsafe conditions.** The causes of all incidents should be reviewed and corrective action taken, if necessary.
- **Emergency drill and evaluation.** Periodic drills for all potential internal and external disasters should be conducted. Drills should address the potential accident or disaster event before it occurs and test the preparedness of the hematology personnel for an emergency situation. Planning for disaster events and practicing the response to a disaster event reduce the panic that results when the correct response procedure is not followed.
- **Emergency management plan.** Emergencies, sometimes called disasters (anything that prevents normal operation of the laboratory) do not occur only in the hospital-based laboratories. Freestanding laboratories, physician office laboratories, and university laboratories can be affected by emergencies that occur in the building or in the community. Emergency planning is crucial to being able to experience an emergency situation and recover enough to continue the daily operation of the laboratory. In addition to the safety risk assessment, a hazard vulnerability analysis should be conducted. Hazard vulnerability analysis helps to identify all of the potential emergencies that may have an impact on the laboratory. Emergencies such as a utility failure—loss of power, water, or telephones—can have a great impact on the laboratory’s ability to perform procedures. Emergencies in the community, such as a terrorist attack, plane crash, severe weather, flood, or civil disturbances, can affect the laboratory employee’s ability to get to work and can affect transportation of crucial supplies or equipment. When the potential emergencies are identified, policies and procedures should be developed and practiced so that the laboratory employee knows the backup procedures and can implement them quickly during an emergency or disaster situation. The emergency management plan should cover the four phases of response to an emergency, as follows:
  1. **Mitigation** includes measures to prevent or reduce the adverse effects of the emergency.
The key to prevention of accidents and laboratory-acquired infections is a well-defined safety program that also includes:

- Safety committee/department safety meetings to communicate safety policies to the employees.
- Review of equipment and supplies purchased for the laboratory for code compliance and safety features.
- Annual evaluation of the safety program for review of goals and performance as well as a review of the regulations to assess compliance in the laboratory.

An example of an emergency management plan is shown in Box 2-2.

**Mitigation Tools**
- Fire alarm pull box
- Emergency code to notify workers
- Smoke detectors
- Fire/smoke doors
- Audible and visual alarms
- Fire exit lights
- Sprinkler system

**Response Activities**
- Fire response plan implementation
- Assignment of specific tasks during the actual event

**Recovery Activities**
- Communication of “all clear”
- Documentation of response to the fire
- Damage assessment
- Financial accounting of response activities
- Replenishment of supplies
- Stress debriefing for employees

**Preparedness Activities**
- Training of workers
- Fire drills
- Fire response procedure development
- Annual and monthly fire extinguisher checks

2. **Preparedness** includes the design of procedures, identification of resources that may be used, and training in the procedures.
3. **Response** includes the actions that will be taken when responding to the emergency.
4. **Recovery** includes the procedures to assess damage, evaluate response, and replenish supplies so that the laboratory can return to normal operation.

An example of an emergency management plan is shown in Box 2-2.

**Summary**

- The responsibility of a medical laboratory professional is to perform analytical procedures accurately, precisely, and safely.
- Safe practices must be incorporated into all laboratory procedures and should be followed by every employee.
- The laboratory must adopt standard precautions that require that all human blood, body fluids, and unfixed tissues be treated as if they were infectious.
- One of the most important safety practices is hand washing.
- Occupational hazards in the laboratory include fire, chemical and electrical hazards, and needle puncture.
- Some commonsense rules of safety are as follows:
  - Be knowledgeable about the procedures being performed. If in doubt, ask for further instructions.
- Wear protective clothing and use protective equipment when required.
- Clean up spills immediately, if the substance is low hazard and the spill is small; otherwise, contact hazardous materials team (internal or vendor) for spill reporting and appropriate spill management.
- Keep workstations clean and corridors free from obstruction.
- Report injuries and unsafe conditions. Review accidents and incidents to determine their fundamental cause. Take corrective action to prevent further injuries.
- Maintain a proactive safety management program.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

**Review Questions**

Answers can be found in the Appendix.

1. Standard precautions apply to all of the following except:
   a. Blood
   b. Cerebrospinal fluid
   c. Semen
   d. Concentrated acids

2. The most important practice in preventing the spread of disease is:
   a. Wearing masks during patient contact
   b. Proper hand washing
   c. Wearing disposable laboratory coats
   d. Identifying specimens from known or suspected HIV- and HBV-infected patients with a red label
3. The appropriate dilution of bleach to be used in laboratory disinfection is:
   a. 1:2
   b. 1:5
   c. 1:10
   d. 1:100

4. How frequently should fire alarms and sprinkler systems be tested?
   a. Weekly
   b. Monthly
   c. Quarterly
   d. Annually

5. Where should alcohol and other flammable chemicals be stored?
   a. In an approved safety can or storage cabinet away from heat sources
   b. Under a hood and arranged alphabetically for ease of identification in an emergency
   c. In a refrigerator at 2°C to 8°C to reduce volatilization
   d. On a low shelf in an area protected from light

6. The most frequent cause of needle punctures is:
   a. Patient movement during venipuncture
   b. Improper disposal of phlebotomy equipment
   c. Inattention during removal of needle after venipuncture
   d. Failure to attach needle firmly to syringe or tube holder

7. Under which of the following circumstances would a SDS be helpful?
   a. A phlebotomist has experienced a needle puncture with a clean needle.
   b. A fire extinguisher failed during routine testing.
   c. A laboratory employee has asked whether she needs to be concerned about working with a given reagent.
   d. During a safety inspection, an aged microscope power supply is found to have a frayed power cord.

8. It is a busy evening in the City Hospital hematology department. One staff member called in sick, and there was a major auto accident that has one staff member tied up in the blood bank all evening. Mary, the medical laboratory scientist covering hematology, is in a hurry to get a stat sample on the analyzer but needs to pour off an aliquot for another department. She is wearing gloves and a lab coat. She carefully covers the stopper of the well-mixed ethylenediaminetetraacetic acid (EDTA) tube with a gauze square and tilts the stopper toward her so it opens away from her. She pours off about 1 mL into a prelabeled tube, replaces the stopper of the EDTA tube, and puts it in the sample rack and sets it on the conveyor. She then brings the poured sample off to the other department. How would you assess Mary’s safety practice?
   a. Mary was careful and followed all appropriate procedures.
   b. Mary should have used a shield when opening the tube.
   c. Mary should have poured the sample into a sterile tube.
   d. Mary should have wiped the tube with alcohol after replacing the stopper.

9. What class fire extinguisher would be appropriate to use on a fire in a chemical cabinet?
   a. Class A
   b. Class B
   c. Class C
   d. Class D

10. According to OSHA standards, laboratory coats must be all of the following except:
    a. Water resistant
    b. Made of cloth fabric that can be readily laundered
    c. Long-sleeved
    d. Worn fully buttoned

11. Which one of the following would NOT be part of a safety management plan?
    a. Job safety analysis
    b. Risk assessment of potential safety hazards
    c. Mechanism for reporting accidents
    d. Budget for engineering controls and personal protective equipment

REFERENCES

Objectives

After completion of this chapter, the reader will be able to:

1. Describe the application of standard precautions to the collection of blood specimens.
2. List collection equipment used for venipuncture and skin puncture.
3. Correlate tube stopper color with additive, if any, and explain the purpose of the additive and use of that tube type for laboratory tests.
4. Explain reasons for selection of certain veins for venipuncture and name the veins of choice in the antecubital fossa in order of preference.
5. Describe the steps recommended by the Clinical and Laboratory Standards Institute for venipuncture, including the recommended order of draw for tubes with additives.
6. Describe complications encountered in blood collection and the proper response of the phlebotomist.
7. Describe the steps recommended by the Clinical and Laboratory Standards Institute for skin puncture, including collection sites for infants, children, and adults, and the order of draw for tubes with additives.
8. Describe components of quality assurance in specimen collection.
11. Recognize deviations from the recommended venipuncture practice in a written scenario and describe corrective procedures.
12. State the most important step in the phlebotomy procedure.
13. List reasons for inability to obtain a blood specimen.
14. Summarize legal issues that need to be considered in blood specimen collection and handling.

Case Studies

After studying the material in this chapter, the reader should be able to respond to the following case studies:

Case 1
A phlebotomist asks an outpatient, “Are you Susan Jones?” After the patient answers yes, the phlebotomist proceeds by labeling the tubes and drawing the blood. What is wrong with this scenario?

Case 2
A patient must have blood drawn for a complete blood count (CBC), potassium level, prothrombin time (PT), and type and screen. The phlebotomist draws blood into the following tubes in this order:
1. Serum separation tube
2. Light blue stopper tube for PT
3. Lavender stopper tube for CBC
4. Green stopper tube for the potassium
Which of the results will be affected by the incorrect order of draw? Explain.

*The author extends appreciation to Carole A. Mullins, whose work in prior editions provided the foundation for this chapter.
SAFETY

Standard precautions must be followed in the collection of blood, and all specimens must be treated as potentially infectious for bloodborne pathogens. Regulations of the Occupational Safety and Health Administration (OSHA) that took effect on March 6, 1992, outlined in detail what must be done to protect health care workers from exposure to bloodborne pathogens, such as the pathogens that cause hepatitis C, hepatitis B, hepatitis D, syphilis, malaria, and human immunodeficiency virus (HIV) infection.1

Bloodborne pathogens may enter the body through an accidental injury by a sharp object, such as a contaminated needle, a scalpel, broken glass, or any other object that can pierce the skin. Cuts, skin areas with dermatitis or abrasions, and mucous membranes of the mouth, eyes, and nose may also provide a portal of entry. Indirect transmission can occur when a person touches a contaminated surface or object and then touches the mouth, eyes, nose, or nonintact skin without washing the hands. Hepatitis B virus can survive on inanimate or dried surfaces for at least 1 week.2

Hand washing is the most important practice to prevent the spread of infectious diseases. The phlebotomist should wash his or her hands with soap and running water between patients and every time gloves are removed. An alcohol-based hand rub may be used if hands are not visibly contaminated.3 Antimicrobial wipes or towelettes are less effective for hand sanitation.3

Gloves are essential personal protective equipment and must be worn during blood collection procedures. When gloves are removed, no blood from the soiled gloves should come in contact with the hands. Glove removal is covered in detail in Chapter 2. Contaminated sharps and infectious wastes should be placed in designated puncture-resistant containers. The red or red-orange biohazard sign (Figure 2-2) indicates that a container holds potentially infectious materials. Biohazard containers should be easily accessible and should not be overfilled.

RESPONSIBILITY OF THE PHLEBOTOMIST IN INFECTION CONTROL

Because phlebotomists interact with patients and staff throughout the day, they potentially can infect numerous people. Phlebotomists should become familiar with and observe infection control and isolation policies. Violations of policies should be reported. A phlebotomist must maintain good personal health and hygiene, making sure to have clean clothes, clean hair, and clean, short fingernails. Standard precautions must be followed at all times, with special attention to the use of gloves and hand washing.

PHYSIOLOGIC FACTORS AFFECTING TEST RESULTS

Certain physiologic variables under the control of the patient or the phlebotomist may introduce preanalytical variation in laboratory test results. Examples of these factors include posture (supine or erect), diurnal rhythms, exercise, stress, diet (fasting or not), and smoking (Box 3-1).4-8 The phlebotomist must adhere to the specific schedule for timed specimen collections and accurately record the time of collection.

**BOX 3-1 Some Physiologic Factors That Can Contribute to Preanalytical Variation in Test Results**

**Posture**
Changing from a supine (lying) to a sitting or standing position results in a shift of body water from inside the blood vessels to the interstitial spaces. Larger molecules, such as protein, cholesterol, and iron cannot filter into the tissues, and their concentration increases in the blood.4,5

**Diurnal Rhythm**
*Diurnal rhythm* refers to daily body fluid fluctuations that occur with some constituents of the blood. For example, levels of cortisol, thyroid-stimulating hormone, and iron are higher in the morning and decrease in the afternoon.4,5 Other test values, such as the eosinophil count, are lower in the morning and increase in the afternoon.4,5

**Exercise**
Exercise can increase various constituents in the blood such as creatinine, total protein, creatine kinase, myoglobin, aspartate aminotransferase, white blood cell count, and HDL-cholesterol.6 The extent and duration of the increase depend on the intensity, duration, and frequency of the exercise and the time the blood specimen was collected postexercise.

**Stress**
Anxiety and excessive crying in children can cause a temporary increase in the white blood cell count.4

**Diet**
*Fasting* means no food or beverages except water for 8 to 12 hours before a blood draw. If a patient has eaten recently (less than 2 hours earlier), there will be a temporary increase in glucose and lipid content in the blood. In addition, the increased lipids may cause turbidity (lipemia) in the serum or plasma, affecting some tests that require photometric measurement, such as the hemoglobin concentration and coagulation tests performed on optical detection instruments.

**Smoking**
Patients who smoke before blood collection may have increased white blood cell counts and cortisol levels.7,8 Long-term smoking can lead to decreased pulmonary function and result in increased hemoglobin levels.
This chapter only covers an overview of blood specimen collection; sources that provide detailed information are listed in the reference section.

Equipment for Venipuncture

Tourniquet
A tourniquet is used to provide a barrier against venous blood flow to help locate a vein. A tourniquet can be a disposable elastic strap, a heavier Velcro strap, or a blood pressure cuff. The tourniquet should be applied 3 to 4 inches above the venipuncture site and left on for no longer than 1 minute before the venipuncture is performed. Latex-free tourniquets are available for individuals with a latex allergy.

Collection Tubes
The most common means of collecting blood specimens is through the use of an evacuated tube system. The system includes an evacuated tube, which can be either plastic or glass; a needle; and an adapter that is used to secure the needle and the tube. When the needle is inserted into a vein and a tube is inserted into the holder, the back of the needle pierces the stopper, allowing the vacuum pressure in the tube to automatically draw blood into the tube. For safety, OSHA recommends the use of plastic tubes whenever possible. Most glass tubes are coated with silicone to help decrease the possibility of hemolysis and to prevent blood from adhering to the sides of the tube. Evacuated tubes are available in various sizes and may contain a variety of premeasured additives.

Manufacturers of evacuated tubes in the United States follow a universal color code in which the stopper color indicates the type of additive contained in the tube. Figure 3-1 provides a summary of various types of evacuated collection tubes.

Additives in Collection Tubes

Clot activators. Blood specimens for serum testing must first be allowed to clot for 30 to 60 minutes prior to centrifugation and removal of the serum. A clot activator accelerates the clotting process and decreases the specimen preparation time. Examples of clot activators include glass or silica particles (activates factor XII in the coagulation pathway) and thrombin (an activated clotting factor). The Vacutainer® Blood Collection System (BD Medical, Franklin Lakes, NJ) allows single-handed activation after the venipuncture is performed by pushing the safety shield forward with the thumb until it is over the needle and an audible click is heard. The BD Eclipse needle is used with a single-use needle holder. After the safety shield is activated, the entire assembly is discarded intact into a sharps container.

Separator Gel. Separator gel is an inert material that undergoes a temporary change in viscosity during the centrifugation process; this enables it to serve as a separation barrier between the liquid (serum or plasma) and cells. Because this gel may interfere with some testing, serum or plasma from these tubes cannot be used with certain instruments or for blood bank procedures.

Needles
Venipuncture needles are sterile and are available in a variety of lengths and gauges (bore or opening size). Needles used with evacuated tube systems screw into a plastic needle holder and are double pointed. The end of the needle that is inserted into the vein is longer and has a point with a slanted side or bevel. A plastic cap covers this end of the needle and is removed prior to insertion. The end of the needle that pierces the stopper of the evacuated tube is shorter and is covered by a rubber sleeve in multiple-sample needles. The rubber sleeve prevents blood from dripping into the holder when changing tubes (Figure 3-2). Needles used with syringes are discussed below.

The gauge number of a needle is inversely related to the bore size: the smaller the gauge number, the larger the bore. Needles for drawing blood range from 19 to 23 gauge. The most common needle size for adult venipuncture is 21 gauge with a length of 1 inch. The advantage of using a 1-inch needle is that it provides better control during venipuncture.

Needle Holders

Needles and holders are designed to comply with OSHA’s revised Occupational Exposure to Bloodborne Pathogens Standard (effective April 18, 2001) and its requirement for implementation of safer medical devices. Needles and holders have safety features to prevent accidental needle sticks. Needle holders are made to fit a specific manufacturer’s needles and tubes and should not be interchanged. The holders are disposable and must be discarded after a single use with the needle still attached as required by OSHA.

The following are some examples of safety needles and holders:

1. The Vacutainer® Eclipse™ Blood Collection System (BD Medical, Franklin Lakes, NJ) allows single-handed activation after the venipuncture is performed by pushing the safety shield forward with the thumb until it is over the needle and an audible click is heard. The BD Eclipse needle is used with a single-use needle holder. After the safety shield is activated, the entire assembly is discarded intact into a sharps container.

2. The Jelco multisample blood collection needle used with the Venipuncture Needle-Pro® Device (Smiths Medical ASD,
# BD Vacutainer® Venous Blood Collection Tube Guide

For the full array of BD Vacutainer® Blood Collection Tubes, visit www.bd.com/vacutainer.

Many are available in a variety of sizes and draw volumes (for pediatric applications). Refer to our website for full descriptions.

<table>
<thead>
<tr>
<th>BD Vacutainer® Tubes with BD Hemogard™ Closure</th>
<th>BD Vacutainer® Tubes with Conventional Stopper</th>
<th>Additive</th>
<th>Inversions of Blood Collection*</th>
<th>Laboratory Use</th>
<th>Your Lab's Draw Volume/Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold</td>
<td>Red/Gray</td>
<td>• Clot activator and gel for serum separation</td>
<td>5</td>
<td>For serum determinations in chemistry. May be used for routine blood donor screening and diagnostic testing of serum for infectious diseases. Tube inversions ensure mixing of clot activator with blood. Blood clotting time: 30 minutes.</td>
<td></td>
</tr>
<tr>
<td>Light Green</td>
<td>Green/Gray</td>
<td>• Lithium heparin and gel for plasma separation</td>
<td>B</td>
<td>For plasma determinations in chemistry. Tube inversions ensure mixing of anticoagulant (heparin) with blood to prevent clotting.</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>Red</td>
<td>• Silicon coated (glass) • Clot activator, Silicon coated (plastic)</td>
<td>0/5</td>
<td>For serum determinations in chemistry. May be used for routine blood donor screening and diagnostic testing of serum for infectious diseases. Tube inversions ensure mixing of clot activator with blood. Blood clotting time: 30 minutes.</td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td>• Thrombin-based clot activator for gel for serum separation</td>
<td>5 to 6</td>
<td>For stat serum determinations in chemistry. Tube inversions ensure mixing of clot activator with blood. Blood clotting time: 5 minutes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Royal Blue</td>
<td>• Clot activator (plastic serum) • K$_2$EDTA (plastic)</td>
<td>B</td>
<td>For trace-element, toxicity, and nutritional-chemistry determinations. Special stopper formulation provides low levels of trace elements (see package insert). Tube inversions ensure mixing of either clot activator or anticoagulant (EDTA) with blood.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>• Sodium heparin • Lithium heparin</td>
<td>B</td>
<td>For plasma determinations in chemistry. Tube inversions ensure mixing of anticoagulant (heparin) with blood to prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>• Potassium oxalate/sodium fluoride • Sodium fluoride/Na$_2$EDTA (serum tube)</td>
<td>B</td>
<td>For glucose determinations. Oxalate and EDTA anticoagulants will give plasma samples. Sodium fluoride is the antiglycolytic agent. Tube inversions ensure proper mixing of additive with blood.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tan</td>
<td>• K$_2$EDTA (plastic)</td>
<td>B</td>
<td>For lead determinations. This tube is certified to contain less than 0.1 µg/mL (ppm) lead. Tube inversions prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td>• Sodium polyacrylate sulfonate (SPS) • Acid citrate dehydrogenase additives (ACD) Solution A - 22.0 g/L tromethamine citrate, 8.0 g/L citric acid, 24.5 g/L dehydrogenase Solution B - 13.2 g/L tromethamine, 4.8 g/L citric acid, 14.7 g/L dehydrogenase</td>
<td>B</td>
<td>SPS for blood culture specimen collections in microbiology. ACD for use in blood bank studies, HLA phenotyping, and DNA and paternity testing. Tube inversions ensure mixing of anticoagulant with blood to prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lavender</td>
<td>• Liquid K$_2$EDTA (glass) • Spray-coated K$_3$EDTA (plastic)</td>
<td>B</td>
<td>K$_2$EDTA and K$_3$EDTA for whole blood hematology determinations. K$_2$EDTA may be used for routine immunohematology testing, and blood donor screening.*** Tube inversions ensure mixing of anticoagulant (EDTA) with blood to prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>• K$_2$EDTA and gel for plasma separation</td>
<td>B</td>
<td>For use in molecular diagnostic test methods (such as, but not limited to, polymerase chain reaction [PCR] and/or branched DNA [bDNA] amplification techniques.) Tube inversions ensure mixing of anticoagulant (EDTA) with blood to prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pink</td>
<td>• Spray-coated K$_2$EDTA (plastic)</td>
<td>B</td>
<td>For whole blood hematology determinations. May be used for routine immunohematology testing and blood donor screening.*** Designed with special cross-match label for patient information required by the AABB. Tube inversions prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light Blue</td>
<td>• Buffered sodium citrate 0.105 M (~3.2%) glass 0.109 M (~3.2%) plastic</td>
<td>3-4</td>
<td>For coagulation determinations. CTAD for selected platelet function assays and routine coagulation determination. Tube inversions ensure mixing of anticoagulant (citrate) to prevent clotting.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear</td>
<td>• None (plastic)</td>
<td>D</td>
<td>For use as a discard tube or secondary specimen tube.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** BD Vacutainer® Tubes for pediatric and partial draw applications can be found on our website.

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* Figure depicted, do not draw

** The performance characteristics of these tubes have not been established for infectious disease testing in general. Therefore, users must evaluate the use of these tubes for their specific assay/method/diagnostic system combination and specimen storage conditions.

*** The performance characteristics of these tubes have not been established for immunohematology testing in general. Therefore, users must evaluate the use of these tubes for their specific assay/method/diagnostic system combination and specimen storage conditions.

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Figure 3-1 Vacutainer® tube guide. (Courtesy and © Becton, Dickinson and Company.)
Norwell, MA) allows the Needle-Pro® sheath to be snapped over the needle by pushing it against a flat, firm surface after the venipuncture is completed. The entire device is discarded into the sharps container (Figure 3-3).

3. The Greiner Bio-One (Monroe, NC) VACUETTE® QUICKSHIELD has a sheath that locks into place over the needle after use. The QUICKSHIELD Complete PLUS is a system that incorporates a holder with an attached VACUETTE® Visio PLUS multisample needle. The flash window in the needle hub indicates when a successful venipuncture has been achieved (Figure 3-4).

**Winged Blood Collection Set (Butterfly)**

A winged blood collection set or butterfly consists of a short needle with plastic wings connected to thin tubing (Figure 3-5). The other end of the tubing can be connected to a needle holder for an evacuated tube, a syringe, or a blood culture bottle with the use of special adapters. Winged blood collection sets are useful in collecting specimens from children or other patients from whom it is difficult to draw blood. They also have sheathing devices to minimize the risk of needle stick injury. Examples include MONOJECT™ ANGEL WING™ Blood Collection Set (Covidien, Mansfield, MA), Vacutainer® Safety-Lok™ and Vacutainer® Push Button Blood Collection Set (BD Medical, Franklin Lakes, NJ), VACUETTE® Safety Blood Collection Set (Greiner Bio-One, Monroe, NC), and Jelco Saf-T Wing® Blood Collection set (Smiths Medical ASD, Norwell, MA).

**Syringes**

A syringe consists of a barrel, graduated in milliliters, and a plunger. Syringe needles have a point at one end and an open hub at the other end that attaches to the barrel of the syringe. Syringes are available with different types of needle attachments and in different sizes. It is important to attach the needle securely to the syringe to prevent air from entering the system.
Syringes may be useful in drawing blood from pediatric, geriatric, or other patients with tiny, fragile, or “rolling” veins that would not be able to withstand the vacuum pressure from evacuated tubes. With a syringe, the amount of pressure exerted is controlled by the phlebotomist by slowly pulling back the plunger. Syringes may also be used with winged infusion sets.

If only one tube of blood is needed, the phlebotomist fills the syringe barrel with blood, removes the needle from the arm, activates the needle safety device, removes and discards the needle in a sharps container, and attaches the hub of the syringe to a transfer device to transfer the blood into an evacuated tube. An example is the BD Vacutainer® Blood Transfer Device with Luer adapter. If multiple tubes are needed, the phlebotomist can use a closed blood collection system such as the Jelco Saf-T Holder® with male Luer adapter with Saf-T Wing® butterfly needle (Smiths Medical ASD) (Figure 3-6). With this system, the butterfly needle tubing branches into a Y shape and attaches to the syringe on one side and an evacuated tube in a holder on the other side. Clamps in the tubing control the flow of blood from the arm to the syringe and then from the syringe to the evacuated tube. To prevent hemolysis when using transfer devices, only the tube’s vacuum (and not the plunger) should be used to transfer the blood from the syringe into the evacuated tube.

**Selection of a Vein for Routine Venipuncture**

The superficial veins of the antecubital fossa (bend in the elbow) are the most common sites for venipuncture. There are two anatomical patterns of veins in the antecubital fossa⁴,⁹ (Figure 3-7). In the “H” pattern, the three veins that are used, in the order of preference, are (1) the median cubital vein, (2) the basilic vein, and (3) the cephalic vein. In the “M” pattern, the preferred vein for venipuncture is the median cubital vein in the H pattern and the median vein in the M pattern.

**Solutions for Skin Antisepsis**

The most common skin antiseptic is 70% isopropyl alcohol in a commercially prepared pad. The phlebotomist cleans the phlebotomy site in a circular motion, beginning in the center and working outward. The area is allowed to air-dry before the venipuncture is performed so that the patient does not experience a burning sensation after needle insertion and to prevent contamination of the specimen with alcohol. The phlebotomist must use a non-alcohol-based antiseptic to collect blood for a legal blood alcohol level.⁹ When a sterile site is prepared for collection of specimens for blood culture, a two-step procedure with a 30- to 60-second scrub is used in which cleansing with 70% isopropyl alcohol is followed by cleansing with 1% to 10% povidone-iodine pads, tincture of iodine, chlorhexidine compounds, or another isopropyl alcohol prep.⁹ Some health care facilities use a one-step application of chlorhexidine gluconate/isopropyl alcohol or povidone-70% ethyl alcohol.⁹ Whatever method is used, the antiseptic agent should be in contact with the skin for at least 30 seconds to minimize the risk of accidental contamination of the blood culture.

**Figure 3-7** Superior veins of the anterior right arm in the antecubital fossa (two views). A, “H” pattern of veins. B, “M” pattern of veins. The preferred vein for venipuncture is the median cubital vein in the H pattern and the median vein in the M pattern. (Adapted from McCall RE, Tankersley CM. Phlebotomy Essentials, ed. 5, Philadelphia, 2012, Lippincott, Williams & Wilkins.)
which connects the basilic and cephalic veins in the antecubital fossa; (2) the cephalic vein, located on the outside (lateral) aspect of the antecubital fossa on the thumb side of the hand; and (3) the basilic vein, located on the inside (medial) aspect of the antecubital fossa. In the “M” pattern, the order of preference is the (1) median vein, (2) accessory cephalic vein, and (3) the basilic vein. The cephalic and basilic veins should only be used if the median cubital or median veins are not prominent after checking both arms. The basilic vein is the last choice due to the increased risk of injury to the median nerve and/or accidental puncture of the brachial artery, both located in close proximity to the basilic vein.9

If necessary, the phlebotomist should have the patient make a fist after application of the tourniquet; the veins should become prominent. The patient should not pump the fist because it may affect some of the test values. The phlebotomist should palpate (examine by touching) the vein with his or her index finger to determine vein depth, direction, and diameter. If a vein cannot be located in either arm, it may be necessary to examine the veins on the dorsal surface of the hand.

The veins in the feet should not be used without physician permission. The policy in some institutions is to request that a second phlebotomist attempt to locate a vein in the arm or the hand before a vein in the foot is used. The veins in the inner wrist should never be used due to the high risk of injury to tendons and nerves in that area.9

**Venipuncture Procedure**

The phlebotomist uses standard precautions, which include washing hands and applying gloves at the beginning of the procedure and removing gloves and washing hands at the end of the procedure. The Clinical and Laboratory Standards Institute (CLSI) recommends the following steps:9

1. Prepare the accession (test request) order.
2. Greet the patient and identify the patient by having the patient verbally state his or her full name and confirm with the patient’s unique identification number, address, and/or birth date. Ensure the same information is on the request form.
3. Sanitize hands.
4. Verify that any dietary restrictions have been met (e.g., fasting, if appropriate) and check for latex sensitivity.
5. Assemble supplies and appropriate tubes for the requested tests. Verify paperwork and tube selection.
6. Reassure and position the patient.
7. If necessary to help locate a vein, request that the patient clench his or her fist.
8. Apply the tourniquet and select an appropriate venipuncture site, giving priority to the median cubital or median vein. Ensure the tourniquet is on for no longer than 1 minute.
10. Cleanse the venipuncture site with 70% isopropyl alcohol using concentric circles from the inside to outside. Allow skin to air-dry.
11. Inspect the equipment and needle tip for burrs and bends.
12. Perform the venipuncture by anchoring the vein with the thumb 1 to 2 inches below the site and inserting the needle, bevel up, with an angle less than 30 degrees between the needle and the skin. Collect tubes using the correct order of draw, and invert each tube containing any additive immediately after collection. CLSI recommends a particular order of draw when collecting blood in multiple tubes from a single venipuncture.9 Its purpose is to avoid possible test result error because of cross-contamination from tube additives. The recommended order of draw is as follows: (Box-3-2)

   a. Blood culture tube (yellow stopper)
   b. Coagulation tube (light blue stopper)
   c. Serum tube with or without clot activator or gel (red, gold, red-gray marbled, orange, or yellow-gray stopper)
   d. Heparin tube (green or light green stopper)
   e. EDTA tube (lavender or pink stopper)
   f. Sodium fluoride tube with or without EDTA or oxalate (gray stopper)

13. Release and remove the tourniquet as soon as blood flow is established or after no longer than 1 minute.
14. Ensure that the patient’s hand is open.
15. Place gauze lightly over the puncture site without pressing down.
16. After the last tube has been released from the back of the multisample needle, remove the needle and activate the safety device according to the manufacturer’s directions.
17. Apply direct pressure to the puncture site using a clean gauze pad.
18. Bandage the venipuncture site after checking to ensure that bleeding has stopped.
19. If a syringe has been used, fill the evacuated tubes using a syringe transfer device.
20. Dispose of the puncture equipment and other biohazardous waste.
21. Label the tubes with the correct information. The minimal amount of information that must be on each tube is as follows:
   a. Patient’s full name
   b. Patient’s unique identification number
   c. Date of collection
   d. Time of collection (military time)
   e. Collector’s initials or code number

**Note:** Compare the labeled tube with the patient’s identification bracelet or have the patient verify that the information on the labeled tube is correct whenever possible.

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**Box 3-2 Order of Draw for Venipuncture**9

1. Blood culture tube (yellow stopper)
2. Coagulation tube (light blue stopper)
3. Serum tube with or without activator (red, gold, red-gray marbled, orange, or yellow-gray stopper)
4. Heparin tube (green or light green stopper)
5. EDTA tube (lavender or pink stopper)
6. Sodium fluoride with or without EDTA or oxalate (gray stopper)

EDTA, ethylenediaminetetraacetic acid
22. Carry out any special handling requirements (e.g., chilling or protecting from light).
23. Cancel any phlebotomy-related dietary restrictions and thank the patient.
24. Send the properly labeled specimens to the laboratory.

The most crucial step in the process is patient identification. The patient must verbally state his or her full name, or someone must identify the patient for the phlebotomist. In addition, at least one additional identifier needs to be checked such as the address, birth date, or the unique number on the patient’s identification bracelet (for hospitalized patients). The phlebotomist must match the patient’s full name and unique identifier with the information on the test requisition. Any discrepancies must be resolved before the venipuncture can continue. Failure to confirm proper identification can result in a life-threatening situation for the patient and possible legal ramifications for the facility. The phlebotomist must also label all tubes immediately after the blood specimen has been drawn, with the label attached to the tube, before leaving the patient’s side.

Coagulation Testing

If only a light blue stopper coagulation tube is to be drawn for determination of the prothrombin time or activated partial thromboplastin time, the first tube drawn may be used for testing. It is no longer necessary to draw a 3-mL discard non-additive tube before collecting for routine coagulation testing. The phlebotomist must fill tubes for coagulation testing to full volume (or to the minimum volume specified by the manufacturer) to maintain a 9:1 ratio of blood to anticoagulant. Underfilling coagulation tubes results in prolonged test values. When a winged blood collection set is used to draw a single light blue stopper tube, the phlebotomist must first partially fill a nonadditive tube or another light blue stopper tube to clear the dead air space in the tubing before collecting the tube to be used for coagulation testing. For special coagulation testing, however, a second-drawn light blue stopper tube may be required.9 Chapter 42 covers specimen collection for hemostasis testing in more detail.

Venipuncture in Children

Pediatric phlebotomy requires experience, special skills, and a tender touch. Excellent interpersonal skills are needed to deal with distraught parents and with crying, screaming, or frightened children. Ideally, only experienced phlebotomists should draw blood from children; however, the only way to gain experience is through practice. Through experience, one learns what works in different situations. Smaller gauge (22- to 23-gauge) needles are employed.9 Use of a syringe or winged blood collection set may be advantageous for accessing small veins in young children. The child’s arm should be immobilized as much as possible so that the needle can be inserted successfully into the vein and can be kept there if the child tries to move. Use of special stickers or character bandages as rewards may serve as an incentive for cooperation; however, the protocol of the institution with regard to their distribution must be followed.

Complications Encountered in Venipuncture

Ecchymosis (Bruise)

Bruising is the most common complication encountered in obtaining a blood specimen. It is caused by leakage of a small amount of blood in the tissue around the puncture site. The phlebotomist can prevent bruising by applying direct pressure to the venipuncture site with a gauze pad. Bending the patient’s arm at the elbow to hold the gauze pad in place is not effective in stopping the bleeding and may lead to bruising.

Hematoma

A hematoma results when leakage of a large amount of blood around the puncture site causes the area to rapidly swell. If swelling begins, the phlebotomist should remove the needle immediately and apply pressure to the site with a gauze pad for at least 2 minutes. Hematomas may result in bruising of the patient’s skin around the puncture site. Hematomas can also cause pain and possible nerve compression and permanent damage to the patient’s arm. Hematomas most commonly occur when the needle goes through the vein or when the bevel of the needle is only partially in the vein (Figure 3-8, B and C) and when the phlebotomist fails to remove the tourniquet before removing the needle or does not apply enough pressure to the site after venipuncture. Hematomas can also form after inadvertent puncture of an artery.

Fainting (Syncope)

Fainting is also a common complication encountered. Before drawing blood, the phlebotomist should ask the patient whether he or she has had any prior episodes of fainting during or after blood collection. The CLSI does not recommend the use of ammonia inhalants to revive the patients because they may trigger an adverse response that could lead to patient injury.9 The phlebotomist should follow the protocol at his or her facility. If the patient begins to faint, the phlebotomist should remove and discard the needle immediately, apply pressure to the site with a gauze pad, lower the patient’s head, and loosen any constrictive clothing. The phlebotomist should also notify the designated first-aid providers at the facility. The incident should be documented.

Hemoconcentration

Hemoconcentration is an increased concentration of cells, larger molecules, and analytes in the blood as a result of a shift in water balance. Hemoconcentration can be caused by leaving the tourniquet on the patient’s arm for too long. The tourniquet should not remain on the arm for longer than 1 minute. If it is left on for a longer time because of difficulty in finding a vein, it should be removed for 2 minutes and reapplied before the venipuncture is performed.9

Hemolysis

The rupture of red blood cells with the consequent escape of hemoglobin—a process termed hemolysis—can cause the plasma or serum to appear pink or red. Hemolysis can occur if
the phlebotomist used too small a needle during a difficult draw; drew the blood through an existing hematoma; pulled back too quickly on the plunger of a syringe; forced blood into a tube from a syringe by pushing the plunger; mixed a tube too vigorously; or contaminated the specimen with alcohol or water at the venipuncture site or in the tubes. Hemolysis also can occur physiologically as a result of hemolytic anemias. Hemolyzed specimens can alter test results, such as levels of potassium, lactate dehydrogenase, and aspartate aminotransferase, which can result in patient treatment errors. 10

**Petechiae**

Petechiae are small red spots indicating that small amounts of blood have escaped into the skin. Petechiae indicate a possible hemostasis abnormality and should alert the phlebotomist to be aware of possible prolonged bleeding.

**Allergies**

Some patients may be allergic to skin antiseptic substances and adhesive bandages and tape. The phlebotomist should use hypoallergenic tape or apply pressure manually until the bleeding has stopped completely. The phlebotomist should also determine if the patient has a latex sensitivity before the phlebotomy procedure.

**Nerve Damage**

The phlebotomist must select the appropriate veins for venipuncture and should not blindly probe the arm with the needle or try to laterally relocate the needle. If a nerve has been affected, the patient may complain about shooting or sharp pain, tingling, or numbness in the arm. The phlebotomist should immediately remove and discard the needle, apply pressure with a gauze pad, and collect the blood from the other arm.

**Seizures**

Patients occasionally experience seizures because of a preexisting condition or as a response to the needle stick. If a seizure occurs, the phlebotomist should immediately remove and discard the needle, apply pressure with a gauze pad, and notify the nurse or designated first-aid providers at the facility. The phlebotomist should also ensure the patient’s safety by preventing injury from nearby objects.

**Vomiting**

If the patient begins vomiting, the phlebotomist should provide the patient an appropriate container and tissues, notify the nurse or designated first-aid providers at the facility, and ensure the patient’s head is positioned so that he or she does not aspirate vomit.

**Venipuncture in Special Situations**

**Edema**

Swelling caused by an abnormal accumulation of fluid in the intercellular spaces of the tissues is termed edema. The most common cause is infiltration of the tissues by the solution running through an incorrectly positioned intravenous catheter. Edematous sites should be avoided for venipuncture because the veins are hard to find and the specimens may become contaminated with tissue fluid.

**Obesity**

In obese patients, veins may be neither readily visible nor easy to palpate. Sometimes the use of a blood pressure cuff can aid in locating a vein. The cuff should not be inflated any higher than 40 mm Hg and should not be left on the arm for longer than 1 minute.9 The phlebotomist should not probe blindly in the patient’s arm because nerve damage may result.
Burned, Damaged, Scarred, and Occluded Veins

Burned, damaged, scarred, and occluded veins should be avoided because they do not allow the blood to flow freely and may make it difficult to obtain an acceptable specimen.

Intravenous Therapy

Drawing blood from an arm with an intravenous (IV) infusion should be avoided if possible; the phlebotomist should draw the blood from the opposite arm without the IV. If there is no alternative, blood should be drawn below the IV with the tourniquet also placed below the IV site. Prior to venipuncture, the phlebotomist should ask an authorized caregiver to stop the infusion for 2 minutes before the specimen is drawn. The phlebotomist should note on the requisition and the tube that the specimen was obtained from an arm into which an IV solution was running, indicating the arm and the location of the draw relative to the IV.4,9 The phlebotomist should always follow the protocol established at his or her facility.

Mastectomy Patients

The CLSI requires physician consultation before blood is drawn from the same side as a prior mastectomy (removal of the breast), even in the case of bilateral mastectomies.9 The pressure on the arm that is on the same side as the mastectomy from a tourniquet or blood pressure cuff can lead to pain or lymphostasis from accumulating lymph fluid. The other arm on the side without a mastectomy should be used.

Inability to Obtain a Blood Specimen

Failure to Draw Blood

One reason for failure to draw blood is that the vein is missed, often because of improper needle positioning. The needle should be inserted completely into the vein with the bevel up and at an angle of less than 30 degrees.9 Figure 3-8 shows reasons for unsatisfactory flow of blood. It is sometimes possible to reposition the needle in the vein by slightly withdrawing or advancing the needle, but only an experienced phlebotomist should attempt this. The phlebotomist should never attempt to relocate the needle in a lateral direction because such manipulation can cause pain and risk a disabling nerve injury to the patient.

Occasionally an evacuated tube has insufficient vacuum, and insertion of another tube yields blood. Keeping extra tubes within reach during blood collection can avoid a re-collection when the problem is a technical issue associated with the tube.

Each institution should have a policy covering the proper procedure when a blood specimen cannot be collected. If two unsuccessful attempts at collection have been made, the CLSI recommends that the phlebotomist seek the assistance of another practitioner with blood collection expertise.9 Another individual can make two attempts to obtain a specimen. If a second person is unsuccessful, the physician should be notified.

Patient Refusal

The patient has the right to refuse to give a blood specimen. If gentle urging does not persuade the patient to allow blood to be drawn, the phlebotomist should alert the nurse, who will either talk to the patient or notify the physician. The phlebotomist must not force an uncooperative patient to have blood drawn; it can be unsafe for the phlebotomist and for the patient. In addition, forcing a patient of legal age and sound mind to have blood drawn against his or her wishes can result in charges of assault and battery or unlawful restraint.

If the patient is a child and the parents offer to help hold the child, it is usually acceptable to proceed. Any refusals or problems should be documented for legal reasons.

Missing Patient

For hospitalized patients, if the patient is not in his or her room, the absence should be reported to the nursing unit so that the nurses are aware that the specimen was not obtained.

SKIN PUNCTURE

Skin puncture is the technique of choice to obtain a blood specimen from newborns and pediatric patients. In adults skin puncture may be used in patients who are severely burned and whose veins are being reserved for therapeutic purposes; in patients who are extremely obese; and in elderly patients with fragile veins.

Blood obtained from skin puncture is a mixture of blood from venules, arterioles, capillaries, and interstitial and intracellular fluids.9 After the puncture site is warmed, the specimen more closely resembles arterial blood. The phlebotomist should note that the specimen was obtained by skin puncture because those specimens may generate slightly different test results.13 For example, higher glucose values are found in specimens obtained by skin puncture compared with those obtained by venipuncture, and this difference can be clinically significant.13 It is especially important to note the specimen type when a glucose tolerance test is performed or when glucometer results are compared with findings from venous specimens.

Collection Sites

The site of choice for skin puncture in infants under 1 year of age is the lateral (outside) or medial (inside) plantar (bottom) surface of the heel (Figure 3-9, A). In children older than 1 year of age and in adults, the palmar surface of the distal portion of the third (middle) or fourth (ring) finger on the nondominant hand may be used.13 The puncture on the finger should be made perpendicular to the fingerprint lines (Figure 3-9, B). Fingers of infants should not be punctured because of the risk of serious bone injury.

Warming the site can increase the blood flow sevenfold.13 The phlebotomist should warm the site with a commercial heel warmer or a warm washcloth to a temperature no greater than 42° C and for no longer than 3 to 5 minutes.13 The phlebotomist should clean the skin puncture site with 70% isopropyl alcohol and allow it to air-dry. Povidone-iodine should not be used because of possible specimen contamination, which could falsely elevate levels of potassium, phosphorus, or uric acid.13
Precautions with Skin Puncture

The finger or heel must be securely immobilized. Heel punctures in infants should not be made more than 2 mm deep because of the risk of bone injury and possible infection (osteomyelitis).13 In premature infants, a puncture device that makes an incision with even less depth is preferred. The first drop of blood should be wiped away with a clean gauze pad to prevent contamination of the specimen with tissue fluid and to facilitate the free flow of blood.13

Equipment for Skin Puncture

Devices for skin puncture contain sterile lancets that puncture or sterile blades that make a small incision in the skin. The lancet or blade is spring-loaded in the device, and when activated by the phlebotomist, pierces the skin. Devices are single-use, disposable, and have retractable blades in compliance with OHSA safety standards.11 Devices are available for newborns, children, and adults that produce punctures or incisions of varying depths in the skin.

Containers for collecting blood from skin puncture include capillary tubes and microcollection tubes.13 Capillary tubes of various sizes are available with or without heparin. OSHA recommends the use of plastic tubes or Mylar-coated glass tubes to avoid injury by broken glass and exposure to bloodborne pathogens. Microcollection tubes are preferred and are available with or without additives. The cap colors on microcollection tubes correspond with the color coding system for evacuated tubes. The order of draw, however, is different for microcollection tubes (Box 3-3). The EDTA microcollection tube should be filled first to ensure adequate volume and accurate hematology results, especially for platelets, which tend to aggregate at the site of puncture.13 Skin puncture specimens should be labeled with the same information as required for evacuated tubes. Examples of skin puncture equipment are shown in Figure 3-10.

Skin Puncture Procedure

The phlebotomist uses standard precautions that include washing hands and applying gloves at the beginning of the procedure and removing gloves and washing hands at the end of the procedure. CLSI recommends the following steps:13

1. Prepare the accession (test request) order.
2. Greet the patient (and parents); identify the patient by having the patient (or parent in the case of a child) verbally

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Figure 3-9 Areas for skin puncture: A, Heel of infant less than 1 year old. Puncture is made on the lateral or medial plantar surface of the heel, in the shaded area demarcated by lines from the middle of the big toe to the heel, and from between the fourth and fifth toe to the heel.12,13 B, Finger. Puncture is made on the palmar surface of the distal portion of the third or fourth finger, perpendicular to the fingerprint lines.

Figure 3-10 Examples of equipment used for skin puncture. A, Various puncture devices. B, Various microcollection tubes. (A, B Courtesy Dennis J. Ernst, MT[ASCP], Director, Center for Phlebotomy Education, Inc.)
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state his or her full name and confirm with patient’s identification number, address, and/or birth date. Ensure that the same information is on the requisition form.

3. Position the patient and the parents (or individual designated to hold an infant or small child) as necessary.

4. Verify that any dietary restrictions have been met (e.g., fasting), and check for latex sensitivity.

5. Wash hands and put on gloves.

6. Assemble supplies and appropriate tubes for the requested tests. Check paperwork and tube selection.

7. Select the puncture site.

8. Warm the puncture site.

9. Cleanse the puncture site with 70% isopropyl alcohol using concentric circles, working from the inside to outside. Allow skin to air-dry.

10. Open and inspect the sterile disposable puncture device, and perform the puncture while firmly holding the heel or finger. Discard the device in the appropriate sharps container.

11. Wipe away the first drop of blood with a clean, dry gauze pad. This removes any residual alcohol and any tissue fluid contamination.

12. Make blood films if requested.

13. Collect blood in the appropriate collection tubes and mix as needed. If an insufficient specimen has been obtained because the blood flow has stopped, repeat the puncture at a different site with all new equipment. CLSI recommends the following order of draw:13 (Box 3-3)
   a. Tube for blood gas analysis
   b. Slides, unless made from a specimen in the EDTA microcollection tube
   c. EDTA microcollection tube
   d. Other microcollection tubes with anticoagulants
   e. Serum microcollection tubes

14. Apply pressure and elevate the puncture site until bleeding has stopped.

15. Label each specimen with the required information and indicate skin puncture collection.

   NOTE: Compare the labeled tubes with the identification bracelet for inpatients; have outpatients verify that the information on the labeled tubes is correct, whenever possible.

16. Handle the specimens appropriately.

17. Discard all puncture equipment and biohazardous materials appropriately.

**BOX 3-3** Order of Draw for Skin Puncture13

1. Tube for blood gas analysis
2. Slides, unless made from a specimen in the EDTA microcollection tube
3. EDTA microcollection tube
4. Other microcollection tubes with anticoagulants
5. Serum microcollection tubes

EDTA, ethylenediaminetetraacetic acid

18. Remove gloves and wash hands.

19. Deliver the properly labeled specimens to the laboratory.

**Preparation of Peripheral Blood Films**

Peripheral blood films can be made directly from skin puncture blood or from a tube of EDTA-anticoagulated venous blood. With a skin puncture, the phlebotomist must remember to wipe away the first drop of blood and use the second drop to make the blood film. Chapter 16 covers preparation of blood films in detail.

**QUALITY ASSURANCE IN SPECIMEN COLLECTION**

To ensure accurate patient test results, it is essential that the blood collection process, which includes specimen handling, be monitored. Patient diagnosis and medical care are based on the outcomes of these tests. The following areas should be monitored.

**Technical Competence**

The individual performing phlebotomy should be trained properly in all phases of blood collection. Certification by an appropriate agency is recommended. Continuing education is required to keep current on all the changes in the field. Competency should be assessed and documented on an annual basis for each employee performing phlebotomy.

**Collection Procedures**

Periodic review of collection procedures is essential to maintaining the quality of specimens. This includes a review of policies on the allowable number of blood collection attempts for unsuccessful blood draws, procedures for what to do when the patient is unavailable for a blood draw, or when the patient refuses a draw. Proper patient preparation and correct patient identification are crucial. The correct tube or specimen container must be used.

**Anticoagulants and Other Additives**

The phlebotomist must follow the manufacturer’s instructions with regard to mixing all tubes with additives to ensure proper specimen integrity and prevent formation of microclots in the anticoagulated tubes. All tubes should be checked for cracks, expiration dates, and discoloration or cloudiness, which could indicate contamination. New lot numbers of tubes must be checked to verify draw and fill accuracy. When blood is collected in the light blue stopper tube for coagulation, a 9:1 ratio of blood to anticoagulant must be maintained to ensure accurate results. Specimens must be stored and handled properly before testing.

**Requirements for a Quality Specimen**

Requirements for a quality specimen are as follows:
1. Patient properly identified
2. Patient properly prepared for draw
3. Specimens collected in the correct order and labeled correctly
4. Correct anticoagulants and other additives used
5. Specimens properly mixed by inversion, if required
6. Specimens not hemolyzed
7. Specimens requiring patient fasting collected in a timely manner
8. Timed specimens drawn at the correct time

Collection of Blood for Blood Culture
Each facility should monitor its blood culture contamination rate and keep that rate lower than 3% as recommended by the CLSI and the American Society for Microbiology. Higher blood culture contamination rates should prompt an investigation of the causes and implementation of the appropriate corrective action. False-positive blood culture results lead to unnecessary testing and treatment for patients and increased costs for the institution. A 2012 CDC-funded Laboratory Medicine Best Practices systematic review and meta-analysis concluded that the use of well-trained phlebotomy teams and proper venipuncture technique was an effective way to reduce blood culture contamination rates.

Quality Control and Preventive Maintenance for Specimen Processing and Storage Equipment
Thermometers used in refrigerators and freezers in which specimens are stored should be calibrated annually, or only thermometers certified by the National Bureau of Standards should be used. Centrifuges should be maintained according to the manufacturer’s instructions for cleaning and timing verification.

Reasons for Specimen Rejection
A laboratory result is only as good as the integrity of the specimen provided. Specimens are rejected for conditions that may result in identification errors or inaccurate results. Box 3-4 lists some reasons for specimen rejection.

SPECIMEN HANDLING
Proper handling of specimens begins with the initiation of the test request and ends when the specimen is tested. Accurate test results depend on what happens to the specimen during that time. This pretesting period is called the preanalytical phase of the total testing process (Chapter 5).

Blood collected into additive tubes must be inverted to mix the additive and blood according to manufacturer’s instructions. Shaking can result in hemolysis of the specimen and lead to specimen rejection or inaccurate test results. Specimens should be transported in an upright position to ensure complete clot formation and reduce agitation, which can also result in hemolysis.

Exposure of the blood specimen to light can cause falsely decreased values for bilirubin, beta-carotene, vitamin A, and porphyrins. For certain tests, the specimens need to be chilled, not frozen, and should be placed in an ice-water bath to slow down cellular metabolism. Examples of these tests include ammonia, lactic acid, parathyroid hormone, and gastrin. Other tests, such as the cold agglutinin titer, require that specimens be kept warm to ensure accurate results. If the specimen is refrigerated before the serum is removed, the antibody in the serum will bind to the red blood cells, thus falsely decreasing the serum cold agglutinin titer. To ensure accurate results, cells and serum must be separated within 2 hours of collection for tests such as those measuring glucose, potassium, and lactate dehydrogenase.

The CLSI provides recommendations to laboratories for the maximum time uncentrifuged specimens are stable at room temperature for various tests based on studies in the literature.

LEGAL ISSUES IN PHLEBOTOMY
There are many daily practices in health care that, if performed without reasonable care and skill, can result in a lawsuit. Facilities have been and will continue to be held legally accountable for the actions of those who collect blood for diagnostic testing. Two areas of particular concern to phlebotomists are breach of patient confidentiality and patient misidentification. Unless there is a clinical need to know or a patient has given written permission, no one has a right to patient information. A patient will not be misidentified if correct procedures for specimen collection are followed. Phlebotomists often are called to testify in court in cases involving blood alcohol levels. The phlebotomist is asked about patient identification procedures and skin antisepsis. Only alcohol-free antiseptics should be used for skin antisepsis in such cases. Soap and water may be used if no other cleaners are available.

To minimize the risk of legal action, the phlebotomist should do the following:
1. Follow up on all incident reports.
2. Participate in continuing education.
3. Become certified in the profession.
4. Acknowledge the extent of liability coverage.
5. Follow established procedures.
6. Always exhibit professional, courteous behavior.
7. Always obtain proper consent.
8. Respect and honor the Patients’ Bill of Rights.
9. Maintain proper documentation.

**Box 3-4 Reasons for Specimen Rejection**
- Test order requisition and the tube identification do not match.
- Tube is unlabeled, or the labeling, including patient identification number, is incorrect.
- Specimen is hemolyzed.
- Specimen was collected at the wrong time.
- Specimen was collected in the wrong tube.
- Specimen was clotted, and the test requires whole blood or plasma.
- Specimen was contaminated with intravenous fluid.
- Specimen is lipemic.*

*Lipemic specimens cannot be used for certain tests; however, the phlebotomist has no control over this aspect. Collection of a specimen after patient fasting may be requested to try to reduce the potential for lipemia.
Summarize

- Laboratory test results are only as good as the integrity of the specimen tested.
- Standard precautions must be followed in the collection of blood to prevent exposure to bloodborne pathogens.
- Some physiologic factors affecting test results include posture, diurnal rhythm, exercise, stress, diet, and smoking.
- U.S. manufacturers of evacuated tubes follow a universal color coding system in which the stopper color indicates the type of additive contained in the tube.
- The gauge numbers of needles relate inversely to bore size: the smaller the gauge number, the larger the bore. Needle safety devices are required for venipuncture equipment.
- For venipuncture in the antecubital fossa, the median cubital vein (H-shaped vein pattern) or median vein (M-shaped vein pattern) is preferred to avoid accidental arterial puncture and nerve damage. If those veins are not available after checking both arms, the cephalic, then the basilic veins are the second and third choices.
- CLSI guidelines should be followed for venipuncture and skin puncture.
- Sites for skin puncture include the lateral or medial plantar surface of the heel (infants), or the palmar surface of the distal portion of the third or fourth finger on the nondominant hand (children and adults). Heel punctures are used for infants less than 1 year old; the puncture must be less than 2 mm deep to avoid injury to the bone.
- Common complications of blood collection include bruising, hematoma, and fainting.
- Each institution should establish a policy covering proper procedure when a blood specimen cannot be obtained.
- Following established procedures and documenting all incidents minimize the risk of liability when performing phlebotomy.

Now that you have completed this chapter, go back and read again the case studies at the beginning and respond to the questions presented.

Review Questions

Answers can be found in the Appendix.

1. Which step in the CLSI procedure for venipuncture is part of standard precautions?
   a. Wearing gloves
   b. Positively identifying the patient
   c. Cleansing the site for the venipuncture
   d. Bandaging the venipuncture site

2. Select the needle most commonly used in standard venipuncture in an adult:
   a. One inch, 18 gauge
   b. One inch, 21 gauge
   c. One-half inch, 23 gauge
   d. One-half inch, 25 gauge

3. For a complete blood count (hematology) and measurement of prothrombin time (coagulation), the phlebotomist collected blood into lavender stopper and green stopper tubes. Are these specimens acceptable?
   a. Yes, EDTA is used for hematologic testing and heparin is used for coagulation testing.
   b. No, although EDTA is used for hematologic testing, citrate, not heparin, is used for coagulation testing.
   c. No, although heparin is used for hematologic testing, citrate, not EDTA, is used for coagulation testing.
   d. No, hematologic testing requires citrate and coagulation testing requires a clot, so neither tube is acceptable.

4. The vein of choice for performing a venipuncture is the:
   a. Basilic, because it is the most prominent vein in the antecubital fossa
   b. Cephalic or accessory cephalic, because it is the least painful site
   c. Median or median cubital, because it has the lowest risk of damaging nerves in the arm
   d. One of the hand veins, because they are most superficial and easily accessed

5. The most important step in phlebotomy is:
   a. Cleansing the site
   b. Identifying the patient
   c. Selecting the proper needle length
   d. Using the correct evacuated tube

6. The venipuncture needle should be inserted into the arm with the bevel facing:
   a. Down and an angle of insertion between 15 and 30 degrees
   b. Up and an angle of insertion less than 30 degrees
   c. Down and an angle of insertion greater than 45 degrees
   d. Up and an angle of insertion between 30 and 45 degrees
7. Failure to obtain blood by venipuncture may occur because of all of the following except:
   a. Incorrect needle positioning
   b. Tying the tourniquet too tightly
   c. Inadequate vacuum in the tube
   d. Collapsed vein

8. What is the recommended order of draw when the evacuated tube system is used?
   a. Gel separator, nonadditive, coagulation, and blood culture
   b. Additive, nonadditive, gel separator, and blood culture
   c. Nonadditive, blood culture, coagulation, and other additives
   d. Blood culture, coagulation, nonadditive, and gel separator or other additives

9. Which one of the following is an acceptable site for skin puncture on infants:
   a. Back curvature of the heel
   b. Lateral or medial plantar surface of the heel
   c. Plantar surface of the heel close to the arch of the foot
   d. Middle of the plantar surface of the heel

10. An anticoagulant is an additive placed in evacuated tubes to:
    a. Make the blood clot faster
    b. Dilute the blood before testing
    c. Prevent the blood from clotting
    d. Ensure the sterility of the tube

11. Which one of the following is a reason for specimen rejection:
    a. Clot in a red stopper tube
    b. Specimen collected for blood cortisol in the morning
    c. Specimen in lavender stopper tube grossly hemolyzed
    d. Room number is missing from the specimen tube label

12. One legal area of concern for the phlebotomist is:
    a. Breach of patient confidentiality
    b. Failure to obtain written consent for phlebotomy
    c. Entering a patient’s room when the family is present
    d. Asking an outpatient for his or her full name in the process of identification

REFERENCES

**CASE STUDY**

After studying the material in this chapter, the reader should be able to respond to the following case study:

A Wright-stained peripheral blood film focuses under 10× and 40× but does not come into focus under the 100× oil objective. What steps should be taken to identify and correct this problem?

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**PRINCIPLES OF MICROSCOPY**

In the compound microscope, a magnified intermediate image of the illuminated specimen is formed in the optical tube by each objective lens. This image is then magnified again and viewed through the eyepiece as an enlarged virtual image that appears to be located about 10 inches from the eye (Figure 4-1). Microscopists must focus their eyes in that more distant plane, rather than trying to focus at the distance of the microscope stage.

An example of a simple microscope is a magnifying lens that enlarges objects that are difficult to view with the unaided eye. Movie theater projection units incorporate this system efficiently.

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**OBJECTIVES**

After completion of this chapter, the reader will be able to:

1. Given a diagram of a brightfield light microscope, identify the component parts.
2. Explain the function of each component of a brightfield light microscope.
3. Define achromatic, plan achromatic, parfocal, and parcentric as applied to lenses and microscopes; explain the advantages and disadvantages of each; and recognize examples of each from written descriptions of microscope use and effects.
4. Explain the purpose of and proper order of steps for adjusting microscope light using Koehler illumination.
5. Describe the proper steps for viewing a stained blood film with a brightfield light microscope, including use of oil immersion lenses, and recognize deviations from these procedures.
6. Describe the proper care and cleaning of microscopes and recognize deviations from these procedures.
7. Given the magnification of lenses in a compound microscope, calculate the total magnification.
8. Given a problem with focusing a blood film using a brightfield light microscope, suggest possible causes and their correction.
9. For each of the following, describe which components of the microscope differ from those of a standard light microscope, what the differences accomplish, and what are the uses and benefits of each type in the clinical laboratory:
   - Phase-contrast microscope
   - Polarized light microscope
   - Darkfield microscope

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**OUTLINE**

**Principles of Microscopy**
- Component Parts and Their Functions
- Operating Procedure with Koehler Illumination
- Considerations
- Immersion Oil and Types
- Care of the Microscope
- Basic Troubleshooting
- Other Microscopes Used in the Clinical Laboratory
  - Phase-Contrast Microscope
  - Polarized Light Microscope
  - Darkfield Microscope

**Microscopes available today reflect improvement in every aspect from the first microscope of Anton van Leeuwenhoek (1632-1723).** Advanced technology as applied to microscopy has resulted in computer-designed lens systems, sturdier stands, perfected condensers, and built-in illumination systems. Microscopes can be fitted with multiple viewing heads for teaching or conferences, or they can be attached to a computer to allow an object to be projected onto a monitor or a large screen. Regular care and proper cleaning ensure continued service from this powerful diagnostic instrument. The references listed at the end of this chapter address the physical laws of light and illumination as applied to microscopy.
The *compound microscope* employs two separate lens systems, objective and eyepiece, the product of which produces the final magnification. Standard microscopes use brightfield illumination in which light passes through the thin specimen.

**COMPONENT PARTS AND THEIR FUNCTIONS**

Component parts and the function of each part of the microscope are summarized as follows (Figure 4-2):

1. The *eyepieces, or oculars*, usually are equipped with 10× lenses (degree of magnification is 10×). The lenses magnify the intermediate image formed by the objective lenses in the optical tube; they also limit the area of visibility. Microscopes may have either one or two adjustable eyepieces. All eyepieces should be used correctly for optimal focus (see section on operating procedure). Eyepieces should not be interchanged with the eyepieces of the same model or other models of microscopes, because the eyepieces in a pair are optically matched.

2. The *interpupillary control* is used to adjust the lateral separation of the eyepieces for each individual. When it is properly adjusted, the user should be able to focus both eyes comfortably on the specimen and visualize one clear image.

3. The *optical tube* connects the eyepieces with the objective lens. The intermediate image is formed in this component. The standard length is 160 mm, which, functionally, is the distance from the real image plane (eyepieces) to the objective lenses.

4. The *neck, or arm*, provides a structural site of attachment for the revolving nosepiece.

5. The *stand* is the main vertical support of the microscope. The stage assembly, together with the condenser and base, is supported by the stand.

6. The *revolving nosepiece* holds the objectives and allows for easy rotation from one objective lens to another. The working distance (WD) between the objectives and the slide varies with the make and model of the microscope.

7. There are usually three or four *objective lenses* (Figure 4-3), each with a specific power of magnification. Engraved on the barrel of each objective lens is the power of magnification and the numerical aperture (NA). The NA is related to the angle of light collected by the objective; in essence, it indicates the light-gathering ability of the objective lens. Functionally, the larger the NA, the greater the resolution or the ability to distinguish between fine details of two closely situated objects.

Four standard powers of magnification and NA used in the hematology laboratory are 10×/0.25 (low power), 40×/0.65 or 45×/0.66 (high power, dry), 50×/0.90 (oil immersion), and 100×/1.25 (oil immersion). The smaller the magnification, the larger the viewing field; the larger the magnification, the smaller the viewing field. Total magnification is calculated by multiplying the magnification of the eyepiece by the magnification of the objective lens; for example, 10× (eyepiece) multiplied by 100× (oil immersion) is 1000× total magnification.

Microscopes employed in the clinical laboratory are used with achromatic or plan achromatic objective lenses, whose
function is to correct for chromatic and spheric aberrations. Chromatic aberrations are caused by the spheric surface of the lens, which acts as a prism. As the various wavelengths pass through the lens, each focuses at a different point, which gives rise to concentric rings of color near the periphery of the lens. Spheric aberrations result as light waves travel through the varying thicknesses of the lens, blurring the image. The achromatic objective lens brings light of two colors into focus, partially correcting for the aberrations. When achromatic objective lenses are used, the center of the field is in focus, whereas the periphery is not. A plan achromatic lens provides additional corrections for curvature of the field, which results in a flat field with uniform focus. Plan achromatic lenses sometimes are referred to as flat field lenses. Critical microscopy applications may require a plan apochromatic lens, which brings light of three colors into focus and almost completely corrects for chromatic aberration. This type of objective lens is more expensive and is rarely needed for routine laboratory use.

A set of lenses with corresponding focal points all in the same plane is said to be parfocal. As the nosepiece is rotated from one magnification to another, the specimen remains in focus, and only minimal fine adjustment is necessary.

8. The stage supports the prepared microscope slide to be reviewed. A spring assembly secures the slide to the stage.

9. The focus controls (or adjustments) can be incorporated into one knob or can be two separate controls. When a single knob is used, moving it in one direction engages the coarse control, whereas moving it in the opposite direction engages the fine control. One gradation interval of turning is equivalent to 2 μm. Many microscopes are equipped with two separate adjustments: one coarse and one fine. The order of usage is the same: engage the coarse adjustment first and then fine-tune with the fine adjustment.

10. The condenser, consisting of several lenses in a unit, may be permanently mounted or vertically adjustable with a rack-and-pinion mechanism. It gathers, organizes, and directs the light through the specimen. Attached to and at the bottom of the condenser is the aperture diaphragm, an adjustable iris containing numerous leaves that control the angle and amount of the light sent through the specimen. The angle, also expressed as an NA, regulates the balance between contrast (ability to enhance parts within a cell) and resolution (ability to differentiate fine details of two closely situated objects). The best resolution is achieved when the iris is used fully open, but there is some sacrifice of image contrast. In practice, this iris is closed only enough to create a slight increase in image contrast. Closing it beyond this point leads to a loss of resolution.

Some microscopes are equipped with a swing-out lens immediately above or below the main condenser lens. This lens is used to permit a wider field of illumination when the NA of the objective lens is less than 0.25 (e.g., the 4×0.12 objective lens). If the swing-out lens is above the main condenser, it should be out for use with the 4× objective lens and in for lenses with magnification of 10× and higher. If it is below the condenser, it should be in for use with the 4× objective lens and out for lenses of magnification of 10× and higher. The 4× objective is not used routinely for examination of peripheral blood films.

The stage and condenser (Figure 4-4) consist of a swing-out lens, an aperture diaphragm, a control for vertical adjustment of the condenser, and two centering screws for adjustment of the condenser.

11. The condenser top lens can swing out of position.

12. The stage controls located under the stage move it along an x- or a y-axis.
The field diaphragm is located below the condenser within the base. When it is open, it allows a maximally sized circle of light to illuminate the slide. Almost closing the diaphragm, when low power is used, assists in centering the condenser apparatus by the use of two centering screws. Some microscopes have permanently centered condensers, whereas in others the screws are used for this function. The glass on top of the field diaphragm protects the diaphragm from dust and mechanical damage.

14. Microscopes depend on electricity as the primary source for illumination power. There are two types of brightfield illumination: (1) critical illumination, in which the light source is focused at the specimen, which results in increased but uneven brightness; and (2) the Koehler (or Köhler) system, in which the light source and the condenser are properly aligned. The end result of Koehler illumination is a field of evenly distributed brightness across the specimen. This is especially important when using the oil objectives or when taking photomicrographs. Tungsten-halogen light bulbs are used most frequently as the illumination source. They consist of a tungsten filament enclosed in a small quartz bulb that is filled with a halogen gas. Tungsten possesses a high melting point and gives off bright yellowish light. A blue (daylight) filter should be used to eliminate the yellow color produced by tungsten. The rheostat or light control knob or lever turns on the light and should be used to regulate the brightness of the light needed to visualize the specimen. The aperture diaphragm control lever should never be used for this purpose, because closing it reduces resolving ability.

OPERATING PROCEDURE WITH KOEHLER ILLUMINATION

The procedure outlined here applies to microscopes with a nonfixed condenser. The following steps should be performed at the start of each laboratory session in which the oil objectives will be used:

1. Connect the microscope to the power supply.
2. Turn on the light source with the power switch.
3. Open the condenser aperture and field diaphragms.
4. Revolve the nosepiece until the 10× objective lens is directly above the stage.
5. Place a stained blood film on the stage and focus on it, using the fixed eyepiece, while covering the other eye. (Do not simply close the other eye, because this would necessitate adjustment of the pupil when you focus with the other eyepiece.)
6. Adjust the interpupillary control so that looking through both eyepieces yields one clear image.
7. Using the adjustable eyepiece and covering the opposite eye, focus on the specimen. Start with the eyepiece all the way out, and adjust inward. If using two adjustable eyepieces, focus each individually.
8. Raise the condenser to its upper limit.
9. Focus the field so that the cells become sharp and clear. Concentrate on one cell and place it in the center of the field.
10. Close the field (lower) diaphragm. Look through the eyepieces. A small circle of light should be seen. If the light is not in the center of the field, center it by using the two centering screws located on the condenser. This step is essential, because an off-center condenser will result in uneven distribution of light. Adjust the vertical height of the substage condenser so that you see a sharp image of the field diaphragm, ringed by a magenta halo. If the condenser is raised too much, the halo is orange; if it is lowered too far, the halo is blue.
11. Reopen the field diaphragm until it is nearly at the edge of the field, and fine-tune the centering process.
12. Open the field diaphragm slightly until it just disappears from view.
13. Remove one eyepiece and, while looking through the microscope (without the eyepiece), close the condenser aperture diaphragm completely. Reopen the condenser aperture diaphragm until the leaves just disappear from view. Replace the eyepiece.
14. Rotate the nosepiece until the 40× objective lens is above the slide. Adjust the focus (the correction should be minimal) and find the cell that you had centered. If it is slightly off center, center it again with the stage x-y control. Note the greater amount of detail that you can see.
15. Move the 40× objective out of place. Place a drop of immersion oil on top of the slide. Rotate the nosepiece until the 100× objective lens is directly above the slide. Avoid moving a non–oil immersion objective through the drop of oil. Adjust the focus (the correction should be minimal) and observe the detail of the cell: the nucleus and its chromatin pattern; the cytoplasm and its color and texture. The objective lens should dip into the oil slightly.

Considerations

1. When revolving the nosepiece from one power to another, rotate it in such a direction that the 10× and 40× objective lenses never come into contact with the oil on a slide. If oil
inadvertently gets onto the high dry objective, clean the objective immediately.

2. *Paracentric* refers to the ability to center a cell in question in the microscopic field and rotate from one magnification power to another while retaining the cell close to the center of the viewing field. Recentering of the cell at each step is minimal. Most laboratory microscopes have this feature.

3. In general, when the 10× and 40× objective lenses are used, the light intensity should be low. When the 50× and 100× objective lenses are used, increase the intensity of the light by adjusting only the rheostat (light control knob or lever) or by varying neutral density filters. Neutral density filters are used to reduce the amplitude of light and are available in a variety of densities.

4. Do not change the position of the condenser or the aperture diaphragm control lever to regulate light intensity when viewing specimens with the oil immersion objectives. The condenser should always be in its upward position as set during the Koehler illumination adjustment. The aperture diaphragm may be adjusted to achieve proper contrast of the features of the specimen being viewed.

5. After setting the Koehler illumination, when a new slide is to be examined, always bring the specimen into focus with the 10× objective first, and then move to the higher magnifications.

### IMMERSION OIL AND TYPES

Immersion oil is required to increase the refractive index when either the 50× or the 100× oil immersion objective lens is used. The refractive index is the speed at which light travels in air divided by the speed at which light travels through a substance. This oil, which has the same properties as glass, allows the objective lens to collect light from a wide NA, which provides high resolution of detail.

Three types of immersion oil, differing in viscosity, are employed in the clinical laboratory:

1. **Type A** has very low viscosity and is used in fluorescence and darkfield studies.
2. **Type B** has high viscosity and is used in brightfield and standard clinical microscopy. In hematology, this oil is routinely used.
3. **Type C** has very high viscosity and is used with inclined microscopes with long-focus objective lenses and wide condenser gaps.

   Bubbles in the oil tend to act as prisms and consequently reduce resolution. Bubbles may be created when oil is applied to the slide. They are caused by lowering the objective immediately into the oil. Sweeping the objective from right to left in the oil eliminates bubbles.

### CARE OF THE MICROSCOPE

Care of the microscope involves the following details:

1. When not in use for an extended period of time, always cover the microscope to protect it from dust.

2. Before use, inspect the component parts. If dust is found, use an air syringe, a camel hair brush, or a soft lint-free cloth to remove it. Using lens paper directly on a dirty lens without first removing the dust may scratch the lens. Do not use laboratory wipes or facial tissue to clean the lenses.

3. Avoid placing fingers on the lens surface. Fingerprints affect the contrast and resolution of the image.

4. Use solvent sparingly. The use of xylene is discouraged, because it contains a carcinogenic component (benzene). Xylene is also a poor cleaning agent, leaving an oily film on the lens. Lens cleaner or 70% isopropyl alcohol employed sparingly on a cotton applicator stick can be used to clean the objective lenses. Alcohol should be kept away from the periphery of the lenses, because alcohol can dissolve the cement and seep into the back side of the lens.

5. When fresh oil is added to residual oil on the 100× objective lens, there may be loss of contrast. Clean off all residual oil first.

6. Do not use water to clean lenses. If no lens cleaner is available, use a clean microfiber cloth.

7. When transporting the microscope, place one hand under the base as support and one hand firmly around the arm.

In addition to daily care of the microscope, semiannual or annual maintenance with thorough cleaning should be done by a professional. Microscope professionals may recognize and correct problems with mechanics or optics before they are detected by the microscope user. They can correct problems such as sticking of stage controls or incorrect optical alignment that can lead to physical problems like carpal tunnel syndrome and headaches.

### BASIC TROUBLESHOOTING

Most common problems are related to inability to focus. Once the operator has ensured that he or she is not trying to obtain a “flat field” using an objective lens that is not plan achromatic, the following checklist can aid in identifying the problem:

- Eyepieces
  - Clean?
  - Securely assembled?

- Objective lens
  - Screwed in tightly?
  - Dry objective free of oil?

- Condenser
  - Adjusted to proper height?
  - Free of oil?

- Slide
  - Correct side up?

- Coverslip
  - Correct side of blood film?
  - Only one coverslip on slide?

- Light source
  - Free of mounting media?

- Fingertips on bulb?
  - Bulb in need of changing?

- Light source aligned correctly?
OTHER MICROSCOPES USED IN THE CLINICAL LABORATORY

Phase-Contrast Microscope
The ability to view a stained specimen by the use of brightfield microscopy is affected by two features: (1) the ability of the specimen to absorb the light hitting it, and (2) the degree to which light waves traveling through the specimen remain in phase.

Specimens that are transparent or colorless, such as unstained cells, are not clearly visualized with brightfield microscopy. Phase-contrast microscopy, through the installation of an annular diaphragm in the condenser, together with a phase-shifting element, creates excellent contrast of a cell against its surrounding background.

The principle of phase contrast is related to the index of refraction and the thickness of a specimen, which produce differences in the optical path. Light passing through a transparent specimen travels slightly slower than light that is unobstructed. The difference is so small that it is not noticeable to the viewer. When a transparent phase plate is placed into the microscope, however, the change in phase can be increased to half a wavelength, which makes the otherwise transparent objective visible.

This phase difference produces variation in light intensity from bright to dark, creating contrast in the image. Often the objects appear to have “haloes” surrounding them.

In hematology, phase-contrast microscopy is employed in counting platelets in a hemacytometer, since they are difficult to visualize and count using brightfield microscopy. It also can be used to view formed elements in unstained urine sediments.

Polarized Light Microscope
Polarized light microscopy is another contrast-enhancing technique used to identify substances such as crystals in urine and other body fluids (Chapter 18). With brightfield microscopy, light vibrates in all directions. If a polarizer (filter) is placed in the light path, the light vibrates in only one direction or plane, which creates polarized light. To convert a brightfield microscope to a polarizing one, two filters are needed. One filter (the polarizer) is placed below the condenser and allows only light vibrating in the east-west direction perpendicular to the light path to pass through the specimen. The second filter (the analyzer) is placed between the objective and the eyepiece and allows only light vibrating in a north-south direction to pass to the eyepiece. When the transmission axes of these two filters are oriented at right angles, no light can pass through the pair to the eyepieces. When polarized light (vibrating in an east-west direction) passes through an optically active substance such as a monosodium urate crystal, however, the light is refracted into two beams, one vibrating in the original direction (east-west) and one vibrating in a plane 90 degrees to it (i.e., north-south). The refracted light vibrating in the north-south direction can pass through the second filter (the analyzer) and is visible at the eyepiece. The magnified crystal appears white against a black background. If a first-order red compensator filter also is placed in the light path below the stage, the background becomes pink-red, and the crystal appears yellow or blue, depending on its physical orientation relative to the incident light path (east-west). Some crystals can be specifically identified based on their unique birefringent (doubly refractive) characteristics when polarizing microscopy is used (Figures 18-22 and 18-23).

Darkfield Microscope
Darkfield microscopy is a contrast-enhancing technique that employs a special condenser. The condenser sends light upward toward the specimen in a hollow cone. Because of the high angle of this cone, none of the illuminating rays enters the objective lens. Without the specimen in place, the field would appear black because of the absence of light. When the specimen is in place, and if fine detail exists in the specimen, light is diffracted in all directions. This diffracted light is picked up by the objective lens and appears as bright detail on a black background. Darkfield microscopy is helpful in microbiology in the identification of spirochetes.

SUMMARY

- The compound microscope, through the use of an objective lens in the optical tube, forms an intermediate image of the illuminated specimen. The image is then magnified and viewed through the eyepiece lenses.
- The numerical aperture or NA, which is engraved on the barrel of objective lenses, designates the light-gathering ability of the lens. The larger the NA, the greater the resolution.
- Achromatic lenses maintain the center of the field in focus, whereas plan achromatic lenses correct for the curvature of a field, providing a flat field uniform focus.
- The condenser gathers the light and directs it through a thin specimen.
- Koehler illumination establishes a field of evenly distributed brightness across the specimen; the microscope should be adjusted for proper Koehler illumination with each use.
- Only the rheostat (light control knob or lever) should be used to regulate the light intensity needed to visualize a specimen. Light intensity should not be regulated by adjusting the position of the aperture diaphragm or the height of the condenser when using the oil immersion lenses.
PART I
Introduction to Hematology

- The aperture diaphragm control lever may be adjusted to achieve proper contrast of the features of the specimen being viewed.
- The use of oil immersion for the 50× and 100× oil immersion objectives improves the resolution of the image; type B oil is typically used with brightfield microscopy in hematology.
- Microscopes should be carefully handled and maintained. Solvents should not be used to clean lenses; lens cleaner or 70% isopropyl alcohol is recommended.
- Phase-contrast microscopy relies on the effect of index of refraction and the thickness of the specimen; these two features affect light by retarding a fraction of the light waves, resulting in a difference in phase. This allows transparent or colorless objects to become visible.
- Polarizing microscopes use two polarizing filters to cancel the light passing through the specimen. If the object is able to polarize light, as are some crystals, the light passing through is rotated and the object becomes visible.
- Darkfield microscopes use condensers that send light to the specimen at a high angle, directing the light away from the objective lens. If the specimen has fine detail, it causes the light to bend back toward the objective, which allows it to be viewed against an otherwise dark background.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the question presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Use of which one of the following type of objective lens causes the center of the microscope field to be in focus, whereas the periphery is blurred?
   a. Plan achromatic
   b. Achromatic
   c. Plan apochromatic
   d. Flat field

2. Which of the following gathers, organizes, and directs light through the specimen?
   a. Eyepiece
   b. Objective lens
   c. Condenser
   d. Optical tube

3. After focusing a specimen by using the 40× objective, the laboratory professional switches to a 10× objective. The specimen remains in focus at 10×. Microscopes with this characteristic are described as:
   a. Parfocal
   b. Paracentric
   c. Compensated
   d. Parachromatic

4. Which objective has the greatest degree of color correction?
   a. Achromatic
   b. Plan apochromatic
   c. Bichromatic
   d. Plan achrromatic

5. In adjusting the microscope light using Koehler illumination, which one of the following is true?
   a. Condenser is first adjusted to its lowest position
   b. Height of the condenser is adjusted by removing the eyepiece
   c. Image of the field diaphragm iris is used to center the condenser
   d. Closing the aperture diaphragm increases the resolution of the image

6. The total magnification obtained when a 10× eyepiece and a 10× objective lens are used is:
   a. 1×
   b. 10×
   c. 100×
   d. 1000×

7. After a microscope has been adjusted for Koehler illumination, and the specimen is being viewed with an oil immersion objective lens, light intensity should never be regulated by adjusting the:
   a. Rheostat
   b. Neutral density filter
   c. Light control knob
   d. Condenser

8. The recommended cleaner for removing oil from objectives is:
   a. 70% alcohol or lens cleaner
   b. Xylene
   c. Water
   d. Benzene
9. Which of the following types of microscopy is valuable in
   the identification of crystals that are double refractive?
   a. Compound brightfield
   b. Darkfield
   c. Polarizing
   d. Phase-contrast

10. A laboratory science student has been reviewing a hematol-
    ogy slide using the 10× objective to find a suitable portion
    of the slide for examination. He moves the 10× objective
    out of place, places a drop of oil on the slide, rotates
    the nosepiece so that the 40× objective passes through
    the viewing position, and continues to rotate the 100× oil
    objective into viewing position. This practice should be
    corrected in which way?
    a. The stage of a parfocal microscope should be lowered
       before the objectives are rotated.
    b. The 100× oil objective should be in place for viewing
       before the oil is added.
    c. The drop of oil should be in place and the 100× objective
       lowered into the oil, rather than swinging the objective
       into the drop.
    d. The objectives should be rotated in the opposite direction
       so that the 40× objective does not risk entering the oil.

11. Darkfield microscopes create the dark field by:
    a. Using two filters that cancel each other out, one above
       and the other below the condenser
    b. Angling the light at the specimen so that it misses the
       objective unless something in the specimen bends it
       backward
    c. Closing the condenser diaphragm entirely, limiting
       light to just a tiny ray in the center of the otherwise dark
       field
    d. Using a light source above the specimen and collecting
       light reflected from the specimen, rather than transmit-
       ted through the specimen, so that when there is no
       specimen in place, the field is dark

REFERENCES
3. Olympus Microscopy Resource Center: Anatomy of the Micro-
   scope. Available at: http://www.olympusmicro.com/primer/
   Accessed 06.04.14.

ADDITIONAL RESOURCES
PART I
Introduction to Hematology

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Describe the procedures to validate and document a new or modified laboratory assay.
2. Compare a new or modified assay to a reference using statistical tests to establish accuracy.
3. Select appropriate statistical tests for a given application and interpret the results.
4. Define and compute precision using standard deviation and coefficient of variation.
5. Determine assay linearity using graphical representations and transformations.
6. Discuss analytical limits and analytical sensitivity and specificity.
7. Explain Food and Drug Administration clearance levels for laboratory assays.
8. Compute a reference interval and a therapeutic range for a new or modified assay.
9. Interpret internal quality control using controls and moving averages.
10. Explain the benefits of participation in periodic external quality assessment.
11. Measure and describe assay clinical efficacy.
12. Interpret relative and absolute risk ratios.
13. Interpret receiver operating characteristic curves.
14. Describe methods to enhance and assess laboratory staff competence.
15. Describe a quality assurance plan to control for preanalytical and postanalytical variables.
16. List the agencies that regulate hematology and hemostasis quality.

OUTLINE
Statistical Significance and Expressions of Central Tendency and Dispersion
- Statistical Significance
- Computing the Mean
- Determining the Median
- Determining the Mode
- Computing the Variance
- Computing the Standard Deviation
- Computing the Coefficient of Variation
Validation of a New or Modified Assay
- Accuracy
- Statistical Tests
- Precision
- Linearity
- Lower Limit of Detection
- Analytical Specificity
- Levels of Laboratory Assay Approval
- Documentation and Reliability
Lot-to-Lot Comparisons
Development of the Reference Interval and Therapeutic Range
Internal Quality Control
- Controls
- Moving Average of the Red Blood Cell Indices
- Delta Checks
External Quality Assessment
Assessing Diagnostic Efficacy
- The Effects of Population Incidence and Odds Ratios on Diagnostic Efficacy

CASE STUDY
After studying the material in this chapter, the reader should be able to respond to the following case study:

On an 8:00 AM assay run, the results for three levels of a preserved hemoglobin control specimen are 2 g/dL higher than the upper limit of the target interval. The medical laboratory scientist reviews δ-check data on the hemoglobin results for the last 10 patients in sequence and notices that the results are consistently 1.8 to 2.2 g/dL higher than results generated the previous day.

1. What do you call the type of error detected in this case?
2. Can you continue to analyze patient specimens as long as you subtract 2 g/dL from the results?
3. What aspect of the assay should you first investigate in troubleshooting this problem?

In medical laboratory science, quality implies the ability to provide accurate, reproducible assay results that offer clinically useful information. Because physicians base 70% of their clinical decision making on laboratory results, assay results must be reliable. Reliability requires vigilance and effort on the part of all laboratory staff members. An experienced medical laboratory scientist who is a quality assurance and quality control specialist often directs this effort.

*The author acknowledges David McGlasson, M.S., MLS (ASCP)CM, Clinical Research Scientist, Wilford Hall Ambulatory Surgical Center, JBSA, San Antonio, Texas, for his assistance in preparing this chapter.
CHAPTER 5  Quality Assurance in Hematology and Hemostasis Testing

Of the terms quality control and quality assurance, quality assurance is the broader concept, encompassing preanalytical, analytical, and postanalytical variables (Box 5-1). Quality control processes are employed to document assay validity, accuracy, and precision, including external quality assessment, reference interval preparation and publication, and lot-to-lot validation.

Preanalytical variables, listed further in this chapter in Table 5-11, are addressed in Chapter 3, which discusses blood specimen collection, and in Chapter 42, which includes a section on coagulation specimen management. Postanalytical variables are discussed briefly at the end of this chapter and are listed in Table 5-12. Quality assurance further encompasses laboratory assay utilization and physician test ordering patterns, nicknamed “pre-pre” analytical variables, and the appropriate application of laboratory assay results, sometimes called “post-post” analytical variables. There exists a combined 17% medical error rate associated with the pre-pre and post-post analytical phases of laboratory test utilization and application, prompting laboratory directors and scientists to develop clinical query systems that guide clinicians in laboratory assay selection. Clinical query systems are enhanced by reflex assay algorithms developed in collaboration with the affiliated medical and surgical staff. Equally important, a system of narrative reports that accompany and augment numerical laboratory assay output, authored by medical laboratory scientists and directors, is designed to assist physicians with case management. A discussion of pre-pre and post-post analytical variables extends beyond the scope of this textbook but may be found in the references listed at the end of this chapter.

Quality control relies on the initial computation of central tendency and dispersion.

STATISTICAL SIGNIFICANCE AND EXPRESSIONS OF CENTRAL TENDENCY AND DISPERSION

Statistical Significance

When applying a statistical test such as the Student’s t-test of means or the analysis of variance (ANOVA), the statistician begins with a null hypothesis. The null hypothesis states that there is no difference between or among the means or variances of the populations being compared. The alternative (research) hypothesis is the logical opposite of the null hypothesis. For example, the null hypothesis may state there is no difference between t-test means, but the alternative hypothesis states that the null hypothesis is rejected and a statistical difference between the means does indeed exist (Table 5-2). In medical research, the null and alternative hypotheses may go unstated but are always implied.

The power of a statistical test is defined as its ability to reject the null hypothesis when the null hypothesis is indeed false. Power is expressed as \( p \), which stands for the probability that the test is able to detect an effect. The \( p \) scale ranges from 0 to 1. Power is determined by the sample size (number of data points, \( n \)), the design of the research study, and the study’s ability to control for extraneous variables.

The conventional levels for significance, or for rejecting the null hypothesis, are \( p \leq 0.05 \) (5%) or \( p \leq 0.01 \) (1%). In the former instance, there exists a 5% chance that the effect has occurred by chance alone; in the latter instance, there exists a more stringent 1% chance. Often researchers combine the statistical results, and thus the powers of several studies, to compute a common \( p \)-value, a process called meta-analysis.

BOX 5-1  Examples of Components of Quality Assurance

1. Preanalytical variables: selection of assay relative to patient need; implementation of assay selection; patient identification and preparation; specimen collection equipment and technique; specimen transport, preparation, and storage; monitoring of specimen condition
2. Analytical variables: laboratory staff competence; assay and instrument selection; assay validation, including linearity, accuracy, precision, analytical limits, and specificity; internal quality control; external quality assessment
3. Postanalytical variables: accuracy in transcription and filing of results; content and format of laboratory report, narrative report; reference interval and therapeutic range; timeliness in communicating critical values; patient and physician satisfaction; turnaround time; cost analysis; physician application of laboratory results
The term significant has a specific meaning based on the p-value, and it should not be generalized to imply practical or clinical significance. A statistical test result may indicate a statistically significant difference that is based on a selected study condition and power, but the difference may not possess practical importance because the clinical difference may be inconsequential. Experience and clinical judgment help when analyzing data, as does asking the question “Will this result generate a change in the prognosis, diagnosis, or treatment plan?”

Computing the Mean

The arithmetic mean ( \( \bar{x} \) ), or average, of a data series is the sum (Σ) of the individual data values divided by the number (n) of data points. A data series that represents a single population, for instance, a series of prothrombin time results from a population, is called a sample. Often clinical laboratory personnel apply the terms sample and specimen interchangeably. A specimen may be defined as a single data point within a data series (sample). In the clinical laboratory, of course, a specimen often means a tube of blood or a piece of tissue collected from a patient, which provides a single data point. The sum of sample data values above the mean is equal to the sum of the data values below the mean; however, the actual numbers of points above and below the mean are not necessarily equal.

The mean is a standard expression of central tendency employed in most scientific applications; however, it is profoundly affected by outliers and is unreliable in a skewed population. This is the formula for computing the arithmetic mean:

\[
\text{Mean (} \bar{x} \text{)} = \frac{\sum x_i}{n} ;
\]

where \( \bar{x} \) = mean; \( \sum x \) = sum of data point values; and n = number of data points

The geometric mean is the \( n \) root of the product of \( n \) individual data points and is used to compute means of unlike data series. The geometric mean of the prothrombin time reference interval is used to compute the prothrombin time international normalized ratio (Chapter 43). This is the formula for computing the geometric mean:

\[
\text{Geometric mean of } n \text{ instances of } a = \sqrt[n]{a_1 a_2 ... a_n} .
\]

Determining the Median

The median is the data point that separates the upper half from the lower half of a data series (sample). To find the median, arrange the data series in numerical order and select the central data point. If the data series has an even number of data points, the median is the mean of the two central points. The median is a robust expression of central tendency in a skewed distribution because it minimizes the effects of outliers.

Determining the Mode

The mode of a data series (sample) is the data point that appears most often in the sample. The mode is not a true measure of central tendency because there is often more than one mode in a data series. For instance, a typical white blood cell histogram may be trimodal, with three modes, one each for lymphocytes, monocytes, and neutrophils. Conversely, in a Gaussian, “normal” sample, in which the data points are distributed symmetrically, the mean, median, and mode coincide at a single data point.

Computing the Variance

Variance (\( \sigma^2 \)) expresses the deviation of each data point from its expected value, usually the mean of the data series (sample) from which the data point is drawn. The difference between each data point from the mean is squared, the squared differences are summed, and the sum of squares is divided by \( n - 1 \). Variance is expressed in the units of the variable squared as follows:

\[
\sigma^2 = \frac{\sum (x_i - \bar{x})^2}{n - 1},
\]

where \( \sigma^2 \) = sample variance; \( x_i \) = value of each data point; \( \bar{x} \) = mean; and \( n \) = number of data points

Computing the Standard Deviation

Standard deviation (SD), a commonly used measure of dispersion, is the square root of the variance and is the mean distance of all the data points in a sample from the sample mean (Figure 5-1). The larger the SD of a sample, the greater the deviation from the mean. In clinical analyses, the SD of an assay is an expression of its quality based on its inherent dispersion or variability. The formula for SD is:

\[
SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}} ,
\]

where SD = standard deviation; \( x_i \) = each data point value; \( \bar{x} \) = mean; and \( n \) = number of observations

SD states the confidence, or degree of random error, for statistical conclusions. Dispersion is typically expressed as \( \bar{x} \pm 2 \) SD or the 95.5% confidence interval (CI). Data points that are over 2 SD from the mean are outside the 95.5% CI and may be considered abnormal. The dispersion of data points within \( \bar{x} \pm 2 \) SD is considered the expression of random or chance variation. Typically, \( \bar{x} \pm 2 \) SD is used to establish biological reference intervals (normal ranges), provided the frequency of the data points is “Gaussian,” or normally distributed, meaning symmetrically distributed about the mean.

Computing the Coefficient of Variation

The coefficient of variation (CV) is the normalized expression of the SD, ordinarily articulated as a percentage (CV%). CV% is the most commonly used measure of dispersion in laboratory medicine. CV% is expressed without units (except percentage), thus making it possible to compare data sets that use different units. The computation formula is:

\[
\text{CV\%} = 100 \times \frac{SD}{\bar{x}} ,
\]

where CV% = coefficient of variation expressed as a percentage; SD = standard deviation; and \( \bar{x} \) = mean
VALIDATION OF A NEW OR MODIFIED ASSAY

All new laboratory assays and all assay modifications require validation. Validation is an activity comprised of procedures to determine accuracy, specificity, precision, limits, and linearity. The results of these procedures are faithfully recorded and made available to on-site assessors upon request.

Accuracy

Accuracy is the measure of agreement between an assay value and the theoretical “true value” of its analyte (Figure 5-1). Some statisticians prefer to define accuracy as the magnitude of error separating the assay result from the true value. By comparison, precision is the expression of reproducibility or dispersion about the mean, often expressed as SD or CV%, as discussed in a subsequent section, “Precision.” Accuracy is easy to define but difficult to establish and maintain; precision is relatively easy to measure and maintain.

For many analytes, laboratory professionals employ primary standards to establish accuracy. A primary standard is a material of known, fixed composition that is prepared in pure form, often by determining its mass on an analytical balance. The practitioner dissolves the weighed standard in an aqueous solution, prepares suitable dilutions, calculates the anticipated concentration for each dilution, and assigns the calculated concentrations to assay outcomes. For example, he or she may obtain pure glucose, weigh 100 mg, dilute it in 100 mL of buffer, and assay an aliquot of the solution using photometry. The resulting absorbance would then be assigned the value of 100 mg/dL. The practitioner may repeat this procedure using a series of four additional glucose solutions at 20, 60, 120, and 160 mg/dL to produce a five-point standard curve. The curve may be reassayed several times to generate means for each concentration. Standard curve generation is automated, however laboratory professionals retain the ability to generate curves manually when necessary. The assay is then employed.

Figure 5-1 The values generated by repeated assays of an analyte are graphed as a frequency distribution. Incremental values are plotted on the horizontal (x) scale and number of times each value was obtained (frequency) on the vertical (y) scale. In this example, the values are normally distributed about their mean (symmetric, Gaussian distribution). Results from an accurate assay generate a mean that closely duplicates the reference target value. Results from a precise assay generate small dispersion about the mean, whereas imprecision is reflected in a broad curve. The ideal assay is both accurate and precise.
on human serum or plasma, with absorbance compared with the standard curve to generate a result. The matrix of a primary standard need not match the matrix of the patient specimen; the standard may be dissolved in an aqueous buffer, whereas the test specimen may be human serum or plasma.

To save time and resources, the laboratory professional may employ a secondary standard, perhaps purchased, that the vendor has previously calibrated to a primary standard. The secondary standard may be a preserved plasma preparation at a certified known concentration. The laboratory professional merely thaws or reconstitutes the secondary standard and incorporates it into the test series during validation or revalidation. Manufacturers often match secondary standards as closely as possible to the test specimen’s matrix, for instance, serum to serum, plasma to plasma, and whole blood to whole blood. Primary and secondary standards are seldom assayed during routine patient specimen testing, only during calibration or when the assay tends to be unstable.

Regrettably, in hematology and hemostasis, where the analytes are often cell suspensions or enzymes, there are just a handful of primary standards: cyanmethemoglobin, fibrinogen, factor VIII, protein C, antithrombin, and von Willebrand factor. For scores of analytes, the hematology and hemostasis practitioner relies on calibrators. Calibrators for hematology may be preserved human blood cell suspensions, sometimes supplemented with microlatex particles or nucleated avian red blood cells (RBCCs) as surrogates for hard-to-preserve human white blood cells (WBCs). In hemostasis, calibrators may be frozen or lyophilized plasma from healthy human donors. For most of these analytes, it is impossible to prepare “weighed-in” standards; instead, calibrators are assayed using reference methods (“gold standards”) at selected independent expert laboratories. For instance, a vendor may prepare a 1000-L lot of preserved human blood cell suspension, assay for the desired analytes within their laboratory (“in-house”), and send aliquots to five laboratories that employ well-controlled reference instrumentation and methods. The vendor obtains blood count results from all five, averages the results, compares them to their in-house values, and publishes the averages as the reference calibrator values. The vendor then distributes sealed aliquots to customer laboratories with the calibrator values published in the accompanying package inserts. Vendors often market calibrators in sets of three or five, spanning the range of assay linearity or the range of potential clinical results.

As with secondary standards, vendors attempt to match their calibrators as closely as possible to the physical properties of the test specimen. For instance, human preserved blood used to calibrate complete blood count (CBC) analytes generated by an automated cell counter is prepared to closely match the matrix of fresh anticoagulated patient blood specimens, despite the need for preservatives, refrigeration, and sealed packaging. Vendors submit themselves to rigorous certification by governmental or voluntary standards agencies in an effort to verify and maintain the validity of their products.

The laboratory practitioner assays the calibration material using the new or modified assay and compares results with the vendor’s published results. When new results parallel published results within a selected range, for example ±10%, the results are recorded and the assay is validated for accuracy. If they fail to match, the new assay is modified or a new reference interval and therapeutic range is prepared.

Medical laboratory professionals may employ locally collected fresh blood from a healthy donor as a calibrator; however, the process for validation and certification is laborious, so few attempt it. The selected specimens are assayed using reference instrumentation and methods, calibration values are assigned, and the new or modified assay is calibrated (adjusted) from these values.

New or modified assays may also be compared to reference methods. A reference method may be a previously employed, well-controlled assay or an assay currently being used by a neighboring laboratory. Several statistics are available to compare results of the new or modified assay to a reference method, including the Student’s t-test, analysis of variance (ANOVA), linear regression, Pearson correlation coefficient, and the Bland-Altman plot.

### Statistical Tests

#### Comparing Means of Two Data Series Using Student’s t-Test

The Student’s t-test compares the sample mean of a new or modified assay to the sample mean of a reference assay. In a standard t-test the operator assumes that population distributions are normal (Gaussian), the SDs are equal, and the assays are independent. Often laboratory professionals use the more robust paired t-test in which the new and reference assays are performed using specimens from the same donors (aliquots). Laboratory scientists also choose between the one-tailed and two-tailed t-test, depending on whether the population being sampled has one (high or low) versus two (high and low) critical values. For instance, when assaying plasma for glucose, clinicians are concerned about both elevated and reduced glucose values, so the laboratory professional would use the two-tailed t-test; however, when assaying for bilirubin, clinical concern focuses only on elevated bilirubin concentrations, so the laboratory professional would apply the more robust one-tailed t-test in method comparison studies.

The laboratory professional generates t-test data by entering the paired data sets side by side into columns of a spreadsheet and applying an automated t-test formula. The program generates the number, mean, and variance for each data series (sample; \( n_1, n_2; \bar{X}_1, \bar{X}_2; \sigma_1^2, \sigma_2^2 \)), and the “degrees of freedom” (df) for the test: \( df = n_1 + n_2 - 2 \). The operator selects the appropriate critical value (p), often \( p \leq 0.05 \). The computer uses df and p to compare the computed t-value to the standard table of critical t-values (Table 5-1) and reports the corresponding p-value. If the p-value is less than 0.05, the means of the two samples are unequal and the result is “statistically significant.” For instance, if the two assays are each performed on aliquots from 10 donors, the df is 18. If the computed t-value is 2.10 or higher, the means are unequal at \( p \leq 0.05 \). Applying a stricter critical value, the computed t-value would have to be 2.88 or higher for the means to be considered unequal at \( p \leq 0.01 \). Table 5-2 illustrates a typical t-test result.

When the t-test indicates that two sample means are not unequal, the operator may choose to implement the new or modified assay. However, statistically, if two means are adjudged “not
unequal," that is not the same as "equal." To increase the power
of the validation, the scientist often chooses to compute the
Pearson correlation coefficient and to apply linear regression
and the Bland-Altman plot.

Using Analysis of Variance to Compare Variances of
More Than Two Data Series
ANOVA accomplishes the same outcomes as the t-test; how-
ever, ANOVA may be applied to more than two series of data.
A laboratory scientist may often choose to compare two, three,
or four new methods with a reference method. The ANOVA
computes variance ($\sigma^2$) for each group (between-group $\sigma^2$),
an overall $\sigma^2$ (within-group $\sigma^2$), and an F-statistic (similar to the
t-statistic) based on the within-group $\sigma^2$. The F-statistic is com-
pared with a table of critical F-statistic values to determine
significance analogous to the t-statistic as shown in Table 5-1.

Like the Student’s t-test, ANOVA is available on computer
spreadsheets. The operator enters the data in one column per
data series (group or sample) and applies the ANOVA formula.
The test reports between-groups df (the number of groups – 1)
and the within-group df (total of observations – 1 per group).
The test also computes and reports the sum of squares within
and between groups, the total sum of squares, the mean
squares within and between groups, and the F-statistic. Spread-
sheet programs compare the F-statistic to the table of critical
F-values and report the p-value, which the operator then com-
pares to the selected p-value limit to determine significance.

In the first example of a two-tail t-test, the difference in the means does not rise to statistical significance, the computed p-value
exceeds the selected p-value, and the means are "not unequal." In the second example, the selected p-value exceeds the
computed p-value, and the means are unequal.

$\bar{x}$, mean; SD, standard deviation; n, number of data points.

### Table 5-1
Excerpt from the Standard Table of Critical t-Values for a Two-tailed Test

<table>
<thead>
<tr>
<th>df</th>
<th>$p \leq 0.05$</th>
<th>$p \leq 0.01$</th>
<th>df</th>
<th>$p \leq 0.05$</th>
<th>$p \leq 0.01$</th>
<th>df</th>
<th>$p \leq 0.05$</th>
<th>$p \leq 0.01$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.30</td>
<td>9.93</td>
<td>12</td>
<td>2.18</td>
<td>3.06</td>
<td>22</td>
<td>2.07</td>
<td>2.82</td>
</tr>
<tr>
<td>4</td>
<td>2.78</td>
<td>4.60</td>
<td>14</td>
<td>2.15</td>
<td>2.98</td>
<td>24</td>
<td>2.06</td>
<td>2.80</td>
</tr>
<tr>
<td>6</td>
<td>2.45</td>
<td>3.71</td>
<td>16</td>
<td>2.12</td>
<td>2.92</td>
<td>26</td>
<td>2.06</td>
<td>2.78</td>
</tr>
<tr>
<td>8</td>
<td>2.31</td>
<td>3.36</td>
<td>18</td>
<td>2.10</td>
<td>2.88</td>
<td>28</td>
<td>2.05</td>
<td>2.76</td>
</tr>
<tr>
<td>10</td>
<td>2.23</td>
<td>3.17</td>
<td>20</td>
<td>2.09</td>
<td>2.85</td>
<td>30</td>
<td>2.04</td>
<td>2.75</td>
</tr>
</tbody>
</table>

The operator matches the degrees of freedom (df) with the test and looks up the critical t-value at the selected level of signifi-
cance, often $p \leq 0.05$ or $p \leq 0.01$. If the computed t-value exceeds the critical value from the table, the null hypothesis is
rejected and the difference between method means is statistically significant.

### Table 5-2
Typical Student’s t-Test Results

<table>
<thead>
<tr>
<th>Example</th>
<th>Reference Method</th>
<th>New or Modified Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>0.45</td>
<td>0.46</td>
</tr>
<tr>
<td>SD</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Null hypothesis</td>
<td>$\bar{x}$ of control = $\bar{x}$ of test</td>
<td></td>
</tr>
<tr>
<td>Selected p-value</td>
<td>0.01</td>
<td>—</td>
</tr>
<tr>
<td>Computed p-value</td>
<td>0.085</td>
<td>—</td>
</tr>
<tr>
<td>Two-tail t</td>
<td>0.99</td>
<td>Null hypothesis is supported, means are not unequal</td>
</tr>
<tr>
<td>Critical two-tail t</td>
<td>2.88</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Example</th>
<th>Reference Method</th>
<th>New or Modified Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>SD</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Null hypothesis</td>
<td>$\bar{x}$ of control = $\bar{x}$ of test</td>
<td></td>
</tr>
<tr>
<td>Selected p-value</td>
<td>0.01</td>
<td>—</td>
</tr>
<tr>
<td>Computed p-value</td>
<td>0.008</td>
<td>—</td>
</tr>
<tr>
<td>Two-tail t</td>
<td>3.89</td>
<td>Null hypothesis is rejected, means are unequal</td>
</tr>
<tr>
<td>Critical two-tail t</td>
<td>2.88</td>
<td>—</td>
</tr>
</tbody>
</table>
The following regression equation:

\[ y = a + bx \]

Comparing Data Series Using Linear Regression

If a series of five calibrators is used, results may be analyzed by entering the following formula into a spreadsheet program that offers an automatic regression line and the y-axis:

\[ r = \frac{\sum xy/n - \bar{x}\bar{y}}{SD_x SD_y}, \]

where \( r \) is the Pearson correlation coefficient; \( \Sigma xy \) is sum of the products of each pair of scores; \( n \) is the number of values; \( \bar{x} \) is mean of the X distribution; \( \bar{y} \) is mean of the Y distribution; \( SD_x \) is SD of the X distribution; and \( SD_y \) is SD of the Y distribution.

Pearson \( r \)-values from 0 to +1.0 represent positive correlation; 1.0 equals perfect correlation. Laboratorians employ the Pearson formula to assess the range of values from two like assays or to compare assay results to previously assigned standard or calibrator results. Most operators set an \( r \)-value of 0.975 (or \( r^2 \)-value of 0.95) as the lower limit of correlation; any Pearson \( r \)-value less than 0.975 is considered invalid because it indicates unacceptable variability of the reference method.

When the Pearson \( r \)-value result indicates the adequacy of the range of values, the linear regression \( r \)-value equation described in the next section is applied. Linear regression finds the line that best predicts \( x \) from \( y \) but its equation does not account for dispersion. The Pearson correlation coefficient formula quantifies how \( x \) and \( y \) vary together while documenting dispersion.

### Comparing Data Series Using Linear Regression

If a series of five calibrators is used, results may be analyzed by the following regression equation:

\[ y = a + bx \]

\[ \text{Slope} (b) = n \sum xy - (\sum x)(\sum y) / n \sum x^2 - (\sum x)^2 \]

\[ \text{Intercept} (a) = \sum y - b(\sum x) / n \]

where \( x \) and \( y \) are the variables; \( a \) is the intercept between the regression line and the y-axis; \( b \) is the slope of the regression line; \( n \) is the number of values or elements; \( X \) is first calibrator value; \( Y \) is second calibrator value; \( \Sigma XY \) is sum of the product of first and second calibrator values; \( \Sigma X \) is sum of first calibrator values; \( \Sigma Y \) is sum of second calibrator values; and \( \Sigma x^2 \) is sum of squared first calibrator values.

Perfect correlation generates a slope of 1 and a y intercept of 0. Local policy based on total error calculation establishes limits for slope and y intercept; for example, many laboratory directors reject a slope of less than 0.9 or an intercept of more than 10% above or below zero (Figure 5-2).

Slope measures proportional systematic error; the higher the analyte value, the greater the deviation from the line of identity. Proportional errors are caused by malfunctioning instrument components or a failure of some part of the testing process. The magnitude of the error increases with the concentration or activity of the analyte. An assay with proportional error may be invalid.

Intercept measures constant systematic error (or bias, in laboratory vernacular), a constant difference between the new and reference assay regardless of assay result magnitude. A laboratory director may choose to adopt a new assay with systematic error but must modify the published reference interval.

Regression analysis gains sufficient power when 40 or more patient specimens are tested using both the new and reference assay in place of or in addition to calibrators. Data may be entered into a spreadsheet program that offers an automatic regression equation.

### Comparing Data Series Using the Bland-Altman Difference Plot

Linear regression and the Pearson correlation coefficient are essential tests of accuracy and performance; however, both are influenced by dispersion. The Bland-Altman difference plot, also known as the Tukey mean-difference plot, provides a graphical representation of agreement between two assays. Similar to the \( t \)-test, Pearson correlation, and linear regression,
paired assay results are tabled in automated spreadsheet columns. This formula is applied:

\[ S(x,y) = \frac{(S_1 + S_2)}{2}, \text{ and } S_1 - S_2, \]

where \( S \) = individual coordinates

The operator computes the mean of the assays and the signed difference between the values. A chart is prepared with the means plotted on the y-axis and the numerical or % differences on the y-axis. Difference limits are provided, characteristically at \( \bar{x} \pm 2 \text{ SD} \) (Figure 5-3). The plot visually illustrates the magnitude of the differences. In a normal distribution, 95.5% of the values are expected to fall within the limits; when more than 5% of data points fall outside the limits, the assay is rejected.

**Precision**

Unlike the determination of accuracy, assessment of precision (dispersion, reproducibility, variation, random error) is a simple validation effort, because it merely requires performing a series of assays on a single specimen or lot of reference material (Figure 5-1).\(^2\) Precision studies always assess both *within-day* and *day-to-day* variation about the mean and are usually performed on three to five calibration specimens, although they may also be performed using a series of patient specimens. To calculate within-day precision, the scientist assays a single specimen at least 20 consecutive times using one reagent batch and one instrument run. For day-to-day precision, 20 assays are required on at least 10 runs on 10 consecutive days. The day-to-day precision study employs the same source specimen and instrument but separate aliquots. Day-to-day precision accounts for the effects of different operators, reagents, and environmental conditions such as temperature and barometric pressure.

The collected data from within-day and day-to-day sequences are reduced by formula to the mean and a measure of dispersion such as standard deviation or, most often, coefficient of variation in percent (CV%), as described in “Statistical Significance and Expressions of Central Tendency and Dispersion”. The CV% documents the degree of dispersion or random error generated by an assay, a function of assay stability.
CV% limits are established locally. For analytes based on primary standards, the within-run CV% limit may be 5% or less, and for hematology and hemostasis assays, 10% or less; however, the day-to-day run CV% limits may be as high as 30%, depending on the stability and complexity of the assay. Although accuracy, linearity, and analytical specificity are just as important, medical laboratory professionals often equate the quality of an assay with its CV%. The best assay, of course, is one that combines the smallest CV% with the greatest accuracy.

Precision for visual light microscopy leukocyte differential counts on stained blood films is immeasurably broad, particularly for low-frequency eosinophils and basophils. Most visual differential counts are performed by reviewing 100 to 200 leukocytes. Although impractical, it would take differential counts of 800 or more leukocytes to improve precision to measurable though inadequate levels. Automated differential counts generated by profiling instruments, however, provide CV% levels of 5% or lower because these instruments count thousands of cells.

**Linearity**

Linearity is the ability to generate results proportional to the calculated concentration or activity of the analyte. The laboratory professional dilutes a high-end calibrator or elevated patient specimen to produce at least five dilutions spanning the full range of the assay. The dilutions are then assayed. Computed and assayed results for each dilution are paired and plotted on a linear graph,
produce the final concentration. Laboratory personnel never report results that fall below or above the linear limits, because accuracy is compromised in the nonlinear regions of the assay. Lower limits are especially important when counting platelets or assaying coagulation factors. For example, the difference between 1% and 3% coagulation factor VIII activity affects treatment options and the potential for predicting coagulation factor inhibitor formation. Likewise, the difference between a platelet count of 10,000/μL and 5000/μL affects the decision to treat with platelet concentrate.

**Lower Limit of Detection**

Linearity studies are coupled with the lower limit of detection study. A “zero calibrator,” or blank, is assayed 20 times, and the mean and standard deviation are computed from the results. The lower limit of detection is determined from the computed standard deviation. The limit is three standard deviations above the mean of blank assay results. This cutoff prevents false-positive results generated by low-end assay interference, commonly called noise. The manufacturer or distributor typically performs limit assays and provides the results on the package insert; however, local policies often require that results of the manufacturer’s limit studies be confirmed.

**Analytical Specificity**

Analytical specificity is the ability of an assay to distinguish the analyte of interest from anticipated interfering substances within the specimen matrix. The laboratory practitioner “spikes” identical specimens with potential interfering substances and measures the effects of each upon the assay results. Analytical specificity is determined by the manufacturer and need not be confirmed at the local laboratory unless there is suspicion of interference from a particular substance not assayed by the manufacturer. Manufacturer specificity data are transferred from the package insert to the laboratory validation report.

**Levels of Laboratory Assay Approval**

The U.S. Food and Drug Administration (FDA) categorizes assays as cleared, analyte-specific reagent (ASR) assays, research use only (RUO), and laboratory-developed (home-brew) assays. FDA-cleared assays are approved for the detection of specific analytes and should not be used for non-cleared (off-label) applications. ASRs that are bundled with other ASRs or other general reagents and labeled with an intentional use are subject to premarket review requirements. RUO kits may be used on a trial basis, but the institution or a clinical trial typically bears their expense, not the third-party payer or the patient. The FDA monitors in-house assays by regulating the main components, which include, but are not limited to, ASRs, locally prepared reagents, and laboratory instrumentation. Details are given in Table 5-4.

**Documentation and Reliability**

Validation is recorded on standard forms available from commercial sources, for example, Data Innovations LLC EP Evaluator®. Validation records are stored for 7 to 10 years in readily accessible databases and made available to laboratory assessors upon request.

Precision and accuracy records document assay reliability over specified periods. The recalibration interval may be once every 6 months or in accordance with operators’ manual recommendations. Recalibration is necessary whenever reagent lots are updated unless the laboratory professional can demonstrate that the reportable range is unchanged using lot-to-lot comparison. When control results demonstrate a shift or consistently fall outside action limits, or when an instrument

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**TABLE 5-4** Categories of Laboratory Assay Approval by the United States Food and Drug Administration

<table>
<thead>
<tr>
<th>Assay Category</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA-cleared assay</td>
<td>The local institution may use package insert data for linearity and specificity but must establish accuracy and precision.</td>
</tr>
<tr>
<td>Analyte-specific reagent</td>
<td>Manufacturer may provide individual reagents but not in kit form, and may not provide package insert validation data. Local institution must perform all validation steps.</td>
</tr>
<tr>
<td>Research use only</td>
<td>Local institution must perform all validation steps. Research use only assays are intended for clinical trials, and carriers are not required to pay.</td>
</tr>
<tr>
<td>Laboratory-developed</td>
<td>Assays devised locally, Food and Drug Administration evaluates using criteria developed for FDA-cleared assay kits.</td>
</tr>
</tbody>
</table>
is repaired, the laboratory professional repeats the validation procedure.\textsuperscript{25}

Regularly scheduled validity rechecks, lot-to-lot comparisons, instrument preventive maintenance, staff competence, and scheduled performance of internal quality control and external quality assessment procedures ensure continued reliability and enhance the value of a laboratory assay to the patient and physician.

**LOT-TO-LOT COMPARISONS**

Laboratory managers reach agreements with vendors to seques-
ter kit and reagent lots, thereby ensuring infrequent lot changes, optimistically no more than once a year.\textsuperscript{26} The new reagent lot must arrive approximately a month before the laboratory runs out of the old lot so that lot-to-lot comparisons may be completed and differences resolved, if necessary. The scientist uses control or patient specimens and prepares a range of analyte dilutions, typically five, spanning the limits of linearity. If the reagent kits provide controls, these are also included, and all are assayed using the old and new reagent lots. Results are charted as illustrated in Table 5-5.

Action limits vary by laboratory, but many managers reject the new lot when more than one specimen (data point pair) generates a variance greater than 10% or when all variances are positive or negative. In the latter case, the new lot may be rejected or it may be necessary to use the lot but develop a new reference interval and therapeutic range.

For several analytes, lot-to-lot comparisons include revalid-
ation of the analytical measurement range (AMR) or report-
able range. AMR is the range of results a method produces without any specimen pre-treatment, such as dilution, and is similar to a linearity study.

**DEVELOPMENT OF THE REFERENCE INTERVAL AND THERAPEUTIC RANGE**

Once an assay is validated, the laboratory professional develops the reference interval (reference range, normal range).\textsuperscript{25} Most laboratory professionals use the vernacular phrase normal range; however, reference interval is preferred by statisticians. Using strict mathematical definitions, range encompasses all assay results from largest to smallest, whereas interval is a statistic that trims outliers.

To develop a reference interval, the laboratory professional carefully defines the desired healthy population and recruits representative donors who meet the criteria to provide blood specimens. The definition may, for example, exclude smokers, women taking oral contraceptives, and people using specified over-the-counter or prescription medications. Donors may be paid. There should be an equal number of males and females, and the chosen healthy donors should match the institution’s population demographics in terms of age and race. When practi-
cal, large-volume blood specimens are collected, aliquotted, and placed in long-term storage. For instance, plasma aliquots for coagulation reference interval development are stored indefinitely at \(-70^\circ\) C. It may be impractical to develop local reference intervals for infants, children, or geriatric populations. In these cases the laboratory director may choose to use published (textbook) intervals.\textsuperscript{27} In general, although published reference intervals are available for educational and general discussion purposes, local laboratories must generate their own reference intervals for adults to most closely match the demographics of the area served by their institution.

The minimum number of subject specimens (data points) required to develop a reference interval may be determined using statistical power computations; however, practical limitations prevail.\textsuperscript{28} For a new assay with no currently established reference interval, a minimum of 120 data points is necessary. In most cases, however, the assay manufacturer provides a reference interval on the package insert, and the local laboratory practitioner need only assay 30 specimens, approximately 15 male and 15 female, to validate the manufacturer’s reference interval, a process called transference. Likewise, the practitioner may refer to published reference intervals and, once they are locally validated, transfer them to the institution’s report form.

Scientists assume that the population specimens employed to generate reference intervals will produce frequency distributions (in laboratory vernacular, histograms) that are normal bell-shaped (Gaussian) curves (Figure 5-5). In a Gaussian frequency distribution the mean is at the center; the mean, median, and mode coincide; and the dispersion about the mean is identical in both directions. In many instances, however, biologic frequency distributions are “log-normal” with a “tail” on the high end. For example, laboratory professionals assumed for years that the visual reticulocyte percentage reference interval in adults is 0.5% to 1.5%; however, repeated analysis of healthy populations in several locations has established the interval to be 0.5% to 2%, owing to a subset of healthy donors whose reticulocyte counts fall at the high end of the interval.\textsuperscript{29} Scientists may choose to live with a log-normal distribution, or they may transform it by reploting the curve using a semilog or log-log graphic display. The decision to transform may arise locally but eventually becomes adopted as a national practice standard.

In a normal distribution, the mean (\(\mu\)) is computed by di-
viding the sum of the observed values by the number of data

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Old Lot Value</th>
<th>New Lot Value</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>7</td>
<td>6</td>
<td>-14.3%</td>
</tr>
<tr>
<td>Low middle value</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Middle</td>
<td>20.5</td>
<td>19.4</td>
<td>-5%</td>
</tr>
<tr>
<td>High middle</td>
<td>31</td>
<td>27</td>
<td>-12.9%</td>
</tr>
<tr>
<td>High</td>
<td>48</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Old kit control 1</td>
<td>9</td>
<td>11</td>
<td>22%</td>
</tr>
<tr>
<td>Old kit control 2</td>
<td>22</td>
<td>24</td>
<td>9%</td>
</tr>
<tr>
<td>New kit control 1</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>New kit control 2</td>
<td>24</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

Negative % difference indicates the new lot value is below the old lot (reference) value. The new lot is rejected because the low and high middle value results differ by more than 10%.

**TABLE 5-5 Example of a Lot-to-Lot Comparison**
of the test values from the healthy population are included within developing the reference interval, laboratory directors often use and the mean, mode, and median coincide. The segments of the population distribution representing $\pm 1$, $\pm 2$, and $\pm 3$ standard deviations are illustrated. In developing the reference interval, laboratory directors often use $\pm 2$ standard deviations to establish the 95.5% confidence interval. This means that 95.46% of the test values from the healthy population are included within $\pm 2$ standard deviations. Consequently, 4.54%, or approximately 1 in 20 test results from theoretically healthy donors, fall outside the interval, half (2.27%) above and half below.

![Normal (Gaussian) distribution](image)

**Figure 5-5** Normal (Gaussian) distribution. When the test values obtained for a given subject population are normally distributed, the mean is at the peak and the mean, mode, and median coincide. The segments of the population distribution representing $\pm 1$, $\pm 2$, and $\pm 3$ standard deviations are illustrated. In developing the reference interval, laboratory directors often use $\pm 2$ standard deviations to establish the 95.5% confidence interval. This means that 95.46% of the test values from the healthy population are included within $\pm 2$ standard deviations. Consequently, 4.54%, or approximately 1 in 20 test results from theoretically healthy donors, fall outside the interval, half (2.27%) above and half below.

Because failure to observe new intervals and ranges may result in diagnosis and treatment errors.

### INTERNAL QUALITY CONTROL

**Controls**

Laboratory managers prepare, or more often purchase, assay controls. Although it may appear similar, a control is wholly distinct from a calibrator. Indeed, cautious laboratory directors may insist that controls be purchased from distributors different from those who supply their calibrators. As discussed in the section “Validation of a New or Modified Assay,” calibrators are used to adjust instrumentation or to develop a standard curve. Calibrators are assayed by a reference method in expert laboratories, and their assigned value is certified. Controls are used independently of the calibration process so that systematic errors caused by deterioration of the calibrator or a change in the analytical process can be detected through internal quality control. This process is continuous and is called *calibration verification*. Compared with calibrators, control materials are inexpensive and are prepared from the same matrix as patient specimens except for preservatives, lyophilization, or freezing necessary to prolong shelf life. Controls provide known values and are sampled alongside patient specimens to accomplish within-run assay validation. In nearly all instances, two controls are required per test run: one within the reference interval and one above or below the reference interval. For some assays there is reason to select controls whose values are just outside the upper or lower limit of the reference interval, “slightly” abnormal. In institutions that perform continuous runs, the controls should be run at least once per shift, for instance, at 7 AM, 3 PM, and 11 PM. In laboratories where assay runs are discrete events, two controls are assayed with each run.

Control results must fall within predetermined dispersal limits, typically $\pm 2$ SD. Control manufacturers provide limits; however, local laboratory practitioners must validate and transfer manufacturer limits or establish their own, usually
by computing standard deviation from the first 20 control assays. Whenever the result for a control is outside the established limits, the run is rejected and the cause is found and corrected. The steps for correction are listed in Table 5-6.

Control results are plotted on a Levey-Jennings chart that displays each data point in comparison to the mean and limits (Figure 5-6). The Levey-Jennings chart assumes that the control results distribute in a Gaussian manner and provide limits at 1, 2, and 3 SD above and below the mean. In addition to being analyzed for single-run errors, the data points are examined for sequential errors over time (Figure 5-7). Both single-run and long-term control variation are a function of assay dispersion or random error and reflect the CV% of an assay.

Dr. James Westgard has established a series of internal quality control rules that are routinely applied to long-term deviations, called the Westgard rules. The rules were developed for assays that employ primary standards, but a few Westgard rules

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Reassay</td>
</tr>
<tr>
<td></td>
<td>When a limit of ±2 standard deviations is used, 5% of expected assay results fall above or below the limit.</td>
</tr>
<tr>
<td>2.</td>
<td>Prepare new control and reassay</td>
</tr>
<tr>
<td></td>
<td>Controls may deteriorate over time when exposed to adverse temperatures or subjected to conditions causing evaporation.</td>
</tr>
<tr>
<td>3.</td>
<td>Prepare fresh reagents and reassay</td>
</tr>
<tr>
<td></td>
<td>Reagents may have evaporated or become contaminated.</td>
</tr>
<tr>
<td>4.</td>
<td>Recalibrate instrument</td>
</tr>
<tr>
<td></td>
<td>Instrument may require repair.</td>
</tr>
</tbody>
</table>

Figure 5-6 Levey-Jennings chart illustrating acceptable control results. Control results from 19 runs in 20 days all fall within the action limits established as ±2 standard deviations (s). Results distribute evenly about the mean.

Figure 5-7 Levey-Jennings chart that illustrates a systematic error or Westgard 10X condition (shift, Table 5-7). Control results from 21 runs in 22 days all fall within the action limits established as ±2 standard deviations (s); however, the final 11 control results are above the mean. When 10 consecutive control results fall on one side of the mean, the assay has been affected by a systematic error (shift). The operator troubleshoots and recalibrates the assay.

<table>
<thead>
<tr>
<th>TABLE 5-7 Westgard Rules Employed in Hematology and Hemostasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>13s A single control value is outside the ±3 SD limit.</td>
</tr>
<tr>
<td>22s Two control values are outside the ±2 SD limit.</td>
</tr>
<tr>
<td>Rs Two consecutive control values within a run are more than 4 SD apart.</td>
</tr>
<tr>
<td>41s Four consecutive control values within a run exceed the mean by ±1 SD.</td>
</tr>
<tr>
<td>10X Also called a “shift.” A series of 10 consecutive control values remain within the dispersal limits but are consistently above or below the mean.</td>
</tr>
<tr>
<td>7T Also called a “trend.” A series of at least 7 control values trend in a consistent direction.</td>
</tr>
</tbody>
</table>

10X or 7T may indicate an instrument calibration issue that has introduced a constant systematic error (bias). Shifts or trends may be caused by deterioration of reagents, pump fittings, or light sources. Abrupt shifts may reflect a reagent or instrument fitting change.

In all cases, assay results are rejected and the error is identified using the steps in Table 5-6.

that are the most useful in hematology and hemostasis laboratories are provided in Table 5-7, along with the appropriate actions to be taken.

**Moving Average (X̄₈) of the Red Blood Cell Indices**

In 1974, Dr. Brian Bull proposed a method of employing patient RBC indices to monitor the stability of hematology analyzers, recognizing that the RBC indices mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin
concentration (MCHC) remain constant on average despite individual patient variations. Each consecutive sequence of 20 patient RBC index assay results is collected and treated by the moving average formula (see reference), which accumulates, “smooths,” and “trims” data to reduce the effect of outliers. Each trimmed 20-specimen mean, $\bar{X}_r$, is plotted on a Levey-Jennings chart and tracked for trends and shifts using Westgard rules. The formula has been automated and embedded in the circuitry of all hematology analyzers, which provide a Levey-Jennings chart for MCV, MCH, and MCHC. The moving average concept has been generalized to WBC and platelet counts and to some clinical chemistry analytes, albeit with moderate success.

To begin, 500 consecutive specimens are analyzed for the mean MCV, MCH, and MCHC. A Levey-Jennings chart is prepared using ±3% of the mean or one SD as the action limits, and subsequent data accumulation commences in groups of 20.

The moving average method requires a computer to calculate the averages, does not detect within-run errors, and is less sensitive than the use of commercial controls in detecting systematic shifts and trends. It works well in institutions that assay specimens from generalized populations that contain minimal numbers of sickle cell or oncology patients. A population that has a high percentage of abnormal hematologic results, as may be seen in a tertiary care facility, may generate a preponderance of moving average outliers. Moving average systems do not replace the use of control specimens but provide additional means to detect shifts and trends.

**Delta Checks**

The δ-check system compares a current analyte result with the result from the most recent previous analysis for the same patient. Certain patient values remain relatively consistent over time unless there is an intervention. A result that fails a δ-check, often a 20% deviation, is investigated for intervention such as a transfusion or surgery, or a profound change in the patient’s condition subsequent to the previous analysis. If there is no ready explanation, the failed δ-check may indicate an analytical error or mislabeled specimen. Results that fail a δ-check are sequestered until the cause is found. Laboratory directors may require δ-checks on MCV, RDW, HGB, PLT, PT, INR, and PTT. Action limits for δ-checks are based on clinical impression and are assigned by hematology and hemostasis laboratory directors in collaboration with clinicians and laboratory staff. Computerization is essential, and δ-checks are designed only to identify gross errors, not changes in random error, or shifts or trends. There is no regulatory requirement for δ-checks.

**EXTERNAL QUALITY ASSESSMENT**

External quality assessment further validates the accuracy of hematology and hemostasis assays by comparing results from identical aliquots of specimens distributed at regular intervals among laboratories nationwide or worldwide. The aliquots are often called survey or proficiency testing specimens and include preserved human donor plasma and whole blood, stained peripheral blood films and bone marrow smears, and photomicrographs of cells or tissues.

In most proficiency testing systems, target (true or reference) values for the test specimens are established in-house by their manufacturer or distributor and are then further validated by preliminary distribution to a handful of “expert” laboratories. Separate target values may be assigned for various assay methods and instruments, as feasible.

Laboratories that participate in external quality assessment are directed to manage the survey specimens using the same principles as those employed for patient specimens—survey specimens should not receive special attention. Turnaround is swift, and results are sent electronically to the provider.

In addition to establishing a target value, agencies that administer surveys reduce the returned data to statistics, including the mean, median, and standard deviation of all participant results. Provided the survey is large enough, the statistics may be computed individually for the various instruments and assay methods. The statistics collected from participants should match the predetermined targets. If they do not, the agency troubleshoots the assay and assigns the most reliable statistics, usually the group mean and standard deviations.

The agency provides a report to each laboratory, illustrating its result in comparison with the target value and appending a comment if the laboratory result exceeds the established limits, usually ±2 standard deviations from the mean. If the specimen is a blood or bone marrow smear, a photomicrograph, or a problem that requires a binary (positive/negative, yes/no) response, the local laboratory comment is compared with expert opinion and consensus.

Although a certain level of error is tolerated, error rates that exceed established limits result in corrective recommendations or, in extreme circumstances, loss of laboratory accreditation or licensure.

There are a number of external quality assessment agencies; however, the College of American Pathologists (CAP, cap.org) and the American Proficiency Institute (API, api-pt.com) provide the largest survey systems. Survey packages are provided for laboratories offering all levels of service. API and CAP are nongovernmental agencies; however, survey participation is necessary to meet the accreditation requirements of the Joint Commission (jointcommission.org) and to qualify for Medicare reimbursement. The North American Specialized Coagulation Laboratory Association (nascola.org) provides survey systems for specialty coagulation laboratories in the United States and Canada and is affiliated with the ECAT (external quality control of diagnostic assays and tests, ecat.nl) Foundation External Quality Assessment Program of the Netherlands, which provides survey materials throughout Europe. Many state health agencies provide proficiency testing surveys, requiring laboratories to participate as a condition of licensure.

**ASSESSING DIAGNOSTIC EFFICACY**

Since the 1930s, surgeons have used the bleeding time test to predict the risk of intraoperative hemorrhage. The laboratory scientist, technician, or phlebotomist activates an automated
lancet to make a 5-mm long, 1-mm deep incision in the volar surface of the forearm and uses a clean piece of filter paper to meticulously absorb drops of blood in 30-second intervals. The time interval from initial incision to bleeding cessation is recorded, normally 2 to 9 minutes. The test is simple and logical, and experts have claimed for over 50 years that if the incision bleeds for longer than 9 minutes, there is a risk of surgical bleeding. In the 1990s clinical researchers compared within-range and prolonged bleeding times with instances of intraoperative bleeding and found to their surprise that prolonged bleeding time results predicted fewer than 50% of intraoperative bleeds.36 Many bleeds occurred despite a bleeding time shorter than 9 minutes. Thus, the positive predictive value of the bleeding time for intraoperative bleeding was less than 50%, which is the probability of turning up heads in a coin toss. Today the bleeding time test is widely agreed to have no clinical efficacy and is obsolete, though still available.

Like the bleeding time test, many time-honored hematology and hemostasis assays gain credibility on the basis of logic and expert opinion. Now, however, besides being valid, accurate, linear, and precise, a new or modified assay must be diagnostically effective.38 To compute diagnostic efficacy, the laboratory professional obtains a series of specimens from healthy subjects, volunteers who do not have the particular disease or condition being measured, called controls; and from patients who conclusively possess a disease or condition. The patients’ diagnosis is based on downstream clinical outcomes, discharge notes, or the results of valid existing laboratory tests, excluding the new assay. The new assay is then applied to specimens from both the healthy control and disease patient groups to assess its efficacy.

In a perfect world, the laboratory scientist sets the discrimination threshold at the 95.5% confidence interval limit (±2 SD) of the mean. When this threshold, also called the limit or “cut point,” is used, the test hopefully yields a positive result, meaning a level elevated beyond the upper limit or reduced below the lower limit, in every instance of disease and a negative result, within the reference interval, in all subjects (controls) without the disease. In reality, there is always some overlap: a “gray area” in which some positive test results are generated from non-disease specimens (false positives) and some negative results are generated from specimens taken from patients with proven disease (false negatives). False positives cause unnecessary anxiety, follow-up expense, and erroneous diagnostic leads—worrysome, expensive, and time consuming, but seldom fatal. False negatives fail to detect the disease and may delay treatment which can be potentially life threatening. The laboratory scientist employs diagnostic efficacy computations to establish the effectiveness of laboratory assays and to minimize both false-positive and false-negative results (Table 5-8). Diagnostic efficacy testing includes determination of diagnostic sensitivity and specificity, positive and negative predictive value, and receiver operating characteristic analysis.

To start a diagnostic efficacy study, the scientist selects control specimens from healthy subjects and specimens from patients proven to have the disease or condition addressed by the assay. To make this discussion simple, assume that 50 specimens of each are chosen. All are assayed, and the results are shown in Table 5-9.

The scientist next computes diagnostic sensitivity and specificity and positive and negative predictive value as shown in Table 5-10. These values are then used to consider the conditions in which the assay may be effectively used.

The Effects of Population Incidence and Odds Ratios on Diagnostic Efficacy
Epidemiologists describe population events using the terms prevalence and incidence. Prevalence describes the total number of events or conditions in a broadly defined population, for instance, the total number of patients with chronic heart disease in the United States. Prevalence quantitates the burden of a disease on society but is not qualified by time intervals and does not predict disease risk.

Incidence describes the number of events occurring within a randomly selected number of subjects representing a population, over a defined time, for instance, the number of new cases of heart disease per 100,000 U.S. residents per year. Incidence numbers are non-cumulative. Incidence can be further defined, for instance by the number of heart disease cases per 100,000 nonsmokers, 100,000 women, or 100,000 people ages 40 to 50. Scientists use incidence, not prevalence, to select

<table>
<thead>
<tr>
<th>Table 5-8 Diagnostic Efficacy Definitions and Binary Display</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>True positive</strong></td>
</tr>
<tr>
<td><strong>False positive</strong></td>
</tr>
<tr>
<td><strong>True negative</strong></td>
</tr>
<tr>
<td><strong>False negative</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5-9 Diagnostic Efficacy Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay is negative</strong></td>
</tr>
<tr>
<td><strong>Individuals Unaffected by the Disease or Condition</strong></td>
</tr>
<tr>
<td>True negative: 40</td>
</tr>
<tr>
<td>False negative: 5</td>
</tr>
</tbody>
</table>

Data on specimens from 50 individuals who are unaffected by the disease or condition and 50 individuals who are affected by the disease or condition.
laboratory assays for specific applications such as screening or confirmation.

For all assays, as diagnostic sensitivity rises, specificity declines. A screening test is an assay that is applied to a large number of subjects within a convenience sample where the participant’s condition is unknown, for example, lipid profiles offered in a shopping mall. Assays that possess high sensitivity and low specificity make effective screening tests, although they produce a number of false positives. For instance, if the condition being studied has an incidence of 0.0001 (1 in 10,000 per year) and the false-positive rate is a modest 1%, the assay will produce 99 false-positive results for every true-positive result. Clearly such a test is useful only when the consequence of a false-positive result is minimal and follow-up confirmation is readily available.

Conversely, as specificity rises, sensitivity declines. Assays with high specificity provide effective confirmation when used in follow-up to positive results on screening assays. High-sensitivity assays produce a number of false negatives and should not be used as initial screens. A positive result on both a screening assay and a confirmatory assay provides a definitive conclusion. A positive screening result followed by a negative confirmatory test result generates a search for alternative diagnoses.

Laboratory assays are most effective when chosen to assess patients with high clinical pretest probability. In such instances, the incidence of the condition is high enough to mitigate the effects of false positives and false negatives. For instance, when a physician orders hemostasis testing for patients who are experiencing easy bruising, there is a high pretest probability, which raises the assays’ diagnostic efficacy. Conversely, ordering hemostasis assays as screens of healthy individuals prior to elective surgery introduces a low pretest probability and reduces the efficacy of the test profile, raising the relative rate of false positives.

Epidemiologists further assist laboratory professionals by designing prospective randomized control trials to predict the relative odds ratio (or relative risk ratio, RRR) and the absolute odds ratio (or absolute risk ratio, ARR) of an intervention that is designed to modify the incidence of an event within a selected population, as illustrated in the following example.38

You design a 5-year study in which you select 2000 obese smokers ages 40 to 60 who have no heart disease. You randomly select 1000 for intervention: periodic laboratory assays for inflammatory markers, with follow-up aspirin for those who have positive assay results. The 1000 controls are tested with the same lab assays but are given a placebo that resembles aspirin. The primary endpoint is acute myocardial infarction (AMI). No one dies or drops out, and at the end of five years, 10 of the 1000 intervention arm have suffered AMIs. The control arm ratio is 50 of the 1000 controls and 50 of the 1000 members of the intervention group had suffered AMIs. The control arm ratio is 100/1000 = 0.1; the intervention arm ratio is 50/1000 = 0.05; and the RRR is 0.05/0.1 = 0.5 (50%). You predict from your study that the odds (RRR) of having a heart attack are cut in half by the intervention. You repeat the study using 2000 slim nonsmokers ages 20 to 40. In this sample, 10 of the 1000 controls and 5 in the intervention group suffer AMIs, the computation is 0.01/0.005 = 0.5, same as in the obese smoker group, thus enabling you to generalize your results to slim nonsmokers. RRR has been used extensively to support widespread medical interventions, often without regard to control arm incidence or the risks associated with generalizing to non-studied populations.

You go on to compute the ARR, which is the absolute value of the arithmetic difference in the event rates of the control and intervention arms.39 In our example using the obese smokers

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Definition</th>
<th>Formula</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic sensitivity</td>
<td>Proportion with the disease who have a positive test result</td>
<td>Sensitivity (%) = TP/(TP + FN) × 100</td>
<td>45/(45 + 5) × 100 = 90%</td>
</tr>
<tr>
<td>Diagnostic specificity</td>
<td>Proportion without the disease who have a negative test result</td>
<td>Specificity (%) = TN/(TN + FP) × 100</td>
<td>40/(40 + 10) × 100 = 80%</td>
</tr>
<tr>
<td>Positive predictive value (PPV)</td>
<td>Proportion with a disease who have a positive test result compared with all individuals who have a positive test result</td>
<td>PPV (%) = TP/(TP + FP) × 100</td>
<td>45/(45 + 10) × 100 = 82%</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td>Proportion without a disease who have a negative test result compared with all individuals who have a negative test result</td>
<td>NPV (%) = TN/(TN + FN) × 100</td>
<td>40/(40 + 5) × 100 = 89%</td>
</tr>
</tbody>
</table>

FN, false negative; FP, false positive; TN, true negative; TP, true positive.

*Using data from Table 5-9.
group, the ARR = 0.1 – 0.05 = 0.05, or 5% (not 50%, as reported in the example using RRR above). The ARR is often expressed as the number necessary to treat (NNT), the inverse of ARR. In our example, for everyone whose AMI is prevented by the laboratory test and subsequent treatment, you would have to treat 20 total donors over a 5-year period. Further, if you reduce the ARR to an annual rate, 0.05/5 years = 0.01, or a 1% annual reduction. You conclude from your study that 100 interventions per year are required to prevent one AMI.

Finally, your RRR and ARR are not discrete integers but means computed from samples of 1000, so they must include an expression of dispersion, usually ±2 SD or a 95.5% confidence interval. Suppose the 95.5% confidence interval for the RRR turns out to be relatively broad: -0.1 to +1.1. A ratio of 1 implies no effect from the intervention, that is, the rate of change in the intervention arm is equal to the rate of change in the control arm. Given that the 95.5% confidence interval embraces the number 1, the intervention has failed to provide any benefit.

In summary, once a laboratory assay is verified to be accurate and precise, it must then be revealed to possess diagnostic efficacy and to provide for effective intervention as determined by favorable RRR and ARR or NNT values. Application of the receiver operating characteristic (ROC) curve may help achieve these goals.

**RECEIVER OPERATING CHARACTERISTIC CURVE**

A ROC curve is a further refinement of diagnostic efficacy testing that may be employed to determine the decision limit (cutoff, threshold) for an assay when the assay generates a continuous variable. In diagnostic efficacy testing as described in the previous section, the ±2 SD limits of the reference interval are used as the thresholds for discriminating a positive from a negative test result. Often the “true” threshold varies from the ±2 SD limit. Using ROC analysis, the limit is adjusted by increments of 1 (or other increments depending upon the analytical range), and the true-positive and false-positive rates are recomputed for each new threshold level using the same formulas provided in the section named “Assessing Diagnostic Efficacy.” The limit that is finally selected is the one that provides the largest true-positive and smallest false-positive rate (Figure 5-8). The operator generates a line graph plotting true positives on the y-axis and false positives on the x-axis. Measuring the area under the curve (a computer-based calculus function) assesses the overall efficacy of the assay. If the area under the curve is 0.5, the curve is at the line of identity between false and true positives and provides no discrimination. Most agree that a clinically useful assay should have an area under the curve of 0.85 or higher.

**ASSAY FEASIBILITY**

Most laboratory managers and directors review assay feasibility before launching complex validation, efficacy, reference interval, and quality control initiatives. Feasibility studies include a review of assay throughput (number of assays per unit time), dwell time (length of assay interval from specimen sampling to report), cost per test, cost/benefit ratio, turnaround time, and the technical skill required to perform the assay. To select a new instrument, the manager reviews issues of operator safety, footprint, overhead, compatibility with laboratory utilities and information system, the need for middleware, frequency and duration of breakdowns, and distributor support and service.

**LABORATORY STAFF COMPETENCE**

Staff integrity and professional staff competence are the keys to assay reliability. In the United States, California, Florida, Georgia, Hawaii, Louisiana, Montana, Nevada, New York, North Dakota, Rhode Island, Tennessee, West Virginia, and Puerto Rico enforce licensure laws. In these states, only licensed laboratory professionals may be employed in medical center or reference laboratories. Legislatures in Alaska, Illinois, Massachusetts, Minnesota, Missouri, Vermont, and Virginia have considered and rejected licensure bills, the bills having been opposed by competing health care specialty associations and for-profit entities. In non-licensure states, conscientious laboratory directors employ only nationally certified professionals. Certification is available from the American Society for Clinical Pathology Board of Certification in Chicago, Illinois. Studies of laboratory errors and outcomes demonstrate that laboratories that employ only licensed or certified professionals produce the most reliable assay results.

Competent laboratory staff members continuously watch for and document errors by inspecting the results of internal validation and quality control programs and external quality assessment. Error is inevitable, and incidents should be documented and highlighted for quality improvement and instruction. When error is associated with reprimand, the opportunity for improvement may be lost to cover-up. Except in cases of negligence, the analysis of error without blame is consistently practiced in an effort to improve the quality of laboratory service.

**Proficiency Systems**

Laboratory managers and directors assess and document professional staff skills using proficiency systems. The hematology laboratory manager may, for instance, maintain a collection of normal and abnormal blood films, case studies, or laboratory assay reports that technicians and scientists are required to examine at regular intervals. Personnel who fail to reproduce the target values on examination of the blood film are provided remedial instruction. The proficiency set may also be used to assess applicants for laboratory positions. Proficiency testing systems are available from external quality assessment agencies, and proficiency reports are made accessible to laboratory assessors.

**Continuing Education**

The American Society for Clinical Pathology Board of Certification and state medical laboratory personnel licensure boards require technicians and scientists to participate in and document
**Figure 5-8**  
A, Receiver operating characteristic curve. The false-positive and true-positive rates for each discrimination threshold from 70% to 80% are computed and graphed as paired variables on a linear scale, false-positive rate on the horizontal (x) scale and true-positive rate on the vertical (y) scale. The assay has acceptable discrimination between affected and non-affected individuals; with an area under the curve (AUC) of 0.85, and 73% is the threshold that produces the most desirable false-positive and true-positive rates.  
B, This assay has unacceptable discrimination between affected and unaffected individuals, with an AUC of 0.70. It is difficult to find the threshold that produces the most desirable false-positive and true-positive rates.  
C, This assay, with an AUC of 0.50, has no ability to discriminate.  
*FP*, false positive; *TP*, true positive.
continuing education for periodic recertification or relicensure. Educators and experts deliver continuing education in the form of journal articles, case studies, online seminars (webinars), and seminars and workshops at professional meetings. Medical centers offer periodic internal continuing education opportunities (in-service education) in the form of grand rounds, lectures, seminars, and participative educational events. Presentation and discussion of current cases are particularly effective. Continuing education maintains the critical skills of laboratory personnel and provides opportunities to learn about new clinical and technical approaches. The Colorado Association for Continuing Medical Laboratory Education (camlc.org), the American Society for Clinical Laboratory Science (ascls.org), the American Society for Clinical Pathology (ascp.org), the American Society of Hematology (hematology.org), the National Hemophilia Foundation (hemophilia.org), and the Fritsma Factor (fritsma-factor.com) are examples of the scores of organizations that direct their activities toward quality continuing education in hematology and hemostasis.

The medical laboratory science profession stratifies professional staff responsibilities by educational preparation. In the United States, professional levels are defined as the associate (2-year) degree level, or medical laboratory technician; bachelor (4-year) degree level, or medical laboratory scientist; and the levels of advanced degrees: master’s degree or doctorate in clinical laboratory science and related sciences. Many colleges and universities offer articulation programs that enable professional personnel to advance their education and responsibility levels. Several of these institutions provide undergraduate and graduate distance-learning opportunities. A current list is maintained by the National Accrediting Agency for Clinical Laboratory Sciences (naacls.org), and the American Society for Clinical Laboratory Science publishes the Directory of Graduate Programs for Clinical Laboratory Practitioners, 5th ed. Enlightened employers encourage personnel to participate in advanced educational programs, and many provide resources for this purpose. Education contributes to quality laboratory services.

### QUALITY ASSURANCE PLAN: PREANALYTICAL AND POSTANALYTICAL

In addition to keeping analytical quality control records, U.S. regulatory agencies such as the Centers for Medicare and Medicaid Services (cms.gov) require laboratory directors to maintain records of preanalytical and postanalytical quality assurance and quality improvement efforts. Although not exhaustive, Table 5-11 lists and characterizes a number of examples of preanalytical quality efforts, and Table 5-12 provides a review of postanalytical components. All quality assurance plans provide objectives, sources of authority, scope of services, an activity calendar, corrective action, periodic evaluation, standard protocol, personnel involvement, and methods of communication.

API Paperless Proficiency Testing™ and CAP Q-PROBES® are subscription services that provide model quality assurance programs. Experts in quality assurance continuously refine the consensus of appropriate indicators of laboratory medicine quality. Quality assurance programs search for events that provide improvement opportunities.

### AGENCIES THAT ADDRESS HEMATOLOGY AND HEMOSTASIS QUALITY

The following are agencies that are concerned with quality assurance in hematology and hemostasis laboratory testing:

- Data Innovations North America (datainnovations.com), 120 Kimball Avenue, Suite 100, South Burlington, VT 05403: Quality assurance management software: instrument management middleware, laboratory production management software, EP Evaluator®, reference interval tables, allowable total error tables.

<table>
<thead>
<tr>
<th>TABLE 5-11 Preanalytical Quality Assurance Components and the Laboratory’s Responsibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preanalytical Component</strong></td>
</tr>
<tr>
<td>Test orders</td>
</tr>
<tr>
<td>Test request forms</td>
</tr>
<tr>
<td>Stat orders and timeliness</td>
</tr>
<tr>
<td>Specimen collection</td>
</tr>
<tr>
<td>Specimen transport</td>
</tr>
<tr>
<td>Specimen management</td>
</tr>
</tbody>
</table>
**TABLE 5-12 Postanalytical Quality Assurance Components and the Laboratory’s Responsibility**

<table>
<thead>
<tr>
<th>Postanalytical Component</th>
<th>Laboratory Staff Responsibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication of reports</td>
<td>Are results accurately transcribed into the information system? Are they reviewed for errors by additional laboratory staff? If autoverification is in effect, are the correct parameters employed? Do reports provide reference intervals? Do they flag abnormal results? Are result narratives appended when necessary? Does the laboratory staff conduct in-service education to support test result interpretation? Are critical values provided to nursing and physician staff? Are verbal reports confirmed with feedback? Are anomalous findings resolved?</td>
</tr>
<tr>
<td>Timeliness</td>
<td>Are turnaround times recorded and analyzed? Are laboratory reports being posted to patient charts in a timely fashion?</td>
</tr>
<tr>
<td>Patient satisfaction</td>
<td>Does the institution include laboratory care in patient surveys? Was specimen collection explained to the patient?</td>
</tr>
</tbody>
</table>

- American Proficiency Institute (API, api-pt.com), 1159 Business Park Drive, Traverse City, MI 49686. Laboratory proficiency testing and quality assurance programs, continuing education programs and summaries, tutorials, special topics library.
- College of American Pathologists (CAP, cap.org), 325 Waukegan Road, Northfield, IL 60093. Laboratory accreditation, proficiency testing, and quality assurance programs; laboratory education, reference resources, and e-lab solutions.
- Joint Commission (jointcommission.org), One Renaissance Boulevard, Oakbrook Terrace, IL 60181. Medical center-wide accreditation and certification programs.
- Laboratory Medicine Quality Improvement (cdc.gov/osels/lspppo/Laboratory_Medicine_Quality_Improvement/index.html), an initiative of the U.S. Centers for Disease Control and Prevention.

**SUMMARY**

- Hematology and hemostasis laboratory quality assurance relies on basic statistics describing measures of central tendency, measures of dispersion, and significance.
- Each new assay or assay modification must be validated for accuracy, precision, linearity, specificity, and lower limit of detection ability. In the hematology and hemostasis laboratory, accuracy validation usually requires a series of calibrators. Accuracy is established using the Student’s t-test, ANOVA, Pearson product-moment correlation, linear regression, and the Bland-Altman distribution.
- Precision is established by using repeated within-day and day-to-day assays, then computing the mean, standard deviation, and coefficient of variation of the results.
- Vendors usually provide assay linearity, specificity, and lower limit of detection; however, laboratory managers may require that these parameters be revalidated locally.
- Internal quality control is accomplished by assaying controls with each test run. Control results are compared with action limits, usually the mean of the control assay ±2 SD. When a specified number of control values are outside the limits, the use of the assay is suspended and the practitioner begins troubleshooting. Control results are plotted on Levey-Jennings charts and examined for shifts and trends. Internal quality control is enhanced through the use of the moving average algorithm and δ-checks.
- All conscientious laboratory directors subscribe to an external quality assessment system, also known as proficiency testing or proficiency surveys. External quality assessment enables the director to compare selected assay results with other laboratory results, nationally and internationally, as a further check of accuracy. Maintaining a good external quality assessment record is essential to laboratory accreditation. Most U.S. states require external quality assessment for laboratory licensure.
- All laboratory assays are analyzed for diagnostic efficacy, including diagnostic sensitivity and specificity, their true-positive and true-negative rates, and positive and negative predictive values. Highly sensitive assays may be used for population screening but may poorly discriminate between the healthy and diseased population. Specific assays may be used to confirm a condition, but generate a number of false negatives. Assays are chosen on the basis of the value of their intervention, based on relative or
absolute risk ratios. Diagnostic efficacy computations expand to
close receiver operating characteristic curve analysis.
• Conscientious laboratory managers hire only certified or licensed
medical laboratory scientists and technicians and provide regular
individual proficiency tests that are correlated with in-service edu-
cation. They encourage staff members to participate in continuing
education activities and in-house discussion of cases. Quality lab-
atories provide resources for staff to pursue higher education.

Now that you have completed this chapter, go back and
read again the case study at the beginning and respond
to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. What procedure is employed to validate a new assay?
   a. Comparison of assay results to a reference method
   b. Test for assay precision
   c. Test for assay linearity
   d. All of the above

2. You validate a new assay using linear regression to compare
   assay calibrator results with the distributor's published cali-
   brator results. The slope is 0.99 and the y intercept is +10%.
   What type of error is present?
   a. No error
   b. Random error
   c. Constant systematic error
   d. Proportional systematic error

3. Which is a statistical test comparing means?
   a. Bland-Altman
   b. Student's t-test
   c. ANOVA
   d. Pearson

4. The acceptable hemoglobin control value range is 13 ± 0.4
   g/dL. The control is assayed five times and produces the
   following five results:
   12.0 g/dL 12.3 g/dL 12.0 g/dL 12.2 g/dL 12.1 g/dL
   These results are:
   a. Accurate but not precise
   b. Precise but not accurate
   c. Both accurate and precise
   d. Neither accurate nor precise

5. A WBC count control has a mean value of 6000/μL and
   a standard deviation of 300/μL. What is the 95.5% confi-
dence interval?
   a. 3000 to 9000/μL
   b. 5400 to 6600/μL
   c. 5500 to 6500/μL
   d. 5700 to 6300/μL

6. The ability of an assay to distinguish the targeted analyte
   from interfering substances within the specimen matrix is
called:
   a. Analytical specificity
   b. Analytical sensitivity
   c. Clinical specificity
   d. Clinical sensitivity

7. The laboratory purchases reagents from a manufacturer
   and develops an assay using standard references. What FDA
category is this assay?
   a. Cleared
   b. Home-brew
   c. Research use only
   d. Analyte-specific reagent

8. A laboratory scientist measures prothrombin time for
   plasma aliquots from 15 healthy males and 15 healthy
   females. She computes the mean and 95.5% confidence
   interval and notes that they duplicate the manufacturer's
   statistics within 5%. This procedure is known as:
   a. Confirming linearity
   b. Setting the reference interval
   c. Determining the therapeutic range
   d. Establishing the reference interval by transference

9. You purchase a preserved whole blood specimen from a
   distributor who provides the mean values for several com-
   plete blood count analytes. What is this specimen called?
   a. Normal specimen
   b. Calibrator
   c. Control
   d. Blank
10. You perform a clinical efficacy test and get the following results:

<table>
<thead>
<tr>
<th>Unaffected by Disease or Condition</th>
<th>Affected by Disease or Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay is negative</td>
<td>40</td>
</tr>
<tr>
<td>Assay is positive</td>
<td>10</td>
</tr>
</tbody>
</table>

What is the number of false-negative results?
- a. 40
- b. 10
- c. 5
- d. 45

11. What agency provides external quality assurance (proficiency) surveys and laboratory accreditation?
- a. Clinical Laboratory Improvement Advisory Committee (CLIAC)
- b. Centers for Medicare and Medicaid Services (CMS)
- c. College of American Pathologists (CAP)
- d. Joint Commission

12. What agency provides continuing medical laboratory education?
- a. Colorado Association for Continuing Medical Laboratory Education (CACMLE)
- b. Clinical Laboratory Improvement Advisory Committee (CLIAC)
- c. Centers for Medicare and Medicaid Services (CMS)
- d. College of American Pathologists (CAP)

13. Regular review of blood specimen collection quality is an example of:
- a. Postanalytical quality assurance
- b. Preanalytical quality assurance
- c. Analytical quality control
- d. External quality assurance

14. Review of laboratory report integrity is an example of:
- a. Preanalytical quality assurance
- b. Analytical quality control
- c. Postanalytical quality assurance
- d. External quality assurance

15. When performing a receiver operating curve analysis, what parameter assesses the overall efficacy of an assay?
- a. Area under the curve
- b. Performance limit (threshold)
- c. Positive predictive value
- d. Negative predictive value

16. You require your laboratory staff to annually perform manual lupus anticoagulant profiles on a set of plasmas with known values. This exercise is known as:
- a. Assay validation
- b. Proficiency testing
- c. External quality assessment
- d. Pre-pre analytical variable assay

REFERENCES

Knowledge of the normal structure, composition, and function of cells is fundamental to the understanding of blood cell pathophysiology covered in later chapters. From the invention of the microscope and the discovery of cells in the 1600s to the present-day highly sophisticated analysis of cell ultrastructure with electron microscopy and other technologies, a remarkable body of knowledge is available about the structure of cells and their varied organelles. Complementing these discoveries were other advances in technology that enabled detailed understanding of the biochemistry, metabolism, and genetics of cells at the molecular level. Today, highly sophisticated analysis of cells using flow cytometry, cytogenetics, and molecular genetic testing (Chapters 30, 31, and 32) has become the standard of care in diagnosis and management of many malignant and non-malignant blood cell diseases. This new and ever-expanding knowledge has revolutionized the diagnosis and treatment of hematologic diseases resulting in a dramatic improvement in patient survival for many conditions that previously had a dismal prognosis. With all these advances, however, the visual examination of blood cells on a peripheral blood film by light microscopy still remains the hallmark for the initial evaluation of hematologic abnormalities.

This chapter will provide an overview of the structure, composition, and function of the components of the cell, the hematopoietic microenvironment, the cell cycle and its regulation, and the process of cell death by apoptosis and necrosis.

