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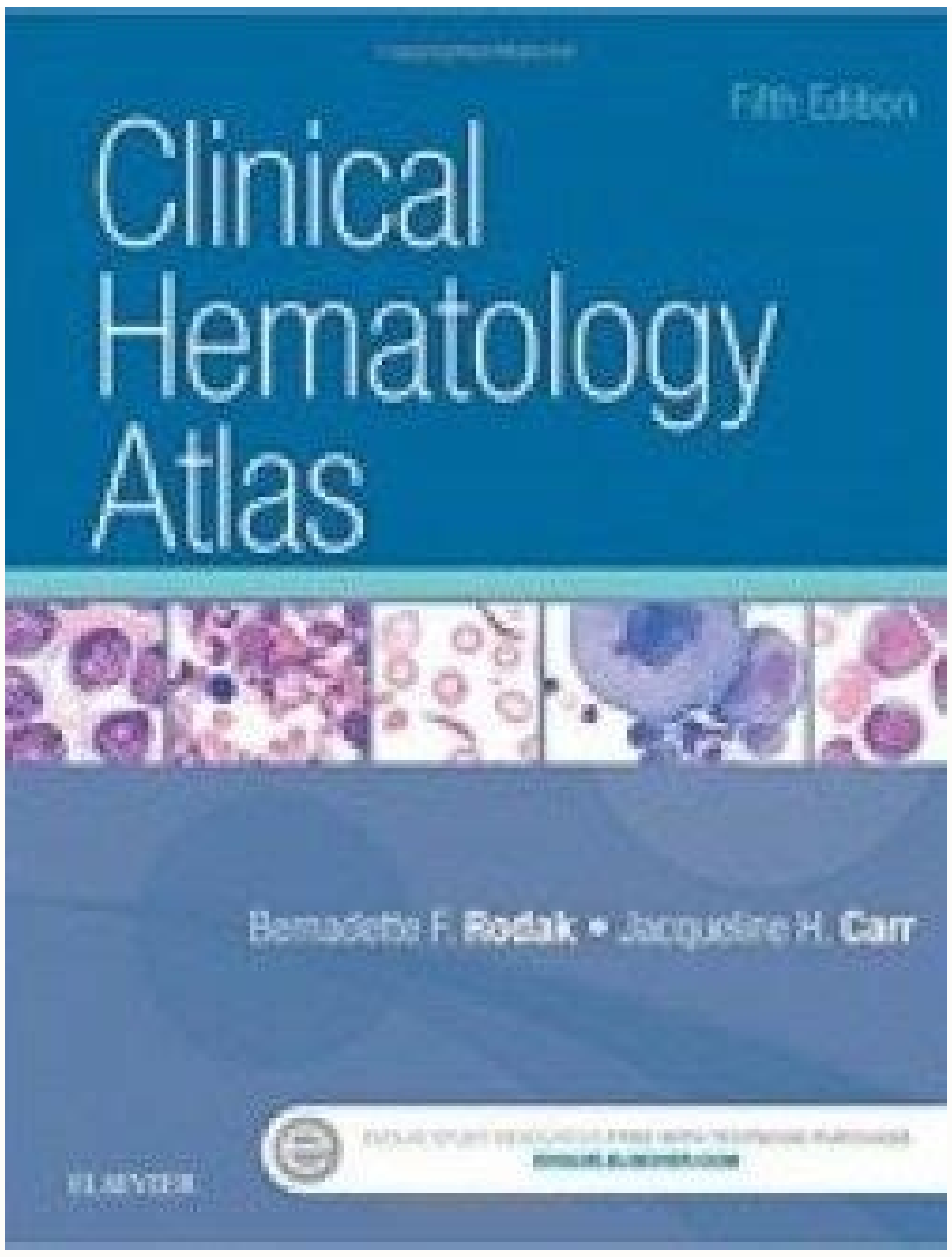

Fifth Edition

Clinical Hematology Atlas

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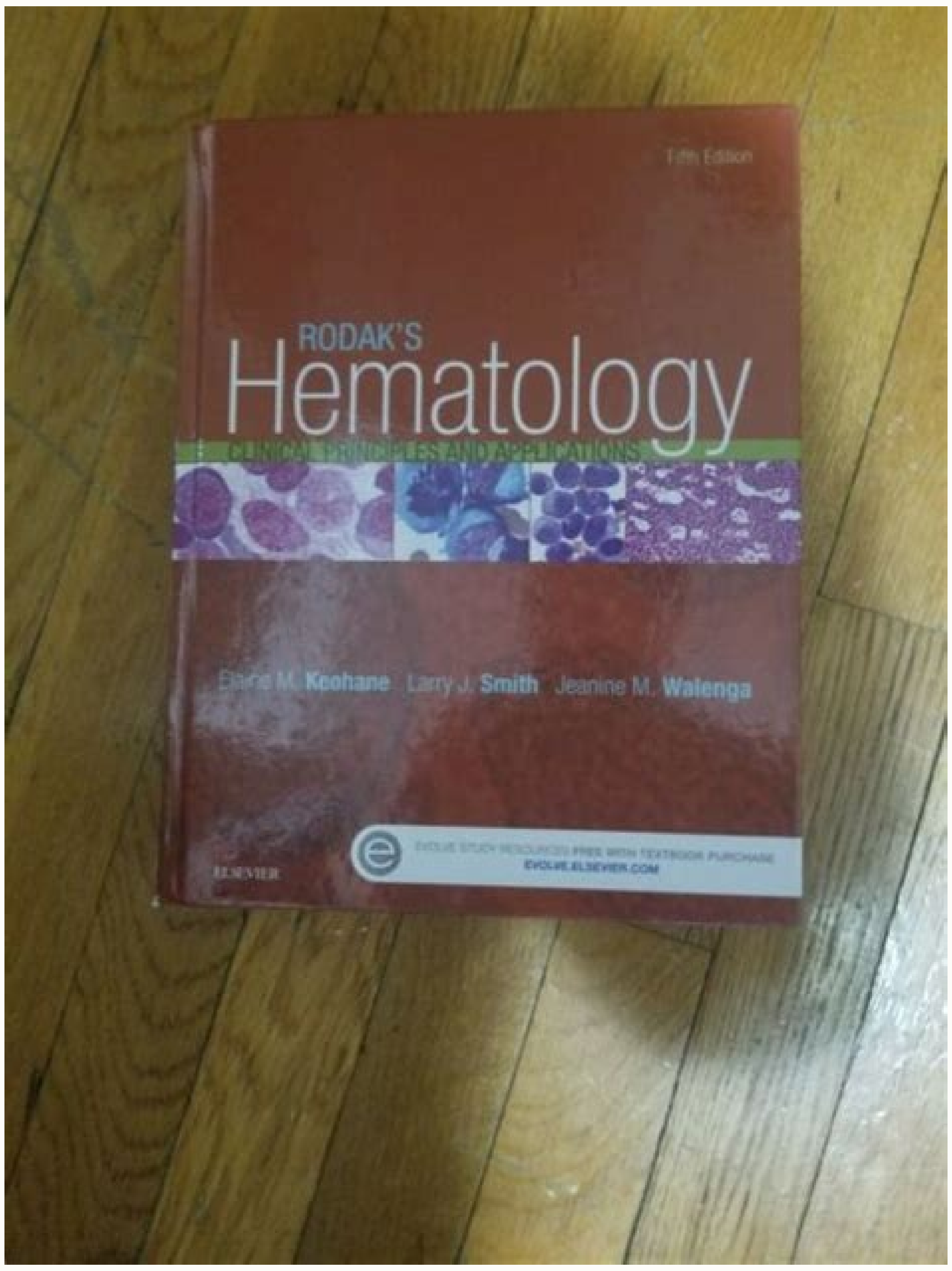

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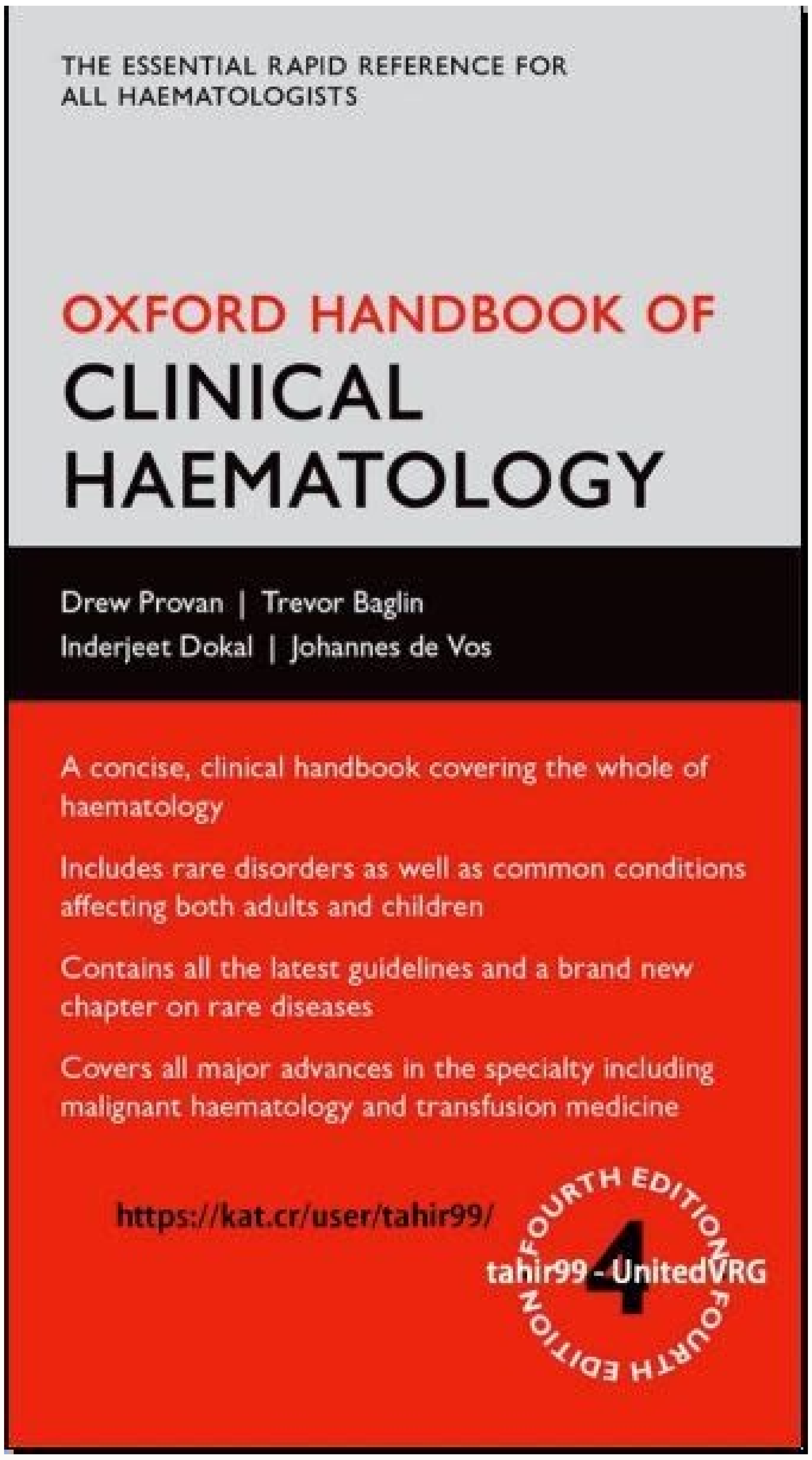
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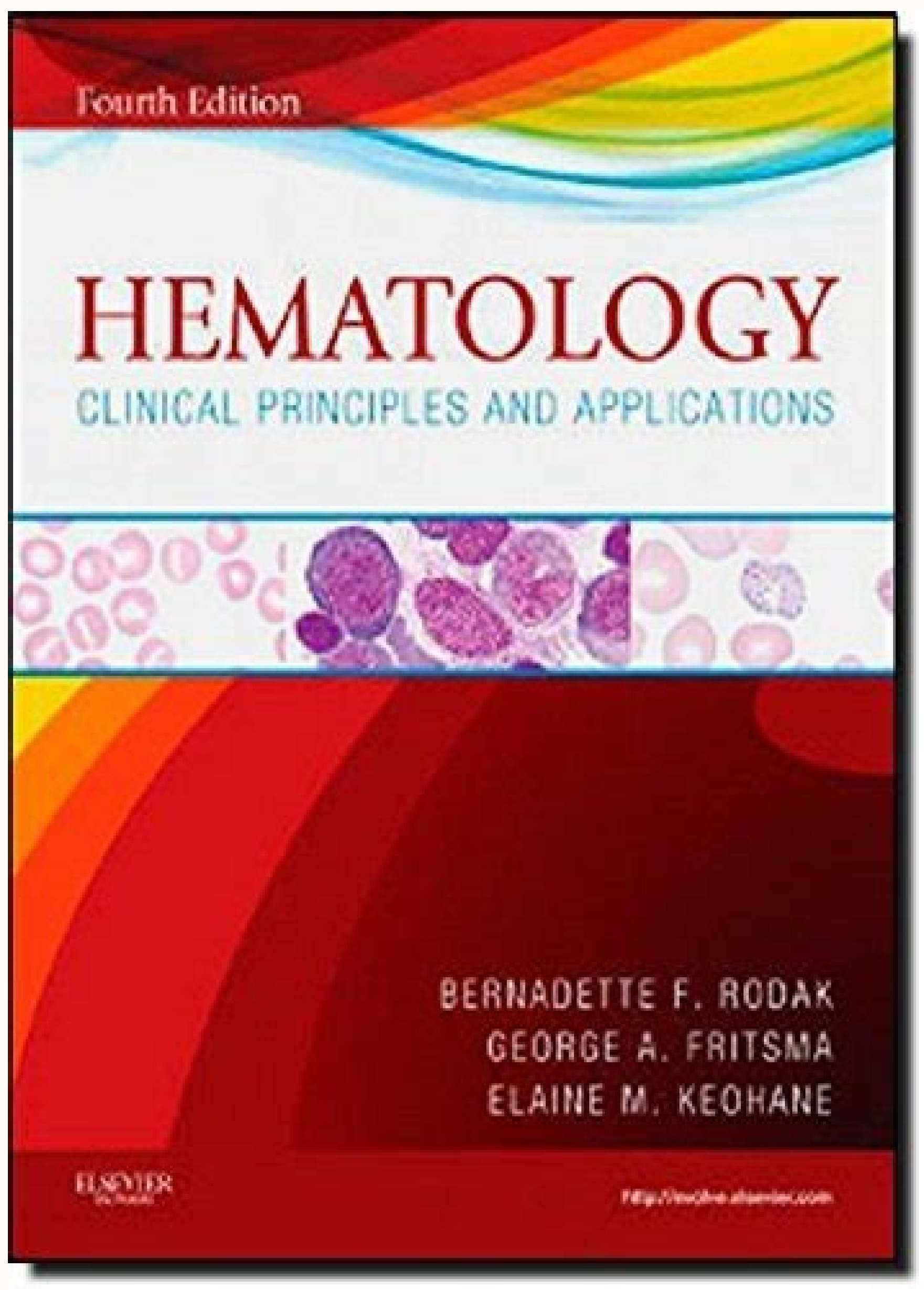

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Hematology/Hemostasis Reference Ranges Unless otherwise noted, data for reference range tables were compiled from multiple sources and may vary slightly from ranges listed within chapters. Each laboratory must establish its particular ranges based on its instrumentation, methodology, and demographics of the population it serves. Hematology Reference Ranges (Adult) Assay Adult Male Adult Female Common Units SI Units RBC HGB (Hb) 4.60-6.00 14.0-18.0 (140-180) 40-54 (0.40-0.54) 80-100 26-32 32-36 11.5-14.5 25-75 0.5-1.5 0 4.5-11.5 4.00-5.40 12.0-15.0 (120-150) 35-49 (0.35-0.49) 80-100 26-32 32-36 11.5-14.5 25-75 0.5-1.5 0 4.5-11.5 × 106/µL g/dL × 1012/L (g/L) % (L/L) fL pg % % × 103/µL % /100 WBC × 103/µL fL pg g/dL % × 109/L HCT MCV MCH MCHC RDW Retics (automated) Visual retics* NRBCs WBCs SEGs (PMNs) BANDs ANC LYMPHs MONOs EOs BASOs PLTs MPV Erythrocyte mass Erythrocyte sedimentation rate, Westergren 1 hour /100 WBC × 109/L % ABS % ABS × 103/µL × 109/L 50-70 0-5 2.3-8.1 0-0.6 2.3-8.1 0.8-4.8 0.45-1.3 0-0.4 0-0.1 50-70 0-5 2.3-8.1 0-0.6 2.3-8.1 0.8-4.8 0.45-1.3 0-0.4 0-0.1 × 103/µL × 103/µL × 103/µL × 103/µL × 103/µL × 103/µL × 103/µL × 103/µL fL × 109/L × 109/L × 109/L × 109/L × 109/L × 109/L × 109/L × 109/L fL 18-42 2-11 1-3 0-2 150-450 6.8-10.2 50 years: 0-20 mm 18-42 2-11 1-3 0-2 150-450 6.8-10.2 50 years: 0-30 mm *Reticulocyte counts performed by manual methods. HGB, Hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; PLTs, platelets; PMNs, polymorphonuclear neutrophils; Segs, segmented neutrophils; Lymphs, lymphocytes; Monos, monocytes; Eos, eosinophils; Basos, basophils; ANC, absolute neutrophil count, includes segs and bands; Retic, reticulocyte. Tests Commonly Used to Assess Anemia (same for male and female, except noted) Test Reference Interval Serum Iron Total iron-binding capacity Percent transferrin saturation Serum ferritin, male Serum ferritin, female Vitamin B12 Serum folate RBC folate HGB A (electrophoresis) HGB A2 (electrophoresis) HGB F, Adult HGB F, Newborn Haptoglobin Free serum hemoglobin 50-160 µg/dL 250-400 µg/dL 20-55 15-400 ηg/mL 10-106 ηg/mL 200-850 pg/mL 2.0-10.0 ηg/mL >120 ηg/mL 95-100% 0-3.5% 0-2.0% 65-90% 31-209 mg/dL 0-10 mg/dL RBC, Red blood cell; HGB, hemoglobin. Hematology Reference Ranges (Pediatric) Assay 0-1 d 2-4 d 5-7 d 8-14 d 15-30 d 1-2 mo 3-5 mo 6-11 mo 1-3 y 4-7 y 8-13 y RBC HGB (Hb) 4.10-6.10 16.5-21.5 (165-215) 48-68 95-125 30-42 30-34 * 1.5-5.8 2-24 9.0-37.0 37-67 3-11 18-38 3-14 1-4 0-2 3.7-30 4.36-5.96 16.4-20.8 (164-208) 48-68 98-118 30-42 30-34 * 1.3-4.7 5-9 8.0-24.0 30-60 3-9 16-46 4-17 1-5 0-2 2.6-17.0 4.20-5.80 15.2-20.4 (152-204) 50-64 100-120 30-42 30-34 * 0.2-1.4 0-1 5.0-21.0 27-51 1-9 24-54 2-15 2-6 0-2 1.5-12.6 4.00-5.60 15.0-19.6 (150-196) 46-62 95-115 30-42 30-34 * 0-1.0 0 5.0-21.0 22-46 0-5 30-62 3-14 1-5 0-2 1.2-11.6 3.20-5.00 12.2-18.0 (122-180) 38-53 93-113 28-40 30-34 * 0.2-1.0 0 5.0-21.0 20-40 0-5 41-61 2-11 1-5 0-2 1.0-9.5 4.00-5.20 10.2-15.2 (102-152) 34-48 80-94 23-31 32-36 11.5-14.5 0.5-1.5 0 5.0-17.0 29-65 0-5 29-65 2-11 1-4 0-2 1.5-11.0 4.00-5.40 12.0-15.0 (120-150) 35-49 80-94 26-32 32-36 11.5-14.5 0.5-1.5 0 4.5-13.5 23-53 0-5 23-53 2-11 1-4 0-2 1.6-9.5 HCT MCV MCH MCHC RDW RETICs NRBCs WBC SEGs/PMNs BANDs LYMPHs MONOs EOs BASOs ANC PLTs 3.40-5.00 10.6-16.4 (106-164) 32-50 83-107 27-37 31-36 * 0.8-2.8 0 6.0-18.0 20-40 0-5 42-72 2-11 1-4 0-2 1.2-8.1 150-450 3.65-5.05 10.4-16.0 (104-160) 35-51 78-102 25-35 32-36 * 0.5-1.5 0 6.0-18.0 18-38 0-5 45-75 2-11 1-4 0-2 1.1-7.7 3.60-5.20 10.4-15.6 (104-156) 35-51 76-92 23-31 32-36 11.5-14.5 0.5-1.5 0 6.0-18.0 20-40 0-5 48-78 2-11 1-4 0-2 1.2-8.1 3.40-5.20 9.6-15.6 (96-156) 38-48 78-94 23-31 32-36 11.5-14.5 0.5-1.5 0 5.5-17.5 22-46 0-5 37-73 2-11 1-4 0-2 1.2-8.9 Common Units SI Units × 106/µL g/dL × 1012/L (g/L) % fL pg % % % /100 WBC × 103/µL % % % % % × 103/µL × 103/µL L/L fL pg g/dL % % /100 WBC × 109/L % % % % % % × 109/L × 109/L *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. From Riley Hospital for Children, Indiana University Health, Indianapolis, IN. HGB, Hemoglobin; PMNs, polymorphonuclear neutrophils; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red blood cell distribution width; Lymphs, lymphocytes; Monos, monocytes; Eos, eosinophils; ANC, absolute neutrophil count, includes segs and band; Segs, segmented neutrophils; Retic, reticulocyte count; PLTs, platelets; Basos, basophils; NRBC, nucleated red blood cells. Bone Marrow Aspirate WBC Differential Blasts Promyelocytes N. myelocytes N. metamyelocytes N. bands N. segmented (polymorphonuclear) Eosinophils Basophils Lymphocytes Plasma cells Monocytes Histiocytes (macrophages) Range (%) 0-3 1-5 6-17 3-20 9-32 7-30 0-3 0-1 5-18 0-1 0-1 0-1 N, Neutrophilic; NB, normoblast; M:E, myeloid:erythroid; lpf, low power field. Erythrocyte Series Range (%) Pronormoblasts Basophilic NB Polychromatophilic NB Orthochromic NB 0-1 1-4 10-20 6-10 Other M:E ratio Megakaryocytes 1.5-3.3:1 2-10/lpf REGISTER TODAY! To access your Student Resources, visit: Register today and gain access to: Glossary Provides definitions for essential hematology terms. Weblinks Links to places of interest on the web specifically for hematology. BERNADETTE F. RODAK, MS, MLS Professor Clinical Laboratory Science Program Department of Pathology and Laboratory Medicine Indiana University School of Medicine Indianapolis, Indiana GEORGE A. FRITSMA, MS, MLS Manager The Fritsma Factor, Your Interactive Hemostasis Resource Birmingham, Alabama ELAINE M. KEOHANE, PhD, MLS Professor Department of Clinical Laboratory Sciences University of Medicine and Dentistry of New Jersey School of Health Related Professions Newark, New Jersey 3251 Riverport Lane St. Louis, Missouri 63043 HEMATOLOGY: CLINICAL PRINCIPLES AND APPLICATIONS ISBN: 978-1-4377-0692-5 Copyright © 2012, 2007, 2002, 1995 by Saunders, an imprint of Elsevier Inc. Chapter 47: "Coagulation Instrumentation" by David L. McGlasson is Public Domain. All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the publisher. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. 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www.sabre.org To my husband, Bob, for his continued patience, understanding, encouragement, and love. And to Annie, who missed her walk times and is glad to have them back. BFR To my wife, Margaret, and children, Greg and Teri, for their love, support, and loyalty. GAF To my students for being great teachers, and to Camryn, Riley, Harper, Jackie, Alana, and Kenny for reminding me about the important things in life. EMK This page intentionally left blank Reviewers Shamina Davis, MS, MT(ASCP) Amy Kapanka, MS, MT(ASCP)SC Faculty, Bachelor's of Applied Technology College of Applied Technologies and General Studies Office of Applied Technology University of Texas at Brownsville Brownsville, Texas MLT Program Director and Professor Hawkeye Community College Waterloo, Iowa David Hoblit, MD Faculty, Department of Pathology University of North Texas Health Science Center Instructor, Department of Clinical Laboratory Science Tarleton State University Fort Worth, Texas E. 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Finch, CHSP, CHMM, MS, BS, MT(ASCP) System Director, Environment of Care/ISO Administrator Detroit Medical Center Detroit, Michigan Safety in the Hematology Laboratory Contributors George A. Fritsma, MS, MLS Mark E. Lasbury, MS, PhD Manager The Fritsma Factor, Your Interactive Hemostasis Resource Birmingham, Alabama An Overview of Clinical Laboratory Hematology Quality Assurance in Hematology and Hemostasis Testing Energy Metabolism and Membrane Physiology of the Erythrocyte Platelet Production, Structure, and Function Bone Marrow Examination Normal Hemostasis and Coagulation Hemorrhagic Coagulation Disorders Thrombosis Risk Testing Laboratory Evaluation of Hemostasis Monitoring Antithrombotic Therapies Assistant Scientist Department of Pathology and Laboratory Medicine Indiana University School of Medicine Indianapolis, Indiana Molecular Diagnostics in the Clinical Laboratory Margaret G. 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Keohane, PhD, MLS Professor Department of Clinical Laboratory Sciences University of Medicine and Dentistry of New Jersey School of Health Related Professions Newark, New Jersey Bone Marrow Failure Intrinsic Defects Leading to Increased Erythrocyte Destruction Extrinsic Defects Leading to Increased Erythrocyte Destruction— Nonimmune Causes Extrinsic Defects Leading to Increased Erythrocyte Destruction— Immune Causes Thalassemias ix Susan J. Leclair, PhD, MLS Chancellor Professor Department of Medical Laboratory Science University of Massachusetts Dartmouth Dartmouth, Massachusetts Acute Leukemias Sharral Longanbach, SH(ASCP) Lynn B. Maedel, MS, MLS(ASCP)SH Executive Director Colorado Association for Continuing Medical Laboratory Education, Inc. (CACMLE) Denver, Colorado Adjunct Faculty Michigan State University East Lansing, Michigan Examination of the Peripheral Blood Film and Correlation with the Complete Blood Count David L. 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Rodak, MS, MLS Professor Clinical Laboratory Science Program Department of Pathology and Laboratory Medicine Indiana University School of Medicine Indianapolis, Indiana Care and Use of the Microscope Cytochemistry Myelodysplastic Syndromes Acute Leukemias Anne Stiene-Martin, PhD Professor Emeritus Clinical Laboratory Science Program Department of Clinical Science College of Health Sciences University of Kentucky Lexington, Kentucky Leukocyte Development, Kinetics, and Functions Nonmalignant Leukocyte Disorders Gail H. Vance, MD Professor Indiana University School of Medicine Department of Medical and Molecular Genetics Indiana University—Purdue University at Indianapolis Clarian Health Hospitals Staff Physician Indianapolis, Carmel and Lafayette, Indiana Cytogenetics Preface The science of clinical laboratory hematology provides for the analysis of normal and pathologic peripheral blood cells, hematopoietic (blood-producing) tissue, and the cells in cerebrospinal and serous fluid. Laboratory hematology also analyzes the cells and plasma enzymes essential to clinical hemostasis. Although the hematology laboratory is closely aligned with clinical hematology, an internal medicine subspecialty of oncology, it provides fundamental information for all patients requiring the services of the specialties of medicine and surgery. Hematology laboratory assay results help predict, diagnose, establish the prognosis for, and monitor the treatment for a variety of medical disorders. Likewise hematology results are used to establish the need for surgery, monitor the effects of surgery, and monitor treatments that support surgical procedures. Clinical laboratory hematology has been enhanced by profound changes, as reflected in the numerous updates in this, the fourth edition of Hematology: Clinical Principles and Applications. Automation and digital data management have revolutionized the way blood specimens are transported and stored, the way laboratory assays are ordered, the way laboratory assay results are validated and reported, and the clinical science of laboratory result interpretation. Molecular diagnosis has augmented and in many instances replaced long-indispensable laboratory assays. Hematological disorders have been reclassified on the basis of phenotypic, cytogenetic, and point mutation analyses. Diagnoses that once depended on the analysis of cell morphology and cytochemical stains now rely on flow cytometry, cytogenetic testing, fluo rescence in situ hybridization, end-point and real-time polymerase chain reaction assays, gene sequencing, and microarrays. Therapeutic regimen monitoring has shifted to the management of biologic response modifiers in place of traditional chemotherapy and minimal residual disease characterization at the molecular and not the cellular level. Hemostasis has grown to encompass thrombophilia testing, methods that reliably monitor newly available antiplatelet and anticoagulant drugs, molecular analysis, and a shift from clot-based to functional and chromogenic assays. Some specific advances that appear in this fourth edition of Hematology: Clinical Principles and Applications include updated stem cell differentiation models based on increased understanding of cell signaling mechanisms and anemia classifications that incorporate updated cell membrane structure and receptors, cytochemical growth factors, and single nucleotide polymorphism analyses. The chapters on hemolytic anemia are reorganized for enhanced clarity. The discussion of white blood cell disorders includes details of the updated World Health Organization leukemia and lymphoma classifications that apply molecular techniques to detect BCR/ABL, JAK2, gene rearrangement, and newly described genetic markers. These chapters have been rewritten to reflect the WHO classification and to blend time-honored morphologic techniques with flow cytometry and molecular analyses. The hemostasis chapters now include references to emerging platelet analysis techno logies and methods for monitoring the antiplatelet drugs eptifibitide, abciximab, integrilin, clopidogrel, prasugrel, and aspirin. The authors have updated anticoagulant monitoring descriptions to include chromogenic substrate analyses and new means for monitoring direct thrombin inhibitors and the oral anticoagulants rivaroxaban, dabigatran, and apixiban, currently in clinical trials. Sections on automated coagulometers and point-of-care coagulation testing are rewritten for clarity and timeliness. ORGANIZATION Hematology: Clinical Principles and Applications is organized into 8 parts, 47 chapters, a detailed appendix, and a fully rewritten glossary. Part I: Introduction to Hematology Chapters 1 to 5 preview the science of clinical laboratory hematology and discuss current approaches to medical laboratory scientist and patient safety, peripheral blood specimen collection, care and use of the light microscope, and hematology and hemostasis quality assurance. Updates to the quality assurance chapter include discussions of new assay validation, accuracy and precision exercises, moving averages, lot-to-lot validations, clinical efficacy analyses, and receiver-operating characteristic analyses. Part II: Hematopoiesis Chapters 6 to 13, richly illustrated with color-balanced photomicrographs, describe the components of a hematologic cell and the biological and genetic details of erythropoiesis (red blood cell production), leukopoiesis (white blood cell production), and megakaryopoiesis (platelet production). Chapters 9 to 11 examine mature red blood cell metabolism; iron metabolism; the production, structure, and function of hemoglobin; and red blood cell senescence and destruction. Chapter 12 discusses the production of WBCs and their kinetic functions. xi xii Preface Chapter 13 provides detail on platelet production, structure, and function including adhesion, aggregation, and activation. Part III: Routine Laboratory Evaluation of Blood Cells Chapter 14 describes time-honored manual hematologic procedures that are employed daily such as manual cell counts, hemoglobin, and hematocrit determinations and compares them to current point-of-care technology. Chapter 15 describes peripheral blood film preparation examination and peripheral blood cell morphology. Chapter 16 follows up with bone marrow aspirate and biopsy collection, preparation, examination, and reporting. Chapter 17 describes the methods for analyzing the normal and pathological cells of cerebrospinal fluid, joint fluid, transudates, and exudates, illustrated with many excellent photomicrographs. Part IV: Hematopathology: Erythrocyte Disorders Chapter 18 provides an overview of anemia, which is the most common hematologic disorder, and integrates peripheral blood and bone marrow morphology with red blood cell indices, reticulocyte counts, and interpretation of pathologic red blood cell morphology. Chapters 19 to 21 describe disorders of iron and DNA metabolism, and bone marrow failure. Chapters 22 to 25, fully revised, provide the hemolytic anemias (anemias of shortened red blood cell life span) that are caused by intrinsic or extrinsic defects. Chapters 26 and 27 provide updates in pathophysiology and address the latest advancements in the diagnosis and treatment of hemoglobinopathies, such as sickle cell disease, and the thalassemias. Part V: Leukocyte Disorders Chapter 28 addresses nonmalignant systemic disorders that are reflected in the distribution of peripheral blood white blood cells. These include bacterial and viral infections, systemic disorders reflected in abnormal white blood cell morphology, infectious mononucleosis, and the blood cell effects of benign lymphoproliferative disorders. There are many new, color-balanced digital photomicrographs. Chapter 29 introduces the pathologic mechanisms of white blood cell neoplasms (malignancy) and Chapters 30 to 33 provide updated details on time-honored but still effective cyto chemistry; cytogenetic procedures, which now include fluorescence in situ hybridization; and molecular diagnostics, with emphasis on end-point and real-time polymerase chain reaction, microarrays, and gene sequencing. Chapter 33 describes the technology of flow cytometry and its diagnostic applications, includes the detection of current and emerging phenotypic markers. The chapter provides numerous examples of scatterplot results in normal and leukemic conditions. Chapters 34 to 37 provide the latest worldwide classifications and pathophysiologic models for myeloproliferative neoplasms, myelodysplastic syndromes, acute lymphoblastic and myeloblastic leukemias, chronic lymphocytic leukemia, and solid tumor lymphoid neoplasms such as lymphoma and myeloma. Part VI: Hematology in Selected Populations Chapter 38, newly written, provides valuable laboratory information on the hematology of the pediatric and geriatric populations. Part VII: Cell-Counting Automation Chapter 39 reviews current automated hematology analyzers. Part VIII: Hemostasis and Thrombosis Chapter 40 provides the coagulation cascade mechanism and relates it to current cell-based hemostasis models. Chapter 41 details hemorrhagic disorders, newly expanded to include management of the acute coagulopathy of trauma and shock. Chapter 42 describes laboratory tests that predict and monitor thrombotic diseases of the arteries and veins. Chapters 43 and 44 describe quantitative and qualitative platelet disorders, and Chapter 45 details laboratory assays of platelets and the coagulation mechanism. Chapter 46 expands on Chapter 45 with details for monitoring antithrombotic drugs, including the latest oral anticoagulants and a variety of antiplatelet drugs, and Chapter 47 reviews automated coagulation analyzers and bench-top point of care instrumentation. READERS Hematology: Clinical Principles and Applications is written for Medical Laboratory Scientists, Medical Laboratory Technicians, and the faculty of Medical Laboratory Science and Medical Laboratory Technician educational programs. It is a valuable 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 Hematology: Clinical Principles and Applications is logically organized and reflects the practical clinical expertise of its authors. The text is enhanced by full-color, color-balanced digital photomicrographs, figures and line art, and detailed text boxes and tables. Figures and tables are enhanced with stand-alone captions. Pedagogy is supported by learning objectives, real-life introductory case studies with open-ended discussion questions, comprehensive summaries, and study questions at the end of each chapter. Students preparing for examinations may use the learning objectives, review questions, and chapter summaries to guide their test preparation. The appendix provides examples of material safety data sheets, answers to the case study questions and review questions, and an extensive, fully updated glossary. NEW TO THIS EDITION • Editor Bernadette Rodak, MS, MLS Indiana University School of Medicine, who has been the lead editor for the first three editions, and George A. Fritsma, MS, MLS, University of Alabama at Birmingham Department of Preface • • • • • Pathology, co-editor of the third edition, are joined for the fourth edition by Elaine M. Keohane, PhD, MLS, University of Medicine and Dentistry of New Jersey Department of Clinical Laboratory Sciences. Co-editor George A. Fritsma, proprietor of the blog The Fritsma Factor, Your Interactive Hemostasis Reference, sponsored by Precision BioLogic, Dartmouth, Nova Scotia, rewrote Chapter 5, Quality Assurance in Hematology and Hemostasis Testing; and Chapter 16, Bone Marrow Examination. Prof. Fritsma continues as editor of Part VIII: Hemostasis and Thrombosis. Chapter 47, Coagulation Instrumentation, has been completely updated by internationally recognized hemostasis researcher David McGlasson, Wilford Hall USAF Hospital, San Antonio, Texas. Substantial updates were made to Chapter 29, Introduction to Leukocyte Neoplasms, by Peter D. Emanuel, MD, Director, Winthrop P. Rockefeller Cancer Institute, Professor of Medicine, Kent Westbrook, MD Endowed Chair, University of Arkansas for Medical Sciences, Little Rock, Arkansas. Dr. Keohane provided substantial updates to Chapters 23 to 25, on the Hemolytic Anemias. Substantial updates in Chapter 20, Anemias Caused by Defects of DNA Metabolism, and Chapter 38, Pediatric and Geriatric Hematology, by Linda H. Goossen, PhD, MT (ASCP), College of Health Professions, Grand Valley State University, Grand Rapids, Michigan. PEDAGOGY Each chapter contains: • Learning objectives for all cognitive domains. • One or two case studies at the beginning of each chapter that pique the readers' interest and provide open-ended discussion questions. xiii • 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 are written to match the chapter objectives; they are given in the format used by certification examinations. EVOLVE ANCILLARIES For the Instructor • Test bank: An ExamView test bank of 850 multiple-choice questions features rationales, cognitive levels, and page number references to the text. Available for in-class review or for test development. • Instructor's manual: The instructor's manual includes one chapter for every chapter in the text and contains key terms, objectives, outlines, and study questions. This can be used in preparation for classes and during lecture. • Case studies: 54 case studies feature photomicrographs, images, and discussion questions. These can be used in class for review of material, for practical exams, or for students' class presentations. • PowerPoint presentations: One PowerPoint presentation per chapter can be used "as is" or as a template to prepare lectures. • Image Collection: All of the images from the book are available as electronic files that can be downloaded into PowerPoint presentations. These can be used during lecture to illustrate important concepts. For the Student • Glossary: The glossary is available on the Evolve site and can be used as a quick reference to look up unfamiliar terms electronically. • Weblinks: Links to places of interest on the web specifically for hematology. Acknowledgments Editors Elaine Keohane and George Fritsma respectfully acknowledge Bernadette F. 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Leclair and Bernadette F. Rodak CHAPTER 37 Mature Lymphoid Neoplasms, 558 Magdalena Czader PART VI Hematology in Selected Populations CHAPTER 38 Pediatric and Geriatric Hematology, 580 Linda H. Goossen Larry D. Brace CHAPTER 45 Laboratory Evaluation of Hemostasis, 734 George A. Fritsma CHAPTER 46 Monitoring Antithrombotic Therapies, 765 George A. Fritsma CHAPTER 47 Coagulation Instrumentation, 783 David L. McGlasson APPENDIX Material Safety Data Sheet, 796 Answers, 801 Glossary, 816 CHAPTER 1 PART I Introduction to Hematology An Overview of Clinical Laboratory Hematology 1 1 George A. Fritsma OUTLINE History Red Blood Cells Hemoglobin, Hematocrit, and Red Blood Cell Indices Reticulocytes White Blood Cells Platelets Complete Blood Count Blood Film Examination Endothelial Cells Coagulation Advanced Hematology Procedures Additional Hematology Procedures Hematology Quality Assurance and Quality Control T he average human possesses 5 L 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 other components, provides coagulation enzymes that protect vessels from trauma and maintain the circulation. Plasma transports and nourishes blood cells. There are three families of blood cells: red blood cells (RBCs), or erythrocytes; white blood cells (WBCs), or leukocytes; and platelets, or thrombocytes.1 Hematology is the study of blood cells. By expertly staining, counting, analyzing, and recording the appearance, phenotype, and genotype of all three types of cells, the medical laboratory 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. HISTORY Early scientists such as Athanasius Kircher in 1657 described "worms" in the blood, and Anton van Leeuwenhoek in 1674 gave an account of RBCs,2 but it was not until the late 1800s that Giulio Bizzozero described platelets as "petites plaques."3 The development of Wright stain by James Homer Wright in 1902 opened a new world of visual blood examination through the microscope. Although many automated instruments now differentiate and enumerate blood cells, Wright's Romanowskytype stain (polychromatic, a mixture of acidic and basic dyes), and refinements thereof, remains the heart of blood cell identification.4 In the present-day hematology laboratory, RBC, WBC, and platelet appearance is analyzed visually using 500× to 1000× light microscopy examination of cells fixed to a glass microscope slide and stained with Wright or Wright-Giemsa stain (see Chapter 15). The scientific term for cell appearance is morpho logy, which encompasses cell color, size, shape, cytoplasmic inclusions, and nuclear condensation. RED BLOOD CELLS RBCs are anucleate biconcave cells filled with a reddish protein, hemoglobin (Hb, HGB), which transports oxygen and carbon dioxide (see Chapter 10). RBCs appear pink to red and measure 6 to 8 µm in diameter with a zone of pallor covering one third of their center (Figure 1-1, A), reflecting their biconcavity (see Chapters 8 and 9). Since before 1900, physicians and medical laboratory scientists 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 (see Chapters 14 and 18). Polycythemia means an increased RBC count reflecting increased body RBC mass, a condition that leads to hyperviscosity (see Chapter 34). To count RBCs, laboratory scientists carefully pipetted a tiny aliquot of whole blood and mixed it with 0.85% (normal) saline. This saline concentration matches the osmolality of normal blood; consequently, the suspended RBCs closely retained their native 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 and is still available from clinical laboratory supply companies. 1 2 PART I Introduction to Hematology B C D A F E G H Figure 1-1 A, Normal erythrocyte (red blood cell, RBC). B, Normal polymorphonuclear neutrophil (PMN, segmented neutrophil, seg). C, Normal band neutrophil (band). D, Normal eosinophil (EO). E, Normal basophil (baso). F, Normal lymphocyte (lymph). G, Normal monocyte (mono). H, Normal platelet. The diluted blood was transferred to a counting chamber or hemacytometer (see Figure 14-1). The medical laboratory scientist 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 (see Chapter 14), and reported the RBC count in cells per microliter (mcL), milliliter (mL, also called cubic centimeter, or cc), or liter (L). Visual RBC counting was developed before 1900 and, although never accurate, 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 (see Chapter 39).5 The Coulter principle of direct current electrical impedance is still used for RBC counting in many automated hematology profiling instruments. Happily, the widespread availability of automated cell counters has replaced visual RBC counting. Hemoglobin, Hematocrit, and Red Blood Cell Indices RBCs also are assayed for hemoglobin concentration and hematocrit (Hct, see Chapters 10 and 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 (hemiglobincyanide), and the solution is placed in a photometer with incident light at 540 nm wavelength. The color intensity is compared with that of a reagent blank and a known standard and is mathematically converted to hemoglobin concentration. Drabkin reagent is used in manual and most automated applications, although some automated hematology profiling instruments use a formulation of the ionic surfactant (detergent) sodium dodecyl sulfate to reduce environmental cyanide. Hematocrit is the ratio of the volume of RBCs to the volume of whole blood and is determined by transferring blood to a graduated plastic tube, centrifuging, measuring the column of RBCs, and dividing by the total length of RBCs plus plasma. The normal ratio approaches 50% (see inside front cover for reference ranges). 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. The laboratory scientist may use the three numerical results, RBC count, hemoglobin, and hematocrit, to compute the RBC indices mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC, see Chapter 14). The MCV, although a measure of volume, reflects RBC dia meter on a Wright-stained blood film; the MCHC reflects RBC staining intensity or degree of pallor. The MCH expresses the mass of hemoglobin and closely reflects 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 but cannot be provided using manual RBC measurements. In addition to aiding diagnosis, the RBC indices provide stable measurements for internal quality control (see Chapter 5). Laboratory scientists routinely use 1000× visual examination (see Chapter 4) to review RBC morphology, commenting consistently on RBC diameter, color or hemoglobinization, shape, and the presence of cytoplasmic inclusions (see Chapter 18). All these parameters—RBC count, hemoglobin, hematocrit, indices, and RBC morphology—are used to detect, diagnose, assess the severity of, and monitor the treatment of anemia, polycythemia, and numerous systemic conditions that affect RBCs. Automated hematology profiling instruments are used in nearly all laboratories to generate these data, although examination of the Wright-stained blood film is still essential. Reticulocytes In the Wright-stained film, 1% to 2% of RBCs exceed the 6- to 8-µm average diameter and stain slightly blue-gray. These are polychromatophilic erythrocytes, newly released from the RBC production site, the bone marrow (see Chapters 8 and 16). Polychromatophilic erythrocytes are closely observed because they indicate bone marrow regeneration during blood loss and certain anemias (see Chapters 23 to 25). Methylene blue dyes, called nucleic acid stains or vital stains, are used to differentiate and count these young RBCs. Vital stains are dyes absorbed by live cells. Young RBCs contain ribonucleic acid (RNA) and are called reticulocytes when the CHAPTER 1 An Overview of Clinical Laboratory Hematology RNA is highlighted using vital stains. Reticulocyte counting was (and remains) a tedious and imprecise visual chore 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 a particularly sensitive measure of bone marrow function, the immature reticulocyte count or immature reticulocyte fraction. To everyone's regret, it is still necessary to confirm instrument counts visually from time to time, which requires that medical laboratory scientists retain this skill. WHITE BLOOD CELLS WBCs, or leukocytes, are not really blood cells; they are a loosely related grouping of cell families dedicated to protecting their host from infection and injury (see Chapters 12 and 28). WBCs "hitch a ride" in the blood from their source, usually bone marrow or lymphoid tissue, to their tissue destination. They are so named because they are nearly colorless in an unstained cell suspension. In chronic leukemia, an extreme increase in the WBC count imparts a milky appearance to the blood (see Chapters 34 and 36). WBCs may be counted visually using a microscope, hemacytometer, and a Thoma pipette. The technique is the same as RBC counting, but the typical dilution is 1 : 20, and the diluent is composed of dilute acetic acid in normal saline. The acid causes RBCs to lyse or rupture; without it, RBCs would obscure the WBCs, whose count ranges from 4500 to 11,500/mcL. Visual WBC counting has been largely replaced by automated hematology profiling instruments, but is accurate and useful in situations in which no automation is available. Laboratory scientists who analyze body fluids such as cerebrospinal fluid employ visual WBC counting every day. A decreased WBC count (fewer than 4500/mcL) is called leukopenia, and an increased WBC count (more than 11,500/ mcL) is called leukocytosis, but the WBC count alone has modest clinical value. The laboratory scientist must differentiate the families of WBCs transported in the blood by using a Wrightstained blood film and light microscopy (see Chapter 15). The types of WBCs are as follows: • Polymorphonuclear neutrophils (PMNs, or segmented neutrophils or segs; see Figure 1-1, B). Segs are phagocytic cells whose sole purpose is to engulf and destroy bacteria that have been earlier labeled as harmful by the immune system. An increase in segs is called neutrophilia and often signals bacterial infection. A decrease is called neutropenia and often is caused by long-term drug administration or a viral infection. • Band neutrophils (bands; see Figure 1-1, C). Bands are part of the seg family; they are simply less differentiated or less "mature" than segs. A band increase also signals bacterial infection and is customarily called a left shift. The cytoplasm of segmented neutrophils and bands contains submicroscopic, pink-staining granules filled with bactericidal secretions. 3 • Eosinophils (EOs; see Figure 1-1, D). Eosinophils are cells with bright orange, regular cytoplasmic granules filled with antihistamine. An elevated eosinophil count is called eosinophilia and signals a response to allergy or parasitic infection. • Basophils (basos; see Figure 1-1, E). Basophils are cells with dark purple, irregular cytoplasmic granules that obscure the nucleus. An elevated basophil count is basophilia. Basophilia is rare and often signals a hematologic disease, such as leukemia. • Segs, bands, eosinophils, and basophils are collectively called granulocytes because of their prominent cytoplasmic granules, although their functions differ. The distribution of eosinophils and basophils in blood is so small compared with that of PMNs that the terms eosinopenia and basopenia are theoretical and unused. Leukemia is uncontrolled proliferation of WBCs. Leukemia may be chronic, for example chronic myelogenous (granulocytic) leukemia, or acute, such as acute myeloblastic leukemia. There are several forms of granulocytic leukemias (see Chapters 34 to 36), categorized by their respective genetic aberrations (see Chapters 31 and 32), and laboratory scientists are responsible for their identification using Wright-stained bone marrow smears, cytochemical stains, cytogenetics, and molecular diagnostic technology (see Chapters 16, 30, 31, and 32). • Lymphocytes (lymphs, see Figure 1-1, F). Lymphocytes comprise a complex system of cells that provide for host immunity. Lymphocytes recognize foreign antigens and mount antibody (humoral) and cell-mediated antagonistic responses. On a Wright-stained 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. An abnormally low lymphocyte count is lymphopenia or lymphocytopenia and is associated with long-term drug therapy or immunodeficiency. Lymphocytes are also implicated in leukemia; chronic lymphocytic leukemia is prevalent in people older than 70 years, whereas acute lymphoblastic leukemia is the most common form of childhood leukemia (see Chapters 36 to 38). Laboratory scientists classify lymphocytic leukemias, of which there are several, largely based on Wright-stained blood films and flow cytometry (see Chapter 33). • Monocytes (monos, see Figure 1-1, G). The mono 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 in the body, more abundant than RBCs or skin cells, although they are a minor component of the blood film differential count. They occupy every body cavity; some are motile and some immobilized. Their task is to identify and phagocytose (engulf) foreign particles and assist the lymphocytes in mounting an immune response through the assembly and presentation of immunogenic epitopes. On a Wright-stained film, monos have a slightly larger diameter than other WBCs, gray cytoplasm, and a lobulated nucleus. An increase in the number of monocytes may signal a hematologic 4 PART I Introduction to Hematology disease, such as leukemia, and is called monocytosis. Scientists seldom document a decreased mono count, so the theoretical term monocytopenia is seldom used. The monocyte-macrophage cell line is another source for leukemias, for instance, chronic or acute monocytic leukemia (see Chapter 36). PLATELETS Platelets, or thrombocytes, are true blood cells that maintain blood vessel integrity by instigating vessel wall repairs (see 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 cells that control hemostasis, a series of cellular and plasma-based mechanisms that seals wounds, repairs vessel walls, and maintains vascular patency. Platelets are only 2 to 4 µm in diameter, round or oval, anucleate, and slightly granular (see Figure 1-1, H). Their diminutive 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 (miscarriage). The medical laboratory professional counts platelets using the same technique used in counting WBCs on a hemacyto meter, although the counting is usually confined to the center square millimeter of the hemacytometer. Owing to their minuscule volume, platelets are hard to distinguish visually in a hemacytometer, and phase microscopy provides for easier identification (see Chapter 4). Automated profiling instruments have largely replaced visual platelet counting and provide greater accuracy (see Chapter 39). 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 (see Chapters 13 and 43). Elevated platelet counts, called thrombocytosis, signal inflammation or trauma but carry small 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 (see Chapter 34). 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 (see Chapter 43). Thrombocytopenia accounts for the majority of hemorrhage-related emergency department visits. Accurate platelet counting is essential to patient safety for diagnosis of thrombocytopenia in many disorders or therapeutic regimens. COMPLETE BLOOD COUNT The medical laboratory scientist may collect a blood specimen for the complete blood count (CBC); a phlebotomist, nurse, physician, or patient care technician may also collect the specimen (see Chapters 3, 14, and 45). In all cases, the scientist is responsible for the integrity of the specimen and ensures that it is free of clots, hemolysis, and inappropriate anticoagulantto-specimen ratios known as short draws. The medical laboratory scientist also ensures that the specimen is fresh enough for accurate analysis (see Chapter 5) and then accurately registers the specimen in the work list, a process known as specimen accession. Accession may be automated, relying on bar code or radio-frequency identification technology. Although all laboratory scientists are equipped to perform visual RBC, WBC, and platelet counts using dilution pipettes, hemacytometers, and microscopes, most laboratories employ profiling instruments to generate the CBC parameters listed in Box 1-1. The more sophisticated profiling instruments provide comments on RBC, WBC, and platelet morphology (see Chapter 39). 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, the scientist performs a "reflex" blood film examination (see Chapter 15). The blood film examination is a specialized, demanding, and fundamental CBC activity. Nevertheless, if all profiling BOX 1-1 Measurements Generated by Automated Hematology Profiling Instruments RBC Parameters RBC count Hb Hct MCV MCH MCHC RDW Retic WBC Parameters WBC count Seg count: % and absolute Band count: % and absolute Lymph count: % and absolute Mono count: % and absolute EO and baso counts: % and absolute Platelet Parameters Platelet count MPV baso, Basophil; EO, eosinophil; Hb, hemoglobin; Hct, hematocrit; Lymph, lymphocyte; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; Mono, monocyte; MPV, mean platelet volume; Seg, segmented neutrophil; RBC, red blood cell; RDW, RBC distribution width; Retic, reticulocyte; WBC, white blood cell. CHAPTER 1 An Overview of Clinical Laboratory Hematology instrument results are normal, the blood film examination is omitted from the CBC. Indeed, the blood film examination is waived in 50% to 80% of cases, depending on the facility's case mix. Acute care facilities report more normal CBCs than specialized or tertiary care facilities, in which the percentage of blood film examinations may exceed 50%. Physicians may request a blood film examination on the basis of clinical suspicion even when the profiling instrument results are within normal limits (WNL). BLOOD FILM EXAMINATION To accomplish a blood film examination, the medical laboratory scientist 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 (see Chapter 15). The scientist affixes the slide to the microscope stage and examines the RBCs and platelets for abnormalities of shape, diameter, color, or inclusions using the 50× or 100× oil immersion lens to generate 500× or 1000× magnification (see Chapter 4). The WBC count and platelet count are estimated for comparison with the instrument counts, and all abnormalities are carefully recorded. The scientist 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"). This individualized activity is highly reliant on the scientist's skill, visual acuity, and integrity, and it provides extensive diagnostic information. Medical laboratory scientists pride themselves on their technical and analytical skills in performing the blood film examination. The results of the CBC, including all profiling and blood film examination parameters and interpretive comments, are provided on a single page or computer screen 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 by medical laboratory scientists in the hematology laboratory. Nevertheless, endothelial cells are important in maintaining normal blood flow, in snaring platelets during times of injury, and in enabling WBCs to escape from the vessel to the surrounding tissue when called upon. Increasingly refined laboratory methods will enable us to examine fully at least the secretions (cytokines) of these important cells. COAGULATION Most hematology laboratories provide a blood coagulation testing department (see Chapters 41, 42, and 45). Plasma coagulation is one component of hemostasis; another is platelets, as reviewed previously. 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 six to eight enzymes 5 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 manifold and complex, and the coagulation section of the hematology laboratory provides a series of plasma-based laboratory assays reflecting complex interactions of hematologic cells with plasma proteins (see Chapters 40 and 45). The medical laboratory scientist 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 (see Chapters 3 and 45). 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 (see Chapter 45). The prothrombin time and partial thromboplastin time are particularly high-volume assays. These tests assess each arm of the coagulation pathway for deficiencies and are used to monitor anticoagulant therapy. Another 30 to 40 low-volume assays are available in specialized or tertiary care facilities. The specialized or tertiary care coagulation laboratory with its interpretive complexities is a haven for 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, 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 bone marrow collection and prepare, stain, and microscopically review bone marrow smears (see Chapter 16). Bone marrow aspirates and biopsy specimens are collected and stained to analyze nucleated cells that are precursors to blood cells. Cells of the erythroid series are precursors to RBCs (see Chapter 8); myeloid series cells mature to form bands and segmented neutrophils, eosinophils, and basophils (see Chapter 12); and megakaryocytes produce platelets (see Chapter 13). Laboratory scientists, clinical pathologists, and hematologists review Wright-stained aspirate smears for morphologic abnormalities, high or low bone marrow cell concentration, and inappro priate cell line distributions. An increase in the erythroid cell line may indicate bone marrow compensation for abnormally increased RBC consumption during blood loss (see Chapter 18). The biopsy specimen, enhanced by hematoxylin and eosin (H&E) staining, may reveal abnormalities in bone marrow architecture indicating leukemia, aplastic anemia, or one of a host of other hematologic disorders. Results of examination of bone marrow aspirates and biopsy specimens are compared with CBC results generated from the peripheral blood to establish commonalities and develop pattern-based diagnoses. 6 PART I Introduction to Hematology In the bone marrow laboratory, cytochemical stains may be employed to differentiate abnormal myeloid, erythroid, and lymphoid cells (see Chapter 30). 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 and are gradually being replaced by flow cytometry immunophenotyping, cytogenetics, and molecular diagnosis (see Chapters 31 to 34). Since 1980, immunostaining methods have enabled us to identify cell lines, particularly lymphocyte precursors, with certainty. An example of immunostaining is an immunebased dye bound to antibodies to coagulation factor VIII, which is present in megakaryocytes and may be diagnostic for megakaryoblastic leukemia. 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 developed from research applications (see Chapter 39). 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 (see Chapter 39). 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. The qualitative flow cytometry laboratory is indispensable to leukemia and lymphoma diagnosis. Molecular diagnostic techniques enhance and even replace some of the advanced hematologic methods. Polymerase chain reaction, real-time polymerase chain reaction, microarrays, and sequencing systems enable laboratory scientists to detect mutations such as BCR/ABL fusion, JAK2, and the t(15;17) translocation that signal specific forms of leukemia and establish their therapeutic profile and prognosis (see Chapters 32 and 34). ADDITIONAL HEMATOLOGY PROCEDURES Laboratory scientists provide several specific manual wholeblood 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 spherocytic or warm autoimmune hemolytic anemia (see Chapters 23 and 25). Likewise, the glucose-6-phosphate dehydrogenase assay tests for an inherited RBC enzyme deficiency causing severe episodic hemolytic anemia (see Chapter 23). The sickle cell solubility screening assay and its follow-up test, Hb electrophoresis, are used to detect and diagnose sickle cell anemia and other inherited qualitative hemoglobin abnormalities and thalassemias (see Chapters 26 and 27). One of the oldest hematology tests, the erythrocyte sedimentation rate, detects inflammation and roughly estimates its intensity (see Chapter 14). Finally, the hematology laboratory scientist reviews the cellular counts, distribution, and morphology in body fluids other than blood (see Chapter 17). 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 speedy turnaround, because cells in these hostile environments rapidly lose their integrity. The conditions leading to a need for body fluid withdrawal are invariably acute. HEMATOLOGY QUALITY ASSURANCE AND QUALITY CONTROL Medical laboratory scientists employ particularly complex quality control systems in the hematology laboratory (see Chapter 5). Owing to the unavailability of weighed standards, the measurement of cells and biologic systems defies chemical standardization and requires elaborate validation, matrix effect examination, linearity, and reference interval determinations. An internal standard methodology known as the moving average also arose in hematology laboratory applications.6 Laboratory scientists compare methods through clinical efficacy calculations that produce clinical sensitivity, specificity, and positive and negative predictive values for each assay. Scientists must monitor specimen integrity and test ordering patterns and ensure the integrity 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 1. Rhan DH: Examination of the blood. In Lichtman MA, Beutler E, Kipps TJ, et al, editors: Williams hematology, ed 7, New York, 2006, McGraw-Hill Medical. 2. Wintrobe MM: Hematology, the blossoming of a science, Philadelphia, 1985, Lea & Febiger. 3. Bizzozero J: Über einem neuen formbestandtheil des blutes und dessen rolle bei der Thrombose und der Blutgerinnung. Virchows Arch Pathol Anat Physiol Klin Med 90:261-332, 1882. 4. Woronzoff-Dashkoff KK: The Wright-Giemsa stain. Secrets revealed. Clin Lab Med 22:15-23, 2002. 5. Blades AN, Flavell HC: Observations on the use of the Coulter model D electronic cell counter in clinical haematology. J Clin Pathol 16:158-163, 1963. 6. Gulati GL, Hyun BH: Quality control in hematology. Clin Lab Med 6:675-688, 1986. Safety in the Hematology Laboratory 2 Sheila A. Finch* OUTLINE OBJECTIVES Standard Precautions After completion of this chapter, the reader will be able to: 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 1. Define standard precautions. 2. List infectious materials included in standard precautions. 3. Describe the safe practices required in the Occupational Exposure to Bloodborne Pathogens Standard. 4. Identify occupational hazards that exist in the hematology laboratory. 5. Identify the requirements of the Occupational Exposure to Hazardous Chemicals in Laboratories Standard. 6. Discuss the development of a safety management program. 7. Describe the principles of a fire prevention program, including details such as the frequency of testing equipment. 8. Name the most important practice to prevent the spread of infection. 9. Given a written laboratory scenario, assess it for safety hazards and recommend corrective action. 10. Select the proper class of fire extinguisher for a given type of fire. 11. Define material safety data sheets (MSDSs), list information contained on MSDSs, and determine when MSDSs would be used in laboratory activity. 12. Name the specific practice during which most needle stick injuries occur. 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 that should be taken. 1. A hematology technologist was observed removing gloves and immediately left the laboratory for a meeting. The medical laboratory professional did not remove the laboratory coat. 2. Food was found in the specimen refrigerator. 3. Syringes were found in the proper sharps container. On further investigation, 50% of the attached needles were recapped. 4. Hematology technologists were seen in the lunchroom wearing laboratory coats. 5. Fire extinguishers were found every 75 feet of the laboratory. 6. Fire extinguishers were inspected quarterly and annually. 7. One fire drill was conducted in the last 8 months. 8. Unlabeled bottles were found at the workstation. 9. A 1 : 10 solution of bleach was found near the electronic cell counter. Further investigation revealed that the bleach solution was made 6 months ago. 10. Gloves were worn by the staff receiving specimens. 11. Material safety data sheets were obtained by fax. 12. Chemicals were stored alphabetically. 13. Fifty percent of the staff interviewed had not participated in a fire drill. *The author acknowledges the assistance of Debra Walters, safety officer at Indiana University Health, Indianapolis, Ind., for review of this chapter. 7 8 PART I Introduction to Hematology M 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 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 healthcare 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 the following potentially infectious materials: 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. Past practice was to label specimens from patients known to have infectious diseases; however, experience has shown that patients without visible symptoms can harbor infectious diseases. Labeling such specimens also jeopardizes patient confidentiality. Adopting standard precautions lessens the risk of healthcare 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, 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 hemato logy 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. Handwashing 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 handwashing 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 (see Figure 2-1, B). d. Dry hands with a paper towel (see Figure 2-1, C). Use the paper towel to turn off the faucet handles (see Figure 2-1, D). Hands must be washed: a. Whenever there is visible contamination with blood or body fluids b. After completion of work c. After gloves are removed and between glove changes d. Before leaving the laboratory e. Before and after eating and drinking, smoking, applying cosmetics or lip balm, changing a contact lens, and using the lavatory f. 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 worker's mouth and all mucous membranes. 4. Food and drink, including oral medications and tolerancetesting 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 (see Chapter 3). Documentation in the exposure control plan should identify the specific procedure by 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 leak-proof (Figure 2-3). 8. Procedures such as removing caps when checking for clots, filling hemacytometer chambers, making slides, discarding CHAPTER 2 Safety in the Hematology Laboratory A B C D 9 Figure 2-1 Proper handwashing 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. 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 worker. The most common forms of personal protective equipment are described in the following: 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. 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. 10 PART I Introduction to Hematology A B C D 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, Mass.) All protective clothing should be removed before the worker leaves 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 would be laundered by the employee. 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 finger sticks are performed. One of the limitations of gloves is that they do not prevent needle sticks or other puncture wounds. Provision of gloves to laboratory workers must accommodate latex allergies. Alternative gloves must be readily accessible to any laboratory worker with a latex 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).1 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 the cell counter, and centrifuging specimens are examples of tasks that could result in creation of aerosol mist. 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), placed into a special selfsealing leak-proof 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 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 someone wearing gloves, a CHAPTER 2 Safety in the Hematology Laboratory A 11 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 2 1 2 cups of bleach to 1 gallon of water to achieve the recommended concentration of chlorine of 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 B Figure 2-4 Examples of safety shields. A, Face shield. B, Adjustable swing arm shield. (Courtesy Steve Kasper.) 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 would consist at 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 Laboratory workers 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 epidemio logy 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. Specific disposal guidelines are specific to the state disposal standards. When two 12 PART I Introduction to Hematology A B C D Figure 2-5 Removal of gloves. A, Using one hand, grasp the outside of the other glove and slowly pull it off the hand, turning it inside out as you remove it. B, Scrunch the removed glove into a ball. C, Place the index and middle finger of the ungloved hand on the inside of the other glove. D, Pull the second glove off of the hand, turning it inside out as it is removed and enclosing the balled-up glove. (From Bonewit-West K: Clinical procedures for medical assistants, ed 7, St Louis, 2008, Saunders.) regulations conflict it is recommended that the more stringent standard be 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 fire. 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 TABLE 2-1 Fire Extinguisher Classifications Class/Type of Extinguisher A B C ABC Type of Fire Use class A extinguishers on fires involving ordinary combustibles such as wood, cloth, or paper. Use class B extinguishers on fires involving flammable liquids, gases, or grease. Use class C extinguishers on energized (plugged-in) electrical fires. Examples are fires involving household appliances, computer equipment, fuse boxes, or circuit breakers. ABC extinguishers are multipurpose extinguishers that handle type A, B, and C fires. 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 workers should be trained to recognize the class of extinguisher and to 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 systems (alarms, 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. Workers 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 is by the state fire marshall. All laboratory staff members should participate in the fire drills. Proper documentation should be maintained to show that all phases of the fire response plan were activated. If patients are in the hemato logy 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. 8. Written storage requirements for any flammable or com bustible chemicals stored in the laboratory. Chemicals should be arranged according to hazard class and not alphabetically. 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 extinguishers and pull 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 work with facilities to conduct fire safety programs. 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 standard requires laboratories to develop a chemical hygiene plan that outlines safe work practices to minimize exposures to hazardous chemicals. The full text of this standard can be found in 29 CFR (Code of Federal Regulations) 1910.1450. 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 CHAPTER 2 Safety in the Hematology Laboratory 13 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 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. Material safety data sheets (MSDSs) are written by the manufacturers of chemicals to provide information on the chemicals that cannot be put on a label. When an MSDS is received in the laboratory, it must be retained and reviewed with laboratory personnel. The MSDS provides information on the following: a. Manufacturer—name, address, emergency phone number, date prepared b. Hazardous ingredients—common names, worker exposure limits c. Physical and chemical characteristics—boiling point, vapor pressure, evaporation rate, appearance, and odor under normal conditions d. Physical hazards—data on fire and explosion hazard and ways to handle the hazards 14 PART I Introduction to Hematology e. Reactivity—stability of the chemical and with what chemicals it will react f. Health hazards—signs and symptoms of exposure, such as eye irritation, nausea, dizziness, and headache g. Documentation on the MSDS of whether the reagent or a component of the reagent is considered a carcinogen, teratogen, or mutagen, so that process and procedures are adopted to limit risk of exposure h. Precautions for safe handling and use—what to do if the chemical spills, how to dispose of the chemical, and what equipment is required for cleanup i. Control measures—how to reduce the exposure to the chemical and protective clothing or equipment, such as respirator, gloves, and eye protection, that should be used in handling the chemical. See Appendix A for a sample MSDS. Use this sample to review the information that can be found on the MSDS. An MSDS management system should be considered to track the incoming MSDSs received in the laboratory. When new or revised MSDSs are received, a notice should be posted to alert the hematology staff that new or revised MSDSs have been received. MSDSs may be obtained electronically by means of computer, fax, Internet, or CD-ROM. If an electronic device is used in the laboratory to receive and store MSDSs, 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 MSDSs must be accessible to medical staff. MSDSs 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 is 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 threepronged 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. Needle Puncture Needle puncture is a serious occupational hazard for laboratory workers. Needle-handling procedures should be written and followed, with special attention to phlebotomy procedures and disposal of contaminated needles (see 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 workers when performing venipunctures or capillary punctures. Wall-mounted needle disposal con tainers also are available and make disposal convenient. As mentioned previously, all needle punctures should be reported to the health services or proper authorities within the institution. 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 CHAPTER 2 Safety in the Hematology Laboratory BOX 2-1 Government Regulatory Agencies Providing Laboratory Safety Standards • Department of Labor: 29 Code of Federal Regulations Parts 1900-1910 Hazard Communication Standard (right to know)—29 CFR 1910.1200 Occupational Exposure to Bloodborne Pathogens Standard—29 CFR 1910.1030 Occupational Exposure to Hazardous Chemicals in Laboratories Standard—29 CFR 1910.1450 Formaldehyde Standard—29 CFR 1910.1048 Air Contaminants: Permissible Exposure Limits—29 CFR 1910.1000 Occupational Noise Level Standard—29 CFR 1910.95 Hazardous Waste Operations and Emergency Response Standard—29 CFR 1910.120 Personal Protective Equipment—29 CFR 1910.132 Eye and Face Protection—29 CFR 1910.133 Respiratory Protection—29 CFR 1910.134 • • • 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. can be frustrating, 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 that explain the steps to be taken for all of the occupational and • • • • 15 environmental hazards that exist in the hematology laboratory. 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 (identification) assessment of all the potential safety, occupational, or environmental hazards that exist in the laboratory. The assessment 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 worker. 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. 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. Drills should address the potential accident or disaster before it occurs and test the preparedness of the hematology workers for an emergency situation. Planning for the accident and practicing the response to the accident reduces the panic that results when the correct response 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 workers' 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 worker knows the backup procedures and can implement them quickly during an emergency or disaster 16 PART I Introduction to Hematology situation. The emergency management plan should cover the four phases of response to an emergency, as follows: 1. Mitigation—measures to reduce the adverse effects of the emergency 2. Preparedness—design of procedures, identification of resources that may be used, and training in the procedures 3. Response—actions that will be taken when responding to the emergency 4. Recovery—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. The Clinical and Laboratory Standards Institute document "Planning for Challenges to Clinical Laboratory Operations During a Disaster" describes detailed actions to prepare for an emergency or disaster. The document also lists some valuable websites for additional resources.3 • 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—review of goals and performance as well as a review of the regulations to assess compliance in the laboratory. BOX 2-2 Emergency Management Activities: Planning for Response to a Fire 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 Preparedness Activities Training of workers Fire drills Fire response procedure development Annual and monthly fire extinguisher checks 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 workers SUMMARY • The responsibility of a medical laboratory professional is to perform analytic 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, which 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 handwashing. • Occupational hazards in the laboratory include fire, chemical, and electrical hazards and needle puncture. • Some common-sense 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 substance is low hazard and 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. R E V I E W Q UESTIONS 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 handwashing c. Wearing disposable laboratory coats d. Identifying specimens from known or suspected HIV- and HBV-infected patients with a red label CHAPTER 2 Safety in the Hematology Laboratory 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 an MSDS be helpful? a. A phlebotomist has experienced a needle puncture with a clean needle. b. A fire extinguisher failed during routine testing. c. A pregnant laboratory staff member 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. 17 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 gown. 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 runs 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. A class C fire extinguisher would be appropriate to use on a fire in a chemical cabinet. a. True b. False 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 REFERENCES 1. Garza D, Becan-McBride K: Phlebotomy handbook, ed 7, Upper Saddle River, NJ, 2005, Pearson Prentice Hall. 2. Department of Labor: 29 Code of Federal Regulations Parts 1900-1910. Federal Register, July 1, 1998. Available at: http:// www.access.gpo.gov/nara/cfr/waisidx_98/29cfrv5__98.html. Accessed June 5, 2010. 3. Hearn TL, Astles JR, Kaplan LA, et al: Planning for challenges to clinical laboratory operations during a disaster, a report. X4- R, vol 23, no 29. Available at: orders/free/x04rf.pdf. Accessed June 4, 2010. ADDITIONAL RESOURCES The Joint Commission Manual: Environment of Care Standards, 2009. National Fire Protection Association: Laboratories Using Chemicals, NFPA 45. Resource for occupational hazards found in the laboratory and other related regulations. Available at: SLTC/etools/hospital/lab/lab.html. Resource for four phases of emergency planning and up-to-date disaster information. Available at: . Resource for regulatory health guidelines. Available at: http:// www.osha.gov/SLTC/healthguidelines. National Institute for Occupational Safety and Health pocket guide for chemical hazards. Available at: npg. 3 Specimen Collection* Carole A. Mullins OUTLINE OBJECTIVES Safety Responsibility of the Phlebotomist in Infection Control Physiologic Factors Affecting Test Results Venipuncture After completion of this chapter, the reader will be able to: Collection Equipment for Venipuncture Routine Venipuncture Procedure Venipuncture Procedure in Children and Infants Complications Encountered in Blood Collection Skin Punctures Collection Sites Skin Puncture Technique Devices for Collecting Blood by Skin Puncture Skin Puncture Procedure Preparation of Blood Smears Quality Assurance in Specimen Collection Technical Competence Collection Procedures Anticoagulants and Preservatives Requirements for a Quality Specimen Blood Collection Attempts Collection of Specimens for Blood Culture Quality Control and Preventive Maintenance on Specimen Collection Instruments Reasons for Specimen Rejection 1. Describe the application of standard precautions to the collection of blood samples. 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 in the laboratory. 4. Discuss selection of a vein for venipuncture and name the vein that is preferred in most instances. 5. List in order the steps recommended by the Clinical and Laboratory Standards Institute for venipuncture in adults, including recommended order of collection for tubes with various additives. 6. Discuss complications encountered in blood collection and proper response of the phlebotomist. 7. Explain appropriate use of skin puncture equipment and procedure to be followed, including venipuncture sites for infants, children, and adults. 8. Discuss essentials of quality assurance in specimen collection. 9. List reasons for specimen rejection. 10. Given the description of a specimen and its collection, determine specimen acceptability. 11. Recognize deviations from the recommended venipuncture practice in a written scenario and describe corrective procedures. 12. Name the most important step in the venipuncture procedure. 13. List reasons for inability to obtain a blood specimen. 14. Summarize legal issues that need to be considered in specimen acquisition. 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 (K+) level, prothrombin time (PT), and type and screen. The phlebotomist draws blood into the following tubes in this order: 1. SST or serum separation tube 2. Light blue tube for PT 3. Lavender tube for CBC 4. Green tube for K+ Which of the results will be affected by the incorrect order of draw? Explain. Specimen Handling Legal Issues in Phlebotomy *The editors thank Dennis J. Ernst, MT(ASCP), director, Center for Phlebotomy Education, Inc., for his review of this chapter. 