**CELL ORGANIZATION**

Cells are the structural units that constitute living organisms (Figures 6-1 and 6-2). Cells have specialized functions and contain the components necessary to perform and perpetuate these
functions. Regardless of shape, size, or function, human cells contain:

- A plasma membrane that separates the cytoplasm and cellular components from the extracellular environment;
- A membrane-bound nucleus (with the exception of mature red blood cells and platelets); and
- Other unique subcellular structures and organelles that support the various cellular functions.¹

Table 6-1 summarizes the cellular components and their functions, which are explained in more detail later.

**PLASMA MEMBRANE**

The plasma membrane serves as a semipermeable outer boundary separating the cellular components from their surrounding environment. The cell membrane serves four basic functions: (1) it provides a physical but flexible barrier to contain and protect cell components from the extracellular environment; (2) it regulates and facilitates the interchange of substances with the environment by endocytosis, exocytosis, and selective permeability (using various membrane channels and transporters); (3) it establishes electrochemical gradients between the interior and exterior of the cell; and (4) it has receptors that allow the cell to respond to a multitude of signaling molecules through signal transduction pathways.²

Relevant to hematology, the membrane is also the location of cell surface glycoprotein and glycolipid molecules (surface markers or antigens) used for blood cell identity. Each type of blood cell expresses a unique repertoire of surface markers at different stages of differentiation.³ Monoclonal antibodies are used to identify a blood cell’s surface antigens using flow cytometry (Chapter 32). An international nomenclature was developed, called the cluster of differentiation, or CD, system, in which a CD number was assigned to each identified blood cell surface antigen.⁴ Over 350 CD antigens have been identified on blood
cells. The CD nomenclature allows scientists, clinicians, and laboratory practitioners to communicate in a universal language for hematology research and diagnostic and therapeutic practice.

In addition to the plasma membrane, many components found within the cell (e.g., the mitochondria, Golgi apparatus, nucleus, and endoplasmic reticulum) have similarly constructed membrane systems. The red blood cell membrane has been the most widely studied and serves as an example of a cell membrane (Figure 9-2).

To accomplish its many requirements, the cell membrane must be resilient and elastic. It achieves these qualities by being a fluid structure of proteins floating in lipids. The lipids are phospholipids and cholesterol arranged in two layers. The phosphate end of the phospholipid and the hydroxyl radical of cholesterol are polar—charged hydrophilic (water-soluble) structures that orient toward the extracellular and cytoplasmic surfaces of the cell membrane. The fatty acid chains of the phospholipids and the steroid

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**TABLE 6-1** Summary of Cellular Components and Functions

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Location</th>
<th>Appearance and Size</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>Outer boundary of cell</td>
<td>Lipid bilayer consisting of phospholipids, cholesterol, proteins; glycolipids and glycoproteins form a glycocalyx</td>
<td>Provides physical barrier for cell; facilitates and restricts cellular exchange of substances; maintains electrochemical gradient and receptors for signal transduction</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Within cell</td>
<td>Round or oval; varies in diameter; composed of DNA and proteins</td>
<td>Controls cell division and functions; and contains genetic code</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>Within nucleus</td>
<td>Usually round or irregular in shape; 2-4 μm in diameter; composed of ribosomal RNA and the genes coding it, and accessory proteins; there may be one to several within the nucleus</td>
<td>Synthesizes ribosomal RNA and assembles ribosome subunits</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>Free in cytoplasm; also on outer surface of rough endoplasmic reticulum</td>
<td>Macromolecular complex composed of protein and ribosomal RNA; composed of large and small subunits</td>
<td>Synthesizes proteins</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum</td>
<td>Membranous network throughout cytoplasm</td>
<td>Membrane-lined tubules that branch and connect to nuclear membrane; studded with ribosomes</td>
<td>Synthesizes most membrane-bound proteins</td>
</tr>
<tr>
<td>Smooth endoplasmic reticulum</td>
<td>Membranous network throughout cytoplasm</td>
<td>Membrane-lined tubules contiguous with rough endoplasmic reticulum; does not have ribosomes</td>
<td>Synthesizes phospholipids and steroids; detoxifies drugs; stores calcium</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>Next to nucleus and rough endoplasmic reticulum</td>
<td>System of stacked, membrane-bound, flattened sacs</td>
<td>Modifies and packages macromolecules for other organelles and for secretion</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Randomly distributed in cytoplasm</td>
<td>Round or oval structures; 3-14 nm in length, 2-10 nm in width; membrane has two layers; inner layer has folds called cristae</td>
<td>Produces most of the cell’s ATP by oxidative phosphorylation</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>Randomly distributed in cytoplasm</td>
<td>Membrane-bound sacs; diameter varies</td>
<td>Contains hydrolytic enzymes that degrade unwanted material in the cell</td>
</tr>
<tr>
<td>Microfilaments</td>
<td>Near nuclear envelope, plasma membrane, and mitotic processes</td>
<td>Double-stranded, intertwined solid structures of actin; 5-7 nm in diameter</td>
<td>Supports cytoskeleton and motility</td>
</tr>
<tr>
<td>Intermediate filaments</td>
<td>Cytoskeleton</td>
<td>Solid structures 8-10 nm in diameter; self-assemble into larger bundles</td>
<td>Provides strong structural support</td>
</tr>
<tr>
<td>Microtubules</td>
<td>Cytoskeleton and centrioles, near nuclear envelope and Golgi apparatus</td>
<td>Hollow cylinder of α- and β-tubulin forming 13 protofilaments; 20-25 nm in diameter</td>
<td>Maintains cell shape, motility, and mitotic process</td>
</tr>
<tr>
<td>Centrosome</td>
<td>Near nucleus</td>
<td>Composed of two centrioles, each having nine sets of triplet microtubules; 150 nm in diameter, 300-500 nm in length</td>
<td>Contains centrioles that serve as insertion points for mitotic spindle fibers</td>
</tr>
</tbody>
</table>
nucleus of cholesterol are non-polar-charged hydrophobic (water-insoluble) structures and are directed toward each other in the center of the bilayer (Figure 13-10). The phospholipids are distributed asymmetrically in the membrane with mostly phosphatidylserine and phosphatidylethanolamine in the inner layer and sphingomyelin and phosphatidylcholine in the outer layer (Chapters 9 and 13). In the outer layer, carbohydrates (oligosaccharides) are covalently linked to some membrane proteins and phospholipids (forming glycoproteins and glycolipids, respectively).

These also contribute to the membrane structure and function.

Membrane Proteins
Cell membranes contain two types of proteins: transmembrane and cytoskeletal. Transmembrane proteins may traverse the entirety of the lipid bilayers in one or more passes and penetrate the plasma and cytoplasmic layers of the membrane. The transmembrane proteins serve as channels and transporters of water, ions, and other molecules between the cytoplasm and the external environment. They also function as receptors and adhesion molecules. Cytoskeletal proteins are found only on the cytoplasmic side of the membrane and form the lattice of the cytoskeleton. The cytoplasmic ends of transmembrane proteins attach to the cytoskeletal proteins at junctional complexes to provide structural integrity to the cell and vertical support in linking the membrane to the cytoskeleton (Figure 9-4).

Inherited mutations in genes coding for transmembrane or cytoskeletal proteins can disrupt membrane integrity, decrease the life span of red blood cells, and lead to a hemolytic anemia. An example is hereditary spherocytosis (Chapter 24).

Membrane Carbohydrates
The carbohydrate chains of the glycoproteins and glycolipids extend beyond the outer cell surface, giving the cell a carbohydrate coat often called the glycocalyx. These carbohydrate moieties function in cell-to-cell recognition and provide a negative surface charge, surface receptor sites, and cell adhesion capabilities. The function of the red blood cell membrane is discussed in detail in Chapter 9.

NUCLEUS

The nucleus is composed of three components: the chromatin, the nuclear envelope, and the nucleoli. It is the control center of the cell and the largest organelle within the cell. The nucleus is composed largely of deoxyribonucleic acid (DNA) and is the site of DNA replication and transcription (Chapter 31). It is responsible for the chemical reactions within the cell and the cell’s reproductive process. The nucleus has an affinity for basic dyes because of the nucleic acids contained within it; it stains deep purple with Wright stain (Chapters 8 and 12).

Chromatin
The chromatin consists of one long molecule of double-stranded DNA in each chromosome that is tightly folded with histone and nonhistone proteins. The first level of folding is the formation of nucleosomes along the length of the DNA molecule (Figure 30-3). Each nucleosome is 11 nm in length and consists of approximately 150 base pairs of DNA wrapped around a histone protein core. The positive charge of the histones facilitates binding with the negatively charged phosphate groups of DNA. The nucleosomes are folded into 30 nm chromatin fibers, and these fibers are further folded into loops, then supercoiled chromatin fibers that greatly condense the DNA (Figure 30-3). This highly structured folding allows the long strands of DNA to be tightly condensed in the nucleus when inactive and enables segments of the DNA to be rapidly unfolded for active transcription when needed. This complex process of gene expression is controlled by transcription factors and other regulatory proteins and processes. Inappropriate silencing of genes needed for blood cell maturation contributes to the molecular pathology of myelodysplastic syndromes and acute leukemias (Chapters 34 and 35).

Morphologically, chromatin is divided into two types: (1) the heterochromatin, which is represented by the more darkly stained, condensed clumping pattern and is the transcriptionally inactive area of the nucleus, and (2) the euchromatin, which has diffuse, uncondensed, open chromatin and is the genetically active portion of the nucleus where DNA transcription into mRNA occurs. The euchromatin is loosely coiled and turns a pale blue when stained with Wright stain. More mature cells have more heterochromatin because they are less transcriptionally active.

Nuclear Envelope
Surrounding the nucleus is a nuclear envelope consisting of two phospholipid bilayer membranes. The inner membrane surrounds the nucleus, and the outer membrane is continuous with an extension of the endoplasmic reticulum. Between the two membranes is a 30- to 50-nm perinuclear space that is continuous with the lumen of the endoplasmic reticulum. Nuclear pore complexes penetrate the nuclear envelope, which allows passage of molecules between the nucleus and the cytoplasm.

Nucleoli
The nucleus contains one to several nucleoli. The nucleolus is the site of ribosomal RNA (rRNA) production and assembly into ribosome subunits. Because the ribosomes synthesize proteins, the number of nucleoli in the nucleus is proportional to the amount of protein synthesis that occurs in the cell. As blood cells mature, protein synthesis decreases, and the nucleoli eventually disassemble.

Nucleoli contain a large amount of rRNA, the genes that code for rRNA (or rDNA), and ribosomal proteins. In ribosome biogenesis, rDNA is first transcribed to rRNA precursors. The rRNA precursors are processed into smaller RNA molecules and subsequently complexed with proteins forming the small and large ribosome subunits. The ribosomal proteins enter the nucleus through the nuclear pores after being synthesized in the cytoplasm. After the ribosome subunits are synthesized and assembled, they are transported...
out of the nucleus through the nuclear pores. Once in the cytoplasm, the large and small ribosome subunits self-assemble into a functional ribosome during protein synthesis (Chapter 31).6

**CYTOPLASM**

The cytoplasmic matrix is a homogeneous, continuous, aqueous solution called the *cytosol*. It is the environment in which the organelles exist and function. These organelles are discussed individually.

**Ribosomes**

Ribosomes are macromolecular complexes composed of a small and large subunit of rRNA and many accessory ribosomal proteins. Ribosomes are found free in the cytoplasm or on the surface of rough endoplasmic reticulum. They may exist singly or form chains (polyribosomes). Ribosomes serve as the site of protein synthesis. This is accomplished with transfer RNA (tRNA) for amino acid transport to the ribosome, and specific messenger RNA (mRNA) molecules. The mRNA provides the genetic code for the sequence of amino acids for the protein being synthesized (Chapter 31). Cells that actively produce proteins have many ribosomes in the cytoplasm which give it a dark blue color (basophilia) when stained with Wright stain. Cytoplasmic basophilia is particularly prominent in RBC precursor cells when hemoglobin and other cell components are actively synthesized (Chapter 8).

**Endoplasmic Reticulum**

The endoplasmic reticulum (Figure 6-3) is a membranous network found throughout the cytoplasm and appears as flattened sheets, sacs, and tubes of membrane. The outer membrane of the nuclear envelope is continuous with the endoplasmic reticulum membrane and it specializes in making and transporting lipids and membrane proteins.

Rough endoplasmic reticulum (RER) has a studded look on its outer surface caused by the presence of ribosomes engaged in the synthesis of mainly membrane-bound proteins.2 Smooth endoplasmic reticulum (SER) is contiguous with the RER, but it does not contain ribosomes. It is involved in synthesis of phospholipids and steroids, detoxification or inactivation of harmful compounds or drugs, and calcium storage and release.2

**Golgi Apparatus**

The Golgi apparatus is a system of stacked, membrane-bound, flattened sacs called *cisternae* that are involved in modifying, sorting, and packaging macromolecules for secretion or delivery to other organelles. It contains numerous enzymes for these activities. The Golgi apparatus is normally located in close proximity to the rough endoplasmic reticulum (RER) and the nucleus. In stained bone marrow smears of developing white blood cell precursors, the Golgi area may be observed as an unstained region next to the nucleus.

Vesicles containing membrane-bound and soluble proteins from the RER enter the Golgi network on the “cis face” and are directed through the stacks where the proteins are modified, as needed, by enzymes for glycosylation, sulfation, or phosphorylation.1,2 Vesicles with processed proteins exit the Golgi on the “trans face” to form lysosomes or secretory vesicles bound for the plasma membrane.1,2

**Mitochondria**

The mitochondrion (Figure 6-4) has a continuous outer membrane. Running parallel to the outer membrane is an inner membrane that invaginates at various intervals, giving the interior a shelllike or ridgelike appearance. These internal ridges, termed *cristae*, are where oxidative enzymes are attached. The convolution of the inner membrane increases the surface area to enhance the respiratory capability of the cell. The interior of the mitochondrion consists of a homogeneous material known as the *mitochondrial matrix*, which contains many enzymes for the extraction of energy from nutrients.

The mitochondria generate most of the adenosine triphosphate (ATP) for the cell. Mitochondrial enzymes oxidize pyruvate and fatty acids to acetyl CoA, and the citric acid cycle oxidizes the acetyl CoA producing electrons for the electron-transport pathway. This pathway generates ATP through oxidative phosphorylation.2
The mitochondria are capable of self-replication. This organelle has its own DNA and RNA for the mitochondrial division cycle. There may be fewer than 100 or up to several thousand mitochondria per cell. The number is directly related to the amount of energy required by the cell.

**Lysosomes**

Lysosomes contain hydrolytic enzymes bound within a membrane and are involved in the cell’s intracellular digestive process. The membrane prevents the enzymes from digesting cellular components and macromolecules. Lysosomal enzymes are active at the acidic pH of the lysosome and are inactivated at the higher pH of the cytosol. This also protects the cell in case lysosomal enzymes are released into the cytoplasm. Lysosomes fuse with endosomes and phagosomes (Chapter 12); this allows the lysosome hydrolytic enzymes to safely digest their contents. With Wright stain, lysosomes are visualized as granules in white blood cells and platelets (Chapters 12 and 13). Lysosomal lipid storage diseases result from inherited mutations in genes for enzymes that catabolize lipids. Gaucher disease and Tay-Sachs disease are examples of these disorders (Chapter 29).

**Microfilaments and Intermediate Filaments**

Actin microfilaments are double-stranded, intertwined solid structures approximately 5 to 7 nm in diameter. They associate with myosin to enable cell motility, contraction, and intracellular transport. They locate near the nuclear envelope or in the proximity of the nucleus and assist in cell division. They also are present near the plasma membrane and provide cytoskeletal support.

Intermediate filaments, with a diameter of approximately 8 to 10 nm, self-assemble into larger bundles. They are the most durable element of the cytoskeleton and provide structural stability for the cells, especially those subjected to more physical stress, such as the epidermal layer of skin. Examples include the keratins and laminas.

**Microtubules**

Microtubules are hollow cylindrical structures that are approximately 25 nm in diameter and vary in length. These organelles are organized from α- and β-tubulin through self-assembly. The tubulin polypeptides form protofilaments, and the microtubule usually consists of 13 protofilaments. This arrangement gives the microtubules structural strength. Tubulins can rapidly polymerize and form microtubules and then rapidly depolymerize when no longer needed by the cell.

Microtubules have several functions. They help support the cytoskeleton to maintain the cell’s shape and are involved in the movement of some intracellular organelles. Microtubules also form the mitotic spindle fibers during mitosis and are the major components of centrioles.

**Centrosomes**

The centrosome consists of two cylinder-shaped centrioles that are typically oriented at right angles to each other. A centriole consists of nine bundles of three microtubules each. They serve as insertion points for the mitotic spindle fibers during mitosis.

**HEMATOPOIETIC MICROENVIRONMENT**

Hematopoiesis occurs predominantly in the bone marrow from the third trimester of fetal life through adulthood (Chapter 7). The bone marrow microenvironment must provide for hematopoietic stem cell self-renewal, proliferation, differentiation, and apoptosis and support the developing progenitor cells. This protective environment is provided by stromal cells, which is a broad term for specialized endothelial cells; reticular adventitial cells (fibroblasts); adipocytes (fat cells); lymphocytes and macrophages; osteoblasts; and osteoclasts. The stromal cells secrete substances that form an extracellular matrix, including collagen, fibronectin, thrombospondin, laminin, and proteoglycans (such as hyaluronate, chondroitin sulfate, and heparan sulfate). The extracellular matrix is critical for cell growth and for anchoring developing blood cell progenitors in the bone marrow. Hematopoietic progenitor cells have many receptors for cytokines and adhesion molecules. One purpose of these receptors is to provide a mechanism for attachment to extracellular matrix. This provides an avenue for cell-cell interaction, which is essential for regulated hematopoiesis.

Stromal cells also secrete many different growth factors required for stem, progenitor, and precursor cell survival (Chapter 7). Growth factors participate in complex processes to regulate the proliferation and differentiation of progenitor and precursor cells. Growth factors must bind to specific receptors on their target cells to exert their effect. Most growth factors are produced by cells in the hematopoietic microenvironment and exert their effects in local cell-cell interactions. One growth factor, erythropoietin, has a hormone-type stimulation in that it is produced in another location (kidney) and exerts its effect on erythroid progenitors in the bone marrow (Chapter 8). An important feature of growth factors is their use of synergism to stimulate a cell to proliferate or differentiate. In other words, several different growth factors work together to generate a more effective response. Growth factors are specific for their corresponding receptors on target cells.

Growth factor receptors are transmembrane proteins. When the growth factor (or ligand) binds the extracellular domain of the receptor, a signal is transmitted to the nucleus in the cell through the cytoplasmic domain. For example, when erythropoietin binds with its receptor, it causes a conformational change in the receptor which activates a kinase (Janus kinase 2 or JAK2) associated with its cytoplasmic domain. The activated kinase in turn activates other intracellular signal transduction molecules that ultimately interact with the DNA in the nucleus to promote expression of genes required for cell growth and proliferation (Figure 33-9).

**CELL CYCLE**

The purpose of the cell cycle is to replicate DNA once and distribute identical chromosome copies equally to two daughter
The cell cycle is a biochemical and morphologic four-stage process through which a cell passes when it is stimulated to divide (Figure 6-5). These stages are G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis). G1 is a period of cell growth and synthesis of components necessary for replication. G1 lasts about 10 hours. In the S stage, DNA replication takes place, a process requiring about 8 hours (Chapter 31). An exact copy of each chromosome is produced and they pair together as sister chromatids. The centrosome is also duplicated during the S stage. In G2, the tetraploid DNA is checked for proper replication and damage (discussed later). G2 takes approximately 4 hours. The time spent in each stage can be variable, but mitosis takes approximately 1 hour. During G0 (quiescence) the cell is not actively in the cell cycle.

The mitosis or M stage involves the division of chromosomes and cytoplasm into two daughter cells. It is divided into six phases (Figure 6-5):

1. Prophase: the chromosomes condense, the duplicated centrosomes begin to separate, and mitotic spindle fibers appear.
2. Prometaphase: the nuclear envelope disassembles, the centrosomes move to opposite poles of the cell and serve as a point of origin of the mitotic spindle fibers; the sister chromatids (chromosome pairs) attach to the mitotic spindle fibers.
3. Metaphase: the sister chromatids align on the mitotic spindle fibers at a location equidistant from the centrosome poles.
4. Anaphase: the sister chromatids separate and move on the mitotic spindles toward the centrosomes on opposite poles.
5. Telophase: the nuclear membrane reassembles around each set of chromosomes and the mitotic spindle fibers disappear.
6. Cytokinesis: the cell divides into two identical daughter cells.

**Regulation of the Cell Cycle**
A regulatory mechanism is needed to prevent abnormal or mutated cells from going through the cell cycle and producing an abnormal clone. The cell cycle is a highly complicated process that can malfunction. There are four major checkpoints in the cell cycle (Figure 6-5). The first is a restriction point late in G1 that checks for the appropriate amount of nutrients and appropriate cell volume. The second checkpoint at the end of G1 (called the G1 DNA damage checkpoint) checks the DNA for damage and makes the cell wait for DNA repair or initiates apoptosis. The third checkpoint, G2 DNA damage checkpoint, takes place after DNA synthesis at the end of G2, and its purpose is to verify that replication took place without error or damage. If abnormal or malformed replication occurred, then mitosis is blocked. The last checkpoint is during mitosis at the time of metaphase (metaphase checkpoint). Here the attachment and alignment of chromosomes on the mitotic spindle and the integrity of the spindle apparatus are checked. Anaphase will be blocked if any defects are detected.

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**Figure 6-5** Stages of the cell cycle. A, Diagrams of cellular morphology and chromosome structure across the cell cycle; B, Time scale of cell cycle stages; C, Length of cell cycle stages in cultured cells. (From Pollard TD, Earnshaw WC. Chapter 40 Introduction to the cell cycle. In: Cell Biology, e2. Philadelphia, 2008, Saunders, An imprint of Elsevier.)
Cell cycle control is under the direction of cyclin and cyclin-dependent kinases (CDKs). The cyclin/CDK complexes phosphorylate key substrates that assist the cell through the cell cycle. Cyclin is named appropriately because the concentration of the cyclin/CDK complex moves the cell through the different stages of the cell cycle. G1 begins with a combination of the cyclin D family (D1, D2, D3) with cyclin4 and cyclin6.10 To transition the cell from G1 to S, cyclin E increases and binds to CDK2, producing the cyclin E/CDK2 complex. In the S stage cyclin E decreases and cyclin A increases and complexes with CDK2, forming cyclin A/CDK2. This complex takes the cell through the S and G2 stage. Cyclin A also partners with CDK1 (cyclin A/CDK1). For mitosis to occur, cyclin B must replace cyclin A and bind to CDK1, forming the cyclin B/CDK1 complex. This complex takes the cell through the intricate process of mitosis.10,11 Inhibitors of the cyclin/CDK complexes also play a primary role in cell cycle regulation.12

Tumor suppressor proteins are needed for the proper function of the checkpoints. One of the first tumor suppressor genes recognized was TP53. It codes for the TP53 protein that detects DNA damage during G1. It can also assist in triggering apoptosis. Many tumor suppressor genes have been described.11 When these genes are mutated or deleted, abnormal cells are allowed to go through the cell cycle and replicate. Some of these cells simply malfunction, but others form neoplasms, often with aggressive characteristics. For example, patients with chronic lymphocytic leukemia have a more aggressive disease with a shorter survival time when their leukemic cells lose TP53 activity either through gene mutation or deletion (Chapter 36). Patients whose leukemia cells have normal TP53 function have a better prognosis.

**CELL DEATH BY NECROSIS AND APOPTOSIS**

Cell death occurs as a normal physiologic process in the body or as a response to injury. Events that injure cells include ischemia (oxygen deprivation), mechanical trauma, toxins, drugs, infectious agents, autoimmune reactions, genetic defects including acquired and inherited mutations, and improper nutrition.12 There are two major mechanisms for cell death: necrosis and apoptosis. Necrosis is a pathologic process caused by direct external injury to cells—for example, from burns, radiation, or toxins.12 Apoptosis is a self-inflicted cell death originating from the activation signals within the cell itself.13 Most apoptosis occurs as a normal physiologic process to eliminate potentially harmful cells (e.g., self-reacting lymphocytes [Chapter 7]), cells that are no longer needed (e.g., excess erythroid progenitors in oxygen-replete states [Chapter 8] or neutrophils after phagocytosis), and aging cells.12 Apoptosis of older terminally differentiated cells balances with new cell growth to maintain needed numbers of functional cells in organs, hematopoietic tissue, and epithelial cell barriers, particularly in skin and the intestines. On the other hand, apoptosis also initiates in response to internal or external pathologic injury to a cell. For example, if DNA damage occurred during the replication phase of the cell cycle and the damage is beyond the capability of the DNA repair mechanisms, the cell will activate apoptosis to prevent its further progression through the cell cycle. Apoptosis can also be triggered in virally infected cells by the virus itself or by the body’s immune response.12 This is one of the mechanisms to remove virally infected cells from the body.

The first morphologic manifestation of necrosis is a swelling of the cell. The cell may be able to recover from minor injury at that point. More severe damage, however, disrupts organelles and membranes; enzymes leak out of lysosomes that denature and digest DNA, RNA, and intracellular proteins; and ultimately the cell lyses.12 An inflammatory response usually accompanies necrosis due to the release of cell contents into the extracellular space.

The morphologic manifestation of apoptosis is shrinkage of the cell. The nucleus condenses and undergoes systematic fragmentation due to cleavage of the DNA between nucleosome subunits (multiples of 180 to 200 base pairs). The plasma membrane remains intact, but the phospholipids lose their asymmetric distribution and “flip” phosphatidylserine (PS) from the inner to the outer leaflet.14 The cytoplasm and nuclear fragments bud off in membrane-bound vesicles. Macrophages, recognizing the PS and other signals on the membranes, rapidly phagocytize the vesicles. Thus, cellular products are not released into the extracellular space and an inflammatory response is not elicited.12 Figure 6-6 and Table 6-2 summarize the differences between necrosis and apoptosis.

Activation of apoptosis occurs through extrinsic and intrinsic pathways. Both pathways involve the activation of proteins called caspases. The extrinsic pathway, also called the death receptor pathway, initiates with the binding of ligand to a death receptor on the cell membrane. Examples of death receptors and their ligands include Fas and Fas ligand, and tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor.15 The binding activates caspase-8. The intrinsic pathway is initiated by intracellular stressors (such as hypoxia, DNA damage, or membrane disruption) that stimulate the release of cytochrome c from mitochondria.17 Cytochrome c binds to apoptotic protease-activating factor-1 (APAF-1) and caspase-9, forming an apoptosisome, which activates caspase-9. Both pathways converge when the “initiator” caspases (8 or 9) activate “executioner” caspases 3, 6, and 7, which leads to apoptosis.13,15

Various cellular proapoptotic and antiapoptotic proteins tightly regulate apoptosis. Examples of antiapoptotic proteins include some members of the BCL-2 family of proteins (such as Bcl-2, Bcl-XL) as well as various growth factors (such as erythropoietin, granulocyte-colony stimulating factor, granulocyte-macrophage-colony stimulating factor, interleukin-3, and FLT3 ligand).14 BAX, BAK, and BID are examples of proapoptotic proteins.14 The ratio of these intracellular proteins plays a primary role in regulating apoptosis. Any dysregulation, mutation, or translocation can cause inhibition or overexpression of apoptotic proteins, which can lead to hematopoietic malignancies or malfunctions.13,14
Necrosis Apoptosis

Cell size
- Enlarged due to swelling
- Reduced due to shrinkage

Nucleus
- Random breaks and lysis (karyolysis)
- Condensation and fragmentation between nucleosomes

Plasma membrane
- Disrupted with loss of integrity
- Intact with loss phospholipid asymmetry

Inflammation
- Enzyme digestion and leakage of cell contents; inflammatory response occurs
- Release of cell contents in membrane-bound apoptotic bodies which are phagocytized by macrophages; no inflammation occurs

Physiologic or pathologic function
- Pathologic; results from cell injury
- Mostly physiologic to remove unwanted cells; pathologic in response to cell injury

The cell contains cytoplasm that is separated from the extracellular environment by a plasma membrane; a membrane-bound nucleus (with the exception of mature red blood cells and platelets); and other unique subcellular structures and organelles.

The plasma membrane is a bilayer of phospholipids, cholesterol, and transmembrane proteins. Glycolipids and glycoproteins on the outer surface form the glycocalyx.
• The cytoplasm contains ribosomes for protein synthesis, which can be free in the cytoplasm or located on rough endoplasmic reticulum (RER). The RER makes most of the membrane proteins. Smooth endoplasmic reticulum (SER) lacks ribosomes; the SER is involved in synthesis of phospholipids and steroids, detoxification or inactivation of harmful compounds or drugs, and calcium storage and release.
• The Golgi apparatus modifies and packages macromolecules for secretion and for other cell organelles. Mitochondria make ATP to supply energy for the cell. Lysosomes contain hydrolytic enzymes involved in the cell’s intracellular digestive process.
• The bone marrow provides a suitable microenvironment for hematopoietic stem cell self-renewal, proliferation, differentiation, and apoptosis. Stromal cells secrete substances that form an extracellular matrix to support cell growth and function and help to anchor developing cells in the bone marrow. Growth factors participate in complex processes to regulate the proliferation and differentiation of hematopoietic stem and progenitor cells.
• The cell cycle involves four active stages: G₁ (gap 1), S (DNA synthesis), G₂ (gap 2), and M (mitosis). The cell cycle is under the direction of cyclins and CDKs. Checkpoints in the cell cycle recognize abnormalities and initiate apoptosis.
• Two major mechanisms for cell death are necrosis and apoptosis. Necrosis is a pathologic process caused by direct external injury to cells, while apoptosis is a self-inflicted cell death originating from the activation signals within the cell itself. Most apoptosis occurs as a normal physiologic process to eliminate unwanted cells, but it can also be initiated in response to internal or external pathologic injury to a cell.

**REVIEW QUESTIONS**

Answers can be found in the Appendix.

1. The organelle involved in packaging and trafficking of cellular products is the:
   a. Nucleus
   b. Golgi apparatus
   c. Mitochondria
   d. Rough endoplasmic reticulum

2. The glycocalyx is composed of membrane:
   a. Phospholipids and cholesterol
   b. Glycoproteins and glycolipids
   c. Transmembrane and cytoskeletal proteins
   d. Rough and smooth endoplasmic reticulum

3. The “control center” of the cell is the:
   a. Nucleus
   b. Cytoplasm
   c. Membrane
   d. Microtubular system

4. The nucleus is composed largely of:
   a. RNA
   b. DNA
   c. Ribosomes
   d. Glycoproteins

5. Protein synthesis occurs in the:
   a. Nucleus
   b. Mitochondria
   c. Ribosomes
   d. Golgi apparatus

6. The shape of a cell is maintained by which of the following?
   a. Microtubules
   b. Spindle fibers
   c. Ribosomes
   d. Centrioles

7. Functions of the cell membrane include all of the following except:
   a. Regulation of molecules entering or leaving the cell
   b. Receptor recognition of extracellular signals
   c. Maintenance of electrochemical gradients
   d. Lipid production and oxidation

8. The energy source for cells is the:
   a. Golgi apparatus
   b. Endoplasmic reticulum
   c. Nucleolus
   d. Mitochondrion

9. Ribosomes are synthesized by the:
   a. Endoplasmic reticulum
   b. Mitochondrion
   c. Nucleolus
   d. Golgi apparatus

10. Euchromatin functions as the:
    a. Site of microtubule production
    b. Transcriptionally active DNA
    c. Support structure for nucleoli
    d. Attachment site for centrioles
11. The cell cycle is regulated by:
   a. Cyclins and CDKs
   b. Protooncogenes
   c. Apoptosis
   d. Growth factors

12. The transition from the G₁ to S stage of the cell cycle is regulated by:
   a. Cyclin B/CDK1 complex
   b. Cyclin A/CDK2 complex
   c. Cyclin D1
   d. Cyclin E/CDK2 complex

13. Apoptosis is morphologically identified by:
   a. Cellular swelling
   b. Nuclear condensation
   c. Rupture of the cytoplasm
   d. Rupture of the nucleus

14. Regulation of the hematopoietic microenvironment is provided by the:
   a. Stromal cells and growth factors
   b. Hematopoietic stem cells
   c. Liver and spleen
   d. Cyclins and caspases

REFERENCES

Hematopoiesis
Richard C. Meagher

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Define hematopoiesis.
2. Describe the evolution and formation of blood cells from embryo to fetus to adult, including anatomic sites and cells produced.
3. Predict the likelihood of encountering active marrow from biopsy sites when given the patient’s age.
4. Relate normal and abnormal hematopoiesis to the various organs involved in the hematopoietic process.
5. Explain the stem cell theory of hematopoiesis, including the characteristics of hematopoietic stem cells, the names of various progenitor cells, and their lineage associations.
6. Discuss the roles of various cytokines and hematopoietic growth factors in differentiation and maturation of hematopoietic progenitor cells, including nonspecific and lineage-specific factors.
7. Describe general morphologic changes that occur during blood cell maturation.
8. Define apoptosis and discuss the relationship between apoptosis, growth factors, and hematopoietic stem cell differentiation.
9. Discuss therapeutic applications of cytokines and hematopoietic growth factors.

HEMATOPOIETIC DEVELOPMENT
Hematopoiesis is a continuous, regulated process of blood cell production that includes cell renewal, proliferation, differentiation, and maturation. These processes result in the formation, development, and specialization of all of the functional blood cells that are released from the bone marrow to the circulation. The hematopoietic system serves as a functional model to study stem cell biology, proliferation, maturation and their contribution to disease and tissue repair. Rationale for this assumption is founded on the observations that mature blood cells have a limited lifespan (e.g., 120 days for RBC), a cell population capable of renewal is present to sustain the system, and the demonstration that the cell renewal population is unique in this capacity. A hematopoietic stem cell is capable of self-renewal (i.e., replenishment) and directed differentiation into all required cell lineages.¹

Hematopoiesis in humans can be characterized as a select distribution of embryonic cells in specific sites that rapidly change during development.² In healthy adults hematopoiesis is restricted primarily to the bone marrow. During fetal development, the restricted, sequential distribution of cells initiates in the yolk sac and then progresses in the aorta-gonad mesonephros (AGM) region (mesoblastic phase), then to the fetal liver (hepatic phase), and finally resides in the bone marrow (medullary phase). Due to the different locations and resulting microenvironmental conditions (i.e., niches) encountered, each of these locations has distinct but related populations of cells.

Mesoblastic Phase
Hematopoiesis is considered to begin around the nineteenth day of embryonic development after fertilization.³ Early in embryonic development, cells from the mesoderm migrate to the yolk sac. Some of these cells form primitive erythroblasts in the central cavity of the yolk sac, while the others (angio­blasts) surround the cavity of the yolk sac and eventually form blood vessels.⁴ ⁷ These primitive but
transient yolk sac erythroblasts are important in early embryogenesis to produce hemoglobin (Gower-1, Gower-2, and Portland) needed for delivery of oxygen to rapidly developing embryonic tissues (Chapter 10).\(^8\) Yolk sac hematopoiesis differs from hematopoiesis that occurs later in the fetus and the adult in that it occurs intravascularly, or within developing blood vessels.\(^8\)

Cells of mesodermal origin also migrate to the aorta-gonad-mesonephros (AGM) region and give rise to hematopoietic stem cells (HSCs) for definitive or permanent adult hematopoiesis.\(^4,7\) The AGM region has previously been considered to be the only site of definitive hematopoiesis during embryonic development. However, more recent evidence suggests that HSC development and definitive hematopoiesis occur in the yolk sac. Metcalf and Moore performed culture experiments using 7.5-day mouse embryos lacking the yolk sac and demonstrated that no hematopoietic cells grew in the fetal liver after several days of culture.\(^9\) They concluded that the yolk sac was the major site of adult blood formation in the embryo.\(^9\) This view is supported by Weissman and colleagues in transplant experiments demonstrating that T cells could be recovered following transplantation of yolk sac into fetuses.\(^10\) However, others have postulated de novo production of HSCs could occur at different times or locations.\(^11\) Reports indicate that Flk1\(^1\) HSCs separated from human umbilical cord blood could generate hematopoietic as well as endothelial cells in vitro.\(^12\) Others have shown that purified murine HSCs generate endothelial cells following in vivo transplantation.\(^13\) More recently, others have challenged the AGM origin of HSCs based on transgenic mouse data showing that yolk sac hematopoietic cells in 7.5-day embryos express Runx1 regulatory elements needed for definitive hematopoiesis.\(^14\) This suggests that the yolk sac contains either definitive HSCs or cells that can give rise to HSCs.\(^14\) The precise origin of the adult HSC remains unresolved.

**Hepatic Phase**

The hepatic phase of hematopoiesis begins at 5 to 7 gestational weeks and is characterized by recognizable clusters of developing erythroblasts, granulocytes, and monocytes colonizing the fetal liver, thymus, spleen, placenta, and ultimately the bone marrow space in the final medullary phase.\(^8\) These varied niches support development of HSCs that migrate to them. However, the contribution of each site to the final composition of the adult HSC pool remains unknown.\(^15,16\) The developing erythroblasts signal the beginning of definitive hematopoiesis with a decline in primitive hematopoiesis of the yolk sac. In addition, lymphoid cells begin to appear.\(^17,18\) Hematopoiesis during this phase occurs extravascularly, with the liver remaining the major site of hematopoiesis during the second trimester of fetal life.\(^8\) Hematopoiesis in the aorta-gonad-mesonephros region and the yolk sac disappear during this stage. Hematopoiesis in the fetal liver reaches its peak by the third month of fetal development, then gradually declines after the sixth month, retaining minimal activity until 1 to 2 weeks after birth\(^8\) (Figure 7-1). The developing spleen, kidney, thymus, and lymph nodes contribute to the hematopoietic process during this phase. The thymus, the first fully developed organ in the fetus, becomes the major site of T cell production, whereas the kidney and spleen produce B cells.

Production of megakaryocytes also begins during the hepatic phase. The spleen gradually decreases granulocytic

![Figure 7-1 Sites of hematopoiesis by age.](image-url)
production and involves itself solely in lymphopoiesis. During the hepatic phase, fetal hemoglobin (Hb F) is the predominant hemoglobin, but detectable levels of adult hemoglobin (Hb A) may be present (Chapter 10).8

Medullary (Myeloid) Phase
Prior to the fifth month of fetal development, hematopoiesis begins in the bone marrow cavity.3 This transition is called medullary hematopoiesis because it occurs in the medulla or inner part of the bone. During the myeloid phase, HSCs and mesenchymal cells migrate into the core of the bone.8 The mesenchymal cells, which are a type of embryonic tissue, differentiate into structural elements (i.e., stromal cells such as endothelial cells and reticular adventitial cells) that support the developing blood cells.19,20 Hematopoietic activity, especially myeloid activity, is apparent during this stage of development, and the myeloid-to-erythroid ratio gradually approaches 3:1 (adult levels).8 By the end of 24 weeks’ gestation, the bone marrow becomes the primary site of hematopoiesis.8 Measurable levels of erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and hemoglobins F and A can be detected.8 In addition, cells at various stages of maturation can be seen in all blood cell lineages.

ADULT HEMATOPOIETIC TISSUE
In adults, hematopoietic tissue is located in the bone marrow, lymph nodes, spleen, liver, and thymus. The bone marrow contains developing erythroid, myeloid, megakaryocytic, and lymphoid cells. Lymphoid development occurs in primary and secondary lymphoid tissue. Primary lymphoid tissue consists of the bone marrow and thymus and is where T and B lymphocytes are derived. Secondary lymphoid tissue, where lymphoid cells respond to foreign antigens, consists of the spleen, lymph nodes, and mucosa-associated lymphoid tissue.

Bone Marrow
Bone marrow, one of the largest organs in the body, is the tissue located within the cavities of the cortical bones. Resorption of cartilage and endosteal bone creates a central space within the bone. Projections of calcified bone, called trabeculae, radiate out from the bone cortex into the central space, forming a three-dimensional matrix resembling a honeycomb. The trabeculae provide structural support for the developing blood cells.

Normal bone marrow contains two major components: red marrow, hematopoietically active marrow consisting of the developing blood cells and their progenitors, and yellow marrow, hematopoietically inactive marrow composed primarily of adipocytes (fat cells), with undifferentiated mesenchymal cells and macrophages. During infancy and early childhood, all the bones in the body contain primarily red (active) marrow. Between 5 and 7 years of age, adipocytes become more abundant and begin to occupy the spaces in the long bones previously dominated by active marrow. The process of replacing the active marrow by adipocytes (yellow marrow) during development is called retrogression and eventually results in restriction of the active marrow in the adult to the sternum, vertebrae, scapulae, pelvis, ribs, skull, and proximal portion of the long bones (Figure 7-2). Hematopoietically inactive yellow marrow is scattered throughout the red marrow so that in adults, there is approximately equal amounts of red and yellow marrow in these areas (Figure 7-3). Yellow marrow is capable of reverting back to active marrow in cases of increased demand on the bone marrow, such as in excessive blood loss or hemolysis.3

The bone marrow contains hematopoietic cells, stromal cells, and blood vessels (arteries, veins, and vascular sinuses). Stromal cells originate from mesenchymal cells that migrate into the central cavity of the bone. Stromal cells include endothelial cells, adipocytes (fat cells), macrophages and lymphocytes, osteoblasts, osteoclasts, and reticular adventitial cells (fibroblasts).3 Endothelial cells are broad, flat cells that form a single continuous layer along the inner surface of the arteries, veins, and vascular sinuses.21 Endothelial cells regulate the flow of particles entering and leaving hematopoietic spaces in the vascular sinuses. Adipocytes are large cells with a single fat vacuole; they play a role in regulating the volume of the marrow in which active hematopoiesis occurs. They also secrete cytokines or growth factors that

![Figure 7-2](image-url) The adult skeleton, in which darkened areas depict active red marrow hematopoiesis.
may positively stimulate HSC numbers and bone homeostasis.\textsuperscript{22,23} Macrophages function in phagocytosis, and both macrophages and lymphocytes secrete various cytokines that regulate hematopoiesis; they are located throughout the marrow space.\textsuperscript{3,24} Other cells involved in cytokine production include endothelial cells and reticular adventitial cells. \textit{Osteoblasts} are bone-forming cells, and \textit{osteoclasts} are bone-resorbing cells. \textit{Reticular adventitial cells} form an incomplete layer of cells on the abluminal surface of the vascular sinuses.\textsuperscript{3} They extend long, reticular fibers into the perivascular space that form a supporting lattice for the developing hematopoietic cells.\textsuperscript{3} Stromal cells secrete a semifluid extracellular matrix that serves to anchor developing hematopoietic cells in the bone cavity. The extracellular matrix contains substances such as \textit{fibronectin}, \textit{collagen}, \textit{laminin}, \textit{thrombospondin}, \textit{tenascin}, and proteoglycans (such as \textit{hyaluronate}, \textit{heparan sulfate}, \textit{chondroitin sulfate}, and \textit{dermatan}).\textsuperscript{3,25} Stromal cells play a critical role in the regulation of hematopoietic stem and progenitor cell survival and differentiation.\textsuperscript{21}

**Red Marrow**

The red marrow is composed of the hematopoietic cells and macrophages arranged in extravascular cords. The cords are located in spaces between the vascular sinuses and are supported by trabeculae of spongy bone.\textsuperscript{3} The cords are separated from the lumen of the vascular sinuses by endothelial and reticular adventitial cells (Figure 7-4). The hematopoietic cells develop in specific niches within the cords. \textit{Erythroblasts} develop in small clusters, and the more mature forms are located adjacent to the outer surfaces of the vascular sinuses\textsuperscript{3} (Figures 7-4 and 7-5); in addition, erythroblasts are found surrounding iron-laden macrophages (Figure 7-6). \textit{Megakaryocytes} are located adjacent to the walls of the vascular sinuses, which facilitates the release of platelets into the lumen of the sinus.\textsuperscript{3} Immature myeloid (granulocytic) cells through the metamyelocyte stage are located deep within the cords. As these maturing granulocytes proceed along their differentiation pathway, they move closer to the vascular sinuses.\textsuperscript{19}

The mature blood cells of the bone marrow eventually enter the peripheral circulation by a process that is not well understood. Through a highly complex interaction between the maturing blood cells and the vascular sinus wall, blood cells pass between layers of adventitial cells that form a discontinuous layer along the abluminal side of the sinus. Under the layer of

![Figure 7-3](image-url)  
**Figure 7-3** Fixed and stained bone marrow biopsy specimen (hematoxylin and eosin stain, ×100). The extravascular tissue consists of blood cell precursors and various tissue cells with scattered fat tissue. A normal adult bone marrow displays 50% hematopoietic cells and 50% fat.

![Figure 7-4](image-url)  
**Figure 7-4** Graphic illustration of the arrangement of a hematopoietic cord and vascular sinus in bone marrow.
adventitial cells is a basement membrane followed by a continuous layer of endothelial cells on the luminal side of the vascular sinus. The adventitial cells are capable of contracting, which allows mature blood cells to pass through the basement membrane and interact with the endothelial layer.

As blood cells come in contact with endothelial cells, they bind to the surface through a receptor-mediated process. Cells pass through pores in the endothelial cytoplasm, are released into the vascular sinus, and then move into the peripheral circulation.3,26

**Marrow Circulation**

The nutrient and oxygen requirements of the marrow are supplied by the nutrient and periosteal arteries, which enter via the bone foramina. The nutrient artery supplies blood only to the marrow.20 It coils around the central longitudinal vein, which passes along the bone canal. In the marrow cavity, the nutrient artery divides into ascending and descending branches that also coil around the central longitudinal vein. The arteriole branches that enter the inner lining of the cortical bone (endosteum) form sinusoids (endosteal beds), which connect to periosteal capillaries that extend from the periosteal artery.3 The periosteal arteries provide nutrients for the osseous bone and the marrow. Their capillaries connect to the venous sinuses located in the endosteal bed, which empty into a larger collecting sinus that opens into the central longitudinal vein.3 Blood exits the marrow via the central longitudinal vein, which runs the length of the marrow. The central longitudinal vein exits the marrow through the same foramen where the nutrient artery enters. Hematopoietic cells located in the endosteal bed receive their nutrients from the nutrient artery.3

**Hematopoietic Microenvironment**

The hematopoietic inductive microenvironment, or niche, plays an important role in nurturing and protecting HSCs and regulating a balance among their quiescence, self-renewal, and differentiation.21,27 As the site of hematopoiesis transitions from yolk sac to liver, then to bone marrow, so must the microenvironmental niche for HSCs. The adult bone marrow HSC niche has received the most attention, although its complex nature makes studying it difficult. Stromal cells form an extracellular matrix in the niche to promote cell adhesion and regulate HSCs through complex signaling networks involving cytokines, adhesion molecules, and maintenance proteins. Key stromal cells thought to support HSCs in bone marrow niches include osteoblasts, endothelial cells, mesenchymal stem cells, CXCL12-abundant reticular cells, perivascular stromal cells, glial cells, and macrophages.28,29

Recent findings suggest that HSCs are predominantly quiescent, maintained in a nondividing state by intimate interactions with thrombopoietin-producing osteoblasts.30 Opposing studies suggest that vascular cells are critical to HSC maintenance through CXCL12, which regulates migration of HSCs to the vascular niche.31 These studies suggest a heterogeneous microenvironment that may impact the HSC differently, depending on location and cell type encountered.32 Given the close proximity of cells within the bone marrow cavity, it is likely
that niches may overlap, providing multiple signals simultaneously and thus ensuring tight regulation of HSCs. Although the cell-cell interactions are complex and multifactorial, understanding these relationships is critical to the advancement of cell therapies based on HSCs such as clinical marrow transplantation.

Recent reviews, which are beyond the scope of this chapter, discuss and help to delineate between transcription factors required for HSC proliferation or function and those that regulate HSC differentiation pathways. The importance of transcription factors and their regulatory role in HSC maturation and redeployment in hematopoietic cell lineage production are demonstrated by their intimate involvement in disease evolution, such as in leukemia. Ongoing study of hematopoietic disease continues to demonstrate the complex and delicate nature of normal hematopoiesis.

Liver

The liver serves as the major site of blood cell production during the second trimester of fetal development. In adults, the hepatocytes of the liver have many functions, including protein synthesis and degradation, coagulation factor synthesis, carbohydrate and lipid metabolism, drug and toxin clearance, iron recycling and storage, and hemoglobin degradation in which bilirubin is conjugated and transported to the small intestine for eventual excretion.

The liver consists of two lobes situated beneath the diaphragm in the abdominal cavity. The position of the liver with regard to the circulatory system is optimal for gathering, transferring, and eliminating substances through the bile duct. Anatomically, the hepatocytes are arranged in radiating plates emanating from a central vein (Figure 7-7). Adjacent to the longitudinal plates of hepatocytes are vascular sinusoids lined with endothelial cells. A small noncellular space separates the endothelial cells of the sinusoids from the plates of hepatocytes. This spatial arrangement allows plasma to have direct access to the hepatocytes for two-directional flow of solutes and fluids.

The lumen of the sinusoids contains Kupffer cells that maintain contact with the endothelial cell lining. Kupffer cells are macrophages that remove senescent cells and foreign debris from the blood that circulates through the liver; they also secrete mediators that regulate protein synthesis in the hepatocytes. The particular anatomy, cellular components, and location in the body enables the liver to carry out many varied functions.

Liver Pathophysiology

The liver is often involved in blood-related diseases. In porphyrias, hereditary or acquired defects in the enzymes involved in heme biosynthesis result in the accumulation of the various intermediary porphyrins that damage hepatocytes, erythrocyte precursors, and other tissues. In severe hemolytic anemias, the liver increases the conjugation of bilirubin and the storage of iron. The liver sequesters membrane-damaged RBCs and removes them from the circulation. The liver can maintain hematopoietic stem and progenitor cells to produce various blood cells (called extramedullary hematopoiesis) as a response to infectious agents or in pathologic myelofibrosis of the bone marrow. It is directly affected by storage diseases of the monocyte/macrophage (Kupffer) cells as a result of enzyme deficiencies that cause hepatomegaly with ultimate dysfunction of the liver (Gaucher disease, Niemann-Pick disease, Tay-Sachs disease; Chapter 29).

Spleen

The spleen is the largest lymphoid organ in the body. It is located directly beneath the diaphragm behind the fundus of the stomach in the upper left quadrant of the abdomen. It is vital but not essential for life and functions as an indiscriminate filter of the circulating blood. In a healthy individual, the spleen contains about 350 mL of blood. The exterior surface of the spleen is surrounded by a layer of peritoneum covering a connective tissue capsule. The capsule projects inwardly, forming trabeculae that divide the spleen into discrete regions. Located within these regions are three types of splenic tissue: white pulp, red pulp, and a marginal zone. The white pulp consists of scattered follicles with germinal centers containing lymphocytes, macrophages, and dendritic cells. Aggregates of T lymphocytes surround arteries that pass through these germinal centers, forming a region called the periarteriolar lymphatic sheath, or PALS. Interspersed along the periphery of the PALS are lymphoid nodules containing primarily B lymphocytes. Activated B lymphocytes are found in the germinal centers.
The marginal zone surrounds the white pulp and forms a reticular meshwork containing blood vessels, macrophages, memory B cells, and CD4^+ T cells. The red pulp is composed primarily of vascular sinuses separated by cords of reticular cell meshwork (cords of Billroth) containing loosely connected specialized macrophages. This creates a sponge-like matrix that functions as a filter for blood passing through the region. As RBCs pass through the cords of Billroth, there is a decrease in the flow of blood, which leads to stagnation and depletion of the RBCs' glucose supply. These cells are subject to increased damage and stress that may lead to their removal from the spleen. The spleen uses two methods for removing senescent or abnormal RBCs from the circulation: culling, in which the cells are phagocytized with subsequent degradation of cell organelles, and pitting, in which splenic macrophages remove inclusions or damaged surface membrane from the circulating RBCs. The spleen also serves as a storage site for platelets. In a healthy individual, approximately 30% of the total platelet count is sequestered in the spleen.

The spleen has a rich blood supply receiving approximately 350 mL/min. Blood enters the spleen through the central splenic artery located at the hilum and branches outward through the trabeculae. The branches enter all three regions of the spleen: the white pulp with its dense accumulation of lymphocytes, the marginal zone, and the red pulp. The venous sinuses, which are located in the red pulp, unite and leave the spleen as splenic veins (Figure 7-8).

**Spleen Pathophysiology**

As blood enters the spleen, it may follow one of two routes. The first is a slow-transit pathway through the red pulp in which the RBCs pass circuitously through the macrophage-lined cords before reaching the sinuses. Plasma freely enters the sinuses, but the RBCs have a more difficult time passing through the tiny openings created by the interendothelial junctions of adjacent endothelial cells (Figure 7-9). The combination of the slow passage and the continued RBC metabolism creates an environment that is acidic, hypoglycemic, and hypoxic. The increased environmental stress on the RBCs circulating through the spleen leads to possible hemolysis.

In the rapid-transit pathway, blood cells enter the splenic artery and pass directly to the sinuses in the red pulp and continue to

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**Figure 7-8** Schematic of the normal spleen. (From Weiss L, Tavossoli M: Anatomical hazards to the passage of erythrocytes through the spleen, Semin Hematol 7:372-380, 1970.)
Lymph Nodes

Figure 7-9 Scanning electron micrograph of the spleen shows erythrocytes (numbered 1 to 6) squeezing through the fenestrated wall in transit from the splenic cord to the sinus. The view shows the endothelial lining of the sinus wall, to which platelets (P) adhere, along with white blood cells, probably macrophages. The arrow shows a protrusion on a red blood cell (×5000). (From Weiss L: A scanning electron microscopic study of the spleen, Blood 43:665, 1974.)

Lymph nodes are organs of the lymphatic system located along the lymphatic capillaries that parallel, but are not part of, the circulatory system. The nodes are bean-shaped structures (1 to 5 mm in diameter) that occur in groups or chains at various intervals along lymphatic vessels. They may be superficial (inguinal, axillary, cervical, supratrochlear) or deep (mesenteric, retroperitoneal). Lymph is the fluid portion of blood that escapes into the connective tissue and is characterized by a low protein concentration and the absence of RBCs. Afferent lymphatic vessels carry circulating lymph to the lymph nodes. Lymph is filtered by the lymph nodes and exits via the efferent lymphatic vessels located in the hilus of the lymph node.

Lymph nodes can be divided into an outer region called the cortex and an inner region called the medulla. An outer capsule forms trabeculae that radiate through the cortex and provide support for the macrophages and lymphocytes located in the node. The trabeculae divide the interior of the lymph node into follicles. After antigenic stimulation, the cortical region of some follicles develop foci of activated B cell proliferation called germinal centers. Follicles with germinal centers are called secondary follicles, while those without are called primary follicles. Located between the cortex and the medulla is a region called the paracortex, which contains predominantly T cells and numerous macrophages. The medullary cords lie toward the interior of the lymph node. These cords consist primarily of plasma cells and B cells. Lymph nodes have three main functions: they are a site of lymphocyte proliferation from the germinal centers, they are involved in the initiation of the specific immune response to foreign antigens, and they filter particulate matter, debris, and bacteria entering the lymph node via the lymph.

Lymph Node Pathophysiology

Lymph nodes, by their nature, are vulnerable to the same organisms that circulate through the tissue. Sometimes increased numbers of microorganisms enter the nodes, overwhelming the macrophages and causing adenitis (infection of the lymph node). More serious is the frequent entry into the lymph nodes of malignant cells that have broken loose from malignant tumors. These malignant cells may grow and metastasize to other lymph nodes in the same group.

Thymus

To understand the role of the thymus in adults, certain formative intrauterine processes that affect function must be considered. First, the thymus tissue originates from endodermal and mesenchymal tissue. Second, the thymus is populated initially by primitive lymphoid cells from the yolk sac and the liver. This increased population of lymphoid cells physically pushes the epithelial cells of the thymus apart; however, their long processes remain attached to one another by desmosomes. In adults, T cell progenitors migrate to the thymus from the bone marrow for further maturation.

At birth, the thymus is an efficient, well-developed organ. It consists of two lobes, each measuring 0.5 to 2 cm in diameter, and is further divided into lobules. The thymus is located in the upper part of the anterior mediastinum at about the level of the great vessels of the heart. It resembles other lymphoid tissue in that the lobules are subdivided into two areas: the cortex (a peripheral zone) and the medulla (a central zone). Both areas are populated with the same cellular components—lymphoid cells, mesenchymal cells, reticular cells, epithelial cells, dendritic cells, and many macrophages—although in different proportions. The cortex

The venous system to exit the spleen. When splenomegaly occurs, the spleen becomes enlarged and is palpable. This occurs as a result of many conditions, such as chronic leukemias, inherited membrane or enzyme defects in RBCs, hemoglobinopathies, Hodgkin disease, thalassemia, malaria, and the myeloproliferative disorders. Splenectomy may be beneficial in cases of excessive destruction of RBCs, such as autoimmune hemolytic anemia when treatment with corticosteroids does not effectively suppress hemolysis or in severe hereditary spherocytosis. Splenectomy also may be indicated in severe refractory immune thrombocytopenic purpura or in storage disorders with portal hypertension. Other causes include thrombosis, vascular stenosis, other vascular deformities such as aneurysm of the splenic artery, and cysts.
Blood Cell Production, Structure, and Function

is characterized by a blood supply system that is unique in that it consists only of capillaries. Its function seems to be that of a “waiting zone” densely populated with progenitor T cells. When these progenitor T cells migrate from the bone marrow and first enter the thymus, they have no identifiable CD4 and CD8 surface markers (double negative), and they locate to the corticomедullary junction. Under the influence of chemokines, cytokines, and receptors, these cells move to the cortex and express both CD4 and CD8 (double positive). Subsequently they give rise to mature T cells that express either CD4 or CD8 surface antigen as they move toward the medulla. Eventually, the mature T cells leave the thymus to populate specific regions of other lymphoid tissue, such as the T cell-dependent areas of the spleen, lymph nodes, and other lymphoid tissues. The lymphoid cells that do not express the appropriate antigens and receptors, or are self-reactive, die in the cortex or medulla as a result of apoptosis and are phagocytized by macrophages. The medulla contains only 15% mature T cells and seems to be a holding zone for mature T cells until they are needed by the peripheral lymphoid tissues.

The thymus also contains other cell types, including B cells, eosinophils, neutrophils, and other myeloid cells.

Gross examination indicates that the size of the thymus is related to age. The thymus weighs 12 to 15 g at birth, increases to 30 to 40 g at puberty, and decreases to 10 to 15 g at later ages. It is hardly recognizable in old age due to atrophy (Figure 7-12). The thymus retains the ability to produce new T cells, however, as has been shown after irradiation treatment that may accompany bone marrow transplantation.

**Thymus Pathophysiology**

Nondevelopment of the thymus during gestation results in the lack of formation of T lymphocytes. Related manifestations seen in patients with this condition are failure to thrive, uncontrollable infections, and death in infancy. Adults with thymic disturbance are not affected because they have developed and maintained a pool of T lymphocytes for life.
HEMATOPOIETIC STEM CELLS
AND CYTOKINES

Stem Cell Theory
In 1961, Till and McCulloch\textsuperscript{46} conducted a series of experiments in which they irradiated spleens and bone marrow of mice, creating a state of aplasia. These aplastic mice were given an intravenous injection of marrow cells. Colonies of HSCs were seen 7 to 8 days later in the spleens of the irradiated (recipient) mice. These colonies were called colony-forming units–spleen (CFU-S). These investigators later showed that these colonies were capable of self-renewal and the production of differentiated progeny. The CFU-S represents what we now refer to as committed myeloid progenitors or colony-forming unit–granulocyte, erythrocyte, monocyte, and megakaryocyte (CFU-GEMM).\textsuperscript{46,47} These cells are capable of giving rise to multiple lineages of blood cells.

Morphologically unrecognizable hematopoietic progenitor cells can be divided into two major types: noncommitted or undifferentiated hematopoietic stem cells, and committed progenitor cells. These two groups give rise to all of the mature blood cells. Originally there were two theories describing the origin of hematopoietic progenitor cells. The monophyletic theory suggests that all blood cells are derived from a single progenitor stem cell called a pluripotent hematopoietic stem cell. The polyphyletic theory suggests that each of the blood cell lineages is derived from its own unique stem cell. The monophyletic theory is the most widely accepted theory among experimental hematologists today.

Hematopoietic stem cells by definition are capable of self-renewal, are pluripotent and give rise to differentiated progeny, and are able to reconstitute the hematopoietic system of a lethally irradiated host. The undifferentiated HSCs can differentiate into progenitor cells committed to either lymphoid or myeloid lineages. These lineage-specific progenitor cells are the common lymphoid progenitor, which proliferates and differentiates into T, B, and natural killer lymphocyte and dendritic lineages; and the common myeloid progenitor, which proliferates and differentiates into individual granulocytic, erythrocytic, monocytic, and megakaryocytic lineages. The resulting limited lineage-specific progenitors give rise to morphologically recognizable, lineage-specific precursor cells (Figure 7-13 and Table 7-1). Despite the limited numbers of HSCs in the bone marrow, 6 billion blood cells per kilogram of body weight are produced each day for the entire life span of an individual.\textsuperscript{3} Most of the cells in normal bone marrow are precursor cells at various stages of maturation.

HSCs are directed to one of three possible fates: self-renewal, differentiation, or apoptosis.\textsuperscript{48} When the HSC divides, it gives rise to two identical daughter cells. Both daughter cells may follow the path of differentiation, leaving the stem cell pool (symmetric division), or one daughter cell may return to the stem cell pool and the other daughter cell may follow the path of differentiation (asymmetric division) or undergo apoptosis. Many theories have been proposed to describe the mechanisms that determine the fate of the stem cell. Till and McCulloch proposed that hematopoiesis is a random process whereby the HSC randomly commits to self-renewal or differentiation.\textsuperscript{46} This model is also called the stochastic model of hematopoiesis. Later studies suggested that the microenvironment in the bone marrow determines whether the HSC will self-renew or differentiate (instructive
Current thinking is that the ultimate decision made by the HSC can be described by both the stochastic and instructive models of hematopoiesis. The initial decision to self-renew or differentiate is probably stochastic, whereas lineage differentiation that occurs later is determined by various signals from the hematopoietic inductive microenvironment in response to specific requirements of the body.

The multilineage priming model suggests that HSCs receive low-level signals from the hematopoietic inductive microenvironment to amplify or repress genes associated with commitment to multiple lineages. The implication is that the cell’s fate is determined by intrinsic and extrinsic factors. Extrinsic regulation involves proliferation and differentiation signals from...
specialized niches located in the hematopoietic inducive microenvironment via direct cell-to-cell or cellular-extracellular signaling molecules. Some of the cytokines released from the hematopoietic inducive microenvironment include factors that regulate proliferation and differentiation, such as KIT ligand, thrombopoietin (TPO), and FLT3 ligand. Intrinsic regulation involves genes such as TAL1, which is expressed in cells in the hemangioblast, a bipotential progenitor cell of mesodermal origin that gives rise to hematopoietic and endothelial lineages; and GATA2, which is expressed in later-appearing HSCs. Both of these genes are essential for primitive and definitive hematopoiesis. In addition to factors involved in differentiation and regulation, there are regulatory signaling factors, such as Notch-1 and Notch-2, that allow HSCs to respond to hematopoietic inducive microenvironment factors, altering cell fate.

As hematopoietic cells differentiate, they take on various morphologic features associated with maturation. These include an overall decrease in cell volume and a decrease in the ratio of nucleus to cytoplasm. Additional changes that take place during maturation occur in the cytoplasm and nucleus. Changes in the nucleus include loss of nucleoli, decrease in the diameter of the nucleus, condensation of nuclear chromatin, possible change in the shape of the nucleus, and possible loss of the nucleus. Changes occurring in the cytoplasm include decrease in basophilia, increase in the proportion of cytoplasm, and possible appearance of granules in the cytoplasm. Specific changes in each lineage are discussed in subsequent chapters.

### Stem Cell Cycle Kinetics

The bone marrow is estimated to be capable of producing approximately 2.5 billion erythrocytes, 2.5 billion platelets, and 1 billion granulocytes per kilogram of body weight daily. The determining factor controlling the rate of production is physiologic need. HSCs exist in the marrow in the ratio of 1 per 1000 nucleated blood cells. They are capable of many mitotic divisions when stimulated by appropriate cytokines. When mitosis has occurred, the cell may reenter the cycle or go into a resting phase, termed G0. Some cells in the resting phase reenter the active cell cycle and divide, whereas other cells are directed to terminal differentiation (Figure 7-14).

From these data, a mitotic index can be calculated to establish the percentage of cells in mitosis in relation to the total number of cells. Factors affecting the mitotic index include the duration of mitosis and the length of the resting state. Normally, the mitotic index is approximately 1% to 2%. An increased mitotic index implies increased proliferation. An exception to this rule is in the case of megaloblastic anemia, in which mitosis is prolonged. An understanding of the mechanism of the generative cycle aids in understanding the mode of action of specific drugs used in the treatment and management of proliferative disorders.

### Stem Cell Phenotypic and Functional Characterization

The identification and origin of HSCs can be determined by immunophenotypic analysis using flow cytometry. The earliest identifiable human HSCs capable of initiating long-term cultures are CD34+, CD38-, HLA-DRlow, Thy1low, and Lin-. This population of marrow cells is enriched in primitive progenitors. The expression of CD38 and HLA-DR is associated with a loss of "stemness." The acquisition of CD33 and CD38 is seen on committed myeloid progenitors, and the expression of CD10 and CD38 is seen on committed lymphoid progenitors. The expression of CD7 is seen on T-lymphoid progenitor cells and natural killer cells, and the expression of CD19 is seen on B-lymphoid progenitors (Chapter 32).

Functional characterization of HSCs can be accomplished through in vitro techniques using long-term culture assays. These involve the enumeration of colony-forming units (e.g., CFU-GEMM) on semisolid media, such as methylcellulose. Primitive progenitor cells, such as the high proliferative potential colony-forming cell and the long-term colony initiating cell, also have been identified. These hematopoietic precursor cells give rise to colonies that can survive for 5 to 8 weeks and be replated. In vivo functional assays also are available and require transplantation of cells into syngeneic, lethally irradiated animals, followed by transference of the engrafted bone marrow cells into a secondary recipient. These systems promote the proliferation and differentiation of HSCs, thus allowing them to be characterized; they may serve as models for developing clinically applicable techniques for gene therapy and hematopoietic stem cell transplantation.

From our rudimentary knowledge of stem cell biology, it has been possible to move from the bench to the bedside with amazing speed and success. Hematopoietic stem cell transplantation (HSCT) is over a half-century old, and we have witnessed tremendous growth in the field due to the reproducibility of clinical procedures to produce similar outcomes. However, caution must be exercised because the cells capable of these remarkable clinical events are still not well defined, the niche that they inhabit is poorly understood, and the signals that they potentially respond to are plentiful and diverse in action. Current treatment of hematologic disorders is based on
fundamental understanding of the biologic principles of HSC proliferation and maturation. The control mechanisms that regulate HSCs, and the requisite processes necessary to manipulate them to generate sufficient numbers for clinical use, remain largely unknown.

Cytokines and Growth Factors
A group of specific glycoproteins called hematopoietic growth factors or cytokines regulate the proliferation, differentiation, and maturation of hematopoietic precursor cells. \(^{51}\) Figure 7-15 illustrates the hematopoietic system and the sites of action of some of the cytokines. These factors are discussed in more detail in subsequent chapters.

Cytokines are a diverse group of soluble proteins that have direct and indirect effects on hematopoietic cells. Classification of cytokines has been difficult because of their overlapping and redundant properties. The terms cytokine and growth factor are often used synonymously; cytokines include interleukins (ILs), lymphokines, monokines, interferons, chemokines, and colony-stimulating factors (CSFs). \(^{52}\) Cytokines are responsible for stimulation or inhibition of production, differentiation, and trafficking of mature blood cells and their precursors. \(^{52}\) Many of these cytokines exert a positive influence on hematopoietic stem cells and progenitor cells with multilineage potential (e.g., KIT ligand, FLT3 ligand, GM-CSF, IL-1, IL-3, IL-6, and IL-11). \(^{52}\) Cytokines that exert a negative influence on hematopoiesis include transforming growth factor-\(\beta\), tumor necrosis factor-\(\alpha\), and the interferons. \(^{49}\)

Hematopoietic progenitor cells require cytokines on a continual basis for their growth and survival. Cytokines prevent hematopoietic precursor cells from dying by inhibiting apoptosis; they stimulate them to divide by decreasing the transit time from G\(_0\) to G\(_1\) of the cell cycle; and they regulate cell differentiation into the various cell lineages.

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**Figure 7-15** Diagram of derivation of hematopoietic cells, illustrating sites of action of cytokines. EPO, Erythropoietin; FLT3L, FLT3 ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1, interleukin-1; IL-3, interleukin-3; IL-5, interleukin-5; IL-6, interleukin-6; IL-7, interleukin-7; IL-11, interleukin-11; KITLG, KIT ligand; M-CSF, macrophage colony-stimulating factor; TPO, thrombopoietin.


Atrophy refers to programmed cell death, a normal physiological process that eliminates unwanted, abnormal, or harmful cells. Atrophy differs from necrosis, which is accidental death from trauma (Chapter 6). When cells do not receive the appropriate cytokines necessary to prevent cell death, apoptosis is initiated. In some disease states, apoptosis is “turned on,” which results in early cell death, whereas in other states apoptosis is inhibited, which allows uncontrolled proliferation of cells.

Research techniques have accomplished the purification of many of these cytokines and the cloning of pure recombinant growth factors, some of which are discussed in detail later in this chapter. The number of cytokines identified has expanded greatly in recent years and will further increase as research continues. This chapter focuses primarily on CSFs, KIT ligand, FLT3 ligand, and IL-3. A detailed discussion is beyond the scope of this text, and the reader is encouraged to consult current literature for further details.

**Colony-Stimulating Factors**

CSFs are produced by many different cells. They have a high specificity for their target cells and are active at low concentrations. The names of the individual factors indicate the predominant cell lines that respond to their presence. The primary target of G-CSF is the granulocytic cell line, and GM-CSF targets the granulocytic-monocytic cell line. The biologic activity of CSFs was first identified by their ability to induce hematopoietic colony formation in semisolid media. In addition, it was shown in cell culture experiments that although a particular CSF may show specificity for one cell lineage, it is often capable of influencing other cell lineages as well. This is particularly true when multiple growth factors are combined. Although GM-CSF stimulates the proliferation of granulocyte and monocyte progenitors, it also works synergistically with IL-3 to enhance megakaryocyte colony formation.

**Early-Acting Multilineage Growth Factors**

Ogawa described early-acting growth factors (multilineage), intermediate-acting growth factors (multilineage), and late-acting growth factors (lineage restricted). KIT ligand, also known as stem cell factor (SCF), is an early-acting growth factor; its receptor is the transmembrane protein, KIT. KIT is a receptor-type tyrosine-protein kinase that is expressed on HSCs and is down-regulated with differentiation. The binding of KIT ligand to the extracellular domain of the KIT receptor triggers its cytoplasmic domain to induce a series of signals that are sent via signal transduction pathways to the nucleus of the HSC, stimulating the cell to proliferate. As HSCs differentiate and mature, the expression of KIT receptor decreases. Activation of the KIT receptor by KIT ligand is essential in the early stages of hematopoiesis.

FLT3 is also a receptor-type tyrosine-protein kinase. KIT ligand and FLT3 ligand work synergistically with IL-3, GM-CSF, and other cytokines to promote early HSC proliferation and differentiation. In addition, IL-3 regulates blood cell production by controlling the production, differentiation, and function of granulocytes and macrophages. GM-CSF induces expression of specific genes that stimulate HSC differentiation to the common myeloid progenitor.

**Interleukins**

Cytokines originally were named according to their specific function, such as lymphocyte-activating factor (now called IL-1), but continued research showed that a particular cytokine may have multiple actions. A group of scientists began calling some of the cytokines interleukins, numbering them in the order in which they were identified (e.g., IL-1, IL-2). Characteristics shared by interleukins include the following:

1. They are proteins that exhibit multiple biologic activities, such as the regulation of autoimmune and inflammatory reactions and hematopoiesis.
2. They have synergistic interactions with other cytokines.
3. They are part of interacting systems with amplification potential.
4. They are effective at very low concentrations.

**LINEAGE-SPECIFIC HEMATOPOIESIS**

**Erythropoiesis**

Erythropoiesis occurs in the bone marrow and is a complex, regulated process for maintaining adequate numbers of erythrocytes in the peripheral blood. The CFU-GEMM gives rise to the earliest identifiable colony of RBCs, called the burst-forming unit–erythroid (BFU-E). The BFU-E produces a large multicelled colony that resembles a cluster of grapes containing brightly colored hemoglobin. These colonies range from a single large cluster to 16 or more clusters. BFU-Es contain only a few receptors for EPO, and their cell cycle activity is not influenced significantly by the presence of exogenous EPO. BFU-Es under the influence of IL-3, GM-CSF, TPO, and KIT ligand develop into colony-forming unit–erythroid (CFU-E) colonies. The CFU-E has many EPO receptors and has an absolute requirement for EPO. Some CFU-Es are responsive to only low levels of EPO and do not have the proliferative capacity of the BFU-E. EPO serves as a differentiation factor that causes the CFU-E to differentiate into pronormoblasts, the earliest visually recognized erythrocyte precursors in the bone marrow.

EPO is a lineage-specific glycoprotein produced in the renal peritubular interstitial cells. In addition, a small amount of EPO is produced by the liver. Oxygen availability in the kidney is the stimulus that activates production and secretion of EPO. EPO exerts its effects by binding to transmembrane receptors expressed by erythroid progenitors and precursors. EPO serves to recruit CFU-E from the more primitive BFU-E compartment, prevents apoptosis of erythroid progenitors, and induces hemoglobin synthesis. Erythropoiesis and EPO’s actions are discussed in detail in Chapter 8.

**Leukopoiesis**

Leukopoiesis can be divided into two major categories: myelopoiesis and lymphopoiesis. Factors that promote differentiation of the CFU-GEMM into neutrophils, monocytes, eosinophils, and basophils include GM-CSF, G-CSF, macrophage colony-stimulating
factor (M-CSF), IL-3, IL-5, IL-11, and KIT ligand. GM-CSF stimulates the proliferation and differentiation of neutrophil and macrophage colonies from the colony-forming unit–granulocyte-monocyte. G-CSF and M-CSF stimulate neutrophil differentiation and monocyte differentiation from the colony-forming unit–granulocyte and colony-forming unit–monocyte. IL-3 is a multilineage stimulating factor that stimulates the growth of granulocytes, monocytes, megakaryocytes, and erythrocyte cells. Eosinophils require GM-CSF, IL-5, and IL-3 for differentiation. The requirements for basophil differentiation are less clear, but it seems to depend on the presence of IL-3 and KIT ligand. Growth factors promoting lymphoid differentiation include IL-2, IL-7, IL-12, and IL-15 and to some extent IL-4, IL-10, IL-13, IL-14, and IL-16. Leukopoiesis is discussed further in Chapter 12.

**Megakaryopoiesis**

Earlier influences on megakaryopoiesis include GM-CSF, IL-3, IL-6, IL-11, KIT ligand, and TPO. The stimulating hormonal factor TPO (also known as MPL ligand), along with IL-11, controls the production and release of platelets. The liver is the main site of production of TPO. Megakaryopoiesis is discussed in Chapter 13.

**TABLE 7-2 Selected Cytokines, Characteristics, Current and Potential Therapeutic Applications**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primary Cell Source</th>
<th>Primary Target Cell</th>
<th>Biological Activity</th>
<th>Current/Potential Therapeutic Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO</td>
<td>Kidney (peritubular</td>
<td>Bone marrow erythroid</td>
<td>Stimulates proliferation of</td>
<td>Anemia of chronic renal disease (in predialysis,</td>
</tr>
<tr>
<td></td>
<td>interstitial cell)</td>
<td>progenitors (BFU-E</td>
<td>erythroid progenitors and</td>
<td>dialysis dependent, and chronic anemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and CFU-E)</td>
<td>prevents apoptosis of</td>
<td>patients)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFU-E</td>
<td>Treatment of anemia in cancer patients on</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Autologous predonation blood collection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anemia in HIV infection to permit use of</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>zidovudine (AZT)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Post autologous hematopoietic stem cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>transplant</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Endothelial cells</td>
<td>Neutrophil precursors</td>
<td>Stimulates granulocyte</td>
<td>Chemotherapy-induced neutropenia</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td></td>
<td>colonies</td>
<td>Stem cell mobilization</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Fibroblasts</td>
<td>Differentiation of progenitors</td>
<td>Peripheral blood/bone marrow transplantation</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>Leukemic myeloblasts</td>
<td>toward neutrophil lineage</td>
<td>Congenital neutropenia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stimlation of neutrophil</td>
<td>Idiopathic neutropenia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>maturation</td>
<td>Cyclic neutropenia</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>T cells</td>
<td>Bone marrow</td>
<td>Promotes antigen</td>
<td>Chemotherapy-induced neutropenia</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>progenitor cells</td>
<td>presentation</td>
<td>Stem cell mobilization</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td>Dendritic cells</td>
<td>T cell homeostasis</td>
<td>Peripheral blood/bone marrow transplantation</td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td>Macrophages</td>
<td>Hematopoietic cell growth</td>
<td>Leukemia treatment</td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td>NKT cells</td>
<td>factor</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>CD4+ T cells</td>
<td>T cells</td>
<td>Cell growth/activation of</td>
<td>Metastatic melanoma</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>NK cells</td>
<td>CD4+ and CD8+</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td></td>
<td>B cells</td>
<td>B cells</td>
<td>T cells</td>
<td>Non-Hodgkin lymphoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monocytes</td>
<td>Suppress T&lt;sub&gt;reg&lt;/sub&gt; responses</td>
<td>Asthma</td>
</tr>
</tbody>
</table>

**THERAPEUTIC APPLICATIONS**

Clinical use of growth factors approved by the U.S. Food and Drug Administration has provided numerous options in the treatment of hematologic malignancies and solid tumors. In addition, growth factors can be used as priming agents to increase the yield of HSCs during apheresis for transplantation protocols. Advances in molecular biology have resulted in cloning of the genes that are responsible for the synthesis of various growth factors and the recombinant production of large quantities of these proteins. Table 7-2 is an overview of selected cytokines and their major functions and clinical applications. Many more examples can be found in the literature.

In addition to the cytokines previously mentioned, it is important to recognize another family of low-molecular-weight proteins known as chemokines (chemotactic cytokines) that complement cytokine function and help to regulate the adaptive and innate immune system. These interacting biological mediators have amazing capabilities, such as controlling growth and differentiation, hematopoiesis, and a number of lymphocyte functions like recruitment, differentiation, and inflammation. The chemokine field has rapidly developed and is beyond the scope of this chapter. Nevertheless, a classification...
### TABLE 7-2 Selected Cytokines, Characteristics, Current and Potential Therapeutic Applications—cont’d

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primary Cell Source</th>
<th>Primary Target Cell</th>
<th>Biological Activity</th>
<th>Current/Potential Therapeutic Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>Activated T cells</td>
<td>hematopoietic stem cells and progenitors</td>
<td>Proliferation of hematopoietic progenitors</td>
<td>Stem cell mobilization; Postchemotherapy/transplantation; Bone marrow failure states</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>T cells</td>
<td>T cells</td>
<td>Costimulation with other cytokines; Cell growth/activation of T cells and B cells; Megakaryocyte maturation; Neural differentiation; Acute phase reactant</td>
<td>Stimulation of platelet production, but not at tolerable doses; Melanoma; Renal cell carcinoma; IL-6 inhibitors may be promising</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>CD4⁺, Th2 T cells</td>
<td>T cells</td>
<td>Inhibits cytokine production; Inhibits macrophages</td>
<td>Target lymphokines in prevention of B cell lymphoma and Epstein-Barr virus lymphomagenesis; Human immunodeficiency virus infection</td>
</tr>
<tr>
<td></td>
<td>CD8⁺ T cells</td>
<td>Macrophages</td>
<td></td>
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<td></td>
<td>Monocytes</td>
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<tr>
<td></td>
<td>Macrophages</td>
<td></td>
<td></td>
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<tr>
<td>IL-12</td>
<td>Macrophages</td>
<td>T cells</td>
<td>T cell, Th1 differentiation</td>
<td>Allergy treatment; Adjuvant for infectious disease therapy; Asthma; Possible role for use in vaccines</td>
</tr>
<tr>
<td>IL-15</td>
<td>Activated CD4⁺ T cells</td>
<td>CD4⁺ T cells</td>
<td>CD4⁺/CD8⁺ T cell proliferation; CD8⁺/NK cell cytotoxicity</td>
<td>Melanoma; Rheumatoid arthritis; Adoptive cell therapy; Generation of antigen-specific T cells</td>
</tr>
<tr>
<td></td>
<td>CD8⁺ T cells</td>
<td>NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-α</td>
<td>Dendritic cells</td>
<td>Macrophages</td>
<td>Antiviral; Enhances MHC expression</td>
<td>Adjuvant treatment for stage II/III melanoma; Hematologic malignancies: Kaposi sarcoma, hairy cell leukemia, and chronic myelogenous leukemia</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>NK cells</td>
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<td></td>
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<td></td>
<td>T cells</td>
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<td></td>
<td>B cells</td>
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<td></td>
<td>Macrophages</td>
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<tr>
<td></td>
<td>Fibroblasts</td>
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<tr>
<td></td>
<td>Endothelial cells</td>
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<tr>
<td></td>
<td>Osteoblasts</td>
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</tbody>
</table>


Experimental studies conducted in the 1990s identified a critical role for CXCL12 and its receptor CXCR4 in the migration of HSCs during early development. Further investigation in adult bone marrow demonstrated that CXCL12 is a key factor in the retention of HSCs within the stem cell niche. It was also shown that inhibiting the CXCL12-CXCR4 interaction permitted release of HSCs into the peripheral circulation for harvesting by apheresis. Plexifor (a CXCR4 antagonist) is currently being used as a single agent and in conjunction with G-CSF in novel mobilization strategies to optimize donor stem cell collection.
Hematopoiesis is a continuous, regulated process of blood cell production that includes cell renewal, proliferation, differentiation, and maturation. These processes result in the formation, development, and specialization of all the functional blood cells.

During fetal development, hematopoiesis progresses through the mesoblastic, hepatic, and medullary phases.

Organs that function at some point in hematopoiesis include the liver, spleen, lymph nodes, thymus, and bone marrow.

The bone marrow is the primary site of hematopoiesis at birth and throughout life. In certain situations, blood cell production may occur outside the bone marrow; such production is termed extra-medullary.

The hematopoietic inductive microenvironment in the bone marrow is essential for regulating hematopoietic stem cell maintenance, self-renewal, and differentiation.

Monophyletic theory suggests that all blood cells arise from a single stem cell called a pluripotent hematopoietic stem cell.

Hematopoietic stem cells (HSCs) are capable of self-renewal. They are pluripotent and can differentiate into all the different types of blood cells. One HSC is able to reconstitute the entire hematopoietic system of a lethally irradiated host.

As cells mature, certain morphologic characteristics of maturation allow specific lineages to be recognized. General characteristics of maturation include decrease in cell diameter, decrease in nuclear diameter, loss of nucleoli, condensation of nuclear chromatin, and decreased basophilia in cytoplasm. Some morphologic changes are unique to specific lineages (e.g., loss of the nucleus in RBCs).

Cytokines or growth factors play a major role in the maintenance, proliferation, and differentiation of HSCs and progenitor cells; they are also necessary to prevent premature apoptosis. Cytokines include interleukins, colony stimulating factors, chemokines, interferons, and others.

Cytokines can exert a positive or negative influence on HSCs and blood cell progenitors; some are lineage specific, and some function only in combination with other cytokines.

Cytokines have provided new options in the treatment of hematologic malignancies and solid tumors. They are also used as priming agents to increase the yield of HSCs during apheresis for transplantation protocols.

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**REVIEW QUESTIONS**

Answers can be found in the Appendix.

1. The process of formation and development of blood cells is termed:
   a. Hematopoiesis
   b. Hematogenesis
   c. Hematocytometry
   d. Hemorrhage

2. During the second trimester of fetal development, the primary site of blood cell production is the:
   a. Bone marrow
   b. Spleen
   c. Lymph nodes
   d. Liver

3. Which one of the following organs is responsible for the maturation of T lymphocytes and regulation of their expression of CD4 and CD8?
   a. Spleen
   b. Liver
   c. Thymus
   d. Bone marrow

4. The best source of active bone marrow from a 20-year-old would be:
   a. Iliac crest
   b. Femur
   c. Distal radius
   d. Tibia

5. Physiologic programmed cell death is termed:
   a. Angiogenesis
   b. Apoptosis
   c. Aneurysm
   d. Apherhematics

6. Which organ is the site of sequestration of platelets?
   a. Liver
   b. Thymus
   c. Spleen
   d. Bone marrow

7. Which one of the following morphologic changes occurs during normal blood cell maturation:
   a. Increase in cell diameter
   b. Development of cytoplasm basophilia
   c. Condensation of nuclear chromatin
   d. Appearance of nucleoli
8. Which one of the following cells is a product of the CLP?
   a. Megakaryocyte
   b. T lymphocyte
   c. Erythrocyte
   d. Granulocyte

9. What growth factor is produced in the kidneys and is used to treat anemia associated with kidney disease?
   a. EPO
   b. TPO
   c. G-CSF
   d. KIT ligand

10. Which one of the following cytokines is required very early in the differentiation of a hematopoietic stem cell?
    a. IL-2
    b. IL-8
    c. EPO
    d. FLT3 ligand

11. When a patient has severe anemia and the bone marrow is unable to effectively produce red blood cells to meet the increased demand, one of the body’s responses is:
    a. Extramedullary hematopoiesis in the liver and spleen
    b. Decreased production of erythropoietin by the kidney
    c. Increased apoptosis of erythrocyte progenitor cells
    d. Increase the proportion of yellow marrow in the long bones

12. Hematopoietic stem cells produce all lineages of blood cells in sufficient quantities over the lifetime of an individual because they:
    a. Are unipotent
    b. Have the ability of self-renewal by asymmetric division
    c. Are present in large numbers in the bone marrow niches
    d. Have a low mitotic potential in response to growth factors

REFERENCES

A 42-year-old premenopausal woman has emphysema. This lung disease impairs the ability to oxygenate the blood, so patients experience significant fatigue and shortness of breath. To alleviate these symptoms, oxygen is typically prescribed, and this patient has a portable oxygen tank she carries with her at all times, breathing through nasal cannulae. Before she began using oxygen, her red blood cell (RBC) count was $5.8 \times 10^{12}$ /L. After oxygen therapy for several months, her RBC count dropped to $5.0 \times 10^{12}$ /L.

1. What physiologic response explains the elevation of the first RBC count?
2. What hormone is responsible? How is its production stimulated? What is the major way in which it acts?
3. What explains the decline in RBC count with oxygen therapy for this patient?
Maturation Process

**Erythroid Progenitors**

As described in Chapter 7, the morphologically identifiable erythroid progenitors develop from two functionally identifiable progenitors, burst-forming unit–erythroid (BFU-E) and colony-forming unit–erythroid (CFU-E), both committed to the erythroblast cell line. Estimates of time spent at each stage suggest that it takes about one week for the BFU-E to mature to the CFU-E and another week for the CFU-E to become a pronormoblast, which is the first morphologically identifiable RBC precursor. While at the CFU-E stage, the cell completes approximately three to five divisions before maturing further. As seen later, it takes approximately another 6 to 7 days for the precursors to become mature enough to enter the circulation, so approximately 18 to 21 days are required to produce a mature RBC from the BFU-E.

**Erythroid Precursors**

Normoblastic proliferation, similar to the proliferation of other cell lines, is a process encompassing replication (i.e., division) to increase cell numbers and development from immature to mature cell stages (Figure 8-1). The earliest morphologically recognizable erythroid progenitor, the pronormoblast, is derived via the BFU-E and CFU-E from the pluripotential stem cells, as discussed in Chapter 7. The pronormoblast is able to divide, with each daughter cell maturing to the next stage of development, the basophilic normoblast. Each of these cells can divide, with each of its daughter cells maturing to the next stage, the polychromatophilic normoblast. Each of these cells also can divide and mature. In the erythrocyte cell line, there are typically three and occasionally as many as five divisions with subsequent nuclear and cytoplasmic maturation of the daughter cells, so from a single pronormoblast, 8 to 32 mature RBCs usually result. The conditions under which the number of divisions can be increased or reduced are discussed later.

The cellular activities at each stage of development described below occur in an orderly and sequential process. It is often likened to a computer program that once activated runs certain processes in a specified order at specified times. The details of the developmental program are becoming clearer, and selected details are provided in these descriptions.

### NORMOBLASTIC MATURATION

**Terminology**

RBCs are formally called erythrocytes. The nucleated precursors in the bone marrow are called erythroblasts. They also may be called normoblasts, which refers to developing nucleated cells (i.e., blasts) with normal appearance. This is in contrast to the abnormal appearance of the developing nucleated cells in megaloblastic anemia, in which the erythroblasts are called megaloblasts because of their large size.

Three nomenclatures are used for naming the erythroid precursors (Table 8-1). The erythroblast terminology is used primarily in Europe. Like the normoblastic terminology used more often in the United States, it has the advantage of being descriptive of the appearance of the cells. Some prefer the rubriblast terminology because it parallels the nomenclature used for granulocyte development (Chapter 12). Normoblastic terminology is used in this chapter.

### TABLE 8-1 Three Erythroid Precursor Nomenclature Systems

<table>
<thead>
<tr>
<th>Normoblastic</th>
<th>Rubriblastic</th>
<th>Erythroblastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronormoblast</td>
<td>Rubriblast</td>
<td>Proerythroblast</td>
</tr>
<tr>
<td>Basophilic normoblast</td>
<td>Prorubricyte</td>
<td>Basophilic erythroblast</td>
</tr>
<tr>
<td>Polychromatic (polychromatophilic) normoblast</td>
<td>Rubricyte</td>
<td>Polychromatic (polychromatophilic) erythroblast</td>
</tr>
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<td>Orthochromic normoblast</td>
<td>Metarubricyte</td>
<td>Orthochromic erythroblast</td>
</tr>
<tr>
<td>Polychromatic (polychromatophilic) erythrocyte*</td>
<td>Polychromatic (polychromatophilic) erythrocyte*</td>
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</tr>
<tr>
<td>Erythrocyte</td>
<td>Erythrocyte</td>
<td>Erythrocyte</td>
</tr>
</tbody>
</table>

*Polychromatic erythrocytes are called reticulocytes when observed with vital stains.
Criteria Used in Identification of the Erythroid Precursors

Morphologic identification of blood cells depends on a well-stained peripheral blood film or bone marrow smear (Chapters 16 and 17). In hematology, a modified Romanowsky stain, such as Wright or Wright-Giemsa, is commonly used. The descriptions that follow are based on the use of these types of stains.

The stage of maturation of any blood cell is determined by careful examination of the nucleus and the cytoplasm. The qualities of greatest importance in identification of RBCs are the nuclear chromatin pattern (texture, density, homogeneity), nuclear diameter, nucleus:cytoplasm (N:C) ratio (Box 8-1), presence or absence of nucleoli, and cytoplasmic color.

As RBCs mature, several general trends affect their appearance. Figure 8-2 graphically represents these trends.

1. The overall diameter of the cell decreases.
2. The diameter of the nucleus decreases more rapidly than does the size of the cell. As a result, the N:C ratio also decreases.
3. The nuclear chromatin pattern becomes coarser, clumped, and condensed. The nuclear chromatin of RBCs is inherently coarser than that of myeloid precursors. It becomes even coarser and more clumped as the cell matures, developing a raspberry-like appearance, in which the dark staining of the chromatin is distinct from the almost white appearance of the parachromatin. This chromatin/parachromatin distinction is more dramatic than in other cell lines. Ultimately, the nucleus becomes quite condensed, with no parachromatin evident at all, and the nucleus is said to be pyknotic.
4. Nucleoli disappear. Nucleoli represent areas where the ribosomes are formed and are seen early in cell development as cells begin actively synthesizing proteins. As RBCs mature, the nucleoli disappear, which precedes the ultimate cessation of protein synthesis.

**BOX 8-1 Nucleus-to-Cytoplasm (N:C) Ratio**

The nucleus-to-cytoplasm (N:C) ratio is a morphologic feature used to identify and stage red blood cell and white blood cell precursors. The ratio is a visual estimate of what area of the cell is occupied by the nucleus compared with the cytoplasm. If the areas of each are approximately equal, the N:C ratio is 1:1. Although not mathematically proper, it is common for ratios other than 1:1 to be referred to as if they were fractions. If the nucleus takes up less than 50% of the area of the cell, the proportion of nucleus is lower, and the ratio is lower (e.g., 1:5 or less than 1). If the nucleus takes up more than 50% of the area of the cell, the ratio is higher (e.g., 3:1 or 3). In the red blood cell line, the proportion of nucleus shrinks as the cell matures and the cytoplasm increases proportionately, although the overall cell diameter grows smaller. In short, the N:C ratio decreases.
5. The cytoplasm changes from blue to gray-blue to salmon pink. Blueness or basophilia is due to acidic components that attract the basic stain, such as methylene blue. The degree of cytoplasmic basophilia correlates with the amount of ribosomal RNA. These organelles decline over the life of the developing RBC, and the blueness fades. Pinkness called *eosinophilia* or *acidophilia* is due to accumulation of more basic components that attract the acid stain eosin. Eosinophilia of erythrocyte cytoplasm correlates with the accumulation of hemoglobin as the cell matures. Thus the cell starts out being active in protein production on the ribosomes that make the cytoplasm basophilic, transitions through a period in which the red of hemoglobin begins to mix with that blue, and ultimately ends with a thoroughly salmon pink color when the ribosomes are gone and only hemoglobin remains.

**Maturation Sequence**

Table 8-2 lists the stages of RBC development in order and provides a convenient comparison. The listing makes it appear that these stages are clearly distinct and easily identifiable. The process of cell maturation is a gradual process, with changes occurring in a generally predictable sequence but with some variation for each individual cell. The identification of a given cell’s stage depends on the preponderance of characteristics, although the cell may not possess all the features of the archetypal descriptions that follow. Essential

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**Figure 8-2** General trends affecting the morphology of red blood cells during the developmental process. A, Cell diameter decreases and cytoplasm changes from blue to salmon pink. B, Nuclear diameter decreases and color changes from purplish-red to a very dark purple-blue. C, Nuclear chromatin becomes coarser, clumped, and condensed. D, Composite of changes during developmental process. (Modified from Diggs LW, Sturm D, Bell A: The morphology of human blood cells, ed 5, Abbott Park, Ill, 1985, Abbott Laboratories.)

<table>
<thead>
<tr>
<th>Table 8-2</th>
<th>Normoblastic Series: Summary of Stage Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell or Stage</strong></td>
<td><strong>Diameter</strong></td>
</tr>
<tr>
<td>Pronormoblast</td>
<td>12–20 μm</td>
</tr>
<tr>
<td>Basophilic normoblast</td>
<td>10–15 μm</td>
</tr>
<tr>
<td>Polychromatnic normoblast</td>
<td>10–12 μm</td>
</tr>
<tr>
<td>Orthochromatnic normoblast</td>
<td>8–10 μm</td>
</tr>
<tr>
<td>Bone marrow polychromatnic erythrocyte*</td>
<td>8–10 μm</td>
</tr>
</tbody>
</table>

*Also called reticulocyte.
features of each stage are in italics in the following descriptions. The cellular functions described subsequently also are summarized in Figure 8-3.

**Pronormoblast (Rubriblast)**

Figure 8-4 shows the pronormoblast.

**Nucleus.** The nucleus takes up much of the cell (N:C ratio of 8:1). The nucleus is round to oval, containing one or two nucleoli. The purple red chromatin is open and contains few, if any, fine clumps.

**Cytoplasm.** The cytoplasm is dark blue because of the concentration of ribosomes. The Golgi complex may be visible next to the nucleus as a pale, unstained area. Pronormoblasts may show small tufts of irregular cytoplasm along the periphery of the membrane.

**Division.** The pronormoblast undergoes mitosis and gives rise to two daughter pronormoblasts. More than one division is possible before maturation into basophilic normoblasts.

**Location.** The pronormoblast is present only in the bone marrow in healthy states.

**Cellular Activity.** The pronormoblast begins to accumulate the components necessary for hemoglobin production. The proteins and enzymes necessary for iron uptake and protoporphyrin synthesis are produced. Globin production begins.

**Length of Time in This Stage.** This stage lasts slightly more than 24 hours.

**Basophilic Normoblast (Prorubricyte)**

Figure 8-5 shows the basophilic normoblast.

**Nucleus.** The chromatin begins to condense, revealing clumps along the periphery of the nuclear membrane and a few in the interior. As the chromatin condenses, the parachromatin areas become larger and sharper, and the N:C ratio decreases to about 6:1. The chromatin stains deep purple-red. Nucleoli may be present early in the stage but disappear later.

**Figure 8-3** Changes in cellular diameter, RNA synthesis and content, DNA synthesis and content, protein and hemoglobin content during red blood cell development. **A,** Red blood cell diameter (solid line) shrinks from the pronormoblast to the reticulocyte stage. **B,** The rate of RNA synthesis (solid line) for protein production is at its peak at the pronormoblast stage and ends in the orthochromic normoblast stage. The RNA accumulates so that the RNA content (dashed line) remains relatively constant into the orthochromic normoblast stage when it begins to degrade, being eliminated by the end of the reticulocyte stage. **C,** The rate of DNA synthesis (solid line) correlates to those stages of development that are able to divide; the pronormoblast, basophilic normoblast, and early polychromatic normoblast stages. DNA content (dashed line) of a given cell remains relatively constant until the nucleus begins to break up and be extruded during the orthochromic normoblast stage. There is no DNA, i.e., no nucleus, in reticulocytes. **D,** The dashed line represents the total protein concentration which declines slightly during maturation. Proteins other than hemoglobin predominate in early stages. The hemoglobin concentration (solid line) begins to rise in the basophilic normoblast stage, reaching its peak in reticulocytes and representing most of the protein in more mature cells. Hemoglobin synthesis is visible as acidophilia (dotted line) that parallels hemoglobin accumulation but is delayed since the earliest production of hemoglobin in basophilic normoblasts is not visible microscopically. (Modified from Granick S, Levere RD: Heme synthesis in erythroid cells. In Moore CV, Brown EB, editors: Progress in hematology, New York, 1964, Grune & Stratton.)
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Cytoplasm. When stained, the cytoplasm may be a deeper, richer blue than in the pronormoblast—hence the name basophilic for this stage.

Division. The basophilic normoblast undergoes mitosis, giving rise to two daughter cells. More than one division is possible before the daughter cells mature into polychromatic normoblasts.

Location. The basophilic normoblast is present only in the bone marrow in healthy states.

Cellular Activity. Detectable hemoglobin synthesis occurs, but the many cytoplasmic organelles, including ribosomes and a substantial amount of messenger ribonucleic acid (RNA; chiefly for hemoglobin production), completely mask the minute amount of hemoglobin pigmentation.

Length of Time in This Stage. This stage lasts slightly more than 24 hours.

Polychromatic (Polychromatophilic) Normoblast (Rubricyte)

Figure 8-6 shows the polychromatic normoblast.

Nucleus. The chromatin pattern varies during this stage of development, showing some openness early in the stage but becoming condensed by the end. The condensation of chromatin reduces the diameter of the nucleus considerably, so the N:C ratio decreases from 4:1 to about 1:1 by the end of the stage. Notably, no nucleoli are present.

Cytoplasm. This is the first stage in which the pink color associated with stained hemoglobin can be seen. The stained color reflects the accumulation of hemoglobin pigmentation over time and concurrent decreasing amounts of RNA. The color produced is a mixture of pink and blue, resulting in a murky gray-blue. The stage’s name refers to this combination of multiple colors, because polychromatophilic means “many color loving.”
**Division.** This is the last stage in which the cell is capable of undergoing mitosis, although likely only early in the stage. The polychromatic normoblast goes through mitosis, producing daughter cells that mature and develop into orthochromic normoblasts.

**Location.** The polychromatic normoblast is present only in the bone marrow in healthy states.

**Cellular Activity.** Hemoglobin synthesis increases, and the accumulation begins to be visible in the color of the cytoplasm. Cellular organelles are still present, particularly ribosomes, which contribute a blue aspect to the cytoplasm. The progressive condensation of the nucleus and disappearance of nucleoli are evidence of progressive decline in transcription of deoxyribonucleic acid (DNA).

**Length of Time in This Stage.** This stage lasts approximately 30 hours.³

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**Orthochromic Normoblast (Metarubricyte)**

Figure 8-7 shows the orthochromic normoblast.

**Nucleus.** The nucleus is completely condensed (i.e., pyknotic) or nearly so. As a result, the N:C ratio is low or approximately 1:2.

**Cytoplasm.** The increase in the salmon-pink color of the cytoplasm reflects nearly complete hemoglobin production. The residual ribosomes react with the basic component of the stain and contribute a slightly bluish hue to the cell, but that fades toward the end of the stage as the organelles are degraded.

**Division.** The orthochromic normoblast is not capable of division due to the condensation of the chromatin.

**Location.** The orthochromic normoblast is present only in the bone marrow in healthy states.

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![Figure 8-6 A](image1) A, Polychromatic normoblast (rubricyte), bone marrow (Wright stain, ×1000). B, Electron micrograph of polychromatic normoblast (×15,575). (B from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders, an imprint of Elsevier Inc.)

![Figure 8-7 A](image2) A, Orthochromic normoblast (metarubricyte), bone marrow (Wright stain, ×1000). B, Electron micrograph of orthochromic normoblast (×20,125). (B from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders, an imprint of Elsevier Inc.)
Cellular Activity. Hemoglobin production continues on the remaining ribosomes using messenger RNA produced earlier. Late in this stage, the nucleus is ejected from the cell. The nucleus moves to the cell membrane and into a pseudopod-like projection. As part of the maturation program, loss of vimentin, a protein responsible for holding organelles in proper location in the cytoplasm, is probably important in the movement of the nucleus to the cell periphery. Ultimately, the nucleus-containing projection separates from the cell by having the membrane seal and pinch off the projection with the nucleus enveloped by cell membrane. Nonmuscle myosin of the membrane is important in this pinching process. The enveloped extruded nucleus, called a pyrenocyte, is then engulfed by bone marrow macrophages. The macrophages recognize phosphatidylserine on the pyenocyte surface as an “eat me” flag. Other organelles are extruded and ingested in similar fashion. Often, small fragments of nucleus are left behind if the projection is pinched off before the entire nucleus is enveloped. These fragments are called Howell-Jolly bodies when seen in peripheral blood cells (Table 19-3 and Figure 19-1) and are typically removed from the cells by the splenic macrophage pitting process once the cell enters the circulation.

Length of Time in This Stage. This stage lasts approximately 48 hours.

Polychromatic (Polychromatophilic) Erythrocyte or Reticulocyte

Figure 8-8 shows the polychromatic erythrocyte.

Nucleus. Beginning at the polychromatic erythrocyte stage, there is no nucleus. The polychromatic erythrocyte is a good example of the prior statement that a cell may not have all the classic features described but may be staged by the preponderance of features. In particular, when a cell loses its nucleus, regardless of cytoplasmic appearance, it is a polychromatic erythrocyte.

Cytoplasm. The cytoplasm can be compared with that of the late orthochromic normoblast in that the predominant color is that of hemoglobin. By the end of the polychromatic erythrocyte stage, the cell is the same color as a mature RBC, salmon pink. It remains larger than a mature cell, however. The shape of the cell is not the mature biconcave disc but is irregular in electron micrographs (Figure 8-8, B).

Division. Lacking a nucleus, the polychromatic erythrocyte cannot divide.

Location. The polychromatic erythrocyte resides in the bone marrow for 1 day or longer and then moves into the peripheral blood for about 1 day before reaching maturity. During the first several days after exiting the marrow, the polychromatic erythrocyte is retained in the spleen for pitting of inclusions and membrane polishing by splenic macrophages, which results in the biconcave discoid mature RBC.

Cellular Activity. The polychromatic erythrocyte completes production of hemoglobin from residual messenger RNA using the remaining ribosomes. The cytoplasmic protein production machinery is simultaneously being dismantled. Endoribonuclease, in particular, digests the ribosomes. The acidic components that attract the basophilic stain decline during this stage to the point that the polychromatophilia is not readily evident in the polychromatic erythrocytes on a normal peripheral blood film stained with Wright stain. A small amount of residual ribosomal RNA is present, however, and can be visualized with a vital stain such as new methylene blue, so called because the cells are stained while alive in suspension (i.e., vital), before the film is made (Box 8-2). The residual ribosomes appear as a mesh of small blue strands, a reticulum, or, when more fully digested, merely blue dots (Figure 8-9). When so stained, the polychromatic erythrocyte is called a reticulocyte. However, the name reticulocyte is often used to refer to the stage immediately preceding the mature
CHAPTER 8  Erythrocyte Production and Destruction

Erythrocyte

Figure 8-10 shows the erythrocyte.

Nucleus.  No nucleus is present in mature RBCs.

Cytoplasm.  The mature circulating erythrocyte is a biconcave disc measuring 7 to 8 μm in diameter, with a thickness of about 1.5 to 2.5 μm. On a stained blood film, it appears as a salmon pink-staining cell with a central pale area that corresponds to the concavity. The central pallor is about one third the diameter of the cell.

Division.  The erythrocyte cannot divide.

Location and Length of Time in This Stage.  Mature RBCs remain active in the circulation for approximately 120 days. Aging leads to their removal by the spleen as described subsequently.

Cellular Activity.  The mature erythrocyte delivers oxygen to tissues, releases it, and returns to the lung to be reoxygenated. The dynamics of this process are discussed in detail in Chapter 10. The interior of the erythrocyte contains mostly hemoglobin, the oxygen-carrying component. It has a surface-to-volume ratio and shape that enable optimal gas exchange to occur. If the cell were to be spherical, it would have hemoglobin at the center of the cell that would be relatively distant from the membrane and would not be readily oxygenated and deoxygenated. With the biconcave shape, even hemoglobin molecules that are toward the center of the cell are not distant from the membrane and are able to exchange oxygen.

The cell’s main function of oxygen delivery throughout the body requires a membrane that is flexible and deformable—that is, able to flex but return to its original shape. The interaction of various membrane components described in Chapter 9 creates these properties. RBCs must squeeze through small spaces such as the basement membrane of the bone marrow venous sinus. Similarly, when a cell enters the red pulp of the spleen, it must squeeze between epithelial cells to move into the venous outflow. Deformability is crucial for RBCs to enter and subsequently remain in the circulation.

ERYTHROKINETICS

Erythrokinetics is the term describing the dynamics of RBC production and destruction. To understand erythrokinetics, it is helpful to appreciate the concept of the erythron. Erythron is the name given to the collection of all stages of erythrocytes throughout the body: the developing precursors in the bone marrow and the circulating erythrocytes in the peripheral blood and the vascular spaces within specific organs such as the spleen. When the term erythron is used, it conveys the concept of a unified functional tissue. The erythron is distinguished from the RBC mass. The erythron is the entirety of erythroid cells in the body, whereas the RBC mass refers only to the cells in circulation. This discussion of erythrokinetics begins by looking at the erythrocytes in the bone marrow and

erythrocyte, even when stained with Wright stain and without demonstrating the reticulum.

A second functional change in polychromatic erythrocytes is the reduced production of receptors for the adhesive molecules that hold developing RBCs in the marrow (see details later). As these receptors decline, cells are freed to leave the marrow.

Length of Time in This Stage.  The cell typically remains a polychromatic erythrocyte for about 3 days, with the first 2 days spent in the marrow and the third spent in the peripheral blood, although possibly sequestered in the spleen.

BOX 8-2  Cellular Basophilia: Diffuse and Punctate

The reticulum of a polychromatic erythrocyte (reticulocyte) is not seen using Wright stain. The residual RNA imparts the bluish tinge to the cytoplasm seen in Figure 8-8, A. Based on the Wright-stained appearance, the reticulocyte is called a polychromatic erythrocyte because it lacks a nucleus and is no longer an erythroblast but has a bluish tinge. When polychromatic erythrocytes are prominent on a peripheral blood film, the examiner uses the comment polychromasia or polychromatophilia. Wright-stained polychromatic erythrocytes are also called diffusely basophilic erythrocytes for their regular bluish tinge. This term distinguishes polychromatic erythrocytes from red blood cells with punctate basophilia, in which the blue appears in distinct dots throughout the cytoplasm. More commonly known as basophilic stippling (Table 19-3 and Figure 19-1), punctate basophilia is associated with some anemias. Similar to the basophilia of polychromatic erythrocytes, punctate basophilia is due to residual ribosomal RNA, but the RNA is degenerate and stains deeply with Wright stain.

Figure 8-9  Reticulocytes at arrows, peripheral blood (new methylene blue stain, ×1000).

Figure 8-10  Erythrocyte.
the factors that affect their numbers, their progressive development, and their ultimate release into the blood.

**Hypoxia—the Stimulus to Red Blood Cell Production**

As mentioned previously, the role of RBCs is to carry oxygen. To regulate the production of RBCs for that purpose, the body requires a mechanism for sensing whether there is adequate oxygen being carried to the tissues. If not, RBC production and the functional efficiency of existing cells must be enhanced. Thus a second feature of the oxygen-sensing system must be a mechanism for influencing the production of RBCs.

The primary oxygen-sensing system of the body is located in peritubular fibroblasts of the kidney. Hypoxia, too little tissue oxygen, is detected by the peritubular cells, which produce erythropoietin (EPO), the major stimulatory cytokine for RBCs. Under normal circumstances, the amount of EPO produced fluctuates very little, maintaining a level of RBC production that is sufficient to replace the approximately 1% of RBCs that normally die each day (see section on erythrocyte destruction).

When there is hemorrhage, increased RBC destruction, or other factors that diminish the oxygen-carrying capacity of the blood (Box 8-3), the production of EPO is increased.

Hypoxia increases EPO production in peritubular cells mainly by transcriptional regulation. The EPO gene has a hypoxia-sensitive region (enhancer) in its 3’ regulatory component. When oxygen tension in the cell decreases, hypoxia-inducible factor-1, a transcription factor, is assembled in the cytoplasm, migrates to the nucleus, and interacts with the 3’ enhancer of the gene. This results in transcription of more EPO messenger RNA molecules, and production of more EPO.

**Erythropoietin**

**Structure.** EPO is a thermostable, nondialyzable, glycoprotein hormone with a molecular weight of 34 kD. It consists of a carbohydrate unit that reacts specifically with RBC receptors and a terminal sialic acid unit, which is necessary for biological activity in vivo. On desialation, EPO activity ceases.

**Box 8-3 Hypoxia and Red Blood Cell Production**

Teleologically speaking, the location of the body’s hypoxia sensor in the kidney is practical, because the kidney receives approximately 20% of the cardiac output with little loss of oxygen from the levels leaving the heart. The location provides early detection when oxygen levels decline. Making the hypoxia sensor the cell that is able to stimulate red blood cell (RBC) production also is practical, because regardless of the cause of hypoxia, having more RBCs should help to overcome it. The hypoxia might result from decreased RBC numbers, as with hemorrhage.

Decreased RBC number, however, is only one cause of hypoxia. Another cause is the failure of each RBC to carry as much oxygen as it should. This can occur because the hemoglobin is defective or because there is not enough hemoglobin in each cell. The hypoxia may be unrelated to the RBCs in any way; poor lung function resulting in diminished oxygenation of existing RBCs is an example.

The kidney’s hypoxia sensor cannot know why there is hypoxia, but it does not matter. Even when there are plenty of RBCs compared with the reference interval, if there is still hypoxia, stimulation of RBC production is warranted because the numbers present are not meeting the oxygen need. An elevation of RBC numbers above the reference interval, erythrocytosis, is seen in conditions such as lung disease and cardiac disease in which the blood is not being well oxygenated. Newborns have higher numbers of RBCs because the fetal hemoglobin in their cells does not unload oxygen to the tissues readily, so newborns are slightly hypoxic compared with adults. To compensate, they make more RBCs.

**Action.** EPO is a true hormone, being produced at one location (the kidney) and acting at a distant location (the bone marrow). It is a growth factor (or cytokine) that initiates an intracellular message to the developing RBCs; this process is called signal transduction. EPO must bind to its receptor on the surface of cells to initiate the signal or message (Figure 33-9). The receptor is a transmembrane homodimer consisting of two identical polypeptide chains. EPO-responsive cells vary in their sensitivity to EPO. Some are able to respond to low levels of EPO, whereas others require higher levels. In healthy circumstances when RBC production needs to proceed at a modest but regular rate, the cells requiring only low levels of EPO respond. If EPO levels rise secondary to hypoxia, however, a larger number of RBCs are produced, and more red blood cells enter the circulation. EPO’s effects are mediated by Janus-activated tyrosine kinase 2 (JAK2) signal transducers that are associated with the cytoplasmic domain of the EPO receptor and ultimately affect gene expression in the RBC nucleus (Figure 33-9).

The binding of EPO, the ligand, to its receptor on erythrocyte progenitors initiates a cascade of intracellular events ("the program") that ultimately leads to cell division, maturation, and more red blood cells entering the circulation. EPO’s effects are mediated by Janus-activated tyrosine kinase 2 (JAK2) signal transducers that are associated with the cytoplasmic domain of the EPO receptor and ultimately affect gene expression in the RBC nucleus (Figure 33-9).

Early Release of Reticulocytes. EPO promotes early release of developing erythroid precursors from the marrow by two mechanisms. EPO induces changes in the adventitial cell layer of the marrow/sinus barrier that increase the width of the spaces for RBC egress into the sinus. This mechanism alone, however, is insufficient for cells to leave the marrow. RBCs are held in the marrow because they express surface membrane receptors for adhesive molecules located on the bone marrow stroma. EPO downregulates the expression of these receptors so that cells can exit the marrow earlier than they normally would. The result is the presence in the circulation of reticulocytes that are still very basophilic because they have not spent as much time degrading their ribosomes or making hemoglobin as they normally would before entering the bloodstream. These are called shift reticulocytes because they have been shifted from the bone marrow early (Figure 8-8, A). Their bluish cytoplasm with Wright stain is evident, so the overall blood picture is said to have polychromasia. Even nucleated RBCs (i.e., normoblasts) can be released early in cases of extreme anemia when the demand for RBCs in the peripheral circulation is great. Releasing cells from the marrow early is a quick fix, so to speak; it is limited in effectiveness because the available precursors in the marrow are depleted within several days and still may not be enough to meet the need in the peripheral blood for more cells. A more sustained response is required in times of increased need for RBCs in the circulation.

**Inhibition of Apoptosis.** A second, and probably more important, mechanism by which EPO increases the number of circulating RBCs is by increasing the number of cells that will be able to mature into circulating erythrocytes. It does this by decreasing apoptosis, the programmed death of RBC progenitors. The mechanism by which EPO inhibits apoptosis is not fully understood. However, the body has a way to prepare for such a need and to maintain a store of mature RBCs in the body for emergencies. RBCs cannot be stored in the body for this sort of eventual use, however, because they have a limited life span. Therefore, instead of storing mature cells for emergencies, the body produces more CFU-Es than needed at all times. When there is a basal or steady-state demand for RBCs, the extra progenitors are available to die. When there is an increased demand for RBCs, however, the RBC progenitors have about an 8- to 10-day head start in the production process. This process of intensional wastage of cells occurs by apoptosis, and it is part of the cell’s genetic program.

**Process of apoptosis.** Apoptosis is a sequential process characterized by, among other things, the degradation of chromatin into fragments of varying size that are multiples of 180 to 185 base pairs long; protein clustering; and activation of transglutamate. This is in contrast to necrosis, in which cell injury causes swelling and lysing with release of cytoplasmic contents that stimulate an inflammatory response (Chapter 6). Apoptosis is not associated with inflammation.

During the sequential process of apoptosis, the following morphologic changes can be seen: condensation of the nucleus, causing increased basophilic staining of the chromatin; nucleolar disintegration; and shrinkage of cell volume with concomitant increase in cell density and compaction of cytoplasmic organelles, while mitochondria remain normal. This is followed by a partition of cytoplasm and nucleus into membrane-bound apoptotic bodies that contain varying amounts of ribosomes, organelles, and nuclear material. The last stage of degradation produces nuclear DNA fragments consisting of multimers of 180 to 185 base pair segments. Characteristic blebbing of the plasma membrane is observed. The apoptotic cell contents remain membrane-bound and are ingested by macrophages, which prevents an inflammatory reaction. The membrane-bound vesicles display so-called "eat me" signals on the membrane surface (discussed later) that promote macrophage ingestion.

**Evasion of apoptosis by erythroid progenitors and precursors.** Thus, under normal circumstances, many red cell progenitors will undergo apoptosis. However, when increased numbers of red cells are needed, apoptosis can be avoided. One effect of EPO is an indirect avoidance of apoptosis by removing an apoptosis induction signal. Apoptosis of RBCs is a cellular process that depends on a signal from either the inside or outside of the cell. Among the crucial molecules in the external messaging system is the death receptor Fas on the membrane of the earliest RBC precursors, while its ligand, FasL, is expressed by
more mature RBCs. When EPO levels are low, cell production should be at a low rate because hypoxia is not present. The excess early erythroid precursors should undergo apoptosis. This occurs when the older FasL-bearing erythroid precursors, such as polychromatophilic normoblasts, cross-link with Fas-marked immature erythroid precursors, such as pronormoblasts and basophilic normoblasts, which are then stimulated to undergo apoptosis. As long as the more mature cells with FasL are present in the marrow, erythropoiesis is subdued. If the FasL-bearing cells are depleted, as when EPO stimulates early marrow release, the younger Fas-positive precursors are allowed to develop, which increases the overall output of RBCs from the marrow. Thus early release of older cells in response to EPO indirectly allows more of the younger cells to mature.

A second mechanism for escaping apoptosis exists for RBC progenitors: direct EPO rescue from apoptosis. This is the major way in which EPO is able to increase RBC production. When EPO binds to its receptor on the CFU-E, one of the effects is to reduce production of Fas ligand. Thus the younger cells avoid the apoptotic signal from the older cells. Additionally, EPO is able to stimulate production of various anti-apoptotic molecules, which allows the cell to survive and mature. The cell that has the most EPO receptors and is most sensitive to EPO rescue is the CFU-E, although the late BFU-E and early pronormoblast have some receptors. Without EPO, the CFU-E does not survive.

The binding of EPO to its transmembrane receptors on erythroid progenitors and precursors activates JAK2 protein associated with its cytoplasmic domain (Figure 33-9). Activated JAK2 then phosphorylates (activates) the signal transduction and activator of transcription (STAT) pathway, leading to the production of the anti-apoptotic molecule Bcl-XL (now called Bcl-2 like protein 1). EPO-stimulated cells develop this molecule on their mitochondrial membranes, preventing release of cytochrome c, an apoptosis initiator. EPO’s effect is mediated by the transcription factor GATA-1, which is essential to red cell survival.

Reduced marrow transit time. Apoptosis rescue is the major way in which EPO increases RBC mass—by increasing the number of erythroid cells that survive and mature to enter the circulation. Another effect of EPO is to increase the rate at which the surviving precursors can enter the circulation. This is accomplished by two means: increased rate of cellular processes and decreased cell cycle times.

EPO stimulates the synthesis of RBC RNA and effectively increases the rate of the developmental “program.” Among the processes that are accelerated is hemoglobin production. As mentioned earlier, another accelerated process is bone marrow egress with the loss of adhesive receptors and the acquisition of egress-promoting surface molecules. The other process that is accelerated is the cessation of division. Cell division takes time and would delay entry of cells to the circulation. This occurs when the older FasL-bearing erythroid precursors, such as polychromatophilic normoblasts, cross-link with Fas-marked immature erythroid precursors, such as pronormoblasts and basophilic normoblasts, which are then stimulated to undergo apoptosis. As long as the more mature cells with FasL are present in the marrow, erythropoiesis is subdued. If the FasL-bearing cells are depleted, as when EPO stimulates early marrow release, the younger Fas-positive precursors are allowed to develop, which increases the overall output of RBCs from the marrow. Thus early release of older cells in response to EPO indirectly allows more of the younger cells to mature.

Measurement of Erythropoietin. Quantitative measurements of EPO are performed on plasma and other body fluids. EPO can be measured by chemiluminescence. Although the reference interval for each laboratory varies, an example reference interval is 4 to 27 mU/L. Increased amounts of EPO in the urine are expected in most patients with anemia, with the exception of patients with anemia caused by renal disease.

Therapeutic Uses of Erythropoietin. Recombinant erythropoietin is used as therapy in certain anemias such as those associated with chronic kidney disease and chemotherapy. It is also used to stimulate RBC production prior to autologous blood donation and after bone marrow transplantation. The indications for EPO therapy are summarized in Table 7-2.

Unfortunately, some athletes illicitly use EPO injections to increase the oxygen-carrying capacity of their blood to enhance endurance and stamina, especially in long-distance running and cycling. The use of EPO is one of the methods of blood doping, and aside from being banned in organized sports events, it increases the RBC count and blood viscosity to dangerously high levels and can lead to fatal arterial and venous thrombosis.

Other Stimuli to Erythropoiesis
In addition to tissue hypoxia, other factors influence RBC production to a modest extent. It is well documented that testosterone directly stimulates erythropoiesis, which partially explains the higher hemoglobin concentration in men than in women. Also, pituitary and thyroid hormones have been shown to affect the production of EPO and so have indirect effects on erythropoiesis.

MICROENVIRONMENT OF THE BONE MARROW

The microenvironment of the bone marrow is described in Chapter 7, and the cytokines essential to hematopoiesis are...
discussed there. Here, the details pertinent to erythropoiesis (i.e., the erythropoietic inductive microenvironment) are emphasized, including the locale and arrangement of erythroid cells and the anchoring molecules involved.

Hematopoiesis occurs in marrow cords, essentially a loose arrangement of cells outside a dilated sinus area between the arterioles that feed the bone and the central vein that returns blood to efferent veins. Erythropoiesis typically occurs in what are called erythroid islands (Figure 7-6). These are macrophages surrounded by erythroid precursors in various stages of development. It was previously believed that these macrophages provided iron directly to the normoblasts for the synthesis of hemoglobin. This was termed the suckling pig phenomenon. However, since developing RBCs obtain iron via transferrin (Chapter 11), no direct contact with macrophages is needed for this. Macrophages are now known to elaborate cytokines that are vital to the maturation process of the RBCs.44-46 RBC precursors would not survive without macrophage support via such stimulation.

A second role for macrophages in erythropoiesis also has been identified. Although movement of cells through the marrow cords is sluggish, developing cells would exit the marrow prematurely in the outflow were it not for an anchoring system within the marrow that holds them there until development is complete. There are three components to the anchoring system: a stable matrix of accessory and stromal cells to which normoblasts can attach, bridging (adhesive) molecules for that attachment, and receptors on the erythrocyte membrane.

The major cellular anchor for the RBCs is the macrophage. Several systems of adhesive molecules and RBC receptors tie the developing RBCs to the macrophages.44 At the same time, RBCs are anchored to the extracellular matrix of the bone marrow, chiefly by fibronectin.9

When it comes time for the RBCs to leave the marrow, they cease production of the receptors for the adhesive molecules.9 Without the receptor, the cells are free to move from the marrow into the venous sinus. Entering the venous sinus requires the RBC to traverse the barrier created by the adventitial cells on the cord side, the basement membrane, and the endothelial cells lining the sinus. Egress through this barrier occurs between adventitial cells, through holes (fenestrations) in the basement membrane, and through pores in the endothelial cells9 (Figure 8-11).24,47,48

**Figure 8-11** Egress of a red blood cell through a pore in an endothelial cell of the bone marrow venous sinus. Arrowheads indicate the endothelial cell junctions. (From DeBruyn PPH: Structural substrates of bone marrow function, Semin Hematol 18:182, 1981.)

SENESCOENCE, which culminates in phagocytosis by macrophages. This is the major way in which RBCs die normally.

**Macrophage-Mediated Hemolysis (Extravascular Hemolysis)**

At any given time, a substantial volume of blood is in the spleen, which generates an environment that is inherently stressful on cells. Movement through the red pulp is sluggish. The available glucose in the surrounding plasma is depleted quickly as the cell flow stagnates, so glycolysis slows. The pH is low, which promotes iron oxidation. Maintaining reduced iron is an energy-dependent process, so factors that promote iron oxidation cause the RBC to expend more energy and accelerate the catabolism of enzymes.

In this hostile environment, aged RBCs succumb to the various stresses. Their deteriorating glycolytic processes lead to reduced ATP production, which is complicated further by diminished amounts of available glucose. The membrane systems that rely on ATP begin to fail. Among these are enzymes that maintain the location and reduction of phospholipids of the membrane. Lack of ATP leads to oxidation of membrane lipids and proteins. Other ATP-dependent enzymes are responsible for maintaining the high level of intracellular potassium while pumping sodium out of the cells. As this system fails, intracellular sodium increases and potassium decreases. The effect is that the selective permeability of the membrane is lost and water enters the cell. The discoid shape is lost and the cell becomes a sphere.

RBCs must remain highly flexible to exit the spleen by squeezing through the so-called splenic sieve formed by the endothelial cells lining the venous sinuses and the basement membrane. Spherical RBCs are rigid and are not able to squeeze through the narrow spaces; they become trapped

**ERYTHROCYTE DESTRUCTION**

All cells experience the deterioration of their enzymes over time due to natural catabolism. Most cells are able to replenish needed enzymes and continue their cellular processes. As a nonnucleated cell, however, the mature erythrocyte is unable to generate new proteins, such as enzymes, so as its cellular functions decline, the cell ultimately approaches death. The average RBC has sufficient enzyme function to live 120 days. Because RBCs lack mitochondria, they rely on glycolysis for production of adenosine triphosphate (ATP). The loss of glycolytic enzymes is central to this process of cellular aging, called
against the endothelial cells and basement membrane. In this situation, they are readily ingested by macrophages that patrol along the sinusoidal lining (Figure 8-12).

Some researchers view erythrocyte death as a nonnucleated cell version of apoptosis, termed eryptosis, that is precipitated by oxidative stress, energy depletion, and other mechanisms that create membrane signals that stimulate phagocytosis. It is highly likely that there is no single signal but rather that macrophages recognize several. Examples of the signals generating continuing research interest include binding of autologous immunoglobulin G (IgG) to band-3 membrane protein clusters, exposure of phosphatidylserine on the exterior (plasma side) of the membrane, and inability to maintain cation balance. Senescent cells and distinguish them from younger cells; thus the older cells are targeted for ingestion and lysis.

When an RBC lyses within a macrophage, the major components are catabolized. The iron is removed from the heme. It can be stored in the macrophage as ferritin until transported out. The globin of hemoglobin is degraded and returned to the metabolic amino acid pool. The protoporphyrin component of heme is degraded through several intermediaries to bilirubin, which is released into the plasma and ultimately excreted by the liver in bile. The details of bilirubin metabolism are discussed in Chapter 23.

**Mechanical Hemolysis (Fragmentation or Intravascular Hemolysis)**

Although most natural RBC deaths occur in the spleen, a small portion of RBCs rupture intravascularly (within the lumen of blood vessels). The vascular system can be traumatic to RBCs, with turbulence occurring in the chambers of the heart or at points of bifurcation of vessels. Small breaks in blood vessels and resulting clots can also trap and rupture cells. The intravascular rupture of RBCs from purely mechanical or traumatic stress results in fragmentation and release of the cell contents into the plasma; this is called fragmentation or intravascular hemolysis.

When the membrane of the RBC has been breached, regardless of where the cell is located when it happens, the cell contents enter the surrounding plasma. Although mechanical lysis is a relatively small contributor to RBC demise under normal circumstances, the body still has a system of plasma proteins, including haptoglobin and hemopexin, to salvage the released hemoglobin so that its iron is not lost in the urine. Hemolysis and the functions of haptoglobin and hemopexin are discussed in Chapter 23.

**SUMMARY**

- RBCs develop from committed erythroid progenitor cells in the bone marrow, the BFU-E and CFU-E.
- The morphologically identifiable precursors of mature RBCs, in order from youngest to oldest, are the pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatophilic normoblast, and polychromatophilic erythrocyte or reticulocyte.
- As erythroid precursors age, the nucleus becomes condensed and ultimately is ejected from the cell, which produces the polychromatophilic erythrocyte or reticulocyte stage. The cytoplasm changes color from blue, reflecting numerous ribosomes, to salmon-pink as hemoglobin accumulates and the ribosomes are degraded. Each stage can be identified by the extent of these nuclear and cytoplasmic changes.
- It takes approximately 18 to 21 days for the BFU-E to mature to an RBC, of which about 6 days are spent as identifiable precursors in the bone marrow. The mature erythrocyte has a life span of 120 days in the circulation.
- Hypoxia of peripheral blood is detected by the peritubular fibroblasts of the kidney, which upregulates transcription of the EPO gene to increase the production of EPO.
- EPO, the primary hormone that stimulates the production of erythrocytes, is able to rescue the CFU-E from apoptosis, shorten the time between mitoses of precursors, release reticulocytes from the marrow early, and reduce the number of mitoses of precursors.
- Apoptosis is the mechanism by which an appropriate normal production level of cells is controlled. Fas, the death receptor, is expressed by young normoblasts, and FasL, the ligand, is expressed by older normoblasts. As long as older cells mature slowly in the marrow, they induce the death of unneeded younger cells.
- EPO rescues cells from apoptosis by stimulating the production of anti-apoptotic molecules that counteract the effects of Fas and FasL and simultaneously decreasing Fas production by young normoblasts.
- Survival of RBC precursors in the bone marrow depends on adhesive molecules, such as fibronectin, and cytokines that are elaborated by macrophages and other bone marrow stromal cells. RBCs are found in erythroid islands, where erythroblasts at various stages of maturation surround a macrophage.
- As RBC precursors mature, they lose adhesive molecule receptors and can leave the bone marrow. Egress occurs between

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**Figure 8-12** Macrophage ingesting a spherocytic erythrocyte. (From Bessis M: Corpuscles, atlas of RBC shapes, New York, 1974, Springer-Verlag.)
adventitial cells but through pores in the endothelial cells of the venous sinus.

- Aged RBCs, or senescent cells, cannot regenerate catabolized enzymes because they lack a nucleus. The semipermeable membrane becomes more permeable to water, so the cell swells and becomes spherocytic and rigid. It becomes trapped in the splenic sieve.
- Extravascular or macrophage-mediated hemolysis accounts for most normal RBC death. The signals to macrophages that initiate RBC ingestion may include binding of autologous IgG, expression of phosphatidylserine on the outer membrane, cation balance changes, and CD47-thrombospondin 1 binding.
- Fragmentation or intravascular hemolysis results when mechanical factors rupture the cell membrane while the cell is in the peripheral circulation. This pathway accounts for a minor component of normal destruction of RBCs.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

### REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Which of the following is an erythrocyte progenitor?
   a. Pronormoblast
   b. Reticulocyte
   c. CFU-E
   d. Orthochromic normoblast

2. Which of the following is the most mature normoblast?
   a. Orthochromic normoblast
   b. Basophilic normoblast
   c. Pronormoblast
   d. Polychromatophilic erythroblast

3. What erythroid precursor can be described as follows: the cell is of medium size compared with other normoblasts, with an N:C ratio of nearly 1:1. The nuclear chromatin is condensed and chunky throughout the nucleus. No nucleoli are seen. The cytoplasm is a muddy, blue-pink color.
   a. Reticulocyte
   b. Pronormoblast
   c. Orthochromic normoblast
   d. Polychromatophilic erythroblast

4. Which of the following is not related to the effects of erythropoietin?
   a. The number of divisions of a normoblast
   b. The formation of pores in sinusoidal endothelial cells for marrow egress
   c. The time between mitoses of normoblasts
   d. The production of antiapoptotic molecules by erythroid progenitors

5. Hypoxia stimulates RBC production by:
   a. Inducing more pluripotent stem cells into the erythroid lineage
   b. Stimulating EPO production by the kidney
   c. Increasing the number of RBC mitoses
   d. Stimulating the production of fibronectin by macrophages of the bone marrow

6. In the bone marrow, RBC precursors are located:
   a. In the center of the hematopoietic cords
   b. Adjacent to megakaryocytes along the adventitial cell lining
   c. Surrounding fat cells in apoptotic islands
   d. Surrounding macrophages in erythroid islands

7. Which of the following determines the timing of egress of RBCs from the bone marrow?
   a. Maturing normoblasts slowly lose receptors for adhesive molecules that bind them to stromal cells.
   b. Stromal cells decrease production of adhesive molecules over time as RBCs mature.
   c. Endothelial cells of the venous sinus form pores at specified intervals of time, allowing egress of free cells.
   d. Periodic apoptosis of pronormoblasts in the marrow cords occurs.

8. What single feature of normal RBCs is most responsible for limiting their life span?
   a. Loss of mitochondria
   b. Increased flexibility of the cell membrane
   c. Reduction of hemoglobin iron
   d. Loss of the nucleus

9. Intravascular or fragmentation hemolysis is the result of trauma to RBCs while in the circulation.
   a. True
   b. False

10. Extravascular hemolysis occurs when:
    a. RBCs are mechanically ruptured
    b. RBCs extravasate from the blood vessels into the tissues
    c. Splenic macrophages ingest senescent cells
    d. Erythrocytes are trapped in blood clots outside the blood vessels
11. A pronormoblast in its usual location belongs to the RBC mass of the body, but not to the erythron.  
   a. True  
   b. False  
12. A cell has an N:C ratio of 4:1. Which of the following statements would describe it?  
   a. The bulk of the cell is composed of cytoplasm.  
   b. The bulk of the cell is composed of nucleus.  
   c. The proportions of cytoplasm and nucleus are roughly equal.  

REFERENCES  
Cyanosis is blue skin coloration, visible in Caucasians, that occurs when the blood does not deliver enough oxygen to the tissues. It is a common sign of heart or lung disease, in which the blood fails to become oxygenated or is distributed improperly throughout the body. In the 1940s, Dr. James Deeny, an Irish physician, was experimenting with the use of vitamin C (ascorbic acid), a potent reducing agent, as a treatment for heart disease. To his disappointment, it was ineffective for nearly all patients. However, he discovered two brothers with the distinction of being truly blue men. When he treated them with vitamin C, each turned a healthy pink. Neither man was determined to have either heart or lung disease.

1. What does it mean to say that vitamin C is a reducing agent?
2. What must be happening if vitamin C was able to cure the cyanosis?
3. What is the significance of finding this condition in brothers?
The erythrocyte (red blood cell, RBC) is the primary blood cell, circulating at 5 million RBCs per microliter of blood on average. It is anucleate and biconcave and has an average volume of 90 fL. The cytoplasm provides abundant hemoglobin, a complex of globin, protoporphyrin, and iron that transports elemental oxygen \((O_2)\) from high partial pressure environments, that is, from lung capillaries to the capillaries of organs and tissues. Hemoglobin, plasma proteins, and additional RBC proteins also transport molecular carbon dioxide \((CO_2)\) and bicarbonate \((HCO_3^-)\) from the tissues to the lungs. Hemoglobin is composed of four globin molecules, each supporting one heme molecule; each heme molecule contains a molecule of iron (Chapters 10 and 11). The biconcave RBC shape supports deformation, enabling the circulating cell to pass smoothly through capillaries, where it readily exchanges \(O_2\) and \(CO_2\) while contacting the vessel wall.

RBCs are produced through erythroid (normoblastic) maturation in bone marrow tissue (Chapter 8). The nucleus, while present in maturing normoblasts, becomes extruded as the cell passes from the bone marrow to peripheral blood. Cytoplasmic ribosomes and mitochondria disappear 24 to 48 hours after bone marrow release, eliminating the cells’ ability to produce proteins or support oxidative metabolism. Adenosine triphosphate (ATP) is produced within the cytoplasm through anaerobic glycolysis (Embden-Meyerhof pathway, EMP) for the lifetime of the cell. ATP drives mechanisms that slow the destruction of protein and iron by environmental peroxides and superoxide anions, maintaining hemoglobin’s function and membrane integrity. Oxidation, however, eventually takes a toll, limiting the RBC circulating life span to 120 days, whereupon the cell becomes disassembled into its reusable components globin, iron, and the phospholipids and proteins of the cell membrane, while the protoporphyrin ring is excreted as bilirubin (Chapters 8 and 23).

This chapter is one of a series of four that present the physiology of normal RBC production, structure, function, and senescence. These include Chapter 8, Erythrocyte Production and Destruction; Chapter 10, Hemoglobin Metabolism; and Chapter 11, Iron Kinetics and Laboratory Assessment. This chapter describes RBC energy production, the protective mechanisms that preserve structure and function, and the structure, function, deformability, and maintenance of the cell membrane. Taken as a unit, these four chapters form the basis for understanding RBC disorders (anemias), as described in Chapters 19 through 28.

### BOX 9-1 Erythrocyte Metabolic Processes Requiring Energy

<table>
<thead>
<tr>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular cationic gradient maintenance</td>
</tr>
<tr>
<td>Maintenance of membrane phospholipid distribution</td>
</tr>
<tr>
<td>Maintenance of skeletal protein deformability</td>
</tr>
<tr>
<td>Maintenance of functional hemoglobin with ferrous iron</td>
</tr>
<tr>
<td>Protecting cell proteins from oxidative denaturation</td>
</tr>
<tr>
<td>Glycolysis initiation and maintenance</td>
</tr>
<tr>
<td>Glutathione synthesis</td>
</tr>
<tr>
<td>Nucleotide salvage reactions</td>
</tr>
</tbody>
</table>

Glucose enters the RBC without energy expenditure via the transmembrane protein Glut-1. Anaerobic glycolysis, the EMP (Figure 9-1), requires glucose to generate ATP, a high-energy phosphate source. Without cytoplasmic glycogen organelles, RBCs lack internal energy stores and rely on plasma glucose for glycolysis-generated ATP. Through the EMP, glucose is catabolized to pyruvate (pyruvic acid), consuming two molecules of ATP per molecule of glucose and maximally generating four molecules of ATP per molecule of glucose, for a net gain of two molecules of ATP.

The sequential list of biochemical intermediates involved in glucose catabolism, with corresponding enzymes, is given in Figure 9-1. Tables 9-1 through 9-3 organize glycolysis into three phases.

The first phase of glycolysis employs glucose phosphorylation, isomerization, and diphosphorylation to yield fructose 1,6-bisphosphate (F1,6-BP). Fructose-bisphosphate aldolase cleaves F1,6-BP to produce glyceraldehyde-3-phosphate (G3P; Figure 9-1 and Table 9-1). Intermediate stages employ, in order, the enzymes hexokinase, glucose-6-phosphate isomerase, and 6-phosphofructokinase. The initial hexokinase and 6-phosphofructokinase steps consume a total of 2 ATP molecules and limit the rate of glycolysis.

The second phase of glucose catabolism converts G3P to 3-phosphoglycerate (3-PG). The substrates, enzymes, and products for this phase of glycolytic metabolism are summarized in Table 9-2. In the first step, G3P is oxidized to 1,3-bisphosphoglycerate (1,3-BPG) through the action of glyceraldehyde-3-phosphate dehydrogenase (G3PD). 1,3-BPG is diphosphorylated by phosphoglycerate kinase, which generates 2 ATP molecules and 3-PG.

The third phase of glycolysis converts 3-PG to pyruvate and generates ATP. Substrates, enzymes, and products are listed in Table 9-3. The product 3-PG is isomerized by phosphoglycerate mutase to 2-phosphoglycerate (2-PG). Enolase (phosphopyruvate hydratase) then converts 2-PG to phosphoenolpyruvate (PEP). Pyruvate kinase (PK) splits off the phosphates, forming 2 ATP molecules and pyruvate. PK activity is allosterically modulated by increased concentrations of F1,6-BP, which enhances the affinity of PK for PEP. Thus, when the F1,6-BP is plentiful, increased activity of PK favors pyruvate production. Pyruvate may diffuse from the erythrocyte or may become a substrate for
Figure 9-1  Glucose metabolism in the erythrocyte. ADP, Adenosine diphosphate; ATP, adenosine triphosphate; G6PD, glucose-6-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NADP, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form).
ATP, adenosine triphosphate; environment, where it oxidizes and destroys heme iron, phosphate by the action of HMP. The HMP detoxifies peroxide, the HMP extends the functional life span (Figure 9-1). The HMP detoxifies peroxide phosphate shunt.

Three alternate pathways, called diversions or shunts, branch from the glycolytic pathway. The three diversions are the hexose monophosphate pathway (HMP) or aerobic glycolysis, the methemoglobin reductase pathway, and the Rapoport-Luebering path.

**Hexose Monophosphate Pathway**

Aerobic or oxidative glycolysis occurs through a diversion of glucose catabolism into the HMP, also known as the pentose phosphate shunt (Figure 9-1). The HMP detoxifies peroxide (H₂O₂), which arises from O₂ reduction in the cell’s aqueous environment, where it oxidizes and destroys heme iron, proteins, and lipids, especially lipids containing thiol groups. By detoxifying peroxide, the HMP extends the functional life span of the RBC. The HMP diverts glucose-6-phosphate (G6P) to ribulose 5-phosphate by the action of glucose-6-phosphate dehydrogenase (G6PD). In the process, oxidized nicotinamide adenine dinucleotide phosphate (NADP) is converted to its reduced form (NADPH). NADPH is then available to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of glutathione reductase. Glutathione is a cysteine-containing tripeptide, and the designation GSH highlights the sulfur in the cysteine moiety. Reduced glutathione becomes oxidized as it reduces peroxide to water and oxygen via glutathione peroxidase.

During steady-state glycolysis, 5% to 10% of G6P is diverted to the HMP. After oxidative challenge, HMP activity may increase up to thirtyfold. The HMP catabolizes G6P to ribulose 5-phosphate and carbon dioxide by oxidizing G6P at carbon 1. The substrates, enzymes, and products of the HMP are listed in Table 9-4.

G6PD provides the only means of generating NADPH for glutathione reduction, and in its absence erythrocytes are particularly vulnerable to oxidative damage (Chapter 24). With normal G6PD activity, the HMP detoxifies oxidative compounds and safeguards hemoglobin, sulfhydryl-containing enzymes, and membrane thiols, allowing RBCs to safely carry O₂. However, in G6PD deficiency, the most common inherited RBC enzyme deficiency worldwide, the ability to detoxify is hampered, resulting in hereditary nonspherocytic anemia.

**Methemoglobin Reductase Pathway**

Heme iron is constantly exposed to oxygen and peroxide. Peroxide oxidizes heme iron from the ferrous (+2) to the ferric (+3) state. The affected hemoglobin molecule is called methemoglobin. Although the HMP prevents hemoglobin oxidation by reducing peroxide, it is not able to reduce methemoglobin once it forms. NADPH is able to do so, but only slowly. The reduction of methemoglobin by NADPH is rendered more efficient in the presence of methemoglobin reductase, also called cytochrome b₅ reductase. Using H⁺ from NADH formed when G3P is converted to 1,3-BPG, cytochrome b₅ reduces HMP acts as an intermediate electron carrier, returning the oxidized ferric iron to its ferrous, oxygen-carrying state. This enzyme accounts for more than 65% of the methemoglobin-reducing capacity within the RBC.

**Rapoport-Luebering Pathway**

A third metabolic shunt generates 2,3-bisphosphoglycerate (2,3-BPG; also called 2,3-diphosphoglycerate or 2,3-DPG). 1,3-BPG is diverted by bisphosphoglycerate mutase to form 2,3-BPG. 2,3-BPG regulates oxygen delivery to tissues by competing with oxygen for the oxygen-binding site of hemoglobin.

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**GLYCOLYSIS DIVERSION PATHWAYS (SHUNTS)**

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**TABLE 9-1 Glucose Catabolism: First Phase**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme</th>
<th>Products</th>
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<tbody>
<tr>
<td>Glucose, ATP</td>
<td>Hexokinase</td>
<td>G6P, ADP</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate isomerase</td>
<td>F6P</td>
</tr>
<tr>
<td>F6P, ATP</td>
<td>6-Phosphofructokinase</td>
<td>F1,6-BP, ADP</td>
</tr>
<tr>
<td>F1,6-BP</td>
<td>Fructose-bisphosphate adolase</td>
<td>DHAP, G3P</td>
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</tbody>
</table>

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**TABLE 9-2 Glucose Catabolism: Second Phase**

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<tr>
<th>Substrates</th>
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<tbody>
<tr>
<td>G3P</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>1,3-BPG</td>
</tr>
<tr>
<td>1,3-BPG, ADP</td>
<td>Phosphoglycerate kinase</td>
<td>3PG, ATP</td>
</tr>
<tr>
<td>1,3-BPG</td>
<td>Bisphosphoglycerate mutase</td>
<td>2,3-BPG</td>
</tr>
<tr>
<td>2,3-BPG</td>
<td>Bisphosphoglycerate phosphatase</td>
<td>3-PG</td>
</tr>
</tbody>
</table>

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**TABLE 9-3 Glucose Catabolism: Third Phase**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PG</td>
<td>Phosphoglycerate mutase</td>
<td>2-PG</td>
</tr>
<tr>
<td>2-PG</td>
<td>Enolase (phosphopyruvate hydratase)</td>
<td>PEP</td>
</tr>
<tr>
<td>PEP, ADP</td>
<td>Pyruvate kinase</td>
<td>Pyruvate, ATP</td>
</tr>
</tbody>
</table>

---

**TABLE 9-4 Glucose Catabolism: Hexose Monophosphate Pathway**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Enzyme</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate dehydrogenase and 6-Phosphogluconolactonase</td>
<td>6-PG</td>
</tr>
<tr>
<td>6-PG</td>
<td>6-Phosphogluconate dehydrogenase</td>
<td>R5P</td>
</tr>
</tbody>
</table>

---

PEP, phosphoenolpyruvate; lactate dehydrogenase with regeneration of the oxidized form of nicotinamide adenine dinucleotide (NAD⁺). The ratio of NAD⁺ to the reduced form (NADH) modulates the activity of this enzyme.
(Chapter 10). When 2,3-BPG binds heme, oxygen is released, which enhances delivery of oxygen to the tissues.

2,3-BPG forms 3-PG by the action of bisphosphoglycerate phosphatase. This diversion of 1,3-BPG to form 2,3-BPG sacrifices the production of two ATP molecules. There is further loss of two ATP molecules at the level of PK, because fewer molecules of PEP are formed. Because two ATP molecules were used to generate 1,3-BPG and production of 2,3-BPG eliminates the production of four molecules, the cell is put into ATP deficit by this diversion. There is a delicate balance between ATP generation to support the energy requirements of cell metabolism and the need to maintain the appropriate oxygenation and deoxygenation status of hemoglobin. Acidic pH and low concentrations of 3-PG maintain the appropriate oxygenation and deoxygenation status of hemoglobin. Acidic pH and low concentrations of 3-PG favor generation of ATP by causing the shunt and retaining 1,3-BPG in the EMP. These conditions and decreased ATP activate bisphosphoglycerate phosphatase, which returns 2,3-BPG to the glycolysis mainstream. In summary, these conditions favor generation of ATP by causing the conversion of 1,3-BPG directly to 3-PG and returning 2,3-BPG to 3-PG for ATP generation downstream by PK.

RBC MEMBRANE

RBC Membrane Deformability

RBCs are biconcave and average 90 fL in volume. Their average surface area is 140 mm², a 40% excess of surface area compared with a 90-fL sphere. This excess surface-to-volume ratio enables RBCs to stretch undamaged up to 2.5 times their resting diameter as they pass through narrow capillaries and through splenic pores 2 μm in diameter; this property is called RBC deformability. The RBC plasma membrane, which is 5 μm thick, is 100 times more elastic than a comparable latex membrane, yet it has tensile (lateral) strength greater than that of steel. The deformable RBC membrane provides the broad surface area and close tissue contact necessary to support the delivery of O₂ from lungs to body tissue and CO₂ from body tissue to lungs.

RBC deformability depends not only on RBC geometry but also on relative cytoplasmic (hemoglobin) viscosity. The normal mean cell hemoglobin concentration (MCHC) ranges from 32% to 36% (Chapter 14 and inside front cover), and as MCHC rises, internal viscosity rises. MCHCs above 36% compromise deformability and shorten the RBC life span because viscous cells become damaged as they stretch to pass through narrow capillaries or splenic pores. As RBCs age, they lose membrane surface area, while retaining hemoglobin. As the MCHC rises, the RBC, unable to pass through the splenic pores, is destroyed by splenic macrophages (Chapter 8).

RBC Membrane Lipids

Besides geometry and viscosity, membrane elasticity (pliancy) also contributes to deformability. The RBC membrane consists of approximately 8% carbohydrates, 52% proteins, and 40% lipids. The lipid portion, equal parts of cholesterol and phospholipids, forms a bilayer universal to all animal cells (Figure 13-10). Phospholipids form an impenetrable fluid barrier as their hydrophilic polar head groups are arrayed upon the membrane’s surfaces, oriented toward both the aqueous plasma and the cytoplasm, respectively. Their hydrophobic nonpolar acyl tails arrange themselves to form a central layer dynamically sequestered (hidden) from the aqueous plasma and cytoplasm. The membrane maintains extreme differences in osmotic pressure, cation concentrations, and gas concentrations between external plasma and the cytoplasm. Phospholipids reseal rapidly when the membrane is torn.

Cholesterol, esterified and largely hydrophobic, resides parallel to the acyl tails of the phospholipids, equally distributed between the outer and inner layers, and evenly dispersed within each layer, approximately one cholesterol molecule per phospholipid molecule. Cholesterol’s β-hydroxyl group, the only hydrophilic portion of the molecule, anchors within the polar head groups, while the rest of the molecule becomes intercalated among and parallel to the acyl tails. Cholesterol confers tensile strength to the lipid bilayer.

The ratio of cholesterol to phospholipids remains relatively constant and balances the need for deformability and strength. Membrane enzymes maintain the cholesterol concentration by regularly exchanging membrane and plasma cholesterol. Deficiencies in these enzymes are associated with membrane abnormalities such as acanthocytosis, as the membrane loses tensile strength (Chapter 24). Conversely, as cholesterol concentration rises, the membrane gains strength but loses elasticity.

The phospholipids are asymmetrically distributed. Phosphatidylcholine and sphingomyelin predominate in the outer layer; phosphatidylserine (PS) and phosphatidylethanolamine form most of the inner layer. Distribution of these four phospholipids is energy dependent, relying on a number of membrane-associated enzymes, whimsically termed flippases, floppases, and scramblases, for their positions. When phospholipid distribution is disrupted, as in sickle cell anemia and thalassemia (Chapters 27 and 28) or in RBCs that have reached their 120-day life span, PS, the only negatively charged phospholipid, redistributes (flips) to the outer layer. Splenic macrophages possess receptors that bind PS and destroy senescent and damaged RBCs.

Membrane phospholipids and cholesterol may also redistribute laterally so that the RBC membrane may respond to stresses and deform within 100 milliseconds of being challenged by the presence of a narrow passage, such as when arriving at a capillary. Redistribution becomes limited as the proportion of cholesterol increases. Plasma bile salt concentration also affects cholesterol exchange. In liver disease with low bile salt concentration, membrane cholesterol concentration becomes reduced. As a result, the more elastic cell membrane shows a “target cell” appearance when the RBCs are layered on a glass slide (Figure 19-1).

Glycolipids (sugar-bearing lipids) make up 5% of the external half of the RBC membrane. They associate in clumps or rafts and support carbohydrate side chains that extend into the aqueous plasma to anchor the glycolalx. The glycolalx is a layer of carbohydrates whose net negative charge prevents microbial attack and protects the RBC from mechanical damage caused by adhesion to neighboring RBCs or to the endothelium. Glycolipids may bear copies of carbohydrate-based blood group antigens, for example, antigens of the ABH and the Lewis blood group systems.
RBC Membrane Proteins

Although cholesterol and phospholipids constitute the principal RBC membrane structure, transmembrane (integral) and cytoskeletal (skeletal, peripheral) proteins make up 52% of the membrane structure by mass.19 A proteomic study reveals there are at least 300 RBC membrane proteins, including 105 transmembrane proteins. Of the purported 300 membrane proteins, about 50 have been characterized and named, some with a few hundred copies per cell, and others with over a million copies per cell.20

Transmembrane Proteins

The transmembrane proteins serve a number of RBC functions, as listed in Table 9-5.21 Through glycosylation they support surface carbohydrates, which join with glycolipids to make up the protective glycocalyx.22 They serve as transport and adhesion sites and signaling receptors. Any disruption in transport protein function changes the osmotic tension of the cytoplasm, which leads to a rise in viscosity and loss of deformability. Any change affecting adhesion proteins permits RBCs to adhere to one another and to the vessel walls, promoting fragmentation (vesiculation), reducing membrane flexibility, and shortening the RBC life span. Signaling receptors bind plasma ligands and trigger activation of intracellular signaling proteins, which then initiate various energy-dependent cellular activities, a process called signal transduction.

Blood Group Antigens.

Transmembrane proteins support carbohydrate-defined blood group antigens.23 For instance, band 3 (anion transport) and Glut-1 (glucose transport) support the majority of ABH system carbohydrate determinants by virtue of their high copy numbers.24 ABH system determinants are also found on several low copy number transmembrane proteins. Certain transmembrane proteins provide peptide epitopes. For instance, glycophorin A carries the peptide-defined M and N determinants, and glycophorin B carries the Ss determinants, which together comprise the MNSs system.25,26

The Rh system employs two multipass transmembrane lipoproteins and a multipass glycoprotein, each of which crosses the membrane 12 times.27-30 The two lipoproteins present the D and CcEe epitopes, respectively, but expression of the D and

### Table 9-5 Names and Properties of Selected Transmembrane RBC Proteins

<table>
<thead>
<tr>
<th>Transmembrane Protein</th>
<th>Band</th>
<th>Molecular Weight (D)</th>
<th>Copies per Cell (×10^3)</th>
<th>% of Total Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaporin 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Water transporter</td>
</tr>
<tr>
<td>Band 3 (anion exchanger 1)</td>
<td>3</td>
<td>90,000–102,000</td>
<td>1200</td>
<td>27%</td>
<td>Anion transporter, supports ABH antigens</td>
</tr>
<tr>
<td>Ca^2+ -ATPase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ca^2+ transporter</td>
</tr>
<tr>
<td>Duffy</td>
<td></td>
<td>35,000–43,000</td>
<td></td>
<td></td>
<td>G protein-coupled receptor, supports Duffy antigens</td>
</tr>
<tr>
<td>Glut-1</td>
<td>4.5</td>
<td>45,000–75,000</td>
<td></td>
<td>5%</td>
<td>Glucose transporter, supports ABH antigens</td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>PAS-1</td>
<td>36,000</td>
<td>500–1000</td>
<td>85% of glycophorins</td>
<td>Transports negatively charged sialic acid, supports MN determinants</td>
</tr>
<tr>
<td>Glycophorin B</td>
<td>PAS-4</td>
<td>20,000</td>
<td>100–300</td>
<td>10% of glycophorins</td>
<td>Transports negatively charged sialic acid, supports Ss determinants</td>
</tr>
<tr>
<td>Glycophorin C</td>
<td>PAS-2</td>
<td>14,000–32,000</td>
<td>50–100</td>
<td>4% of glycophorins</td>
<td>Transports negatively charged sialic acid, supports Gerbich system determinants</td>
</tr>
<tr>
<td>ICAM-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Integrin adhesion</td>
</tr>
<tr>
<td>K^+–Cl^– cotransporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urea transporter</td>
</tr>
<tr>
<td>Kell</td>
<td></td>
<td>93,000</td>
<td></td>
<td></td>
<td>Zn^{2+}-binding endopeptidase, Kell antigens</td>
</tr>
<tr>
<td>Kidd</td>
<td></td>
<td>46,000–50,000</td>
<td></td>
<td></td>
<td>Urea transporter</td>
</tr>
<tr>
<td>Na^+,K^+-ATPase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urea transporter</td>
</tr>
<tr>
<td>Na^+–K^+–2Cl^− cotransporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na^+–Cl^− cotransporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na^+–K^+ cotransporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rh</td>
<td></td>
<td>30,000–45,500</td>
<td></td>
<td></td>
<td>D and CcEe antigens</td>
</tr>
<tr>
<td>RhAG</td>
<td></td>
<td>50,000</td>
<td></td>
<td></td>
<td>Necessary for expression of D and CcEe antigens; gas transporter, probably of CO2</td>
</tr>
</tbody>
</table>

ATPase, Adenosine triphosphatase; Duffy, Duffy blood group system protein; ICAM, intracellular adhesion molecule; Kell, Kell blood group system protein; PAS, periodic acid–Schiff dye; RBC, red blood cell; Rh, Rh blood group system protein; RhAG, Rh antigen expression protein.
Figure 9-2  Representation of the human red blood cell membrane. The transmembrane proteins assemble in one of two complexes defined by their anchorage to skeletal protein ankyrin and skeletal protein 4.1. Band 3 is the most abundant transmembrane protein. In the ankyrin complex band 3 and protein 4.2 anchor to ankyrin, which is bound to the spectrin backbone. In the 4.1 complex, band 3, Rh, and other transmembrane proteins bind the complex of dematin, adducin, actin, tropomyosin, and tropomodulin through protein 4.1. CD47, Signaling receptor; Duffy, Duffy blood group system protein; GPA, glycophorin A; GPC, glycophorin C; Kell, Kell blood group system protein; LW, Landsteiner-Weiner blood group system protein; Rh, Rh blood group system protein; RhAG, Rh antigen expression protein; XK, X-linked Kell antigen expression protein.
CcEe antigens requires the separately inherited glycoprotein RhAG, which localizes near the Rh lipoproteins in the ankyrin complex. Loss of the RhAG glycoprotein prevents expression of both the D and CcEe antigens (Rh-null) and is associated with RBC morphologic abnormalities (Chapter 24). Additional blood group antigens localize to the 4.1 complex or specialized proteins.28-30

The GPI Anchor and Paroxysmal Nocturnal Hemoglobinuria. A few copies of the phospholipid phosphatidylinositol (PI), not mentioned in the RBC Membrane Lipids section, reside in the outer, plasma-side layer of the membrane. PI serves as a base upon which a glycan core of sugar molecules is synthesized, forming the glycosylphosphatidylinositol (GPI) anchor. Over 30 surface proteins bind to the GPI anchor including decay-accelerating factor (DAF, or CD55) and membrane inhibitor of reactive lysis (MIRL, or CD59).31,32 These proteins appear to float on the surface of the membrane as they link to the GPI anchor. The phosphatidylinositol glycan anchor biosynthesis class A (PIGA) gene codes for a glycosyltransferase required to add N-acetylglucosamine to the PI base early in the biosynthesis of the GPI anchor on the membrane. In paroxysmal nocturnal hemoglobinuria (Chapter 24), an acquired mutation in the PIGA gene affects the cells’ ability to synthesize the GPI anchor. Without the GPI anchor on the membrane, CD55 and CD59 become deficient, and the cells are susceptible to complement-mediated hemolysis.