18 CHAPTER 3 Specimen Collection SAFETY Standard precautions must be followed in the collection of blood, and all specimens must be treated as potentially infectious for bloodborne pathogens (e.g., hepatitis B virus, hepatitis C virus, and human immunodeficiency virus [HIV]). Regulations of the Occupational Health and Safety Administration (OSHA) that took effect on March 6, 1992, outlined in detail what must be done to protect healthcare workers from exposure to bloodborne pathogens, such as the pathogens that cause hepatitis C, hepatitis B, hepatitis D, syphilis, malaria, and HIV infection.1 Bloodborne pathogens may enter the body via an accidental injury by a sharp object, such as a contaminated needle, a scalpel, broken glass, or anything else that can pierce the skin. Cuts, skin areas with dermatitis, abrasions, and mucous membranes of the mouth, eyes, and nose may 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 at room temperature for at least 1 week.2 Handwashing is the most important practice to prevent the spread of infectious diseases. The phlebotomist should wash his or her hands with a nonabrasive soap and running water between patients and every time gloves are removed. If handwashing facilities are unavailable, an antiseptic hand cleanser or an antiseptic towelette may be used as a temporary measure. Gloves are essential protective equipment and must be worn when venipunctures are performed. When gloves are removed, it is important that no substances from the soiled gloves 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 redorange biohazard sign (see 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 fingernails. Standard precautions must be followed at all times, with special attention to the use of gloves and handwashing. PHYSIOLOGIC FACTORS AFFECTING TEST RESULTS Certain physiologic factors specific to the patient may affect results of laboratory testing. These factors include posture 19 BOX 3-1 Physiologic Factors Affecting 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 cannot filter into the tissues and concentrate in the blood. There are significant increases in test values for lipids, enzymes, and proteins. Diurnal Rhythm Diurnal pertains to daylight, and diurnal rhythm refers to daily body fluid fluctuations that occur. Levels of certain hormones, such as cortisol and adrenocorticotropic hormone, decrease in the afternoon. Other test values, such as iron and eosinophil levels, increase in the afternoon. Exercise Muscle activity elevates creatinine, protein, creatine kinase, aspartate transaminase, and lactate dehydrogenase test values. Research also suggests that exercise activates coagulation and fibrinolysis and increases platelet and white blood cell counts.3 Stress Anxiety can cause a temporary increase in white blood cells.2 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. As a result, the serum or plasma may appear cloudy or turbid (lipemic), which interferes with testing, especially for tests such as glucose level, sodium level, and complete blood counts. Smoking Patients who smoke before blood collection may have increased white blood cell counts and cortisol levels. Long-term smoking can lead to decreased pulmonary function and result in increased hemoglobin levels. Skin puncture samples may be more difficult to obtain as a result of impaired circulation. (supine or erect), diurnal rhythms (day or night), exercise, stress, diet (fasting or not), and smoking (Box 3-1).3-5 It is important that the phlebotomist adhere to requests for specimens to be drawn at a specific time and record the time of collection. VENIPUNCTURE This chapter offers only an overview of specimen collection; texts that give detailed information are listed in the references. Collection 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 20 PART I Introduction to Hematology elastic strap, a heavier Velcro strap, or a blood pressure cuff. The tourniquet should be applied 2 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 a tube, which can be either plastic or glass; a needle; and an adapter, which is used to secure the needle and 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. All tubes come in various sizes and may contain a variety of premeasured additives. Although there are several manufacturers of evacuated tubes in the United States all 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 collection tube types. Additives in Collection Tubes Antiglycolytic agent. An antiglycolytic agent inhibits the use of glucose by blood cells. Such inhibition may be necessary if testing for glucose level is delayed. Examples of antiglycolytic agents are sodium fluoride and lithium iodoacetate. Tubes containing sodium fluoride alone yield serum. Sodium fluoride is combined with potassium oxalate or potassium ethylenediaminetetraacetic acid (K2EDTA), both anticoagulants, to yield plasma for more rapid testing. Anticoagulant agent. An anticoagulant prevents blood from clotting. The mechanism by which clotting is prevented varies with the anticoagulant. EDTA, citrate, and oxalate remove calcium by forming insoluble salts, whereas heparin prevents the conversion of prothrombin to thrombin. If calcium is removed or thrombin is not formed, coagulation does not occur. Examples of anticoagulants are EDTA, sodium citrate, and lithium or sodium heparin. Tubes must be inverted gently several times to ensure proper mixing immediately after collection, according to the manufacturer's instructions. Clot activator. A clot activator helps initiate or enhance the clotting mechanism. Clot activators include glass or silica particles that provide increased surface area for platelet activation and a clotting factor such as thrombin. Separator gel. Separator gel is an inert material that undergoes a temporary change in viscosity during the centrifugation process, which 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. evacuated tube holder or to be attached to the tips of syringes. Evacuated tube needles are double pointed and have a long, narrow, pointed end that punctures the patient's skin and is covered by a plastic cap, and a short beveled point at the other end that punctures the rubber stopper of the evacuated tube and is covered by a rubber sleeve. The rubber sleeve prevents blood from dripping into the holder when tubes are changed (Figure 3-2). On syringes, "single-sample" needles are used. The end of the needle that is inserted into the vein has a point with a slanted side (bevel), which must be facing up when the needle is inserted. Needle tips should be examined for burrs or bends before a venipuncture is performed. Gauge numbers are related inversely to the bore size: the smaller the gauge number, the larger the bore. Needle gauges for drawing blood range from 20 gauge to 25 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. Needle Holders Several new needles and holders have been designed to comply with the revised Occupational Exposure to Bloodborne Pathogens Standard (effective April 18, 2001) and its requirement for implementation of safer medical devices. These needle holders have safety features to prevent the possibility of needle sticks. Needle holders are made to fit a specific manufacturer's needles and tubes and, for best results, should not be interchanged. The holders are disposable and must be discarded after a single use with the needle still attached as per OSHA requirements.6 Examples of these needles and holders are the following: 1. The Vacutainer Eclipse Blood Collection System (Becton, Dickinson and Company, Franklin Lakes, N.J.) allows single-handed activation after the venipuncture is performed by pushing the safety shield forward with the thumb until an audible click is heard. The Becton Dickinson Eclipse needle should be used with a single-use tube holder. After the safety shield is activated, the entire assembly should be discarded intact into the sharps container. 2. The Jelco multisample blood collection needle used with the Venipuncture Needle-Pro Device (Smiths Medical ASD, Norwell, Mass.) 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 disposed of into the sharps container (Figure 3-3). 3. Greiner Bio-One (Monroe, N.C.) offers the VACUETTE QUICKSHIELD, which has a sheath that locks into place after use, and the QUICKSHIELD Complete PLUS, a system that incorporates a holder with the VACUETTE Visio PLUS multisample needle attached. The flash window in the needle hub indicates when a successful venipuncture has been achieved (Figure 3-4). Winged Infusion Sets (Butterflies) Needles Sterile needles come in a variety of lengths and gauges (bore or opening size). Needles are made to be screwed into the A butterfly is an intravenous device that consists of a short needle and a thin tube with attached plastic wings (Figure 3-5). The butterfly can be connected to evacuated tube holders, CHAPTER 3 Specimen Collection 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. ® BD Vacutainer Tubes with ™ BD Hemogard Closure ® BD Vacutainer Tubes with Conventional Stopper Gold Red/ Gray Light Green Green/ Gray Red Red Orange Gray/ Yellow Royal Blue Green Green Gray Gray Additive Inversions at Blood Collection* 5 For serum determinations in chemistry. May be used for routine blood donor screening and diagnostic testing of serum for infectious disease.** Tube inversions ensure mixing of clot activator with blood. Blood clotting time: 30 minutes. • Lithium heparin and gel for plasma separation 8 For plasma determinations in chemistry. Tube inversions ensure mixing of anticoagulant (heparin) with blood to prevent clotting. • Silicone coated (glass) • Clot activator, Silicone coated (plastic) 0 5 For serum determinations in chemistry. May be used for routine blood donor screening and diagnostic testing of serum for infectious disease.** Tube inversions ensure mixing of clot activator with blood. Blood clotting time: 60 minutes. • Thrombin 8 For stat serum determinations in chemistry. Tube inversions ensure mixing of clot activator (thrombin) with blood to activate clotting. • Clot activator (plastic serum) • K2EDTA (plastic) 8 For trace-element, toxicology, 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. • Sodium heparin • Lithium heparin 8 8 For plasma determinations in chemistry. Tube inversions ensure mixing of anticoagulant (heparin) with blood to prevent clotting. • Potassium oxalate/ sodium fluoride • Sodium fluoride/Na2 EDTA • Sodium fluoride (serum tube) 8 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. • K2EDTA (plastic) 8 For lead determinations. This tube is certified to contain less than .01 µg/mL(ppm) lead. Tube inversions prevent clotting. • Sodium polyanethol sulfonate (SPS) • Acid citrate dextrose additives (ACD): Solution A 22.0 g/L trisodium citrate, 8.0 g/L citric acid, 24.5 g/L dextrose Solution B 13.2 g/L trisodium citrate, 4.8 g/L citric acid, 14.7 g/L dextrose 8 SPS for blood culture specimen collections in microbiology. • Liquid K3EDTA (glass) • Spray-coated K2EDTA (plastic) 8 8 K2EDTA and K3EDTA for whole blood hematology determinations. K2EDTA may be used for routine immunohematology testing, and blood donor screening.*** Tube inversions ensure mixing of anticoagulant (EDTA) with blood to prevent clotting. • K2EDTA with gel 8 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. • Spray-coated K2EDTA (plastic) 8 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. 3-4 For coagulation determinations. CTAD for selected platelet function assays and routine coagulation determination. Tube inversions ensure mixing of anticoagulant (citrate) to prevent clotting. 8 8 8 Tan Yellow Lavender 8 ACD for use in blood bank studies, HLA phenotyping, and DNA and paternity testing. 8 Tube inversions ensure mixing of anticoagulant with blood to prevent clotting. White Pink Pink Light Blue Light Blue Laboratory Use • Clot activator and gel for serum separation • Buffered sodium citrate 0.105 M (≈3.2%) glass 0.109 M (3.2%) plastic • Citrate, theophylline, adenosine, dipyridamole (CTAD) 3-4 Your Lab's Draw Volume/Remarks Clear • None (plastic) New Clear 0 For use as a discard tube or secondary specimen tube. Red/ Light Gray ® Note: BD Vacutainer Tubes for pediatric and partial draw applications can be found on our website. BD Diagnostics Preanalytical Systems 1 Becton Drive Franklin Lakes, NJ 07417 USA BD Global Technical Services: 1.800.631.0174 [email protected] BD Customer Service: 1.888.237.2762 www.bd.com/vacutainer BD, BD Logo and all other trademarks are property of Becton, Dickinson and Company. © 2008 BD * Invert gently, do not shake ** The performance characteristics of these tubes have not been established for infectious disease testing in general; therefore, users must validate the use of these tubes for their specific assay-instrument/reagent system combinations and specimen storage conditions. *** The performance characteristics of these tubes have not been established for immunohematology testing in general; therefore, users must validate the use of these tubes for their specific assay-instrument/reagent system combinations and specimen storage conditions. Printed in USA Figure 3-1 Vacutainer tube guide. (Courtesy and © Becton, Dickinson and Company.) 08/08 VS5229-9 21 22 PART I Introduction to Hematology Figure 3-2 Multisample needle. The rubber sleeve prevents blood from dripping into the holder when tubes are changed. (Courtesy and © Becton, Dickinson and Company.) A 1 2 3 Figure 3-4 QUICKSHIELD Complete PLUS with flash window. Blood in the flash window indicates successful venipuncture. (Courtesy Greiner Bio-One GmbH.) B Figure 3-3 A, Jelco Needle-Pro. B, Use of Jelco Needle-Pro. (1) Attach needle. (2) Remove cap and draw blood from patient. (3) Press sheath on flat surface. (Courtesy Smiths Medical ASD, Norwell Mass.) syringes, or blood culture bottles with the use of special adapters. Butterflies are useful in collecting specimens from children or other patients from whom it is difficult to draw blood. Butterflies now come with resheathing devices to minimize the risk of needle stick injury (e.g., MONOJECT ANGEL WING Blood Collection [Covidien, Mansfield, Mass.], Vacutainer brand SAFETY-LOK and Vacutainer Push Button Blood Collection Set [Becton Dickinson], and VACUETTE Safety Blood Collection Set [Greiner Bio-One]). Syringes Syringes consist of a barrel that is graduated in milliliters and a plunger. Syringe needles have a point at one end and an open Figure 3-5 Butterfly Saf-T Wing. (Courtesy Smiths Medical ASD, Norwell, Mass.) CHAPTER 3 Specimen Collection Saf-T Holder Side clamp directs blood flow Sample syringe Luer 23 center and working outward. It is important to allow the area to air-dry before the venipuncture is performed so that the patient does not experience a burning sensation and contamination of the specimen is prevented. When a sterile site is prepared for collection of specimens for blood culture, a twostep procedure is used in which cleansing with isopropyl alcohol is followed by cleansing with iodine. Some healthcare facilities use a one-step application of chlorhexidine gluconate/ isopropyl alcohol. To avoid contamination when legal blood alcohol level is to be measured, benzalkonium chloride (Zephiran) or a nonalcohol antiseptic is used. Selection of a Vein for Routine Venipuncture A Å É Ç B Figure 3-6 A, Jelco closed blood collection system. (Courtesy Smiths Medical ASD, Norwell, Mass.) B, Device for transferring blood from syringe to vacuum tube. (1) Draw sample with syringe. (2) Close clamp. (3) Insert tube to transfer blood from sample syringe to tube. To fill additional tubes, open clamp, draw syringe again, close clamp, and transfer. (Courtesy Smiths Medical ASD, Norwell, Mass.) hub at the other end that attaches to the barrel. Syringes come 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. Syringes may also be used with winged infusion sets. If only one tube of blood is needed, the syringe needle is shielded, removed, and discarded in a sharps container, and a syringe blood transfer device (Becton Dickinson) or Saf-T Holder with male Luer adapter (Smiths Medical ASD) is attached to the syringe and a vacuum tube is inserted into the transfer device. The blood is transferred from the syringe into the tube using the tube's vacuum (Figure 3-6). Solutions for Skin Preparation The most common skin cleanser is 70% isopropyl alcohol. It can be applied using a commercially prepared alcohol pad or a cotton ball or piece of gauze soaked in alcohol. The site should be cleaned with a circular motion, beginning in the The superficial veins of the antecubital fossa (bend in the elbow) are the most common sites for venipuncture. The three primary veins that are used are (1) the cephalic vein, located on the upper forearm on the thumb side of the hand; (2) the basilic vein, located on the inside (medial) aspect of the antecubital fossa; and (3) the median cubital vein, which connects the basilic and cephalic veins in the antecubital fossa. The median cubital vein is the vein of choice (Figure 3-7). If necessary, the phlebotomist should have the patient make a fist after application of the tourniquet; the vein should become prominent. The patient should not do any pumping of 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 side of the wrist and 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 before a vein in one of these three alternate sites is used. Routine Venipuncture Procedure The phlebotomist should practice standard precautions, which include applying gloves and washing hands at the beginning of the procedure and removing gloves and washing hands at the end of the procedure. The following steps are recommended by the Clinical and Laboratory Standards Institute (CLSI)4: 1. Prepare the accession order. 2. Identify the patient by having the patient verbally state his or her name and confirm with one of the patient's unique identification numbers (i.e., medical records number, birth date, or Social Security number). 3. Verify that any dietary restrictions have been met (e.g., fasting, if appropriate) and check for any sensitivity to latex. 4. Assemble supplies. 5. Reassure the patient. 6. Position the patient. 7. Verify paperwork and tube selection. 8. If necessary, to help in locating the vein, ensure that the patient's hand is closed. 9. Select an appropriate venipuncture site, giving priority to the median cubital vein, and put on gloves. 24 PART I Introduction to Hematology Cephalic vein Median cubital Antecubital fossa Anterior Cephalic vein Posterior Basilic vein Basilic vein Figure 3-7 Veins of the forearm (two views). 10. Cleanse the venipuncture site with 70% isopropyl alcohol, working in concentric circles from the inside to outside. Allow skin to air-dry. 11. Apply the tourniquet 2 to 4 inches above the selected puncture site for no longer that 1 minute. 12. Inspect the needle and equipment. 13. Perform the venipuncture by anchoring the vein with the thumb 1 to 2 inches below the site and inserting the needle, bevel up, with a 15- to 30-degree angle between the needle and the skin. Collect tubes using the correct order of draw, and invert each tube containing any additive immediately after collection. A particular order of draw is recommended when drawing multiple specimens from a single venipuncture. Its purpose is to avoid possible test result error because of cross-contamination from tube additives. The recommended order of draw is as follows: a. Blood culture or sterile tubes (i.e., yellow stopper) b. Coagulation tube (i.e., light blue stopper) c. Serum tube with or without clot activator or gel (i.e., red, gold, or red-gray marbled stopper) d. Heparin tubes (i.e., green or light green stopper) e. EDTA tubes (i.e., lavender stopper) f. Oxalate/fluoride tubes (i.e., gray stopper) 14. Release and remove the tourniquet as soon as blood flow is established or after no longer than 1 minute. 15. Ensure that the patient's hand is open. 16. Place gauze lightly over the puncture site without pressing down. 17. After the last tube has been released from the back of the multisample needle, remove the needle and activate the safety device as per manufacturer's directions. 18. Apply direct pressure to the puncture site. 19. Bandage the venipuncture site after checking to ensure that bleeding has stopped. 20. If a syringe has been used, fill the tubes using a syringe transfer device. 21. Dispose of the puncture equipment and other biohazardous waste. 22. 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. 23. Carry out any special handling requirements (i.e., chilling or protecting from light). 24. Cancel any phlebotomy-related dietary restrictions and thank the patient. 25. 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 name, or someone must identify the patient for the phlebotomist. A hospitalized patient also must be identified by his or her identification bracelet. The patient's name and unique identification number must match the information on the test requisition. Any discrepancies must be resolved before the procedure can continue. Failure to confirm proper identification can result in a lifethreatening situation for the patient and possible legal ramifications for the facility. All tubes should be labeled immediately CHAPTER 3 Specimen Collection after the blood specimen has been drawn, with the label attached to the tube before the phlebotomist leaves the patient's side. Coagulation Testing If only a coagulation tube is to be drawn for determination of 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 into a nonadditive tube before collecting for routine coagulation testing. Tubes for coagulation testing must be filled to the correct level to maintain a 9 : 1 ratio of blood to anticoagulant to ensure accurate test results. Underfilling of tubes results in prolonged test values. When a butterfly is used to draw a single blue-topped tube, a nonadditive tube or another light blue tube must be used to clear the dead space in the tubing before collecting the tube to be used for testing. For special coagulation testing, however, the second or third tube drawn should be used.4 Venipuncture Procedure in Children and Infants Pediatric phlebotomy requires experience, special skills, and a tender touch. Excellent interpersonal skills are needed to deal with distraught parents and with crying, screaming, and scared 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. Frequently, smaller-gauge (23-gauge or 25-gauge) needles are employed. Use of syringes or butterflies may be advantageous with some infants' veins. No matter what equipment is used, the real secret to a successful venipuncture is a compassionate assistant. 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 Blood Collection Ecchymosis (Bruise) Bruising is the most common complication encountered in obtaining a blood specimen. It is caused by leakage of a small amount of fluid around the tissue. The phlebotomist can prevent bruising by applying direct pressure to the venipuncture site, instead of having the patient bend the arm at the elbow. Syncope (Fainting) Fainting is the second most common complication encountered. Before drawing blood, the collector always should ask the patient whether he or she has had any prior episodes of fainting during or after blood collection. The CLSI recommends that ammonia inhalants no longer be used because they may trigger an asthma attack or a sudden, exaggerated response that could lead to patient injury. The phlebotomist should follow the protocol at his or her facility. 25 If the patient begins to faint, the phlebotomist should remove the needle immediately, lower the patient's head, and apply cold compresses to the back of the patient's neck and loosen any constrictive clothing. The patient should take some deep breaths and be offered some orange juice or cold water to drink. The patient should sit for at least 30 minutes before leaving. The incident always should be documented. Hematoma A hematoma results when leakage of a large amount of fluid around the puncture site causes the area to swell. If swelling begins, the needle should be removed immediately and pressure applied to the site for at least 2 minutes. Hematomas may result in bruising of the patient's skin around the puncture site if adequate pressure is not maintained. Blood that leaks out of the vein under the patient's skin may clot and result in nerve compression and permanent damage to the patient's arm. A hematoma most commonly occurs when the needle goes through the vein, when the bevel of the needle is only partially in the vein, and when the phlebotomist fails to apply enough pressure after venipuncture. 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 slanted side (bevel) up, at an angle of 15 to 30 degrees. Figure 3-8 shows reasons for unsatisfactory flow of blood. It is sometimes possible to enter the vein by redirecting the needle, but only an experienced phlebotomist should attempt this, because such manipulation can cause discomfort and 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 recollection when the problem is a technical issue associated with the tubes (i.e., inadequate vacuum). Petechiae Petechiae are small red spots indicating that small amounts of blood have escaped into the skin epithelium. Petechiae indicate a possible coagulation problem and should alert the phlebotomist to be aware of possible prolonged bleeding. 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 the 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 26 PART I Introduction to Hematology Vein Skin A Correct needle position Vein D Skin B Skin Bevel resting on vein wall Hematoma Needle inserted through vein Skin E Vein Veins Needle too near vein valve Hematoma Skin C Partial needle insertion Skin F Vein Vein Collapsed vein Figure 3-8 Proper and improper needle insertion for venipuncture. than the patient's diastolic pressure and should not be left on the arm for longer than 1 minute. It is not advisable to probe blindly in the patient's arm because muscle or nerve damage may result. Intravenous Therapy Drawing blood from an arm with an intravenous catheter should be avoided if possible. The arm opposite the arm with the intravenous line should be used. If there is no alternative, blood should be drawn below the catheter with the tourniquet placed below the catheter site. It is preferable to have the nurse stop the infusion for 2 minutes before the specimen is drawn. The CLSI recommends that 5 mL of blood be drawn for discard before samples to be used for testing are obtained. It is important to note on the requisition and the tube that the specimen was obtained from an arm into which an intravenous solution was running.2,4 The phlebotomist always should follow the protocol established at his or her facility. tube too hard; or if contamination by alcohol or water occurs at the venipuncture site or in the tubes. Hemolysis also can occur physiologically as a result of hemolytic anemias or severe renal problems. Testing hemolyzed specimens can alter test results, such as levels of potassium and enzymes, which can have a negative impact on patient outcome. 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. Seizures and Tremors Patients occasionally experience seizures because of a preexisting condition or as a response to the needle stick. If a seizure occurs, the needle should be removed immediately. The patient's safety should be ensured by preventing injury from nearby objects. Hemoconcentration Vomiting and Choking Hemoconcentration is an increased concentration of 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. It is recommended that the tourniquet not remain on for longer than 1 minute before venipuncture. 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.5 If the patient begins vomiting, the patient's head must be positioned so that he or she does not aspirate any vomit. During vomiting or choking, the phlebotomist should prevent the patient from hitting his or her head. Hemolysis The rupture of red blood cells (RBCs) 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 too small a needle was used during a difficult draw; if the phlebotomist pulls back too quickly on the plunger of a syringe, forces blood into a tube from a syringe, or shakes a Allergies Some patients may be allergic to skin antiseptic substances other than alcohol. Adhesive bandages and tape also may cause an allergic reaction. Hypoallergenic tape should be used or pressure applied manually until the bleeding has stopped completely. Sensitivity to latex should be determined before any phlebotomy procedure. Mastectomy Patients The CLSI requires physician permission before blood is drawn from the same side as a prior mastectomy (removal of the CHAPTER 3 Specimen Collection breast), even in the case of bilateral mastectomies. 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 should be used whenever possible. 27 Puncture across fingerprints Inability to Obtain a Blood Specimen Each institution should have a policy covering 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 caregiver with blood collection expertise. Another individual can make two attempts to obtain a specimen. If a second person is unsuccessful, the physician should be notified. 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 try to 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 all right to proceed. Any refusals or problems should be documented for legal reasons. 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 PUNCTURES Skin punctures often are performed in newborns; in pediatric patients younger than 1 year; in adults who are severely burned and whose veins are being reserved for therapeutic purposes; and in elderly patients with fragile veins. When peripheral circulation is poor, however, accurate results may not be obtained with specimens acquired by skin puncture. Capillary blood is actually a mixture of venous blood, arterial blood, and tissue fluid. When the puncture site is warmed, the specimen more closely resembles arterial blood. Because capillary specimens may generate slightly different test results, a notation should be made when the specimen is obtained by skin puncture.7 White blood cell counts in specimens obtained by skin puncture may be 15% to 20% higher than the counts in venous specimens.8 Clinically significantly higher glucose values are found in specimens obtained by skin puncture compared with those obtained by venipuncture.7 This is especially important to note when a glucose tolerance test is performed or when glucometer results are compared with findings from venous samples. Collection Sites In most patients, skin punctures may be performed on the heel or finger. In infants, the finger should not be punctured because Lateral side of heel Medial side of heel Posterior tibial artery A B Figure 3-9 Areas for skin puncture specimens: heel (A) and finger (B). the lancets could cause serious injury to the bones of the fingers. The site of choice in infants is the lateral (outside) or medial (inside) surface of the plantar side (bottom) of the heel, although there have been some problems with puncturing the posterior tibial artery when the medial heel surface is used (Figure 3-9, A). In older children and adults, the palmar surface of the distal portion of the third (middle) or fourth (ring) finger on the nondominant hand may be used.7 The puncture on the finger should be made perpendicular to the fingerprint lines when a puncture device with a blade is used (Figure 3-9, B). Warming can increase the blood flow sevenfold. The site can be warmed with a warm washcloth or a commercial heel warmer. The site should be warmed to a temperature no greater than 42° C for no longer than 2 to 5 minutes, unless the collection is for capillary blood gas analysis. The skin puncture site should be cleansed with 70% isopropyl alcohol and allowed to air-dry. Povidone-iodine should not be used because of possible blood contamination, which would produce falsely elevated levels of potassium, phosphorus, or uric acid. Skin Puncture Technique The finger or heel must be securely immobilized. Punctures should not be made more than 2 mm deep because of the risk of bone injury and possible infection (osteomyelitis). For heel punctures on premature infants, it is advisable to use a puncture device with even less depth. Most devices on the market for performing skin punctures come in varying depths. The use of plastic tubes or Mylar-coated glass tubes is recommended by OSHA to avoid broken glass and exposure to biohazardous materials. The phlebotomist should not puncture an area that is swollen or bruised or already has been punctured. The first drop of blood should be wiped away to prevent contamination of the specimen with tissue fluid and to facilitate the free flow of blood.7 28 PART I Introduction to Hematology Figure 3-10 Examples of equipment used for collection of skin puncture specimens. (Courtesy Dennis J. Ernst, MT[ASCP], Director, Center for Phlebotomy Education, Inc.) Devices for Collecting Blood from Skin Puncture Devices for collecting blood from skin puncture include capillary tubes and microcollection tubes.7 Capillary tubes of various sizes are available with or without heparin added. Microcollection tubes are available with or without additives, and the cap colors on the tubes correspond with the colors on vacuum tubes. The order of drawing is different for microcollection tubes. 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. Other tubes containing anticoagulants should be collected next, followed by serum tubes.5 Labeling for capillary specimens should contain the same information as for vacuum tubes. Examples of skin puncture equipment are shown in Figure 3-10. Skin Puncture Procedure Standard precautions, which include applying gloves and washing hands at the beginning of the procedure and removing gloves and washing hands at the end of the procedure, should be implemented. The following steps are recommended by the CLSI7: 1. Obtain and examine the requisition form. 2. Assemble equipment and supplies. 3. Greet the patient (and parents); identify the patient by having the patient verbally state his or her name and confirm with patient's identification number (i.e., medical records number, birth date, or Social Security number). 4. Verify that any dietary restrictions have been met (e.g., fasting), and check for any sensitivity to latex. 5. Position the patient and the parents or designated holder as necessary. 6. Put on gloves. 7. Organize equipment and supplies. 8. Select the puncture site. 9. Warm the puncture site. Warming increases the blood flow sevenfold. Use a commercial heel warmer or warm washcloth (40° C to 42° C) for 2 to 5 minutes. 10. Cleanse the puncture site with 70% isopropyl alcohol using concentric circles, working from the inside to outside. Allow skin to air-dry. 11. Perform the puncture. Puncture depth should not exceed 2 mm. 12. Wipe away the first drop of blood. This removes any residual alcohol and any tissue fluid contamination. 13. Make blood smears if requested. 14. Collect the specimens and mix as needed. If an insufficient sample has been obtained because the blood flow has stopped, repeat the puncture at a different site with all new equipment. Order of collection of specimens is as follows: a. Tube for blood gas analysis b. Slides, unless made from sample in the EDTA micro collection tube c. EDTA microcollection tube d. Other microcollection tubes with anticoagulants (i.e., green or gray) e. Serum microcollection tubes 15. Elevate the puncture site and apply pressure until bleeding has stopped. 16. Label the specimens with the required information. NOTE: Compare the labeled tube with the identification bracelet for inpatients; have outpatients verify that the information on the labeled tube is correct, whenever possible. 17. Handle the specimen appropriately. 18. Thank the patient and parents. 19. Dispose of all puncture equipment and biohazardous materials. 20. Complete paperwork and indicate "skin puncture collection." 21. Deliver the properly labeled specimens to the laboratory. PREPARATION OF BLOOD SMEARS Blood smears can be made directly from capillary blood or from venous blood by the wedge or coverslip method. In either method, the phlebotomist must remember to wipe away the first drop of blood and use the second drop to make the smear, if blood from a finger or heel stick is used (see Chapter 15). 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 in specimen collection. Technical Competence The individual performing phlebotomy should be trained properly in all phases of blood collection. Certification is recommended. Continuing education is encouraged 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. Proper patient preparation and correct patient identification are crucial. The correct tube or specimen container must be used. Anticoagulants and Preservatives The manufacturer's instructions must be followed with regard to mixing of all tubes with additives to ensure accurate test results and prevent formation of microclots in the tubes. All tubes should be checked for cracks and expiration dates. The additives should be observed for 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 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 preservatives 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 Blood Collection Attempts One individual should not attempt to obtain a specimen successfully from a given patient more than twice. If two individuals have each tried twice without success, the physician should be contacted. There should be written procedures for what to do when the patient is unavailable for a blood draw or when the patient refuses a draw. Collection of Specimens for Blood Culture Each facility should monitor its blood culture contamination rate and keep that rate lower than 3% as recommended by the American Association of Microbiology.9-11 Failure to do so could indicate a problem in the quality of all procedures being performed. Quality Control and Preventive Maintenance for Specimen Collection Instruments 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. If bleeding times are to be measured, the blood CHAPTER 3 Specimen Collection 29 BOX 3-2 Reasons for Specimen Rejection • The test order requisition and the tube identification do not match. • The tube is unlabeled, or the labeling, including patient identification number, is incorrect. • The specimen is hemolyzed. • The specimen was collected at the wrong time. • The specimen was collected in the wrong tube. • The specimen was clotted, and the test requires whole blood. • The specimen was contaminated with intravenous fluid. • The 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. pressure cuff should be checked for leaks and accuracy. 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 specimen provided. At times it may be suspected that a specimen will not yield accurate results and must be rejected. Box 3-2 lists reasons for specimen rejection. SPECIMEN HANDLING Proper handling of specimens begins with the initiation of the test request and ends when the specimen is finally tested. Accurate test results depend on what happens to the specimen during that time. This pretesting period is referred to as the preanalytical phase of the total testing process. Routine specimens must be adequately inverted to mix the additive and blood. 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 result in hemolysis. Exposure to light can cause falsely decreased values in tests measuring parameters such as bilirubin, carotene, RBC folate, and urine porphyrin levels. For certain tests, the specimens need to be chilled, not frozen, and should be placed in an icewater bath to slow down cellular metabolism. These tests include blood gas analysis, ammonia concentration, lactic acid level, and certain coagulation tests. Other tests require that specimens be kept warm to ensure accurate results. The cold agglutinin test is one such test; if the specimen is refrigerated before the serum is removed, the antibody is reabsorbed onto the RBCs. Most specimens for routine testing should be delivered to the laboratory within 45 minutes to 1 hour of collection for processing. To ensure accurate results, less time is recommended for tests such as those measuring glucose, potassium, cortisol, and some enzymes. The CLSI recommends that the maximum time limit for separating serum and plasma from cells be 2 hours from the time of collection. 30 PART I Introduction to Hematology LEGAL ISSUES IN PHLEBOTOMY There are many daily practices in healthcare 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 never 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. No antiseptics containing alcohol 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. SUMMARY • Laboratory test results are only as good as the specimen tested. • Standard precautions must be followed in the collection of blood to prevent exposure to bloodborne pathogens. • Physiologic factors affecting test results include posture, diurnal rhythm, exercise, stress, diet, and smoking. • Although there are several U.S. manufacturers of evacuated tubes, all 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. • The three primary veins used for phlebotomy are the cephalic, basilic, and medial veins. • CLSI guidelines should be followed for venipuncture and skin puncture. • Common complications of blood collection include bruising, fainting, and hematoma. • 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. R E V I E W Q UESTIONS 1. The vein of choice for performing a venipuncture is the: a. Basilic b. Cephalic c. Median cubital d. Femoral 2. 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 3. 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 4. The needle should be inserted into the arm with the bevel facing: a. Down b. Up c. To either side d. Makes no difference 5. What is the proper angle of needle insertion for phlebotomy? a. 5 degrees b. 15 to 30 degrees c. 35 degrees d. 45 degrees 6. What is the recommended order of drawing 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 7. Acceptable sites for skin puncture on infants are: a. Middle of the heel b. Lateral or medial surface of the bottom of the heel c. Inside of the heel, close to the arch of the foot, and any of the toes, close to the tip d. Middle of the bottom of the heel CHAPTER 3 Specimen Collection 8. 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 9. You are evaluating a new phlebotomist on his venipuncture performance. He completed the following steps in the order listed: • Asked the outpatient his name and date of birth and compared those with the requisition • Applied a tourniquet and selected a prominent vein in the center of the antecubital fossa • Released the tourniquet, collected needed equipment, and assembled it all • Cleansed the venipuncture site and allowed it to dry • Applied the tourniquet, unsheathed the needle, stretched the skin below the proposed venipuncture site, and inserted the needle into the selected vein within the cleansed area • Pushed the tube onto the holder while holding the needle still, withdrew the tube from the holder, removed the needle from the arm, and engaged the safety device • Immediately applied a gauze pad to the site and released the tourniquet • Applied pressure to the site while gently mixing the specimen • Verified that the patient was not bleeding and applied a bandage • Labeled the tube appropriately 31 What is your assessment of the phlebotomist's technique? a. All steps were performed acceptably in the proper order. b. The phlebotomist should have labeled the tubes before collecting the specimen. c. The phlebotomist should have cleansed the arm while the tourniquet was in place. d. The phlebotomist should have removed the tourniquet before removing the needle from the arm. 10. For a complete blood count (hematology) and measurement of prothrombin time (coagulation), the phle botomist collected blood into a lavender-topped and a green-topped tube. 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. 11. 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 REFERENCES 1. Rules and regulations: bloodborne pathogens. Fed Reg 56:64175-64182, 1991. 2. McCall RE, Tankersley CM: Phlebotomy essentials, ed 4, Philadelphia, 2008, Lippincott Williams & Wilkins. 3. Christensen RD, Hill HR: Exercise-induced changes in the blood concentration of leukocyte populations in teenage athletes. Am J Pediatr Hematol Oncol 9:140-142, 1987. 4. Clinical and Laboratory Standards Institute: Procedures for the collection of diagnostic blood specimens by venipuncture; approved standard, ed 6, CLSI document H3-A6, Wayne, Pa, 2007, Clinical and Laboratory Standards Institute. 5. Garza D, Becan-McBride K: Phlebotomy handbook, ed 7, Upper Saddle River, NJ, 2005, Pearson Prentice Hall. 6. 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Hall KK, Lyman JA: Updated review of blood culture contamination. Clin Microbiol Rev 19:788-802, 2006. ADDITIONAL RESOURCES Center for Phlebotomy Education, . BD Vacutainers (Becton Dickinson). Available at: . com/vacutainer/. Lab Tests Online, . 4 Care and Use of the Microscope Bernadette F. Rodak OUTLINE OBJECTIVES Principles of Microscopy Component Parts and Function of Each Part Operating Procedure with Koehler Illumination After completion of this chapter, the reader will be able to: Considerations Immersion Oil and Types Care of the Microscope Basic Troubleshooting Microlocator Slide Other Microscopes Used in the Clinical Laboratory Phase-Contrast Microscope Polarized Light Microscope Darkfield Microscope 1. Given either a diagram or an actual brightfield light microscope, identify the component parts. 2. Explain the function of each component of a brightfield light microscope. 3. Define achromatic, planachromatic, 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 adjusting microscope light using a procedure such as Koehler illumination. 5. List in proper order the steps for adjusting a brightfield light microscope using Koehler illumination. 6. Using the procedure provided in the text and a brightfield light microscope with appropriate components, properly adjust a brightfield light microscope by the use of Koehler illumination. 7. 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. 8. Using the procedure described in the text and a brightfield light microscope with appropriate lenses, focus a stained blood film with dry and oil immersion objectives. 9. Describe the proper care and cleaning of microscopes and recognize deviations from these procedures. 10. Using the procedures described in the text and a microscope, properly clean the microscope after routine use. 11. Given the magnification of lenses in a compound microscope, calculate the total magnification. 12. Given a problem with focusing a blood film using a brightfield light microscope, suggest possible causes and their correction. 13. 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 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 M icroscopes available today reflect improvement in every aspect from the first microscope of Anton van Leeuwenhoek (1632-1723).1 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 32 objective. What steps should be taken to identify and correct this problem? 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. PRINCIPLES OF MICROSCOPY In the compound microscope, an intermediate image of the illuminated specimen is formed by the objective lens in the CHAPTER 4 Care and Use of the Microscope Retina 33 standard microscopes, the brightfield illumination system, which passes light directly through the transparent specimen, is used. COMPONENT PARTS AND FUNCTION OF EACH PART Lens of eye Eyepiece Image formed by objective 250 mm (10 inches) Objective lens Specimen Condenser Virtual image Figure 4-1 Compound microscope. (From Abramowitz M: The microscope and beyond, vol 1, Lake Success, NY, 1985, Olympus Corp, p 2. Reprinted courtesy Eastman Kodak Company, Rochester, NY.) optical tube. This image is then magnified and viewed through the oculars (Figure 4-1). 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. The compound microscope employs two separate lens systems, the product of which produces the final magnification. In Component parts and the function of each part of the microscope are summarized as follows (Figure 4-2): 1. The oculars, or eyepieces, usually are equipped with 10× lenses (degree of magnification is 10×). The lenses magnify the intermediate image formed by the objective lens in the optical tube; they also limit the area of visibility. Microscopes may have either one or two adjustable oculars. All oculars should be used correctly for optimal focus (see section on operating procedure). Oculars should not be interchanged with the oculars of other models of microscopes. The oculars in a pair are optically matched. 2. The interpupillary control is used to adjust the lateral separation of the oculars 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 oculars 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 (oculars) 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 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 ocular by the magnification of the objective lens; for example, 10× (ocular) multiplied by 100× (oil immersion) is 1000× total magnification. Microscopes employed in the clinical laboratory are used with achromatic or planachromatic objective lenses, 34 PART I Introduction to Hematology 1. Eyepieces or oculars 2. Interpupillary control 3. Optical tube 1. Eyepieces or oculars 2. Interpupillary control 3. Optical tube 4. Neck/arm 6. Revolving nosepiece 7. Objective lens 8. Stage 10. Condenser 11. Aperture diaphragm control lever 12. Stage controls 9. Focus controls 13. Field diaphragm 14. Light source 5. Stand Figure 4-2 Components of a microscope. (Courtesy Commercial Imaging & Design, Inc., Royal Oak, Mich.) NA 1.25 Magnification 100 Tube length 160 Figure 4-3 Microscope objective lens. NA, Numerical aperture. (Courtesy Commercial Imaging & Design, Inc., Royal Oak, Mich.) 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 planachromatic lens, which is more expensive, also corrects for curvature of the field, which results in a flat field with uniform focus.2 Planachromatic lenses sometimes are referred to as flat field lenses. For critical microscopy, a planapochromatic lens, which brings light of three colors into focus and almost completely corrects for chromatic aberration, may be used. This type of objective lens is fairly 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).3 If the swing-out lens is above CHAPTER 4 Care and Use of the Microscope 35 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 (1) a control lever for a swing-out lens, (2) an aperture diaphragm control lever, (3) a control for vertical adjustment of the condenser, and (4) a condenser aperture diaphragm. 11. The control lever swings the condenser top lens out of position. 12. The stage controls located under the stage move it along an x- or a y-axis. 13. 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 is focused at the condenser aperture diaphragm. The end result of Koehler illumination is a field of evenly distributed brightness across the specimen. 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.4,5 The light control knob turns on the light and should be used to regulate the brightness of the light needed to visualize the specimen. The aperture diaphragm should never be used for this purpose, because closing it reduces resolving ability.3 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 using the microscope: 1. Connect the microscope to the power supply. 2. Turn on the light source. 3. Open all diaphragms. 4. Revolve the nosepiece until the 10× objective lens is directly above the stage. 5. Adjust the interpupillary control so that looking through both oculars yields one clear image. 36 PART I Introduction to Hematology 2. Aperture diaphragm control lever 4. Condenser diaphragm 1. Swing-out lens 3. Vertical adjustment of condenser Figure 4-4 Condenser. (Courtesy Commercial Imaging & Design, Inc., Royal Oak, Mich.) 6. Place a stained blood film on the stage and focus on it, using the fixed ocular, 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 ocular.) 7. Using the adjustable ocular and covering the opposite eye, focus on the specimen. Start with the ocular all the way out, and adjust inward. If using two adjustable oculars, 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 oculars. 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 condenser so that you see a sharp image of the field diaphragm, ringed by a magenta halo. If the substage 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 the image is nearly at the edge of the field, and fine-tune the centering process. 12. Open the diaphragm slightly until the image just disappears. 13. Remove one ocular and, while looking through the microscope (without the ocular), close the condenser diaphragm completely. Reopen the condenser diaphragm until the leaves just disappear from view. Replace the ocular. 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. Parcentric 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× CHAPTER 4 Care and Use of the Microscope objective lenses are used, increase the intensity of the light by adjusting only the light control knob 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.3 4. Do not change the position of the condenser or the aperture lever to regulate light intensity. The condenser should always be in its upward position. The aperture lever is used only to achieve proper contrast of the features of the specimen being viewed. 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.5 CARE OF THE MICROSCOPE Care of the microscope involves the following details: 1. When not in use for an extended period of time, the microscope always should be covered or protected 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.6 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. 37 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. Your condensed breath on the lens surface may be useful in cleaning slightly soiled lenses. 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 planachromatic, the following checklist can aid in identifying the problem: • Oculars 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 smear? Only one coverslip on slide? Free of mounting media? • Light source Fingerprints on bulb? Bulb in need of changing? Light source aligned correctly? MICROLOCATOR SLIDE Some microscope stages include locator values for x and y on the stage, which allows the operator to take coordinates of a cell so that it can be located easily for review. When locator values are not incorporated into the stage, a microlocator slide or microslide field finder can be used. The field finder slide is a commercially manufactured standard glass slide with a precise etched coordinate grid running along the x and y axes. Figure 4-5 shows one example of such a slide. 1. When a cell of interest is located on a prepared slide, the cell should be centered under a high dry or oil objective and 38 PART I Introduction to Hematology 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 Figure 4-5 Microlocator slide. then under the 10× objective lens. Note whether the feathered edge of the blood film faces right or left. 2. Carefully remove the slide from the spring assembly, taking care not to disturb the position of the stage. 3. Place the microlocator slide onto the stage. 4. View the microlocator through the ocular and record the letter-number combination. 5. To relocate the cell, place the microlocator onto the stage and place the recorded letter-number combination in the center of the stage. 6. Remove the locator slide carefully and replace it with the initial blood film. The cell of interest should be in the field. 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 un obstructed. 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 ( ). Polarized light microscopy is another contrast-enhancing technique used to identify substances such as crystals in urine and other body fluids (see Chapter 17). With brightfield micro scopy, 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 ocular and allows only light vibrating in a north-south direction to pass to the ocular. When the transmission axes of these two filters are oriented at right angles, no light can pass through the pair to the ocular. 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 northsouth direction can pass through the second filter (the analyzer) and is visible at the ocular. 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 (see Figures 17-20 and 17-21). Darkfield Microscope Darkfield microscopy is a contrast-enhancing technique that employs a special condenser. The condenser sends light up toward the specimen in a hollow cone. Because of the high angle of this cone, none of the illuminating rays enter 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. When combined with the use of fluorochrome dyes, darkfield fluorescence microscopy can be used to identify lymphocyte subsets. CHAPTER 4 Care and Use of the Microscope 39 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 oculars. • The NA, which is engraved on the objective lenses, designates the light-gathering ability of the lens. The higher the NA, the greater the resolution. • Achromatic lenses maintain the center of the field in focus, whereas planachromatic lenses also correct for the curvature of a field, providing uniform focus. • The condenser gathers the light and directs it across a specimen. • Koehler illumination establishes a field of evenly distributed brightness across the specimen. • 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 lenses to cancel the light passing through the sample. If the object is able to polarize light, as are some crystals, the light passing through is rotated and becomes visible. • Darkfield microscopes use condensers that send light to the sample a high angle, directing the light away from the objective lens. If the sample 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. R E V I E W Q UESTIONS 1. Use of which of the following type of objective lens causes the center of the microscope field to be in focus, whereas the periphery is blurred? a. Planachromatic b. Achromatic c. Planapochromatic d. Flat field 2. Which of the following gathers, organizes, and directs light through the specimen? a. Ocular 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. Parcentric c. Compensated d. Parachromatic 4. Which objective has the greatest degree of color correction? a. Achromatic b. Planapochromatic c. Bichromatic d. Planachromatic 5. The total magnification obtained when a 10× ocular and a 10× objective lens are used is: a. 1× b. 10× c. 100× d. 1000× 6. After a microscope has been adjusted for Koehler illu mination, light intensity should never be regulated by using the: a. Rheostat b. Neutral density filter c. Koehler magnifier d. Condenser 7. The recommended cleaner for removing oil from objectives is: a. 70% alcohol or lens cleaner b. Xylene c. Water d. Benzene 8. Which of the following types of microscopy is valuable in the identification of crystals that are able to rotate light? a. Compound brightfield b. Darkfield c. Polarizing d. Phase-contrast 40 PART I Introduction to Hematology 9. A laboratory science student has been reviewing a hematology 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. 10. 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 sample so that it misses the objective unless something in the sample 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 sample and collecting light reflected from the sample, rather than transmitted through the sample, so that when there is no sample in place, the field is dark REFERENCES 1. Asimov I: Understanding physics: light, magnetism, and electricity, London, 1966, George Allen & Unwin. 2. Microscope terms. Available at: microscope-terms-t-5.html. Accessed June 2, 2010. 3. Olympus Microscopy Resource Center: Anatomy of the Microscope—Köhler Illumination. Available at: . olympusmicro.com/primer/anatomy/kohler.html. Accessed June 2, 2010. 4. Microscope Illumination. Olympus Microscope primer. Available at: illumination.html. 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Fritsma OUTLINE OBJECTIVES Validation of a New or Modified Assay After completion of this chapter, the reader will be able to: Accuracy Precision Linearity Limits 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 1. Validate and document a new or modified laboratory assay. 2. Define accuracy and calculate accuracy using linear regression to compare to a reference. 3. Compute precision using standard deviation and coefficient of variation. 4. Determine assay linearity using graphical representations and transformations. 5. Discuss analytical limits and analytical specificity. 6. Recount Food and Drug Administration clearance levels for laboratory assays. 7. Compute a reference interval and a therapeutic range for a new or modified assay. 8. Perform internal quality control using controls and moving averages. 9. Participate in periodic external quality assessment. 10. Measure and publish assay clinical efficacy. 11. Compute receiver operating characteristic curves. 12. Periodically assess laboratory staff competence. 13. Provide continuing education programs for laboratory staff. 14. Prepare a quality assurance plan to control for preanalytical and postanalytical variables. 15. List the agencies that regulate hematology and hemostasis quality. Controls Moving Average ( X B ) of the Red Blood Cell Indices Delta Checks CASE STUDY After studying the material in this chapter, the reader should be able to respond to the following case study: External Quality Assessment Measurement of Clinical Efficacy Receiver Operating Characteristic Curve Assay Feasibility Laboratory Staff Competence On an 8:00 AM assay run, the results for three levels of a preserved hemoglobin control sample are 2 g/dL higher than the upper limit of the target interval. The medical laboratory scientist reviews δ-check data on the last 10 patient results 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 samples 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? Proficiency Systems Continuing Education Quality Assurance Plan: Preanalytical and Postanalytical Agencies that Address Hematology and Hemostasis Quality 41 42 PART I Introduction to Hematology BOX 5-1 Examples of Components of Quality Assurance • Preanalytical variables: selection of assay relative to patient need; implementation of assay selection; patient identification and preparation; specimen collection technique; specimen collection apparatus used; specimen transport, preparation, and storage; monitoring of specimen condition • 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 • 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 I n clinical laboratory science, quality implies the ability to provide accurate, reproducible assay results that offer clinically useful information.1 Because physicians base 70% of their clinical decision making on laboratory results, the results must be reliable. Reliability requires vigilance and effort on the part of all laboratory staff members, and this effort is often directed by an experienced medical laboratory scientist who is a quality assurance and quality control specialist. Of the terms quality control and quality assurance, quality assurance is the broader concept, encompassing preanalytical, analytical, and postanalytical variables (Box 5-1).2 Quality control processes document assay validity, accuracy, and precision, and should include external quality assessment, reference interval preparation and publication, and lot-to-lot validation.3 Preanalytical variables are addressed in Chapter 3, which discusses blood specimen collection, and in Chapter 45, which includes a section on coagulation specimen management. Postanalytical variables are discussed at the end of this chapter. The control of analytical variables begins with laboratory assay validation. VALIDATION OF A NEW OR MODIFIED ASSAY All new laboratory assays and all assay modifications require validation.4 Validation is comprised of procedures to determine accuracy, specificity, precision, limits, and linearity.5 The results of these procedures are faithfully recorded and made available to on-site assessors upon request.6 Accuracy Accuracy is the measure of concurrence or difference between an assay value and the theoretical "true value" of an analyte (Figure 5-1). Some statisticians prefer to define accuracy as the extent of error between the assay result and the true value. Accuracy is easy to define but difficult to establish and maintain. For many analytes, laboratory scientists employ primary standards to standardize assays and establish accuracy. A primary standard is a material of known, fixed composition that is prepared in pure form, often by weighing on an analytical balance. The scientist dissolves the weighed standard in an aqueous solution, prepares suitable dilutions, calculates the anticipated concentration in each dilution, and assigns the calculated concentrations to assay outcomes. For example, the scientist 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 scientist 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 re-assayed several times to generate means for each concentration. The assay is then employed on human 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 scientist 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 delivered at a certified known concentration. The scientist 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 sample's matrix, for instance, plasma to plasma, whole blood to whole blood. Neither primary nor secondary standards are assayed during routine patient sample testing, only during calibration. Unfortunately, in hematology and hemostasis, in which 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.7 For scores of analytes, the hematology and hemostasis laboratory scientist relies on calibrators. Calibrators for hematology may be preserved human blood cell suspensions, sometimes supplemented with microlatex particles or nucleated avian red blood cells (RBCs) as surrogates for hardto-preserve human white blood cells (WBCs). In hemostasis, calibrators may be frozen or lyophilized plasma from healthy human donors. For most 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 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 the 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 CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing Gaussian, accurate, and precise 43 Gaussian, inaccurate, but precise Frequency Frequency Dispersion Target Target Gaussian, accurate, but imprecise Gaussian, but inaccurate, and imprecise Frequency Value Frequency Value Target Target Value Value 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 (gaussian distribution). An accurate assay generates a mean that closely duplicates the reference target value. A precise assay generates small dispersion about the mean, whereas imprecision is reflected in a broad curve. The ideal assay is both accurate and precise. 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 sample. For instance, human preserved blood used to calibrate complete blood count analytes 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 scientist 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 scientists may employ locally collected fresh normal blood as a calibrator; however, the process for validation and certification is laborious, so few attempt it. The selected specimens are assayed using reference equipment and methods, calibration values are assigned, and the new or modified assay is calibrated from these values. The Student t-test is often the statistic employed to match the means of the reference and of the new assay. Often the reference equipment and methods are provided by a nearby laboratory. Determination of Accuracy by Regression Analysis If a series of five calibrators is used, results may be analyzed by the following regression equation: y = a + bx ∑ XY − (∑ X )(∑ Y )⎤⎦ Intercept (a ) = ⎡⎣∑ Y − b (∑ X )⎤⎦ n Slope (b ) = ⎡⎣n ⎡⎣n ⎣ ∑ X − (∑ X ) ⎤⎦ 2 2 44 PART I Introduction to Hematology 130 130 r = 1.0 Y intercept = 0.0 125 120 120 115 110 105 Line of identity 100 New Assay 3 New Assay 1 115 110 105 100 95 95 90 90 85 85 80 80 80 85 90 95 80 100 105 110 115 120 125 130 Reference 130 85 90 95 100 105 110 115 120 125 130 Reference 130 r = 1.0 Y intercept = 5.0 125 120 r = 0.89 Y intercept = 5.0 125 120 115 New Assay 4 115 New Assay 2 r = 0.89 Y intercept = 0.0 125 110 105 100 110 105 100 95 95 90 90 85 85 80 80 80 85 90 95 100 105 110 115 120 125 130 Reference 80 85 90 95 100 105 110 115 120 125 130 Reference Figure 5-2 Linear regression comparing four new assays with a reference method. Assay 1 is a perfect match. The y intercept of assay 2 is 5.0, which illustrates a constant systematic error, or bias. The regression (r) value for assay 3 is 0.89, which illustrates a proportional systematic error. New assay 4 has both bias and proportional error. where x and y are the variables; a = intercept between the regression line and the y-axis; b = slope of the regression line; n = number of values or elements; X = first score; Y = second score; ΣXY = sum of the product of first and second scores; ΣX = sum of first scores; ΣY = sum of second scores; ΣX2 = sum of squared first scores. Perfect correlation generates a slope of 1 and a y intercept of 0. Local policy 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 100 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. Precision Unlike 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 sample or lot of reference material.8 Precision studies always assess both within-day and day-to-day variation about the mean and are usually performed on three to five calibration samples, although they may also be performed using a series of patient samples. To calculate within-day precision, the scientist assays a sample at least 20 consecutive times using one reagent batch and one instrument run. For day-today precision, at least 10 runs on 10 consecutive days are required. The day-to-day precision study employs the same 45 CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing sample source and instrument but separate aliquots. Day-today 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%): Mean ( x ) = ∑χ ; 180 160 170.6 146.2 140 Assayed, percent 120 100 100.4 80 60 56.1 40 n 30.1 20 where (Σx) = the sum of the data values and n = the number of data points collected ∑ (x − x ) 2 Standard deviation ( s) = n −1 s CV% = 100 x The CV% documents the degree of 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 upon the complexity of the assay. Although accuracy, linearity, and analytical specificity are just as important, medical laboratory scientists often equate the quality of an assay with its CV%. The best assay, of course, is one that combines the lowest 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.9 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 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 scientist dilutes a high-end calibrator or patient sample to produce at least five dilutions spanning the full range of assay. The dilutions are then assayed. Computed and assayed results for each dilution are paired and plotted on a linear graph, x scale and y scale, respectively. The line is inspected visually for nonlinearity at the highest and lowest dilutions (Figure 5-3). The acceptable range of linearity is established inboard based on the values at which linearity loss is evident. Although formulas exist for computing the limits of linearity, visual inspection is the accepted practice. Nonlinear graphs may be transformed using semilog or log-log graphs when necessary. Patient samples with results above the linear range must be diluted and reassayed. Results from diluted samples that fall 0 0 20 40 60 80 100 120 140 160 180 200 Computed, percent Figure 5-3 Determination of linearity. Prepare at least five dilutions of the standard or calibrator. Dilutions must span the expected range of analyte measurements. Compute the concentration of the analyte for each of the five dilutions. Plot the assayed values on the y scale and the computed concentrations on the x scale. Select the linear range by visual inspection, including the dilutions for which assayed values vary in a linear manner. In this example, the limits of linearity are 56.1% to 146.2%. Assay results that fall outside these limits are inaccurate. within the linear range are valid; however, they must be multiplied by the dilution. Laboratory scientists 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% 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/mcL and 5000/mcL affects the decision to treat with platelet concentrate. Limits Linearity studies are coupled with the lower limit of detection study. A "zero calibrator," or blank, is assayed 20 times and the standard deviation is 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 assays. This cutoff prevents false-positive results generated by low-end assay interference, commonly called noise. Limit assays are typically performed by the manufacturer or distributor and the results provided 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 sample matrix. The laboratory scientist "spikes" identical samples with potential interfering substances and measures the effects of each upon the assay results. Specificity is always determined by the manufacturer and need not be confirmed at the local provider's site unless there is suspicion 46 PART I Introduction to Hematology TABLE 5-1 Categories of Laboratory Assay Approval Assay Category Food and Drug Administration cleared Analyte-specific reagent Research use only Home brew TABLE 5-2 Example of a Lot-to-Lot Comparison Comment The local institution may use package insert data for linearity and specificity but must establish accuracy and precision. 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. Local institution must perform all validation steps. Research use only assays are intended for clinical trials, and carriers are not required to pay. Assays devised locally, all validation studies are performed locally. of interference from a particular substance not assayed by the manufacturer. Manufacturer specificity data are transferred from the package insert to the local 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) assays, and home-brew assays. FDA-cleared assays are approved for the detection of specific analytes and should not be used for off-label applications. Details are given in Table 5-1. Documentation and Reliability Validation is recorded on standard forms. The Clinical Laboratory Standards Institute (CLSI) and David G. Rhoads Associates ( provide automated electronic forms. Validation records are stored in prominent laboratory locations and made available to laboratory assessors upon request. Precision and accuracy maintained over time provide assay reliability. 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 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 is repaired, the validation procedure is repeated. 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 assure 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 sequester kit and reagent lots, which ensures infrequent lot changes, Sample Low Low middle Middle High middle High Old kit control 1 Old kit control 2 New kit control 1 New kit control 2 Old Lot Value New Lot Value 7 12 20.5 31 48 9 22 10 24 6 12 19.4 27 48 11 24 10 24 % Difference −14% −5% −20% 8% 8% optimistically no more than once a year. 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 samples 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 5-2. Action limits vary by location, but many managers reject the new lot when more than one sample 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. DEVELOPMENT OF THE REFERENCE INTERVAL AND