Nomenclature. Numerical naming, for instance, band 3, protein 4.1, and protein 4.2, derives from historical (pre-proteomics) protein identification techniques that distinguished 15 membrane proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as illustrated in Figure 9-3.33 Bands migrate through the gel, with their velocity a property of their molecular weight and net charge, and are identified using Coomassie blue dye. The glycophorins, with abundant carbohydrate side chains, are stained using periodic acid–Schiff (PAS) dye.

Band 3, protein 4.2, and RhAG, members of the ankyrin complex, link their associated proteins and the bilayer membrane to the cytoskeletal proteins through ankyrin.34-36 Likewise, glycophorin C, Rh, and blood group Duffy link the 4.1 complex through protein 4.1.37 The 4.1 anchorage also includes the more recently defined proteins adducin and dematin, which link with band 3 and Glut-1, respectively.38

Cytoskeletal Proteins
The principal cytoskeletal proteins are the filamentous α-spectrin and β-spectrin (Table 9-6), which assemble to form an antiparallel heterodimer held together with a series of laterally bound subunits.39 Antiparallel means that the carboxyl (COOH) end of one strand associates with the amino (NH₂) end of the other, and the two heterodimers associate head-to-head to form a tetramer (Figure 9-2). The spectrins form a hexagonal lattice (Figure 9-4) that is immediately adjacent to the cytoplasmic membrane lipid layer and provides lateral or horizontal membrane stability.40 Because the skeletal proteins do not penetrate the bilayer, they are also called peripheral proteins.

Figure 9-3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of RBC membrane proteins stained with Coomassie blue dye. Lane A illustrates numerical band naming based on migration. Lane B names and illustrates the positions of some of the major proteins. (Adapted from Costa FF, Agre P, Watkins PC, et al: Linkage of dominant hereditary spherocytosis to the gene for the erythrocyte membrane-skeleton protein ankyrin, N Engl J Med 323:1046, 1990.)

Spectrin Stabilization. The secondary structure of both α- and β-spectrin features triple-helical repeats of 106 amino acids each; 20 such repeats make up α-spectrin, and 16 make up β-spectrin.41 Essential to the cytoskeleton are the previously mentioned ankyrin, protein 4.1, adducin and dematin, and, in addition, actin, tropomyosin, and tropomodulin (Figure 9-4).35 A single helix at the amino terminus of α-spectrin consistently binds a pair of helices at the carboxyl terminus of the β-spectrin chain, forming a stable triple helix that holds together the ends of the heterodimers.42 Joining these ends are actin and protein 4.1. Actin forms short filaments of 14 to 16 monomers whose length is regulated by tropomyosin. Adducin and tropomodulin cap the ends of actin, and dematin appears to stabilize actin in a manner that is the subject of current investigation.43

Membrane Deformation. Spectrin dimer bonds that appear along the length of the molecules dissociate and reassocia-
These flexible interactions plus the spectrin-actin-protein 4.1 junctions between the tetramers are key regulators of membrane elasticity and mechanical stability, and abnormalities in any of these proteins result in deformation-induced membrane fragmentation. For instance, hereditary elliptocytosis (ovalocytosis) arises from one of several autosomal dominant mutations affecting spectrin dimer-to-dimer lateral bonds or the spectrin–ankyrin–protein 4.1 junction. In hereditary elliptocytosis, the membrane fails to rebound from deformation, and RBCs progressively elongate to form visible elliptocytes, which causes a mild to severe hemolytic anemia. Conversely, autosomal dominant mutations that affect the integrity of band 3, ankyrin, protein 4.2, or α- or β-spectrin are associated with hereditary spherocytosis (Chapter 24). In these cases there are too few vertical anchorages to maintain membrane stability. The lipid membrane peels off in small fragments called “blebs,” or vesicles, whereas the cytoplasmic volume remains intact. This generates spherocytes with a reduced membrane-to-cytoplasm ratio.

**Osmotic Balance and Permeability**

The RBC membrane is impermeable to cations Na⁺, K⁺, and Ca²⁺. It is permeable to water and the anions bicarbonate (HCO₃⁻) and chloride (Cl⁻), which freely exchange between plasma and RBC cytoplasm. Aquaporin 1 (Table 9-5) is a transmembrane protein that forms pores or channels whose
surface charges create inward water flow in response to internal osmotic changes.

The ATP–dependent cation pumps Na⁺-ATPase and K⁺-ATPase (Table 9-5) regulate the concentrations of Na⁺ and K⁺, maintaining intracellular-to-extracellular ratios of 1:12 and 25:1, respectively.\textsuperscript{50,51} Ca²⁺-ATPase extrudes calcium, maintaining low intracellular levels of 5 to 10 μmol/L. Calmodulin, a cytoplasmic Ca²⁺-binding protein, controls the function of Ca²⁺-ATPase.\textsuperscript{52} These enzymes, in addition to aquaporin, maintain osmotic balance.

The cation pumps consume 15% of RBC ATP production. ATP loss or pump damage permits Ca²⁺ and Na⁺ influx, with water following osmotically. The cell swells, becomes spheroid, and eventually ruptures. This phenomenon is called colloid osmotic hemolysis.

Sickle cell disease provides an example of increased cation permeability. When crystallized sickle hemoglobin deforms the cell membranes, internal levels of Na⁺, K⁺, and especially Ca²⁺ rise, which results in hemolysis.\textsuperscript{52}

### REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Which RBC process does not require energy?
   a. Oxygen transport
   b. Cytoskeletal protein deformability
   c. Preventing the peroxidation of proteins and lipids
   d. Maintaining cytoplasm cationic electrochemical gradients

2. What pathway anaerobically generates energy in the form of ATP?
   a. Hexose monophosphate pathway
   b. Rapoport-Luebering pathway
   c. Embden-Meyerhof pathway
   d. 2,3-BPG pathway

---

### SUMMARY

- Glucose enters the RBC with no energy expenditure via the transmembrane protein Glut-1.
- The anaerobic Embden-Meyerhof pathway (EMP) metabolizes glucose to pyruvate, consuming two ATP molecules. The EMP subsequently generates four ATP molecules per glucose molecule, a net gain of two.
- The hexose-monophosphate pathway (HMP) pathway aerobically converts glucose to pentose and generates NADPH. NADPH reduces glutathione. Reduced glutathione reduces peroxides and protects proteins, lipids, and heme iron from oxidation.
- The methemoglobin reductase pathway converts ferric heme iron (valence 3⁺ iron, methemoglobin) to reduced ferrous (valence 2⁺ form), which binds O₂.
- The Rapoport-Luebering pathway generates 2,3-BPG and enhances O₂ delivery to tissues.
- The RBC membrane is a lipid bilayer whose hydrophobic components are sequestered from aqueous plasma and cytoplasm. The membrane provides a semipermeable barrier separating plasma from cytoplasm and maintaining an osmotic differential.
- RBC membrane phospholipids are asymmetrically distributed. Phosphatidylcholine and sphingomyelin predominate in the outer layer; phosphatidylethanolamine and phosphatidyethanolamine form most of the inner layer.
- Enzymatic plasma to membrane exchange maintains RBC membrane cholesterol.
- Acanthocytosis and target cells are associated with abnormalities in the concentration or distribution of membrane cholesterol and phospholipids.
- RBC transmembrane proteins channel ions, water, and glucose and anchor cell membrane receptors. They also provide the vertical support connecting the lipid bilayer to the underlying cytoskeleton to maintain membrane integrity.
- RBC cytoplasm K⁺ concentration is higher than plasma K⁺, whereas Na⁺ and Ca²⁺ concentrations are lower. Disequilibria are maintained by membrane enzymes K⁺-ATPase, Na⁺-ATPase, and Ca²⁺-ATPase. Pump failure leads to Na⁺ and water influx, cell swelling, and lysis.
- The shape and flexibility of the RBC, which are essential to its function, depend on the cytoskeleton. The cytoskeleton is derived from a group of peripheral proteins on the interior of the lipid membrane. The major structural proteins are α- and β-spectrin, which are bound together and connected to transmembrane proteins by ankyrin, actin, protein 4.1, adducin, tropomodulin, dematin, and band 3. Cytoskeletal proteins provide the horizontal or lateral support for the membrane.
- Hereditary spherocytosis arises from defects in proteins that provide vertical support for the membrane. Hereditary elliptocytosis is due to defects in cytoskeletal proteins that provide horizontal support for the membrane.
- Membrane proteins are extracted using sodium dodecyl sulfate, separated using polyacrylamide gel electrophoresis, and stained with Coomassie blue. They are numbered from the point of application; lower numbers correlate to high protein molecular weight and lower net charge.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.
3. Which is true concerning 2,3-BPG?
   a. The least abundant of RBC organophosphates
   b. Enhances O\textsubscript{2} release from hemoglobin
   c. Source of RBC glucose
   d. Source of RBC ATP

4. To survive, the RBC must detoxify peroxides. What hexose-monophosphate shunt product(s) accomplishes detoxification?
   a. ATP
   b. 2,3-BPG
   c. Pyruvic and lactic acid
   d. NADPH and reduced glutathione

5. Which of the following helps maintain RBC shape?
   a. Membrane phospholipids
   b. Cytoskeletal proteins
   c. GPI anchor
   d. Glycocalyx

6. The glycolipids of the RBC membrane:
   a. Provide flexibility.
   b. Carry RBC antigens.
   c. Constitute ion channels.
   d. Attach the cytoskeleton to the lipid layer.

7. RBC membranes block passage of most large molecules such as proteins, but allow passage of small molecules such as the cations Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+}. What is the term for this membrane property?
   a. Semipermeable
   b. Deformable
   c. Intangible
   d. Flexible

8. RBC membrane phospholipids are arranged:
   a. In a hexagonal lattice.
   b. In chains beneath a protein exoskeleton.
   c. In two layers whose composition is asymmetric.
   d. So that hydrophobic portions are facing the plasma.

9. RBC membrane cholesterol is replenished from the:
   a. Plasma.
   b. Mitochondria.
   c. Cytoplasm.
   d. EMB pathway.

10. The hemoglobin iron ion may become oxidized to the +3 valence state by several pathological mechanisms. What portion of the Embden-Meyerhof pathway reduces iron to the physiologic +2 valence state?
    a. Methemoglobin reductase pathway
    b. Hexose monophosphate pathway
    c. Rapoport-Luebering pathway
    d. The 2,3-BPG shunt

11. Which of the following is an example of a transmembrane or integral membrane protein?
    a. Glycophorin A
    b. Ankyrin
    c. Spectrin
    d. Actin

12. Abnormalities in the horizontal and vertical linkages of the transmembrane and cytoskeletal RBC membrane proteins may be seen as:
    a. Shape changes.
    b. Methemoglobin increase.
    c. Reduced hemoglobin content.
    d. Enzyme pathway deficiencies.

REFERENCES

Hemoglobin Metabolism

Elaine M. Keohane*

CASE STUDY
After studying the material in this chapter, the reader should be able to respond to the following case study:

Hemoglobin and hemoglobin fractionation and quantification using high performance liquid chromatography (HPLC) were performed on a mother and her newborn infant, both presumed to be healthy. The assays were part of a screening program to establish reference intervals. The mother’s hemoglobin concentration was 14 g/dL, and the newborn’s was 20 g/dL. The mother’s hemoglobin fractions were quantified as 97% Hb A, 2% Hb A2, and 1% Hb F by HPLC. The newborn’s results were 88% Hb F and 12% Hb A.

1. Were these hemoglobin results within expected reference intervals?
2. Why were the mother’s and the newborn’s hemoglobin concentration so different?
3. What is the difference between the test to determine the hemoglobin concentration and the test to analyze hemoglobin by HPLC?
4. Why were the mother’s and newborn’s hemoglobin fractions so different?

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Describe the components and structure of hemoglobin.
2. Describe steps in heme synthesis that occur in the mitochondria and the cytoplasm.
3. Name the genes and the chromosome location and arrangement for the various polypeptide chains of hemoglobin.
4. Describe the polypeptide chains produced and the hemoglobins they form in the embryo, fetus, newborn, and adult.
5. List the three types of normal hemoglobin in adults and their reference intervals.
6. Describe mechanisms that regulate hemoglobin synthesis.
7. Describe the mechanism by which hemoglobin transports oxygen to the tissues and transports carbon dioxide to the lungs.
8. Explain the importance of maintaining hemoglobin iron in the ferrous state (Fe^{2+}).
9. Explain the significance of the sigmoid shape of the oxygen dissociation curve.
10. Correlate right and left shifts in the oxygen dissociation curve with conditions that can cause shifts in the curve.
11. Differentiate T and R forms of hemoglobin and the effect of oxygen and 2,3-bisphosphoglycerate on those forms.
12. Explain the difference between adult Hb A and fetal Hb F and how that difference impacts oxygen affinity.
13. Compare and contrast the composition and the effect on oxygen binding of methemoglobin, carboxyhemoglobin, and sulfhemoglobin.

OUTLINE

Hemoglobin Structure
- Heme Structure
- Globin Structure
- Complete Hemoglobin Molecule

Hemoglobin Biosynthesis
- Heme Biosynthesis
- Globin Biosynthesis
- Hemoglobin Assembly

Hemoglobin Ontogeny
Regulation of Hemoglobin Production
- Heme Regulation
- Globin Regulation
- Systemic Regulation of Erythropoiesis

Hemoglobin Function
- Oxygen Transport
- Carbon Dioxide Transport
- Nitric Oxide Transport

Dyshemoglobins
- Methemoglobin
- Sulpherhemoglobin
- Carboxyhemoglobin

Hemoglobin Measurement

*The author extends appreciation to Mary Coleman, whose work in prior editions provided the foundation for this chapter.
Hemoglobin (Hb) is one of the most studied proteins in the body due to the ability to easily isolate it from red blood cells (RBCs). It comprises approximately 95% of the cytoplasmic content of RBCs. The body very efficiently carries hemoglobin in RBCs, which provides protection from denaturation in the plasma and loss through the kidneys. Free (non-RBC) hemoglobin, generated from RBCs through hemolysis, has a short half-life outside of the RBCs. When released into the plasma, it is rapidly salvaged to preserve its iron and amino acid components; when salvage capacity is exceeded, it is excreted by the kidneys (Chapter 23). The concentration of hemoglobin within RBCs is approximately 34 g/dL, and its molecular weight is approximately 64,000 Daltons. Hemoglobin's main function is to transport oxygen from the lungs to tissues and transport carbon dioxide from the tissues to the lungs for exhalation. Hemoglobin also contributes to acid-base balance by binding and releasing hydrogen ions and transports nitric oxide (NO), a regulator of vascular tone. 1,3

This chapter covers the structure, biosynthesis, ontogeny, regulation, and function of hemoglobin. The formation, composition, and characteristics of several dyshemoglobins—namely, methemoglobin, carboxyhemoglobin, and sulfhemoglobin—are also discussed at the end of the chapter.

HEMOGLOBIN STRUCTURE

Hemoglobin is the first protein whose structure was described using x-ray crystallography. The hemoglobin molecule is a globular protein consisting of two different pairs of polypeptide chains and four heme groups, with one heme group imbedded in each of the four polypeptide chains (Figure 10-1).

Heme Structure

Heme consists of a ring of carbon, hydrogen, and nitrogen atoms called protoporphyrin IX, with a central atom of divalent ferrous iron (Fe²⁺) (Figure 10-2). Each of the four heme groups is positioned in a pocket of the polypeptide chain near the surface of the hemoglobin molecule. The ferrous iron in each heme molecule reversibly combines with one oxygen molecule. When the ferrous ions are oxidized to the ferric state (Fe³⁺), they no longer can bind oxygen. Oxidized hemoglobin is also called methemoglobin and is discussed later in this chapter.

Globin Structure

The four globin chains comprising each hemoglobin molecule consist of two identical pairs of unlike polypeptide chains, 141 to 146 amino acids each. Variations in amino acid sequences give rise to different types of polypeptide chains. Each chain is designated by a Greek letter (Table 10-1). 1,3

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Number of Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
<td>141</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
<td>146</td>
</tr>
<tr>
<td>γₐ</td>
<td>Gamma A</td>
<td>146 (position 136: alanine)</td>
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<tr>
<td>γ₂</td>
<td>Gamma G</td>
<td>146 (position 136: glycine)</td>
</tr>
<tr>
<td>δ</td>
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<td>141</td>
</tr>
<tr>
<td>θ</td>
<td>Theta</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* * *

**Table 10-1 Globin Chains**

α₁β₂ bonds and α₂β₁ bonds are located between the dimers, α₁β₁ bonds are in the front, α₂β₂ bonds are behind.
Each globin chain is divided into eight helices separated by seven nonhelical segments (Figure 10-3). The helices, designated A to H, contain subgroup numberings for the sequence of the amino acids in each helix and are relatively rigid and linear. Flexible nonhelical segments connect the helices, as reflected by their designations: NA for the sequence between the N-terminus and the A helix, AB between the A and B helices, and so forth, with BC, CD, DE, EF, FG, GH, and finally HC between the H helix and the C-terminus.

**Complete Hemoglobin Molecule**

The hemoglobin molecule can be described by its primary, secondary, tertiary, and quaternary protein structures. The *primary structure* refers to the amino acid sequence of the polypeptide chains. The *secondary structure* refers to chain arrangements in helices and nonhelices. The *tertiary structure* refers to the arrangement of the helices into a pretzel-like configuration.

Globin chains loop to form a cleft pocket for heme. Each chain contains a heme group that is suspended between the E
and F helices of the polypeptide chain (Figure 10-3).²,³ The iron atom at the center of the protoporphyrin IX ring of heme is positioned between two histidine radicals, forming a proximal histidine bond within F8 and, through the linked oxygen, a close association with the distal histidine residue in E7.³ Globin chain amino acids in the cleft are hydrophobic, whereas amino acids on the outside are hydrophilic, which renders the molecule water soluble. This arrangement also helps iron remain in its divalent ferrous form regardless of whether it is oxygenated (carrying an oxygen molecule) or deoxygenated (not carrying an oxygen molecule).

The quaternary structure of hemoglobin, also called a tetramer, describes the complete hemoglobin molecule. The complete hemoglobin molecule is spherical, has four heme groups attached to four polypeptide chains, and may carry up to four molecules of oxygen (Figure 10-4). The predominant adult hemoglobin, Hb A, is composed of two α-globin chains and two β-globin chains. Strong α₁–β₁ and α₂–β₂ bonds hold the dimers in a stable form. The α₁–β₂ and α₂–β₁ bonds are important for the stability of the quaternary structure in the oxygenated and deoxygenated forms (Figure 10-1).¹,²

A small percentage of Hb A is glycated. Glycation is a post-translational modification formed by the nonenzymatic binding of various sugars to globin chain amino groups over the life span of the RBC. The most characterized of the glycated hemoglobins is Hb A₁c, in which glucose attaches to the N-terminal valine of the β chain.¹ Normally, about 4% to 6% of Hb A circulates in the A₁c form. In uncontrolled diabetes mellitus, the amount of A₁c is increased proportionally to the mean blood glucose level over the preceding 2 to 3 months.

HEMOGLOBIN BIOSYNTHESIS

Heme Biosynthesis

Heme biosynthesis occurs in the mitochondria and cytoplasm of bone marrow erythroid precursor cells, beginning with the pronormoblast through the circulating polychromatophilic (also known as polychromatophilic) erythrocyte (Chapter 8). As they lose their ribosomes and mitochondria (location of the citric/tricarboxylic acid cycle), mature erythrocytes can no longer make hemoglobin.⁵

Heme biosynthesis begins in the mitochondria with the condensation of glycine and succinyl coenzyme A (CoA) catalyzed by aminolevulinate synthase to form aminolevulinic acid (ALA) (Figure 10-5).⁵ In the cytoplasm, aminolevulinic acid dehydratase (also known as porphobilinogen synthase) converts ALA to porphobilinogen (PBG). PBG undergoes several transformations in the cytoplasm from hydroxymethylbilane to coproporphyrinogen III. This pathway then continues in the mitochondria until, in the final step of production of heme, Fe²⁺ combines with...
with protoporphyrin IX in the presence of ferrochelatase (heme synthase) to make heme.5

Transferrin, a plasma protein, carries iron in the ferric (Fe³⁺) form to developing erythroid cells (Chapter 11). Transferrin binds to transferrin receptors on erythroid precursor cell membranes and the receptors and transferrin (with bound iron) are brought into the cell in an endosome (Figure 11-5). Acidification of the endosome releases the iron from transferrin. Iron is transported out of the endosome and into the mitochondria where it is reduced to the ferrous state, and is united with protoporphyrin IX to make heme. Heme leaves the mitochondria and is joined to the globin chains in the cytoplasm.

**Globin Biosynthesis**

Six structural genes code for six globin chains. The α- and ζ-globin genes are on the short arm of chromosome 16; the ε-, γ-, δ-, and β-globin gene cluster is on the short arm of chromosome 11.
In the human genome, there is one copy of each globin gene per chromatid, for a total of two genes per diploid cell, with the exception of $\alpha$ and $\gamma$. There are two copies of the $\alpha$- and $\gamma$-globin genes per chromatid, for a total of four genes per diploid cell.

The production of globin chains takes place in erythroid precursors from the pronormoblast through the circulating polychromatic erythrocyte, but not in the mature erythrocyte. Transcription of the globin genes to messenger ribonucleic acid (mRNA) occurs in the nucleus, and translation of mRNA to the globin polypeptide chain occurs on ribosomes in the cytoplasm. Although transcription of the $\alpha$-globin genes produces more mRNA than the $\beta$-globin gene, there is less efficient translation of the $\alpha$-globin mRNA. Therefore, the $\alpha$ and $\beta$ chains are produced in approximately equal amounts. After translation is complete, the chains are released from the ribosomes in the cytoplasm.

Hemoglobin Assembly

After their release from ribosomes, each globin chain binds to a heme molecule, then forms a heterodimer (Figure 10-5). The non-$\alpha$ chains have a charge difference that determines their affinity to bind to the $\alpha$ chains. The $\alpha$ chain has a positive charge and has the highest affinity for a $\beta$ chain due to its negative charge. The $\gamma$-globin chain has the next highest affinity, followed by the $\delta$-globin chain. Two heterodimers then combine to form a tetramer. This completes the hemoglobin molecule.

Two $\alpha$ and two $\beta$ chains form Hb A, the major hemoglobin present from 6 months of age through adulthood. Hb $A_2$ contains two $\alpha$ and two $\delta$ chains. Owing to a mutation in the promoter region of the $\delta$-globin gene, production of the $\delta$ chain polypeptide is very low. Consequently, Hb $A_2$ comprises less than 3.5% of total hemoglobin in adults. Hb F contains two $\alpha$ and two $\gamma$ chains. In healthy adults, Hb F comprises 1% to 2% of total hemoglobin, and it is present only in a small proportion of the RBCs (uneven distribution). These RBCs with Hb F are called F or A/F cells.

The various amino acids that comprise the globin chains affect the net charge of the hemoglobin tetramer. Electrophoresis and high performance liquid chromatography (HPLC) are used for fractionation, presumptive identification, and quantification of normal hemoglobins and hemoglobin variants (Chapter 27). Molecular genetic testing of globin gene DNA provides definitive identification of variant hemoglobins.

Hemoglobin Ontogeny

Hemoglobin composition differs with prenatal gestation time and postnatal age. Hemoglobin changes reflect the sequential activation and inactivation (or switching) of the globin genes, progressing from the $\zeta$- to the $\alpha$-globin gene on chromosome 16 and from the $\epsilon$- to the $\gamma$-, $\delta$-, and $\beta$-globin genes on chromosome 11. The $\zeta$- and $\epsilon$-globin chains normally appear only during the first 3 months of embryonic development. These two chains, when paired with the $\alpha$ and $\gamma$ chains, form the embryonic hemoglobins (Figure 10-6). During the second and third trimesters of fetal life and at birth, the $\epsilon$- and $\zeta$-globin chains are inactivated, and the $\alpha$ and $\gamma$ chains are produced.

Figure 10-6 Timeline of globin chain production from intrauterine life to adulthood. See also Table 10-2.
TABLE 10-2 Normal Hemoglobins

<table>
<thead>
<tr>
<th>Stage</th>
<th>Globin Chain</th>
<th>Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrauterine</td>
<td>ζ&lt;sub&gt;2&lt;/sub&gt; + ε&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Gower-1</td>
</tr>
<tr>
<td>Early embryogenesis (product of yolk sac erythroblasts)</td>
<td>α&lt;sub&gt;2&lt;/sub&gt; + ε&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Gower-2</td>
</tr>
<tr>
<td></td>
<td>ζ&lt;sub&gt;2&lt;/sub&gt; + γ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Portland</td>
</tr>
<tr>
<td>Begins in early embryogenesis; peaks during third trimester and begins to decline just before birth</td>
<td>α&lt;sub&gt;2&lt;/sub&gt; + γ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>F</td>
</tr>
</tbody>
</table>

| Birth | α<sub>2</sub> + γ<sub>2</sub> | F, 60% to 90% |
| | α<sub>2</sub> + β<sub>2</sub> | A, 10% to 40% |

| Two Years through Adulthood | α<sub>2</sub> + γ<sub>2</sub> | F, 1% to 2% |
| | α<sub>2</sub> + δ<sub>2</sub> | A<sub>δ</sub>, <3.5% |
| | α<sub>2</sub> + β<sub>2</sub> | A, >95% |

birth, Hb F (α<sub>2</sub>γ<sub>2</sub>) is the predominant hemoglobin. By 6 months of age and through adulthood, Hb A (α<sub>2</sub>β<sub>2</sub>) is the predominant hemoglobin, with small amounts of Hb A<sub>2</sub> (α<sub>2</sub>δ<sub>2</sub>) and Hb F. Table 10-2 presents the reference intervals for the normal hemoglobin fractions at various ages.

Mechanisms that control the switching from γ chain production to β chain production (γ-β switching) are discussed in the next section.

REGULATION OF HEMOGLOBIN PRODUCTION

Heme Regulation
The key rate-limiting step in heme synthesis is the initial reaction of glycine and succinyl CoA to form ALA, catalyzed by ALA synthase (Figure 10-5). Heme inhibits the transcription of the ALA synthase gene, which leads to a decrease in heme production (a negative feedback mechanism). Heme inhibits other enzymes in the biosynthesis pathway, including ALA dehydrase and PBG deaminase. A negative feedback mechanism by heme or substrate inhibition by protoporphyrin IX is believed to inhibit the ferrochelatase enzyme. Conversely, an increased demand for heme induces an increased synthesis of ALA synthase.

Globin Regulation
Globin synthesis is highly regulated so that there is a balanced production of globin and heme. This is critical because an excess of globin chains, protophophyrin IX, or iron can accumulate and damage the cell, reducing its life span.

Globin production is mainly controlled at the transcription level by a complex interaction of deoxiribonucleic acid (DNA) sequences (cis-acting promoters, enhancers, and silencers) and soluble transcription factors (trans-acting factors) that bind to DNA or to one another to promote or suppress transcription. Initiation of transcription of a particular globin gene requires (1) the promoter DNA sequences immediately before the 5’ end or the beginning of the gene; (2) a key transcription factor called Krüppel-like factor 1 (KLF1); (3) a number of other transcription factors (such as GATA1, Ikaros, TAL1, p45-NF-E2, and LDB1); and (4) an enhancer region of DNase 1 hypersensitive nucleic acid sequences located more than 20 kilobases upstream (before the 5’ start site of the gene) from the globin gene called the locus control region or LCR. For example, to activate transcription of the β-globin gene in the β-globin gene cluster on chromosome 11, the LCR, the promoter for the β-globin gene, and various transcription factors join together to form a three-dimensional active chromosome hub (ACH), with KLF1 playing a key role in connecting the complex. Because the LCR is located a distance upstream from the β-globin gene complex, a loop of DNA is formed when the LCR and β-globin gene promoter join together in the chromosome hub. The other globin genes in the cluster (ε-, γ-, and δ-) are maintained in the inactive state in the DNA loop, so only the β-globin gene is transcribed.

Krüppel-like factor 1 also plays a key regulatory role in the switch from γ chain to β chain production (γ-β switching) that begins in late fetal life and continues through adulthood. The KLF1 is an exact match for binding to the DNA promoter sequences of the β-globin gene, while the γ-globin gene promoter has a slightly different sequence. This results in a preferential binding to and subsequent activation of transcription of the β-globin gene. KLF1 also regulates the expression of repressors of γ-globin gene transcription, such as BCL11A and MYB.

Globin synthesis is also regulated during translation when the mRNA coding for the globin chains associates with ribosomes to produce the polypeptide. Many protein factors are required to control the initiation, elongation, and termination steps of translation. Heme is an important regulator of globin mRNA translation at the initiation step by promoting the activation of a translation initiation factor and inactivating its repressor. Conversely, when the heme level is low, the repressor accumulates and inactivates the initiation factor, thus blocking translation of the globin mRNA.

Systemic Regulation of Erythropoiesis
When there is an insufficient quantity of hemoglobin or if the hemoglobin molecule is defective in transporting oxygen, tissue hypoxia occurs. The hypoxia is detected by the peritubular cells of the kidney, which respond by increasing the production of erythropoietin (EPO). EPO increases the number of erythrocytes produced and released into the periphery; it also accelerates the rate of synthesis of erythrocyte components, including hemoglobin (Chapter 8).

Although each laboratory must establish its own reference intervals based on their instrumentation, methodology, and
patient population, in general, reference intervals for hemoglobin concentration are as follows:

<table>
<thead>
<tr>
<th>Gender</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>14 to 18 g/dL (140 to 180 g/L)</td>
</tr>
<tr>
<td>Women</td>
<td>12 to 15 g/dL (120 to 150 g/L)</td>
</tr>
<tr>
<td>Newborns</td>
<td>16.5 to 21.5 g/dL (165 to 215 g/L)</td>
</tr>
</tbody>
</table>

Reference intervals for infants and children vary according to age group. Individuals living at high altitudes have slightly higher levels of hemoglobin as a compensatory mechanism to provide more oxygen to the tissues in the oxygen-thin air. Tables on the inside front cover of this text provide reference intervals for all age-groups.

**HEMOGLOBIN FUNCTION**

**Oxygen Transport**

The function of hemoglobin is to readily bind oxygen molecules in the lung, which requires high oxygen affinity; to transport oxygen; and to efficiently unload oxygen to the tissues, which requires low oxygen affinity. During oxygenation, each of the four heme iron atoms in a hemoglobin molecule can reversibly bind one oxygen molecule. Approximately 1.34 mL of oxygen is bound by each gram of hemoglobin.1

The affinity of hemoglobin for oxygen relates to the partial pressure of oxygen (PO2), often defined in terms of the amount of oxygen needed to saturate 50% of hemoglobin, called the P50 value. The relationship is described by the oxygen dissociation curve of hemoglobin, which plots the percent oxygen saturation of hemoglobin versus the PO2 (Figure 10-7). The curve is sigmoidal, which indicates low hemoglobin affinity for oxygen at low oxygen tension and high affinity for oxygen at high oxygen tension.

Cooperation among hemoglobin subunits contributes to the shape of the curve. Hemoglobin that is completely deoxygenated has little affinity for oxygen. However, with each oxygen molecule that is bound, there is a change in the conformation of the tetramer that progressively increases the oxygen affinity of the other heme subunits. Once one oxygen molecule binds, the remainder of the hemoglobin molecule quickly becomes fully oxygenated.2 Therefore, with the high oxygen tension in the lungs, the affinity of hemoglobin for oxygen is high, and hemoglobin becomes rapidly saturated with oxygen. Conversely, with the relatively low oxygen tension in the tissues, the affinity of hemoglobin for oxygen is low, and hemoglobin rapidly releases oxygen.

Normally, a PO2 of approximately 27 mm Hg results in 50% oxygen saturation of the hemoglobin molecule. If there is a shift of the curve to the right, a lower oxygen affinity is seen. With a shift in the curve to the right, a lower oxygen affinity is seen.

In addition to the PO2 shifts of the curve to the left or right occur if there are changes in the pH of the blood. In the tissues, a lower pH shifts the curve to the right and reduces the affinity of hemoglobin for oxygen, and the hemoglobin more readily releases oxygen. A shift in the curve due to a change in pH (or hydrogen ion concentration) is termed the Bohr effect. It facilitates the ability of hemoglobin to exchange oxygen and carbon dioxide (CO2) and is discussed later.

The concentration of 2,3-bisphosphoglycerate (2,3-BPG, formerly 2,3-diphosphoglycerate) also has an effect on oxygen affinity. In the deoxygenated state, the hemoglobin tetramer assumes a tense or T conformation that is stabilized by the binding of 2,3-BPG between the β-globin chains (Figure 10-8). The formation of salt bridges between the phosphates of 2, 3-BPG and positively charged groups on the globin chains further stabilizes the tetramer in the T conformation.1 The binding of 2, 3-BPG shifts the oxygen dissociation curve to the right, favoring the release of oxygen.1 In addition, a lower pH and higher PCO2 in the tissues further shifts the curve to the right, favoring the release of oxygen.1

As hemoglobin binds oxygen molecules, a change in conformation of the hemoglobin tetramer occurs with a change in hydrophobic interactions at the α1β2 contact point, a disruption of the salt bridges, and release of 2, 3-BPG.1 A 15-degree rotation of the α1β1 dimer, relative to the α2β2 dimer, occurs...
along the α1β2 contact point. When the hemoglobin tetramer is fully oxygenated, it assumes a relaxed or R state (Figure 10-8).

Clinical conditions that produce a shift of the oxygen dissociation curve to the left include a lowered body temperature due to external causes; multiple transfusions of stored blood with depleted 2,3-BPG; alkalosis; and the presence of hemoglobin variants with a high affinity for oxygen. Conditions producing a shift of the curve to the right include increased body temperature; acidosis; the presence of hemoglobin variants with a low affinity for oxygen; and an increased 2,3-BPG concentration in response to hypoxic conditions, such as high altitude, pulmonary insufficiency, congestive heart failure, and severe anemia (Chapter 19).

The sigmoidal oxygen dissociation curve generated by normal hemoglobin contrasts with myoglobin’s hyperbolic curve (Figure 10-7). Myoglobin, present in cardiac and skeletal muscle, is a 17,000-Dalton, monomeric, oxygen-binding heme protein. It binds oxygen with greater affinity than hemoglobin. Its hyperbolic curve indicates that it releases oxygen only at very low partial pressures, which means it is not as effective as hemoglobin in releasing oxygen to the tissues at physiologic oxygen tensions. Myoglobin is released into the plasma when there is damage to the muscle in myocardial infarction, trauma, or severe muscle injury, called rhabdomyolysis. Myoglobin is normally excreted by the kidney, but levels may become elevated in renal failure. Serum myoglobin levels aid in diagnosis of myocardial infarction in patients who have no underlying trauma, rhabdomyolysis, or renal failure. Myoglobin in the urine produces a positive result on the urine dipstick test for blood; this must be differentiated from a positive result caused by hemoglobin.

Hb F (fetal hemoglobin, the primary hemoglobin in newborns) has a P50 of 19 to 21 mm Hg, which results in a left shift of the oxygen dissociation curve and increased affinity for oxygen relative to that of Hb A. This increased affinity for oxygen is due to its weakened ability to bind 2,3-BPG. There is only one amino acid difference in a critical 2,3-BPG binding site between the γ chain and the β chain that accounts for this difference in binding.

In fetal life, the high oxygen affinity of Hb F provides an advantage by allowing more effective oxygen withdrawal from the maternal circulation. At the same time, Hb F has a disadvantage in that it delivers oxygen less readily to tissues. The bone marrow in the fetus and newborn compensates by producing more RBCs to ensure adequate oxygenation of the tissues. This response is mediated by erythropoietin (Chapter 8). Consequently, the RBC count, hemoglobin concentration, and hematocrit of a newborn are higher than adult values (values are on the inside front cover), but they gradually decrease to normal physiologic levels by 6 months of age as the γ-β switching is completed and most of the Hb F is replaced by Hb A.

**Carbon Dioxide Transport**

A second crucial function of hemoglobin is the transport of carbon dioxide. In venous blood, the carbon dioxide diffuses into the red blood cells and combines with water to form carbonic acid (H2CO3). This reaction is facilitated by the RBC enzyme carbonic anhydrase. Carbonic acid then dissociates to release H⁺ and bicarbonate (HCO3⁻) (Figure 10-9).

The H⁺ from the second reaction binds oxygenated hemoglobin (HbO2), and the oxygen is released from the hemoglobin due to the Bohr effect. The oxygen then diffuses out of the cell into the tissues. As the concentration of the negatively charged bicarbonate increases, it diffuses across the RBC membrane into the plasma. Chloride (Cl⁻), also negatively charged, diffuses from the plasma into the cell to maintain electroneutrality across the membrane; this is called the chloride shift (Figure 10-9).

In the lungs, oxygen diffuses into the cell and binds to deoxygenated hemoglobin (Hb) due to the high oxygen tension. H⁺ is released from hemoglobin and combines with bicarbonate to form carbonic acid. Carbonic acid is converted to water and CO2; the latter diffuses out of the cells and is expelled by the lungs. As more bicarbonate diffuses into the cell to produce carbonic acid, chloride diffuses back out into
The plasma. Approximately 85% of the CO₂ produced in the tissues is transported by hemoglobin as H⁺. In this capacity, hemoglobin provides a buffering effect by binding and releasing H⁺. A small percentage of CO₂ remains in the cytoplasm and the remainder binds to the globin chains as a carbamino group.

**Nitric Oxide Transport**

A third function of hemoglobin involves the binding, inactivation, and transport of nitric oxide (NO). Nitric oxide is secreted by vascular endothelial cells and causes relaxation of the vascular wall smooth muscle and vasodilation. When released, free nitric oxide has a very short half-life, but some enters the RBCs and can bind to cysteine in the β chain of hemoglobin, forming S-nitrosohemoglobin. Some investigators propose that hemoglobin preserves and transports nitric oxide to hypoxic microvascular areas, which stimulates vasodilation and increases blood flow (hypoxic vasodilation). In this way, hemoglobin may work with other systems in regulating local blood flow to microvascular areas by binding and inactivating nitric oxide (causing vasoconstriction and decreased blood flow) when oxygen tension is high and releasing nitric oxide (causing vasodilation and increased blood flow) when oxygen tension is low. This theory is not universally accepted, and the roles of hemoglobin, endothelial cells, and nitric oxide in regulating blood flow and oxygenation of the microcirculation are still being investigated.

**DYSHEMOGLOBINS**

Dyshemoglobins (dysfunctional hemoglobins that are unable to transport oxygen) include methemoglobin, sulhemoglobin, and carboxyhemoglobin. Dyshemoglobins form and may accumulate to toxic levels, after exposure to certain drugs or environmental chemicals or gases. The offending agent modifies the structure of the hemoglobin molecule, preventing it from binding oxygen. Most cases of dyshemoglobinemia are acquired; a small fraction of methemoglobinemia cases are hereditary.

**Methemoglobin**

Methemoglobin (MetHb) is formed by the reversible oxidation of heme iron to the ferric state (Fe³⁺). Normally, a small amount of methemoglobin is continuously formed by oxidation of iron during the normal oxygenation and deoxygenation of hemoglobin. However, methemoglobin reduction systems, predominantly the NADH-cytochrome b₅ reductase 3 (NADH-methemoglobin reductase) pathway, normally limit its accumulation to only 1% of total hemoglobin (Chapter 9 and Figure 9-1).

Methemoglobin cannot carry oxygen because the oxidized ferric iron cannot bind it. An increase in the methemoglobin level results in decreased delivery of oxygen to the tissues. Individuals with methemoglobin levels less than 25% are generally asymptomatic. If the methemoglobin level increases to more than 30% of total hemoglobin, cyanosis (bluish discoloration of skin and mucous membranes) and symptoms of hypoxia (dyspnea, headache, vertigo, change in mental status) occur. Levels of methemoglobin greater than 50% can lead to coma and death.

An increase in methemoglobin, called methemoglobinemia, can be acquired or hereditary. The acquired form, also called toxic methemoglobinemia, occurs in normal individuals after exposure to an exogenous oxidant, such as nitrates, primaquine, dapsone, or benzocaine. As the oxidant overwhelms the hemoglobin reduction systems, the level of methemoglobin increases, and the patient may exhibit cyanosis and symptoms of hypoxia. In many cases, withdrawal of the offending oxidant is sufficient for a recovery, but if the level of methemoglobin increases to 30% or more of total hemoglobin, intravenous methylene blue is administered. The methylene blue reduces...
the methemoglobin ferric iron to the ferrous state through the NADPH-methemoglobin reduction pathway that involves glutathione reductase and glucose-6-phosphate dehydrogenase (Figure 9-1). In life-threatening cases, exchange transfusion may be required.12

Hereditary causes of methemoglobinemia are rare and include mutations in the gene for NADH-cytochrome b5 reductase (CYB5R3), resulting in a diminished capacity to reduce methemoglobin, and mutations in the α-, β-, or γ-globin gene, resulting in a structurally abnormal polypeptide chain that favors the oxidized ferric form of iron and prevents its reduction.11,12 The methemoglobin produced by the latter group is called M hemoglobin or Hb M. (Chapter 27). Hb M is inherited in an autosomal dominant pattern, with methemoglobin comprising 30% to 50% of total hemoglobin.12 There is no effective treatment for this form of methemoglobinemia.11,12 Cytochrome b5 reductase deficiency is an autosomal recessive disorder, and methemoglobin elevations occur in individuals who are homozygous or compound heterozygous for a CYB5R3 mutation.11,12 Most individuals with Hb M or homozygous cytochrome b5 reductase deficiency maintain methemoglobin levels below 50%; they have cyanosis but only mild symptoms of hypoxia that do not require treatment.11-13 Individuals heterozygous for the CYB5R3 mutation have normal levels of methemoglobin but develop methemoglobinemia, cyanosis, and hypoxia when exposed to an oxidant drug or chemical.12,14

Methemoglobin is assayed by spectral absorption analysis instruments such as the CO-oximeter. Methemoglobin shows an absorption peak at 630 nm.12 With high levels of methemoglobin, the blood takes on a chocolate brown color and does not revert back to the normal red color after oxygen exposure.12,13 The methemoglobin in Hb M disease has different absorption peaks, depending on the variant.11 Hemoglobin electrophoresis, high performance liquid chromatography, and DNA mutation testing are used for identification of Hb M variants. Cytochrome b5 reductase 3 deficiency is diagnosed by enzyme assays and DNA mutation testing.11

Sulfhemoglobin
Sulfhemoglobin is formed by the irreversible oxidation of hemoglobin by drugs (such as sulfonilamides, phenacetin, nitrates, and phenylhydrazine) or exposure to sulfur chemicals in industrial or environmental settings.11,12 It is formed by the addition of a sulfur atom to the pyrrole ring of heme and has a greenish pigment.11 Sulfhemoglobin is ineffective for oxygen transport, and patients with elevated levels present with cyanosis. Sulfhemoglobin cannot be converted to normal Hb A; it persists for the life of the cell. Treatment consists of prevention by avoidance of the offending agent.

Sulfhemoglobin has a similar peak to methemoglobin on a spectral absorption instrument. The sulfhemoglobin spectral curve, however, does not shift when cyanide is added, a feature that is used to distinguish it from methemoglobin.11

Carboxyhemoglobin
Carboxyhemoglobin (COHb) results from the combination of carbon monoxide (CO) with heme iron. The affinity of carbon monoxide for hemoglobin is 240 times that of oxygen.11 Once one molecule of carbon monoxide binds to hemoglobin, it shifts the hemoglobin-oxygen dissociation curve to the left, further increasing its affinity and severely impairing release of oxygen to the tissues.11,15 Carbon monoxide has been termed the silent killer because it is an odorless and colorless gas, and victims may quickly become hypoxic.15

Some carboxyhemoglobin is produced endogenously, but it normally comprises less than 2% of total hemoglobin.15 Exogenous carbon monoxide is derived from the exhaust of automobiles, tobacco smoke, and from industrial pollutants, such as coal, gas, and charcoal burning. In smokers, COHb levels may be as high as 15%.15 As a result, smokers may have a higher hematocrit and polycythemia to compensate for the hypoxia.11,14

Exposure to carbon monoxide may be coincidental, accidental, or intentional (suicidal). Many deaths from house fires are the result of inhaling smoke, fumes, or carbon monoxide.15 Even when heating systems in the home are properly maintained, accidental poisoning with carbon monoxide may occur. Toxic effects, such as headache, dizziness, and disorientation, begin to appear at blood levels of 20% to 30% COHb.13,14 Levels of more than 40% of total hemoglobin may cause coma, seizure, hypotension, cardiac arrhythmias, pulmonary edema, and death.11,15

Carboxyhemoglobin may be detected by spectral absorption instruments at 540 nm.12 It gives blood a cherry red color, which is sometimes imparted to the skin of victims.15 A diagnosis of carbon monoxide poisoning is made if the COHb level is greater than 3% in nonsmokers and greater than 10% in smokers.15 Treatment involves removal of the patient from the carbon monoxide source and administration of 100% oxygen.11 The use of hyperbaric oxygen therapy is controversial.15 It is primarily used to prevent neurologic and cognitive impairment after acute carbon monoxide exposure in patients whose COHb level exceeds 25%.15

HEMOGLOBIN MEASUREMENT

The cyanmethemoglobin method is the reference method for hemoglobin assay.16 A lying agent present in the cyanmethemoglobin reagent frees hemoglobin from RBCs. Free hemoglobin combines with potassium ferricyanide contained in the cyanmethemoglobin reagent, which converts the hemoglobin iron from the ferrous to the ferric state to form methemoglobin. Methemoglobin combines with potassium cyanide to form the stable pigment cyanmethemoglobin. The cyanmethemoglobin color intensity, which is proportional to hemoglobin concentration, is measured at 540 nm spectrophotometrically and compared with a standard (Chapter 14). The cyanmethemoglobin method is performed manually but has been adapted for use in automated instruments.

Many instruments now use sodium lauryl sulfate (SLS) to convert hemoglobin to SLS-methemoglobin. This method does not generate toxic wastes (Chapter 15).

Hemoglobin electrophoresis and HPLC are used to separate the different types of hemoglobins such as Hb A, A, and F (Chapters 27 and 28).
• The hemoglobin molecule is a tetramer composed of two pairs of unlike polypeptide chains. A heme group (protoporphyrin IX + Fe^{2+}) is bound to each of the four polypeptide chains.
• Hemoglobin, contained in RBCs, carries oxygen from the lungs to the tissues. Oxygen binds to the ferrous iron in heme. Each hemoglobin tetramer can bind four oxygen molecules.
• Six structural genes code for the six globin chains of hemoglobin. The α- and ζ-globin genes are on chromosome 16; the ε-, γ-, δ-, and β-globin gene cluster is on chromosome 11. There is one copy of the δ-globin gene and one β-globin gene per chromosome, for a total of two genes per diploid cell. There are two copies of the α- and γ-globin genes per chromosome, for a total of four genes per diploid cell.
• The three hemoglobins found in normal adults are Hb A, Hb A\(_2\), and Hb F. Hb A (α\(_2ββ\)) is composed of two αβ heterodimers, is the predominant hemoglobin of adults. Hb F (α\(_2γγ\)) is the predominant hemoglobin in the fetus and newborn. Hb A\(_2\) (α\(_2δδ\)) is present from birth through adulthood, but at low levels.
• Hemoglobin ontogeny describes which hemoglobins are produced by the erythroid precursor cells from the fetal period through birth to adulthood.
• Complex genetic mechanisms regulate the sequential expression of the polypeptide chains in the embryo, fetus, and adult. Heme provides negative feedback regulation on protoporphyrin and globin chain production.
• The hemoglobin-oxygen dissociation curve is sigmoid owing to cooperativity among the hemoglobin subunits in binding and releasing oxygen.
• 2,3-BPG produced by the glycolytic pathway facilitates the delivery of oxygen from hemoglobin to the tissues. The Bohr effect is the influence of pH on the release of oxygen from hemoglobin.
• In the tissues, carbon dioxide diffuses into the RBCs and combines with water to form carbonic acid (H\(_2\)CO\(_3\)). The carbonic acid is then converted to bicarbonate and hydrogen ions (HCO\(_3^-\) and H\(^+\)). Most of the carbon dioxide is carried by hemoglobin as H\(^+\).
• Methemoglobin, sulfhemoglobin, and carboxyhemoglobin cannot transport oxygen. They can accumulate to toxic levels due to exposure to certain drugs, industrial or environmental chemicals, or gases. A small fraction of methemoglobinemia cases are hereditary. Cyanosis occurs in patients with increased levels of methemoglobin or sulfhemoglobin.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

Answers can be found in the Appendix.

1. A hemoglobin molecule is composed of:
   a. One heme molecule and four globin chains
   b. Ferrous iron, protoporphyrin IX, and a globin chain
   c. Protoporphyrin IX and four globin chains
   d. Four heme molecules and four globin chains

2. Normal adult Hb A contains which polypeptide chains?
   a. α and β
   b. α and δ
   c. α and γ
   d. α and ε

3. A key rate-limiting step in heme synthesis is suppression of:
   a. Aminolevulinate synthase
   b. Carbonic anhydrase
   c. Protoporphyrin IX reductase
   d. Glucose 6-phosphate dehydrogenase

4. Which of the following forms of hemoglobin molecule has the lowest affinity for oxygen?
   a. Tense
   b. Relaxed

5. Using the normal hemoglobin-oxygen dissociation curve in Figure 10-7 for reference, predict the position of the curve when there is a decrease in pH.
   a. Shifted to the right of normal with decreased oxygen affinity
   b. Shifted to the left of normal with increased oxygen affinity
   c. Shifted to the right of normal with increased oxygen affinity
   d. Shifted to the left of normal with decreased oxygen affinity

6. The predominant hemoglobin found in a healthy newborn is:
   a. Gower-1
   b. Gower-2
   c. A
   d. F

7. What is the normal distribution of hemoglobins in healthy adults?
   a. 80% to 90% Hb A, 5% to 10% Hb A\(_2\), 1% to 5% Hb F
   b. 80% to 90% Hb A, 5% to 10% Hb A, 1% to 5% Hb F
   c. >95% Hb A, <5% Hb A\(_2\), 1% to 2% Hb F
   d. >90% Hb A, 5% Hb F, <5% Hb A\(_2\)
8. Which of the following is a description of the structure of oxidized hemoglobin?
   a. Hemoglobin carrying oxygen on heme; synonymous with oxygenated hemoglobin
   b. Hemoglobin with iron in the ferric state (methemoglobin) and not able to carry oxygen
   c. Hemoglobin with iron in the ferric state so that carbon dioxide replaces oxygen in the heme structure
   d. Hemoglobin carrying carbon monoxide; hence "oxidized" refers to the single oxygen

9. In the quaternary structure of hemoglobin, the globin chains associate into:
   a. α tetramers in some cells and β tetramers in others
   b. A mixture of α tetramers and β tetramers
   c. α dimers and β dimers
   d. Two αβ dimers

10. How are the globin chain genes arranged?
    a. With α genes and β genes on the same chromosome, including two α genes and two β genes
    b. With α genes and β genes on separate chromosomes, including two α genes on one chromosome and one β gene on a different chromosome
    c. With α genes and β genes on the same chromosome, including four α genes and four β genes
    d. With α genes and β genes on separate chromosomes, including four α genes on one chromosome and two β genes on a different chromosome

11. The nature of the interaction between 2,3-BPG and hemoglobin is that 2,3-BPG:
    a. Binds to the heme moiety, blocking the binding of oxygen
    b. Binds simultaneously with oxygen to ensure that it stays bound until it reaches the tissues, when both molecules are released from hemoglobin
    c. Binds to amino acids of the globin chain, contributing to a conformational change that inhibits oxygen from binding to heme
    d. Oxidizes hemoglobin iron, diminishing oxygen binding and promoting oxygen delivery to the tissues

REFERENCES


Iron Kinetics and Laboratory Assessment

Kathryn Doig*

OUTLINE
Iron Chemistry
Iron Kinetics
Systemic Body Iron Regulation
Iron Transport
Cellular Iron Absorption and Disposition
Iron Recycling
Dietary Iron, Bioavailability, and Demand
Laboratory Assessment of Body Iron Status
Serum Iron (SI)
Total Iron-Binding Capacity (TIBC)
Percent Transferrin Saturation
Prussian Blue Staining Ferritin
Soluble Transferrin Receptor (sTfR)
Hemoglobin Content of Reticulocytes
Soluble Transferrin Receptor/Log Ferritin
Thomas Plot
Zinc Protoporphyrin

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Describe the essential metabolic processes in which iron participates.
2. State whether body iron is regulated by excretion or absorption.
3. Describe the compartments in which body iron is distributed, including the relative amounts in each site.
4. Trace a molecule of iron from its absorption through the enterocyte to transport into mitochondria and then recycling via macrophages, including the names of all proteins with which it interacts and that control its kinetics.
5. Name the ionic form and number of molecules of iron that bind to one molecule of apotransferrin.
6. Explain how hepcidin regulates body iron levels.
7. Explain how individual cells absorb iron.
8. Explain how individual cells regulate the amount of iron they absorb.
9. Describe the role of each of the following in the kinetics of iron:
   a. Divalent metal transporter 1 (DMT1)
   b. Ferroportin
   c. Transferrin (Tf)
   d. Transferrin receptor (TfR)
   e. Hepcidin
10. For the proteins listed in objective 9, distinguish those that are involved in the regulation of iron within individual cells versus those involved in systemic body iron regulation.
11. Recognize the names of proteins involved in hepatocyte iron sensing and regulation of hepcidin production.
12. List factors that increase and decrease the bioavailability of iron.
13. Name foods high in iron, both heme-containing and ionic.
14. For each of the following assays, describe the principle of the assay and the iron compartment assessed:
   a. Total serum iron (SI)
   b. Total iron-binding capacity (TIBC)
   c. Percent transferrin saturation
   d. Serum ferritin
   e. Soluble transferrin receptor (sTfR)
   f. Measures of the hemoglobin content of reticulocytes
   g. Prussian blue staining of tissues and cells
   h. Zinc protoporphyrin (ZPP)
15. Plot given patient values on a Thomas plot and interpret the patient’s iron status.
16. Calculate the percent transferrin saturation when given total serum iron and TIBC.
17. When given reference intervals, interpret the results of each of the assays in objective 14 plus a Thomas plot and sTfR/log ferritin and recognize results consistent with decreased, normal, and increased iron status.
18. Identify instances in which sTfR, hemoglobin content of reticulocytes, sTfR/log ferritin, and Thomas plots may be needed to improve diagnosis of iron deficiency.

CASE STUDY
After studying the material in this chapter, the reader will be able to respond to the following case study:

In 1995, Garry, Koehler, and Simon assessed changes in stored iron in 16 female and 20 male regular blood donors aged 64 to 71. They measured hemoglobin, hematocrit, serum ferritin concentration, and % transferrin saturation in specimens from the donors, who gave an average of 15 units (approximately 485 mL/unit) of blood over 3.5 years. The investigators collected comparable data

*The author extends appreciation to Mary Coleman, whose coverage of iron metabolism in the prior editions provided the foundation for this chapter.
CASE STUDY—cont’d

After studying the material in this chapter, the reader will be able to respond to the following case study:

from nondonors. Of the donors, 10 women and 6 men took a dietary supplement providing approximately 20 mg of iron per day. In addition, mean dietary iron intake was 18 mg/day for the women and 20 mg/day for the men. Over the period of the study, mean iron stores in women donors decreased from 12.53 to 1.14 mg/kg of body weight. Mean iron stores in male donors declined from 12.45 to 1.92 mg/kg. Nondonors’ iron stores remained unchanged. Based on hemoglobin and hematocrit results, no donors became anemic. As iron stores decreased, the calculated iron absorption rose to 3.55 mg/day for the women and 4.10 mg/day for the men—more than double the normal rate for both women and men.

1. Why did the donors’ iron stores decrease?
2. Why did the donors’ iron absorption rate rise? Explain using the names of all proteins involved.
3. Name the laboratory test(s) performed in the study used to evaluate directly the iron storage compartment?
4. What is the diagnostic value of the % transferrin saturation? What iron compartment does it assess?

Among the metals that are required for metabolic processes, none is more important than iron. It is critical to energy production in all cells, being at the center of the cytochromes of mitochondria. Oxygen needed for energy production is carried attached to iron by the hemoglobin molecule in red blood cells. Iron is so critical to the body that there is no mechanism for active excretion, just minimal daily loss with exfoliated skin and hair and intestinal epithelia. Iron is even recycled to conserve as much as possible in the body. To insure against times when iron may be scarce in the diet, the body stores iron as well.

The largest percentage of body iron, nearly 65% of it, is held within hemoglobin in red blood cells of various stages (Table 11-1) while about 25% of body iron is in storage, mostly within macrophages and hepatocytes. The remaining 10% is divided among the muscles, the plasma, the cytochromes of cells, and various iron-containing enzymes within cells. A more functional approach to thinking about body iron distribution conceives of the iron as distributed in three compartments (Table 11-1). The functional compartment contains all iron that is functioning within cells. Though most of this is the iron in hemoglobin, the iron in myoglobin (in muscles) and cytochromes (in all cells) is part of the functional compartment. The storage compartment is the iron that is not currently functioning but is available when needed. The major sources of this stored iron are the macrophages and hepatocytes, but every cell except mature red blood cells, stores some iron. The third compartment is the transport compartment of iron that is in transit from one body site to another in the plasma.

Although the reactivity of iron ions makes them central to energy production processes, it also makes them dangerous to the stability of cells. Thus the body regulates iron carefully at the level of the whole body and also within individual cells, maintaining levels that are necessary for critical metabolic processes, while avoiding the dangers of excess iron accumulation. The conditions that develop when this balance is perturbed are described in Chapter 20. The routine tests used to assess body iron status are discussed here.

IRON CHEMISTRY

The metabolic functions of iron depend on its ability to change its valence state from reduced ferrous (Fe$^{2+}$) iron to the oxidized ferric (Fe$^{3+}$) state. Thus it is involved in oxidation and reduction reactions such as the electron transport within mitochondrial cytochromes. In cells, ferrous iron can react with peroxide via the Fenton reaction, forming highly reactive oxygen molecules.

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 = \text{Fe}^{3+} + \text{OH}^- + \text{OH}^.$$  

The resulting hydroxyl radical (OH·), also known as a free radical, is especially reactive as a short-lived but potent oxidizing agent, able to damage proteins, lipids, and nucleic acids. As will be described in the section on iron kinetics, there are various mechanisms within cells to reduce the potential for this type of damage.

IRON KINETICS

Systemic Body Iron Regulation

Figure 11-1 provides an overview of systemic body iron regulation that can be a reference throughout the section on iron

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Form and Anatomical Site</th>
<th>Percent of Total Body Iron</th>
<th>Typical Iron Content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional</td>
<td>Hemoglobin iron in the blood</td>
<td>~68</td>
<td>2.400</td>
</tr>
<tr>
<td></td>
<td>Myoglobin iron in muscles</td>
<td>~10</td>
<td>0.360</td>
</tr>
<tr>
<td></td>
<td>Peroxidase, catalase, cytochromes, riboflavin enzymes in all cells</td>
<td>~3</td>
<td>0.120</td>
</tr>
<tr>
<td>Storage</td>
<td>Ferritin and hemosiderin mostly in macrophages and hepatocytes; small amounts in all cells except mature red blood cells</td>
<td>~18</td>
<td>0.667</td>
</tr>
<tr>
<td>Transport</td>
<td>Transferrin in plasma</td>
<td>&lt;1</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Iron Kinetics and Laboratory Assessment

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The total amount of iron available to all body cells, systemic body iron, is regulated by absorption into the body because there is no mechanism for excretion. Ferrous iron in the lumen of the small intestine is carried across the luminal side of the enterocyte by divalent metal transporter 1 (DMT1) (Figure 11-2). Once iron has been absorbed into enterocytes, it requires another transporter, ferroportin, to carry it across the basolaminal enterocyte membrane into the bloodstream, thus truly absorbing it into the body. Ferroportin is the only known protein that exports iron across cell membranes. When the body has adequate stores of iron, the hepatocytes sense that and will increase production of hepcidin, a protein able to bind to ferroportin, leading to its inactivation. As a result, iron absorption into the body decreases. When the body iron decreases, hepcidin will also decrease so that absorption and recycling are again activated. Thus homeostasis of iron is maintained by modest fluctuations in liver hepcidin production in response to body iron status. The regulation of systemic body iron is summarized in Figure 11-3.

The mechanism by which the hepatocytes are able to sense iron levels and produce hepcidin is highly complex, with multiple stimulatory pathways likely involved. Although this system is not yet fully elucidated, a number of critical molecules have been identified. One postulated system for the interaction of these molecules is modeled in Figure 11-4. The proteins involved include, at least, the hemochromatosis receptor (HFE), transferrin receptor 2, hemojuvelin, bone morphogenic protein (BMP) and its receptor (BMPR), and sons of mothers against decapentaplegic (SMAD). Table 11-2 lists their functions. The importance of these various molecules to iron kinetics has been demonstrated via mutations, both natural in humans and induced in mice, that lead to either iron overload or iron deficiency. Testing for these mutations is increasing in molecular diagnostic laboratories. The diseases associated with the known human mutations are described in Chapter 20.

Iron Transport

Iron exported from the enterocyte into the blood is ferrous and must be converted to the ferric form for transport in the blood. Hephaestin, a protein on the basolaminal enterocyte membrane, is able to oxidize iron as it exits the enterocyte. Once oxidized, the iron is ready for plasma transport, carried by a specific protein, apotransferrin (ApoTf). Once iron binds, the molecule is known as transferrin (Tf). Apotransferrin binds two molecules of ferric iron.

Cellular Iron Absorption and Disposition

Individual cells regulate the amount of iron they absorb to minimize the adverse effects of free radicals. This is accomplished by relying on an iron-specific carrier to move it into the cell by a process called receptor-mediated endocytosis (Figure 11-5). Cell membranes possess a receptor for transferrin, transferrin receptor 1 (TfR1). TfR1 has the highest affinity for diferric Tf at the physiologic pH of the plasma and extracellular fluid. When the TfR1 molecules bind Tf, they move and cluster together in the membrane. Once a critical mass accumulates, the membrane begins to invaginate, progressing...
Iron: the body’s most precious metal. Denver, 2013, Colorado Association for

Iron recycling is central to regulating systemic body iron, macrophages and hepatocytes, typically contain the most. Ferric iron is stored in a cage-like protein called apoferritin. Once iron binds, it is known as ferritin. One ferritin molecule can bind more than 40,000 iron ions. Ferritin can be mobilized for use during times of iron need by lysosomal degradation of the protein. Partially degraded ferritin is known as hemosiderin and is considered to be less metabolically available than ferritin, though greater understanding of ferritin chemistry may revise this view.

In order to regulate the amount of iron inside the cell and avoid free radicals, cells are able to control the amount of TfR1 on their surface. The process depends on an elegant system of iron-sensitive cytoplasmic proteins that are able to affect the posttranscriptional function of the mRNA for TfR1. The result is that when iron stores inside the cell are sufficient, production of TfR1 declines. Conversely, when iron stores inside the cell are low, TfR1 production increases. This is useful diagnostically to detect iron deficiency because a truncated form of the TfR1 is sloughed from cells and is measurable in serum as soluble transferrin receptor (sTfR). The serum sTfR levels reflect the number of TfRs expressed on cells. So increases in sTfR can indicate increases in membrane TfR that result from low intracellular iron as seen in iron deficiency anemia.

Red blood cells deserve special mention in regard to cellular iron regulation. Because production of hemoglobin requires them to acquire far more iron than other cells, special mechanisms exist that allow them to circumvent the usual limitations on iron accumulation. The complete understanding of these processes has yet to be developed, but hypotheses suggest that the iron may bypass the cytoplasmic iron-sensing system, moving directly into the mitochondria from the endosome.

Iron Recycling

When cells die, their iron is recycled. Multiple mechanisms salvage iron from dying cells. The largest percentage of recycled iron comes from red blood cells. Senescent (aging) red blood cells are ingested by macrophages in the spleen. The hemoglobin is degraded, with the iron being held by the macrophages as ferritin. Like enterocytes, macrophages possess ferroportin in their membranes. This allows macrophages to be iron exporters so that the salvaged iron can be used by other cells. The exported iron is bound to plasma apotransferrin, just as if it were newly absorbed from the intestine.

Haptoglobin and hemopexin are plasma proteins able to salvage free hemoglobin or heme, respectively, preventing them from urinary loss at the glomerulus and returning the iron to the liver. Like macrophages, hepatocytes are important to iron salvage. They also possess ferroportin so that the salvaged iron can be exported to transferrin and ultimately to other body cells. These salvage pathways are described in greater detail in Chapter 23.

DIETARY IRON, BIOAVAILABILITY, AND DEMAND

Under normal circumstances, the only source of iron for the body is from the diet. Foods containing high levels of iron
include red meats, legumes, and dark green leafy vegetables.\textsuperscript{12} Although some foods may be high in iron, that iron may not be readily absorbed and thus is not bioavailable. Iron can be absorbed as either ionic iron or nonionic iron in the form of heme. Ionic iron must be in the ferrous (Fe\textsuperscript{2+}) form for absorption into the enterocyte via the luminal membrane carrier, divalent metal transporter (DMT1). However, most dietary iron is ferric, especially from plant sources. As a result, it is not readily absorbed. Furthermore, other dietary compounds can bind iron and inhibit its absorption. These include oxalates, phytates, phosphates, and calcium.\textsuperscript{12} Release from these binders and reduction to the ferrous form are enhanced by gastric acid, acidic foods (e.g., citrus), and an enterocyte luminal membrane protein, duodenal cytochrome B (DcytB). Thus, although the U.S. diet contains on the order of 10 to 20 mg of iron/day, only 1 to 2 mg is absorbed.\textsuperscript{13} This amount is adequate for most men, but menstruating women, pregnant and lactating women, and growing children usually need additional iron supplementation to meet their increased need for iron. Chapter 20 discusses this further. Heme with its bound iron is more readily absorbed than ionic iron.\textsuperscript{13} Thus meat, with heme in both myoglobin of muscle and hemoglobin of blood, is the most bioavailable source of dietary iron. The means by which heme is absorbed by enterocytes is not entirely clear. Although one carrier has been identified, it is actually more efficient at carrying folic acid.\textsuperscript{14,15} So the primary heme carrier protein is still being sought.

**LABORATORY ASSESSMENT OF BODY IRON STATUS**

Disease occurs when body iron levels are either too low or too high (Chapter 20). The tests used to assess body iron status are able to detect both conditions. They include the traditional or classic iron studies: serum iron (SI), total iron-binding capacity (TIBC), percent transferrin saturation, and Prussian blue staining of tissues. More recently, ferritin assays have been included among the routine tests. For special circumstances

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**Figure 11-3** Summary of body iron regulation. When body iron stores drop sufficiently low that plasma iron also drops, the liver’s iron-sensing system is activated and hepcidin production is decreased. As a result, ferroportin in the enterocyte and macrophage membranes will transport iron into the circulation. Plasma iron will rise and body stores will be restored. The hepatocyte iron-sensing system recognizes this and produces hepcidin. Hepcidin inactivates ferroportin in the enterocyte and macrophage membranes so that less iron is absorbed and recycled. The resulting drop in iron stores and plasma iron is detected by the liver, repeating the cycle. (From Doig K: Iron: the body’s most precious metal. Denver, 2013, Colorado Association for Continuing Medical Laboratory Education, Inc., p. 18.)
**Figure 11-4.** Hepatic iron-sensing systems leading to hepcidin production. One system of iron sensing by hepatocytes involves the release of the hemochromatosis receptor (HFE) from transferrin receptor 1 (TfR1) when the latter binds transferrin (Tf). The freed HFE then binds to transferrin receptor 2 (TfR2), initiating a membrane signal that phosphorylates sons of mothers against decapentaplegic (SMAD) proteins that move to the nucleus and upregulate hepcidin gene expression. A second system activated when body iron is high in secretion of bone morphogenic protein 6 (BMP6) by liver macrophages. BMP6 binds with its receptor, BMPR, and coreceptor, hemajuvelin (HJV), to phosphorylate SMAD and ultimately increase hepcidin production. Matriptase 2 can inactivate HJV and is an important mechanism to reduce hepcidin production when body iron is low. (From Camaschella C, Silvestri L: Molecular mechanisms regulating hepcidin revealed by hepcidin disorders. The Scientific World 11: 1357-1366, 2011 and Doig K: Iron: the body’s most precious metal. Denver, 2013, Colorado Association for Continuing Medical Laboratory Education, Inc., p. 17.)

when the results of routine assays are equivocal or too invasive, newer assays include the soluble transferrin receptor (sTfR) and hemoglobin content of reticulocytes. The results of these measured parameters can be combined to calculate an sTfR/log ferritin ratio or graph a Thomas plot. Finally, zinc protoporphyrin is another assay with special application in sideroblastic anemia. Diagnostically, the tests can be organized to assess each of the iron compartments as indicated in Table 11-3.

**Serum Iron (SI)**
Serum iron can be measured colorimetrically using any of several reagents such as ferrozine. The iron is first released from transferrin by acid, and then the reagent is allowed to react with the freed iron, forming a colored complex that can be detected spectrophotometrically. Reference intervals are reported separately for men, women, and children, and will vary from laboratory to laboratory and from method to method. The serum iron level has limited utility on its own because of its high within-day and between-day variability; it also increases after recent ingestion of iron-containing foods and supplements. To avoid the apparent diurnal variation, the standard practice has been to collect the specimen fasting and early in the morning when levels are expected to be highest. However, this practice has recently been questioned. A diurnal variation in hepcidin has been detected that may explain some of the serum iron variability and may still support the early-morning phlebotomy practice. A typical reference interval is provided in Table 11-3.

**Total Iron-Binding Capacity (TIBC)**
The amount of iron in plasma or serum will be limited by the amount of transferrin that is available to carry it. To assess this, transferrin is maximally saturated by addition of excess ferric iron to the specimen. Any unbound iron is removed by precipitation with magnesium carbonate powder. Then the basic iron method as described above is performed on the absorbed serum, beginning with the release of the iron from transferrin. The amount of iron detected represents all the binding sites available on transferrin—that is, the total iron-binding capacity (TIBC). It is expressed as an iron value, although it is actually an indirect measure of transferrin. A typical reference interval is provided in Table 11-3.
TABLE 11-2 Functions and Locations of Proteins Involved in Body Iron Sensing and Hepcidin Production

<table>
<thead>
<tr>
<th>Protein</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemochromatosis protein (HFE)</td>
<td>Hepatocyte membrane</td>
<td>A protein that is bound to transferrin receptor 1 (TfR1) until released by the binding of transferrin to TfR1</td>
</tr>
<tr>
<td>Transferrin receptor 2 (TfR2)</td>
<td>Hepatocyte membrane</td>
<td>A hepatocyte transferrin receptor that is able to bind freed HFE to initiate an internal cell signal for hepcidin production</td>
</tr>
<tr>
<td>Bone morphogenic protein (BMP)</td>
<td>Secreted product of macrophages</td>
<td>The ligand secreted by macrophages that initiates signal transduction when it binds to its receptor in a cell membrane</td>
</tr>
<tr>
<td>Bone morphogenic protein receptor (BMPR)</td>
<td>Hepatocyte (and other cells) membrane</td>
<td>A common membrane receptor initiating signal transduction within a cell when its ligand (BMP) binds</td>
</tr>
<tr>
<td>Hemojuvelin (HJV)</td>
<td>Hepatocyte membrane</td>
<td>A coreceptor acting with BMPR for signal transduction, leading to hepcidin production</td>
</tr>
<tr>
<td>Sons of mothers against decapentaplegic</td>
<td>Hepatocyte (and other cells) cytoplasm</td>
<td>A second messenger of signal transduction activated by BMPR-HJV complex, able to migrate to the nucleus and upregulate hepcidin gene expression</td>
</tr>
</tbody>
</table>

Figure 11-5 Cellular iron regulation. A critical mass of transferrin receptor 1 (TIR) with bound transferrin (Tf) will initiate an invagination of the membrane that ultimately fuses to form an endosome. Hydrogen ion (H+) inside the endosome releases the iron from Tf, and once reduced, it is transported into the cytosol by divalent metal transporter 1 (DMT1). In the cytosol, iron may be stored as ferritin or transferred to the mitochondria, where it is transported across the membrane by mitoferrin (not shown). The TIR with apotransferrin is returned to the cell membrane, where the ApoTf releases and the TIR is available to bind more Tf for iron transport into the cell. (From http://walz.med.harvard.edu/Research/Iron_Transport/Tf-Tf-Figure-1.gif)
### Percent Transferrin Saturation

Since the TIBC represents the total number of sites for iron binding and the SI represents the number bound with iron, the degree to which the available sites are occupied by iron can be calculated. The percent of transferrin saturated with iron is calculated as:

\[
% \text{ transferrin saturation} = \frac{\text{SI}}{\text{TIBC}} \times 100\%
\]

It is important that both the SI and TIBC be expressed in the same units, but it does not matter which units are used in the calculation. A typical reference interval is provided in Table 11-3. A convenient rule of thumb evident from the table is that about one third \((1/3)\) of transferrin is typically saturated with iron.

### Prussian Blue Staining

Prussian blue is actually a chemical compound with the formula \(\text{Fe}_7(\text{CN})_{18}\). The compound forms during the staining process, which uses acidic potassium ferrocyanide as the reagent/stain. The ferric iron in the tissue reacts with the reagent, forming the Prussian blue compound that is readily seen microscopically as dark blue dots. Tissues can be graded or scored semiquantitatively by the amount of stain that is observed. Prussian blue stain is considered the gold standard for assessment of body iron. Staining is conducted routinely when bone marrow or liver biopsies are taken for other purposes. Although ferric iron reacts with the reagent, ferritin is not detected, likely due to the intact protein cage. However, hemosiderin stains readily.

### Ferritin

As mentioned above, until the development of serum ferritin assays, the only way to truly assess body iron stores was to take a sample of bone marrow and stain it with Prussian blue. Such an invasive procedure prevented regular assessment of body iron. The development of the serum immunoassay for ferritin provided a convenient assessment of body iron stores. Though ferritin is an intracellular protein, it is secreted by macrophages into plasma for reasons that are not yet understood. The level of serum ferritin has been shown to correlate highly with stored iron as indicated by Prussian blue stains of bone marrow. Typical reference intervals are provided in Table 11-3.

There is a significant drawback in the interpretation of serum ferritin results. Ferritin is an acute phase protein or acute phase reactant (APR). The APRs are proteins that are produced, mostly by the liver, during the acute (i.e., initial) phase of inflammation, especially during infections. They include cytokines that are nonspecific, but also other proteins with the apparent intent to suppress bacteria. Since bacteria need iron, the body’s production of ferritin during the acute phase seems to be an attempt to sequester the iron away from the bacteria. Thus increases in ferritin can be induced without an increase in the amount of systemic body iron. These rises may not be outside the reference interval but still high enough to elevate a patient’s ferritin above what it would otherwise be. Ferritin values between 20 and 100 ng/mL are most equivocal, making it difficult to recognize true iron deficiency when an inflammatory condition is also present. Therefore, the predictive value of a ferritin result within the reference interval is weak. However, only a decreased level of stored body iron can lower ferritin levels below the reference interval, so the predictive value of a low ferritin result is high for iron deficiency.

### Soluble Transferrin Receptor (sTfR)

As described above, cells regulate the amount of TfR on their membrane based on the amount of intracellular iron. When the latter drops, the cell expresses more TfR on the membrane. A truncated form of the receptor is shed into the plasma and can be detected with immunoassay. Thus increases in the sTfR

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**TABLE 11-3** Assessment of Body Iron Status

<table>
<thead>
<tr>
<th>Laboratory Assay</th>
<th>Typical Adult Male Reference Interval</th>
<th>Diagnostic Use and Compartment Assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum iron level</td>
<td>50–160 µg/dL</td>
<td>Indicator of available transport iron</td>
</tr>
<tr>
<td>Serum transferrin level (TIBC)</td>
<td>250–400 µg/dL</td>
<td>Indirect indicator of iron stores</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>20%–55%</td>
<td>Indirect indicator of iron stores with transport iron</td>
</tr>
<tr>
<td>Serum ferritin level</td>
<td>40–400 ng/mL</td>
<td>Indicator of iron stores</td>
</tr>
<tr>
<td>Bone marrow or liver biopsy with Prussian blue staining</td>
<td>Normal iron stores visualized</td>
<td>Visual qualitative assessment of tissue iron stores</td>
</tr>
<tr>
<td>Soluble transferrin receptor (sTfR) level</td>
<td>1.15–2.75 mg/L</td>
<td>Indicator of functional iron available in cells</td>
</tr>
<tr>
<td>sTfR/log ferritin index</td>
<td>0.63–1.8</td>
<td>Indicator of functional iron available in cells</td>
</tr>
<tr>
<td>RBC zinc protoporphyrin level</td>
<td>&lt;80 µg/dL of RBCs</td>
<td>Indicator of functional iron available in red blood cells</td>
</tr>
<tr>
<td>Hemoglobin content of reticulocytes</td>
<td>27–34 pg/cell</td>
<td>Indicator of functional iron available in developing red blood cells</td>
</tr>
</tbody>
</table>

RBC, Red blood cell; TIBC, total iron-binding capacity.

---

**Laboratory Assay**

- Serum iron level
- Serum transferrin level (TIBC)
- Transferrin saturation
- Serum ferritin level
- Bone marrow or liver biopsy with Prussian blue staining
- Soluble transferrin receptor (sTfR) level
- sTfR/log ferritin index
- RBC zinc protoporphyrin level
- Hemoglobin content of reticulocytes

**Typical Adult Male Reference Interval**

- Serum iron level: 50–160 µg/dL
- Serum transferrin level (TIBC): 250–400 µg/dL
- Transferrin saturation: 20%–55%
- Serum ferritin level: 40–400 ng/mL
- Bone marrow or liver biopsy with Prussian blue staining: Normal iron stores visualized
- Soluble transferrin receptor (sTfR) level: 1.15–2.75 mg/L
- sTfR/log ferritin index: 0.63–1.8
- RBC zinc protoporphyrin level: <80 µg/dL of RBCs
- Hemoglobin content of reticulocytes: 27–34 pg/cell

**Diagnostic Use and Compartment Assessed**

- Indicator of available transport iron
- Indirect indicator of iron stores
- Indirect indicator of iron stores with transport iron
- Indicator of iron stores
- Visual qualitative assessment of tissue iron stores
- Indicator of functional iron available in cells
- Indicator of functional iron available in cells
- Indicator of functional iron available in red blood cells
- Indicator of functional iron available in developing red blood cells
reflect either increases in the amounts of TfR on individual cells, as in iron deficiency, or an increase in the number of cells each with a normal number of TfRs. The latter occurs during instances of rapid erythropoiesis, such as a response to hemolytic anemia. Typical reference intervals are provided in Table 11-3.

**Hemoglobin Content of Reticulocytes**
Chapter 15 describes how some hematology instruments are able to report a value for the amount of hemoglobin in reticulocytes; it is analogous to the mean cell hemoglobin (MCH), but just for reticulocytes. Because, under normal conditions, the number of circulating reticulocytes represents the status of erythropoiesis in the prior 24-hour period, the amount of hemoglobin in reticulocytes provides a near real-time assessment of iron available for hemoglobin production. The hemoglobin content of reticulocytes will drop when iron for erythropoiesis is restricted. A representative adult reference interval is provided in Table 11-3. Separate reference intervals may be provided for children and infants.

**Soluble Transferrin Receptor/Log Ferritin**
Although ferritin and sTfR values alone can point to iron deficiency, the ratio of sTfR to ferritin or sTfR to log ferritin improves the identification of iron deficiency when values are equivocal. Because the sTfR rises in iron deficiency and the ferritin (and its log) drops, these ratios are especially useful when one of the parameters has changed but is not outside the reference interval. A typical reference interval is provided in Table 11-3.

**Thomas Plot**
Thomas and Thomas demonstrated that when the sTfR/log ferritin is plotted against the hemoglobin content of reticulocytes, a four-quadrant plot results that can improve the identification of iron deficiency (Figure 11-6). In instances where there is true iron deficiency, the sTfR will rise and the ferritin will drop so that the sTfR/log ferritin will be high and the hemoglobin content of reticulocytes will be low; patient results will plot to the lower right quadrant. In instances where the ferritin may be falsely elevated by inflammation, the sTfR/log ferritin will be normal despite reduced availability of iron for hemoglobin production—thus a low hemoglobin content in reticulocytes. In this instance, patient values will plot to the lower left quadrant called functional iron deficiency because the systemic body stores are adequate but not available for transport and use by cells. As iron deficiency develops, other cells are starved before erythrocytes; production of hemoglobin in reticulocytes remains at a normal level for as long as possible. However, the body’s other iron-starved cells will increase sTfR production and systemic iron stores of ferritin will be depleted, thus elevating the sTfR/log ferritin value. These early iron-deficient patients’ results will plot to the upper right quadrant called latent iron deficiency. By incorporating several different assessments of iron status, the use of the Thomas plot, as it is called, can improve the identification of iron deficiency in instances when other tests are equivocal. Chapter 20 will elucidate further the impact of various diseases on the parameters of the Thomas plot.

**Zinc Protoporphyrin**
Zinc protoporphyrin (ZPP) accumulates in red blood cells when iron is not incorporated into heme and zinc binds instead to protoporphyrin IX. It is easily detected by fluorescence. Although ZPP will rise during iron-deficient erythropoiesis, the value of this test is greatest when the activity of the ferrochelatase is impaired, as in lead poisoning (Chapter 20).
• Iron is so critical for transport and use of oxygen that the body conserves and recycles it, and it does not have a mechanism for its active excretion. Free radical production by iron ions severely damages cells and thus demands regulation. The body adjusts its iron levels by intestinal absorption, depending on need.
• Iron is absorbed into enterocytes as ferrous iron by the divalent metal transporter 1 (DMT1) on the luminal side of the cells. Heme can also be absorbed. Iron is exported into the plasma via ferroportin, a protein carrier in the enterocyte basolaminal membrane.
• Iron is carried in the plasma in ferric form attached to transferrin. Each molecule of apotransferrin can bind two molecules of iron. Apotransferrin with bound iron is called transferrin.
• Individual cells absorb iron when ferric transferrin binds to transferrin receptor 1 (TfR1) on their surfaces. Bound receptors cluster and invaginate the membrane to form an endosome. Iron released by acid within the endosome is exported into the plasma and ultimately into the mitochondria for incorporation into cytochromes and heme. Alternatively, it can also be stored as ferritin in the cytosol. The iron-depleted endosome fuses with the cell membrane, releasing the apotransferrin and thus allowing the TfR1 on the cell membrane to bind more ferric transferrin.
• Macrophages ingest dying red blood cells. They salvage and store the iron derived from heme.
• Hepatocytes sense body iron status through the interaction of the hemochromatosis receptor, transferrin receptor 2, hemouvelin, bone morphogenic protein, and SMAD.
• When the hepatocyte iron-sensing system detects that body iron levels are high, the hepatocyte secretes hepcidin. Hepcidin inactivates ferroportin in enterocyte, macrophage, and hepatocyte membranes, reducing the absorption of new iron and the release of stored iron. When the hepatocyte senses low body iron, hepcidin secretion is reduced, and ferroportin is active for intestinal iron absorption and macrophage and hepatocyte iron export into the plasma.
• Individual cells adjust the number of transferrin receptors on their surface to regulate the amount of iron they absorb; receptor numbers rise when the cell needs additional iron but decrease when the iron in the cell is adequate. Truncated soluble transferrin receptors are also shed into the plasma in proportion to their number on cells.
• Cells store iron as ferritin when they have an excess. Iron can be released from ferritin when needed by degradation of the protein by lysosomes. Partially degraded ferritin can be detected in cells as stainable hemosiderin. Ferritin is secreted into the plasma by macrophages in proportion to the amount of iron that is in storage. Ferritin is elevated in plasma by the acute phase response, unrelated to amounts of stored iron.
• Dietary iron is most bioavailable as heme from meat sources. Plant sources typically supply ferric iron that must be released from iron-binding compounds and reduced before absorption.
• Most body iron is found in hemoglobin or stored as ferritin. Less than 10% of all body iron is found in muscles, plasma, cytochromes, and iron-dependent enzymes throughout body cells.
• Laboratory tests for assessment of iron status include total serum iron, total iron-binding capacity, percent transferrin saturation, serum ferritin, soluble transferrin receptor, tissue staining for hemosiderin, zinc protoporphyrin, and the hemoglobin content of reticulocytes. Additional parameters derived from these, the Thomas plot and sTIR/log ferritin, are particularly useful for the recognition of iron deficiency when other test results are equivocal.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

### REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Iron is transported in plasma via:
   a. Hemosiderin
   b. Ferritin
   c. Transferrin
   d. Hemoglobin

2. What is the major metabolically available storage form of iron in the body?
   a. Hemosiderin
   b. Ferritin
   c. Transferrin
   d. Hemoglobin

3. The total iron-binding capacity (TIBC) of the serum is an indirect measure of which iron-related protein?
   a. Hemosiderin
   b. Ferritin
   c. Transferrin
   d. Hemoglobin

4. For a patient with classic iron study values that are equivocal for iron deficiency, which of the following tests would be most helpful in determining whether iron deficiency is present or not?
   a. Zinc protoporphyrin
   b. Peripheral blood sideroblast assessment
   c. Soluble transferrin receptor
   d. Mean cell hemoglobin
5. What membrane-associated protein in enterocytes transports iron from the intestinal lumen into the enterocyte?
   a. Transferrin
   b. Ferroportin
   c. DMT1
   d. Ferrochelatase

6. Iron is transported out of macrophages, hepatocytes, and enterocytes by what membrane protein?
   a. Transferrin
   b. Ferroportin
   c. DMT1
   d. Ferrochelatase

7. Below are several of the many steps in the process from absorption and transport of iron to incorporation into heme. Place them in proper order.
   i. Transferrin picks up ferric iron.
   ii. Iron is transferred to the mitochondria.
   iii. DMT1 transports ferrous iron into the enterocyte.
   iv. Ferroportin transports iron from enterocyte to plasma.
   v. The transferrin receptor transports iron into the cell.
   a. v, iv, i, ii, iii
   b. iii, ii, iv, i, v
   c. ii, i, v, iii, iv
   d. iii, iv, i, v, ii

8. What is the fate of the transferrin receptor when it has completed its role in the delivery of iron to a cell?
   a. It is recycled to the plasma membrane and released into the plasma.
   b. It is recycled to the plasma membrane, where it can bind its ligand again.
   c. It is catabolized and the amino acids are returned to the metabolic pool.
   d. It is retained in the endosome for the life span of the cell.

9. The transfer of iron from the enterocyte into the plasma is REGULATED by:
   a. Transferrin
   b. Ferroportin
   c. Hephaestin
   d. Hepcidin

10. What is the percent transferrin saturation for a patient with total serum iron of 63 μg/dL and TIBC of 420 μg/dL?
    a. 6.7%
    b. 12%
    c. 15%
    d. 80%

11. Referring to Figure 11-6, into which quadrant of a Thomas plot would a patient’s results fall with the following test results:
    Soluble transferrin receptor: increased above reference interval
    Ferritin: decreased below reference interval
    Hemoglobin content of reticulocytes: within the reference interval
    a. Normal iron status
    b. Latent iron deficiency
    c. Functional iron deficiency
    d. Iron deficiency

12. A physician is concerned that a patient is developing iron deficiency from chronic intestinal bleeding due to aspirin use for rheumatoid arthritis. The iron studies on the patient show the following results:

<table>
<thead>
<tr>
<th>Laboratory Assay</th>
<th>Adult Reference Intervals</th>
<th>Patient Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ferritin level</td>
<td>12–400 ng/mL</td>
<td>25 ng/mL</td>
</tr>
<tr>
<td>Serum iron level</td>
<td>50–160 μg/dL</td>
<td>45 μg/dL</td>
</tr>
<tr>
<td>Total iron-binding capacity (TIBC)</td>
<td>250–400 μg/dL</td>
<td>405 μg/dL</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>20%–55%</td>
<td>CALCULATE IT</td>
</tr>
</tbody>
</table>

How would these results be interpreted?
   a. Latent iron deficiency
   b. Functional iron deficiency
   c. Iron deficiency
   d. Equivocal for iron deficiency

REFERENCES


Leukocyte Development, Kinetics, and Functions

Woodlyne Roquiz, Sameer Al Diffalha, Ameet R. Kini*

OUTLINE

Granulocytes
  Neutrophils
  Eosinophils
  Basophils
  Mast Cells
Mononuclear Cells
  Monocytes
  Lymphocytes

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the pathways and progenitor cells involved in the derivation of leukocytes from the hematopoietic stem cell to mature forms.
2. Name the different stages of neutrophil, eosinophil, and basophil development and describe the morphology of each stage.
3. Discuss the important functions of neutrophils, eosinophils, and basophils.
4. Describe the morphology of promonocytes, monocytes, macrophages, T and B lymphocytes, and immature B cells (hematogones).
5. Discuss the functions of monocytes, macrophages, T cells, B cells, and natural killer cells in the immune response.
6. Compare the kinetics of neutrophils and monocytes.
7. Discuss in general terms how the various types of lymphocytes are produced.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 5-year-old girl presents with shortness of breath and wheezing. The patient gives a history of similar symptoms in the last 6 months. After the patient was given albuterol to control her acute symptoms, long-term control of her disease was achieved through the use of corticosteroids, along with monoclonal antibodies to IL-5.

1. Which leukocytes are important in mediating the clinical symptoms in this patient?
2. A complete blood count with differential was performed on this patient. What are the typical findings in such patients?
3. How did monoclonal antibodies to IL-5 help in controlling her disease?

Leukocytes (also known as white blood cells, or WBCs) are so named because they are relatively colorless compared to red blood cells. The number of different types of leukocytes varies depending on whether they are being viewed with a light microscope after staining with a Romanowsky stain (5 or 6 types) or are identified according to their surface antigens using flow cytometry (at least 10 different types). For the purposes of this chapter, the classic, light microscope classification of leukocytes will be used.

Granulocytes are a group of leukocytes whose cytoplasm is filled with granules with differing staining characteristics and whose nuclei are segmented or lobulated. Individually, they include eosinophils, with granules containing basic proteins that stain with acid stains such as eosin; basophils, with granules that are acidic and stain with basic stains such as methylene blue; and neutrophils, with granules that react with both acid and basic stains, which gives them a pink to lavender color. Because nuclear segmentation is quite prominent in mature neutrophils, they have also been called polymorphonuclear cells, or PMNs.

Mononuclear cells are categorized into monocytes and lymphocytes. These cells have nuclei that are not segmented but are round, oval, indented, or folded. Leukocytes develop from hematopoietic stem cells (HSCs) in the bone marrow, where most undergo differentiation and maturation (Figure 12-1), and then are released into the circulation. The number of circulating leukocytes varies with sex, age, activity, time of day, and ethnicity; it

*The authors acknowledge the contributions of Anne Stiene-Martin, author of this chapter in the previous edition.
also differs according to whether or not the leukocytes are reacting to stress, being consumed, or being destroyed, and whether or not they are being produced by the bone marrow in sufficient numbers. Reference intervals for total leukocyte counts vary among laboratories, depending on the patient population and the type of instrumentation being used, but a typical reference interval is $4.5 \times 10^9/L$ to $11.5 \times 10^9/L$ for adults.

The overall function of leukocytes is in mediating immunity, either innate (nonspecific), as in phagocytosis by neutrophils, or specific (adaptive), as in the production of antibodies by lymphocytes and plasma cells. The term kinetics refers to the movement of cells through developmental stages, into the circulation, and from the circulation to the tissues and includes the time spent in each phase of the cell’s life. As each cell type is discussed in this chapter, developmental stages, kinetics, and specific functions will be addressed.

Figure 12-1 Diagram of hematopoiesis showing the derivation pathways of each type of blood cell from a hematopoietic stem cell.

GRANULOCYTES

Neutrophils

Neutrophils are present in the peripheral blood in two forms according to whether the nucleus is segmented or still in a band shape. Segmented neutrophils make up the vast majority of circulating leukocytes.

Neutrophil Development

Neutrophil development occurs in the bone marrow. Neutrophils share a common progenitor with monocytes, known as the granulocyte-monocyte progenitor (GMP). The major cytokine responsible for the stimulation of neutrophil production is granulocyte colony-stimulating factor, or G-CSF.

There are three pools of developing neutrophils in the bone marrow (Figure 12-2): the stem cell pool, the proliferation
pool, and the maturation pool.\textsuperscript{4,7} The stem cell pool consists of hematopoietic stem cells (HSCs) that are capable of self-renewal and differentiation.\textsuperscript{8} The proliferation (mitotic) pool consists of cells that are dividing and includes (listed in the order of maturation) common myeloid progenitors (CMPs), also known as colony-forming units–granulocyte, erythrocyte, monocyte, and megakaryocyte (CFU-GEMMs); granulocyte-macrophage progenitors (GMPs); myeloblasts; promyelocytes; and myelocytes. The third marrow pool is the maturation (storage) pool consisting of cells undergoing nuclear maturation that form the marrow reserve and are available for release: metamyelocytes, band neutrophils, and segmented neutrophils.

HSCs, CMPs, and GMPs are not distinguishable with the light microscope and Romanowsky staining and may resemble early type I myeloblasts or lymphoid cells. They can, however, be identified through surface antigen detection by flow cytometry.

Myeloblasts make up 0% to 3% of the nucleated cells in the bone marrow and measure 14 to 20 \textmu{}m in diameter. They are frequently subdivided into type I, type II, and type III myeloblasts. The type I myeloblast has a high nucleus-to-cytoplasm (N:C) ratio of 8:1 to 4:1 (the nucleus occupies most of the cell, with very little cytoplasm), slightly basophilic cytoplasm, fine nuclear chromatin, and two to four visible nucleoli. Type I blasts have no visible granules when observed under light microscopy with Romanowsky stains. The type II myeloblast shows the presence of dispersed primary (azurophilic) granules in the cytoplasm; the number of granules does not exceed 20 per cell (Figure 12-3). Type III myeloblasts have a darker chromatin and a more purple cytoplasm, and they contain more than 20 granules that do not obscure the nucleus. Type III myeloblasts are rare in normal bone marrows, but they can be seen in certain types of acute myeloid leukemias. Recently, Mufti and colleagues\textsuperscript{9} proposed combining type II and type III blasts into a single category of “granular blasts” due to the difficulty in distinguishing type II blasts from type III blasts.

Promyelocytes comprise 1% to 5% of the nucleated cells in the bone marrow. They are relatively larger than the myeloblast cells and measure 16 to 25 \textmu{}m in diameter. The nucleus is round to oval and is often eccentric. A paranuclear halo or “hof” is usually seen in normal promyelocytes but not in the malignant promyelocytes of acute promyelocytic leukemia (described in Chapter 35). The cytoplasm is evenly basophilic and full of primary (azurophilic) granules. These granules are the first in a series of granules to be produced during neutrophil maturation (Box 12-1).\textsuperscript{10} The nucleus is similar to that described earlier for myeloblasts except that chromatin clumping (heterochromatin) may be visible, especially around the edges of the nucleus. One to three nucleoli can be seen but may be obscured by the granules (Figure 12-4).

Neutrophil myelocytes make up 6% to 17% of the nucleated cells in the bone marrow and are the final stage in which cell division (mitosis) occurs. During this stage, the production of primary granules ceases, and the cell begins to manufacture secondary (specific) neutrophil granules. This stage of neutrophil development is sometimes divided into early and late myelocytes. Early myelocytes may look very similar to the promyelocytes (described earlier) in size and nuclear characteristics except that patches of grainy pale pink cytoplasm representing secondary granules begin to be evident in the area of the Golgi apparatus. This has been referred to as the dawn of neutrophilia. Secondary neutrophilic granules slowly spread through the cell until its cytoplasm is more lavender-pink than blue. As the cell divides, the number of primary granules per cell is decreased, and their membrane chemistry changes so that they are much less visible. Late myelocytes are somewhat smaller than promyelocytes (15 to 18 \textmu{}m), and the nucleus has considerably more heterochromatin. Nucleoli are difficult to see by light microscopy (Figure 12-5).

Neutrophil metamyelocytes constitute 3% to 20% of nucleated marrow cells. From this stage forward, the cells are no longer capable of division, and the major morphologic change is in the shape of the nucleus. The nucleus is indented (kidney bean shaped or peanut shaped), and the chromatin is increasingly clumped. Nucleoli are absent. Synthesis of tertiary granules (also known as gelatinase granules) may begin during this stage. The size of the metamyelocyte is slightly smaller than that of the myelocyte (14 to 16 \textmu{}m). The cytoplasm contains very little residual ribonucleic acid (RNA) and therefore little or no basophilia (Figure 12-6).

Neutrophil bands make up 9% to 32% of nucleated marrow cells and 0% to 5% of the nucleated peripheral blood cells. All evidence of RNA (cytoplasmic basophilia) is absent, and tertiary granules continue to be formed during this stage. Secondary granules (also known as secretory vesicles) may begin to be formed during this stage. The nucleus is highly clumped, and the nuclear indentation that began in the metamyelocyte stage now exceeds one half the diameter of the nucleus, but actual segmentation has not yet occurred (Figure 12-7). Over the past 70 years, there has been considerable controversy over the definition of a band and the differentiation between bands and segmented forms. There have been three schools of thought concerning identification of bands, from the most...
conservative—holding that the nucleus in a band must have the same diameter throughout its length—to the most liberal—requiring that a filament between segments be visible before a band becomes a segmented neutrophil. The middle ground states that when doubt exists, the cell should be called a segmented neutrophil. An elevated band count was thought to be useful in the diagnosis of patients with infection. However, the clinical utility of band counts has been called into question, and most laboratories no longer perform routine band counts. The Clinical and Laboratory Standards Institute (CLSI) recommends that bands should be included within the neutrophil counts and not reported as a separate count.

### BOX 12-1 Neutrophil Granules

#### Primary (Azurophilic) Granules
Formed during the promyelocyte stage
Last to be released (exocytosis)
Contain:
- Myeloperoxidase
- Acid β-glycerophosphatase
- Cathepsins
- Defensins
- Elastase
- Proteinase-3
- Others

#### Secondary (Specific) Granules
Formed during myelocyte and metamyelocyte stages
Third to be released
Contain:
- β2-Microglobulin
- Collagenase
- Gelatinase
- Lactoferrin
- Neutrophil gelatinase-associated lipocalin
- Transcobalamin I
- Others

#### Tertiary Granules
Formed during metamyelocyte and band stages
Second to be released
Contain:
- Gelatinase
- Collagenase
- Lysozyme
- Acetyltransferase
- β2-Microglobulin

#### Secretory Granules (Secretory Vesicles)
Formed during band and segmented neutrophil stages
First to be released (fuse to plasma membrane)
Contain (attached to membrane):
- CD11b/CD18
- Alkaline phosphatase
- Vesicle-associated membrane-2
- CD10, CD13, CD14, CD16
- Cytochrome b₅₅₈
- Complement 1q receptor
- Complement receptor-1

---

**Figure 12-3** A. Type I myeloblast (arrow). Note that no granules are visible in the cytoplasm. B. Type II myeloblast (arrow) with a few azure granules in the cytoplasm. C. Electron micrograph of a myeloblast. (C from Rodak BF, Carr JH. Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders, Elsevier.)
category, due to the difficulty in reliably distinguishing bands from segmented neutrophils.\textsuperscript{12}

Segmented neutrophils make up 7\% to 30\% of nucleated cells in the bone marrow. Secretory granules continue to be formed during this stage. The only morphologic difference between segmented neutrophils and bands is the presence of between two and five nuclear lobes connected by threadlike filaments (Figure 12-8). Segmented neutrophils are present in the highest numbers in the peripheral blood of adults (50\% to 70\% of leukocytes in relative numbers and 2.3 to 8.1 × 10^9/L in absolute terms). As can be seen from the table on the inside front cover, pediatric values are quite different; relative percentages can be as low as 18\% of leukocytes in the first few months of life and do not begin to climb to adult values until after 4 to 7 years of age.

**Neutrophil Kinetics**

Neutrophil kinetics involves the movement of neutrophils and neutrophil precursors between the different pools in the bone marrow, the peripheral blood, and tissues. Neutrophil production has been calculated to be on the order of between 0.9 and 1.0 × 10^9 cells/kg per day.\textsuperscript{13}

The proliferative pool contains approximately 2.1 × 10^9 cells/kg, whereas the maturation pool contains roughly 5.6 × 10^9 cells/kg or a 5-day supply.\textsuperscript{13} The transit time from the HSC to the myeloblast has not been measured. The transit time from myeloblast through myelocyte has been estimated to be roughly 6 days, and the transit time through the maturation pool is approximately 4 to 6 days.\textsuperscript{4,13,14} Granulocyte release from the bone marrow is stimulated by G-CSF.\textsuperscript{2,3}
Once in the peripheral blood, neutrophils are divided randomly into a circulating neutrophil pool (CNP) and a marginated neutrophil pool (MNP). The neutrophils in the MNP are loosely localized to the walls of capillaries in tissues such as the liver, spleen, and lung. There does not appear to be any functional differences between neutrophils of either the CNP or the MNP, and cells move freely between the two peripheral pools.\(^{15}\) The ratio of these two pools is roughly equal overall;\(^{4,16}\) however, marginated neutrophils in the capillaries of the lungs make up a considerably larger portion of peripheral neutrophils.\(^{17}\) The half-life of neutrophils in the blood is relatively short at approximately 7 hours.\(^{4,18}\)

Integrins and selectins are of significant importance in allowing neutrophils to marginate as well as exit the blood and enter the tissues by a process known as **diapedesis**.\(^{19,20}\) Those neutrophils that do not migrate into the tissues eventually undergo programmed cell death or apoptosis and are removed by macrophages in the spleen.\(^{21}\)

Once neutrophils are in the tissues, their life span is variable, depending on whether or not they are responding to infectious or inflammatory agents. In the absence of infectious or inflammatory agents, the neutrophil’s life span is measured in hours. Some products of inflammation and infection tend to prolong the neutrophil’s life span through anti-apoptotic signals, whereas others such as MAC-1 trigger the death and phagocytosis of neutrophils.\(^{20}\)

**Neutrophil Functions**

Neutrophils are part of the innate immune system. Characteristics of innate immunity include destruction of foreign organisms that is not antigen specific; no protection against reexposure to the same pathogen; reliance on the barriers provided by skin and mucous membranes, as well as phagocytes such as neutrophils and monocytes; and inclusion of a humoral component known as the **complement system**.

The major function of neutrophils is phagocytosis and destruction of foreign materials and microorganisms. The process involves seeking (chemotaxis, motility, and diapedesis) and destruction (phagocytosis and digestion).

Neutrophil recruitment to an inflammatory site begins when chemotactic agents bind to neutrophil receptors. Chemotactic agents may be produced by microorganisms, by damaged cells, or by other leukocytes such as lymphocytes or other phagocytes. The first neutrophil response is to roll along endothelial cells of the blood vessels using stronger adhesive molecules than those used by nonstimulated marginated neutrophils. Rolling consists of transient adhesive
contacts between neutrophil selectins and adhesive molecules on the surface of endothelial cells. At the same time, secretory granules containing additional adhesive molecules are fused to the neutrophil’s plasma membrane. β2 integrins such as CD11b/CD18 from secretory granules contribute to tight stationary binding between neutrophils and endothelial cells. This is followed by diapedesis or transmigration of neutrophils either between or through endothelial cells—a process that is also mediated by integrins and integrin-associated proteins. Tertiary granules containing gelatinase and collagenase are released by transmigrating neutrophils. Gelatinase degrades denatured collagen as well as types IV and V collagen and activates chemokines such as interleukin-8 (IL-8). 22 Neutrophils then migrate in a directional manner toward the area of greatest concentration of chemotactic agents.

Once at the site of infection or inflammation, neutrophils begin the process of phagocytosis (Box 12-2). They utilize their enormous inventory of surface receptors either to directly recognize the pathogen, apoptotic cell, or particle, or to recognize opsonic molecules attached to the foreign particle such as antibodies or complement components. With recognition comes attachment and engulfment, in which cytoplasmic pseudopodia surround the particle, forming a phagosome within the neutrophil cytoplasm.23 Formation of the phagosome allows the reduced nicotinamide adenine dinucleotide (NADH) oxidase complex within the phagosome membrane to assemble; this leads to the generation of reactive oxygen species such as hydrogen peroxide, which is converted to hypochlorite by myeloperoxidase. Likewise, a series of metabolic changes culminate in the fusion of primary and/or secondary granules to the phagosome and the release of numerous bactericidal molecules into the phagosome.24 This combination of reactive oxygen species and non-oxygen-dependent mechanisms is generally able to destroy most pathogens.

In addition to emptying their contents into phagosomes, secondary and primary granules may fuse to the plasma membrane, which results in release of their contents into the extracellular matrix. These molecules can then act as chemotactic agents for additional neutrophils and as stimulating agents for macrophages to phagocytize dead neutrophils, as well as inflammatory agents that may cause tissue damage.

A second function of neutrophils is the generation of neutrophil extracellular traps, or NETs.25,26 NETs are extracellular threadlike structures believed to represent chains of nucleosomes from unfolded nuclear chromatin material (DNA). These structures have enzymes from neutrophil granules attached to them and have been shown to be able to trap and kill gram-positive and gram-negative bacteria as well as fungi.

**BOX 12-2 Phagocytosis**

**Recognition and Attachment**

Phagocyte receptors recognize and bind to certain foreign molecular patterns and opsonins such as antibodies and complement components.

**Ingestion**

Pseudopodia are extended around the foreign particle and enclose it within a “phagosome” (engulfment).

The phagosome is pulled toward the center of the cell by polymerization of actin and myosin and by microtubules.

**Killing and Digestion**

**Oxygen Dependent**

Respiratory burst through the activation of NADPH oxidase. H$_2$O$_2$ and hypochlorite are produced.

**Oxygen Independent**

The pH within the phagosome becomes alkaline and then neutral, the pH at which digestive enzymes work.

Primary and secondary lysosomes (granules) fuse to the phagosome and empty hydrolytic enzymes and other bactericidal molecules into the phagosome.

**Formation of Neutrophil Extracellular Traps**

Nuclear and organelle membranes dissolve, and activated cytoplasmic enzymes attach to DNA.

The cytoplasmic membrane ruptures, and DNA with attached enzymes is expelled so that the bacteria are digested in the external environment.

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form).
NETs are generated at the time that neutrophils die as a result of antibacterial activity. The term NETosis has been used to describe this unique form of neutrophil cell death that results in the release of NETs.

A third and final function of neutrophils is their secretory function. Neutrophils are a source of transcobalamin I or R binder protein, which is necessary for the proper absorption of vitamin B₁₂. In addition, they are a source of a variety of cytokines.

**Eosinophils**

Eosinophils make up 1% to 3% of nucleated cells in the bone marrow. Of these, slightly more than a third are mature, a quarter are eosinophilic metamyelocytes, and the remainder are eosinophilic promyelocytes or eosinophilic myelocytes. Eosinophils account for 1% to 3% of peripheral blood leukocytes, with an absolute number of up to $0.4 \times 10^9/L$ in the peripheral blood.

**Eosinophil Development**

Eosinophil development is similar to that described earlier for neutrophils, and evidence indicates that eosinophils arise from the common myeloid progenitor (CMP).²⁷,²⁸ Eosinophil lineage is established through the interaction between the cytokines IL-3, IL-5, and GM-CSF and three transcription factors (GATA-1, PU.1, and c/EBP). IL-5 is critical for eosinophil growth and survival.²⁹ Whether or not there exist myeloblasts that are committed to the eosinophil line has not been established. Eosinophilic promyelocytes can be identified cytochemically due to the presence of Charcot-Leyden crystal protein in their primary granules. The first maturation phase that can be identified as eosinophilic using light microscopy and Romanowsky staining is the early myelocyte.

*Eosinophil myelocytes* are characterized by the presence of large (resolvable at the light microscope level), pale, reddish-orange secondary granules, along with azure granules in blue cytoplasm. The nucleus is similar to that described for neutrophil myelocytes. Transmission electron micrographs of eosinophils reveal that many secondary eosinophil granules contain an electron-dense crystalline core (Figure 12-9).³⁰

**Eosinophil metamyelocytes and bands** resemble their neutrophil counterparts with respect to their nuclear shape. Secondary granules increase in number, and a third type of granule is generated called the *secretory granule* or *secretory vesicle*. The secondary granules become more distinct and refractory. Electron microscopy indicates the presence of two other organelles: lipid bodies and small granules (Box 12-3).³¹

*Mature eosinophils* usually display a bilobed nucleus. Their cytoplasm contains characteristic refractile, orange-red secondary granules (Figure 12-10). Electron microscopy of mature eosinophils reveals extensive secretory vesicles, and their number increases considerably when the eosinophil is stimulated or activated.³⁰

**Eosinophil Kinetics**

The time from the last myelocyte mitotic division to the emergence of mature eosinophils from the marrow is about 3.5 days. The mean turnover of eosinophils is approximately $2.2 \times 10^9$ cells/kg per day. There is a large storage pool of eosinophils in the marrow consisting of between 9 and $14 \times 10^8$ cells/kg.³¹

Once in the circulation, eosinophils have a circulating half-life of roughly 18 hours;³² however, the half-life of eosinophils is prolonged when eosinophilia occurs. The tissue destinations of eosinophils under normal circumstances appear to be underlying columnar epithelial surfaces in the respiratory, gastrointestinal, and genitourinary tracts. Survival time of eosinophils in human tissues ranges from 2 to 5 days.³³

**Eosinophil Functions**

Eosinophils have multiple functions. Eosinophil granules are full of a large number of previously synthesized proteins, including cytokines, chemokines, growth factors, and cationic proteins. There is more than one way for eosinophils to degranulate. By classical exocytosis, granules move to the plasma membrane, fuse with the plasma membrane, and empty their contents into the extracellular space. Compound exocytosis is a second mechanism in which granules fuse together within the eosinophil prior to fusing with the plasma membrane. A third method is known as *piecemeal degranulation*, in which secretory vesicles remove specific proteins from the secondary granules.

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**Figure 12-9** A, Eosinophil myelocyte. Note the rounded nucleus and the cytoplasm in which there are numerous large, pale eosinophil granules. B, Electron micrograph of eosinophil granules showing the central crystalline core in some of the granules. (A, B from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders, Elsevier.)
well as cytokine production, and they also produce nerve growth factor that promotes mast cell survival and activation. Eosinophil production is increased in infection by parasitic helminths, and in vitro studies have shown that the eosinophil is capable of destroying tissue-invading helminths through the secretion of major basic protein and eosinophil cationic protein as well as the production of reactive oxygen species. There is also a suggestion that eosinophils play a role in preventing reinfection. Finally, eosinophilia is a hallmark of allergic disorders, of which asthma has been the best studied. The number of eosinophils in blood and sputum correlates with disease severity. This has led to the suggestion that the eosinophil is one of the causes of airway inflammation and mucosal cell damage through secretion or production of a combination of basic proteins, lipid mediators, reactive oxygen species, and cytokines such as IL-5. Eosinophils have also been implicated in airway remodeling (increase in thickness of the airway wall) through eosinophil-derived fibrogenic growth factors. Treatment with an anti-IL-5 monoclonal antibody has been shown to reduce exacerbations in certain asthmatic patients. Eosinophil accumulation in the gastrointestinal tract occurs in allergic disorders such as food allergy, allergic colitis, and inflammatory bowel disease such as Crohn’s disease and ulcerative colitis.

**Basophils**

Basophils and mast cells are two cells with morphologic and functional similarities; however, basophils are true leukocytes because they mature in the bone marrow and circulate in the blood as mature cells with granules, whereas mast cell precursors leave the bone marrow and use the blood as a transit system to gain access to the tissues where they mature. Basophils are discussed first. Basophils are the least numerous of the WBCs, making up between 0% and 2% of circulating leukocytes and less than 1% of nucleated cells in the bone marrow.

**Basophil Development**

Basophils are derived from progenitors in the bone marrow, where they differentiate under the influence of a number of
cytokines, including IL-3.\textsuperscript{42,43} Due to their very small numbers, the stages of basophil maturation are very difficult to observe and have not been well characterized. Basophils will therefore be described simply as immature basophils and mature basophils.

Immature basophils have round to somewhat lobulated nuclei with only slightly condensed chromatin. Nucleoli may or may not be apparent. The cytoplasm is blue and contains large blue-black secondary granules (Figure 12-11). Primary azure granules may or may not be seen. Basophil granules are water soluble and therefore may be dissolved if the blood film is washed too much during the staining process.

Mature basophils contain a lobulated nucleus that is often obscured by its granules. The chromatin pattern, if visible, is clumped. Actual nuclear segmentation with visible filaments occurs rarely. The cytoplasm is colorless and contains large numbers of the characteristic large blue-black granules. If any granules have been dissolved during the staining process, they often leave a reddish-purple rim surrounding what appears to be a vacuole (Figure 12-12).

**Basophil Kinetics**

Basophil kinetics is poorly understood because of their very small numbers. According to a recent study, the life span of a mature basophil is 60 hours.\textsuperscript{44} This life span of basophils is relatively longer than that of the other granulocytes. This has been attributed to the fact that when they are activated by the cytokine IL-3, anti-apoptotic pathways are initiated that cause the prolongation of the basophil life span.\textsuperscript{15}

**Basophil Functions**

Basophil functions are also poorly understood because of the small numbers of these cells and the lack of animal models such as basophil-deficient animals. However, the recent development of a conditional basophil-deficient mouse model promises to enhance the understanding of basophil function.\textsuperscript{46} In the past, basophils have been regarded as the “poor relatives” of mast cells and minor players in allergic inflammation because, like mast cells, they have immunoglobulin E (IgE) receptors on their surface membranes that, when cross-linked by antigen, result in granule release.\textsuperscript{51} Today, something of a reawakening has occurred regarding basophils and their functions in both innate and adaptive immunity. Basophils are capable of releasing large quantities of subtype 2 helper T cell (T\textsubscript{H}2) cytokines such as IL-4 and IL-13 that regulate the T\textsubscript{H}2 immune response.\textsuperscript{48,49} Basophils also induce B cells to synthesize IgE.\textsuperscript{50} Whereas mast cells are the effectors of IgE-mediated chronic allergic inflammation, basophils function as initiators of the allergic inflammation through the release of preformed cytokines.\textsuperscript{47} Basophil activation is not restricted to antigen-specific IgE cross-linking, but it can be triggered in nonsensitized individuals by a growing list of parasitic antigens, lectins, and viral superantigens binding to nonspecific IgE antibodies.\textsuperscript{51}

The contents of basophil granules are not well known. Box 12-4 provides a short list of some of the substances released by activated basophils. Moreover, mature basophils are evidently capable of synthesizing granule proteins based on activation signals. For example, basophils can be induced to produce a mediator of allergic inflammation known as granzyme B.\textsuperscript{52} Mast cells can induce basophils to produce and release retinoic acid, a regulator of immune and resident cells in allergic diseases.\textsuperscript{53} Basophils also play a role in angiogenesis through the expression of vascular endothelial growth factor (VEGF) and its receptors.\textsuperscript{54}

Along with eosinophils, basophils are involved in the control of helminth infections. They promote eosinophilia, are associated with the differentiation of alternatively activated
macrophages in the lung, and contribute to efficient worm expulsion. Finally, data from the basophil-deficient mouse model indicate that basophils play a nonredundant role in mediating acquired immunity against ticks.

**Mast Cells**

Mast cells are not considered to be leukocytes. They are tissue effector cells of allergic responses and inflammatory reactions. A brief description of their development and function is included here because (1) their precursors circulate in the peripheral blood for a brief period on their way to their tissue destinations, and (2) mast cells have several phenotypic and functional similarities with both basophils and eosinophils.

Mast cell progenitors (MCPs) originate from the bone marrow and spleen. The progenitors are then released to the blood before finally reaching tissues such as the intestine and lung, where they mediate their actions. The major cytokine responsible for mast cell maturation and differentiation is KIT ligand (stem cell factor). Once the MCP reaches its tissue destination, complete maturation into mature mast cells occurs under the control of the local microenvironment (Figure 12-13).

Mast cells function as effector cells in allergic reactions through the release of a wide variety of lipid mediators, proteases, proteoglycans, and cytokines as a result of cross-linking of IgE on the mast cell surface by specific allergens. Mast cells can also be activated independently of IgE, which leads to inflammatory reactions. Mast cells can function as antigen-presenting cells to induce the differentiation of T<sub>H</sub>2 cells; therefore, mast cells act in both innate and adaptive immunity. In addition, mast cells can have anti-inflammatory and immunosuppressive functions, and thus they can both enhance and suppress features of the immune response.

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**MONONUCLEAR CELLS**

**Monocytes**

Monocytes make up between 2% and 11% of circulating leukocytes, with an absolute number of up to 1.3 × 10⁹/L.

**Monocyte Development**

Monocyte development is similar to neutrophil development because both cell types are derived from the granulocyte-monocyte progenitor (GMP) (see Figure 12-1). Macrophage colony-stimulating factor (M-CSF) is the major cytokine responsible for the growth and differentiation of monocytes. The morphologic stages of monocyte development are monoblasts, promonocytes, and monocytes. Monoblasts in normal bone marrow are very rare and are difficult to distinguish from myeloblasts based on morphology. Malignant monoblasts in acute monoblastic leukemia are described in Chapter 35. Therefore, only promonocytes and monocytes are described here.

Promonocytes are 12 to 18 μm in diameter, and their nucleus is slightly indented or folded. The chromatin pattern is delicate, and at least one nucleolus is apparent. The cytoplasm is blue and contains scattered azure granules that are fewer and smaller than those seen in promyelocytes (Figure 12-14).
Electron microscopic and cytochemical studies have shown that monocyte azure granules are heterogeneous with regard to their content of lysosomal enzymes, peroxidase, nonspecific esterases, and lysozyme.

Monocytes appear to be larger than neutrophils (diameter of 15 to 20 μm) because they tend to stick to and spread out on glass or plastic. Monocytes are slightly immature cells whose ultimate goal is to enter the tissues and mature into macrophages, osteoclasts, or dendritic cells.

The nucleus may be round, oval, or kidney shaped, but more frequently is deeply indented (horseshoe shaped) or folded on itself. The chromatin pattern is looser than in the other leukocytes and has sometimes been described as lacelike or stringy. Nucleoli are generally not seen with the light microscope; however, electron microscopy reveals nucleoli in roughly half of circulating monocytes. Their cytoplasm is blue-gray, with fine azure granules often referred to as azure dust or a ground-glass appearance. Small cytoplasmic pseudopods or blebs may be seen. Cytoplasmic and nuclear vacuoles may also be present (Figure 12-15). Based on flow cytometry immunophenotyping, three subsets of human monocytes have been described: the classical, intermediate, and nonclassical monocytes. The roles of these monocyte subsets in health and disease are currently being characterized.

**Box 12-5**

Monocyte/Macrophage Functions

Functions of monocytes/macrophages are numerous and varied. They can be subdivided into innate immunity, adaptive immunity, and housekeeping functions.

- **Innate immunity:** Monocytes/macrophages recognize a wide range of bacterial pathogens by means of pattern recognition receptors (toll-like receptors) that stimulate inflammatory cytokine production and phagocytosis. Macrophages can synthesize nitric oxide, which is cytotoxic against viruses, bacteria, fungi, protozoa, helminths, and tumor cells. Monocytes and macrophages also have Fc receptors and complement receptors. Hence, they can phagocytize foreign organisms or materials that have been coated with antibodies or complement components.

- **Adaptive immunity:** Both macrophages and dendritic cells degrade antigen and present antigen fragments on their surfaces.
(antigen-presenting cells). Because of this, they interact with and activate both T lymphocytes and B lymphocytes to initiate the adaptive immune response. Dendritic cells are the most efficient and potent of the antigen-presenting cells.

- **Housekeeping functions:** These include removal of debris and dead cells at sites of infection or tissue damage, destruction of senescent red blood cells and maintenance of a storage pool of iron for erythropoiesis, and synthesis of a wide variety of proteins, including coagulation factors, complement components, interleukins, growth factors, and enzymes.\(^{70}\)

**Lymphocytes**

Lymphocytes are divided into three major groups: T cells, B cells, and natural killer (NK) cells. T and B cells are major players in adaptive immunity. NK cells make up a small percentage of lymphocytes and are part of innate immunity. Adaptive immunity has three characteristics: it relies on an enormous number of distinct lymphocytes, each having surface receptors for a different specific molecular structure on a foreign antigen; after an encounter with a particular antigen, memory cells are produced that will react faster and more vigorously to that same antigen upon reexposure; and self-antigens are “ignored” under normal circumstances (referred to as tolerance).\(^{70}\)

Lymphocytes can be subdivided into two major categories: those that participate in humoral immunity by producing antibodies and those that participate in cellular immunity by attacking foreign organisms or cells directly. Antibody-producing lymphocytes are called B lymphocytes or simply B cells because they develop in the bone marrow. Cellular immunity is accomplished by two types of lymphocytes: T cells, so named because they develop in the thymus, and NK cells, which develop in both the bone marrow and the thymus.\(^{71-73}\)

Lymphocytes are different from the other leukocytes in several ways, including the following:

1. Lymphocytes are not end cells. They are resting cells, and when stimulated, they undergo mitosis to produce both memory and effector cells.

2. Unlike other leukocytes, lymphocytes recirculate from the blood to the tissues and back to the blood.

3. B and T lymphocytes are capable of rearranging antigen receptor gene segments to produce a wide variety of antibodies and surface receptors.

4. Although early lymphocyte progenitors such as the common lymphoid progenitor originate in the bone marrow, T and NK lymphocytes develop and mature outside of the bone marrow.

For these reasons, lymphocyte kinetics is extremely complicated, not well understood, and beyond the scope of this chapter.

Lymphocytes make up between 18% and 42% of circulating leukocytes with an absolute number of \(0.8 \text{ to } 4.8 \times 10^9/L\).

**Lymphocyte Development**

For both B and T cells, development can be subdivided into antigen-independent and antigen-dependent phases. Antigen-independent lymphocyte development occurs in the bone marrow and thymus (sometimes referred to as central or primary lymphatic organs), whereas antigen-dependent lymphocyte development occurs in the spleen, lymph nodes, tonsils, and mucosa-associated lymphoid tissue such as the Peyer’s patches in the intestinal wall (sometimes referred to as peripheral or secondary lymphatic organs).
B lymphocytes develop initially in the bone marrow and go through three stages known as pro-B, pre-B, and immature B cells. It is during these stages that immunoglobulin gene rearrangement occurs so that each B cell produces a unique immunoglobulin antigen receptor. The immature B cells, which have not yet been exposed to antigen (antigen-naive B cells), leave the bone marrow to migrate to secondary lymphatic organs, where they take up residence in specific zones such as lymph node follicles. These immature B cells, also known as hematogones, have a homogeneous nuclear chromatin pattern and extremely scanty cytoplasm (Figure 12-17). These cells are normally seen in newborn peripheral blood and bone marrow and in regenerative bone marrows. Leukemic cells from patients with acute lymphoblastic leukemia (ALL) can sometimes resemble hematogones, but the leukemic cells can be distinguished from hematogones by flow cytometry immunophenotyping.

It is in the secondary lymphatic organs or in the blood where B cells may come in contact with antigen, which results in cell division and the production of memory cells as well as effector cells. Effector B cells are antibody-producing cells known as plasma cells and plasmacytoid lymphocytes (Figure 12-18).

Approximately 3% to 21% of circulating lymphocytes are B cells. Resting B lymphocytes cannot be distinguished morphologically from resting T lymphocytes. Resting lymphocytes are small (around 9 μm in diameter), and the N:C ratio ranges from 5:1 to 2:1. The chromatin is arranged in blocks, and the nucleolus is rarely seen, although it is present (Figure 12-19).

T lymphocytes develop initially in the thymus—a lymphoepithelial organ located in the upper mediastinum. Lymphoid progenitor cells migrate from the bone marrow to the thymic cortex, where, under the regulation of cytokines produced by thymic epithelial cells, they progress through stages known as pro-T, pre-T, and immature T cells. During these phases they undergo antigen receptor gene rearrangement to produce T cell receptors that are unique to each T cell. T cells whose receptors react with self-antigens are allowed to undergo apoptosis. In addition, T cells are subdivided into two major categories, depending on whether or not they have CD4 or CD8 coexpressed on their surface.

Figure 12-17 Immature B lymphocyte or hematogone (arrow). Note the extremely scanty cytoplasm. This was taken from the bone marrow of a newborn infant.

Figure 12-18 A, Plasma cell. B, Plasmacytoid lymphocyte. These are effector cells of the B lymphocyte lineage.

Figure 12-19 A, Small resting lymphocyte. B, Electron micrograph of a small lymphocyte. (B from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders, Elsevier.)
CD8 antigen on their surfaces. Immature T cells then proceed to the thymic medulla, where further apoptosis of self-reactive T cells occurs. The remaining immature T cells (or antigen-naive T cells) then leave the thymus and migrate to secondary lymphatic organs, where they take up residence in specific zones such as the paracortical areas. T cells comprise 51% to 88% of circulating lymphocytes.

T cells in secondary lymphatic organs or in the circulating blood eventually come in contact with antigen. This results in cell activation and the production of either memory cells or effector T cells, or both (Figure 12-20). The transformation of resting lymphocytes into activated forms is the source of so-called medium and large lymphocytes that have increased amounts of cytoplasm and usually make up only about 10% of circulating lymphocytes. The morphology of effector T cells varies with the subtype of T cell involved, and they are often referred to as reactive or variant lymphocytes.

NK cells are a heterogeneous group of cells with respect to their surface antigens. The majority are CD56+CD16+CD3−CD7− large granular lymphocytes. The mature NK cell is relatively large compared with other resting lymphocytes because of an increased amount of cytoplasm. Its cytoplasm contains azurophilic granules that are peroxidase negative. Approximately 4% to 29% of circulating lymphocytes are NK cells.

**Figure 12-20** Three cells representing lymphocyte activation. A small resting lymphocyte (A) is stimulated by antigen and begins to enlarge to form a medium to large lymphocyte (B). The nucleus reverts from a clumped to a delicate chromatin pattern with nucleoli (C). The cell is capable of dividing to form effector cells or memory cells.

**Figure 12-21** A large granular lymphocyte that could be either a cytotoxic T lymphocyte or a natural killer lymphocyte.

**Lymphocyte Functions**

Functions can be addressed according to the type of lymphocyte. B lymphocytes are essential for antibody production. In addition, they have a role in antigen presentation to T cells and may be necessary for optimal CD4 activation. B cells also produce cytokines that regulate a variety of T cell and antigen-presenting cell functions.79

*T* lymphocytes can be divided into CD4+ T cells and CD8+ T cells. CD4+ effector lymphocytes are further subdivided into T_{H1}, T_{H2}, T_{H17}, and T_{reg} (CD4+CD25+ regulatory T) cells. T_{H1} cells mediate immune responses against intracellular pathogens. T_{H2} cells mediate host defense against extracellular parasites, including helminths. They are also important in the induction of asthma and other allergic diseases. T_{H17} cells are involved in the immune responses against extracellular bacteria and fungi. T_{reg} cells play a role in maintaining self-tolerance by regulating immune responses.80,81

CD8+ effector lymphocytes are capable of killing target cells by secreting granules containing granzyme and perforin or by activating apoptotic pathways in the target cell.82 These cells are sometimes referred to as *cytotoxic T lymphocytes*.

NK lymphocytes function as part of innate immunity and are capable of killing certain tumor cells and virus-infected cells without prior sensitization. In addition, NK cells modulate the functions of other cells, including macrophages and T cells.83

**SUMMARY**

- Granulocytes are classified according to their staining characteristics and the shape of their nuclei. Neutrophils are a major component of innate immunity as phagocytes; eosinophils are involved in allergic reactions and helminth destruction; and basophils function as initiators of allergic reactions, helminth destruction, and immunity against ticks.

- Neutrophil development can be subdivided into specific stages, with cells at each stage having specific morphologic characteristics (myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and segmented neutrophil). Various granule types are produced during neutrophil development, each with specific contents.

- Eosinophil development can also be subdivided into specific stages, although eosinophilic myeloblasts are not recognizable and eosinophil promyelocytes are rare.

- Basophil development is difficult to describe, and basophils have been divided simply into immature and mature basophils.


- Mononuclear cells consist of monocytes and lymphocytes. Monocytes are precursors to tissue cells such as osteoclasts, macrophages, and dendritic cells. As a group, they perform several functions as phagocytes.
- Monocyte development can be subdivided into the promonocyte, monocyte, and macrophage stages, each with specific morphologic characteristics.
- The majority of lymphocytes are involved in adaptive immunity. B lymphocytes and plasma cells produce antibodies against foreign organisms or cells, and T lymphocytes mediate the immune response against intracellular and extracellular invaders. Both B and T lymphocytes produce memory cells for specific antigens so that the immune response is faster if the same antigen is encountered again.
- Lymphocyte development is complex, and morphologic divisions are not practical because a large number of lymphocytes develop in the thymus. Benign B-lymphocyte precursors (hematogones) as well as B-lymphocyte effector cells (plasma cells and plasmacytoid lymphocytes) have been described. NK lymphocytes and cytotoxic T cells also have a distinct and similar morphology.

### REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Neutrophils and monocytes are direct descendants of a common progenitor known as:
   a. CLP
   b. GMP
   c. MEP
   d. HSC

2. The stage in neutrophilic development in which the nucleus is indented in a kidney bean shape and the cytoplasm has secondary granules that are lavender in color is the:
   a. Band
   b. Myelocyte
   c. Promyelocyte
   d. Metamyelocyte

3. Type II myeloblasts are characterized by:
   a. Presence of fewer than 20 primary granules per cell
   b. Basophilic cytoplasm with many secondary granules
   c. Absence of granules
   d. Presence of a folded nucleus

4. Which one of the following is a function of neutrophils?
   a. Presentation of antigen to T and B lymphocytes
   b. Protection against reexposure by same antigen
   c. Nonspecific destruction of foreign organisms
   d. Initiation of delayed hypersensitivity response

5. Which of the following cells are important in immune regulation, allergic inflammation, and destruction of tissue invading helminths?
   a. Neutrophils and monocytes
   b. Eosinophils and basophils
   c. T and B lymphocytes
   d. Macrophages and dendritic cells

6. Basophils and mast cells have high-affinity surface receptors for which immunoglobulin?
   a. A
   b. D
   c. E
   d. G

7. Which of the following cell types is capable of differentiating into osteoclasts, macrophages, or dendritic cells?
   a. Neutrophils
   b. Lymphocytes
   c. Monocytes
   d. Eosinophils

8. Macrophages aid in adaptive immunity by:
   a. Degrading antigen and presenting it to lymphocytes
   b. Ingesting and digesting organisms that neutrophils cannot
   c. Synthesizing complement components
   d. Storing iron from senescent red cells

9. Which of the following is the final stage of B cell maturation after activation by antigen?
   a. Large, granular lymphocyte
   b. Plasma cell
   c. Reactive lymphocyte
   d. Immunoblast

10. The following is unique to both B and T lymphocytes and occurs during their early development:
    a. Expression of surface antigens CD4 and CD8
    b. Maturation in the thymus
    c. Synthesis of immunoglobulins
    d. Rearrangement of antigen receptor genes
REFERENCES


Platelet Production, Structure, and Function

George A. Fritsma

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Diagram megakaryocyte localization in bone marrow.
2. List the transcription products that trigger and control megakaryocytopoiesis and endomitosis.
3. Diagram terminal megakaryocyte differentiation, the proplatelet process, and thrombocytopoiesis.
4. Describe the ultrastructure of resting platelets in the circulation, including the plasma membrane, tubules, microfibrils, and granules.
5. List the important platelet receptors and their ligands.
6. Recount platelet function, including adhesion, aggregation, and secretion.
7. Reproduce the biochemical pathways of platelet activation, including integrins, G proteins, the eicosanoid, and the diacylglycerol-inositol triphosphate pathway.

OUTLINE

MEGAKARYOCYTOPIOESIS
    Megakaryocyte Differentiation and Progenitors
    Endomitosis
    Terminal Megakaryocyte Differentiation
    Megakaryocyte Membrane Receptors and Markers
    Thrombocytopoiesis (Proplatelet Shedding)
    Hormones and Cytokines of Megakaryocytopoiesis

Platelets
    Platelet Ultrastructure
        Resting Platelet Plasma Membrane
        Surface-Connected Canalicular System
        Dense Tubular System
        Platelet Plasma Membrane Receptors That Provide for Adhesion
        The Seven-Transmembrane Receptors
        Additional Platelet Membrane Receptors
        Platelet Cytoskeleton: Microfilaments and Microtubules
        Platelet Granules: α-Granules, Dense Granules, and Lysosomes
    Platelet Activation
        Adhesion: Platelets Bind Elements of the Vascular Matrix
        Aggregation: Platelets Irreversibly Cohere
        Secretion: Activated Platelets Release Granular Contents
    Platelet Activation Pathways
        G Proteins
        Eicosanoid Synthesis
        Inositol Triphosphate Diacylglycerol Activation Pathway

CASE STUDY

After studying this chapter, the reader should be able to respond to the following case study:

A 35-year-old woman noticed multiple pinpoint red spots and bruises on her arms and legs. The hematologist confirmed the presence of petechiae, purpura, and ecchymoses on her extremities and ordered a complete blood count, prothrombin time, and partial thromboplastin time. The platelet count was 35 × 10^9/L, the mean platelet volume was 13.2 fL, and the diameter of platelets on the Wright-stained peripheral blood film appeared to exceed 6 μm. Other complete blood count parameters and the coagulation parameters were within normal limits. A Wright-stained bone marrow aspirate smear revealed 10 to 12 small unlobulated megakaryocytes per low-power microscopic field.

1. Do these signs and symptoms indicate systemic (mucocutaneous) or anatomical (soft tissue) bleeding?
2. What is the probable cause of the bleeding?
3. Is the thrombocytopenia the result of inadequate bone marrow production?
4. List the growth factors involved in recruiting megakaryocyte progenitors.

MEGAKARYOCYTOPIOESIS

Platelets are nonnucleated blood cells that circulate at 150 to 400 × 10^9/L, with average platelet counts slightly higher in women than in men and slightly lower in both sexes when over 65 years old. Platelets trigger primary hemostasis upon exposure to subendothelial collagen or endothelial cell inflammatory proteins at the time of blood vessel injury. On a Wright-stained wedge-preparation blood film, platelets are distributed throughout the red blood cell monolayer at 7 to 21 cells per 100× field. On the blood film they have an average diameter of 2.5 μm, corresponding to a mean platelet volume (MPV) of 8 to 10 fL when measured by impedance in a buffered isotonic suspension, as determined
using laboratory profiling instruments. Platelets arise from unique bone marrow cells called megakaryocytes. Megakaryocytes are the largest cells in the bone marrow and are polyploid, possessing multiple chromosome copies. On a Wright-stained bone marrow aspirate smear, each megakaryocyte is 30 to 50 μm in diameter with a multilobulated nucleus and abundant granular cytoplasm. Megakaryocytes account for less than 0.5% of all bone marrow cells, and on a normal Wright-stained bone marrow aspirate smear the microscopist may identify two to four megakaryocytes per \(10^3\) low-power field (Chapter 7).

In healthy intact bone marrow tissue, megakaryocytes, under the influence of an array of stromal cell cytokines, cluster with hematopoietic stem cells in vascular niches adjacent to venous sinusoid endothelial cells (Figure 13-1). Responding to the growth factor thrombopoietin (TPO), megakaryocyte progenitors are recruited from common myeloid progenitors (Chapter 7) and subsequently differentiate through several maturation stages. They extend proplatelet processes, projections that resemble strings of beads, through or between the endothelial cells and into the venous sinuses, releasing platelets from the tips of the processes into the circulation. Megakaryocytes are also found in the lungs.

**Megakaryocyte Differentiation and Progenitors**

Megakaryocyte progenitors arise from the common myeloid progenitor under the influence of the transcription gene product, GATA-1, regulated by cofactor FOG1 (Box 13-1). Megakaryocyte differentiation is suppressed by another transcription gene product, MYB, so GATA-1 and MYB act in opposition to balance megakaryocytopoiesis in one arm with differentiation to the red blood cell line in another arm, called erythropoiesis. From the common myeloid progenitor there arise three megakaryocyte lineage-committed progenitor stages, defined by their in vitro culture colony characteristics (Figure 7-13). In order of differentiation, these are the least mature burst-forming unit (BFU-Meg), the intermediate colony-forming unit (CFU-Meg), and the more mature progenitor, the light-density CFU (LD-CFU-Meg).

All three progenitor stages resemble lymphocytes and cannot be distinguished by Wright-stained light microscopy. The BFU-Meg and CFU-Meg are diploid and participate in normal mitosis, maintaining a viable pool of megakaryocyte progenitors. Their proliferative properties are reflected in their ability to form hundreds (BFU-Megs) or scores (CFU-Megs) of colonies in culture (Figure 13-2). The third stage, LD-CFU-Meg, loses its capacity to divide but retains its DNA replication and cytoplasmic maturation, a partially characterized form of mitosis unique to megakaryocytes known as endomitosis.

**Endomitosis**

Endomitosis is a form of mitosis that lacks telophase and cytokinesis (separation into daughter cells). As GATA-1 and
FOG1 transcription slows, another transcription factor, RUNX1, mediates the switch from mitosis to endomitosis by suppressing the Rho/ROCK signaling pathway, which suppresses the assembly of the actin cytoskeleton. In response to the reduced Rho/ROCK signal, inadequate levels of actin and myosin (muscle fiber–like molecules) assemble in the cytoplasmic constrictions where separation would otherwise occur, preventing cytokinesis. Subsequently, under the influence of yet another transcription factor, NF-E2, DNA replication proceeds to the production of 8N, 16N, or even 32N ploidy with duplicated chromosome sets. Some megakaryocyte nuclei replicate five times, reaching 128N; this level of ploidy is unusual, however, and may signal hematologic disease.

Megakaryocytes employ their multiple DNA copies to synthesize abundant cytoplasm, which differentiates into platelets. A single megakaryocyte may shed 2000 to 4000 platelets, a process called thrombopoiesis or thrombocytopenia. In an average-size healthy human there are $10^9$ megakaryocytes producing $10^{11}$ platelets per day, a total turnover rate of 8 to 9 days. In instances of high platelet consumption, such as immune thrombocytopenic purpura, platelet production rises by as much as tenfold.

**Terminal Megakaryocyte Differentiation**

As endomitosis proceeds, megakaryocyte progenitors leave the proliferative phase and enter terminal differentiation, a series of stages in which microscopists become able to recognize their unique Wright-stained morphology in bone marrow aspirate films (Figure 13-3) or hematoxylin and eosin–stained bone marrow biopsy sections (Table 13-1).

Morphologists call the least differentiated megakaryocyte precursor the MK-I stage or megakaryoblast. Although they no
TABLE 13-1 Features of the Three Terminal Megakaryocyte Differentiation Stages

<table>
<thead>
<tr>
<th>Feature</th>
<th>MK-I</th>
<th>MK-II</th>
<th>MK-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of precursors</td>
<td>20</td>
<td>25</td>
<td>55</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>14—18</td>
<td>15—40</td>
<td>30—50</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Round</td>
<td>Indented</td>
<td>Multilobed</td>
</tr>
<tr>
<td>Nucleoli</td>
<td>2—6</td>
<td>Variable</td>
<td>Not visible</td>
</tr>
<tr>
<td>Chromatin</td>
<td>Homogeneous</td>
<td>Moderately condensed</td>
<td>Deeply and variably condensed</td>
</tr>
<tr>
<td>Nucleus-to-cytoplasm ratio</td>
<td>3:1</td>
<td>1:2</td>
<td>1:4</td>
</tr>
<tr>
<td>Mitosis</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Endomitosis</td>
<td>Present</td>
<td>Ends</td>
<td>Absent</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Basophilic</td>
<td>Basophilic and granular</td>
<td>Azurophilic and granular</td>
</tr>
<tr>
<td>α-Granules</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Dense granules</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Demarcation system</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

MK-I, Megakaryoblast; MK-II, promegakaryocyte; MK-III, megakaryocyte.

Figure 13-4 The megakaryoblast (MK-I) resembles the myeloblast and pronormoblast (rubriblast); identification by morphology alone is inadvisable. This megakaryoblast has cytoplasmic “blebs” that resemble platelets.

longer look like lymphocytes, megakaryoblasts cannot be reliably distinguished from bone marrow myeloblasts or pronormoblasts (also named rubriblasts) using light microscopy (Figure 13-4). The morphologist may occasionally see a vague clue: plasma membrane blebs, blunt projections from the margin that resemble platelets. The megakaryoblast begins to develop most of its cytoplasmic ultrastructure, including procoagulant-laden α-granules, dense granules (dense bodies), and the demarcation system (DMS).9

The contents and functions of α-granules and dense granules are described in the subsequent sections on mature platelet ultrastructure and function. The DMS is a series of membrane-lined channels that invade from the plasma membrane and grow inward to subdivide the entire cytoplasm. The DMS is biologically identical to the megakaryocyte plasma membrane and ultimately delineates the individual platelets during thrombocytopoiesis.

Nuclear lobularity first becomes apparent as an indentation at the 4N replication stage, rendering the cell identifiable as an MK-II stage, or *promegakaryocyte*, by light microscopy. The morphologist seldom makes the effort to distinguish MK-I, MK-II, and MK-III stages during a routine examination of a bone marrow aspirate smear.

The promegakaryocyte reaches its full ploidy level by the end of the MK-II stage. At the most abundant MK-III stage, the megakaryocyte is easily recognized at 10× magnification on the basis of its 30- to 50-μm diameter (Figures 13-5 and 13-6). The nucleus is intensely indented or lobulated, and the degree of lobulation is imprecisely proportional to ploidy.
When necessary, ploidy levels are measured using mepacrine, a nucleic acid dye in megakaryocyte flow cytometry. The chromatin is variably condensed with light and dark patches. The cytoplasm is azurophilic (lavender), granular, and platelet-like because of the spread of the DMS and α-granules. At full maturation, platelet shedding, or thrombocytopoiesis, proceeds.

**Megakaryocyte Membrane Receptors and Markers**

In specialty and tertiary care laboratories, scientists and technicians employ immunostaining of fixed tissue, flow cytometry with immunologic probes, and fluorescent in situ hybridization (FISH) with genetic probes to identify visually indistinguishable megakaryocyte progenitors in hematologic disease. There are several flow cytometric megakaryocyte membrane markers, including MPL, which is the TPO receptor site present at all maturation stages, and the stem cell and common myeloid progenitor marker CD34. The CD34 marker disappears as differentiation proceeds. The platelet membrane glycoprotein IIb/IIIa (CD41, a marker located on the IIb portion) first appears on megakaryocyte progenitors and remains present throughout maturation, along with immunologic markers CD36, CD42, CD61, and CD62. Cytoplasmic coagulation factor VIII, von Willebrand factor (VWF), and fibrinogen, may be detected by immunostaining in the fully developed megakaryocyte (Table 13-2).

**Thrombocytopoiesis (Platelet Shedding)**

Figure 13-7 shows platelet shedding, termed thrombocytopoiesis. One cannot find reliable evidence for platelet budding or shedding simply by examining megakaryocytes in situ, even in well-structured bone marrow biopsy preparations. However, in megakaryocyte cultures examined by transmission electron microscopy, the DMS dilates, longitudinal bundles of tubules form, proplatelet processes develop, and transverse constrictions appear throughout the proplatelet processes. In the bone marrow environment, processes are believed to pierce through or between sinusoid-lining endothelial cells, extend into the venous blood, and shed platelets. Thrombocytopoiesis leaves behind naked megakaryocyte nuclei to be consumed by marrow macrophages.

**Hormones and Cytokines of Megakaryocytopoiesis**

The growth factor TPO is a 70,000 Dalton molecule that possesses 23% homology with the red blood cell–producing hormone erythropoietin (Table 13-3). Messenger ribonucleic acid (mRNA) for TPO has been found in the kidney, liver, stromal cells, and smooth muscle cells, though the liver has the most copies and is considered the primary source. TPO circulates as a hormone in plasma and is the ligand that binds the megakaryocyte and platelet membrane receptor protein identified above, MPL, named for v-mpl, a viral oncogene associated with murine myeloproliferative leukemia. The plasma concentration of TPO is inversely proportional to platelet and megakaryocyte mass, implying that membrane binding and consequent removal of TPO by platelets is the primary platelet count control mechanism. Investigators have used both in vitro and in vivo experiments to show that TPO, in synergy with other cytokines, induces stem cells to differentiate into megakaryocyte progenitors and that it further induces the differentiation of megakaryocyte progenitors into megakaryoblasts and megakaryocytes. TPO also induces the proliferation and maturation of megakaryocytes and induces thrombocytopoiesis, or platelet release (Table 13-3). Synthetic TPO mimetics (anallogues) elevate the platelet count in patients being treated for a variety of cancers, including acute leukemia. One commercial MPL receptor agonist, romiplostim (NPlate™, Amgen Inc., Thousand Oaks, CA, FDA cleared in 2008), is a nonimmunogenic oligopeptide that is also effective in raising the platelet count in immune thrombocytopoietic purpura. A second nonpeptide MPL receptor agonist, eltrombopag (Promacta® and Revolade®, Glaxo Smith

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**TABLE 13-2 Markers at Each Stage of Megakaryocyte Maturation Detected by Flow Cytometry, Immunostaining, Fluorescence In Situ Hybridization, or Cytochemical Stain**

<table>
<thead>
<tr>
<th></th>
<th>BFU-Meg</th>
<th>CFU-Meg</th>
<th>LD-CFU-Meg</th>
<th>MK-I</th>
<th>MK-II</th>
<th>MK-III</th>
<th>PLTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPL, TPO receptor by FCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34: stem cell marker by FCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD41: β3 portion of αIIbβ3; peroxidase by TEM cytochemical stain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• CD42; GP Iib portion of VWF receptor, by FCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• PF4 by FCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• VWF by immunostaining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The arrows indicate at which stage of differentiation the marker appears and at which stage it disappears. BFU-Meg, Burst-forming unit–megakaryocyte; CFU-Meg, colony-forming unit–megakaryocyte; LD-CFU-Meg, low-density colony-forming unit–megakaryocyte; MK-I, megakaryoblast; MK-II, promegakaryocyte; MK-III, megakaryocyte; FCM, flow cytometry; TEM, transmission electron microscopy; GP, glycoprotein; PF4, platelet factor 4; TPO, thrombopoietin; VWF, von Willebrand factor.
PLATELETS

The proplatelet process sheds platelets, cells consisting of granular cytoplasm with a membrane but no nuclear material, into the venous sinus of the bone marrow. Their diameter in the monolayer of a Wright-stained peripheral blood wedge film averages 2.5 μm. MPV, as measured in a buffered isotonic suspension flowing through the impedance-based detector cell of a clinical profiling instrument, ranges from 8 to 10 fL (Figure 13-1). A frequency distribution of platelet volume is log-normal, however, which indicates a subpopulation of large platelets (Figure 15-14). Heterogeneity in the MPV of normal healthy humans reflects random variation in platelet release volume and is not a function of platelet age or vitality, as many authors claim.22

TABLE 13-3  Hormones and Cytokines That Control Megakaryocytopoiesis

<table>
<thead>
<tr>
<th>Cytokine/ Hormone</th>
<th>Differentiation to Progenitors</th>
<th>Differentiation to Megakaryocytes</th>
<th>Late Maturation</th>
<th>Thrombocytopoiesis</th>
<th>Clinical Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>Available</td>
</tr>
<tr>
<td>IL-3</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>IL-11</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Available</td>
</tr>
</tbody>
</table>

Cytokines and hormones that have been shown to interact synergistically with TPO and IL-3 include IL-6 and IL-11; stem cell factor, also called kit ligand or mast cell growth factor; granulocyte-macrophage colony–stimulating factor; granulocyte colony–stimulating factor; and erythropoietin. Substances that inhibit megakaryocyte production include platelet factor 4, β-thromboglobulin, neutrophil-activating peptide 2, and IL-8. IL, Interleukin; TPO, thrombopoietin.
Circulating, resting platelets are biconvex, although the platelets in blood collected using the anticoagulant ethylenediaminetetraacetic acid (EDTA, lavender closure tubes) tend to "round up." On a Wright-stained wedge-preparation blood film, platelets appear circular to irregular, lavender, and granular, although their small size makes them hard to examine for internal structure. In the blood, their surface is even, and they flow smoothly through veins, arteries, and capillaries. In contrast to leukocytes, which tend to roll along the vascular endothelium, platelets cluster with the erythrocytes near the center of the blood vessel. Unlike erythrocytes, however, platelets move back and forth with the leukocytes from venules into the white pulp of the spleen, where both become sequestered in dynamic equilibrium.

The normal peripheral blood platelet count is 150 to 400 × 10^9/L. The count decreases after 65 years old to 122 to 350 × 10^9/L in men and 140 to 379 × 10^9/L in women. This count represents only two thirds of available platelets because the spleen sequesters an additional one third. Sequestered platelets are immediately available in times of demand—for example, in acute inflammation or after an injury, after major surgery, or during plateletpheresis. In hypersplenism or splenomegaly, increased sequestration may cause a relative thrombocytopenia. Under conditions of hemostatic need, platelets answer cellular and humoral stimuli by becoming irregular and sticky, extending pseudopods, and adhering to neighboring structures or aggregating with one another.

Reticulated platelets, sometimes known as stress platelets, appear in compensation for thrombocytopenia (Figure 13-9). Reticulated platelets are markedly larger than ordinary mature circulating platelets; their diameter in peripheral blood films exceeds 6 μm, and their MPV reaches 12 to 14 fL. Like ordinary platelets, they round up in EDTA, but in citrated (blue-closure tubes) whole blood, reticulated platelets are cylindrical and beaded, resembling fragments of megakaryocyte proplatelet processes. Reticulated platelets carry free ribosomes and fragments of rough endoplasmic reticulum, analogous to red blood cell reticulocytes, which triggers speculation that they arise from early and rapid proplatelet extension and release. Nucleic acid dyes such as thiazole orange bind the RNA of the endoplasmic reticulum. This property is exploited by profiling instruments to provide a quantitative evaluation of reticulated platelet production under stress, a measurement that may be more useful than the MPV. Platelet dense granules, however, may interfere with this measurement, falsely raising the reticulated platelet count by taking up nucleic acid dyes. Reticulated platelets are potentially prothrombotic, and may be associated with increased risk of cardiovascular disease.

**PLATELET ULTRASTRUCTURE**

Platelets, although anucleate, are strikingly complex and are metabolically active. Their ultrastructure has been studied using scanning and transmission electron microscopy, flow cytometry, and molecular sequencing.

**Resting Platelet Plasma Membrane**

The platelet plasma membrane resembles any biological membrane: a bilayer composed of proteins and lipids, as diagrammed in Figure 13-10. The predominant lipids are phospholipids, which form the basic structure, and cholesterol, which distributes asymmetrically throughout the phospholipids. The phospholipids form a bilayer with their polar heads oriented toward aqueous environments—toward the plasma externally and the cytoplasm internally. Their fatty acid chains, esterified to carbons 1 and 2 of the phospholipid triglyceride backbone, orient toward each other, perpendicular to the plane of the membrane, to form a hydrophobic barrier sandwiched within the hydrophilic layers.

The neutral phospholipids phosphatidylcholine and sphingomyelin predominate in the plasma layer; the anionic or polar phospholipids phosphatidylserine, phosphatidyethanolamine, and phosphatidylserine predominate in the inner, cytoplasmic layer. These phospholipids, especially phosphatidylserine, support platelet activation by supplying arachidonate, an unsaturated fatty acid that becomes converted to the eicosanoids prostaglandin and thromboxane A2 during platelet activation. Phosphatidylserine flips to the outer surface upon activation and is the charged phospholipid surface on which the coagulation enzymes, especially coagulation factor complex VIII and IX and coagulation factor complex X and V, assemble.

Esterified cholesterol moves freely throughout the hydrophobic internal layer, exchanging with unesterified cholesterol from the surrounding plasma. Cholesterol stabilizes the membrane, maintains fluidity, and helps control the transmembranous passage of materials.

Anchored within the membrane are glycoproteins and proteoglycans; these support surface glycosaminoglycans, oligosaccharides, and glycolipids. The platelet membrane surface, called the glyocalyx, also absorbs albumin, fibrinogen, and other plasma proteins, in many instances transporting them to storage organelles within using a process called endocytosis.

![Figure 13-9](image-url) A "stress" or "reticulated" platelet. The stress platelet may appear in compensation for thrombocytopenia, which produces early and rapid proplatelet extension and release. The diameter of reticulated platelets exceeds 6 μm. Reticulated platelets carry free ribosomes and fragments of rough endoplasmic reticulum, detectable in flow cytometry using nucleic acid dyes.
At 20 to 30 nm, the platelet glycocalyx is thicker than the analogous surface layer of leukocytes or erythrocytes. This thick layer is adhesive and responds readily to hemostatic demands. The platelet carries its functional environment with it, meanwhile maintaining a negative surface charge that repels other platelets, other blood cells, and the endothelial cells that line the blood vessels.

The plasma membrane is selectively permeable, and the membrane bilayer provides phospholipids that support platelet activation internally and plasma coagulation externally. The anchored glycoproteins support essential plasma surface–oriented glycosylated receptors that respond to cellular and humoral stimuli, called ligands or agonists, transmitting their stimulus through the membrane to internal activation organelles.

**Surface-Connected Canalicular System**
The plasma membrane invades the platelet interior, producing its unique surface-connected canalicular system (SCCS; Figures 13-11 and 13-12). The SCCS twists spongelike throughout the platelet, enabling the platelet to store additional quantities of the same hemostatic proteins found on the glycocalyx and raising its capacity manyfold. The glycocalyx is less developed in the SCCS and lacks some of the glycoprotein receptors present on the platelet surface. However, the SCCS is the route for endocytosis and for secretion of α-granule contents upon platelet activation.

**Dense Tubular System**
Parallel and closely aligned to the SCCS is the dense tubular system (DTS), a condensed remnant of the rough endoplasmic reticulum (Figures 13-11 and 13-12). Having abandoned its usual protein production function upon platelet release, the DTS sequesters Ca²⁺ and bears a series of enzymes that support platelet activation. These enzymes include phospholipase A₂, cyclooxygenase, and thromboxane synthetase, which support the eicosanoid synthesis pathway that produces thromboxane A₂, and phospholipase C, which supports production of inositol triphosphate (IP₃) and diacylglycerol (DAG). The DTS is the “control center” for platelet activation.

**Platelet Plasma Membrane Receptors That Provide for Adhesion**
The platelet membrane supports more than 50 categories of receptors, including members of the cell adhesion molecule (CAM) integrin family, the CAM leucine-rich repeat family, the CAM immunoglobulin gene family, the CAM selectin family, the seven-transmembrane receptor (STR) family, and some miscellaneous receptors. Table 13-4 lists the receptors that support the initial phases of platelet adhesion and aggregation.

Several integrins bind collagen, enabling the platelet to adhere to the injured blood vessel lining. Integrins are heterodimeric (composed of two dissimilar proteins) CAMs that integrate their ligands, which they bind on the outside of the cell, with the internal cytoskeleton, triggering activation. GP Ia/IIa, or, using integrin terminology, α₂β₁, is an integrin that binds the subendothelial collagen that becomes uncovered in the damaged blood vessel wall, promoting adhesion of the platelet to the vessel wall (Figures 13-11 and 13-12). Likewise, α₁β₃ and α₅β₁ bind the adhesive endothelial cell proteins laminin and fibronectin, which further promotes platelet adhesion. Another collagen-binding receptor is GP VI, a member of the immunoglobulin gene family, so named because the genes of its members have multiple immunoglobulin-like domains.
unclassified platelet receptor GP IV is a key collagen receptor that also binds the adhesive protein thrombospondin.\textsuperscript{33}

Another adhesion receptor is GP Ib/IX/V, a leucine-rich-repeat family CAM, named for its members’ multiple leucine-rich domains. GP Ib/IX/V arises from the genes GP1BA, GP1BB, GP5, and GP9. It is composed of two molecules each of GP Ibα, GP Ibβ, and GP IX, and one molecule of GP V. These total seven noncovalently bound subunits. The two copies of subunit GP Ibα bind VWF and support platelet tethering (deceleration), necessary in capillaries and arterioles where blood flow shear rates exceed 1000 s\textsuperscript{-1}. The accompanying GP Ibβ molecules cross the platelet membrane and interact with actin-binding protein to provide “outside-in” signaling. Two molecules of GP IX and one of GP V help assemble the four GP Ib molecules. Mutations in GP Ibα, GP Ibβ, or GP IX (but not GP V) are associated with a moderate-to-severe mucocutaneous bleeding disorder, Bernard-Soulier syndrome (Chapter 41). Additionally, VWF deficiency is the basis for the most common inherited bleeding disorder, von Willebrand disease (VWD). VWD also is associated with mucocutaneous bleeding, although the disorder is technically a plasma protein (VWF) deficiency, not a platelet abnormality.\textsuperscript{34}

The subunits of the integrin GP IIb/IIIa (α\textsubscript{IIb}β\textsubscript{3}), are separate and inactive (α\textsubscript{IIb} and β\textsubscript{3}) as they are distributed across the
plasma membrane, the SCCS, and the internal layer of α-granule membranes. These form their active heterodimer, αIIbβ3, only when they encounter an “inside-out” signaling mechanism triggered by collagen binding to GP VI. Although various agonists may activate the platelet, αIIbβ3 is a physiologic requisite because it binds fibrinogen, generating interplatelet cohesion, called platelet aggregation. Mutations in αIIb or β3 cause a severe inherited mucocutaneous bleeding disorder, Glanzmann thrombasthenia (Chapter 41). The αIIbβ3 integrin also binds VWF, vitronectin, and fibronectin, all adhesive proteins that share the target arginine-glycine-aspartate (RGD) amino acid sequence with fibrinogen.

### The Seven-Transmembrane Repeat Receptors
Thrombin, thrombin receptor activation peptide (TRAP), adenosine diphosphate (ADP), epinephrine, and the eicosanoid synthesis pathway (also called the prostaglandin or the cyclooxygenase pathway) product thromboxane A2 (TXA2) all function individually or together to activate platelets (Figures 13-11 and 13-12). These platelet “agonists” are ligands for the seven-transmembrane repeat receptors (STRs), so named for their unique membrane-anchoring structure. The STRs have seven hydrophobic anchoring domains supporting an external binding site and an internal terminus that interacts with G proteins for outside-in platelet signaling. The STRs are listed in Table 13-5.

Thrombin cleaves two STRs, protease-activated receptor 1 (PAR1) and PAR4, that together have a total of 1800 membrane copies on an average platelet. Thrombin cleavage of either of these two receptors activates the platelet through G-proteins that in turn activate at least two internal physiologic pathways, described subsequently. Thrombin also interacts with platelets by binding or digesting two CAMs in the leucine-rich repeat family, GP Ibα and GP V, both of which are parts of the GP Ib/IX/V VWF adhesion receptor.

There are about 600 copies of the high-affinity ADP receptors P2Y1 and P2Y12 per platelet. These STRs also activate the platelet through the G-protein signaling pathways. TPα and TPβ bind TXA2. This interaction produces more TXA2 from the platelet, a G-protein based autocrine (self-perpetuating) system that activates neighboring platelets. Epinephrine binds α2-adrenergic sites that also couple to G-proteins and open up membrane calcium channels.

### Table 13-4 Glycoprotein Platelet Membrane Receptors That Participate in Adhesion and the Initiation of Aggregation by Binding Specific Ligands

<table>
<thead>
<tr>
<th>Electrophoresis Nomenclature</th>
<th>Current Nomenclature</th>
<th>Ligand</th>
<th>Cluster Designation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP Ia/IIa</td>
<td>Integrin: α3β1</td>
<td>Collagen</td>
<td>CD29, CD49b</td>
<td>Avidity is upregulated via “inside-out” activation that depends on collagen binding to GP VI.</td>
</tr>
<tr>
<td></td>
<td>Integrin: α6β1</td>
<td>Vitronectin</td>
<td>CD29, CD49e</td>
<td></td>
</tr>
<tr>
<td>GP VI</td>
<td>CAM of the immunoglobulin gene family</td>
<td>Collagen</td>
<td>CD29, CD49f</td>
<td>Key collagen receptor, triggers activation, release of agonists that increase the avidity of integrins α2β1 and α6β1.</td>
</tr>
<tr>
<td>GP Ib/IX/V</td>
<td>CAM of the leucine-rich repeat family</td>
<td>VWF and thrombin bind GP Ibα; thrombin cleaves a site on GP V</td>
<td>CD42a, CD42b, CD42c, CD42d</td>
<td>GP Ib/IX/V is a 2:2:2:1 complex of GP Ibα and Ibβ, GP IX, and GP V. There are 25,000 copies on the resting platelet membrane surface, 5% to 10% on the α-granule membrane, but few on the SCCS membrane. GP Ibα is the VWF-specific site. Fifty percent of GP Ibα/Ibβ is cleared from the membrane on activation. Bernard-Soulier syndrome mutations are identified for all but GP V. Bound to subsurface actin-binding protein.</td>
</tr>
<tr>
<td>GP IIIa/IIIla</td>
<td>Integrin: α6β1</td>
<td>Fibrinogen, VWF</td>
<td>CD41, CD61</td>
<td>GP Ib and IIIa are distributed on the surface membrane, SCCS, and α-granule membranes (30%). Heterodimer forms on activation.</td>
</tr>
</tbody>
</table>

CAM, Cell adhesion molecule; GP, glycoprotein; SCCS, surface-connected canicular system; VWF, von Willebrand factor.
**TABLE 13-5 Platelet STR Receptor-Ligand Interaction Coupled to Signaling**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>G Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1</td>
<td>Thrombin</td>
<td>Coupled to G₁ protein that reduces cAMP; coupled to G₆ and G₁₂ proteins that increase IP₃ and DAG</td>
</tr>
<tr>
<td>PAR4</td>
<td>Thrombin</td>
<td>Coupled to G₁ protein that reduces cAMP; coupled to G₆ and G₁₂ proteins that increase IP₃ and DAG</td>
</tr>
<tr>
<td>P₂Y₁₁</td>
<td>ADP</td>
<td>Coupled to G₁₂ protein that increases IP₃ and DAG</td>
</tr>
<tr>
<td>P₂Y₁₃</td>
<td>ADP</td>
<td>Coupled to G₁₂ protein that increases IP₃ and DAG</td>
</tr>
<tr>
<td>TPα and TPβ</td>
<td>TXA₂</td>
<td>Coupled to G₁₂ protein that increases IP₃ and DAG</td>
</tr>
<tr>
<td>α₂-adrenergic</td>
<td>Epinephrine</td>
<td>Coupled to G₁ protein that reduces cAMP; potentiates effects of ADP, thrombin, and TXA₂</td>
</tr>
<tr>
<td>IP</td>
<td>PGI₂</td>
<td>Coupled to G₁ protein that increases cAMP to inhibit activation</td>
</tr>
</tbody>
</table>

**Additional Platelet Membrane Receptors**

About 15 clinically relevant receptors were discussed in the preceding paragraphs. The platelet supports many additional receptors. The CAM immunoglobulin family includes the ICAMs (CD50, CD54, CD102), which play a role in inflammation and the immune reaction; PECAM (CD31), which mediates platelet-to-blood cell and platelet-to-endothelial cell adhesion; and FcyIIA (CD32), a low-affinity receptor for the immunoglobulin Fc portion that plays a role in a dangerous condition called heparin-induced thrombocytopenia (Chapter 39). P-selectin (CD62) is an integrin that facilitates platelet binding to endothelial cells, leukocytes, and one another. P-selectin is found on the α-granule membranes of the resting platelet but migrates via the SCCS to the surface of activated platelets. P-selectin or CD62 quantification by flow cytometry is a successful clinical means for measuring in vivo platelet activation.

**Platelet Cytoskeleton: Microfilaments and Microtubules**

A thick circumferential bundle of microtubules maintains the platelet’s discoid shape. The *circumferential microtubules* (Figures 13-11 and 13-12) parallel the plane of the outer surface of the platelet and reside just within, although not touching, the plasma membrane. There are 8 to 20 tubules composed of multiple subunits of tubulin that disassemble at refrigerator temperature or when treated with colchicine. When microtubules disassemble in the cold, platelets become round, but upon warming to 37° C, they recover their original disc shape. On cross section, microtubules are cylindrical, with a diameter of 25 nm. The circumferential microtubules could be a single spiral tubule.42 Besides maintaining the platelet shape, microtubules move inward on activation to enable the expression of α-granule contents. They also reassemble in long parallel bundles during platelet shape change to provide rigidity to pseudopods.

In the narrow area between the microtubules and the membrane lies a thick meshwork of microfilaments composed of actin (Figures 13-11 and 13-12). Actin is contractile in platelets (as in muscle) and anchors the plasma membrane glycoproteins and proteoglycans. Actin also is present throughout the platelet cytoplasm, constituting 20% to 30% of platelet protein. In the resting platelet, actin is globular and amorphous, but as the cytoplasmic calcium concentration rises, actin becomes filamentous and contractile.

The cytoplasm also contains intermediate filaments, ropelike polymers 8 to 12 nm in diameter, of *desmin* and *vimentin*. The intermediate filaments connect with actin and the tubules, maintaining the platelet shape. Microtubules, actin microfilaments, and intermediate microfilaments control platelet shape change, extension of pseudopods, and secretion of granule contents.

**Platelet Granules: α-Granules, Dense Granules, and Lysosomes**

There are 50 to 80 α-granules in each platelet. Unlike the nearly opaque dense granules, α-granules stain medium gray in osmium-dye transmission electron microscopy preparations (Figures 13-11 and 13-12). The α-granules are filled with proteins, some endocytosed, some synthesized within the megakaryocyte and stored in platelets (Table 13-6). Several α-granule proteins are membrane bound. As the platelet becomes activated, α-granule membranes fuse with the SCCS. Their contents flow to the nearby microenvironment, where they participate in platelet adhesion and aggregation and support plasma coagulation.43

There are two to seven dense granules per platelet. Also called dense bodies, these granules appear later than α-granules in megakaryocyte differentiation and stain black (opaque) when treated with osmium in transmission electron microscopy (Figures 13-11 and 13-12). Small molecules are probably endocytosed and are stored in the dense granules; these are listed in Table 13-7. In contrast to the α-granules, which employ the SCCS, dense granules migrate to the plasma membrane and release their contents directly into the plasma upon platelet activation. Membranes of dense granules support the same integral proteins as the α-granules—P-selectin, α₃B₃, and GP Ib/IX/V, for instance—which implies a common source for the membranes of both types of granules.44
TABLE 13-6 Representative Platelet α-Granule Proteins

<table>
<thead>
<tr>
<th>Proteins Present in Platelet Cytoplasm</th>
<th>Coagulation Proteins</th>
<th>Noncoagulation Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocytosed Proteins</td>
<td>Fibronectin</td>
<td>Albumin</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>Megakaryocyte-synthesized Proteins</td>
<td>Factor V</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Thrombospondin</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>VWF</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteins Present in α-Granules but Not Cytoplasm</th>
<th>Endocytosed Proteins</th>
<th>α-Granule Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megakaryocyte-Synthesized Proteins</td>
<td>β-thromboglobulin</td>
<td>EGF</td>
</tr>
<tr>
<td></td>
<td>HMWK</td>
<td>Multimerin</td>
</tr>
<tr>
<td>PAI-1</td>
<td>PDC1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasminogen</td>
<td>PDGF</td>
</tr>
<tr>
<td></td>
<td>PF4</td>
<td>TGF-β</td>
</tr>
<tr>
<td></td>
<td>Protein C inhibitor</td>
<td>VEGF/NPF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Platelet Membrane-Bound Proteins</th>
<th>Restricted to α-granule membrane</th>
<th>P-selectin</th>
<th>GMP33</th>
</tr>
</thead>
<tbody>
<tr>
<td>In α-granule and plasma membrane</td>
<td>GP IIb/IIIa</td>
<td>cap1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GP IV</td>
<td>CD9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GP Ib/IX/V</td>
<td>PECAM-1</td>
<td></td>
</tr>
</tbody>
</table>

EGF, Endothelial growth factor; GMP, guanosine monophosphate; GP, glycoprotein; HMWK, high-molecular-weight kininogen; Ig, immunoglobulin; PAI-1, plasminogen activator inhibitor-1; PDC1, platelet-derived collagenase inhibitor; PDGF, platelet-derived growth factor; PECAM-1, platelet-endothelial cell adhesion molecule-1; PF4, platelet factor 4; TGF-β, transforming growth factor-β; VEGF/VPF, vascular endothelial growth factor/vascular permeability factor; VWF, von Willebrand factor; cap1, adenyl cyclase–associated protein.

TABLE 13-7 Dense Granule (Dense Body) Contents

<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Nonmetabolic, supports neighboring platelet aggregation by binding to ADP receptors P2Y1, P2Y13</td>
</tr>
<tr>
<td>ATP</td>
<td>Function unknown, but ATP release is detectable upon platelet activation</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Vasoconstrictor that binds endothelial cells and platelet membranes</td>
</tr>
<tr>
<td>Ca²⁺ and Mg²⁺</td>
<td>Divalent cations support platelet activation and coagulation</td>
</tr>
</tbody>
</table>

ADP, Adenosine diphosphate; ATP, adenosine triphosphate; P2Y1 and P2Y13, members of the purinergic receptor family (receptors that bind purines).

Platelets also have a few lysosomes, similar to those in neutrophils, 300-nm-diameter granules that stain positive for arylsulfatase, β-glucuronidase, acid phosphatase, and catalase. The lysosomes probably digest vessel wall matrix components during in vivo aggregation and may also digest autophagic debris.

**PLATELET ACTIVATION**

Although the following discussion seems to imply a linear and stepwise process, adhesion, aggregation, and secretion are often simultaneous.⁴⁵,⁴⁶

**Adhesion: Platelets Bind Elements of the Vascular Matrix**

As blood flows, vessel walls create stress, or shear force, measured in units labeled s⁻¹. Shear forces range from 500 s⁻¹ in venules and veins to 5000 s⁻¹ in arterioles and capillaries and up to 40,000 s⁻¹ in stenosed (hardened) arteries (Figure 13-13). In vessels where the shear rate is over 1000 s⁻¹, platelet adhesion and aggregation require a defined sequence of events that involves collagen, tissue factor, phospholipid, VWF, and a number of platelet CAMs, ligands, and activators (Figure 13-14).⁴⁷

Injury to the blood vessel wall disrupts the collagen of the extracellular matrix (ECM).⁴⁸ Damaged endothelial cells release VWF from cytoplasmic storage organelles (Figure 13-15).⁴⁹ VWF, whose molecular weight ranges from 800,000 to 2,000,000 Daltons “unrolls” like a carpet and adheres to the injured site. Though VWF circulates as a globular protein, it become fibrillar as it unrolls and exposes sites that partially bind the GPIbα portion of the platelet membrane GP Ib/IX/V leucine-rich receptor. This is a reversible binding process that “tethers” or decelerates the platelet. Platelet and VWF interactions remain localized by a liver-secreted plasma enzyme, ADAMTS-13, also called VWF-cleaving protease, that digests “unused” VWF.

At high shear rates, the VWF-GP Ibα tethering reaction is temporary, and the platelet rolls along the surface unless GP VI comes in contact with the exposed ECM collagen.⁵⁰ When type I fibrillar collagen binds platelet GP VI, the receptor, which is anchored in the membrane by an Fn receptor–like molecule, triggers internal platelet activation pathways, releasing TXA₂ and ADP, an “outside-in” reaction.⁵¹ These agonists attach to their respective receptors: TPα and TPβ for TXA₂, and P2Y₁ and P2Y₁₂ for ADP, triggering an “inside-out” reaction that raises the affinity of integrin α₂β₁ for collagen. The combined effect of GP Ib/IX/V, GP VI, and α₂β₁ causes the platelet to become firmly affixed to the damaged surface, where it subsequently loses its discoid shape and spreads.⁵²

The internal platelet activators TXA₂ and ADP are also secreted from the platelet to the microenvironment, where they activate neighboring platelets through their respective receptors. Further, they provide inside-out activation of integrin α₃β₃, the key receptor site for fibrinogen, which assists in platelet aggregation.

**Aggregation: Platelets Irreversibly Cohere**

In addition to collagen exposure and VWF secretion, blood vessel injury releases constitutive (integral) tissue factor from endothelial cells. Tissue factor triggers the production of thrombin, which reacts with platelet STRs PAR1 and PAR4. This further activation generates the “collagen and thrombin activated” or COAT platelet, integral to the cell-based coagulation model.
factor complexes. As platelet aggregation continues, membrane integrity is lost, and a syncytium of platelet cytoplasm forms as the platelets exhaust internal energy sources.

Platelet aggregation is a key part of primary hemostasis, which in arteries may end with the formation of a “white clot,” a clot composed primarily of platelets and VWF (Figure 13-17). Although aggregation is a normal part of vessel repair, white clots often imply inappropriate platelet activation in seemingly uninjured arterioles and arteries and are the pathological basis for arterial thrombotic events, such as acute myocardial infarction, peripheral artery disease, and strokes. The risk of these cardiovascular events rises in proportion to the numbers and avidity of platelet membrane $\alpha_2\beta_1$ and GP VI.53

The combination of polar phospholipid exposure on activated platelets, platelet fragmentation with cellular microparticle dispersion, and secretion of the platelet’s $\alpha$-granule and dense granule contents triggers secondary hemostasis, called coagulation (see Chapter 37). Fibrin and red blood cells deposit around and within the platelet syncytium, forming a bulky “red clot” (Figure 13-18). The red clot is essential to wound repair, but it may also be characteristic of inappropriate coagulation in venules and veins, resulting in deep vein thrombosis and pulmonary emboli.

**Secretion: Activated Platelets Release Granular Contents**

Outside-in platelet activation through ligand (agonist) binding to integrins, STRs (such as ADP binding to P2Y$_{12}$), and the immunoglobulin gene product GP VI triggers actin filament contraction. Intermediate filaments also contract, moving the circumferential microtubules inward and compressing the granules. Contents of $\alpha$-granules and lysosomes flow through the SCS, while dense granules migrate to the plasma membrane, where their contents are secreted (Figure 13-11). The dense granule contents are vasoconstrictors and platelet agonists that amplify primary hemostasis; most of the $\alpha$-granule

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**Figure 13-13** Normal blood flow in intact vessels. RBCs and platelets flow near the center, and WBCs marginate and roll. Endothelial cells and the ECM provide several properties that suppress hemostasis. EC, Endothelial cell; ECM, extracellular matrix; FB, fibroblast; PLT, platelet; RBC, red blood cell; SMC, smooth muscle cell; WBC, white blood cell.

**Figure 13-14** Initial platelet activation leading to platelet adhesion. The glycoprotein (GP) 1b$\alpha$ portion of the GP Ib/IX/V von Willebrand factor (VWF) receptor site binds VWF (1) and GP VI binds collagen (2). The bound GP VI initiates the release of thromboxane A$_2$ (TXA$_2$) and adenosine diphosphate (ADP, 3), which activate $\alpha_2\beta_1$, an additional collagen receptor (4), stabilizing platelet adhesion, and $\alpha_IIb\beta_3$, the arginine-glycine-aspartate (RGD) receptor site (5) that binds fibrinogen and VWF to support platelet aggregation.

---

**Figure 13-15** Normal ECM suppresses hemostasis:
- Prostacyclin
- Heparan sulfate
- Tissue factor pathway inhibitor
- Nitric oxide
- Thrombomodulin

---

**Figure 13-16** described in Chapter 37 (Figure 13-16). Meanwhile, integrin $\alpha_IIb\beta_3$ assembles from its resting membrane units $\alpha_IIb$ and $\beta_3$, binding RGD sequences of fibrinogen and VWF and supports platelet-to-platelet aggregation. P-selectin from the $\alpha$-granule membranes moves to the surface membrane to further promote aggregation. Platelets lose their shape and extend pseudopods. Membrane phospholipids redeploy with the more polar molecules, especially phosphatidylserine, flipping to the outer layer, establishing a surface for the assembly of coagulation factor complexes. As platelet aggregation continues, membrane integrity is lost, and a syncytium of platelet cytoplasm forms as the platelets exhaust internal energy sources.

Platelet aggregation is a key part of primary hemostasis, which in arteries may end with the formation of a “white clot,” a clot composed primarily of platelets and VWF (Figure 13-17). Although aggregation is a normal part of vessel repair, white clots often imply inappropriate platelet activation in seemingly uninjured arterioles and arteries and are the pathological basis for arterial thrombotic events, such as acute myocardial infarction, peripheral artery disease, and strokes. The risk of these cardiovascular events rises in proportion to the numbers and avidity of platelet membrane $\alpha_2\beta_1$ and GP VI.53

The combination of polar phospholipid exposure on activated platelets, platelet fragmentation with cellular microparticle dispersion, and secretion of the platelet’s $\alpha$-granule and dense granule contents triggers secondary hemostasis, called coagulation (see Chapter 37). Fibrin and red blood cells deposit around and within the platelet syncytium, forming a bulky “red clot” (Figure 13-18). The red clot is essential to wound repair, but it may also be characteristic of inappropriate coagulation in venules and veins, resulting in deep vein thrombosis and pulmonary emboli.
contents are coagulation proteins that participate in secondary hemostasis (Tables 13-6 and 13-7).

By presenting polar phospholipids on their membrane surfaces, platelets provide a localized cellular milieu that supports coagulation. Phosphatidylserine is the polar phospholipid on which two coagulation pathway complexes assemble: factor IX/VIII (tenase) and factor X/V (prothrombinase), both supported by ionic calcium secreted by the dense granules. The α-granule contents fibrinogen, factors V and VIII, and VWF (which binds and stabilizes factor VIII) are secreted and increase the localized concentrations of these essential coagulation proteins. Their presence further supports the action of tenase and prothrombinase. Platelet secretions provide for cell-based, controlled, localized coagulation. Table 13-8 lists some additional α-granule secretion products that, although not proteins of the coagulation pathway, indirectly support hemostasis. The lists in Tables 13-6, 13-7, and 13-8 are not exhaustive because more and more platelet granule contents continue to be identified through platelet research activities.

**PLATELET ACTIVATION PATHWAYS**

**G Proteins**

G proteins control cellular activation for all cells (not just platelets) at the inner membrane surface (Figure 13-19). G proteins are αβγ heterotrimers (proteins composed of three dissimilar peptides) that bind guanosine diphosphate (GDP) when inactive. Membrane receptor-ligand (agonist) binding promotes GDP release and its replacement with guanosine triphosphate (GTP). The Ga portion of the three-part G molecule briefly dissociates, exerts enzymatic guanosine triphosphatase activity, and hydrolyzes the bound GTP to GDP, releasing a phosphate radical. The G protein resumes its resting state, but the hydrolysis step provides the necessary phosphorylation trigger to energize the eicosanoid synthesis or the IP3-DAG pathway (Table 13-9).

**Eicosanoid Synthesis**

The eicosanoid synthesis pathway, alternatively called the prostaglandin, cyclooxygenase, or thromboxane pathway, is one of two essential platelet activation pathways triggered by G proteins.
**Figure 13-17** In arteries and arterioles, the “white clot” consists of platelets and von Willebrand factor. Though primarily a protective mechanism, the white clot may occlude the vessel, causing acute myocardial infarction, stroke, or peripheral artery disease. EC, Endothelial cell; ECM, extracellular matrix; SMC, smooth muscle cell; FB, fibroblast; PLT, platelet; RBC, red blood cell; WBC, white blood cell; VWF, von Willebrand factor; lines indicate collagen.

**Figure 13-18** In veins and venules, the bulky “red clot” consists of platelets, von Willebrand factor, fibrin, and RBCs. Though a protective mechanism, the red clot may occlude the vessel, causing venous thromboembolic disease. EC, Endothelial cell; ECM, extracellular matrix; SMC, smooth muscle cell; FB, fibroblast; PLT, platelet; RBC, red blood cell; WBC, white blood cell; VWF, von Willebrand factor; lines indicate collagen.

**TABLE 13-8** Selected α-Granule Proteins and Their Properties

<table>
<thead>
<tr>
<th>α-Granule Protein</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-derived growth factor</td>
<td>Supports mitosis of vascular fibroblasts and smooth muscle cells</td>
</tr>
<tr>
<td>Endothelial growth factor</td>
<td>Supports mitosis of vascular fibroblasts and smooth muscle cells</td>
</tr>
<tr>
<td>Transforming growth factor-β</td>
<td>Supports mitosis of vascular fibroblasts and smooth muscle cells</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>Heparin neutralization, suppresses megakaryocytopenosis</td>
</tr>
<tr>
<td>β-thromboglobulin</td>
<td>Found nowhere but platelet α-granules</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Fibrinolysis promotion</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>Fibrinolysis control</td>
</tr>
<tr>
<td>α2-Antiplasmin</td>
<td>Fibrinolysis control</td>
</tr>
<tr>
<td>Protein C inhibitor</td>
<td>Coagulation control</td>
</tr>
</tbody>
</table>
Blood Cell Production, Structure, and Function

The platelet membrane’s inner leaflet is rich in phosphatidylinositol, a phospholipid whose number 2 carbon binds numerous types of unsaturated fatty acids, but especially 5,8,11,14-eicosatetraenoic acid, commonly called arachidonic acid. Membrane receptor-ligand binding and the consequent G-protein activation triggers phospholipase A2, a membrane enzyme that cleaves the ester bond connecting the number 2 carbon of the triglyceride backbone with arachidonic acid. Cleavage releases arachidonic acid to the cytoplasm, where it becomes the substrate for cyclooxygenase, anchored in the DTS. Cyclooxygenase converts arachidonic acid to prostaglandin G2 and prostaglandin H2, and then thromboxane synthetase acts on prostaglandin H2 to produce TXA2. TXA2 binds membrane receptors TPα or TPβ, decelerating adenylate cyclase activity and reducing cAMP concentrations, which mobilizes ionic calcium from the DTS (Figure 13-21). The rising cytoplasmic calcium level causes contraction of actin microfilaments and platelet activation.

The cyclooxygenase pathway in endothelial cells incorporates the enzyme prostacyclin synthetase in place of the thromboxane synthetase in platelets. The eicosanoid pathway end point for the endothelial cell is prostaglandin I2, or prostacyclin, which infiltrates the platelet and binds its IP receptor site. Prostacyclin binding accelerates adenylate cyclase, increasing cAMP, and sequesters ionic calcium to the DTS. The endothelial cell pathway suppresses platelet activation in the intact blood vessel through this mechanism, creating a dynamic equilibrium.
TXA₂ has a half-life of 30 seconds, diffuses from the platelet, and becomes spontaneously reduced to thromboxane B₂, a stable, measurable plasma metabolite. Efforts to produce a clinical assay for plasma thromboxane B₂ have been unsuccessful, because special specimen management is required to prevent ex vivo platelet activation with unregulated release of thromboxane B₂ subsequent to collection. Thromboxane B₂ is acted on by a variety of liver enzymes to produce an array of soluble urine metabolites, including 11-dehydrothromboxane B₂, which is stable and measurable.⁵⁴,⁵⁵

**Inositol Triphosphate–Diacylglycerol Activation Pathway**

The IP₃-DAG pathway is the second G protein–dependent platelet activation pathway (Figure 13-22). G-protein activation triggers the enzyme phospholipase C. Phospholipase C
cleaves membrane phosphatidylinositol 4,5-bisphosphate to form IP$_3$ and DAG, both second messengers for intracellular activation. IP$_3$ promotes release of ionic calcium from the DTS, which triggers actin microfilament contraction. IP$_3$ may also activate phospholipase A$_2$. DAG triggers a multistep process: activation of phosphokinase C, which triggers phosphorylation of the protein pleckstrin, which regulates actin microfilament contraction.

Internal platelet activation pathways, like internal pathways of all metabolically active cells, are often called second messengers because they are triggered by a primary ligand-receptor binding event. Second messengers include G proteins, the eicosanoid synthesis pathway, the IP$_3$-DAG pathway, adenylate cyclase, cAMP, and intracellular ionic calcium. This discussion has been limited to activation pathways whose aberrations cause hemostatic disease. The reader is referred to cell physiology texts for a comprehensive discussion of cellular activation pathways.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

**REVIEW QUESTIONS**

Answers can be found in the Appendix.

1. The megakaryocyte progenitor that undergoes endomitosis is:
   a. MK-I
   b. BFU-Meg
   c. CFU-Meg
   d. LD-CFU-Meg

2. The growth factor that is produced in the kidney and induces growth and differentiation of committed megakaryocyte progenitors is:
   a. IL-3
   b. IL-6
   c. IL-11
   d. TPO
3. What platelet organelle sequesters ionic calcium and binds a series of enzymes of the eicosanoid pathway?
   a. G protein
   b. Dense granules
   c. DTS
   d. SCCS

4. What platelet membrane receptor binds fibrinogen and supports platelet aggregation?
   a. GP Ib/IX/V
   b. GP Ib/IIa
   c. GP Ia/IIa
   d. P2Y1

5. What platelet membrane phospholipid flips from the inner surface to the plasma surface on activation and serves as the assembly point for coagulation factors?
   a. Phosphatidylethanolamine
   b. Phosphatidylinositol
   c. Phosphatidylcholine
   d. Phosphatidylserine

6. What is the name of the eicosanoid metabolite produced from endothelial cells that suppresses platelet activity?
   a. TXA2
   b. Arachidonic acid
   c. Cyclooxygenase
   d. Prostacyclin

7. Which of the following molecules is stored in platelet dense granules?
   a. Serotonin
   b. Fibrinogen
   c. PF4
   d. Platelet-derived growth factor

8. What plasma protein is essential to platelet adhesion?
   a. VWF
   b. Factor VIII
   c. Fibrinogen
   d. P-selectin

9. Reticulated platelets can be enumerated in peripheral blood to detect:
   a. Impaired production in disease states
   b. Abnormal organelles associated with diseases such as leukemia
   c. Increased platelet production in response to need
   d. Inadequate rates of membrane cholesterol exchange with the plasma

10. Platelet adhesion refers to platelets:
    a. Sticking to other platelets
    b. Releasing platelet granule constituents
    c. Providing the surface for assembly of coagulation factors
    d. Sticking to surfaces such as subendothelial collagen

REFERENCES


1. State the dimensions of the counting area of a Neubauer ruled hemacytometer.

2. Describe the performance of manual cell counts for white blood cells, red blood cells, and platelets, including types of diluting fluids, typical dilutions, and typical areas counted in the hemacytometer.

3. Calculate dilutions for cell counts when given appropriate data.

4. Calculate hemacytometer cell counts when given numbers of cells, area counted, and dilution.

5. Correct white blood cell counts for the presence of nucleated red blood cells.

6. Describe the principle of the cyanmethemoglobin assay for determination of hemoglobin.

7. Calculate the values for a standard curve for cyanmethemoglobin determination when given the appropriate data, describe how the standard curve is constructed, and use the standard curve to determine hemoglobin values.

8. Describe the procedure for performing a microhematocrit.

9. Identify sources of error in routine manual procedures discussed in this chapter and recognize written scenarios describing such errors.

10. Compare red blood cell count, hemoglobin, and hematocrit values using the rule of three.

11. Calculate red blood cell indices (mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration) when given appropriate data, and interpret the results relative to the volume and hemoglobin content and concentration in the red blood cells.

12. Describe the principle and procedure for performing a manual reticulocyte count and the clinical value of the test.

13. Given the appropriate data, calculate the relative, absolute, and corrected reticulocyte counts and the reticulocyte production index; interpret results to determine the adequacy of the bone marrow erythropoietic response in an anemia.

14. Describe the procedure for performing the Westergren erythrocyte sedimentation rate and state its clinical utility.

15. Describe the aspects of establishing a point-of-care testing program, including quality management and selection of instrumentation.

16. Discuss the advantages and disadvantages of point-of-care testing as they apply to hematology tests.

17. Describe the principles of common instruments used for point-of-care testing for hemoglobin level, hematocrit, white blood cell counts, and platelet counts.

**OBJECTIVES**

After completion of this chapter, the reader will be able to:

**CASE STUDIES**

After studying the material in this chapter, the reader should be able to respond to the following case studies:

**Case 1**

The following results are obtained for a patient with normocytic, normochromic red blood cells on a peripheral blood film:

- RBC count = 4.63 \times 10^{12}/L
- HGB = 15 g/dL
- HCT = 40% (0.40 L/L)

Continued
Clinical laboratory hematology has evolved from simple observation and description of blood and its components to a highly automated, extremely technical science, including examination at the molecular level. However, some of the more basic tests have not changed dramatically over the years. This chapter provides an overview of these basic tests and presents the manual and semiautomated methods that can be used in lieu of automated instrumentation. Included in this chapter is a discussion of point-of-care testing in hematology.

**MANUAL CELL COUNTS**

Although most routine cell-counting procedures in the hematology laboratory are automated, it may be necessary to use manual methods when counts exceed the linearity of an instrument, when an instrument is nonfunctional and there is no backup, in remote laboratories in Third World countries, or in a disaster situation when testing is done in the field. Although the discussion in this chapter concerns whole blood, body fluid cell counts are also often performed using manual methods. Chapter 18 discusses the specific diluents and dilutions used for body fluid cell counts. Chapter 15 discusses automated cell-counting instrumentation in detail.

Manual cell counts are performed using a hemacytometer, or counting chamber, and manual dilutions made with calibrated, automated pipettes and diluents (commercially available or laboratory prepared). The principle for the performance of cell counts is essentially the same for white blood cells (WBCs), red blood cells (RBCs), and platelets; only the dilution, diluting fluid, and area counted vary. Any particle (e.g., sperm) can be counted using this system.

**Equipment**

**Hemacytometer**

The manual cell count uses a hemacytometer, or counting chamber. The most common one is the Levy chamber with improved Neubauer ruling. It is composed of two raised surfaces, each with a 3 mm \( \times \) 3 mm square counting area or grid (total area 9 mm\(^2\)), separated by an H-shaped moat. As shown in Figure 14-1, this grid is made up of nine 1 mm \( \times \) 1 mm squares. Each of the four corner (WBC) squares is subdivided further into 16 squares, and the center square subdivided into 25 smaller squares. Each of these smallest squares is 0.2 mm \( \times \) 0.2 mm which is \( \frac{1}{25} \) of the center square or 0.04 mm\(^2\). A coverslip is placed on top of the counting surfaces. The distance between each counting surface and the coverslip is 0.1 mm; thus the total volume of one entire grid or counting area on one side of the hemacytometer is 0.9 mm\(^3\). Hemacytometers and coverslips must meet the specifications of the National Bureau of Standards, as indicated by the initials “NBS” on the chamber. When the dimensions of the hemacytometer are thoroughly understood, the area counted can be changed to facilitate the counting of samples with extremely low or high counts.

**Calculations**

The general formula for manual cell counts is as follows and can be used to calculate any type of cell count:

\[
\text{Total count} = \frac{\text{cells counted} \times \text{dilution factor}}{\text{area (mm}^2\text{)} \times \text{depth (0.1)}}
\]

Or

\[
\text{Total count} = \frac{\text{cells counted} \times \text{dilution factor} \times \text{10}^*}{\text{area (mm}^2\text{)}}
\]

\* Reciprocal of depth.
5. Mix again by inversion and fill a plain microhematocrit tube.
6. Charge both sides of the hemacytometer by holding the microhematocrit tube at a 45-degree angle and touching the tip to the coverslip edge where it meets the chamber floor.
7. After charging the hemacytometer, place it in a moist chamber (Box 14-1) for 10 minutes before counting the cells to give them time to settle. Care should be taken not to disturb the coverslip.
8. While keeping the hemacytometer in a horizontal position, place it on the microscope stage.
9. Lower the condenser on the microscope and focus by using the low-power (10×) objective lens (100× total magnification). The cells should be distributed evenly in all of the squares.
10. For a 1:20 dilution, count all of the cells in the four corner squares, starting with the square in the upper left-hand corner (Figure 14-1). Cells that touch the top and left lines should be counted; cells that touch the bottom and right lines should be ignored (Figure 14-2). See Figure 14-3 for the appearance of WBCs in the hemacytometer using the low-power objective lens of a microscope.

**PROCEDURE**

1. Clean the hemacytometer and coverslip with alcohol and dry thoroughly with a lint-free tissue. Place the coverslip on the hemacytometer.
2. Make a 1:20 dilution by placing 25 µL of well-mixed blood into 475 µL of WBC diluting fluid in a small test tube.
3. Cover the tube and mix by inversion.
4. Allow the dilution to sit for 10 minutes to ensure that the red blood cells have lysed. The solution will be clear once lysis has occurred. WBC counts should be performed within 3 hours of dilution.

**BOX 14-1 How to Make a Moist Chamber**

A moist chamber may be made by placing a piece of damp filter paper in the bottom of a Petri dish. An applicator stick broken in half can serve as a support for the hemacytometer.
11. Repeat the count on the other side of the counting chamber. The difference between the total cells counted on each side should be less than 10%. A greater variation could indicate an uneven distribution, which requires that the procedure be repeated.

12. Average the number of WBCs counted on the two sides. Using the average, calculate the WBC count using one of the equations given earlier.

**Example Using the First Equation**

When a 1:20 dilution is used, the four large squares on one side of the chamber yield counts of 23, 26, 22, and 21. The total count is 92. The four large squares on the other side of the chamber yield counts of 28, 24, 22, and 26. The total count is 100. The difference between sides is less than 10%.

The average number of cells of the two sides of the chamber is 96. Using the average in the formula:

\[
\text{WBC count} = \frac{\text{cells counted \times dilution factor}}{\text{area counted (mm}^2\text{) \times depth}}
\]

\[
= \frac{96 \times 20}{4 \times 0.1}
\]

\[
= 4800/\text{mm}^3 \text{ or } 4800/\mu\text{L or } 4.8 \times 10^9/\mu\text{L or } 4.8 \times 10^7/\text{L}
\]

Alternately, a 1:100 dilution may be used counting the number of cells in the entire counting area (9 large squares, 9 mm²) on both sides of the chamber (Table 14-1). As an example, if an average of 54 cells were counted in the entire counting area on both sides of the chamber:

\[
\text{WBC count} = \frac{\text{cells counted \times dilution factor}}{\text{area counted (mm}^2\text{) \times depth}}
\]

\[
= \frac{54 \times 100}{9 \times 0.1}
\]

\[
= 6000/\text{mm}^3 \text{ or } 6000/\mu\text{L or } 6.0 \times 10^9/\mu\text{L or } 6.0 \times 10^7/\text{L}
\]

General reference intervals for males and females in different age groups can be found on the inside front cover of this text. Reference intervals may vary slightly according to the population tested and should be established for each laboratory.

**Sources of Error and Comments**

1. The hemacytometer and coverslip should be cleaned properly before they are used. Dust and fingerprints may cause difficulty in distinguishing the cells.

2. The diluting fluid should be free of contaminants.

**Table 14-1 Manual Cell Counts with Most Common Dilutions, Counting Areas**

<table>
<thead>
<tr>
<th>Cells Counted</th>
<th>Diluting Fluid</th>
<th>Dilution</th>
<th>Objective</th>
<th>Area Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells</td>
<td>1% ammonium oxalate</td>
<td>1:20</td>
<td>10x</td>
<td>4 mm²</td>
</tr>
<tr>
<td>or</td>
<td>3% acetic acid</td>
<td>1:100</td>
<td>10x</td>
<td>9 mm²</td>
</tr>
<tr>
<td>or</td>
<td>1% hydrochloric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells</td>
<td>Isotonic saline</td>
<td>1:100</td>
<td>40x</td>
<td>0.2 mm² (5 small squares of center square)</td>
</tr>
<tr>
<td>Platelets</td>
<td>1% ammonium oxalate</td>
<td>1:100</td>
<td>40x</td>
<td>1 mm²</td>
</tr>
</tbody>
</table>
3. If the count is low, a greater area may be counted (e.g., 9 mm²) to improve accuracy.
4. The chamber must be charged properly to ensure an accurate count. Uneven flow of the diluted blood into the chamber results in an irregular distribution of cells. If the chamber is overfilled or underfilled, the chamber must be cleaned and recharged.
5. After the chamber is filled, allow the cells to settle for 10 minutes before counting.
6. Any nucleated red blood cells (NRBCs) present in the sample are not lysed by the diluting fluid. The NRBCs are counted as WBCs because they are indistinguishable when seen on the hemacytometer. If five or more NRBCs per 100 WBCs are observed on the differential count on a stained peripheral blood film, the WBC count must be corrected for these cells. This is accomplished by using the following formula:

\[ \text{Uncorrected WBC count} \times \frac{100}{\text{Number of NRBCs per 100 WBCs} + 100} \]

Report the result as the “corrected” WBC count.
7. The accuracy of the manual WBC count can be assessed by performing a WBC estimate on a Wright-stained peripheral blood film made from the same specimen (Chapter 16).

**Platelet Count**
A platelet count is the number of platelets in 1 liter (L) or 1 microliter (µL) of whole blood. Platelets adhere to foreign objects and to each other, which makes them difficult to count. They also are small and can be confused easily with dirt or debris. In this procedure, whole blood, with EDTA as the anticoagulant, is diluted 1:100 with 1% ammonium oxalate to lyse the nonnucleated red blood cells. The platelets are counted in the 25 small squares in the large center square (1 mm²) of the hemacytometer. Platelets should be counted on each side of the hemacytometer, and the difference between the totals should be less than 10%.

6. Calculate the platelet count by one of the equations given earlier. Using the first equation as an example, if 200 platelets were counted in the entire center square,

\[ \frac{200 \times 100}{1 \times 0.1} = 200,000/\text{mm}^3 \text{ or } 200,000/\mu \text{L} \]

or \[200 \times 10^3/\mu \text{L} \text{ or } 200 \times 10^7/L\]

7. The accuracy of the manual platelet count should be verified by performing a platelet estimate on a Wright-stained peripheral blood film made from the same specimen (Chapter 16).

**Sources of Error and Comments**
1. Inadequate mixing and poor collection of the specimen can cause the platelets to clump on the hemacytometer. If the problem persists after redilution, a new specimen is needed. A skin puncture specimen is less desirable because of the tendency of the platelets to aggregate or form clumps.
2. Dirt in the pipette, hemacytometer, or diluting fluid may cause the counts to be inaccurate.
3. If fewer than 50 platelets are counted on each side, the procedure should be repeated by diluting the blood to 1:20. If more than 500 platelets are counted on each side, a 1:200 dilution should be made. The appropriate dilution factor should be used in calculating the results.
4. If the patient has a normal platelet count, the 5 small, red blood cell squares (Figure 14-1) may be counted. Then, the area is 0.2 mm² on each side.
5. The phenomenon of “platelet satellitosis” may occur when EDTA anticoagulant is used. This refers to the adherence of platelets around neutrophils, producing a ring or satellite effect (Figure 16-1). Using sodium citrate as the anticoagulant should correct this problem. Because of the dilution in the citrate evacuated tubes, it is necessary to multiply the obtained platelet count by 1.1 for accuracy (Chapter 16).

**Red Blood Cell Count**
Manual RBC counts are rarely performed because of the inaccuracy of the count and questionable necessity. Use of other, more accurate manual RBC procedures, such as the microhematocrit and hemoglobin concentration, is desirable when automation is not available.

Table 14-1 contains information on performing manual WBC, platelet, and RBC counts.
Disposable Blood Cell Count Dilution Systems

Capillary pipette and diluent reservoir systems are commercially available for WBC and platelet counts. One such system is LeukoChek™ (Biomedical Polymers, Inc., Gardner, MA). It consists of a capillary pipette (calibrated to accept 20 µL of blood) that fits into a plastic reservoir containing 1.98 mL of 1% buffered ammonium oxalate (Figure 14-4). Blood from a well-mixed EDTA-anticoagulated specimen or from a skin puncture is allowed to enter the pipette by capillary action to the fill volume. The blood is added to the reservoir making a 1:100 dilution. After mixing the reservoir and allowing 10 minutes for lysis of the red blood cells, the reverse end of the capillary pipette is placed in the reservoir cap making a dropper. The first 3 or 4 drops of the diluted sample is discarded, and the capillary pipette is used to charge the hemacytometer.

Both WBC and platelet counts can be done from the same diluted sample. WBCs are counted in all 9 large squares (9 mm²) using low power (100× total magnification). Platelets are counted in the 25 small squares in the center square (1 mm²) using high power (400× total magnification). The standard formula is used to calculate the cell counts.

Body Fluid Cell Counts

Body fluid cell counts are discussed in detail in Chapter 18.

HEMOGLOBIN DETERMINATION

The primary function of hemoglobin within the red blood cell is to carry oxygen to and carbon dioxide from the tissues. The cyanmethemoglobin (hemoglobin-cyanide) method for hemoglobin determination is the reference method approved by the Clinical and Laboratory Standards Institute.²

Principle

In the cyanmethemoglobin method, blood is diluted in an alkaline Drabkin solution of potassium ferricyanide, potassium cyanide, sodium bicarbonate, and a surfactant. The hemoglobin is oxidized to methemoglobin (Fe³⁺) by the potassium ferricyanide, K₃Fe(CN)₆. The potassium cyanide (KCN) then converts the methemoglobin to cyanmethemoglobin:

\[
\text{Hemoglobin (Fe}^{2+}) + K_3\text{Fe(CN)}_6 \rightarrow \text{methemoglobin (Fe}^{3+}) + KCN \rightarrow \text{cyanmethemoglobin}
\]

The absorbance of the cyanmethemoglobin at 540 nm is directly proportional to the hemoglobin concentration. Sulfhemoglobin is not converted to cyanmethemoglobin; it cannot be measured by this method. Sulfhemoglobin fractions of more than 0.05 g/dL are seldom encountered in clinical practice, however.³

PROCEDURE

1. Create a standard curve, using a commercially available cyanmethemoglobin standard.
   a. When a standard containing 80 mg/dL of hemoglobin is used, the following dilutions should be made:

<table>
<thead>
<tr>
<th>Hemoglobin Concentration (g/dL)</th>
<th>Blank</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanmethemoglobin standard (mL)</td>
<td>0</td>
<td>1.5</td>
<td>3</td>
<td>4.5</td>
<td>6</td>
</tr>
<tr>
<td>Cyanmethemoglobin reagent (mL)</td>
<td>6</td>
<td>4.5</td>
<td>3</td>
<td>1.5</td>
<td>0</td>
</tr>
</tbody>
</table>

   b. Transfer the dilutions to cuvettes. Set the wavelength on the spectrophotometer to 540 nm and use the blank to set to 100% transmittance.

   c. Using semilogarithmic paper, plot percent transmittance on the y-axis and the hemoglobin concentration on the x-axis. The hemoglobin concentrations of the control and patient samples can be read from this standard curve (Figure 14-5).

   d. A standard curve should be set up with each new lot of reagents. It also should be checked when alterations are made to the spectrophotometer (e.g., bulb change).

2. Controls should be run with each batch of samples. Commercial controls are available.
3. Using the patient’s whole blood anticoagulated with EDTA or heparin or blood from a capillary puncture, make a 1:251 dilution by adding 0.02 mL (20 μL) of blood to 5 mL of cyanmethemoglobin reagent. The pipette should be rinsed thoroughly with the reagent to ensure that no blood remains. Follow the same procedure for the control samples.

4. Cover and mix well by inversion or use a vortex mixer. Let stand for 10 minutes at room temperature to allow full conversion of hemoglobin to cyanmethemoglobin.

5. Transfer all of the solutions to cuvettes. Set the spectrophotometer to 100% transmittance at the wavelength of 540 nm, using cyanmethemoglobin reagent as a blank.

6. Using a matched cuvette, continue reading the % transmittance of the patient samples and record the values.

7. Determine the hemoglobin concentration of the control samples and the patient samples from the standard curve. General reference intervals can be found on the inside cover of this text.

Sources of Error and Comments

1. Cyanmethemoglobin reagent is sensitive to light. It should be stored in a brown bottle or in a dark place.

2. A high WBC count (greater than \(20 \times 10^9/L\)) or a high platelet count (greater than \(700 \times 10^9/L\)) can cause turbidity and a falsely high result. In this case, the reagent-sample solution can be centrifuged and the supernatant measured.

3. Lipemia also can cause turbidity and a falsely high result. It can be corrected by adding 0.01 mL of the patient’s plasma to 5 mL of the cyanmethemoglobin reagent and using this solution as the reagent blank.

4. Cells containing Hb S and Hb C may be resistant to hemolysis, causing turbidity; this can be corrected by making a 1:2 dilution with distilled water (1 part diluted sample plus 1 part water) and multiplying the results from the standard curve by 2.

5. Abnormal globulins, such as those found in patients with plasma cell myeloma or Waldenström macroglobulinemia, may precipitate in the reagent. If this occurs, add 0.1 g of potassium carbonate to the cyanmethemoglobin reagent. Commercially available cyanmethemoglobin reagent has been modified to contain KH₂PO₄ salt, so this problem is not likely to occur.

6. Carboxyhemoglobin takes 1 hour to convert to cyanmethemoglobin and theoretically could cause erroneous results in samples from heavy smokers. The degree of error is probably not clinically significant, however.

7. Because the hemoglobin reagent contains cyanide, it is highly toxic and must be used cautiously. Consult the safety data sheet (Chapter 2) supplied by the manufacturer. Acidification of cyanide in the reagent releases highly toxic hydrogen cyanide gas. A licensed waste disposal service should be contracted to discard the reagent; reagent-sample solutions should not be discarded into sinks.

8. Commercial absorbance standards kits are available to calibrate spectrophotometers.

9. Handheld systems are commercially available to measure the hemoglobin concentration. An example is the HemoCue® (HemoCue, Inc., Brea, CA) (Figure 14-19) in which hemoglobin is converted to azidemethemoglobin and is read photometrically at two wavelengths (570 nm and 880 nm).
This method avoids the necessity of sample dilution and interference from turbidity. It is discussed later in the section on point-of-care testing. Another method that has been used in some automated instruments involves the use of sodium lauryl sulfate (SLS) to convert hemoglobin to SLS-methemoglobin. This method does not generate toxic wastes.\(^6\)-\(^9\)

### MICROHEMATOCRIT

The hematocrit is the volume of packed red blood cells that occupies a given volume of whole blood. This is often referred to as the packed cell volume (PCV). It is reported either as a percentage (e.g., 36\%) or in liters per liter (0.36 L/L).

### PROCEDURE

1. Fill two plain capillary tubes approximately three quarters full with blood anticoagulated with EDTA or heparin. Mylar-wrapped tubes are recommended by the National Institute for Occupational Safety and Health to reduce the risk of capillary tube injuries.\(^10\) Alternatively, blood may be collected into heparinized capillary tubes by skin puncture. Wipe any excess blood from the outside of the tube.

2. Seal the end of the tube with the colored ring using nonabsorbent clay. Hold the filled tube horizontally and seal by placing the dry end into the tray with sealing compound at a 90-degree angle. Rotate the tube slightly and remove it from the tray. The plug should be at least 4 mm long.\(^10\)

3. Balance the tubes in a microhematocrit centrifuge with the clay ends facing the outside away from the center, touching the rubber gasket.

4. Tighten the head cover on the centrifuge and close the top. Centrifuge the tubes at 10,000 \(\text{g}\) to 15,000 \(\text{g}\) for the time that has been determined to obtain maximum packing of red blood cells, as detailed in Box 14-2. Do not use the brake to stop the centrifuge.

5. Determine the hematocrit by using a microhematocrit reading device (Figure 14-6). Read the level of red blood cell packing; do not include the buffy coat (WBCs and platelets) when taking the reading (Figure 14-7).

6. The values of the duplicate hematocrits should agree within 1\% (0.01 L/L).\(^10\)

---

**Figure 14-6** Microhematocrit reader.

**Figure 14-7** Capillary tube with anticoagulated whole blood after it has been centrifuged. Notice the layers containing plasma, the buffy coat (white blood cells and platelets), and the red blood cells.
General reference intervals according to sex and age can be found on the inside front cover of this text.

**Sources of Error and Comments**

1. Improper sealing of the capillary tube causes a decreased hematocrit reading as a result of leakage of blood during centrifugation. A higher number of red blood cells are lost compared with plasma due to the packing of the cells in the lower part of the tube during centrifugation.

2. An increased concentration of anticoagulant (short draw in an evacuated tube) decreases the hematocrit reading as a result of red blood cell shrinkage.

3. A decreased or increased result may occur if the specimen was not mixed properly.

4. The time and speed of the centrifugation and the time when the results are read are important. Insufficient centrifugation or a delay in reading results after centrifugation causes hematocrit readings to increase. Time for complete packing should be determined for each centrifuge and rechecked at regular intervals. When the microhematocrit centrifuge is calibrated, one of the samples used must have a hematocrit of 50% or higher.

5. The buffy coat of the sample should not be included in the hematocrit reading because this falsely elevates the result.

6. A decrease or increase in the readings may be seen if the microhematocrit reader is not used properly.

7. Many disorders, such as sickle cell anemia, macrocytic anemias, hypochromic anemias, spherocytosis, and thalassemia, may cause plasma to be trapped in the red blood cell layer even if the procedure is performed properly. The trapping of the plasma causes the microhematocrit to be 1% to 3% (0.01 to 0.03 L/L) higher than the value obtained using automated instruments that calculate or directly measure the hematocrit and are unaffected by the trapped plasma.

8. A temporarily low hematocrit reading may result immediately after a blood loss because plasma is replaced faster than are the red blood cells.

9. The fluid loss associated with dehydration causes a decrease in plasma volume and falsely increases the hematocrit reading.

10. Proper specimen collection is an important consideration. The introduction of interstitial fluid from a skin puncture or the improper flushing of an intravenous catheter causes decreased hematocrit readings.

The READACRIT centrifuge (Becton, Dickinson and Company, Franklin Lakes, NJ) uses precalibrated capillary tubes and has built-in hematocrit scales, which eliminates the need for separate reading devices (Figure 14-8). The use of SUREPREP Capillary Tubes (Becton, Dickinson) eliminates the use of sealants. They have a factory-inserted plug that seals automatically when the blood touches the plug.

**RULE OF THREE**

When samples are analyzed by automated or manual methods, a quick visual check of the results of the hemoglobin and hematocrit can be done by applying the “rule of three.” This rule applies only to samples that have normocytic normochromic red blood cells. The value of the hematocrit should be three times the value of the hemoglobin plus or minus 3: 

\[
\text{HGB} \times 3 = \text{HCT} \pm 3 (0.03 \text{ L/L})
\]

It should become habit for the analyst to multiply the hemoglobin by 3 mentally for every sample; a value discrepant with this rule may indicate abnormal red blood cells, or it may be the first indication of error.

For example, the following results are obtained from patients:

**Case 1**

- HGB = 12 g/dL
- HCT = 36% (0.36 L/L)

According to the rule of three,

\[
(12) \times 3 = (36) 
\]

An acceptable range for hematocrit would be 33% to 39%. These values conform to the rule of three.

**Case 2**

- HGB = 9 g/dL
- HCT = 32%

According to the rule of three,

\[
(9.0) \times 3 = (27) \text{ versus actual value of 32}
\]

An acceptable range for hematocrit would be 24% to 30%, so these values do not conform to the rule of three.
Mean Cell Volume

The MCV is the average volume of the red blood cell, expressed in femtoliters (fL), or \(10^{-15}\) L:

\[
MCV = \frac{HCT \times 10}{RBC \text{ count (} \times 10^{12}/L\text{)}},
\]

For example, if the HCT = 45% and the RBC count = 5 \(\times 10^{12}/L\), the MCV = 90 fL.

The reference interval for MCV is 80 to 100 fL. RBCs with an MCV of less than 80 fL are microcytic; those with an MCV of more than 100 fL are macrocytic.

Mean Cell Hemoglobin

The MCH is the average weight of hemoglobin in a red blood cell, expressed in picograms (pg), or \(10^{-12}\) g:

\[
MCH = \frac{HGB \text{ (g/dL) } \times 10}{RBC \text{ count (} \times 10^{12}/L\text{)}},
\]

For example, if the hemoglobin = 16 g/dL and the RBC count = 5 \(\times 10^{12}/L\), the MCH = 32 pg.

The reference interval for adults is 26 to 32 pg. The MCH generally is not considered in the classification of anemias.

Mean Cell Hemoglobin Concentration

The MCHC is the average concentration of hemoglobin in each individual red blood cell. The units used are grams per deciliter (formerly given as a percentage):

\[
MCHC = \frac{HGB \text{ (g/dL) } \times 100}{HCT \text{ (%)}}
\]

For example, if the HGB = 16 g/dL and the HCT = 48%, the MCHC = 33.3 g/dL.

Values of normochromic red blood cells range from 32 to 36 g/dL; values of hypochromic cells are less than 32 g/dL, and values of “hyperchromic” cells are greater than 36 g/dL. Hypochromic red blood cells occur in thalassemias, iron deficiency, and other conditions listed in Table 14-2. The term “hyperchromic” is a misnomer: a cell does not really contain more than 36 g/dL of hemoglobin, but its shape may have become spherocytic, which makes the cell appear full. An MCHC between 36 and 38 g/dL should be checked for spherocytes. An MCHC

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**RED BLOOD CELL INDICES**

The mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) are the RBC indices. These are calculated to determine the average volume and hemoglobin content and concentration of the red blood cells in the sample. In addition to serving as a quality control check, the indices may be used for initial classification of anemias. Table 14-2 provides a summary of the RBC indices, morphology, and correlation with various anemias. The morphologic classification of anemia on the basis of MCV is discussed in detail in Chapter 19.

**TABLE 14-2 Red Blood Cell Indices, Red Blood Cell Morphology, and Disease States**

<table>
<thead>
<tr>
<th>MCV (fL)</th>
<th>MCHC (g/dL)</th>
<th>Red Blood Cell Morphology</th>
<th>Found in</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;80</td>
<td>&lt;32</td>
<td>Microcytic; hypochromic</td>
<td>Iron deficiency anemia, anemia of inflammation, thalassemia, Hb E disease and trait, sideroblastic anemia</td>
</tr>
<tr>
<td>80–100</td>
<td>32–36</td>
<td>Normocytic; normochromic</td>
<td>Hemolytic anemia, myelophtisis anemia, bone marrow failure, chronic renal disease</td>
</tr>
<tr>
<td>&gt;100</td>
<td>32–36</td>
<td>Macrocytic; normochromic</td>
<td>Megaloblastic anemia, chronic liver disease, bone marrow failure, myelodysplastic syndrome</td>
</tr>
</tbody>
</table>

*Hb, Hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume.*
greater than 38 g/dL should be investigated for an error in hemoglobin value (see Sources of Error and Comments in the section on hemoglobin determination). Another cause for a markedly increased MCHC could be the presence of a cold agglutinin. Incubating the specimen at 37° C for 15 minutes before analysis usually produces accurate results. Cold agglutinin disease is discussed in more detail in Chapter 26.

5. To improve accuracy, have another laboratorian count the other film; counts should agree within 20%.
6. Calculate the % reticulocyte count:

\[
\text{Reticulocytes} \% = \frac{\text{number of reticulocytes} \times 100}{1000 \ (\text{RBCs counted})}
\]

For example, if 15 reticulocytes are counted,

\[
\text{Reticulocytes} \% = \frac{15 \times 100}{1000} = 1.5\%
\]

Or the number of reticulocytes counted can be multiplied by 0.1 (100/1000) to obtain the result.

**Miller Disc**

Because large numbers of red blood cells should be counted to obtain a more precise reticulocyte count, the Miller disc was designed to reduce this labor-intensive process. The disc is composed of two squares, with the area of the smaller square measuring \(\frac{1}{9}\) the area of the larger square. The disc is inserted into the eyepiece of the microscope, and the grid in Figure 14-10 is seen. RBCs are counted in the smaller square, and reticulocytes are counted in the larger square. Selection of the counting area is the same as described earlier. A minimum of 112 cells should be counted in the small square, because this is equivalent to 1008 red cells in the large square and satisfies the College of American Pathologists (CAP) hematology standard for a manual reticulocyte count based on at least 1000 red cells. The calculation formula for percent reticulocytes is

\[
\text{Reticulocytes} \% = \frac{\text{no. reticulocytes in square A}}{\text{(large square) \times 100}} \times \frac{1}{9}
\]

\[
\text{no. RBCs in square B, \ (small square) \times 9}
\]

**Figure 14-9** Reticulocytes with new methylene blue vital stain (peripheral blood \(\times 1000\)). Reticulocytes are nonnucleated red blood cells with two or more blue-stained filaments or particles.

**Figure 14-10** Miller ocular disc counting grid as viewed through a microscope. The area of square B is \(\frac{1}{9}\) the area of square A. Alternatively, square B may be in the center of square A.
For example, if 15 reticulocytes are counted in the large square and 112 red blood cells are counted in the small square,

\[ \text{Reticulocytes} \% = \frac{15 \times 100}{112 \times 9} = 1.5\% \]

**Equation Reference Interval**

General reference intervals can be found on the inside front cover of this text.

**Sources of Error and Comments**

1. If a patient is very anemic or polycythemic, the proportion of dye to blood should be adjusted accordingly.
2. An error may occur if the blood and stain are not mixed before the films are made. The specific gravity of the reticulocytes is lower than that of mature red blood cells, and reticulocytes settle at the top of the mixture during incubation.
3. Moisture in the air, poor drying of the slide, or both may cause areas of the slide to appear refractile, and these areas could be confused with reticulocytes. The RNA remnants in a reticulocyte are not refractile.
4. Other red blood cell inclusions that stain supravitally include Heinz, Howell-Jolly, and Pappenheimer bodies (Table 19-3). Heinz bodies are precipitated hemoglobin, usually appear round or oval, and tend to adhere to the cell membrane (Figure 14-11). Howell-Jolly bodies are round nuclear fragments and are usually singular. Pappenheimer bodies are iron in the mitochondria whose presence can be confirmed with an iron stain, such as Prussian blue. This stain is discussed in Chapter 17.
5. If a Miller disc is used, it is important to heed the “edge rule” as described in the WBC count procedure and illustrated in Figure 14-2. A significant bias is observed if the rule is ignored.

**Absolute Reticulocyte Count**

**Principle**

The absolute reticulocyte count (ARC) is the actual number of reticulocytes in 1 liter (L) or 1 microliter (μL) of blood.

**Calculations**

\[ \text{ARC} = \frac{\text{reticulocytes}\% \times \text{RBC count} \times (\times 10^{12}/L)}{100} \]

For example, if a patient’s reticulocyte count is 2% and the RBC count is \(2.20 \times 10^{12}/L\), the ARC is calculated as follows (note that the calculated result has to be converted from \(10^{12}/L\) to \(10^{9}/L\)):

\[ \text{ARC} = \frac{2 \times (2.20 \times 10^{12}/L)}{100} = 44 \times 10^{9}/L \]

The absolute reticulocyte count can also be reported as the number of cells per μL. Using the example above, the RBC count in μL (\(2.20 \times 10^{9}/μL\)) is used in the formula, and the ARC result is \(44 \times 10^{3}/μL\).

**Corrected Reticulocyte Count**

**Principle**

In specimens with a low hematocrit, the percentage of reticulocytes may be falsely elevated because the whole blood contains fewer red blood cells. A correction factor is used, with the average normal hematocrit considered to be 45%.

**Calculation**

\[ \text{Corrected reticulocyte count}\% = \frac{\text{reticulocyte}\% \times \text{patient HCT}\%}{45} \]

**Reference Interval**

Values between \(20 \times 10^9/L\) and \(115 \times 10^9/L\) are within the reference interval for most populations.

**Reticulocyte Production Index**

**Principle**

Reticulocytes that are released from the marrow prematurely are called *shift reticulocytes*. These reticulocytes are “shifted” from the bone marrow to the peripheral blood earlier than usual to compensate for the mild anemia. Instead of losing their reticulum in 1 day, as do most normal circulating reticulocytes, these cells take 2 to 3 days to lose their reticula. When erythropoiesis is evaluated, a correction should be made for the presence of shift reticulocytes if polychromasia is reported in the red blood cell morphology. Most normal (nonshift) reticulocytes become mature red blood cells within 1 day after entering the bloodstream and thus represent 1 day’s production of red blood cells in the bone marrow.
Cells shifted to the peripheral blood prematurely stay longer as reticulocytes and contribute to the reticulocyte count for more than 1 day. For this reason, the reticulocyte count is falsely increased when polychromasia is present, because the count no longer represents the cells maturing in just 1 day. On many automated instruments, this mathematical adjustment of the reticulocyte count has been replaced by the measurement of immature reticulocyte fraction (Chapter 15). The major instrument manufacturers offer are analyzers that can be used to verify the laboratory's accuracy and precision when manual counts are performed. The control samples are treated in the same manner as the patient samples. The control is available at three levels. The control samples are treated in the same manner as the patient samples. The control is available at three levels. The control samples are treated in the same manner as the patient samples. The control is available at three levels.

The patient's hematocrit is used to determine the appropriate correction factor (reticulocyte maturation time in days):

<table>
<thead>
<tr>
<th>Patient’s Hematocrit Value (%)</th>
<th>Correction Factor (Maturation Time, Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40–45</td>
<td>1</td>
</tr>
<tr>
<td>35–39</td>
<td>1.5</td>
</tr>
<tr>
<td>25–34</td>
<td>2</td>
</tr>
<tr>
<td>15–24</td>
<td>2.5</td>
</tr>
<tr>
<td>&lt;15</td>
<td>3</td>
</tr>
</tbody>
</table>

**Calculation**

The reticulocyte production index (RPI) is calculated as follows:

\[
RPI = \frac{\text{reticulocyte} \times [\text{HCT}]/45}{\text{maturation time}}
\]

Or

\[
RPI = \frac{\text{corrected reticulocyte count}}{\text{maturation time}}
\]

For example, for a patient with a reticulocyte count of 7.8% and a HCT of 30%, and with polychromasia noted, the previous table indicates a maturation time of 2 days. Thus

\[
RPI = \frac{7.8 \times [30/45]}{2} = 2.6
\]

**Reference Interval**

An adequate bone marrow response usually is indicated by an RPI that is greater than 3. An inadequate erythropoietic response is seen when the RPI is less than 2.

**Reticulocyte Control**

Several commercial controls are now available for monitoring manual and automated reticulocyte counts [e.g., Retic-Chex II, Streck Laboratories, Omaha, NE; Liquichek Reticulocyte Control (A), Bio-Rad Laboratories, Hercules, CA]. Most of the controls are available at three levels. The control samples are treated in the same manner as the patient samples. The control can be used to verify the laboratory’s accuracy and precision when manual counts are performed.

**Automated Reticulocyte Counts**

The major instrument manufacturers offer are analyzers that perform automated reticulocyte counts. All of the analyzers evaluate reticulocytes using optical scatter or fluorescence after the red blood cells are treated with fluorescent dyes or nucleic acid stains to stain residual RNA in the reticulocytes. The percentage and the absolute count are provided. These results are statistically more valid because of the large number of cells counted. Other reticulocyte parameters that are offered on some automated instruments include a maturation index/im mature reticulocyte fraction or IRF (reflecting the proportion of the more immature reticulocytes in the sample), the reticuocyte hemoglobin concentration, and reticulocyte indices (such as the mean reticulocyte volume and distribution width). The IRF may be especially useful in detecting early erythropoietic activity after chemotherapy or hematopoietic stem cell transplantation. The reticulocyte hemoglobin is useful to detect early iron deficiency (Chapter 20). Automated reticulocyte counting is discussed in Chapter 15.

**ERYTHROCYTE SEDIMENTATION RATE**

The erythrocyte sedimentation rate (ESR) is ordered with other tests to detect and monitor the course of inflammatory conditions such as, rheumatoid arthritis, infections, or certain malignancies. It is also useful in the diagnosis of temporal arteritis and polymyalgia rheumatica. The ESR, however, is not a specific test for inflammatory diseases and is elevated in many other conditions such as plasma cell myeloma, pregnancy, anemia, and older age. It is also prone to technical errors that can falsely elevate or decrease the sedimentation rate. Because of its low specificity and sensitivity, the ESR is not recommended as a screening test to detect inflammatory conditions in asymptomatic individuals. Other tests for inflammation, such as the C-reactive protein level, may be a more predictable and reliable alternative to monitor inflammation.

**Principle**

When anticoagulated blood is allowed to stand at room temperature undisturbed for a period of time, the red blood cells settle toward the bottom of the tube. The ESR is the distance in millimeters that the red blood cells fall in 1 hour. The ESR is affected by red blood cell, plasma, and mechanical and technical factors. Red blood cells have a net negative surface charge and tend to repel one another. The repulsive forces are partially or totally counteracted if there are increased quantities of positively charged plasma proteins. Under these conditions the red blood cells settle more rapidly as a result of the formation of rouleaux (stacking of red blood cells). Examples of macromolecules that can produce this reaction are fibrinogen, β-globulins, and pathologic immunoglobulins.

Normal red blood cells have a relatively small mass and settle slowly. Certain diseases can cause rouleaux formation, in which the plasma fibrinogen and globulins are altered. This alteration changes the red blood cell surface, which leads to stacking of the red blood cells, increased red blood cell mass, and a more rapid ESR. The ESR is directly proportional to the red blood cell mass and inversely proportional to plasma viscosity. Several methods, both manual and automated, are available for measuring the ESR. Only the most commonly used methods are discussed here.
Modified Westergren Erythrocyte Sedimentation Rate

The most commonly used method today is the modified Westergren method. One advantage of this method is that the taller column height allows the detection of highly elevated ESRs. It is the method recommended by the International Council for Standardization in Hematology and the Clinical and Laboratory Standards Institute. 15,19

PROCEDURE
1. Use well-mixed blood collected in EDTA and dilute at four parts blood to one part 3.8% sodium citrate or 0.85% sodium chloride (e.g., 2 mL blood and 0.5 mL diluent). Alternatively, blood can be collected directly into special sedimentation test tubes containing sodium citrate. Standard coagulation test tubes are not acceptable, because the dilution is nine parts blood to one part sodium citrate. 15
2. Place the diluted sample in a 200-mm column with an internal diameter of 2.55 mm or more.
3. Place the column into the rack and allow to stand undisturbed for 60 minutes at room temperature (18 to 25 °C). Ensure that the rack is level.
4. Record the number of millimeters the red blood cells have fallen in 1 hour. The buffy coat should not be included in the reading. Read the tube from the bottom of the plasma layer to the top of the sedimented red blood cells (Figure 14-12). Report the result as the ESR, 1 hour = x mm. 15

Wintrobe Erythrocyte Sedimentation Rate

When the Wintrobe method was first introduced, the specimen used was oxalate-anticoagulated whole blood. This was placed in a 100-mm column. Today, EDTA-treated or citrated whole blood is used with the shorter column. The shorter column height allows a somewhat increased sensitivity in detecting mildly elevated ESRs.

PROCEDURE
1. Use fresh blood collected in EDTA anticoagulant. A minimum of 2 mL of whole blood is needed.
2. After mixing the blood thoroughly, fill a Pasteur pipette using a rubber pipette bulb.
3. Place the filled pipette into the Wintrobe tube until the tip reaches the bottom of the tube.
4. Carefully squeeze the bulb and expel the blood into the Wintrobe tube while pulling the Pasteur pipette up from the bottom of the tube. There must be steady, even pressure on the bulb to expel blood into the tube as well as continuous movement of the pipette up the tube to prevent the introduction of air bubbles into the column of blood.
5. Fill the Wintrobe tube to the 0 mark.
6. Place the tube into a Wintrobe rack (tube holder) and allow to stand undisturbed for 1 hour at room temperature. The rack must be perfectly level and placed in a draft-free room.
7. Record the number of millimeters the red blood cells have fallen. Read the tube from the bottom of the plasma meniscus to the top of the sedimented red cells. The result is reported in millimeters per hour.

Reference Interval
Reference intervals according to sex and age can be found on the inside front cover of this text. Table 14-3 lists some of the factors that influence the ESR.

Sources of Error and Comments
1. If the concentration of anticoagulant is increased, the ESR will be falsely low as a result of sphering of the RBCs, which inhibits rouleaux formation.
2. The anticoagulants sodium or potassium oxalate and heparin cause the red blood cells to shrink and falsely elevate the ESR.
3. A significant change in the temperature of the room alters the ESR.
4. Even a slight tilt of the pipette causes the ESR to increase.
5. Blood specimens must be analyzed within 4 hours of collection if kept at room temperature (18 to 25 °C). 15 If the specimen is allowed to sit at room temperature for more than 4 hours, the red blood cells start to become
### TABLE 14-3 Factors Affecting the Erythrocyte Sedimentation Rate (ESR)

<table>
<thead>
<tr>
<th>Category</th>
<th>Increased ESR</th>
<th>Decreased ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood proteins and lipids</td>
<td>Hypercholesterolemia</td>
<td>Hyperalbuminemia</td>
</tr>
<tr>
<td></td>
<td>Hyperfibrinogenemia</td>
<td>Hyperglycemia</td>
</tr>
<tr>
<td></td>
<td>Hypergamma globulinemia</td>
<td>Hypofibrinogenemia</td>
</tr>
<tr>
<td></td>
<td>Hypoalbuminemia</td>
<td>Hypogamma globulinemia</td>
</tr>
<tr>
<td></td>
<td>Hyperalbuminemia</td>
<td>Increased bile salts</td>
</tr>
<tr>
<td></td>
<td>Hyperglycemia</td>
<td>Increased phospholipids</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>Anemia</td>
<td>Acanthocytosis</td>
</tr>
<tr>
<td></td>
<td>Macrocytosis</td>
<td>Anisocytosis (marked)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hemoglobin C</td>
</tr>
<tr>
<td>White blood cells</td>
<td>Leukemia</td>
<td>Microcytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polycythemia</td>
</tr>
<tr>
<td>Drugs</td>
<td>Dextran</td>
<td>Sickle cells</td>
</tr>
<tr>
<td></td>
<td>Heparin</td>
<td>Spherocytosis</td>
</tr>
<tr>
<td></td>
<td>Penicillamine</td>
<td>Thalassemia</td>
</tr>
<tr>
<td></td>
<td>Procainamide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin A</td>
<td></td>
</tr>
<tr>
<td>Clinical conditions</td>
<td>Acute heavy metal poisoning</td>
<td>Cachexia</td>
</tr>
<tr>
<td></td>
<td>Acute bacterial infections</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td></td>
<td>Collagen vascular diseases</td>
<td>Newborn status</td>
</tr>
<tr>
<td></td>
<td>Diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>End-stage renal failure</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gout</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Malignancy</td>
<td></td>
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<tr>
<td></td>
<td>Menstruation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multiple myeloma</td>
<td></td>
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<tr>
<td></td>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rheumatic fever</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Syphilis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temporal arteritis</td>
<td></td>
</tr>
<tr>
<td>Specimen handling</td>
<td>Refrigerated sample not returned to room temperature</td>
<td>Clotted blood sample</td>
</tr>
<tr>
<td>Technique</td>
<td>High room temperature</td>
<td>Bubbles in ESR column</td>
</tr>
<tr>
<td></td>
<td>Tilted ESR tube</td>
<td>Low room temperature</td>
</tr>
<tr>
<td></td>
<td>Vibration</td>
<td>Narrow ESR column diameter</td>
</tr>
</tbody>
</table>


spherical, which may inhibit the formation of rouleaux. Blood specimens may be stored at 4° C up to 24 hours prior to testing, but must be rewarmed by holding the specimen at ambient room temperature for at least 15 minutes prior to testing.15

6. Bubbles in the column of blood invalidate the test results.
7. The blood must be filled properly to the zero mark at the beginning of the test.
8. A clotted specimen cannot be used.
9. The tubes must not be subjected to vibrations on the lab bench which can falsely increase the ESR.
10. Hematologic disorders that prevent the formation of rouleaux (e.g., the presence of sickle cells and spherocytes) decrease the ESR.
11. The ESR of patients with severe anemia is of little diagnostic value, because it will be falsely elevated.
Disposable Kits
Disposable commercial kits are available for ESR testing (Figure 14-13). Several kits include safety caps for the columns that allow the blood to fill precisely to the zero mark. This safety cap makes the column a closed system and eliminates the error involved in manually setting the blood to the zero mark.

Automated Erythrocyte Sedimentation Rate
There are several automated ESR systems available using the traditional Westergren and Wintrobe methods, as well as alternate methods such as centrifugation. The Ves-Matic system (Diesse, Inc., Hialeah, FL) is a bench-top analyzer designed to determine ESR by use of an optoelectronic sensor, which measures the change in opacity of a column of blood as sedimentation of blood progresses. Blood is collected in special Ves-Tec or Vacu-Tec tubes, which contain sodium citrate and are compatible with the Vacutainer system. These tubes are used directly in the instrument (Figure 14-14). Acceleration of sedimentation is achieved by positioning the tubes at an 18-degree angle in relation to the vertical axis. Results comparable with Westergren 1-hour values are obtained in 20 minutes.20

Another automated ESR analyzer is the Sedimat 15 (Polymedco, Cortlandt Manor, NY), which uses the principle of infrared measurement. It is capable of testing one to eight samples randomly or simultaneously and provides results in 15 minutes (Figure 14-15).

The ESR STAT PLUS system (HemaTechnologies, Lebanon, NJ) is based on centrifugation. The advantages of this method are a smaller required sample volume and shorter testing time, which makes it more suitable for a pediatric patient population. The disadvantage of this method is the number of exacting preanalytical steps that must be strictly followed to prevent erroneous results. Compliance with these steps may be difficult to achieve consistently in a busy hematology laboratory.21

ADDITIONAL METHODS
Additional manual and semi-automated methods are included in other chapters that are relevant to their clinical application. Examples include: Chapter 24 for the osmotic fragility test and qualitative and quantitative assays for glucose-6-phosphate dehydrogenase and pyruvate kinase activity; Chapter 27 for the solubility test for Hb S, hemoglobin electrophoresis (alkaline and acid pH), and unstable hemoglobin test; and Chapter 28 for the vital stain for hemoglobin H and the Kleihauer-Betke acid elution test for Hb F distribution in the RBCs.

POINT-OF-CARE TESTING
Point-of-care testing offers the ability to produce rapid and accurate results that help facilitate faster treatment, which can decrease patient length of stay. This testing is rarely performed by trained laboratory personnel; most often, it is carried out by nurses. Manufacturers have created analyzers with nonlaboratory operators in mind, but results obtained using these systems are still affected by preanalytic and analytical variables. The laboratory’s partnership with nursing is the key to success in any hospital’s point-of-care program.

Point-of-care testing is defined as diagnostic testing at or near the site of patient care. The Clinical Laboratory Improvement Amendments of 1988 (CLIA) introduced the concept of “testing site neutrality,” which means that regardless of where the diagnostic testing is performed or who performs the test, all testing sites must follow the same regulatory requirements based on the “complexity” of the test. Under CLIA, point-of-care testing (including physician-performed microscopy) is classified as “waived” or “moderately complex.” Tests are classified as waived if they are determined to be “simple tests with an insignificant risk of an erroneous result.” Point-of-care testing is commonly performed in hospital inpatient units, outpatient clinics, surgery centers, emergency departments, long-term care facilities, and dialysis units. For waived point-of-care testing, facilities are required to obtain a certificate of waiver, pay the appropriate fees, and follow the manufacturers’ testing instructions.22 For any point-of-care program to be successful, certain key elements must be present. Clear administrative responsibility, well-written procedures, a training program, quality control, proficiency testing, and equipment maintenance are essential for success. The first step is appointing a laboratory point-of-care testing coordinator. This person
not only is the “go-to” person but is also an important liaison between the laboratory and nursing staff. The second step to ensuring a successful program is to create a multidisciplinary team with authority to impact all aspects of the POC program. This committee would have the authority to oversee the integrity and quality of the existing POC program and institute changes or new testing as needed. It is also important to have administrative support to help remove barriers.

A point-of-care testing program must incorporate all of the following. A written policy should be developed that defines the program. This policy should outline who is responsible for each part of the program. The policy should also indicate where the testing is to be performed and who is going to perform the testing. Testing procedures should be written that clearly state how to perform the tests and that address how to handle critical values and/or any discrepant results. The program must be monitored. An ongoing evaluation of the point-of-care testing is vital for success.

When the instrument to be used in the point-of-care testing program is being selected, it is helpful to invite the vendors to demonstrate their equipment. An equipment display that is available for hands-on use by the operators can be very helpful in selection of the appropriate instrumentation. Patient correlation studies are very useful in choosing equipment that best covers the patient population for that particular institution. Point-of-care operators need handheld analyzers that are lightweight, accurate, fast, and that require little specimen material. The point-of-care testing system should also address the following laboratory concerns:

- What is the range of measurement?
- How well does the test system correlate with laboratory instrumentation?
- Can it be interfaced to the laboratory information system?
- Does it give reliable results?
- Does the company supply excellent technical support?
- Is it affordable?

Paramount to point-of-care testing is patient safety. It is important to maintain good practices, and with waived testing, this often comes down to the basics. Such basics include proper and appropriate specimen collection, proper identification of the patient and specimen, proper storage of reagents, and good documentation of patient test results (use of point-of-care interfaces is beneficial), as well as proper performance of any necessary instrument maintenance. Laboratory oversight
is sometimes absent, and basic safety precautions necessary for waived tests can be easily overlooked, often due to a lack of understanding, lack of training, and high personnel turnover rates. Patient safety, risk management, and error reduction are primary goals of all health care facilities. All testing personnel should be properly trained in best practices to avoid exposure. The individual responsible for oversight—whether laboratory or nonlaboratory—must avoid taking safety for granted. All applicable standards (including those of the Occupation Safety and Health Administration, Centers for Disease Control and Prevention, The Joint Commission, CAP, CLIA, and so forth) should be implemented and easily accessible. Because the number of waived tests has grown significantly since waived tests were first defined by CLIA, it is paramount that standard safety precautions and the basic steps outlined earlier be implemented to ensure that patient safety is not sacrificed in the unique situation of CLIA-waived testing.

### Point-of-Care Tests

Various point-of-care instruments are available to measure parameters such as hemoglobin level and hematocrit, and some perform a complete blood count.

#### Hematocrit

The most common methods for determining the hematocrit include the microhematocrit centrifuge, conductometric methods, and calculation by automated cell counters (Chapter 15).

Centrifuge-based microhematocrit systems have been available for years, and the results obtained correlate well with the results produced by standard cell counters. Nonlaboratorians and inexperienced operators, however, may be unaware of the error that can be introduced by insufficient centrifugation time and inaccurate reading of the microhematocrit tube (see comments in the Microhematocrit section). Examples of centrifuge-based devices are the Hematastat II (Separation Technology, Inc., Altamonte Springs, FL) and STAT Crit (Wampole Laboratories, Cranbury, NJ).

The i-STAT 1 (Abbott Laboratories, Abbott Park, IL)\(^4\) (Figure 14-16) and the Epoc (Epocal, Inc., Ottawa, ON) (Figure 14-17)\(^5\) use the conductivity method to determine the hematocrit. Plasma conducts electrical current, whereas WBCs act as insulators. In the i-STAT system, before the measured sample conductance is converted into the hematocrit value, corrections are applied for the temperature of the sample, the size of the fluid segment being measured, and the relative conductivity of the plasma component. The first two corrections are determined from the measured value of the calibrant conductance and the last correction from the measured concentrations of sodium and potassium in the sample.\(^4\)

Sources of Error and Comments. Conductivity of a whole blood sample is dependent on the amount of electrolytes in the plasma portion. Conductivity does not distinguish red blood cells from other nonconductive elements such as proteins, lipids, and WBCs that may be present in the sample.

A low total protein level will falsely decrease the hematocrit. The presence of lipids can interfere with the hematocrit measurement. An increased WBC count will falsely increase the hematocrit. The presence of cold agglutinins can falsely decrease the hematocrit.\(^4\)

#### Other Instruments.

Other instruments that measure the hematocrit include the following:

- ABL 77 (Radiometer, Westlake, OH)
- IRMA (ITC, a subsidiary of Thoratec Corporation, Edison, NJ)
- Gem Premier (Instrumentation Laboratory Company, Lexington, MA) (Figure 14-18)

#### Hemoglobin Concentration

In point-of-care testing, hemoglobin concentration is measured by modified hemoglobinometers or by oximeters integrated
with a blood gas analyzer. The HemoCue hemoglobinometer (HemoCue, Inc., Brea, CA) uses a small cuvette that contains a lysing agent and reagents to form a hemoglobin azide, which is measured by a photometer at two wavelengths (570 nm and 880 nm) (Figure 14-19). This eliminates interference from turbidity in the sample. Results obtained with the instrument compare well with those produced by reference methods, but a major source of error is mixture of blood with tissue fluid during skin puncture collection. The AVOX 1000E (ITC) measures total hemoglobin by a spectrophotometric method. The STAT-Site MHbg Meter (Stanbio Laboratory, Boerne, TX) uses the azidemethemoglobin principle and reflectance photometry to measure reflected light in the test area. The test card is composed of molded plastic with a fluid well that contains numerous pads impregnated with specific chemical reagents. A drop of whole blood is applied to the center of the well and reacts with the chemicals in the pad to produce a specific color that is measured from the bottom of the card.

**Cell and Platelet Counts**

Traditional cell-counting methods can be employed at the point of care for the analysis of WBCs, RBCs, and platelets. The Ichor Hematology Analyzer (Helena Laboratories, Beaumont, TX) performs a complete blood count along with platelet aggregation. Another option for cell quantitation and differentiation employs a buffy coat analysis method. Quantitative buffy coat analysis (QBC STAR, manufactured by QBC Diagnostics, Inc., Philipsburg, PA) involves centrifugation in specialized capillary tubes designed to expand the buffy coat layer. The components (platelets, mononuclear cells, and granulocytes) can be measured with the assistance of fluorescent dyes and a measuring device.

**SUMMARY**

- Although most laboratories are highly automated, the manual tests discussed in this chapter, such as the cyanmethemoglobin method of hemoglobin determination and centrifuge-based measurement of the microhematocrit, are used as a part of many laboratories’ quality control and backup methods of analysis.
- The hemacytometer allows counts of any type of cell or particle (e.g., WBCs or platelets) to be performed.
- The reference method for hemoglobin determination is based on the absorbance of cyanmethemoglobin at 540 nm. When a spectrophotometer is used, a standard curve is employed to obtain the results.
- The microhematocrit is a measure of packed red blood cell volume.
- The rule of three specifies that the value of the hematocrit should be three times the value of the hemoglobin plus or minus 3 (%) or 0.03 (L/L). A value discrepant with this rule may indicate abnormal red blood cells or it may be the first indication of error.
- RBC indices—the mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC)—are calculated to determine the average volume, hemoglobin content, and hemoglobin concentration of red blood cells. The indices give an indication of possible causes of an anemia.
- The reticulocyte count, which is used to assess the erythropoietic activity of the bone marrow, is accomplished through the use of supravital stains (e.g., new methylene blue) or by flow cytometric methods.
- The erythrocyte sedimentation rate (ESR), a measure of the settling of red blood cells in a 1-hour period, depends on the red blood cells’ ability to form rouleaux. It is used to detect and monitor conditions...
with inflammation such as rheumatoid arthritis, infections, and some malignancies. It is subject to many physiologic and technical errors.

- Point-of-care testing is often performed by nonlaboratory personnel. It is defined as diagnostic laboratory testing at or near the site of patient care.
- CLIA introduced the concept of “testing site neutrality,” which means that it does not matter where diagnostic testing is performed or who performs the test; all testing sites must follow the same regulatory requirements based on the “complexity” of the test.
- Tests are classified as waived if they are determined to be “simple tests with an insignificant risk of an erroneous result.” Most, but not all, point-of-care testing is waived.

- For a point-of-care testing program to be successful, key elements such as clear administrative responsibility, well-written procedures, quality control, proficiency testing, and equipment maintenance must be present.
- Paramount to point-of-care testing is patient safety.

Now that you have completed this chapter, read again the case studies at the beginning and respond to the questions presented.

### REVIEW QUESTIONS

1. A 1:20 dilution of blood is made with 3% glacial acetic acid as the diluent. The four large corner squares on both sides of the hemacytometer are counted, for a total of 100 cells. What is the total WBC count \((\times 10^9/L)\)?
   a. 0.25
   b. 2.5
   c. 5
   d. 10

2. The total WBC count is \(20 \times 10^9/L\). Twenty-five NRBCs per 100 WBCs are observed on the peripheral blood film. What is the corrected WBC count \((\times 10^9/L)\)?
   a. 0.8
   b. 8
   c. 16
   d. 19

3. If potassium cyanide and potassium ferricyanide are used in the manual method for hemoglobin determination, the final product is:
   a. Methemoglobin
   b. Azide methemoglobin
   c. Cyanmethemoglobin
   d. Myoglobin

4. Which of the following would not interfere with the result when hemoglobin determination is performed by the cyanmethemoglobin method?
   a. Increased lipids
   b. Elevated WBC count
   c. Lyse-resistant RBCs
   d. Fetal hemoglobin

5. A patient has a hemoglobin level of 8.0 g/dL. According to the rule of three, what is the expected range for the hematocrit?
   a. 21% to 24%
   b. 23.7% to 24.3%
   c. 24% to 27%
   d. 21% to 27%

6. Calculate the MCV and MCHC for the following values:
   \[ \begin{array}{cc}
   \text{RBCs} & 5.00 \times 10^{12}/L \\
   \text{HGB} & 9 \text{ g/dL} \\
   \text{HCT} & 30\% \\
   \end{array} \]
   \[ \begin{array}{cc}
   \text{MCV (fL)} & 85 \\
   \text{MCHC (g/dL)} & 35 \\
   \end{array} \]

7. What does the reticulocyte count assess?
   a. Inflammation
   b. Response to infection
   c. Erythropoietic activity of the bone marrow
   d. Ability of red blood cells to form rouleaux

8. For a patient with the following test results, which measure of bone marrow red blood cell production provides the most accurate information?
   \[ \begin{array}{cc}
   \text{Observed reticulocyte count} & 5.3\% \\
   \text{HCT} & 35\% \\
   \text{Morphology—moderate polychromasia} & \\
   \end{array} \]
   a. Observed reticulocyte count
   b. Corrected reticulocyte count
   c. RPI
   d. ARC

9. Given the following values, calculate the RPI:
   \[ \begin{array}{cc}
   \text{Observed reticulocyte count} & 6\% \\
   \text{HCT} & 30\% \\
   \end{array} \]
   a. 2
   b. 3
   c. 4
   d. 5
10. Which of the following would be associated with an elevated ESR value?
   a. Microcytosis
   b. Polycythemia
   c. Decreased globulins
   d. Inflammation

REFERENCES


ADDITIONAL RESOURCES


Point of Care.net, http://www.pointofcare.net
http://us.instrumentationlaboratory.com
www.abbottpointofcare.com
www.hemocue.com
www.itcmed.com
www.radiometer.com
www.stanbio.com
www.Helena.com
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Automated Blood Cell Analysis

Sharral Longanbach and Martha K. Miers

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Explain the different principles of automated blood cell counting and analysis.
2. Describe how the general principles are implemented on different instruments.
3. Identify the parameters directly measured on the four analyzers discussed.
4. Explain the derivation of calculated or indirectly measured parameters for the same four analyzers.
5. Explain the derivation of the white blood cell differential count on the different instruments discussed.
6. Interpret and compare patient data, including white blood cell, red blood cell, and platelet histograms or cytograms or both, obtained from the four major hematology instruments.
7. Explain the general principles of automated reticulocyte counting.
8. Identify sources of error in automated cell counting and determine appropriate corrective action.

GENERAL PRINCIPLES OF AUTOMATED BLOOD CELL ANALYSIS

Since the 1980s, automated blood cell analysis has virtually replaced manual hemoglobin, hematocrit, and cell counting, due to its greater accuracy and precision, with the possible exception of phase platelet counting in certain circumstances. Hematology analyzers are marketed by multiple instrument manufacturers. These analyzers typically provide the eight standard hematology parameters (complete blood count [CBC]), plus a three-part, five-part, or six-part differential leukocyte count in less than 1 minute on 200 μL or less of whole blood. Automation allows more efficient workload management and more timely diagnosis and treatment of disease.

Electronic Impedance

The impedance principle of cell counting is based on the detection and measurement of changes in electrical resistance produced by cells as they traverse a small aperture. Cells suspended in an electrically conductive diluent such as saline are pulled...
through an aperture (orifice) in a glass tube. In the counting chamber, or transducer assembly, low-frequency electrical current is applied between an external electrode (suspended in the cell dilution) and an internal electrode (housed inside the aperture tube). Electrical resistance between the two electrodes, or impedance in the current, occurs as the cells pass through the sensing aperture, causing voltage pulses that are measurable (Figure 15-1).\(^5\) Oscilloscope screens on some instruments display the pulses that are generated by the cells as they interrupt the current. The number of pulses is proportional to the number of cells counted. The height of the voltage pulse is directly proportional to the volume of the cell, which allows discrimination and counting of cells of specific volumes through the use of threshold circuits. Pulses are collected and sorted (channelized) according to their amplitude by pulse height analyzers. The data are plotted on a frequency distribution graph, or volume distribution histogram, with relative number on the y-axis and volume (channel number equivalent to a specific volume) on the x-axis. The histogram produced depicts the volume distribution of the cells counted. Figure 15-2 illustrates the construction of a frequency distribution graph. Volume thresholds separate the cell populations on the histogram, and the count is the cells enumerated between the lower and upper set thresholds for each population. Volume distribution histograms may be used for the evaluation of one cell population or subgroups within a population.\(^5\) The use of proprietary lytic reagents to control shrinkage and lysis of specific cell types, as in the older Coulter S-Plus IV, STKR, and Sysmex E-5000 models, allows separation and quantitation of white blood cells (WBCs) into three populations (lymphocytes, mononuclear cells, and granulocytes) for the three-part differential on one volume distribution histogram.\(^6\)\(^-\)\(^8\)

Several factors may affect volume measurements in impedance or volume displacement instruments. Aperture diameter is crucial, and the red blood cell (RBC)/platelet aperture is smaller than the WBC aperture to increase platelet counting sensitivity. On earlier systems, protein buildup occurred, decreasing the diameter of the orifice, slowing the flow of cells, and increasing their relative electrical resistance. Protein buildup results in lower cell counts, which result in falsely elevated cell volumes. Impedance instruments once required frequent manual aperture cleaning, but current instruments incorporate burn circuits or other internal cleaning systems to prevent or slow protein buildup.\(^5\)\(^-\)\(^9\) Carryover of cells from one sample to the next also is minimized by these internal cleaning systems. Coincident passage of more than one cell at a time through the orifice causes artificially large pulses, which results in falsely increased cell volumes and falsely decreased cell counts. This count reduction, or coincident passage loss, is statistically predictable (and mathematically correctable) because of its direct relationship to cell concentration and the effective volume of the aperture.\(^7\)\(^-\)\(^9\) Coincidence correction typically is
completed by the analyzer computer before final printout of cell counts from the instrument. Other factors affecting pulse height include orientation of the cell in the center of the aperture and deformability of the RBC, which may be altered by decreased hemoglobin content.\(^{10,11}\) Recirculation of cells back into the sensing zone creates erroneous pulses and falsely elevates cell counts. A backwash or sweep-flow mechanism prevents recirculation of cells back into the sensing zone, and anomalously shaped pulses are edited out electronically.\(^{6,7,9}\)

The use of hydrodynamic focusing avoids many of the potential problems inherent in a rigid aperture system. The sample stream is surrounded by a sheath fluid as it passes through the central axis of the aperture. Laminar flow allows the central sample stream to narrow sufficiently to separate and align the cells into single file for passage through the sensing zone.\(^{12-14}\) The outer sheath fluid minimizes protein buildup and plugs, eliminates recirculation of cells back into the sensing zone with generation of spurious pulses, and reduces pulse height irregularity because off-center cell passage is prevented and better resolution of the blood cells is obtained. Coincident passage loss also is reduced because blood cells line up one after another in the direction of the flow.\(^{15}\) Laminar flow and hydrodynamic focusing are discussed further in Chapter 32.

### Radiofrequency

Low-voltage DC impedance, as described previously, may be used in conjunction with RF resistance, or resistance to a high-voltage electromagnetic current flowing between both electrodes simultaneously. Although the total volume of the cell is proportional to the change in DC, the cell interior density is proportional to pulse height or change in the RF signal. Conductivity, as measured by this high-frequency electromagnetic probe, is attenuated by nucleus-to-cytoplasm ratio, nuclear density, and cytoplasmic granulation. DC and RF voltage changes may be detected simultaneously and separated by two different pulse processing circuits.\(^{15,16}\) Figure 15-3 illustrates the simultaneous use of DC and RF current.

Two different cell properties, such as low-voltage DC impedance and RF resistance, can be plotted against each other to create a two-dimensional distribution cytogram or scatterplot (Figure 15-4). Such plots display the cell populations as clusters, with the number of dots in each cluster representing the concentration of that cell type. Computer cluster analysis can determine absolute counts for specific cell populations. The use of multiple methods by a given instrument for the determination of at least two cell properties allows the separation of WBCs into a five-part differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). DC and RF detection are two methods used by the Sysmex analyzers to perform WBC differentials.\(^{15,16}\)

### Optical Scatter

Optical scatter may be used as the primary methodology or in combination with other methods. In optical scatter systems (flow cytometers), a hydrodynamically focused sample stream is directed through a quartz flow cell past a focused light source (Figure 32-3). The light source is generally a tungsten-halogen lamp or a helium-neon laser (light amplification by stimulated emission of radiation). Laser light, termed monochromatic light because it is emitted at a single wavelength, differs from brightfield light in its intensity, its coherence (i.e., it travels in phase), and its low divergence or spread. These characteristics allow for the detection of interference in the laser beam and enable enumeration and differentiation of cell types.\(^{12,17}\) Optical scatter may be used to study RBCs, WBCs, and platelets.

As the cells pass through the sensing zone and interrupt the beam, light is scattered in all directions. Light scatter results from the interaction between the processes of absorption, diffraction (bending around corners or the surface of a cell),

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**Figure 15-3** Radiofrequency/direct current (RF/DC) detection method, showing simultaneous use of DC and RF in one measurement system on the Sysmex SE-9500. (From TOA Medical Electronics Company: Sysmex SE-9500 operator’s manual [CN 461-2464-2], Kobe, Japan, 1997, TOA Medical Electronics Co.)

**Figure 15-4** Illustration of cell volume measurement with direct current (DC) voltage change versus measurement of cell nuclear volume/complexity with change in the radiofrequency (RF) signal. The two measurements can be plotted against each other to form a two-dimensional distribution scatterplot. (From TOA Medical Electronics Company: Sysmex SE-9500 operator’s manual [CN 461-2464-2], Kobe, Japan, 1997, TOA Medical Electronics Co.)
refraction (bending because of a change in speed), and reflection (backward scatter of rays caused by an obstruction). 10 The detection of scattered rays and their conversion into electrical signals is accomplished by photodetectors (photodiodes and photomultiplier tubes) at specific angles. Lenses fitted with blocker bars to prevent nonscattered light from entering the detector are used to collect the scattered light. A series of filters and mirrors separate the varying wavelengths and present them to the photodetectors. Photodiodes convert light photons to electronic signals proportional in magnitude to the amount of light collected. Photomultiplier tubes are used to collect the weaker signals produced at a 90-degree angle and multiply the photoelectrons into stronger, useful signals. Analogue-to-digital converters change the electronic pulses to digital signals for computer analysis. 12,17

Forward-angle light scatter (0 degrees) correlates with cell volume, primarily because of diffraction of light. Orthogonal light scatter (90 degrees), or side scatter, results from refraction and reflection of light from larger structures inside the cell and correlates with degree of internal complexity. Forward low-angle scatter (2 to 3 degrees) and forward high-angle scatter (5 to 15 degrees) also correlate with cell volume and refractive index or with internal complexity. 17,19 Differential scatter is the combination of this low-angle and high-angle forward light scatter and is primarily used on Siemens systems for cellular analysis. The angles of light scatter measured by the different flow cytometers are manufacturer and method specific.

Scatter properties at different angles may be plotted against each other to generate two-dimensional cytograms or scatterplots, as on the Abbott CELL-DYN instruments. 20,21 Optical scatter may also be plotted against absorption, as on the Siemens systems, 22,23 or against volume, as on the larger Beckman Coulter systems. 9 Computer cluster analysis of the cytograms may yield quantitative and qualitative information.

### Overview

Hematology blood cell analyzers are produced by multiple manufacturers, including but not limited to, Abbott Laboratories (Abbott Park, IL); 24 HORIBA Medical (Irvine, CA); 25 Siemens Healthcare Diagnostics, Inc. (Deerfield, IL); 26 Beckman Coulter, Inc. (Brea, CA); 27 and Sysmex Corporation (Kobe, Japan). 28 The following discussion is limited to instrumentation produced by four of these suppliers. Emphasis is not placed on sample size or handling, speed, level of automation, or comparison of instruments or manufacturers. Likewise, technology continues to improve, and the newest (or most recent) models produced by a manufacturer may not be mentioned. Instead, a detailed description of primary methods used by these manufacturers is given to show the application of, and clarify further, the principles presented earlier and to enable the medical laboratory scientist or technician to interpret patient data, including instrument-generated histograms and cytograms. Table 15-1 summarizes methods used for the hemogram, reticulocyte, nucleated red blood cell, and WBC differential count determination on four major hematology instruments.

Hematology analyzers have some common basic components, including hydraulics, pneumatics, and electrical systems. The hydraulics system includes an aspirating unit, dispensers, diluters, mixing chambers, apertureaths or flow cells or both, and a hemoglobinometer. The pneumatics system generates the vacuums and pressures required for operating the valves and moving the sample through the hydraulics system. The electrical system controls operational sequences of the total system and includes electronic analyzers and computing circuitry for processing the data generated. Some older-model instruments have oscilloscope screens that display the electrical pulses in real time as the cells are counted. A data display unit receives information from the analyzer and prints results, histograms, or cytograms.

Specimen handling varies from instrument to instrument based on degree of automation, and systems range from discrete analyzers to walkaway systems with front-end load capability. Computer functions also vary, with the larger instruments having extensive microprocessor and data management capabilities. Computer software capabilities include automatic start-up and shutdown, with internal diagnostic self-checks and some maintenance; quality control, with automatic review of quality control data, calculations, graphs, moving averages, and storage of quality control files; patient data storage and retrieval, with δ checks (Chapter 5), critical value flagging, and automatic verification of patient results based on user-defined algorithms; host query with the laboratory or hospital information system to allow random access discrete testing capability; analysis of animal specimens; and even analysis of body fluids.

### Beckman Coulter Instrumentation

Beckman Coulter, Inc., manufactures an extensive line of hematology analyzers, including the smaller Ac-T series that provide complete RBC, platelet, and WBC analysis with a five-part differential. The LH 780 system, part of the LH 700 series, provides a fully automated online reticulocyte analysis. 27 The LH series also has the capability to perform CD4 and CD8 counts. 29 Coulter instruments typically have two measurement channels in the hydraulics system for determining the hemogram data. The RBC and WBC counts and hemoglobin are considered to be measured directly. The aspirated whole-blood sample is divided into two aliquots, and each is mixed with an isotonic diluent. The first dilution is delivered to the RBC aperture chamber, and the second is delivered to the WBC aperture chamber. In the RBC chamber, RBCs and platelets are counted and discriminated by electrical impedance as the cells are pulled through each of three sensing apertures (50 μm in diameter, 60 μm in length). Particles 2 to 20 fl are counted as platelets, and particles greater than 36 fl are counted as RBCs. In the WBC chamber, a reagent to lyse RBCs and release hemoglobin is added before WBCs are counted simultaneously by impedance in each of three sensing apertures (100 μm in diameter, 75 μm in length). Alternatively, some models employ consecutive counts in the same RBC or WBC aperture. After
counting cycles are completed, the WBC dilution is passed to the hemoglobinometer for determination of hemoglobin concentration (light transmittance read at a wavelength of 525 nm). Electrical pulses generated in the counting cycles are sent to the analyzer for editing, coincidence correction, and digital conversion. Two of the three counts obtained in the RBC and the WBC baths must match within specified limits for the counts to be accepted by the instrument.\(^5,9\) This multiple counting procedure prevents data errors resulting from aperture obstructions or statistical outliers and allows for excellent reproducibility on the Beckman Coulter instruments.

Pulse height is measured and categorized by pulse height measurement and fluorescence detection for basos, differential lysis, low-angle light scatter, and absorbsance. 64 channels are used for platelet analysis. Volume-distribution histograms of WBC, RBC, and platelet populations are generated. The RBC mean cell volume (MCV) is the average volume of the RBCs taken from the volume distribution data. The hematocrit (HCT), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) are calculated from measured and derived values. The RBC distribution width (RDW) is calculated directly from the histogram as the coefficient of variation (CV) of the RBC volume distribution, with a reference interval of 11.5% to 14.5%.\(^5\) The RDW is an index of anisocytosis, but it may be falsely skewed because it reflects the ratio of the standard deviation (SD) to MCV. That is, an RBC distribution histogram with normal divergence but a decreased

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Beckman Coulter UniCel DxCi 800</th>
<th>Sysmex XN Series</th>
<th>Abbott CELL-DYN Sapphire</th>
<th>Siemens ADVIA 2120i</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>Impedance volume and conductivity and five-angle light scatter measurement</td>
<td>Fluorescent staining; forward light scatter and side fluorescent light detection</td>
<td>Light scatter (primary count); impedance (secondary count)</td>
<td>Light scatter and absorption</td>
</tr>
<tr>
<td>RBC</td>
<td>Impedance</td>
<td>Impedance</td>
<td>Impedance</td>
<td>Low-angle and high-angle laser light scatter</td>
</tr>
<tr>
<td>HGB</td>
<td>Modified cyanmethemoglobin (525 nm)</td>
<td>Sodium lauryl sulfate-HGB (555 nm)</td>
<td>Modified cyanmethemoglobin (540 nm)</td>
<td>Modified cyanmethemoglobin (546 nm)</td>
</tr>
<tr>
<td>HCT</td>
<td>(RBC × MCV)/10</td>
<td>Cumulative RBC pulse height detection</td>
<td>(RBC × MCV)/10</td>
<td>(RBC × MCV)/10</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean of RBC volume distribution histogram</td>
<td>(HCT/RBC) × 10</td>
<td>Mean of RBC volume distribution histogram</td>
<td>Mean of RBC volume distribution histogram</td>
</tr>
<tr>
<td>MCH</td>
<td>(HGB/RBC) × 10</td>
<td>(HGB/RBC) × 10</td>
<td>(HGB/RBC) × 10</td>
<td>(HGB/RBC) × 10</td>
</tr>
<tr>
<td>MCHC</td>
<td>(HGB/HCT) × 100</td>
<td>(HGB/HCT) × 100</td>
<td>(HGB/HCT) × 100</td>
<td>(HGB/HCT) × 100</td>
</tr>
<tr>
<td>Platelet count</td>
<td>Impedance volume and conductivity and five-angle light scatter measurement</td>
<td>Impedance; light scatter; fluorescent staining; forward light scatter, and side fluorescent light detection</td>
<td>Dual angle light scatter analysis; impedance count for verification; optional CD61 monoclonal antibody count</td>
<td>Low-angle and high-angle light scatter; refractive index</td>
</tr>
<tr>
<td>RDW</td>
<td>RDW as CV (%) of RBC histogram or RDW-SD (fL)</td>
<td>RDW-SD (fL) or RDW-CV (%)</td>
<td>Relative value, equivalent to CV</td>
<td>CV (%) of RBC histogram</td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>Supravital staining; impedance volume and conductivity and light scatter measurement</td>
<td>Fluorescent staining; forward light scatter and side fluorescent light detection</td>
<td>Fluorescent staining; low-angle scatter, and fluorescent light detection</td>
<td>Supravital staining (oxazine 750); low-angle and high-angle light scatter and absorbance</td>
</tr>
<tr>
<td>NRBC*</td>
<td>Impedance volume and conductivity and five-angle light scatter measurement</td>
<td>Fluorescent staining; forward light scatter and side fluorescent light detection</td>
<td>Red fluorescent dye staining; forward light scatter and fluorescent light detection</td>
<td>Multi-angle light scatter measurements in the two WBC differential channels</td>
</tr>
<tr>
<td>WBC differential</td>
<td>Impedance volume and conductivity and five-angle light scatter measurement</td>
<td>Fluorescent staining; forward light scatter, and side fluorescent light detection</td>
<td>Multi-angle polarized scatter separation (MAPSS) and three-color fluorescence detection</td>
<td>Peroxidase staining, light scatter and absorption; for basos, differential lysis, low-angle and high-angle laser light scatter</td>
</tr>
</tbody>
</table>

CV, Coefficient of variation; DC, direct current; HCT, hematocrit; HGB, hemoglobin; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; NRBC, nucleated red blood cell count; RBC, red blood cell (or count); RDW, RBC distribution width; SD, standard deviation; VCS, volume, conductivity, scatter; WBC, white blood cell (or count).

*Instruments auto-correct the WBC count for the presence of nucleated RBCs.
MCV may imply a high RDW, falsely indicating increased anisocytosis. MCV and RDW are used by the instrument to flag possible anisocytosis, microcytosis, and macrocytosis.9

Platelets are counted within the range of 2 to 20 fl, and a volume-distribution histogram is constructed. If the platelet volume distribution meets specified criteria, a statistical least-squares method is applied to the raw data to fit the data to a log-normal curve. The curve is extrapolated from 0 to 70 fl, and the final count is derived from this extended curve. This fitting procedure eliminates interference from particles in the noise region, such as debris, and in the larger region, such as small RBCs. The mean platelet volume (MPV), analogous to the RBC MCV, also is derived from the platelet histogram. The reference interval for the MPV is about 6.8 to 10.2 fl. The MPV increases slightly with storage of the specimen in ethylenediaminetetraacetic acid (EDTA).5

Many older-model Beckman Coulter instruments, such as the STKR, and the newer, smaller models, such as the Ac-T series, provide three-part leukocyte subpopulation analysis, which differentiates WBCs into lymphocytes, mononuclear cells, and granulocytes. In the WBC channel, a special lysing reagent causes differential shrinkage of the leukocytes, which allows the different cells to be counted and volumetrically sized based on their impedance. A WBC histogram is constructed from the channelized data. Particles between approximately 35 and 90 fl are considered lymphocytes; particles between 90 and 160 fl are considered mononuclears (monocytes, blasts, immature granulocytes, and reactive lymphocytes); and particles between 160 and 450 fl are considered granulocytes. This allows the calculation of relative and absolute numbers for these three populations (Figure 15-5).6 Proprietary computerized algorithms further allow flagging for increased eosinophils or basophils or both and interpretation of the histogram differential, including flagging for abnormal cells, such as reactive lymphocytes and blasts.7 When cell populations overlap or a distinct separation of populations does not exist, a region alarm (R flag) may be triggered that indicates the area of interference on the volume-distribution histogram. An R1 flag represents excess signals at the lower threshold region of the WBC histogram and a questionable WBC count. This interference is visualized as a high takeoff of the curve and may indicate the presence of nucleated RBCs, clumped platelets, unlysed RBCs, or electronic noise.5,7

More recent Beckman Coulter instruments, the LH 700 Series and UniCel DxH series, generate hemogram data (including the WBC count) as before but use Coulter’s proprietary VCS (volume, conductivity, scatter) technology in a separate channel to evaluate WBCs for the determination of a five-part differential. The VCS technology includes the volumetric sizing of cells by impedance, conductivity measurements of cells, and laser light scatter, all performed simultaneously for each cell. After RBCs are lysed and WBCs are treated with a stabilizing reagent to maintain them in a near-native state, a hydrodynamically focused sample stream is directed through the flow

Figure 15-5 Printouts from the Coulter STKR showing the interpretive differential. A, Note the three distinct white blood cell (WBC) populations, Gaussian or normal distribution of red blood cells (RBCs), and right-skewed or log-normal distribution of platelets. B, Note the left shift in the WBC histogram with possible interference at the lower threshold region. R2 flag indicates interference and loss of valley owing to overlap or insufficient separation between the lymphocyte and mononuclear populations at the 90-fl region. RM flag indicates interference at more than one region. Eosinophil data have been suppressed. Also note the abnormal platelet volume distribution with a low platelet count. Manual 200-cell differential counts on the same samples: A, 52.5% neutrophils (47% segmented neutrophils, 5.5% bands), 41.5% lymphocytes, 4.0% monocytes, 1% basophils, 0.5% metamyelocytes, 0.5% reactive lymphocytes; B, 51% neutrophils (23% segmented neutrophils, 28% bands), 12% lymphocytes, 9.5% monocytes, 1% metamyelocytes, 1.5% myelocytes, 25% reactive lymphocytes, and 17 nucleated RBCs/100 WBCs.
cell past the sensing zone. Low-frequency DC measures cell volume, whereas a high-frequency electromagnetic probe measures conductivity, an indicator of cellular internal content. The conductivity signal is corrected for cellular volume, which yields a unique measurement called opacity. Each cell also is scanned with monochromatic laser light that reveals information about the cell surface, such as structure, shape, and reflectivity. Beckman Coulter’s unique rotated light scatter detection method, which covers a 10-degree to 70-degree range, allows for separation of cells with similar volume but different scatter characteristics. Beckman Coulter’s newest analyzer, the UniCel DxH 800, uses volume and conductivity as well as five additional parameters: axial light loss (AL2), low-angle light scatter (LALS), median-angle light scatter (MALS), lower median-angle light scatter (LMALS), and upper median-angle light scatter (UMALS). Using the data collected by the parameters listed above, the instrument applies data transformation, the process by which populations of cells are separated, allowing the determination of major populations as well as the enhancement of subpopulations of cells. Once those populations are established, a technique called the watershed concept searches for those populations and aids in determining counts as well as flagging based on all the populations found for that sample.

This combination of technologies provides a three-dimensional plot or cytograph of the WBC populations, which are separated by computer cluster analysis. Two-dimensional scatterplots of the measurements represent different views of the cytograph. The scatterplot of volume (y-axis) versus light scatter (x-axis) shows clear separation of lymphocytes, monocytes, neutrophils, and eosinophils. Basophils are hidden behind the lymphocytes but are separated by conductivity owing to their cytoplasmic granularity. Single-parameter histograms of volume, conductivity, and light scatter also are available.

Two types of WBC flags (alarms or indicators of abnormality) are generated on all hematology analyzers that provide a WBC differential count: (1) user defined, primarily set for distributional abnormalities, such as eosinophilia or lymphocytopenia (based on absolute eosinophil or lymphocyte counts); and (2) instrument specific, primarily suspect flags for morphologic abnormalities. For distributional flags, the user establishes reference intervals and programs the instrument to flag each parameter as high or low. Suspect flags indicating the possible presence of abnormal cells are triggered when cell populations fall outside expected regions or when specific statistical limitations are exceeded. Instrument-specific suspect flags on the Coulter UniCel DxH 800 system and LH 700 series include immature granulocytes/bands, blasts, variant lymphocytes, nucleated RBCs, and platelet clumps. The UniCel DxH 800 also utilizes the International Society for Laboratory Hematology (ISLH) consensus rules in addition to the user defined and system defined flags for complete data analysis. In addition to the flags listed above, inadequate separation of cell populations may disallow reporting of differential results by the instrument and may elicit a review slide message.

The UniCel DxH 800 system utilizes VCS as well as digital signal processing from five light scatter angles for clear cellular resolution. On the LH 700 series, Coulter utilizes an IntelliKinetics application. This application is used to ensure consistency with the kinetic reactions. It provides the instrument the best signals for analysis independent of laboratory environment variations. Compared with earlier models Coulter IntelliKinetics provides better separation of cell populations for WBCs and reticulocytes, which enables better analysis by the system algorithms.

The UniCel DxH 800 also includes the number of nucleated RBCs as part of the standard CBC report. They are identified, counted, and subtracted from the white blood cell count using volume, conductivity, and the same five light scatter measurement described above. The AL2 measurement (which reflects the amount of light absorbed as it passes through the flow cell) initially separates the nucleated RBCs from the WBCs. Algorithms are applied using the scatter from the other angles to electronically separate and count the nucleated RBCs. Two scatterplots display the nucleated RBC data by plotting axial light loss (AL2) on the x-axis against low-angle light scatter (RLALS) and upper median-angle light scatter (UMALS) on y-axis.

Figure 15-6 represents a standard patient printout from the Beckman Coulter UniCel DxH 800.

**Sysmex Instrumentation**

Sysmex Corporation, formerly TOA Medical Electronics Company, Ltd., manufactures a full line of hematology analyzers that provide complete RBC, platelet, and WBC analysis with three-part differential; the larger XT-1800i (SF-3000 and SE-9000) that performs a CBC with five-part differential; and the XE series and the newest XN series that also provide a fully automated reticulocyte count. The newest XN series is modular. The series is scalable, and multiple modules can be combined onto one platform. Each module contains the XN-CBC and XN-DIFF with other options available, including XN-BF, the body fluid application. Included standard on the CBC and DIFF modules are NRBCs, RET-He (reticulocyte hemoglobin), and IRF (immature reticulocyte fraction). The platelet analysis on the XT also utilizes a fluorescent count, in addition to the impedance count and optical count, called the PLT-F, performed by optical measurement. The PLT-F can be performed on each sample or set up as a reflex based on the laboratory’s PLT criteria. The method uses a fluorocell fluorescent dye (oxazine) combined with an extended PLT counting volume and time. The PLTs can be differentiated from other cells based on differences in intensity of the fluorescence combined with forward scattered light. The WBC, RBC, platelet counts, hemoglobin, and hematocrit are considered to be measured directly. Three hydraulic subsystems are used for determining the hemogram: the WBC channel, the RBC/platelet channel, and a separate hemoglobin channel. In the WBC and RBC transducer chambers, diluted WBC and RBC samples are aspirated through the different apertures and counted using the impedance (DC detection) method for counting and volumetrically sizing cells. Two unique features enhance the impedance technology: in the RBC/platelet channel, a sheathed stream with hydrodynamic focusing is used to direct cells through the aperture, which reduces coincident
passage, particle volume distortion, and recirculation of blood cells around the aperture; and in the WBC and RBC/platelet channels, floating thresholds are used to discriminate each cell population.8,15,16

As cells pass through the apertures, signals are transmitted in sequence to the analogue circuit and particle volume distribution analysis circuits for conversion to cumulative cell volume distribution data. Particle volume distribution curves are constructed, and optimal position of the autodiscrimination level (i.e., threshold) is set by the microprocessor for each cell population. The lower platelet threshold is automatically adjusted in the 2- to 6-fL volume range, and the upper threshold is adjusted in the 12- to 30-fL range, based on particle volume distribution. Likewise, the RBC lower and upper thresholds may be set in the 25- to 75-fL and 200- to 250-fL volume ranges. This floating threshold circuitry allows for discrimination of cell populations on a sample-by-sample basis. Cell counts are based on pulses between the lower and upper autodiscriminator levels, with dilution ratio, volume counted, and coincident passage error accounted for in the final computer-generated numbers. In the RBC channel, the floating discriminator is particularly useful in separating platelets from small RBCs. The hematocrit also is determined from the RBC/platelet channel, based on the principle that the pulse height generated by the RBC is proportional to cell volume. The hematocrit is the RBC cumulative pulse height and is considered a true

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**Table 15-6** Coulter UniCel DxH 800. The DxH 800 printout displays the CBC, DIFF and reticulocyte data for the same patient in Figures 15-7, 15-9, and 15-12. A, CBC data; B, Differential with the nucleated red blood cells (NRBCs); C, Reticulocyte data, including the IRF (immature reticulocyte fraction); D, Impedance histograms for the WBC, RBC, and PLT; E, Advanced two-dimensional optical scatterplots for WBCs, NRBCs, and reticulocytes; F, Suspect area in which any sample or system flags will display.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Flags</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>9.143</td>
<td></td>
<td>10^3/uL</td>
</tr>
<tr>
<td>UWBC</td>
<td>9.143</td>
<td></td>
<td>10^3/uL</td>
</tr>
<tr>
<td>RBC</td>
<td>3.931</td>
<td></td>
<td>10^6/uL</td>
</tr>
<tr>
<td>HGB</td>
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<td>g/dL</td>
</tr>
<tr>
<td>HCT</td>
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</tr>
<tr>
<td>MCV</td>
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<td></td>
<td>fL</td>
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<tr>
<td>MCH</td>
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<td></td>
<td>pg</td>
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<tr>
<td>MCHC</td>
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<td>g/dL</td>
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<tr>
<td>RDW</td>
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<tr>
<td>RDW-SD</td>
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<tr>
<td>PLT</td>
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<td>10^3/uL</td>
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<tr>
<td>MPV</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>BA</td>
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</tr>
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<tr>
<td>IRF</td>
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relative percentage volume of erythrocytes. In the hemoglobin flow cell, hemoglobin is oxidized and binds to sodium lauryl sulfate (SLS) forming a stable SLS–hemoglobin complex, which is measured photometrically at 555 nm.

The following indices are calculated in the microprocessor using directly measured or derived parameters: MCV, MCH, MCHC, RDW-SD, RDW-CV, MPV, and plateletcrit. RDW-SD is the RBC arithmetic distribution width measured at 20% of the height of the RBC curve, reported in femtoliters, with a reference interval of 37 to 54 fl. RDW-CV is the RDW reported as a CV. Plateletcrit is the platelet volume ratio, analogous to the hematocrit. MPV is calculated from the plateletcrit and platelet count just as erythrocyte MCV is calculated from the hematocrit and RBC count. The proportion of platelets greater than 12 fl in the total platelet count may be an indicator of possible platelet clumping, giant platelets, or cell fragments.

The XE series has the capability to run the platelet counting in the optical mode, which eliminates common interferences found with impedance counting. In the optical mode, the, immature platelet fraction or IPF, can be measured to provide additional information concerning platelet kinetics in cases of thrombocytopenia.

The SE-9000/9500 uses four detection chambers to analyze WBCs and obtain a five-part differential: the DIFE, IMI (immature myeloid information), EO, and BASO chambers. The high-end instrumentation such as the XE-series and the XN series has a six-part differential: neutrophils, lymphocytes, monocytes, eosinophils, basophils, and immature granulocytes. Every differential performed generates a percentage and absolute number for immature granulocytes, thus providing valuable information about the complete differential. In the DIFF detection chamber, RBCs are hemolyzed and WBCs are analyzed simultaneously by low-frequency DC and high-frequency current (DC/RF detection method). A scattergram of RF detection signals (γ-axis) versus DC detection signals (x-axis) allows separation of the WBCs into lymphocytes, monocytes, and granulocytes. Floating discriminators determine the optimal separation between these populations. Granulocytes are analyzed further in the IMI detection chamber to determine immature myeloid information. RBCs are lysed, and WBCs other than immature granulocytes are selectively shrunk by temperature and chemically controlled reactions. Analysis of the treated sample using the DC/RF detection method allows separation of immature cells on the IMI scattergram. A similar differential shrinkage and lysis method is also used in the EO and BASO chambers. That is, eosinophils and basophils are counted by impedance (DC detection) in separate chambers in which the RBCs are lysed, and WBCs other than eosinophils or basophils are selectively shrunk by temperature and chemically controlled reactions. Eosinophils and basophils are subtracted from the granulocyte count derived from the DIFF scattergram analysis to determine the neutrophil count. User-defined distributional flags may be set, and instrument-specific suspect flags, similar to those described for the Beckman Coulter LH 700 series, are triggered for the possible presence of morphologic abnormalities. A POSITIVE or NEGATIVE interpretive message is displayed.

In the XN-1000, fluorescent flow cytometry is used for the WBC count, WBC differential, and enumeration of nucleated RBCs. In the WDF channel, RBCs are lysed, WBC membranes are perforated, and the DNA and RNA in the WBCs are stained with a fluorescent dye. Plotting side scatter on the x-axis and side fluorescent light on the y-axis enables separation and enumeration of neutrophils, eosinophils, lymphocytes, monocytes, and immature granulocytes. In the WNR channel, the RBCs are lysed including nucleated RBCs, and WBC membranes are perforated. A fluorescent polymethine dye stains the nucleus and organelles of the WBCs with high fluorescence intensity and stains the released nuclei of the nucleated RBCs with low intensity. Plotting side fluorescent light on the x-axis and forward scatter on the y-axis enables separation and enumeration of the total WBC count, basophils, and nucleated RBCs. The WBC count is automatically corrected when nucleated RBCs are present in the sample. A WPC channel detects blasts and abnormal lymphocytes in a similar manner using a lysing agent and fluorescent dye and plotting side scatter on the x-axis and side fluorescent light on the y-axis. Figure 15-7 shows a patient report from the Sysmex XN-1000 analyzing the same patient specimen for which data are given in Figure 15-6.

**Abbott Instrumentation**

Instruments offered by Abbott Laboratories include the smaller CELL-DYN Emerald, which provides complete RBC, platelet, and WBC analysis with three-part differential, and the larger CELL-DYN Sapphire and the midrange CELL-DYN Ruby, both of which provide a CBC with five-part differential and random fully automated reticulocyte analysis. The CELL-DYN 4000 system has three independent measurement channels for determining the hemogram and differential: an optical channel for WBC count and differential data, an impedance channel for RBC and platelet data, and a hemoglobin channel for hemoglobin determination. The WBC, RBC, hemoglobin, and platelet parameters are considered to be measured directly. A 60- to 70-μm aperture is used in the RBC/platelet transducer assembly for counting and volumetrically sizing of RBCs and platelets by the electronic impedance method.

A unique von Behrens plate is located in the RBC/platelet counting chamber to minimize the effect of recirculating cells. Pulses are collected and sorted in 256 channels according to their amplitudes: particles between 1 and 35 fl are included in the initial platelet data, and particles greater than 35 fl are counted as RBCs. Floating thresholds are used to determine the best separation of the platelet population and to eliminate interferences, such as noise, debris, or small RBCs, from the count. Coincident passage loss is corrected for in the final RBC and platelet counts. RBC pulse editing is applied before MCV derivation to compensate for aberrant pulses produced by nonaxial passage of RBCs through the aperture. The MCV is the average volume of the RBCs derived from RBC volume distribution data. Hemoglobin is measured directly using a modified hemoglobin cyanide method that measures absorbance at 540 nm. Hematocrit, MCH, and MCHC are calculated from the directly measured or derived parameters. The RDW, equivalent to CV, is a relative value, derived from the RBC histogram by using the
The platelet analysis is based on a two-dimensional optical platelet count using fluorescent technology, the same technology used for direct nucleated RBC counting by adding a red fluorescence to the sample to stain nucleated red cells. Further analysis of platelets and platelet aggregates can be performed by using an automated CD61 monoclonal antibody to generate an immunoplatelet count. Other indices available include MPV and plateletcrit.

The WBC count and differential are derived from the optical channel using CELL-DYN’s patented multiangle polarized scatter separation (MAPSS) technology with three-color fluorescent technology. A hydrodynamically focused sample stream is directed through a quartz flow cell past a focused light source, an argon ion laser. Scattered light is measured at multiple angles: 0-degree forward light scatter measurement is used for determination of cell volume, 90-degree orthogonal light scatter measurement is used for determination of cellular lobularity, 7-degree narrow-angle scatter measurement is used to correlate with cellular complexity, and 90-degree depolarized light scatter measurement is used for evaluation of cellular granularity. Orthogonal light scatter is split, with one
portion directed to a 90-degree photomultiplier tube and the other portion directed through a polarizer to the 90-degree depolarized photomultiplier tube. Light that has changed polarization (depolarized) is the only light that can be detected by the 90-degree depolarized photomultiplier tube. Various combinations of these four measurements are used to differentiate and quantify the five major WBC subpopulations: neutrophils, lymphocytes, monocytes, eosinophils, and basophils.20,40,44 Figure 15-8 illustrates CELL-DYN’s MAPSS technology.

The light scatter signals are converted into electrical signals, sorted into 256 channels on the basis of amplitude for each angle of light measured, and graphically presented as scatterplots. Scatter information from the different angles is plotted in various combinations: 90 degrees/7 degrees, or lobularity versus complexity; 0 degrees/7 degrees, volume versus complexity; and 90 degrees depolarized/90 degrees, granularity versus lobularity. Lobularity or 90-degree scatter (y-axis) plotted against complexity or 7-degree scatter (x-axis) yields separation of mononuclear and segmented (polymorphonuclear neutrophil) subpopulations. Basophils cluster with the mononuclears in this analysis, because the basophil granules dissolve in the sheath reagent, and the degranulated basophil is a less complex cell. Each cell in the two clusters

Figure 15-8  A, Multiangle polarized scatter separation (MAPSS) technology. Cells are measured and characterized by plotting light scatter from four different angles. B, Mononuclear and polymorphonuclear scatter with MAPSS technology. It plots 10 degree scatter (complexity) on the x-axis and 90 degree scatter (lobularity) on the y-axis. The system uses algorithms to further separate the two populations, displaying mononuclear on the lower left and polymorphonuclear on the upper right. C, Separation and plotting of the polymorphonuclear cells into neutrophils and eosinophils based on MAPSS technology. It plots 90 degree scatter (lobularity) on the x-axis and 90 degree depolarized (90 D) scatter on the y-axis. The system uses algorithms to further separate the two populations of cells. D, Scatter of all WBC populations by MAPSS technology plotting 10 degree scatter (complexity) on the x-axis and 0 degree scatter (size or volume) on the y-axis. On the newer instruments, a 7-degree angle for complexity is now used instead of the 10-degree angle. The change reflects use of the midrange of the angle instead of the end range; however, it still provides the same information. (From Abbott Laboratories: CELL-DYN 3700 system operator’s manual [914032C], Abbott Park, IL, 2000.)
is identified as a mononuclear or segmented neutrophil for further evaluation.

The mononuclear subpopulation is plotted on a 0-degree/7-degree scatterplot, with volume on the y-axis and complexity on the x-axis. Three populations (lymphocytes, monocytes, and basophils) are seen clearly on this display. Nucleated RBCs, unlysed RBCs, giant platelets, and platelet clumps fall below the lymphocyte cluster on this scatterplot and are excluded from the WBC count and differential. Information from the WBC impedance channel also is used in discriminating these particles.

The segmented neutrophil subpopulation is plotted on a 90-degree depolarized/90-degree scatterplot, with granularity or 90-degree depolarized scatter on the y-axis and lobularity or 90-degree scatter on the x-axis. Because of the unique nature of eosinophil granules, eosinophils scatter more 90-degree depolarized light, which allows clear separation of eosinophils and neutrophils on this display. Dynamic thresholds are used for best separation of the different populations in the various scatterplots. Each cell type is identified with a distinct color, so that after all classifications are made and volume (0-degree scatter) is plotted on the y-axis against complexity (7-degree scatter) on the x-axis, each cell population can be visualized easily by the operator on the data terminal screen. Other scatterplots (90 degrees/0 degrees, 90 degrees depolarized/0 degrees, 90 degrees depolarized/7 degrees) are available and may be displayed at operator request. On earlier instruments, the 7-degree angle measurement for complexity was referred to as the 10-degree angle. The change reflects use of the midrange of the angle instead of the end range; however, it still provides the same information.15-46 As on the previously described instruments, user-defined distributional flags may be set, and instrument-specific suspect flags may alert the operator to the presence of abnormal cells.20,45 Figure 15-9 represents a patient printout from the CELL-DYN Sapphire analyzing the same patient specimen for which data are given in Figures 15-6 and 15-7.

### Siemens Healthcare Diagnostics Instrumentation

Siemens Healthcare Diagnostics Inc. manufactures the ADVIA 2120 and 2120i, the next generation of the ADVIA 120.22,33,47 Siemens has simplified the hydraulics and operations of the analyzer by replacing multiple complex hydraulic systems with a unified fluids circuit assembly, or Unifluids technology. The ADVIA 2120, 2120i, and 120 provide a complete hemogram and WBC differential, while also providing a fully automated reticulocyte count.22,23

Four independent measurement channels are used in determining the hemogram and differential: RBC/platelet channel, hemoglobin channel, and peroxidase (PEROX) and basophil-lobularity (BASO) channels for WBC and differential data. WBC, RBC, hemoglobin, and platelets are measured directly. Hemoglobin is determined using a modified cymnemoglobin method that measures absorbance in a colorimeter flow cuvette at approximately 546 nm. The RBC/platelet method uses flow cytometric light scattering measurements determined as cells, in a sheath-stream, pass through a flow cell by a laser optical assembly (laser diode light source). RBCs and platelets are isovolumetrically spheres before entering the flow cell to eliminate optical orientation noise. Laser light scattered at two different angular intervals—low angle (2 to 3 degrees), correlating with cell volume, and high angle (5 to 15 degrees), correlating with internal complexity (i.e., refractive index or hemoglobin concentration)—is measured simultaneously (Figure 15-10). This differential scatter technique, in combination with isovolumetric spherering, eliminates the adverse effect of variation in cellular hemoglobin concentration on the determination of RBC volume (as seen by differences in cellular deformability affecting the pulse height generated on impedance instruments).10,48

### Peroxidase (PEROX) Channel

In the PEROX channel, RBCs are lysed, and WBCs are stained for their peroxidase activity. The following reaction is catalyzed by cellular peroxidase, which converts the substrate to a dark

The segmented neutrophil subpopulation is plotted on a 90-degree depolarized/90-degree scatterplot, with granularity or 90-degree depolarized scatter on the y-axis and lobularity or 90-degree scatter on the x-axis. Because of the unique nature of eosinophil granules, eosinophils scatter more 90-degree depolarized light, which allows clear separation of eosinophils and neutrophils on this display. Dynamic thresholds are used for best separation of the different populations in the various scatterplots. Each cell type is identified with a distinct color, so that after all classifications are made and volume (0-degree scatter) is plotted on the y-axis against complexity (7-degree scatter) on the x-axis, each cell population can be visualized easily by the operator on the data terminal screen. Other scatterplots (90 degrees/0 degrees, 90 degrees depolarized/0 degrees, 90 degrees depolarized/7 degrees) are available and may be displayed at operator request. On earlier instruments, the 7-degree angle measurement for complexity was referred to as the 10-degree angle. The change reflects use of the midrange of the angle instead of the end range; however, it still provides the same information.15-46 As on the previously described instruments, user-defined distributional flags may be set, and instrument-specific suspect flags may alert the operator to the presence of abnormal cells.20,45 Figure 15-9 represents a patient printout from the CELL-DYN Sapphire analyzing the same patient specimen for which data are given in Figures 15-6 and 15-7.

### Siemens Healthcare Diagnostics Instrumentation

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Four independent measurement channels are used in determining the hemogram and differential: RBC/platelet channel, hemoglobin channel, and peroxidase (PEROX) and basophil-lobularity (BASO) channels for WBC and differential data. WBC, RBC, hemoglobin, and platelets are measured directly. Hemoglobin is determined using a modified cymnemoglobin method that measures absorbance in a colorimeter flow cuvette at approximately 546 nm. The RBC/platelet method uses flow cytometric light scattering measurements determined as cells, in a sheath-stream, pass through a flow cell by a laser optical assembly (laser diode light source). RBCs and platelets are isovolumetrically spheres before entering the flow cell to eliminate optical orientation noise. Laser light scattered at two different angular intervals—low angle (2 to 3 degrees), correlating with cell volume, and high angle (5 to 15 degrees), correlating with internal complexity (i.e., refractive index or hemoglobin concentration)—is measured simultaneously (Figure 15-10). This differential scatter technique, in combination with isovolumetric spherering, eliminates the adverse effect of variation in cellular hemoglobin concentration on the determination of RBC volume (as seen by differences in cellular deformability affecting the pulse height generated on impedance instruments).10,48 The Mie theory of light scatter of dielectric spheres is applied to plot scatter-intensity signals from the two angles against each other for a cell-by-cell RBC volume (y-axis) versus hemoglobin concentration (x-axis) cytogram or RBC map (Figure 15-11).

Independent histograms of RBC volume and hemoglobin concentration also are plotted. On the ADVIA 2120 and 120 platelets are counted and volumetrically sized using a two-dimensional (low-angle and high-angle) platelet analysis, which allows better discrimination of platelets from interfering particles, such as RBC fragments and small RBCs. Larger platelets can be included in the platelet count.23,47

Several parameters and indices are derived from the measurements described in the previous paragraph. MCV and MPV are the mean of the RBC volume histogram and the platelet volume histogram. Hematocrit, MCH, and MCHC are mathematically computed using RBC, hemoglobin, and MCV values. RDW is calculated as the CV of the RBC volume histogram, whereas hemoglobin distribution width (HDW), an analogous index, is calculated as the SD of the RBC hemoglobin concentration histogram. The reference interval for HDW is 2.2 to 3.2 g/dL. Cell hemoglobin concentration mean (CHCM), analogous to MCHC, is derived from cell-by-cell direct measures of hemoglobin concentration. Interferences with the hemoglobin colorimetric method, such as lipemia or icterus, affect the calculated MCHC but do not alter measured CHCM. CHCM generally is not reported as a patient result but is used by the instrument as an internal check for the MCHC and is available to the operator for calculating the cellular hemoglobin if interferences are present. Unique RBC flags derived from CHCM include hemoglobin concentration variance (HC VAR), hypochromia (HYPO), and hyperchromia (HYPER).22,23

Siemens hematology analyzers determine WBC count and a six-part WBC differential (neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells [LUCs]) by cytochemistry and optical flow cytometry, using the PEROX and BASO channels. LUCs include reactive or variant lymphocytes and blasts.

### Peroxidase (PEROX) Channel

In the PEROX channel, RBCs are lysed, and WBCs are stained for their peroxidase activity. The following reaction is catalyzed by cellular peroxidase, which converts the substrate to a dark
precipitate in peroxidase-containing cells (neutrophils, monocytes, and eosinophils):

\[ \text{H}_2\text{O}_2 \; + \; 4\text{-chloro-1-naphthol} \rightarrow \text{cellular} \; \text{peroxidase} \rightarrow \text{dark precipitate} \]

A portion of the cell suspension is fed to a sheath-stream flow cell where a tungsten-halogen darkfield optics system is used to measure absorbance (proportional to the amount of peroxidase in each cell) and forward scatter (proportional to the volume of each cell). Absorbance is plotted on the x-axis of the cytogram, and scatter is plotted on the y-axis.\(^{22,23}\) A total WBC count (WBC-PEROX) is obtained from the optical signals in this channel and is used as an internal check of the primary WBC count obtained in the basophil-lobularity channel (WBC-BASO). If significant interference occurs in the WBC-BASO count, the instrument substitutes the WBC-PEROX value.\(^ {23}\)

Computerized cluster analysis allows classification of the different cell populations, including abnormal clusters such as nucleated RBCs and platelet clumps. Nucleated RBCs are analyzed for every sample using four counting algorithms, which permits the system to choose the most accurate count based on internal rules and conditions. Neutrophils and eosinophils contain the most peroxidase and cluster to the right on
the cytogram. Monocytes stain weakly and cluster in the midregion of the cytogram. Lymphocytes, basophils, and LUCs (including variant or reactive lymphs and blasts) contain no peroxidase and appear on the left of the cytogram, with LUCs appearing above the lymphocyte area. Basophils cluster with the small lymphocytes and require further analysis for classification.22,23,49

**Basophil-Lobularity (BASO) Channel**

In the BASO channel, cells are treated with a reagent containing a nonionic surfactant in an acidic solution. Basophils are particularly resistant to lysis in this temperature-controlled reaction, whereas RBCs and platelets lyse and other leukocytes (nonbasophils) are stripped of their cytoplasm. Laser optics, using the same two-angle (2 to 3 degrees and 5 to 15 degrees) forward scattering system of the RBC/platelet channel, is used to analyze the treated cells. High-angle scatter (proportional to nuclear complexity) is plotted on the x-axis, and low-angle scatter (proportional to cell volume) is plotted on the y-axis. Cluster analysis allows for identification and quantification of the individual cellular populations. The intact basophils are identifiable by their large low-angle scatter. The remaining nuclei are classified as mononuclear, segmented, and blast cell nuclei based on their nuclear complexity (shape and cell density) and high-angle scatter.22,23

Basophils fall above a horizontal threshold on the cytogram. The stripped nuclei fall below the basophils, with segmented cells to the right and mononuclear cells to the left along the x-axis. Blast cells uniquely cluster below the mononuclear cells. Lack of distinct separation between the segmented and mononuclear clusters indicates WBC immaturity or suspected left shift. As indicated earlier, this channel provides the primary WBC count, the WBC-BASO. Relative differential results (in percent) are computed by dividing absolute numbers of the different cell classifications by the total WBC count.22,23

The nucleated RBC method is based on the physical characteristics of volume and density of the nucleated RBC nuclei. These characteristics allow counting in both WBC channels on the ADVIA 2120, and algorithms are applied to determine the absolute number and percentage of nucleated RBCs. Information from the PEROX and BASO channels is used to generate differential morphology flags indicating the possible presence of reactive lymphocytes, blasts, left shift, immature granulocytes, nucleated RBCs, or large platelets or platelet clumps.22,23,49

Figure 15-12 shows a patient printout from the ADVIA 2120i analyzing the same patient specimen for which data are given in Figures 15-6, 15-7, and 15-9.
Reticulocyte counting is the last of the manual cell-counting procedures to be automated and has been a primary focus of hematology analyzer advancement in recent years. The imprecision and inaccuracy in manual reticulocyte counting are due to multiple factors, including stain variability, slide distribution error, statistical sampling error, and interobserver error. All of these potential errors, with the possible exception of stain variability, are correctable with automated reticulocyte counting. Increasing the number of RBCs counted produces increased precision. This was evidenced in the 1993 College of American Pathologists pilot reticulocyte proficiency survey (Set RT-A, Sample RT-01) on which the CV for results obtained using flow cytometry was 35% compared with 8.3% for results obtained using one of the automated methods. Precision of automated methods has continued to improve. The manual reticulocyte results for one specimen in the 2000 Reticulocyte Survey Set RT/RT2-A showed a CV of 28.7%, whereas the CV was 2.8% for results obtained using one of the automated methods. Automated reticulocyte analyzers may count 32,000 RBCs compared with 1000 cells in the routine manual procedure.

Available automated reticulocyte analyzers include flow cytometry systems such as the FACS system from Becton, Dickinson and Company (Franklin Lakes, NJ) or the Coulter EPICS system; the Sysmex R-3500, R-500, XE-2100, XE-5000, and XN-series systems; the CELL-DYN 3500R, 3700, and 4000 systems; the Coulter LH 750 systems and the UniCel DxH800; and the Siemens ADVIA 2120, 2120i, and 120. All of these analyzers evaluate reticulocytes based on optical scatter or fluorescence after the RBCs are treated with fluorescent dyes or nucleic acid stains to stain residual RNA in the reticulocytes. Because neither the FACS nor EPICS system is generally available in the routine hematology laboratory, the discussion here is limited to the other analyzers.

The Sysmex R-3000/3500 is a stand-alone reticulocyte analyzer that uses auramine O, a supravital fluorescent dye, and measures forward scatter and side fluorescence as the cells, in a sheath-stream, pass through a flow cell by an argon laser. The signals are plotted on a scattergram with forward scatter intensity, which correlates with volume, plotted against fluorescence intensity, which is proportional to RNA content. Automatic discrimination separates the populations into mature RBCs and reticulocytes. The reticulocytes fall into low-fluorescence, middle-fluorescence, or high-fluorescence regions, with the less intense regions designated as reticulocytes.
mature reticulocytes showing higher fluorescence. The immature reticulocyte fraction (IRF) is the sum of the middle-fluorescence and high-fluorescence ratios and indicates the ratio of immature reticulocytes to total reticulocytes in a sample. The XE-5000, the XT-2000i and the XN series also determine the reticulocyte count and IRF by measuring forward scatter and side fluorescence. They also have a parameter called RET-He (reticulocyte hemoglobin equivalent) that measures the hemoglobin content of the reticulocytes. It uses a proprietary polymethine dye to fluorescently stain the reticulocyte nuclei. This is similar to the reticulocyte hemoglobin content (CHr) parameter on the ADVIA 2120i (discussed below). Platelets, which also are counted, fall below a lower discriminator line. The Sysmex SE-9500/9000 + RAM-1 module uses the same flow cytometry methodology for reticulocyte counting as the R-3500. Off-line sample preparation is not required. The smaller Sysmex R-500 uses flow cytometry with a semiconductor laser as the light source and polymethine required. The smaller Sysmex R-500 uses flow cytometry methodology for reticulocyte detection to allow fully automated, random access reticulocyte testing. The RBCs are stained with a proprietary polymethine dye to fluorescently stain the reticulocyte nuclei. This is similar to the reticulocyte hemoglobin equivalent (CD4K530) that binds stoichiometrically to nucleic acid and emits green light as the cells, in a sheath-stream, pass through a flow cell by an argon ion laser. Platelets and reticulocytes are separated based on intensity of green fluorescence (scatter measured at 7 degrees and 90 degrees), and the reticulocyte count along with the IRF is determined. Beckman Coulter also has incorporated reticulocyte methods into its primary cell-counting instruments: LH 700 series systems and the UniCel DxH800. The Coulter method uses a new methylene blue dye and the VCS technology described earlier. Volume is plotted against light scatter (DF 5 scatter-plot) and against conductivity (DF 6 scatter-plot), which correlates with opacity of the RBC. Stained reticulocytes show greater optical scatter and greater opacity than mature RBCs. Relative and absolute reticulocyte counts are reported, along with mean reticulocyte volume and maturation index or IRF: The Siemens ADVIA 2120, 2120i, and 120 systems enumerate reticulocytes in the same laser optics flow cell used in the RBC/platelet and BASO channels described earlier. The reticulocyte reagent isovolumetrically spheres the RBCs and stains the reticulocytes with oxazine 750, a nucleic acid-binding dye. Three detectors measure low-angle scatter (2 to 3 degrees), high-angle scatter (5 to 15 degrees), and absorbance simultaneously as the cells pass through the flow cell. Three cytograms are generated: high-angle scatter versus absorption, low-angle scatter versus high-angle scatter (Mie cytogram or RBC map), and volume versus hemoglobin concentration. The absorption cytogram allows separation and quantitation of reticulocytes, with additional subdivision into low-absorbing, medium-absorbing, and high-absorbing cells based on amount of staining. The sum of the medium-absorbing and high-absorbing cells reflects the IRF. Volume and hemoglobin concentration for each cell are derived from the RBC map by applying Mie scattering theory. Unique reticulocyte indices (MCVr, CHCMr, RDWr, HDWr, CHr, and CHDWr) are provided. The CHr or reticulocyte hemoglobin content of each cell is calculated as the product of the cell volume and the cell hemoglobin concentration. A single-parameter histogram of CHr is constructed, with a corresponding distribution width (CHDWr) calculated. These reticulocyte indices are not reported on the routine patient printout but are available to the operator. Figure 15-13 is a reticulocyte printout from an ADVIA 120, showing the cytograms and reticulocyte indices.

Automation of reticulocyte counting has allowed increased precision and accuracy and has greatly expanded the analysis of immature RBCs, providing new parameters and indices that may be useful in the diagnosis and treatment of anemias. The IRF, first introduced to indicate immature reticulocytes, shows an early indication of erythropoiesis. The IRF and the absolute reticulocyte count can be used to distinguish types of anemias. Anemias with increased marrow erythropoiesis, such as hemolytic anemia, have a high total reticulocyte count and increased IRF, while chronic renal disease has decreased absolute count and an IRF indicating decreased marrow erythropoiesis. An increased IRF and normal to decreased absolute reticulocyte count indicates an early response to therapy in nutritional anemias. Utilization of both tests is a reliable indicator of changes in erythropoietic activity and may prove to be a valuable therapeutic monitoring tool in patients. The reticulocyte maturity measurements also may be useful in evaluating bone marrow suppression during chemotherapy, monitoring hematopoietic regeneration after bone marrow or stem cell transplantation, monitoring renal transplant engraftment, and assessing efficacy of anemia therapy. The reticulocyte hemoglobin content, CHr (Advia) and Ret-He (Sysmex), provides an assessment of the availability of iron for erythropoiesis (Chapters 11 and 20). The additional reticulocyte indices derived on the ADVIA 2120 and 120 are valuable in following the response to erythropoietin therapy, and the CHr in particular has proved useful in the early detection and diagnosis of iron deficient erythropoiesis in children. The National Kidney Foundation KDOQI (Kidney Disease Outcomes Quality Initiative) recommends the addition of the reticulocyte hemoglobin content to the CBC, in addition to the reticulocyte count and ferritin level to assess the iron status in patients with chronic kidney disease. Widespread use of the new parameters may be limited by the availability of instrumentation.
Implementing automation in the hematology laboratory requires critical evaluation of the instrument’s methods and limitations, and the performance goals for the individual laboratory. The Clinical and Laboratory Standards Institute (CLSI) has approved a standard for validation, verification, and quality assurance of automated hematology analyzers. This standard provides guidelines for instrument calibration and assessment of performance criteria, including accuracy, precision, linearity, sensitivity, and specificity. The clinical accuracy (sensitivity and specificity) of the methods should be such that the instrument appropriately identifies patients who have disease and patients who do not have disease. Quality control systems should reflect the laboratory’s established performance goals and provide a high level of assurance that the instrument is working within its specified limits.

**Calibration**

Calibration is crucial in defining the accuracy of the data produced (Chapter 5). Calibration, or the process of electronically correcting an instrument for analytical bias (numerical difference from the “true” value), may be accomplished by appropriate use of reference methods, reference materials, or commercially prepared calibrators. Because few instruments are precalibrated by the manufacturer, calibration must be performed at initial installation and verified at least every 6 months under the requirements of the Clinical Laboratory Improvement Act of 1988. Periodic recalibration may be required after major instrument repair requiring optical alignment or part replacement.

Whole-blood calibration using fresh whole-blood specimens requires the use of reference methods, materials, and procedures to determine “true” values. The International Committee for Standardization in Haematology has established guidelines for selecting a reference blood cell counter for this purpose, but the cyanmethemoglobin method remains the only standard available in hematology for calibration and quality control. Whole-blood calibration, which historically has been considered the preferred method for calibration of multi-channel hematology analyzers, has been almost completely replaced by the use of commercial calibrators assayed using...
reference methods. Calibration bias is possible with the use of these calibrators because of inherent differences in stabilized and preserved cell suspensions. It is essential that calibrations be carried out properly and verified by comparison with reference methods or review of quality control data after calibration and by external comparison studies such as proficiency testing.

**Instrument Limitations**

The continual improvement of automated technologies has resulted in greater sensitivity and specificity of instrument flagging with detection of possible interferences in the data. The parallel improvement in instrument walk-away capabilities has increased the importance of the operator’s awareness and understanding of instrument limitations, however, and of his or her ability to recognize factors that may interfere and cause erroneous laboratory results. Limitations and interferences may be related to methodology or to inherent problems in the blood specimen.

Each instrument has limitations related to methodology that are defined in instrument operation manuals and in the literature. A common limitation of impedance methods is an instrument’s inability to distinguish cells reliably from other particles or cell fragments of the same volume. Cell fragments may be counted as platelets in specimens from chemotherapy-treated patients with increased WBC fragility. Likewise, schistocytes or small RBCs may interfere with the platelet count. Larger platelet clumps may be counted as WBCs, which results in a falsely decreased platelet count and potentially increases the WBC count. Micromegakaryocytes may be counted as nucleated RBCs or WBCs. RBCs containing variant hemoglobins such as Hb S or Hb C are often resistant to lysis, and the unlysed cells can be falsely counted as nucleated RBCs or WBCs and interfere in the hemoglobin reaction. This phenomenon has become more apparent with the use of milder diluent and lysing reagents in the analyzers with automated WBC differential technology. Non-lysis also may be seen in specimens from patients with severe liver disease, those undergoing chemotherapy treatment, and neonates (due to increased levels of Hb F) on the older Sysmex instruments. The ADVIA 2120 and 120 reports the WBC-BASO as the primary WBC count. An extended lyse cycle may be used on the CELL-DYN 3500, and the newer instruments are able to provide a correct WBC impedance count when lysteresistant RBCs are present.

The suppression of automated data, particularly WBC differential data, may occur when internal instrument checks fail or cast doubt on the validity of the data. Instruments from some manufacturers release results with specific error codes or flagging for further review. The suppression of automated differential data ensures that a manual differential count is performed, whereas the release of data with appropriate flagging mandates the need for careful review of the data and possibly a blood film examination. This suggests a difference in philosophy among the manufacturers and affects the work flow in different ways. More importantly, each laboratory must establish its own criteria for directed blood film review based on established performance goals, instrument flagging, and inherent instrument limitations.

**Specimen Limitations**

Limitations resulting from inherent specimen problems include those related to the presence of cold agglutinins, icterus, and lipemia. Cold agglutinins manifest as a classic pattern of increased MCV (frequently greater than 130 fL), markedly decreased RBC count, and increased MCHC (frequently greater than 40 g/dL). Careful examination of the histograms or cytograms from the instruments may yield clues to this abnormality. Icterus and lipemia directly affect hemoglobin measurements and related indices. Table 15-2 summarizes conditions that cause interference on some hematology analyzers and offers suggestions for manually obtaining correct patient results. As instrumentation advances, instrumentation software can adjust or correct for some of the conditions listed. Historically, a nucleated RBC flag required examination of a blood film to enumerate the nucleated RBCs and correct the WBC. All four major vendors offer online nucleated RBC enumeration and WBC correction, although the laboratory must validate the results. Lipemia interferes with the hemoglobin reading by falsely elevating the hemoglobin and associated indices. The Siemens technology uses direct measurement of the CHCM parameter, which allows back-calculation of the hemoglobin unaffected by lipemia and thus eliminates the need for the manual method of saline replacement in lipemic specimens. These two examples involving nucleated RBCs and lipemia illustrate instrument advances, and continued future improvements in technology will eliminate or decrease the need for manual intervention to obtain accurate results.

Specimen age and improper specimen handling can have profound effects on the reliability of hematology test results. These factors have even greater significance as hospitals move toward greater use of off-site testing by large reference laboratories. Specific problems with older specimens include increased WBC fragility, swelling and possible lysis of RBCs, and the deterioration of platelets. Stability studies should be performed before an instrument is used, and specific guidelines should be established for specimen handling and rejection.

**CLINICAL UTILITY OF AUTOMATED BLOOD CELL ANALYSIS**

The use of automated hematology analyzers has directly affected the availability, accuracy, and clinical usefulness of the CBC and WBC differential count. Some parameters that are available on hematology analyzers, but cannot be derived manually, have provided further insight into various clinical conditions. The RDW, a quantitative estimate of erythrocyte anisocytosis, can be used with the MCV for initial classification of anemia. Although the classification scheme is not absolute, a low MCV with a high RDW suggests iron deficiency, while a high MCV and high RDW suggests a folate/vitamin B12 deficiency or myelodysplasia (Chapter 19). The immature reticulocyte fraction and the immature platelet fraction provide an early indication of engraftment success after hematopoietic stem cell transplant. The reticulocyte hemoglobin content (Chr and Ret-He) provides an assessment of the iron available for hemoglobin synthesis. It is useful in the early diagnosis of
Iron deficiency, functional iron deficiency, as well as an early indicator of recovery after iron therapy.61,62,77 The MPV may be useful in distinguishing thrombocytopenia due to idiopathic thrombocytopenia purpura (high MPV), inherited macrothrombocytopenia (higher MPV), or bone marrow suppression (low MPV).76,79 High MPV values are also associated with higher-risk cardiovascular disease and may have use in assessing a patient’s risk of thrombosis.79,80 However the use of the MPV in these conditions has been hampered by the varying ability of instruments to accurately measure MPV in patients with macroplatelets (they are underestimated in impedance methods), the lack of standardization of MPV cut-off values in various conditions, and the lack of well-controlled prospective studies to prove clinical utility.79 In addition to method variations, anticoagulation and storage time also influence the MPV, which further impacts the utility.79 In addition to method variations, anticoagulation and storage time also influence the MPV, which further impacts the utility.79

Automation of the WBC differential has had a significant impact on the laboratory work flow because of the labor-intensive
nature of the manual differential count. The three-part differential count on earlier instruments generally proved suitable as a screening leukocyte differential count to identify specimens that required further workup or a manual differential count. Partial differential counts, however, do not substitute for a complete differential count in populations with abnormalities. Abnormal cells such as blasts and nucleated RBCs in low concentrations may not be detected by the instruments but likewise may be missed by the routine 100-cell manual/visual differential count. The CELL-DYN Sapphire, with its added fluorescent detection technology, has been shown to have high sensitivity and specificity for flagging nucleated RBCs and platelet clumps. As technology continues to improve, blood film review to confirm the presence of platelet clumps or nucleated RBCs and to correct leukocyte counts for interference from platelet clumps or nucleated RBCs is becoming unnecessary, especially for nucleated RBCs, because the four major vendors now count and correct the WBC for nucleated RBCs on their high-end analyzers.

Instrument evaluations based on the Clinical and Laboratory Standards Institute H20-A2 standard on reference leukocyte differential counting using an 800-cell or 400-cell manual leukocyte differential count as the reference method have shown acceptable correlation coefficients for all WBC types, with the possible exception of monocytes. However, further studies using monoclonal antibodies as the reference method for counting monocytes suggest that automated analyzers yield a more accurate assessment of monocytosis than do manual methods.

Histograms and cytograms, along with instrument flagging, provide valuable information in the diagnosis and treatment of RBC and WBC disorders. Multiple reports indicate the usefulness of histograms and cytograms in the characterization of various abnormal conditions, including RBC disorders such as cold agglutination and WBC diseases such as leukemias and myelodysplastic disorders.

Manufacturers are developing integrated hematology workstations for the greatest automation and laboratory efficiency. The Beckman Coulter LH 1500 Automation Series is Beckman’s solution to integrated hematology. The line can be customized to have two to four LH analyzers as well as SlideMakers and SlideStainers based on the laboratory’s needs for efficiency and automation. The Sysmex Total Hematology Automation System (HST series) robotically links the SE-9000, R-3500 (automated reticulocyte analyzer), and SP-100 (automatic slide maker/stainer). The HST line links two XE-2100 units and one SP-100 instrument for complete automation or systemization of hematology testing. The SE-Alpha is a smaller version that links the SE-9000 and SP-100. The Siemens ADVIA LabCell links the ADVIA 2120i to the track, and the Autoslide (automatic slide maker/stainer) links to the ADVIA 2120i. Finally, as a result of increasing customer needs, manufacturers have added body fluid counting to their high-end instrumentation. The Beckman Coulter UniCel DxH 800 system, the LH 780, and the Sysmex XN series and XE-5000 count WBCs and RBCs in body fluids; the ADVIA 2120i counts WBCs and RBCs in body fluids and, in addition to cerebrospinal fluid WBC and RBC cell counts, performs a differential count on cerebrospinal fluid.