THERAPEUTIC RANGE Once an assay is validated, the laboratory scientist develops the reference interval (reference range, normal range).10 Most practitioners tend to use the vernacular term normal range; however, reference interval is the preferred term. According to the 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 scientist carefully defines the desired normal 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 over-the-counter or prescription medications. Donors may be paid. There should be an equal number of males and females, and the normal subjects should match the institution's population demographics in terms of age and race. When practical, large-volume blood specimens are collected, aliquoted, and placed in long-term storage. For instance, plasma aliquots for coagulation reference interval development are stored at –70° 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. In general, although published normal values are available for educational CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing 47 68.26% 95.46% 0.14% 0.14% 99.73% -3s -2s -1s x +1s +2s +3s Figure 5-4 Normal (gaussian) distribution. When the test values obtained for a given subject population are normally distributed, the mean is at the peak. The segments of the population distribution representing ±1, ±2, and ±3 standard deviations are illustrated. In developing the reference interval, laboratory directors often use ±2 standard deviations to establish the 95.5% confidence interval. This means that 95.46% of the test values from the normal (healthy) population are included within ±2 standard deviations. Consequently, 4.54%, or approximately 1 in 20 normal test results, fall outside the interval, half (2.27%) above and half below. and general discussion purposes, local laboratories must generate their own reference intervals to most closely match the demographics of the area served by their institution. The minimum number of subject samples required to develop a reference interval may be determined using statistical power computations; however, practical limitations prevail.11 For a completely new assay with no currently established reference interval, a minimum of 120 samples is necessary. In most cases, however, the assay manufacturer provides a reference interval on the package insert, and the local laboratory scientist need only assay 30 samples, 15 male and 15 female, to validate the manufacturer's reference interval, a process called transference. Likewise, the scientist may refer to published reference intervals and, once they are locally validated, transfer them to the institution's report form. It is assumed that the population sample subjects employed to generate reference intervals will produce frequency distributions (in laboratory vernacular, histograms) that are normal bellshaped (gaussian) curves (Figure 5-4). In a gaussian frequency distribution the mean is at the center 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, it has been assumed for years that the visual reticulocyte percentage reference interval in adults is 0.5% to 1.5%; however, repeated analysis of normal populations in several locations has established it at 0.5% to 2%, owing to a subset of normal subjects whose reticulocyte counts fall at the high end of the range. Scientists may choose to live with a log-normal distribution or they may transform it by replotting 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 ( x ) is computed by dividing the sum of the observed values by the number of data points, n, as shown in the equation on page 45. The standard deviation is calculated using the second formula in that equation. A typical reference interval is computed as ±2 standard deviations and assumes that the distribution is normal. The limits at ±2 standard deviations encompass 95.46% of normal results, known as the 95% confidence interval. This implies that 4.54% of theoretically normal results fall outside the interval. A standard deviation computed from a nongaussian distribution may turn out to be too narrow to reflect the true reference interval and may thus encompass fewer than 95% of theoretical normal values and generate a number of false positives. Assays with high CV% values have high levels of random error reflected in a broad curve; low CV% assays with "tight" dispersal have smaller random error and generate a narrow curve, as illustrated in Figure 5-1. The breadth of the curve may also reflect biologic variation in values of the analyte. A few hematology and hemostasis assays are used to monitor drug therapy. For instance, the international normalized ratio (INR) for prothrombin time is used to monitor the effects of oral Coumadin (warfarin) therapy, and the therapeutic range is universally established at an INR of 2 to 3. On the other hand, the therapeutic range for monitoring treatment with unfractionated heparin using the partial thromboplastin time (PTT) assay must be established locally by performing regression of the PTT results in seconds against the results of the chromogenic anti-Xa heparin assay, whose therapeutic range is established empirically as 0.3 to 0.7 international heparin units. The PTT therapeutic range is called the Brill-Edwards curve and is described in Chapter 45. If assay revalidation or lot-to-lot comparison reveals a systematic change caused by reagent or kit modifications, a new reference interval (and therapeutic range, when applicable) is established. The laboratory director must warn the hospital staff of reference interval and therapeutic range changes, 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 PART I Introduction to Hematology TABLE 5-3 Steps Used to Correct an Out-of-Control Assay Run Step Description 1. Reassay When a limit of ±2 standard deviations is used, 5% of expected assay results fall above or below the limit. Controls may deteriorate over time when exposed to adverse temperatures or subjected to conditions causing evaporation. Reagents may have evaporated or become contaminated. Instrument may require repair. 2. Prepare new control and reassay 3. Prepare fresh reagents and reassay 4. Recalibrate instrument 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.14 The rules were developed for assays that employ primary standards, but a few Westgard rules that are the most useful in hematology and hemostasis laboratories are provided in Table 5-4, along with the appropriate actions to be taken.15 Normal distribution +3s +2s +1s Mean 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.12 Compared with calibrators, control materials are inexpensive and are comprised of the same matrix as patient samples except for preservatives or freezing that provide a long shelf life. Controls provide known values and are sampled directly alongside patient specimens to accomplish within-run assay validation. In nearly all instances, two controls are required per test run, one in the normal range and the other in an expected abnormal range. For some assays there is reason to select controls whose values are near the interface of normal and 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 ±2 standard deviations. Control manufacturers provide limits; however, local laboratory scientists 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 limits the run is rejected and the cause is found and corrected. The steps for correction are listed in Table 5-3. Control results are plotted on a Levey-Jennings chart that displays each data point in comparison to the mean and limits (Figure 5-5).13 The Levey-Jennings chart assumes that the control results distribute in a gaussian manner and provide limits at 1, 2, and 3 standard deviations about the mean. In addition to being analyzed for single-run errors, the data points are examined for sequential errors over time (Figure 5-6). Both 0 -1s -2s -3s 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 Day Control Figure 5-5 Normal Levey-Jennings plot. 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. Shift +3s +2s +1s Mean 48 0 -1s -2s -3s 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 Day Control Figure 5-6 Levey-Jennings plot that illustrates a systematic error or shift. 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. CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing TABLE 5-4 Westgard Rules Employed in Hematology and Hemostasis 12s or 22s 13s R4s Shift Trend A single control assay or two control assays are outside the ±2 standard deviation limit. Assay results are held until the error is identified using the steps in Table 5-3. Variations of this rule are 13s and 41s. A single control assay is outside the ±3 standard deviation limit. Assay results are held until the error is identified using the steps in Table 5-3. Two consecutive control values are more than 4 standard deviations apart. Assay results are held until the error is identified using the steps in Table 5-3. A series of at least 10 control values remain within the dispersal limits but are consistently above or below the mean. Use of the assay is suspended until the cause is found; often it is an instrument calibration issue that has introduced a constant systematic error (bias). A series of at least 10 control values changes in a consistent direction. Use of the assay is suspended until the cause is found; often it is an instrument calibration issue that has introduced a systematic proportional error. In hematology, shifts may be caused by deterioration of reagents, pump fittings, or light sources. Abrupt shifts may reflect a reagent or instrument fitting change. 49 control samples but provides additional protection against 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.17 Patient values remain relatively consistent over time unless there is an intervention. A result that fails a δ check is investigated for intervention 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. Not all assays are amenable to δ checking; only analytes known to show little intraindividual variation are checked. These include MCV and RBC distribution width. In hemostasis, the prothrombin time and INR are checked. Action limits for δ checks are based on clinical impression and are assigned by hematology and hemostasis laboratory directors in collaboration with the house 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 Moving Average ( X B) 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.16 Each consecutive sequence of 20 patient RBC index assay results is collected and treated by the moving average formula, which accumulates, "smoothes," and trims data to reduce the effect of outliers. Each trimmed 20-sample mean, X B, is plotted on a LeveyJennings 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 LeveyJennings graph 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 samples are analyzed for the mean MCV, MCH, and MCHC. A Levey-Jennings chart is prepared using ±3% of the mean or one standard deviation 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 less well in institutions that assay samples from specialized populations, such as sickle cell or oncology populations, for which the index values may include a number of outliers. It does not replace the use of External quality assessment further validates the accuracy of hematology and hemostasis assays by comparing results from identical aliquots of samples distributed at regular intervals among laboratories nationwide or worldwide. The aliquots are often called survey or proficiency testing samples and include preserved human donor plasma and whole blood, stained blood and bone marrow smears, and photomicrographs of cells or tissues. In most proficiency testing systems, target (true or reference) values for the test samples 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 samples using the same sample-handling principles as those employed for patient specimens—survey samples 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 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 50 PART I Introduction to Hematology 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 action 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) provides the largest survey systems, CAP accreditation, and CAP Surveys/ EXCEL proficiency testing. Survey packages are provided for laboratories offering all levels of service. CAP is a nongovernmental agency; however, survey participation is necessary to meet the accreditation requirements of the Joint Commission (formerly the Joint Commission on Accreditation of Healthcare Organizations) and to qualify for Medicare reimbursement. The North American Specialized Coagulation Laboratory Association 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) 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. MEASUREMENT OF CLINICAL EFFICACY Since 1940 and before, surgeons have used the bleeding time test to predict the risk of intraoperative hemorrhage. The laboratory scientist or phlebotomist activates an automated lancet to make a 5-mm long, 1-mm deep incision in the volar surface of the forearm and, using a filter paper to soak up the blood, times the interval from the initial incision to bleeding cessation, normally 2 to 9 minutes. The test is simple and logical, and for over 50 years experts have claimed that if the incision bleeds for longer than 9 minutes, there is a risk of surgical bleeding. In the 1990s clinical researchers compared normal and prolonged bleeding times with instances of intraoperative bleeding and found to their surprise that prolonged bleeding time predicted only 50% of intraoperative bleeds.18,19 The other 50% occurred despite a normal bleeding time. Thus the positive predictive value of the bleeding time for intraoperative bleeding was 50%, the same as the probability of obtaining heads (or tails) in tossing a coin. Today the bleeding time test is widely agreed to have no clinical relevance and is obsolete. Like the bleeding time test, many time-honored hematology and hemostasis assays gain credibility on the basis of expert opinion. Now, however, besides being valid, accurate, linear, and precise, a new or modified assay must be clinically effective.20 To compute clinical efficacy, the scientist uses a series of samples from normal healthy subjects, called controls, and patients who conclusively possess a disease or condition. The patient's diagnosis is based on clinical outcomes, discharge diagnosis notes, or the results of existing laboratory tests, excluding the new assay. The new assay is then applied to samples from both the normal and disease groups to assess its efficacy. In a perfect world, the laboratory scientist sets the discrimination threshold at the 95% confidence interval limit (±2 standard deviations) of the reference interval. When this threshold is used, the test will hopefully yield a positive result (e.g., elevated or reduced level) in every instance of disease and a negative result (within the reference interval) in all normal control subjects. In reality, there is always overlap: a "gray area" in which some positive test results are generated by normal samples (false positives) and some negative results are generated by samples from patients with disease (false negatives). False positives cause unnecessary anxiety, follow-up expense, and erroneous diagnostic leads—worrisome, expensive, and time consuming, but not fatal. False negatives fail to detect the disease and may delay treatment, which is potentially lifethreatening. The laboratory scientist employs clinical efficacy computations to establish laboratory assay efficacy and minimize both false positives and false negatives (Table 5-5). Clinical efficacy testing includes determination of diagnostic sensitivity and specificity, and positive and negative predictive value, as well as receiver operating characteristic analysis. To start a clinical efficacy study the scientist selects normal control samples and samples from people proven to have the disease or condition addressed by the assay. To make the discussion easy, assume that 50 samples of each are chosen. All are assayed, and the results turn out as shown in Table 5-6. The scientist next computes clinical sensitivity and speci ficity and positive and negative predictive value as shown in Table 5-7. TABLE 5-5 Clinical Efficacy Definitions and Binary Display True positive False positive True negative False negative Assay is negative Assay is positive Assay correctly identifies a disease or condition in those who have it. Assay incorrectly identifies disease when none is present. Assay correctly excludes a disease or condition in those without it. Assay incorrectly excludes disease when it is present. Normal (Control Sample) Disease or Condition (Patient Sample) True negative False positive False negative True positive TABLE 5-6 Clinical Efficacy Study Assay is negative Assay is positive Normal (Control Sample) Disease or Condition (Patient Sample) True negative: 45 False positive: 5 False negative: 5 True positive: 45 CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing 51 TABLE 5-7 Clinical Efficacy Computations Statistic Definition Formula Example Proportion with the disease who have a Sensitivity (%) = TP/(TP + FN) × 100 45/(45 + 5) × 100 = 90% positive test result Distinguish clinical sensitivity from analytical sensitivity. Analytical sensitivity is a measure of the smallest increment of the analyte that can be distinguished by the assay. Specificity Proportion without the disease who have a Specificity (%) = TN/(TN + FP) × 100 45/(45 + 5) × 100 = 90% negative test result Distinguish clinical specificity from analytical specificity. Analytical specificity is the ability of the assay to distinguish the analyte from interfering substances. Positive predictive value (PPV) Proportion with a disease who have a PPV (%) = TP/(TP + FP) × 100 45/(45 + 5) × 100 = 90% positive test result compared with all subjects who have a positive test result The positive predictive value predicts the probability that an individual with a positive assay result has the disease or condition. Negative predictive value (NPV) Proportion without a disease who have a NPV (%) = TN/(TN + FN) × 100 45/(45 + 5) × 100 = 90% negative test result compared with all subjects who have a negative test result The negative predictive value predicts the probability that an individual with a negative assay result does not have the disease or condition. Sensitivity FN, False negative; FP, false positive; TN, true negative; TP, true positive. For all assays, as sensitivity rises, specificity decreases. Assays with 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 a prevalence of 0.0001 (1 in 10,000) and the false-positive rate is 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. Likewise, as specificity rises, sensitivity goes down. Assays with high specificity make effective confirmatory assays when used in follow-up to positive results on screening assays. Highspecificity 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 used for patients with high clinical pretest probability. In such instances, the prevalence of the condition is high enough to mitigate the effects of false positives and false negatives. When a physician orders hemostasis testing for patients who are experiencing easy bruising, there is a high pretest probability, which raises the tests' clinical efficacy. Ordering hemostasis tests routinely for healthy individuals prior to elective surgery presumes a low pretest probability and reduces the efficacy of the test profile. RECEIVER OPERATING CHARACTERISTIC CURVE A receiver operating characteristic (ROC) curve or ROC analysis is a further refinement of clinical efficacy testing that may be employed when the assay being validated generates a continuous variable.21 In clinical efficacy testing as described earlier, the ±2 standard deviation limit of the reference interval is used as the threshold for discriminating a positive from a negative test result. Often the "true" threshold varies from the ±2 standard deviation limit. Using ROC analysis, the discrimination threshold is adjusted by increments of 1, and the true-positive and false-positive rates are recomputed for each new threshold level. The threshold that is selected is the one that provides the largest true-positive and smallest false-positive rate (Figure 5-7). A line graph is generated plotting true positives on the y-axis and false positives on the x-axis. The overall efficacy of the assay is assessed by measuring the area under the curve. 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, and quality control initiatives. Feasibility study includes a review of assay throughput (number of assays per unit time), cost per test, 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, 12 states have medical laboratory personnel licensure laws. In these states, only licensed laboratory professionals may be employed in 52 PART I Introduction to Hematology 1.00 0.90 0.80 True-positive rate Threshold FP rate TP rate 70% 0.02 0.51 71% 0.05 0.62 72% 0.10 0.80 73% 0.15 0.85 74% 0.30 0.89 75% 0.35 0.91 76% 0.38 0.93 77% 0.44 0.96 78% 0.47 0.96 79% 0.51 0.98 80% 0.57 0.98 0.70 0.60 0.50 0.40 AUC ~0.85 0.30 0.20 0.10 0.00 0.00 0.20 A 0.40 0.60 0.80 1.00 0.80 1.00 0.80 1.00 False-positive rate 1.00 0.90 FP rate TP rate 0.02 0.31 0.05 0.45 0.10 0.55 0.15 0.65 0.30 0.79 0.35 0.81 0.42 0.83 0.44 0.86 0.47 0.86 0.51 0.89 0.57 0.90 0.80 True-positive rate Cutoff 70% 71% 72% 73% 74% 75% 76% 77% 78% 79% 80% 0.70 0.60 0.50 0.40 AUC ~0.70 0.30 0.20 0.10 0.00 0.00 0.20 B 0.40 0.60 False-positive rate 1.00 0.90 FP rate TP rate 0.05 0.30 0.10 0.35 0.15 0.40 0.20 0.45 0.25 0.50 0.30 0.55 0.35 0.60 0.40 0.65 0.45 0.70 0.50 0.75 0.55 0.80 0.80 True-positive rate Cutoff 70% 71% 72% 73% 74% 75% 76% 77% 78% 79% 80% 0.70 0.60 0.50 0.40 AUC ~0.50 0.30 0.20 0.10 0.00 0.00 C 0.20 0.40 0.60 False-positive rate Figure 5-7 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 is acceptable, 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 is unacceptable, 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 clinical efficacy. FP, False positive; TP, true positive. CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing medical center or reference laboratories. In nonlicensure 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 outcomes and laboratory errors demonstrate that laboratories that employ only licensed or certified professionals produce the most reliable assay results.22,23 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 such incidents should be used for quality improvement and instruction. When error is associated with liability, the opportunity for improvement is often lost to obfuscation. The analysis of error without blame may be 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 staff 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 licensure boards require professional personnel to participate in and document continuing education for periodic recertification or relicensure. Continuing education is delivered in the form of journal articles, case studies, distance learning seminars, and 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 local cases is particularly effective. Continuing education maintains the critical skills of laboratory scientists and provides opportunities to learn about new clinical and technical approaches. The Colorado Association for Continuing Medical Laboratory Education ( ), the American Society for Clinical Laboratory Science (http:// www.ascls.org), the American Society for Clinical Pathology ( ), the American Society of Hematology ( ), the National Hemophilia Foundation ( ), CLOT-ED ( . clot-ed.com), and the Fritsma Factor ( . com) are examples of scores of organizations that direct their activities toward quality continuing education in hematology and hemostasis. The medical laboratory science profession stratifies pro fessional staff responsibilities by educational preparation. In 53 the United States, professional levels are defined as the asso ciate (2-year) degree level, or medical laboratory technician; bachelor (4-year) degree level, or medical laboratory scientist; and the level of advanced degree, masters 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—for example, the University of Cincinnati, University of North Dakota, Weber State University, and University of Medicine and Dentistry in New Jersey—provide distance learning opportunities. 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. agencies require laboratory directors to maintain records of preanalytical and postanalytical quality assurance and quality improvement efforts.24 Although not exhaustive, Table 5-8 lists and characterizes a number of examples of preanalytical quality efforts, and Table 5-9 provides a review of postanalytical components. All quality assurance plans include objectives, sources of authority, scope of services, an activity calendar, corrective action, periodic evaluation, standard protocol, personnel involvement, and methods of communication.25 CAP Q-PROBES is a subscription service that provides a model quality assurance program. Experts in quality assurance continuously refine the consensus of appropriate indicators of laboratory medicine quality. The Q-PROBES program searches 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: • Clinical and Laboratory Standards Institute (CLSI) (http:// www.clsi.org), 940 West Valley Road, Suite 1400, Wayne, PA 19087. Produces guidelines and standards for laboratory practice. Hemostasis documents include H21-A5, H30-A2, and H47–H58. Hematology standards include H02-A4, H07-A3, and H22–H46. Clinical efficacy method evaluation, mostly EP suffix standards; quality assurance and quality management systems, mostly GP suffix standards are available. • Center for Medicare and Medicaid Services (CMS) (http:// www.cms.hhs.gov), 7500 Security Boulevard, Baltimore, MD 21244. Administers the laws and rules developed from the Clinical Laboratory Improvement Amendments of 1988. Establishes Current Procedural Terminology (CPT) codes, reimbursement rules, and test complexity. • College of American Pathologists (CAP) ( . org/apps/cap.portal), 325 Waukegan Road, Northfield, IL 54 PART I Introduction to Hematology TABLE 5-8 Preanalytical Quality Assurance Components and the Laboratory's Responsibility Preanalytical Component Laboratory Staff Responsibility Test orders Conduct continuous utilization reviews to ensure that physician laboratory orders are comprehensive and appropriate to patient condition. Inform physician about laboratory test availability and ways to avoid unnecessary orders. Reduce unnecessary repeat testing. Are requisition forms legible? Can you confirm patient identity? Are physician orders promptly and correctly interpreted and transcribed? Is adequate diagnostic, treatment, and patient preparation information provided to assist the laboratory in appropriately testing and interpreting results? Do turnaround time expectations match clinical necessity and ensure that stat orders are reserved for medical emergencies? Is the patient correctly identified, prepared, and available for specimen collection? Is fasting and therapy status appropriate for laboratory testing? Is the tourniquet correctly applied and released at the right time? Are venipuncture sites appropriately cleansed? Are timed specimens collected at the specified intervals? Are specimen tubes collected in the specified order? Are additive tubes properly mixed? Are specimen tubes labeled correctly? Are specimens delivered intact, sealed, and in a timely manner? Are they maintained at the correct temperature? Are specimens centrifuged correctly? Are tests begun within specified time frames? Are samples stored properly? Are coagulation specimens platelet-poor when specified? Test request forms Stat orders and timeliness Specimen collection Specimen transport Specimen management TABLE 5-9 Postanalytical Quality Assurance Components and the Laboratory's Responsibility Postanalytical Component Laboratory Staff Responsibility Publication of reports 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? Are turnaround times recorded and analyzed? Are laboratory reports being posted to patient charts in a timely fashion? Does the institution include laboratory care in patient surveys? Was specimen collection explained to the patient? Timeliness Patient satisfaction 60093. Laboratory accreditation, proficiency testing, and quality assurance programs; laboratory education, reference resources, and e-lab solutions. • Joint Commission ( ), One Renaissance Boulevard, Oakbrook Terrace, IL 60181. Accreditation and certification programs. SUMMARY • 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, although it may be accomplished by using a number of patient specimens and comparing results with those obtained using a reference method. In all cases, accuracy is established using the Student t-test and linear regression. • Precision is established by using repeated within-day and day-today assays, then computing the mean, standard deviation, and coefficient of variation of the results. • Assay linearity, specificity, and lower limit of detection are usually provided by the vendor; however, many laboratory managers 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 standard deviations. If the control value is outside the limits, use of the assay is suspended and the scientist begins troubleshooting. Control results are plotted on Levey-Jennings charts and examined for drift and trends. Internal quality control is enhanced through the use of the moving average algorithm. • 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 55 CHAPTER 5 Quality Assurance in Hematology and Hemostasis Testing essential to laboratory accreditation. Most U.S. states require external quality assessment for laboratory licensure. • All laboratory assays are analyzed for clinical efficacy, sensitivity, and specificity, including their true-positive and true-negative rates, and positive and negative predictive values. Highly sensitive assays may be used for population screening, but may lack good discrimination. Specific assays may be used to confirm a condition, but generate a number of false negatives. Clinical efficacy computations expand to include receiver operating characteristic curve analysis. • Thoughtful laboratory managers hire only certified or licensed medical laboratory scientists and technicians and provide regular individual proficiency tests that are correlated with in-service education. Staff are encouraged to participate in continuing education activities and in-house discussion of cases. Quality laboratories provide resources for staff to pursue higher education. • The laboratory director maintains a protocol for assessing and improving upon preanalytical and postanalytical variables and finds means to communicate enhancements to other members of the health care team. Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented. R E V I E W Q UESTIONS 1. You validate a new assay using linear regression to compare assay calibrator results with the distributor's published calibrator results. The slope is 0.95 and y intercept is +10%. What type of error is present? a. No error b. Random error c. Constant systematic error d. Proportional systematic error 2. 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. 3. A WBC count control has a mean value of 6000/mcL and a standard deviation of 300/mcL. What is the 95.5% confidence interval? a. 3000 to 9000/mcL b. 5400 to 6600/mcL c. 5500 to 6500/mcL d. 5700 to 6300/mcL 4. The ability of an assay to distinguish the targeted analyte from interfering substances within the sample matrix is called: a. Analytical specificity. b. Analytical sensitivity. c. Clinical specificity. d. Clinical sensitivity. 5. 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 6. A laboratory scientist measures prothrombin time for plasma aliquots from 15 normal males and 15 normal 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. 7. You purchase a preserved whole blood specimen from a distributor who provides the mean values for several complete blood count analytes. What is this specimen called? a. Normal specimen b. Calibrator c. Control d. Blank 8. You perform a clinical efficacy test and get the following results: Normal (Control Sample) Disease or Condition (Patient Sample) Assay is negative 40 5 Assay is positive 10 45 What is the number of false-negative results? a. 40 b. 10 c. 5 d. 45 56 PART I Introduction to Hematology 9. What agency provides external quality assurance (proficiency) surveys and laboratory accreditation? a. College of American Pathologists (CAP) b. Clinical Laboratory Improvement Advisory Committee (CLIAC) c. Center for Medicare and Medicaid Services (CMS) d. Joint Commission 10. What agency provides continuing medical laboratory education? a. Clinical Laboratory Improvement Advisory Committee (CLIAC) b. Center for Medicare and Medicaid Services (CMS) c. Colorado Association for Continuing Medical Laboratory Education (CACMLE) d. College of American Pathologists (CAP) 11. Regular review of blood specimen collection quality is an example of: a. Preanalytical quality assurance. b. Analytical quality control. c. Postanalytical quality assurance. d. External quality assurance. 12. 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. REFERENCES 1. Westgard JO: Assuring the right quality right: good laboratory practices for verifying the attainment of the intended quality of test results, Madison, Wisc, 2008, Westgard QC. 2. Clinical and Laboratory Standards Institute (CLSI): Continuous quality improvement: integrating five key quality system components; approved guideline, ed 2, CLSI document GP22-A2. Wayne, Pa, 2004, CLSI. 3. Clinical and Laboratory Standards Institute (CLSI): Procedures for the collection of diagnostic blood specimens by venipuncture; approved standard, ed 6, CLSI document H3-A6. Wayne, Pa, 2004, CLSI. 4. Westgard JO: Basic method validation, ed 3, Madison, Wisc, 2008, Westgard QC. 5. 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Wayne, Pa, 2004, CLSI. 11. Cembrowski GS, Martindale RA: Quality control and statistics. In Bishop ML, Fody EP, Schoeff LE, editors: Clinical chemistry: principles, procedures, correlations, ed 5, Philadelphia: 2004 Lippincott Williams & Wilkins, chap 3, pp 48-89. 12. Bachner P: Quality assurance in hematology. In Howanitz JF, Howanitz JH, editors: Laboratory quality assurance, New York, 1987, McGraw-Hill, pp 214–243. 13. Levey S, Jennings ER: The use of control charts in the clinical laboratory. Am J Clin Pathol 20:1059–1066, 1950. 14. Westgard JO, Quam EF, Barry PL, et al: Basic QC practices, ed 2, Madison, Wisc, 2002, Westgard QC. 15. Westgard JO, Barry PL, Hunt MR, et al: A multi-rule Shewhart chart for quality control in clinical chemistry. Clin Chem 27:493–501, 1981. 