Selection of a hematology analyzer for an individual laboratory requires careful evaluation of the laboratory’s needs and close scrutiny of several important instrument issues, including instrument specifications and system requirements, methods used, training requirements, maintenance needs, reagent usage, data management capabilities, staff response, and short-term and long-term expenditures. All instruments claim to improve laboratory efficiency through increased automation that results in improved work flow and faster turnaround time or through the addition of new parameters that may have clinical efficacy. All four major vendors offer a slide maker/stainer that can be connected directly to their high-end analyzers. The slide makers/stainers can be programmed to make blood films for every specimen or to make films based on the laboratory’s internal criteria for a film review. This reduces, but does not completely eliminate, the use of manual peripheral blood film review. Automated slide makers/stainers connect only to high-end analyzers and as such are not suitable for some laboratories. Each laboratory must assess its own efficiency needs to determine if a slidemaker and stainer is a value-added instrument to the laboratory.

The instrument selected should suit the workload and patient population and should have a positive effect on patient outcomes. The instrument selected for a cancer center may be different from that selected for a community hospital. Ultimately, however, the instrument decision may be swayed by individual preferences.

**SUMMARY**

- Automated cell counting provides greater accuracy and precision compared to manual cell-counting methods.
- The primary principles of operation, electronic impedance and optical scatter, are used by most automated hematology analyzers. Radiofrequency (RF) is sometimes used in conjunction with electronic impedance.
- The electronic impedance method detects and measures changes in electrical resistance between two electrodes as cells pass through a sensing aperture. The measurable voltage changes are plotted on frequency distribution graphs, or histograms, that allow the evaluation of cell populations based on cell volume.
- RF resistance uses high-voltage electromagnetic current. Measurable changes in the RF signal are proportional to cell interior density, or conductivity. Impedance and conductivity can be plotted against each other on a two-dimensional distribution cytogram.
or scatterplot, which allows the evaluation of cell populations using cluster analysis.

- Optical scatter systems (flow cytometers) use detection of interference in a laser beam or light source to differentiate and enumerate cell types.
- Major manufacturers of hematology instrumentation include Beckman Coulter, Inc.; Sysmex Corporation; Abbott Diagnostics; and Siemens Healthcare Diagnostics, Inc. Table 15-1 summarizes the methods used for the hemogram, and reticulocyte, nucleated RBC, and WBC differential counts in the newer instruments.
- Reticulocyte analysis has been incorporated into the primary cell-counting instruments of all major manufacturers. All use either fluorescent or other dyes that stain nucleic acid in reticulocytes before the cells are counted using fluorescence or absorbance and light scatter.
- Each instrument has limitations related to methodology that may result in instrument flagging of specific results or suppression of automated data. Likewise, inherent specimen problems may result in instrument flagging that indicates possible rejection of automated results.
- Automated hematology analyzers have had a significant impact on laboratory work flow, particularly automation of the WBC differential. In addition, newer parameters that can now be measured, such as the immature reticulocyte fraction (IRF) and the reticulocyte hemoglobin concentration (RET-He and CHr), have documented clinical utility.

**REVIEW QUESTIONS**

Answers can be found in the Appendix.

Examine the histograms/scatterplots obtained from four major instruments for the same patient specimen (Figure 15-14, A-D). Compare the results, and respond to questions 1 to 4 based on the results.

1. Which printout lets the end user know at a glance that the results are acceptable and no manual work needs to be performed?
   a. CELL-DYN Sapphire
   b. UniCel DxH 800
   c. ADVIA 2120i
   d. Sysmex XN-series

2. Which instrument printout has a system flag on the platelet count?
   a. CELL-DYN Sapphire
   b. UniCel DxH 800
   c. ADVIA 2120i
   d. XN-series

3. What do you suspect is the cause of the variation in platelet counting among the four instruments?
   a. Different instruments have different levels of sensitivity.
   b. All instruments use the same principle for counting platelets.
   c. Some instruments are susceptible to false-positive platelet flagging under certain conditions.
   d. Different instruments use different thresholds to capture and count platelets.

4. Based on the overall flagging for this specimen on each instrument, should a manual differential count be performed for this patient?
   a. Yes, because immature granulocytes are present in the sample.
   b. Yes, because the WBC scatterplots are abnormal.
   c. No, because each differential count is complete with no system or morphology flags.
   d. No, because each differential count is complete with no system or morphology flags.

5. A patient peripheral blood film demonstrates agglutinated RBCs, and the CBC shows an elevated MCHC. What other parameters will be affected by the agglutination of the RBCs?
   a. MCV will be decreased and the RBC count will be increased.
   b. MCV will be decreased and the RBC count will be decreased.
   c. MCV will be increased and the RBC count will be decreased.
   d. MCV will be increased and the RBC count will be increased.

6. Match the cell-counting methods listed with the appropriate definition:
   - ___ Impedance  a. Uses diffraction, reflection, and refraction of light waves
   - ___ RF  b. Uses high-voltage electrical waves to measure the internal complexity of cells
   - ___ Optical scatter  c. Involves detection and measurement of changes in electrical current between two electrodes

7. Low-voltage DC is used to measure:
   a. Cell nuclear volume
   b. Total cell volume
   c. Cellular complexity in the nucleus
   d. Cellular complexity in the cytoplasm

8. Orthogonal light scatter is used to measure:
   a. Cell volume
   b. Internal complexity of the cell
   c. Cellular granularity
   d. Nuclear density
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Figure 15-14 Composite scatterplots/histograms obtained from four major instruments. A, Coulter UniCel DxH 800; B, ADVIA 2120i;
Sample comments:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
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</tr>
<tr>
<td>RBC</td>
<td>5.42</td>
</tr>
<tr>
<td>HGB</td>
<td>16.0</td>
</tr>
<tr>
<td>HCT</td>
<td>50.8 +</td>
</tr>
<tr>
<td>MCV</td>
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</tr>
<tr>
<td>MCH</td>
<td>29.5</td>
</tr>
<tr>
<td>MCHC</td>
<td>31.5</td>
</tr>
<tr>
<td>PLT &amp;F</td>
<td>277</td>
</tr>
<tr>
<td>RDW-SD</td>
<td>46.7</td>
</tr>
<tr>
<td>RDW-CV</td>
<td>13.6</td>
</tr>
<tr>
<td>MPV</td>
<td>10.9</td>
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<tr>
<td>NRBC</td>
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<td>NEUT</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>BASO</td>
<td>0.07</td>
</tr>
<tr>
<td>IG</td>
<td>0.02</td>
</tr>
<tr>
<td>RET</td>
<td>1.53</td>
</tr>
<tr>
<td>IRF</td>
<td>8.8</td>
</tr>
<tr>
<td>RET-He</td>
<td>34.5</td>
</tr>
<tr>
<td>IPF</td>
<td>7.4</td>
</tr>
</tbody>
</table>

WBC-BF [10^3/μL]
RBC-BF [10^6/μL]
MN [10^3/μL]
PMN [10^9/μL]
TC-BF# [10^9/μL]

WBC IP Message
RBC IP Message
PLT IP Message

**Figure 15-14, cont’d** C, Sysmex XN-1000;
9. On the Beckman Coulter instruments, hematocrit is a calculated value. Which of the following directly measured parameters is used in the calculation of this value?
   a. RDW
   b. Hemoglobin
   c. MCV
   d. MCHC

10. Match each instrument listed with the technology it uses to determine WBC differential counts.

   ___ Abbott CELL-DYN Sapphire
   ___ Siemens ADVIA 2120i
   ___ Sysmex XN-1000
   ___ Beckman Coulter UniCel DxH 800

   a. Volume, conductivity, and five angles of light scatter
   b. MAPSS technology and three-color fluorescence
   c. Peroxidase-staining absorbance and light scatter
   d. Detection of forward and side scattered light and fluorescence
REFERENCES


A healthy-looking 56-year-old man had an automated CBC performed as part of a preoperative evaluation. Results are shown here. Refer to reference intervals provided on the inside front cover of this book.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>15.8 \times 10^3/\text{L}</td>
</tr>
<tr>
<td>RBC</td>
<td>4.93 \times 10^{12}/\text{L}</td>
</tr>
<tr>
<td>HGB</td>
<td>14.8 \text{g/dL}</td>
</tr>
<tr>
<td>HCT</td>
<td>45.1%</td>
</tr>
<tr>
<td>MCV</td>
<td>91.5 \text{fl}</td>
</tr>
<tr>
<td>MCH</td>
<td>30 \text{pg}</td>
</tr>
<tr>
<td>MCHC</td>
<td>32.8 \text{g/dL}</td>
</tr>
<tr>
<td>RDW</td>
<td>14.2%</td>
</tr>
<tr>
<td>PLT</td>
<td>34 \times 10^9/\text{L}</td>
</tr>
<tr>
<td>MPV</td>
<td>6.6 \text{fl}</td>
</tr>
</tbody>
</table>

The peripheral blood film was examined, and the only abnormal finding was “platelets in clumps.”

1. Describe the blood picture succinctly, using proper terminology for red blood cells, white blood cells, and platelets.
2. What automated results should be questioned?
3. What is the best course of action to handle this problem?
A well-made, well-stained, and carefully examined peripheral blood film can provide valuable information regarding a patient's health. More can be learned from this test than from many other routinely performed hematologic tests. White blood cell (WBC) and platelet count estimates can be achieved, relative proportions of the different types of WBCs can be obtained, and the morphology of all three cell lines can be evaluated for abnormalities. Although routine work is now handled by the sophisticated automated instruments found in most hematology laboratories, skilled and talented laboratory professionals are still essential to the reporting of reliable test results. Accurate peripheral film evaluation is quite likely to be needed for some time.

The peripheral film evaluation is the capstone of a panel of tests called the complete blood count (CBC) or hemogram. The CBC includes enumeration of cellular elements, quantitation of hemoglobin, and statistical analyses that provide a snapshot of cell appearances. These results can be derived using the manual methods and calculations described in Chapter 14 or using the automated instruments described in Chapter 15. Regardless of method, the numerical values should be consistent with the assessment derived by examining the cells microscopically. Careful examination of the data in a systematic way ensures that all relevant results are noted and taken into consideration in the diagnosis.

This chapter begins with a discussion of the preparation and assessment of the blood film, followed by a systematic approach to review of the CBC, including blood film evaluation. Such an evaluation can be applied in the hematology chapters that follow.

**PERIPHERAL BLOOD FILMS**

**Specimen Collection**

**Sources of Specimens**

Essentially all specimens received for routine testing in the hematology section of the laboratory have been collected in lavender (purple)–topped tubes (Chapter 3). These tubes contain disodium or tripotassium ethylenediaminetetraacetic acid (EDTA), which anticoagulates the blood by chelating the calcium that is essential for coagulation. Liquid tripotassium EDTA is often preferred to the powdered form because it mixes more easily with blood. High-quality blood films can be made from the blood in the EDTA tube, provided that they are made within 2 to 3 hours of drawing the specimen. Blood films from EDTA tubes that remain at room temperature for more than 5 hours often have unacceptable blood cell artifacts (echinocytic red blood cells [RBCs], spherocytes, necrobiotic leukocytes, and vacuolated neutrophils). Vacuolization of monocytes normally occurs almost immediately with EDTA but causes no evaluation problems.

The main advantages of making films from blood in the EDTA tube are that multiple slides can be made if necessary and they do not have to be prepared immediately after the blood is drawn. In addition, EDTA generally prevents platelets from clumping on the glass slide, which makes the platelet estimate more accurate during film evaluation. There are purists, however, who believe that anticoagulant-free blood is still the specimen of choice for evaluation of blood cell morphology. Although some artifacts can be avoided in this way, samples made from unanticoagulated blood pose other problems.

Under certain conditions, use of a different anticoagulant or no anticoagulant may be helpful. Some patients’ blood undergoes an in vitro phenomenon called platelet satellitosis when anticoagulated with EDTA. The platelets surround or adhere to neutrophils, which potentially causes pseudothrombocytopenia when counting is done by automated methods (Figure 16-1). In addition, spuriously low platelet counts and falsely increased WBC counts (pseudoleukocytosis) can result from EDTA-induced platelet clumping. Pseudoleukocytosis occurs when platelet agglutinates are similar in size to WBCs and automated analyzers cannot distinguish the two. The platelet clumps are counted as WBCs instead of platelets. Platelet-specific autoantibodies that react best at room temperature are one of the mechanisms known to cause this phenomenon. In these circumstances, the examination of a blood film becomes an important quality control strategy, identifying these phenomena so that they can be corrected before the results are reported to the patient's chart or health care provider.

Problems such as these can be eliminated by recollecting specimens in sodium citrate tubes (light blue top) and ensuring that the proper ratio of nine parts blood to one part anticoagulant is observed (a properly filled tube). These new specimens can be analyzed in the usual way by automated instruments. Platelet counts and WBC counts from sodium citrate specimens must be corrected for the dilution of blood with the anticoagulant, however. In a full-draw tube, the blood is nine tenths of the total tube volume (2.7 mL of blood and 0.3 mL of sodium citrate). The "dilution factor" is the reciprocal of the dilution (i.e., 10/9 or 1.1). The WBC and platelet counts are multiplied by 1.1 to obtain accurate counts. All other CBC parameters should be reported from the original EDTA tube specimen and slide.

Another source of blood for films is from finger and heel punctures. In general, the films are made immediately at the patient’s side. There are, however, a few limitations to this procedure. First, some platelet clumping must be expected if...
films are made directly from a drop of finger-stick or heel-stick blood or if blood is collected in heparinized microhematocrit tubes. Generally, this clumping is not enough to interfere with platelet estimates if the films are made promptly before clotting begins in earnest. Second, only a few films can be made directly from blood from a skin puncture before the site stops bleeding. If slides are made quickly and correctly, however, cell distribution and morphology should be adequate. These problems with finger and heel sticks can be eliminated with the use of EDTA microcollection tubes, such as Microtainer® Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) (Chapter 3).

Peripheral Film Preparation

Types of Films

Manual Wedge Technique. The wedge film technique is probably the easiest to master. It is the most convenient and commonly used method for making peripheral blood films. This technique requires at least two 3-inch × 1-inch (75-mm × 25-mm) clean glass slides. High-quality, beveled-edge microscopic slides with chamfered (beveled) corners for good lateral borders are recommended. A few more slides may be kept handy in case a good-quality film is not made immediately. One slide serves as the film slide, and the other is the pusher or spreader slide. They can then be reversed. It is also possible to make good wedge films by using a hemacytometer coverslip attached to a handle (pinch clip or tongue depressor) as the spreader.

A drop of blood (about 2 to 3 mm in diameter) from a finger, heel, or microhematocrit tube (nonheparinized for EDTA-anticoagulated blood or heparinized for capillary blood) is placed at one end of the slide. The drop also may be delivered using a Diff-Safe dispenser (Alpha Scientific Corporation, Malvern, PA). The Diff-Safe dispenser is inserted through the rubber stopper of the EDTA tube, which eliminates the need to remove the stopper. The size of the drop of blood is important: too large a drop creates a long or thick film, and too small a drop often makes a short or thin blood film. The pusher slide, held securely in the dominant hand at about a 30- to 45-degree angle (Figure 16-2, A), is drawn back into the drop of blood, and the blood is allowed to spread across the width of the slide (Figure 16-2, B). It is then quickly and smoothly pushed forward to the end of the slide to create a wedge film (Figure 16-2, C). It is important that the whole drop be picked up and spread. Moving the pusher slide forward too slowly accentuates poor leukocyte distribution by pushing larger cells, such as monocytes and granulocytes, to the very end and sides of the film. Maintaining an even, gentle pressure on the slide is essential. It is also crucial to keep the same angle all the way to the end of the film. When the hematocrit is higher than normal (i.e., >60%), as is found in patients with polycythemia or in newborns, the angle should be lowered (i.e., 25 degrees) so the film is not too short and thick. For extremely low hematocrits, the angle may need to be raised. If two or three films are made, the best one is chosen for staining, and the others are disposed of properly. Some laboratories require two good films and save one unstained in case another slide is required.

The procedure just described is for a push-type wedge preparation. It is called push because the spreader slide is pulled into the drop of blood, and the film is made by pushing the blood along the slide. The same procedure can be modified to produce a pulled film. In this procedure, the spreader slide is pushed into the drop of blood and pulled along the length of the slide to make the film. Although this method is much less commonly used, it also provides a satisfactory wedge preparation and may be easier for some individuals to perform. Other variations on the wedge technique include using the 3-inch side of the slide as the spreader slide or balancing the spreader slide on the fingers to avoid placing too much pressure on it. Learning to make consistently good blood films takes a lot of practice but can be accomplished if one is patient and persistent.

Features of a well-made wedge peripheral blood film

1. The film is two thirds to three fourths the length of the slide (Figure 16-3).
2. The film is finger shaped, very slightly rounded at the feather edge, not bullet shaped; this provides the widest area for examination.
3. The lateral edges of the film are visible.
4. The film is smooth without irregularities, holes, or streaks.
5. When the slide is held up to the light, the thin portion (feather edge) of the film has a "rainbow" appearance.
6. The whole drop of blood is picked up and spread.

Figure 16-4, A-H, shows unacceptable films.
Figure 16-5 Sysmex SP-10 (Sysmex America, Inc., Lincolnshire, IL) is an automated slide making and staining system.

Figure 16-3 Well-made peripheral blood film.

Figure 16-4 Unacceptable peripheral blood films. Slide appearances associated with the most common errors are shown, but note that a combination of causes may be responsible for unacceptable films. A, Chipped or rough edge on spreader slide. B, Hesitation in forward motion of spreader slide. C, Spreader slide pushed too quickly. D, Drop of blood too small. E, Drop of blood not allowed to spread across the width of the slide. F, Dirt or grease on the slide; may also be due to elevated lipids in the blood specimen. G, Uneven pressure on the spreader slide. H, Time delay; drop of blood began to dry.

Automated Slide Making and Staining. The Sysmex SP-10 (Sysmex Corporation of America, Mundelein, IL) is an automated slide-making and -staining system (Figure 16-5). After the instrument has performed a CBC for a specimen, a conveyor moves the racked tube to the SP-10, where the bar code is read. User-definable, onboard rules built into the system determine whether a slide is required. Criteria for a manual slide review are determined by each laboratory based on its patient population. Based on the hematocrit reading, the system adjusts the size of the drop of blood used and the angle and speed of the spreader slide in making a wedge preparation. After each blood film is prepared, the spreader slide is automatically cleaned and is ready for the next blood film to be made. Films can be produced approximately every 30 seconds. Patient identification information, such as name, number, and date for the specimen, is printed on the slide. The slide is dried, loaded into a cassette, and moved to the staining position. Based on the laboratory’s desired stain protocol, stain and then buffer and rinse are added at designated times. When staining is complete, the slide is moved to a dry position, then to a collection area where it can be picked up for microscopic evaluation. Films made off-line, such as bone marrow smears and cytospin preparations, may be stained using this system as well. Other blood analyzer manufacturers, such as Beckman Coulter (Brea, CA), also have automated slide making and staining instruments.

Drying of Films
Regardless of film preparation method, before staining, all blood films and bone marrow smears should be dried as quickly as possible to avoid drying artifact. In some laboratories, a small fan is used to facilitate drying. Blowing breath on a slide is counterproductive because the moisture in breath causes RBCs to become echinocytic (crenated) or to develop water artifact (also called drying artifact).

Staining of Peripheral Blood Films
Pure Wright stain or a Wright-Giemsa stain (Romanowsky stain)13 is used for staining peripheral blood films and bone marrow smears. These are considered polychrome stains because they contain both eosin and methylene blue. Giemsa stains also contain methylene blue azure. The purpose of staining blood films is simply to make the cells more visible and to allow their morphology to be evaluated. Consistent day-to-day staining quality is essential.

Methanol in the stain fixes the cells to the slide. Actual staining of cells or cellular components does not occur until the buffer is added. The oxidized methylene blue and eosin form a thiazine-eosinate complex, which stains neutral components. Because staining reactions are pH dependent, the buffer that is added to the stain should be 0.05 M sodium phosphate (pH 6.4) or aged distilled water (distilled water placed in a glass bottle for at least 24 hours; pH 6.4 to 6.8). Free methylene blue is basic and stains acidic (and basophilic) cellular components, such as ribonucleic acid (RNA). Free eosin is acidic and stains basic (and eosinophilic) components, such as hemoglobin and eosinophilic granules. Neutrophils are so named because they have cytoplasmic granules that have a neutral pH and pick up some staining characteristics from both stains. The slides must be completely dry before staining or the thick part of the blood film may come off the slide in the staining process.

Water or drying artifact has long been a nuisance to hematology laboratories. It has several appearances. It can give a
moth-eaten look to the RBCs, or it may appear as a heavily demarcated central pallor. It also may appear as refractive (shiny) blotches on the RBCs. Other times, it manifests simply as echinocytes (crenation) seen in the areas of the slide that dried most slowly.

Multiple factors contribute to this problem. Humidity in the air as the slide dries may add to the punched-out, moth-eaten, or echinocytic appearance of the RBCs. It is difficult to avoid drying artifact on films from extremely anemic patients because of the very high ratio of plasma to RBCs. Water absorbed from the air into the alcohol-based stain also can contribute. Drying the slide as quickly as possible helps, and keeping a stopper tightly on the stain bottle keeps moisture out. In some laboratories, slides are fixed in pure, anhydrous methanol before staining to help reduce water artifact. More recently, stain manufacturers have used 10% volume-to-volume methanol to minimize water or drying artifact.

**Wright Staining Methods**

**Manual Technique.** Traditionally, Wright staining has been performed over a sink or pan with a staining rack. Slides are placed on the rack, film side facing upward. The Wright stain may be filtered before use or poured directly from the bottle through a filter onto the slide. It is important to flood the slide completely. The stain should remain on the slide at least 1 to 3 minutes to fix the cells to the glass. Then an approximately equal amount of buffer is added to the slide. Surface tension allows very little of the buffer to run off. A metallic sheen (or green “scum”) should appear on the slide if mixing is correct (Figure 16-6). More buffer can be added if necessary. The mixture is allowed to remain on the slide for 3 minutes or more (bone marrow smears take longer to stain than peripheral blood films). The timing may be adjusted to produce the best staining characteristics. When staining is complete, the slide is rinsed with a steady but gentle stream of neutral-pH water, the back of the slide is wiped to remove any stain residue, and the slide is air-dried in a vertical position.

Use of the manual Wright staining technique is desirable for staining peripheral blood films containing very high WBC counts, such as the films from leukemic patients. As with bone marrow smears, the time can be easily lengthened to enhance the staining required by the increased numbers of cells. Understaining is common when a leukemia slide is placed on an automated slide stainer. The main disadvantages of the manual technique are the increased risk of spilling the stain and the longer time required to complete the procedure. This technique is best suited for low-volume laboratories.

**Automated Slide Stainers.** Numerous automated slide stainers are commercially available. For high-volume laboratories, these instruments are essential. Once they are set up and loaded with slides, staining proceeds without operator attention. In general, it takes about 5 to 10 minutes to stain a batch of slides. The processes of fixing/staining and buffering are similar in practice to those of the manual method. The slides may be automatically dipped in stain and then in buffer and a series of rinses (Midas III, EM Science, Gibbstown, NJ) (Figure 16-7) or propelled along a platen surface by two conveyor spirals (Hema-Tek, Siemens Healthcare Diagnostics, Inc., Deerfield, IL) (Figure 16-8). In the Hema-Tek device, stain, buffer, and rinse are pumped through holes in the platen surface, flooding the slide at the appropriate time. Film quality and color consistency are usually good with any of these
instruments. Some commercially prepared stain, buffer, and rinse packages do vary from lot to lot or manufacturer to manufacturer, so testing is recommended. Some disadvantages of the dip-type batch stainers are (1) stat slides cannot be added to the batch once the staining process has begun, and (2) working or aqueous solutions of stain are stable for only 3 to 6 hours and need to be made often. Stat slides can be added at any time to the Hema-Tek stainer, and stain packages are stable for about 6 months.

**Quick Stains.** Quick stains, as the name implies, are fast and easy. The whole process takes about 1 minute. The stain is purchased in a bottle as a modified Wright or Wright-Giemsa stain. The required quantity can be filtered into a Coplin jar or a staining dish, depending on the quantity of slides to be stained. Aged, distilled water is used as the buffer. Stained slides are given a final rinse under a gentle stream of tap water and allowed to air-dry. It is helpful to wipe off the back of the slide with alcohol to remove any excess stain. Quick stains are convenient and cost effective for low-volume laboratories, such as clinics and physician office laboratories, or whenever rapid turnaround time is essential. Quality is often a concern with quick stains. With a little time and patience in adjusting the staining and buffering times, however, color quality can be acceptable.

**Features of a Well-Stained Peripheral Blood Film.** Properly staining a peripheral blood film is just as important as making a good film. Macroscopically, a well-stained blood film should be pink to purple. Microscopically, the RBCs should appear orange to salmon pink, and WBC nuclei should be purple to blue. The cytoplasm of neutrophils should be pink to tan with violet or lilac granules. Eosinophils should have bright orange refractile granules. Faulty staining can be troublesome for reading the films, causing problems ranging from minor shifts in color to the inability to identify cells and assess morphology. Trying to interpret a poorly prepared or poorly stained blood film is extremely frustrating. If possible, a newly stained film should be studied. Hints for troubleshooting poorly stained blood films are provided in Box 16-1.

The best staining results are obtained on fresh slides because the blood itself acts as a buffer in the staining process. Slides stained after 1 week or longer turn out too blue. In addition, specimens that have increased levels of proteins (i.e., globulins) produce bluer-staining blood films, even when freshly stained.

**Peripheral Film Examination**
Microscopic blood film review is essential whenever instrument analysis indicates that specimen abnormalities exist. The laboratory professional evaluates the platelet and WBC count and differential, along with WBC, RBC, and platelet morphology.

**Macroscopic Examination**
Examining the film before placing it on the microscope stage sometimes can give the evaluator an indication of abnormalities or test results that need rechecking. For example, a film that is bluer overall than normal may indicate that the patient has increased blood proteins, as in plasma cell myeloma, and that rouleaux may be seen on the film. A grainy appearance to the film may indicate RBC agglutination, as found in cold hemagglutinin diseases. In addition, holes all over the film could mean that the patient has increased lipid levels, and some of the automated CBC parameters should be rechecked for interferences from lipemia. Markedly increased WBC counts and platelet counts can be detected from the blue specks out at the feather edge. Valuable information might be obtained before the evaluator looks through the microscope.

**Microscopic Examination**
The microscope should be adjusted correctly for blood film evaluation. The light from the illuminator should be properly centered, the condenser should be almost all the way up and adjusted correctly for the magnification used, and the iris diaphragm should be opened to allow a comfortable amount of light to the eye. Many individuals prefer to use a neutral density filter over the illuminator to create a whiter light from a tungsten light source. If the microscope has been adjusted for Koehler illumination, all these conditions should have been met (Chapter 4).

**10× Objective Examination.** Blood film evaluation begins using the 10× or low-power objective lens (total magnification = 100×). Not much time needs to be spent at this magnification. However, it is a common error to omit this step altogether and go directly to the higher-power oil immersion lens. At the low-power magnification, overall film quality, color, and distribution of cells can be assessed. The feather edge and lateral edges should be checked quickly for WBC

<table>
<thead>
<tr>
<th>BOX 16-1 Troubleshooting Poorly Stained Blood Films</th>
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<tr>
<td><strong>First Scenario</strong></td>
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<tr>
<td><strong>Problems</strong></td>
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<tr>
<td>RBCs appear gray.</td>
</tr>
<tr>
<td>WBCs are too dark.</td>
</tr>
<tr>
<td>Eosinophil granules are gray, not orange.</td>
</tr>
<tr>
<td><strong>Causes</strong></td>
</tr>
<tr>
<td>Stain or buffer too alkaline (most common)</td>
</tr>
<tr>
<td>Inadequate rinsing</td>
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<td>Prolonged staining</td>
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<td>Heparinized blood sample</td>
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<tr>
<th><strong>Second Scenario</strong></th>
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</thead>
<tbody>
<tr>
<td><strong>Problems</strong></td>
</tr>
<tr>
<td>RBCs are too pale or are red.</td>
</tr>
<tr>
<td>WBCs are barely visible.</td>
</tr>
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<td><strong>Causes</strong></td>
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<tr>
<td>Stain or buffer too acidic (most common)</td>
</tr>
<tr>
<td>Underbuffering (too short)</td>
</tr>
<tr>
<td>Over-rinsing</td>
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RBC, Red blood cell; WBC, white blood cell.
distribution. The presence of more than four times the number of cells per field at the edges or feather compared with the monolayer area of the film indicates that the film is unacceptable (i.e., a “snowplow” effect), and the film should be remade. Under the 10× objective, it is possible to check for the presence of fibrin strands; if they are present, the sample should be rejected, and another one should be collected. RBC distribution can be noted as well. Rouleaux formation or RBC agglutination is easy to recognize at this power. The film can be scanned quickly for any large abnormal cells, such as blasts, reactive lymphocytes, or even unexpected parasites. Finally, the area available for suitable examination can be assessed.

**40× High-Dry or 50× Oil Immersion Objective Examination.** The next step is using the 40× high-dry objective lens (total magnification = 400×) or, as many laboratories now use, a 50× oil immersion objective instead. At either of these magnifications, it is easy to select the correct area of the film in which to begin the differential count and to evaluate cellular morphology. The WBC estimate also can be performed at these powers. To perform a WBC estimate, the evaluator selects an area in which the RBCs are separated from one another with minimal overlapping (where only two or three RBCs can overlap). Depending on which lens is used (40× or 50×), the procedure is the same; only the multiplication factor changes. Count the WBCs in 10 fields and find the average number of WBCs/field. The average number of WBCs per high-power field (40×) is multiplied by 2000 (if a 50× oil lens is used, multiply by 3000) to get an approximation of the WBC count per mm3 or 109/L.

**Example:** If an average of three WBCs were observed per field:

- Using the 40× high-dry objective, the WBC estimate is 6000/μL or 6.0 × 10⁹/L.
- Using a 50× oil immersion objective, the WBC estimate is 9000/μL or 9.0 × 10⁹/L.

**100× Oil Immersion Objective Examination.** The 100× oil immersion objective provides the highest magnification on most standard binocular microscopes (10× eyepiece × 100× objective lens = 1000× magnification). The WBC differential count generally is performed using the 100× oil immersion objective. Performing the differential normally includes counting and classifying 100 WBCs to obtain percentages of WBC types. The RBC, WBC, and platelet morphology evaluation and the platelet estimate also are executed under the 100×

**BOX 16-2 Performing a White Blood Cell Estimate**

1. Select an area of the blood film in which most RBCs are separated from one another with minimal overlapping of RBCs.
2. Using the 40× high-dry or 50× oil immersion objective, count the number of WBCs in 10 consecutive fields, and calculate the average number of WBCs per field.
3. To obtain the WBC estimate per μL of blood, multiply the average number of WBCs per field by 2000* (if using the 40× high-dry objective) or 3000* (if using the 50× oil immersion objective).
4. Compare the instrument WBC count with the WBC estimate from the blood film.

**Example:** If an average of three WBCs were observed per field:

- Using a 40× high-dry objective, the WBC estimate is 6000/μL or 6.0 × 10⁹/L.
- Using a 50× oil immersion objective, the WBC estimate is 9000/μL or 9.0 × 10⁹/L.

**BOX 16-3 Validation of the White Blood Cell and Platelet Estimation Factor**

1. Perform automated white blood cell (WBC) and platelet (PLT) counts on 30 consecutive fresh patient blood specimens. Make sure the counts are in control.
2. Prepare and stain one peripheral blood film for each specimen.
3. Using the 40× high-dry or 50× oil immersion objective for the WBC estimate and the 100× oil immersion objective for the PLT estimate, select an area of the blood film in which most RBCs are separated from one another with minimal overlapping of RBCs.
4. Count the number of WBCs or PLTs in 10 consecutive fields using the magnification specified in step 3, and calculate the average number of WBCs and PLTs per field.
5. For each of the 30 specimens, divide the automated WBC count by the average number of WBCs per field (40× or 50×); divide the automated PLT count by the average number of PLTs per field (100×).
6. Add the numbers obtained in step 5 for the WBCs, and divide by 30 (the number of observations in this analysis) to obtain the average ratio of the WBC count-to-WBCs per 40× or 50× field; add the numbers obtained in step 5 for the PLTs, and divide by 30 (the number of observations in this analysis) to obtain the average ratio of the PLT count-to-PLTs per 100× field.
7. Round the number calculated in step 6 to the nearest whole number to obtain an estimation factor for WBCs and PLTs at the specified magnification.

**NOTE:** Because of the variation in the field diameter among different microscopes, an estimation factor should be determined for each microscope in use, and that number should be used to obtain the WBC and PLT estimates when using that microscope.

Oil immersion objective lens. At this magnification segmented neutrophils can be readily differentiated from bands. RBC inclusions, such as Howell-Jolly bodies, and WBC inclusions, such as Döhle bodies, can be seen easily if present. Reactive or abnormal cells are enumerated under the 100× objective as well. If present, nucleated RBCs (NRBCs) are counted and reported as NRBCs per 100 WBCs (Chapter 14).

**50× Oil Immersion Objective Examination.** The WBC differential and morphology examinations described for the 100× oil immersion objective also can be accomplished by experienced morphologists using a 50× oil immersion objective. The larger field of view allows more cells to be evaluated faster. Examination at this power is especially efficient for validating or verifying instrument values when a total microscopic assessment of the film is not needed. Particular cell features that may require higher magnification can be assessed by moving the parfocal 100× objective into place and then returning to the 50× objective to continue the differential. As previously mentioned, the WBC estimate also can be performed with the 50× objective, but the multiplication factor is 3000. The observer should conform to the estimation protocol of the particular laboratory.

**Optimal Assessment Area.** The tasks described, especially for the 100×, 40×, and 50× objectives, need to be performed in the best possible area of the peripheral blood film. That occurs between the thick area, or “heel,” where the drop of blood was initially placed and spread, and the very thin feather edge. In the ideal area, microscopically, the RBCs are uniformly and singly distributed, with few touching or overlapping, and have their normal biconcave appearance (central pallor) (Figure 16-9, A). An area that is too thin, in which there are holes in the film or the RBCs look flat, large, and distorted, is unacceptable (Figure 16-9, B). A too-thick area also distorts the RBCs by piling them on top of one another like rouleaux (Figure 16-9, C). WBCs are similarly distorted, which makes morphologic evaluation more difficult and classification potentially incorrect. When the correct area of a specimen from a patient with a normal RBC count is viewed, there are generally about 200 to 250 RBCs per 100× oil immersion field.

Although already discussed in Chapter 4, a common problem encountered with the oil immersion objective lens is worth mentioning again. If the blood film was in focus under the 10× and 40× objectives but is impossible to bring into focus under the 100× objective, the slide is probably upside down. The 100× objective does not have sufficient depth of field to focus through the slide. The oil must be completely removed before the film is put on the stage right-side up.

**Performance of the White Blood Cell Differential.** Fewer manual differentials are performed today because of the superior accuracy of automated differentials and because of cost and time constraints. When indicated, however, the manual differential always should be performed in a systematic manner. When the correct area has been selected, use of a back-and-forth serpentine, or “battlement,” track pattern is preferred to minimize distribution errors (Figure 16-10) and ensure that each cell is counted only once. One hundred WBCs are counted and classified through the use of push-down button counters (Figure 16-11, A) or newer computer-interfaced key pads (Figure 16-11, B). To increase accuracy, it is advisable to count at least 200 cells when the WBC count is higher than 40 × 10^9/L. If the WBC count is 100 × 10^9/L or greater, it would be more precise and accurate to count 300 or 400 cells. Results are reported as percentages—for example, 54% segmented neutrophils, 6% bands, 28% lymphocytes, 9% monocytes, 3% eosinophils. The evaluator always should check to ensure that the sum of the percentages is 100.
Performing 100-cell differentials on extremely low WBC counts can become tedious and time consuming, even when the 50× oil immersion objective is used. In some laboratories, the WBCs are concentrated by centrifugation, and buffy coat smears are made. This practice is helpful for examining the morphology of the cells; however, it is not recommended for performing differentials because of possible errors in cell distribution from centrifugation. In other laboratories evaluators may perform a 50-cell differential and multiply the results by 2 to get a percentage. The accuracy of this practice is questionable, and it should be avoided if possible. In some laboratories the buffy coat smear is examined for the presence of blasts, but no differential is performed. It is essential to include the side margins of the blood film in any differential so that the larger cells, such as monocytes, reactive lymphocytes, and immature cells, are not missed.

In addition to counting the cells, the evaluator assesses their appearance. If present, WBC abnormalities such as toxic granulation, Döhle bodies, reactive lymphocytes, and Auer rods (Chapters 29 and 35) are evaluated and reported. Unfortunately, the exact method by which these are reported varies from laboratory to laboratory. Reactive lymphocytes may be reported as a separate percentage of the 100 cells, as a percentage of the total number of lymphocytes, or semiquantitatively ("occasional" to "many"). Toxic granulation generally is reported as "present" or is sometimes reported semiquantitatively ("slight" to "marked," or 1+ to 3+). Standardization of this process has been difficult, but laboratorians must look to simplify blood film morphology reports to help ensure better accuracy, consistency, and clinical relevance. Simply reporting "present" is becoming preferable to the older "semiquantitative" reporting of morphology abnormalities. Regardless of reporting format, each laboratory should establish criteria for reporting microscopic cell morphology.

Because the differential alone provides only partial information, reported in relative percentages, the absolute cell counts are calculated for each cell type in some laboratories. Automated differentials already include this information.

Automation has also been applied to the processes of microscopic cell location and identification. These automated systems are especially dependent on the quality and consistency of the blood film and stain in order for the digital systems to recognize and identify the cells. Once cells are located, a digital image is made and the cell is classified using sophisticated computerized visual recognition systems. Digital images of the classified cells are presented to the operator, who can override the instrument's identification, if needed, or add identifications for cells that the instrument could not classify (Figure 16-12).
**Red Blood Cell Morphology.** Evaluation of RBC morphology is an important part of the blood film examination and includes an assessment of cell size (microcytosis, macrocytosis), variability in size (anisocytosis), cell color (hypochromia), cell shape (poikilocytosis), and cellular inclusions (Chapter 19). Some laboratories use specific terminology for reporting the degree of abnormal morphology, such as “slight,” “moderate,” or “marked,” or use a scale from 1+ to 3+. Other laboratories more recently have gone to a more simple report, using the term *present* for morphologic abnormalities that are clinically significant. Still other laboratories provide a summary statement regarding the overall RBC morphology that is consistent with the RBC indices and histogram. The latter methods are becoming more popular with the increased computer interfacing in most laboratories. Regardless of the reporting method used, the microscopic RBC morphology assessment should be congruent with the information given by the automated hematology analyzer. If not, further investigation is needed.

**Platelet Estimate.** As previously mentioned, the platelet estimate is performed under the 100× oil immersion objective lens. In an area of the film where the RBCs barely touch, with a few overlapping, the number of platelets in 10 oil immersion fields is counted. The average number of platelets per oil immersion field times 20,000 approximates the platelet count per μL or mm³. For example, 12 to 16 platelets per oil immersion field equals about 280,000 platelets/μL or mm³ (280 × 10⁹/L) and is considered adequate. Box 16-4 contains a summary of the procedure for the platelet estimate. In situations in which the patient is anemic or has erythrocytosis, however, the relative proportion of platelets to RBCs is altered. In these instances, a more involved formula for platelet estimates may be used:

\[
\frac{\text{Average no. of platelets/field} \times \text{total RBC count}}{200 \text{ RBCs/field}}
\]

**BOX 16-4 Performing a Platelet Estimate**

1. Select an area of the blood film in which most RBCs are separated from one another with minimal overlapping of RBCs.
2. Using the 100× oil immersion objective, count the number of platelets in 10 consecutive fields, and calculate the average number of platelets per field.
3. To obtain the platelet estimate per μL of blood, multiply the average number of platelets per field by 20,000.°
4. Compare the instrument platelet count with the platelet estimate from the blood film.

Example: If an average of 20 platelets were observed per 100× oil immersion field, the platelet estimate is 400,000/μL or mm³ (400 × 10⁹/L).

In instances of significant anemia or erythrocytosis, use the following formula for the platelet estimate:

\[
\frac{\text{Average no. of platelets/field} \times \text{total RBC count}}{200 \text{ RBCs/field}}
\]

°A platelet estimation factor of 20,000 is provided as a general guideline. A platelet estimation factor should be determined and validated for each microscope in use.

Regardless of whether or not an “official” estimate is made, verification of the instrument platelet count should be included in the overall examination for internal quality control purposes. Blood film examination also includes an assessment of the morphology of the platelets, including size as well as granularity and overall appearance.

As mentioned in Chapter 4, and worth mentioning again, immersion oils with different viscosities do not mix well. If slides are taken to another microscope for review, oil should be wiped off first.

**SUMMARIZING COMPLETE BLOOD COUNT**

To this point, this chapter has focused on slide preparation and performance of a differential cell count. The differential is only the capstone, however, of a panel of tests collectively called the complete blood count, or CBC, that includes many of the routine tests described in Chapter 14. Now that the testing for the component parts has been described, interpretation of the results for the total panel can be discussed.

The CBC has evolved over time to the typical test panel reported today, including assessment of WBCs, RBCs, and platelets. The CBC provides information about the hematopoietic system, but because abnormalities of blood cells can be caused by diseases of other organ systems, the CBC also plays a role in screening of those organs for disease. The CBC provides such valuable information about a patient’s health status that it is among the most frequently ordered laboratory tests performed by medical laboratory scientists and laboratory technicians.

The process of interpreting the CBC test results has two phases. In phase 1, the numbers and descriptions generated by the testing are summarized using appropriate terminology. This summary provides a verbal picture of the numbers that is easy to communicate to the physician, other health care provider, or another laboratorian. It is much more convenient to be able to say, “The patient has a microcytic anemia” than to say, “The hemoglobin was low, and the mean cell volume was also low.” Phase 2 of interpretation is to recognize a pattern of results consistent with various diseases and to be able to narrow the diagnosis for the given patient or perhaps even to pinpoint it so that appropriate follow-up testing or treatment can be recommended.

The following discussion focuses on phase 1 of CBC interpretation—how to collect the pertinent information and summarize it. Phase 2 of the interpretation is the essence of the remaining chapters of this book on various hematologic conditions or other metabolic conditions that have an impact on the hematologic system.

**Organization of Complete Blood Count Results**

Today, most laboratorians perform CBCs using sophisticated automated analyzers as described in Chapter 15, but the component tests can be performed using the manual methods described in Chapter 14. The blood film assessment described
in this chapter is also part of a CBC. As previously mentioned, the CBC “panel” is essentially divided into WBC, RBC, and platelet parameters.

For phase 2 interpretation, it is sometimes important to look at all three groupings of the CBC results; at other times, only one or two may be of interest. If a patient has an infection, the WBC parameters may be the only ones of interest. If the patient has anemia, all three sets—WBCs, RBCs, and platelets—may require assessment. Generally, all the parameters interpreted together provide the best information, so a complete summary of the results should be generated.

**Assessing Hematology Results Relative to Reference Intervals**

Proper performance of the phase 1 summary of test results requires comparison of the patient values with the reference intervals. The table of reference intervals for the CBC on the inside cover of this text shows that there are different reference intervals for men and women, particularly for the RBC parameters. There are also different intervals for children of different ages, with the WBC changes the most notable. It is important to select the appropriate set of reference intervals in hematology for the gender and age of the patient.

CBC results can be reported in standard international (SI) units or in common units. For example the following results in SI units (WBC count = $7.2 \times 10^9$/L, RBC count = $4.20 \times 10^{12}$/L, HGB = 128 g/L, HCT = 0.41 L/L, and PLT = $237 \times 10^9$/L) are equivalent to the following results in common units (WBC count = $7.2 \times 10^5$/μL, RBC count = $4.20 \times 10^{9}$/μL, HGB = 128 g/dL, HCT = 41%, and PLT = $237 \times 10^3$/μL). The older mm$^3$ for cell count units is equivalent to μL. (Chapter 14). Since either system may be used on laboratory reports, the laboratory should be able to easily interconvert CBC results between the systems. SI units are used for the cell counts in this chapter.

Several strategies can help in determining the significance of the results. First, if the results are very far from the reference interval, it is more likely that they are truly outside the interval and represent a pathologic process. Second, if two or more diagnostically related parameters are slightly or moderately outside the interval in the same direction (both high or both low), this suggests that the results are clinically significant and associated with some pathologic process. Because some healthy individuals always have results slightly outside the reference interval, the best comparison for their results is not the reference interval but their own results from a prior time when they were known to be healthy.

**Summarizing White Blood Cell Parameters**

The WBC-related parameters of a routine CBC include the following:

1. Total WBC count (WBCs $\times 10^9$/L)
2. WBC differential count values expressed as percentages, called *relative counts*
3. WBC differential count values expressed as the actual number of each type of cell (e.g., neutrophils $\times 10^9$/L), called *absolute counts*
4. WBC morphology

- **Step 1**
  Start by ensuring that there is an accurate WBC count. Compare the WBC histogram and/or scatterplot to the respective cell counts to make sure they correlate with one another. Today’s automated instruments are able to eliminate nucleated RBCs that falsely increase the WBC count. However, manual WBC results must be corrected mathematically to eliminate the contribution of the NRBC (Chapter 14).

- **Step 2**
  Look at the total WBC count. When the count is elevated, it is called leukocytosis. When the WBC count is low, it is called leukopenia. As described later, increases and decreases of WBCs are associated with infections and conditions such as leukemias. Because there is more than one type of WBC, increases and decreases in the total count are usually due to changes in one of the subtypes—for example, neutrophils or lymphocytes. Determining which one is the next step.

- **Step 3**
  Examine the relative differential counts for a preliminary assessment of which cell lines are affected. The relative differential count is reported in percentages. The proportion of each cell type can be described by its relative number (i.e., percent) and compared with its reference interval. Then it is described using appropriate terminology, such as a relative neutrophilia, which is an increase in neutrophils, or a relative lymphopenia, which is a decrease in lymphocytes. The terms used for increases and decreases of each cell type are provided in Table 16-1.

If the total WBC count or any of the relative values are outside the reference interval, further analysis of the WBC differential is needed. If the proportion of one of the cell types increases, then the proportion of others must decrease because the proportions are relative to one another. The second cell type may not have changed in actual number at all, however. The way to assess this accurately is with absolute differential counts.

- **Step 4**
  If not reported by the instrument, absolute counts can be calculated easily using the total WBC count and the relative differential. Multiply each relative cell count (i.e., percentage) by the total WBC count and by so doing determine the absolute count for each cell lineage.

### **TABLE 16-1 Terminology for Increases and Decreases in White Blood Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Increases</th>
<th>Decreases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>Neutrophilia</td>
<td>Neutropenia</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Eosinophilia</td>
<td>N/A</td>
</tr>
<tr>
<td>Basophil</td>
<td>Basophilia</td>
<td>N/A</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>Lymphocytosis</td>
<td>Lymphopenia (lymphocytopenia)</td>
</tr>
<tr>
<td>Monocyte</td>
<td>Monocytosis</td>
<td>Monocytopenia</td>
</tr>
</tbody>
</table>

N/A, Not applicable because the reference interval begins at or near zero.
Examine the set of WBC parameters from a CBC shown in Table 16-2. On first inspection, one may look at the WBC count and recognize that a leukocytosis is present, but it is important to determine what cell line is causing the increased count. In this case, the cells are all within reference intervals relative to one another. There is no indication as to which cell line could be causing the increase in total numbers of WBCs.

When each relative number (e.g., neutrophils at 0.67 or 67%) is multiplied by the total WBC count (13.6 \( \times 10^9/L \)), the absolute numbers indicate that the neutrophils are elevated (9.1 \( \times 10^9/L \) compared with the reference interval provided). The acronym for absolute neutrophil count is ANC. The ANC is a very useful parameter for assessing neutropenia and neutrophilia. The absolute lymphocyte count (3.5 \( \times 10^9/L \)) is still within the reference interval. Given this information, these results can be described as showing a leukocytosis with only an absolute neutrophilia, and the overall increase in the WBC count is due to an increase only in neutrophils. This description provides a concise summary of the WBC counts without the need to refer to every type of cell.

Box 16-5 extends this concept to a convention used to describe the neutrophilic cells, and Box 16-6 addresses the clinical utility of reporting % bands as a separate category.

When the absolute numbers of each of the individual cell types are totaled, the sum equals the WBC count (slight differences may occur due to rounding, as in the example). This is a method for checking whether the absolute calculations are correct. Absolute counts may be obtained directly from automated analyzers, which count actual numbers (i.e., produce absolute counts) and calculate relative values. Some laboratories do not report the absolute counts, so being able to calculate them is important.

As will be evident in later chapters, the findings in this particular example point toward a bacterial infection. Had there been an absolute lymphocytosis, a viral infection would be likely.

### BOX 16-5 Summarizing Neutrophilia When Young Cells Are Present

A subtle convention in assessing the differential counts has to do with the presence of young cells of the neutrophilic series (e.g., bands). They typically are grouped together with the mature neutrophils in judging whether neutrophilia is present. For example, look at the following differential and the reference intervals provided in parentheses:

- White blood cells—10.8 \( \times 10^9/L \)
- Neutrophils (also called segmented neutrophils)—65 (48% to 70%)
- Bands—18 (0% to 10%)
- Lymphocytes—13 (18% to 42%)
- Monocytes—3 (1% to 10%)
- Eosinophils—1 (1% to 4%)
- Basophils—0 (0% to 2%)

Although the mature neutrophils are within the reference interval, the bands exceed their reference interval. The total of the two, 83 (65% + 18%), exceeds the upper limit of neutrophilic cells even when the two intervals are combined, 80 (70% + 10%), so these results would be described as a neutrophilia even though the neutrophil value itself is within the reference interval. See steps 4 and 5 under Summarizing White Blood Cell Parameters for how to communicate the increase in bands.

### Step 5

Each cell line should be examined for immature cells. Young WBCs are not normally seen in the peripheral blood, and they may indicate infections or malignancies such as leukemia. For neutrophilic cells, there is a unique term that refers to the presence of increased numbers of bands or cells younger than bands in the peripheral blood: left shift or shift to the left (Box 16-7).
When young lymphocytic or monocytic cells are present, they can be reported in the differential as prolymphocytes, lymphoblasts, promonocytes, or monoblasts. When observed, young eosinophils and basophils are typically just called immature and are not specifically staged. For example, eosinophilic metamyelocytes are counted as eosinophils.

**Step 6**
Any abnormalities of appearance are reported in the morphology section of the report. For WBCs, abnormal morphologic features that would be noted include changes in overall cellular appearance, such as cytoplasmic toxic granulation and nuclear abnormalities such as hypersegmentation. The clinical significance of these types of changes is discussed in Chapter 29.

To summarize the WBC parameters, begin with an accurate total WBC count, followed by the relative differential, or preferably the absolute counts, noting whether any abnormal young cells are present in the blood. Finally, note the presence of any abnormal morphology or inclusions.

**Summarizing Red Blood Cell Parameters**
RBC parameters are as follows:
1. RBC count (RBCs \( \times 10^{12} \) /L)
2. HGB (g/dL)
3. HCT (% or L/L)
4. Mean cell volume (MCV, fl)
5. Mean cell hemoglobin (MCH, pg)
6. Mean cell hemoglobin concentration (MCHC, g/dL)
7. RBC distribution width (RDW, %)
8. Morphology

**Step 1**
Examine the hemoglobin (or hematocrit) for anemia or polycythemia. Anemia is the more common condition. If the RBC morphology is relatively normal, three times the hemoglobin approximates the hematocrit; this is called the rule of three (Chapter 14). If the rule of three holds, the expectation is that the following assessments will find normal RBC parameters. If the rule of three fails and all test results are reliable, further assessment should uncover some patient RBC abnormalities. Remember that the rule of three only holds true when overall red blood cell morphology is normocytic and normochromic.

Hemoglobin concentration (HGB) is a more reliable indicator of anemia than is the hematocrit, because the hematocrit can be influenced by the size of the RBCs. Hemoglobin concentration is a more direct indicator of the ability of the blood to carry oxygen.

**Step 2**
When the hemoglobin and hematocrit values have been inspected and the rule of three applied, the next RBC parameter that should be evaluated is the MCV (Chapter 14). This value provides the average RBC volume. The MCV should be correlated with the RBC histogram from the instrument and morphologic appearance of the cells using the classification first introduced by Wintrobe a century ago (Table 16-3).

The MCV is expected to be within the established reference interval (approximately 80 to 100 fl), and the RBC histogram and morphology are expected to be normal (normocytic). For a patient with anemia, classifying the anemia morphologically by the MCV narrows the range of possible causes to microcytic, normocytic, or macrocytic anemias (Chapter 19).

**Step 3**
Examine the MCHC to evaluate how well the cells are filled with hemoglobin. Remember that MCHC is a concentration and takes into consideration the volume of the red blood cells when considering decreased or normal color. If the MCHC is within the reference interval, the cells are considered normal or normochromic and display typical central pallor of one third the volume of the cell. If the MCHC is below the reference interval, the cells are called hypochromic, which literally means “too little color.” This correlates with a larger central pallor (hypochromia) when the cells are examined on a Wright-stained blood film.

It is possible for the MCHC to be elevated in two situations, but this does not correlate with hyperchromia. In fact, the term hyperchromia is never used. A slight elevation may be seen when

<table>
<thead>
<tr>
<th>Mean Cell Volume Value</th>
<th>Wintrobe Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within reference interval (80–100 fl)</td>
<td>Normocytic</td>
</tr>
<tr>
<td>Lower than reference interval (&lt;80 fl)</td>
<td>Microcytic</td>
</tr>
<tr>
<td>Higher than reference interval (&gt;100 fl)</td>
<td>Macrocytic</td>
</tr>
</tbody>
</table>

**TABLE 16-3 Interpretation of Mean Cell Volume Values Using the Wintrobe Terminology**

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**BOX 16-6 Clinical Utility of Band Counts**
An elevated band count was thought to be useful in the diagnosis of patients with infection. However, the clinical utility of band counts has been called into question, and most laboratories no longer perform routine band counts. The Clinical and Laboratory Standards Institute (CLSI) recommends that bands and neutrophils be counted together and placed in a single category rather than in separate categories because it is difficult to reliably differentiate bands from segmented neutrophils.

**BOX 16-7 Origin of the Phrase Left Shift**
The origin of the phrase left shift is a 1920s publication by Josef Arneth in which neutrophil maturity was correlated with segment count. A graphical representation was made, and the fewer the segments, the farther left was the median—hence left shift. This was called the Arneth count or Arneth-Schilling count and was abandoned around the time Arneth died in 1955, but the term left shift lived on to describe increased numbers of immature cells as an indicator of infection.
cells are spherocytic (MCHC over 36 g/dL). They retain roughly normal volume but have decreased surface area. So the hemoglobin is slightly more concentrated than usual, and the cells look darker with no central pallor. A more dramatic increase in MCHC (values even as high as 60) can be caused by analytical problems, often associated with patient sample problems that falsely elevate hemoglobin measurement. Common problems of this type include interference from lipemia, icterus, or grossly elevated WBC counts. Each of these interferes with the spectrophotometric measurement of hemoglobin, thus falsely elevating the hemoglobin and affecting the calculation of the MCHC (Chapter 14). It is worth noting that the MCHC is often best used as an internal quality control parameter.

**Step 4**

The RDW is determined from the histogram of RBC volumes. Briefly, when the volumes of the RBCs are about the same, the histogram is narrow (Figure 16-13, A). If the volumes are variable (more small cells, more large cells, or both), the histogram becomes wider. The width of the histogram, the RDW, is reflected statistically as a coefficient of variation (CV) or a standard deviation (SD). Most analyzer manufacturers provide a CV and an SD, and the operator can select which to report. Figure 16-13, A-D, depicts RBC histograms demonstrating normal, microcytosis, macrocytosis, anisocytosis, and dimorphic red cell populations.

Therefore, the RDW provides information about the presence and degree of anisocytosis (variation in RBC volume). What is important is increased values only, not decreased values. If an RDW-CV reference interval is 11.5% to 14.5% and a patient has an RDW-CV of 20.6%, the patient has a more heterogeneous RBC population with more variation in cell volume (anisocytosis). If the RDW is elevated, a notation about anisocytosis is expected in the morphologic evaluation of the blood film. Using the MCV along with the RDW provides the most helpful information (Chapter 19).

**Step 5**

Examine the morphology for pertinent abnormalities. Whenever anemia is indicated by the RBC parameters reported by the analyzer, and potential abnormal RBC morphology is suggested by the indices and the rule of three, a Wright-stained peripheral blood film must be reviewed. Abnormalities include abnormal volume, abnormal shape, inclusions, immature RBCs, abnormal color, and abnormal arrangement (Chapter 19). The blood film also serves as quality control, because the morphologic characteristics seen through the microscope (e.g., microcytosis, anisocytosis) should be congruent with the results provided by the analyzer. When they do not agree, further investigation is necessary.

If everything in the morphology is normal, by convention, no notation regarding morphology is included. Therefore, any notation in the morphology section requires scrutiny. All RBC-related abnormal morphologic findings should be noted, including specific poikilocytosis (abnormal shape) and the presence of RBC inclusions, such as Howell-Jolly bodies. One of the major challenges is in determining when the amount or degree of an abnormality is worth noting at all (i.e., when it should be reported). Laboratories strive for standardization of reporting. All laboratories must have good standardized morphology criteria and competent staff whose evaluations are consistent with one another and with the standardized criteria used in that facility. Generally a semiquantitative method is used that employs terms such as slight, moderate, and marked or the numbers 1+, 2+, and 3+. Numerical
ranges representing the reporting unit are defined; for example, three to six spherocytes per oil immersion field might be reported as 2+ spherocytes. Although this practice has been followed for many years, this semiquantitative method may not best serve the needs of medical staff, and many laboratories are moving toward simplifying their reports to state present for morphologic abnormalities.

The presence of immature RBCs suggests that the bone marrow is attempting to respond to an anemia. Polychromasia on the peripheral blood film indicates bone marrow response. This is manifested by the bluer color of reticulocytes that have entered the bloodstream earlier than usual in the body’s attempt to improve oxygen-carrying capacity. If the anemia is severe, NRBCs also may be present. As noted earlier, it is also important to recognize NRBCs because they may falsely elevate the WBC count and may be an indication of an underlying disease process.

A better way to assess replacement erythropoiesis is with the reticulocyte count and subsequent calculation of the reticulocyte production index, if appropriate. The reticulocyte count is not normally part of the CBC, although it is now performed on the same analyzers. If the reticulocyte count is available with the CBC, its interpretation can improve the assessment of young RBCs (Chapter 14).

**Step 6**
Examine the RBC count and MCH. On a practical level, the RBC count is not the parameter used to judge anemia, because there are some types of anemia, such as the thalassemias (Chapter 28), in which the RBC count is normal or even elevated. Thus the assessment of anemia would be missed by relying only on the RBC count. However, this inconsistency (low hemoglobin and high RBC count) is often helpful diagnostically.

The MCH follows the MCV; that is, smaller cells necessarily hold less hemoglobin, whereas larger cells can hold more. For this reason it is less often used than the MCV and MCHC. In the instances in which the MCH does not follow the MCV, the MCHC detects the discrepancy between size and hemoglobin content of the cell. The MCH is not crucial to the assessment of anemia when the other parameters are provided. In summary, when evaluating the RBC parameters of the CBC, examine the hemoglobin first, then the MCV, RDW, and MCHC. Finally, take note of any abnormal morphology.

**Summarizing Platelet Parameters**
The platelet parameters of the CBC are as follows:
1. Platelet count (platelets × 10^9/L)
2. Mean platelet volume (MPV, fl)
3. Morphology

**Step 1**
The platelet count should be examined for increases (thrombocytosis) or decreases (thrombocytopenia) outside the established reference interval. A patient who has unexplained bruising or bleeding may have a decreased platelet count. The platelet count should be assessed along with the WBC count and hemoglobin to determine whether all three are decreased (pancytopenia) or increased (pancytosis). Pancytopenia is clinically significant because it can indicate a possible developing acute leukemia (Chapter 35) or aplastic anemia (Chapter 22). Pancytosis frequently is associated with a diagnosis of polycythemia vera (Chapter 33).

**Step 2**
Compare the instrument-generated MPV with the MPV reference interval, 6.9 to 10.2 fl, and with the platelet diameter observed on the peripheral blood film. An elevated MPV should correspond with increased platelet diameter, just as an elevated MCV reflects macrocytosis. In platelet consumption disorders such as immune thrombocytopenic purpura, an elevated MPV, accompanied by platelets 6 μm or larger in diameter (giant platelets), reflects bone marrow release of early “stress” or “reticulated” platelets, evidence for bone marrow compensation (Chapter 13 and Figure 13-9). Comparing platelet diameter by visual inspection with MPV is a recommended quality control step; however, the MPV has a wide percent CV, which reflects interindividual variation and platelet swelling in EDTA and reduces its clinical effectiveness. Some instruments identify and record a reticulated platelet count using nucleic acid dye, analogous to a reticulocyte count. Figure 16-14 demonstrates platelet histograms with a normal platelet population (A) and one with giant platelets (B).

**Step 3**
Examine platelet morphology and platelet arrangement. Although the MPV can recognize abnormally large platelets, the morphologic evaluation also notes this. Some laboratories distinguish between large platelets (two times normal size) and giant platelets (more than twice as large as normal) or compare platelet size to RBC size.

**Figure 16-14** Platelet histograms. A, Normal with MPV of 8.0 fl; B, Platelet population with abnormal histogram and MPV of 9.1 fl. Although the MPV is within the reference interval, the histogram shows an increase in the number of platelets with a volume between 10 and 20 fl (curve above baseline) representing giant platelets. Reference interval for MPV: 6.8-10.2 fl. MPV, Mean platelet volume.
Additional morphologic descriptors include terms for reporting granularity, which is most important if missing, and in this case the platelets are described as "hypogranular" or "agranular." Sometimes the abnormalities are too variable to classify, and the platelets are described simply as "bizarre" or dysplastic. In some cases, platelets can be clumped or adherent to WBCs, and these arrangements should be noted. As described previously, corrective actions can be taken to derive accurate platelet and WBC counts when these arrangements are observed on the film. Summarizing platelet parameters includes reporting total number, platelet size by either instrument MPV or morphologic evaluation, and platelet appearance.

Box 16-8 gives an example of how the entire CBC can be summarized using the steps described. When results of the CBC are properly summarized and no information has been overlooked, there is confidence that the phase 2 interpretation of the results will be reliable. Adopting a methodical approach to examining each parameter ensures that the myriad information available from the CBC can be used effectively and efficiently in patient care. Box 16-9 summarizes the systematic approach to CBC interpretation.

**BOX 16-8 Applying a Systematic Approach to Complete Blood Count Summarization**

A specimen from an adult male patient yields the following CBC results (refer to the reference intervals inside the front cover of this book):

- WBCs—3.20 × 10⁹/L
- RBCs—2.10 × 10¹²/L
- HGB—8.5 g/dL
- HCT—26.3%
- MCV—125 fL
- MCH—40.5 pg
- MCHC—32.3 g/dL
- RDW—20.6%
- PLT—115 × 10⁹/L

**Differential in percentages:**

- Neutrophils—43
- Bands—2
- Lymphocytes—45
- Monocytes—10

**Morphology:**

- Hypersegmentation of neutrophils, anisocytosis, macrocytes, oval macrocytes, occasional teardrop, Howell-Jolly bodies, and basophilic stippling

The step-by-step assessment of the WBCs indicates that the WBC count is accurate and can be interpreted as leukopenia. Although the relative differential values are all within the reference intervals, calculation of absolute counts indicates an absolute neutropenia and lymphopenia. No unexpected young WBCs are noted, but the morphology indicates hypersegmentation of neutrophils. For the RBCs, the hemoglobin concentration (8.5) indicates that this person is anemic. Inspection of the MCV indicates macrocytosis because the MCV is increased to more than 100 fL, the upper limit of the reference interval. Examination of the MCHC shows it to be normal, so for these results, the RBC morphology would be described as macrocytic, normochromic. The elevated RDW indicates substantial anisocytosis. There is no mention of polychromasia, so no young RBCs are seen. The morphologic description supports the interpretation of the RDW with mention of anisocytosis due to macrocytosis and poikilocytosis characterized by oval macrocytes and occasional teardrop cells. Howell-Jolly bodies and basophilic stippling are significant RBC inclusions. The platelet count indicates thrombocytopenia. Although MPV is not reported, the platelets are of normal size and show no morphologic abnormalities because there are no notations in the morphologic descriptions.

CBC, Complete blood count; HCT, hematocrit; HGB, hemoglobin; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; MPV, mean platelet volume; RBC, red blood cell; RDW, RBC distribution width; WBC, white blood cell.

**BOX 16-9 Systematic Approach to Complete Blood Count Interpretation**

**White Blood Cells**

**Step 1:** Ensure that the WBC count is accurate. Review WBC histogram and/or scatterplot and correlate with counts. The presence of nucleated RBCs may require correction of the WBC count.

**Step 2:** Compare the patient’s WBC count with the laboratory’s established reference interval.

**Steps 3 and 4:** Examine the differential information (relative and absolute) on variations in the distribution of WBCs.

**Step 5:** Make note of immature cells in any cell line reported in the differential that should not appear in normal peripheral blood.

**Step 6:** Make note of any morphologic abnormalities and correlate film findings with the numerical values.

**Red Blood Cells**

**Step 1:** Examine the HGB concentration first to assess anemia.

**Step 2:** Examine the MCV to assess cell volume.

**Step 3:** Examine the MCHC to assess cell HGB concentration in RBC.

**Step 4:** Examine the RDW to assess anisocytosis. (Correlate both MCV and RDW with RBC histogram.)

**Step 5:** Examine the morphologic description and correlate with the numerical values. Look for evidence of a reticulocyte response.

**Step 6:** Review remaining information.

**Platelets**

**Step 1:** Examine the total platelet count.

**Step 2:** Examine the MPV to assess platelet size.

**Step 3:** Examine platelet morphology and correlate with the numerical values.

HGB, Hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; MPV, mean platelet volume; RBC, red blood cell; RDW, RBC distribution width; WBC, white blood cell.
• Although fewer manual peripheral blood film evaluations are performed today, much valuable information still can be obtained from a well-made and well-stained film.
• The specimen of choice for routine hematology testing is whole blood collected in a lavender/purple topped tube. The tube additive is EDTA, which chelates plasma calcium.
• Only rarely does EDTA create problems in analyzing certain individuals’ blood. EDTA-induced platelet clumping or satellitosis causes automated analyzers to report falsely decreased platelet counts (pseudothrombocytopenia) and falsely increased WBC counts (pseudoleukocytosis). This problem must be recognized through blood film examinations and the proper course of action followed to produce accurate results.
• Several methods exist for making peripheral blood films; however, the manual wedge film technique is used most frequently.
• Learning to make consistently good blood films takes practice. The basic technique can be modified as needed to accommodate specimens from patients with very high or very low hematocrits.
• The stain used routinely in hematology is Wright or Wright-Giemsa stain. Staining of all cellular elements occurs when the pH-specific buffer is added to the stain already on the slide. Staining reactions depend on the pH of the cellular components.
• Wright staining is done manually, by automated techniques, or by quick stains, depending on the laboratory and the number of slides to be processed.
• Peripheral blood films and bone marrow smears always should be evaluated in a systematic manner, beginning with the 10× objective lens and finishing with the 100× oil immersion objective lens. Leukocyte differential and morphologic evaluation, RBC and platelet morphologic evaluation, and platelet number estimate all are included.
• Use of a systematic approach to CBC interpretation ensures that all valuable information is assessed and nothing is overlooked. The systematic approach to CBC interpretation is summarized in Box 16-9.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

Answers can be found in the Appendix.

1. A laboratory science student consistently makes wedge-technique blood films that are too long and thin. What change in technique would improve the films?
   a. Increasing the downward pressure on the pusher slide
   b. Decreasing the acute angle of the pusher slide
   c. Placing the drop of blood closer to the center of the slide
   d. Increasing the acute angle of the pusher slide

2. When a blood film is viewed through the microscope, the RBCs appear redder than normal, the neutrophils are barely visible, and the eosinophils are bright orange. What is the most likely cause?
   a. The slide was overstained.
   b. The stain was too alkaline.
   c. The buffer was too acidic.
   d. The slide was not rinsed adequately.

3. A stained blood film is held up to the light and observed to be bluer than normal. What microscopic abnormality might be expected on this film?
   a. Rouleaux
   b. Spherocytosis
   c. Reactive lymphocytosis
   d. Toxic granulation

4. A laboratorian using the 40× objective lens sees the following numbers of WBCs in 10 fields: 8, 4, 7, 5, 4, 7, 8, 6, 4, 6. Which of the following WBC counts most closely correlates with the estimate?
   a. 1.5 × 10^9/L
   b. 5.9 × 10^9/L
   c. 11.8 × 10^9/L
   d. 24 × 10^9/L

5. A blood film for a very anemic patient with an RBC count of 1.25 × 10^12/L shows an average of seven platelets per oil immersion field. Which of the following values most closely correlates with the estimate per microliter?
   a. 14,000
   b. 44,000
   c. 140,000
   d. 280,000

6. A blood film for a patient with a normal RBC count has an average of 10 platelets per oil immersion field. Which of the following values best correlates with the estimate per microliter?
   a. 20,000
   b. 100,000
   c. 200,000
   d. 400,000
7. What is the absolute count \((10^9/L)\) for the lymphocytes if the total WBC count is \(9.5 \times 10^9/L\) and there are 37% lymphocytes?
   a. 3.5
   b. 6.5
   c. 13
   d. 37

8. Which of the following blood film findings indicates EDTA-induced pseudothrombocytopenia?
   a. The platelets are pushed to the feathered end.
   b. The platelets are adhering to WBCs.
   c. No platelets at all are seen on the film.
   d. The slide has a bluish discoloration when examined macroscopically.

9. Which of the following is the best area to review or perform a differential on a stained blood film?
   a. Red blood cells are all overlapped in groups of three or more.
   b. Red blood cells are mostly separated, with a few overlapping.
   c. Red blood cells look flattened, with none touching.
   d. Red blood cells are separated and holes appear among the cells.

10. Use the reference intervals provided inside the front cover of this text. Given the following data, summarize the following blood picture:
    WBC: 86.3 \(\times 10^9/L\)
    HGB: 9.7 g/dL
    HCT: 24.2%
    MCV: 87.8 fl.
    MCHC: 33.5%
    PLT: 106 \(\times 10^9/L\)
    a. Leukocytosis, normocytic-normochromic anemia, thrombocytopenia
    b. Microcytic-hypochromic anemia, thrombocytopenia
    c. Neutrophilia, macrocytic anemia, thrombocytosis
    d. Leukocytosis, thrombocytopenia

REFERENCES

Bone Marrow Examination

George A. Fritsma*

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Diagram bone marrow architecture and locate hematopoietic tissue.
2. List indications for bone marrow examinations.
3. Specify sites for bone marrow aspirate and biopsy.
4. Assemble supplies for performing and assisting in bone marrow specimen collection.
5. Assist the physician with bone marrow sample preparation subsequent to collection.
6. List the information gained from bone marrow aspirates and biopsy specimens.
7. Perform a bone marrow aspirate smear and core biopsy specimen examination.
8. List the normal hematopoietic and stromal cells of the bone marrow and their anticipated distribution.
9. Perform a bone marrow differential count and compute the myeloid-to-erythroid ratio.
10. Characterize features of hematopoietic and metastatic tumor cells.
11. Prepare specimens for and assist in performing specialized confirmatory bone marrow studies.
12. Prepare a systematic written bone marrow examination report.

OUTLINE

Bone Marrow Anatomy and Architecture
Indications for Bone Marrow Examination
Bone Marrow Specimen Collection Sites
Bone Marrow Aspiration and biopsy
Preparation
Core Biopsy
Aspiration
Patient Care
Managing the Bone Marrow Specimen
Direct Aspirate Smears
Anticoagulated Aspirate Smears
Crush Smears
Imprints (Touch Preparations)
Concentrate (Buffy Coat) Smears
Histologic Sections (Cell Block)
Bone Marrow Smear Dyes
Examining Bone Marrow Aspirate or Imprint
Low-Power (100×) Examination
High-Power (500×) Examination
Prussian Blue Iron Stain Examination
Examining the Bone Marrow Core Biopsy Specimen
Definitive Bone Marrow Studies
Bone Marrow Examination Reports

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A patient came for treatment complaining of weakness, fatigue, and malaise. Complete blood count results were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB concentration</td>
<td>7.5 gm/dL</td>
</tr>
<tr>
<td>HCT</td>
<td>21%</td>
</tr>
<tr>
<td>RBC count</td>
<td>2.5 × 10^{12} /L</td>
</tr>
<tr>
<td>WBC count</td>
<td>30 × 10^9 /L</td>
</tr>
<tr>
<td>Platelet count</td>
<td>540 × 10^9 /L</td>
</tr>
<tr>
<td>Segmented neutrophils</td>
<td>21 × 10^9 /L (70%)</td>
</tr>
<tr>
<td>Immature neutrophils</td>
<td>6 × 10^9 /L (20%)</td>
</tr>
<tr>
<td>Basophils</td>
<td>1.5 × 10^9 /L (5%)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.3 × 10^9 /L (1%)</td>
</tr>
</tbody>
</table>

Bone marrow was hypercellular with 90% myeloid precursors and 10% erythroid precursors. There were 15 megakaryocytes per 10× microscopic objective field.

1. What bone marrow finding provides information on blood cell production?
2. What is the myeloid-to-erythroid ratio in this patient, and what does it indicate?
3. What megakaryocyte distribution is normally seen in a bone marrow aspirate?
In adults, bone marrow accounts for 3.4% to 5.9% of body weight, contributes 1600 to 3700 g or a volume of 30 to 50 mL/kg, and produces roughly 6 billion blood cells per kilogram per day in a process called hematopoiesis. At birth, nearly all the bones contain red hematopoietic marrow (Chapter 7). In the fifth to seventh year, adipocytes (fat cells) begin to replace red marrow in the long bones of the hands, feet, legs, and arms, producing yellow marrow, and by late adolescence hematopoietic marrow is limited to the lower skull, vertebrae, shoulder, pelvic girdle, ribs, and sternum (Figure 7-2). Although the percentage of bony space devoted to hematopoiesis is considerably reduced, the overall volume remains constant as the individual matures. Yellow marrow reverts to hematopoiesis, increasing red marrow volume, in conditions such as chronic blood loss or hemolytic anemia that raise demand.

The arrangement of red marrow and its relationship to the central venous sinus are illustrated in Figure 7-3. Hematopoietic tissue is enmeshed in spongy trabeculae (bony tissue) surrounding a network of sinuses that originate at the endosteum (vascular layer just within the bone) and terminate in collecting venules. Adipocytes occupy approximately 50% of red hematopoietic marrow space in a 30- to 70-year-old adult, and fatty metamorphosis increases approximately 10% per decade after age 70.

### TABLE 17-1 Indications for Bone Marrow Examination

<table>
<thead>
<tr>
<th>Indication</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoplasia diagnosis</td>
<td>Acute leukemias</td>
</tr>
<tr>
<td></td>
<td>Myeloproliferative neoplasms such as chronic leukemias, myelofibrosis</td>
</tr>
<tr>
<td></td>
<td>Myelodysplastic neoplasms such as refractory anemia</td>
</tr>
<tr>
<td></td>
<td>Lymphoproliferative disorders such as acute lymphoblastic leukemia</td>
</tr>
<tr>
<td></td>
<td>Immunoglobulin disorders such as plasma cell myeloma, macroglobulinemia</td>
</tr>
<tr>
<td></td>
<td>Metastatic tumors</td>
</tr>
<tr>
<td>Neoplasia diagnosis and staging</td>
<td>Hodgkin and non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>Marrow failure: cytopenias</td>
<td>Hypoplastic or aplastic anemia</td>
</tr>
<tr>
<td></td>
<td>Pure red cell aplasia</td>
</tr>
<tr>
<td></td>
<td>Idiosyncratic drug-induced marrow suppression</td>
</tr>
<tr>
<td></td>
<td>Myelodysplastic syndromes such as refractory anemia</td>
</tr>
<tr>
<td></td>
<td>Marrow necrosis secondary to tumor</td>
</tr>
<tr>
<td></td>
<td>Marrow necrosis secondary to severe infection such as parvovirus B19 infection</td>
</tr>
<tr>
<td></td>
<td>Immune versus amegakaryocytic thrombocytopenia</td>
</tr>
<tr>
<td></td>
<td>Sickle cell crisis</td>
</tr>
<tr>
<td></td>
<td>Differentiation of megaloblastic, iron deficiency, sideroblastic, hemolytic, and blood loss anemia</td>
</tr>
<tr>
<td></td>
<td>Estimation of storage iron to assess for iron deficiency</td>
</tr>
<tr>
<td></td>
<td>Infiltrative processes or fibrosis</td>
</tr>
<tr>
<td>Metabolic disorders</td>
<td>Gaucher disease</td>
</tr>
<tr>
<td></td>
<td>Mast cell disease</td>
</tr>
<tr>
<td>Infections</td>
<td>Granulomatous disease</td>
</tr>
<tr>
<td></td>
<td>Miliary tuberculosis</td>
</tr>
<tr>
<td></td>
<td>Fungal infections</td>
</tr>
<tr>
<td></td>
<td>Hemophagocytic syndromes</td>
</tr>
<tr>
<td>Monitoring of treatment</td>
<td>After chemotherapy or radiation therapy to assess minimal residual disease</td>
</tr>
<tr>
<td></td>
<td>After stem cell transplantation to assess engraftment</td>
</tr>
</tbody>
</table>

Because the procedure is invasive, the decision to collect and examine a bone marrow specimen requires clinical judgment and the application of inclusion criteria. With the development of cytogenetic chromosome studies, flow cytometry, immunohistochemistry, and molecular diagnostics, peripheral blood may often provide information historically available only from bone marrow, reducing the demand for marrow specimens. On the other hand, these advanced techniques also augment bone marrow–based diagnosis and thus potentially raise the demand for bone marrow examinations in assessment of conditions not previously diagnosed through bone marrow examination.

Table 17-1 summarizes indications for bone marrow examination. Bone marrow examinations may be used to diagnose and stage hematologic and nonhematologic neoplasia, to determine the cause of cytopenias, and to confirm or exclude metabolic or infectious conditions suspected on the basis of clinical symptoms and peripheral blood findings.

Each bone marrow procedure is ordered after thorough consideration of clinical and laboratory information. For instance, bone marrow examination is most likely unnecessary in anemia when the cause is apparent from red blood cell (RBC) indices, serum iron and ferritin levels, or vitamin B₁₂ and folate.
levels. Multilineage abnormalities, circulating blasts in adults, and unexpected pancytopenia usually prompt marrow examination. Bone marrow puncture is prohibited in patients with coagulopathies such as hemophilia or vitamin K deficiency, although thrombocytopenia (low platelet count) is not an absolute contraindication. Special precautions such as bridging therapy may be necessary to prevent uncontrolled bleeding when a bone marrow procedure is performed on a patient receiving antithrombotic therapy, for instance, Coumadin or heparin.

**BONE MARROW SPECIMEN COLLECTION SITES**

Bone marrow specimen collection is a collaboration between a medical laboratory scientist and a skilled specialty physician, often a pathologist or hematologist. Prior to bone marrow collection, a medical laboratory practitioner or phlebotomist collects peripheral blood for a complete blood count with blood film examination. During bone marrow collection, the laboratory scientist assists the physician by managing the specimens and producing initial preparations for examination.

Red marrow is gelatinous and amenable to sampling. Most bone marrow specimens consist of an aspirate (obtained by bone marrow aspiration) and a core biopsy specimen (obtained by trephine biopsy), both examined with light microscopy using 100× and 500× magnification. The aspirate is examined to identify the types and proportions of hematologic cells and to look for morphologic variance. The core biopsy specimen demonstrates bone marrow architecture: the spatial relationship of hematologic cells to fat, connective tissue, and bony stroma. The core biopsy specimen is also used to estimate cellularity.

The core biopsy specimen is particularly important for evaluating diseases that characteristically produce focal lesions, rather than diffuse involvement of the marrow. Hodgkin lymphoma, non-Hodgkin lymphoma, multiple myeloma, metastatic tumors, amyloid, and granulomas produce predominantly focal lesions. Granulomas, or granulomatous lesions, are cell accumulations that contain Langerhans cells—large, activated granular macrophages that look like epithelial cells. Granulomas signal chronic infection. The biopsy specimen also allows for morphologic evaluation of bony spicules, which may reveal changes associated with hyperparathyroidism or Paget disease.

Bone marrow collection sites include the following:

- **Posterior superior iliac crest** (spine) of the pelvis (Figure 17-1). In both adults and children, this site provides adequate red marrow that is isolated from anatomical structures that are subject to injury. This site is used for both aspiration and core biopsy.

- **Anterior superior iliac crest** (spine) of the pelvis. This site has the same advantages as the posterior superior iliac crest, but the cortical bone is thicker. This site may be preferred for a patient who can only lie supine.

- **Sternum**, below the angle of Lewis at the second intercostal space. In adults, the sternum provides ample material for aspiration but is only 1 cm thick and cannot be used for core biopsy. It is possible for the physician to accidentally transfix the sternum and enter the pericardium within, damaging the heart or great vessels.

- **Anterior medial surface of the tibia** in children younger than age 2. This site may be used only for aspiration.

- **Spinous process of the vertebrae, ribs, or other red marrow-containing bones.** These locations are available but are rarely used unless one is the site of a suspicious lesion discovered on a radiograph.

Adverse outcomes are seen in less than 0.05% of marrow collections. Infections and reactions to anesthetics may occur, but the most common side effect is hemorrhage associated with platelet function disorder or thrombocytopenia.

**BONE MARROW ASPIRATION AND BIOPSY**

**Preparation**

Less than 24 hours prior to bone marrow collection, the medical laboratory scientist or phlebotomist collects venous peripheral blood for a complete blood count and blood film examination using a standard collection procedure. Peripheral blood collection is often accomplished immediately before bone marrow specimen collection. The peripheral blood specimen is seldom collected after bone marrow collection to avoid stress-related white blood cell (WBC) count elevation.

Most institutions purchase or assemble disposable sterile bone marrow specimen collection trays that provide the following:

- Surgical gloves.
- Shaving equipment.
- Antiseptic solution and alcohol pads.
- Drape material.
- Local anesthetic injection, usually 1% lidocaine, not to exceed 20 mL per patient.
- No. 11 scalpel blade for skin incision.
• Disposable Jamshidi biopsy needle (Care Fusion, McGaw Park, IL; Figure 17-2) or Westerman-Jensen needle (Becton, Dickinson and Company, Franklin Lakes, NJ; Figure 17-3). Both provide an obturator, core biopsy tool, and stylet. A Snarecoil biopsy needle also is available (Kendall Company, Mansfield, MA). The Snarecoil has a coil mechanism at the needle tip that allows for capture of the bone marrow specimen without needle redirection (Figure 17-4).

• Disposable 14- to 18-gauge aspiration needle with obturator. Alternatively, the University of Illinois aspiration needle may be used for sternal puncture. The University of Illinois needle provides a flange that prevents penetration of the sternum to the pericardium.

Figure 17-2 Disposable sterile Jamshidi bone marrow biopsy and aspiration needle. The outer puncture cannula is advanced to the medulla with the obturator in place to prevent bone coring. The physician removes the obturator and slides the core biopsy needle through the cannula and into the medulla with the expulsion stylus removed. The core biopsy needle is removed from the puncture needle with the specimen in place. The specimen is expelled using the stylus. (Courtesy Care Fusion, McGaw Park, IL.)

Figure 17-3 Bone marrow biopsy technique using the Westerman-Jensen needle.

Figure 17-4 Snarecoil bone marrow biopsy needle. The coil mechanism resides within the biopsy needle as illustrated in the magnified image. The coil is turned to draw the marrow specimen into the needle. (Courtesy Tyco Healthcare/Kendall, Mansfield, MA.)
The physician slowly withdraws the blades so that the cannula, and advances the blades into the medullary cavity. The obturator prevents coring of skin or bone. Reciprocating rotation promotes the forward advancement of the cannula until the resistance weakens, which indicates penetration through the cortex to the medullary cavity of the bone. The obturator is removed, and a 10- to 20-mL syringe is attached. The physician withdraws the plunger to create negative pressure and aspirates 1.0 to 1.5 mL of marrow into the syringe. Collecting more than 1.5 mL dilutes the hematopoietic marrow with sinusoidal (peripheral) blood. The physician detaches the syringe and passes it immediately to the laboratory scientist, who expels the material onto a series of clean and sterile microscopic slides or coverslips. The physician may attach a second syringe to aspirate an additional specimen for cytogenetic analysis, molecular diagnosis, or immunophenotyping using flow cytometry. The needle is then withdrawn, and pressure is applied to the wound.

If no marrow is obtained, the physician returns the obturator to the needle, advances the needle, attaches a fresh syringe, and tries again. The syringe and needle are retracted slightly and the process is repeated. If this attempt is unsuccessful, the physician removes the needle and syringe, applies pressure, and begins the procedure at a new site. If the marrow is fibrotic, acellular, or packed with leukemic cells, the first and second aspiration may be unsuccessful, known as a dry tap. In this case, a biopsy is necessary, and the laboratory scientist may observe cell morphology using a slide imprint, or touch preparation.

**Patient Care**

Subsequent to bone marrow biopsy or aspiration, the physician applies a pressure dressing and advises the patient to remain in the same position for 60 minutes to prevent bleeding.

**MANAGING THE BONE MARROW SPECIMEN**

**Direct Aspirate Smears**

The medical laboratory scientist receives the aspirate syringe from the physician at the bedside and immediately transfers drops of the marrow specimen onto six to eight ethanol-washed microscope slides. Marrow clots rapidly, so good organization is essential. Using spreader slides, the scientist spreads the drop into a wedge-shaped smear ½ to ¾ the length of the slide, similar to a peripheral blood film. Bony spicules 0.5 to 1.0 mm in diameter and larger fat globules follow behind the spreader and become deposited on the slide. In the direct smear preparation the scientist avoids crushing the spicules. The scientist may lightly fan the smears to promote rapid drying in an effort to preserve cell morphology.

In the syringe, the specimen consists of peripheral blood with suspended light-colored bony spicules and fat globules. The scientist evaluates the syringe blood for spicules: more spicules mean a specimen with more cells to identify and categorize. If the specimen has few fat globules or spicules, the scientist may alert the physician to collect an additional specimen.
Anticoagulated Aspirate Smears
Anticoagulated specimens are a more leisurely alternative to direct aspirate smears. The scientist expresses the aspirate from the syringe into a vial containing K3EDTA and subsequently pipettes the anticoagulated aspirate to clean glass slides, spreading the aspirate using the same approach as in direct smear preparation. All anticoagulants distort cell morphology, but K3EDTA generates the least distortion.

Crush Smears
To prepare crush smears, the medical laboratory scientist expels a portion of the aspirate to a Petri dish or watch glass covered with a few milliliters of K3EDTA solution and spreads the aspirate over the surface with a sterile applicator. Individual bony spicules are transferred using applicators, forceps, or micropipettes (preferred) to several ethanol-washed glass slides. The scientists places additional glass slides directly over the specimens at right angles and presses gently to crush the spicules. The slides are separated laterally to create two rectangular smears, which the scientist may fan to encourage rapid drying.

Some scientists prefer to transfer aspirate directly to the slide, subsequently tilting the slide to drain off peripheral blood while retaining spicules. Once drained, the spicules are then crushed with a second slide as described earlier.

The scientist may add one drop of 22% albumin to the EDTA solution, particularly if the specimen is suspected to contain prolymphocytes or lymphoblasts, which tend to rupture. The albumin reduces the occurrence of “smudge” or “basket” cells often seen in lymphoid marrow lesions.

The crush preparation procedure may also be performed using ethanol-washed coverslips in place of slides. The coverslip method demands adroit manipulation but may yield better morphologic information, because the smaller coverslips generate less cell rupture during separation. Use of glass slides offers the opportunity for automated staining, whereas coverslip preparations must first be affixed smear side up to slides and then stained manually (Chapter 16).

Imprints (Touch Preparations)
Core biopsy specimens and clotted marrow may be held in forceps and repeatedly touched to a washed glass slide or coverslip so that cells attach and rapidly dry. The scientist lifts directly upward to prevent cell distortion. Imprints are valuable when the specimen has clotted or there is a dry tap: the cell morphology may closely replicate aspirate morphology, although few spicules are transferred.

Concentrate (Buffy Coat) Smears
Buffy coat smears are useful when there are sparse nucleated cells in the direct marrow smear or when the number of nucleated cells is anticipated to be small, as in aplastic anemia. The scientist transfers approximately 1.5 mL of K3EDTA-anticoagulated marrow specimen to a narrow-bore glass or plastic tube such as a Wintrobe hematocrit tube. The tube is centrifuged at 2500 g for 10 minutes and examined for four layers.

The top layer is yellowish fat and normally occupies 1% to 3% of the column. The second layer, plasma, varies in volume, depending on the amount of peripheral blood in the specimen. The third layer consists of nucleated cells and is called the myeloid-erythroid (ME) layer. The ME layer is normally 5% to 8% of the total column. The bottom layer is RBCs, and its volume, like that of the plasma layer, depends on the amount of peripheral blood present. The scientist records the ratio of the fat and ME layers using millimeter gradations on the tube.

Once the column is examined, the scientist aspirates a portion of the ME layer with a portion of plasma and transfers the suspension to a Petri dish or watch glass. Marrow smears are subsequently prepared using the crush smear technique.

The concentrated buffy coat smear compensates for hypocellular marrow and allows for examination of large numbers of nucleated cells without interference from fat or RBCs. On the other hand, cell distribution is distorted by the procedure. Therefore, the scientist does not estimate numbers of different cell types or maturation stages on a buffy coat smear.

Histologic Sections (Cell Block)
After the scientist has prepared aspirate smears and has distributed aliquots of marrow for cytogenetic, molecular, and immunophenotypic studies, the remaining core biopsy specimen, spicules, or clotted specimen is submitted for histologic examination. The specimen is suspended in 10% formalin, Zenker glacial acetic acid, or B5 fixative for approximately 2 hours.

The fixed specimen is subsequently centrifuged, and the pellet is decalcified and wrapped in an embedding bag or lens paper and placed in a paraffin-embedding cassette. A histotechnologist sections the embedded specimen, applies hematoxylin and eosin (H&E) dye, and examines the section.

Marrow Smear Dyes
Marrow aspirate smears are stained with Wright or Wright-Giemsa dyes using the same protocols as for peripheral blood film staining. Some laboratory managers increase staining time to compensate for the relative thickness of marrow smears compared with peripheral blood films.

Marrow aspirate smears and core biopsy specimens may also be stained using a ferric ferricyanide (Prussian blue) solution to detect and estimate marrow storage iron or iron metabolism abnormalities (Chapter 20). Further, a number of cytochemical dyes may be used for cell identification or differentiation (Table 17-2).

EXAMINING BONE MARROW ASPIRATE OR IMPRINT

Box 17-1 describes the uses of low- and high-power objectives in examining bone marrow aspirate direct smears or imprints.

Low-Power (100x) Examination
Once the bone marrow aspirate direct smear or imprint is prepared and stained, the scientist or pathologist begins the microscopic examination using the low-power (10×) dry lens, which, when linked with 10× oculars, provides a total 100× magnification. Most bone marrow examinations are
TABLE 17-2 Cytochemical Dyes Used to Identify Bone Marrow Cells and Maturation Stages

<table>
<thead>
<tr>
<th>Cytochemical Dye</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase (MPO)</td>
<td>Detects myelocytic cells by staining cytoplasmic granular contents</td>
</tr>
<tr>
<td>Sudan black B (SBB)</td>
<td>Detects myelocytic cells by staining cytoplasmic granular contents</td>
</tr>
<tr>
<td>Periodic acid–Schiff (PAS)</td>
<td>Detects lymphocytic cells and certain abnormal erythrocytic cells by staining of cytoplasmic glycogen</td>
</tr>
<tr>
<td>Esterases</td>
<td>Distinguish myelocytic from monocytic maturation stages (several esterase substrates)</td>
</tr>
<tr>
<td>Tartrate-resistant acid phosphatase (TRAP)</td>
<td>Detects tartrate-resistant acid phosphatase granules in hairy cell leukemia</td>
</tr>
</tbody>
</table>

BOX 17-1 Bone Marrow Aspirate Microscopic Smear Examination: Low and High Power

**Low Power: 10x Objective (100x total magnification)**
- Assess peripheral blood dilution
- Find bony spicules and areas of clear cell morphology
- Observe fat-to-marrow ratio, estimate cellularity
- Search for tumor cells in clusters
- Examine and estimate megakaryocytes

**High Power: 50x and 100x objective (500x and 1000x total magnification)**
- Observe myelocytic and erythrocytic maturation
- Distinguish abnormal distribution of cells or cell maturation stages
- Perform differential count on 300 to 1000 cells
- Compute myeloid-to-erythroid ratio

performed using a teaching report format that employs projection or multiead microscopes to allow observation by residents, fellows, medical laboratory students, and attending staff. The microscopist locates the bony spicules, aggregations of bone and hematopoietic cells, which stain dark blue (Figure 17-5). In imprints, spicules are sparse or absent, and the search is for hematopoietic cells in the absence of spicules. Within these areas the microscopist selects intact and nearly contiguous nucleated cells for examination, avoiding areas of distorted morphology or areas diluted with sinusoidal blood.

Near the spicules, cellularity is estimated by observing the proportion of hematopoietic cells to adipocytes (clear fat areas). For anterior or posterior iliac crest marrow, 50% cellularity is normal for patients aged 30 to 70 years. In childhood, cellularity is 80%, and after age 70, cellularity becomes reduced. For those older than age 70, a rule of thumb is to subtract patient age from 100% and add ±10%. Thus, for a 75-year-old, the anticipated cellularity is 15% to 35%. By comparing with the age-related normal cellularity values, the microscopist classifies the observed area as hypocellular, normocellular, or hypercellular. If a core biopsy specimen was collected, it provides a more accurate estimate of cellularity than an aspirate smear, because in aspirates there is always some dilution of hematopoietic tissue with peripheral blood. In the absence of leukemia, lymphocytes should total fewer than 30% of nucleated cells; if more are present, the marrow specimen has been substantially diluted and should not be used to estimate cellularity.

Using the 10× objective, the microscopist searches for abnormal, often molded, cell clusters (syncytia) of metastatic tumor cells or lymphoblasts. Tumor cell nuclei often stain darkly (hyperchromatic), and vacuoles are seen in the cytoplasm. Tumor cell clusters are often found near the edges of the smear.

Although myelocytic cells and erythrocytic cells are best examined using 500× magnification, they may be more easily distinguished from each other using the 10× objective. The erythrocytic maturation stages stain more intensely, and their margins are more sharply defined, features more easily distinguished at lower magnification.

The microscopist evaluates megakaryocytes using low power (Figure 17-6). Megakaryocytes are the largest cells in the bone marrow, 30 to 50 μm in diameter, with multilobed nuclei (Chapter 13). Although in special circumstances microscopists may differentiate three megakaryocyte maturation stages—megakaryoblast, promegakaryocyte, and megakaryocyte (MK-I to MK-III)—a total megakaryocyte estimate is generally satisfactory. In a well-prepared aspirate or biopsy specimen, the microscopist observes 2 to 10 megakaryocytes per low-power field. Deviations yield important information and are reported as decreased or increased megakaryocytes. Bone marrow megakaryocyte estimates are essential to the evaluation of peripheral blood thrombocytopenia and thrombocytosis; for instance, in immune thrombocytopenia, marrow megakaryocytes proliferate markedly.

Abnormal megakaryocytes may be small, lack granularity, or have poorly lobulated or hyperlobulated nuclei. Indications of abnormality may be visible using low power; however, conclusive descriptions require 500× or even 1000× total magnification.
PART III  Laboratory Evaluation of Blood Cells

High-Power (500×) Examination

Having located a suitable examination area, the microscopist places a drop of immersion oil on the specimen and switches to the 50× objective, providing 500× total magnification. All of the nucleated cells are reviewed for morphology and normal maturation. Besides megakaryocytes, cells of the myelocytic (Figures 17-7 through 17-10) and erythrocytic (rubricytic, normoblastic; Figure 17-11) series should be present, along with eosinophils, basophils, lymphocytes, plasma cells, monocytes, and histiocytes. Chapters 7, 8, and 12 provide detailed cell and cell maturation stage descriptions. Table 17-3 names all normal marrow cells and provides their expected percentages.

The microscopist searches for maturation gaps, misdistribution of maturation stages, and abnormal morphology. Although the specimen is customarily reviewed using the 50× oil immersion objective, the 100× oil immersion objective is frequently employed to detect small but significant morphologic abnormalities in the nuclei and cytoplasm of suspect cells.

Figure 17-6 Bone marrow aspirate smear showing megakaryocyte with budding platelets at the plasma membrane (Wright stain, 1000×). Megakaryocytes are counted at 100× magnification, but if there is abnormal morphology, cells are examined at 500× or 1000×.

Figure 17-7 Bone marrow aspirate smear. Myelocytic stages include a myeloblast (MyBl), promyelocyte (ProMy), and myelocyte (Myel). The lymphocyte (Lymph) diameter illustrates its size relative to the myelocytic stages. The source of the lymphocyte is sinus blood (Wright stain, 1000×).

Figure 17-8 Bone marrow aspirate smear. Myelocytic stages include a myeloblast (MyBl), promyelocytes (ProMy), a myelocyte (Myel), and metamyelocyte (Meta). One orthochromic normoblast (OrthoN) and one lymphocyte (Lymph) are present (Wright stain, 1000×).

Figure 17-9 Bone marrow aspirate smear. Myelocytic stages include myelocytes (Myel), a metamyelocyte (Meta), and neutrophilic bands (Wright stain, 1000×).

Figure 17-10 Bone marrow aspirate smear illustrating neutrophilic bands and segmented neutrophils (SEGs) (Wright stain, 1000×).

Many laboratory directors require a differential count of 300 to 1000 nucleated cells. These seemingly large totals are rapidly reached in a well-prepared bone marrow smear at 500× magnification and compensate statistically for the anticipated uneven distribution of spicules and hematopoietic cells. The microscopist counts cells and maturation stages surrounding several spicules to maximize the opportunity for
detecting disease-related cells. Some laboratory directors eschew the differential in favor of a thorough examination of the smear.

Many microscopists choose not to differentiate the four nucleated erythrocytic maturation stages, and others may combine three of the four—basophilic, polychromatophilic, and orthochromic normoblasts—in a single total, counting only pronormoblasts separately. In normal marrow, most erythrocytic precursors are either polychromatophilic or orthochromic normoblasts, and differentiation yields little additional information. On the other hand, differentiation may be helpful in megaloblastic, iron deficiency, or refractory anemias.

The microscopist may infrequently find osteoblasts and osteoclasts (Figure 17-12). Osteoblasts are responsible for bone formation and remodeling, and they derive from endosteal (inner lining) cells. Osteoblasts resemble plasma cells with eccentric round to oval nuclei and abundant blue, mottled cytoplasm, but they lack the prominent Golgi apparatus characteristic of plasma cells. Osteoblasts are usually found in clusters resembling myeloma cell clusters. Their presence in marrow aspirates and core biopsy specimens is incidental; they do not signal disease, but they may create confusion.

Osteoclasts are nearly the diameter of megakaryocytes, but their multiple, evenly spaced nuclei distinguish them from multilobed megakaryocyte nuclei (Figure 17-13). Osteoclasts appear to derive from myeloid progenitor cells and are responsible for bone resorption, acting in concert with osteoblasts. Osteoclasts are recognized more often in core biopsy specimens than in aspirates.

Adipocytes, endothelial cells that line blood vessels, and fibroblast-like reticular cells complete the bone marrow stroma (Chapter 7). Stromal cells and their extracellular matrix provide

---

**TABLE 17-3** Anticipated Distribution of Cells and Cell Maturation Stages in Aspirates or Imprints

<table>
<thead>
<tr>
<th>Cell or Cell Maturation Stage</th>
<th>Distribution</th>
<th>Cell or Cell Maturation Stage</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblasts</td>
<td>0%–3%</td>
<td>Pronormoblasts/rubriblasts</td>
<td>0%–1%</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>1%–5%</td>
<td>Basophilic normoblasts/prorubricytes</td>
<td>1%–4%</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>6%–17%</td>
<td>Polychromatophilic normoblasts/rubricytes</td>
<td>10%–20%</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>3%–20%</td>
<td>Orthochromic normoblasts/metarubricytes</td>
<td>6%–10%</td>
</tr>
<tr>
<td>Neutrophilic bands</td>
<td>9%–32%</td>
<td>Lymphocytes</td>
<td>5%–18%</td>
</tr>
<tr>
<td>Segmented neutrophils</td>
<td>7%–30%</td>
<td>Plasma cells</td>
<td>0%–1%</td>
</tr>
<tr>
<td>Eosinophils and eosinophilic precursors</td>
<td>0%–3%</td>
<td>Monocytes</td>
<td>0%–1%</td>
</tr>
<tr>
<td>Basophils and mast cells</td>
<td>0%–1%</td>
<td>Histocytes</td>
<td>0%–1%</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>2–10 visible per low-power field</td>
<td>Myeloid-to-erythroid ratio</td>
<td>1.5:1–3.3 : 1</td>
</tr>
</tbody>
</table>

---

**Figure 17-11** Bone marrow aspirate smear showing an island of erythrocytic precursors with polychromatophilic and orthochromic normoblasts (Wright stain, 1000×).

**Figure 17-12** Bone marrow aspirate smear showing a cluster of osteoblasts that superficially resemble plasma cells. Osteoblasts have round to oval eccentric nuclei and mottled blue cytoplasm that is devoid of secretory granules. They may have a clear area within the cytoplasm but lack the well-defined central Golgi complex of the plasma cell (Wright stain, 1000×).
the suitable microenvironment for the maturation and proliferation of hematopoietic cells but are seldom examined for diagnosis of hematologic or systemic disease. Finally, Langerhans cells, giant cells with “palisade” nuclei found in granulomas, signal chronic inflammation.

Once the differential is completed, the myeloid-to-erythroid (M:E) ratio is computed from the total of myeloid to the total of nucleated erythroid cell stages. Excluded from the M:E ratio are lymphocytes, plasma cells, monocytes, histiocytes, nonnucleated erythrocytes, and nonhematopoietic stromal cells.

**Prussian Blue Iron Stain Examination**

A Prussian blue (ferric ferricyanide) iron stain is commonly used on the aspirate smear. Figure 17-14 illustrates normal iron, absence of iron, and increased iron stores in aspirate smears. The iron stain may be used for core biopsy specimens, but decalciﬁying agents used to soften the biopsy specimen during processing may leach iron, which gives a false impression of decreased or absent iron stores. For this reason, the aspirate is favored for the iron stain if sufﬁcient spicules are present.

**EXAMINING THE BONE MARROW CORE BIOPSY SPECIMEN**

The standard dye for the core biopsy specimen is H&E. Other dyes and their purposes are listed in Table 17-4. Bone marrow core biopsy specimen and imprint (touch preparation) examinations are essential when the aspiration procedure yields a dry tap, which may be the result of hypoplastic or aplastic anemia, ﬁbrosis, or tight packing of the marrow cavity with leukemic cells. The key advantage of the core biopsy specimen is preservation of bone marrow architecture so that cells, tumor clusters (Figure 17-15), and maturation stages may be examined relative to stromal elements. The disadvantage is that individual hematopoietic cell morphology is obscured.

The microscopist ﬁrst examines the core biopsy specimen preparation using the 10× objective (100× total magnification) to assess cellularity. Because the sample is larger, the core biopsy specimen provides a more accurate estimate of cellularity than the aspirate. The microscopist compares cellular areas with the clear-appearing adipocytes, using a method identical to that employed in examination of aspirate smears to assess cellularity. All ﬁelds are examined because cells distribute unevenly. Examples of hypocellular and hypercellular core biopsy
sections are provided for comparison with normocellular marrow in Figure 17-16.

Megakaryocytes are easily recognized by their outsized diameter and even distribution throughout the biopsy. They exhibit the characteristic lobulated nucleus, although nuclei of the more mature megakaryocytes are smaller and more darkly stained in H&E preparations than on a Wright-stained aspirate. Their cytoplasm varies from light pink in younger cells to dark pink in older cells (Figure 17-17). Owing to the greater sample volume, microscopists assess megakaryocyte numbers more accurately by examining a core biopsy section than an aspirate smear. Normally there are 2 to 10 megakaryocytes per \(10 \times\) field, the same as in an aspirate smear or imprint.

Using the \(50 \times\) oil immersion objective, the microscopist next observes cell distribution relative to bone marrow stroma. For instance, in people older than age 70, normal lymphocytes may form small aggregates in nonparatrabecular regions, whereas malignant lymphoma cell clusters are often paratrabecular. In addition, normal lymphocytes remain as discrete cells, whereas lymphoma cells are pleomorphic and syncytial.

If no aspirate or imprint smears could be prepared, the core biopsy specimen may be stained using Wright, Giemsa, or Wright-Giemsa dyes to make limited observations of cellular morphology. In Wright- or Giemsa-stained biopsy sections, myeloblasts and promyelocytes have oval or round nuclei with cytoplasm that stains blue (Figure 17-18). Neutrophilic myelocytes and metamyelocytes have light pink cytoplasm. Mature segmented neutrophils (SEGs) and neutrophilic bands (BANDs) are recognized by their smaller diameter and darkly stained C-shaped nuclei (BANDs) or nuclear segments (SEGs). The cytoplasm of BANDs and SEGs may be light pink or may seem unstained (Figure 17-19).

The cytoplasm of eosinophils stains red or orange, which makes them the most brightly stained cells of the marrow. Basophils cannot be recognized on marrow biopsy specimens fixed with Zenker glacial acetic acid solution.

The microscopist may find it difficult to differentiate myelocytic cells from erythrocytic cells in biopsy specimens other than to observe that the latter tend to cluster with more mature normoblasts and often surround histiocytes. Polychromatophilic and orthochromic normoblasts, the two most common erythrocytic maturation stages, have centrally placed, round nuclei that may be recognized using the \(10 \times\) objective, although their individual morphology cannot be seen.

Lymphocytes are among the most difficult cells to recognize in the core biopsy specimen, unless they occur in clusters. Mature lymphocytes exhibit speckled nuclear chromatin in a small, round nucleus, along with a scant amount of blue cytoplasm (Figure 17-21). Immature lymphocytes (prolymphocytes) have larger round or lobulated nuclei but still only a small rim of blue cytoplasm.

Plasma cells are difficult to distinguish from myelocytes in H&E-stained sections but are recognized using Wright-Giemsa dye as cells with eccentric dark nuclei and blue cytoplasm and a prominent pale central Golgi apparatus (Figure 17-22). Characteristically, plasma cells are located adjacent to blood vessels.

### Table 17-4 Dyes Used in Examination of Bone Marrow Core Biopsy Specimens

<table>
<thead>
<tr>
<th>Dye</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin and eosin (H&amp;E)</td>
<td>Evaluate cellularity and hematopoietic cell distribution, locate abnormal cell clusters.</td>
</tr>
<tr>
<td>Prussian blue (ferric ferrocyanide) iron stain</td>
<td>Evaluate iron stores for deficiency or excess iron. Decalcification may remove iron from fixed specimens; thus ethylenediaminetetraacetic acid chelation or the aspirate smear is preferred for iron store estimation.</td>
</tr>
<tr>
<td>Reticulin and trichrome dyes</td>
<td>Examine for marrow fibrosis.</td>
</tr>
<tr>
<td>Acid-fast stains</td>
<td>Examine for acid-fast bacilli, fungi, or bacteria in granulomatous disease.</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Examine for acid-fast bacilli, fungi, or bacteria in granulomatous disease.</td>
</tr>
<tr>
<td>Immunohistochemical dyes</td>
<td>Establish the identity of malignant cells with dye-tagged monoclonal antibodies specific for tumor surface markers.</td>
</tr>
<tr>
<td>Wright or Wright-Giemsa dyes</td>
<td>Observe hematopoietic cell structure. Cell identification is less accurate in a biopsy specimen than in an aspirate smear.</td>
</tr>
</tbody>
</table>

![Figure 17-15](image1.png) Bone marrow aspirate smear showing a cluster or syncytia of tumor cells. Nuclei are irregular and hyperchromatic, and cytoplasm is vacuolated. Cytoplasmic margins are poorly delineated (Wright stain, \(500 \times\)).
A, Representative core biopsy section showing normal cellularity, approximately 50% fat and 50% hematopoietic cells (hematoxylin and eosin, 50×). B, Hypocellular core biopsy specimen with only fat and connective tissue cells from a patient with aplastic anemia (hematoxylin and eosin stain, 100×). C, Hypercellular core biopsy specimen from a patient with chronic myelogenous leukemia. There is virtually 100% cellularity with no fat visible (hematoxylin and eosin stain, 100×). (B courtesy Dennis P. O’Malley, MD, director, Immunohistochemistry Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

Figure 17-17 Core biopsy section containing many large lobulated megakaryocytes and increased blasts (Giemsa stain, 400×).

Figure 17-18 Core biopsy section infiltrated by blasts with blue cytoplasm and a few myelocytes with pink cytoplasm (Giemsa, 400×).

Figure 17-19 Core biopsy section showing myelocytes, metamyelocytes, bands, segmented neutrophils, and bright red-orange eosinophils (Giemsa stain, 400×).
DEFINITIVE BONE MARROW STUDIES

Although in many cases the aspirate smear and biopsy specimen are diagnostic, additional studies may be needed. Such studies and their applications are given in Table 17-5. These studies require additional bone marrow volume and specialized specimen collection. Information on Prussian blue iron stains and cytochemistry was provided earlier. Each study is described in the chapter referenced in Table 17-5.

BONE MARROW EXAMINATION REPORTS

The components of a bone marrow report should be generated systematically and are given in Table 17-6. An example of a bone marrow examination report is provided in Figure 17-23.

### TABLE 17-5 Definitive Studies Performed on Selected Bone Marrow Specimens

<table>
<thead>
<tr>
<th>Bone Marrow Study</th>
<th>Application</th>
<th>Specimen</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron stain</td>
<td>Identification of iron deficiency, iron overload</td>
<td>Fresh marrow aspirate</td>
<td>20</td>
</tr>
<tr>
<td>Cytochemical studies</td>
<td>Diagnosis of leukemias and lymphomas</td>
<td>Fresh marrow aspirate</td>
<td>29, 33, 35, 36</td>
</tr>
<tr>
<td>Cytogenetic studies</td>
<td>Diagnosis of acute leukemias via deletions, translocations, and polysomy; remission studies</td>
<td>1 mL marrow in heparin</td>
<td>30</td>
</tr>
<tr>
<td>Molecular studies</td>
<td>Polymerase chain reaction for diagnostic point mutations; minimal residual disease studies</td>
<td>1 mL marrow in EDTA</td>
<td>31</td>
</tr>
<tr>
<td>Fluorescence in situ hybridization</td>
<td>Staining for diagnostic mutations; minimal residual disease studies</td>
<td>Fresh marrow aspirate</td>
<td>31</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Immunophenotyping, usually of malignant hematopoietic cells, clonality; minimal residual disease studies</td>
<td>1 mL marrow in heparin, EDTA, or ACD</td>
<td>32</td>
</tr>
</tbody>
</table>

ACD, Acid-citrate dextrose; EDTA, ethylenediaminetetraacetic acid.
PART III  Laboratory Evaluation of Blood Cells

Laboratory of Bone Marrow Pathology Report

<table>
<thead>
<tr>
<th>Name</th>
<th>Room number and Unit</th>
<th>Patient number</th>
<th>Specimen number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>Age</td>
<td>Sex</td>
<td>Race</td>
</tr>
</tbody>
</table>

Address

City, State, Zip

Site | Aspirate | Biopsy | Markers | Cytogenetics and Molecular Studies

Attending Physician

Pathologist

Clinical Diagnosis

Acute leukemia

Pathology Diagnosis

Acute myelomonocytic leukemia (FAB M4) with mild eosinophilia. M98613, M47150, D 40800

Differential

<table>
<thead>
<tr>
<th>Date</th>
<th>PB</th>
<th>Marrow</th>
<th>Marrow Range (percent)</th>
<th>PB</th>
<th>Marrow</th>
<th>Marrow Range (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blast, unclassified</td>
<td>4</td>
<td>77</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>3</td>
<td>5</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>1</td>
<td>8</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>4</td>
<td>5-21</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>6</td>
<td>22</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Band neutrophils</td>
<td>6</td>
<td>22</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Segmented neutrophils</td>
<td>40</td>
<td>4</td>
<td>9-27</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3</td>
<td>4</td>
<td>9-27</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Basophils</td>
<td>3</td>
<td>4</td>
<td>9-27</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>21</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>21</td>
<td>0-2</td>
<td>50</td>
<td>2</td>
<td>3-24</td>
</tr>
<tr>
<td>WBC</td>
<td>2,300</td>
<td>PLT</td>
<td>89,000</td>
<td>MPV</td>
<td>8</td>
<td>HGB</td>
</tr>
</tbody>
</table>


Figure 17-23  Example of a bone marrow examination report, including reference intervals, peripheral blood complete blood count results, marrow differential, and narrative. PB, Peripheral blood; BMA, bone marrow aspirate; BMBX, bone marrow biopsy specimen; DX, diagnosis.
TABLE 17-6 Components of a Bone Marrow Examination Report

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient history</td>
<td>Patient identity and age, narration of symptoms, physical findings, findings in kindred, treatment</td>
</tr>
<tr>
<td>Complete blood count (CBC)</td>
<td>Peripheral blood CBC collected no more than 24 hours before the bone marrow puncture, includes hemogram and peripheral blood film examination</td>
</tr>
<tr>
<td>Cellularity</td>
<td>Hypocellular, normocellular, or hypercellular classification based on ratio of hematopoietic cells to adipocytes</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>Estimate using 10× objective (100× magnification), compare with reference interval and comment on morphology</td>
</tr>
<tr>
<td>Maturation</td>
<td>Narrative characterizing the maturation of the myelocytic and erythrocytic (normoblastic, rubricytic) series</td>
</tr>
<tr>
<td>Additional hematologic cells</td>
<td>Narrative describing numbers and morphology of eosinophils, basophils, mast cells, lymphocytes, plasma cells, monocytes, and histiocytes if appropriate, with reference intervals</td>
</tr>
<tr>
<td>Stromal cells</td>
<td>Narrative describing numbers and morphology of osteoblasts, osteoclasts, bony trabeculae, fibroblasts, adipocytes, and endothelial cells; appearance of sinuses; presence of amyloid, granulomas, fibrosis, necrosis</td>
</tr>
<tr>
<td>Differential count</td>
<td>Numbers of all cells and cell stages observed after performing a differential count on 300 to 1000 cells and comparing results with reference intervals</td>
</tr>
<tr>
<td>Myeloid-to-erythroid ratio</td>
<td>Computed from nucleated hematologic cells less lymphocytes, plasma cells, monocytes, and histiocytes</td>
</tr>
<tr>
<td>Iron stores</td>
<td>Categorization of findings as increased, normal, or decreased iron stores</td>
</tr>
<tr>
<td>Diagnostic narrative</td>
<td>Summary of the recorded findings and additional laboratory chemical, microbiologic, and immunoassay tests</td>
</tr>
</tbody>
</table>

**SUMMARY**

- Adult hematopoietic tissue is located in the flat bones and the ends of the long bones. Hematopoiesis occurs within the spongy trabeculae of the bone adjacent to vascular sinuses.
- Bone marrow collection is a safe but invasive procedure performed by a pathologist or hematologist in collaboration with a medical laboratory scientist to obtain specimens used to diagnose hematologic and systemic disease and to monitor treatment.
- The necessity for a bone marrow examination should be evaluated in light of all clinical and laboratory information. In anemias for which the cause is apparent from the RBC indices, a bone marrow examination is not required. Examples of indications for bone marrow examination include multilineage abnormalities in the peripheral blood, pancytopenia, circulating blasts, and staging of lymphomas and carcinomas.
- A peripheral blood specimen is collected for a complete blood count no more than 24 hours before the bone marrow is collected, and the results of the CBC are reported with the bone marrow examination results.
- Bone marrow may be collected from the posterior or anterior iliac crest or sternum using sterile disposable biopsy and aspiration needles and canulas. The site and equipment depend on how old the patient is and whether both an aspirate and a biopsy specimen are desired.
- The medical laboratory scientist receives the bone marrow specimen and prepares aspirate smears, crush preparations, imprints, anticoagulated bone marrow smears, and fixed biopsy sections, and specimens for confirmatory studies.
- The medical laboratory scientist and pathologist collaborate with residents, fellows, attending physicians, and medical laboratory science students to stain and review bone marrow aspirate smears, biopsy sections, and confirmatory procedure results.
- Confirmatory procedures include cytochemistry, cytogenetics, and immunophenotyping by flow cytometry; fluorescence in situ hybridization; and molecular diagnostics.
- The medical laboratory scientist and pathologist determine cellularity and megakaryocyte distribution, then perform a differential count of 300 to 1000 bone marrow hematopoietic cells and compute the M:E ratio, comparing the results with reference intervals.
- The pathologist characterizes features of hematopoietic disease, metastatic tumor cells, and abnormalities of the bone marrow stroma and prepares a systematic written bone marrow examination report including a diagnostic narrative.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

**REVIEW QUESTIONS**

Answers can be found in the Appendix.

1. Where is most hematopoietic tissue found in adults?
   - a. Liver
   - b. Lungs
   - c. Spleen
   - d. Long bones

2. What is the preferred bone marrow collection site in adults?
   - a. Second intercostal space on the sternum
   - b. Anterior or posterior iliac crest
   - c. Any of the thoracic vertebrae
   - d. Anterior head of the femur
3. The aspirate should be examined under low power to assess all of the following except:
   a. Cellularity
   b. Megakaryocyte numbers
   c. Morphology of abnormal cells
   d. Presence of tumor cell clusters

4. What is the normal M:E ratio range in adults?
   a. 1.5:1 to 3.3:1
   b. 5.1:1 to 6.2:1
   c. 8.6:1 to 10.2:1
   d. 10:1 to 12:1

5. Which are the most common erythrocytic stages found in normal marrow?
   a. Pronormoblasts
   b. Pronormoblasts and basophilic normoblasts
   c. Basophilic and polychromatophilic normoblasts
   d. Polychromatophilic and orthochromic normoblasts

6. What cells, occasionally seen in bone marrow biopsy specimens, are responsible for the formation of bone?
   a. Macrophages
   b. Plasma cells
   c. Osteoblasts
   d. Osteoclasts

7. What is the largest hematopoietic cell found in a normal bone marrow aspirate?
   a. Osteoblast
   b. Myeloblast
   c. Pronormoblast
   d. Megakaryocyte

8. Which of the following is not an indication for a bone marrow examination?
   a. Pancytopenia (reduced numbers of RBCs, WBCs, and platelets in the peripheral blood)
   b. Anemia with RBC indices corresponding to low serum iron and low ferritin levels
   c. Detection of blasts in the peripheral blood
   d. Need for staging of Hodgkin lymphoma

9. In a bone marrow biopsy specimen, the RBC precursors were estimated to account for 40% of the cells in the marrow, and the other 60% were granulocyte precursors. What is the M:E ratio?
   a. 4:6
   b. 1.5:1
   c. 1:1.5
   d. 3:1

10. On a bone marrow core biopsy sample, several large cells with multiple nuclei were noted. They were located close to the endosteum, and their nuclei were evenly spaced throughout the cell. What are these cells?
    a. Megakaryocytes
    b. Osteoclasts
    c. Adipocytes
    d. Fibroblasts

11. The advantage of a core biopsy bone marrow sample over an aspirate is that the core biopsy specimen:
    a. Can be acquired by a less invasive collection technique
    b. Permits assessment of the architecture and cellular arrangement
    c. Retains the staining qualities of basophils owing to the use of Zenker fixative
    d. Is better for the assessment of bone marrow iron stores with Prussian blue stain

REFERENCES

Body Fluid Analysis in the Hematology Laboratory

Bernadette F. Rodak*

OUTLINE

Performing Cell Counts on Body Fluids
Preparing Cytocentrifuge Slides
Cerebrospinal Fluid
  Gross Examination
  Cell Counts
  Differential Cell Counts
Serous Fluid
  Transudates Versus Exudates
  Gross Examination
  Differential Cell Counts
Synovial Fluid
  Gross Examination
  Differential Cell Counts
  Crystals
Bronchoalveolar Lavage Specimens
  Procedure and Precautions
  Differential Cell Counts

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the method for performing cell counts on body fluids.
2. Given a description of a body fluid for cell counting, choose the appropriate diluting fluid, select a counting area, and calculate and correct (if necessary) the counts.
3. Using the proper terminology, discuss the gross appearance of body fluids, including its significance and its practical use in determining cell count dilutions.
4. Discuss the advantages and disadvantages of cytocentrifuge preparations.
5. Differentiate between traumatic spinal tap and cerebral hemorrhage on the basis of cell counts and the appearance of uncentrifuged and centrifuged specimens.
6. Identify from written descriptions normal cells found in cerebrospinal, serous, and synovial fluids.
7. Describe the characteristics of benign versus malignant cells in body fluids, and recognize written descriptions of each.
8. Differentiate exudates and transudates based on formation (cause), specific gravity, protein concentration, appearance, and cell concentration.
9. Identify crystals in synovial fluids from written descriptions, including polarization characteristics.
10. Describe the process of obtaining bronchoalveolar lavage (BAL) samples, including safety precautions for analysis; state the purpose of BAL; and recognize types of cells that normally would be found in BAL specimens.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 33-year-old semiconscious woman was brought to the emergency department by her husband. The previous day she had complained of a headache and had left work early. When she got home, she took aspirin and took a brief nap, and she reported she felt better that evening. Her husband stated that the next morning “she couldn’t talk,” so he brought her to the emergency department. A spinal tap was performed. The fluid that arrived in the laboratory was cloudy. The WBC count was 10.6 \times 10^9/L. Most of the cells seen on the cytocentrifuge slide were neutrophils.

1. When multiple tubes of CSF are obtained, which tube should be used for cell counts?
2. What dilution should be made to obtain a satisfactory cytocentrifuge slide?
3. What should you look for on the cytocentrifuge slide?
4. What is the most likely diagnosis for this patient?

*The author would like to acknowledge Leilani Collins for her work on this chapter in the previous edition.
The analysis of body fluids, including nucleated blood cell count and differential count, can provide valuable diagnostic information. This chapter is not intended as a comprehensive treatment of all body fluids, but it covers cell counting and morphologic hematology. The fluids discussed in this chapter include cerebrospinal fluid (CSF), serous or body cavity fluids (pleural, pericardial, and peritoneal fluids), and synovial (joint) fluids. Bronchoalveolar lavage (BAL) specimens are discussed briefly.

**Performing Cell Counts on Body Fluids**

Examination of all fluids should include observation of color and turbidity, determination of cell counts, and white blood cell (WBC) evaluation. Blood cell counts should be performed and cytocentrifuge slides should be prepared as quickly as possible after collection of the specimen, because WBCs begin to deteriorate within 30 minutes after collection. It is important to mix the specimen gently but thoroughly before every manipulation (i.e., counting cells, preparing any dilution, and preparing cytocentrifuge slides). Cell counts on fluids usually are performed using a hemacytometer (Chapter 14); however, many automated instruments now are capable of performing blood cell counts on fluids. Even specimens with low counts can be reported as long as the WBC count is above the limits of linearity of the instrument and no cellular interference flags are noted. Each instrument manufacturer should provide a statement of intended use that defines which body fluids have been approved by a regulatory agency for testing on the instrument. Care should be taken to observe the operating limits of these instruments—that is, the analytical measurement range (AMR) and the volume limits for the fluid received. Red blood cell (RBC) counts on serous and synovial fluids have little clinical value; relevant clinical information is obtained merely from the appearance of the fluid (grossly bloody, bloody, slightly bloody).

Cell counts are performed with undiluted fluid if the fluid is clear. If the fluid is hazy or bloody, appropriate dilutions should be made to permit accurate counts of WBCs and RBCs. The smallest reasonable dilution should be made. The diluting fluid for RBCs is isotonic saline. Diluting fluids for WBCs include glacial acetic acid and methylene blue to stain the nuclei of the WBCs. Acetic acid cannot be used for synovial fluids because synovial fluid contains hyaluronic acid, which coagulates in acetic acid. A small amount of hyaluronidase powder (a pinch, or what can be picked up between two wooden sticks) or one drop of 0.05% hyaluronidase in phosphate buffer per milliliter of fluid should be added to the synovial fluid sample to liquefy it before performing cell counts or preparing cytocentrifuge slides. Dilutions should be based on the turbidity of the fluid or on the number of cells seen on the hemacytometer when using an undiluted sample.

A WBC count of approximately 200/μL or an RBC count of approximately 400/μL causes a fluid to be slightly hazy. If the fluid is blood-tinged to slightly bloody, the RBCs can be counted using undiluted fluid, but it is advisable to use a small (1:2) dilution with Türk solution (or similar) to lyse the RBCs and provide an accurate WBC or nucleated cell count. If the fluid is bloody, a 1:200 dilution with isotonic saline for RBCs and either a 1:2 or a 1:20 dilution with Türk solution for nucleated cells should be used to obtain an accurate count. When performing dilutions for blood cell counts, a calibrated pipette should be used, such as MLA pipettes or the Ovation BioNatural pipettes (VistaLab Technologies, Inc. Brewster, NY).

The number of squares to be counted on the hemacytometer should be determined on the basis of the number of cells present. In general, all nine squares on both sides of the hemacytometer should be counted. If the number of cells is high, however, fewer squares may be counted. Each square equals 1 mm². The formula for calculating the number of cells (Chapter 14) is:

\[
\text{Cells counted} \times \text{depth factor} \times \text{dilution factor} = \frac{\text{Area counted (mm}^2\text{)}}{}
\]

Guidelines for counting are summarized in Table 18-1.

**Preparing Cytocentrifuge Slides**

The cytocentrifuge enhances the ability to identify the types of cells present in a fluid. This centrifuge spins at a low speed, which minimizes distortion of the cellular elements and provides a “button” of cells that are concentrated into a small area. The cytocentrifuge assembly consists of a cytofunnel, filter paper to absorb excess fluid, and a glass slide. These three components are fastened together in a clip assembly, a few drops of well-mixed specimen are dispensed into the cytofunnel, and the entire assembly is centrifuged slowly. The cells are deposited onto the slide, and excess fluid is absorbed into the filter paper, which produces a monolayer of cells in a small button (Figure 18-1).

Although there is some cell loss into the filter paper, this is not selective, and an accurate representation of the types of cells present in a fluid is provided. There also may be some distortion of cells as a result of the centrifugation process or crowding of cells when high cell counts are present. To minimize distortion resulting from overcrowding of cells, appropriate dilutions should be made with normal saline before centrifugation. The basis for this dilution should be the WBC count or the nucleated cell count. A nucleated cell count of 200/μL or fewer provides a good basis for the differential. If the WBC count is extremely elevated, a larger dilution may be necessary; however, an RBC count of 5000/μL would not cause significant nucleated cell distortion. If a fluid has a nucleated cell count of 2000/μL and an RBC count of 10,000/μL, a 1:10 dilution should be made, which produces a nucleated cell count of 200/μL and an RBC count of 1000/μL for the cytocentrifuge slide. If the RBC count of a fluid is greater than 1 million/μL³, it is best to make a “push” slide to perform the differential. In this case, the differential should be performed on the cells “pushed out” on the end of the smear instead of in the body of the
smear, because that is where the larger, and possibly more significant, cells would be deposited.

If a consistent amount of fluid is used when cytocentrifuge slides are prepared, a consistent yield of cells can be expected; this can be used to confirm the WBC or nucleated cell count. For example, if five drops of fluid (undiluted or diluted) are always used to prepare cytocentrifuge slides, a 100-cell differential count should be obtainable if the WBC or nucleated cell count is equal to or greater than 3×10^6/L. In all cases, the entire cell button should be scanned before the differential count is performed to ensure that significant clumps of cells are not overlooked. The area of the cell button that is used for performing the differential count is not important, but if the number of nucleated cells present is small, use of a “systematic meander” starting at one side of the button and working toward the other side is best. In case the number of cells recovered is small, the area around the cell button should be marked on the back of the slide with a wax pencil, or premarked slides should be used to prepare cytocentrifuge slides (Figure 18-1).

### CEREBROSPINAL FLUID

CSF is the only fluid that exists in quantities sufficient to sample in healthy individuals. CSF is present in volumes of 100 to 150 mL in adults, 60 to 100 mL in children, and 10 to 60 mL in newborns. This fluid bathes the brain and spinal column and serves as a cushion to protect the brain, as a circulating nutrient medium, as an excretory channel for nervous tissue metabolism, and as lubrication for the central nervous system. CSF is collected by lumbar puncture using either the L3-4 or L4-5 interspace (Figure 18-2).

### Gross Examination

Normal CSF is nonviscous, clear, and colorless. A cloudy or hazy appearance may indicate the presence of WBCs (greater than 200/μL), RBCs (greater than 400/μL), or microorganisms. Bloody fluid may be caused by a traumatic tap, in which blood is acquired as the puncture is performed, or by a pathologic hemorrhage within the central nervous system. If more than one tube is received, the tubes can be observed for clearing from tube to tube. If the first tube contains blood but the remaining tubes are clear or progressively clearer, the blood is the result of a traumatic puncture. If all tubes are uniformly bloody, the probable cause is a subarachnoid hemorrhage. When a bloody sample is received, an aliquot should be centrifuged, and the color of the supernatant should be observed and reported. A clear, colorless supernatant indicates a traumatic

---

**TABLE 18-1 Guidelines for Counting Fluids**

<table>
<thead>
<tr>
<th>Test</th>
<th>Clear (0–70)</th>
<th>Hazy (&gt;200)</th>
<th>Blood-Tinged</th>
<th>Cloudy (1:2 Türk)</th>
<th>Cloudy (1:20 in Türk solution)</th>
<th>Cloudy (1:2 in Türk solution)</th>
<th>Cloudy (1:20 in isotonic saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs</td>
<td>0–200/μL</td>
<td>&gt;200/μL</td>
<td>Unknown</td>
<td>High</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Dilution for counting</td>
<td>None</td>
<td>1:2 Türk</td>
<td>1:2 Türk</td>
<td>1:20 in Türk solution</td>
<td>1:2 in Türk solution</td>
<td>1:20 in isotonic saline</td>
<td></td>
</tr>
<tr>
<td>No. squares to count on hemacytometer</td>
<td>9 or 4</td>
<td>9 or 4</td>
<td>9 or 4</td>
<td>9 or 4</td>
<td>9 or 4</td>
<td>9 or 4</td>
<td>9 or 4</td>
</tr>
<tr>
<td>RBCs</td>
<td>0–400/μL</td>
<td>Unknown</td>
<td>&gt;400/μL</td>
<td>Unknown</td>
<td>&gt;6000/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution for counting</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>1:200 in isotonic saline</td>
</tr>
<tr>
<td>No. squares to count on hemacytometer</td>
<td>9 large</td>
<td>9 large</td>
<td>9 or 4 large</td>
<td>4 large or 5 small</td>
<td>5 small</td>
<td>5 small</td>
<td></td>
</tr>
<tr>
<td>Cytospin dilution (0.25 mL [5 drops] of fluid)*</td>
<td>Undiluted</td>
<td>Dilute with saline to 100–200/μL nucleated cell count</td>
<td>Dilute with saline to 100–200/μL nucleated cell count</td>
<td>Dilute with saline to 100–200/μL nucleated cell count</td>
<td>Dilute by nucleated cell count</td>
<td>Dilute by nucleated cell count</td>
<td></td>
</tr>
</tbody>
</table>

RBC: Red blood cell; WBC: white blood cell.

*Expected cell yield (WBC count for number of cells recovered on slide): 0/μL for 0–70, 1-2/μL for 12–100, >3/μL for >100.
Cell Counts

When multiple tubes of spinal fluid are collected, the cell count is generally performed on tube 3, or the tube with the lowest possibility of peripheral blood contamination. Tube 1 is used for chemistry and immunology, and tube 2 is used for microbiology. Normal cell counts in CSF are 0 to 5 WBCs/μL and 0 RBCs/μL in adults, and 0 to 30 WBCs/μL and 0 RBCs/μL in neonates. If a high RBC count is obtained, one may determine whether the source of WBCs is peripheral blood contamination by using the peripheral blood ratio of 1 WBC per 500 to 900 RBCs. If peripheral blood cell counts are known, the number of blood WBCs added to the CSF sample can be calculated using the following formula:

$$\text{WBC}_{\text{added}} = \frac{\text{WBC}_{\text{peripheral}} \times \frac{\text{RBC}_{\text{CSF}}}{\text{RBC}_{\text{peripheral}}}}{1000}$$

where WBCperipheral is the WBC count for peripheral blood, RBCCSF is the RBC count for CSF, and RBCperipheral is the RBC count for peripheral blood. The corrected or true CSF WBC count (WBCCSF) is calculated as follows:

$$\text{True WBC}_{\text{CSF}} = \text{CSF hemacytometer count} - \text{WBCs added}$$

Some laboratories have questioned the value of an RBC count on CSF and report only the WBC count. A high WBC count may be found in fluid from patients with infective processes, such as meningitis. In general, WBC counts are much higher (in the thousands) in patients with tap, whereas a yellowish or pinkish yellow tinge may indicate a subarachnoid hemorrhage. This yellowish color sometimes is referred to as xanthochromia, but because not all xanthochromia is pathologic, the Clinical and Laboratory Standards Institute recommends avoiding the term and simply reporting the actual color of the supernatant (Figure 18-3 and Table 18-2).
bacterial meningitis than in patients with viral meningitis (in the hundreds).\textsuperscript{10-12} The predominant cell type present on the cytocentrifuge slide (neutrophils or lymphocytes), however, is a better indicator of the type of meningitis—bacterial or viral. Elevated WBC or nucleated cell counts also may be obtained in patients with inflammatory processes and malignancies.

**Differential Cell Counts**

The cells normally seen in CSF are lymphocytes and monocytes (Figure 18-4). In adults, the predominant cells are lymphocytes, and in newborns, the predominant cells are monocytes.\textsuperscript{8,9} Neutrophils are not normal in CSF but may be seen in small numbers because of concentration techniques. When the WBC count is elevated and large numbers of neutrophils are seen, a thorough and careful search should be made for bacteria because organisms may be present in very small numbers early in bacterial meningitis (Figure 18-5). In viral meningitis, the predominant cells seen are lymphocytes, including reactive or viral lymphocytes and plasmacytoid lymphocytes (Figure 18-6). However, early in the course of the illness, neutrophils may predominate.\textsuperscript{10-12} Eosinophils and basophils may be seen in response to the presence of foreign materials such as shunts, in parasitic infections, or in allergic reactions (Figure 18-7).\textsuperscript{8,9} When nucleated RBCs are seen, bone marrow contamination resulting from accidental puncture of the vertebral body during spinal tap should be suspected and reported. In the case of bone marrow contamination, other immature neutrophils and megakaryocytes also may be seen. When there is obvious bone marrow contamination, the WBC differential is likely to be equivalent to that of the bone marrow and not that of the CSF.

Ependymal and choroid plexus cells, lining cells of the central nervous system, may be seen. These are large cells with abundant cytoplasm that stains lavender with Wright stain. They most often appear in clumps, and although they are not diagnostically significant, it is important not to confuse them with malignant cells (Figure 18-8).

Cartilage cells may be seen if the vertebral body is accidentally punctured. These cells usually occur singly, are medium to large, and have cytoplasm that stains wine red with a deep wine red nucleus with Wright stain (Figure 18-9).

Siderophages are macrophages (i.e., monocytes or histiocytes) that have ingested RBCs and, as a result of the breakdown of the RBCs, contain hemosiderin. Hemosiderin appears as large, rough-shaped, dark blue or black granules in the cytoplasm of the macrophage. These cells also may contain bilirubin or hematoidin crystals, which are golden yellow and are a result of further breakdown of the ingested RBCs. The presence of siderophages indicates a pathologic

<table>
<thead>
<tr>
<th>Traumatic Tap</th>
<th>Pathologic Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear supernatant</td>
<td>Colored or hemolyzed supernatant</td>
</tr>
<tr>
<td>Clearing from tube to tube</td>
<td>Same appearance in all tubes</td>
</tr>
<tr>
<td>Bone marrow contamination</td>
<td>Erythrophages</td>
</tr>
<tr>
<td>Cartilage cells</td>
<td>Siderophages (may have bilirubin crystals)</td>
</tr>
</tbody>
</table>

**Figure 18-4** Monocyte (left) and lymphocyte (right) seen in normal cerebrospinal fluid (×1000).

**Figure 18-5** Neutrophils with bacteria in cerebrospinal fluid from a patient with bacterial meningitis (Wright stain, ×1000). (From Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders.)

**Figure 18-6** Reactive (viral) lymphocytes in cerebrospinal fluid from a patient with viral meningitis (×1000).
leukemic cells (i.e., blast forms) in the CSF of patients with leukemia. Patients with lymphoma, myeloma, and chronic myelogenous leukemia in blast crisis also may have blast cells in the CSF. These blast cells have the characteristics of blast forms in the peripheral blood, including a high nucleus-to-cytoplasm ratio, a fine stippled nuclear chromatin pattern, and prominent nucleoli. They are usually large cells that stain basophilic with Wright stain and have a fairly uniform appearance (Figure 18-11). If a traumatic tap has occurred and the patient has a high blast count in the peripheral blood, the blasts seen in the CSF may be the result of peripheral blood contamination and not central nervous system involvement. The possibility of peripheral blood contamination should be reported and the tap should be repeated in a few days.

Malignant cells resulting from metastases to the central nervous system may be found. The most common primary tumors that metastasize to the central nervous system in adults are breast, lung, and gastrointestinal tract tumors and melanoma. In children, metastases to the central nervous system are related to Wilms tumor, Ewing sarcoma, neuroblastoma, and embryonal rhabdomyosarcoma. Malignant cells are usually large with a high nucleus-to-cytoplasm ratio and hemorrhage. Siderophages appear approximately 48 hours after hemorrhage and may persist for 2 to 8 weeks after the hemorrhage has occurred (Figure 18-10).

A high percentage of patients with acute lymphoblastic leukemia or acute myeloid leukemia have central nervous system involvement. It is always important to look carefully for
between the membranes of an organ and the sac in which it is housed. Pleural fluid is found in the space between the lungs and the pleural sac; pericardial fluid, in the space between the heart and the pericardial sac; and peritoneal fluid, between the intestine and the peritoneal sac (Figure 18-13). An accumulation of fluid in a cavity is termed an effusion. When an effusion is in the peritoneal cavity, it also may be referred to as ascites or ascitic fluid. It would be difficult to remove these fluids from a healthy individual; the presence of these fluids in detectable amounts indicates a disease state.

**Transudates Versus Exudates**

As noted, the accumulation of a large amount of fluid in a cavity is called an effusion. Effusions are subdivided further into transudates and exudates to distinguish whether disease is present within or outside the body cavity. In general, transudates develop as part of systemic disease processes, such as congestive heart failure, whereas exudates indicate disorders associated with bacterial or viral infections, malignancy, pulmonary embolism, or systemic lupus erythematosus. Several parameters can be measured to determine whether an effusion is a transudate or an exudate (Table 18-4).

**Gross Examination**

Transudates should appear straw-colored and clear. A cloudy or hazy fluid may indicate an exudate from an infectious process; a bloody fluid, trauma, or malignancy; and a milky fluid, effused chyle in the pleural cavity.

**Differential Cell Counts**

The cells found in normal serous fluid are lymphocytes, histiocytes (macrophages), and mesothelial cells. Neutrophils commonly are seen in the fluid sent to the laboratory for analysis but would not be present in normal fluid. When neutrophils are seen, they have more segments and longer filaments than in peripheral blood (Figure 18-14).

### Table 18-3 Characteristics of Benign and Malignant Cells

<table>
<thead>
<tr>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasional large cells</td>
<td>Many cells may be very large.</td>
</tr>
<tr>
<td>Light to dark staining</td>
<td>May be very basophilic.</td>
</tr>
<tr>
<td>Rare mitotic figures</td>
<td>May have several mitotic figures.</td>
</tr>
<tr>
<td>Round to oval nucleus; nuclei are uniform in size with varying amounts of cytoplasm.</td>
<td>May have irregular or jagged nuclear shape.</td>
</tr>
<tr>
<td>Nuclear edge is smooth.</td>
<td>Edges of nucleus may be indistinct and irregular.</td>
</tr>
<tr>
<td>Nuclear is intact.</td>
<td>Nucleus may be disintegrated at edges.</td>
</tr>
<tr>
<td>Nucleoli are small, if present.</td>
<td>Nucleoli may be large and prominent.</td>
</tr>
<tr>
<td>In multinuclear cells (mesothelial), all nuclei have similar appearance (size and shape).</td>
<td>Multinuclear cells have varying sizes and shapes of nuclei.</td>
</tr>
<tr>
<td>Moderate to small N:C ratio</td>
<td>May have high N:C ratio.</td>
</tr>
<tr>
<td>Clumps of cells have similar appearance among cells, are in the same plane of focus, and may have “windows” between cells.</td>
<td>Clumps of cells contain cells of varying sizes and shapes, are “three-dimensional” (require focusing up and down to see all cells), and have dark-staining borders.</td>
</tr>
</tbody>
</table>

N:C, Nucleus-to-cytoplasm.
Mesothelial cells are the lining cells of body cavities and are shed into these cavities constantly. These are large (12- to 30-μm) cells and have a “fried egg” appearance with basophilic cytoplasm, oval nucleus with smooth nuclear borders, stippled nuclear chromatin pattern, and one to three nucleoli. Mesothelial cells may vary in size, may be multinucleated (including giant cells with 20 to 25 nuclei), and may have frayed cytoplasmic borders, cytoplasmic vacuoles, or both. They may occur singly, in small or large clumps, or in sheets. When they occur in clumps, there are usually “windows” between the cells. The nucleus-to-cytoplasm ratio is 1:2 to 1:3, and this is generally consistent despite the variability in cell size. They tend to have a similar appearance to each other on a slide. Mesothelial cells are seen in most effusions, and their numbers are increased in sterile inflammations and decreased in tuberculous pleurisy and bacterial infections (Figure 18-15).8

Macrophages appear as monocytes or histiocytes in serous fluids and may contain RBCs (erythrophages) or siderotic granules (siderophages), or they may appear as signet ring cells when lipid has been ingested and the resulting large vacuole pushes the nucleus to the periphery of the cell (Figure 18-16).

Eosinophils and basophils are not normally seen. These may be present in large numbers, however, as a result of allergic reaction or sensitivity to foreign material.
When large numbers of neutrophils are seen, a thorough search should be made for bacteria. If possible, Gram staining should be performed on a second cytocentrifuge slide to aid in rapid identification if bacteria are found. Table 18-5 lists Gram-stained organisms most commonly seen in body fluids.

Lupus erythematosus cells may be seen in serous fluids of patients with systemic lupus erythematosus, because all the factors necessary for the formation of these cells—presence of the lupus erythematosus factor, incubation, and trauma to the cells—exist in vivo. A lupus erythematosus cell is an intact neutrophil that has engulfed a homogeneous mass of degenerated nuclear material, which displaces the normal nucleus. Lupus erythematosus cells can form in vivo and in vitro in serous and synovial fluids and should be reported (Figure 18-17).

**Table 18-5** Gram-Stained Organisms Most Commonly Seen in Body Fluids

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrospinal</td>
<td>Gram-negative diplococci</td>
</tr>
<tr>
<td></td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td></td>
<td>Gram-negative coccobacilli</td>
</tr>
<tr>
<td></td>
<td>Yeast—stains gram-positive</td>
</tr>
<tr>
<td></td>
<td>Cryptococcus—look for capsule</td>
</tr>
<tr>
<td>Serous (peritoneal, pleural, or pericardial)</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td></td>
<td>Gram-negative bacilli</td>
</tr>
<tr>
<td></td>
<td>Gram-positive bacilli</td>
</tr>
<tr>
<td></td>
<td>Yeast—stains gram-positive</td>
</tr>
<tr>
<td>Synovial (joint)</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td></td>
<td>Gram-negative bacilli</td>
</tr>
<tr>
<td></td>
<td>Gram-negative diplococci</td>
</tr>
<tr>
<td></td>
<td>Gram-negative coccobacilli</td>
</tr>
</tbody>
</table>

Note: If the Gram-stained organisms seen in a fluid are not listed above for that fluid, do not report Gram stain results. Save the slide for review.
Malignant cells are seen in serous fluids from primary or metastatic tumors. They have the characteristics of malignant cells found in CSF (Figure 18-18). Figure 18-19 presents a flow chart for examination of serous fluids.

### SYNOVIAL FLUID

#### Gross Examination

Synovial fluid is normally present in very small amounts in the synovial cavity surrounding joints. When fluid is present in amounts large enough to aspirate, there is a disease process in the joint. Figure 18-20 demonstrates placement of the needle for synovial fluid collection from a knee. Normally this fluid is straw-colored and clear. Synovial fluid contains hyaluronic acid, which makes it very viscous. A small amount (pinch) of hyaluronidase powder should be added to all joint fluids to liquefy them before cell counts are performed or cytocentrifuge slides are prepared. If a crystal analysis is to be performed, an aliquot of fluid should be removed for this purpose before the hyaluronidase is added.

#### Differential Cell Counts

Cells found in normal synovial fluid are lymphocytes, monocytes/histiocytes, and synovial cells. Synovial cells line the synovial cavity and are shed into the cavity. They resemble mesothelial cells but are usually present in smaller numbers (Figure 18-21).

Lupus erythematous cells may be present in synovial fluid just as in serous fluid. Malignant cells are rarely seen in synovial fluid, but when present resemble tumor cells seen in serous fluids or CSF.

Many neutrophils are present in synovial fluid in acute inflammation of joints. As always, a careful search should be made for bacteria when many neutrophils are seen.

#### Crystals

Intracellular and extracellular crystals may be present in synovial fluid. Crystal examination may be performed by placing a drop of

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**Figure 18-17** Lupus erythematous cell (arrow) in pleural fluid (×1000).

**Figure 18-18** A, Clump of tumor cells in pleural fluid (×200). B, Tumor cells and mitotic figure in pleural fluid (×1000). C, Adenocarcinoma cells in pleural fluid (×200). D, Tumor cells in peritoneal fluid (×200). Note cannibalism.
CHAPTER 18  Body Fluid Analysis in the Hematology Laboratory

Serous fluids  
(Pleural, peritoneal, pericardial, ascitic)

Gross appearance (color, clarity)

Cell counts

WBC

Türk (1:2) or (1:20)

RBC

Undiluted or (1:200) dilution with saline

Cytospin slide

If dilution is necessary, base dilution on NCC. Dilute to 100–200/mm³

Normal

Lymphocytes
Histiocytes/monocytes
Mesothelial cells

Segemented leukocytes

Look for bacteria

Perform gram stain on cytocentrifuge slide

Tumor

Send to cytology laboratory

Report results

Figure 18-19  Flowchart for examination of serous fluid. NCC, Nucleated cell count; RBC, red blood cell; WBC, white blood cell.

Figure 18-20  Schematic of knee demonstrating placement of the needle for synovial fluid aspiration. (From Applegate E: The anatomy and physiology learning system, ed 4, Philadelphia, 2011, Saunders.)

Figure 18-21  Synovial cells in synovial fluid (×400). Note similarity to mesothelial cells.
fluid on a slide and adding a coverslip or by examining a cytocentrifuge preparation. However, the specimen should be fresh, without hyaluronidase added. All synovial fluids should be examined carefully for crystals using a polarizing microscope with a red compensator. The crystals most commonly seen in synovial fluids are cholesterol, calcium pyrophosphate, and monosodium urate.

Cholesterol crystals are large, flat, extracellular crystals with a notched corner. They are seen in patients with chronic effusions, particularly patients with rheumatoid arthritis. Calcium pyrophosphate crystals are seen in pseudogout. These crystals are intracellular and are small rhomboid, platelike, or rodlike crystals. The crystals are weakly birefringent when polarized (i.e., they do not appear bright when polarized). When the red compensator is used, calcium pyrophosphate crystals appear blue when the longitudinal axis of the crystal is parallel to the \( y \)-axis (Figure 18-22).

Monosodium urate crystals are seen in gout. They are large needlelike crystals that may be intracellular or extracellular. These crystals are strongly birefringent when polarized. When the red compensator is used, monosodium urate crystals appear yellow when the longitudinal axis of the crystal is parallel to the \( y \)-axis (Figure 18-23).

**Figure 18-22** Intracellular calcium pyrophosphate crystals in synovial fluid (\( \times \)1000). A, Wright stain. B, Polarized with red compensator. (B courtesy of George Girgis, MT[ASCP], Indiana University Health, Indianapolis, IN.)

**Figure 18-23** Intracellular (A) and extracellular (B) monosodium urate crystals in synovial fluid (\( \times \)1000). A, Wright stain. B, Polarized with red compensator. (A from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Saunders. B courtesy of George Girgis, MT[ASCP], Indiana University Health, Indianapolis, IN.)

**BRONCHOALVEOLAR LAVAGE SPECIMENS**

**Procedure and Precautions**

BAL specimens are not naturally occurring fluids; they are produced when the BAL procedure is performed. The procedure consists of introducing warmed saline into the lungs in 50-mL aliquots and then withdrawing it. The specimen received in the laboratory is the withdrawn fluid. The purpose of the procedure is to determine types of organisms and cells that are present in areas of the lung that are otherwise inaccessible. This procedure is performed on patients with severe lung dysfunction. The specimen should always undergo an extensive microbiologic workup and often cytologic examination. It is common to see bacteria, yeast, or both on cytocentrifuge slides prepared from these specimens. Because samples are obtained from the interior of the lung and may contain airborne organisms, care should be taken to avoid aerosol production. Samples should be mixed and containers opened under a biologic safety hood, and a mask should be worn when performing cell counts. Because the risk of performing cell counts and preparing cytocentrifuge slides on BAL specimens outweighs the clinical relevance of the information obtained, some
hematology laboratories no longer perform this procedure and defer to information reported from the microbiology laboratory.

Cell counts and cytocentrifuge preparations are performed as with any body fluid. Significant cell deterioration occurs within 30 minutes of collection, with the neutrophils disintegrating most rapidly.

**Differential Cell Counts**

The cell types most commonly seen in BAL specimens are neutrophils, monohistiocytes (macrophages), and lymphocytes. Mesothelial cells are not seen in BAL specimens because these cells line the body cavities and not the interior of the lung. Pneumocytes, which can resemble mesothelial cells or adenocarcinoma, may be seen in patients with adult respiratory distress syndrome.

Ciliated epithelial cells can be seen and should be reported because they indicate that the sample was obtained from the upper respiratory tract instead of deeper in the lung. These are columnar cells, with the nucleus at one end of the cell, elongated cytoplasm, and cilia at the opposite end of the cell from the nucleus. They can occur in clusters. If the sample is not aged when the cell count is performed, these cells are in motion in the hemacytometer, because they can be propelled by their cilia (Figure 18-25).

Histiocytes laden with carbonaceous material are seen in patients who use tobacco. These cells resemble siderophages in other fluids, but the carbonaceous material is black, brown, or blue-black and is more dropletlike (Figure 18-26).

*Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) may be seen in specimens from patients infected with human immunodeficiency virus. The *P. jiroveci* organisms appear as clumps of amorphous material. Close examination of the clumps may reveal cysts (Figure 18-27).
Figure 18-26  Histocytes with carbonaceous material in bronchoalveolar lavage fluid (×40).

Figure 18-27  Cyst of *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) in bronchoalveolar lavage fluid (×100).

**SUMMARY**

- Cell counts and differential counts performed on body fluid specimens are valuable diagnostic tools.
- Calibrated methods must be used when performing cell counts to provide accurate counts.
- To optimize cell morphologic features, specimens should not be overdiluted or underdiluted when cytocentrifuge slides are prepared.
- Normal cell types in any fluid are lymphocytes, macrophages (monocytes, histiocytes), and lining cells (ependymal cells in CSF, mesothelial cells in serous fluids, synovial cells in joint fluids).

- Bacteria and yeast may be seen in any fluid.
- Malignant cells may be seen in any fluid but are rare in synovial fluid.
- Synovial fluid should be examined for crystals using a compensated polarizing microscope.
- BAL specimens are not a true body fluid, but examination of cells present may provide diagnostic information.

*Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.*

**REVIEW QUESTIONS**

Answers can be found in the Appendix.

*Refer to the following scenario to answer questions 1 and 2:* A spinal fluid specimen is diluted 1:2 with Türk solution to perform the nucleated cell count. A total of 6 nucleated cells are counted on both sides of the hemacytometer, with all nine squares counted on both sides. Undiluted fluid is used to perform the RBC count. A total of 105 RBCs is counted on both sides of the hemacytometer, with four large squares on both sides counted.

1. The nucleated cell count is ____/μL.
   a. 3  
   b. 7  
   c. 13  
   d. 66

2. The RBC count is ____/μL.
   a. 131  
   b. 263  
   c. 1050  
   d. 5830

3. Based on the cell counts, the appearance of the fluid is:
   a. Turbid  
   b. Hemolyzed  
   c. Clear  
   d. Cloudy

4. All of the following cells are normally seen in CSF, serous fluids, and synovial fluids except:
   a. Lining cells  
   b. Neutrophils  
   c. Lymphocytes  
   d. Monocytes/histiocytes (macrophages)
5. Spinal fluid was obtained from a 56-year-old woman. On receipt in the laboratory, the fluid was noted to be slightly bloody. When a portion of the fluid was centrifuged, the supernatant was clear. The cell counts were 5200 RBCs/µL and 24 WBCs/µL. On the cytocentrifuge preparation, several nucleated RBCs were seen. The differential was 52% lymphocytes, 20% neutrophils, 22% monocytes, 4% myelocytes, and 2% blasts. What is the most likely explanation for these results?
   a. Bone marrow contamination
   b. Bacterial meningitis
   c. Peripheral blood contamination
   d. Leukemic infiltration in the central nervous system

6. A 34-year-old woman with a history of breast cancer developed a pleural effusion. The fluid obtained was bloody and had a nucleated cell count of 284/µL. On the cytocentrifuge preparation, there were several neutrophils and a few monocytes/histiocytes. There were also several clusters of large, dark-staining cells. These cell clumps appeared “three-dimensional” and contained some mitotic figures. What is the most likely identification of the cells in clusters?
   a. Mesothelial cells
   b. Metastatic tumor cells
   c. Cartilage cells
   d. Pneumocytes

7. A serous fluid with a clear appearance, specific gravity of 1.010, protein concentration of 1.5 g/dL, and fewer than 500 mononuclear cells/µL would be considered:
   a. Infectious
   b. An exudate
   c. A transudate
   d. Sterile

8. On the cytocentrifuge slide prepared from a peritoneal fluid sample, many large cells are seen, singly and in clumps. The cells have a “fried egg” appearance and basophilic cytoplasm, and some are multinucleated. These cells should be reported as:
   a. Suspicious for malignancy
   b. Macrophages
   c. Large lymphocytes
   d. Mesothelial cells

Refer to the following scenario to answer questions 9 and 10: A 56-year-old man came to the physician’s office with complaints of pain and swelling in his left big toe. Fluid aspirated from the toe was straw-colored and cloudy. The WBC count was 2543/µL. The differential consisted mainly of neutrophils and monocytes/histiocytes. Intracellular and extracellular crystals were seen on the cytocentrifuge slide. The crystals were needle-shaped and, when polarized with the use of the red compensator, appeared yellow on the y-axis.

9. The crystals are:
   a. Cholesterol
   b. Hyaluronidase
   c. Monosodium urate
   d. Calcium pyrophosphate

10. This patient’s painful toe was caused by:
    a. Gout
    b. Infection
    c. Inflammation
    d. Pseudogout

REFERENCES

A 45-year-old female phoned her physician and complained of fatigue, shortness of breath on exertion, and general malaise. She requested “B12 shots” to make her feel better. The physician asked the patient to schedule an appointment so that she could determine the cause of the symptoms before offering treatment. A point-of-care hemoglobin determination performed in the office was 9.0 g/dL.

The physician then requested additional laboratory tests, including a CBC with a peripheral blood film examination and a reticulocyte count.

1. Why did the physician want the patient to come to the office before she prescribed therapy?
2. How do the mean cell volume and reticulocyte count help determine the classification of the anemia?
3. Why is the examination of the peripheral blood film important in the investigation of an anemia?
Red blood cells (RBCs) perform the vital physiologic function of oxygen delivery to tissues. Hemoglobin within the RBCs has the remarkable capacity to bind oxygen in the lungs and then release it appropriately in the tissues. The term anemia is derived from the Greek word anaimia, meaning “without blood.” A decrease in hemoglobin concentration or number of RBCs results in decreased oxygen delivery to tissue, resulting in tissue hypoxia. Anemia is a common condition affecting an estimated 1.62 billion people worldwide. Anemia should not be thought of as a disease but rather as a manifestation of an underlying disease or deficiency. Therefore, causes of anemia should be thoroughly investigated. This chapter provides an overview of the mechanisms, diagnosis, and classification of anemia. In the following chapters, each anemia is discussed in detail.

**DEFINITION OF ANEMIA**

A functional definition of anemia is a decrease in the oxygen-carrying capacity of the blood. It can arise if there is insufficient hemoglobin or the hemoglobin has impaired function. The former is the more frequent cause.

Anemia is defined operationally as a reduction in the hemoglobin content of blood that can be caused by a decrease in RBCs, hemoglobin, and hematocrit below the reference interval for healthy individuals of similar age, sex, and race, under similar environmental conditions. The reference intervals are derived from large pools of “healthy” individuals; however, the definition of healthy is different for each of these groups. Thus these pools of “healthy” individuals may lack the heterogeneity required to be universally applied to any one of these populations of individuals. This fact has led to the development of different reference intervals for individuals of different sex, age, and race.