16. Bull BS, Elashoff RM, Heilbron DC, et al: A study of various estimators for the derivation of quality control procedures from patient erythrocyte indices. Am J Clin Pathol 61:473– 481, 1974. 17. 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Westgard JO, Ehrmeyer SS, Darcy TP: CLIA final rule for quality systems, quality assessment issues and answers, Madison, Wisc, 2004, Westgard QC. 25. Howanitz PJ, Hoffman GG, Schifman RB, et al: A nationwide quality assurance program can describe standards for the practice of pathology and laboratory medicine. Qual Assur Health Care 3:245–256, 1992. PART II Hematopoiesis Cellular Structure and Function 6 Keila B. Poulsen OUTLINE OBJECTIVES Cell Organization Cell Membrane After completion of this chapter, the reader will be able to: Membrane Proteins Membrane Carbohydrates Nucleus Chromatin Nuclear Envelope Nucleoli Cytoplasm Golgi Complex Endoplasmic Reticulum Ribosomes Mitochondria Lysosomes Microfilaments Microtubules Centrioles 1. Describe the general function and chemical composition of cellular membranes. 2. List and describe the components of the nucleus, including staining qualities visible by light microscopy. 3. Correlate the nuclear structures to the activities of the cell. 4. Name and describe the ultrastructural appearance of the cytoplasmic organelles found in the cell and staining qualities by light microscopy, if appropriate. 5. Correlate the cytoplasmic structures to the activities of the cell. 6. Describe the function of the hematopoietic inductive microenvironment. 7. Associate stages in the cell cycle with activities of the cell. 8. Correlate regulatory proteins with the stage of the cell cycle. 9. Discuss the function of checkpoints in the cell cycle and where in the cycle they occur. 10. Differentiate between apoptosis and necrosis. Hematopoietic Microenvironment Cell Cycle Regulation of the Cell Cycle Apoptosis T he numerous multichannel instruments available to assist in the clinical diagnostic process have revolutionized the study of hematology. The technologies of light scatter, electrical impedance, and conductivity have added parameters and scatter plots whose significance is yet to be fully realized and clinically applied, but morphologic examination of the peripheral blood film by light microscopy remains the hallmark for clinical evaluation of patients with hematologic abnormalities. The study of cells under the microscope was greatly enhanced when Paul Ehrlich (1854-1915) developed staining techniques to better differentiate the various normal and abnormal cells present in human blood. The development of the electron microscope revolutionized the ability to study and understand the internal components of the cell.1 CELL ORGANIZATION Cells are structural units that constitute living organisms (Figures 6-1 and 6-2). Many cells have specialized functions and contain the components necessary to perform and perpetuate these functions. Regardless of shape, size, or function, most cells have three basic parts: unit membranes, the cytoplasm, and the nucleus. Each of these basic parts has components or subdivisions that assist in their varied functions. Table 6-1 summarizes the cellular components and functions, which are explained in more detail later. CELL MEMBRANE The cell membrane serves as a semipermeable outer boundary separating the cellular components from their surrounding environment. The cell membrane serves three basic functions: (1) it restricts and facilitates the interchange of substances with the environment by selective permeability, endocytosis, exocytosis, and locomotion; (2) it detects hormonal signals facilitating cell-to-cell recognition; and (3) it is the location of surface markers for cell identity.2 Monoclonal antibodies are used to identify a cell's surface markers. The nomenclature 57 58 PART II Hematopoiesis Microfilaments Glycogen aggregates Golgi complex Nuclear envelope Chromatin Nuclear pore Centriole Rough endoplasmic reticulum Microtubule Perinucleolar chromatin Vacuole Nucleolus Mitochondria Euchromatin Lysosome Heterochromatin Smooth endoplasmic reticulum Free ribosomes Figure 6-1 Cell organization. Nuclear pore Golgi body Nucleus Nucleolus Lysosomes Mitochondria Rough endoplastic reticulum Figure 6-2 Electron micrograph with labeled organelles. (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) CHAPTER 6 Cellular Structure and Function 59 TABLE 6-1 Summary of Cellular Components and Functions Organelle Location Appearance and Size Function Comments Membranes: plasma, nuclear, mitochondrial, endoplasmic reticulum Outer boundary of cell, nucleus, endoplasmic reticulum, mitochondria, and other organelles Separates various cellular components; facilitates and restricts cellular exchange of substances Membrane must be resilient and flexible Nucleus Within cell Nucleolus Within nucleus Control center of cell and contains genetic blueprint Site of synthesis and procession of ribosomal RNA Golgi body Next to nucleus Governs cellular activity and transmits information for cellular control Appearance varies with activity of cells; larger when cell is actively involved in protein synthesis Well developed in cells with large secretion responsibilities Endoplasmic reticulum Randomly distributed throughout cytoplasm Ribosomes Free in cytoplasm; outer surface of rough endoplasmic reticulum Usually a lipid bilayer consisting of proteins, cholesterol, phospholipids, and polysaccharides; membrane thickness varies with cell or organelle Usually round or oval but varies depending on cell; varies in size; composed of DNA Usually round or irregular in shape; 2-4 µm in size; composed of RNA; there may be 1-4 within nucleus System of stacked, membrane-bound, flattened sacs; horseshoe shaped; varies in size Membrane-lined tubules that branch and connect to nucleus and plasma membrane Small granule (100-300 Å); composed of protein and nucleic acid Mitochondria Randomly distributed in cytoplasm Lysosomes Randomly distributed in cytoplasm Microfilaments Near nuclear envelope and within proximity of mitotic process Cytoskeleton, near nuclear envelope and component part of centriole near Golgi body In centrosome near nucleus Microtubules Centriole Round or oval structures; 3-14 nm in length, 2-10 nm in width; membrane has 2 layers; inner layer has folds called cristae Membrane-bound sacs; size varies Small, solid structure approximately 5 nm in diameter Hollow cylinder with protofilaments surrounding outside tube; 20-25 nm in diameter, variable length Cylinder; 150 nm in diameter, 300-500 nm in length uses the letters CD (cluster designation) and a number following the CD. This common terminology assists in unifying classification in clinical practice, research efforts, and the literature (see Chapter 33). 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 widely studied and serves as an example of a cell membrane (see Figure 9-2). To accomplish its many requirements, this cell membrane must be resilient and elastic. It achieves these qualities by being Involved in modifying and packaging macromolecules for secretion Stores and transports fluids and chemicals Site of production of proteins, such as enzymes and blood proteins Cell's "powerhouse"; make adenosine triphosphate, energy source for cell Two types: smooth with no ribosomes; rough with ribosomes on surface Large proteins are synthesized from polyribosomes (chains of ribosomes) Active cells have more present than do inactive cells Contain hydrolytic enzymes for cellular digestive system Support cytoskeleton and motility If membrane breaks, hydrolytic enzymes can destroy cell Maintains cell shape, motility, and mitotic process Produced from tubulin polymerization; make up mitotic spindles and part of structure of centriole Composed of nine sets of triplet microtubules Serves as insertion point for mitotic spindle fibers Consist of actin and myosin (contractile proteins) a fluid structure of globular proteins floating in lipids. The lipids comprise 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) lipids oriented toward the inner and outer surfaces of the cell membrane. The fatty acid portion of the phospholipid and the steroid nucleus of cholesterol are non– polar-charged hydrophobic (water-insoluble) lipids directed toward each other in the center of the cell membrane. Other lipids, such as lipoproteins and lipopolysaccharides, contribute to the membrane structure. 60 PART II Hematopoiesis Membrane Proteins Most proteins present in the cell membrane are called glycoproteins and are found floating in the lipid bilayers.2,3 Two types of proteins, integral and peripheral, have been described in the cell membrane. Integral proteins may traverse the entirety of the lipid bilayers and penetrate the outside of the membrane or only the cytoplasmic side of the membrane. These transmembrane proteins are thought to serve as a communication and transport system between the cell's interior and the external environment. Peripheral proteins are found only on the inner cytoplasmic side of the membrane and form the cell's cyto skeleton. Peripheral proteins also are attached to the cyto plasmic ends of integral proteins to form a reticular network for maintaining structural integrity and holding the integral proteins in a fixed position. Membrane Carbohydrates Membrane carbohydrates occur in combination with proteins (glycoproteins) and lipids (glycolipids). The carbohydrate portion usually extends 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.2 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. It is responsible for the chemical reactions within the cell and the cell's reproductive process. The nucleus has an affinity for the basic dyes because of the nucleic acids contained within it. Chromatin The chromatin consists of nucleic acids and proteins. The proteins are the histones, which are negatively charged, and the nonhistones, which are positively charged. Chromatin has been divided into two types: (1) the heterochromatin, which is represented by the more darkly stained, condensed clumping pattern and is the genetically inactive area of the nucleus, and (2) the euchromatin, which has diffuse, uncondensed chromatin and is the genetically active portion of the nucleus where ribonucleic acid (RNA) transcription occurs. This genetic material is loosely coiled and turns a pale blue when stained with Wright stain. Nuclear Envelope Surrounding the nucleus is a nuclear envelope consisting of an inner and an outer membrane. The outer membrane is continuous with an extension of the endoplasmic reticulum. Between the two membranes is a diaphragm approximately 50 nm in thickness that is continuous with the lumen of the endoplasmic reticulum. Nuclear pores penetrate the nuclear envelope, which allows communication between the nucleus and the cytoplasm. The number of these pores decreases as the cell matures. Nucleoli The nucleus contains one to several nucleoli. The number is directly proportional to the amount of protein synthesis that occurs in the cell. These organelles contain a large amount of ribosomal RNA, DNA, and other proteins in a loose fibrillar form. The nucleolus is the site of synthesis of the cell's ribosomes. Ribosomes consist of two subunits, a large and a small one. The subunits are produced in the nucleolus and are transported through the nuclear pores for ribosomal assembly and protein synthesis. CYTOPLASM The cytoplasmic matrix is a homogeneous, continuous, aqueous solution called cytosol. It is the environment in which the organelles join and function. These organelles are discussed individually. Golgi Complex The Golgi complex is a system of stacked, membrane-bound, flattened sacs referred to as cisternae that are involved in modifying, sorting, and packaging macromolecules for secretion or delivery to other organelles. The number of stacked cisternae ranges from 6 to 30 per cell, depending on the cell type. The Golgi complex is normally located next to the nucleus. The Golgi complex is horseshoe shaped and usually located in close proximity to the endoplasmic reticulum. The concave aspect has numerous enzymes for synthetic activities. The convex side is the "maturing surface" and is where the various products are packaged.2 Membrane-bound vesicles are closely associated with the stacks of cisternae. Some of the vesicles are coated and bud off for transport to other areas of the cell. The Golgi complex directs traffic in the cell. The exact mechanism of macromolecule modification and sorting is still being defined, although it is clearly a responsibility of the Golgi complex. Endoplasmic Reticulum The endoplasmic reticulum (Figure 6-3) is a lacelike network found throughout the cytoplasm of cells and appears as flattened sheets, sacs, and tubes of membrane. The endoplasmic reticulum subdivides the cytoplasm into various compartments. The outer membrane of the nuclear envelope is in continuity with the endoplasmic reticulum membrane and specializes in making and transporting lipid and membrane proteins. Rough endoplasmic reticulum has a studded look on its outer surface caused by the presence of ribosomes engaged in the synthesis of proteins. The amount of endoplasmic reticulum found within a cell is proportional to the protein production required by the cell. More endoplasmic reticulum is necessary for increased protein synthesis. Smooth endoplasmic reticulum does not contain ribosomes and may serve as storage CHAPTER 6 Cellular Structure and Function 61 Smooth endoplasmic reticulum Rough endoplasmic reticulum Ribosomes Figure 6-3 Endoplasmic reticulum. Outer membrane Oxidation enzymes Matrix space Inner membrane Figure 6-4 Mitochondrion. sites for the newly synthesized protein. Also, it has been suggested as a site for steroid hormone production and synthesis of lipid substances. Its function varies with the type of cell. Additional activities of the smooth endoplasmic reticulum include detoxification or inactivation of harmful compounds or drugs.4 Ribosomes Ribosomes are small particles composed of nearly equal amounts of protein and ribosomal RNA. Ribosomes are found free in the cytoplasm, on the surface of rough endoplasmic reticulum, and in the nucleus and nucleoli of a cell. These bodies may exist singly (monoribosome) or form chains (polyribosomes). The more ribosomes present within the cell, the more protein production and the more basophilia observed with Wright staining. Ribosomes serve as the site of protein synthesis. This is accomplished with the assistance of transfer RNA, for amino acid transport to the ribosome, and messenger RNA, which provides the necessary information for the sequencing order of the amino acids for each protein.5 Mitochondria The existence of mitochondria (Figure 6-4) within the cell has been known since the nineteenth century, and their function is now clearly defined. Structurally, the mitochondrion has a continuous outer membrane. Running parallel to the outer membrane is an inner membrane that invaginates at various intervals, giving the interior a shelflike or ridgelike appearance. These internal ridges, termed cristae mitochondriales, are where oxidative enzymes are attached. The two membranes differ chemically; the inner membrane has a higher protein content and a lower lipid content. The convolution of the inner 62 PART II Hematopoiesis 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 are responsible for the metabolic processes of energy-producing reactions and electron transfer– oxidative reactions. The oxidative systems described within the mitochondria are the Krebs cycle, the fatty acid cycle, and the respiratory chain.6 Also present in the mitochondria are proteins, phosphorylase, ribosomes, and DNA.7 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. Mitochondria do not stain with Wright stain, but instead give a negative image or clear impression against the stained cytoplasm. When in a hyperactive state, these organelles become so swollen that they appear as short white rods against a blue cytoplasmic background. 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 attacking the protein, nucleic acids, mucopolysaccharides, lipids, and glycogen within the cell.8 The enzymes become active when lysosomes bind to the phagocytic vacuole and the membrane ruptures, which allows the escape of the hydrolytic enzymes into the phagosome. With Wright stain, lysosomes are visualized as granules. When stained, many of the granules appear azurophilic, unless they are too small to be visualized under the light microscope. Special staining techniques are required to indicate the presence of the smaller granules. Microfilaments Microfilaments are solid structures approximately 5 nm in diameter and consist of actin and myosin proteins. These fibrils or groups of fibrils are located near the nuclear envelope or in the proximity of the nucleus and assist in cell division. They also are present near the membrane for cytoskeletal support and motility. Intermediate filaments are the most durable element of the cytoskeleton and provide structural stability for the cell, especially during stress.9 Intermediate filaments also are detectable and identifiable as cancer markers.4 Microtubules Microtubules are approximately 25 nm in diameter and vary in length. These organelles are organized from tubulin through self-assembly. The tubulin polypeptides form protofilaments. Usually 13 protofilaments are lined up in parallel rows of hollow spheres.9,10 This arrangement gives the microtubules structural strength. A variety of conditions cause microtubules to become disorganized and disappear, especially after mitosis. Tubulin can polymerize and reform the microtubule as needed. Microtubules have several functions. Their contribution to the cytoskeleton helps maintain the cell's shape and the movement of some intracellular organelles. The microtubule makes up the mitotic spindle fibers and the centrioles during mitosis.11 In the peripheral smear, the microtubules and microfilaments are not visible, but under special conditions the mitotic spindles may cluster and form Cabot rings.7 Centrioles Centrioles are paired structures consisting of nine bundles of three microtubules each. They are shaped like cylinders and serve as insertion points for the mitotic spindle fibers during metaphase and anaphase of mitosis. The cylinders are 150 nm in diameter and 300 to 500 nm in length. The long axes are typically at right angles to one another. HEMATOPOIETIC MICROENVIRONMENT Hematopoiesis occurs predominantly in the bone marrow after birth. This is a suitable environment for hematopoietic stem cells, progenitor cells, and marrow precursors. The marrow environment must provide for stem cell self-renewal, proliferation, differentiation, and apoptosis. This protective environment is made up of stromal cells, which is a broad term for specialized fibroblasts, reticulum cells, endothelial cells, adipocytes, lymphocytes, and macrophages. Many extracellular molecules are secreted from these cells, including collagen, glycoproteins, (fibronectin and thrombospondin), and glycosaminoglycans. These help form the extracellular matrix that is so critical for the support of cell growth and function in the bone marrow microenvironment. 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. Several adhesion subgroups of receptors help perform this function. Some examples of the subgroups are integrins, immunoglobulins, and selectins.12 Stromal cells also secrete many different growth factors required for stem, progenitor, and precursor cell survival. 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. An important feature of growth factors is their use of synergism to stimulate a cell to proliferate or differentiate.12 In other words, several different growth factors work together to generate a more effective response. Growth factors are very specific for their corresponding receptors on target cells. The receptors are typically glycoproteins that traverse the membrane. The intracellular portion of the receptors associate with protein tyrosine kinase and the Janus kinase family, which initiates the cell signaling CHAPTER 6 Cellular Structure and Function pathways. This will ultimately interact with the DNA in the nucleus for gene expression and proliferation.13,14 CELL CYCLE The cell cycle is a biochemical and morphologic four-stage process through which a cell passes when it is stimulated to divide (see Figure 31-3). 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 9 hours. In G2, the nucleus contains tetraploid DNA, and the cell volume seems to help trigger the onset of mitosis. DNA is also checked for damage. G2 takes approximately 4 hours. The time spent in each stage can be variable, but mitosis takes approximately 1 hour. During mitosis cell division occurs. During G0 (quiescence) the cell is not actively in the cell cycle. When the cell receives the signal to proliferate, a number of biochemical reactions take place. Cell cycle control is under the direction of cyclin and cyclin-dependent kinases (CDKs). This cyclin/CDK complex can phosphorylate key substrates that assist the cell through 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) partnered with CDK4 and CDK6. 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 complexes with CDK2 (cyclin A/CDK2). This combination takes the cell through the S stage. At the end of S stage, cyclin A starts to activate CDK1 and partners with it. The cyclin A/CDK1 complex takes the cell through the S stage into G2. For mitosis to occur, cyclin B must replace cyclin A and bind to CDK1 in the cyclin B/CDK1 complex. This complex takes the cell through the intricate process of mitosis.13 Mitosis is divided into five phases. Interphase is the first phase and correlates with G1, S, and G2. Next is prophase, during which the cell condenses the chromosomes. In prometaphase the centrosomes move to opposite poles. In metaphase, the chromosomes are aligned on microtubular spindles. Anaphase is when the sister chromatids segregate. During telophase, the chromatids move to opposite poles and the cell divides. Nuclear division is referred to as karyokinesis and cytoplasmic division is cytokinesis. The purpose of the cell cycle is to replicate DNA once during the S stage and distribute identical chromosome copies equally to both daughter cells during mitosis.4 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 complicated and involved process that has numerous stages and biochemical reactions that can malfunction. There are several checkpoints or restriction points in the cell cycle at which the level of nutrients and DNA is evaluated. The first checkpoint is late in G1. The checkpoint makes the cell wait for DNA repair to occur or it initiates 63 apoptosis (programmed cell death). A second DNA check takes place after DNA synthesis. This checkpoint occurs during 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. Here the attachment of chromosomes to the mitotic spindle, the integrity of the spindle apparatus, and the alignment of the spindles are checked. Tumor suppressor genes play a significant role in the proper function of the checkpoints. One of the first tumor suppressor genes recognized was p53. The p53 gene detects DNA damage during G1. It can also assist in triggering apoptosis. Multiple tumor suppressor genes have been described.15,16 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.17 Inhibitors of the cyclin/CDK complexes also play a primary role in cell cycle regulation.13 APOPTOSIS Since the concept of programmed cell death or apoptosis was first described many far-reaching purposes of this process have been recognized. Not only does apoptosis limit the number of cells produced, but its role includes control of organ growth, which prevents the progression of malignant and abnormal cells through the cell cycle. Apoptosis also serves as a defense mechanism by removing cells affected by a virus.18 APOPTOSIS (single cell) Normal cell Nuclear changes Cytoplasmic fragmentation Macrophage Apoptotic bodies Figure 6-5 Apoptosis manifests as single cell death. The nucleus condenses and karyorrhexis (fragmentation) occurs. The cell fragments (apoptotic bodies) containing parts of the nucleus and functioning cytoplasmic organelles are ultimately engulfed by phagocytic cells, such as macrophages. (From Damjanov I: Pathology for the health professions, ed 3, St Louis, 2006, Saunders.) 64 PART II Hematopoiesis There are two major mechanisms for cell death. One is apoptosis and the other is necrosis. Apoptosis is internal, whereas necrosis is caused by external influences. Apoptosis occurs due to the activation of intracellular proteins and triggering of a regulated cell death. Caspases are the intracellular proteins that are activated, and this activation occurs through two different pathways. One pathway involves membrane proteins such as Fas ligand and tumor necrosis factor. This is the death receptor pathway. The second pathway entails the mitochondrial release of cytochrome c, which in turn binds to Apaf-1. The cytochrome c/Apaf-1 complex is referred to as an apoptosome and results in activation of caspase-9, which triggers a cascade leading to apoptosis. This pathway may be the one involved in cellular response to chemotherapy or irradiation.12 The initiation of apoptosis typically occurs from three possible sources. The first is an abnormal hematopoietic microenvironment in the bone marrow and/or decreased levels of growth factors (see Chapter 7). The second is stimulation of the death receptor pathway. The third is cell-damaging stress. Many proteins have been described that are proapoptotic, and several others have been identified that act as inhibitors to apoptosis. The BCL-2 family of proteins are antiapoptotic and the BAX, BAK, and BAD are examples of proapoptotic proteins. The ratio of these intracellular proteins plays a primary role in the mechanism for regulating apoptosis. Any dysregulation, mutation, or translocation can cause inhibition or overexpression of apoptotic proteins, which can lead to hematopoietic malignancies or malfunctions.12,17 The morphologic manifestation of apoptosis is shrinkage of the cell. The nucleus and cytoplasm condense. The borders of the cell begin to bud off. Macrophages eliminate the cell through phagocytosis (Figure 6-5). No inflammatory response occurs. The morphologic manifestation of necrosis is a swelling of the cell with damage of the plasma membrane and lysis of the cell. An inflammatory response then occurs.17 SUMMARY • Cells are building blocks of the living organism and provide the basis for all life processes. • The nucleus serves as a control center: it directs, transmits information, and maintains the cell. • The nucleolus is the site of synthesis and processing of ribosomal RNA. • The Golgi complex modifies and packages macromolecules for secretion. • Mitochondria make adenosine triphosphate to supply energy for the cell. • Lysosomes contain hydrolytic enzymes involved in the cell's intracellular digestive process. • 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 cyclin and CDKs. • Checkpoints in the cell cycle recognize abnormalities and initiate apoptosis. R E V I E W Q UESTIONS 1. The organelle involved in packaging and trafficking of cellular products is the: a. Nucleus b. Golgi complex c. Mitochondria d. Rough endoplasmic reticulum 2. The most common type of protein found in the cell membrane is: a. Lipoprotein b. Mucoprotein c. Glycoprotein d. Nucleoprotein 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 complex 6. The shape of a cell is maintained by which of the following? a. Microtubules b. Spindle fibers c. Ribosomes d. Centrioles CHAPTER 6 Cellular Structure and Function 7. Functions of the cell membrane include all of the following except: a. Interchange of substances b. Cell-to-cell recognition c. Cellular identification through receptors d. Lipid production 65 11. The cell cycle is regulated by: a. Cyclins and CDKs b. Protooncogenes c. Apoptosis d. Growth factors 8. The energy source for cells is the: a. Golgi complex b. Endoplasmic reticulum c. Nucleolus d. Mitochondrion 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 9. Ribosomes are synthesized by the: a. Endoplasmic reticulum b. Mitochondrion c. Nucleolus d. Golgi apparatus 13. Apoptosis is morphologically identified by: a. Cellular swelling b. Nuclear condensation c. Rupture of the cytoplasm d. Rupture of the nucleus 10. Euchromatin functions as the: a. Site of microtubule production b. Genetically active DNA c. Support structure for nucleoli d. Attachment site for centrioles REFERENCES 1. Wintrobe MM: Blood, pure and eloquent, New York, 1980, McGraw-Hill. 2. Guyton AC, Hall JE: Textbook of medical physiology, ed 11, Philadelphia, 2006, Saunders. 3. Steinberg MH, Benz EJ, Adewoye AH, et al: Pathobiology of the human erythrocyte and its hemoglobins. 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J Virol 71(3):1739-1746, 1997. 7 Hematopoiesis Larry Smith OUTLINE OBJECTIVES Hematopoietic Development After completion of this chapter, the reader will be able to: Mesoblastic Phase (Yolk Sac Phase) Hepatic Phase Medullary (Myeloid) Phase Adult Hematopoietic Tissue Bone Marrow Red Marrow Marrow Circulation Hematopoietic Microenvironment Liver Spleen Lymph Nodes Thymus 1. Define hematopoiesis. 2. Discuss 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 names of various stem cells and progenitor cells and their lineage associations. 6. Discuss the roles of hematopoietic growth factors in differentiation and maturation sequences of hematopoietic progenitor cells, including nonspecific and lineage-specific factors. 7. Describe general morphologic changes that occur during cell maturation. 8. Define apoptosis and discuss the relationship between apoptosis, growth factors, and stem cell differentiation. 9. Discuss the roles of various cytokines and hema topoietic growth factors in the process of hematopoiesis. 10. Discuss therapeutic applications of cytokines and hematopoietic growth factors. Stem Cells and Cytokines Stem Cell Theory Stem Cell Cycle Kinetics Stem Cell Phenotypic and Functional Characterization Cytokines and Growth Factors Colony-Stimulating Factors Stem Cell Factor, Flt3 Ligand, Granulocyte/ Monocyte ColonyStimulating Factor, Interleukin-3, and Interleukin-6 Interleukins Lineage-Specific Hematopoiesis Erythropoiesis Leukopoiesis Megakaryopoiesis Analytical and Therapeutic Applications 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. In adults, all of these processes are restricted primarily to the bone marrow. During fetal development, hematopoiesis occurs in different areas of the developing fetus. This process has been divided into three phases: the mesoblastic phase, the hepatic phase, and the medullary phase. Mesoblastic Phase (Yolk Sac Phase) Hematopoiesis is considered to begin around the nineteenth day of embryologic development after fertilization. Progenitor cells of mesenchymal origin migrate from the aorta-gonad-mesonephros region of the developing aorta-splanchnopleure to the yolk sac.1 The cells arising from the aorta-gonadmesonephros region give rise to hematopoietic stem cells (HSCs), but not to primitive erythroblasts. The primitive erythroblasts found in the yolk sac arise from mesodermal cells, which initially line the cavity of the yolk sac. These primitive cells migrate from the periphery into the central cavity of the yolk sac, where they develop into primitive erythroblasts.2-5 The remaining cells surrounding the cavity of the yolk sac are called angioblasts and form the future blood vessels.2-5 The yolk sac phase of hematopoiesis is characterized by the development of primitive erythroblasts that produce measurable amounts of hemoglobins, including Portland, Gower-1, and Gower-2 (see Chapter 10). Yolk sac hematopoiesis does not contribute significantly to definitive hematopoiesis.4 This phase of hematopoiesis occurs intravascularly, or within a developing blood vessel. Hepatic Phase The hepatic phase of hematopoiesis begins at 4 to 5 gestational weeks and is characterized by recognizable clusters of developing erythroblasts, granulocytes, and monocytes.5 The developing erythroblasts signal the beginning of definitive hematopoiesis with a decline in primitive hematopoiesis of 66 CHAPTER 7 Hematopoiesis 67 Cellularity (%) 100 Bone marrow 80 Yolk sac Vertebra Liver 60 1 2 Sternum 3 40 Femur Rib Tibia 20 Spleen Lymph nodes 0 1 2 3 4 5 6 Fetal months 7 8 9 10 Birth 20 30 40 Age in years 50 60 Sites of hematopoiesis 1 Mesoblastic 2 Hepatic 3 Myeloid Figure 7-1 Sites of hematopoiesis. the yolk sac. In addition, lymphoid cells begin to appear.6,7 Hematopoiesis during this phase occurs extravascularly, with the liver remaining the major site of hematopoiesis during fetal life and retaining activity until 1 to 2 weeks after birth. Hematopoiesis in the aorta-gonad-mesonephros region and the yolk sac disappears during this stage. Hematopoiesis in the fetal liver reaches its peak by the third month of development (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 production and involves itself solely in lymphopoiesis. During the hepatic phase, detectable levels of hemoglobin (Hb) F, Hb A, and Hb A2 may be present.8 Medullary (Myeloid) Phase During the fifth month of fetal development, hematopoiesis begins in the developing bone marrow cavity. This transition is called medullary hematopoiesis because it occurs in the medulla or inner part of the bone marrow. During this phase, mesenchymal cells, which are a type of embryonic tissue, migrate into the core of the bone and differentiate into skeletal and hematopoietic blood cells.9,10 Hematopoietic activity, especially myeloid activity, is apparent during this stage of development, and the myeloid-to-erythroid ratio approaches the adult level of 3 : 1 by 21 weeks of gestation.11 By the end of the sixth month, the bone marrow becomes the primary site of hematopoiesis. Measurable levels of erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), fetal hemoglobin, Hb A2, and adult hemoglobin can be detected. In addition, cells at various stages of maturation can be seen in all three lineages. ADULT HEMATOPOIETIC TISSUE In adults, hematopoietic tissue is involved in the proliferation and maturation of blood cells. Numerous organs and tissues contribute to this process, including the bone marrow, lymph nodes, spleen, liver, and thymus. The bone marrow contains developing erythroid, myeloid, megakaryocytic, and lymphoid cells. The tissues where lymphoid development occurs are divided into primary and secondary lymphoid tissue. Primary lymphoid tissue consists of the bone marrow and thymus and is where T and B cells are derived. Secondary lymphoid tissue, where lymphoid cells become competent, consists of the spleen and lymph nodes and gut-associated lymphoid tissue. Bone Marrow Bone marrow, one of the largest organs in the body, is defined as the tissue located within the cavities of the cortical bones. These cavities consist of trabecular bone resembling a honeycomb. Normal bone marrow located within these cavities consists of two types of marrow: (1) red marrow, which is hematopoietically active marrow, and (2) yellow marrow, representing hematopoietically inactive marrow composed primarily of adipocytes (fat cells). Red marrow in adults is found in the sternum, skull, scapulae, vertebrae, ribs, pelvic bones, and proximal ends of the long bones. Normal adult bone marrow has approximately equal amounts of red and yellow marrow. 