Examples of hematologic reference intervals for the adult and pediatric populations are included on the inside cover of this text. They are listed according to age and sex, but race, environmental, and laboratory factors can also influence the values. Each laboratory must determine its own reference intervals based on its particular instrumentation, the methods used, and the demographics and environment of its patient population. For the purpose of the discussion in this chapter, a patient is considered anemic if the hemoglobin value falls below those listed in these tables.

**PATIENT HISTORY AND CLINICAL FINDINGS**

The history and physical examination are important components in making a clinical diagnosis of anemia. A decrease in oxygen delivery to tissues decreases the energy available to individuals to perform day-to-day activities. This gives rise to the classic symptoms associated with anemia, fatigue and shortness of breath. To elucidate the reason for a patient’s anemia, one starts by obtaining a good history that requires carefully questioning the patient, particularly with regard to diet, drug ingestion, exposure to chemicals, occupation, hobbies, travel, bleeding history, race or ethnic group, family history of disease, neurologic symptoms, previous medication, previous episodes of jaundice, and various underlying disease processes that result in anemia. Therefore, a thorough discussion is required to elicit any potential cause of the anemia. For example, iron deficiency can lead to an interesting symptom called pica. Patients with pica have cravings for unusual substances such as ice (pagophagia), cornstarch, or clay. Alternatively, individuals with anemia may be asymptomatic, as can be seen in mild or slowly progressive anemias.

Certain features should be evaluated closely during the physical examination to provide clues to hematologic disorders, such as skin (for petechiae), eyes (for pallor, jaundice, and hemorrhage), and mouth (for mucosal bleeding). The examination should also search for sternal tenderness, lymphadenopathy, cardiac murmurs, splenomegaly, and hepatomegaly. Jaundice is important for the assessment of anemia, because it may be due to increased RBC destruction, which suggests a hemolytic component to the anemia. Measuring vital signs is also a crucial component of the physical evaluation. Patients experiencing a rapid fall in hemoglobin concentration typically have tachycardia (fast heart rate), whereas if the anemia is long-standing, the heart rate may be normal due to the body’s ability to compensate for the anemia (discussed below).

Moderate anemias (hemoglobin concentration of 7 to 10 g/dL) may cause pallor of conjunctivae and nail beds but may not produce clinical symptoms if the onset of anemia is slow. However, depending on the patient’s age and cardiovascular state, symptoms such as dyspnea, vertigo, headache, muscle weakness, and lethargy can occur. Severe anemias (hemoglobin concentration of less than 7 g/dL) usually produce tachycardia, hypotension, and other symptoms of volume loss, in addition to the symptoms listed earlier. Thus, severity of the anemia is gauged by the degree of reduction in hemoglobin, cardiopulmonary adaptation, and the rapidity of progression of the anemia.

**PHYSIOLOGIC ADAPTATIONS**

Anemia resulting from acute blood loss, such as with severe hemorrhage, can lead to profound changes in physiological processes that ensure adequate perfusion of vital organs and maintenance of homeostasis. In cases of severe blood loss, such as in trauma, blood volume decreases and hypotension develops, resulting in decreased blood supply to the brain and heart. As an immediate adaptation, there is sympathetic overdrive that results in increasing heart rate, respiratory rate, and cardiac output. In severe anemia, blood is preferentially shunted to organs that are key to survival, including the brain, muscle, and heart. This results in oxygen being preferentially supplied to vital organs even in the presence of reduced oxygen-carrying capacity. In addition, tissue hypoxia triggers an increase in RBC 2,3-bisphosphoglycerate that shifts the oxygen dissociation curve to the right (decreased oxygen affinity of hemoglobin) and results in increased delivery of oxygen to tissues (Chapter 10). This is also a significant mechanism in chronic anemias that enables patients with low levels of hemoglobin to remain relatively asymptomatic. Thus with persistent anemia, the body develops physiologic adaptations to increase the oxygen-carrying capacity of a reduced amount of hemoglobin, which improves oxygen delivery to tissue. With persistent...
and severe anemia, however, the strain on the heart can ultimately lead to cardiac failure.

Reduced delivery of oxygen to tissues caused by reduced hemoglobin concentration elicits an increase in erythropoietin secretion by the kidneys. Erythropoietin stimulates the RBC precursors in the bone marrow, which leads to the release of more RBCs into the circulation (Chapter 8).

It should be noted that with rapid blood loss, the hemoglobin and hematocrit may be initially unchanged because there is balanced loss of plasma and cells. However, as the drop in blood volume is compensated for by movement of fluid from the extracellular to the intravascular compartment or by administration of resuscitation fluid, there will be a dilution of RBCs and anemia. Box 19-1 summarizes the body’s physiologic adaptations to anemia.

**MECHANISMS OF ANEMIA**

The life span of the RBC in the circulation is about 120 days. In a healthy individual with no anemia, each day, approximately 1% of the RBCs are removed from circulation due to senescence, but the bone marrow continues to produce RBCs to replace those lost. Hematopoietic stem cells differentiate into erythroid (RBC) precursor cells, and the bone marrow releases reticulocytes (immature anucleated RBCs) that mature into RBCs in the peripheral circulation. Adequate RBC production requires several nutritional factors such as iron, vitamin B₁₂, and folate. Globin (polypeptide chain) synthesis must also function normally. In conditions with excessive bleeding or hemolysis, the bone marrow must increase RBC production to compensate for the increased RBC loss. Therefore, the maintenance of a stable hemoglobin concentration requires the production of functionally normal RBCs in sufficient numbers to replace the amount lost.⁴

**Ineffective and Insufficient Erythropoiesis**

*Erythropoiesis* is the term used for marrow erythroid proliferative activity. Normal erythropoiesis occurs in the bone marrow and is under the control of the hormone *erythropoietin* (produced by the kidney) and other growth factors and cytokines (Chapters 7 and 8).⁷,⁸ When erythropoiesis is effective, the bone marrow is able to produce functional RBCs that replace the daily loss of RBCs.

*Ineffective erythropoiesis* refers to the production of erythroid precursor cells that are defective. These defective precursors often undergo apoptosis (programmed cell death) in the bone marrow before they have a chance to mature to the reticulocyte stage and be released into the peripheral circulation. Several conditions, such as megaloblastic anemia (deficient DNA synthesis due to vitamin B₁₂ or folate deficiency), thalassemia (deficient globin chain synthesis), and sideroblastic anemia (deficient protoporphyrin synthesis) involve ineffective erythropoiesis as a mechanism of anemia. In these anemias, the peripheral blood hemoglobin is low, which triggers an increase in erythropoietin leading to increased erythropoietic activity. Although the RBC production rate is high, it is ineffective in that many of the defective RBC precursors undergo destruction in the bone marrow. The end result is a decreased number of circulating RBCs resulting in anemia.³,¹¹

*Insufficient erythropoiesis* refers to a decrease in the number of erythroid precursors in the bone marrow, resulting in decreased RBC production and anemia. Many factors can lead to the decreased RBC production, including a deficiency of iron (inadequate intake, malabsorption, excessive loss from chronic bleeding); a deficiency of erythropoietin (renal disease); or loss of the erythroid precursors due to an autoimmune reaction (aplastic anemia, acquired pure red cell aplasia) or infection (parvovirus B19). Infiltration of the bone marrow with granulomas (sarcoidosis) or malignant cells (acute leukemia) can also suppress erythropoiesis.⁴,⁷

**Blood Loss and Hemolysis**

Anemia can also develop as a result of acute blood loss (such as a traumatic injury) or chronic blood loss (such as an intermittent bleeding colonic polyp). Increased hemolysis results in a shortened RBC life span, thus increasing the risk for anemia. Chronic blood loss induces iron deficiency as a cause of anemia. With acute blood loss and excessive hemolysis, the bone marrow takes a few days to increase production of RBCs.⁴,⁷,⁸ This response may be inadequate to compensate for a sudden excessive RBC loss as in traumatic hemorrhage or in conditions with a high rate of hemolysis and shortened RBC survival. Numerous causes of hemolysis exist, including intrinsic defects in the RBC membrane, enzyme systems, or hemoglobin, or extrinsic causes such as antibody-mediated processes, mechanical fragmentation, or infection-related destruction.⁴,⁷,⁸

**LABORATORY DIAGNOSIS OF ANEMIA**

**Complete Blood Count with Red Blood Cell Indices**

To detect the presence of anemia, the medical laboratory professional performs a complete blood count (CBC) using an automated hematology analyzer to determine the RBC count, hemoglobin concentration, hematocrit, RBC indices, white blood cell count, and platelet count. The RBC indices include the mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) (Chapter 14).¹³ The most important of these indices is the
MCV, a measure of the average RBC volume in femtoliters (fL). Reference intervals for these determinations are listed on the inside front cover of the text. Automated hematology analyzers also provide the red cell distribution width (RDW), an index of variation of cell volume in a red blood cell population (discussed below). A reticulocyte count should be performed for every patient with anemia. As with RBCs, automated analyzers provide accurate measurements of reticulocyte counts.

The RBC histogram provided by the automated analyzer is an RBC volume frequency distribution curve with the relative number of cells plotted on the ordinate and RBC volume (fL) on the abscissa. In healthy individuals, the distribution is approximately Gaussian. Abnormalities include a shift in the curve to the left (population of smaller cells or microcytosis) or to the right (larger cell population or macrocytosis). A widening of the curve caused by a greater variation of RBC volume about the mean can occur due to a population of RBCs with different volumes (anisocytosis). The histogram complements the peripheral blood film examination in identifying variable RBC populations. A discussion of histograms with examples can be found in Chapters 15 and 16.

The RDW is the coefficient of variation of RBC volume expressed as a percentage.\(^4\) It indicates the variation in RBC volume within the population measured and an increased RDW correlates with anisocytosis on the peripheral blood film. Automated analyzers calculate the RDW by dividing the standard deviation of the RBC volume by the MCV and then multiplying by 100 to convert to a percentage. The usefulness of the RDW is discussed later.

**Reticulocyte Count**

The reticulocyte count serves as an important tool to assess the bone marrow's ability to increase RBC production in response to an anemia. Reticulocytes are young RBCs that lack a nucleus but still contain residual ribonucleic acid (RNA) to complete the production of hemoglobin. Normally, they circulate peripherally for only 1 day while completing their development. The adult reference interval for the reticulocyte count is 0.5% to 2.5% expressed as a percentage of the total number of RBCs.\(^2\) The newborn reference interval is 1.5% to 6.0%, but these values change to approximately those of an adult within a few weeks after birth.\(^3\) An absolute reticulocyte count is determined by multiplying the percent reticulocytes by the RBC count. The reference interval for the absolute reticulocyte count is 20 to 115 \(\times 10^9/L\), based on an adult RBC count within the reference interval.\(^5,7\) A patient with a severe anemia may seem to be producing increased numbers of reticulocytes if only the percentage is considered. For example, an adult patient with \(1.5 \times 10^12/L\) RBCs and 3% reticulocytes has an absolute reticulocyte count of \(45 \times 10^9/L\). The percentage of reticulocytes is above the reference interval, but the absolute reticulocyte count is within the reference interval. For the degree of anemia, however, both of these results are inappropriately low. In other words, production of reticulocytes within the reference interval is inadequate to compensate for an RBC count that is approximately one third of normal.

Two successive corrections are made to the reticulocyte count to obtain a better representation of RBC production. First, to obtain a corrected reticulocyte count, one corrects for the degree of anemia by multiplying the reticulocyte percentage by the patient’s hematocrit and dividing the result by 45 (the average normal hematocrit). If the reticulocytes are released prematurely from the bone marrow and remain in the circulation 2 to 3 days (instead of 1 day), the corrected reticulocyte count must be divided by maturation time to determine the reticulocyte production index (RPI) (Table 19-1). The RPI is a better indication of the rate of RBC production than is the corrected reticulocyte count.\(^4\) The reticulocyte count and derivation of RPI is discussed in Chapter 14.

In addition, state-of-the-art automated hematology analyzers determine the fraction of immature reticulocytes among the total circulating reticulocytes, called the immature reticulocyte fraction (IRF). The IRF is helpful in assessing early bone marrow response after treatment for anemia and is covered in Chapter 15.

Analysis of the reticulocyte count plays a crucial role in determining whether an anemia is due to an RBC production defect or to premature hemolysis and shortened survival defect. If there is shortened RBC survival, as in the hemolytic anemias, the bone marrow tries to compensate by increasing RBC production to release more reticulocytes into the peripheral circulation. Although an increased reticulocyte count is a hallmark of the hemolytic anemias, it can also be observed over time in acute blood loss.\(^5,7,8\) Chronic blood loss, on the other hand, does not lead to an appropriate increase in the reticulocyte count, but rather leads to iron deficiency and a subsequent low reticulocyte count. Thus an inappropriately low reticulocyte count results from decreased production of normal RBCs, due to either insufficient or ineffective erythropoiesis.

**TABLE 19-1 Formulas for Reticulocyte Counts and Red Blood Cell Indices**

<table>
<thead>
<tr>
<th>Test</th>
<th>Formula</th>
<th>Adult Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute reticulocyte count (\times 10^9/L)</td>
<td>(\text{[reticulocytes (%)}/100 \times \text{RBC count (\times 10^{12}L)})</td>
<td>20–115 (\times 10^9/L)</td>
</tr>
<tr>
<td>Corrected reticulocyte count (%)</td>
<td>(\text{reticulocytes (%)} \times \text{patient's HCT (%)/45})</td>
<td>---</td>
</tr>
<tr>
<td>Reticulocyte production index (RPI)</td>
<td>(\text{corrected reticulocyte count/maturation time})</td>
<td>In anemic patients, RPI should be &gt;3</td>
</tr>
<tr>
<td>Mean cell volume (MCV) (fL)</td>
<td>(\text{HCT (%)} \times 10/\text{RBC count (\times 10^{12}L)})</td>
<td>80–100 fL</td>
</tr>
<tr>
<td>Mean cell hemoglobin (MCH) (pg)</td>
<td>(\text{HGB (g/dL)} \times 10/\text{RBC count (\times 10^{12}L)})</td>
<td>26–32 pg</td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration (MCHC) (g/dL)</td>
<td>(\text{HGB (g/dL)} \times 100/\text{HCT (%)})</td>
<td>32–36 g/dL</td>
</tr>
</tbody>
</table>

\(\text{HGB, Hemoglobin; HCT, hematocrit; RBC, red blood cell.}\)
**Peripheral Blood Film Examination**

An important component in the evaluation of an anemia is examination of the peripheral blood film, with particular attention to RBC diameter, shape, color, and inclusions. The peripheral blood film also serves as a quality control to verify the results produced by automated analyzers. Normal RBCs on a Wright-stained blood film are nearly uniform, ranging from 6 to 8 µm in diameter. Small or microcytic cells are less than 6 µm in diameter, and large or macrocytic RBCs are greater than 8 µm in diameter. Certain shape abnormalities of diagnostic value (such as sickle cells, spherocytes, schistocytes, and oval macrocytes) and RBC inclusions (such as malarial parasites, basophilic stippling, and Howell-Jolly bodies) can be detected only by studying the RBCs on a peripheral blood film (Tables 19-2 and 19-3). Examples of abnormal shapes and inclusions are provided in Figure 19-1.

Finally, a review of the white blood cells and platelets may help show that a more generalized bone marrow problem is leading to the anemia. For example, hypersegmented neutrophils can be seen in vitamin B₁₂ or folate deficiency, whereas blast cells and decreased platelets may be an indication of acute leukemia. Chapter 16 contains a complete discussion of

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**TABLE 19-2 Description of Red Blood Cell (RBC) Abnormalities and Commonly Associated Disease States**

<table>
<thead>
<tr>
<th>RBC Abnormality</th>
<th>Cell Description</th>
<th>Commonly Associated Disease States</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisocytosis</td>
<td>Abnormal variation in RBC volume or diameter</td>
<td>Hemolytic, megaloblastic, iron deficiency anemia</td>
</tr>
<tr>
<td>Macrocyte</td>
<td>Large RBC (&gt;8 µm in diameter), MCV &gt;100 fl</td>
<td>Megaloblastic anemia, myelodysplastic syndrome, chronic liver disease, bone marrow failure, reticulocytosis</td>
</tr>
<tr>
<td>Oval macrocyte</td>
<td>Large oval RBC</td>
<td>Megaloblastic anemia</td>
</tr>
<tr>
<td>Microcyte</td>
<td>Small RBC (&lt;6 µm in diameter), MCV &lt;80 fl</td>
<td>Iron deficiency anemia, anemia of chronic inflammation, sideroblastic anemia, thalassemia/hb E disease and trait</td>
</tr>
<tr>
<td>Poikilocytosis</td>
<td>Abnormal variation in RBC shape</td>
<td>Severe anemia; certain shapes helpful diagnostically</td>
</tr>
<tr>
<td>Spherocyte</td>
<td>Small, round, dense RBC with no central pallor</td>
<td>Hereditary spherocytosis, immune hemolytic anemia</td>
</tr>
<tr>
<td>Elliptocyte, ovalocyte</td>
<td>Elliptical (cigar-shaped), oval (egg-shaped), RBC</td>
<td>Hereditary elliptocytosis or ovalocytosis, iron deficiency anemia, thalassemia major, myelophthisis anemias</td>
</tr>
<tr>
<td>Stomatocyte</td>
<td>RBC with slit-like area of central pallor</td>
<td>Hereditary stomatocytosis, Rh deficiency syndrome, acquired stomatocytosis (liver disease, alcoholism), artifact</td>
</tr>
<tr>
<td>Sickle cell</td>
<td>Thin, dense, elongated RBC pointed at each end; may be curved</td>
<td>Sickle cell anemia, sickle cell β-thalassemia</td>
</tr>
<tr>
<td>Hb C crystal</td>
<td>Hexagonal crystal of dense hemoglobin formed within the RBC membrane</td>
<td>Hb C disease</td>
</tr>
<tr>
<td>Hb SC crystal</td>
<td>Fingerlike or quartz-like crystal of dense hemoglobin protruding from the RBC membrane</td>
<td>Hb SC disease</td>
</tr>
<tr>
<td>Target cell (codocyte)</td>
<td>RBC with hemoglobin concentrated in the center and around the periphery resembling a target</td>
<td>Liver disease, hemoglobinopathies, thalassemia</td>
</tr>
<tr>
<td>Schistocyte (schizocyte)</td>
<td>Fragmented RBC due to rupture in the peripheral circulation</td>
<td>Microangiopathic hemolytic anemia* (along with microspherocytes), macroangiopathic hemolytic anemia**, extensive burns (along with microspherocytes)</td>
</tr>
<tr>
<td>Helmet cell (keratocyte)</td>
<td>RBC fragment in shape of a helmet</td>
<td>Same as schistocyte</td>
</tr>
<tr>
<td>Folded cell</td>
<td>RBC with membrane folded over</td>
<td>Hb C disease, Hb SC disease</td>
</tr>
<tr>
<td>Acanthocyte (spur cell)</td>
<td>Small, dense RBC with few irregularly spaced projections of varying length</td>
<td>Severe liver disease (spur cell anemia), neuroacanthocytosis (abetalipoproteinemia, McLeod syndrome)</td>
</tr>
</tbody>
</table>
### TABLE 19-3 Erythrocyte Inclusions: Description, Composition, and Some Commonly Associated Disease States*

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Appearance in Supravital Stain</th>
<th>Appearance in Wright Stain</th>
<th>Inclusion Composed of</th>
<th>Associated Diseases/Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse basophilia</td>
<td>Dark blue granules and filaments in cytoplasm (seen in reticulocytes)</td>
<td>Blush tinge throughout cytoplasm; also called polychromasia (seen in polychromatic erythrocytes)</td>
<td>RNA</td>
<td>Hemolytic anemia After treatment for iron, vitamin B₁₂, or folate deficiency</td>
</tr>
<tr>
<td>Basophilic stippling</td>
<td>Dark blue-purple, fine or coarse punctate granules distributed throughout cytoplasm</td>
<td>Dark blue-purple, fine or coarse punctate granules distributed throughout cytoplasm</td>
<td>Precipitated RNA</td>
<td>Lead poisoning Thalassemia Hemoglobinopathies Megaloblastic anemia Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Howell-Jolly body</td>
<td>Dark blue-purple dense, round granule; usually one per cell; occasionally multiple</td>
<td>Dark blue-purple dense, round granule; usually one per cell; occasionally multiple</td>
<td>DNA (nuclear fragment)</td>
<td>Hyposplenism Postsplenectomy Megaloblastic anemia Hemolytic anemia Thalassemia Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Heinz body</td>
<td>Round, dark blue-purple granule attached to inner RBC membrane</td>
<td>Not visible</td>
<td>Denatured hemoglobin</td>
<td>Glucose-6-phosphate dehydrogenase deficiency Unstable hemoglobins Oxidant drugs/chemicals</td>
</tr>
<tr>
<td>Pappenheimer bodies***</td>
<td>Irregular clusters of small, light to dark blue granules, often near periphery of cell</td>
<td>Irregular clusters of small, light to dark blue granules, often near periphery of cell</td>
<td>Iron</td>
<td>Sideroblastic anemia Hemoglobinopathies Thalassemias Megaloblastic anemia Myelodysplastic syndrome Hyposplenism Postsplenectomy</td>
</tr>
<tr>
<td>Cabot ring</td>
<td>Rings or figure-eights</td>
<td>Blue rings or figure-eights</td>
<td>Remnant of mitotic spindle</td>
<td>Megaloblastic anemia Myelodysplastic syndromes</td>
</tr>
<tr>
<td>Hb H</td>
<td>Fine, evenly dispersed, dark blue granules; imparts “golf ball” appearance to RBCs</td>
<td>Not visible</td>
<td>Precipitate of β-globin chains of hemoglobin</td>
<td>Hb H disease</td>
</tr>
</tbody>
</table>

**Hb, Hemoglobin.**

*Inclusions of hemoglobin crystals (Hb S, Hb C, Hb SC) are covered in Table 19-2.

**Such as new methylene blue.

***Stain dark blue and are called siderotic granules when observed in Prussian blue stain.
Figure 19-1  Red blood cells (RBCs): varied RBC shapes and inclusions. Hb, Hemoglobin. (Modified from Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St Louis, 2013, Elsevier, Saunders.)
the peripheral blood film evaluation. Information from the blood film examination always complements the data from the automated hematology analyzer.

**Bone Marrow Examination**

The cause of many anemias can be determined from the history, physical examination, and results of laboratory tests on peripheral blood. When the cause cannot be determined, however, or the differential diagnosis remains broad, a bone marrow aspiration and biopsy may help in establishing the cause of anemia. A bone marrow examination is indicated for a patient with an unexplained anemia associated with or without other cytopenias, fever of unknown origin, or suspected hematologic malignancy. A bone marrow examination evaluates hematopoiesis and can determine if there is an infiltration of abnormal cells into the bone marrow. Important findings in the bone marrow can point to the underlying cause of the anemia include abnormal cellularity (e.g., hypocellularity in aplastic anemia); evidence of ineffective erythropoiesis and megaloblastic changes (e.g., folate/vitamin B12 deficiency or myelodysplastic syndrome); lack of iron on iron stains of the bone marrow (the gold standard for diagnosis of iron deficiency); and the presence of granulomas, fibrosis, infectious agents, and tumor cells that may be inhibiting normal erythropoiesis. Chapter 17 discusses bone marrow procedures and bone marrow examination in detail.

Other tests that can assist in the diagnosis of anemia can be performed on the bone marrow sample as well, including immunophenotyping of membrane antigens by flow cytometry (Chapter 32), cytogenetic studies (Chapter 30), and molecular analysis to detect specific genetic mutations and chromosome abnormalities in leukemia cells (Chapter 31).

**Other Laboratory Tests**

Other laboratory tests that can assist in establishing the cause of anemia include routine urinalysis (to detect hemoglobinuria or an increase in urobilinogen) with a microscopic examination (to detect hematuria or hemosiderin) and analysis of stool (to detect occult blood or intestinal parasites). Also, certain chemistry studies are very useful, such as serum haptoglobin, lactate dehydrogenase, and unconjugated bilirubin (to detect excessive hemolysis) and renal and hepatic function tests. With more patients having undergone gastric bypass surgery for obesity, certain rare deficiencies such as insufficient copper have become more common as another nutritional deficiency that can cause anemia.

After the hematologic laboratory studies are completed, the anemia may be classified based on reticulocyte count, MCV, and peripheral blood film findings. Iron studies (including serum iron, total iron-binding capacity, transferrin saturation, and serum ferritin) are valuable if an inappropriately low reticulocyte count and a microcytic anemia are present. Serum vitamin B12 and serum folate assays are helpful in investigating a macrocytic anemia with a low reticulocyte count, whereas a direct antiglobulin test can differentiate autoimmune hemolytic anemias from hemolytic anemias due to other causes. Because of the numerous potential etiologies of anemia, the specific cause needs to be determined to initiate appropriate therapy.

**APPRAOCH TO EVALUATING ANEMIAS**

The approach to the patient with anemia begins with taking a complete history and performing a physical examination. For example, new-onset fatigue and shortness of breath suggest an acute drop in the hemoglobin concentration, whereas minimal or lack of symptoms suggests a long-standing condition where adaptive mechanisms have compensated for the drop in hemoglobin. A strict vegetarian may not be getting enough vitamin B12 in the diet, whereas an individual with alcoholism may not be getting enough folate. A large spleen may be an indication of hereditary spherocytosis, whereas a stool positive for occult blood may indicate iron deficiency. Thus a complete history and physical examination can yield information to narrow the possible cause or causes of the anemia and thus lead to a more rational and cost-effective approach to ordering the appropriate diagnostic tests.

The first step in the laboratory diagnosis of anemia is detecting its presence by the accurate measurement of the hemoglobin, hematocrit, MCV and RBC count and comparison of these values with the reference interval for healthy individuals of the same age, sex, race, and environment. Knowledge of previous hematologic values is valuable as a reduction of 10% or more in these values may be the first clue that an abnormal condition may be present.

There are numerous causes of anemia, so a rational algorithm to initially evaluate this condition utilizing the above-mentioned tests is required. A reticulocyte count and a peripheral blood film examination are of paramount importance in evaluating anemia.

The remainder of this chapter discusses the importance of individual RBC measurements, the MCV, reticulocyte count, and RDW, and how they assist in classifying anemias so as to arrive at a specific diagnosis. Two widely used classification schemes for anemias relate to the morphology of red cells and the pathophysiological condition responsible for the patient’s anemia.

**Morphologic Classification of Anemia Based on Mean Cell Volume**

The MCV is an extremely important tool and is key in the morphologic classification of anemia. Microcytic anemia is characterized by an MCV of less than 80 fL with small RBCs (less than 6 µm in diameter). Microcytosis is often associated with hypochromia, characterized by an increased central pallor in the RBCs and an MCHC of less than 32 g/dL. Microcytic anemias are caused by conditions that result in reduced hemoglobin synthesis. Heme synthesis is diminished in iron deficiency, iron sequestration (chronic inflammatory states), and defective protoporphyrin synthesis (sideroblastic anemia, lead poisoning). Globin chain synthesis is defective in thalassemia and in Hb E disease. Iron deficiency is the most common cause of microcytic anemia; the low iron level is insufficient for maintaining normal erythropoiesis. Although iron deficiency anemia is characterized by abnormal iron studies, the early stages of iron deficiency do not result in microcytosis or anemia and are manifested only by reduced iron stores. The causes of iron deficiency vary in infants, children, adolescents, and adults, and it is imperative to find the cause before beginning treatment (Chapter 20).
Macrocytic anemia is characterized by an MCV greater than 100 fL with large RBCs (greater than 8 μm in diameter). Macrocytic anemias arise from conditions that result in megaloblastic or nonmegaloblastic red cell development in the bone marrow. Megaloblastic anemias are caused by conditions that impair synthesis of deoxyribonucleic acid (DNA), such as vitamin B₁₂ and folate deficiency or myelodysplasia. Nuclear maturation lags behind cytoplasmic development as a result of the impaired DNA synthesis. This asynchrony between nuclear and cytoplasmic development results in larger cells. All cells of the body are ultimately affected by the defective production of DNA (Chapter 21). Pernicious anemia is one cause of vitamin B₁₂ deficiency, whereas malabsorption secondary to inflammatory bowel disease is one cause of folate deficiency. A megaloblastic anemia is characterized by oval macrocytes and hypersegmented neutrophils in the peripheral blood and by megaloblasts or large nucleated RBC precursors in the bone marrow. The MCV in megaloblastic anemia can be markedly increased (up to 150 fL), but modest increases (100 to 115 fL) occur as well.

Nonmegaloblastic forms of macrocytic anemias are also characterized by large RBCs, but in contrast to megaloblastic anemias, they are typically related to membrane changes owing to disruption of the cholesterol-to-phospholipid ratio. These macrocytic cells are mostly round, and the marrow nucleated RBCs do not display megaloblastic maturation changes. Macrocytic anemias are often seen in patients with chronic liver disease, alcohol abuse, and bone marrow failure. It is rare for the MCV to be greater than 115 fL in these nonmegaloblastic anemias.

Normocytic anemia is characterized by an MCV in the range of 80 to 100 fL. The RBC morphology on the peripheral blood film must be examined to rule out a dimorphic population of microcytes and macrocytes that can yield a normal MCV. The presence of a dimorphic population can also be verified by observing a bimodal distribution on the RBC histogram produced by an automated hematology analyzer (Chapters 15 and 16). Some normocytic anemias develop due to the premature destruction and shortened survival of RBCs (hemolytic anemias), and they are characterized by an elevated reticulocyte count. The hemolytic anemias can be further divided into those due to intrinsic causes (membrane defects, hemoglobinopathies, and enzyme deficiencies) and those due to extrinsic causes (immune and nonimmune RBC injury). A direct antiglobulin test helps differentiate immune-mediated destruction from the other causes. In the other hemolytic anemias, reviewing the peripheral blood film is vital for determining the cause of the hemolysis (Table 19-2, Table 19-3, and Figure 19-1). Hemolytic anemias are discussed in Chapters 23 to 26.

Other normocytic anemias develop due to a decreased production of RBCs and are characterized by a decreased reticulocyte count. Figure 19-2 presents an algorithm for initial morphologic classification of anemia based on the MCV.

Figure 19-2 Algorithm for morphologic classification of anemia based on mean cell volume (MCV). Anemia of chronic liver disease is multifactorial and can be normocytic. ↑, Increased; ↓, decreased; Hb, hemoglobin; N, normal; retic, absolute reticulocyte count.
Morphologic Classification of Anemias and the Reticulocyte Count

The absolute reticulocyte count is useful in initially classifying anemias into the categories of decreased or ineffective RBC production (decreased reticulocyte count) and excessive RBC loss (increased reticulocyte count). Using the morphologic classification in the first category, when the reticulocyte count is decreased, the MCV can further classify the anemia into three subgroups: normocytic anemias, microcytic anemias, and macrocytic anemias. The excessive RBC loss category includes acute hemorrhage and the hemolytic anemias with shortened RBC survival. Figure 19-3 presents an algorithm that illustrates how anemias can be classified based on the absolute reticulocyte count and MCV.

Morphologic Classification and the Red Blood Cell Distribution Width

The RDW can help determine the cause of an anemia when used in conjunction with the MCV. Each of the three MCV categories mentioned previously (normocytic, microcytic, macrocytic) can also be subclassified by the RDW as homogeneous (normal RDW) or heterogeneous (increased or high RDW), according to Bessman and colleagues. For example, a decreased MCV with an increased RDW is suggestive of iron deficiency (Table 19-4). This classification is not absolute, however, because there can be an overlap of RDW values among some of the conditions in each MCV category.

Pathophysiologic Classification of Anemias and the Reticulocyte Count

In a pathophysiologic classification of anemia, related conditions are grouped by the mechanism causing the anemia. In this classification scheme, the anemias caused by decreased RBC production have inappropriately low reticulocyte counts (e.g., disorders of DNA synthesis and aplastic anemia) and are distinguished from other anemias caused by increased RBC destruction (intrinsic and extrinsic abnormalities of RBCs) or blood loss, which have increased reticulocyte counts. Some anemias have more than one pathophysiologic mechanism. Box 19-2 presents a pathophysiologic classification of anemia based on the causes of the abnormality and gives one or more examples of an anemia in each classification.

![Figure 19-3](image-url)  
*Figure 19-3* Algorithm for evaluating causes of anemia based on absolute reticulocyte count and mean cell volume (MCV). The list of anemias contains examples; there are numerous other causes not listed. Anemia of chronic liver disease is multifactorial and can be normocytic. DIC, Disseminated intravascular coagulation; Hb, hemoglobin; HUS, hemolytic uremic syndrome; RBC, red blood cell; TTP, thrombotic thrombocytopenic purpura.
TABLE 19-4  Morphologic Classification of Anemia Based on Red Blood Cell Mean Volume (MCV) and Red Blood Cell Distribution Width (RDW)*

<table>
<thead>
<tr>
<th>MEAN CELL VOLUME</th>
<th>Decreased</th>
<th>Normal</th>
<th>Increased</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDW Normal</td>
<td>α- or β-thalassemia trait</td>
<td>Anemia of chronic inflammation</td>
<td>Aplastic anemia</td>
</tr>
<tr>
<td></td>
<td>Anemia of chronic inflammation</td>
<td>Anemia of renal disease</td>
<td>Chronic liver disease</td>
</tr>
<tr>
<td></td>
<td>Hb E disease/trait</td>
<td>Acute hemorrhage</td>
<td>Alcoholism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hereditary spherocytosis</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>RDW Increased</td>
<td>Iron deficiency</td>
<td>Early iron, folate, or vitamin B₁₂ deficiency</td>
<td>Folate or vitamin B₁₂ deficiency</td>
</tr>
<tr>
<td></td>
<td>Sickle cell–β-thalassemia</td>
<td>Mixed deficiency of iron + vitamin B₁₂ or folate</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sickle cell anemia</td>
<td>Cold agglutinin disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hb SC disease</td>
<td>Chronic liver disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myelodysplastic syndrome</td>
<td>Chemotherapy</td>
</tr>
</tbody>
</table>

*Hb, hemoglobin; MCV, mean cell volume; RDW, red blood cell distribution width.

*This classification scheme is not absolute because there can be overlap of RDW values among some of the conditions in each MCV category.


BOX 19-2  Pathophysiologic Classification of Anemias

Anemia Caused by Decreased Production of Red Blood Cells

Hematopoietic stem cell failure: acquired and inherited aplastic anemia

Functional impairment of erythroid precursors:

• Disturbance of DNA synthesis: megaloblastic anemia
• Disturbance of hemoglobin synthesis: iron deficiency anemia, thalassemia, sideroblastic anemia, anemia of chronic inflammation
• Disturbance of proliferation and differentiation of erythroid precursors: anemia of renal failure, anemia associated with marrow infiltration

Anemia Caused by Increased Red Blood Cell Destruction or Loss

Intrinsic abnormality

• Membrane defect: hereditary spherocytosis, hereditary elliptocytosis, pyropoikilocytosis, paroxysmal nocturnal hemoglobinuria
• Enzyme deficiency: glucose-6-phosphate dehydrogenase deficiency, pyruvate kinase deficiency
• Globin abnormality: sickle cell anemia, other hemoglobinopathies

Extrinsic abnormality

• Immune causes: warm-type autoimmune hemolytic anemia, cold agglutinin disease, paroxysmal cold hemoglobinuria, hemolytic transfusion reaction, hemolytic disease of the fetus and newborn
• Nonimmune red blood cell injury: microangiopathic hemolytic anemia (thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, HELLP syndrome, disseminated intravascular coagulation), macroangiopathic hemolytic anemia (traumatic cardiac hemolysis), infectious agents (malaria, babesiosis, bartonellosis, clostridial sepsis), other injury (chemicals, drugs, venoms, extensive burns)
• Blood loss: acute blood loss anemia

HELLP: Hemolysis, elevated liver enzymes, and low platelets syndrome.

The list of anemias is not all-inclusive; numerous other conditions are not listed.


SUMMARY

• Anemia is defined conventionally as a decrease in RBCs, hemoglobin, and hematocrit below the reference interval for healthy individuals of the same age, sex, and race, under similar environmental conditions.
• Diagnosis of anemia is based on history, physical examination, symptoms, and laboratory test results.
• Many anemias have common manifestations. Careful questioning of the patient may reveal contributing factors, such as diet, medications, occupational hazards, and bleeding history.
• A thorough physical examination is valuable in determining the cause of anemia. Some of the areas that should be evaluated are
skin, nail beds, eyes, mucosa, lymph nodes, heart, and size of the spleen and liver.

- Moderate anemias (hemoglobin concentration between 7 and 10 g/dL) may not manifest clinical symptoms if the onset is slow. Severe anemias (hemoglobin concentration of less than 7 g/dL) usually produce pallor, dyspnea, vertigo, headache, weakness, lethargy, hypotension, and tachycardia.

- Laboratory procedures helpful in the initial diagnosis of anemia include the complete blood count (CBC) with RBC indices and the red blood cell distribution width (RDW), reticulocyte count, and examination of the peripheral blood film with emphasis on RBC morphology. Examination of a peripheral blood film is very important in the diagnosis of hemolytic anemias.

- Bone marrow examination is not usually required for diagnosis of anemia but is indicated in cases of unexplained anemia, fever of unknown origin, or suspected hematologic malignancy. Other tests are indicated based on the RBC indices, history, and physical examination, such as serum iron, total iron-binding capacity, and serum ferritin (for microcytic anemias), and serum folate and vitamin B12 (for macrocytic anemias).

- The reticulocyte count and mean cell volume (MCV) play crucial roles in investigation of the cause of an anemia.

- The morphologic classification of anemias is based on the MCV and includes normocytic, microcytic, and macrocytic anemias. The MCV, when combined with the reticulocyte count and the RDW, also can aid in classification of anemia.

- Major subgroups of the pathophysiologic classification include anemias caused by decreased RBC production and those caused by increased RBC destruction or loss. Anemias may have more than one pathophysiologic cause.

- The cause of anemia should be determined before treatment is initiated.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

### REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Which of the following patients would be considered anemic with a hemoglobin value of 14.5 g/dL? Refer to reference intervals inside the front cover of this text.
   a. An adult man
   b. An adult woman
   c. A newborn boy
   d. A 10-year-old girl

2. Common clinical symptoms of anemia include:
   a. Splenomegaly
   b. Shortness of breath and fatigue
   c. Chills and fever
   d. Jaundice and enlarged lymph nodes

3. Which of the following are important to consider in the patient’s history when investigating the cause of an anemia?
   a. Diet and medications
   b. Occupation, hobbies, and travel
   c. Bleeding episodes in the patient or in his or her family members
   d. All of the above

4. Which one of the following is reduced as an adaptation to long-standing anemia?
   a. Heart rate
   b. Respiratory rate
   c. Oxygen affinity of hemoglobin
   d. Volume of blood ejected from the heart with each contraction

5. An autoimmune reaction destroys the hematopoietic stem cells in the bone marrow of a young adult patient, and the amount of active bone marrow, including RBC precursors, is diminished. The RBC precursors that are present are normal in appearance, but there are too few to meet the demand for circulating red blood cells, and anemia develops. The reticulocyte count is low. The mechanism of the anemia would be described as:
   a. Effective erythropoiesis
   b. Ineffective erythropoiesis
   c. Insufficient erythropoiesis

6. What are the initial laboratory tests that are performed for the diagnosis of anemia?
   a. CBC, iron studies, and reticulocyte count
   b. CBC, reticulocyte count, and peripheral blood film examination
   c. Reticulocyte count and serum iron, vitamin B12, and folate assays
   d. Bone marrow study, iron studies, and peripheral blood film examination

7. An increase in which one of the following suggests a shortened life span of RBCs and hemolytic anemia?
   a. Hemoglobin
   b. Hematocrit
   c. Reticulocyte count
   d. Red cell distribution width
8. Which of the following is detectable only by examination of a peripheral blood film?
   a. Microcytosis
   b. Anisocytosis
   c. Hypochromia
   d. Poikilocytosis

9. Schistocytes, ovalocytes, and acanthocytes are examples of abnormal changes in RBC:
   a. Volume
   b. Shape
   c. Inclusions
   d. Hemoglobin concentration

10. Refer to Figure 19-3 to determine which one of the following conditions would be included in the differential diagnosis of an anemic adult patient with an absolute reticulocyte count of 20 × 10⁹/L and an MCV of 65 fl.
   a. Aplastic anemia
   b. Sickle cell anemia
   c. Iron deficiency
   d. Folate deficiency

11. Which one of the following conditions would be included in the differential diagnosis of an anemic adult patient with an MCV of 125 fl and an RDW of 20% (reference interval 11.5% to 14.5%)? Refer to Table 19-4.
   a. Aplastic anemia
   b. Sickle cell anemia
   c. Iron deficiency
   d. Vitamin B₁₂ deficiency

REFERENCES
An 85-year-old slender, frail white woman was hospitalized for diagnosis and treatment of anemia suspected during a routine examination by her physician. The physician noted that she appeared pale and inquired about fatigue and tiredness. Although the patient generally felt well, she admitted to feeling slightly tired when climbing stairs. A point-of-care hemoglobin performed in the physician’s office showed a dangerously low value of 3.5 g/dL, so the patient was hospitalized for further evaluation. Her hospital CBC results are as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient Value</th>
<th>Reference Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (× 10^9/L)</td>
<td>8.5</td>
<td>4.5–11</td>
</tr>
<tr>
<td>RBCs (× 10^12/L)</td>
<td>1.66</td>
<td>4.3–5.9</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>3.0</td>
<td>13.9–16.3</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>11.0</td>
<td>39–55</td>
</tr>
</tbody>
</table>

**CASE STUDY**

After studying the material in this chapter, the reader should be able to respond to the following case study:

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient Value</th>
<th>Reference Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV (fL)</td>
<td>66.3</td>
<td>80–100</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>18.1</td>
<td>26–32</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>27.3</td>
<td>32–36</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>20</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>Platelets (× 10^9/L)</td>
<td>165.0</td>
<td>150–450</td>
</tr>
<tr>
<td>WBC differential</td>
<td>Unremarkable</td>
<td></td>
</tr>
<tr>
<td>RBC morphology</td>
<td>Marked anisocytosis, marked poikilocytosis, marked hypochromia, marked microcytosis</td>
<td></td>
</tr>
</tbody>
</table>
CASE STUDY—cont’d
After studying the material in this chapter, the reader should be able to respond to the following case studies:

2. What causes of anemia should you consider based on the results of the CBC?
3. Assuming that this patient has not been diagnosed with anemia at any other time during her life, can any of the conditions listed in the answer to question 2 be eliminated?
4. Assuming that the patient is otherwise healthy and experiencing only the common declines of sight, hearing, and mobility associated with aging, are any of the conditions listed in the answer to question 2 more likely than the others?
5. What additional testing would you recommend? What results do you expect for this patient?

GENERAL CONCEPTS IN ANEMIA

Anemia may result whenever red blood cell (RBC) production is impaired, RBC life span is shortened, or there is frank loss of cells. The anemias associated with iron and heme typically are categorized as anemias of impaired production resulting from the lack of raw materials for hemoglobin assembly. Depending on the cause, lack of available iron results in iron deficiency anemia or the anemia of chronic inflammation. Inadequate production of protoporphyrin leads to diminished production of heme and thus hemoglobin, but with a relative excess of iron. The result is sideroblastic anemia. These causes are discussed in this chapter. Inadequate globin production results in the thalassemias, which are discussed separately in Chapter 28.

As described more extensively in Chapter 11, iron is absorbed from the diet in the small intestine, carried by transferrin to a cell in need, and brought into the cell, where it is held as ferritin until incorporated into its final functional molecule. That functional molecule may be a heme-based cytochrome, muscle myoglobin, or, in the case of developing RBCs, hemoglobin. Iron may be unavailable for incorporation into heme because of inadequate stores of body iron or impaired mobilization. The anemia associated with inadequate stores is termed iron deficiency anemia, whereas the anemia resulting from impaired iron mobilization is known as anemia of chronic inflammation because of its association with chronic inflammatory conditions, such as rheumatoid arthritis. When the iron supply is adequate and mobilization is unimpaired but an RBC defect or impairment prevents production of protoporphyrin or incorporation of iron into it, the resulting anemia is termed sideroblastic, which refers to the presence of nonheme iron in the developing RBCs. One other form of anemia develops when the porphyrin component of hemoglobin is in short supply as with several of the porphyrias, diseases of impaired porphyrin production. Tests of body iron status are critical in diagnosis of these conditions, so the reader is referred to Chapter 11, where these tests are described in detail.

IRON DEFICIENCY ANEMIA

Etiology
Iron deficiency anemia develops when the intake of iron is inadequate to meet a standard level of demand, when the need for iron expands without compensated intake, when there is impaired absorption, or when there is chronic loss of hemoglobin from the body.

Inadequate Intake
Iron deficiency anemia can develop when the erythron is slowly starved for iron. Each day, approximately 1 mg of iron is lost from the body, mainly in the mitochondria of desquamated skin and sloughed intestinal epithelium. Because the body tenaciously conserves all other iron from senescent cells, including RBCs, daily replacement of 1 mg of iron from the diet maintains iron balance and supplies the body's need for RBC production as long as there is no other source of loss. When the iron in the diet is consistently inadequate, over time the body's stores of iron become depleted. Ultimately, RBC production slows as a result of the inability to produce hemoglobin. With approximately 1% of cells dying naturally each day, the anemia becomes apparent when the production rate is insufficient for replacement of lost cells.

Increased Need
Iron deficiency can also develop when the level of iron intake is inadequate to meet the needs of an expanding erythron. This is the case in periods of rapid growth, such as infancy (especially in prematurity), childhood, and adolescence. For example, although both infants and adult men need about 1 mg/day of iron, that corresponds to a much higher amount per kilogram of body weight for the infant. Pregnancy and nursing place similar demands on the mother's body to provide iron for the developing fetus or nursing infant in addition to her own iron needs. In each of these instances, what had previously been an adequate intake of iron for the individual becomes inadequate as the need for iron increases.

Impaired Absorption
Even when the diet is adequate in iron, the inability to absorb iron through the enterocyte into the blood over time will result in a deficiency of iron in the body. The impairments may be pathologic, as with malabsorption due to celiac disease. Others may be inherited mutations of iron regulatory proteins, like the mutations of the matriptase 2 protein (Chapter 11) that lead to a persistent production of hepcidin, causing ferroportin in the enterocyte to be inactivated, thus preventing iron absorption in the intestine. In addition, diseases that decrease stomach acidity impair iron absorption by decreasing
the capacity to reduce dietary ferric iron to the absorbable ferrous form. Some loss of acidity accompanies normal aging, but gastrectomy or bariatric surgeries can impair iron absorption dramatically. Medications such as antacids can inhibit absorption, and others may even bind the iron in the intestine, preventing its absorption.

**Chronic Blood Loss**
A fourth way iron deficiency develops is with chronic hemorrhage or hemolysis that results in the loss of small amounts of heme iron from the body over a prolonged period of time. Eventually anemia develops when the iron loss continually exceeds iron intake and the storage iron is exhausted. Excessive heme iron can be lost through chronic gastrointestinal bleeding from ulcers, gastritis due to alcohol or aspirin ingestion, tumors, parasitosis, diverticulitis, ulcerative colitis, or hemorrhoids. In women, prolonged menorrhagia (heavy menstrual bleeding) or conditions such as fibroid tumors or uterine malignancies can also lead to heme iron loss. Heme iron can also be lost excessively through the urinary tract with kidney stones, tumors, or chronic infections. Individuals with chronic intravascular hemolytic processes, such as paroxysmal nocturnal hemoglobinuria, can develop iron deficiency due to the loss of iron in hemoglobin passed into the urine.

**Pathogenesis**
Iron deficiency anemia develops slowly, progressing through stages that physiologically blend into one another but are useful delineations for understanding disease progression. As shown in Figure 20-1, iron is distributed among three compartments: the storage compartment, principally as ferritin in the bone marrow macrophages and liver cells; the transport compartment of serum transferrin; and the functional compartment of hemoglobin, myoglobin, and cytochromes. Hemoglobin and intracellular ferritin and hemosiderin constitute nearly 90% of the total distribution of iron (Table 11-1).

For a period of time as an increase in demand or increased loss of iron exceeds iron intake, essentially normal iron status continues. The body strives to maintain iron balance by accelerating absorption of iron from the intestine through a decrease in the production of hepcidin in the liver. This state of declining body iron with increased absorption is not apparent in routine laboratory test results or patient symptoms. The individual appears healthy. As the negative iron balance continues, however, a stage of iron depletion develops.

**Stage 1**
Stage 1 of iron deficiency is characterized by a progressive loss of storage iron. RBC development is normal, however, because the body’s reserve of iron is sufficient to maintain the transport and functional compartments through this phase. There is no evidence of iron deficiency in the peripheral blood because RBCs survive 120 days, and the patient does not experience symptoms of anemia. Serum ferritin levels are low, however, which indicates the decline in stored iron, and this also could

![Figure 20-1](http://example.com/image.jpg) Development of iron deficiency anemia. ↑ Increased; ↓ decreased; N, normal; TIBC, total iron-binding capacity. (Adapted from Suominen P, Punnonen K, Rajamäki A, et al: Serum transferrin receptor and transferrin receptor–ferritin index identify healthy subjects with subclinical iron deficits, Blood 92:2934–2939, 1998; reprinted with permission.)
be detected in an iron stain of the bone marrow. Without evidence of anemia, however, these tests would not be performed because individuals appear healthy. The prevalence of stage 1 iron deficiency in the United States has been estimated at 14.4% for 1- to 2-year-olds, 3.7% for 3- to 5-year-olds, 9.3% for 12- to 19-year-old females, and 9.2% for 20- to 49-year-old females. This stage is sometimes called latent or subclinical iron deficiency because the iron stores are inadequate but the hemoglobin value remains normal, and thus the deficiency is unlikely to be recognized.

**Stage 2**
Stage 2 of iron deficiency is defined by the exhaustion of the storage pool of iron (Figure 20-1). For a time, RBC production continues as normal, relying on the iron available in the transport compartment. Quickly the hemoglobin content of reticulocytes begins to decrease, which reflects the onset of iron deficient erythropoiesis, but because the bulk of the circulating RBCs were produced during the period of adequate iron availability, the overall hemoglobin measurement is still normal. Thus anemia is still not evident, although an individual's hemoglobin may begin dropping, and the RBC distribution width (RDW) may begin increasing as some smaller RBCs are released from the bone marrow. Other iron-dependent tissues, such as muscles, may begin to be affected, although the symptoms may be nonspecific. The serum iron and serum ferritin levels decrease, whereas total iron-binding capacity (TIBC), an indirect measure of transferrin, increases. Free erythrocyte protoporphyrin (FEP), the porphyrin into which iron is inserted to form heme, begins to accumulate. Transferrin receptors increase on the surface of iron-starved cells as they try to capture as much available iron as possible. They also are shed into the plasma, so the soluble transferrin receptor levels increase measurably. Prussian blue stain of the bone marrow in stage 2 shows essentially no stored iron, and iron deficient erythropoiesis is evident (subsequent description). Hepcidin, though not commonly measured clinically, would be measurably decreased. The hemoglobin content of reticulocytes would begin to drop, demonstrating iron-restricted erythropoiesis. As in stage 1, iron deficiency in stage 2 is still subclinical, and testing is not likely to be undertaken.

**Stage 3**
Stage 3 of iron deficiency is frank anemia. The hemoglobin concentration and hematocrit are low relative to the reference intervals. Depletion of storage iron and diminished levels of transport iron (Figure 20-1) prevent normal development of RBC precursors. The RBCs become microcytic and hypochromic (Figure 20-2) as their ability to produce hemoglobin is restricted. As expected, serum ferritin levels are exceedingly low. Results of other iron studies (see later) are also abnormal, and the free erythrocyte protoporphyrin and soluble transferrin receptor levels continue to increase. The hemoglobin content of reticulocytes will continue to drop. If measured, erythropoietin would be elevated, while hepcidin would be decreased.

In this phase, the patient experiences the nonspecific symptoms of anemia, typically fatigue, weakness, and shortness of breath, especially with exertion. Pallor is evident in light-skinned individuals but also can be noted in the conjunctivae, mucous membranes, or palmar creases of dark-skinned individuals. More severe signs are not seen as often in the United States but include a sore tongue (glossitis) due to iron deficiency in the rapidly proliferating epithelial cells of the alimentary tract and inflamed cracks at the corners of the mouth (angular cheilosis). Koilonychia (spooning of the fingernails) may be seen if the deficiency is long-standing. Patients also may experience cravings for nonfood items, called *pica*. The cravings may be for things such as dirt, clay, laundry starch, or, most commonly, ice (craving for the latter is called *pagophagia*).

As should be evident from this discussion, numerous individuals may be iron deficient while appearing healthy. Until late in stage 2, they may experience no symptoms at all and are unlikely to come to medical attention. Even in stage 3, frankly anemic patients may not seek medical care, because the body is able to compensate remarkably for slowly developing anemia (Chapter 19), like that in the patient in the case study at the beginning of this chapter. Because results of routine screening tests included in the CBC do not become abnormal until late in stage 2 or early in stage 3, most patients are not diagnosed until relatively late in the progression of the iron depletion.

**Epidemiology**
From the previous discussion, it is apparent that certain groups of individuals are more prone to develop iron deficiency anemia. Menstruating women are at especially high risk. Their monthly loss of blood increases their routine need for iron, which often is not met with the standard U.S. diet. For adolescent girls, this is compounded by increased iron needs associated with growth. If
women of childbearing age do not receive proper iron supplementation, pregnancy and nursing can lead to a loss of nearly 900 mg of iron,\textsuperscript{7} which further depletes iron stores. Succeeding pregnancies can exacerbate the problem, leading to iron deficient fetuses.\textsuperscript{8}

Growing children also are at high risk. Growth requires iron for the cytochromes of all new cells, myoglobin for new muscle cells, and hemoglobin in the additional RBCs needed to supply oxygen for a larger body. The increasing need for iron as the child grows can be coupled with dietary inadequacies, especially in circumstances of poverty or neglect. Cow’s milk is not a good source of iron, and infants need to be placed on iron-supplemented formula by about age 6 months, when their fetal stores of iron become depleted.\textsuperscript{5} This assumes that the infants were able to establish adequate iron stores by drawing iron from their mothers in utero. Even though breast milk is a better source of iron than cow’s milk,\textsuperscript{7} it is not a consistent source.\textsuperscript{8} Therefore, iron supplementation is also recommended for breastfed infants after 6 months of age.\textsuperscript{5}

Iron deficiency is relatively rare in men and postmenopausal women because the body conserves iron so tenaciously, and these individuals lose only about 1 mg/day. Gastrointestinal disease, such as ulcers, tumors, or hemorrhoids, should be suspected in iron deficient patients in either of these groups if the diet is known to be adequate in iron. Regular aspirin ingestion and alcohol consumption can lead to gastritis and chronic bleeding. Elderly individuals, particularly those living alone, may not eat a balanced diet, so pure dietary deficiency is seen among these individuals. In some elderly individuals, the loss of gastric acidity with age can impair iron absorption. Iron deficiency is associated with infection by hookworms, \textit{Necator americanus} and \textit{Ancylostoma duodenale}. The worm attaches to the intestinal wall and literally sucks blood from the gastric vessels. Iron deficiency is also associated with infection with other parasites, such as \textit{Trichurus trichiura}, \textit{Schistosoma mansoni}, and \textit{Schistosoma haematobium}, in which the heme iron is lost from the body due to intestinal or urinary bleeding.

Soldiers subjected to prolonged maneuvers and long-distance runners also can develop iron deficiency. Exercise-induced hemoglobinuria, also called march hemoglobinuria, develops when RBCs are hemolyzed by foot-pounding trauma and iron is lost as hemoglobin in the urine.\textsuperscript{9} The amount lost in the urine can be so little that it is not apparent on visual inspection of the urine. Nevertheless, in rare cases, the cumulative iron loss can lead to anemia if the foot-pounding trauma is recurrent and especially severe.

**Laboratory Diagnosis**

Iron deficiency can be readily diagnosed in later stages using routine tests. Detection in the early stages requires sophisticated tests, but individuals are unlikely to be referred for such studies because there is virtually no physiologic evidence of the declining iron state. Nevertheless, early iron deficiency might be suspected in an individual in a high-risk group, and appropriate testing can be ordered.\textsuperscript{9} The tests for iron deficiency can be grouped into three general categories: screening, diagnostic, and specialized. The principles are discussed in more detail in Chapter 11.

**Screening for Iron Deficiency Anemia**

When iron deficient erythropoiesis is under way, the CBC results begin to show evidence of anisocytosis, microcytosis, and hypochromia (Figure 20-2). The classic picture of iron deficiency anemia in stage 3 includes a decreased hemoglobin level. An RDW greater than 15% is expected and may precede the decrease in hemoglobin.\textsuperscript{7} For patients in high-risk groups, the elevated RDW can be an early and sensitive indicator of iron deficiency that is provided in a routine CBC.\textsuperscript{31} As the hemoglobin level continues to fall, microcytosis and hypochromia become more prominent, with progressively declining values for mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). The RBC count ultimately becomes decreased, as does the hematocrit. Polychromasia may be apparent early, although it is not a prominent finding. A low absolute reticulocyte count confirms a diminished rate of effective erythropoiesis because this is a nonregenerative anemia.\textsuperscript{12} Poikilocytosis, including occasional target cells and elliptocytes, may be present, although no particular shape is characteristic or predominant. Thrombocytosis may be present, particularly if the iron deficiency results from chronic bleeding, but this is not a diagnostic parameter. White blood cells (WBCs) are typically normal in number and appearance. Iron deficiency should be suspected when the CBC findings show a hypochromic, microcytic anemia with an elevated RDW but no consistent shape changes to the RBCs.

**Diagnosis of Iron Deficiency**

Iron studies remain the backbone for diagnosis of iron deficiency. They include assays of serum iron, total iron-binding capacity (TIBC), transferrin saturation, and serum ferritin; Chapter 11 covers the principle of each assay and technical considerations affecting test performance and interpretation. Serum iron is a measure of the amount of iron bound to transferrin (transport protein) in the serum. TIBC is an indirect measure of transferrin and the available binding sites for iron in the plasma. The percent of transferrin saturated with iron can be calculated from the total iron and the TIBC:

$$\text{Transferrin saturation (\% sat) = \frac{\text{serum iron (\mu g/dL) x 100}}{\text{TIBC (\mu g/dL)}}}$$

Ferritin is not truly an extracellular protein because it provides an intracellular storage repository for metabolically active iron. However, ferritin is present in serum, and serum levels reflect the levels of iron stored within cells. Serum ferritin is an easily accessible surrogate for stainable bone marrow iron. The iron studies are used collectively to assess the iron status of an individual. Table 20-1 shows that, as expected, serum ferritin and serum iron values are decreased in iron deficiency anemia, a state called sideropenia. Transferrin levels increase when the hepatocytes detect low iron levels, and research shows that this is a transcriptional and posttranslational response to low iron levels.\textsuperscript{13} The result is a decline in the iron...
The amount of hemoglobin in reticulocytes can be assessed on some automated hematology analyzers (Chapter 15). The hemoglobin content of reticulocytes is analogous to the MCH, but for reticulocytes only. The MCH is the average weight of hemoglobin per cell across the entire RBC population. Some of the RBCs are nearly 120 days old, whereas others are just 1 to 2 days old. If iron deficiency is developing, the MCH does not change until a substantial proportion of the cells are iron deficient, and the diagnosis is effectively delayed for weeks or months after iron deficient erythropoiesis begins. Measuring the hemoglobin content of reticulocytes enables detection of iron-restricted erythropoiesis within days as the first iron deficient cells leave the bone marrow. It is a sensitive indicator of iron deficiency. Even in stage 2 of iron deficiency, before anemia is apparent, the hemoglobin content of reticulocytes will be low.

**Specialized Tests**

Other tests, although not commonly used for the diagnosis of iron deficiency, show abnormalities that become important in the differential diagnosis of similar conditions. Test results for the accumulated porphyrin precursors to heme are elevated (Table 20-1). Free erythrocyte protoporphyrin accumulates when iron is unavailable. In the absence of iron, free erythrocyte protoporphyrin may be preferentially chelated with zinc to form zinc protoporphyrin (ZPP). The FEP and zinc chelate can be assayed fluorometrically, although they are not particularly valuable in the diagnosis of iron deficiency. Soluble transferrin receptors (sTfR) also can be assayed using immunoassay. Levels increase as the disease progresses, and individual cells seek to take in as much iron as possible.

A bone marrow assessment is not indicated for suspected uncomplicated iron deficiency. A therapeutic trial of iron (see Response to Treatment) provides a less invasive and less expensive diagnostic assessment. However, marrow examination for iron is routinely performed when a bone marrow sample is indicated for other reasons. With routine stains, the iron deficient bone marrow appears hyperplastic early in the progression of the disease, with a decreased myeloid-to-erythroid ratio as a result of increased erythropoiesis. As the disease progresses, hyperplasia subsides, and the profound deficiency of iron leads to slowed RBC production. Polychromatic normoblasts (i.e., rubricytes) show the most dramatic morphologic changes (Figure 20-3). Nuclear-cytoplasmic asynchrony is evident, with cytoplasmic maturation lagging behind nuclear maturation. Without the pink provided by hemoglobin, the cytoplasm remains bluish after the nucleus has begun to condense. The cell membranes appear irregular and are usually described as “shaggy.”

**Treatment and Its Effects**

**Treatment**

The first therapy for iron deficiency is to treat any underlying contributing cause, such as hookworms, tumors, or ulcers. As in the treatment of simple nutritional deficiencies or increased need, dietary supplementation is necessary to replenish the body’s iron stores. Oral supplements of ferrous sulfate (3 tablets/day

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### TABLE 20-1 Results of Iron Studies in Microcytic, Hypochromic Anemias

<table>
<thead>
<tr>
<th>Test</th>
<th>Iron Deficiency</th>
<th>Thalassemia Minor</th>
<th>Anemia of Chronic Inflammation</th>
<th>Sideroblastic Anemia</th>
<th>Lead Poisoning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ferritin</td>
<td>↓</td>
<td>↑/N</td>
<td>↑/N</td>
<td>↑</td>
<td>N</td>
</tr>
<tr>
<td>Serum iron</td>
<td>↓/N</td>
<td>↑/N</td>
<td>↓</td>
<td>↑</td>
<td>Variable</td>
</tr>
<tr>
<td>TIBC</td>
<td>↑</td>
<td>N</td>
<td>↓</td>
<td>↓/N</td>
<td>N</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>↓</td>
<td>↑/N</td>
<td>↓/N</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>FEP/ZPP</td>
<td>↑</td>
<td>N</td>
<td>↑</td>
<td>↑</td>
<td>↑ (marked)</td>
</tr>
<tr>
<td>BM iron (Prussian blue reaction)</td>
<td>No stainable iron</td>
<td>↑/N</td>
<td>↑/N</td>
<td>↑</td>
<td>N</td>
</tr>
<tr>
<td>Sideroblasts in BM</td>
<td>None</td>
<td>N</td>
<td>None/very few</td>
<td>↑ (ring)</td>
<td>N (ring)</td>
</tr>
<tr>
<td>Other special tests</td>
<td>↑ Hb A₂ (β-thalassemia minor)</td>
<td>Specific tests for inflammatory disorders or cancer</td>
<td>↑ ALA in urine</td>
<td>↑ Whole-blood lead levels</td>
<td></td>
</tr>
</tbody>
</table>

†, Increased; ↓, decreased; ALA, aminolevulinic acid; BM, bone marrow; FEP/ZPP, free erythrocyte protoporphyrin/zinc protoporphyrin; Hb, hemoglobin; N, normal.
containing 65 mg of elemental iron) are the standard prescription. The supplements should be taken on an empty stomach to maximize absorption. Many patients experience side effects such as nausea and constipation, however, which leads to poor patient compliance. Vigilance on the part of the health care providers is important to ensure that patients complete the course of iron replacement, which usually lasts 6 months or longer. In rare cases in which intestinal absorption of iron is impaired (for example, in conditions like matriptase-2) parenteral administration of iron is necessary. When optimal treatment with iron is initiated, the effects are quickly evident. Reticulocyte counts (relative and absolute) begin to increase within 5 to 10 days. The anticipated rise in hemoglobin appears in 2 to 3 weeks, and levels should return to normal for the individual by about 2 months after the initiation of adequate treatment. The peripheral blood film and indices still reflect the microcytic RBC population for several months, with a biphasic population including the younger normocytic cells. The normocytic population eventually predominates. Iron therapy must continue for another 3 to 4 months to replenish the storage pool and prevent a relapse.

It is common and reasonable for care providers to assume that iron deficiency is due to dietary deficiency because that is the case in most instances of iron deficiency. Thus supplementation should correct it. If the patient has been adherent to the therapeutic regimen, the failure to respond to iron treatment points to the need for further investigation. The patient may be experiencing continued occult loss of blood or inadequate absorption, justifying additional diagnostics. Alternatively, causes of hypochromic, microcytic anemia unrelated to iron deficiency, such as thalassemia, should be considered.

**Response to Treatment**

When optimal treatment with iron is initiated, the effects are quickly evident. Reticulocyte hemoglobin content will correct within 2 days. Reticulocyte counts (relative and absolute) begin to increase within 5 to 10 days. The anticipated rise in hemoglobin appears in 2 to 3 weeks, and levels should return to normal for the individual by about 2 months after the initiation of adequate treatment. The peripheral blood film and indices still reflect the microcytic RBC population for several months, with a biphasic population including the younger normocytic cells. The normocytic population eventually predominates. Iron therapy must continue for another 3 to 4 months to replenish the storage pool and prevent a relapse.

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**ANEMIA OF CHRONIC INFLAMMATION**

Anemia is commonly associated with systemic diseases, including chronic inflammatory conditions such as rheumatoid arthritis, chronic infections such as tuberculosis or human immunodeficiency virus infection, and malignancies. Cartwright was the first to suggest that although the underlying diseases seem quite disparate, the associated anemia may be from a single cause, proposing the concept of anemia of chronic disease. This anemia represents the most common anemia among hospitalized patients.

**Etiology**

Although the anemia associated with chronic systemic disorders was originally called *anemia of chronic disease*, chronic blood loss is not among the conditions leading to the anemia of chronic disease. Chronic blood loss results in clear-cut iron deficiency. Anemia of chronic disease is more correctly termed *anemia of chronic inflammation* (ACI), because inflammation is the unifying factor among the three aforementioned general types of conditions in which this anemia is seen. The central feature of anemia of chronic inflammation is sideropenia in the face of abundant iron stores. The cause is now understood to be largely impaired ferrokinetics.

The apparent inconsistency of decreased serum iron but abundant iron stores is explained by the role of hepcidin in regulation of body iron (Chapter 11). Hepcidin is a hormone produced by hepatocytes to regulate body iron levels, particularly absorption of iron in the intestine and release of iron from macrophages. Hepcidin interacts with and causes the degradation of the transmembrane protein ferroportin, which exports iron from enterocytes into the plasma, reducing the amount of iron absorbed into the blood from the intestine. Hepcidin and hepatocyte release of iron also increases. When systemic iron levels are high, hepcidin increases, enterocytes export less iron into the plasma, and macrophages and hepatocytes retain iron.

Hepcidin is an acute phase reactant. During inflammation, the liver increases the synthesis of hepcidin in response to interleukin-6 produced by activated macrophages. This increase occurs regardless of systemic iron levels in the body. As a result, during inflammation, there is a decrease in iron absorption from the intestine and iron release from macrophages and hepatocytes (Figure 20-4). Although there is plenty of iron in the body, it is unavailable to developing RBCs because it is sequestered in the macrophages and hepatocytes.

This response of hepcidin during inflammation is likely a nonspecific defense against invading bacteria. If the body can sequester iron, it reduces the amount of iron available to bacteria and contributes to their demise. Although this response of hepcidin is not harmful during disorders of short duration, chronically high levels of hepcidin sequester iron for long periods, which leads to diminished production of RBCs.

A second acute phase reactant seems to contribute to anemia of chronic inflammation, although probably to a much smaller extent than hepcidin. Lactoferrin is an iron-binding protein in the granules of neutrophils. Its avidity for iron is greater than that of transferrin. Lactoferrin is important intracellularly for phagocytes to prevent phagocytized bacteria from using intracellular iron for their metabolic processes. During infection and inflammation, however, neutrophil lactoferrin also is released into the plasma. There it scavenges available iron, at the expense of transferrin. When it is carrying iron, the lactoferrin becomes bound to macrophages and liver cells that salvage the iron. RBCs are deprived of this source of plasma iron, however, because they do not have lactoferrin receptors.

Finally, a third acute phase reactant, ferritin, contributes to the anemia of chronic inflammation. Increased levels of
ferritin in the plasma also bind some iron. Because developing RBCs do not have a ferritin receptor, this iron is also unavailable for incorporation into hemoglobin.

The result of these effects is that although iron is present in abundance in bone marrow macrophages, its release to developing erythrocytes is slowed. This can be seen histologically with iron stains that show iron in macrophages but not in erythroblasts (Figure 17-14, C). The effect on the developing RBCs is essentially no different from that in mild iron deficiency because they are effectively deprived of the iron. Like iron deficiency anemia, this is iron-restricted erythropoiesis.

Production of inflammatory cytokines (such as tissue necrosis factor–α and interleukin-1 from activated macrophages and interferon-γ from activated T-cells) also impairs proliferation of erythroid progenitor cells, diminishes their response to erythropoietin, and decreases production of erythropoietin by the kidney (Figure 20-4). Although these mechanisms contribute to the anemia of chronic inflammation, the impaired ferrokinetics is the more significant cause of the anemia.

Laboratory Diagnosis

The peripheral blood picture in anemia of chronic inflammation is that of a mild anemia, with hemoglobin concentration usually 8 to 10 g/dL and without reticulocytosis. The cells are usually normocytic and normochromic, although microcytosis and hypochromia develop in about one third of patients and may represent coexistent iron deficiency. The inflammatory condition leading to the anemia also may cause leukocytosis, thrombocytosis, or both. Iron studies (Table 20-1) show low serum iron and TIBC values. Because hepatocyte production of transferrin is regulated by intracellular iron levels, the low TIBC (an indirect measure of transferrin) reflects the abundant iron stores in the body. The transferrin saturation may be normal or low. The serum ferritin level is usually increased beyond the value that would be expected for the same patient in the absence of the inflammatory condition. It may not be outside the reference interval, but it is nevertheless increased. The failure to incorporate iron into heme results in elevation of free erythrocyte protoporphyrin, although this test typically is not used diagnostically. The hemoglobin content of reticulocytes will be decreased, reflecting the iron restricted hematopoiesis, but the soluble transferrin receptor will be normal, reflecting normal intracellular iron. The bone marrow shows hypoproliferation of the RBCs, consistent with the lack of reticulocytes in the peripheral blood. Prussian blue stain of the bone marrow confirms abundant stores of iron in macrophages, although not in RBC precursors, but bone marrow examination is not usually required in the diagnostic evaluation.

Patients with iron deficiency anemia who have an inflammatory condition present a special diagnostic dilemma. The iron deficiency may be missed because of the increase in serum ferritin levels associated with the inflammation. Serum ferritin values generally in the 30 to 100 ng/mL range are most equivocal. Iron deficiency anemia and anemia of chronic inflammation may be distinguished in such situations, or their coexistence can be verified, by measuring soluble transferrin receptors (sTfRs) in the serum. These receptors are sloughed from cells into the plasma. As noted earlier, levels increase during iron deficiency anemia but remain essentially normal during anemia of chronic inflammation.

Additional modifications to the use of the sTfR have been developed to better distinguish iron deficiency, latent iron deficiency, and ACI. The principles of these assays and calculations are discussed in Chapter 11 and the measurement of the hemoglobin content of reticulocytes is discussed in Chapter 15. It is expected that the sTfR/log ferritin will rise most dramatically in iron deficiency as the numerator rises and the denominator falls; in ACI, both remain essentially normal, and thus a normal
Disorders of Iron Kinetics and Heme Metabolism

Chapter 20

Figure 20-6

Ring sideroblasts (arrows) in bone marrow shown with Prussian blue stain (×1000).

Anemia

Biochemical or clinical evidence of inflammation

Rule out other causes of anemia

Transferrin saturation < 15%

Ferritin < 30 ng/mL

Ferritin 30-100 ng/mL

Ferritin > 100 ng/mL

Soluble Transferrin Receptor (sTfR)

sTfR/log ferritin > 2

sTfR/log ferritin < 1

Iron deficiency anemia

Anemia of inflammation with true iron deficiency

Anemia of inflammation

Figure 20-5

Algorithm for diagnosis and differentiation of iron deficiency anemia and the anemia of chronic inflammation (disease). Patients with iron deficiency may exhibit normal ferritin levels in the range of 30 to 100 ng/mL when there is a rise of ferritin due to coexistent anemia of chronic inflammation (disease). Use of the soluble transferrin receptor assay in conjunction with ferritin can help distinguish these conditions or establish their coexistence. sTfR, Soluble transferrin receptor in mg/L. (Adapted from Weiss G, Goodnough LT. Anemia of chronic disease. N Eng J Med 352, 2005, p. 1020.)

Treatment

Therapeutic administration of erythropoietin can correct anemia of chronic inflammation, but iron must be administered concurrently because stored body iron remains sequestered and unavailable. The anemia is typically not severe, however, and this costly treatment is warranted only in select patients. The best course of treatment is effective control or alleviation of the underlying condition.

SIDEROBLASTIC ANEMIAS

Just as anemia can result from inadequate supplies of iron for production of hemoglobin, diseases that interfere with the production of adequate amounts of protoporphyrin also can produce anemia. (Chapter 10 covers heme synthesis.) As in iron deficiency, the anemia may be microcytic and hypochromic. In contrast to iron deficiency, however, iron is abundant in the bone marrow. A Prussian blue stain of the bone marrow shows normoblasts with iron deposits in the mitochondria surrounding the nucleus. Its presence in the mitochondria shows that the iron is awaiting incorporation into heme. These

ring sideroblasts are the hallmark of the sideroblastic anemias (Figure 20-6).

The sideroblastic anemias are a diverse group of diseases that include hereditary and acquired conditions (Box 20-1). Among the hereditary forms, X-linked and autosomal varieties of this condition are known. Some patients experience at least modest improvement of anemia with pharmacologic doses of pyridoxine to stimulate heme synthesis. Pyridoxine is a cofactor in the first step of porphyrin synthesis (Figure 10-5) in which glycine is condensed with succinyl coenzyme A to form aminolevulinic acid.

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**BOX 20-1 Disorders Included in Sideroblastic Anemias**

**Hereditary**
- X-linked
- Autosomal

**Acquired**
- Primary sideroblastic anemia (refractory)
- Secondary sideroblastic anemias caused by drugs and bone marrow toxins
  - Antitubercular drugs
  - Chloramphenicol
  - Alcohol
  - Lead
  - Chemotherapeutic agents

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**Lead Poisoning**

The acquired conditions leading to sideroblastic anemia constitute a diverse group in themselves. Certain drugs, such as chloramphenicol or isoniazid, can induce sideroblastic anemia. Other toxins, including heavy metals, also have been implicated. Among these, lead is a significant public health concern. Adults may be exposed at work to leaded compounds. Adults and children living in older homes can be exposed to lead from paints produced before the 1970s. They are at risk if dust is created during renovations or paint is permitted to peel. Toddlers and crawling infants are at special risk from getting dust on their hands and placing them in their mouths. Although anyone can experience lead poisoning, it is of special concern in children because the metal affects the central nervous system and the hematologic system, leading to impaired mental development as well as anemia.

Children and adults with lead poisoning, a peripheral neuropathy can be seen. The fluorescence of some accumulated compounds in the urine is diagnostic for lead poisoning. Removal of the drug or toxin is usually successful for the treatment of acquired sideroblastic anemias. In the case of lead, salts of ethylenediaminetetraacetic acid (EDTA) are often used to chelate the lead present in the body so it can be excreted in the urine.

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**Porphyrias**

Lead poisoning is an example not only of an acquired sideroblastic anemia but also of an acquired porphyria. The porphyrias are diseases characterized by impaired production of the porphyrin component of heme. The impairments to heme synthesis may be acquired, as with lead poisoning, or hereditary. The term *porphyria* is most often used to refer to the hereditary conditions that impair production of protoporphyrin.

Among the inherited disorders, single deficiencies of most enzymes in the synthetic pathway for heme have been identified. Although even the autosomal dominant conditions are relatively rare, the disease has been influential historically. The intermarrying European monarchies of past centuries were plagued with some variants of the porphyrias in which psychosis is a prominent clinical feature.

When an enzyme in heme synthesis is missing, the products from earlier stages in the pathway accumulate in cells that actively produce heme, such as erythrocytes and hepatocytes. The excess porphyrins leak from the cells as they age or die, and may be excreted in urine or feces, which allows diagnosis. The accumulated products also deposit in body tissues. Some of the accumulated products are fluorescent. Their deposition in skin can lead to photosensitivity with severe burns upon exposure to sunlight. Accumulation during childhood leads to fluorescence of developing teeth and bones. Only three of the porphyrias have hematologic manifestations; the others have a greater effect on liver cells. Even in those with hematologic effects, the hematologic impact is relatively minimal, and photosensitivity is a greater clinical problem. The fluorescence of some accumulated compounds can be used diagnostically—for example, to measure free erythrocyte protoporphyrin (Chapter 11). In a sample of bone marrow, the erythroblasts will be bright red under a fluorescent microscope. Table 20-2 summarizes the deficient enzymes, affected genes, inheritance, and clinical and

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**Anemia, when present in lead poisoning, is most often normocytic and normochromic; however, with chronic exposure to lead, a microcytic, hypochromic clinical picture may be seen. The degree of anemia in adults may not be dramatic, but in children it may be more profound. The reticulocyte count in acute poisoning may be quite elevated, which suggests that the anemia has a hemolytic component. The presence of a hemolytic component is supported by studies showing impairment of the pentose-phosphate shunt by lead, which makes the cells sensitive to oxidant stress as in glucose-6-phosphate dehydrogenase deficiency (Chapter 24). Although the bone marrow may show erythroid hyperplasia, consistent with the elevated reticulocyte count, in some patients it may be hypoplastic. Basophilic stippling is a classic finding associated with lead toxicity. Lead inhibits pyrimidine 5’-nucleotidase, an enzyme involved in the breakdown of ribosomal ribonucleic acid (RNA) in reticulocytes. This causes undegraded ribosomes to aggregate, forming basophilic stippling. The size of the aggregates in lead poisoning is typically large, so the stippling is heavier than that seen in many anemias and thus represents truly punctate basophilia. Because basophilic stippling is also seen in other anemias, this is not a pathognomonic finding but an expected finding, and whenever basophilic stippling is seen, lead poisoning should be under consideration.

Removal of the drug or toxin is usually successful for the treatment of acquired sideroblastic anemias. In the case of lead, salts of ethylenediaminetetraacetic acid (EDTA) are often used to chelate the lead present in the body so it can be excreted in the urine.
**TABLE 20-2 Erythropoietic Porphyrias**

<table>
<thead>
<tr>
<th>Enzyme affected</th>
<th>Congenital Erythropoietic Porphyria (CEP)</th>
<th>Erythropoietic Protoporphyria (EPP)</th>
<th>X-linked Erythropoietic Protoporphyria (XLEPP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uroporphyrinogen III synthase deficiency</td>
<td>Ferrochelatase deficiency</td>
<td>ALA-synthase 2 (gain of function)</td>
<td></td>
</tr>
<tr>
<td>UROS</td>
<td>FECH</td>
<td>ALAS2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inheritance</th>
<th>Autosomal recessive</th>
<th>Autosomal dominant</th>
<th>X-linked dominant</th>
</tr>
</thead>
</table>

| Clinical features | Photosensitivity, hemolytic anemia | Photosensitivity; anemia is mild if present | Photosensitivity; mild microcytic, hypochromic anemia with reticulocyte response is possible |

<table>
<thead>
<tr>
<th>Laboratory features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells</td>
</tr>
<tr>
<td>Protoporphyrin</td>
</tr>
<tr>
<td>Uroporphyrin</td>
</tr>
<tr>
<td>Coproporphyrin</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>Porphobilinogen</td>
</tr>
<tr>
<td>Uroporphyrin</td>
</tr>
<tr>
<td>Coproporphyrin</td>
</tr>
<tr>
<td>Feces</td>
</tr>
<tr>
<td>Protoporphyrin</td>
</tr>
<tr>
<td>Coproporphyrin</td>
</tr>
<tr>
<td>Confirmatory tests</td>
</tr>
<tr>
<td>↓↓↓ Uroporphyrinogen III synthase activity</td>
</tr>
<tr>
<td>Genetic testing</td>
</tr>
</tbody>
</table>

↑, Minimally increased levels; ↑↑, moderately increased levels; ↑↑↑, markedly increased levels; ↓↓↓, moderately decreased levels; ↓↓↓, markedly decreased levels.


Laboratory features that can be used in the diagnosis of the hematologically significant porphyrias.

**IRON OVERLOAD**

Chapter 11 describes the body’s tenacity in conserving iron. For some individuals, this tenacity becomes the basis for disease related to excess iron accumulations in nearly all cells. Iron overload may be primary, as in hereditary hemochromatosis, or secondary to chronic anemias and their treatments. In both cases, the toxic effects of excess iron lead to serious health problems as lipids, proteins, and heme iron become oxidized.

**Etiology**

Excess accumulation of iron results from acquired or hereditary conditions in which the body’s rate of iron acquisition exceeds the rate of loss, which is usually about 1 mg/day. Regardless of the source of the iron, the body’s first reaction is to store excess iron in the form of ferritin and, ultimately, hemosiderin within cells. Eventually the storage system is overwhelmed, and, as described later, parenchymal cells are damaged in organs such as the liver, heart, and pancreas.

Accumulation of excess iron may be an acquired condition. It occurs when there is a need for repeated transfusions, as in the treatment of anemias such as sickle cell anemia and β-thalassemia major (Chapters 27 and 28). The iron present in the transfused RBCs exceeds the usual 1 mg/day of iron typically added to the body’s stores by a healthy diet. This is sometimes called *transfusion-related hemosiderosis.*

Hemochromatosis may develop as a result of mutations to genes for the proteins of iron metabolism (Table 11-2) so that the feedback regulation of iron is impaired and the body continues to absorb iron, even when stores are full. An autosomal recessive disease was recognized for many years before modern methods allowed a molecular investigation of its cause. In the mid-1990s, a mutation was identified affecting the hereditary hemochromatosis (HFE) protein, and shortly after that, additional mutations of the same gene were identified. With reliable and specific molecular tests, individuals with the phenotypic disease (i.e., excess iron deposition in tissues) were soon discovered who did not have the HFE mutations. Trying to explain their diseases led to discovery of the other proteins that we now know are involved in iron metabolism. What has emerged is a picture of hereditary hemochromatosis as a general phenotype that can be produced by various genotypes when a gene for an iron regulatory protein is mutated (Table 20-3). The biologic default is to absorb and store iron, and the regulatory mechanisms typically dampen that process. Failure of normal regulation due to mutations leads to excessive absorption and storage, causing the diseases collectively known as the *hereditary hemochromatoses.* Although substantial understanding of the proteins involved in iron kinetics has emerged in the past 2 decades, likely more remains to be discovered.
Table 20-3 describes each of the known forms of hemochromatosis, its mutated protein, age of onset, and the nature of the mutation and its effect. As mutations of HFE remain the most common, that form of the disease will be discussed here with some references to differences seen in other forms of the disease. However, in general, all the hereditary hemochromatoses involve mutated proteins that impair hepcidin regulation of ferroportin activity.

Homzygous hereditary hemochromatosis involving the HFE gene occurs in approximately 5 of 1000 northern Europeans.\textsuperscript{46} Heterozygosity approaches 13%.\textsuperscript{46} The first two mutations known to produce the hereditary hemochromatosis phenotype involve HFE, a gene on the short arm of chromosome 6 that encodes an HLA class I–like molecule that is closely linked to HLA-A.\textsuperscript{33} The most common of the two mutations substitutes tyrosine for cysteine at position 282 (Cys282Tyr or C282Y), while the other substitutes aspartate for histidine at position 63 (His63Asp or H63D).\textsuperscript{45} Other mutations are now known.\textsuperscript{45} The normal HFE protein binds \( \beta_2 \)-microglobulin intracellularly.\textsuperscript{46} This binding is necessary for the HFE to appear on the cell surface, where it interacts with transferrin receptor 1 (TfR1). HFE is bound to TfR1 until TfR1 binds transferrin, and then the HFE is released. It subsequently binds to transferrin receptor 2 (TfR2), which initiates a signal for hepcidin production, ultimately reducing iron absorption. The mutated HFE molecule either does not bind \( \beta_2 \)-microglobulin and thus does not reach the cell surface or it does not bind TfR1 or TfR2 if it does reach the cell surface.\textsuperscript{47} In either case, the result is that when HFE protein is mutated, the TfR2-mediated signal to produce hepcidin is diminished. Without hepcidin, ferroportin in the intestinal enterocyte membrane is continually active and absorbing iron, even when the body stores are replete.

**Pathogenesis**

The processes described previously lead to increased amounts of iron in parenchymal cells throughout the body. The first cellular reaction to excess iron is to form ferritin and ultimately hemosiderin, essentially a degenerate and non–metabolically active form of ferritin. When cells exhaust the capacity to store iron as hemosiderin, free iron (ferrous) accumulates intracellularly. In the presence of oxygen, ferrous iron initiates the generation of superoxide and other free radicals, which results in the peroxidation of membrane lipids (Chapter 11).\textsuperscript{48} The membranes affected include not only the cell membranes but also mitochondrial, nuclear, and lysosomal membranes. Cell respiration is compromised, and lysosomal enzymes are released intracellularly. Vitamins E and C can act to moderate the effects and interrupt the chain reaction, but in iron overload, even these protective mechanisms are overwhelmed. The ultimate result is cell death due to irreversible membrane damage.

Because all cells except mature RBCs require iron and have the cellular machinery for iron acquisition, most cells have the potential for iron damage. The tissues most obviously affected are the skin, where deposition of hemosiderin gives the skin a golden color; the liver, where cirrhosis-induced jaundice and subsequent cancer develop; and the pancreas, where damage results in diabetes mellitus. Hence the traditional characterization

<table>
<thead>
<tr>
<th>Feature</th>
<th>Hemochromatosis, Type 1 (HFE-Associated Hereditary Hemochromatosis)</th>
<th>Hemochromatosis, Type 2A Juvenile (HFE2-Related Juvenile Hemochromatosis)</th>
<th>Hemochromatosis, Type 2B Juvenile (HAMP-Related Juvenile Hemochromatosis)</th>
<th>Hemochromatosis, Type 3 (TFR2-Related Hereditary Hemochromatosis)</th>
<th>Hemochromatosis, Type 4 (Ferroportin-Related Iron Overload)</th>
<th>Hemochromatosis, Type 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected gene</td>
<td>HFE</td>
<td>HFE2 (HJV)</td>
<td>HAMP</td>
<td>TFR2</td>
<td>SLC40A1</td>
<td>FTH1</td>
</tr>
<tr>
<td>Mutated protein</td>
<td>Hereditary hemochromatosis protein</td>
<td>Hemojuvelin</td>
<td>Hepcidin</td>
<td>Transferrin receptor protein 2</td>
<td>Solute carrier family 40 member 1 (Ferroportin-1)</td>
<td>Ferritin heavy chain</td>
</tr>
<tr>
<td>Normal function of affected protein</td>
<td>Inhibits TfR1-mediated iron uptake; regulates hepcidin expression</td>
<td>Regulates hepcidin expression</td>
<td>Downregulates ferroportin-mediated iron transport in macrophages and enterocytes</td>
<td>Provides hepatocyte iron uptake; regulates hepcidin expression</td>
<td>Transports iron out of enterocytes and macrophages</td>
<td>Iron storage</td>
</tr>
<tr>
<td>Age of onset of symptoms (yr)</td>
<td>30–40</td>
<td>Teens–20</td>
<td>Teens–20</td>
<td>20–40, mild</td>
<td>30–40</td>
<td>*</td>
</tr>
</tbody>
</table>

\( \text{TfR1, Transferrin receptor 1; \ TfR2, transferrin receptor 2.} \)

* Found in three members of a Japanese family.


of hemochromatosis is “bronzed diabetes.” The heart muscle also is especially vulnerable to excessive iron deposition, which leads to congestive heart failure. Early diagnosis and treatment (see later) can prevent the development of these secondary effects of iron overload. Hepatocellular carcinoma occurs more frequently in patients with hemochromatosis. Mutations of the p53 tumor suppressor gene seem to contribute to the pathogenesis of the carcinoma, and there is some evidence that the free radicals produced by the iron cause the mutations in the p53 gene.

The disease (i.e., hemochromatosis phenotype) is rarer than the C282Y gene frequency predicts, so the penetrance of the genes is not heavy. Other factors also affect the development of clinical disease, including the particular mutation, zygosity, presence of other physiologic conditions, diet, and other environmental influences.

In classic hereditary hemochromatosis (HFE mutations), individuals usually harbor 20 to 30 g of iron by the time their disease becomes clinically evident at the age of 40 to 60 years. This is more than 10 times the amount of stored iron in normal individuals and represents just 1 to 2 mg/day of excess iron absorbed over many years. In the juvenile forms of the disease associated with mutations to the hepcidin or hemojuvelin genes, the process of iron accumulation is accelerated, so these effects may appear as early as the teenage years.

In the slower-developing diseases, phenotypic expression of the tissue damage in hereditary hemochromatosis is more common in men, although the gene frequency of HFE mutations is not higher in men. This is because the blood loss associated with menstruation and childbirth forestalls the effects of excess iron in affected women, and they usually develop clinical symptoms later in life than affected men. In each sex, homozygous individuals develop disease faster than heterozygotes.

The amount of iron available in the diet for absorption affects the rate at which disease can develop. Substances that can promote iron absorption even in normal individuals, such as ascorbic acid and alcohol, also affect absorption in individuals with hemochromatosis. In transfusion-related hemosiderosis, the frequency of transfusions over time affects the rate of development of clinical disease.

**Laboratory Diagnosis**

Laboratory testing in hemochromatosis serves four purposes. It can be used to screen for the condition, diagnose the cause of organ damage, pinpoint the mutation for family genetic counseling, and monitor treatment. Elevations of transferrin saturation or serum ferritin can be used as a screening test for hereditary hemochromatosis. Although screening was estimated to be performed cost-effectively in populations with a disease prevalence of at least 3 per 1000, large-scale screening programs remain controversial, even in Europe, where the incidence of the disease is high.

Individuals with undiagnosed hereditary hemochromatosis may come to medical attention because of organ function problems leading to nonspecific physical complaints (e.g., abdominal pain), or the disease may be discovered incidentally with routine laboratory testing. Abnormal results on common tests of liver function (e.g., elevated alanine transaminase levels) may be among the first laboratory findings that lead a physician to order further testing to identify the cause. Because inflammation is minimal, however, a finding of diminished levels of the liver’s synthetic products, such as albumin, may be more helpful. If hereditary hemochromatosis is among the disorders being considered to explain organ dysfunction, serum iron, transferrin saturation, and serum ferritin testing are warranted because elevations in these values are among the earliest findings in most forms of hemochromatosis. Genetic testing for known mutations provides confirmation of the diagnosis for most patients with hereditary hemochromatosis. It is especially valuable for testing nonaffected family members who can be counseled in lifestyle changes to prevent the phenotype from developing or for whom early treatment interventions can prevent organ damage.

Whether hemochromatosis is acquired or hereditary, the serum ferritin level provides an assessment of the degree of iron overload and can be monitored after treatment is initiated to reduce iron stores. Hemoglobin concentration and hematocrit are inexpensive tests that can also be used to monitor treatment, as described later.

Determination of the actual extent of tissue damage is beyond the scope of the clinical laboratory. Liver biopsy with assessment of iron staining and degree of scarring in liver specimens is essential to determining the degree of organ damage.

**Treatment**

The treatment of secondary tissue damage, such as liver cirrhosis and heart failure, follows standard protocols. Treatment of the underlying condition leading to excess iron accumulation is also needed. Hereditary hemochromatosis and transfusion-related hemosiderosis require different treatment approaches. In forms of hereditary hemochromatosis, withdrawal of blood by phlebotomy provides a simple, inexpensive, and effective means of removing iron from the body. The regimen calls for weekly phlebotomy early in treatment to remove about 500 mL of blood per treatment. Maintenance phlebotomies are required about every 3 months for life. Hemoglobin levels are monitored, and a mild anemia is sought and maintained. Such monitoring is an easy and inexpensive substitute for iron studies because, as explained in the discussion of iron deficiency, iron stores must be exhausted before anemia develops.

Individuals who rely on transfusions to maintain hemoglobin levels and prevent anemia cannot be treated with phlebotomy. Instead, iron-chelating drugs are used to bind excess iron in the body for excretion. Desferrioxamine is the classic treatment, although it is not without side effects. The drug typically is injected subcutaneously to maximize exposure time for iron binding. When absorbed into the bloodstream with its bound iron, it is readily excreted in the urine. Recently, oral iron chelators have been developed. Although they also have side effects, the convenience of oral administration with the potential for improved patient outcomes may lead to a greater reliance on this form of treatment.
• Impaired iron kinetics or heme metabolism can result in microcytic, hypochromic anemias.
• Three conditions affecting iron kinetics can result in microcytic, hypochromic anemias: iron deficiency, anemia of chronic inflammation, and sideroblastic anemias, especially lead poisoning. The RBCs in thalassemias also may be microcytic and hypochromic, and this condition must be differentiated from the anemias of disordered iron metabolism.
• Iron deficiency results from inadequate iron intake, increased need, decreased absorption, or excessive loss. All four of these situations create a relative deficit of body iron that over time results in a microcytic, hypochromic anemia.
• Infants, children, and women of childbearing age are at greatest risk for iron deficiency anemia. If iron deficiency anemia is present in men and postmenopausal women, the possibility of gastrointestinal bleeding should be investigated because it is the primary, although not the only, cause of iron loss.
• Iron deficiency is suspected when the CBC shows microcytic, hypochromic RBCs and elevated RDW but no consistent morphologic abnormality. The diagnosis is confirmed with iron studies showing low levels of serum iron and serum ferritin, elevated TIBC, and decreased transferrin saturation.
• Inadequate dietary iron is treated by oral supplementation, and with good patient adherence, the anemia should be corrected within 3 months. Gastrointestinal distress resulting from iron supplements can make patient adherence a significant concern. Other causes of iron deficiency must be treated by eliminating the underlying cause or with intravascular iron administration.
• The anemia of chronic inflammation (ACI) is associated with chronic infections such as tuberculosis, chronic inflammatory conditions such as rheumatoid arthritis, and tumors. It may be a microcytic, hypochromic anemia, but most often it is a mild normocytic, normochromic anemia.
• In ACI, increased levels of hepcidin, an acute phase reactant, decrease iron absorption in the intestines and sequester iron in macrophages and hepatocytes. Bone marrow macrophages show abundant stainable iron, whereas developing RBCs show inadequate iron. Inflammatory cellular products also impair the production and action of erythropoietin.
• Iron studies in the anemia of chronic inflammation show decreased serum iron level, decreased TIBC, decreased or normal transferrin saturation, and normal or increased ferritin level.
• Sideroblastic anemias develop when the synthesis of protoporphyrin or the incorporation of iron into protoporphyrin is blocked. The result is accumulation of iron in the mitochondria of developing RBCs. When stained using Prussian blue, the iron appears in deposits around the nucleus of the developing cells in the bone marrow. These cells are called ring sideroblasts.
• Protoporphyrin synthesis and iron incorporation into protoporphyrin can be blocked when any of the enzymes of the heme synthetic pathway are deficient or impaired. Deficiencies of these enzymes may be hereditary, as in the porphyrias, or acquired, as in heavy metal poisoning. The most common of the latter conditions is lead poisoning.
• Iron studies in sideroblastic anemias show elevated levels of serum iron and serum ferritin, decreased or normal TIBC, and increased transferrin saturation. Test values for the accumulating products of the heme synthetic pathway, such as zinc protoporphyrin (ZPP) or free erythrocyte protoporphyrin (FEP), are also elevated.
• Lead interferes with several steps in heme synthesis, preventing iron incorporation into heme and resulting in a normocytic, normochromic anemia, although with long-term exposure it can be microcytic and hypochromic. Lead also impairs glucose-6-phosphate dehydrogenase, which adds a hemolytic component to the anemia.
• Children are especially vulnerable to the effects of lead on the central nervous system, which may result in irreversible brain damage. Treatment consists of removing the source of lead from the patient’s environment and, if necessary, chelating drug therapy to facilitate excretion of lead in the urine.
• Because the body has no mechanism for iron excretion, iron overload can occur when transfusions are used to sustain patients with chronic anemias such as β-thalassemia major (called transfusion-related hemosiderosis).
• A defective HFE gene can lead to hereditary hemochromatosis by decreased hepcidin production. Affected men develop symptoms earlier in life than women; homozygotes develop more severe disease than heterozygotes.
• Mutations of other genes affecting iron regulation can produce a phenotype similar to that of hereditary hemochromatosis. When the hepcidin or hemosiderin gene is mutated, the disease develops early in life, affecting even teenagers.
• Free iron becomes available in cells when ferritin and hemosiderin become saturated. Free iron causes tissue damage by creating free radicals that lead to cell membrane damage and perhaps mutations. The liver, pancreas, skin, and heart muscle are especially damaged by excess iron deposition.
• Elevated transferrin saturation can be an indicator of hemochromatosis that can be diagnosed fully using genetic testing to identify mutated genes.
• Hereditary hemochromatosis and similar diseases are treated by lifelong periodic phlebotomy to induce a mild iron deficiency anemia and keep body iron levels low. Transfusion-related hemosiderosis must be treated with iron-chelating drugs.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.
Answers can be found in the Appendix.

1. The mother of a 4-month-old infant who is being breastfed sees her physician for a routine postpartum visit. She expresses concern that she may be experiencing postpartum depression because she does not seem to have any energy. Although the physician is sympathetic to the patient's concern, she orders a CBC and iron studies seeking an organic explanation for the patient's symptoms. The results are as follows:
   
   CBC: all results within reference intervals except
   RDW = 15%
   Serum iron: decreased
   TIBC: increased
   % transferrin saturation: decreased
   Serum ferritin: decreased

   Correlate the patient's laboratory and clinical findings. What can you conclude?
   a. The results of the iron studies reveal findings consistent with a thalassemia that was apparently previously undiagnosed.
   b. The patient is in stage 2 of iron deficiency, before frank anemia develops.
   c. The results of the iron studies are inconsistent with the CBC results, and a laboratory error should be suspected.
   d. There is no evidence of a hematologic explanation for the patient's symptoms.

2. A bone marrow biopsy was performed as part of the cancer staging protocol for a patient with Hodgkin lymphoma. Although no evidence of spread of the tumor was apparent in the marrow, other abnormal findings were noted, including a slightly elevated myeloid-to-erythroid ratio. WBC and RBC morphology appeared normal, however. The Prussian blue stain showed abundant stainable iron in the marrow macrophages. The patient's CBC revealed a hemoglobin of 10.8 g/dL, but RBC indices were within reference intervals. RBC morphology was unremarkable. These findings would be consistent with:
   a. Anemia of chronic inflammation
   b. Sideroblastic anemia
   c. Thalassemia
   d. Iron deficiency anemia

3. Predict the iron study results for the patient with Hodgkin lymphoma described in question 2.

<table>
<thead>
<tr>
<th>Serum Iron Level</th>
<th>TIBC</th>
<th>% Transferrin Saturation</th>
<th>Serum Ferritin Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Decreased</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>b. Increased</td>
<td>Normal</td>
<td>Increased</td>
<td>Normal</td>
</tr>
<tr>
<td>c. Increased</td>
<td>Increased</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>d. Decreased</td>
<td>Decreased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

4. A 35-year-old white woman went to her physician complaining of headaches, dizziness, and nausea. The headaches had been increasing in severity over the past 6 months. This was coincident with her move into an older house built about 1900. She had been renovating the house, including stripping paint from the woodwork. Her CBC results showed a mild hypochromic, microcytic anemia, with polychromasia and basophilic stippling noted. Which of the following tests would be most useful in confirming the cause of her anemia?
   a. Serum lead level
   b. Serum iron level and TIBC
   c. Absolute reticulocyte count
   d. Prussian blue staining of the bone marrow to detect iron stores in macrophages

5. In men and postmenopausal women whose diets are adequate, iron deficiency anemia most often results from:
   a. Increased need associated with aging
   b. Impaired absorption in the gastric mucosa
   c. Chronic gastrointestinal bleeding
   d. Diminished resistance to hookworm infections

6. Which one of the following individuals is at greatest risk for the development of iron deficiency anemia?
   a. A 15-year-old boy who eats mainly fast food and junk food
   b. A 37-year-old woman who has never been pregnant and has amenorrhea
   c. A 63-year-old man with reactivation of tuberculosis from his childhood
   d. A 40-year-old man who lost blood during surgery to repair a fractured leg

7. Which of the following individuals is at the greatest risk for the development of anemia of chronic inflammation?
   a. A 15-year-old girl with asthma
   b. A 40-year-old woman with type 2 diabetes mellitus
   c. A 65-year-old man with hypertension
   d. A 30-year-old man with severe rheumatoid arthritis

8. In what situation will increased levels of free erythrocyte protoporphyrin be present?
   a. Gain of function mutation to one of the enzymes in the heme synthesis pathway
   b. A mutation that prevents heme attachment to globin so that protoporphyrin remains free
   c. Any condition that prevents iron incorporation into protoporphyrin IX
   d. When red blood cells lyse, freeing their contents into the plasma
9. In the pathogenesis of the anemia of chronic inflammation, hepcidin levels:
   a. Decrease during inflammation and reduce iron absorption from enterocytes
   b. Increase during inflammation and reduce iron absorption from enterocytes
   c. Increase during inflammation and increase iron absorption from enterocytes
   d. Decrease during inflammation and increase iron absorption from enterocytes

10. Sideroblastic anemias result from:
   a. Sequestration of iron in hepatocytes
   b. Inability to incorporate heme into apohemoglobin
   c. Sequestration of iron in myeloblasts
   d. Failure to incorporate iron into protoporphyrin IX

11. In general, the hereditary hemochromatoses result from mutations that impair:
   a. The manner in which developing red cells acquire and manage iron
   b. The hepcidin-ferroportin iron regulatory system
   c. The TfR-Tf endocytic iron acquisition process for body cells other than blood cells
   d. The function of divalent metal transporter in enterocytes and macrophages

12. In the erythropoietic porphyrias, mild anemia may be accompanied by what distinctive clinical finding?
   a. Gallstones
   b. Impaired night vision
   c. Unintentional nighttime leg movements
   d. Heightened propensity for sunburn

Also review Chapter 11, questions 4 and 12 to 14 pertinent to the diagnostic value of various tests of iron status.

REFERENCES


Anemias Caused by Defects of DNA Metabolism

Linda H. Goossen

Etiology
Physiologic Roles of Vitamin B12 and Folate
Defect in Megaloblastic Anemia Due to Deficiency in Folate and Vitamin B12
Other Causes of Megaloblastosis
Systemic Manifestations of Folate and Vitamin B12 Deficiency
Causes of Vitamin Deficiencies
Folate Deficiency
Vitamin B12 Deficiency
Laboratory Diagnosis
Screening Tests
Specific Diagnostic Tests
Macrocytic Nonmegaloblastic Anemias
Treatment

OBJECTIVES
After completion of this chapter, the reader will be able to:

1. Discuss the relationships among macrocytic anemia, megaloblastic anemia, and pernicious anemia, and classify anemias appropriately within these categories.
2. Discuss the physiologic roles of folate and vitamin B12 in DNA production and the general metabolic pathways in which they act.
3. Describe the absorption and distribution of vitamin B12, including carrier proteins and the biologic activity of various vitamin-carrier complexes.
4. Describe the biochemical basis for development of anemia with deficiencies of vitamin B12 and folate, and explain the cause of the accompanying megaloblastosis.
5. Recognize individuals at risk for megaloblastic anemia by virtue of age, dietary habits, or physiologic circumstance such as pregnancy, drug regimens, or pathologic conditions.
6. Recognize complete blood count, reticulocyte count, red and white blood cell morphologies, and bone marrow findings consistent with megaloblastic anemia.
7. Given the results of tests measuring levels of serum vitamin B12, serum methylmalonic acid, serum folate, plasma or serum homocysteine, and antibodies to intrinsic factor, determine the likely cause of a patient’s deficiency.
8. Recognize results of bilirubin and lactate dehydrogenase tests that are consistent with megaloblastic anemia and explain why the test values are elevated in this condition.

CASE STUDY
After studying the material in this chapter, the reader should be able to respond to the following case study:

During a holiday visit, the children of a 76-year-old man noticed that he seemed more forgetful than usual and that he had difficulty walking. Concerned about the possibility of a mild stroke, the children insisted that he see his physician. The physician diagnosed a peripheral neuropathy affecting the father’s ability to walk. In addition, the physician noted that he was pale and slightly jaundiced and ordered routine hematologic studies. The results were as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Patient Value</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT (%)</td>
<td>27.0</td>
<td>40–54</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>121.6</td>
<td>80–100</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>38.3</td>
<td>26–32</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.5</td>
<td>32–36</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>18</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>Platelets (×10^9/L)</td>
<td>115</td>
<td>150–450</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>1.8</td>
<td>0.5–2.5</td>
</tr>
</tbody>
</table>

WBC differential: unremarkable with the exception of hypersegmentation of neutrophils
RBC morphology: moderate anisocytosis, moderate poikilocytosis, macrocytes, oval macrocytes, few teardrop cells
Impaired deoxyribonucleic acid (DNA) metabolism causes systemic effects by impairing production of all rapidly dividing cells of the body. These are chiefly the cells of the skin, the epithelium of the gastrointestinal tract, and the hematopoietic tissues. Because these all must be replenished throughout life, any impairment of cell production is evident in these tissues first. Patients may experience symptoms in any of these systems, but the blood provides a ready tissue for analysis. The hematologic effects, especially megaloblastic anemia, have come to be recognized as the hallmark of the diseases affecting DNA metabolism.

**ETIOLOGY**

The root cause of megaloblastic anemia is impaired DNA synthesis. The anemia is named for the very large cells of the bone marrow that develop a distinctive morphology (see section on laboratory diagnosis) due to a reduction in the number of cell divisions. Megaloblastic anemia is one example of a macrocytic anemia. Box 21-1 shows the classification of macrocytic anemias. Understanding the etiology of megaloblastic anemia requires a review of DNA synthesis with particular attention to the roles of vitamin B₁₂ (cobalamin) and folic acid (folate).

**Physiologic Roles of Vitamin B₁₂ and Folate**

Vitamin B₁₂ (cobalamin) is an essential nutrient consisting of a tetrapyrrole (corrin) ring containing cobalt that is attached to 5,6-dimethylbenzimidazolyl ribonucleotide (Figure 21-1). Vitamin B₁₂ is a coenzyme in two biochemical reactions in humans. One is isomerization of methylmalonyl coenzyme A (CoA) to succinyl CoA, which requires vitamin B₁₂ (in the adenosylcobalamin form) as a cofactor and is catalyzed by the enzyme methylmalonyl CoA mutase (Figure 21-2). In the absence of vitamin B₁₂, the impaired activity of methylmalonyl CoA mutase leads to a high level of serum methylmalonic acid (MMA), which is useful for the diagnosis of vitamin B₁₂ deficiency (discussed in the section on laboratory diagnosis). The second reaction is the transfer of a methyl group from 5-methyltetrahydrofolate (5-methyl THF) to homocysteine, which thereby generates methionine. This reaction is catalyzed by the enzyme methionine synthase and uses vitamin B₁₂.
in the methylcobalamin form) as a coenzyme (discussed later in this section). Methylcobalamin is synthesized through reduction and methylation of vitamin B12. This reaction represents the link between folate and vitamin B12 coenzymes and appears to account for the requirement for both vitamins in normal erythropoiesis.1,2

Folate is the general term used for any form of the vitamin folic acid. Folic acid is the synthetic form in supplements and fortified food. Folates consist of a pteridine ring attached to para-aminobenzoate with one or more glutamate residues (Figure 21-3). The function of folate is to transfer carbon units in the form of methyl groups from donors to receptors. In this capacity, folate plays an important role in the metabolism of amino acids and nucleotides. Deficiency of the vitamin leads to impaired cell replication and other metabolic alterations.

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**Figure 21-1** Structure of vitamin B12 (cobalamin) and its analogs, hydroxycobalamin and cyanocobalamin (forms often found in food and supplements) and methylcobalamin and 5′-deoxyadenosylcobalamin (coenzyme forms). The basic structure of cobalamin includes a tetrpyrrole (corrin) ring with a central cobalt atom linked to 5,6-dimethylbenzimidazolyl ribonucleotide. (From Scott JM, Browne P: Megaloblastic anemia. In Caballero B, Allen L, Prentice A, editors: Encyclopedia of human nutrition, ed 2, Oxford, 2006, Academic Press, p 113.)

**Figure 21-2** Role of vitamin B12 in the metabolism of methylmalonyl coenzyme A (CoA). Vitamin B12, in the 5′-deoxyadenosylcobalamin form, is a coenzyme in the isomerization of methylmalonyl CoA to succinyl CoA. The reaction is catalyzed by the enzyme methylmalonyl CoA mutase.

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**Figure 21-3** Structure of synthetic folic acid and the naturally occurring forms of the vitamin. (From Scott JM, Browne P: Megaloblastic anemia. In Caballero B, Allen L, Prentice A, editors: Encyclopedia of human nutrition, ed 2, Oxford, 2006, Academic Press, p 114.)
Folate circulates in the blood predominantly as 5-methyl THF. Folate has an important role in DNA synthesis. As shown in Figure 21-4, within the cytoplasm of the cell, a methyl group is transferred from 5-methyl THF to homocysteine, converting it to methionine and generating tetrahydrofolate (THF). This reaction is catalyzed by methionine synthase and requires vitamin B_{12} as a cofactor. THF is then converted to 5,10-methylene THF by the donation of a methyl group from serine. The methyl group of 5,10-methylene THF is then transferred to deoxyuridine monophosphate (dUMP), which converts it to deoxythymidine monophosphate (dTMP) and converts 5,10-methylene THF to dihydrofolate (DHF). This reaction is catalyzed by thymidylate synthase. dTMP is a precursor of deoxythymidine triphosphate (dTTP), which is used to synthesize DNA. THF is regenerated by the conversion of DHF to THF by the enzyme dihydrofolate reductase. A deficiency of vitamin B_{12} prevents the production of THF from 5-methyl THF; as a result, folate becomes metabolically trapped as 5-methyl THF. This constitutes the "folate trap."
When excisions at opposing DNA strand sites coincide, double-strand breaks occur. Repeated DNA strand breaks lead to fragmentation of the DNA strand. The resulting DNA double-strand breaks occur. Repeated DNA strand breaks when excisions at opposing DNA strand sites coincide, lead to fragmentation of the DNA strand. This abnormal blood cell development is called ineffective hematopoiesis. The dependency of DNA production on folate has been used in cancer chemotherapy (Box 21-2).

In addition to the increased apoptosis of erythroid progenitor cells in the bone marrow discussed above, the remaining erythroid cells are larger than normally seen during the final stages of erythropoiesis, and their nuclei are immature-appearing compared with the cytoplasm. In contrast to the normally dense chromatin of comparable normoblasts, the nuclei of megaloblastic erythroid precursors have an open, finely stippled, reticular pattern. The nuclear changes seen in the megaloblastic cells are related to cell cycle delay, prolonged resting phase, and arrest in nuclear maturation. Electron microscopy has revealed that reduced synthesis of histones is also responsible for morphologic changes in the chromatin of megaloblastic erythroid precursors. Ribonucleic acid (RNA) function is not affected by vitamin B12 or folate deficiency because RNA contains uracil instead of thymidine nucleotides, so cytoplasmic development progresses normally. The slower maturation rate of the nucleus compared with the cytoplasm is called nuclear-cytoplasmic asynchrony. Together, the accumulation of cells with nuclei at earlier stages of development and cells with increased size and immaturity result in the appearance of erythroid cells in the bone marrow that are pathognomonic of megaloblastic anemia. Because ineffective hematopoiesis affects all three blood cell lineages, pancytopenia is also evident, with certain distinctive cellular changes (see section on laboratory diagnosis).

**Other Causes of Megaloblastosis**

Vitamin B12 and folate deficiency are not the only causes of megaloblastic erythrocytes. Dysplastic erythroid cells in megaloblastic syndrome (MDS) can also have megaloblastoid features (Chapter 34). In MDS, however, the macrocytic erythrocytes and their progenitors characteristically show delayed cytoplasmic and nuclear maturation, including cytoplasmic vacuole formation, nuclear budding, multinucleation, and nuclear fragmentation, and thus may be distinguished from the megaloblastic RBCs seen in the vitamin deficiencies. In addition, nuclear-cytoplasmic asynchrony and megaloblastic RBCs may be seen in congenital dyserythropoietic anemia (CDA) types I and III (Chapter 22). The CDAs are rare conditions that usually manifest in childhood and may be distinguished from the acquired causes of megaloblastosis by clinical history and morphologic differences. In CDA I, internuclear chromatin bridging of erythroid cells or binucleated forms are observed, and in CDA III, giant multinucleated erythroblasts are present. Another rare condition in which RBC precursors have a megaloblastic appearance is acute erythroid leukemia, previously classified as FAB M6 (Chapter 35). In this condition, the cells are macrocytic, and the immature appearance of the nuclear chromatin is similar to the more open appearance of the chromatin in megaloblasts. There are usually other aberrant findings in erythroid leukemia, including an increase of myeloblasts in the bone marrow; however, an experienced morphologist can discern the subtle differences. Reverse transcriptase inhibitors, used to treat human immunodeficiency virus (HIV) infections, interfere with DNA production and may also lead to megaloblastic changes.

Although the conditions described in this section are characterized by megaloblastic morphology, they are due to acquired or inherited mutations in progenitor cells or interference with DNA synthesis and are refractive to therapy with vitamin B12 or folic acid.
loss of vibratory sense, especially in the lower limbs.\(^{13}\) Neuropsychiatric symptoms may also be present, including personality changes and psychosis. These symptoms seem to be the result of demyelination of the spinal cord and peripheral nerves, but the relationship of this demyelination to vitamin B\(_12\) deficiency is unclear. The roles of increases in tumor necrosis factor-\(\alpha\), a neurotoxic agent, and decreases in epidermal growth factor, a neurotrophic agent, in the development of neuropsychologic symptoms in vitamin B\(_12\)-deficient patients are being researched.\(^{12,13}\)

At one time, folate deficiency was believed to be more benign clinically than vitamin B\(_12\) deficiency. Later research suggested that low levels of folate and the resulting high homocysteine levels were risk factors for cardiovascular disease.\(^{14}\) More recent research has provided mixed results, with studies both refuting this association\(^{15,16}\) as well as substantiating the association between high circulating homocysteine levels and the risk of cardiovascular disease.\(^{17}\) Several studies suggest that high folate levels provide a cardioprotective effect in diabetic patients and certain ethnic populations.\(^{15,18,19}\) The evidence at this time is unclear as to whether persistent suboptimal folate status may have a significant long-term health impact. In addition, there is evidence of depression, peripheral neuropathy, and psychosis related to folate deficiency.\(^{20-22}\) Folate levels appear to influence the effectiveness of treatments for depression.\(^{23}\)

Folate deficiency during pregnancy can result in impaired formation of the fetal nervous system, resulting in neural tube defects such as spina bifida,\(^{24}\) despite the fact that the fetus accumulates folate at the expense of the mother. Pregnancy requires a considerable increase in folate to fulfill the requirements related to rapid fetal growth, uterine expansion, placental maturation, and expanded blood volume.\(^3\) Insuring adequate folate levels in women of childbearing age is particularly important because many women are likely to be unaware of their pregnancy during the first crucial weeks of fetal development. Fortification of the U.S. food supply with folic acid in grain and cereal products was mandated by the Food and Drug Administration in 1998 to lower the risk of neural tube defects in the unborn.

### Causes of Vitamin Deficiencies

In general, vitamin deficiencies may arise because the vitamin is in relatively short supply, because use of the vitamin is impaired, or because of excessive loss. Folate deficiency can be caused by all of these mechanisms.

**Folate Deficiency**

**Inadequate Intake**

Folate is synthesized by microorganisms and higher plants. Folate is ubiquitous in foods, but a generally poor diet can result in deficiency. Good sources of folate include leafy green vegetables, dried beans, liver, beef, fortified breakfast cereals, and some fruits, especially oranges.\(^{3,25}\) Folic acid is heat labile, and overcooking of foods can diminish their nutritional value.\(^3\)

**Increased Need**

Increased need for folate occurs during pregnancy and lactation when the mother must supply her own needs plus those of the fetus or infant. Infants and children also have increased need for folate during growth.\(^3\)

### Impaired Absorption

Food folates must be hydrolyzed in the gut before absorption in the small intestine; however, only 50% of what is ingested is available for absorption.\(^1\) A rare autosomal recessive deficiency of a folate transporter protein (PCFT) severely decreases intestinal absorption of folate.\(^{25,26}\) Once across the intestinal cell, most folate is transported in the plasma as 5-methyl THF unbound to any specific carrier.\(^{15}\) Its entry into cells, however, is carrier mediated.\(^{27}\)

Folate absorption may also be impaired by intestinal disease, especially sprue and celiac disease. Sprue is characterized by weakness, weight loss, and steatorrhea (fat in the feces), which is evidence that the intestine is not absorbing food properly. It is seen in the tropics (tropical sprue), where its cause is generally considered to be overgrowth of enteric pathogens.\(^{28}\) Celiac disease (nontropical sprue) has been traced to intolerance of the gluten in some grains\(^{28}\) (gluten-induced enteropathy) and can be controlled by eliminating wheat, barley, and rye products from the diet. Surgical resection of the small intestine and inflammatory bowel disease can also decrease folate absorption.

### Impaired Use of Folate

Numerous drugs impair folate metabolism (Box 21–3).\(^{29,30}\) Antiepileptic drugs are particularly known for this,\(^{31}\) and the result is macrocytosis with frank megaloblastic anemia. In most instances, folic acid supplementation is sufficient to override the impairment and allow the patient to continue therapy.\(^{32}\) Because folate deficiency results in inhibition of cell replication, several anticancer drugs, including methotrexate, are folate inhibitors.\(^3\)

### Excessive Loss of Folate

Physiologic loss of folate occurs through the kidney. The amount is small and not a cause of deficiency. Patients undergoing renal dialysis lose folate in the dialysate, however; thus supplemental folic acid is routinely provided to these individuals to prevent megaloblastic anemia.\(^{15}\)

### Box 21-3 Some Drugs That May Lead to Impaired Use of Folate

**Impair Folate Metabolism**

- Methotrexate (Trexall): antirarthritic, chemotherapeutic
- 5-Fluorouracil (Adrucil): antimicrobial
- Hydroxyurea (Hydrea): antimitabolite
- Pyrimethamine (Daraprim): antibacterial
- Pentamidine (Pentam): antimicrobial
- Phenytoin (Dilantin): anticonvulsant
- Trimethoprim (Primsol): antimicrobial

**Impair Folate Absorption**

- Metformin (Glucophage): oral antidiabetic
- Cholestyramine (Questran): cholesterol lowering
Vitamin B\textsubscript{12} Deficiency

Inadequate Intake

While true dietary deficiency of vitamin B\textsubscript{12} is rare, this condition is possible for strict vegetarians (vegans) who do not eat meat, eggs, or dairy products. Although it is an essential vitamin for animals, plants cannot synthesize vitamin B\textsubscript{12}, and it is not available from vegetable sources. The best dietary sources are animal products such as liver, dairy products, fish, shellfish, and eggs. In contrast to the heat-labile folate, vitamin B\textsubscript{12} is not destroyed by cooking.

Increased Need

Increased need for vitamin B\textsubscript{12} occurs during pregnancy, lactation, and growth. Due to the vigorous cell replication, what would otherwise be a diet adequate in vitamin B\textsubscript{12} can become inadequate during these periods.

Impaired Absorption

Vitamin B\textsubscript{12} in food is released from food proteins primarily in the acid environment of the stomach, aided by pepsin, and is subsequently bound by a specific salivary protein, haptocorrin, also known as R protein or transcobalamin I (Figure 21-5). In the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure21-5.png}
\caption{Normal absorption of vitamin B_{12}. Dietary vitamin B_{12} (cobalamin, CBL) is food protein (P) bound. In the stomach, pepsin and hydrochloric acid (HCl) secreted by parietal cells release CBL from P. CBL then binds haptocorrin (R protein, R), released from salivary glands, and remains bound until intestinal pancreatic proteases, including trypsin, catalyze its release. Parietal cells secrete intrinsic factor (IF), which binds CBL in the duodenum. Cubilin-amnionless (cubam) and megalin receptors in ileal enterocytes bind CBL-IF and release the CBL. Enterocytes produce transcobalamin (TC), which binds CBL and transports it through the portal circulation. Bone marrow pronormoblast membrane TC receptors (TC-R) bind CBL-TC and release the CBL, which is converted to methylcobalamin (methyl-CBL). Methyl-CBL is a coenzyme that supports homocysteine-methionine conversion. Hepatocyte TC-R receptors bind CBL-TC and release the CBL, which is moved to storage organelles or excreted through the biliary system.}
\end{figure}
small intestine, vitamin B₁₂ is released from haptocorrin by the action of pancreatic proteases, including trypsin. It is then bound by intrinsic factor, which is produced by the gastric parietal cells. Vitamin B₁₂ binding to intrinsic factor is required for absorption by ileal enterocytes that possess receptors for the complex. These receptors are cubilin-amnionless complex, collectively known as cubam, which binds the vitamin B₁₂–intrinsic factor complex, and megalin, a membrane transport protein.5,33–36 Once in the enterocyte, the vitamin B₁₂ is then freed from intrinsic factor and bound to transcobalamin (previously called transcobalamin II) and released into the circulation. In the plasma, only 10% to 30% of the vitamin B₁₂ is bound to transcobalamin; the remaining 75% is bound to transcobalamin I and III, referred to as the haptocorrins.35,37 The vitamin B₁₂–transcobalamin complex, termed holotranscobalamin (holoTC), is the metabolically active form of vitamin B₁₂. Holotranscobalamin binds to specific receptors on the surfaces of many different types of cells and enters the cells by endocytosis, with subsequent release of vitamin B₁₂ from the carrier.38 The body maintains a substantial reserve of absorbed vitamin B₁₂ in hepatocytes.4

The absorption of vitamin B₁₂ can be impaired by (1) failure to separate vitamin B₁₂ from food proteins in the stomach, (2) failure to separate vitamin B₁₂ from haptocorrin in the intestine, (3) lack of intrinsic factor, (4) malabsorption, and (5) competition for available vitamin B₁₂.

### Failure to Separate Vitamin B₁₂ from Food Proteins.

A condition known as food-cobalamin malabsorption is characterized by hypochlorhydria and the resulting inability of the body to release vitamin B₁₂ from food or intestinal transport proteins for subsequent binding to transcobalamin. Food-cobalamin malabsorption is caused primarily by atrophic gastritis or atrophy of the stomach lining that often occurs with increasing age.39 Because histamine 2 receptor blockers and proton pump inhibitors lower gastric acidity, the long-term use of these drugs for the treatment of ulcers and gastroesophageal reflux disease, and gastric bypass surgery also induce food-cobalamin malabsorption.19

### Failure to Separate Vitamin B₁₂ from Haptocorrin.

Lack of gastric acidity or lack of trypsin as a result of chronic pancreatic disease can prevent vitamin B₁₂ absorption because the vitamin remains bound to haptocorrin in the intestine and unavailable to intrinsic factor.11

### Lack of Intrinsic Factor.

Lack of intrinsic factor constitutes a significant cause of impaired vitamin B₁₂ absorption. It is most commonly due to autoimmune disease, as in pernicious anemia, but can also result from the loss of parietal cells with Helicobacter pylori infection, total or partial gastrectomy, or hereditary intrinsic factor deficiency.

**Pernicious anemia.** Pernicious anemia is an autoimmune disorder characterized by impaired absorption of vitamin B₁₂ due to a lack of intrinsic factor.40 This condition is called pernicious anemia because the disease was fatal before its cause was discovered. The incidence per year is roughly 25 new cases per 100,000 persons older than 40 years of age.3 Pernicious anemia most often manifests in the sixth decade or later, but can also be found in children. Patients with pernicious anemia have an increased risk of developing gastric tumors.4

In pernicious anemia, autoimmune lymphocyte-mediated destruction of gastric parietal cells severely reduces the amount of intrinsic factor secreted in the stomach. Pathologic CD4 T cells inappropriately recognize and initiate an autoimmune response against the H⁺/K⁺–adenosine triphosphatase embedded in the membrane of the parietal cells.41 A chronic inflammatory infiltration follows, which extends into the wall of the stomach.40 Over a period of years and even decades, there is progressive development of atrophic gastritis resulting in the loss of the parietal cells with their secretory products, H⁺ and intrinsic factor. The loss of H⁺ production in the stomach constitutes achlorhydria. Low gastric acidity was previously an important diagnostic criterion for pernicious anemia. Serum gastrin levels can be markedly elevated due to the gastric achlorhydria.1 The absence of intrinsic factor can also be detected using the Schilling test. However, because the test requires a 24-hour urine collection and the use of radioactive cobalt in vitamin B₁₂ to trace absorption, safer diagnostic tests are currently used (see section on laboratory diagnosis).

Another feature of the autoimmune response in pernicious anemia is the production of antibodies to intrinsic factor42 and gastric parietal cells43 that are detectable in serum. The most common antibody to intrinsic factor blocks the site on intrinsic factor where vitamin B₁₂ binds,40 which inhibits the formation of the intrinsic factor–vitamin B₁₂ complex and prevents the absorption of the vitamin. These blocking antibodies are present in serum or gastric fluid in about 90% of patients with pernicious anemia.5 Parietal cell antibodies are detectable in the serum of about 90% of patients with pernicious anemia.5,40

**Other causes of lack of intrinsic factor.** A lack of intrinsic factor may also be related to H. pylori infection. Left untreated, colonization of the gastric mucosa with H. pylori progresses until the parietal cells are entirely destroyed, a process involving both local and systemic immune processes.44,45 In addition, partial or total gastrectomy, which results in removal of intrinsic factor–producing parietal cells, invariably leads to vitamin B₁₂ deficiency.

Impaired absorption of vitamin B₁₂ can also be caused by hereditary intrinsic factor deficiency. This is a rare autosomal recessive disorder characterized by the absence or nonfunctionality of intrinsic factor. In contrast to the acquired forms of pernicious anemia, histology and gastric acidity are normal.35

**Malabsorption.** General malabsorption of vitamin B₁₂ can be caused by the same conditions interfering with folate absorption, such as celiac disease, tropical sprue, and inflammatory bowel disease.

**Inherited Errors of Vitamin B₁₂ Absorption and Transport.** Imerslund-Gräsbeck syndrome is a rare autosomal recessive condition caused by mutations in the genes for either cubulin or amnionless. This defect results in decreased endocytosis of the intrinsic factor–vitamin B₁₂ complex by ileal enterocytes. Transcobalamin deficiency is another rare autosomal recessive condition resulting in a deficiency of physiologically available vitamin B₁₂.35,46
Competition for Vitamin $B_{12}$

Competition for available vitamin $B_{12}$ in the intestine may come from intestinal organisms. The fish tapeworm *Diphyllobothrium latum* is able to split vitamin $B_{12}$ from intrinsic factor, rendering the vitamin unavailable for host absorption. Also, blind loops, portions of the intestines that are stenotic as a result of surgery or inflammation, can become overgrown with intestinal bacteria that compete effectively with the host for available vitamin $B_{12}$. In both of these cases, the host is unable to absorb sufficient vitamin $B_{12}$, and megaloblastic anemia results.

**LABORATORY DIAGNOSIS**

The tests used in the diagnosis of megaloblastic anemia include screening tests and specific diagnostic tests to identify the specific vitamin deficiency and perhaps its cause.

**Screening Tests**

Five tests used to screen for megaloblastic anemia are the complete blood count (CBC), reticulocyte count, white blood cell (WBC) manual differential, serum bilirubin, and lactate dehydrogenase.

**Complete Blood Count and Reticulocyte Count**

Slight macrocytosis often is the earliest sign of megaloblastic anemia. Patients with uncomplicated megaloblastic anemia are expected to have decreased hemoglobin and hematocrit values, pancytopenia, and reticulocytopenia. Megaloblastic anemia develops slowly, and the degree of anemia is often severe when first detected. Hemoglobin values of less than 7 or 8 g/dL are not unusual. When the hematocrit is less than 20%, erythroblasts with megaloblastic nuclei, including an occasional promegaloblast, may appear in the peripheral blood. The mean cell volume (MCV) is usually 100 to 150 fl and commonly is greater than 120 fl, although coexisting iron deficiency, thalassemia trait, or inflammation can prevent macrocytosis. The mean cell hemoglobin (MCH) is elevated by the increased volume of the cells, but the mean cell hemoglobin concentration (MCHC) is usually within the reference interval because hemoglobin production is unaffected. The red blood cell distribution width (RDW) is also elevated.

The characteristic morphologic findings of megaloblastic anemia in the peripheral blood include oval macrocytes (enlarged oval RBCs) (Figure 21-6) and hypersegmented neutrophils with six or more lobes (Figure 21-7). Impaired cell production results in a low absolute reticulocyte count, especially in light of the severity of the anemia, and polychromasia is not observed on the peripheral blood film. Additional morphologic changes may include the presence of teardrop cells, RBC fragments, and microspherocytes. These smaller cells further increase the RDW. The presence of schistocytes sometimes leads to a paradoxically lower mean cell volume (MCV) than is seen in less severe cases. These erythrocyte changes reflect the severity of the dyserythropoiesis and should not be taken as evidence of microangiopathic hemolysis. Nucleated RBCs, Howell-Jolly bodies, basophilic stippling, and Cabot rings may also be observed.

**White Blood Cell Manual Differential**

Hypersegmentation of neutrophils is essentially pathognomonic for megaloblastic anemia. It appears early in the course of the disease and may persist for up to 2 weeks after treatment is initiated. Hypersegmented neutrophils noted in the WBC differential report are a significant finding and require a reporting rule that can be applied consistently because even healthy individuals may have an occasional one. One such rule is to report hypersegmentation when there are at least 5 five-lobed neutrophils per 100 WBCs or at least 1 six-lobed neutrophil is noted. Some laboratories perform a lobe count on 100 neutrophils and then calculate the mean. In megaloblastic anemia, the mean lobe count should be greater than 3.4. The cause of the hypersegmentation is not understood, despite
considerable investigation. More recent advances in the understanding of growth factors and their impact on transcription factors may yet solve this mystery. Nevertheless, a search for neutrophil hypersegmentation on a peripheral blood film constitutes an inexpensive yet sensitive screening test for megaloblastic anemia.

**Bilirubin and Lactate Dehydrogenase Levels**

Although generally considered a nutritional anemia, megaloblastic anemia is in one sense a hemolytic anemia. Because many RBC precursors die during division in the bone marrow, many RBCs never enter the circulation (ineffective hematopoiesis), so a decrease in reticulocytes occurs in the peripheral blood. The usual signs of hemolysis are evident in the serum, including an elevation in the levels of total and indirect bilirubin and lactate dehydrogenase (predominantly RBC derived).

The constellation of findings including macrocytic anemia, moderate to marked pancytopenia, reticulocytopenia, oval macrocytes, hypersegmented neutrophils, plus increased levels of total and indirect bilirubin and lactate dehydrogenase justifies further testing to confirm a diagnosis of megaloblastic anemia and determine its cause. Occasionally, the classic findings may be obscured by coexisting conditions such as iron deficiency, which makes the diagnosis more challenging. Most hematologic aberrations do not appear until vitamin deficiency is fairly well advanced (Box 21-4).

**Specific Diagnostic Tests**

**Bone Marrow Examination**

Modern tests for vitamin deficiencies and autoimmune antibodies have made bone marrow examination an infrequently used diagnostic test for megaloblastic anemia. Nevertheless, it remains the reference confirmatory test to identify the megaloblastic appearance of the developing RBCs.

*Megablastic,* in contrast to *macrocytic,* anemia refers to specific morphologic changes in the developing RBCs. The cells are characterized by a nuclear-cytoplasmic asynchrony in which the cytoplasm matures as expected with increasing pinkness as hemoglobin accumulates. The nucleus lags behind, however, appearing younger than expected for the degree of maturity of the cytoplasm (Figure 21-8). This asynchrony is most striking at the stage of the polychromatic normoblast. The cytoplasm appears pinkish-blue as expected for that stage, but the nuclear chromatin remains more open than expected, similar to that in the nucleus of a basophilic normoblast. Overall, the marrow is hypercellular, with a myeloid-to-erythroid ratio of about 1:1 by virtue of the increased erythropoietic activity. The hematopoiesis is ineffective, however, and although cell production in the bone marrow is increased, the apoptosis of cells in the marrow results in peripheral pancytopenia.

The WBCs are also affected in megaloblastic anemia and appear larger than normal. This is most evident in metamyelocytes and bands, because in the usual development of neutrophils, the cells should be getting smaller at these stages. The effect creates “giant” metamyelocytes and bands (Figure 21-9).

**BOX 21-4  Sequence of Development of Megaloblastic Anemias**

1. Decrease in vitamin levels
2. Hypersegmentation of neutrophils in peripheral blood
3. Oval macrocytes in peripheral blood
4. Megaloblastosis in bone marrow
5. Anemia

Figure 21-8  Erythroid precursors in megaloblastic anemia. Note nuclear-cytoplasmic asynchrony (bone marrow, ×500). (From Rodak BF, Carr JH: Clinical hematology atlas, ed 4, St. Louis, 2013, Elsevier, Saunders.)

Figure 21-9  Giant band (early) in megaloblastic anemia (bone marrow, original magnification ×1000).
Megakaryocytes do not show consistent changes in megaloblastic anemia. They may be either increased or decreased in number and may show diminished lobulation. The latter finding is not consistently seen, however, and even when present, it is difficult to assess.

**Assays for Folate, Vitamin B₁₂, Methylmalonic Acid, and Homocysteine**

Although bone marrow aspiration is confirmatory for megaloblastosis, the invasiveness of the procedure and its expense mean that other testing is performed more often than a bone marrow examination. Furthermore, the confirmation of megaloblastic morphology in the marrow does not identify its cause. Tests for serum levels of folate and vitamin B₁₂ are readily available using immunoassay; serum vitamin B₁₂ may also be assayed by competitive binding chemiluminescence. However, there are a number of interferences with these assays that can cause false increased and decreased results (Box 21-5); reflexive testing to methylmalonic acid and homocysteine (covered below) can increase diagnostic accuracy. RBC folate levels may also be measured. Unlike serum folate levels, which fluctuate with diet, RBC folate values are stable and may be a more accurate reflection of true folate status; however, current RBC folate tests have less than optimal sensitivity and specificity and have not been validated in actual patients with normal and deficient folate levels. Thus the serum folate level is preferred over RBC folate level in the United States as the initial test for evaluation of folate deficiency.

Some laboratories conduct a reflexive assay for methylmalonic acid if vitamin B₁₂ levels are low. As indicated previously, in addition to playing a role in folate metabolism, vitamin B₁₂ is a cofactor in the conversion of methylmalonyl CoA to succinyl CoA by the enzyme methylmalonyl CoA mutase (Figure 21-2). If vitamin B₁₂ is deficient, methylmalonyl CoA accumulates. Some of it hydrolyzes to methylmalonic acid, and the increase can be detected in serum and urine. Because methylmalonic acid is also elevated in patients with impaired renal function, the test is not specific, and thus increased levels cannot be definitively related to vitamin B₁₂ deficiency. Methylmalonic acid is assayed by gas chromatography–mass spectrometry.

Homocysteine levels are affected by deficiencies in either folate or vitamin B₁₂. 5-Methyl THF donates a methyl group to homocysteine in the generation of methionine. This reaction uses vitamin B₁₂ as a coenzyme (Figure 21-4). Thus a deficiency in either folate or vitamin B₁₂ results in elevated levels of homocysteine. Total homocysteine can be measured in either plasma or serum. Homocysteine may be assayed by gas chromatography–mass spectrometry, high-performance liquid chromatography, or fluorescence polarization immunoassay. Homocysteine levels are also elevated in patients with renal failure and dehydration. Figure 21-10 presents an algorithm of the analysis of these analytes in the diagnosis of vitamin B₁₂ and folate deficiency.

**Gastric Analysis and Serum Gastrin**

Gastric analysis may be used to confirm achlorhydria, an expected finding in pernicious anemia. Achlorhydria occurs in other conditions, however, including natural aging. When other causes of vitamin B₁₂ deficiency have been eliminated, a finding of achlorhydria is supportive, although not diagnostic, of pernicious anemia. The H⁺ concentration is determined by pH measurement.

As a result of the gastric achlorhydria, serum gastrin levels can be markedly elevated. Serum gastrin is measured by immunoassay, including chemiluminescent immunomessays.

**Antibody Assays**

Antibodies to intrinsic factor and parietal cells can be detected in the serum of most patients with pernicious anemia. Various immunoassays can detect intrinsic factor–blocking antibodies; parietal cell antibodies can be detected by indirect fluorescent antibody techniques or enzyme-linked immunosorbent assays. Anti-IF antibodies are highly specific and confirmatory for pernicious anemia, but their absence does not rule out the condition. The test for parietal cell antibodies is nonspecific and not clinically useful for the diagnosis of pernicious anemia.

Figure 21-11 presents an algorithm for the diagnosis of pernicious anemia using tests for serum vitamin B₁₂, methylmalonic...
Figure 21-10  Algorithm for the use of assays for serum folate, vitamin B₁₂, methylmalonic acid, and homocysteine in the diagnosis of vitamin B₁₂ and folate deficiency.5,44 ↑, Increased; MCV, mean cell volume; MMA, methylmalonic acid; N, within reference interval.

Figure 21-11  Algorithm for the diagnosis of pernicious anemia. (Adapted from Klee GG. Cobalamin and folate evaluation: Measurement of methylmalonic acid and homocysteine vs vitamin B₁₂ and folate. Clin Chem 2000;46, p. 1281.)
Holotranscobalamin Assay
Holotranscobalamin is the metabolically active form of vitamin B₁₂. Until recently, methods for measuring holotranscobalamin were manual and not suitable for use in clinical laboratories. Newer, more rapid immunoassays using monoclonal antibodies specific for holotranscobalamin have been developed in the past several years that are both sensitive and specific.⁵⁴,⁵⁵ Recent studies suggest the specificity of holotranscobalamin to detect vitamin B₁₂ deficiency is low; thus the adoption of holotranscobalamin in routine clinical testing is not supported.⁵³

Deoxyuridine Suppression Test
The principle of the deoxyuridine suppression test is that the preincubation of normal bone marrow with deoxyuridine will suppress the subsequent incorporation of labeled thymidine into DNA because the normal cells can successfully methylate the uridine into thymidine. However, in patients with either a vitamin B₁₂ or a folate deficiency, this suppression is abnormally low. By adding either vitamin B₁₂ or folate to the test cells, one can determine whether the inadequate suppression is caused by vitamin B₁₂ or folate deficiency.⁵⁶-⁵⁸ Although micromethods have been developed, the necessity of using bone marrow tissue and the complexity of the test make it impractical for clinical testing.

Stool Analysis for Parasites
When vitamin B₁₂ is found to be deficient, a stool analysis for eggs or proglottids of the fish tapeworm D. latum may be part of the diagnostic workup. Table 21-1 contains a summary of laboratory tests used to diagnose vitamin B₁₂ and folate deficiency.

MACROCYTIC NONMEGALOBLASTIC ANEMIAS
The macrocytic nonmegaloblastic anemias are macrocytic anemias in which DNA synthesis is unimpaired. The macrocytosis tends to be mild; the MCV usually ranges from 100 to 110 fL and rarely exceeds 120 fL. Patients with nonmegaloblastic, macrocytic anemia lack hypersegmented neutrophils and oval macrocytes in the peripheral blood and megaloblasts in the bone marrow. Macrocytosis may be physiologically normal, as in the newborn (Chapter 45), or the result of pathology, as in liver disease, chronic alcoholism, or bone marrow failure. Reticulocytosis is a

| TABLE 21-1 Laboratory Tests Used to Diagnose Vitamin B₁₂ and Folate Deficiency |
|---------------------------------|---------------------------------|-------------------------|
| **Screening tests**             | **Folate Deficiency**           | **Vitamin B₁₂ Deficiency** |
| Complete blood count            | ↓ HGB, HCT, RBCs, WBCs, PLTs    | Same as Folate Deficiency |
| Manual differential count       | ↑ MCV, MCH                      | Same as Folate Deficiency |
| Absolute reticulocyte count     | ↓                               | ↓                       |
| Serum total and indirect bilirubin| ↑                               | ↑                       |
| Serum lactate dehydrogenase     | ↑                               | ↑                       |
| **Specific diagnostic tests**   | **Bone marrow examination**     | **Erythroid hyperplasia (ineffective)** |
| Serum vitamin B₁₂               | N                               | Presence of megaloblasts |
| Serum folate                    | ↓                               | ↑                       |
| RBC folate                      | ↑                               | ↑                       |
| Serum methylmalonic acid        | N                               | ↑                       |
| Serum/plasma homocysteine       | ↑                               | ↑                       |
| Antibodies to intrinsic factor and gastric parietal cells | Absent | Present in pernicious anemia |
| Serum gastrin                   | N                               | Can be markedly elevated in pernicious anemia |
| Gastric analysis                | N                               | Achlorhydria in pernicious anemia |
| Holotranscobalamin assay        | N                               | ↓                       |
| Stool analysis for parasites    | Negative                        | D. latum may be the cause of deficiency |

† Increased; ↓ decreased; HGB, hemoglobin; HCT, hematocrit; MCH, mean cell hemoglobin; MCV, mean cell volume; N, within reference interval; PLT, platelet; RBC, red blood cell; WBC, white blood cell.
*Bone marrow examination and gastric analysis are not usually required for diagnosis.
†Without vitamin B₁₂, the cell is unable to produce intracellular polyglutamated tetrahydrofolate; therefore, 5-methyltetrahydrofolate leaks out of the cell, which results in a decreased level of intracellular folate.
‡Holotranscobalamin level is also decreased in transcobalamin deficiency.
common cause of macrocytosis. Figure 21-12 presents an algorithm for the preliminary investigation of macrocytic anemias.

**TREATMENT**

Treatment should be directed at the specific vitamin deficiency established by the diagnostic tests and should include addressing the cause of the deficiency (e.g., better nutrition, treatment for *D. latum*), if possible. Vitamin B₁₂ is administered intramuscularly to treat pernicious anemia to bypass the need for intrinsic factor. High-dose oral vitamin B₁₂ treatment is increasingly popular in the treatment of pernicious anemia.³⁹,⁵⁹,⁶⁰ Regardless of the treatment modality, those with pernicious anemia or malabsorption must have lifelong vitamin replacement therapy. Folic acid can be administered orally. The inappropriate treatment of vitamin B₁₂ deficiency with folic acid improves the anemia but does not correct or stop the progress of the neurologic damage, which may advance to an irreversible state.³ Thus proper diagnosis prior to treatment is important. Iron is often supplemented concurrently to support the rapid cell production that accompanies effective treatment.

When proper treatment is initiated, the body’s response is prompt and brisk and can be used to confirm the accuracy of the diagnosis. The bone marrow morphology will begin to revert to a normoblastic appearance within a few hours of treatment. A substantial reticulocyte response is apparent at about 1 week, with hemoglobin increasing toward normal levels in about 3 weeks.³¹ Hypersegmented neutrophils disappear from the peripheral blood within 2 weeks of initiation of treatment.³ Thus with proper treatment, hematologic parameters may return to normal within 3 to 6 weeks.

![Algorithm for preliminary investigation of macrocytic anemias.](image)
SUMMARY

- Impaired DNA synthesis affects all rapidly dividing cells of the body, including the skin, gastrointestinal tract, and bone marrow. The effect on hematologic cells results in megaloblastic anemia.
- Vitamin B₁₂ and folate are needed for the production of thymidine nucleotides for DNA synthesis. Deficiencies of either vitamin impair DNA replication, halt cell division, and increase apoptosis, which results in ineffective hematopoiesis and megaloblastic morphology of erythrocyte precursors.
- Vitamin B₁₂ deficiency is associated with peripheral neuropathies and neuropsychiatric abnormalities as a result of demyelination of nerves in the peripheral and central nervous system. Peripheral neuropathy and depression also may accompany folate deficiency. Folate deficiency in early pregnancy can lead to neural tube defects in the fetus.
- Lack of vitamin B₁₂ leads to the accumulation of methylmalonic acid (MMA) and homocysteine. Folate deficiency, in particular, leads to elevation of homocysteine levels and possible risk of coronary artery disease.
- Folate deficiency may result from inadequate intake, increased need with growth or pregnancy, impaired absorption, impaired use, or excessive loss. The action of folate can be impaired by drugs such as those used to treat epilepsy or cancer. Renal dialysis patients experience significant folate loss to the dialysate.
- Vitamin B₁₂ deficiency arises from inadequate intake, increased need, or inadequate absorption. Inadequate intake of vitamin B₁₂, although possible, is uncommon because vitamin B₁₂ is ubiquitous in animal products. Pregnancy, lactation, and growth create increased need for vitamin B₁₂.
- Absorption of vitamin B₁₂ depends on production of intrinsic factor by parietal cells of the stomach. Vitamin B₁₂ bound to transcobalamin—holotranscobalamin—is the metabolically active form of the vitamin in the circulation.
- Impaired absorption of vitamin B₁₂ can be caused by several mechanisms. Decrease in gastric acid production or lack of trypsin in the intestine causes vitamin B₁₂ to be excreted in the stool rather than absorbed. Malabsorption can be caused by intestinal diseases, such as sprue, celiac disease, and inflammatory bowel disease. Competition for vitamin B₁₂ can develop from an intestinal parasite (D. latum) or bacteria in intestinal blind loops. Lack of intrinsic factor may result from loss of gastric parietal cells with pernicious anemia, H. pylori infection, gastrectomy, or inherited intrinsic factor deficiency.
- Pernicious anemia is vitamin B₁₂ deficiency resulting from an autoimmune disease that causes destruction of gastric parietal cells. H⁺ and intrinsic factor secretion is lost. Antibodies to parietal cells or intrinsic factor, or both, are detectable in the serum.
- Classic CBC findings in megaloblastic anemia include decreased hemoglobin, hematocrit, and RBC count; leukopenia; thrombocytopenia; decreased absolute reticulocyte count; elevated MCV (usually greater than 120 fl); elevated RDW and MCH; MCHC within the reference interval; and oval macrocytes and hypersegmented neutrophils observed on the peripheral blood film. Additional abnormal laboratory test findings may include elevated levels of total and indirect serum bilirubin and lactate dehydrogenase due to the intramedullary hemolysis of megaloblastic erythroid precursors.
- The bone marrow in megaloblastic anemia is hyperplastic with increased erythropoiesis; however, it is ineffective due to increased apoptosis of developing cells. RBC precursors show nuclear-cytoplasmic asynchrony, with the nuclear maturation lagging behind the cytoplasmic maturation. Giant metamyelocytes and bands are evident.
- The cause of megaloblastic anemia is determined using specific immunoassays for serum folate and vitamin B₁₂. Immunoassays for antibodies to intrinsic factor and parietal cells can aid in the diagnosis of pernicious anemia. Additional tests for gastrointestinal disease or parasites may be needed.
- Treatment of megaloblastic anemia is directed at correcting the cause of the deficiency and supplementing the missing vitamin.
- For pernicious anemia, lifelong supplementation with vitamin B₁₂ is necessary.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Which of the following findings is consistent with a diagnosis of megaloblastic anemia?
   a. Hyposegmentation of neutrophils
   b. Decreased serum lactate dehydrogenase level
   c. Absolute increase in reticulocytes
   d. Increased MCV

2. A patient has a clinical picture of megaloblastic anemia. The serum folate level is decreased, and the serum vitamin B₁₂ level is 600 pg/mL (reference interval is 200–900 pg/mL). What is the expected value for the methylmalononic acid assay?
   a. Increased
   b. Decreased
   c. Within the reference interval
3. Which one of the following statements characterizes the relationships among macrocytic anemia, megaloblastic anemia, and pernicious anemia?
   a. Macrocytic anemias are megaloblastic.
   b. Macrocytic anemia is pernicious anemia.
   c. Megaloblastic anemia is macrocytic.
   d. Megaloblastic anemia is pernicious anemia.

4. Which of the following CBC findings is most suggestive of a megaloblastic anemia?
   a. MCV of 103 fl
   b. Hypersegmentation of neutrophils
   c. RDW of 16%
   d. Hemoglobin concentration of 9.1 g/dL

5. In the following description of a bone marrow smear, find the statement that is inconsistent with the expected picture in megaloblastic anemia.
   "The marrow appears hypercellular with a myeloid-to-erythroid ratio of 1:1 due to prominent erythroid hyperplasia. Megakaryocytes appear normal in number and appearance. The WBC elements appear larger than normal, with especially large metamyelocytes, although they otherwise appear morphologically normal. The RBC precursors also appear large. There is nuclear-cytoplasmic asynchrony, with the nucleus appearing more mature than expected for the color of the cytoplasm."d.
   a. Erythroid nuclei that are more mature than cytoplasm
   b. Larger than normal WBC elements
   c. Larger than normal RBCs
   d. Normal appearance of megakaryocytes

6. Which one of the following findings would be inconsistent with elevated titers of intrinsic factor blocking antibodies?
   a. Hypersegmentation of neutrophils
   b. Low levels of methylmalonic acid
   c. Macrocytic RBCs
   d. Low levels of vitamin B₁₂

7. Which of the following is the most metabolically active form of absorbed vitamin B₁₂?
   a. Transcobalamin
   b. Intrinsic factor–vitamin B₁₂ complex
   c. Holotranscobalamin
   d. Haptocorrin–vitamin B₁₂ complex

8. Folate and vitamin B₁₂ work together in the production of:
   a. Amino acids
   b. RNA
   c. Phospholipids
   d. DNA

9. The macrocytosis associated with megaloblastic anemia results from:
   a. Reduced numbers of cell divisions with normal cytoplasmic development
   b. Activation of a gene that is typically active only in megakaryocytes
   c. Reduced concentration of hemoglobin in the cells so that larger cells are needed to provide the same oxygen-carrying capacity
   d. Increased production of reticulocytes in an attempt to compensate for the anemia

10. Which one of the following groups has the highest risk for pernicious anemia?
    a. Malnourished infants
    b. Children during growth periods
    c. Persons older than 60 years of age
    d. Pregnant women

REFERENCES

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 16-year-old female presented to her pediatrician with jaundice. Her pediatrician checked liver enzyme and bilirubin levels, which were elevated. Hepatitis A, B, and C serologies were all negative. She was referred to a gastroenterologist, who diagnosed her with autoimmune hepatitis. With immunomodulatory treatment, her hepatitis improved. However, over the next several months, she noticed increasing fatigue and bruising. She also developed heavier menses, with menstrual cycles lasting up to 2 weeks in duration. Physical examination revealed pallor and scattered ecchymoses with petechiae on her chest and shoulders with no other abnormalities. Complete blood count results were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (×10⁹/L)</td>
<td>2.0</td>
<td>4.5–11.0</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>7.9</td>
<td>12.0–15.0</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>104</td>
<td>80–100</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>15</td>
<td>150–450</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.6</td>
<td>0.5–2.5</td>
</tr>
<tr>
<td>Reticulocytes (×10⁹/L)</td>
<td>16</td>
<td>20–115</td>
</tr>
<tr>
<td>Neutrophils (×10⁹/L)</td>
<td>0.5</td>
<td>2.3–8.1</td>
</tr>
<tr>
<td>Lymphocytes (×10⁹/L)</td>
<td>0.4</td>
<td>0.8–4.8</td>
</tr>
</tbody>
</table>

Serum vitamin B₁₂ and folate levels were within reference intervals. Bone marrow aspirate revealed mild dyserythropoiesis but normal myelopoiesis and megakaryopoiesis. Iron stain revealed normal stores. A bone marrow biopsy specimen was moderately hypocellular (15%) with a reduction in all three cell lines. There was no increase in reticulin or blasts. Cytogenetic testing revealed a normal karyotype, and results of flow cytometry for paroxysmal nocturnal hemoglobinuria (PNH) cells was negative.

1. What term is used to describe a decrease in all cell lines in the peripheral blood?
2. Which anemia of bone marrow failure should be considered?
3. How would an increase in either reticulin or blasts alter the preliminary diagnosis?
4. How would the severity of this patient’s condition be classified?
5. What treatment modality would be considered for this patient?

*The authors acknowledge the contributions of Elaine M. Keohane, author of this chapter in the previous edition*
Bone marrow failure is the reduction or cessation of blood cell production affecting one or more cell lines. Pancytopenia—or decreased numbers of circulating red blood cells (RBCs), white blood cells (WBCs), and platelets—is seen in most cases of bone marrow failure, particularly in severe or advanced stages.

The pathophysiology of bone marrow failure includes (1) the destruction of hematopoietic stem cells due to injury by drugs, chemicals, radiation, viruses, or autoimmune mechanisms; (2) premature senescence and apoptosis of hematopoietic stem cells due to genetic mutations; (3) ineffective hematopoiesis due to stem cell mutations or vitamin B₁₂ or folate deficiency; (4) disruption of the bone marrow microenvironment that supports hematopoiesis; (5) decreased production of hematopoietic growth factors or related hormones; and (6) the loss of normal hematopoietic tissue due to infiltration of the marrow space with abnormal cells.

The clinical consequences of bone marrow failure vary, depending on the extent and duration of the cytopenias. Severe pancytopenia can be rapidly fatal if untreated. Some patients may initially be asymptomatic, and their cytopenia may be detected during a routine blood examination. Thrombocytopenia can result in bleeding and increased bruising. Decreased RBCs and hemoglobin can result in fatigue, pallor, and cardiovascular complications. Sustained neutropenia increases the risk of life-threatening bacterial or fungal infections.

This chapter focuses on aplastic anemia, a bone marrow failure syndrome resulting from damaged or defective stem cells (mechanisms 1 and 2 listed earlier). Bone marrow failure resulting from other mechanisms may present similarly to aplastic anemia, and differentiation is discussed later. Because there are many mechanisms involved in the various bone marrow failure syndromes, accurate diagnosis is essential to ensure appropriate treatment.

### Aplastic Anemia

Aplastic anemia is a rare but potentially fatal bone marrow failure syndrome. In 1888, Ehrlich provided the first case report of aplastic anemia involving a patient with severe anemia, neutopenia, and a hypocellular marrow on postmortem examination.¹ The name *aplastic anemia* was given to the disease by Vaquez and Aubertin in 1904.² The characteristic features of aplastic anemia include pancytopenia, reticulocytopenia, bone marrow hypocellularity, and depletion of hematopoietic stem cells (Box 22-1). Approximately 80% to 85% of aplastic anemia cases are acquired, whereas 15% to 20% are inherited.³ Box 22-2 provides etiologic classifications.⁴,⁵

#### Acquired Aplastic Anemia

Acquired aplastic anemia is classified into two major categories: idiopathic and secondary. Idiopathic acquired aplastic anemia has no known cause. Secondary acquired aplastic anemia is associated with an identified cause. Approximately 70% of all aplastic anemia cases are idiopathic, whereas 10% to 15% are secondary.⁴ Idiopathic and secondary acquired aplastic anemia have similar clinical and laboratory findings. Patients may initially present with macrocytic or normocytic anemia and reticulocytopenia. Pancytopenia may develop slowly or progress at a rapid rate, with complete cessation of hematopoiesis.

**Incidence**

In North America and Europe, the annual incidence is approximately 1 in 500,000.⁶ In Asia and East Asia, the incidence is two to three times higher than in North America or Europe, which may be due to environmental and/or genetic differences.⁷ Aplastic anemia can occur at any age, with peak incidence at 15 to 25 years and the second highest frequency at greater than 60 years.⁴,⁶ There is no gender predisposition.⁶
**Etiology**

As the name indicates, the cause of idiopathic aplastic anemia is unknown. Secondary aplastic anemia is associated with exposure to certain drugs, chemicals, radiation, or infections. Cytotoxic drugs, radiation, and benzenes are responsible for 10% of secondary aplastic anemia cases and suppress the bone marrow in a predictable, dose-dependent manner. Depending on the dose and exposure duration, the bone marrow generally recovers after withdrawal of the agent. Alternatively, approximately 70% of cases of secondary aplastic anemia occur due to idiosyncratic reactions to drugs or chemicals. In idiosyncratic reactions, the bone marrow failure is unpredictable and unrelated to dose. Documentation of a responsible factor or agent in these cases is difficult, because evidence is primarily circumstantial and symptoms may occur months or years after exposure. Some drugs associated with idiosyncratic secondary aplastic anemia are listed in Box 22-3.

Generally, idiosyncratic secondary aplastic anemia is a rare event and is likely due to a combination of genetic and environmental factors in susceptible individuals. Currently, there are no readily available tests that predict individual susceptibility to these idiosyncratic reactions. However, genetic variations in immune response pathways or metabolic enzymes may play a role. There is an approximately twofold higher incidence of HLA-DR2 and its major serologic split, HLA-DR15, in aplastic anemia patients compared to the general population, but the relationship of this finding to disease pathophysiology has not been elucidated. There are also reports that genetic polymorphisms in enzymes that metabolize benzene increase susceptibility to toxicity, even at low exposure levels. These include polymorphisms in glutathione S-transferase (GST) enzymes (GSTT1 and GSTM1), myeloperoxidase, nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), quinine oxidoreductase 1, and cytochrome oxidase P450 2E1. A deficiency in GST due to the GSTT1 null genotype is overrepresented in whites, Hispanics, and Asians with aplastic anemia, with a frequency of 30%, 28%, and 75%, respectively. White patients with aplastic anemia also have a higher frequency (22%) of the GSTM1/GSTT1 null genotype than the general population. GST is important for metabolism and neutralization of chemical toxins, and deficiencies of this enzyme may increase the risk of aplastic anemia. Further study is required to assess how these genetic variations, and other yet undiscovered factors, contribute to aplastic anemia.