68 PART II Hematopoiesis A central space is created within the cavities of these bones by resorption of cartilage and endosteal bone. Mesenchymal cells migrate into the space and eventually differentiate into three cell types, which give rise to the blood and bone marrow matrix cells (reticular cells and adipose tissue). Reticular cells are formed on the exterior surfaces of the venous sinuses and extend long, narrow branches into the perivascular space, creating a meshlike network; this provides a supportive skeletal network for developing hematopoietic cells, macrophages, and mast cells.10 During infancy and early childhood, the bone marrow consists primarily of 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 adipose tissue (yellow marrow) during development is called retrogression and eventually results in restriction of the active marrow to the flat bones, sternum, vertebrae, pelvis, ribs, skull, and proximal portion of the long bones (Figure 7-2). Areas located within the bone marrow cavity where red marrow has been replaced by yellow marrow consist of an mixture of adipocytes, undifferentiated mesenchymal cells, and macrophages. Inactive yellow marrow is also scattered throughout active red marrow and is capable of reverting back to active marrow in cases of increased demand on the bone marrow.8 Such cases might be excessive blood loss or increased erythrocyte destruction in the bone marrow by toxic chemicals or irradiation. Red Marrow The red marrow is composed of extravascular cords that contain all of the developing blood cell lineages, stem and progenitor cells, adventitial cells, and macrophages (Figures 7-3 and 7-4). The cords are separated from the lumen of the sinusoids by endothelial and adventitial cells and are located between the trabeculae of spongy bone. Trabeculae are projections of calcified bone radiating out from the cortical bone into the marrow space and provide support for the developing marrow. The hematopoietic cells tend to develop in specific niches within the cords. Normoblasts develop in small clusters adjacent to the outer surfaces of the vascular sinuses (Figure 7-5); in addition, some normoblasts are found surrounding iron-laden macrophages (Figure 7-6). Megakaryocytes are located close to the vascular walls of the sinuses, which facilitates the release of Hematopoietic cords Central sinus Skull Central artery Bone Proximal end of large bones Sternum Vertebrae Endosteum Axial skeleton Megakaryocyte Iliac crest Erythrocytic cells Granulocytes Adventitial cells Fat cell Figure 7-2 The adult skeleton, in which darkened areas depict active red marrow hematopoiesis. Endothelial cells Figure 7-3 Graphic illustration of the arrangement of the extravascular area in hematopoietic tissue. 69 CHAPTER 7 Hematopoiesis Hematopoietic tissue Adventitial cells Bone Megakaryocyte Adipocytes Adventitial cells Arteriole Adipocytes Processes of adventitial cells Erythroid precursors Bone Figure 7-4 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% tissue and 50% fat. platelets into the lumen of the sinusoids. 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.9 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 sinus wall, blood cells pass between layers of adventitial cells that form a discontinuous layer along the abluminal side of the bone marrow sinus. Adjacent to the layer of adventitial cells is a basement membrane followed by a continuous layer of endothelial cells on the luminal side of the bone marrow sinus. These adventitial cells are described as reticular cells and extend long cytoplasmic processes into the marrow cords. The extensions of these reticular filaments form a meshwork that provides support for the developing hematopoietic cells. 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 via a receptor-mediated process. Cells pass Figure 7-5 Fixed and stained bone marrow biopsy specimen (hematoxylin and eosin stain, ×400). Hematopoietic tissue reveals areas of granulopoiesis (lighter-staining cells) and erythropoiesis (darker-staining nuclei). One megakaryocyte can be seen. Adventitial cells and their processes give support to the hematopoietic cells; they also guard apertures of the basement membrane. through pores in the endothelial cytoplasm and are released into the circulation.12 Marrow Circulation The nutrient and gas 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.10 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 (endo steum) form sinusoids (endosteal beds), which connect to perio steal capillaries that extend from the periosteal artery.13 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.14 Blood exits the marrow via the central longitudinal vein, which runs the length of the marrow. The central longitudinal vein exits the marrow 70 PART II Hematopoiesis are located throughout the marrow space.17 Other cells involved in cytokine production include endothelial cells, adipocytes, and fibroblasts. Osteoblasts are bone-forming cells, and osteo clasts are bone-resorbing cells. Reticular cells are associated with the formation of reticular fibers that form a lattice that supports the vascular sinuses and developing hematopoietic cells. Stromal cells are believed to be derived from fibroblasts. They play a role in support and regulation of hematopoietic stem/ progenitor cell survival and differentiation.15 The extracellular matrix of the bone marrow contains proteoglycans or glycosaminoglycans, fibronectin, collagen, laminin, hemonectin, and thrombospondin.15 Proteoglycans are expressed on the endothelial cell surface and mediate progenitor binding to the stroma. Fibronectin, collagen, laminin, hemonectin, and thrombospondin function as adhesion molecules, promoting the adhesion of HSCs to the extracellular matrix. Liver Figure 7-6 Bone marrow aspirate smear (Wright-Giemsa stain, ×500). Macrophage with extensive iron-laden cytoplasm, surrounded by developing erythroid precursors. through the same foramen where the nutrient artery enters. Hematopoietic cells located in the endosteal bed receive their nutrients from the nutrient artery. Hematopoietic Microenvironment The hematopoietic inductive microenvironment plays an important role in stem cell differentiation and proliferation.15,16 It is responsible for supplying a semifluid matrix, which serves as an anchor for the developing hematopoietic cells. The matrix is responsible for maintaining differentiation and proliferation and provides a supporting tissue in the hematopoietic inductive microenvironment. Stromal cells in the matrix are of several types: (1) endothelial cells, (2) adipocytes, (3) macrophages, (4) osteoblasts, (5) osteoclasts, and (6) reticular cells (fibroblasts). Endothelial cells are broad flat cells that form a single continuous layer along the inner surface of the bone marrow sinus.15 They regulate the flow of particles entering and leaving hematopoietic spaces. Adipocytes are large cells with a single fat vacuole; they secrete various steroids that influence erythro poiesis and maintain bone integrity.14 They also play a role in regulating the volume of the marrow in which active hematopoiesis occurs.14 Macrophages function in phagocytosis and secretion of various cytokines that regulate hematopoiesis and The liver plays a significant role in hematopoiesis beginning around the second trimester and serves as the major site of blood cell production during the hepatic stage of hemato poiesis. In adults, the liver has many cellular production functions, including synthesizing various transport proteins, storing essential minerals and vitamins that are used in the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), conjugating bilirubin from hemoglobin degradation, and transporting bilirubin 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 via the bile.18 Anatomically, liver cells are arranged in radiating hepatic lobules emanating from a central vein (Figure 7-7). Adjacent to the longitudinal lobes of the liver and separated only by a small space are sinusoids, which are lined by two types of cells: Kupffer cells and epithelial cells. Kupffer cells are macrophages, removing cellular and foreign debris from the blood that circulates through the liver; they also are responsible for protein synthesis.19 The epithelial cells are arranged in the lining so as to be separated from one another by a noncellular area; this arrangement allows plasma to have direct access to the hepatocytes. This unusual organization of the liver and its location in the body enables it to be involved in many varied functions. Liver Pathophysiology The liver is often involved in blood-related diseases. In por phyrias, the liver exhibits enzymatic deficiencies that result in the accumulation of the various intermediary porphyrins. In severe hemolytic anemias and red blood cell (RBC) dysplasias, the conjugation of bilirubin and the storage of iron are increased. The liver sequesters membrane-damaged RBCs and removes them from the circulation. The liver is capable of extramedullary hematopoietic production in case of bone marrow shutdown.14 It is directly affected by storage diseases of the monocyte/macrophage (Kupffer) cells as a result of enzymatic deficiencies that cause hepatomegaly with ultimate CHAPTER 7 Hematopoiesis Fatstoring cells Central vein Sinusoid Distributing Hepatic vein artery Portal vein Inlet venule Liver plates Bile duct 71 Sinusoid endothelial cell Kupffer cells Bile canaliculi Figure 7-7 Three-dimensional schematic of the normal liver. dysfunction of the liver (Gaucher disease, Niemann-Pick disease, Tay-Sachs disease; see Chapter 28). Spleen The spleen is the largest lymphoid organ in the body. The spleen 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.18 The exterior surface of the spleen is surrounded by a layer of peritoneum and inwardly by 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: (1) white pulp, (2) red pulp, and (3) a marginal zone. The white pulp consists of scattered follicles with germinal centers containing lymphocytes, macrophages, and dendritic cells. Aggregates of lymphocytes surround splenic arteries that pass through these germinal centers. Adjacent to the splenic arteries is a region called the periarteriolar lymphatic sheath. This area consists of lymphoid nodules containing primarily B lymphocytes. Activated B lymphocytes are found in the germinal centers.19 The marginal zone surrounds the white pulp and forms a reticular meshwork containing blood vessels, macrophages, and specialized B cells. The red pulp is composed primarily of vascular sinusoids and sinuses separated by cords of tissue (cords of Billroth) containing specialized macrophages that are loosely connected to the dendritic process, creating a spongelike region that functions as a filter for blood passing through the region.19 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 72 PART II Hematopoiesis White pulp Lymphatic nodule (containing germinal center) Splenic cords (of red pulp) Periarterial lymphatic sheath Trabecular vein Venous sinus Marginal zone Capsule Pulp vein Venous sinus in red pulp Lymphatic vessel Central artery Periarterial lymphatic sheath Lymphatic nodule Venous sinus (contiguous to white pulp) 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.) removal from the spleen. The spleen uses two methods for removing senescent RBCs from the circulation: (1) culling, in which the cells are phagocytosed with subsequent degradation of cell organelles, and (2) pitting, in which splenic macrophages remove inclusions or damaged surface membrane from the circulating RBCs. The spleen synthesizes immunoglobulin M in the germinal centers, and it serves as a storage site for platelets. In a healthy individual, approximately 30% of the total platelet count is sequestered in the spleen.20 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 (Figures 7-8 and 7-9).21 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 macrophagelined cords before reaching the sinuses. Plasma freely reaches the sinuses, but the RBCs have a more difficult time passing through the tiny openings created by the interendothelial junc tions of adjacent endothelial cells. 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 the venous system to exit the spleen. When spleno megaly occurs, the spleen becomes enlarged and is palpable. This occurs as a result of many conditions, such as chronic leukemias, genetic defects in RBCs, hemoglobinopathies, Hodgkin disease, thalassemia, malaria, and the myeloproliferative disorders. Often splenectomy is beneficial in cases of excessive destruction of RBCs, such as severe hereditary spherocytosis, storage disorders, and autoimmune hemolytic anemias, when treatment with corticosteroids does not effectively suppress hemolysis.22,23 Splenectomy also may be indicated in severe CHAPTER 7 Hematopoiesis 73 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 "hairy" white 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.) cases of agnogenic myeloid metaplasia associated with splenomegaly, severe refractory hemolytic anemia, thrombocytopenia, or qualitative platelet function defect syndromes.22,23 After splenectomy, platelet and leukocyte counts increase transiently.22 In sickle cell anemia, repeated splenic infarcts caused by sickled RBCs trapped in the small-vessel circulation of the spleen cause tissue damage and necrosis, which often results in autosplenectomy (see Chapter 26). Hypersplenism is an enlargement of the spleen resulting in some degree of pancytopenia despite the presence of a hyperactive bone marrow. The most common cause is congestive splenomegaly secondary to cirrhosis of the liver and portal hypertension. Other causes include thrombosis, vascular stenosis, other vascular deformities such as aneurysm of the splenic artery, and cysts.24 Lymph Nodes 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.25 Similar in structure to the spleen, lymph nodes consist of an outer capsule that forms trabeculae and provides support for macrophages and the predominant population of lymphocytes. Lymph nodes can be divided into two basic regions: an outer region called the cortex and an inner region called the medulla. Trabeculae radiate through the cortex and the medulla, dividing the interior of the lymph node into specific areas (Figure 7-10). In the cortical region, these areas are known as cortical nodules and contain follicles. These follicles contain foci of B-cell proliferation termed germinal centers.9,18 The cortical nodules are arranged in circles along the outer cortex region of the lymph node. 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 B lymphocytes and plasma cells.23 Lymph nodes have three main functions: (1) they play a role in the formation of new lymphocytes from the germinal centers, (2) they are involved in the processing of specific immunoglobulins, and (3) 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 74 PART II Hematopoiesis Afferent lymphatic vessel Trabecula Secondary follicle Follicle (primary) Medullary sinus Paracortical area (T cells) Capsule Efferent lymphatic vessel Medullary cord (plasma cells) Figure 7-10 Histologic structure of a normal lymph node. The cortical layer is composed mainly of lymphatic nodules, whose germinal centers can be clearly seen. 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 establish new growths, which 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 lymphocytes 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 each other by desmosomes. At birth, the thymus is an efficient, well-developed organ. It consists of two lobules, each measuring 0.5 to 2 cm in diameter. It is located in the upper part of the anterior mediastinum at about the level of the great vessels of the heart.9,18 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) (Figure 7-11). Both areas are populated with the same cellular components—lymphocytes, mesenchymal cells, reticular cells, and many macrophages—although in different proportions. The cortex 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," which is densely populated with progenitor lymphoid cells that migrated from the bone marrow. These cells have no identifiable surface markers when they enter the thymus but give rise to T cells that later express surface antigens and move toward the medulla. Eventually, they 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 that are deficient in immunocompetent T lymphocytes. It is theorized that the cytoplasmic processes of the epithelial reticular cells contain secretory products, thymic hormones, thymic factor, and thymic humoral hormones (proteins/ peptides extracted from the thymus) that promote differentiation of pre-T (nonmarked) from mature T lymphocytes. The cells that are not marked die in the cortex as a result of apoptosis and are phagocytosed by macrophages before release. The medulla contains only 5% mature T lymphocytes and seems to be a holding zone for conditioned cells until the cells are needed by the peripheral lymphoid tissues.26 The thymus also contains myriad cell types, including B cells, dendritic cells, eosinophils, neutrophils, and myeloid cells.19 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, having atrophied (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, CHAPTER 7 Hematopoiesis Cortex Medulla Epithelial cells and Macrophage dendritic cells 75 Hassall's corpuscle Figure 7-11 Schematic diagram of the edge of a lobule of the thymus, showing cells of the cortex and medulla. (From Abbas AK, Lichtman AH, Pober JS: Cellular and molecular immunology, Philadelphia, 1991, Saunders.) Trachea Thyroid Thyroid Trachea Thymus Thymus Lungs Heart Lungs Heart A B Figure 7-12 Differences in the size of the thymus of the infant (A) and the adult (B). 76 PART II Hematopoiesis 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. STEM CELLS AND CYTOKINES Stem Cell Theory Hematopoiesis is a dynamic and complex developmental process of blood cell production. Blood cell production that occurs during the mesoblastic stage of development is referred to as primitive hematopoiesis, whereas definitive hematopoiesis begins during the fetal liver stage and continues through adult life. In 1961 Till and McCulloch27 conducted a series of experiments in which they irradiated spleens and bone marrows 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 (CFUGEMM).27,28 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: (1) noncommitted or undifferentiated stem cells, and (2) multipotential 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 monophy letic theory suggests that all blood cells are derived from a single progenitor stem cell called a pluripotential stem cell. The polyphy letic 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. Stem cells by definition can be characterized as follows: (1) they are capable of self-renewal, (2) they give rise to differentiated progeny, and (3) they 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 consist of (1) the common lym phoid progenitor, which proliferates and differentiates into lymphocytes of T, B, and natural killer lineages; and (2) the common myeloid progenitor, which proliferates and differentiates into individual granulocytic, erythrocytic, monocytic, and megakaryocytic lineages. The resulting limited lineage-specific precursors 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, more than 1 to 5 × 109 each of erythrocytes and leukocytes are produced each hour for the entire life span of an individual.15 Most of the cells in TABLE 7-1 Culture-Derived Colony-Forming Units (CFUs) Abbreviation Cell Line CFU-GEMM CFU-E CFU-Meg CFU-M CFU-GM CFU-BASO CFU-EO CFU-G CFU-pre-T CFU-pre-B Granulocyte, erythrocyte, megakaryocyte, monocyte Erythrocyte Megakaryocyte Monocyte Granulocyte, monocyte Myeloid to basophil Myeloid to eosinophil Myeloid to neutrophil T lymphocyte B lymphocyte normal bone marrow are precursor cells at various stages of maturation. HSCs are directed to one of three possible fates: (1) selfrenewal, (2) differentiation, or (3) apoptosis.29 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 HSCs randomly commits to self-renewal or differentiation.27 This model is also called the stochastic model of hematopoiesis. Later studies suggested that the microenvironment in the bone marrow determines whether the stem cell will self-renew or differentiate (instructive model of hematopoiesis).29 Current thinking is that the ultimate decision made by the stem cell 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 signals from the hematopoietic inductive microenvironment to amplify or repress genes associated with commitment to multiple lineages that are expressed only at low levels. 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 inductive microenvironment via direct cell-to-cell or cellular-extracellular signaling molecules.29 Some of the cytokines released from the hematopoietic inductive micro-environment include factors that regulate proliferation and differentiation, such as stem cell factor (SCF), thrombopoietin (TPO), and Flt3 ligand. Intrinsic regulation involves genes such as SCL (TAL1), which is expressed in cells in the heman gioblast, a bipotential progenitor cell of mesodermal origin that gives rise to hematopoietic and endothelial lineages, 77 CHAPTER 7 Hematopoiesis Long-term selfrenewing stem cell Short-term selfrenewing stem cell Multipotent progenitor hematopoietic stem cell Common myeloid progenitor Granulocyte-monocyte progenitor Myeloblast Neutrophil Common lymphoid progenitor Eosinophil-basophil progenitor Megakaryocyte-erythrocyte progenitor Monoblast Myeloblast Myeloblast Monocyte Eosinophil Basophil Macrophage Pronormoblast Erythrocyte Mast cell Dendritic cell Megakaryoblast Megakaryocyte Platelets Pre-B B lymphoblast B cell Pre-T Natural killer cell T lymphoblast T cell Plasma cell Figure 7-13 Diagram of hematopoiesis shows derivation of cells from the multipotent stem cell. and GATA2, which is expressed in later-appearing HSCs. Both of these genes are essential for primitive and definitive hematopoiesis.29 In addition to factors involved in differentiation and regulation, there are regulatory signaling factors, such as Notch-1 and Notch-2, which allow HSCs to respond to hematopoietic inductive microenvironment factors, altering cell fate.30 As hematopoietic cells differentiate, they take on various morphologic features associated with maturation. These include an overall decrease in cell size 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 (1) loss of nucleoli, (2) decrease in the size of the nucleus, (3) condensation of chromatin, (4) possible change in the shape of the nucleus, and (5) possible loss of the nucleus. Changes occurring in the cytoplasm include (1) decrease in basophilia, (2) increase in the proportion of cytoplasm, and (3) 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 3 billion erythrocytes, 2.5 billion platelets, and 1.5 billion granulocytes per kilogram of body weight daily. The determining factor controlling the rate of production is physio logic need. Stem cells exist in the marrow in the ratio of 1 per 1000 nucleated blood cells. Stem cells 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 one additional 78 PART II Hematopoiesis G1 G0 M S G2 Figure 7-14 Cell cycle schematic. G0, Resting stage; G1, RNA and protein synthesis; S, DNA synthesis; G2, premitotic phase; M, mitosis. time, 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.31 An understanding of the mechanism of the generative cycle aids in under standing the mode of action of specific drugs used in the treatment and maintenance management of proliferative disorders. Stem Cell Phenotypic and Functional Characterization The identification and origin of stem cells can be determined by immunophenotypic analysis using flow cytometry. The earliest identifiable human HSCs capable of initiating longterm cultures are CD34+, CD38−, HLA-DRlow, Thy1low, and Lin−.30 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,30 and the expression of CD10 and CD38 is seen on committed lymphoid progenitors.30 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 (see Chapter 33).30 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, colony-forming unit–granulocyte-monocyte, burst- forming unit–erythroid) 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.30 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.30 These systems allow the ability of the stem cells to proliferate and differentiate to be characterized and may serve as models for developing clinically applicable techniques for gene therapy and stem cell transplantation. Cytokines and Growth Factors A group of specific glycoproteins called hematopoietic growth factors or cytokines regulates the proliferation, differentiation, and maturation of hematopoietic precursor cells. 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 colonystimulating factors (CSFs).32 Cytokines are responsible for stimulation or inhibition of production, differentiation, and trafficking of mature blood cells and their precursors.33 Many of these cytokines exert a positive influence on stem cells and progenitor cells with multilineage potential (e.g., IL-1, IL-3, IL-6, IL-9, IL-11, GM-CSF, and kit ligand).33 Cytokines that exert a negative influence on hematopoiesis include transforming growth factor-β, tumor necrosis factor-α, and the interferons.30 Hematopoietic precursor cells require growth factors on a continual basis for their growth and survival. Growth factors prevent hematopoietic precursor cells from dying by inhibiting apoptosis (or programmed cell death); they stimulate them to divide by decreasing the transit time from G0 to G1 of the cell cycle; and they regulate cell differentiation into the various cell lineages. Apoptosis refers to programmed cell death, a normal physiologic process that eliminates unwanted, abnormal, or harmful cells. Apoptosis differs from necrosis, which is accidental death from trauma. 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 others the mechanisms involved in apoptosis fail to initiate this process, which allows uncontrolled proliferation of cells.33,34 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 in the appropriate section of this chapter. The number of growth factors recognized has expanded greatly in recent years and will further increase as research continues. This chapter focuses primarily on CSFs, kit ligand, Flt3 ligand, IL-3, and IL-6. A detailed discussion is beyond the scope of this text, and the reader is encouraged to consult current literature for further details. CHAPTER 7 Hematopoiesis 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.32 The names of the individual factors indicate the predominant cell lines that respond to their presence: The primary target of G-CSF is the granulocyte, and GM-CSF targets the granulocyte-monocyte 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.35 Although G-CSF stimulates the proliferation of granulocyte progenitors, it also works synergistically with IL-3 to enhance megakaryocyte colony formation.35 Stem Cell Factor, Flt3 Ligand, Granulocyte-Monocyte Colony-Stimulating Factor, Interleukin-3, and Interleukin-6 Ogawa36 described early-acting growth factors (multilineage), intermediate-acting growth factors (multilineage), and lateacting growth factors (lineage restricted). Kit ligand, also known as SCF, is an early-acting growth factor; its cell surface receptor is the product of the c-kit gene. The receptor c-kit is a type I tyrosine kinase receptor that is expressed on HSCs and is downregulated with differentiation. The binding of SCF to its receptor (c-kit) induces a series of signals that are sent via signal transduction pathways from the cytoplasmic receptor of the HSC to the nucleus of the HSC, stimulating the cell to proliferate. As HSCs differentiate and mature, the expression of c-kit decreases. Activation of kit ligand by SCF is essential in the early stages of hematopoiesis.33,37 Flt3 seems to act at an even earlier stage of HSC development than SCF. Flt3 stands for c-fms-like tyrosine kinase.38 SCF and Flt3 work synergistically with IL-3, G-CSF, and GM-CSF to promote early HSC proliferation. Other early-acting CSFs that are multilineage include IL-3, GM-CSF, and IL-6. IL-3 regulates blood cell production by controlling the production, differentiation, and function of granulocytes and macrophages.39 GM-CSF induces expression of specific genes and stimulates HSC proliferation, differentiation, and functional activation.40 IL-6 is a pleiotropic growth factor with stimulatory effects on myeloid and lymphoid cell lineages.41 Interleukins Cytokines originally were named according to their specific function, such as lymphocyte-activating factor (now called IL1), 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). Figure 7-15 illustrates the hematopoietic system and the sites of action of some of the cytokines. These factors are discussed in 79 more detail in subsequent chapters. 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 and growth factors. 3. They are part of interacting systems with amplification potential. 4. They are effective at very low concentrations. Table 7-2 lists the sources of cytokines and sites of activity. 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 multiclustered 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.28 The CFU-E has many EPO receptors and has an absolute requirement for EPO. Some CFU-Es are responsive to low levels of EPO and do not have the proliferative capacity of the BFU-E.42 EPO is a lineage-specific glycoprotein that prevents apoptosis of erythroid precursors. EPO is produced in the renal peri tubular interstitial cells or renal tubular cells.42 In addition, a small amount of EPO is produced by the liver.37 EPO induces hemoglobin synthesis and serves as a differentiation factor causing the CFU-E to differentiate into pronormoblasts, the earliest visually recognized erythrocyte precursors in the bone marrow.43 Erythropoiesis is discussed in detail in Chapter 8. Leukopoiesis Leukopoiesis can be divided into two major categories: myelo poiesis and lymphopoiesis. Factors that promote differentiation of the CFU-GEMM into neutrophils, monocytes, eosinophils, and basophils include GM-CSF, G-CSF, monocyte colonystimulating 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.42 IL-3 is a multilineage stimulating factor that stimulates the growth of granulocytes, monocytes, megakaryocytes, and erythroid cells. Eosinophils require GM-CSF, IL-5, and IL-3 80 PART II Hematopoiesis Long-term selfrenewing stem cell Short-term selfrenewing stem cell SCF, IL-1, 3, 6 Multipotent progenitor hematopoietic stem cell FL, SCF, GM-CSF FL, SCF IL-1, 3, 6, 11 FL, SCF, GM-CSF Common myeloid progenitor IL-3 Granulocyte-monocyte progenitor G-CSF Myeloblast Neutrophil IL-7 SCF, GM-CSF SCF, GM-CSF IL-3 IL-3, 5 Eosinophil-basophil progenitor Megakaryocyte-erythrocyte progenitor M-CSF EPO Monoblast Myeloblast Myeloblast Monocyte Eosinophil Basophil Macrophage Common lymphoid progenitor Mast cell Pre-B SCF FL IL-7 Pre-T FL, IL-7 Natural killer cell TPO Pronormoblast Erythrocyte Dendritic cell FL IL-7 Megakaryoblast Megakaryocyte Platelets B lymphoblast B cell T lymphoblast T cell Plasma cell Figure 7-15 Diagram of derivation of hematopoietic cells, illustrating sites of activity of CSFs and ILs. EPO, Erythropoietin; FL, Flt ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-monocyte colony-stimulating factor; M-CSF, monocyte colony-stimulating factor; SCF, stem cell factor; TPO, thrombopoietin; IL-1, interleukin-1; IL-3, interleukin-3; IL-5, interleukin-5; IL-6, interleukin-6; IL-7, interleukin-7; IL-11, interleukin-11. 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-16.38 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.34 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.44,45 Megakaryopoiesis is discussed in Chapter 13. ANALYTICAL AND THERAPEUTIC APPLICATIONS Clinical use of growth factors approved by the U.S. Food and Drug Administration has contributed 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 a concise overview of some growth factors, physiologic roles, and applications. Many more examples can be found in the literature. CHAPTER 7 Hematopoiesis 81 TABLE 7-2 Selected Cytokines, Sources, Target, and Clinical Application Cytokine Source Target Clinical Application IL-1 Monocytes/macrophages, endothelial cells, fibroblasts, epithelial cells, T cells T cells Fibroblasts, endothelial cells, basophils Used in cases of inflammation, such as fever, and as a defense mechanism during infection43,46 T, B, NK cells IL-3 T cells, monocytes/macrophages, mast cells IL-4 T cells, B cells, macrophages, basophils, mast cells T cells Monocytes/macrophages, endothelial cells, T cells, fibroblasts, osteoblasts BM stromal cells, spleen, thymus Macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes, T and B cells Macrophages, myeloid progenitors, T and B cells, mast cells, fibroblasts T and B cells, eosinophils Fibroblasts, myeloid precursors, T and B cells, mast cells, fibroblasts Pre-B cells, early T cells, CLL, Sézary cells, ALL Enhances migration of cells with antitumor activity into tissues; induces release of IL-6, tumor necrosis factor, and interferon, which may have antitumor effects47 Used in treatment of refractory anemia with and without ringed sideroblasts; also used to stimulate megakaryopoiesis48 Used to potentiate antitumor activity, especially in cases of gastric carcinoma49 IL-2 IL-5 IL-6 IL-7 IL-8 Chemoattractant for neutrophils, T cells, eosinophils IL-9 Monocytes/macrophages, fibroblasts, neutrophils, endothelial cells, synovial cells, chondrocytes T cells IL-10 T and B cells, macrophages Lymphoid and myeloid cells IL-11 BM stromal cells, fibroblasts Progenitor cells and stromal cells IL-12 Macrophages T, NK cells IL-13 T cells, basophils, stromal cells Monocytes, B lymphocytes IL-14 T cells, T and B lymphoma cells Pre-B, T, NK cells IL-15 Monocytes/macrophages, epithelial cells, skeletal muscle cells, BM and thymic stromal cells T, B, and NK cells, mast cells IL-16 IL-17 CD8+ T cells, mast cells CD4+ cells T and B cells Macrophages IL-18 G-CSF Monocytes/macrophages, liver Endothelial cells, placenta, monocytes/ macrophages, fibroblasts M-CSF B and T cells, endothelial cells, monocytes/macrophages, fibroblasts, epithelial cells, osteoblasts Tumor cells, pathogens All granulocytes, macrophages, endothelial cells, fibroblasts, leukemic myeloblasts Monocytes/macrophages, osteoblasts T cells, macrophages, mast cells Pleiotropic cytokine with wide range of biologic activities in immune regulation and hematopoiesis Enhances allocytolytic activity of lymphokine-activated killer cells50; is considered to be a promoter of thymopoiesis and immune recovery51 Active in neutrophil chemotaxis and degranulation; acts as a principal mediator of inflammation that then can be acted on by IL-452 Potent lymphoid cell factor, stimulating growth of certain T cells and mast cells53,54 Synergistic activity of IL-4 and IL-10 causes antiinflammatory effect on human placental cells in vitro55 Has shown activity in preclinical studies in association with myelosuppression, cancer therapy, neutropenia and thrombocytopenia56; stimulates growth and survivability of certain B and T cells57 Used in stimulating proliferation of peripheral blood mononuclear cells and tumor-infiltrating lymphocytes in melanoma patients57; capable of reversing immunosuppression mediated by the neoplastic agent paclitaxel58 Pleiotropic cytokine that has important antiinflammatory and immunoregulatory activities50 CD4+ cell growth factor; proinflammatory; enhances lymphocyte chemotaxis59 rhIL-15 has clinical applications in immunodeficiency diseases and BM transplantation, where it might be advantageous to accelerate peripheral expansion and promote localization of T cells60 Chemoattracts CD4 T cells61 May be useful in further delineating mechanisms of G-CSF regulation50 Stimulates granulocyte production and granulocyte activation62; may be used to treat neutropenia and to mobilize CD34+ stem cells in the peripheral blood63 Used to stimulate monocytes/macrophages production and activity62 continued 82 PART II Hematopoiesis TABLE 7-2 Selected Cytokines, Sources, Target, and Clinical Application—cont'd Cytokine Source Target Clinical Application GM-CSF B, T, and NK cells, osteoblasts, endothelial cells, fibroblasts, monocytes/macrophages May be used to treat neutropenia in patients receiving chemotherapy and in those undergoing BM transplantation64 EPO Kidney, liver All granulocytes, megakaryocytes, stem cells, BFU-E, monocytes/ macrophages, leukemic myeloblasts BFU-E, CFU-E TPO Kidney, liver, spleen, BM stroma, muscle, brain Megakaryocytes, platelets, c-mpl+ blasts c-kit ligand Fibroblasts, BM stroma, hepatocytes Primitive progenitors Flt3 ligand BM fibroblasts and stroma cells Acts synergistically with other growth factors to stimulate T cells, B cells, NK cells, and dendritic cells67 Used to stimulate growth and proliferation of erythroid precursors; used clinically to treat anemia associated with renal failure, chemotherapy, and BM infiltration by cancer64 Essential for maturation of megakaryocytes and enhances platelet production; used to speed recovery of blood platelets after cytoreductive therapies65 Also referred to as stem cell factor/steel factor; used to stimulate myeloid, erythroid, and lymphoid progenitors66 Stimulates primitive progenitor cells68 ALL, Acute lymphocytic leukemia; BFU-E, burst-forming unit–erythroid; BM, bone marrow; CFU-E, colony-forming unit–erythroid; CLL, chronic lymphocytic leukemia; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-monocyte colony-stimulating factor; IL, interleukin; M-CSF, monocyte colony-stimulating factor; mono/macro, monocytes/macrophages; NK, natural killer; rhIL, recombinant human interleukin; TPO, thrombopoietin. SUMMARY • Hematopoiesis is the formation, development, and specialization of all functional blood cells. • 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 extramedullary. • The microenvironment in the bone marrow is essential for stem cell differentiation and proliferation. • Monophyletic theory suggests that all blood cells arise from a common progenitor. • As cells mature, certain morphologic characteristics of maturation allow specific lineages to be recognized. General characteristics • • • • • of maturation include decrease in cell size, decrease in nuclear size, 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 and growth factors play a major role in determining differentiation of stem cells. Cytokines include ILs, CSFs, interferons, and others. Some cytokines exert influence on stem cells with multilineage potential, some are lineage specific, and some function only in combination with other cytokines. Cytokines are necessary to prevent premature apoptosis. Cytokines have contributed new options in the treatment of bone marrow malignancies, leukemias, and aplastic anemias. R E V I E W Q UESTIONS 1. The process of formation and development of blood cells is termed: a. Hematopoiesis b. Hematemesis c. Hematocytometry d. Hematorrhea 2. During midfetal life, the primary source of blood cells is the: a. Bone marrow b. Spleen c. Lymph nodes d. Liver CHAPTER 7 Hematopoiesis 3. Which of the following organs is responsible for the conditioning of T lymphocytes? 