Acquired aplastic anemia occurs occasionally as a complication of infection with Epstein-Barr virus, human immunodeficiency virus (HIV), hepatitis virus, and human parvovirus B19. A history of acute non-A, non-B, or non-C hepatitis 1 to 3 months before the onset of pancytopenia is found in 2% to 10% of patients with acquired aplastic anemia. The acquired aplastic anemia in these cases may be mediated by such mechanisms as interferon gamma and cytokine release.

Aplastic anemia associated with pregnancy is a rare occurrence, with fewer than 100 cases reported in the literature.

**BOX 22-3 Selected Drugs Reported to Have a Rare Association with Idiosyncratic Secondary Aplastic Anemia**

**Antiarthritics**
- Gold compounds
- Penicillamine

**Antibiotics**
- Chloramphenicol
- Sulfonamides

**Anticonvulsants**
- Carbamazepine
- Hydantoins
- Phenacemide

**Antidepressants**
- Dothiepin
- Phenothiazine

**Antidiabetic Agents**
- Chlorpropamide
- Tolbutamide
- Carbutamide

**Anti-inflammatories (nonsteroidal)**
- Diclofenac
- Fenbufen
- Fenoprofen
- Ibuprofen
- Indomethacin
- Naproxen
- Phenylbutazone
- Piroxicam
- Sulindac

**Antiprotozoals**
- Chloroquine
- Quinacrine

**Antithyroidals**
- Methimazole
- Methylthiouracil

**Carbonic Anhydrase Inhibitors**
- Methazolamide
- Mesalazine
- Acetazolamide

Approximately 10% of individuals with acquired aplastic anemia have a concomitant autoimmune disease and approximately 10% develop hemolytic or thrombotic manifestations of paroxysmal nocturnal hemoglobinuria (PNH). The overlap between acquired aplastic anemia and PNH is discussed later.
Pathophysiology

The primary lesion in acquired aplastic anemia is a quantitative and qualitative deficiency of hematopoietic stem cells. Stem cells of patients with acquired aplastic anemia have diminished colony formation in methylcellulose cultures. The hematopoietic stem and early progenitor cell compartment is identified by expression of CD34 surface antigens. The CD34⁺ cell population in the bone marrow of patients with acquired aplastic anemia can be 10% or lower than that seen in healthy individuals. In addition, these CD34⁺ cells have increased expression of Fas receptors that mediate apoptosis and increased expression of apoptosis-related genes.

The bone marrow stromal cells are functionally normal in acquired aplastic anemia. They produce normal or even increased quantities of growth factors and are able to support the growth of CD34⁺ cells from healthy donors in culture and in vivo after transplantation. Individuals with aplastic anemia also have elevated serum levels of erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, serum levels of FLT3 ligand, a growth factor that stimulates proliferation of stem and progenitor cells, is up to 200 times higher in patients with severe aplastic anemia compared to healthy controls. However, despite their elevated levels, growth factors are generally unsuccessful in correcting the cytopenias found in acquired aplastic anemia.

The severe depletion of hematopoietic stem and progenitor cells from the bone marrow may be due to direct damage to stem cells, immune damage to stem cells, or other unknown mechanisms. Direct damage to stem and progenitor cells results from DNA injury following exposure to cytotoxic drugs, chemicals, radiation, or viruses.

Immune damage to stem cells results from exposure to drugs, chemicals, viruses, or other agents that cause an autoimmune cytotoxic T-lymphocytic destruction of stem and progenitor cells. An autoimmune pathophysiology was first suggested in the 1970s when aplastic anemia patients undergoing pretransplant immunosuppressive conditioning had an improvement in cell counts. Further evidence supporting an autoimmune pathophysiology include (1) elevated blood and bone marrow cytokotic (CD8⁺) T lymphocytes with an oligoclonal expansion of specific T-cell clones; (2) increased T cell production of such cytokines as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which inhibit hematopoiesis and induce apoptosis; (3) upregulation of T-bet, a transcription factor that binds to the promoter of the IFN-γ gene; (4) increased TNF-α receptors on CD34⁺ cells; and (5) improvement in cytopenias after immunosuppressive therapy (IST). Approximately two thirds of patients with acquired aplastic anemia respond to IST. The nonresponders may have a severely depleted stem cell compartment or other pathophysiologic factors contributing to their cytopenias.

Possible autoimmune mechanisms include mutation of stem cell antigens and disruption of immune regulation. Young and co-workers showed that environmental exposures may alter self-proteins, induce expression of abnormal or novel antigens, or induce an immune response that cross-reacts with self-antigens. Solomou and co-workers demonstrated that CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells are decreased in aplastic anemia. These regulatory T cells normally suppress autoreactive T cells, and a deficit of these cells may facilitate an autoimmune reaction. Furthermore, a number of individuals with aplastic anemia have single nucleotide polymorphisms in IFN-γ/874 TT, TNF-α/–308 AA, transforming growth factor-β1/–509 TT, and interleukin-6/–174 GG. These polymorphisms result in cytokine overproduction and may impart a genetic susceptibility to aplastic anemia as well as contribute to its severity.

The specific antigens responsible for triggering and sustaining the autoimmune attack on stem cells are unknown. Candidate antigens have been identified from aplastic anemia patient sera, including kinectin, diazepam-binding inhibitor-related protein, and moesin. These proteins are expressed in hematopoietic progenitor cells, but their role in the pathogenesis of aplastic anemia requires further investigation.

Approximately one third of patients with acquired aplastic anemia have shortened telomeres in their peripheral blood granulocytes compared with age-matched controls. Telomeres protect the ends of chromosomes from damage and erosion, and cells with abnormally short telomeres undergo proliferation arrest and premature apoptosis. Telomerase is an enzyme complex that repairs and maintains the telomeres. Approximately 10% of patients with acquired aplastic anemia and shortened telomeres have a mutation in the telomerase complex gene for either the ribonucleic acid (RNA) template (TERC) or the reverse transcriptase (TERT). The cause for shortened telomeres in the other 90% of patients may be due to stress hematopoiesis or other yet unidentified mutations. In stress hematopoiesis, there is an increase in progenitor cell turnover, and the telomeres become shorter with each cell division.

Approximately 4% of patients with acquired aplastic anemia and shortened telomeres have mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene. The SBDS gene product is involved in ribosome biogenesis, and its relationship to telomere maintenance is currently unknown. The cause for shortened telomeres in the other 90% of patients may be due to stress hematopoiesis or other yet unidentified mutations. In stress hematopoiesis, there is an increase in progenitor cell turnover, and the telomeres become shorter with each cell division.

Correct differentiation between acquired and inherited aplastic anemia has important implications for appropriate treatment and prognosis. Immunosuppressive therapy is not nearly as effective in inherited aplastic anemia as it is in acquired aplastic anemia. Furthermore, hematopoietic stem cell transplantation (HSCT), the only known curative treatment for DKC and SBDS and a treatment option for acquired aplastic anemia, should not be performed with human leukocyte antigen (HLA)-matched siblings who test positive for the same genetic mutation.
immunosuppressive therapy. Defective telomere maintenance may be another pathophysiologic mechanism of stem cell injury, imparting susceptibility to aplastic anemia after an environmental insult.

**Clinical Findings**

Symptoms vary in acquired aplastic anemia, ranging from asymptomatic to severe. Patients usually present with symptoms of insidious-onset anemia, with pallor, fatigue, and weakness. Severe and prolonged anemia can result in serious cardiovascular complications, including tachycardia, hypotension, cardiac failure, and death. Symptoms of thrombocytopenia are also varied and include petechiae, bruising, epistaxis, mucosal bleeding, menorrhagia, retinal hemorrhages, intestinal bleeding, and intracranial hemorrhage. Fever and bacterial or fungal infections are unusual at initial presentation but may occur after prolonged periods of neutropenia. Splenomegaly and hepatomegaly are typically absent.

**Laboratory Findings**

Pancytopenia is typical, although initially only one or two cell lines may be decreased. The absolute neutrophil count is decreased, and the absolute lymphocyte count may be normal or decreased. The hemoglobin is usually less than 10 g/dL, the mean cell volume (MCV) is increased or normal, and the percent and absolute reticulocyte counts are decreased. Table 22-1 lists the diagnostic criteria for aplastic anemia by degree of severity.

Neutrophils, monocytes, and platelets are decreased in the peripheral blood, and the red blood cells are macrocytic or normocytic (Figure 22-1). Toxic granulation may be observed in the neutrophils, but the RBCs and platelets are usually normal in appearance. Leukemic blasts and other immature blood cells are characteristically absent. The serum iron level and percent transferrin saturation are increased, which reflects decreased iron use for erythropoiesis. Liver function test results may be abnormal in cases of hepatitis-associated aplastic anemia.

Approximately two thirds of patients have small numbers (less than 25%) of PNH clones in the peripheral blood, but only 10% of patients develop a sufficient number of PNH cells to have the clinical and biochemical manifestations of PNH disease. PNH is characterized by an acquired stem cell mutation resulting in lack of the glycosylphosphatidylinositol (GPI)-linked proteins CD55 and CD59. The absence of CD55 and CD59 on the surface of the RBCs renders them more susceptible to complement-mediated cell lysis. It is important to test for PNH in acquired aplastic anemia because of the increased risk of hemolytic and/or thrombotic complications (Chapter 24). Historically, PNH diagnosis depended on the Ham acid hemolysis test: patients’ cells were placed in acidified serum, and a positive result demonstrated lysis of RBCs. However, this test was poorly sensitive, because complement-mediated hemolysis was detected only in the presence of large numbers of circulating PNH cells. Currently, flow cytometric analysis for CD59 on RBCs and CD24 and CD14 on granulocytes and monocytes is used as a more sensitive diagnostic method and has replaced the Ham test in nearly all laboratories.

Bone marrow aspirate and biopsy specimens have prominent fat cells with areas of patchy marrow cellularity. Biopsy samples are required for accurate quantitative assessment of marrow cellularity, and severe hypocellularity is a characteristic feature of aplastic anemia (Figure 22-2). Erythroid, granulocytic, and megakaryocytic cells are decreased or absent. Dyserythropoiesis may be present, but there is typically no dysplasia of the granulocyte or platelet cell lines. Blasts and other abnormal cell infiltrates are characteristically absent. Reticulin staining is usually normal.

In patients receiving immunosuppressive therapy, the risk of developing an abnormal karyotype is 14% at 5 years and

<table>
<thead>
<tr>
<th>TABLE 22-1 Diagnostic Criteria for Aplastic Anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAA</strong></td>
</tr>
<tr>
<td>Bone marrow</td>
</tr>
<tr>
<td>Hypocellular bone marrow plus at least two of the following:</td>
</tr>
<tr>
<td>Neutrophils (×10^9/L)</td>
</tr>
<tr>
<td>Platelets (×10^9/L)</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

HGB, Hemoglobin; HCT, hematocrit; MAA, moderate aplastic anemia; SAA, severe aplastic anemia; VSAA, very severe aplastic anemia; g, grams; dL, deciliter; L, liter.

*Or 25% to 50% cellularity with <30% residual hematopoietic cells.
20% at 10 years. Cytogenetic analysis using conventional culture techniques often underestimates the incidence of karyotype abnormalities because of bone marrow hypocellularity and scarcity of cells in metaphase. Alternatively, interphase fluorescence in situ hybridization (FISH) using deoxyribonucleic acid (DNA) probes for specific chromosome abnormalities may be used. In comparison to conventional cytogenetic analysis, FISH has greater sensitivity in the detection of chromosome abnormalities and can also be performed using nondividing cells. In a study performed by Kearns and colleagues, FISH detected monosomy 7 or trisomy 8 in 26% of aplastic anemia patients who had a normal karyotype by conventional cytogenetic testing.

Patients with inherited aplastic anemia may be misdiagnosed with acquired aplastic anemia if symptoms manifest in late adolescence or adulthood or if the patients lack the typical clinical and physical characteristics of an inherited marrow failure syndrome (e.g., abnormal thumbs, short stature). Consideration of inherited aplastic anemia syndromes in the differential diagnosis of acquired aplastic anemia is essential, because these conditions require a different therapeutic approach. The inherited aplastic anemia syndromes are discussed later in the chapter.

**Treatment and Prognosis**

Severe acquired aplastic anemia requires immediate attention to prevent serious complications. If a causative agent is identified, its use should be discontinued. Blood product replacement should be given judiciously to avoid alloimmunization. Platelets should not be transfused at levels greater than 10,000/µL, unless the patient is bleeding.

One of the most important early decisions is determining whether the patient is a candidate for hematopoietic stem cell transplantation (HSCT). HSCT is the treatment of choice for patients with severe aplastic anemia who are younger than 40 years of age and have a human leukocyte antigen (HLA)-identical sibling. Unfortunately, only 20% to 30% of patients meet these criteria. Therefore, IST, consisting of antithymocyte globulin and cyclosporine, is used for patients older than 40 years of age and for patients without an HLA-identical sibling. Antithymocyte globulin decreases the number of activated T cells, and cyclosporine inhibits T-cell function, thereby suppressing the autoimmune reaction against the stem cells. Approximately two thirds of patients initially respond to IST; unfortunately, 30% to 40% relapse. For patients with severe acquired aplastic anemia who are not responsive to IST, a second course of IST or an HSCT from an HLA-matched unrelated donor is an option, but survival is not as high as with HSCT from an HLA-identical sibling. The response rate for a second course of IST is approximately 65% for those who experienced relapse and 30% for those whose disorder was initially refractive to IST. Individuals with PNH cells (CD55−CD59−) are almost twice as likely to respond to IST than are those who lack these cells. In addition, the presence of both PNH cells and HLA-DR2 increases the likelihood of response by 3.5-fold. Granulocyte colony-stimulating factor (G-CSF), other hematopoietic growth factors, and steroids do not increase overall survival or improve the response rate; therefore, they are not recommended for routine use.

Other supportive therapy includes antibiotic and antifungal prophylaxis in cases of prolonged neutropenia. Patients with mild to moderate aplastic anemia may not require treatment but must be monitored periodically for pancytopenia and abnormal cells.

The overall outcome for patients with acquired aplastic anemia has dramatically improved in the past 2 decades. Among patients who receive an HSCT from an HLA-identical sibling, 91% of children and 74% of adults achieve 10-year overall survival. Those percentages decrease slightly to 75% of children and 63% of adults when the bone marrow transplant is from an HLA-matched unrelated donor. In patients treated with IST, 75% of children and 63% of adults achieve 10-year survival. Additional outcomes in the IST-treated patients include a 10-year risk of developing hemolytic or thrombotic PNH and a 10% to 20% risk of myelodysplastic syndrome (MDS) or leukemia. Development of monosomy 7 predicts poor outcome, with a greater likelihood...
of unresponsiveness to IST and progression to MDS or leukemia.56

Inherited Aplastic Anemia
In comparison with acquired aplastic anemia, patients with inherited aplastic anemia present at an earlier age and may have characteristic physical stigmata. The three inherited diseases for which bone marrow failure and pancytopenia are a consistent feature are Fanconi anemia, dyskeratosis congenita, and Shwachman-Bodian-Diamond syndrome.

Fanconi Anemia
Fanconi anemia (FA) is a chromosome instability disorder characterized by aplastic anemia, physical abnormalities, and cancer susceptibility. In 1927, Dr. Guido Fanconi first described this syndrome in three brothers with skin pigmentation, short stature, and hypogonadism.57 FA has a prevalence of 1 to 5 cases per million.56 The carrier rate is 1 in 300 in the United States and Europe, with a threefold higher prevalence in Ashkenazi Jews and South African Africans.37 FA is the most common of the inherited aplastic anemias.

Clinical Findings. Patients with FA have variable features and symptoms. Physical malformations may be present at birth, though hematologic abnormalities may not appear until older childhood or adulthood. Furthermore, only two thirds of patients have physical malformations.3,5,8 These anomalies vary considerably, though there is a higher frequency of skeletal abnormalities (thumb malformations, radial hypoplasia, microcephaly, hip dislocation, and scoliosis); skin pigmentation (hyperpigmentation, hypopigmentation, café-au-lait plaques, microcephaly, hip dislocation, and scoliosis); skin pigmentation (hyperpigmentation, hypopigmentation, café-au-lait lesions); short stature; and abnormalities of the eyes, kidneys, and genitals.56-58 Low birth weight and developmental delay are also common.

The symptoms associated with pancytopenia usually become apparent at 5 to 10 years of age, though some patients may not present until adulthood.3,5,7 Individuals with FA also have an increased cancer risk. This includes an increased incidence of leukemia in childhood and solid tumors (e.g., oral, esophageal, anogenital, cervical) in adulthood.59 In approximately 5% of cases, a malignancy is diagnosed before the FA is recognized.59

Genetics and Pathophysiology. There are currently 15 reported genes associated with FA: FANCA, FANCB, FANCC, FANCD1 (also called BRCA2), FANCD2, FANCE, FANCF, FANCG (also called XRCC9), FANCI, FANCJ (also called BRIP1/BACH1), FANCL, FANCM, FANCN (also called PALB2), FANCO (also called RAD51C), and FANCP (also called SLX4).56 Patients with FA typically have biallelic mutations or deletions in one of these genes. The mode of inheritance is autosomal recessive except for FANCB, which is X-linked recessive. Mutations in the FANCA gene occur with the highest frequency.3,5,8 The relationship between mutations in the FA genes and disease pathology is not clear. Cells are highly susceptible to chromosome breakage after exposure to DNA cross-linking agents. FA cells may also have accelerated telomere shortening and apoptosis, a late S-phase cell cycle delay, hypersensitivity to oxidants, and cytokine dysregulation.3,5,6,5,8

The range of FA protein function is not completely known, but these proteins participate in a highly elaborate DNA damage response pathway. The FA pathway consists of a nuclear core complex, a protein ID complex, and effector proteins.58,60 The FA proteins A, B, C, E, F, G, L, and M form the nuclear core complex; proteins D2 and I form the ID complex; and the effector proteins are D1, J, N, O, and P.56,58,60 The core complex facilitates the monoubiquitylation and activation of the ID complex. The ID complex then localizes with effector DNA repair proteins at foci of DNA damage to effect DNA repair.58,60

Laboratory Findings. Laboratory results are similar to those in acquired aplastic anemia, with pancytopenia, reticulocytopenia, and a hypocellular bone marrow. Macrocytic RBCs are often the first detected abnormality, and thrombocytopenia usually precedes the development of the other cytopenias.56 Fetal hemoglobin (Hb F) may be strikingly elevated, and α-fetoprotein is also increased.56

Chromosomal breakage analysis is the diagnostic test for Fanconi anemia.56 Patients’ peripheral blood lymphocytes are cultured with the DNA cross-linking agents diepoxybutane (DEB) or mitomycin C (MMC). Compared to normal lymphocytes, FA cells have a greater number of characteristic chromosome breaks and ring chromosomes, indicating increased fragility.3,56 Caution must be made in interpreting peripheral blood results, because they may be negative in the 10% to 15% of FA patients who have somatic mosaicism due to a reversion of one abnormal allele to the normal type.5,66 To confirm the diagnosis in these cases, chromosome breakage studies can be performed on cultured skin fibroblasts from a skin biopsy specimen.56,61

Treatment and Prognosis. More than 90% of FA patients develop bone marrow failure by 40 years of age.59 Furthermore, one third of patients develop MDS and/or acute myeloid leukemia (AML) by a median age of 14 years, and 25% develop solid tumors by a median age of 26 years.59,62 Squamous cell carcinomas of the head and neck, anogenital region, and skin are the most common solid tumors, followed by tumors of the liver, brain, and kidney.55 Patients with FA have an increased risk of developing vulvar carcinoma (4300-fold), esophageal cancer (2300-fold), AML (800-fold), and head/neck cancer (700-fold) compared with the general population.62 Approximately 3% of patients develop more than one type of malignancy.62 Left untreated, death by 20 years of age secondary to bone marrow failure or malignancy is common. Patients with mutations in the FANCC gene experience bone marrow failure at a particularly young age and have the poorest survival.62 Increased telomere shortening in FA cells is associated with more severe pancytopenia and a higher risk of malignancy. However, the precise role of telomere shortening in the evolution of bone marrow failure and cancer is currently unclear.64
Supportive treatment for cytopenia includes transfusions and administration of cytokines (G-CSF and GM-CSF).36,57 The only curative treatment is HSCT, preferably from an HLA-identical sibling. It is important to screen donor siblings for FA prior to transplant. Patients should also have decreased intensity pretreatment conditioning because of their underlying chromosomal instability.56,62 Gene therapy has been attempted in clinical trials but has not been successful.

**Dyskeratosis Congenita**

Dyskeratosis congenita (DKC) is a rare inherited bone marrow failure syndrome with fewer than 600 known cases worldwide.56,65

**Clinical Findings.** DKC is characterized by mucocutaneous abnormalities, bone marrow failure, and pancytopenia. The typical clinical presentation involves a triad of abnormal skin pigmentation, dystrophic nails, and oral leukoplakia. Skin and nail findings usually appear before 10 years of age.3,56 Median age of diagnosis is 15 years.66 By 30 years of age, 80% to 90% of patients have bone marrow abnormalities.3 Patients can also manifest a wide range of multisystem abnormalities, including pulmonary fibrosis, liver disease, developmental delay, short stature, microcephaly, prematurely gray hair or hair loss, immunodeficiency, dental caries, and periodontal disease.66 Patients have a 40% risk of cancer by 50 years of age, most commonly AML, MDS, and epithelial malignancies.55

**Genetics and Pathophysiology.** DKC chromosomes have very short telomeres, and inherited defects in the telomerase complex are implicated in the pathophysiology.56 The telomerase complex synthesizes telomere repeats to elongate chromosome ends, maintaining the telomere length needed for cell survival.

There are currently eight different genes implicated in DKC, and it can be inherited in three different patterns: X-linked recessive, autosomal dominant, and autosomal recessive.3,56,66 The best-characterized form results from one or more mutations on the long arm of the X-chromosome on the **DKC1** gene dyskerin. Dyskerin is a ribonucleoprotein involved in RNA processing, and it associates with TERC (telomerase RNA component) in the telomerase complex. The autosomal dominant form is due to mutations in the genes that encode TERC, TERT (telomerase enzyme), or TINF2 (component of the shelterin complex that regulates telomere length).66 In the autosomal recessive form, mutations in TERT, NHP2, NOP10, WRAP53, and CTC1 have been identified.66 The proteins encoded by these genes are also involved in telomere maintenance. Although the exact pathophysiologic mechanisms are still unknown, the shortened telomeres in DKC cause premature death in the rapidly dividing cells in the bone marrow and epithelium and likely lead to genomic instability and a predisposition to cancer.1,3,56,67

**Laboratory Findings.** Pancytopenia and macrocytic RBCs are typical peripheral blood findings. The fetal hemoglobin level may also be increased. Only about 40% of patients have an identified mutation in one of the eight known telomerase complex genes.67 A new flow fluorescence in situ hybridization (FISH) test for detection of very short telomeres in WBC subsets has been proposed as a diagnostic test for those with suspected DKC who lack mutations in known genes.67 Patients with FA, SBDS, and acquired aplastic anemia may also have cells with shortened telomeres, though they are not found in multiple WBC subsets.67 In contrast, DKC cells often have shortened telomeres in several WBC subsets, including naïve T cells and B cells.

**Treatment and Prognosis.** Median survival for patients with DKC is 42 years.55 Approximately 60% to 70% of deaths are due to bone marrow failure complications. Ten percent to 15% of deaths result from severe pulmonary disease, and 10% of deaths result from malignancies.3,56 Treatment with bone marrow transplantation has not been optimal because of the high incidence of fatal pulmonary fibrosis and vascular complications.56,66 Although androgen therapy produces a transient response in 50% to 70% of patients, it does not halt the progression of the bone marrow failure.3

**Shwachman-Bodian-Diamond Syndrome**

Shwachman-Bodian-Diamond syndrome (SBDS) is an inherited multisystem disorder characterized by pancreatic insufficiency, cytopenia, skeletal abnormalities, and a predisposition for hematologic malignancies. The incidence has been estimated to be approximately 8.5 cases per 1 million live births.56

**Clinical Findings.** Patients with SBDS have peripheral blood cytopenia and decreased pancreatic enzyme secretion.45 The pancreatic insufficiency causes gastrointestinal malabsorption, which typically presents in early infancy.3 Patients have neutropenia and immune dysfunction and are at increased risk of severe infections and sepsis.45,68 Nearly all SBDS patients have delayed bone maturation, and approximately 50% have failure to thrive and short stature.45,69

**Genetics and Pathophysiology.** SBDS is an autosomal recessive disorder, and 90% of patients have biallelic mutations in the **SBDS** gene.3,45,68 The **SBDS** gene is involved in ribosome metabolism and mitotic spindle stability,70 but its relationship to the disease manifestations is currently unknown. There are quantitative and qualitative deficiencies in CD34+ cells, dysfunctional bone marrow stromal cells, increased apoptosis and mitotic spindle destabilization in hematopoietic cells, and short telomeres in peripheral blood granulocyes.3,45,68,70

**Laboratory Findings.** Nearly all patients with SBDS have neutropenia (less than 1.5 \times 10^9 neutrophils/L).71 Half of the patients also develop anemia or thrombocytopenia, and one fourth develop pancytopenia.71 The RBCs are usually normocytic but can be macrocytic, and approximately two thirds of patients have elevated Hb F.45,71 The bone marrow is
usually hypocellular but can be normal or even hypercellular. Due to the pancreatic insufficiency, 72-hour fecal fat testing shows increased fat excretion, and serum trypsinogen and isoamylase levels are decreased compared with age-related reference intervals. In comparison to cystic fibrosis, which can have a similar malabsorption presentation, patients with SBDS have normal sweat chloride tests. Testing for the SBDS gene mutation is commercially available and should be done in suspected patients and their parents.

**Treatment and Prognosis.** In some cases no treatment of hematologic features is required. However, if needed, treatment consists of G-CSF for neutropenia, transfusion support for anemia and thrombocytopenia, and enzyme replacement for pancreatic insufficiency. The risk of AML and MDS is approximately 19% at 20 years and 36% at 30 years. Allogeneic bone marrow transplantation is recommended in cases of severe pancytopenia, AML, or MDS. Unfortunately, despite supportive care and attempted curative therapy, 5-year overall survival is 60% to 65%, with many deaths occurring from severe infections and malignancy. Poor outcomes after HSCT occur due to graft failure, transplant-related toxicities, and recurrent leukemia.

**Differential Diagnosis**
A distinction must be made between acquired aplastic anemia, inherited aplastic anemia, and other causes of pancytopenia, including PNH, MDS, megaloblastic anemia, and leukemia. The importance of a correct diagnosis is clear, as diagnostic conclusions dictate therapeutic management and prognosis. The distinguishing features of these conditions are listed in Tables 22-2 and 22-3.

Alternative diagnoses include lymphoma, myelofibrosis, and mycobacterial infections, which also may present with pancytopenia. However, these diagnoses often can be distinguished with a careful history, physical exam, and laboratory testing. Review of a peripheral blood film by an experienced morphologist is important. If needed, bone marrow evaluation and molecular testing for chromosome abnormalities and gene mutations can further distinguish these diagnoses. Anorexia nervosa also may present with pancytopenia. In these cases, the bone marrow is hypocellular and has a decreased number of fat cells. The cytopenias revert with correction of the underlying disease.

**TABLE 22-2** Differentiation of Aplastic Anemia from Other Causes of Pancytopenia

<table>
<thead>
<tr>
<th>Condition</th>
<th>Peripheral Blood</th>
<th>Bone Marrow</th>
<th>Laboratory Test Results</th>
<th>Clinical Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Failure of Bone Marrow to Produce Blood Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>No immature WBCs or RBCs; ↓ reticulocytes; MCV ↑ or normal</td>
<td>Hypocellular; blasts and abnormal cells absent; reticulin normal; RBC dyspoiesis may be present; WBC and platelet dyspoiesis absent</td>
<td>Acquired: PNH cells* may be present; chromosome abnormalities may be present</td>
<td>Splenomegaly absent</td>
</tr>
<tr>
<td><strong>Increased Destruction of Blood Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNH</td>
<td>Reticulocytes ↑; MCV normal or ↑: nucleated RBCs present or absent</td>
<td>Erythroid hyperplasia; may be hypocellular</td>
<td>PNH cells* present; hemoglobinuria +/−; chromosome abnormalities may be present</td>
<td>Splenomegaly absent; thrombosis may be present</td>
</tr>
<tr>
<td><strong>Ineffective Hematopoiesis</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>Variable pancytopenia; reticulocytes ↓; MCV normal or ↑: blasts and abnormal WBCs, RBCs, and platelets may be present</td>
<td>Hypercellular; 20% of cases hypocellular; dyspoiesis in one or more cell lines present; blasts and immature cells present; reticulin ↑</td>
<td>Chromosome abnormalities usually present</td>
<td>Splenomegaly uncommon</td>
</tr>
<tr>
<td>Megaloblastic anemias</td>
<td>MCV ↑; oval macrocytes; hypersegmented neutrophils</td>
<td>Hypercellular with megaloblastic features</td>
<td>Serum vitamin B12 or folate or both ↓</td>
<td>Splenomegaly absent</td>
</tr>
<tr>
<td><strong>Bone Marrow Infiltration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>Blasts present</td>
<td>Hypercellular: blasts ↑; reticulin ↑</td>
<td>Chromosome abnormalities may be present</td>
<td>Splenomegaly may be present</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>Hairy cells present; monocytes ↓</td>
<td>Hairy cells and fibrosis present; reticulin ↑</td>
<td>Hairy cells* present; TRAP + (60–70% of cases)</td>
<td>Splenomegaly present</td>
</tr>
</tbody>
</table>

*Hairy cells are detected by flow cytometry by their lack of expression of CD59; PNH granulocytes and monocytes lack expression of CD24, CD16, and CD14 (Chapter 24).
### TABLE 22-3 Key Characteristics of Inherited/Congenital Bone Marrow Failure Anemias

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genetics</th>
<th>Peripheral Blood</th>
<th>Bone Marrow</th>
<th>Laboratory Test Results</th>
<th>Clinical Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Due to Bone Marrow Hypoplasia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>AR, XLR</td>
<td>Pancytopenia; reticulocytes ↓; MCV ↑</td>
<td>Hypocellular with ↓ in all cell lines</td>
<td>Chromosome breakage with DEB/MMC; Hb F may be ↑</td>
<td>Physical malformations may be present; risk of cancers, leukemia, myelodysplastic syndrome</td>
</tr>
<tr>
<td>DKC</td>
<td>AR, XLR, AD</td>
<td>Pancytopenia; reticulocytes ↓; MCV ↑</td>
<td>Hypocellular with ↓ in all cell lines</td>
<td>75% have mutations in TERC, TERT, DKC1, TINF2, NHP2, NOP10, WRAP53, or CTC1; Hb F may be ↑; very short telomeres in lymphocyte subsets</td>
<td>Physical malformations may be present; pulmonary disease; risk of cancers, leukemia, myelodysplastic syndrome</td>
</tr>
<tr>
<td>SBDS</td>
<td>AR</td>
<td>Neutropenia; pancytopenia (25% of cases); reticulocytes ↓; MCV normal or ↑</td>
<td>Hypocellular, normocellular, or hypercellular</td>
<td>90% have mutations in SBDS gene; serum trypsinogen and isoaamylase ↓ for age; Hb F may be ↑</td>
<td>Pancreatic insufficiency; physical malformations may be present; risk of infections, leukemia, myelodysplastic syndrome</td>
</tr>
<tr>
<td>DBA</td>
<td>AD (50% of cases)</td>
<td>Anemia; reticulocytes ↓; MCV ↑</td>
<td>Erythroid hypoplasia</td>
<td>Erythrocyte adenosine deaminase ↑; Hb F may be ↑; 25% have mutations in RPS19 gene; another 25% have mutations in RPS7, RPS10, RPS17, RPS24, RPS26, RPL5, RPL11, or RPL35A</td>
<td>Physical malformations may be present; risk of cancers, leukemia, myelodysplastic syndrome</td>
</tr>
<tr>
<td><strong>Due to Ineffective Hematopoiesis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDA I</td>
<td>AR</td>
<td>Anemia; reticulocytes ↓; MCV ↑; poik, baso stipp, Cabot rings</td>
<td>Hypercellular; RBC precursors megaloblastoid with inter-nuclear chromatin bridges and &lt;5% binucleated forms</td>
<td>Mutations in CDAN1 gene; spongy, “Swiss cheese” heterochromatin in erythroblasts by electron microscopy</td>
<td>Physical malformations may be present; iron overload; splenomegaly; hepatomegaly</td>
</tr>
<tr>
<td>CDA II</td>
<td>AR</td>
<td>Anemia; reticulocytes ↓; MCV normal; poik, baso stipp</td>
<td>Hypercellular; RBC precursors normoblastic with 10% to 35% binucleated forms</td>
<td>Mutations in SEC23B gene; positive Ham test result (rarely done)</td>
<td>Physical malformations may be present; iron overload; jaundice; gallstones; splenomegaly</td>
</tr>
<tr>
<td>CDA III</td>
<td>AD</td>
<td>Mild anemia; reticulocytes ↓; MCV ↑; poik, baso stipp</td>
<td>Hypercellular; RBC precursors megaloblastoid with giant multinucleated forms with up to 12 nuclei</td>
<td>Mutations in KIF23 gene</td>
<td>Treatment usually not needed</td>
</tr>
</tbody>
</table>

↑ Increased; ↓ decreased; AR, autosomal recessive; AD, autosomal dominant; XLR, X-linked recessive; baso stipp, basophilic stippling; CDA, congenital dyserythropoietic anemia; DBA, Diamond-Blackfan anemia; DKC, dyskeratosis congenita; DEB, diepoxybutane; FA, Fanconi anemia; MCV, mean cell volume; MMC, mitomycin C; poik, polikilocytosis; RBC, red blood cell; SBDS, Shwachman-Bodian-Diamond syndrome; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Hb F, fetal hemoglobin.

*Genes identified as of 2013; genetic discovery is ongoing.

### OTHER FORMS OF BONE MARROW FAILURE

**Pure Red Cell Aplasia**

Pure red cell aplasia (PRCA) is a rare disorder of erythropoiesis characterized by a selective and severe decrease in erythrocyte precursors in an otherwise normal bone marrow. Patients have severe anemia (usually normocytic), reticulocytopenia, and normal WBC and platelet counts. PRCA may be acquired or congenital. It is important to distinguish between acquired and congenital forms, as they require different therapeutic approaches.

**Acquired Pure Red Cell Aplasia**

Acquired PRCA may occur in children or adults and can be acute or chronic. Primary PRCA may be idiopathic or
autoimmune-related. Secondary PRCA may occur in association with an underlying thymoma, hematologic malignancy, solid tumor, infection, chronic hemolytic anemia, collagen vascular disease, or exposure to drugs or chemicals. Therapy is first directed at treatment of the underlying condition, but immunosuppressive therapy may be considered if the PRCA is not responsive. Cyclosporine is associated with a higher response rate (65% to 87%) than corticosteroids (30% to 62%) and is better suited for long-term maintenance if needed.

The acquired form of PRCA in young children is also known as transient erythroblastopenia of childhood (TEC). A history of viral infection is found in half of patients, which is thought to trigger an immune mechanism that targets red cell production. The anemia is typically normocytic, and Hb F and erythrocyte adenosine deaminase levels usually are normal. Red cell transfusion support is the mainstay of therapy if the child is symptomatic from anemia. Normalization of erythropoiesis occurs within weeks in the vast majority patients. There may be a genetic predisposition to TEC in some families.

**Congenital Pure Red Cell Aplasia: Diamond-Blackfan Anemia**

Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplastic disorder of early infancy with an estimated incidence of 7 to 10 cases per million live births. Mutations have been identified in nine genes that encode structural ribosome proteins: RPS7, RPS10, RPS17, RPS19, RPS24, and RPS26 in the 40S subunit and RPL5, RPL11, and RPL35A in the 60S subunit. Approximately 25% of patients have a mutation in the RPS19 gene, and mutations in the other eight genes account for another 25% of cases. Many mutations are still unidentified, and it is of interest that an additional 15% to 20% of cases can be accounted for by haplo-deletions of these same RPS genes. Mutations in these ribosomal proteins disrupt ribosome biogenesis in DBA, but the pathophysiologic mechanisms leading to the clinical manifestations are currently unknown. Nearly 50% of DBA cases are linked to an autosomal dominant inheritance pattern, but sporadic mutations have also been reported.

Over 90% of patients show signs of the disorder during the first year of life, with a median age of 8 weeks; however, some patients with DBA are asymptomatic until adulthood. Approximately half of patients have characteristic physical anomalies, including craniofacial dysmorphisms, short stature, and neck and thumb malformations.

The characteristic peripheral blood finding is a severe macrocytic anemia with reticulocytopenia. The WBC count is normal or slightly decreased, and the platelet count is normal or slightly increased. Bone marrow examination distinguishes DBA from the hypocellular marrow in aplastic anemia, because there is normal cellularity of myeloid cells and megakaryocytes and hypoplasia of erythroid cells. The karyotype in DBA is normal. In most cases, Hb F and erythrocyte adenosine deaminase are increased; these findings distinguish DBA from TEC, in which these levels are normal.

**TABLE 22-4 Distinguishing Characteristics of Diamond-Blackfan Anemia and Transient Erythroblastopenia of Childhood.**

<table>
<thead>
<tr>
<th>Test Result</th>
<th>DBA</th>
<th>TEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte ADA increased at diagnosis</td>
<td>85%</td>
<td>5%</td>
</tr>
<tr>
<td>MCV increased at diagnosis</td>
<td>80%</td>
<td>5%</td>
</tr>
<tr>
<td>MCV increased in remission</td>
<td>80%</td>
<td>0%</td>
</tr>
<tr>
<td>Hb F increased at diagnosis</td>
<td>50%–85%</td>
<td>1%–2%</td>
</tr>
<tr>
<td>Hb F increased in remission</td>
<td>50%–85%</td>
<td>0%</td>
</tr>
</tbody>
</table>

DBA, Diamond-Blackfan anemia; TEC, transient erythroblastopenia of childhood; ADA, adenosine deaminase; MCV, mean corpuscular volume; Hb F, fetal hemoglobin.

Therapy includes RBC transfusions and corticosteroids. Although 50% to 75% of patients respond to corticosteroid therapy, side effects are severe with long-term use, including immunosuppression and growth delay. Overall survival is 75% at 40 years. Bone marrow transplantation improves outcomes, with greater than 90% overall survival in patients younger than 10 years old transplanted with a matched-related donor, and 80% in those with a matched unrelated donor.

**Congenital Dyserythropoietic Anemia**

The congenital dyserythropoietic anemias (CDAs) are a heterogeneous group of rare disorders characterized by refractory anemia, reticulocytopenia, hypercellular bone marrow with markedly ineffective erythropoiesis, and distinctive dysplastic changes in bone marrow erythroblasts. Megaloblastoid development occurs in some types, but it is not related to vitamin B12 or folate deficiency. Granulopoiesis and thrombopoiesis are normal. The anemia varies from mild to moderate, even among affected siblings. Secondary hemosiderosis arises from chronic intramedullary and extramedullary hemolysis, as well as increased iron absorption associated with ineffective erythropoiesis. Iron overload develops even in the absence of blood transfusions. Jaundice, cholelithiasis, and splenomegaly are also common findings. CDAs do not progress to aplastic anemia or hematologic malignancies.

Symptoms of CDA usually occur in childhood or adolescence but may first appear in adulthood. CDA is classified into three major types: CDA I, CDA II, and CDA III. There are rare variants that do not fall into these categories, and they have been assigned to four other groups: CDA IV through CDA VII. Whether CDA types IV through VII actually are separate entities is a matter of some controversy. This merely may be a reflection of the insensitive tests to classify CDA disorders. Further gene mutation studies should clarify this issue.

CDA I is inherited in an autosomal recessive pattern and is characterized by a mild to severe chronic anemia. Over 150 cases have been reported. CDA I is caused by mutations in the CDAN1 gene on chromosome 15, which encodes codanin-1, a cell-cycle regulated nuclear protein. The exact role of codanin-1 in the pathophysiology of CDA I is unknown. Malformations of fingers or toes, brown skin pigmentation, and neurologic defects are found more frequently in CDA I than
in the other CDA subtypes. The hemoglobin usually ranges from 6.5 g/dL to 11.5 g/dL, with a mean of 9.5 g/dL. RBCs are macrocytic and may exhibit marked poikilocytosis, basophilic stippling, and Cabot rings. The erythroblasts are megaloblastoid and characteristically have internuclear chromatin bridges or nuclear stranding (Figure 22-3). There are less than 5% binucleated erythroblasts. The characteristic feature of the CDA I erythroblast is a spongy heterochromatin with a “Swiss cheese” appearance. Treatment includes interferon-α and iron chelation.

CDA II is the most common subtype and is inherited in an autosomal recessive pattern. More than 300 cases have been reported. It results from mutations in the SEC23B gene on chromosome 20. SEC23B encodes a component of the coat protein complex (COPII) that forms vesicles for transport of secretory proteins from the endoplasmic reticulum to the Golgi apparatus. Its exact role in the pathophysiology of CDA II is unknown. The anemia in CDA II is mild to moderate, with hemoglobins ranging from 9 g/dL to 12 g/dL and a mean hemoglobin of 11 g/dL. On peripheral blood film, RBCs are normocytic with anisocytosis, poikilocytosis, and basophilic stippling. The bone marrow has normoblastic erythropoiesis, with 10% to 35% binucleated forms and rare multinucleated forms. Occasional pseudo-Gaucher cells are also evident. Circulating RBCs hemolyze with the Ham acidified serum test but not with the sucrose hemolysis test. For this reason, CDA II is also known as HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum). The Ham test is no longer routinely used for CDA II confirmation, given the difficulty of appropriate quality control and the relative lack of testing availability in most laboratories. RBCs also agglutinate with anti antisera and show abnormal migration of band 3 using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Treatment includes splenectomy and iron chelation.

CDA III is the least common of the CDA subtypes, with about 60 cases reported in the literature, the majority being from one Swedish family. This familial autosomal dominant form is associated with mutations in the KIF23 gene, which codes for a protein involved in cytokinesis. The nonfamilial or sporadic form is extremely rare, with fewer than 20 cases reported. The anemia is mild, and the hemoglobin is usually in the range of 8 to 14 g/dL, with a mean of 12 g/dL. RBCs are macrocytic, and poikilocytosis and basophilic stippling are evident. The bone marrow has megaloblastic changes, and giant erythroblasts with up to 12 nuclei are a characteristic feature. Patients rarely require RBC transfusions, and iron overload is not observed.

Myelophthisic Anemia

Myelophthisic anemia is due to the infiltration of abnormal cells into the bone marrow and subsequent destruction and replacement of normal hematopoietic cells. Metastatic solid tumor cells (particularly from lung, breast, and prostate), leukemic cells, fibroblasts, and inflammatory cells (found in miliary tuberculosis and fungal infections) have been implicated. Cytopenia results from the release of substances such as cytokines and growth factors that suppress hematopoiesis and destroy stem, progenitor, and stromal cells. With disruption of normal bone marrow architecture by the infiltrating cells, the marrow releases immature hematopoietic cells. Furthermore, because of the unfavorable bone marrow environment, stem and progenitor cells migrate to the spleen and liver and establish extramedullary hematopoietic sites. Since blood cell production in the liver and spleen is inefficient, these extramedullary sites also release immature cells into the circulation.

The severity of anemia is mild to moderate, with normocytic erythrocytes and reticulocytopenia. Peripheral blood findings include teardrop erythrocytes and nucleated RBCs, as well as immature myeloid cells and megakaryocyte fragments (Figure 22-4). The infiltrating abnormal cells are detected in a bone marrow aspirate or biopsy specimen.

Anemia of Chronic Kidney Disease

Anemia is a common complication of chronic kidney disease (CKD), with a positive correlation between anemia and renal disease severity. Coresh and colleagues reported that between 1999 and 2004, approximately 26 million adults over...
20 years of age in the United States had CKD. The primary cause of anemia in CKD is inadequate renal production of erythropoietin. Without erythropoietin, the bone marrow lacks adequate stimulation to produce RBCs. Another contributor to the anemia of CKD is uremia, which inhibits erythropoiesis and increases RBC fragility. Furthermore, patients experience chronic blood loss and iron deficiency from hemodialysis and frequent blood draws. Chronic inflammation and a restricted diet may also limit the iron available for erythropoiesis.

Anemia of CKD is normocytic and normochromic with reticulocytopenia. Burr cells are common peripheral blood film findings in cases complicated by uremia. Anemia in CKD can lead to cardiovascular complications, kidney failure, and suboptimal quality of life. The Kidney Disease Outcomes Quality Initiative of the National Kidney Foundation recommends annual hemoglobin testing in patients with CKD and investigation of the anemia if the hemoglobin is less than 13.5 g/dL in adult men and less than 12 g/dL in adult women. Treatment includes recombinant human erythropoietin or other erythropoiesis-stimulating agents (ESAs), with a goal hemoglobin range of 11 g/dL to 12 g/dL. Maintaining the hemoglobin above 13 g/dL is not recommended because of the increased risk of cardiovascular and thromboembolic complications. Successful ESA therapy requires adequate iron stores, so plasma ferritin level and percent transferrin saturation should also be monitored. Iron is administered with ESA therapy to maintain the transferrin saturation above 20% and the plasma ferritin level above 100 ng/mL for non-dialysis-dependent patients and above 200 ng/mL for hemodialysis-dependent patients. Iron therapy is not routinely recommended for ferritin levels above 500 ng/mL.

Patients may become hyporesponsive to ESA therapy because of functional iron deficiency (FID). In FID, the bone marrow is unable to release iron rapidly enough to accommodate the accelerated erythropoiesis. The transferrin saturation remains below 20%, but the serum ferritin level is normal or increased, indicating adequate iron stores. Patients with FID are unable to reach or maintain the target hemoglobin, even with high ESA doses. However, patients are able to reach the target hemoglobin after intravenous iron therapy. Researchers have proposed diagnostic criteria for FID in CKD: decreased reticulocyte hemoglobin content, increased soluble transferrin receptor, and greater than 10% hypochromic RBCs in the peripheral blood. Other causes of ESA hyporesponsiveness include chronic inflammatory disease, infection, malignancy, aplastic anemia, antibody-mediated pure red cell aplasia, thalassemia, multiple myeloma, and the presence of hemoglobin H or hemoglobin S variants.

**SUMMARY**

- Bone marrow failure is the reduction or cessation of blood cell production affecting one or more cell lines. Pancytopenia (decreased RBCs, WBCs, and platelets) is a common finding. Sequelae of pancytopenia include weakness and fatigue, infections, and bleeding.
- Aplastic anemia may be acquired or inherited. Acquired aplastic anemia may be idiopathic or secondary to drugs, chemical exposures, radiation, or viruses. Acquired aplastic anemia may also occur with conditions such as paroxysmal nocturnal hemoglobinuria, autoimmune diseases, and pregnancy.
- Bone marrow failure in acquired aplastic anemia occurs from destruction of hematopoietic stem cells by direct toxic effects of a drug, autoimmune T-cell targeting of stem cells, or other unknown mechanisms. The autoimmune reactions are rare adverse events after exposure to drugs, chemicals, or viruses. They are idiosyncratic in that they are unpredictable, and severity is unrelated to the dose or duration of exposure.
- Aplastic anemia is classified as nonsevere, severe, or very severe, based on bone marrow hypocellularity, absolute neutrophil count, platelet count, hemoglobin level, and reticulocyte count (Table 22-1). The severity classification helps to guide treatment decisions.
- Preferred treatment for severe and very severe acquired aplastic anemia is hematopoietic stem cell transplant (HSCT) for younger patients with an HLA-identical sibling. For those without a matched sibling donor and for those who are not HSCT candidates, immunosuppressive therapy with antithymocyte globulin and cyclosporine is recommended.
- Fanconi anemia (FA), dyskeratosis congenita (DKC), and Shwachman-Bodian-Diamond syndrome (SBDS) are inherited forms of aplastic anemia with progressive bone marrow failure, and patients may present with characteristic physical malformations. FA is inherited in an autosomal recessive or X-linked pattern, and mutations in 15 genes have been identified. A positive chromosome breakage study with diepoxybutane is diagnostic. DKC can be X-linked, autosomal dominant, or autosomal recessive, and mutations in eight genes have been identified. SBDS is autosomal recessive and is associated with mutations in the SBDS gene.
- Telomerase complex defects play a role in the pathophysiology of inherited aplastic anemias and some acquired aplastic anemias. The defects result in the inability of telomerase to elongate telomeres at the ends of chromosomes, which leads to premature hematopoietic stem cell senescence and apoptosis.
- Pure red cell aplasia is a disorder of erythrocyte production. Acquired transient erythroblastopenia of childhood (TEC) and Diamond-Blackfan anemia (DBA) are disparate subtypes with distinct etiologies, clinical features, and courses (Table 22-4). Mutations in nine different ribosomal protein genes have been identified in DBA.
- Patients with congenital dyserythropoietic anemia (CDA) exhibit refractory anemia, reticulocytopenia, secondary hemosiderosis, and distinct abnormalities of erythroid precursors. Three major subtypes are recognized: CDA I, CDA II, and CDA III.
- Myeloproliferative anemia results from the replacement of normal bone marrow with abnormal cells. The main cause of anemia of chronic kidney disease is inadequate production of erythropoietin by the kidneys.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.
Answers can be found in the Appendix.

1. The clinical consequences of pancytopenia include:
   a. Pallor and thrombosis
   b. Kidney failure and fever
   c. Fatigue, infection, and bleeding
   d. Weakness, hemolysis, and infection

2. Idiopathic acquired aplastic anemia is due to a(n):
   a. Drug reaction
   b. Benzene exposure
   c. Inherited mutation in stem cells
   d. Unknown cause

3. The pathophysiologic mechanism in acquired idiosyncratic aplastic anemia is:
   a. Replacement of bone marrow by abnormal cells
   b. Destruction of stem cells by autoimmune T cells
   c. Defective production of hematopoietic growth factors
   d. Inability of bone marrow stroma to support stem cells

4. Based on the criteria in Table 22-1, what is the aplastic anemia classification of a 15-year-old female with a bone marrow cellularity of 10%, hemoglobin of 7 g/dL, absolute neutrophil count of $0.1 \times 10^9/L$, and platelet count of $10^3 \times 10^9/L$?
   a. Nonsevere
   b. Moderate
   c. Severe
   d. Very severe

5. The most consistent peripheral blood findings in severe aplastic anemia are:
   a. Hairy cells, monocytopenia, and neutropenia
   b. Macrocytosis, thrombocytopenia, and neutropenia
   c. Blasts, immature granulocytes, and thrombocytopenia
   d. Polychromasia, nucleated RBCs, and hypersegmented neutrophils

6. The treatment that has shown the best success rate in young patients with severe aplastic anemia is:
   a. Immunosuppressive therapy
   b. Long-term red blood cell and platelet transfusions
   c. Administration of hematopoietic growth factors and androgens
   d. Stem cell transplant with an HLA-identical sibling

7. The test that is most useful in differentiating FA from other causes of pancytopenia is:
   a. Bone marrow biopsy
   b. Ham acidified serum test
   c. Diepoxybutane-induced chromosome breakage
   d. Flow cytometric analysis of CD55 and CD59 cells

8. Mutations in genes that code for the telomerase complex may induce bone marrow failure by causing which one of the following?
   a. Resistance of stem cells to normal apoptosis
   b. Autoimmune reaction against telomeres in stem cells
   c. Decreased production of hematopoietic growth factors
   d. Premature death of hematopoietic stem cells

9. Diamond-Blackfan anemia differs from inherited aplastic anemia in that in the former:
   a. Reticulocyte count is increased
   b. Fetal hemoglobin is decreased
   c. Only erythropoiesis is affected
   d. Congenital malformations are absent

10. Which anemia should be suspected in a patient with refractory anemia, reticulocytopenia, hemosiderosis, and binucleated erythrocyte precursors in the bone marrow?
   a. Fanconi anemia
   b. Dyskeratosis congenita
   c. Acquired aplastic anemia
   d. Congenital dyserythropoietic anemia

11. The primary pathophysiologic mechanism of anemia associated with chronic kidney disease is:
   a. Inadequate production of erythropoietin
   b. Excessive hemolysis
   c. Hematopoietic stem cell mutation
   d. Toxic destruction of stem cells

REFERENCES


This chapter presents an overview of the hemolytic process and provides a foundation that is applicable in the following chapters on red blood cell (RBC) disorders. The term *hemolysis* or *hemolytic disorder* refers to increased rate of destruction (i.e., lysis) of RBCs, shortening their life span. The reduced number of cells results in reduced tissue oxygenation and increased erythropoietin production by the kidney. When the patient is otherwise healthy, the bone marrow responds by accelerating erythrocyte production, which leads to reticulocytosis. A hemolytic process is present without anemia if the bone marrow is able to compensate by accelerating RBC production sufficiently to replace the RBCs lost through hemolysis. Healthy
bone marrow can increase its production of RBCs by six to eight times normal; therefore, significant RBC destruction must occur before an anemia develops. A hemolytic anemia results when the rate of RBC destruction exceeds the increased rate of RBC production.

### CLASSIFICATION

Many anemias have a hemolytic component, including the anemia associated with vitamin B₁₂ or folate deficiency and the anemia of chronic inflammation, renal disease, and iron deficiency. In these conditions, the hemolysis alone does not cause anemia, and so they are not typically classified as hemolytic disorders. Rather, these anemias develop as a result of the inability of the bone marrow to increase production of RBCs. Because hemolysis is not the primary underlying cause, these disorders are considered anemias with a secondary hemolytic component.

When hemolysis is the primary feature, the anemias can be classified as follows:
- Acute versus chronic
- Inherited versus acquired
- Intrinsic versus extrinsic
- Intravascular versus extravascular
- Fragmentation versus macrophage-mediated

Every hemolytic condition can be classified according to each of these descriptors. Table 23-1 shows this and provides a noncomprehensive list of hemolytic anemias. This chapter focuses on the mechanism of hemolysis—that is, the distinction between fragmentation and macrophage-mediated hemolytic conditions. The other classifying schemes are summarized here briefly for application in the chapters that follow.

**Acute versus chronic hemolysis** delineates the clinical presentation. Acute hemolysis has a rapid onset and is isolated (sudden), episodic, or paroxysmal, as in paroxysmal cold hemoglobinuria or paroxysmal nocturnal hemoglobinuria. Patients with paroxysmal cold hemoglobinuria experience hemolysis after exposure to cold, and patients with paroxysmal nocturnal hemoglobinuria may experience intermittent episodes of hemolysis. A hemolytic transfusion reaction is an example of a single acute incident. Whatever the cause, acute hemolysis either disappears or subsides between episodes, during which time the patient’s condition may return to normal.

Chronic hemolysis may not be evident if the bone marrow is able to compensate, but it may be punctuated over time with hemolytic crises that cause anemia. Glucose-6-phosphate dehydrogenase deficiency is such a condition. RBC life span is chronically shortened, but bone marrow compensation prevents anemia. When the cells are challenged with oxidizing agents such as antimalarial drugs, a dramatic acute hemolytic event occurs. When the drug is withdrawn, compensation returns.

Other chronic conditions result in anemia that is so severe that the bone marrow cannot generate cells fast enough to compensate for the anemia. Thalassemia is an example of such a condition. Although red blood cell production is brisk, each cell possesses an inadequate complement of one type of globin chain, and functional hemoglobin production is decreased.

### TABLE 23-1 Classification of Selected Hemolytic Anemias by Primary Cause and Type of Hemolysis

<table>
<thead>
<tr>
<th>Predominantly Fragmentation (Intravascular) Hemolysis</th>
<th>Predominantly Macrophage-Mediated (Extravascular) Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agents from Outside the RBC</strong></td>
<td></td>
</tr>
<tr>
<td>Immune hemolysis: cold antibody</td>
<td>Immune hemolysis: warm antibody</td>
</tr>
<tr>
<td>Microangiopathic hemolysis</td>
<td>Drugs</td>
</tr>
<tr>
<td>Infectious agents, as in malaria</td>
<td></td>
</tr>
<tr>
<td>Thermal injury</td>
<td></td>
</tr>
<tr>
<td>Chemicals/drugs</td>
<td></td>
</tr>
<tr>
<td>Venoms</td>
<td></td>
</tr>
<tr>
<td>Prosthetic heart valve</td>
<td></td>
</tr>
<tr>
<td><strong>Membrane Abnormalities</strong></td>
<td></td>
</tr>
<tr>
<td>Spur cell anemia of severe liver disease</td>
<td>Hereditary membrane defects</td>
</tr>
<tr>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td></td>
</tr>
<tr>
<td><strong>Abnormalities of the RBC Interior</strong></td>
<td></td>
</tr>
<tr>
<td>Enzyme defects such as G6PD deficiency</td>
<td></td>
</tr>
<tr>
<td>Globin abnormalities such as sickle cell, thalassemia</td>
<td></td>
</tr>
</tbody>
</table>

Green text indicates acute or episodic hemolysis.
Red text indicates chronic hemolysis.
Some conditions may exhibit mixed presentations under certain circumstances. It is evident that most hereditary conditions lead to chronic hemolysis, whereas acquired conditions are more often acute. Furthermore, the intrinsic red blood cell defects typically are due to hereditary conditions, whereas extrinsic factors typically lead to acquired hemolytic disorders. G6PD, Glucose-6-phosphate dehydrogenase; RBC, red blood cell.
overall. As a result, the oxygen-carrying capacity of the blood is chronically low. Cells lyse in thalassemia because excess normal globin chains precipitate inside the erythroid cells, which leads to hemolysis and exacerbates chronically reduced hemoglobin production.

Inherited hemolytic conditions, such as thalassemia, are passed to offspring by mutant genes from the parents. Acquired hemolytic disorders develop in individuals who were previously hematologically normal but acquire an agent or condition that lyzes RBCs. Infectious diseases such as malaria are an example.

The hemolytic disorders are also classified as involving intrinsic or extrinsic RBC defects, with the latter caused by the action of external agents. This is the classification scheme used for subsequent chapters in this book. Examples of intrinsic hemolytic disorders are abnormalities of the RBC membrane, enzymatic pathways, or the hemoglobin molecule. With intrinsic defects, if the RBCs of the affected patient were to be transfused into a healthy individual, they would still have a shortened life span because the defect is in the RBC. If normal RBCs are transfused into a patient who has an intrinsic defect, the transfused cells have a normal life span because the transfused cells are normal.

Extrinsic hemolytic conditions are those that arise from outside the RBC, typically substances in the plasma or conditions affecting the anatomy of the circulatory system. Even though malaria protozoa and other infectious agents are within the RBC, they are classified as extrinsic because the RBC was normal until it was invaded by an outside agent. An antibody against RBC antigens and a prosthetic heart valve are examples of noninfectious extrinsic agents that can damage RBCs. In extrinsic hemolysis, cross-transfusion studies have shown that the patient’s RBCs have a normal life span in the bloodstream of a healthy individual, but normal cells are lysed more rapidly in the patient’s circulation. These studies confirm that something outside the RBCs is the cause of the hemolysis. (Of course, in the case of intracellular parasites, the cross-transfusion study is not applicable.) Most intrinsic defects are inherited; most extrinsic ones are acquired (Table 23-1). A few exceptions exist, such as paroxysmal nocturnal hemoglobinuria, an acquired disorder involving an intrinsic defect (Chapter 24).

Intrinsic disorders are subclassified as membrane defects, enzyme defects, and hemoglobinopathies. Extrinsic hemolysis may be immunohemolytic, traumatic, or microangiopathic, or may be caused by infectious agents, chemical agents (drugs and venoms), or physical agents (Table 23-1).

Another classification scheme is based on the site of hemolysis and related to the general mechanism of lysis. Intravascular hemolysis occurs by fragmentation. Although this takes place most often within the bloodstream, RBCs can lyse by fragmentation in the spleen and bone marrow as well. Macrophage-mediated hemolysis occurs when RBCs are engulfed by macrophages and lysed by their digestive enzymes. The designation extravascular, meaning outside the vessels, can refer either to lysis within the macrophage and not in the bloodstream or to the fact that most of the macrophages are in tissues, chiefly the spleen and the liver, and thus are outside the vasculature. Commonly the terms fragmentation and intravascular are used interchangeably, as are macrophage-mediated and extravascular.

The mechanistic classifying scheme is useful because screening laboratory tests rely on the differences in the hemolytic processes. However, the exact cause of the hemolysis must still be determined by targeted testing for appropriate treatment to be implemented.

HEMOLYSIS

Normal Bilirubin Metabolism

Detection of hemolysis depends partly on detection of RBC breakdown products. A prominent product is bilirubin. The process of normal bilirubin production is described to clarify the relationship between hemolysis and increased bilirubin levels.

The story of bilirubin production is, in part, a story of iron salvage. The body salvages and recycles iron like a precious metal. There is also a process for recycling the amino acids of the globin chains to build new proteins. The protoporphyrin component, however, is catabolized and excreted but facilitates dietary fat absorption in the process. Bilirubin is the excretory product derived from the protoporphyrin component of heme.

RBCs live approximately 120 days. During this time, they undergo various metabolic and chemical changes, which result in a loss of deformability. Under normal circumstances, macrophages of the mononuclear phagocyte system (or reticuloendothelial system) recognize these changes and phagocytize the aged erythrocytes (Chapter 8), creating a macrophage-mediated hemolytic process. The organs involved include the spleen, bone marrow, liver, lymph nodes, and circulating monocytes, but it is primarily the macrophages in spleen and liver that process senescent RBCs.

The majority of RBC degradation occurs inside macrophages as enzymes of the macrophage granules lyse the phagocytized erythrocytes (Figure 23-1). Hemoglobin is hydrolyzed into heme and globin; the latter is further degraded into amino acids that return to the amino acid pool. Iron is released from the heme, returned to the plasma via ferroportin, bound to its protein carrier molecule (transferrin), and recycled to needy cells. The remaining protoporphyrin is degraded through a series of biochemical reactions in different tissues and organs.
Figure 23-2 illustrates protoporphyrin catabolism. While protoporphyrin is inside the macrophage, heme oxygenase acts on it, breaking the protoporphyrin ring to yield a linear molecule, biliverdin. The lungs excrete a by-product of that reaction, carbon monoxide. The green biliverdin is reduced to bilirubin, a nonpolar yellow molecule that is secreted into the plasma (Box 23-1). This form of bilirubin is called unconjugated for reasons that will be evident shortly. Because it is hydrophobic, this form of bilirubin must bind to albumin to be transported in plasma to the liver. In the liver sinusoid, the bilirubin dissociates from the albumin. The bilirubin is then transported across the hepatocyte membrane by a process that is not yet clear. It may be carrier independent or involve organic anion transporter (OAT) proteins.2,3 Once inside the hepatocyte, the unconjugated bilirubin is joined (i.e., conjugated) with two molecules of glucuronic acid by glucuronyl transferase to form bilirubin diglucuronide. The addition of the two sugar acid molecules makes the molecule polar and water soluble. Bilirubin diglucuronide is also called conjugated bilirubin or direct bilirubin (Box 23-2). Thus the bilirubin originally released from macrophages that lacks these sugars is termed unconjugated bilirubin or indirect bilirubin (Box 23-2).

**BOX 23-1 Visualizing the Color Changes of Hemoglobin Degradation**

The degradation of heme can be seen in bruises in fair-skinned individuals or in the sclera of the eye after a vascular bleed. The same process that macrophages facilitate can occur in tissues. At first, the extravasated but deoxygenated blood gives the injury the purple-red appearance of hemoglobin. As the hemoglobin is degraded, the color changes to a greenish hue due to biliverdin, but ultimately it becomes yellow due to bilirubin.

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**Figure 23-2** Catabolism of heme to bilirubin. In cells containing heme oxygenase, iron is removed from heme, and the protoporphyrin ring is opened up to form an intermediate, biliverdin. Biliverdin is converted to unconjugated bilirubin by biliverdin reductase. The unconjugated bilirubin is secreted into the plasma and binds to albumin for transport to the liver. When unconjugated bilirubin enters the hepatocyte, glucuronyl transferase adds two molecules of glucuronic acid to form bilirubin diglucuronide, also called conjugated bilirubin.
Conjugated bilirubin is excreted into the hepatic bile duct, continues down the common bile duct, and goes into the intestines (Figure 23-3). There it assists with the emulsification of fats for absorption from the diet. Conjugated bilirubin is oxidized by gut bacteria into various water-soluble compounds, collectively called urobilinogen. Most urobilinogen is oxidized further to ster-cobilin and similar compounds that give the brown color to stool, which is the ultimate route for excretion of protoporphyrin.

Because they are water soluble, some conjugated bilirubin and urobilinogen molecules are absorbed from the intestines...
into the plasma by osmosis (Figure 23-3). The portal circulation (the blood vessels that surround the intestines to absorb nutrients) collects these bile products. The portal circulation carries blood directly to the liver, so most of the absorbed conjugated bilirubin and urobinigen is recycled directly into the bile again. Some remains in the plasma, however, and is filtered by the renal glomerulus and excreted in the urine. Conjugated bilirubin is virtually undetectable in urine, but a measurable amount of urobinigen can be expected normally. The yellow color of urine is not due to the urobinigen, which is colorless. It is due to urobilin, a derivative of urobinigen that is also water-soluble.

Normal Plasma Hemoglobin Salvage During Fragmentation Hemolysis

Fragmentation hemolysis is the result of trauma to the RBC membrane that causes a breach sufficient for the cell contents, chiefly hemoglobin, to spill directly into plasma (Box 23-3). Approximately 10% to 20% of normal RBC destruction is via fragmentation, secondary to turbulence and anatomic restrictions in the vasculature.

Because hemoglobin is filtered by the kidney, some iron could be lost daily with even normal amounts of fragmentation hemolysis. In addition, free hemoglobin, and especially free heme, can cause oxidative damage to cells. Several mechanisms exist to salvage hemoglobin iron and prevent oxidation reactions (Box 23-4) and are collectively called the hemoglobin-hemopexin-methemalbumin system (Figure 23-4).

When free in the plasma, hemoglobin exists mostly as α/β dimers that rapidly complex to a liver-produced plasma protein called haptoglobin. This is the first mechanism of hemoglobin iron salvage. By binding to haptoglobin, hemoglobin avoids filtration at the glomerulus, and the iron is saved from urinary loss. Haptoglobin binds a hemoglobin dimer in a conformation that is very similar to the complementary dimer in the native hemoglobin structure, so the conformation of the complex has been termed a pseudodimer. In this complex, the hemes are sequestered, as they are in intact hemoglobin, so that cells are protected from their oxidative properties. As the plasma is carried to various tissues, the complex is taken up by macrophages, principally those in the liver, spleen, bone marrow, and lung. In these tissues, the macrophages express CD163, the haptoglobin scavenger receptor, on their membranes. Once the hemoglobin-haptoglobin complex binds to CD163, the entire complex is internalized into the macrophage in a lysosome. Inside the lysosome, iron is salvaged, the globin is catabolized, and the protoporphyrin is converted to unconjugated bilirubin, just as though an intact RBC had been ingested by the macrophage. The haptoglobin is also degraded within the lysosome.

The level of haptoglobin in plasma is typically adequate to salvage only the small amount of plasma hemoglobin generated each day. If hemolysis is accelerated, haptoglobin is depleted because the liver’s production does not increase in response to the increased consumption of haptoglobin. See Excessive Fragmentation (Intravascular) Hemolysis below.

A secondary mechanism of iron salvage and oxidation prevention involves hemopexin (Figure 23-4). The iron in free plasma hemoglobin rapidly becomes oxidized, forming methemoglobin. The hemep molecule (actually methem or hemin) dissociates from the globin and binds to another liver-produced plasma protein, hemopexin. This binding also saves the iron from urinary loss and prevents oxidant injury to cells and tissues. Hemopexin-methem binds to hepatocyte CD91 receptors, the lipoprotein receptor-related protein (LRP1), and is internalized. The fate of the internalized heme remains an area of research because some studies suggest that intact heme can be incorporated into needed proteins within the hepatocyte, like cytochromes, and others suggest it is broken down to bilirubin with reuse of the iron. It appears that under normal circumstances, the bulk of the hemopexin is recycled to the plasma from the hepatocyte.

A third mechanism of iron salvage is the methem-albumin system. Albumin acts as a carrier for many molecules, including metheme. This is just a temporary holding state for the metheme, merely by virtue of the high concentration of albumin in plasma. But metheme is rapidly transferred to hemopexin, which has a higher binding affinity for metheme than does albumin. The hemopexin-metheme complex then travels to the liver for processing.

If the previous systems are overloaded, the excess (met) hemoglobin and metheme will be filtered into the urine. Normally, this is a negligible amount so that the kidney is not significantly involved in normal iron salvage. Yet, it has been known for some time that the proximal tubular cells can reabsorb iron, since iron staining of tubular cells in urinary sediment...
Figure 23-4. Normal fragmentation hemolysis. 1, Normally a small number of red blood cells lyse within the circulation, forming schistocytes and releasing hemoglobin (Hb) into the plasma, mostly as αβ dimers. 2, The plasma protein haptoglobin (Hpt) binds a hemoglobin dimer in a complex. 3, The hemoglobin-haptoglobin complex binds to CD163 on the surface of macrophages in various organs. 4, The complex is internalized into the macrophage, where the hemoglobin dimer is released. The hemoglobin dimer is degraded to heme, the iron is released, and the protoporphyrin ring is converted to unconjugated bilirubin. 5, The haptoglobin is degraded. 6, The unconjugated bilirubin released into the plasma is bound to albumin and processed through the liver as in Figure 23-3 (steps 2-8). 7, When free hemoglobin is released into the plasma with fragmentation, the iron is rapidly oxidized, forming methemoglobin, and the heme (metheme) molecule dissociates from the globin. 8, The plasma protein hemopexin (Hpx) binds free metheme into a complex. 9, The hemopexin-metheme complex binds to CD91 on the surface of hepatocytes. 10, The complex is internalized into the hepatocyte. 11, The iron is released from the metheme, and the protoporphyrin ring is converted to unconjugated bilirubin, ready for conjugation and further processing, as in Figure 23-3 (steps 4-8). 12, Hemopexin is recycled to the plasma. Note that metheme can also bind to albumin, forming metheme-albumin (not shown), but this complex is temporary because metheme is rapidly transferred to hemopexin. (Adapted from Brunzel NA. Fundamentals of Urine and Body Fluid Analysis. 3e. St. Louis, Elsevier, 2013.)

during periods of excessive hemolysis demonstrates the presence of hemosiderin. The full picture of renal handling of iron and related proteins is an area of active research yet to be fully elucidated. However, an emerging picture suggests that under normal circumstances, a small amount of filtered transferrin is salvaged by transferrin receptor on the apical surface of proximal tubular cells. This may be the normal source of iron for those cells' metabolic needs. Ferroportin has been identified on the basolateral membrane of proximal tubular cells, suggesting that renal cells are then able to transfer additional salvaged iron back into
the plasma. Once again, these systems evolved to manage the amount and “form” of iron present in normal urinary filtrate. During excessive fragmentation hemolysis, other forms of iron are presented to and processed by the kidney.

**EXCESSIVE MACROPHAGE-MEDIATED (EXTRAVASCULAR) HEMOLYSIS**

Many hemolytic anemias are a result of increased macrophage-mediated hemolysis (Figure 23-5), during which more than the usual number of RBCs are removed from the circulation daily. Under normal circumstances, senescent RBCs display surface markers that identify them to macrophages as aged cells requiring removal (Chapter 8). Pathologic processes also lead to expression of the same markers, so cells are recognized and removed. If the number of affected cells increases beyond the quantity normally removed each day due to senescence, and if the bone marrow cannot compensate, then anemia develops. As an example, Heinz bodies, aggregates of denatured hemoglobin formed in various anemias, bind to the inner surface of the RBC membrane, producing changes to the exterior of the membrane that can be detected by macrophages. When excessive oxidation of hemoglobin causes increased formation of Heinz bodies, the cells are removed from the circulation prematurely by macrophages. A similar process occurs when intracellular parasites are present or when complement or immunoglobulins are on the surface of the RBC.

When an RBC is ingested by a macrophage, it is lysed within a phagolysosome, and the contents are processed entirely within the macrophage as described previously. The contents of the RBC are not detected in plasma because it is lysed inside the macrophage, and the contents are degraded there—hence the designation *extravascular hemolysis*. Since defective cells display markers like those of senescent RBCs, macrophage-mediated hemolysis of defective cells occurs most often in the spleen and liver, where the macrophages possess receptors for those markers.

Sometimes the macrophage ingests a portion of the membrane, leaving the remainder to reseal. Little, if any, cytoplasmic volume is lost, but with less membrane, the cell becomes a

---

**Figure 23-5** Excess macrophage-mediated hemolysis. 1, More than the usual number of red blood cells are ingested each day by macrophages. 2, An increased amount of unconjugated bilirubin is produced, released into the plasma, and binds to albumin. 3, When increased unconjugated bilirubin is presented to the liver, an increased amount of conjugated bilirubin is made and excreted into the intestine. 4, When an increased amount of conjugated bilirubin is present in the intestine, an increased amount of urobilinogen is formed and excreted in the stool. 5, Increased urobilinogen in the intestine results in increased urobilinogen reabsorbed into the plasma. 6, Increased urobilinogen in the plasma results in increased urobilinogen filtered and excreted in the urine. (Adapted from Brunzel NA. Fundamentals of Urine and Body Fluid Analysis. 3e. St. Louis, Elsevier, 2013.)
spherocyte, the characteristic shape change associated with macrophage-mediated hemolysis. Although the spherocyte may enter the circulation, its survival is shortened because of its rigidity and inability to traverse the splenic sieve during subsequent passages through the red pulp. It may become trapped against the basement membrane of the splenic sinus and be fully ingested by a macrophage, or it may lyse mechanically due to its rigidity and in so doing contribute a fragmentation component to what is otherwise a macrophage-mediated process.

In macrophage-mediated hemolytic anemias, the total plasma bilirubin level rises as RBCs lyse prematurely. The rise of the total bilirubin is due to the increase of the unconjugated fraction (Figure 23-5). As long as the liver is healthy, it processes the increased load of unconjugated bilirubin, producing more than the usual amount of conjugated bilirubin that enters the intestine. Increased urobilinogen forms in the intestines and is subsequently absorbed by the portal circulation and excreted by the kidney. As a result, increased urobilinogen is detectable in the urine. Although there is an increase in unconjugated bilirubin in the plasma, none of it appears in the urine because it is bound to albumin and cannot pass through the glomerulus. These findings are summarized in Table 23-2.

**EXCESSIVE FRAGMENTATION (INTRAVASCULAR) HEMOLYSIS**

Although fragmentation hemolysis is a minor component of normal RBC destruction, it can be a major feature of pathologic processes. Dramatic examples of fragmentation hemolysis are the traumatic, physical lysis of RBCs caused by prosthetic heart valves and the exit of mature intracellular RBC parasites, such as malaria protozoa, by bursting out of the cell. In these instances, the fragmentation destruction of RBCs can cause profound anemias.

Excessive fragmentation hemolysis is characterized by the appearance in the plasma of the contents of the red blood cell, chiefly hemoglobin, and thus the development of (met) hemoglobinemia. As a result, the salvage proteins form complexes with their ligands (Figure 23-6, A, B), and hemoglobin-haptoglobin, metheme-hemopexin, and metheme-albumin are detectable, if measured. The levels of free haptoglobin will drop, since more than the usual amounts of the complex will form and be taken up by macrophages (Table 23-2). The endocytosed protein is not recycled to the plasma and there is no compensatory increase in production, so the plasma is depleted of haptoglobin. The levels of free hemopexin can also decrease even though it is normally recycled. It appears that the hepatic recycling system can become saturated when there are high levels of metheme to be salvaged. During these circumstances, hemopexin then gets degraded within the hepatocyte, and plasma levels fall. Still the drop in hemopexin is not as dramatic as the decline of haptoglobin, since some recycling continues.

In roughly the same time frame that hemoglobin appears in the plasma, it can also appear in the urine (hemoglobinuria) (Table 23-2) if the amount of liberated hemoglobin and heme exceeds the salvage capacity of the plasma proteins. Increased amounts of iron-containing proteins are then absorbed into the proximal tubular cells (Figure 23-6, C).

The mechanisms by which renal cells are able to reabsorb more than usual amounts of iron, heme, or iron-containing proteins are still emerging. At least one mechanism is the megalin-cubilin receptor endocytosis system. These receptors are not specific for heme/iron-containing compounds. They

<table>
<thead>
<tr>
<th>Test Specimen</th>
<th>Result</th>
<th>Fragmentation</th>
<th>Macrophage-Mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Total bilirubin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Indirect (unconjugated) bilirubin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Direct (conjugated) bilirubin</td>
<td>WRI</td>
<td>WRI</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase activity</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Haptoglobin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Free hemoglobin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Hemopexin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Urine</td>
<td>Urobilinogen</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Free hemoglobin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Methemoglobin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Prussian blue staining of urine sediment</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Anticoagulated whole blood</td>
<td>Hemoglobin, hematocrit, RBC count</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Schistocytes</td>
<td>Often present</td>
<td>Often present</td>
</tr>
<tr>
<td></td>
<td>Spherocytes</td>
<td>Often present</td>
<td>Often present</td>
</tr>
<tr>
<td>Special tests</td>
<td>Endogenous carbon monoxide</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Erythrocyte life span</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑, Typically increased; ↓, typically only slightly increased, minor component; ↓, typically decreased; RBC, red blood cell; WRI, within the reference interval.

**TABLE 23-2 Comparison of Laboratory Findings Indicating Accelerated Red Blood Cell Destruction in Fragmentation Versus Macrophage-Mediated Hemolysis**
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Figure 23-6  A, Excess fragmentation hemolysis: the role of macrophages. 1, When an increased number of red blood cells lyse by fragmentation, more than the usual amount of hemoglobin (Hb) is released into the plasma, mostly as αβ dimers. 2, Haptoglobin (Hpt) binds the increased hemoglobin dimers, forming more than usual numbers of complexes. 3, The hemoglobin-haptoglobin complexes are taken up by macrophages bearing the CD163 receptor in various organs. 4, An increased amount of hemoglobin dimers is released from the complexes. The hemoglobin is degraded to heme, the iron is released, and the protoporphyrin ring is converted to unconjugated bilirubin. The increased amount of unconjugated bilirubin is then transported to the liver and processed as with excess macrophage mediated hemolysis (Figure 23-5, steps 2-6). 5, Degradation of haptoglobin is accelerated as compared to normal.
Figure 23-6, cont’d B, Excess fragmentation hemolysis: the role of the liver. 1, If the amount of hemoglobin released from lysing red blood cells exceeds the capacity of haptoglobin, the unbound free hemoglobin is rapidly oxidized, forming methemoglobin, and the metheme molecule dissociates from the globin. 2, Hemopexin binds to metheme, and the complex is captured by the CD91 receptor on hepatocytes. 3, The complex is internalized by the hepatocyte, the iron is released from the metheme, and the protoporphyrin ring is converted to unconjugated bilirubin and ultimately to conjugated bilirubin to be processed, as in Figure 23-5, steps 3-6. 4, Although a small amount of hemopexin is recycled to the plasma, most is degraded. Metheme can also temporarily bind to albumin, forming metheme-albumin (not shown), but metheme is rapidly transferred to hemopexin.
Unconjugated bilirubin

Heme degradation

Liver

Conjugated bilirubin

Kidney

Intestine

Urobilinogen

Fecal urobilinogen: ↑↑

Urine urobilinogen: ↑↑

Urine bilirubin: negative

Hemoglobinuria

Some reabsorbed into circulation

Red blood cell

Schistocyte

Heme or metheme

Unconjugated bilirubin

Normal amount of bilirubin

Normal amount of urobilinogen

Heme or metheme

Ferroportin

Proximal tubular cell

Circulation

Filtrate

Hb

Cb

Cb-Hb

Hb

Fe

Hemosiderin

Ferritin

Proximal tubular cell

Figure 23-6, cont’d
For legend see next page
Figure 23-6, cont’d  C, Excess fragmentation hemolysis: the role of the kidney. 1, When excess red blood cells lyse by fragmentation and other systems are saturated, free (met)hemoglobin enters the urinary filtrate. 2, Cubilin (Cb) on the luminal side of the proximal tubular cells binds proteins for reabsorption, including hemoglobin. 3, Cubilin carries hemoglobin into the proximal tubular cells. 4, The hemoglobin is degraded to heme, the iron is released, and the protoporphyrin ring is converted to unconjugated bilirubin and secreted into the plasma (5) in the same manner as in the macrophages (Figure 23-1). 6, When the amount of hemoglobin exceeds the capacity of the proximal tubular cells to absorb it from the filtrate, hemoglobinuria occurs. D, Fate of iron removed from salvaged hemoglobin in the kidney. 1, Iron (Fe) salvaged from absorbed hemoglobin can be transported into the circulation by ferroportin on the basolaminal side of the tubular cell. In the plasma it will be bound to transferrin (Tf) for transport. 2, Iron in excess of what can be transported into the circulation is stored as ferritin, and some is converted to hemosiderin. If the tubular cell is sloughed into the filtrate and appears in the urine sediment, the hemosiderin can be detected using the Prussian blue stain. (A-C, Adapted from Brunzel NA. Fundamentals of Urine and Body Fluid Analysis. 3e. St. Louis, Elsevier, 2013.)

are responsible for nonspecific but very efficient reabsorption of proteins from the urinary filtrate in the proximal tubule. Each has been shown to bind hemoglobin and myoglobin.18 Additionally, megalin can bind lactoferrin, and cubilin can bind transferrin.18 The kinetics of such nonspecific competitive reabsorption favors the iron-containing proteins when they are present in high concentrations. A mechanism for reabsorption of free (met)heme has not been clearly identified. Proximal tubular cells are able to dismantle heme from hemoglobin via heme-oxygenase-1,19 contributing to elevations of unconjugated bilirubin in plasma and thus freeing the iron for export to the plasma by ferroportin. It is reasonable to expect that a specific heme receptor in the kidney may yet be identified.

Proximal tubular cell iron in excess of what can be transported into the circulation is stored as ferritin, and some is converted to hemosiderin (Figure 23-6, D). If the tubular cell is sloughed into the filtrate and appears in the urine sediment, the hemosiderin can be detected using the Prussian blue stain. This provides evidence that hemoglobin has been salvaged from the filtrate.

Elevated levels of plasma indirect bilirubin and urinary urobilinogen are also measurable, although not immediately, because time is needed to produce these products. The time course of these findings assists with the differential diagnosis. Following an acute onset, a rise in reticulocytes several days later would also be seen.

**CLINICAL FEATURES**

The clinical findings typical of hemolysis may be prominent if the hemolytic process is the primary cause of anemia. For patients in whom hemolysis is secondary, however, other clinical features may be more noticeable. If hemolysis is sufficient to result in anemia, patients experience the general symptoms of fatigue, dyspnea, and dizziness to a degree that is consistent with the severity and rate of development of the anemia. The associated signs of pallor and tachycardia can be expected.

Increase in plasma bilirubin gives a yellow tinge, not only to the plasma but also to body tissues. It is readily evident in the sclera of the eyes and the skin of fair-skinned individuals. Jaundice refers to the yellow color of the skin and sclera, whereas icterus describes plasma and tissues. An increase in plasma bilirubin and subsequent jaundice can occur in other conditions besides hemolysis, such as hepatitis or gallstones. If the jaundice is the result of hemolysis, it is called hemolytic jaundice or prehepatic jaundice, which reflects the predominance of unconjugated bilirubin in plasma. The lipid solubility of unconjugated bilirubin also leads to deposition in the brain when hemolysis affects newborns (Chapter 26), since the blood-brain barrier is not fully developed. This can lead to a type of brain damage called kernicterus, which refers to the yellow coloring (icterus) of the brain tissue.

The frequency or constancy of jaundice provides clues to the cause. In glucose-6-phosphate dehydrogenase deficiency, for example, jaundice is periodic, appearing following a crisis. In thalassemia, jaundice is chronic. Jaundice may not be present at all if hemolysis is minimal and the liver is able to process the additional bilirubin, as is often the case in hereditary elliptocytosis.

Some signs differentiate chronic from acute hemolysis. Splenomegaly can develop, particularly with chronic macrophage-mediated hemolytic processes. Gallstones (cholelithiasis) can occur whenever hemolysis is chronic; the constantly increased amount of bilirubin in the bile leads to the formation of the stones.20 When hemolysis is chronic in children, the persistent compensatory bone marrow hyperplasia can lead to bone deformities because the bones are still forming (Figure 28-3). For patients in whom an acquired, acute hemolytic process develops, the associated malaise, aches, vomiting, and possible fever may cause it to be confused with an acute infectious process. Profound prostration and shock may develop, particularly with acute fragmentation hemolysis. Flank pain, oliguria, or anuria develops, which leads to acute renal failure.

Other clinical features may offer a clue as to whether hemolysis is macrophage-mediated or due to fragmentation. In particular, brown urine, associated with (met)hemoglobinuria, points to a fragmentation hemolytic process.

**LABORATORY FINDINGS**

In patients with the clinical features of hemolytic anemia, laboratory tests typically show evidence of increased erythrocyte destruction and the compensatory increase in the rate of erythropoiesis. Other tests that are specific to a particular diagnosis also may be indicated.
Tests of Accelerated Red Blood Cell Destruction

Visual examination of the plasma and urine may suggest fragmentation hemolysis. The presence of methemoglobin, methemalbumin and hemopexin-heme impart a coffee-brown color to plasma, strongly suggestive of fragmentation hemolysis. When these compounds are present in urine, the color is more often described as root beer- or beer-colored. In a properly collected blood specimen, the normal physiologic fragmentation hemolysis produces a plasma hemoglobin level of less than 1 mg/dL. Plasma does not become visibly red/brown until the plasma hemoglobin level is at 50 mg/dL. Typical values during hemolytic processes may be as low as 15 mg/dL, so an increase in plasma hemoglobin may not always be visible.

Hemoglobin/heme from fragmentation hemolysis can be detected in urine when the capacity of the plasma salvage systems is exceeded and the hemoglobin/heme is filtered into the urine. The urinalysis test strip for blood can be positive even when the hemoglobin is not present in a high enough concentration to change the color of the urine significantly. Since the product entering the urine is free hemoglobin/heme, the sediment will be negative for red blood cells. However, renal tubular cells sloughed into the filtrate during the period of hemoglobinuria can demonstrate deposits of hemosiderin (iron), resulting from absorbed hemoglobin, when stained with Prussian blue stain.

For a patient with a hemolytic process, a complete blood count (CBC) may provide clues to the cause. Spherocytes can be expected to be seen with macrophage-mediated hemolysis (Table 23-2), whereas fragmented cells, or schistocytes, are noted with fragmentation hemolysis. Other clues to the particular cause of the hemolysis may be present in the morphology, such as ring forms in malaria, sickle cells in sickle cell anemia, target cells and microcytes in thalassemia, and spherocytes in hereditary spherocytosis or immune-related hemolysis.

In either fragmentation or macrophage-mediated hemolysis, the increased rate of hemoglobin catabolism results in increased amounts of plasma unconjugated bilirubin and carbon monoxide. If liver function is normal, conjugated bilirubin is formed and excreted as urobilinogen in the stool, and the serum level of conjugated bilirubin remains within the reference interval. No bilirubin is detected in the urine because unconjugated bilirubin is bound to albumin and the complex is not filtered by the glomerulus. The urinary urobilinogen level may be increased, however, because there is increased urobilinogen in the stool, and more than usual amounts are absorbed by the portal circulation. In some patients, serum indirect (unconjugated) bilirubin values can be misleadingly low because the amount of bilirubin in the blood depends on the rate of RBC catabolism as well as hepatic function. If the rate of hemolysis is low and liver function is normal, the total serum bilirubin level can be within the reference interval. Quantitative measurements of fecal urobilinogen, however, would demonstrate an increase.

A substantial decline in the serum haptoglobin level indicates fragmentation hemolysis. In what is mostly a macrophage-mediated hemolysis, there can still be a minor component of fragmentation lysis involving spherical cells that are fragile, so a modest decline in haptoglobin level can be seen. In short, whenever the level of hemoglobin in the plasma increases, the haptoglobin level declines. In one study, a low haptoglobin level indicated an 87% probability of hemolytic disease. Haptoglobin measurement is, however, prone to both false-positive and false-negative results. Low values suggest hemolysis but may be due instead to impaired synthesis of haptoglobin caused by liver disease. Alternatively, a patient with hemolysis may have a relatively normal haptoglobin level if there is also a complicating infection or inflammation, because haptoglobin is an acute phase reactant. Although quantitation of hemopexin may also demonstrate a low value, it is not often measured, relying instead on the more dramatic haptoglobin decline to detect fragmentation hemolysis.

Tests to determine the rate of endogenous carbon monoxide production have been developed, because carbon monoxide is produced in the first step of heme breakdown by heme oxygenase. Values of 2 to 10 times the normal rate have been detected in some patients with hemolytic anemia, but testing for carbon monoxide production is not typically required for clinical diagnosis.

Other laboratory test results are incidentally abnormal. Serum lactate dehydrogenase activity is often increased in patients with fragmentation hemolysis due to the release of the enzyme from ruptured RBCs, but other conditions, such as myocardial infarction or liver disease, also can cause increases. Although enzyme isofrom fractionation could be used to identify lactate dehydrogenase of RBC origin, this test is generally not needed. Rather, when other results point to fragmentation hemolysis, one should expect an increase in the level of serum lactate dehydrogenase and other RBC enzymes and should not be misled into assuming that there is liver damage (Table 23-2).

General evidence of reduced RBC survival can be gleaned by measuring glycated hemoglobin (by the Hb A1c test). Glycated hemoglobin increases over the life of a cell as it is exposed to plasma glucose. Glycated hemoglobin level is usually decreased in chronic hemolytic disease because the cells have less exposure to plasma glucose before lysis. The mean reference interval for glycated hemoglobin in one report was 6.7%; in a hemolytic process, the mean value decreased to 3.9%. The magnitude of the decrease is related to the magnitude of the hemolytic process over the previous 4- to 8-week period. Glycated hemoglobin level is not a reliable indicator of shortened RBC survival in patients with diabetes mellitus because of the increased rate of glycation with elevated blood glucose levels. Thus glycated hemoglobin measurement is more useful for diagnosis or monitoring blood glucose control in diabetic patients, rather than detection of hemolysis.

A more exact RBC survival assay uses random labeling of blood with chromium radioisotope. This is the reference method for RBC survival studies published by the International Committee for Standardization in Haematology. A sample of blood is collected, mixed with the isotope, and returned to the patient. The labeled cells are of all ages, reflecting normal peripheral blood. This method differs from cohort labeling in which RBCs are labeled with radioactive iron or heavy nitrogen.
as they are produced in the bone marrow, so the labeled cells are generally of the same age. In both methods, the disappearance of the label from the blood is measured over time. As measured using the random chromium labeling technique, the normal half-time of chromium is 25 to 32 days.\(^\text{26}\) A half-time of 20 to 25 days suggests mild hemolysis; 15 to 20 days, moderate hemolysis; and less than 15 days, severe hemolysis.\(^\text{26}\) There are clinical instances when an estimation of red cell survival would be useful for assessing erythropoietin treatment.\(^\text{27}\) However, both cohort and random techniques have significant limitations,\(^\text{28}\) in addition to being time consuming, expensive, and requiring the use of radioactive isotopes. Therefore, these methods are not often used clinically but are used for research, particularly in the search for improved methods of determining red blood cell survival.

**Tests of Increased Erythropoiesis**

If the bone marrow is healthy, the hypoxia associated with hemolysis leads to increased erythropoiesis. Recognition of this increase may be a first clue to the presence of a hemolytic process. Laboratory findings indicating increased erythropoiesis include an increase in circulating reticulocytes and, in severe cases, nucleated red blood cells (Table 23-3). These findings are persistently present in chronic hemolytic disease and are evident within 3 to 6 days after an acute hemolytic episode. Increased erythropoiesis is not unique to hemolytic anemias and is not diagnostic. Similar results are expected after hemorrhage and with successful specific therapy for anemia caused by iron, folate, or vitamin \(B\)\(_{12}\) deficiency. An assessment of erythropoiesis can determine the effectiveness of the bone marrow response, however, and should be factored into the differential diagnosis (see Differential Diagnosis).

**Complete Blood Count and Morphologic Features**

Peripheral blood film evaluation is crucial. An increase in polychromatic RBCs (reticulocytes) and nucleated RBCs represents bone marrow compensation for hemolysis or blood loss. Spherocytes are expected with excessive fragmentation hemolysis, while schistocytes may be seen with macrophage-mediated processes. Additional morphologic changes to red blood cells may point toward the etiology of the hemolysis (see below).

An increase in the mean cell volume (MCV) is usually seen with extreme compensatory reticulocytosis resulting from the larger, prematurely released “shift” reticulocytes. The increase must be assessed by comparison with the value seen early in hemolysis, before the shift reticulocytes have emerged. The MCV may not increase above the reference interval but rather may be above the baseline value for that patient. Exceptions occur if the hemolytic condition itself involves smaller cells that counter the increased volume of the reticulocytes. In hereditary spherocytosis, microspherocytes are the cause of the anemia, and the MCV may be within the reference interval even when larger shift reticulocytes are generated—hence the importance of a baseline value for comparison. In other circumstances, such as severe burns, numerous schistocytes or microspherocytes may outnumber the reticulocytes so that the MCV remains low.

Leukocytosis and thrombocytosis may accompany acute hemolytic anemia and are considered reactions to the hemolytic process. Conversely, conditions that directly cause leukocytosis, such as sepsis, might cause hemolysis. Low platelet counts in association with other signs of hemolysis may indicate a platelet-consuming microangiopathic process, such as disseminated intravascular coagulopathy.

**Reticulocyte Count**

The reticulocyte count is the most commonly used test to detect accelerated erythropoiesis and is expected to rise during hemolysis or hemorrhage. Assuming the bone marrow is healthy and there are adequate raw materials, all measures of reticulocyte production should rise: absolute reticulocyte count, relative reticulocyte count, reticulocyte production index, and the immature reticulocyte fraction. The association of reticulocytosis with hemolysis is so strong that if an anemic patient has an elevated reticulocyte count and hemorrhage is ruled out, a cause of hemolysis should be investigated. The reticulocyte increase usually correlates well with the severity of the hemolysis. Exceptions occur during aplastic crises of some hemolytic anemias and in some immunohemolytic anemias with hypoplastic marrow, which suggests that the autoantibodies were directed against the bone marrow RBC precursors and circulating erythrocytes.\(^\text{21}\) Chapters 14 and 19 describe the interpretation of reticulocyte indices in patients with anemia.

**Bone Marrow Examination**

Bone marrow examination is usually not necessary to diagnose hemolytic anemia. If conducted, however, bone marrow examination will reveal erythroid hyperplasia that results in peripheral blood reticulocytosis. As the erythroid component (the denominator) of the myeloid-to-erythroid ratio increases, the overall ratio decreases. (The myeloid-to-erythroid ratio is defined in Chapter 17.) As always, the cellularity of the bone marrow should be determined on a core biopsy specimen, rather than an aspirated specimen, for a more accurate judgment.

**Laboratory Tests to Determine Specific Hemolytic Processes**

As noted above, the appearance of spherocytes or schistocytes on a peripheral blood film can point to a hemolytic cause for anemia. Other abnormalities found on the film,

### TABLE 23-3 Hematologic Findings Indicating Accelerated Red Blood Cell Production

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Findings</th>
</tr>
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<tbody>
<tr>
<td>Anticoagulated peripheral blood</td>
<td>Increased absolute reticulocyte count, immature reticulocyte fraction, reticulocyte production index</td>
</tr>
<tr>
<td></td>
<td>Rising mean cell volume (compared with baseline)</td>
</tr>
<tr>
<td>Polychromasia, nucleated RBCs</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Erythroid hyperplasia</td>
</tr>
</tbody>
</table>

\(RBC,\) Red blood cell.
such as elliptocytes, acanthocytes, burr cells, sickle cells, target cells, agglutination, erythrophagocytosis, or parasites, may help reveal the specific disorder causing the hemolysis (Table 23-4).

Other tests dealing with specific types of hemolytic anemia are discussed in subsequent chapters. They include the direct antiglobulin test, osmotic fragility test, eosin-5-maleimide binding test, Heinz body test, RBC enzyme studies, serologic tests, and immunophenotyping.

**DIFFERENTIAL DIAGNOSIS**

The differential diagnosis of hemolytic anemias incorporates several intersecting lines of deduction. The first is to establish the hemolytic nature of the anemia. A rapid decrease in hemoglobin concentration (e.g., 1 g/dL per week) from levels previously within the reference interval can signal hemolysis when hemorrhage and hemodilution have been ruled out. Jaundice and reticulocytosis provide additional confirmation of a hemolytic cause for an anemia of at least several days’ duration. When only the indirect (unconjugated) fraction of the total serum bilirubin is elevated, hemolytic jaundice is confirmed. An elevated urinary urobilinogen level strengthens the conclusion.

The rapid decrease in hemoglobin during an acute hemolytic episode, however, is usually apparent before reticulocytosis and bilirubinemia develop. For acute hemolysis, hemoglobinemia and hemoglobinuria are expected with fragmentation causes; therefore, their absence suggests a macrophage-mediated cause. RBC morphology and haptoglobin levels can assist in differentiating fragmentation from a macrophage-mediated cause. Figure 23-7 is a graphic representation of the general time line of the events in acute fragmentation and macrophage-mediated hemolysis. In chronic hemolysis, persistence of hemoglobinemia, hemoglobinuria, decreased serum haptoglobin level, indirect bilirubinemia, and reticulocytosis can be expected, depending on the mechanism of the hemolysis.

Hemolytic anemias must be differentiated from other anemias associated with bilirubinemia, reticulocytosis, or both. Anemia with reticulocytosis but without bilirubinemia is expected during recovery from hemorrhage not treated with transfusion or with effective treatment of deficiencies such as iron deficiency. Anemia that results from hemorrhage into a body cavity is characterized by reticulocytosis during recovery and bilirubinemia due to catabolism of the hemoglobin in the
TABLE 23-5  Differential Diagnosis of Hemolytic Anemias Versus Other Causes of Indirect Bilirubinemia and Reticulocytosis

<table>
<thead>
<tr>
<th>Hemoglobin Level</th>
<th>Indirect Bilirubinemia</th>
<th>Reticulocytosis</th>
<th>Spherocytes or Schistocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolytic anemia—acute, fragmentation</td>
<td>Rapidly dropping</td>
<td>Delayed</td>
<td>Delayed</td>
</tr>
<tr>
<td>Hemolytic anemia—acute, macrophage-mediated</td>
<td>Rapidly dropping</td>
<td>Delayed</td>
<td>Delayed</td>
</tr>
<tr>
<td>Hemolytic anemia—chronic, fragmentation</td>
<td>Persistently low</td>
<td>Persistent</td>
<td>Persistent</td>
</tr>
<tr>
<td>Hemolytic anemia—chronic, macrophage-mediated</td>
<td>Persistently low</td>
<td>Persistent</td>
<td>Persistent</td>
</tr>
<tr>
<td>Acute hemorrhage</td>
<td>Rapidly dropping</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Hemodilution</td>
<td>Rapidly dropping</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Recovery from hemorrhage</td>
<td>Rising</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Treated anemia (iron, vitamin B₁₂, or folate deficiency)</td>
<td>Rising</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Hemorrhage into a body cavity</td>
<td>Rapidly dropping</td>
<td>Delayed</td>
<td>Delayed</td>
</tr>
<tr>
<td>Ineffective erythropoiesis (e.g., megaloblastic anemia)</td>
<td>Dropping</td>
<td>Persistent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

hemorrhaged cells. The RBC morphology should remain normal throughout the event. Anemias associated with ineffective erythropoiesis, such as megaloblastic anemia, are essentially hemolytic, with the cell death occurring in the bone marrow. Bilirubinemia and elevated serum lactate dehydrogenase levels are to be expected, but the reticulocyte count is low. Because most of the RBCs never reach the periphery, such anemias are typically classified as anemias of diminished production rather than as hemolytic anemias. As summarized in Table 23-5, the differential diagnosis in each of these instances may rely on negative results of tests for increased cell destruction or accelerated production.

**SUMMARY**

- A hemolytic disorder is a condition in which there is increased destruction of erythrocytes and a compensatory acceleration in erythrocyte production by the bone marrow.
- A hemolytic anemia develops when the bone marrow is unable to compensate for the shortened survival of the RBCs.
- Hemolytic anemias can be classified as acute or chronic, intravascular or extravascular, acquired or inherited, intrinsic or extrinsic, and fragmentation or macrophage-mediated.
- Normally, most erythrocyte death occurs via macrophages of the spleen and liver. A small amount occurs by fragmentation due to mechanical trauma.
- Hemoglobin from the RBCs is converted to heme and globin within macrophages. Heme is further degraded to iron, carbon monoxide, and unconjugated bilirubin. The bilirubin is secreted into the plasma, where it binds to albumin and is transported to the liver. In the liver, the bilirubin is conjugated with glucuronic acid, excreted as bile into the intestines, and converted to urobilinogen. Some urobilinogen is reabsorbed into the portal circulation and reexcreted through the liver. A small amount of urobilinogen remains in the plasma, and it is excreted by the kidney into the urine.
- In macrophage-mediated (extravascular) hemolytic anemia, there is a delayed increase in unconjugated bilirubin in the plasma and an increase in urobilinogen in the stool and urine. Spherocytes may be seen on the blood film.
- In macrophage-mediated (intravascular) hemolysis include (meth)hemoglobinemia, (meth)hemoglobinuria, and hemosiderinuria. Serum haptoglobin is markedly decreased or absent. Schistocytes may be seen on the blood film.
- Jaundice can result from increased serum unconjugated bilirubin during any hemolytic anemia.
- The major clinical features of chronic inherited hemolytic anemia are varying degrees of anemia, jaundice, splenomegaly, and the development of cholelithiasis. In children, bone abnormalities may develop as a result of accelerated erythropoiesis.
- Laboratory studies providing evidence of hemolytic anemia include tests for increased erythrocyte destruction and compensatory increase in the rate of erythropoiesis. Elevated serum indirect bilirubin level with a normal serum direct bilirubin level suggests accelerated RBC destruction. A moderate to marked decrease in serum haptoglobin level suggests a fragmentation cause of hemolysis. The reticulocyte count is the most commonly used laboratory test to identify accelerated erythropoiesis, including an elevation of the immature reticulocyte fraction. Other tests that are specific to a particular diagnosis also may be needed.
- Hemolytic anemias must be differentiated from other anemias with reticulocytosis, including the post-acute hemorrhage state and recovery from iron, vitamin B₁₂, or folate deficiency, and from those with bilirubinemia, such as with internal bleeding.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.
Answers can be found in the Appendix.

1. The term hemolytic disorder in general refers to a disorder in which there is:
   a. Increased destruction of RBCs after they enter the bloodstream
   b. Excessive loss of RBCs from the body
   c. Inadequate RBC production by the bone marrow
   d. Increased plasma volume with unchanged red cell mass

2. RBC destruction that occurs when macrophages ingest and destroy RBCs is termed:
   a. Extracellular
   b. Macrophage-mediated
   c. Intra-organ
   d. Extrahematopoietic

3. A sign of hemolysis that is typically associated with both fragmentation and macrophage-mediated hemolysis is:
   a. Hemoglobinuria
   b. Hemosiderinuria
   c. Hemoglobinemia
   d. Elevated urinary urobilinogen level

4. An elderly white woman is evaluated for worsening anemia, with a decrease of approximately 0.5 mg/dL of hemoglobin each week. The patient is pale, and her skin and eyes are slightly yellow. She complains of extreme fatigue and is unable to complete the tasks of daily living without napping in midsomning and midafternoon. She also tires with exertion, finding it difficult to climb even five stairs. Which of the features of this description points to a hemolytic cause for her anemia?
   a. Pallor
   b. Yellow skin and eyes
   c. Need for naps
   d. Tiredness on exertion

5. Which of the following tests provides a good indication of accelerated erythropoiesis?
   a. Urine urobilinogen level
   b. Hemosiderin level
   c. Reticulocyte count
   d. Glycated hemoglobin level

6. A 5-year-old girl was seen by her physician several days prior to this visit and was diagnosed with pneumonia. Her mother has brought her to the physician again because the girl’s urine began to darken after the first visit and now is alarmingly dark. The girl has no history of anemia, and there is no family history of any hematologic disorder. The CBC shows a mild anemia, polychromasia, and a few schistocytes. This anemia could be categorized as:
   a. Acquired, fragmentation
   b. Acquired, macrophage-mediated
   c. Hereditary, fragmentation
   d. Hereditary, macrophage-mediated

7. A patient has a personal and family history of a mild hemolytic anemia. The patient has consistently elevated levels of total and indirect serum bilirubin and urinary urobilinogen. The serum haptoglobin level is consistently decreased, whereas the reticulocyte count is elevated. The latter can be seen as polychromasia on the patient’s blood film, along with spherocytes. Which of the findings reported for this patient is inconsistent with a classical diagnosis of fragmentation hemolysis?
   a. Elevated total and indirect serum bilirubin
   b. Elevated urinary urobilinogen
   c. Decreased haptoglobin
   d. Spherocytes on the peripheral film

8. Select the statement that is true about bilirubin metabolism.
   a. Indirect bilirubin is formed in the liver by the addition of two sugar molecules to direct bilirubin.
   b. Macrophages of the spleen liberate bilirubin during hemoglobin catabolism.
   c. Urobilinogen is not water soluble and is not excreted in the urine.
   d. Normally, the major fraction of bilirubin in the blood is the direct (conjugated) form released from macrophages.

9. A patient has anemia that has been worsening over the last several months. The hemoglobin level has been declining slowly, with a drop of about 1.5 g/dL of hemoglobin over about 6 weeks. Polychromasia and anisocytosis are seen on the blood film, consistent with the elevated reticulocyte count and RBC distribution width (RDW). Serum levels of total bilirubin and indirect fractions are normal. Urinary urobilinogen level also is normal. When these findings are evaluated, the conclusion is drawn that the anemia does not have a hemolytic component. Based on the data given here, why was hemolysis ruled out as the cause of the anemia?
   a. The decline in hemoglobin is too gradual to be associated with hemolysis.
   b. The elevation of the reticulocyte count suggests a malignant cause.
   c. Evidence of increased protoporphyrin catabolism is lacking.
   d. Elevated RDW points to an anemia of decreased production.

10. Which of the following sets of test results is typically expected with chronic fragmentation hemolysis?

<table>
<thead>
<tr>
<th>Serum Haptoglobin</th>
<th>Urine Hemoglobin</th>
<th>Urine Sediment Prussian Blue Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Increased</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>b. Decreased</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>c. Decreased</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>d. Increased</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>
REFERENCES


Intrinsic Defects Leading to Increased Erythrocyte Destruction

Elaine M. Keohane

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the intrinsic cell properties that affect red blood cell (RBC) deformability.
2. Explain how defects in vertical and horizontal membrane protein interactions can result in a hemolytic anemia.
3. Compare and contrast the inheritance pattern, membrane proteins mutated, mechanism of hemolysis, typical RBC morphology, and clinical and laboratory findings of hereditary spherocytosis, hereditary elliptocytosis, and hereditary ovalocytosis.
4. Compare and contrast the RBC morphology and laboratory findings of hereditary spherocytosis and immune-associated hemolytic anemias.
5. Explain the principle, interpretation, and limitations of the osmotic fragility and eosin-5'-maleimide (EMA) binding tests in the diagnosis of hereditary spherocytosis.
6. Describe the causes, pathophysiology, RBC morphology, and clinical and laboratory findings of hereditary anemias characterized by stomatocytosis.
7. Describe the causes and pathophysiology of hereditary and acquired conditions characterized by acanthocytosis.
8. Describe the cause, pathophysiology, clinical manifestations, laboratory findings, and treatment for paroxysmal nocturnal hemoglobinuria.
9. Compare and contrast the inheritance pattern, pathophysiology, clinical symptoms, and typical laboratory findings of glucose-6-phosphate dehydrogenase deficiency and pyruvate kinase deficiency.
10. Given the history, symptoms, laboratory findings, and a representative microscopic field from a peripheral blood film for a patient with a suspected intrinsic hemolytic anemia, discuss possible causes of the anemia and indicate the data that support these conclusions.

OUTLINE

Red Blood Cell Membrane Abnormalities

Red Blood Cell Membrane Structure and Function

Hereditary Red Blood Cell Membrane Abnormalities

Acquired Red Blood Cell Membrane Abnormalities

Red Blood Cell Enzymopathies

Glucose-6-Phosphate Dehydrogenase Deficiency

Pyruvate Kinase Deficiency

Other Enzymopathies

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 45-year-old man sought medical attention for the onset of chest pain. Physical examination revealed slight jaundice and splenomegaly. The medical history included gallstones, and there was a family history of anemia. A CBC yielded the following results:

<table>
<thead>
<tr>
<th>Patient Results</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (×10^9/L)</td>
<td>3.6–10.6</td>
</tr>
<tr>
<td>RBCs (×10^12/L)</td>
<td>4.60–6.00</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>14.0–18.0</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40–54</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>80–100</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>26–32</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32–36</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>Platelets (×10^9/L)</td>
<td>150–450</td>
</tr>
</tbody>
</table>

Continued
Intrinsic hemolytic anemias comprise a large group of disorders in which defects in the red blood cells (RBCs) themselves result in premature hemolysis and anemia. Intrinsic disorders can be divided into abnormalities of the RBC membrane, metabolic enzymes, or hemoglobin. Most of these defects are hereditary. This chapter covers defects in the RBC membrane and enzymes causing hemolytic anemia. Chapter 27 covers qualitative hemoglobin disorders, and Chapter 28 covers quantitative hemoglobin disorders.

**RED BLOOD CELL MEMBRANE ABNORMALITIES**

**Red Blood Cell Membrane Structure and Function**

The RBC maintains a biconcave discoid shape that is essential for normal function and survival for 120 days in the peripheral circulation. The key to maintaining this shape is the plasma membrane, a lipid bilayer embedded with proteins and connected to an underlying protein cytoskeleton (Figure 9-2). The insoluble lipid portion serves as a barrier to separate the vastly different ion and metabolite concentrations of the interior of the RBC from its external environment, the blood plasma. The concentration of the constituents in the cytoplasm is tightly regulated by proteins embedded in the membrane that serve as pumps and channels for movement of ions and other material between the RBC’s interior and the blood plasma. Various membrane proteins also act as receptors, RBC antigens, enzymes, and support for the surface carbohydrates to form a protective glycocalyx with the surface glycolipids. The lipid bilayer remains intact because transmembrane proteins embedded in the membrane anchor it to a two-dimensional protein lattice (cytoskeleton) immediately beneath its surface. Together, the transmembrane proteins and underlying cytoskeleton provide structural integrity, cohesion, and mechanical stability to the cell.

In a normal life span of 120 days, RBCs must repeatedly maneuver through very narrow capillaries and squeeze through the splenic sieve (narrow slits or fenestrations in the endothelial cell lining of the splenic sinuses) as they move from the splenic cords to the sinuses. To accomplish this without premature lysis, the RBCs must have deformability, or the ability to repeatedly bend, stretch, distort, and then return to the normal discoid, biconcave shape. The cellular properties that enable RBC deformability include the RBC’s biconcave, discoid geometry; the elasticity (pliancy) of its membrane; and its cytoplasmic viscosity (Chapter 9).

The biconcave, discoid geometry of the RBC is dependent on vertical and horizontal interactions between the transmembrane and cytoskeletal proteins (listed in Tables 9-5 and 9-6). Two transmembrane protein complexes, the ankyrin complex and protein 4.1 complex, provide vertical structural integrity to the cell by anchoring the lipid bilayer to the underlying spectrin cytoskeleton. In the ankyrin complex, ankyrin and protein 4.2 link transmembrane proteins, band 3 and RhAG (the Rh-associated glycoprotein) to the cytoskeleton. In the protein 4.1 complex, protein 4.1 links transmembrane proteins, glycoporphin C, XK, Rh, and Duffy to the cytoskeleton, and adducin and dematin link with transmembrane proteins, band 3 and glucose transport (glut 1) (Figure 9-2). These interactions are called vertical because they are perpendicular to the plane of the cytoskeleton. They prevent loss of membrane and the resultant decrease in the surface area-to-volume ratio of the

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CASE STUDY—cont’d

After studying the material in this chapter, the reader should be able to respond to the following case study:

The peripheral blood film revealed anisocytosis, polychromasia, and spherocytes (Figure 24-1).

1. From the data given, what is a likely cause of the anemia?
2. What additional laboratory tests would be of value in establishing the diagnosis, and what abnormalities in the results of these tests would be expected to confirm your impression?
3. What is the cause of this type of anemia?

![Figure 24-1](Image) Peripheral blood film for the patient in the case study (×500). (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.)
RBC. Two major cytoskeletal proteins, α-spectrin and β-spectrin, interact laterally with each other to form antiparallel heterodimers, which link with other spectrin heterodimers to form tetramers. The spectrin heterodimers also form a spectrin–actin–protein 4.1 junctional complex with accessory proteins (tropomyosin, tropomodulin, dematin, and adducin), thus linking the spectrin tetramers in a two-dimensional lattice. These proteins provide horizontal mechanical stability, which prevents the membrane from fragmenting in response to mechanical stress.

Factors that impact the elasticity of the cell are not as clear. Interactions between the spectrin dimers and their junctional complexes are flexible and allow for movement as the RBCs stretch and bend (Figure 9-4). In addition, the ability of spectrin repeats to unfold and refold is likely to be one of the determinants of membrane elasticity.

The cytoplasmic viscosity depends on the concentration of hemoglobin, as well as the maintenance of the proper cell volume by the normal functioning of various channels and pumps that allow the passage of ions, water, and macromolecules in and out of the cell.

A defect in the RBCs that changes the membrane geometry, its elasticity, or the viscosity of the cytoplasm affects RBC deformability and can result in premature hemolysis and anemia. The RBC membrane is discussed in more detail in Chapter 9; the reader is encouraged to review that chapter when studying defects in the membrane.

**Hereditary Red Blood Cell Membrane Abnormalities**

Most defects in the RBC membrane that can cause hemolytic anemia are hereditary; however, acquired defects also exist. Hereditary RBC membrane defects have historically been classified by morphologic features. The major disorders are hereditary spherocytosis, characterized by spherocytes, and hereditary elliptocytosis, characterized by elliptical RBCs. Hereditary pyropoikilocytosis, a variant of hereditary elliptocytosis, is characterized by marked poikilocytosis and heat sensitivity. Other less common membrane disorders include hereditary ovalocytosis, overhydrated hereditary stomatocytosis, and dehydrated hereditary stomatocytosis (also called hereditary xerocytosis). Hereditary membrane defects can also be classified as those that affect membrane structure (and alter geometry and elasticity) and those that affect membrane transport (and alter cytoplasmic viscosity) (Box 24-1). The membrane structural defects can be further divided into those that affect vertical membrane protein interactions and those that affect horizontal membrane protein interactions. The major hereditary membrane defects are described in Table 24-1.

**Mutations That Alter Membrane Structure**

**Hereditary Spherocytosis.** Hereditary spherocytosis (HS) is a heterogeneous group of hemolytic anemias caused by defects in proteins that disrupt the vertical interactions between transmembrane proteins and the underlying protein cytoskeleton. HS has worldwide distribution and affects 1 in 2000 to 3000 individuals of northern European ancestry. In 75% of families, it is inherited as an autosomal dominant trait and is expressed in heterozygotes who have one affected parent. Homozygotes are rare; such patients present with severe hemolytic anemia but have asymptomatic parents. In approximately 25% of cases, the inheritance is nondominant, with some autosomal recessive cases.

**Pathophysiology.** HS results from gene mutations in which the defective proteins disrupt the vertical linkages between the lipid bilayer and the cytoskeletal network. Various mutations in five known genes can result in the HS phenotype (Table 24-1). Mutations can occur in genes for (1) cytoskeletal proteins, including ANKI, which codes for ankyrin (40% to 65% of cases in the United States and Europe; 5% to 10% of cases in Japan); SPTA1, which codes for α-spectrin (fewer than 5% of cases); SPTB, which codes for β-spectrin (15% to 30% of cases); and EPB42, which codes for protein 4.2 (fewer than 5% of cases in the United States and Europe; 45% to 50% of cases in Japan); and (2) transmembrane protein, SLC4A1, which codes for band 3 (20% to 35% of cases). Less than 10% of cases involve de novo mutations, with most affecting the ankyrin gene. A mutation database for hereditary spherocytosis lists 130 different mutations (ANK1, 52; SLC4A1, 49; SPTA1, 2; SPTB, 19; and EPB42, 8). Because of the vertical interactions of the transmembrane proteins and cytoskeletal protein lattice, a primary mutation in one gene may have a secondary effect on another protein in the membrane. For example, primary mutations in ANKI result in both ankyrin and spectrin deficiencies in the RBC membrane. In approximately 10% of patients, no mutation is identified.

The defects in vertical membrane protein interactions cause RBCs to lose unsupported lipid membrane over time because of local disconnections of the lipid bilayer and underlying cytoskeleton. Essentially, small portions of the membrane form vesicles; the vesicles are released with little loss of cell volume. The RBCs acquire a decreased surface area–to–volume ratio, and the cells become spherical. These cells do not have the deformability of normal biconcave discoid RBCs, and their survival in the spleen is decreased. As the spherocytes attempt to move through the narrow, elliptical fenestrations of the endothelial cells lining the splenic sinusoids, they acquire further membrane loss or become trapped and are rapidly removed by the macrophages of the red pulp of the spleen. In addition, as the RBCs are sequestered in the spleen, the
membrane can acquire yet more damage; lose more lipid membrane, and become more spherical due to splenic conditions.\textsuperscript{4,13,15} The conditioning may be enhanced by the acidic conditions in the spleen and the prolonged contact of the RBCs with macrophages.\textsuperscript{13,15} Low levels of adenosine triphosphate (ATP) and glucose, and phagocyte-produced free radicals, which cause oxidative damage, may also play a role (Figure 24-2).\textsuperscript{13,15}

In HS, RBC membranes also have abnormal permeability to cations, particularly sodium and potassium, which is likely due to disruption of the integrity of the protein cytoskeleton.\textsuperscript{4,17} The cells become dehydrated, but the exact mechanism is not clear. Overactivity of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase may cause a reduction in intracellular cations, causing more water to diffuse out of the cell.\textsuperscript{4,13} This results in an increase in viscosity and cellular dehydration.\textsuperscript{13} The abnormality is not related to defects in cation transport proteins, and dehydration occurs regardless of the type of primary mutation causing the HS.\textsuperscript{17}

### Clinical and Laboratory Findings

Symptomatic HS has three key clinical manifestations: anemia, jaundice, and splenomegaly. Symptoms of HS may first appear in infancy, childhood, or adulthood, or even at an advanced age.\textsuperscript{16} There is wide variation in symptoms. Silent carriers are clinically asymptomatic with normal laboratory findings and usually are identified only if they are the parents of a child with recessively inherited HS.\textsuperscript{4,13,16} Approximately 20% to 30% of patients have...
mild HS and are asymptomatic because an increase in erythropoiesis compensates for the RBC loss. They usually have normal hemoglobin levels but may show subtle signs of HS, with a slight increase in serum bilirubin in the range of 1.0 to 2.0 mg/dL, an increased reticulocyte count of up to 6%, and a few spherocytes on the peripheral blood film. Mild HS may first become evident during pregnancy, during illnesses that cause splenomegaly (such as infectious mononucleosis), or in aging, when the rate of erythropoiesis starts to decline. Approximately 60% of patients have moderate HS with incompletely compensated hemolytic anemia, hemoglobin levels greater than 8 g/dL, serum bilirubin over 2 mg/dL, reticulocyte counts in the range of 6% to 10%, and spherocytes on the peripheral blood film. Jaundice is seen at some time in about half of these patients, usually during viral infections. About 5% to 10% of patients have moderate to severe HS, with hemoglobin levels usually in the range of 6 to 8 g/dL, serum bilirubin between 2 to 3 mg/dL, and reticulocyte counts greater than 10%. About 3% to 5% of patients have severe HS, with hemoglobin levels below 6 g/dL, serum bilirubin over 3 mg/dL, and reticulocyte counts greater than 10%. Patients with severe HS are usually homozygous for HS mutations and require regular transfusions. Splenomegaly is found in about half of young children and in 75% to 95% of older children and adults with HS.

The hallmark of HS is spherocytes on the peripheral blood film. When present in patients with childhood hemolytic anemia and a family history of similar abnormalities, the uniform spherocytes are highly suggestive of HS. Some of these are microspherocytes—small, round, dense RBCs that are filled with hemoglobin and lack a central pallor. Normal-appearing RBCs, along with polychromasia and varying degrees of anisocytosis and poikilocytosis, are present. In addition to spherocytes, occasionally other RBC morphologic variants may be observed in some types of mutations: acanthocytes in some β-spectrin mutations, pincered or mushroom-shaped cells in some cases of band 3 deficiency in patients without splenectomy, and ovalostomatocytes in homozygous EPB4.2 mutations. Note that spherocytes are not specific for HS and can be seen in other hereditary and acquired conditions.

Figure 24-2 Pathophysiology of splenic trapping and destruction of spherocytes. ATP, Adenosine triphosphate; RBC, red blood cell. (Modified from Becker PS, Lux SE: Disorders of the red cell membrane. In Nathan DG, Oski FA, editors: Hematology of infancy and childhood, ed 4, Philadelphia, 1993, Saunders, pp. 529-633.)
leaving the cell because the intracellular and extracellular osmolarity is the same. In a hypotonic solution, more water will enter the cell to dilute the intracellular contents until equilibrium is reached between the cytoplasm and the hypotonic extracellular solution. As this phenomenon occurs, the cells swell. As the RBCs are subjected to increasingly hypotonic solutions, even more water will enter the RBCs until the internal volume is too great and lysis occurs. Because spherocytes already have a decreased surface area-to-volume ratio, they lyse in less hypotonic solutions than normal-shaped, biconcave RBCs and thus have increased osmotic fragility.

In the procedure, a standard volume of fresh, heparinized blood is mixed with NaCl solutions ranging from 0.85% (isotonic saline) to 0.0% (distilled water) in 0.05% to 0.1% increments. After a 30-minute incubation at room temperature, the tubes are centrifuged and the absorbance of the supernatant is measured spectrophotometrically at 540 nm. The percent hemolysis is calculated for each tube as follows:

\[
\% \text{ hemolysis} = \frac{A_{x\%} - A_{0.85\%}}{A_{0.0\%} - A_{0.85\%}} \times 100
\]

\(A_{x\%}\) is the absorbance in the tube being measured, \(A_{0.85\%}\) is the absorbance in the 0.85% NaCl tube (representing 0% hemolysis), and \(A_{0.0\%}\) is the absorbance in the 0.0% NaCl tube (representing 100% hemolysis). The % hemolysis for each % NaCl concentration is plotted, and an osmotic fragility curve is drawn (Figure 24-4). Normal biconcave RBCs show initial hemolysis at 0.45% NaCl, and 100% or complete hemolysis generally occurs between 0.35% and 0.30% NaCl. If the curve is shifted to the left, the patient’s RBCs have increased osmotic fragility, and in this case, initial hemolysis begins at an NaCl concentration greater than 0.5%. Conversely, if the curve is shifted to the right, the RBCs have decreased osmotic fragility.

Additional tests. In patients with a family history of HS, splenomegaly, an increased MCHC and reticulocyte count, and spherocytes on the peripheral blood film, no further special testing is needed for the diagnosis. In cases in which HS is suspected, but the family history and mode of inheritance are not clear, or there are atypical clinical and/or laboratory findings, further special testing is needed to confirm a diagnosis. No one method will detect all cases of HS, so a combination of methods is needed for definitive diagnosis.

Osmotic fragility. The osmotic fragility test demonstrates increased RBC fragility in blood specimens in which the RBCs have decreased surface area-to-volume ratios. Blood is added to a series of tubes with increasingly hypotonic sodium chloride (NaCl) solutions. In each tube, water enters and leaves the RBCs until equilibrium is achieved. In 0.85% NaCl, the amount of water entering the cell is equivalent to the water hyperplasia due to the increased demand for RBCs to replace the circulating spherocytes that are prematurely destroyed, but bone marrow analysis is not required for diagnosis.

Figure 24-3 Spherocytes (peripheral blood, ×1000).

Figure 24-4 Erythrocyte osmotic fragility curve. A, Curve in thalassemia showing two cell populations: one with increased fragility (lower left of curve) and one with decreased fragility (upper right of curve). B, Normal curve. C, Curve indicating increased fragility, as in hereditary spherocytosis.
Decreased osmotic fragility is found in conditions characterized by numerous target cells, such as thalassemia. The existence of a distinct subpopulation of the most fragile cells, those most conditioned by the spleen, is reflected by the presence of a “tail” on the osmotic fragility curve (Figure 24-4). After splenectomy is performed, the osmotic fragility improves, and this subpopulation of conditioned cells disappears.

Incubating the blood at 37 °C for 24 hours before performing the test (called the incubated osmotic fragility test) allows HS cells to become more spherical and is often needed to detect mild cases. Patients who have increased osmotic fragility only when their blood is incubated tend to have mild disease and a low number of spherocytes in the total RBC population. The osmotic fragility test is time-consuming, and it requires a fresh heparinized blood specimen collected without trauma (to avoid hemolysis) and accurately made NaCl solutions. Specimens are stable for 2 hours at room temperature or 6 hours if the specimen is refrigerated. A major drawback of the osmotic fragility test is its lack of sensitivity. In 2011 a study of 150 HS patients by Bianchi and colleagues, the sensitivity for the unincubated test was 68%, with only a modest increase in sensitivity to 81% with the incubated test. In nonsplenectomized HS patients with compensated anemia, those sensitivity figures dropped to only 53% and 64%, respectively. The osmotic fragility test is also nonspecific. A result indicating increased fragility does not differentiate between HS and spherocytosis caused by other conditions, such as burns, immune hemolytic anemias, and other acquired disorders. These disadvantages have led some not to recommend this test for routine use.

Eosin-5′-maleimide (EMA) binding test. The eosin-5′-maleimide (EMA) binding test has been proposed as a more sensitive alternative for confirmation of HS. EMA is a fluorescent dye that binds to transmembrane proteins band 3, Rh, RhAg, and CD47 in the RBC membrane. When measured in a flow cytometer, specimens from HS patients show a lower mean fluorescence intensity (MFI) than RBCs from normal controls and from patients with spherocytes due to immune-mediated hemolysis. The result is reported in % decrease in MFI when the patient specimen is compared to normal controls. The sensitivity and specificity of the EMA-binding assay varies from 93% to 96% and 94% to 99%, respectively. Positive tests can also occur with congenital dyserythropoietic anemia type II, Southeast Asian ovalocytosis, and hereditary pyropoikilocytosis, but these are rare conditions. The EMA binding test offers advantages in that it is suitable for low-volume pediatric specimens, it can be performed within 3 hours, specimens are acceptable for analysis up to 7 days after collection, and gating can be used to eliminate the interference from transfused or fragmented RBCs. However, there is disagreement among laboratories on the % MFI decrease cutoff value for HS, and standardization is needed across laboratories.

Other tests. In atypical HS cases, additional tests may be required to identify the defective proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to identify membrane protein deficiencies by electrophoretic separation of the various proteins in solubilized RBC membranes with quantitation of the proteins by densitometry. Membrane proteins can also be quantitated by radioimmunoassay. Variation in membrane surface area and cell water content can be determined by osmotic gradient ektacytometry. The ektacytometer is a laser-diffraction viscometer that records the laser diffraction pattern of a suspension of RBCs exposed to constant shear stress in solutions of varying osmolality from hypotonic to hypertonic. An RBC osmotic deformability index is calculated and plotted against the osmolality of the suspending solution to generate an osmotic gradient deformability profile. This method, however, is available only in specialized laboratories.

Several other tests have been used for diagnosis of HS, but they are cumbersome to perform; lack sensitivity and specificity, or both; and are not widely used in the United States. The acidified glycerol lysis test measures the amount of hemolysis after patient RBCs are incubated with a buffered glycerol solution at an acid pH. The test has a sensitivity of 95% but is not specific in that other conditions with spherocytes will yield a positive result, including autoimmune hemolytic anemias. In the autohemolysis test, patient’s RBCs and serum are incubated for 48 hours, with and without glucose. Normal controls generally have less than 5.0% hemolysis at the end of the 48-hour incubation period, and they have less than 1.0% hemolysis if glucose is added. Glucose catabolism (anaerobic glycolysis) provides the ATP to drive the cation pumps to help maintain the osmotic balance in the RBCs. In HS, the hemolysis is 10% to 50%, which corrects considerably but not to the reference interval when glucose is added. The test has a sensitivity similar to that of the incubated osmotic fragility test. The hypertonic cryohemolysis test is based on the fact that cells from HS patients are particularly sensitive to cooling at 0 °C in hypertonic solutions. The percent hemolysis is calculated after the patient’s RBCs are incubated in buffered 0.7 mol/L sucrose, first at 37 °C for 10 minutes and then at 0 °C for 10 minutes. Normal cells show 3% to 15% hemolysis, whereas RBCs in HS have greater than 20% hemolysis. Increased hemolysis can also occur in Southeast Asian ovalocytosis, some types of hereditary elliptocytosis, and congenital dyserythropoietic anemia type II.

Molecular techniques for detection of genetic mutations are not usually required. The polymerase chain reaction procedure followed by single-strand conformational polymorphism analysis can identify potential regions in HS genes that may contain a mutation. Once a region has been identified, nucleic acid sequencing can be performed to identify the specific mutation. Genetic diagnosis is likely to become more important in the future.

Complications. Although most patients with HS have a well-compensated hemolytic anemia and are rarely symptomatic, complications may occur that require medical intervention. Patients may experience various crises, classified as hemolytic, aplastic, and megaloblastic. Hemolytic crises are rare and usually associated with viral syndromes. In aplastic crises there is a dramatic decrease in hemoglobin level and reticulocyte count. The crisis usually occurs in conjunction with parvovirus
B19 infection, which suppresses erythropoiesis, and patients can become rapidly and severely anemic, often requiring transfusion. This complication is more common in children, but it can occur in adults. Patients with moderate and severe HS can also develop folic acid deficiency resulting from increased folate utilization to support the chronic erythroid hyperplasia in the bone marrow. This phenomenon is termed megaloblastic crisis and is particularly acute during pregnancy and during recovery from an aplastic crisis. Providing folic acid supplementation to patients with moderate and severe HS avoids this complication. About half of patients, even those with mild disease, also experience cholelithiasis (bilirubin stones in the gallbladder or bile ducts) due to the chronic hemolysis. Chronic ulceration or dermatitis of the legs is a rare complication.

**Treatment.** Mild HS usually requires no treatment. Splenectomy is reserved for moderate to severe cases, and laparoscopy is the recommended method. Splenectomy results in longer RBC survival in the peripheral blood and helps prevent gallstones by decreasing the amount of hemolysis and thus the amount of bilirubin produced. The major drawbacks of splenectomy are the lifelong risk of overwhelming sepsis and death from encapsulated bacteria and an increased risk of cardiovascular disease with age. Prior to splenectomy, patients receive vaccines for pneumococcus, meningococcus, and *H. influenza* type b, and postsplenectomy antibiotics may be recommended. Because infants and young children are especially susceptible to postsplenectomy sepsis, splenectomy is usually postponed until after the age of 6 years. In a nationwide sample of 1657 children (aged 5 to 12 years) with HS who underwent splenectomy, there were no cases of postoperative sepsis and no fatalities from any cause during hospitalization for the surgery. Partial splenectomy has been performed in young children with severe HS, but further evaluation of the risks and benefits of this procedure is needed.

After splenectomy, spherocytes are still apparent on the blood film, and all of the typical changes in RBC morphology seen after splenectomy also are observed, including Howell-Jolly bodies, target cells, and Pappenheimer bodies (Figure 24-5).

**Differential diagnosis.** HS must be distinguished from immune-related hemolytic anemia with spherocytes. Family history and evaluation of family members, including parents, siblings, and children of the patient, help differentiate the hereditary disease from the acquired disorder. The immune disorders with spherocytes are usually characterized by a positive result on the direct antiglobulin test (DAT), whereas the results are negative in HS. The eosin-5'-maleimide (EMA) binding test shows decreased fluorescence typical of HS. Increased osmotic fragility is not diagnostic of HS, because the cells in acquired hemolytic anemia with spherocytes also show increased osmotic fragility. The typical clinical and laboratory findings in HS are summarized in Table 24-2.

**Hereditary Elliptocytosis.** Hereditary elliptocytosis (HE) is a heterogeneous group of hemolytic anemias caused by defects in proteins that disrupt the horizontal or lateral interactions in the protein cytoskeleton. It reportedly exists in all of its forms in 1 in 2000 to 1 in 4000 individuals, but because the majority of cases are asymptomatic, the actual prevalence is not known. The disease is more common in Africa and Mediterranean regions, where there is a high prevalence of malaria. The prevalence in West Africa of certain spectrin mutations associated with HE is between 0.6% and 1.6%. The molecular basis for the association of spherocytosis and malaria is unknown. The inheritance pattern in HE is mainly autosomal dominant, with a small number of autosomal recessive cases.

**Pathophysiology.** HE results from gene mutations in which the defective proteins disrupt the horizontal linkages in the protein cytoskeleton and weaken the mechanical stability of the membrane. The HE phenotype can result from various mutations in at least three genes: SPTA1, which codes for α-spectrin (65% of cases); SPTB, which codes for β-spectrin (30% of cases); and EPB41, which codes for protein 4.1 (5% of cases) (Table 24-1). A mutation database for hereditary elliptocytosis is available and lists 46 different mutations. The spectrin mutations disrupt spectrin dimer interactions and the EPB41 mutations result in weakened spectrin–actin–protein 4.1 junctional complexes. RBCs are biconcave and discoid at first, but become elliptical over time after repeated exposure to the shear stresses in the peripheral circulation. The extent of the disruption of the spectrin dimer interactions seems to be associated with the severity of the clinical manifestations. In severe cases, the protein cytoskeleton is weakened
**TABLE 24-2** Typical Clinical and Laboratory Findings in Hereditary Spherocytosis (HS)\textsuperscript{13,16}

<table>
<thead>
<tr>
<th>Clinical manifestations</th>
<th>Splenomegaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia*</td>
<td></td>
</tr>
<tr>
<td>Jaundice (can be intermittent)</td>
<td></td>
</tr>
<tr>
<td>Mode of inheritance</td>
<td>75% autosomal dominant</td>
</tr>
<tr>
<td>25% nondominant</td>
<td></td>
</tr>
<tr>
<td>Complete blood count results</td>
<td>↓ Hemoglobin*</td>
</tr>
<tr>
<td></td>
<td>↑ Mean cell hemoglobin concentration</td>
</tr>
<tr>
<td></td>
<td>↑ Red cell distribution width</td>
</tr>
<tr>
<td></td>
<td>↑ Reticulocyte count*</td>
</tr>
<tr>
<td></td>
<td>↑ Hyperchromic (hyperdense)</td>
</tr>
<tr>
<td></td>
<td>RBCs**</td>
</tr>
<tr>
<td>Peripheral blood film findings\textsuperscript{1}</td>
<td>Spherocytes</td>
</tr>
<tr>
<td></td>
<td>Polychromasia</td>
</tr>
<tr>
<td>Direct antiglobulin test result</td>
<td>Negative</td>
</tr>
<tr>
<td>Indicators of hemolysis</td>
<td>↓ Serum haptoglobin</td>
</tr>
<tr>
<td></td>
<td>↑ Serum lactate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>↑ Serum indirect bilirubin</td>
</tr>
<tr>
<td>Selected additional tests for atypical cases</td>
<td>Not required for diagnosis of HS with the typical features listed above</td>
</tr>
<tr>
<td></td>
<td>↓ Fluorescence in eosin-5´-maleimide binding test by flow cytometry\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>↑ Osmotic fragility and incubated osmotic fragility tests\textsuperscript{3}</td>
</tr>
<tr>
<td></td>
<td>SDS-PAGE analysis of membrane proteins</td>
</tr>
</tbody>
</table>

*Varies with severity of HS and ability of the bone marrow to compensate for the hemolysis.

**As measured on some automated blood cell analyzers.

\textsuperscript{1}With some rare mutations, acanthocytes, pincered cells, stomatocytes, or ovalocytes may be seen in addition to spherocytes.

\textsuperscript{2}A result within the reference interval does not rule out HS; similar results can be observed in conditions other than HS.

\textsuperscript{3}Increased; ↓ decreased; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Patients with the Leach phenotype lack the Gerbich antigens and glycoprotein C (GPC).\textsuperscript{4} The phenotype is due to a mutation in the genes for GPC and results in the absence of the glycoprotein in the RBC membrane.\textsuperscript{34} The Gerbich antigens are normally expressed on the extracellular domains of GPC and thus are absent in this condition. Heterozygotes have normal RBC morphology, and homozygotes have mild elliptocytosis but no anemia.\textsuperscript{4} The reason for the elliptocyte morphology may be a defect in the interaction between GPC and protein 4.1 in the junctional complex.\textsuperscript{4}

**Clinical and laboratory findings.** The vast majority of patients with HE are asymptomatic, and only about 10% have moderate to severe anemia.\textsuperscript{11} Some may have a mild compensated hemolytic anemia as evidenced by a slight increase in the reticulocyte count and a decrease in haptoglobin level, or develop transient hemolysis in response to other conditions such as viral infections, pregnancy, hypersplenism, or vitamin B\textsubscript{12} deficiency.\textsuperscript{13} Often an asymptomatic patient is diagnosed after a peripheral blood film is examined for another condition. Rarely, heterozygous parents with undiagnosed, asymptomatic HE have offspring who are homozygous or compound heterozygous for their mutation(s) and have moderate to very severe hemolysis. Some of these asymptomatic parents have normal RBC morphology and laboratory tests.\textsuperscript{13}

The characteristic finding in HE is elliptical or cigar-shaped RBCs on the peripheral blood film in numbers that can vary from a few to 100\%\textsuperscript{4} (Figure 24-6). The number of elliptocytes does not correlate with disease severity.\textsuperscript{4} Investigation of elliptocytosis begins by taking a thorough patient and family history and performing a physical examination, and examining the peripheral blood films of the parents.\textsuperscript{3} Other laboratory tests may be needed to rule out other conditions in which elliptocytes may be present, such as iron deficiency anemia, thalassemia, megaloblastic anemia, myelodysplastic syndrome, and primary myelofibrosis.\textsuperscript{4} In these cases, the elliptocytes to such a point that cell fragmentation occurs. As a result, there is membrane loss and a decrease in surface area-to-volume ratio that reduces the deformability of the RBCs. The damaged RBCs become trapped or acquire further damage in the spleen, which results in extravascular hemolysis and anemia. In general, patients who are heterozygous for a mutation are asymptomatic, and their RBCs have a normal life span; those who are homozygous for a mutation or compound heterozygous for two mutations can have moderate to severe anemia that can be life-threatening.\textsuperscript{6,11}

The RBCs in HE patients all show some degree of decreased thermal stability. Cases in which the RBCs show marked RBC fragmentation upon heating were previously classified as hereditary pyropoikilocytosis (HPP). HPP is now considered a severe form of HE that exists in either the homozygous or compound heterozygous state.\textsuperscript{11,13}
Treatment. Asymptomatic HE patients require no treatment. All HE patients who are significantly anemic and show signs of hemolysis respond well to splenectomy. Transfusions are occasionally needed for life-threatening anemia.

Hereditary Ovalocytosis (Southeast Asian Ovalocytosis). Hereditary ovalocytosis or Southeast Asian ovalocytosis (SAO) is a condition caused by a mutation in the gene for band 3 that results in increased rigidity of the membrane and resistance to invasion by malaria. It is common in the malaria belt of Southeast Asia, where its prevalence can reach 30%. The inheritance pattern is autosomal dominant, and all patients identified are heterozygous.

Pathophysiology. SAO is the result of one mutation, a deletion of 27 base pairs in SLC4A1, the gene that codes for band 3. Deletion occurs at the interface between the transmembrane and cytoplasmic domains. The mutation causes an increase in membrane rigidity that may be due to tighter binding of band 3 to ankyrin or decreased lateral mobility of band 3 in the membrane. RBC membranes also have decreased elasticity as measured by ektacytometry and micropipette aspiration. The molecular mechanism responsible for the increased membrane rigidity that results from the band 3 mutation has not yet been elucidated.

Clinical and laboratory findings. In patients with SAO, hemolysis is mild or absent. On a peripheral blood film, typical cells of SAO are oval RBCs with one to two transverse bars or ridges and usually comprise about 30% of the RBCs. No treatment is required for this condition.

Mutations That Alter Membrane Transport Proteins

RBC volume is regulated by various membrane proteins that serve as passive transporters, active transporters, and ion channels. When RBCs lose the ability to regulate volume, the cells are prematurely hemolyzed. Cell volume is determined by the intracellular concentration of cations, particularly sodium. If the total cation content is increased, water enters the cell and increases the cell volume, forming a stomatocyte. If the total cation content is decreased, water leaves the cell, which decreases the cell volume and produces a dehydrated RBC, also called a xerocyte.

Hereditary stomatocytosis comprises a group of heterogeneous conditions in which the RBC membrane leaks monovalent cations. The two major categories are overhydrated hereditary stomatocytosis and dehydrated hereditary stomatocytosis or hereditary xerocytosis. Molecular characterization of these conditions is ongoing and should provide a better means of classification and clearer understanding of their pathophysiology.

Overhydrated Hereditary Stomatocytosis. Overhydrated hereditary stomatocytosis (OHIS) is a very rare hemolytic anemia due to a defect in membrane cation permeability that causes the RBCs to be overhydrated. It is inherited in an autosomal dominant pattern.

Pathophysiology. In OHIS, the RBC membrane is excessively permeable to sodium and potassium at 37 °C. There is an influx of sodium into the cell that exceeds the loss of...
potassium, which results in a net increase in the intracellular cation concentration. As a result, more water enters the cell, and the cell swells and becomes stomatocytic. Because of the water influx, the cytoplasm has decreased density and viscosity. The increase in cell volume without an increase in membrane surface area causes premature hemolysis in the spleen. The exact molecular defect is unknown, but mutations in the RHAG gene that codes for RhAG protein, along with a deficiency of the gene that codes for the Piezo-type mechanosensitive PIEZO1 channel component 1 protein in the RBC membrane. Two mutations have been identified to date. The PIEZO1 protein combines with other proteins to form a pore in a channel to mediate cation transport. The mutations result in an increase in ion channel activity and an increase in cation transport.

Clinical and laboratory findings. OHS can cause moderate to severe hemolytic anemia. The diagnostic features include 5% to 50% stomatocytes on the peripheral blood film (Figure 24-8), macrocytes (MCV of 110 to 150 fL), decreased MCHC (24 to 30 g/dL), reticulocytosis, reduced erythrocyte potassium concentration, elevated erythrocyte sodium concentration, and increased net cation content in the erythrocytes. Because of the increased cell volume, the RBCs have increased osmotic fragility due to a decreased surface area–to–volume ratio. The RBC morphology includes stomatocytes (usually fewer than 10%), target cells, burr cells, desiccated cells with spicules, and RBCs in which the hemoglobin appears to be puddled in discrete areas on the cell periphery. Most patients with DHS/HX do not require treatment. Splenectomy does not improve the anemia and is contraindicated because it increases the risk of thromboembolic complications.

Dehydrated Hereditary Stomatocytosis or Hereditary Xerocytosis. Dehydrated hereditary stomatocytosis (DHS) or hereditary xerocytosis (HX) is an autosomal dominant hemolytic anemia due to a defect in membrane cation permeability that causes the RBCs to be dehydrated. It is the most common form of stomatocytosis.

Pathophysiology. In DHS/HX, the RBC membrane is excessively permeable to potassium. The potassium leaks out of the cell, but this is not balanced by an increase in sodium. Because of the reduced intracellular cation concentration, water is lost from the cell. It is due to mutations in the PIEZO1 gene that codes for the Piezo-type mechanosensitive ion channel component 1 protein in the RBC membrane. Two mutations have been identified to date. The PIEZO1 protein combines with other proteins to form a pore in a channel to mediate cation transport. The mutations result in an increase in ion channel activity and an increase in cation transport.

Clinical and laboratory findings. Patients with DHS/HX generally have mild to moderate anemia, reticulocytosis, jaundice, and mild to moderate splenomegaly. Fetal loss, hydrops fetalis, and neonatal hepatitis can also be features of DHS/HX. The RBCs are dehydrated, as evidenced by the elevated MCHC and decreased osmotic fragility. The RBC morphology includes stomatocytes (usually fewer than 10%), target cells, burr cells, desiccated cells with spicules, and RBCs in which the hemoglobin appears to be puddled in discrete areas on the cell periphery. Most patients with DHS/HX do not require treatment. Splenectomy does not improve the anemia and is contraindicated because it increases the risk of thromboembolic complications.

Other Hereditary Membrane Defects with Stomatocytes

Familial Pseudohyperkalemia. Familial pseudohyperkalemia (FP) is a rare disorder in which excessive potassium leaks out of the RBCs at room temperature in vitro but not at body temperature in vivo. CBC parameters are near the reference intervals, although some patients have a mild anemia. Occasional stomatocytes may be observed on the peripheral blood film. The gene defect associated with this disorder has not yet been identified.

Cryohydrocytosis. Cryohydrocytosis (CHC) is another rare disorder that manifests as a mild to moderate hemolytic anemia with leakage of sodium and potassium from the RBCs and stomatocytosis. The RBCs have marked cell swelling and hemolysis when stored at 4° C for 24 to 48 hours. Mutations in band 3 have been identified in some patients, which cause it to leak cations out of the RBCs, while other patients have a deficiency in membrane stomatin.

Rh Deficiency Syndrome. Rh deficiency syndrome comprises a group of rare hereditary conditions in which the expression of Rh membrane proteins is absent (Rh-null) or decreased (Rh-mod). Cases of the syndrome can be genetically divided into the amorph type (caused by mutations in Rh proteins) and the regulatory type (caused by mutations in a protein that regulates Rh gene expression). Patients with Rh deficiency syndrome present with mild to moderate hemolytic anemia. Stomatocytes and occasional spherocytes may be observed on the peripheral blood film. Symptomatic patients may be treated by splenectomy, which improves the anemia.

Other Hereditary Membrane Defects with Acanthocytes

Acanthocytes (spur cells) are small, dense RBCs with a few irregular projections that vary in width, length, and surface distribution (Figure 24-9). These are distinct from burr cells (echinocytes), which typically have small, uniform projections.

Figure 24-8 Red blood cell morphology in hereditary stomatocytosis (peripheral blood, ×1000).
irregularly spaced projections of varying length (peripheral blood, Figure 24-9). Decreases the fluidity of the RBC membrane and results in the RBC membrane acquiring increased sphingomyelin, which membrane lipids are in equilibrium with plasma lipids, the membrane precursor of the Kell blood group antigens. Men who lack Kx on their RBCs have reduced expression of Kell antigens, reduced RBC deformability, and shortened RBC survival. Patients with MLS have variable acanthocytosis (up to 85%), mild anemia, and late-onset (aged 40 to 60 years), slowly progressive chorea (movement disorders), peripheral neuropathy, myopathy, and neuropsychiatric manifestations. Some female heterozygote carriers may have acanthocytes, but neurologic symptoms are rare. Clinical manifestations in females depend on the proportion of RBCs with the normal X chromosome inactivated versus those with the mutant X chromosome inactivated.

Chorea acanthocytosis (ChAc) is a rare autosomal recessive disorder characterized by chorea, hyperkinesia, cognitive impairments, and neuropsychiatric symptoms. The mean age of onset of the neurologic symptoms is 35 years. In patients with ChAc, 5% to 50% of RBCs on the peripheral blood film are acanthocytes. ChAc is caused by mutations in VPS13A, a gene that codes for chorein, a protein with uncertain cellular functions. Chorein may be involved in trafficking proteins to the cell membrane; consequently, a deficiency of chorein may lead to abnormal membrane protein structure and acanthocyte formation.

**Acquired Red Blood Cell Membrane Abnormalities**

**Acquired Stomatocytosis**

Stomatocytosis occurs frequently as a drying artifact on Wright-stained peripheral blood films. A medical laboratory professional should examine many areas on several films before categorizing the result as stomatocytosis, because in true stomatocytosis such cells should be found in all areas of the blood film. In normal individuals, 3% to 5% of RBCs may be stomatocytes. In wet preparations in which RBCs are diluted in their own plasma and examined under phase microscopy, stomatocytes tend to be bowl shaped or uniconcave, rather than the normal biconcave shape. This technique can eliminate some of the artifactual stomatocytosis, but target cells also may appear bowl shaped in solution. Acute alcoholism and a wide variety of other conditions (such as malignancies and cardiovascular disease) and medications have been associated with acquired stomatocytosis.

**Spur Cell Anemia**

A small percentage of patients with severe liver disease develop a hemolytic anemia with acanthocytosis called spur cell anemia.
The acanthocytes are due to a defect in the lipid component of the membrane. In severe liver disease, there is excess free cholesterol because of the presence of abnormal plasma lipoproteins. An equilibrium is sought between free cholesterol in the plasma and the RBC membrane cholesterol, and cholesterol preferentially accumulates in the outer leaflet of the membrane. The spleen remodels the membrane into the acanthocyte shape. Acanthocytes have long, rigid projections and become entrapped and hemolyzed in the spleen, which results in a rapidly progressive anemia of moderate severity, splenomegaly, and jaundice. Spur cell anemia in end-stage liver disease has a poor prognosis. The anemia may resolve, however, if the patient is able to undergo liver transplantation.

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare chronic intravascular hemolytic anemia caused by an acquired clonal hematopoietic stem cell mutation that results in circulating blood cells that lack glycosylphosphatidylinositol (GPI)–anchored proteins, such as CD55 and CD59. The absence of CD55 and CD59 on the surface of the RBCs renders them susceptible to spontaneous lysis by complement. Because the mutation occurs in a hematopoietic stem cell, the defect is also found in platelets, granulocytes, monocytes, and lymphocytes. PNH is uncommon, with an annual incidence of two to five new cases per million persons in the United States.

Pathophysiology of the Hemolytic Anemia. The GPI anchor consists of a phosphatidylinositol (PI) molecule and a glycan core. The phosphatidylinositol is incorporated in the outer leaflet of the lipid bilayer. The glycan core consists of glucosamine, three mannose residues, and ethanolamine phosphate. At least 24 genes code for enzymes and proteins involved in the biosynthesis of the GPI anchor. GPI-anchored proteins attach to the ethanolamine in the glycan core by an amide bond at their C-termini (Figure 24-10).

Figure 24-10 Glycosylphosphatidylinositol (GPI) anchor for attachment of surface proteins to the cell membrane. Left: The structure of a GPI-anchored protein. The GPI anchor consists of phosphatidylinositol in the outer leaflet of the lipid bilayer, which is connected to a glycan core consisting of glucosamine (GlcN), three mannose (Man) residues, and ethanolamine phosphate (EtN). The protein is linked to the anchor at its C-terminus by an amide bond. The result is a surface protein with a fluid and mobile attachment to the cell surface. The GPI anchor and GPI-linked protein is extracellular. In paroxysmal nocturnal hemoglobinuria (PNH), a mutation occurs in the PIGA gene coding for phosphatidylinositol glycan class A (PIG-A), one of seven subunits of a glycosyl transferase enzyme needed to add N-acetylglucosamine to the inositol (Inos) component of the phosphatidylinositol molecule (see arrow for location of the PNH defect). The mutated PIG-A enzyme subunit inhibits or prevents the first step in the biosynthesis of the GPI anchor. Right: In contrast, a transmembrane protein has an extracellular domain, a short transmembrane domain, and an intracellular domain. (Adapted from Ware RE, Rosse WF: Autoimmune hemolytic anemia. In Nathan DG, Orkin SH, editors: Nathan and Oski’s hematology of infancy and childhood, ed 5, Philadelphia, 1998, Saunders, p. 514.)
In PNH, a hematopoietic stem cell acquires a mutation in the PIGA gene that codes for phosphatidylinositol glycan class A (PIG-A), also known as phosphatidylinositol N-acetylglucosaminyltransferase subunit A. It is one of seven subunits of a glycosyl transferase enzyme needed to add N-acetylglucosamine to phosphatidylinositol. This is the first step in the biosynthesis of the GPI anchor in the endoplasmic reticulum membrane. The PIGA gene is located on the X chromosome, and over 180 different mutations have been identified. Without a fully functional glycosyl transferase enzyme, the hematopoietic stem cell is unable to effectively synthesize the glycan core on phosphatidylinositol in the membrane; therefore, the cell is deficient in membrane GPI anchors. Without GPI anchors, all the progeny of the mutated stem cell are unable to express any of the approximately 16 currently known GPI-anchored proteins found on normal hematopoietic cells. The GPI-anchored proteins are complement regulators, enzymes, adhesion molecules, blood group antigens, or receptors. Relevant to the expression of the hemolysis in PNH, two GPI-anchored proteins are absent or deficient on the RBC membrane: decay-accelerating factor (DAF, or CD55) and membrane inhibitor of reactive lysis (MIRL, or CD59). CD55 and CD59 are complement-inhibiting proteins. CD55 inhibits the complement alternate pathway C3 and C5 convertases, and CD59 prevents the formation of the membrane attack complex. When CD55 and CD59 are absent from the RBC surface, the cell is unable to prevent the activation of complement, and spontaneous and chronic intravascular hemolysis occurs. Out of all the genes needed for GPI anchor synthesis, PIGA is the only one located on the X chromosome. Therefore, only one acquired mutation in a stem cell is needed for the PNH phenotype (males have only one X chromosome, and in females one of the X chromosomes is inactivated).

The PIGA mutant clone coexists with normal hematopoietic stem cells and progenitors, which results in a population of RBCs that is GPI-deficient and a population that is normal. Some patients have a greater expansion of the mutant clone, a higher percentage of circulating GPI-deficient RBCs, and a more severe chronic hemolytic anemia. On the other hand, other patients have minimal expansion of the mutant clone and have a lower percentage of circulating GPI-deficient RBCs. These patients may be asymptomatic and may not require any treatment. It is unclear why there is a greater expansion of the GPI-deficient clone and chronic hemolysis in some patients and not others.

Patients with PNH also display phenotypic mosaicism. The mosaicism results when a single patient is able to harbor normal clones as well as mutant clones with different PIGA mutations. Those different mutations result in variable expression of CD55 and CD59 on the RBCs within an individual patient, giving rise to three RBC phenotypes: type I, type II, and type III. Type I RBCs are phenotypically normal, express normal amounts of CD55 and CD59, and undergo little or no complement-mediated hemolysis. Type II RBCs are the result of a PIGA mutation that causes only a partial deficiency of CD55 and CD59, and these cells are relatively resistant to complement-mediated hemolysis. Type III RBCs are the result of a PIGA mutation that causes a complete deficiency of the GPI anchor, and therefore no CD55 and CD59 proteins are anchored to the RBC surface. Type III RBCs are highly sensitive to spontaneous lysis by complement. The most common RBC phenotype in PNH is a combination of type I and type III cells, while the second most common has all three types. When the severity of the hemolysis in PNH is being assessed, both the relative amount and the type of circulating RBCs are considered.

In addition to hemolysis, patients with PNH may have bone marrow dysfunction that contributes to the severity of the anemia. Many patients have a history of bone marrow failure caused by acquired aplastic anemia or myelodysplastic syndrome that precedes or coincides with the onset of PNH.

Clinical Manifestations. The onset of PNH most frequently occurs in the third or fourth decade, but it can occur in childhood and advanced age. The major clinical manifestations and complications of PNH are those associated with hemolytic anemia, thrombosis, and bone marrow failure (Box 24-2). Anemia is mild to severe, depending on the predominant type of RBC, the degree of hemolysis, and the presence of bone marrow failure. Free hemoglobin released during intravascular hemolytic episodes rapidly scavenges and removes nitric oxide (NO). The decreased NO can manifest as esophageal spasms and dysphagia (difficulty swallowing), erectile dysfunction, abdominal pain, or platelet activation and

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**BOX 24-2** Major Clinical Manifestations and Complications of Paroxysmal Nocturnal Hemoglobinuria

### Related to Intravascular Hemolysis
- Anemia
- Hemoglobinuria
- Chronic renal failure
- Cholelithiasis
- Esophageal spasm, erectile dysfunction

### Related to Thrombosis
- Venous thrombosis
  - Abdominal vein thrombosis: hepatic (Budd-Chiari syndrome), splenic, renal veins
  - Portal hypertension
  - Cerebral vein thrombosis
  - Retinal vein thrombosis and loss of vision
  - Deep vein thrombosis, pulmonary emboli
- Arterial thrombosis (less common)
  - Stroke
  - Myocardial infarction

### Related to Bone Marrow Failure
- Pancytopenia: fatigue, infections, bleeding
- Myelodysplastic syndrome

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thrombosis.45,50 The most common thrombotic manifestation is hepatic vein thrombosis, which obstructs venous outflow from the liver (Budd-Chiari syndrome), a serious, often fatal complication.50

**Laboratory Findings.** Biochemical evidence of intravascular hemolysis includes hemoglobinemia, hemoglobinuria, decreased level of serum haptoglobin, increased levels of serum indirect bilirubin and lactate dehydrogenase, and hemosiderinuria (Chapter 23). Hemolysis can be exacerbated by conditions such as infections, strenuous exercise, and surgery.50 Hemoglobinuria is present in only 25% of patients at diagnosis, but it will occur in most patients during the course of the illness.52 Very few patients report periodic hemoglobinuria at night, a symptom for which the condition was originally named.50 Hemosiderinuria due to chronic intravascular hemolysis (Chapter 23) may be detected with Prussian blue staining of the urine sediment.

Reticulocyte counts are mildly to moderately increased, with less elevation than would be expected in other hemolytic anemias of comparable severity. The MCV may be slightly elevated due to the reticulocytosis. The direct antiglobulin test (DAT) is negative. If the patient does not receive transfusions, iron deficiency develops due to the loss of hemoglobin iron in the urine, and the RBCs become microcytic and hypochromic. Serum iron studies (serum iron, total iron-binding capacity, and serum ferritin) are performed to detect iron deficiency (Chapter 20). Folate deficiency often occurs if there is chronic erythroid hyperplasia and a greater need for folate, which leads to secondary macrocytosis. Pancytopenia may occur if there is concomitant bone marrow failure.

The bone marrow aspirate and biopsy specimen are examined for evidence of an underlying bone marrow failure syndrome, abnormal cells, and cytogenetic abnormalities.52 The bone marrow may be normocellular to hypercellular with erythroid hyperplasia in response to the hemolysis, or it may be hypocellular in concomitant bone marrow failure.50 A finding of dysplasia or certain chromosome abnormalities is helpful in diagnosis of a myelodysplastic syndrome (Chapter 34). An abnormal karyotype is found in 20% of patients with PNH.50

Confirmation of PNH requires demonstration of GPI-deficient cells in the peripheral blood. Flow cytometric analysis (Chapter 32) of RBCs with fluorescence-labeled anti-CD59 determines the proportion of types I, II, and III RBCs, and thus can provide an assessment of the severity of the hemolysis (Figure 24-11, and Figure 32-17).44,50 Type I cells with normal expression of CD59 show the highest intensity level of fluorescence; type II cells with a partial deficiency of CD59 show moderate fluorescence; and type III cells with no CD59 are negative for fluorescence. Patients with a greater proportion of type III cells (complete deficiency of GPI-anchored proteins) are expected to have a high-grade hemolysis.44,52 Patients with a high percentage of type II cells (partial deficiency of GPI-anchored proteins) and a low percentage of type III cells may have only modest hemolysis.44-52 A high-sensitivity two-parameter flow cytometry method using labeled anti-CD59 and anti-CD235a (anti-glycophorin A) was able to detect

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**Figure 24-11** Flow cytometric analysis of peripheral blood cells from a patient with paroxysmal nocturnal hemoglobinuria (PNH). A, Fluorescence intensity of erythrocytes from a healthy control participant after staining with anti-CD59. B, Fluorescence intensity of erythrocytes from an untransfused PNH patient after staining with anti-CD59. Type II cells are "blended" between the type I (normal) and type III cells. C, Fluorescence intensity of granulocytes from a healthy control participant stained with FLAER. D, Fluorescence intensity of granulocytes from the same PNH patient as in B after staining with FLAER. Note that the granulocytes are almost exclusively type III cells. A small population of type I granulocytes is present. FITC, fluorescein isothiocyanate; FLAER, fluorescein-labeled proaerolysin variant. (From Brodsky RA: Paroxysmal nocturnal hemoglobinuria. In Hoffman R, Benz EJ, Jr., Silberstein LE, et al, editors: Hematology: basic principles and practice, ed 6, Philadelphia, 2013, Saunders, an imprint of Elsevier. Figure 29-2, p. 375.)
type III PNH RBCs with a sensitivity of 0.002% (1 in 50,000 normal cells). However, flow cytometry methods to detect PNH RBCs have two major disadvantages. They underestimate the percentage of type III cells because RBCs lacking CD59 undergo rapid complement lysis in the circulation. In addition, these methods cannot accurately determine the percentage of PNH RBCs after recent transfusion.

Because of the inherent problems with flow cytometry methods to detect PNH RBCs, diagnosis of PNH is accomplished by detection of the absence of GPI-anchored proteins on WBCs using multiparameter flow cytometry. The absence of at least two GPI-anchored proteins in two WBC lineages (usually granulocytes and monocytes) is recommended for greater diagnostic accuracy. Methods typically use fluorescent monoclonal antibodies to GPI-anchored proteins (such as CD59, CD55, CD24, CD15, CD33, CD66b, CD14), along with lineage-specific antibodies to non-GPI-anchored proteins (CD15 and CD33 or CD64) to identify granulocytes and monocytes, respectively. An alternative flow cytometric method uses a fluorescein-labeled proaerolysin variant (FLAER). FLAER binds directly to the glycan core of the GPI anchor with a high signal-to-noise ratio. The absence of binding on granulocytes and/or monocytes is indicative of GPI deficiency (Figure 24-11). Using multiparameter flow cytometry with FLAER in combination with monoclonal antibodies to GPI-anchored antigens and lineage-specific antigens increases sensitivity and specificity for detection of GPI-deficient granulocytes and monocytes. A high-sensitivity four-color protocol using FLAER, CD24, CD15, and CD45 for granulocytes and FLAER, CD14, CD64, and CD45 for monocytes was able to detect GPI-deficient granulocytes and monocytes with a sensitivity of 0.01% (1 in 10,000) and 0.04% (1 in 2500), respectively. The results are not affected by recent transfusions, since donor neutrophils and monocytes have a short life span in stored blood; therefore, it can reliably be used to estimate the percentage of GPI-deficient granulocytes and monocytes in recently transfused patients. The high sensitivity is also important for posttherapy monitoring of PNH clones.

The sugar water test (sucrose hemolysis test) and the Ham test (acidified serum lysis test) have insufficient sensitivity for diagnosis of PNH and have been replaced by flow cytometric techniques.

**TABLE 24-3 Classification of Paroxysmal Nocturnal Hemoglobinuria (PNH)**

<table>
<thead>
<tr>
<th>Subcategory</th>
<th>Rate of Intravascular Hemolysis</th>
<th>Bone Marrow</th>
<th>% GPI-AP Deficient Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic PNH*</td>
<td>Marked; visible hemoglobinuria frequent</td>
<td>Normocellular to hypercellular with erythroid hyperplasia and normal or near-normal morphology</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>PNH in the setting of another specified bone marrow disorder†</td>
<td>Mild to moderate; visible hemoglobinuria is intermittent or absent</td>
<td>Evidence of a concomitant bone marrow failure syndrome†</td>
<td>Variable; usually &lt;30%</td>
</tr>
<tr>
<td>Subclinical PNH</td>
<td>No clinical or biochemical evidence of intravascular hemolysis</td>
<td>Evidence of a concomitant bone marrow failure syndrome†</td>
<td>&lt;1%†</td>
</tr>
</tbody>
</table>

*Subclassification proposed by the International PNH Interest Group.*

†Bone marrow failure syndromes include aplastic anemia, refractory anemia/myelodysplastic syndrome, and other myelopathy (e.g., myelofibrosis).

‡Determined by high-sensitivity flow cytometric analysis.

**Classification.** The International PNH Interest Group proposed three subcategories of PNH: classic PNH, PNH in the setting of another specified bone marrow disorder, and subclinical PNH. In classic PNH, there is clinical and biochemical evidence of intravascular hemolysis, reticulocytosis, a cellular bone marrow with erythroid hyperplasia and normal morphology, and a normal karyotype. In addition, more than 50% of the circulating neutrophils are GPI-deficient. In PNH in the setting of another bone marrow disorder, patients have evidence of hemolysis but have a history of or concomitant aplastic anemia, myeloproliferative disorder, or other myelopathy. The number of GPI-deficient neutrophils is variable but is usually less than 30% of the total neutrophils. In subclinical PNH, patients have no clinical or biochemical evidence of hemolysis but have a small subpopulation of GPI-deficient neutrophils that comprise less than 1% of the total circulating neutrophils. This subcategory is found in association with bone marrow failure syndromes. Features of each subgroup are summarized in Table 24-3.

**Treatment.** In 2007, the U.S. Food and Drug Administration approved eculizumab (Soliris®) for the treatment of hemolysis in PNH. Eculizumab is a humanized monoclonal antibody that binds to complement C5, prevents its cleavage to C5a and C5b, and thus inhibits the formation of the membrane attack unit. Eculizumab is the treatment of choice for patients with classic PNH. It results in an improvement of the anemia and a decrease in transfusion requirements. There is also a reduction in the lactate dehydrogenase level in the serum, reflecting a reduction in hemolysis. In a study by Hillman and colleagues (2013) of 195 patients taking eculizumab for a duration of 30 to 66 months, 96.4% of patients did not have an episode of thrombosis, and in 93%, markers of their chronic kidney disease stabilized or even improved. Because of the inhibition of the complement system, patients taking eculizumab have an increased risk for infections with Neisseria meningitidis and need to be vaccinated prior to administration of the drug. Patients continue to have a mild to moderate anemia and reticulocytosis likely due to extravascular hemolysis of RBCs sensitized with C3 (eculizumab does not inhibit complement C3). To address this issue, research is under way to identify therapies that target the...
early events of complement activation, including monoclonal antibodies to C3, but a universal inhibition of C3 may increase the patient’s susceptibility to infections and immune complex disease. A promising new therapy under investigation is a novel recombinant fusion protein (TT30) designed to prevent the formation of C3 convertase only on the membranes of GPI-deficient RBCs.

Eculizumab is not curative and does not address the bone marrow failure complications of PNH. Other treatments for PNH are mainly supportive. Iron therapy is given to help alleviate the iron deficiency caused by the urinary loss of hemoglobin, and folate supplementation is given to replace the folate consumed in accelerated erythropoiesis. Administration of androgens and glucocorticoids to ameliorate anemia is not universally accepted. Anticoagulants are used in the treatment of thrombotic complications. In suitable patients with severe intravascular hemolysis, hematopoietic stem cell transplantation with an HLA-matched sibling donor may be an option and can be a curative therapy, but with an overall survival of only 50% to 60%.

PNH is a disease with significant morbidity and mortality. Prior to eculizumab, thrombosis was the major cause of death, and the median survival after diagnosis was approximately 10 years. Long-term studies of patients on eculizumab therapy are in progress, and early results show a decrease in the debilitating complications and an increase in survival of patients with PNH.

RED BLOOD CELL ENZYMOPATHIES

The major function of the RBCs is to transport oxygen to the tissues over their life span of 120 days. For the RBCs to do that effectively, they need functional enzymes to maintain glycolysis, preserve the shape and deformability of the cell membrane, keep hemoglobin iron in a reduced state, protect hemoglobin and other cellular proteins from oxidative denaturation, and degrade and salvage nucleotides. Deficiencies in RBC enzymes may impair these functions to varying degrees and decrease the life span of the cell. The most important metabolic pathways are the Embden-Meyerhof pathway (anaerobic glycolysis) and the hexose monophosphate (pentose) shunt (Figure 9-1). The most commonly encountered enzymopathies are deficiencies of glucose-6-phosphate dehydrogenase and pyruvate kinase. Other RBC enzymopathies are rare.

Glucose-6-Phosphate Dehydrogenase Deficiency

RBCs normally produce free oxygen radicals (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) during metabolism and oxygen transport, but they have multiple mechanisms to detoxify these oxidants (Chapter 9). Occasionally RBCs are subjected to an increased level of oxidants (oxidant stress) due to exposure to certain oxidizing drugs, foods, chemicals, herbal supplements, and even through reactive oxygen molecules produced in the body during infections. If allowed to accumulate in the RBCs, these reactive oxygen species would oxidize and denature hemoglobin, membrane proteins and lipids, and ultimately cause premature hemolysis. Therefore, the RBCs’ capacity to detoxify oxidants, especially during oxidant stress, is critical to maintain their normal life span.

Glucose-6-phosphate dehydrogenase (G6PD) is one of the important intracellular enzymes needed to protect hemoglobin and other cellular proteins and lipids from oxidative denaturation. G6PD catalyzes the first step in a series of reactions that detoxify hydrogen peroxide formed from oxygen radicals (Figure 24-12). In the hexose monophosphate shunt (Chapter 9), G6PD generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) by converting glucose-6-phosphate to 6-phosphogluconate. In the next step, glutathione reductase uses the NADPH to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH) and NADP. In the final reaction, glutathione peroxidase uses the GSH generated in the previous step to detoxify hydrogen peroxide to water (H$_2$O). GSSG is formed in the reaction and is rapidly transported out of the cell. During oxidant stress, RBCs with normal G6PD activity are able to readily detoxify hydrogen peroxide to prevent cellular damage and safeguard hemoglobin. G6PD is especially critical to the cell because it provides the only means of generating NADPH. Consequently, G6PD-deficient RBCs are particularly vulnerable to oxidative damage and subsequent hemolysis during oxidant stress.

The G6PD gene is located on the X chromosome. It codes for the G6PD enzyme, which assembles into a dimer and tetramer in its functional configuration. With the X-linked inheritance pattern, men can be normal hemizygotes (have the normal allele) or deficient hemizygotes (have a mutant allele). Women can be normal homozygotes (both alleles normal), deficient homozygotes (both alleles have same mutation), compound heterozygotes (each allele has a different mutation), or heterozygotes (have one normal allele and one mutant allele). The G6PD enzyme activity in female heterozygotes lies between normal and deficient due to the random inactivation of one of the X chromosomes in each cell (lyonization). Therefore, the RBCs of female heterozygotes are a mosaic, with some cells...
having normal G6PD activity and some cells having deficient G6PD activity. Because the X inactivation is random, the proportion of normal to G6PD-deficient RBCs varies among different heterozygous women. Some heterozygous women experience acute hemolytic episodes after exposure to oxidants if they have a high proportion of G6PD-deficient RBCs.

G6PD deficiency is the most common RBC enzyme defect, with a prevalence of 5% of the global population, or approximately 400 million people worldwide. The prevalence of G6PD deficiency varies by geographic location: sub-Saharan Africa (7.5%), the Middle East (6.0%), Asia (4.7%), Europe (3.9%), and the Americas (3.4%). In the United States, the prevalence of G6PD deficiency in African American males is approximately 10%. G6PD deficiency has the highest prevalence in geographic areas in which malaria is endemic because of the selective pressure of malaria. Studies in Africa show that G6PD deficiency (A− variant) in hemizygous males confers protection against life-threatening Plasmodium falciparum malaria. This protective effect is not observed in heterozygous females because of their mosaicism of normal and G6PD-deficient RBCs. In a 2010 case-control study in Pakistan, G6PD deficiency (Mediterranean variant) conferred protection against Plasmodium vivax infection, also with a greater protective effect in hemizygous males. This protective effect may be due to parasite susceptibility to excess free oxygen radicals produced in G6PD-deficient RBCs. In addition, significant oxidative damage may occur to the RBCs early after parasite invasion so that these early-infected cells are more readily phagocytized with elimination of the parasite.

A mutation database published in 2012 reported 186 known mutations in the G6PD gene, with over 85% of them being single missense mutations. An amino acid substitution changes the structure of the enzyme and thus affects its function, stability, or both. In addition, more than 400 variant isoenzymes have been identified. The normal or wildtype G6PD variant is designated G6PD-B. Some G6PD variants have significantly reduced enzyme activity, while others have mild or moderately reduced activity or normal activity. The different variants of G6PD have been divided into classes by the World Health Organization, based on clinical symptoms and amount of enzyme activity (Table 24-4).

### Pathophysiology

G6PD-deficient RBCs cannot generate sufficient NADPH to reduce glutathione and thus cannot effectively detoxify the hydrogen peroxide produced upon exposure to oxidative stress. Oxidative damage to cellular proteins and lipids occurs, particularly affecting hemoglobin and the cell membrane. Oxidation converts hemoglobin to methemoglobin and forms sulfhydryl groups and disulfide bridges in hemoglobin peptides. This leads to decreased hemoglobin solubility and precipitation as Heinz bodies. Heinz bodies adhere to the inner RBC membrane, causing irreversible membrane damage (Figure 14-11). Because of the membrane damage and loss of deformability, RBCs with Heinz bodies are rapidly removed from the circulation by intravascular and extravascular hemolysis. Reticulocytes have approximately five times more G6PD activity than older RBCs, because enzyme activity decreases as the cells age. Therefore, during exposure to oxidants, the older RBCs with less G6PD are preferentially hemolyzed.

### Clinical Manifestations

The vast majority of individuals with G6PD deficiency are asymptomatic throughout their lives. However, some patients have clinical manifestations. The clinical syndromes are acute hemolytic anemia, neonatal jaundice (hyperbilirubinemia), and chronic hereditary nonspherocytic hemolytic anemia (HNSHA).

### Acute Hemolytic Anemia

Oxidative stress can precipitate a hemolytic episode, and the main triggers are certain oxidizing drugs or chemicals, infections, and ingestion of fava beans. Hemolysis secondary to drug exposure is the classic manifestation of G6PD deficiency. The actual discovery of

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**TABLE 24-4** Classification of Glucose-6-Phosphate Dehydrogenase Variants by the World Health Organization

<table>
<thead>
<tr>
<th>Class</th>
<th>G6PD Enzyme Activity</th>
<th>Clinical Manifestations</th>
<th>Examples of Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Severely deficient: &lt;1% activity or not detectable</td>
<td>Chronic, hereditary nonspherocytic hemolytic anemia; severity is variable; rare</td>
<td>G6PD-Serres, G6PD-Madrid</td>
</tr>
<tr>
<td>II</td>
<td>Severely deficient: &lt;10% activity</td>
<td>Severe, episodic acute hemolytic anemia associated with infections, certain drugs, and fava beans; not self-limited and may require transfusions during hemolytic episodes</td>
<td>G6PD-Mediterranean, G6PD-Chatham</td>
</tr>
<tr>
<td>III</td>
<td>Mild to moderately deficient: 10%–60% activity</td>
<td>Episodic, acute hemolytic anemia associated with infections and certain drugs; self-limited</td>
<td>G6PD-A, G6PD-Canton</td>
</tr>
<tr>
<td>IV</td>
<td>Mildly deficient to normal: 60%–150% activity</td>
<td>None</td>
<td>G6PD-B (wildtype), G6PD-A (may also manifest as class III)</td>
</tr>
</tbody>
</table>

| V      | Increased: >150% activity | None |

Intrinsic Defects Leading to Increased Erythrocyte Destruction

Glucose-6-Phosphate Dehydrogenase Deficiency

The mechanism of hemolysis is poorly understood, and the hemolysis is not self-limiting. The degree of change in the G6PD level. Most of these patients are diagnosed at birth as having neonatal hyperbilirubinemia, and the hemolysis continues into adulthood. They usually do not have hemoglobinuria, which suggests that the ongoing hemolysis is extravascular as opposed to intravascular. The RBC morphology is unremarkable. These patients also are vulnerable to acute oxidative stress from the same agents as those affecting other G6PD-deficient individuals and may have acute episodes of hemoglobinuria. The severity of HNSHA is extremely variable, likely related to the type of mutation in the G6PD gene.

Chronic Hereditary Nonspherocytic Hemolytic Anemia.

A small percentage of G6PD-deficient patients have chronic HNSHA, as evidenced by persistent hyperbilirubinemia, decreased serum haptoglobin level, and increased serum lactate dehydrogenase level. Most of these patients are diagnosed at birth as having neonatal hyperbilirubinemia, and the hemolysis continues into adulthood. They usually do not have hemoglobinuria, which suggests that the ongoing hemolysis is extravascular as opposed to intravascular. The RBC morphology is unremarkable. These patients also are vulnerable to acute oxidative stress from the same agents as those affecting other G6PD-deficient individuals and may have acute episodes of hemoglobinuria. The severity of HNSHA is extremely variable, likely related to the type of mutation in the G6PD gene.

Laboratory Findings

General Tests for Hemolytic Anemia. The anemia occurring during a hemolytic crisis may range from moderate to extremely severe and is usually normocytic and normochromic. The morphology of G6PD-deficient RBCs is normal except during a hemolytic episode. The degree of change in morphology during a hemolytic episode varies, depending on

BOX 24-3 Drugs Causing Predictable Hemolysis in Glucose-6-Phosphate Dehydrogenase Deficiency

Drugs with strong evidence-based support for an association with drug-induced hemolysis:1

- Dapsone
- Methylthioninium chloride (methylene blue)
- Nitrofurantoin
- Phenazopyridine
- Primaquine
- Rasburicase
- Tolonium chloride (toluidine blue)

Drugs with well-documented case reports for an association with drug-induced hemolysis:2

- Cotrimoxazole
- Quinolones
- Sulfadiazine

the severity of the hemolysis. In some patients, the change is not striking, but in other individuals with severe variants, marked anisocytosis, poikilocytosis, spherocytosis, and schistocytosis may occur. Bite cells (RBCs in which the margin appears indented and the hemoglobin is concentrated) may be observed in rare cases of drug-induced hemolysis but should not be considered a specific feature of G6PD deficiency. Bite cells are absent in acute and chronic hemolytic states associated with common G6PD-deficient variants, and they can also be found in other conditions. Heinz bodies cannot be detected with Wright staining. They can be visualized with supravital stains, such as crystal violet, as dark purple inclusions attached to the inner RBC membrane (Figure 14-11). The reticulocyte count is increased and may reach 30% of RBCs. Consistent with intravascular hemolysis, the serum haptoglobin level is severely decreased, the serum lactate dehydrogenase activity is elevated, and there is hemoglobinemia and hemoglobinuria. The indirect bilirubin level is also elevated. The white blood cell (WBC) count is moderately elevated, and the platelet count varies. Importantly, the direct antiglobulin test (DAT) is negative, indicating that an immune cause of the hemolysis is unlikely (Chapter 26). Table 24-5 contains a summary of the clinical and laboratory findings in G6PD deficiency during an acute hemolytic episode.

**Tests for G6PD Deficiency.** The two major categories of tests for G6PD deficiency are quantitative and qualitative biochemical assays for G6PD activity (phenotypic assays) and DNA-based molecular tests for mutation detection (genotypic assays). Quantitative spectrophotometric assays are the gold standard to determine G6PD activity, make a definitive diagnosis, and assess the severity of the deficiency. The assays are based on the direct measurement of NADPH generated by the patient’s G6PD in the reaction shown in Figure 24-13. The assays require venous blood collected in heparin or ethylenediaminetetraacetic acid (EDTA) anticoagulant. A hemolysate is prepared and incubated with the substrate/cofactor (glucose-6-phosphate/NADP) reagent. The rate of NADPH formation is proportional to G6PD activity and is measured as an increase in absorbance at 340 nm using a spectrophotometer. The activity is typically reported as a ratio of the units of G6PD activity per gram of hemoglobin (IU/g Hb), so a standard hemoglobin assay must be done on the same specimen used for the G6PD assay. Cutoff points to determine G6PD deficiency are usually set at less than 20% of normal activity (usually less than 4.0 IU/g Hb), but this varies by method, laboratory, and population screened.

Qualitative tests are designed as rapid screening tools to distinguish normal from G6PD-deficient patients. G6PD deficiency is defined by various methods as less than 20% to 50% of normal G6PD activity. Similar to quantitative assays, these tests also incubate a lysate of heparin or EDTA-anticoagulated blood with a glucose-6-phosphate/NADP reagent to generate NADPH (Figure 24-13). The endpoint in qualitative tests, however, is visually observed, and the results are reported as “G6PD-deficient” or “normal.” Qualitative tests with deficient or intermediate results are reflexed to the quantitative assay for verification of the G6PD deficiency.

The fluorescent spot test is based on the principle that the NADPH generated in the reaction is fluorescent, while the NADP in the reagent is not fluorescent. Blood and glucose-6-phosphate/NADP reagent are incubated and spotted on filter paper in timed intervals. Specimens with normal G6PD activity appear as moderate to strong fluorescent spots under long-wave ultraviolet (UV) light; specimens with decreased or no activity do not fluoresce or display weak fluorescence compared to a normal control.

Dye-reduction qualitative assays use the same G6PD enzymatic reaction but have a second step in which the NADPH reduces a dye, giving a visually observed color change. An example is BinaxNOW® (Alere/Inverness Medical, Waltham, MA), which is a handheld device that uses the enzyme chromatographic test (ECT) method. Hemolysate is applied to one section of a lateral flow test strip in the device. The specimen migrates to the reaction pad of the strip containing the glucose-6-phosphate/NADP substrate/cofactor and a nitroblue

### Table 24-5 Typcal Clinical and Laboratory Findings in Glucose-6-Phosphate Dehydrogenase Deficiency During Acute Hemolytic Episode

<table>
<thead>
<tr>
<th>History</th>
<th>Recent infection, administration of drugs associated with hemolysis, or ingestion of fava beans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical manifestations</td>
<td>Chills, fever, headache, nausea, back pain, abdominal pain</td>
</tr>
<tr>
<td></td>
<td>Jaundice</td>
</tr>
<tr>
<td></td>
<td>Dark urine</td>
</tr>
<tr>
<td>Complete blood count results</td>
<td>↓ Hemoglobin (moderate to severe)</td>
</tr>
<tr>
<td></td>
<td>↑ Reticulocyte count</td>
</tr>
<tr>
<td>Peripheral blood film findings</td>
<td>Polychromasia</td>
</tr>
<tr>
<td></td>
<td>RBC morphology varies from normal to marked anisocytosis, poikilocytosis, spherocytosis, or schistocytosis, depending on severity</td>
</tr>
<tr>
<td>Direct antiglobulin test result</td>
<td>Negative</td>
</tr>
<tr>
<td>Indicators of hemolysis</td>
<td>↓ Serum haptoglobin (severe)</td>
</tr>
<tr>
<td></td>
<td>↑ Serum lactate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>↑ Serum indirect bilirubin</td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
</tr>
<tr>
<td>Selected additional tests</td>
<td>↓ G6PD activity (mild to severe); may be falsely normal due to reticulocytosis, leukocytosis, thrombocytosis, and in individuals with mild deficiencies</td>
</tr>
<tr>
<td></td>
<td>DNA-based mutation detection usually needed to identify heterozygous females</td>
</tr>
<tr>
<td></td>
<td>Heinz bodies observed on supravital stain</td>
</tr>
</tbody>
</table>

↓ Decreased; ↑ increased; G6PD, glucose-6-phosphate dehydrogenase; RBC, red blood cell.
Glucose-6-phosphate + NADP $\xrightarrow{\text{G6PD}}$ 6-Phosphogluconate + NADPH

Not fluorescent

Fluorescent; $\uparrow$ absorbance at 340 nm

Figure 24-13 Principle of the glucose-6-phosphate dehydrogenase (G6PD) activity assay. G6PD (in patient’s hemolysate) converts glucose-6-phosphate to 6-phosphogluconate with the conversion of oxidized nicotinamide adenine dinucleotide phosphate (NADP) (not fluorescent) to the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (fluorescent). In the quantitative assay, the rate of production of NADPH is proportional to G6PD activity and is measured as an increase in absorbance at 340 nm using a spectrophotometer. In the qualitative assay, the appearance of a fluorescent spot under ultraviolet light when the reaction mixture is spotted on filter paper indicates normal G6PD activity.

tetrazolium dye. If the specimen has normal G6PD activity, the NADPH generated reduces the dye to a formazan product that is visually observed as a brown-black color on the reaction pad. This method has a sensitivity of 98% in detecting deficient specimens with G6PD activity less than 4.0 U/g Hb. In another formazan-based test, the NADPH reduces a tetrazolium monosodium salt (WST-8) substrate, forming a formazan orange-colored product. The dye-reduction methods have an advantage, since they do not need a UV light for visualization. A disadvantage of the BinaxNOW® method, however, is its higher cost compared to other assays. Rapid, point-of-care screening methods that do not require a venipuncture specimen are in development.

Biochemical qualitative screening tests are reliable to identify hemizygous males and homozygous or compound heterozygous mutant females with severe deficiency (less than 20% of normal activity), but lack the sensitivity to detect mild and moderate deficiencies found in some cases of G6PD deficiency and in heterozygous females. They also have subjective endpoints that may affect test reproducibility and accuracy.

Phenotypic assays for G6PD activity have several additional limitations. As covered earlier in the chapter, reticulocytes have higher G6PD activity compared to mature RBCs. Reticulocytosis typically occurs as a response to an acute hemolytic episode and will falsely increase the patient's G6PD activity over baseline values. Patients who are not G6PD deficient are expected to have high G6PD activity during hemolytic episodes. When a patient with reticulocytosis has an unexpectedly normal G6PD activity result, G6PD deficiency should be suspected. To avoid falsely elevated or falsely normal results, biochemical assays for G6PD activity should not be performed during acute hemolysis. The testing should be performed after the reticulocyte and total RBC counts have returned to baseline, which may take 2 weeks to 2 months after the hemolytic episode.

Another limitation of phenotypic assays is that testing cannot be done after recent transfusion because the mixture of donor and patient RBCs does not reflect the patient’s true G6PD activity. WBCs and platelets also contain G6PD activity. In cases of severe anemia, or in severe leukocytosis or thrombocytosis, theuffy coat should be removed from the specimen before preparing the hemolysate for testing.

DNA-based mutation detection (genotyping) is available in larger hospital and reference laboratories. Typically, the DNA is extracted from WBCs isolated from whole blood. Since the vast majority of G6PD mutations involve single nucleotide substitutions, molecular testing is straightforward. Although there are approximately 186 known mutations, rapid PCR-based methods can be used that target specific mutations with high prevalence in a particular geographic area, racial group, or ethnic group. If targeted mutation testing is negative, whole gene DNA sequencing is required.

DNA-based testing is best suited for prenatal testing, family studies, and identification of heterozygous females who typically have indeterminate or normal biochemical (phenotypic) tests owing to their mosaicism of normal and G6PD-deficient cells. DNA-based methods can be done on patients who were recently transfused because donor WBCs have a short half-life in stored blood and will not interfere with the test. In addition, DNA-based tests are not affected by reticulocytosis and can be done during an acute hemolytic episode.

The disadvantage of DNA-based methods is the requirement for technical expertise and specialized equipment. In addition, knowing the genotype of heterozygous females does not predict the clinical phenotype in terms of the proportion of normal and G6PD-deficient cells.

An additional application of tests for G6PD deficiency is screening asymptomatic patients prior to prescribing the drugs listed in Box 24-3. Screening is recommended for patients with a family history of G6PD deficiency or for those who are members of ethnic/racial groups with a high prevalence of G6PD variants.

**Treatment**

Treatment for G6PD-deficient patients with acute hemolysis begins with discontinuing drugs associated with hemolysis. Most hemolytic episodes, especially in individuals with G6PD-A<sup>-</sup>, are self-limited. In patients with more severe types, such as G6PD-Mediterranean, RBC transfusions may be required. Screening is important in populations that have a high incidence of G6PD deficiency. The prevention of acute hemolytic anemia is difficult because multiple causes exist; however, some cases of acute hemolytic anemia are easily preventable, such as by avoidance of fava bean consumption in families in which this sensitivity exists. Neonates in high risk populations should be screened for hyperbilirubinemia associated with G6PD deficiency, and treated immediately to prevent kernicterus and permanent brain damage.

Favism is a relatively dangerous manifestation and potentially fatal in individuals who do not have access to appropriate medical facilities and a transfusion service. Prevention of drug-induced disease is possible by choosing alternate drugs when possible. In cases in which the offending drugs must be used, especially in individuals with G6PD-A<sup>-</sup>, the dosage can
be lowered to decrease the hemolysis to a manageable level. Infection-induced hemolysis is more difficult to prevent but can be detected early in the course of the episode and treated if necessary. Most episodes resolve without treatment but may be severe enough to warrant RBC transfusion. In patients with hemoglobin levels greater than 9 g/dL with persistent hemoglobinuria, close monitoring is important. Neonates with moderate hyperbilirubinemia and jaundice secondary to G6PD deficiency can be treated with phototherapy, but those with severe hyperbilirubinemia may require exchange transfusion.79

**Pyruvate Kinase Deficiency**

Pyruvate kinase (PK) is a rate-limiting key enzyme of the glycolytic pathway of RBCs. It catalyzes the conversion of phosphoenolpyruvate to pyruvate, forming ATP (Figure 9-1). PK deficiency is an autosomal recessive disorder, with an estimated prevalence of 1 per 20,000 in the white population.77 It is the most common form of hereditary nonspherocytic hemolytic anemia and is found worldwide.59,61 PK deficiency is due to a mutation in the PKLR gene that codes for PK in red blood cells and hepatocytes. Over 180 mutations (predominantly missense) have been reported.78 Symptomatic hemolytic anemia occurs in homozygotes or compound heterozygotes. Certain mutations are more common in the United States, parts of Europe, and Asia.69 There is a high prevalence of PK-deficient homozygotes with the same point mutations in two isolated, consanguineous communities in the United States: 27 Amish kindred in Pennsylvania (1436G>A) and 6 children born into polygamist families in a small town in the Midwest (1529G>A).79,80

**Pathophysiology**

The mechanisms causing hemolysis and premature destruction of PK-deficient cells are not completely known. The metabolic consequence of PK deficiency is a depletion of cellular ATP and an increase in 2,3-bisphosphoglycerate (2,3-BPG).81,82 The increase in 2,3-BPG shifts the hemoglobin-oxygen dissociation curve to the right and decreases the oxygen affinity of hemoglobin59 (Chapter 10). This promotes greater release of oxygen to the tissues and enables affected individuals to tolerate lower levels of hemoglobin.59,81,82 ATP depletion also affects the ability of the cell to maintain its shape and membrane integrity.

**Clinical Manifestations**

Individuals with PK deficiency have a wide range of clinical presentations, varying from severe neonatal anemia and hyperbilirubinemia requiring exchange or multiple transfusions to a fully compensated hemolytic process in apparently healthy adults.85 Most patients, however, have manifestations of chronic hemolysis, including anemia, jaundice, splenomegaly, and increased incidence of gallstones (due to the production of excessive bilirubin).59 Rarely, folate deficiency (due to accelerated erythropoiesis), bone marrow aplasia (usually due to parvovirus B19 infection), and skin ulcers can occur.73,82 Pregnancy carries the risk of fetal loss and exacerbation of the anemia in the mother.79 There is an increased risk of iron overload and organ damage that occurs with age, even in the absence of transfusions.73,82,83 The mechanism of dysregulation of iron homeostasis is not clear but may be related to a decrease in or lack of response to hepcidin, the major iron-regulating protein.79

**Laboratory Findings**

The hemoglobin level is variable, depending on the extent of the hemolysis. Reticulocytosis is usually present, but not in proportion to the severity of the anemia, because the reticulocytes are preferentially destroyed in the spleen.59,62 After splenectomy, the number of circulating reticulocytes can increase fivefold.62 In addition to showing anisocytosis, poikilocytosis, and polychromasia, the peripheral blood film reveals a variable number of burr cells, or echinocytes (in the range of 3% to 30%), which increase in number after splenectomy.82 The postsplenectomy peripheral blood film may also show Howell-Jolly bodies, Pappenheimer bodies, and target cells. The WBC and platelet counts are normal or slightly increased. Patients usually have the characteristic laboratory findings of chronic hemolysis, including an increased serum indirect bilirubin level, a decreased serum haptoglobin level, and increased urinary urobilinogen. The osmotic fragility is usually normal, and the direct antiglobulin test is negative.

Tests for PK deficiency include quantitative and qualitative biochemical assays for PK activity (phenotypic assays) and DNA-based molecular tests for mutation detection (genotypic assays). In the quantitative PK assay, a hemolysate is prepared from patient’s anticoagulated blood after careful removal of the WBCs. WBCs have a very high PK level, and contamination of the hemolysate with WBCs falsely increases the result (i.e., in a PK deficiency, the result could be falsely normal).82 The reagents include phosphoenolpyruvate, adenosine diphosphate (ADP), lactate dehydrogenase, and the reduced form of nicotinamide adenine dinucleotide (NADH). In the first step of the reaction, the patient’s PK converts phosphoenolpyruvate to pyruvic acid, and a phosphate is transferred to ADP, forming adenosine triphosphate (ATP). In the second step, lactate dehydrogenase converts the pyruvic acid to lactic acid, and the NADH is converted to its oxidized form, NAD (Figure 24-14). The rate of NAD formation is proportional to PK activity and is measured as a decrease in absorbance at 340 nm using a spectrophotometer. The activity is typically reported as a ratio of the units of PK activity per gram of hemoglobin (IU/g Hb).

More complex techniques may be necessary when some variant forms of PK are suspected.82 Qualitative tests for PK deficiency are used for screening and are based on the same principle as that described earlier, except the hemolysate and reagents are incubated and spotted onto filter paper. The loss of fluorescence is visually evaluated to determine the oxidation of NADH to NAD (Figure 24-14).84

Mutation detection (genotypic testing) can be accomplished by sequencing the exons, flanking regions, and promoter region of the PKLR gene.82 Molecular diagnosis is superior in sensitivity and specificity, is applicable for use in prenatal testing, and enables correlation of certain mutations with disease severity.79,79,82
The enzyme pyruvate kinase catalyzes the following reaction:

1. \[ \text{ADP + Phosphoenolpyruvic acid} \rightarrow \text{ATP + Pyruvic acid} \]

The pyruvic acid formed then takes part in the following reaction:

2. \[ \text{Pyruvic acid + NADH} \rightarrow \text{Lactic acid + NAD} \]

\[ \text{Lactate dehydrogenase} \rightarrow \text{Lactate dehydrogenase} \]

\[ \text{Not fluorescent; } \downarrow \text{absorbance at } 340 \text{ nm} \]

**Figure 24-14** Principle of the pyruvate kinase (PK) activity test. The reagent contains adenosine diphosphate (ADP), phosphoenolpyruvic acid, lactate dehydrogenase, and the reduced form of nicotinamide adenine dinucleotide (NADH). In the first step, PK (in patient’s hemolysate) converts phosphoenolpyruvic acid to pyruvic acid, and a phosphate is transferred to ADP, forming adenosine triphosphate (ATP). In the second step, lactate dehydrogenase converts pyruvic acid to lactic acid with the conversion of NADH (fluorescent) to the oxidized form of nicotinamide adenine dinucleotide (NAD) (not fluorescent). In the quantitative assay, the rate of production of NAD is proportional to PK activity and is measured as a decrease in absorbance at 340 nm using a spectrophotometer. In the qualitative assay, the disappearance of fluorescence under ultraviolet light when the reaction mixture is spotted on filter paper indicates normal PK activity.

**Treatment**

No specific therapy is available for PK deficiency except supportive treatment and RBC transfusion as necessary. Splenectomy is beneficial in severe cases, and after this procedure the hemoglobin level usually increases enough to reduce or eliminate the need for transfusion. \(^73\) Splenectomy, however, results in a lifelong increased risk of sepsis by encapsulated bacteria. Hematopoietic stem cell transplant may be curative for children with severe hemolytic disease who have an unaffected HLA-identical sibling for a donor. \(^79\)

**Other Enzymopathies**

Pyrimidine 5’-nucleotidase type 1 (P5’NT-1) is an enzyme needed for the degradation and elimination of ribosomal ribonucleic acid (RNA) in reticulocytes. P5’NT-1 removes the phosphate from pyrimidine 5’ ribonucleoside monophosphate to form ribonucleoside and inorganic phosphate. These degradation products are then able to diffuse out of the cell. \(^59\) P5’NT-1 deficiency is inherited in an autosomal recessive manner. \(^59,85\) It is the third most common RBC enzyme deficiency that causes hereditary nonspherocytic hemolytic anemia (after G6PD and PK). \(^59,85\) The NT5C3A gene codes for P5’NT-1, and over 20 different mutations have been reported. \(^85\)

Patients who are homozygotes or compound heterozygotes for NT5C3A mutations develop chronic hemolytic anemia. The P5’NT-1-deficient RBCs accumulate pyrimidine ribonucleoside monophosphates, which precipitate and appear as very coarse basophilic stippling in the cell. \(^59,85\) These RBCs ultimately undergo premature hemolysis. Most patients have a mild to moderate anemia with reticulocytosis, jaundice, and splenomegaly. \(^85\) Mental retardation has been reported in some patients. \(^81,85\)

Diagnostic tests include measurement of P5’NT-1 activity and the concentration of intracellular pyrimidine nucleotides in RBCs and DNA-based testing for mutations. \(^85\) Therapy consists of RBC transfusion as needed.

Other RBC enzymopathies are rarely encountered. In addition to PK deficiency, deficiencies of other enzymes of the RBC Embden-Meyerhof pathway that cause hereditary nonspherocytic hemolytic anemia have been described, including hexokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, triosephosphate isomerase, and phosphoglycerate kinase. \(^61\) All of these deficiencies are autosomal recessive conditions, except for phosphoglycerate kinase deficiency, which is X-linked. Mutations in enolase are rare, and their association with hemolytic anemia is uncertain. \(^61\) Deficiencies in glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase are not associated with hemolytic anemia. \(^61\) Phosphoglycerate mutase deficiency results in a depletion of 2,3-bisphosphoglycerate. This causes a shift in the hemoglobin-oxygen dissociation curve to the left and an increased affinity of hemoglobin for oxygen. The resulting tissue hypoxia manifests as a mild erythrocytosis. \(^61\)

**SUMMARY**

- The RBC membrane must have deformability for the RBC to maneuver through the microcirculation and the splenic sieve over its life span of 120 days. The cellular properties that enable deformability are the biconcave, discoid shape of the cell; the viscoelasticity of the membrane; and cytoplasmic viscosity.
- Two transmembrane protein complexes, the ankyrin complex and protein 4.1 complex, provide vertical structural integrity to the cell by anchoring the lipid bilayer to the underlying spectrin skeleton. α-Spectrin, β-spectrin, and their accessory proteins form a two-dimensional lattice to provide horizontal mechanical stability to the membrane.
- Hereditary spherocytosis (HS) is caused by mutations that disrupt the vertical membrane protein interactions, which results in loss of membrane, decrease in surface area-to-volume ratio, and formation of spherocytes that are destroyed in the spleen. Patients with HS have anemia, splenomegaly, jaundice, an increased MCHC, a negative DAT result, biochemical evidence of hemolysis, and spherocytes and polychromasia on the peripheral blood film. RBCs in HS show decreased fluorescence in the eosin-5’-maleimide binding test when measured by flow cytometry, and the osmotic fragility test usually shows increased fragility.
- Hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP) are caused by mutations that disrupt the horizontal interactions in the protein cytoskeleton, which results in loss of mechanical stability of the membrane. Elliptocytes are present on the peripheral blood film. Only 10% of HE patients have moderate or severe anemia. HPP is a severe thermal-sensitive form of HE in which extreme poikilocytosis along with schistocytes, microspherocytes, and elliptocytes are seen on the peripheral blood film.
• Hereditary ovalocytosis, also called Southeast Asian ovalocytosis (SAO), is caused by a mutation in band 3 that increases membrane rigidity. The prevalence is high in Southeast Asia, and hemolysis is mild or absent; typical cells are oval with one to two transverse bars or ridges.

• Hereditary stomatocytosis is a group of disorders characterized by an RBC membrane that leaks cations. In overhydrated hereditary stomatocytosis (OHS), the RBCs have decreased cytoplasmic viscosity and stomatocytes are observed on the peripheral blood film. In dehydrated hereditary stomatocytosis (DHS) or hereditary xerocytosis (HX), the RBCs have increased cytoplasmic viscosity, and the peripheral blood film shows burr cells, target cells, few stomatocytes, and cells with puddled hemoglobin at the periphery. Stomatocytosis may also occur in Rh deficiency syndrome and in a variety of acquired conditions.

• Neuroacanthocytosis comprises a group of inherited disorders characterized by neurologic impairment and the presence of acanthocytes on the peripheral blood film. Major disorders in this group include abetalipoproteinemia, McLeod syndrome, and chorea acanthocytosis. Acquired acanthocytosis can occur in severe liver disease (spur cell anemia).

• Paroxysmal nocturnal hemoglobinuria (PNH) is due to an acquired hematopoietic stem cell mutation that results in the lack of GPI-anchored proteins on blood cell surfaces. CD55 and CD59, complement-regulating proteins, are partially or completely deficient on RBCs, which makes the RBCs susceptible to spontaneous complement lysis. Flow cytometry is a sensitive method to detect the absence of GPI-anchored proteins on cell surfaces. The rate of hemolysis in classic PNH improves after treatment with eculizumab, a complement C5 inhibitor.

• Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common RBC enzymopathy, but the vast majority of patients are asymptomatic. Patients with classes II and III G6PD variants may develop acute hemolytic anemia after infections or after ingestion of certain drugs or fava beans. A small percentage of patients have class I G6PD variant and chronic hereditary nonspherocytic hemolytic anemia (HNSHA).

• Most patients with pyruvate kinase (PK) deficiency have symptoms of hemolysis. Burr cells are commonly observed on the peripheral blood film. PK deficiency is the most common cause of HNSHA.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. In HS a characteristic abnormality in the CBC results is:
   a. Increased MCV
   b. Increased MCHC
   c. Decreased MCH
   d. Decreased platelet and WBC counts

2. The altered shape of the spherocyte in HS is due to:
   a. An abnormal RBC membrane protein affecting vertical protein interactions
   b. Defective RNA synthesis
   c. An extrinsic factor in the plasma
   d. Abnormality in the globin composition of the hemoglobin molecule

3. Which of the following results are consistent with HS?
   a. Increased osmotic fragility, negative DAT result
   b. Decreased osmotic fragility, positive DAT result
   c. Increased osmotic fragility, positive DAT result
   d. Decreased osmotic fragility, negative DAT result

4. The RBCs in HE are abnormally shaped and have unstable cell membranes as a result of:
   a. Abnormal shear stresses in the circulation
   b. Defects in horizontal membrane protein interactions
   c. Mutations in ankyrin
   d. Lack of all Rh antigens in the RBC membrane

5. The peripheral blood film for patients with mild HE is characterized by:
   a. Elliptical RBCs
   b. Oval RBCs with one or two transverse ridges
   c. Overhydrated RBCs with oval central pallor
   d. Densely stained RBCs with a few irregular projections

6. Laboratory test results for patients with HPP include all of the following except:
   a. RBCs that show marked thermal sensitivity at 41° C to 45° C
   b. Marked poikilocytosis with elliptocytes, RBC fragments, and microspherocytes
   c. Low fluorescence when incubated with eosin-5’-maleimide
   d. Increased MCV and normal RDW

7. Acanthocytes are found in association with:
   a. Abetalipoproteinemia
   b. G6PD deficiency
   c. Rh deficiency syndrome
   d. Vitamin B₁₂ deficiency
A patient experiences an episode of acute intravascular hemolysis after taking primaquine for the first time. The physician suspects that the patient may have G6PD deficiency and orders an RBC G6PD assay 2 days after the hemolytic episode begins. How will this affect the test result?

a. No effect
b. False increase due to reticulocytosis
c. False decrease due to hemoglobinemia
d. Absence of enzyme activity

10. The most common defect or deficiency in the anaerobic glycolytic pathway that causes chronic HNSHA is:

a. Pyruvate kinase deficiency
b. Lactate dehydrogenase deficiency
c. Glucose-6-phosphate dehydrogenase deficiency
d. Methemoglobin reductase deficiency

11. Which of the following laboratory tests would be best to confirm PNH?

a. Acidified serum test (Ham test)
b. Osmotic fragility test
c. Flow cytometry for detection of eosin-5′-maleimide binding on erythrocytes
d. Flow cytometry for detection of CD55, CD59, and FLAER binding on neutrophils and monocytes

REFERENCES


Extrinsic Defects Leading to Increased Erythrocyte Destruction—Nonimmune Causes

Elaine M. Keohane

OUTLINE

Microangiopathic Hemolytic Anemia
- Thrombotic Thrombocytopenic Purpura
- Hemolytic Uremic Syndrome
- HELLP Syndrome
- Disseminated Intravascular Coagulation

Macroangiopathic Hemolytic Anemia
- Traumatic Cardiac Hemolytic Anemia
- Exercise-Induced Hemoglobinuria

Hemolytic Anemia Caused by Infectious Agents
- Malaria
- Babesiosis
- Clostridial Sepsis
- Bartonellosis

Hemolytic Anemia Caused by Other Red Blood Cell Injury
- Drugs and Chemicals
- Venoms
- Extensive Burns (Thermal Injury)

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the general pathophysiology and clinical laboratory findings in microangiopathic hemolytic anemia, including the characteristic red blood cell morphology.
2. Compare and contrast the pathophysiology, clinical symptoms, and typical laboratory findings in thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, and disseminated intravascular coagulation.
3. Explain the pathophysiology and typical laboratory features of traumatic cardiac hemolytic anemia and exercise-induced hemoglobinuria.
4. Describe the life cycle of Plasmodium, including the hepatic and erythrocytic cycles, and the insect vector.
5. Explain the pathophysiologic mechanisms in *P. falciparum* infection that lead to anemia and neurologic manifestations.
6. Differentiate the five *Plasmodium* species affecting humans based on the geographic distribution, characteristic morphology on a peripheral blood film, the extent of parasitemia, and the length of the erythrocytic cycle.
7. Describe the proper specimen collection and procedure for performing a thin and thick blood film examination.
8. Compare and contrast *Babesia* species and *Plasmodium* species in terms of geographic distribution, clinical symptoms of infection, and morphology.
9. Describe the pathophysiology, laboratory findings, and peripheral blood morphology in hemolytic anemia due to clostridial sepsis, bartonellosis, drugs, chemicals, venoms, and extensive burns.
10. Given the history, symptoms, laboratory findings, and a representative microscopic field from a peripheral blood film of a patient with suspected extrinsic, nonimmune hemolytic anemia, discuss possible causes of the anemia and indicate the data that support the conclusions.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 24-year-old woman was brought to the emergency department with a 2-day history of fever, chills, excessive sweating, nausea, and general malaise. Because she had recently returned from a 3-week family trip to Ghana in Western Africa, the treating physician ordered a CBC and examination of thin and thick peripheral blood films. The following are the patient’s laboratory results:

<table>
<thead>
<tr>
<th>Patient Results</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (×10^9/L)</td>
<td>4.5–11.0</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>12.0–15.0</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>35–49</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>80–100</td>
</tr>
<tr>
<td>Platelets (×10^9/L)</td>
<td>150–450</td>
</tr>
</tbody>
</table>
Extrinsic hemolytic anemias comprise a diverse group of disorders in which red blood cells (RBCs) are structurally and functionally normal, but a condition outside of the RBCs causes premature hemolysis. The extrinsic hemolytic anemias can be divided into conditions with nonimmune and immune causes. A common feature in the nonimmune extrinsic hemolytic anemias is the presence of a condition that causes physical or mechanical injury to the RBCs. This injury can be caused by abnormalities in the microvasculature (microangiopathic) or the heart and large blood vessels (macroangiopathic), infectious agents, chemicals, drugs, venoms, or extensive burns. The nonimmune disorders causing hemolytic anemia are discussed in this chapter and are summarized in Box 25-1. In immune hemolytic anemia, hemolysis is mediated by antibodies, complement, or both, and these conditions are covered in Chapter 26. Examination of a peripheral blood film is important in suspected extrinsic hemolytic anemias, because observation of abnormal RBC morphology, such as schistocytes, spherocytes, or the presence of intracellular organisms, provides an important clue to the diagnosis.

**MICROANGIOPATHIC HEMOLYTIC ANEMIA**

Microangiopathic hemolytic anemias (MAHAs) are a group of potentially life-threatening disorders characterized by RBC

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**CASE STUDY—cont’d**

After studying the material in this chapter, the reader should be able to respond to the following case study:

1. Identify the inclusions present on the thin and thick peripheral blood films.

   - Inclusions were noted on the thin and thick peripheral blood films (Figures 25-1 and 25-2).

   - Based on the results of the CBC and peripheral blood films, the patient was treated with oral quinine sulfate and doxycycline.

2. What is the likely diagnosis for this patient?

3. What clues in the history support this diagnosis?

4. What other forms might be found on the peripheral blood films in this disease?

5. What are the pathophysiologic mechanisms for the anemia in this disease?

**Figure 25-1** Thin peripheral blood film for the patient in the case study (×1000).

**Figure 25-2** Thick peripheral blood film for the patient in the case study (×1000). (Courtesy Linda Marler, Indiana Pathology Images.)
fragmentation and thrombocytopenia. The RBC fragmentation occurs intravascularly by the mechanical shearing of RBC membranes as the cells rapidly pass through turbulent areas of small blood vessels that are partially blocked by microthrombi or damaged endothelium.1,2 Upon shearing, RBC membranes quickly reseal with minimal escape of hemoglobin, but the resulting fragments (called schistocytes) are distorted and become rigid.1 The spleen clears the rigid RBC fragments from the circulation through the extravascular hemolytic process (Chapter 23).1 Laboratory evidence of the hemolytic anemia includes a decreased hemoglobin level, increased reticulocyte count, increased serum indirect (unconjugated) bilirubin, increased serum lactate dehydrogenase activity, decreased serum haptoglobin level, and increased urine urobilinogen. In some cases, the fragmentation is so severe that intravascular hemolysis occurs with varying amounts of hemoglobinemia, hemoglobinuria, and markedly decreased levels of serum haptoglobin.3 The presence of schistocytes on the peripheral blood film is a characteristic feature of microangiopathic hemolytic anemia. The RBC shearing may also produce helmet cells and, occasionally, microspherocytes. Polychromasia and nucleated RBCs may also be present on the blood film, depending on the severity of the anemia.

Thrombocytopenia is also a feature of microangiopathic hemolytic anemia; it is due to the consumption of platelets in thrombi that form in the microvasculature.1 Thus these disorders are sometimes called thrombotic microangiopathies.4,5 The major microangiopathic hemolytic anemias include thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, and disseminated intravascular coagulation (DIC).1,4,5 TTP and HUS can be difficult to differentiate because they have overlapping clinical and laboratory findings (Box 25-2). Definitive diagnosis, however, is critical because they have different etiologies and require different treatments.3

This chapter provides an overview of these conditions. TTP, HUS, and HELLP syndrome are covered in more detail in Chapter 40. DIC is covered in more detail in Chapter 39.

**Thrombotic Thrombocytopenic Purpura**

Thrombotic thrombocytopenic purpura is a rare, life-threatening disorder characterized by the abrupt appearance of microangiopathic hemolytic anemia, severe thrombocytopenia, and markedly elevated serum lactate dehydrogenase activity.2,6 Neurologic dysfunction, fever, and renal failure may also occur, but they are not consistently present.2,6 TTP is most commonly found in adults in their fourth decade, but it can present at any age.5,6 There is a higher incidence in females than in males.5

TTP is caused by a deficiency of the von Willebrand factor-clearing protease known as a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13).6-9 ADAMTS-13 regulates the size of circulating von Willebrand factor (VWF) by cleaving ultra-long VWF multimers (ULVWF) into shorter segments that have less hemostatic potential.5,10 VWF multimers circulate in a folded conformation so that their cleavage sites for ADAMTS-13 (in the A2 domain) and binding sites for platelets (GP Ibα receptor in the A1 domain) are hidden.5,10 These sites normally become accessible only when the ULVWF multimer is “unrolled or stretched out,” which occurs (1) during its release from endothelial cells, (2) during passage through small blood vessels with very high shear forces, or (3) after binding to collagen in the subendothelium after vascular injury (Chapter 37).5,6,10 Once unrolled, ADAMTS-13 binds to the cleavage sites on the ULVWFs and cuts them into smaller multimers.5,10 Thus ADAMTS-13 serves an important antithrombotic function by preventing VWF from excessively binding and activating platelets.9

When ADAMTS-13 is deficient, however, the hyperreactive ULVWF multimers adhere to the endothelial cells of the microvasculature, where they readily unroll as a result of hydrodynamic shear forces.5,7,10 Platelets are then able to bind to the A1 domains of the ULVWF multimers, and platelet aggregation is triggered.5,6 The platelet-VWF microthrombi accumulate in and block small blood vessels, leading to severe thrombocytopenia; ischemia in the brain, kidney, and other organs; and hemolytic anemia due to RBC rupture as they pass through blood vessels partially blocked by microthrombi.2,3,7 The intravascular hemolysis along with the extensive tissue ischemia result in a striking increase in serum lactate dehydrogenase activity that is characteristic of TTP.5

TTP can be idiopathic, secondary, or inherited. Idiopathic TTP has no known precipitating event.5,6 In idiopathic TTP, autoantibodies to ADAMTS-13 inhibit its activity, causing a severe deficiency.5,8,9 These autoantibodies are usually of the IgG class but can be IgM or IgA.

Secondary TTP can be triggered by infections, pregnancy, surgery, trauma, inflammation, and disseminated malignancy, possibly by depressing the synthesis of ADAMTS-13.5,6 Other conditions may induce an inhibitory reaction to ADAMTS-13, including hematopoietic stem cell transplantation; autoimmune disorders; human immunodeficiency virus (HIV); and

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**BOX 25-2 Typical Laboratory Findings in TTP and HUS**

**Hematologic**
- Decreased hemoglobin
- Decreased platelets
- Increased reticulocyte count

**Peripheral blood film**
- Schistocytes
- Polychromasia
- Nucleated red blood cells (severe cases)

**Biochemical**
- Markedly increased lactate dehydrogenase activity*
- Increased serum total and indirect bilirubin
- Decreased serum haptoglobin level
- Hemoglobinemia
- Hemoglobinuria
- Proteinuria, hematuria, casts†

HUS, Hemolytic uremic syndrome; TTP, thrombotic thrombocytopenic purpura.

*From systemic ischemia and hemolysis; more commonly found in TTP.
†From acute renal failure; more commonly found in HUS.
Haptoglobin level will be reduced. Hydrogenase activity will be markedly elevated and the serum lactate dehydrogenase level does not occur for several days after an acute onset of hemolysis, but the serum indirect bilirubin level is elevated. The serum indirect bilirubin level does not occur for several days after an acute onset of hemolysis, but the serum indirect bilirubin level is elevated. The serum indirect bilirubin level does not occur for several days after an acute onset of hemolysis, but the serum indirect bilirubin level is elevated. The serum indirect bilirubin level does not occur for several days after an acute onset of hemolysis, but the serum indirect bilirubin level is elevated.

Inherited TTP, also called Upshaw-Schulman syndrome, is a severe ADAMTS-13 deficiency caused by mutations in the ADAMTS13 gene. Over 75 different mutations have been identified, and symptomatic individuals are either homozygous for one of the mutations or compound heterozygous for two different mutations. Inherited TTP may present in infancy or childhood with recurrent episodes throughout life; however, some patients may not be symptomatic until adulthood after their system is stressed by pregnancy or a severe infection.

Typical initial laboratory findings in all types of TTP include a hemoglobin level of 8 to 10 g/dL, a platelet count of 10 to 30 × 10⁹/L, and schistocytes on the peripheral blood film. After the bone marrow begins to respond to the anemia, polychromasia and nucleated RBCs may also be present on the blood film. The white blood cell count is often increased, and immature granulocytes may appear. The bone marrow shows erythroid hyperplasia and a normal number of megakaryocytes. Hemoglobinuria occurs when there is extensive intravascular hemolysis. Various amounts of protein, RBCs, and urinary casts may also be present in the urine, depending on the extent of the renal damage. Results of coagulation tests are usually within the reference interval, which differentiates TTP from DIC (covered below). An elevation of the serum indirect bilirubin level does not occur for several days after an acute onset of hemolysis, but the serum lactate dehydrogenase activity will be markedly elevated and the serum haptoglobin level will be reduced.

In idiopathic and inherited TTP, the ADAMTS-13 activity is usually severely reduced to less than 5% to 10% of normal. In secondary TTP, the ADAMTS-13 deficiency is not as severe. ADAMTS-13 autoantibodies can be detected in idiopathic TTP but are absent in inherited TTP.

Approximately 80% to 90% of patients with idiopathic TTP respond favorably to plasma exchange therapy due to the removal of the offending ADAMTS-13 autoantibody and infusion of replacement ADAMTS-13 enzyme from donor plasma. Therefore, it is important that this type of TTP be recognized and quickly treated with plasma exchange therapy to avoid a fatal outcome. Corticosteroids are also administered to suppress the autoimmune response. Approximately one third of patients who respond to plasma exchange experience recurrent episodes of TTP. Rituximab (anti-CD20) is effective in suppressing an autoantibody response in some patients with relapsing TTP. Patients with secondary TTP generally do not respond well to plasma exchange, and the prognosis in these cases is poor, except when the TTP is related to autoimmune disease, pregnancy, or ticlopidine use. Plasma exchange is not required in inherited TTP, which is treated by infusion of fresh frozen plasma to supply the deficient ADAMTS-13 enzyme.

**Hemolytic Uremic Syndrome**

HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure from damage to endothelial cells in the glomerular microvasculature. There are two general types: typical and atypical HUS. Typical HUS (Shiga toxin-associated HUS or stx-HUS) is caused by bacteria that produce Shiga toxin and is preceded by an episode of acute gastroenteritis, often with bloody diarrhea. Atypical HUS (aHUS) is caused by unregulated activation of the alternative complement pathway. Patients with HUS have the typical laboratory findings of microangiopathic hemolytic anemia discussed previously. However, the platelet count is only mildly to moderately decreased, and evidence of renal failure is usually present, including an elevated level of serum creatinine, proteinuria, hematuria, and the presence of hyaline, granular, and RBC casts in the urine. Stx-HUS type comprises 90% of cases of HUS. The most common cause is infection with Shiga toxin-producing *Escherichia coli* (STEC), such as serotype O157:H7, but strains of toxin-producing *Shigella* have also been implicated. Stx-HUS occurs most often in young children but can be found in patients of all ages. Patients initially have acute gastroenteritis, often with bloody diarrhea, and after approximately 5 to 13 days develop oliguria and other symptoms of renal damage. About one fourth of patients also develop neurologic manifestations.

*E. coli* and *Shigella* serotypes implicated in HUS release Shiga toxins (Stx-1 and Stx-2, also called verotoxins) that are absorbed from the intestines into the plasma. The toxins have an affinity for the Gb3 glycolipid receptors (CD77) on endothelial cells, particularly those in the glomerulus and brain. The toxin is transported into the endothelial cells, where it inhibits protein synthesis and causes endothelial cell injury and eventual apoptosis. The Shiga toxin, together with many cytokines secreted as a result of the infection, also induces changes in endothelial cells that are prothrombotic, including expression of tissue factor, adhesion molecules, and secretion of increased amounts of ULVWF multimers. The endothelial
cell damage can cause stenosis (narrowing) of small blood vessels which can be exacerbated by activation of platelets and formation of platelet-fibrin thrombi. The resultant blockages in the microvasculature of the glomeruli results in acute renal failure. Endothelial damage and microthrombi can also occur in the microvasculature of the brain and other organs. There is no specific treatment for stx-HUS, but patients are provided supportive care as needed, including hydration, dialysis, and transfusions. The symptoms usually resolve spontaneously in 1 to 3 weeks, and the prognosis is favorable for most patients.

Atypical HUS comprises about 10% of cases of HUS and can first present in infancy, childhood, or adulthood. The characteristic feature is uncontrolled activation of the alternative complement system, which causes endothelial cell injury, activation of platelets and coagulation factors, and formation of platelet-fibrin thrombi that obstruct the microvasculature in the glomerulus and other organs. Approximately 50% to 70% of aHUS patients have inherited mutations in genes that code for components of the alternative complement pathway or its regulatory proteins. Inactivating mutations have been identified in genes for complement regulatory proteins, including complement factor H, complement factor I, membrane cofactor protein, and thrombomodulin. Activating mutations have been identified in the genes for complement factor B and C3. An acquired form of aHUS is associated with autoantibodies against complement factor H and accounts for approximately 5% to 10% of cases. In the remaining cases, no mutation or autoantibodies have been identified. aHUS may be triggered by hematopoietic stem cell therapy, pregnancy, infection, inflammation, surgery, or trauma. Plasma exchange and plasma infusion have limited efficacy in aHUS. In recent studies, therapy with eculizumab (antibody to C5) has improved platelet counts and renal function in aHUS patients and may become the therapy of choice.

Differential diagnosis of aHUS and TTP is difficult due to the similarities in their clinical presentation and initial laboratory findings. Both are life-threatening disorders that require rapid action to prevent a fatal outcome, with plasma exchange most beneficial for TTP, and eculizumab more likely to benefit patients with aHUS. Assays for ADAMS-13 activity currently lack sufficient sensitivity and specificity and are not available in all laboratories. DNA analysis for complement system gene mutations is available in specialized laboratories but the results are not timely enough to be used in initial therapy decisions. More sensitive, specific, and rapid tests are needed to confirm aHUS and TTP.

**HELLP Syndrome**

HELLP syndrome is a serious complication in pregnancy and is named for its characteristic presentation of hemolysis, elevated liver enzymes, and low platelet count. It occurs in less than 1% of all pregnancies but develops in approximately 10% to 20% of pregnancies with severe preeclampsia, most often in the third trimester. The exact pathogenesis is not known. In preeclampsia, abnormalities in the development of placental vasculature result in poor perfusion and hypoxia. As a result, antiangiogenic proteins are released from the placenta that block the action of placental growth factors, including vascular endothelial growth factor. Continued vascular insufficiency of the placenta results in maternal endothelial cell dysfunction, which leads to platelet activation and fibrin deposition in the microvasculature, particularly in the liver.

Anemia, biochemical evidence of hemolysis, and schistocytes on the peripheral blood film are found as in the other microangiopathies. The platelet count is less than $100 \times 10^9/L$; counts falling below $50 \times 10^9/L$ indicate a worse prognosis. The serum lactate dehydrogenase activity is elevated, which reflects the hepatic necrosis as well as the hemolysis. The serum aspartate aminotransferase activity can be markedly elevated due to the severe hepatocyte injury. The low platelet count and increased serum lactate dehydrogenase and aspartate aminotransferase activity are major diagnostic criteria for the HELLP syndrome and are used to assess the severity of the disease. The prothrombin time and the partial thromboplastin time are within the reference interval, which distinguishes the HELLP syndrome from DIC. Therapy includes delivery of the fetus and placenta as soon as possible, along with supportive care to control seizures, hypertension, and fluid balance. The mortality rate is 3% to 5% for the mother and 9% to 24% for the fetus.

**Disseminated Intravascular Coagulation**

Disseminated intravascular coagulation (DIC) is characterized by the widespread activation of the hemostatic system, resulting in fibrin thrombi formation throughout the microvasculature. The major clinical manifestations are organ damage due to obstruction of the microvasculature and bleeding due to the consumption of platelets and coagulation factors and secondary activation of fibrinolysis. DIC is a complication of many disorders, such as metastatic cancers, acute leukemias, infections, obstetric complications, crush or brain injuries, acute hemolytic transfusion reactions, extensive burns, snake or spider envenomation, and chronic inflammation (Table 39-14). Thrombocytopenia of varying degrees is a consistent finding. Only about half of the patients have schistocytes on the peripheral blood film. The prothrombin time and partial thromboplastin time are prolonged, the fibrinogen level is decreased, and the level of D-dimer is increased in DIC, which distinguish it from the other microangiopathies. Tables 39-15 and 39-16 contain the primary and specialized tests used in the diagnosis of DIC.

**MACROANGIOPATHIC HEMOLYTIC ANEMIA**

**Traumatic Cardiac Hemolytic Anemia**

Mechanical hemolysis can occur in patients with prosthetic cardiac valves due to the turbulent blood flow through and around the implanted devices. The hemolysis is usually mild, and anemia does not generally develop due to compensation by the bone marrow. Severe hemolysis is rare and is usually due to paravalvular leaks in prosthetic cardiac valves. Hemolysis can also occur in patients with cardiac valve disease prior to corrective surgery. The anemia that occurs in severe...
cases is usually normocytic but can be microcytic if iron deficiency develops due to chronic urinary hemoglobin loss.

Depending on the severity of the hemolysis and the ability of the bone marrow to compensate for the reduced RBC lifespan, patients can be asymptomatic or present with pallor, fatigue, and even heart failure. On the peripheral blood film, schistocytes are a characteristic feature due to the mechanical fragmentation of the RBCs (Figure 25-4). The reticulocyte count is increased, but the platelet count is within the reference interval. Serum lactate dehydrogenase activity and levels of serum indirect bilirubin and plasma hemoglobin are elevated, and the serum haptoglobin level is decreased. Hemoglobinuria may be observed in severe hemolysis. Hemosiderinuria and a decreased level of serum ferritin occur with chronic hemoglobinuria due to the urinary loss of iron.

Surgical repair or replacement of the prothesis may be required if the anemia is severe enough to require transfusions. For patients with hemoglobinuria, iron supplementation is required if the anemia is severe enough to require transfusions. Folic acid may also be required, because deficiencies can occur due to the increased erythropoietic activity in the bone marrow.

**Exercise-Induced Hemoglobinuria**

RBC lysis, with an increase in free plasma hemoglobin and a decrease in serum haptoglobin level, has been demonstrated in some individuals after long-distance running and to a lesser extent after intensive cycling and swimming, but frank hemoglobinuria after exercise is a rare occurrence. Exercise-induced hemoglobinuria has been reported mainly in endurance runners but has also been observed after strenuous hand drumming. Various causes have been proposed, including mechanical trauma from the forceful, repeated impact of the feet or hands on hard surfaces; increased RBC susceptibility to oxidative stress; and exercise-induced alterations in membrane cytoskeletal proteins. 

Exercise-induced hemoglobinuria does not usually cause anemia unless the hemoglobinuria is particularly severe and recurrent. Laboratory findings include a decreased level of serum haptoglobin, an elevated level of free plasma hemoglobin, and hemoglobinuria observed after strenuous exercise.

Patients also have a slight increase in mean cell volume (MCV) and reticulocyte count. Schistocytes are not present on the peripheral blood film except in rare cases. Exercise-induced hemoglobinuria is a diagnosis of exclusion, and other possible causes of hemolysis and hemoglobinuria should be investigated and ruled out. There is no treatment for the disorder other than minimizing the physical impact on the feet with padding in shoes, running on softer terrain, or, if hemolysis is severe, discontinuing the activity.

**HEMOLYTIC ANEMIA CAUSED BY INFECTIOUS AGENTS**

**Malaria**

Malaria is a potentially fatal condition caused by infection of RBCs with protozoan parasites of the genus *Plasmodium*. Most human infections are caused by *P. falciparum* and *P. vivax*, but *P. ovale*, *P. malariae*, and a fifth species, *P. knowlesi*, also infect humans. *P. knowlesi*, a natural parasite of macaque monkeys, is easily misdiagnosed as *P. malariae* by microscopy, because the organisms are difficult to distinguish morphologically. Since 2004, hundreds of microscopically identified cases of *P. malariae* infection in Malaysia were actually found to be caused by *P. knowlesi* when polymerase chain reaction (PCR) assays were used, including tests on archival blood films from 1996, With the use of molecular techniques, *P. knowlesi* malaria is now known to be widespread in Malaysia, and cases have been reported in other areas of Southeast Asia.

**Prevalence**

Approximately 3.4 billion people live in areas in which malaria is endemic and are at risk for the disease. Worldwide in 2012, there were an estimated 207 million cases of malaria, with 627,000 deaths, mostly in children younger than 5 years of age. The majority of deaths were in Africa (90%), followed by Southeast Asia (7%) and the Eastern Mediterranean region (3%). The World Health Organization is coordinating a major global effort to control and eliminate malaria. These efforts include implementation of indoor chemical spraying, distribution of millions of insecticide-treated sleeping nets in high-risk areas, and promotion of policies for appropriate treatment in regions of endemic disease. An estimated 42% reduction in mortality rates from malaria occurred worldwide between 2000 and 2012, and the goal is to reduce mortality rates by 75% by 2015.

In 2011, 1925 cases of malaria were diagnosed in the United States, and almost all of the cases were associated with travel to a malaria-endemic country. *P. falciparum* and *P. vivax* cause most of the infections seen in the United States.

**Plasmodium Life Cycle**

Malaria is transmitted to humans by the bite of an infected female *Anopheles* mosquito. During a blood meal, sporozoites from the salivary gland of the mosquito are injected into the skin and migrate into the bloodstream of the human host. The sporozoites rapidly leave the circulating blood and invade hepatic parenchymal cells to begin *exoerythrocytic schizogony*.

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Figure 25-4. Peripheral blood film for a patient with traumatic cardiac hemolytic anemia. Note the presence of schistocytes (×1000).
the parasite’s asexual cycle. After 5 to 16 days (depending on the species), hepatic cells rupture, with each cell releasing tens of thousands of merozoites into the bloodstream to invade circulating RBCs, thus beginning erythrocytic schizogony.35 Inside the erythrocyte, the merozoite grows and metabolizes the hemoglobin. The merozoite becomes a ring form, which grows into a mature trophozoite, then into an immature schizont (chromatin dividing), and finally into a mature schizont that contains merozoites. The merozoites are released from the erythrocytes into the bloodstream and invade other RBCs to continue the asexual cycle. As the infection continues, the cycles often recur at regular intervals as all the individual parasitic cycles become synchronous; this produces paroxysms of fever and chills at a frequency that varies according to malaria species. Resting stages of *P. vivax* and *P. ovale*, called hypnozoites, can remain dormant in the liver and produce a relapse months or years later.36,37

Some merozoites enter RBCs and form male and female gametocytes (sexual stages). Gametocytes are ingested by an *Anopheles* mosquito when it takes in a blood meal. The female gamete is fertilized by the male gamete in the mosquito gut to produce a zygote, which becomes an ookinete that migrates to the outer wall of the mosquito midgut and develops into an oocyst. The oocyst produces sporozoites that are released into the hemocoel and migrate to the salivary glands of the mosquito. When the mosquito takes in a blood meal, the sporozoites are inoculated into the human host.

Other modes of transmission include congenital infection and transmission by blood transfusion, organ transplantation, or sharing of syringes and needles, but malaria acquired by these routes and local mosquito-transmitted malaria occur infrequently in the United States.34

**Pathogenesis**

If an individual is bitten by infected mosquitoes, the clinical outcome may be (1) no infection, (2) asymptomatic parasitemia (the patient has no symptoms, but parasites are present in the blood), (3) uncomplicated malaria (the patient has symptoms and parasitemia but no organ dysfunction), or (4) severe malaria (the patient has symptoms, parasitemia, and major organ dysfunction).33,36 The clinical outcome depends on parasite factors (species, number of sporozoites injected, multiplication rate, virulence, drug resistance), host factors (age, pregnancy status, immune status, previous exposure, genetic polymorphisms, nutrition status, coinfection with other pathogens, and duration of infection), geographic and social factors (endemicity, poverty, and availability of prompt and effective treatment), and other as yet unknown factors.35,36 In areas of high *Plasmodium* transmission, most individuals develop immunity, and the major risk groups for severe malaria are children younger than 5 years of age and women in their first pregnancy.35 Even in these risk groups, severe malaria is infrequent.34 On the other hand, immunity is low or nonexistent in individuals living in regions with low *Plasmodium* transmission and in travelers to regions where malaria is endemic, so all age groups are at risk for severe malaria.35 Most cases of severe malaria are due to *P. falciparum*; however, *P. vivax* and *P. knowlesi* can also cause severe disease.31,35 Infection with *P. malariae* or *P. ovale*, however, is usually uncomplicated and benign. Hyperparasitemia, defined as greater than 2% to 5% of the total RBCs parasitized, is usually present in severe malaria.33 Major complications of severe malaria include respiratory distress syndrome, metabolic acidosis, circulatory shock, renal failure, hepatic failure, hypoglycemia, severe anemia (defined as a hemoglobin level below 5 g/dL),39 poor pregnancy outcome, and cerebral malaria.35 Even with treatment, the fatality rate of severe malaria is 10% to 20%.39

The causes of anemia in malaria include direct lysis of infected RBCs during schizogony, immune destruction of infected and noninfected RBCs in the spleen, and inhibition of erythropoiesis and ineffective erythropoiesis.37,38 The destruction of noninfected RBCs contributes significantly to the anemia. In the invasion process, parasites shed proteins that bind to infected as well as noninfected RBCs.40 These proteins may change the RBC membrane in noninfected cells, allowing adherence of immunoglobulins and complement and thus enhancing their removal by the spleen.40,41 In addition, parasite proteins may also cause oxidative damage, resulting in decreased RBC survival.42

Malaria parasites metabolize hemoglobin, forming toxic hemozoin or malaria pigment. When RBCs lyse in schizogony, the hemozoin and other toxic metabolites are released, which results in an inflammatory response and cytokine imbalance.40 Abnormal levels of tumor necrosis factor-α and interferon-γ result in inhibition of erythropoiesis as well as ineffective erythropoiesis; increased levels of interleukin-6 stimulate hepcidin production in the liver, which decreases the iron available to developing RBCs (Chapter 20).40 In areas where malaria is endemic, poor nutrition and coinfection with hookworm or HIV contribute to the anemia, inflammation, and cytokine imbalance.38,40

*P. falciparum* is unique and particularly lethal in that infected RBCs adhere to endothelial cells in the microvasculature of internal organs, including the brain, heart, lung, liver, kidney, dermis, and placenta.35,36 This contributes to the pathogenesis by obstructing the microvasculature and decreasing oxygen delivery to organs and by protecting the parasite from clearance by the spleen.35,36 In the placental microvasculature, adherence of infected RBCs to endothelial cells results in local inflammation that can cause severe maternal anemia, decreased fetal growth, premature delivery, and increased risk of fetal loss.35,36 In the brain microvasculature, adherence of infected RBCs to endothelial cells can cause lethal cerebral malaria. Infected RBCs express a parasite protein on their membranes, called *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP1*). *PfEMP1* mediates binding of infected RBCs to cell receptors, particularly CD36 on platelets and some endothelial cells.35,43 Reasons why some strains are more likely to cause severe cerebral malaria is a subject of intense research. Adherence of infected RBCs in the brain has been attributed to a complex formed by VWF released by cytokine-stimulated endothelial cells, platelets bound to the VWF, and infected RBCs bound to platelet CD36.44 A recent mechanism was proposed whereby a specific variant of *PfEMP1*, expressed on the surface of RBCs infected with certain strains of *P. falciparum*,
specifically binds to endothelial protein C receptor (EPCR) on endothelial cells lining the microvessels in the brain. \(^{42}\) This binding prevents the activation of protein C (an inhibitor of activated factors V and VIII), creating a local hypercoagulable state in the brain. The result is fibrin deposition and parasite sequestration in the brain microvasculature and symptoms of severe cerebral malaria. \(^{45}\)

The ability of the parasite to invade RBCs affects the extent of the parasitemia and the severity of the disease. \(P.\) vivax and \(P.\) ovale can only invade reticulocytes, and \(P.\) malariae can only invade older RBCs. On the other hand, \(P.\) falciparum and \(P.\) knowlesi are able to invade RBCs of all ages and thus can lead to very high levels of parasitemia. \(^{35,37}\) \(P.\) vivax requires Duffy antigens on RBCs for invasion, so individuals lacking Duffy antigens are resistant to infection with \(P.\) vivax. The expansion of the Duffy-negative population in West Africa seems to be an effective genetic adaptation because \(P.\) vivax infection is almost nonexistent in West Africa.\(^{35}\)

Polymorphisms in genes for the \(\alpha\) and \(\beta\) hemoglobin chains (Hb S, Hb C, Hb E, and \(\alpha\)- and \(\beta\)-thalassemia) and for glucose-6-phosphate dehydrogenase (G6PD) protect individuals from developing severe \(Hb\) S, \(Hb\) C, \(Hb\) E, and \(Hb\) (sickle cell trait) \(^{49}\) may contribute to this protective effect. Over thousands of years, the evolutionary pressure of \(P. falciparum\) has resulted in a higher frequency of these polymorphisms in populations located in high-prevalence malaria regions, including sub-Saharan Africa, the Middle East, and Southeast Asia (Figure 25-3). Polymorphisms in cytokines and cellular receptors are also being investigated as modulators of malaria severity.\(^{35}\)

**Clinical and Laboratory Findings**

The clinical symptoms of malaria are variable and can include fever, chills, rigors, sweating, headache, muscle pain, nausea, and diarrhea. In severe malaria, jaundice, splenomegaly, hepatomegaly, shock, prostration, bleeding, seizures, or coma may occur. \(^{39}\) In patients with chronic malaria or with repeated malarial infections, the spleen may be massively enlarged.

During fevers, the WBC count is normal to slightly increased, but neutropenia may develop during chills and rigors. In chronic malaria with anemia, the reticulocyte count is decreased due to the negative effect of the inflammation on erythropoiesis. In severe malaria, one or more of the following laboratory features are found: metabolic acidosis, decreased serum glucose (less than 40 mg/dL), increased serum lactate, increased serum creatinine, decreased hemoglobin level (less than 5 g/dL), hemoglobinuria, and hyperparasitemia. \(^{39}\)

**Microscopic Examination**

Malarial infection can be diagnosed microscopically by demonstration of the parasites in the peripheral blood. Optimally, blood should be collected before treatment is initiated. \(^{37,50}\) At least two thick and two thin peripheral blood films should be made as soon as possible after collection of venous blood in ethylenediaminetetraacetic acid (EDTA) anticoagulant. \(^{50}\) Alternatively, blood films can be made directly from a capillary puncture. Wright-Giemsa stain is used for visualization of the parasites. \(^{37}\) Thick blood films concentrate the parasites and are ideal for initial screening of peripheral blood.

Blood films are stained with a water-based Wright-Giemsa stain without methanol fixation to lyse the RBCs (Box 25-3). Thin blood films are used for species identification and determination of the percent parasitemia; they are stained after methanol fixation. The percent parasitemia is determined by counting the number of parasitized RBCs (sexual stages) among 500 to 2000 RBCs on a thin peripheral blood film and converting to a percentage. \(^{31}\) At least 300 fields on the thick and thin blood films should be examined with the 100× objective before a negative result is reported. \(^{37,51}\) Multiple samples taken at 8- to 12-hour intervals may be needed because the number of circulating parasites may vary with the timing of the erythrocytic schizogony. \(^{50}\) Microscopy can detect 5 to 20 parasites per microliter of blood, or 0.0001% parasitemia. \(^{37}\) A negative result for a single set of thick and thin peripheral blood films does not rule out a diagnosis of malaria. \(^{37}\) Malarial parasite detection and species identification require experienced laboratory personnel. A platelet lying on top of an erythrocyte in a thin blood film may be confused with a malarial parasite by an inexperienced observer (Figure 25-5).

**BOX 25-3** **Thick Film Preparation for Malaria**

To make a thick film, place three small drops of blood close together near one end of the slide. With one corner of a clean slide, stir the blood for about 30 seconds to mix the three drops over an area approximately 1 to 2 cm in diameter. Allow the film to dry thoroughly. Stain the film using a water-based Giemsa stain. (The water-based stain lyses the red blood cells. Thin blood films are fixed in methyl alcohol to preserve the red blood cells so they do not lyse.) In a thick film, more parasites are seen in each field.

![Peripheral blood film with a platelet on top of a red blood cell (A) compared with an intraerythrocytic *Plasmodium vivax* ring form (B) (×1000).](Image)
**Plasmodium vivax.** *P. vivax* is widely distributed and causes 40% of human malaria cases worldwide. It is the predominant species in Asia and South and Central America, but also occurs in Southeast Asia, Oceania, and the Middle East. It is rare in Africa and virtually absent in West Africa. The early ring forms are delicate with a red chromatin dot and blue-staining cytoplasm. As the trophozoite grows, the RBC becomes enlarged. Schüffner stippling appears in all stages except the early ring forms. The growing trophozoite is ameboid in appearance and has fine light brown hemozoin pigment. The mature trophozoite almost fills the RBC (Figure 25-6). In an immature schizont, red chromatin begins to divide into two or more dots, and the mature schizont has 12 to 24 merozoites (Figure 25-7). Gametocytes are round with blue cytoplasm and light brown pigment; they have either centrally located (microgametocyte) or eccentrically located (macrogametocyte) red chromatin. The length of the erythrocytic cycle is 44 to 48 hours. *P. vivax* is difficult to eradicate because the hypnozoite forms remain dormant in the liver and may cause a relapse.

**Plasmodium ovale.** *P. ovale* is found mainly in West Africa and India. The young ring forms are larger and more ameboid than those of *P. vivax*, and as the trophozoite grows, the RBCs become enlarged, oval, and fringed (Figure 25-8). Schüffner stippling is present in all stages, including early ring forms. In the schizont stage, the RBCs are oval, and the parasite is round and compact. The mature schizont has 8 to 12 merozoites. Gametocytes are smaller than those of *P. vivax* but have a similar appearance. The length of the erythrocytic cycle is 48 hours.

**Plasmodium malariae.** *P. malariae* is found worldwide but at low frequency. The highest prevalence is in East Africa and India. The ring stage is often smaller and wider than that of *P. vivax*, although the two may be indistinguishable. In growing trophozoites, the cytoplasm forms a characteristic narrow band across the cell and contains dark brown pigment (Figure 25-9). RBCs do not become enlarged, and there is no stippling. The mature schizont has 6 to 12 merozoites and often forms a rosette around clumped pigment. Gametocytes
are similar to those of *P. vivax* but are smaller. The length of the erythrocytic cycle is 72 hours.37

**Plasmodium falciparum.** *P. falciparum* is the predominant species in sub-Saharan Africa, Saudi Arabia, Haiti, and the Dominican Republic.52 It is also found in Asia, Southeast Asia, the Philippines, Indonesia, and South America.52 *P. falciparum* can produce high parasitemia (greater than 50% of RBCs infected) due to its ability to invade RBCs of all ages. Only ring forms and crescent- or banana-shaped gametocytes are observed in the peripheral blood. RBCs with trophozoites and schizonts adhere to the endothelial cells in various organs and do not circulate. Ring forms are small and can be easily missed. One or more rings may occupy a given cell, with some rings located at the cell periphery (Figure 25-1). The crescent-shaped gametocytes have deep blue cytoplasm with brownish pigment and red chromatin near the center and are easily recognized on blood films (Figure 25-10). The length of the erythrocytic cycle is 36 to 48 hours.37

**Plasmodium knowlesi.** *P. knowlesi* is widespread in Malaysia, and cases have been reported in Myanmar, the Philippines, and Thailand.52 In the early trophozoite stage, it can look similar to *P. falciparum* with high parasitemia and multiple ring forms in one cell. In the growing trophozoite, the cytoplasm forms a band across the cell, similar to *P. malariae* (Figure 25-11). Mature schizonts have an average of 16 merozoites and do not form rosettes.37 The length of the erythrocytic cycle is only 24 hours, so infected patients can rapidly develop a high level of parasitemia and severe malaria; thus prompt diagnosis and treatment are critical.37 Molecular methods may be required for definitive diagnosis. Figure 25-12 illustrates four species of *Plasmodium*.

**Other Tests for Diagnosis**

Fluorescent dyes may be used to stain *Plasmodium* species; they are sensitive for parasite detection but are not useful for speciation.37 Molecular-based tests (such as PCR) can be used for detection and speciation of malarial parasites and are especially helpful in cases of mixed infections, low parasitemia, and infection with *P. knowlesi*. A rapid antigen test, the BinaxNOW® Malaria Test, has been approved by the Food and Drug Administration for use in the United States.53 It is based on the detection of *P. falciparum* histidine-rich protein II and generic *Plasmodium* aldolase.53 The sensitivity of the test is low when there are fewer than 100 parasites per microliter of blood.37,53 Therefore, thick and thin blood film microscopy should be done with the antigen test.53

**Treatment**

Chloroquine or hydroxychloroquine is used for treatment of all malaria, except for disease caused by strains of *P. falciparum* and *P. vivax* acquired from areas known to harbor chloroquine-resistant organisms.54 For infection with the chloroquine-resistant strains, combination therapy (use of two drugs with different mechanisms of action) is recommended, including atovaquone-proguanil, quinine sulfate plus doxycycline or tetracycline, or...
mefloquine.54 Another choice for resistant P. falciparum is artemether-lumefantrine.54 Primaquine is also administered in P. vivax and P. ovale infections to eradicate the hypnozoites in the liver and prevent relapse.39,54 Prior to administration of primaquine, testing for G6PD deficiency is recommended because patients with moderate to severe deficiency can develop hemolytic anemia after primaquine treatment (Chapter 24).39,54 There is growing concern about the widespread resistance of P. falciparum to chloroquine, the increase in chloroquine-resistant P. vivax strains, and the possible emergence and spread of resistance to other antimalarial drugs.59 Transfusion therapy is used for severe anemia, including exchange transfusion for patients with a level of parasitemia greater than 10% infected RBCs. Because infections with P. falciparum or P. knowlesi have the potential for a rapid, fatal course, patients must be treated without delay after confirmation of the diagnosis. Intensive research is under way to develop an effective vaccine.

**Babesiosis**

Babesiosis is a tick-transmitted disease caused by intraerythrocytic protozoan parasites of the genus Babesia. There are hundreds of species of Babesia, but only a few are known to cause disease in humans. B. microti is the most common cause of babesiosis in the United States, where it was originally called Nantucket fever because the first cluster of cases was found on Nantucket Island, off Massachusetts, in 1969 and the early 1970s.55,56 The sexual cycle of B. microti occurs in the tick, Ixodes scapularis, whereas its asexual cycle primarily occurs in the white-footed mouse, the reservoir host in the United States.57 Humans are incidental hosts and become infected after injection of sporozoites during a blood meal by infected ticks. Other Babesia species, such as B. duncani and B. divergens, can be found sporadically in humans.56,57 Babesia may also be transmitted by transfusion of RBCs from asymptomatic donors. Between 1979 and 2009, 159 cases of transfusion-transmitted babesiosis were identified.58 Congenitally acquired babesiosis has been reported, but it is rare.57,59

**Geographic Distribution**

The areas in which B. microti is endemic in the United States are southern New England, New York State, New Jersey, Wisconsin, and Minnesota.56,57 B. duncani occurs in northern California and Washington State; B. divergens–like organisms are found in Missouri, Kentucky, and Washington state; and B. divergens and B. venatorum occur in Europe.56,60 Isolated cases of babesiosis have also been reported in Asia, Africa, Australia, and South America.56,60

**Clinical Findings**

The incubation period for B. microti infection can range from 1 to 9 weeks.56 Infection is asymptomatic in perhaps a third of individuals, so the exact prevalence is unknown.56,61 In other individuals, B. microti causes a mild to severe hemolytic anemia.56,57 The patient usually experiences fever and nonrespiratory flu-like symptoms, including chills, headache, sweats, nausea, arthralgias, myalgia, anorexia, and fatigue, that last from several weeks to months. Jaundice, splenomegaly, or hepatomegaly may be present. Some individuals progress to severe, life-threatening disease due to acute respiratory failure, congestive heart failure, renal shutdown, liver failure, central nervous system involvement, or disseminated intravascular coagulation. Severe disease may occur at any age, but it is more common in individuals over 50 years of age.1,56 Immune deficiency due to asplenia, malignancy, immunosuppressive drugs, or HIV infection increases the risk of severe disease.1,56,57 The overall mortality rate is less than 10% but is likely higher in immunocompromised individuals.56,62

**Laboratory Findings and Diagnosis**

Evidence of hemolytic anemia is usually present in symptomatic infection, including decreased hemoglobin level, increased reticulocyte count, decreased serum haptoglobin level, and bilirubinemia. Leukopenia, thrombocytopenia, hemoglobinuria,
and proteinuria may also be present, along with abnormal results on renal and liver function tests.56

The diagnosis of babesiosis is made by demonstration of the parasite on Wright-Giemsa–stained thin peripheral blood films. Babesia appear as tiny rings or occasionally as tetrads inside the RBCs. The ring forms may be round, oval, or ameboid; they have a dark purple chromatin dot and a minimal amount of blue cytoplasm surrounding a vacuole. Multiple rings can be found in one RBC (Figures 25-13 and 25-14). Tetrads may also appear in a “Maltese cross” formation. Babesia can be distinguished from *P. falciparum* by the pleomorphism of their ring forms, absence of hemozoin pigment and gametocytes, and occurrence of extraerythrocytic forms. Parasitemia may be low (fewer than 1% of RBCs affected) in early infections, but as many as 80% of RBCs may be infected in asplenic patients.63 In cases of low parasitemia, babesiosis can be diagnosed by detection of IgG and IgM antibodies to *B. microti* by indirect immunofluorescent antibody assay.57 The sensitivity of the assay ranges from 88% to 96%.64 Definitive species identification requires PCR-based methods.56,57

**Treatment**

Babesiosis is treated with combination therapy using azithromycin/ataovaquone or clindamycin/quinine.56,57 Exchange transfusion is used when greater than 10% of RBCs are infected or when there is evidence of major organ failure.56 Asymptomatic infections usually require no treatment.56,57

**Clostridial Sepsis**

Sepsis with massive intravascular hemolysis, which is often fatal, is a rare complication of infection with *Clostridium perfringens*, an anaerobic gram-positive bacillus. *C. perfringens* grows very rapidly (7-minute doubling time) and produces an α-toxin with phospholipase C and sphingomyelinase activity that hydrolyzes RBC membrane phospholipids.65,66 The RBCs become spherical and extremely susceptible to osmotic lysis, which results in sudden, massive hemolysis and dark red plasma and urine.1,65,67 The hematocrit may drop to below 10%.1,67 The intravascular hemolysis can trigger DIC and renal failure. Spherocytes, microspherocytes, and toxic changes to neutrophils can be observed on a peripheral blood film.1,65,67 Some conditions that increase the risk of clostridial sepsis and hemolytic anemia include malignancies (genitourinary, gastrointestinal, and hematologic), solid organ transplantation, postpartum or postabortion infections, biliary surgery, acute cholecystitis, and deep wounds.1,65,68 Rapid therapy with transfusions, antibiotics, and fluid management is required. The prognosis is grave, and many patients die despite intensive treatment.

**Bartonellosis**

Human bartonellosis (Carrión disease) is transmitted by the bite of a female sandfly and is endemic in certain regions of Peru, Ecuador, and Colombia.69,70 It is caused by *Bartonella bacilliformis*, a small, pleomorphic, intracellular coccobacillus that adheres to RBCs and causes hemolysis.1,71 The bacteria produce a protein called *deformin* that forms pits or invaginations in the RBC membrane.1,71 There are two clinical stages: the first stage is characterized by acute hemolytic anemia (called Oroya fever after a city in the Peruvian Andes); the second or chronic verruga stage is characterized by the eruption of skin lesions and warts on the extremities, face, and trunk. The acute phase begins with fever, malaise, headache, and chills, followed by pallor, jaundice, general lymphadenopathy, and, less commonly, hepatosplenomegaly.69,71 Over several days, there is rapid hemolysis, with the hematocrit dropping below 20% in two thirds of patients.69,71 Polychromasia, nucleated RBCs, and mild leukocytosis with a left shift are observed on the peripheral blood film. The mortality rate is approximately 10% for hospitalized patients and 90% for those who are untreated.69,70 Diagnosis is made by blood culture and observation of bacilli or coccobacilli on the erythrocytes on a Wright-Giemsa–stained peripheral blood film. During the acute phase, 80% of the RBCs can be involved.1 Serologic diagnosis with indirect immunofluorescent antibody or immunoblotting has

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**Figure 25-13** Babesia microti ring forms in a peripheral blood film. Note the varying appearance of the ring forms and the presence of multiple ring forms in individual red blood cells (×1000).

**Figure 25-14** Babesia microti ring and tetrad forms in a peripheral blood film (×1000).
a sensitivity of 89%. The acute stage is treated with transfusions and ciprofloxacin or chloramphenicol.

Carrión disease was named for a Peruvian medical student, Daniel Alcides Carrión, who in 1885 inoculated himself with fluid from a wart of a patient in the chronic stage of infection. He subsequently developed fatal hemolytic anemia similar to that in Oroya fever, which linked the two stages of the disease to the same agent.

**HEMOLYTIC ANEMIA CAUSED BY OTHER RED BLOOD CELL INJURY**

**Drugs and Chemicals**

Hemolytic anemia of varying severity may result from drugs or chemicals that cause the oxidative denaturation of hemoglobin, leading to the formation of methemoglobin and Heinz bodies. Examples of agents that can cause hemolytic anemia in individuals with normal RBCs include dapsone, a drug used to treat leprosy and dermatitis herpetiformis, and naphthalene, a chemical found in mothballs. Individuals deficient in G6PD are particularly sensitive to the effects of oxidative agents. For example, primaquine can cause hemolytic anemia in G6PD-deficient individuals (Chapter 24).

The typical laboratory findings include a decrease in hemoglobin level, increase in the reticulocyte count, increase in serum indirect bilirubin, and decrease in serum haptoglobin level. In severe drug- or chemical-induced hemolytic anemia, Heinz bodies (denatured hemoglobin) may be observed in RBCs. Heinz bodies can only be visualized with a supravital stain, and they appear as round, blue granules attached to the inner RBC membrane (Figure 14-11). Exposure to high levels of arsenic hydride, copper, and lead can also cause hemolysis.

**Venoms**

Envenomation from contact with snakes, spiders, bees, or wasps can induce hemolytic anemia in some individuals. The hemolysis can occur acutely or be delayed 1 or more days after a bite or sting. The severity of the hemolysis depends on the amount of venom injected, and in severe cases, renal failure and death can result. Some mechanisms by which venoms can induce hemolysis are direct disruption of the RBC membrane, alteration of the RBC membrane that results in complement-mediated lysis, and initiation of DIC. Hemolytic anemia has been reported after bites from poisonous snakes (e.g., some cobras and pit vipers) and the brown recluse spider (*Loxosceles reclusa* and *Loxosceles laeta*), and after multiple stings (50 or more) by bees or wasps.

**Extensive Burns (Thermal Injury)**

Warming normal RBCs to 49 °C in vitro induces RBC fragmentation and budding. Likewise, patients with extensive burns manifest similar RBC injury with acute hemolytic anemia. Schistocytes, spherocytes, and microspherocytes are observed on the peripheral blood film (Figure 25-15), but the damaged RBCs are usually cleared by the spleen within 24 hours of the burn injury. In addition to resulting from the acute hemolysis, the anemia associated with extensive burns is also caused by blood loss during surgical excision and grafting of the burn wounds, nutritional deficiency, impaired metabolism, and anemia of chronic inflammation. Overheating blood in malfunctioning bloodwarmers prior to transfusion can also result in RBC fragmentation and hemolysis of the donor RBCs.
transplantation, disseminated cancer, pregnancy, and use of certain drugs. Inherited TTP is due to mutations in the ADAMTS13 gene.

- HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Typical HUS (stx-HUS) comprise 90% of cases, is found predominantly in young children, and is caused by toxin-producing strains of E. coli. Atypical HUS (aHUS) is due to inherited mutations in genes coding for complement components and regulators or autoantibodies to complement factor H. It also occurs secondary to organ transplantation, cancer, pregnancy, HIV infection, and some drugs.
- HELLP syndrome is a serious complication of pregnancy presenting with microangiopathic hemolytic anemia, thrombocytopenia, and elevated levels of liver enzymes.
- DIC is due to the widespread intravascular activation of the hemostatic system and formation of fibrin thrombi; coagulation factors and platelets are consumed in the thrombi, and secondary fibrinolysis occurs. Schistocytes are found in about half of the cases.
- Macroangiopathic hemolytic anemia is caused by traumatic cardiac hemolysis (RBC fragmentation from damaged or prosthetic cardiac valves) or exercise-induced hemolysis (mechanical trauma from forceful impact on feet or hands or from strenuous exercise).

The platelet count is normal in both conditions; schistocytes are seen only in traumatic cardiac hemolysis.

- Infections associated with hemolytic anemia due to invasion of RBCs include malaria and babesiosis. Hemolysis in bartonellosis is due to attachment of the bacteria to red blood cells and production of a lytic protein. Hemolysis in clostridial sepsis is due to the production of α-toxin.
- In malaria, severe anemia is due to direct lysis of infected RBCs, immune destruction of infected and uninfected RBCs in the spleen, and inhibition of erythropoiesis.
- Plasmodium (five species) and Babesia organisms are identified by the morphology of their intraerythrocytic stages on a Wright-Giemsa–stained peripheral blood film. Plasmodium species are transmitted to humans by mosquitoes, whereas a tick is the vector for Babesia.
- Hemolytic anemia can also be caused by injury to RBCs by drugs, chemicals, venoms, and extensive burns (thermal injury). In patients with extensive burns, schistocytes, spherocytes, and microspherocytes are observed on the peripheral blood film.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

**Answers can be found in the Appendix.**

4. Which of the following tests yields results that are abnormal in DIC but are usually within the reference interval or just slightly abnormal in TTP and HUS?
   a. Indirect serum bilirubin and serum haptoglobin
   b. Prothrombin time and partial thromboplastin time
   c. Lactate dehydrogenase and aspartate aminotransferase
   d. Serum creatinine and serum total protein

5. Which one of the following laboratory results may be seen in BOTH traumatic cardiac hemolytic anemia and exercise-induced hemoglobinuria?
   a. Schistocytes on the peripheral blood film
   b. Thrombocytopenia
   c. Decreased serum haptoglobin
   d. Hemosiderinuria

6. Which of the following species of Plasmodium produce hypnozoites that can remain dormant in the liver and cause a relapse months or years later?
   a. *P. falciparum*
   b. *P. vivax*
   c. *P. knowlesi*
   d. *P. malariae*
7. Which one of the following is not a mechanism causing anemia in *P. falciparum* infections?
   a. Inhibition of erythropoiesis
   b. Lysis of infected RBCs during schizogony
   c. Competition for vitamin B₁₂ in the erythrocyte
   d. Immune destruction of noninfected RBCs in the spleen

8. Which *Plasmodium* species is widespread in Malaysia, has RBCs with multiple ring forms, has band-shaped early trophozoites, shows a 24-hour erythrocytic cycle, and can cause severe disease and high parasitemia?
   a. *P. falciparum*
   b. *P. vivax*
   c. *P. knowlesi*
   d. *P. malariae*

9. One week after returning from a vacation in Rhode Island, a 60-year-old man experienced fever, chills, nausea, muscle aches, and fatigue of 2 days’ duration. A complete blood count (CBC) showed a WBC count of 4.5 × 10⁹/L, hemoglobin level of 10.5 g/dL, a platelet count of 134 × 10⁹/L, and a reticulocyte count of 2.7%. The medical laboratory scientist noticed tiny ameboid ring forms in some of the RBCs and some tetrad forms in others. These findings suggest:
   a. Bartonellosis
   b. Malaria
   c. Babesiosis
   d. Clostridial sepsis

10. What RBC morphology is characteristically found within the first 24 hours following extensive burn injury?
   a. Macrocytosis and polychromasia
   b. Burr cells and crenated cells
   c. Howell-Jolly bodies and bite cells
   d. Schistocytes and microspherocytes

11. A 36-year-old woman was brought to the emergency department by her husband because she had experienced a seizure. He reported that she had been well until that morning, when she complained of a sudden headache and malaise. She was not taking any medications and had no history of previous surgery or pregnancy. Laboratory studies showed a WBC count of 15 × 10⁹/L, hemoglobin level of 7.8 g/dL, a platelet count of 18 × 10⁹/L, and schistocytes and helmet cells on the peripheral blood film. Chemistry test results included markedly elevated serum lactate dehydrogenase activity and a slight increase in the level of total and indirect serum bilirubin. The urinalysis results were positive for protein and blood, but there were no RBCs in the urine sediment. Prothrombin time and partial thromboplastin time were within the reference interval. When the entire clinical and laboratory picture is considered, which of the following is the most likely diagnosis?
   a. HUS
   b. HELLP syndrome
   c. TTP
   d. Exercise-induced hemoglobinuria

**REFERENCES**


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Extrinsic Defects Leading to Increased Erythrocyte Destruction—Immune Causes

Kim A. Przekop*

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Define immune hemolytic anemia and indicate the types of antibodies involved.

2. Compare and contrast the mechanisms of immune hemolysis mediated by immunoglobulin M (IgM) and IgG antibodies.

3. Describe typical laboratory findings in immune hemolytic anemia and the importance of the direct antiglobulin test (DAT).

4. Compare and contrast four types of autoimmune hemolytic anemia in terms of the immunoglobulin class involved, the temperature for optimal reactivity of the autoantibody, the proteins detected by the DAT on the patient’s red blood cells, the presence or absence of complement activation, the type and site of hemolysis, and the specificity of the autoantibody.

5. Relate results of the DAT to the pathophysiology and clinical findings in autoimmune hemolytic anemia.

6. Describe three mechanisms of drug-induced immune hemolysis.

7. Compare and contrast the pathophysiology of immune hemolysis due to drug-dependent and drug-independent antibodies, including the related laboratory findings.

8. Describe two types of hemolytic transfusion reactions, the usual immunoglobulin class involved, the typical site of hemolysis, and important laboratory findings.

9. Describe the cause, pathophysiology, and laboratory findings in Rh and ABO hemolytic disease of the fetus and newborn (HDFN).

10. Given a patient history and results of a complete blood count, peripheral blood film examination, pertinent biochemical tests on serum and urine, and the direct and indirect antiglobulin tests, determine the type of immune hemolysis.

OUTLINE

Overview of Immune Hemolytic Anemias
Pathophysiology of Immune Hemolysis
Laboratory Findings in Immune Hemolytic Anemia
Autoimmune Hemolytic Anemia
Warm Autoimmune Hemolytic Anemia
Cold Agglutinin Disease
Paroxysmal Cold Hemoglobinuria
Mixed-Type Autoimmune Hemolytic Anemia
Drug-Induced Immune Hemolytic Anemia
Mechanisms of Drug-Induced Immune Hemolysis
Antibody Characteristics
Nonimmune Drug-Induced Hemolysis
Treatment
Alloimmune Hemolytic Anemias
Hemolytic Transfusion Reaction
Hemolytic Disease of the Fetus and Newborn

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 37-year-old man sought medical attention from his general practitioner for malaise, shortness of breath, and difficulty concentrating for the past five days. Physical examination revealed an otherwise healthy adult with tachycardia (faster than normal heart rate). There was no significant past medical history and the patient was not taking any medications. The physician ordered a complete blood count (CBC), urinalysis, comprehensive metabolic panel (CMP), and electrocardiogram (EKG). The following are the patient’s key laboratory results:

<table>
<thead>
<tr>
<th>Patient Results</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (×10⁹/L)</td>
<td>3.6–10.6</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>14.0–18.0</td>
</tr>
</tbody>
</table>

Continued
PART IV  Erythrocyte Disorders

CASE STUDY—cont’d

After studying the material in this chapter, the reader should be able to respond to the following case study:

<table>
<thead>
<tr>
<th>Patient Results</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV (fL)</td>
<td>105.4</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>36.3</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>17.3</td>
</tr>
<tr>
<td>PLT (×10^9/L)</td>
<td>325</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>18.1</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>72</td>
</tr>
<tr>
<td>Bands (%)</td>
<td>10</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.0–36.0</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11.5–14.5</td>
</tr>
<tr>
<td>PLT (×10^9/L)</td>
<td>150–450</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.5–2.5</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>50–70</td>
</tr>
<tr>
<td>Bands (%)</td>
<td>0–5</td>
</tr>
</tbody>
</table>

The EKG was normal. The CBC instrument printout flagged for nucleated red blood cells (and corrected the WBC for them), 3+ anisocytosis, and reticulocytosis. The peripheral blood film had moderate spherocytes, moderate polychromasia, few macrocytes, 3+ anisocytosis, and 15 nucleated red blood cells/100 WBCs (Figure 26-1). Occasional schistocytes and neutrophilia with a slight left shift were also observed on the blood film (not shown in figure). The urinalysis report included 2+ protein, 2+ blood, increased urobilinogen, with 0 to 5 RBCs seen on the microscopic exam. The patient’s serum was moderately icteric, and the total serum bilirubin was increased. The patient was admitted for further testing. The serum haptoglobin was decreased, the serum indirect (unconjugated) bilirubin and lactate dehydrogenase (LD) were elevated, and urine hemosiderin was positive.

A type and screen was ordered. The antibody screen was negative at the immediate spin and 37°C incubation phase but showed 3+ agglutination in the antihuman globulin (AHG) phase for all panel cells and the autocontrol. The direct antiglobulin test (DAT) showed 3+ agglutination with polyspecific AHG and monospecific anti-IgG but was negative with monospecific anti-C3b/C3d (complement). An acid elution was performed on the patient’s RBCs, and the eluate showed 2+ reactions with all panel cells and autocontrol at the AHG phase. The patient was diagnosed with warm autoimmune hemolytic anemia (WAIHA) and started on 1 mg/kg/d prednisone until the hemoglobin reached 10.0 g/dL (2 weeks of treatment). The patient was continued on prednisone for 4 months with slowly decreasing levels of the drug. He also received bisphosphonates, vitamin D, and calcium to combat the ill effects of prednisone. At 3 weeks postdiagnosis, the spherocytes disappeared, the urinalysis was normal, and the DAT was negative.

1. Explain why the WBC count, MCV, RDW, and reticulocyte count results were elevated.
2. Describe the immune mechanism that caused the spherocytosis, and explain why spherocytes have a shortened life span.
3. Relate the chemistry, urinalysis, and blood bank results with the pathophysiology of the patient’s anemia.
4. Explain why the treatment was effective, and explain if the patient’s WAIHA was acute or chronic.

OVERVIEW OF IMMUNE HEMOLYTIC ANEMIAS

Immune hemolytic anemia and nonimmune hemolytic anemia are the two broad categories comprising the extrinsic hemolytic anemias, disorders in which red blood cells (RBCs) are structurally and functionally normal, but a condition outside of the RBCs causes premature hemolysis. The nonimmune extrinsic hemolytic anemias are the result of physical or mechanical injury to the RBCs and are covered in Chapter 25. The immune hemolytic anemias are conditions in which RBC survival is shortened due to an antibody-mediated mechanism. The antibody may be an autoantibody (directed against a self RBC antigen), an alloantibody (directed against an RBC antigen of another person), or an antibody directed against a drug (or its metabolite) taken by the patient. Some antibodies are able to activate the classical complement pathway, which results in the attachment of activated complement proteins to the RBC membrane. RBCs with bound antibody or complement are prematurely removed from the circulation extravascularly by macrophages (due to their receptors for complement and the Fc component of antibody), intravascularly by complement-mediated hemolysis, or by a combination of both mechanisms. Anemia develops when the amount of hemolysis exceeds the ability of the bone marrow to replace the RBCs that are destroyed. The degree of anemia varies from asymptomatic and mild to severe and life-threatening.
Pathophysiology of Immune Hemolysis

In immune hemolysis an antibody binds to an antigen on the surface of RBCs, which signals premature removal of those cells from the circulation through extravascular or intravascular hemolysis (Chapter 23). The two classes or isotypes of antibodies involved in most immune hemolytic anemias are immunoglobulin G (IgG) and M (IgM). IgG is a monomer in a Y-like structure with two identical heavy chains (γ H chains) and two identical light chains (either κ or λ) connected by disulfide bonds. At the top of the Y-like structure are two antigen-binding (Fab) domains, each formed from the N-terminus of the variable domain of one light and one heavy chain. IgG has one Fc domain (the stem of the Y) consisting of the C-terminus of the two heavy chains (Figure 26-2). IgM is a pentamer consisting of five monomeric units connected by disulfide linkages at the C-termini of their heavy chains (μ H chains). Because the composition, structure, and size of IgG and IgM are different, their properties and mechanisms in mediating hemolysis are also different.

The classical complement pathway is an important mediator of immune hemolysis. The major proteins of the classical complement pathway are designated C1 through C9, and their components or fragments are designated with lowercase suffixes. The first protein, C1, has three components: C1q, C1r, and C1s. After an antibody binds to an antigen on the RBC surface, C1q must bind to two adjacent Fc domains to activate the pathway. Theoretically only one IgM molecule is needed for complement activation due to its larger pentameric structure with five Fc domains; however, at least two molecules of monomeric IgG in close proximity are required for C1q attachment. Therefore, IgM antibodies are highly effective in activating complement, whereas IgG antibodies are unable to activate the pathway unless there is a sufficient number of IgG molecules on the RBC surface. In addition, subclasses IgG1 and IgG3 have high binding affinity for C1q, while subclasses IgG2 and IgG4 have minimal ability to bind C1q.

The binding of C1q to adjacent Fc domains requires calcium and magnesium ions and activates C1r, which then activates C1s. This activated C1q-C1r-C1s complex is an enzyme that cleaves C4 and then C2, which results in the binding of a small number of C4bC2a complexes to the RBC membrane. The C4bC2a complex is an active C3 convertase enzyme that cleaves C3 in plasma; the result is the binding of many C3b molecules to C4bC2a on the RBC surface. The last phase of the classical pathway occurs when C4bC2aC3b converts C5 to C5b, which combines with C6, C7, C8, and multiple C9s to form the membrane attack complex (MAC). The MAC resembles a cylinder that inserts into the lipid bilayer of the membrane, forming a pore that allows water and small ions to enter the cell, causing lysis (Figure 26-3). Negative regulators inhibit various complement proteins and complexes in the pathway to prevent uncontrolled activation and excessive hemolysis.

Hemolysis mediated by IgM antibodies requires complement and can result in both extravascular and intravascular hemolysis. When IgM molecules attach to the RBC surface in relatively low density, complement activation results in C3b binding to the membrane, but complement inhibitors prevent full activation of the pathway to the terminal membrane attack complex. C3b-sensitized RBCs are destroyed by extravascular hemolysis, predominantly by macrophages (Kupffer cells) in the liver, which have C3b receptors. Some of the C3b on the RBCs can be cleaved, however, which leaves the C3d fragment on the cell. RBCs sensitized with only C3d are not prematurely removed from circulation because macrophages lack a C3d receptor. In severe cases of immune hemolysis involving heavy sensitization of RBCs with IgM antibody, significantly more complement is activated, which overwhelsms the complement inhibitors. In these cases, complement activation proceeds from C1 to C9 and results in rapid intravascular hemolysis.

Hemolysis mediated by IgG antibodies occurs with or without complement and predominantly by extravascular mechanisms. RBCs sensitized with IgG are removed from circulation by macrophages in the spleen, which have receptors for the Fc

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**BOX 26-1 Classification of Immune Hemolytic Anemias**

<table>
<thead>
<tr>
<th>Autoimmune hemolytic anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm autoimmune hemolytic anemia (WAHA)</td>
</tr>
<tr>
<td>Idiopathic</td>
</tr>
<tr>
<td>Secondary</td>
</tr>
<tr>
<td>Lymphoproliferative disorders</td>
</tr>
<tr>
<td>Nonlymphoid neoplasms</td>
</tr>
<tr>
<td>Collagen-vascular disease</td>
</tr>
<tr>
<td>Immunodeficiency disorders</td>
</tr>
<tr>
<td>Viral infections</td>
</tr>
<tr>
<td>Cold agglutinin disease (CAD)</td>
</tr>
<tr>
<td>Idiopathic</td>
</tr>
<tr>
<td>Secondary</td>
</tr>
<tr>
<td>Acute: infections (Mycoplasma pneumoniae, infectious mononucleosis, other viruses)</td>
</tr>
<tr>
<td>Chronic: lymphoproliferative disorders</td>
</tr>
<tr>
<td>Paroxysmal cold hemoglobinuria (PCH)</td>
</tr>
<tr>
<td>Idiopathic</td>
</tr>
<tr>
<td>Secondary</td>
</tr>
<tr>
<td>Viral infections</td>
</tr>
<tr>
<td>Syphilis</td>
</tr>
<tr>
<td>Mixed-type autoimmune hemolytic anemia</td>
</tr>
<tr>
<td>Drug-induced immune hemolytic anemia (DIHA)</td>
</tr>
<tr>
<td>Drug dependent</td>
</tr>
<tr>
<td>Drug independent</td>
</tr>
<tr>
<td>Alloimmune hemolytic anemias</td>
</tr>
<tr>
<td>Hemolytic transfusion reaction (HTR)</td>
</tr>
<tr>
<td>Hemolytic disease of the fetus and newborn (HDFN)</td>
</tr>
</tbody>
</table>
IgG antibodies are not efficient in activating complement, and intravascular hemolysis by full activation of complement from C1 to C9 is rare (except with anti-P in paroxysmal cold hemoglobinuria). However, if there is a high density of IgG1 or IgG3 bound to antigens on the RBCs, some complement is activated and C3b binds to the membrane. If both IgG and C3b are on the RBC membrane, there is faster clearance from the circulation by macrophages in both the spleen and the liver. Often, IgG-sensitized RBCs are only partially phagocytized by macrophages, which results in the removal of some membrane. Spherocytes are the result of this process, and they are the characteristic cell of IgG-mediated hemolysis. The spherocytes are eventually removed from circulation by entrapment in the red pulp of the spleen (splenic cords), where they are rapidly phagocytized by macrophages (Chapter 8). The mechanisms of immune hemolysis are summarized in Table 26-1.

**Laboratory Findings in Immune Hemolytic Anemia**

Laboratory findings in immune hemolytic anemia are similar to the findings in other hemolytic anemias and include decreased hemoglobin; increased reticulocyte count; increased levels of indirect serum bilirubin and lactate dehydrogenase; and decreased serum haptoglobin level. If the hemolysis is predominantly intravascular, or the extravascular hemolysis is severe, the haptoglobin level will be moderately to severely decreased, plasma hemoglobin will be increased, and the patient may have hemoglobinuria or even hemosiderinuria (in cases of chronic hemolysis) (Chapter 23). The mean cell volume (MCV) may be increased due to the reticulocytosis and RBC agglutination (if present). Leukocytosis and thrombocytosis may occur along with the increased erythroid proliferation in the bone marrow. Findings on the peripheral blood film include polychromasia (due to the reticulocytosis), spherocytes (due to IgG-mediated membrane damage by macrophages), and occasionally RBC agglutination. Nucleated RBCs, fragmented RBCs or schistocytes, and erythrophagocytosis (phagocytes engulfing RBCs) may also be observed on the peripheral blood film.

To determine if the hemolysis is due to an immune mechanism, a direct antiglobulin test (DAT) is performed. The DAT detects in vivo sensitization of the RBC surface by IgG, C3b, or
In the DAT procedure, polyspecific antihuman globulin (AHG) is added to saline-washed patient RBCs. Polyspecific AHG has specificity for the Fc portion of human IgG and complement components C3b and C3d; agglutination will occur if a critical number of any of these molecules is present on the RBC surface (Figure 26-4). If the DAT result is positive with polyspecific AHG, then the cells are tested with monospecific anti-IgG and anti-C3b/C3d to identify the type of sensitization. If IgG is detected on the RBCs, elution procedures are used to remove the antibody from the RBCs for identification.

The specificity of the IgG antibody may be determined by assessing the reaction of the eluate with screening and panel antigens. The specificity of the IgG antibody may be determined by assessing the reaction of the eluate with screening and panel antigens.
reagent RBCs (RBCs genotyped for the major RBC antigens) using the indirect antiglobulin test (IAT) (Figure 26-4). Identification of any circulating alloantibodies or autoantibodies by the indirect antiglobulin test is also important in the investigation.\(^2\) The DAT result may be negative in patients with some immune hemolytic anemias.\(^2\) In addition, other disorders beside immune hemolytic anemia can cause a positive DAT finding.\(^2\) Therefore, diagnosis of immune hemolytic anemia cannot rely solely on the DAT and must take into account the patient history; symptoms; recent medications; previous transfusions; coexisting conditions, including pregnancy; and the results of the applicable hematologic, biochemical, and serologic tests.\(^2,5\)

**AUTOIMMUNE HEMOLYTIC ANEMIA**

Autoimmune hemolytic anemia (AIHA) is a rare disorder characterized by premature RBC destruction and anemia caused by autoantibodies that bind the RBC surface with or without complement activation. AIHA can affect both children and adults, and its annual incidence is estimated to be between 1 and 3 per 100,000 individuals.\(^6\) In children, more males are affected, but in adults, more females are affected.\(^6\) Autoantibodies may arise as a result of immune system dysregulation and loss of immune tolerance, exposure to an antigen similar to an autoantigen, B-lymphocyte neoplasm, or other unknown reason.\(^3,6\) The type, amount, and duration of antigen exposure and genetic and environmental factors may also contribute to the development of autoantibodies.\(^5\) The anemia can be mild or severe, and the onset can be acute or gradual. The severity of the anemia depends on the autoantibody characteristics (titer, ability to react at 37° C, ability to activate complement, and specificity and affinity for the autoantigen), antigen characteristics (density on RBCs, immunogenicity), as well as patient factors (age, ability of the bone marrow to compensate for the hemolysis, function of macrophages, complement proteins and regulators, and underlying conditions).\(^7,8\)

The autoimmune hemolytic anemias may be divided into four major categories based on the characteristics of the autoantibody and the mechanism of hemolysis: warm autoimmune hemolytic anemia, cold agglutinin disease, paroxysmal cold hemoglobinuria, and mixed-type autoimmune hemolytic anemia (Table 26-2).\(^2,5,6\)

**Warm Autoimmune Hemolytic Anemia**

Warm autoimmune hemolytic anemia (WAHA) is the most commonly encountered autoimmune hemolytic anemia, comprising up to 70% of cases.\(^5\) The autoantibodies causing WAHA react optimally at 37° C, and the vast majority of them are IgG.\(^1\) WAHA may be classified as idiopathic or secondary. In patients with idiopathic WAHA, the etiology is unknown. Secondary WAHA may occur in many conditions such as lymphoproliferative diseases (chronic lymphocytic leukemia, B-lymphocytic lymphomas, and Waldenström macroglobulinemia), nonlymphoid
TABLE 26-2 Characteristics of Autoimmune Hemolytic Anemias

<table>
<thead>
<tr>
<th></th>
<th>Warm Autoimmune Hemolytic Anemia</th>
<th>Cold Agglutinin Disease</th>
<th>Paroxysmal Cold Hemoglobinuria</th>
<th>Mixed-Type Autoimmune Hemolytic Anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin class</td>
<td>IgG (rarely IgM, IgA)</td>
<td>IgM</td>
<td>IgG</td>
<td>IgG, IgM</td>
</tr>
<tr>
<td>Optimum reactivity temperature of antibody</td>
<td>37° C</td>
<td>4° C; reactivity extends to &gt;30° C</td>
<td>4° C</td>
<td>4°–37° C</td>
</tr>
<tr>
<td>Sensitization detected by direct antiglobulin test</td>
<td>IgG or IgG + C3d; only C3d uncommon</td>
<td>C3d</td>
<td>C3d</td>
<td>IgG and C3d</td>
</tr>
<tr>
<td>Complement activation</td>
<td>Variable</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>Extravascular primarily</td>
<td>Extravascular; rarely intravascular</td>
<td>Intravascular</td>
<td>Extravascular and intravascular</td>
</tr>
<tr>
<td>Autoantibody specificity</td>
<td>Panreactive or Rh complex; rarely specific Rh or other antigen</td>
<td>I (most), i (some), Pr (rare)</td>
<td>P</td>
<td>Panreactive; unclear specificity</td>
</tr>
</tbody>
</table>

Ig, Immunoglobulin.

neoplasms (thymoma and cancers of the colon, kidney, lung, and ovary), autoimmune disorders (rheumatoid arthritis, scleroderma, polyarteritis nodosa, Sjögren syndrome, and systemic lupus erythematosus), immunodeficiency disorders, and viral infections.

The onset of WAIHA is usually insidious, with symptoms of anemia (fatigue, dizziness, dyspnea), but some cases can be acute and life-threatening with fever, jaundice, splenomegaly, and hepatomegaly, especially in children with WAIHA secondary to viral infections.

Massive splenomegaly, lymphadenopathy, fever, petechiae, ecchymosis, or renal failure in adults suggests an underlying lymphoproliferative disorder.

Although most autoantibodies that cause WAIHA are IgG, rare cases involving IgA autoantibodies as well as cases with fatal outcomes caused by warm-reacting IgM antibodies have been reported.

Hemolysis is predominantly extravascular in WAIHA, and cases of fulminant intravascular hemolysis are rare.

The result of the DAT is positive in over 95% of patients, with approximately 85% of patients having IgG alone or both IgG and C3d on their RBCs, and 10% to 14% having C3d only. Between 1% and 4% of patients have a negative DAT result caused by IgA or IgM autoantibodies that are not detected by the polyspecific AHG; IgG or C3d in an amount below the reagent detection limit; dissociation of IgG antibodies with low avidity during the washing phase of the DAT; or by various technical errors. Therefore, a negative DAT result does not rule out autoimmune hemolytic anemia.

Warm autoantibodies are usually panreactive; that is, they will agglutinate all screening and panel cells, donor RBCs, and the patient’s own RBCs, so the specificity of the autoantibody is not apparent. In some cases Rh complex specificity can be demonstrated. Rarely, a specific autoantibody to an antigen in the Rh blood group system is identified. Autoantibodies to other antigens (such as LW, Jk, K, Di, Ge, Lu, M, N, S, U, En, and Wr) are occasionally identified.

For most patients (approximately 80%) the autoantibody can be detected in the serum. Because the autoantibody is panreactive, it may mask reactions of alloantibodies with RBC panel cells. If an RBC transfusion is necessary, it is crucial to perform tests to determine if clinically significant alloantibodies are also present.

Anemia in WAIHA can be mild or severe, with RBC life span sometimes reduced to 5 days or less. Laboratory findings for serum and urine reflect the predominantly extravascular hemolysis that occurs in IgG-mediated immune hemolysis. Polychromasia and spherocytes are the typical findings on the peripheral blood film. Occasionally the WAIHA is accompanied by immune thrombocytopenic purpura and a decreased platelet count, a condition known as Evans syndrome, which occurs primarily in children.

In symptomatic but non-life-threatening WAIHA, a glucocorticosteroid such as prednisone is the initial treatment of choice. Approximately 70% to 80% of patients show improvement with prednisone, but many adult patients need to be on a long-term maintenance dosage to remain asymptomatic. Osteoporosis, osteonecrosis, and bone fracture, particularly of the lumbar spine, are serious side effects of long-term steroid use and occur in approximately 30% to 50% of patients. The highest loss of bone density occurs early in treatment, even at smaller steroid doses, and the risk of fracture increases by 75% during the first months of treatment. Patients should also receive bisphosphonates, vitamin D, and calcium according to the recommendation of the American College of Rheumatology.

Splenectomy is an option in patients with chronic WAIHA who are refractive to prednisone therapy or require long-term, high-dose prednisone therapy; a favorable response is achieved in 50% to 75% of patients. Immunosuppressive drugs, such as cyclophosphamide or azathioprine, are used for refractory WAIHA, but the side effects may be severe. Rituximab, a monoclonal anti-CD20 antibody that binds to the corresponding antigen found on B cells, has also been used extensively. It causes minimal side effects and produces a response rate of 17% to 100%, according to various published case reports. Hematopoietic stem cell transplantation (HSCT) has been used for severe, life-threatening autoimmune syndromes, including hemolytic anemia and Evans syndrome. Sources of the stem cells have been autologous, HLA-matched sibling, and cord blood, all of which carry lethal risks. In secondary WAIHA, successful management of the underlying condition...
Cold Agglutinin Disease

Cold agglutinins are autoantibodies of the IgM class that react optimally at 4°C and are commonly found in healthy individuals. These nonpathologic cold agglutinins are polyclonal, occur in low titers (less than 1:64 at 4°C), and have no reactivity above 30°C. Most pathologic cold agglutinins are monoclonal, occur at high titers (greater than 1:1000 at 4°C), and are capable of reacting at temperatures greater than 30°C. Because pathologic cold agglutinins can react at body temperature, they may induce cold agglutinin disease (CAD). Cold agglutinins that are able to bind RBC antigens near or at 37°C (high thermal amplitude) cause more severe symptoms. CAD has recently been recognized as a clonal lymphoproliferative B cell disorder. It comprises approximately 15% to 20% of individuals. These nonpathologic cold agglutinins are polyclonal, with a normal distribution of κ and λ light chains. Chronic CAD is a rare disease that typically occurs in middle-aged and elderly individuals, and the autoantibody is usually monoclonal IgM with κ light chains. In a study of 86 patients with chronic CAD by Berentsen and colleagues, the median age at onset was 67 years, with the median age at death reported to be 82 years. Chronic CAD can be idiopathic, with no known cause, or secondary due to lymphoproliferative neoplasms such as B-lymphocytic lymphomas, Waldenström macroglobulinemia, or chronic lymphocytic leukemia.

Clinical manifestations are variable in chronic CAD. Most patients have a mild anemia with a hemoglobin result ranging from 9 to 12 g/dL, but others can develop life-threatening anemia with hemoglobin levels falling below 5 g/dL, especially after exposure to cold temperatures. Individuals often experience fluctuations between mild and severe symptoms, and approximately half of those affected require transfusions over the course of the disease. Symptoms include fatigue, weakness, dyspnea, pallor (due to the anemia), and acrocyanosis. Acrocyanosis is a bluish discoloration of the extremities (fingers, toes, feet, earlobes, nose) due to RBC autoagglutination, which causes local capillary stasis. Some patients also have episodes of hemoglobinuria, especially after exposure to cold temperatures. In contrast, patients with acute CAD may have mild to severe hemolysis that appears abruptly within 2 to 3 weeks after the onset of infectious mononucleosis, other viral infection, or M. pneumoniae infection, but it resolves spontaneously within days to a few weeks.

The DAT result is positive with polyspecific AHG because of the presence of C3d on the RBC surface. The specificity of the cold agglutinin is most often anti-I but can be anti-i or, very rarely, anti-Pr. Virtually all adult RBCs are positive for the I antigen, so anti-I will agglutinate all screening and panel cells, donor RBCs, and the patient’s own RBCs at room temperature and higher, depending on the thermal amplitude of the autoantibody. Anti-I will show weaker or negative reactions with cord RBCs (cord cells are negative for I antigen but positive for i antigen).

A cold agglutinin method is used to determine the titer of the antibody at 4°C. Pathologic cold agglutinins can reach titers of 1:10,000 to 1:1,000,000 at 4°C. Blood specimens for cold agglutinin testing must be maintained at 37°C after collection to prevent the binding of the autoantibody to the patient’s own RBCs, which can falsely decrease the antibody titer in the serum. Alternatively, a sample anticoagulated with EDTA can be warmed for 15 minutes at 37°C to dissociate autoabsorbed antibody prior to determining the titer.

When a high-titer cold agglutinin is present, an EDTA-anticoagulated blood specimen can show visible agglutinates in the tube at room temperature or below. The agglutination can also be observed on a peripheral blood film (Figure 26-5). Blood specimens from patients with cold agglutinins must be warmed to 37°C for 15 minutes before complete blood count analysis by automated hematology analyzers. RBC agglutination grossly elevates the mean cell volume, reduces the RBC count, and has unpredictable effects on other indices (Chapter 15). When the sample is warmed to 37°C, the antibody dissociates from the RBCs, and agglutination usually disappears. If not, a new specimen is collected and maintained at 37°C for...

Figure 26-5 Wright-stained peripheral blood film showing red blood cell agglutination (×500).
the entire time before testing. To avoid agglutination on a peripheral blood film, the slide can also be warmed to 37°C prior to the application of blood. Cold agglutinins can also interfere with ABO typing.

Acute CAD associated with infections is self-limiting, and the cold agglutinin titers are usually less than 1:4000. If the hemolysis is mild, no treatment is required; however, patients with severe hemolysis require transfusion and supportive care. Patients with chronic CAD and mild anemia are regularly monitored and advised to avoid cold temperatures. In chronic CAD with moderate to severe symptoms, rituximab produces partial remission in about half of patients due to its targeting and ultimate destruction of the B-lymphocytes containing the CD-20 antigen, but median remission is less than a year. In a 2010 study by Berentsen and colleagues, a combination of rituximab and cyclophosphamide and chlorambucil is currently the best second-line treatment for CAD and primary AIHA; it can be given multiple times, and children tolerate it well. Plasmapheresis may be used in severe cases but provides only temporary benefit. Corticosteroid therapy and immunosuppressive therapy with cyclophosphamide and chlorambucil are not effective for most patients. Splenectomy is also not effective because C3b-sensitized RBCs in IgM-mediated autoimmune hemolysis are cleared primarily by the liver.

RBC transfusion is reserved for patients with life-threatening anemia or cardiovascular or cerebrovascular symptoms. If transfusion is needed, the presence of clinically significant alloantibodies must be ruled out. In CAD cases involving an autoimmune with wide thermal amplitude, detection of coexisting warm-reactive alloantibodies can be time-consuming and difficult. During transfusion, the patient is kept warm, small amounts of blood are given while the patient is observed for symptoms of a hemolytic transfusion reaction, and a blood warmer is used to minimize the in vivo reactivity of the cold autoantibody.

**Paroxysmal Cold Hemoglobinuria**

Paroxysmal cold hemoglobinuria (PCH) is an acute form of cold-reactive hemolytic anemia. PCH can be idiopathic or secondary. Historically, secondary PCH was associated with late-stage syphilis, but now it is most commonly seen in young children after a viral respiratory infection. PCH is rare in adults. The prevalence of PCH has been reported to be as high as 32% to 40% of children with autoimmune hemolytic anemia, with a median age at presentation of 5 years.

The anti-P autoantibody, also called the Donath-Landsteiner antibody, is a complement-binding IgG hemolysin with specificity for the P antigen on RBCs. The anti-P autoantibody is biphasic in that at cold temperatures it binds to the P antigen on RBCs and partially activates complement (C1 to C4), but full complement activation (C3 through C9) and hemolysis occur only upon warming to 37°C. Exposure to cold temperatures is not required for the hemolytic manifestations in vivo, however, and the reasons for this have yet to be explained. The anti-P autoantibody binds RBC antigen optimally at 4°C and has a thermal amplitude of less than 20°C. At warmer temperatures, the anti-P autoantibody dissociates from the RBCs; the titer is usually less than 1:64.

Children typically present with acute fever; malaise; and back, leg, and/or abdominal pain to 2 weeks after an upper respiratory tract infection. Pallor, jaundice, and dark urine due to hemoglobinuria are frequently present. The abrupt onset of hemolysis causes a rapidly progressing and severe anemia, with hemoglobin levels often dropping below 5 gm/dL.

Reticulocytosis is typical but can be preceded by reticulocytopenia. The peripheral blood film shows polychromasia and spherocytes, but schistocytes, nucleated RBCs, anisocytosis, poikilocytosis, and erythroagglutination can also be observed. At first, leukopenia may be present; later, leukocytosis occurs. In addition, laboratory findings typical for intravascular hemolysis are found. Because the anti-P autoantibody is dissociated from the RBCs at body temperature, the DAT result is usually positive for C3d only.

The classic Donath-Landsteiner test for anti-P is done by collecting blood samples in two tubes, one for the patient test and the other for the patient control. The patient test sample is incubated first at 4°C for 30 minutes (to allow anti-P binding to the P antigen and partial complement activation on the RBCs) and then at 37°C for 30 minutes (to allow full activation of the complement pathway to lysis). The patient control tube is kept at 37°C for both incubations (and a total of 60 minutes). After centrifugation the supernatant is examined for hemolysis. A positive test result for anti-P is indicated by hemolysis in the patient test sample incubated first at 4°C and then at 37°C and no hemolysis in the patient control sample kept at 37°C. In the control tube, the anti-P is not able to bind to antigen at 37°C, so complement is not activated and hemolysis does not occur. Initial test results may be falsely negative due to low complement and/or anti-P levels in the patient sample because of the brisk hemolysis in vivo. Incubating patient serum with complement and papain-treated compatible group O RBCs increases the sensitivity of the test in detecting anti-P. The enzyme treatment provides greater exposure of the P antigen on the RBC surface for antibody binding.

PCH is severe but self-limiting and resolves in several days to a few weeks, with an excellent prognosis. In most patients, the anemia is severe and can be life-threatening, so transfusion is usually needed until the symptoms resolve. Because the anti-P autoantibody reacts only at lower temperatures and P antigen–negative blood is very rare, P-positive blood can be transfused.

**Mixed-Type Autoimmune Hemolytic Anemia**

Mixed-type autoimmune hemolytic anemia occurs very infrequently. In this condition, the patient simultaneously develops an IgG autoantibody with optimal reactivity at 37°C (WAIHA) and a pathologic IgM autoantibody that reacts optimally at 0°C to 10°C but has a thermal amplitude of greater than 30°C (CAD). Patients with WAIHA and a nonpathogenic cold agglutinin (i.e., an agglutinin that does not react at a temperature greater than 20°C) should not be classified as...
having a mixed-type autoimmune hemolytic anemia because the cold agglutinin is not clinically significant.11

The hemolysis results from a combination of extravascular and intravascular mechanisms. The disease course appears to be chronic, with intermittent episodes of severe anemia.5,11 The DAT results can be positive with IgG only, C3d only, or IgG and C3d.2 The warm autoantibody is typically panreactive with unclear specificity, whereas the cold-reacting antibody usually has anti-I specificity.3 Treatment is the same as that described for WAIHA.3

**Mechanisms of Drug-Induced Immune Hemolysis**

Various theories have been proposed to explain the mechanisms of DIIHA.19,24 Three generally accepted mechanisms involve an antibody produced by the patient as a result of exposure to the drug and include drug adsorption, drug–RBC membrane protein immunogenic complex, and RBC autoantibody induction. A fourth mechanism, drug-induced nonimmunologic protein adsorption (NIPA), can result in a positive DAT result, but no drug or RBC antibody is produced by the patient. This mechanism is discussed at the end of this section.

1. **Drug adsorption:** The patient produces an IgG antibody to a drug. When the drug is taken by the patient, the drug binds strongly to the patient’s RBCs (see paragraph below on unifying theory). The IgG drug antibody binds to the drug attached to the RBCs, usually without complement activation. Because the offending antibody is IgG and is strongly attached to the RBCs via the drug, hemolysis is extravascular by splenic macrophages, which remove the antibody- and drug-coated RBCs from the circulation.

2. **Drug–RBC membrane protein immunogenic complex:** A drug binds loosely to an RBC membrane protein to form a drug–RBC protein immunogenic complex or epitope. The patient produces an IgM and/or IgG antibody that binds to the complex on the RBCs, and complement is fully activated, which causes acute intravascular hemolysis.

3. **RBC autoantibody induction:** A drug induces the patient to produce IgG warm-reactive autoantibodies against RBC self-antigens. These autoantibodies react at 37°C, and the laboratory findings are indistinguishable from those in WAIHA. Hemolysis is extravascular and is mediated by macrophages predominantly in the spleen.

Several authors have suggested that all drug-induced immune hemolysis is explained by a single mechanism, known as the unifying theory. This theory proposes that a drug interacts with the RBC membrane and generates multiple immunogenic epitopes that can elicit an immune response to the drug alone, to the drug–RBC membrane protein combination, or to an RBC membrane protein alone.2,21 Diagnosis of DIIHA can only be made if the antibody screen is positive and the RBC eluate contains an antibody.1

**Antibody Characteristics**

Antibodies implicated in DIIHA can be divided into two general types: drug-dependent (most common) and drug-independent antibodies.2,21 Some drugs are able to induce a combination of both types of antibodies.19,24,25

**Drug-Dependent Antibodies**

Drug-dependent antibodies only react in vitro when the suspected drug or its metabolite is present.2,21 There are two types of drug-dependent antibodies:

1. **Antibodies that react only with drug-treated cells:** These are IgG drug antibodies that bind to the drug when it is strongly associated with the RBC surface (drug adsorption mechanism). Because they have bound IgG, the RBCs are cleared from the circulation extravascularly by macrophages in the spleen, and a hemolytic anemia gradually develops. Complement is not usually activated. If the DIIHA is not recognized, the patient may continue to take the drug to a point in which life-threatening anemia develops.2,19 Examples of drugs that elicit antibodies in this category are penicillin and cyclosporin.2,21 Laboratory features include a positive DAT reaction with anti-IgG, whereas the reaction with anti-C3b/C3d is usually negative. In the indirect antiglobulin test, the patient’s serum and an eluate of the patient’s cells react only with drug-treated RBCs and not with untreated RBCs.2

2. **Antibodies that react only in the presence of the drug:** These IgG and/or IgM antibodies bind to the drug or its metabolite only when it is weakly associated in a drug–RBC membrane protein complex (drug–RBC membrane protein immunogenic complex mechanism). The antibodies activate complement and trigger acute intravascular hemolysis that may progress to renal failure.2 Hemolysis occurs abruptly after short periods of drug exposure or upon readministration of the drug.19 Examples of drugs that elicit antibodies in this category are phenacetin, trimethoprim, quinine, and ciprofloxacin.2,19,22,23 Laboratory features include a positive DAT reaction with anti-C3b/C3d and occasionally with anti-IgG. In the indirect antiglobulin test, the patient’s serum reacts with untreated, normal RBCs only in the presence of the drug.

**Drug-Independent Antibodies**

Drug-independent antibodies are IgG, warm-reactive, RBC autoantibodies induced by the drug (RBC autoantibody induction mechanism). These autoantibodies have the same serologic reactivity as those causing WAIHA, and they do not require the presence of the drug for in vitro reactivity. Hemolysis is extravascular, mediated by macrophages predominantly in the
Nonimmune Drug-Induced Hemolysis
In drug-induced nonimmunologic protein adsorption, the patient does not produce an antibody to the drug or to RBCs. The mechanism is also called the membrane modification method, because certain drugs such as high-dose clavulanate and cisplatin can alter the RBC membrane so that numerous proteins, including IgG and complement, adsorb onto the RBC surface.\(^2,^21\) This phenomenon results in a positive DAT finding, but only rarely has hemolysis been reported. The indirect antiglobulin test on the patient’s serum and an eluate of the patient’s RBCs yield negative results.\(^2\)

Treatment
After a DIIHA is recognized and confirmed, the first treatment is to discontinue the drug. Most patients will gradually show improvement within a few days to several weeks.\(^19\) In cases in which a warm-reacting autoimmune antibody is present, the positive DAT result may persist for months after a hematologic recovery. If the anemia is severe, the patient may require RBC transfusion or plasma exchange.\(^19\) Regardless of mechanism, future episodes of DIIHA are prevented by avoidance of the drug.

ALLOIMMUNE HEMOLYTIC ANEMIAS

Hemolytic Transfusion Reaction
One of the most severe and potentially life-threatening complications of blood transfusion is a hemolytic transfusion reaction (HTR) due to immune-mediated destruction of donor cells by an antibody in the recipient. The offending antibody in the recipient may be IgM or IgG, complement may be partially or fully activated or not activated at all, and the hemolysis may be intravascular or extravascular, depending on the characteristics of the antibody. HTRs can have an acute or delayed onset.\(^2\)

Acute Hemolytic Transfusion Reaction
Acute hemolytic transfusion reactions (AHTRs) occur within minutes to hours of the initiation of a transfusion.\(^26\) The most common cause of AHTR is the accidental transfusion of ABO-incompatible donor red blood cells into a recipient. An example is the transfusion of group A red blood cells into a group O recipient. The recipient has preformed, non-RBC stimulated anti-A (IgM) that is capable of fully activating complement to C9 upon binding to the A antigen on donor red blood cells. There is rapid, complement-mediated intravascular hemolysis and activation of the coagulation system. ABO-incompatible transfusions are usually due to clerical error and have been estimated to occur in approximately 1 in 38,000 to 1 in 70,000 RBC transfusions.\(^26\) The severity of AHTR is variable and is affected by the infusion rate and volume of blood transfused.\(^2,^27\) AHTR carries an estimated mortality rate of 2%.\(^26\) AHTRs can occur due to incompatibilities involving other blood group systems, but these are rare.\(^26\)

Symptoms of severe intravascular hemolysis found in ABO-related AHTRs begin within minutes or hours and may include chills, fever, urticaria, tachycardia, nausea and vomiting, chest and back pain, shock, anaphylaxis, pulmonary edema, congestive heart failure, and bleeding due to disseminated intravascular coagulation (DIC). The transfusion should be immediately terminated upon first appearance of symptoms. Treatment is urgent and includes an effort to prevent or correct shock, maintain renal circulation, and control the DIC.\(^27\)

The immediate investigation of a suspected HTR includes a clerical check for errors, an examination of a posttransfusion blood specimen for hemolysis, and performance of the DAT on the RBCs in a posttransfusion specimen.\(^26\) If an AHTR occurred, hemoglobinemia and hemoglobinuria are detectable, and the DAT result is positive. DAT findings may be negative, however, if all the donor cells are lysed.\(^26\) The hemoglobin and serum haptoglobin levels decrease, but the serum indirect bilirubin will not begin to rise until 2 to 3 days after the episode. The ABO and Rh typing, antibody screen, and cross-matching are repeated on the recipient and the donor blood to identify the blood group incompatibility. Coagulation tests such as D-dimer, fibrinogen, factors V and VIII, and platelet count can help reveal and assess the risk of DIC.\(^2\)

Delayed Hemolytic Transfusion Reaction
A delayed hemolytic transfusion reaction (DHTR) may occur days to weeks after transfusion as the titer of alloantibodies increases.\(^2,^27\) Often, the patient has been alloimmunized by a pregnancy or previous transfusion, but the antibody titer was below the level of serologic detection at the time of transfusion. The second exposure to the antigen results in an increase in titer (anamnestic response). The antibody is usually IgG, is reactive at 37° C, and may or may not be able to partially or fully activate complement. The antibodies most often implicated in DHTRs are directed against antigens in the Duffy and Kidd blood groups.\(^26,^27\) The patient’s antibody binds to the transfused RBCs, which leads to extravascular hemolysis, with or without complement activation. The principal signs are an inadequate posttransfusion hemoglobin increase, positive DAT results for IgG and/or C3d, morphologic evidence of hemolysis, and an increase in serum indirect bilirubin.\(^25,^26\) Management of DHTR includes monitoring of kidney function, especially in acutely ill patients.\(^26\)

Hemolytic Disease of the Fetus and Newborn
Hemolytic disease of the fetus and newborn (HDFN) occurs when an IgG alloantibody produced by the mother crosses the placenta into the fetal circulation and binds to fetal RBCs that are positive for the corresponding antigen. The IgG-sensitized fetal RBCs are cleared from the circulation by macrophages in the fetal spleen (extravascular hemolysis), and an anemia gradually develops. There is erythroid hyperplasia in the fetal bone marrow and extramedullary erythropoiesis in the fetal spleen, liver, kidneys, and adrenal glands.\(^28\) Many nucleated
RBCs are released into the fetal circulation. If the anemia is severe in utero, it can lead to generalized edema, ascites, and a condition called hydrops fetalis, which is fatal if untreated.\cite{2,28}\n
Anti-Kell antibodies are an exception because they also cause anemia by suppressing fetal erythropoiesis.\cite{28}\n
In Rh HDFN, which causes the highest number of fetal fatalities, an Rh (D)-negative mother has preformed anti-D antibodies (IgG, reactive at 37°C) from exposure to the D antigen either through immunization in a previous pregnancy with a D-positive baby or from previous transfusion of blood products with D-positive RBCs. In subsequent pregnancies, the anti-D crosses the placenta, and if the fetus is D positive, the anti-D binds to D antigen sites on the fetal RBCs. These anti-D-sensitized fetal RBCs are cleared from the circulation by macrophages in the fetal spleen, and anemia and hyperbilirubinemia develop. Amniocentesis is accurate at predicting severe fetal anemia, but it is an invasive procedure and carries some risk of fetal loss.\cite{28}\n
If severe fetal anemia and HDFN due to anti-D is suspected, a percutaneous umbilical fetal blood sample can be obtained and tested for the hemoglobin level to determine the severity of the anemia; more recently, a noninvasive assessment of anemia can be done by ultrasound measurement of fetal cerebral flow.\cite{2,28}\n
**Laboratory Findings**\n
ABO, Rh typing, and an antibody screen are performed on the mother when the fetus is between 10 and 16 weeks’ gestation, and again at 28 weeks’ gestation.\cite{28} The antibody screen during pregnancy detects antibodies other than those caused by ABO incompatibility.\cite{2} If the antibody screen is positive, an RBC panel is performed to identify the specificity of the antibody. Mothers with initial positive antibody screens are retested for an antibody screen every month until 28 weeks, then every two weeks thereafter; antibody titters are reported from each sample. Titration of the antibody does not predict the severity of HDFN; rather, it helps determine when to monitor for HDFN by additional methods, such as spectrophotometric analysis of amniotic fluid bilirubin.\cite{2,28} After the first affected pregnancy, the antibody titer is no longer useful, and other means of monitoring the fetus are used, such as amniocentesis and ultrasonography.\cite{28}\n
An unimmunized D-negative mother receives antenatal Rh immune globulin (RhIG) at 28 weeks’ gestation and again within 72 hours of delivery of a D-positive infant to prevent alloimmunization to the D antigen.\cite{2} Even one antenatal dose of 200 μg RhIG will reduce by half the risk of the mother developing anti-D antibodies and having a child with HDFN in the next pregnancy.\cite{29} Rh-negative women who experience spontaneous or induced abortion also receive Rh immune globulin.

At delivery, newborn testing is performed on umbilical cord blood. Neonates with Rh HDFN have a decreased hemoglobin level, increased reticulocyte count, and increased level of serum indirect bilirubin. The peripheral blood film shows polychromasia and many nucleated RBCs. ABO (only forward typing), Rh typing, and the DAT are also performed. The DAT result is positive for IgG, and anti-D can be demonstrated in an eluate of the infant’s RBCs.\cite{28}\n
**Treatment for the Affected Infant**\n
Treatment for a fetus affected by HDFN may include intrauterine transfusion, whereby pooled hemolyzed blood is removed via amniocentesis from the fetal abdomen and replaced with a small amount of fresh red blood cells. This procedure can be used to correct fetal anemia and prevent hydrops fetalis.\cite{30} Cordocentesis is also utilized, whereby fresh red blood cells are injected into the umbilical vein. The survival rates of fetuses receiving transfusions are 85% to 90%; the risk of premature death from these procedures varies from 1% to 3%.\cite{30}\n
After delivery, the neonate may need exchange transfusions and phototherapy to reduce the level of serum indirect bilirubin and prevent kernicterus (bilirubin accumulation in the brain).\cite{28} Prolonged postnatal anemia can be due to a slow decrease of maternal antibody in the newborn’s circulation; rare cases of prolonged anemia are documented in infants who received intrauterine transfusions.\cite{31,32}\n
**Hemolytic Disease of the Fetus and Newborn Caused by Other Blood Group Antigens**\n
ABO HDFN is more common than Rh disease and may occur during the first pregnancy. Unlike Rh disease, ABO disease is asymptomatic or produces mild hyperbilirubinemia and anemia. ABO HDFN is seen in some type A or B infants born to type O mothers who produce IgG anti-A and anti-B which are capable of crossing the placenta. The disease is milder than Rh HDFN likely because A and B antigens are poorly developed on fetal and newborn RBCs, and other cells and tissues express A and B antigens which reduces the amount of maternal antibody directed against fetal RBCs. The DAT result for the newborn with ABO HDFN is only weakly positive and may be negative. Spherocytes and polychromasia on the peripheral blood film are typical.\cite{2} Table 26-3 presents a comparison of HDFN caused by ABO and Rh incompatibility.

HDFN can be caused by other IgG antibodies, particularly antibodies to the K, c, and Fy a antigens.\cite{2} HDFN due to other blood group antibodies is rare.\cite{26-31} Antibody screening in the first trimester can assist in identifying rare antibodies that can cause HDFN.\cite{37} Varying degrees of anemia, jaundice, and kernicterus are the adverse clinical outcomes in all forms of HDFN.

**TABLE 26-3 Characteristics of Rh and ABO Hemolytic Disease of the Fetus and Newborn**

<table>
<thead>
<tr>
<th>Blood Groups</th>
<th>Rh</th>
<th>ABO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity of disease</td>
<td>Severe</td>
<td>Mild</td>
</tr>
<tr>
<td>Jaundice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spherocytes on peripheral blood film</td>
<td>Rare</td>
<td>Usually present</td>
</tr>
<tr>
<td>Anemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct antiglobulin test result</td>
<td>Positive</td>
<td>Negative or weakly positive</td>
</tr>
</tbody>
</table>
SUMMARY

- The immune hemolytic anemias are classified into autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, and alloimmune hemolytic anemia.
- Hemolysis mediated by IgM requires complement; hemolysis may be extravascular (mainly in the liver) if complement is partially activated to C3b, or intravascular if complement is fully activated to C9.
- Hemolysis mediated by IgG occurs with or without complement activation; IgG-sensitized RBCs are removed from the circulation by macrophages in the spleen; partial phagocytosis produces spherocytes, which are prematurely trapped in the spleen and phagocytized; IgG- and C3b-sensitized RBCs are removed by macrophages in the spleen and liver.
- Laboratory findings in immune hemolytic anemia include decreased hemoglobin level, increased reticulocyte count, increased levels of serum indirect bilirubin and lactate dehydrogenase, and decreased serum haptoglobin level. The peripheral blood film may show polychromasia, spherocytes (IgG-mediated hemolysis), or RBC agglutination (cold agglutinins). The DAT detects in vivo sensitization of RBCs by IgG and/or C3b/C3d.
- The classification of autoimmune hemolytic anemia includes warm autoimmune hemolytic anemia (WAIHA), cold agglutinin disease (CAD), paroxysmal cold hemoglobinuria (PCH), and mixed-type autoimmune hemolytic anemia.
- WAIHA is the most common form of autoimmune hemolytic anemia and involves IgG autoantibodies with optimum reactivity at 37° C. The anemia varies from mild to severe, and characteristic morphologic features on the peripheral blood film are polychromasia and spherocytes.
- CAD is caused by an IgM autoantibody with optimum reactivity at 4° C and a thermal amplitude of greater than 30° C. RBC agglutination may be observed on a peripheral blood film, and agglutinates may cause interference with the complete blood count analysis on automated hematology analyzers.
- Paroxysmal cold hemoglobinuria (PCH) is due to a biphasic IgG autoantibody with anti-P specificity. The antibody binds to the P antigen on the RBCs and partially activates complement at 4° C; complete complement activation and hemolysis occur upon warming the sample to 37° C.
- In drug-induced immune hemolytic anemia (DIHA), the patient produces antibodies to (1) a drug only, (2) a complex of a drug loosely bound to an RBC membrane protein, or (3) an RBC membrane protein only. In vitro reactions of antibodies in DIHA may be drug-dependent or drug-independent.
- Acute hemolytic transfusion reactions (AHTRs) occur within minutes to hours after the start of an RBC transfusion and most often involve transfusion of ABO-incompatible blood; the hemolysis is predominantly intravascular. Delayed hemolytic transfusion reactions (DHTRs) may occur days or weeks after the transfusion and represent an anamnestic response to a donor red blood cell antigen; the hemolysis is usually extravascular.
- Hemolytic disease of the fetus and newborn (HDFN) occurs when an IgG alloantibody produced by the mother crosses the placenta into the fetal circulation and binds to fetal RBCs that are positive for the corresponding antigen. The IgG-sensitized fetal RBCs are cleared from the circulation by macrophages in the fetal spleen, and an anemia gradually develops; the usual laboratory findings in the neonate are anemia, hyperbilirubinemia, and a positive direct antiglobulin test (DAT) result.
- ABO HDFN is more common than Rh HDFN and produces no symptoms or mild anemia. Rh HDFN due to anti-D results in severe anemia. Antenatal administration of Rh immune globulin to a D-negative mother when the fetus is at 28 weeks’ gestation and within 72 hours after delivery of a D-positive baby prevents immunization to the D antigen.

NOW THAT YOU HAVE COMPLETED THIS CHAPTER, GO BACK AND READ AGAIN THE CASE STUDY AT THE BEGINNING AND RESPOND TO THE QUESTIONS PRESENTED.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. Immune hemolytic anemia is due to a(n):
   a. Structural defect in the RBC membrane
   b. Allo- or autoantibody against an RBC antigen
   c. T cell immune response against an RBC antigen
   d. Obstruction of blood flow by intravascular thrombi

2. The pathophysiology of immune hemolysis with IgM antibodies always involves:
   a. Complement
   b. Autoantibodies
   c. Abnormal hemoglobin molecules
   d. Alloantibodies

3. In hemolysis mediated by IgG antibodies, which abnormal RBC morphology is typically observed on the peripheral blood film?
   a. Spherocytes
   b. Nucleated RBCs
   c. RBC agglutination
   d. Macrocytes

4. The most important finding in the diagnostic investigation of a suspected autoimmune hemolytic anemia is:
   a. Detection of a low hemoglobin and hematocrit
   b. Observation of hemoglobinemia in a specimen
   c. Recognition of a low reticulocyte count
   d. Demonstration of IgG and/or C3d on the RBC surface
5. In autoimmune hemolytic anemia, a positive DAT is evidence that an:
   a. IgM antibody is in the patient’s serum
   b. IgG antibody is in the patient’s serum
   c. IgM antibody is sensitizing the patient’s red blood cells
   d. IgG antibody is sensitizing the patient’s red blood cells

6. Which of the following is NOT a mechanism of drug-induced hemolytic anemia?
   a. Drug adsorption on red blood cell membrane
   b. Drug–RBC membrane protein immunogenic complex
   c. RBC autoantibody induction
   d. IgM autoantibody sensitization of RBCs after exposure to the cold

7. Which of the following describes a penicillin-induced AIHA?
   a. Extravascular hemolysis, positive DAT with IgG, gradual anemia
   b. Intravascular, possible renal failure, positive DAT with C3d
   c. Rare hemolysis, positive DAT with IgG
   d. Intravascular hemolysis, positive DAT with IgG

8. Which one of the following statements is true about DHTR:
   a. It is usually due to an ABO incompatibility
   b. Hemoglobinemia and hemoglobinuria frequently occur
   c. It is due to an anamnestic response after repeat exposure to a blood group antigen
   d. The DAT yields a positive result for C3d only

9. Chronic secondary CAD is most often associated with:
   a. Antibiotic therapy
   b. M. pneumoniae infection
   c. B cell malignancies
   d. Infectious mononucleosis

10. A 63-year-old man is being evaluated because of a decrease in hemoglobin of 5 gm/dL after a second cycle of fludarabine for treatment of chronic lymphocytic leukemia. The patient’s DAT result is strongly positive for IgG only and antibody testing on his serum and an eluate of his RBCs yield positive results with all panel cells and the patient’s own cells. This suggests which mechanism of immune hemolysis for this patient?
   a. Drug-RBC membrane protein complex
   b. Drug adsorption
   c. RBC autoantibody induction
   d. Drug-induced nonimmunologic protein adsorption

11. A Group A Rh-negative mother gave birth to a Group O Rh-positive baby. The baby is at risk for HDFN if:
   a. This was the mother’s first pregnancy
   b. The mother has IgG ABO antibodies
   c. The mother was previously immunized to the D antigen
   d. The mother received Rh immune globulin prior to delivery

REFERENCES