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 (hip) b. Femur (thigh) c. Distal radius (forearm) d. Tibia (shin) 5. Physiologic programmed cell death is termed: a. Angiogenesis b. Apoptosis c. Aneurysm d. Apohematics 6. Which organ is the site of sequestration of platelets? a. Liver b. Thymus c. Spleen d. Bone marrow 83 7. The term for the earliest hematopoietic cell is: a. Prehematopoietic blast b. Pluripotential stem cell c. CFU-GEMM d. Cytokinetic precursor 8. Which of the following cells is not a product of the CFU-GEMM? a. Megakaryocyte b. Lymphocyte c. Erythrocyte d. Granulocyte 9. Which of the following hematopoietic growth factors is produced in the kidney? a. EPO b. TPO c. GM-CSF d. M-CSF 10. A multilineage cytokine among the ILs is: a. IL-1 b. IL-2 c. IL-3 d. IL-4 REFERENCES 1. Marcos MA, Godlin I, Cumano A, et al: Developmental events from hemopoietic stem cells to B-cell populations and Ig repertoires. Immunol Rev 137:155-171, 1994. 2. Schoemans H, Verfaillie CM: Cellular biology of hemato poiesis. In Hoffman R, Benz EJ, Shattil SJ, et al, editors: Hematology basic principles and practice, ed 5, New York, 2009, Churchill Livingstone, pp 200-212. 3. Peault B: Hematopoietic stem cell emergence in embryonic life: developmental hematology revisited. J Hematotherapy 5:369-378, 1996. 4. Tavian M, Coulombel L, Luton D, et al: Aorta-associated CD34+ hematopoietic cells in the early human embryo. Blood 87:67-72, 1996. 5. Charbord P, Tavian M, Coulombel L, et al: Early ontogeny of the human hematopoietic system [in French], C R Seances Soc Biol Fil 189:601-609 (abstract), 1995. 6. 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Karsunky H, Miriam M, Cozzio A, et al: Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and-myeloid committed progenitors to Flt3+ dendritic cells in vivo. J Environ Monit 198:305-313, 2003. 68. Gratwohl A, John L, Baldomero H, et al: FLT-3 ligand provides hematopoietic protection from total body irradiation in rabbits. Blood 92:765-769, 1998. 8 Erythrocyte Production and Destruction Kathryn Doig OUTLINE OBJECTIVES Normoblastic Maturation After completion of this chapter, the reader will be able to: Terminology Maturation Process Criteria Used in Identification of the Erythroid Precursors Maturation Sequence Erythrokinetics Hypoxia—the Stimulus to Red Blood Cell Production Other Stimuli to Erythropoiesis Microenvironment of the Bone Marrow Erythrocyte Destruction Macrophage-Mediated Hemolysis (Extravascular Hemolysis) Mechanical Hemolysis (Intravascular Hemolysis) 1. List and describe the erythroid precursors in order of maturity, including the morphologic characteristics, cellular activities, normal location, and length of time in the stage for each. 2. Correlate the erythroblast, normoblast, and rubriblast nomenclatures for red blood cell (RBC) stages. 3. Name the stage of erythroid development when given a written description of the morphology of a cell in a Wright-stained bone marrow smear. 4. List and compare the cellular organelles of immature and mature erythrocytes and describe their specific functions. 5. Name the erythrocyte progenitors and distinguish them from precursors. 6. Explain the nucleus-to-cytoplasm (N : C) ratio. Describe the appearance of a cell when given the N : C ratio or estimate the N : C ratio from the appearance of a cell. 7. Explain how reticulocytes can be recognized in a Wright-stained peripheral blood film. 8. Define and differentiate the terms polychromasia, diffuse basophilia, punctate basophilia, and basophilic stippling. 9. Discuss the differences between the reticulum of reticulocytes and punctate basophilic stippling in composition and conditions for viewing. 10. Define and differentiate erythron and RBC mass. 11. Explain how hypoxia stimulates RBC production. 12. Describe the general chemical composition of erythropoietin (EPO) and name the site of production. 13. Discuss the various mechanisms by which EPO contributes to erythropoiesis. 14. Define and explain apoptosis resulting from Fas/FasL interactions and how this regulatory mechanism applies to erythropoiesis. 15. Explain the effect of bcl-xL and the general mechanism by which it is stimulated in red blood cell progenitors. 16. Describe the features of the bone marrow that contribute to establishing the microenvironment necessary for the proliferation of RBCs, including location and arrangement relative to other cells, with particular emphasis on the role of fibronectin. 17. Discuss the role of macrophages in RBC development. 18. Explain how RBCs enter the bloodstream and how premature entry is prevented and, when appropriate, promoted. 19. Describe the characteristics of senescent RBCs and explain why RBCs age. 20. Explain and differentiate the two normal mechanisms of erythrocyte destruction, including location and process. CASE STUDY After studying the material in this chapter, the reader should be able to respond to the following case study: A 42-year-old premenopausal woman has emphysema. This lung disease impairs the ability to oxygenate the blood, so patients experience significant fatigue and tiredness. 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, a complete blood count showed her red blood 86 cell (RBC) count to be 5.8 × 1012/L. After she has received oxygen for several months, her RBC count is 5.0 × 1012/L. 1. What 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? CHAPTER 8 Erythrocyte Production and Destruction T he red blood cell (RBC), or erythrocyte, provides a classic example of the biologic principle that cells have specialized functions and that their structures are specific for those functions. The erythrocyte has one true function: to carry oxygen from the lung to the tissues where the oxygen is released. This is accomplished by the attachment of the oxygen to hemoglobin (Hb), the major cytoplasmic component of mature RBCs. The role of the RBC in returning carbon dioxide to the lungs and buffering the pH of the blood is important but is quite secondary to its oxygen-carrying function. To protect this essential life function, the mechanisms controlling development, production, and normal destruction of RBCs are fine-tuned to avoid interruptions in oxygen delivery, even under adverse conditions such as blood loss with hemorrhage. This chapter and subsequent chapters discussing iron, RBC metabolism, membrane structure, and hemoglobin constitute the foundation for understanding the body's response to diminished oxygen-carrying capacity of the blood, called anemia. Although in some ways a simple cell, and thus highly studied, the mammalian erythrocyte is unique among animal cells in having no nucleus in its mature, functional state. While amphibians and birds possess RBCs similar to those of mammals, the nonmammalian cells retain their nuclei throughout the cells' lives. The implications of this unique mammalian adaptation are significant for cell function and life span. 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 white blood cell development. Normoblastic terminology is used in this chapter. Maturation Process Erythroid Progenitors As described in Chapter 7, the morphologically identifiable erythrocyte precursors develop from two functionally identifiable progenitors, burst-forming unit–erythroid (BFU-E) and colony-forming unit–erythroid (CFU-E), committed to the erythroid cell line. Estimates of time spent at each stage suggest that it takes about 1 week for the BFU-E to mature to the CFU-E and another week for the CFU-E to become a pronormoblast,1 which is the first morphologically identifiable RBC precursor. While at the CFU-E stage, the cell completes approximately 87 TABLE 8-1 Three Erythroid Precursor Nomenclature Systems Nomenclature Maturation Stages Normoblastic Pronormoblast Basophilic normoblast Polychromatic (polychromatophilic) normoblast Orthochromic normoblast Polychromatic (polychromatophilic) erythrocyte Erythrocyte Rubriblastic Prorubricyte Rubricyte Metarubricyte Polychromatic (polychromatophilic) erythrocyte Erythrocyte Proerythroblast Basophilic erythroblast Polychromic (polychromatophilic) erythroblast Orthochromic erythroblast Polychromatic (polychromatophilic) erythrocyte Erythrocyte Rubriblastic Erythroblastic three to five divisions before maturing further.1 As seen later, it takes approximately another 6 days for the precursors to become mature cells ready 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 erythrocyte precursor, the pronormoblast, is derived via the BFU-E and CFU-E from the multipotential 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 divisions2 with subsequent nuclear and cytoplasmic maturation of the daughter cells, so that from a single pronormoblast, eight mature RBCs usually result. The conditions under which the number of divisions can be increased or reduced are discussed later. Criteria Used in Identification of the Erythroid Precursors Morphologic identification of blood cells depends on a wellstained peripheral blood film or bone marrow smear (see Chapter 16). In hematology, a modified Romanowsky stain, such as Wright or Wright-Giemsa, is commonly used. The 88 PART II Hematopoiesis Multipotential stem cell Pronormoblast Basophilic normoblast Polychromatophilic normoblast Orthochromic normoblast Shift reticulocyte – BM Reticulocyte – PB Erythrocyte Figure 8-1 Typical production of erythrocytes from two pronormoblasts illustrating three mitotic divisions. BM, Bone marrow; PB, peripheral blood circulation. 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. 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, as if it is made of rope rather than yarn. 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 myeloid cells. 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 as cells begin actively synthesizing proteins. As RBCs mature, the nucleoli disappear, which precedes the ultimate cessation of protein synthesis. 5. The cytoplasm changes from blue to gray to 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 CHAPTER 8 Erythrocyte Production and Destruction A B C 89 D 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 dark 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.) developing RBC, and the blueness fades. Pinkness or eosino philia is due to 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 quite basophilic, transitions through a period in which the red of hemoglobin begins to mix with that blue, and ultimately ends with a thoroughly pink-red 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. Similar to the maturing of children into adults, however, the process of cell maturation is a gradual process, with changes occurring in a generally predictable sequence but with some variation for each individual. 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 90 PART II Hematopoiesis TABLE 8-2 Normoblastic Series: Summary of Stage Morphology Cell or Stage Diameter Nucleus-to-Cytoplasm Ratio Pronormoblast Basophilic normoblast Polychromatic normoblast Orthochromic normoblast Shift (stress) reticulocyte (polychromatic erythrocyte) Polychromatic erythrocyte 12-20 µm 10-15 µm 10-12 µm 8-10 µm 8-10 µm 8.0-8.5 µm 8 : 1 6 : 1 4 : 1 1 : 2 No nucleus No nucleus Nucleoli 1-2 0-1 0 0 0 0 % in Bone Marrow Bone Marrow Transit Time 1% 1-4% 10-20% 5-10% 1% 1% 24 hr 24 hr 30 hr 48 hr 48-72 hr* 24-48 hr* *Transit time in peripheral blood. 50 hr 30 hr 50 hr ProBasophilic PolyOrthochromic normoblast normoblast chromatic normoblast normoblast A Average cell diameter (µm) 40 hr Reticulocyte 12 10 8 6 B Rate of RNA synthesis C Rate of DNA synthesis RNA content DNA content 34 D Hemoglobin concentration 24 (pg) 14 Total protein concentration/cell 4 Acidophilia Bone marrow Figure 8-3 Timeline of cellular diameter, RNA and DNA synthesis, and hemoglobin concentration during erythropoiesis. (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.) descriptions that follow. Essential 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 (high N : C ratio). The nucleus is round to oval, containing one or two nucleoli. The chromatin is open and contains few, if any, fine clumps. Cytoplasm. The cytoplasm is quite 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 typically present only in the bone marrow. Cellular Activity. The pronormoblast is beginning to accumulate the components necessary for hemoglobin production. The proteins and enzymes necessary for iron uptake and protoporphyrin synthesis are produced. Globin production begins.3 CHAPTER 8 Erythrocyte Production and Destruction A 91 A B Figure 8-4 A, Pronormoblast (rubriblast), bone marrow (Wright stain, ×1000). B, Electron micrograph of pronormoblast (×15,575). (B from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) Length of Time in This Stage. This stage lasts slightly more than 24 hours.3 Basophilic Normoblast (Prorubricyte) B Figure 8-5 A, Basophilic normoblast (prorubricyte), bone marrow (Wright stain, ×1000). B, Electron micrograph of basophilic normoblast (×15,575). (B from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) 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. 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 staining reaction is one of a deep purple-red. Nucleoli may be present early in the stage but disappear later. Cytoplasm. When stained, the cytoplasm may be a deeper, richer blue than in the pronormoblast—hence the name basophilic for this stage. Location. The basophilic normoblast is typically present only in the bone marrow. Cellular Activity. Detectable hemoglobin synthesis occurs,3 but the large number of 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.3 92 PART II Hematopoiesis 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 typically present only in the bone marrow. Cellular Activity. Hemoglobin synthesis is increasing 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). A Length of Time in This Stage. This stage lasts approximately 24 hours.3 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 quite low. B Figure 8-6 A, Polychromatic normoblast (rubricyte), bone marrow (Wright stain, ×1000). B, Electron micrograph of polychromatic normoblast (×15,575). (B from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) Polychromatic (Polychromatophilic) Normoblast (Rubricyte) Cytoplasm. The pink-orange 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. The prefix ortho means "the same" and refers to the fact that the cell's color is the same color as the eosin stain, which is red. Division. The orthochromic normoblast is not capable of division due to the condensation of the chromatin. Location. The orthochromic normoblast is typically present only in the bone marrow. 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 that the N : C ratio is about 4 : 1. Notably, no nucleoli are present. Cytoplasm. This is the first stage in which the redness 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 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 cell develops pseudopod-like projections into which the nucleus squeezes. Ultimately, the projection separates from the cell, with the nucleus enveloped by cell membrane.4 The extruded nucleus is then engulfed by splenic macrophages. 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 (see Table 18-3) and are typically removed from the circulating cells by the splenic pitting process when the cell enters the circulation. CHAPTER 8 Erythrocyte Production and Destruction A 93 A B B Figure 8-7 A, Orthochromic normoblast (metarubricyte), bone marrow (Wright stain, ×1000). B, Electron micrograph of orthochromic normoblast (×20,125). (B from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) Figure 8-8 A, Polychromatic erythrocyte (shift reticulocyte), peripheral blood (Wright stain, ×1000). B, Scanning electron micrograph of polychromatic erythrocyte (×5000). (B from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) Length of Time in This Stage. This stage lasts approximately 48 hours.3 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, like a lumpy potato (Figure 8-8, B). It requires the polishing activity of the spleen to assist the RBC into its final discoid shape. Polychromatic (Polychromatophilic) Erythrocyte 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 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 easily with that of the orthochromic normoblast in that the predominant color is that of hemoglobin. By the end of the polychromatic Division. Lacking a nucleus, the polychromatic erythrocyte cannot divide. Location. The polychromatic erythrocyte resides in the marrow for 1 day or longer and then moves into the peripheral blood, where it circulates about 1 day. During the first several days after exiting the marrow, the polychromatic erythrocyte is retained in the spleen for pitting and polishing by splenic macrophages, which results in the biconcave discoid mature RBC.5 94 PART II Hematopoiesis 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 (see Table 18-3), 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. 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 over this stage to the point that the polychromatophilia is not readily evident in the polychromatic erythrocytes on a normal peripheral blood smear 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 smear 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 renamed the reticulocyte. 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).6-8 As these receptors decline, the cells are freed to leave the marrow. Length of Time in This Stage. The cell typically remains a polychromatic erythrocyte for about 2 days,3 with the first day spent in the marrow and the second spent in the peripheral blood, although possibly sequestered in the spleen. 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 smear, it appears as a salmon pink- or red-staining cell with a central pale area that Figure 8-9 Reticulocyte, peripheral blood (new methylene blue stain, ×1000). corresponds to the concavity. The 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.9 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, and a small proportion of water. 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. 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 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. Flexibility is crucial for RBCs to enter and subsequently remain in the circulation. CHAPTER 8 Erythrocyte Production and Destruction 95 B A Figure 8-10 A, Mature erythrocytes, peripheral blood (Wright stain, ×1000). B, Scanning electron micrograph of mature erythrocytes. 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 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 enough 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 interstitial cells of the kidney.10,11 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 is fairly consistent, 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 can be increased. The mechanism by which hypoxia increases EPO production in peritubular cells is mainly transcriptional regulation. There is a hypoxia-sensitive region of the 3′ regulatory portion of the EPO gene that promotes gene transcription.12 A hypoxiainducible factor, a transcription factor, is assembled in the cytoplasm when oxygen tension in the cells is decreased.13 It migrates to the nucleus and interacts with the 3′ enhancer for the EPO gene and upregulates the gene expression. With hypoxia, more messenger RNA molecules are produced, which results in more EPO production. Erythropoietin Structure. EPO is a thermostable, nondialyzable, glycoprotein hormone with a molecular weight of 34 kD.14 It consists of a carbohydrate unit that reacts specifically with 96 PART II Hematopoiesis BOX 8-3 Hypoxia and Red Blood Cell Production Teleologically speaking, the location of the body's hypoxia sensor in the kidney is practical,1 because the kidney receives approximately 20% of the cardiac output2 with little loss of oxygen. 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 is only one cause of hypoxia, however. Another cause is the failure of each RBC to carry as much oxygen as it should. This can occur because the hemoglobin (Hb) is defective or because there is not enough Hb 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 normal reference values, 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 normal reference values, 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 Hb in their cells does not unload oxygen to the tissues readily, and so newborns are slightly hypoxic compared with adults. To compensate, they make more RBCs. 1. Donnelly S: Why is erythropoietin made in the kidney? The kidney functions as a "critmeter" to regulate the hematocrit, Adv Exp Med Biol 543:73-87, 2003. 2. Stewart P: Physiology of the kidney, Update in Anaesthesia (9):1-3, 1998. Available at: . Accessed May 23, 2010. RBC receptors and a terminal sialic acid unit, which is necessary for biologic activity in vivo.15 On desialation, EPO activity ceases.16 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. Relatively few receptors require binding to initiate intracellular signaling.17 The EPO-responsive cells vary in their sensitivity to EPO.14 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 population of EPOsensitive cells are able to respond. The binding of EPO, the ligand, to its receptor on erythrocyte progenitors initiates a cascade of intracellular events that ultimately leads to more RBCs entering the circulation in a given time. EPO's effects are mediated by intracellular Janus tyrosine kinase signal transducers that in turn affect gene activities in the RBC nucleus.18 The resulting nuclear activities have two major effects: allowing early release of reticulocytes from the bone marrow and preventing apoptotic cell death. In addition, EPO can reduce the time needed for cells to mature in the bone marrow. These processes are described in detail later. The essence is that EPO puts more RBCs into the circulation at a faster rate than occurs without its stimulation. Early release of reticulocytes. EPO promotes early release of developing erythroid precursors from the marrow. Part of that results from EPO-induced changes in the adventitial cell layer of the marrow/sinus membrane.19 Merely increasing the width of the spaces through which cells might move is insufficient for cells to leave the marrow, however. RBCs are held in the marrow because they possess membrane receptors for adhesive molecules. EPO downregulates the production of the receptors on the RBC surface so that cells can exit the marrow earlier than they normally would.6-8 The result is the presence in the circulation of reticulocytes that are still very basophilic, because they have not spent as much time in the marrow as they normally would. These may be called shift reticulocytes because they have been shifted from the bone marrow early (see Figure 8-8, A). 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.20,21 To understand this process, an overview of apoptosis in general is helpful. Apoptosis: programmed cell death. As noted previously, it takes about 18 days to produce an RBC from stimulation of the earliest erythroid progenitor to release from the bone marrow. In times of increased need for RBCs, such as when there is loss from the circulation during hemorrhage, this time lag would be a significant problem. One way to prepare for such a need would be to maintain a store of mature RBCs somewhere in the body for emergencies. RBCs cannot be stored in the body for this sort of eventuality, however, because they have a limited life span. Instead of storing cells for emergencies, the body produces cells more rapidly when needed. It does so by overproduction of RBC progenitors at all times. If they are not needed, which is normal, the extra progenitors are allowed to die. If they are needed, however, they have about an 8- to 10-day head start in the production process. It is like a fast food restaurant preparing extra hamburger patties for the lunch rush just to be sure they have plenty. If the hamburgers are not sold, they are tossed in the garbage, but if they are needed, just the condiments and buns must be added to make them ready to sell, which significantly reduces the time needed to meet the demand. The process of intentional 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 large fragments of 5 to 300 kilobases that are degraded further into smaller monomers of 200 bases; protein clustering; and activation of transglutamase. In contrast to necrosis, in which cell injury causes swelling and lysing with release of cytoplasmic contents that stimulate an inflammatory response, apoptosis is not associated with inflammation.22 During the sequential process of apoptosis, the following morphologic changes can be seen: (1) condensation of the nucleus, causing increased basophilic staining of the chromatin; (2) nucleolar disintegration; and (3) shrinkage of cell volume with concomitant increase in cell density and compaction of cytoplasmic organelles while mitochondria remain normal.23 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 consisting of multimers of 180 base pairs. Characteristic blebbing of the plasma membrane is observed. The apoptotic cell contents remain membrane-bound and are ingested by macrophages, which avoids an inflammatory reaction. Evasion of apoptosis by erythroid progenitors and precursors. 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 messaging system are the death receptor on the earliest RBC precursors, Fas, and its ligand, FasL, which is expressed by more mature RBCs.23,24 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 polychromatic normoblasts, cross-link with Fas-marked immature erythroid precursors, such as pronormoblasts, which are then stimulated to undergo apoptosis.23 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 EPOstimulated early release occurs, the younger Fas-positive precursors are allowed to develop, which increases the overall output of RBCs from the marrow. A second mechanism for escaping apoptosis exists for RBC progenitors: EPO rescue. This is the major way in which EPO is able to increase RBC production. When EPO binds to its receptor, one of the effects is to stimulate antiapoptotic molecules, which allows the cell to survive and mature.25 The cell that has the most EPO receptors and is most sensitive to EPO is the CFU-E, although the late BFU-E and early pronormoblast have some receptors.26 Without EPO, the CFU-E would not survive.27 EPO's effect is mediated by the transcription factor GATA1.28 The two molecules cooperate to promote the production of the anti-apoptotic molecule bcl-xL.25 EPO-stimulated cells develop this molecule on their mitochondrial membranes and are able to resist the FasL activation of apoptosis. CHAPTER 8 Erythrocyte Production and Destruction 97 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. The other effect of EPO is to increase the rate at which the surviving precursors can enter the circulation. This is like a military recruiter who recruits more young people to service and then puts them through a shortened boot camp training so that they are on the battlefield sooner. 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 many processes, especially hemoglobin production.29 The accumulation of hemoglobin is a factor that may control other cellular processes.30 RBCs will continue to cycle through division and maturation until a critical concentration of hemoglobin is achieved,30 typically after three to five divisions. Under the influence of EPO, the messenger RNA increases and the critical concentration of hemoglobin is achieved in two, rather than three, divisions. The cell is released from the marrow earlier than usual. Such cells are larger, due to a lost mitotic division, and do not have time before entering the circulation to dismantle the protein production machinery that gives the bluish tinge to the cytoplasm. These cells are true shift reticulocytes similar to those in Figure 8-8, A, recognizable in the stained peripheral blood film as especially large, bluish cells lacking central pallor. They also are called stress reticulocytes because they exit the marrow early during conditions of bone marrow stress, such as certain anemias. Conversely, if hemoglobin accumulates slowly, a cell may divide more times before achieving the critical hemoglobin concentration to shut off division (Box 8-4). BOX 8-4 Why Are Some Red Blood Cells Small or Large? The decreased volume and diameter of erythrocytes seen in iron deficiency anemia and the thalassemias can be explained by the role of hemoglobin (Hb) in controlling cellular processes. The cell continues the process of division and maturation until a critical concentration of Hb is reached.1 In those anemias in which Hb production is impaired and Hb accumulates more slowly, there is time for more divisions to occur before the critical concentration is reached. More divisions result in smaller cells. Anemias in which this occurs include iron deficiency anemia in which the iron deficit prevents production of the heme component of Hb and thalassemias in which globin production is impaired. The converse also would be true—when cells undergo fewer than three divisions, as is the case under the influence of increased levels of erythropoietin, larger cells result. This is consistent with what is seen as patients recover from hemorrhage and larger cells emerge from the bone marrow. Larger cells also result when DNA production is impaired, leading to fewer than normal numbers of divisions. This is the case with megaloblastic anemia. 1. Stohlman F: Kinetics of erythropoiesis. In Gordon AS, editor: Regulation of hematopoiesis, vol 1, New York, 1970, Appleton-Century-Crofts, pp 318-326. 98 PART II Hematopoiesis The process by which hemoglobin exerts this influence is probably as a transcription factor. The presence of hemoglobin in the nucleus has been recognized for decades,31 and its role as a transcription factor was hypothesized even before the concept of such influential molecules had been fully deduced.30 It seems that hemoglobin is transferred to the nucleus in proportion to its accumulation in the cytoplasm,31 and there it influences other cellular processes. EPO also can reduce the time it takes for cells to mature in the marrow by reducing individual cell cycle time, specifically the length of time that cells spend between mitoses.32 This effect is only about a 20% reduction, however, so that the normal transit time in the marrow of approximately 6 days from pronormoblast to erythrocyte can be shortened by only about 1 day by this effect. With the decreased cell cycle time and fewer mitotic divisions, the time it takes from pronormoblast to reticulocyte can be shortened by about 2 days. If the reticulocyte leaves the marrow early, another day can be saved, and the typical 6-day transit time is reduced to fewer than 4 days under the influence of increased EPO. Measurement of Erythropoietin. Quantitative measurements of EPO are performed on plasma and other body fluids. EPO can be measured by immunoassays of various types, including radioimmunoassay and chemiluminescence. The reference range is 5 to 30 mUnits/mL.1 Increased amounts of EPO are expected in the urine of most patients with anemia, with the exception of patients with anemia caused by renal disease. 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.33 Also, pituitary34 and thyroid35 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 in a dilated sinus area between the arterioles that feed the bone and the venous sinus that returns blood to efferent veins. Erythropoiesis typically occurs in what are called erythroid islands (see Figures 7-5 and 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. Although there is some evidence that macrophages may secrete some ferritin that normoblasts acquire,36 developing RBCs probably obtain most iron via transferrin (see Chapter 11), and no direct contact with macrophages is needed for this. Because macrophages do release ferritin, it is logical to assume that this could account for the intimate arrangement. However, a more credible explanation of the cellular arrangement has been established. Macrophages are now known to elaborate cytokines that are vital to the maturation process of the RBCs.37,38 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: (1) a stable matrix of stromal cells to which normoblasts can attach, (2) bridging (adhesive) molecules for that attachment, and (3) receptors on the erythrocyte membrane. The system is analogous to anchoring a ship, with the anchor corresponding to a stromal cell, the anchor cable corresponding to the adhesive molecules, and the cable post on the ship corresponding to the receptor. 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.36 At the same time, RBCs are anchored to the extracellular matrix of the bone marrow, chiefly by fibronectin.7 When it comes time for the RBCs to leave the marrow, they cease production of the receptors for the adhesive molecules.7 Without the receptor, the cells are free to move from the marrow into the venous sinus. Fibronectin appears to play an important role in the directional migration of red blood cells toward the sinus.8 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 epithelial 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 cells19 (Figure 8-11).39,40 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 senescence, which results in phagocytosis by macrophages. This is the major way in which RBCs die normally. CHAPTER 8 Erythrocyte Production and Destruction 99 Figure 8-12 Macrophage ingesting a sphered erythrocyte. (From Bessis M: Corpuscles, atlas of RBC shapes, New York, 1974, Springer-Verlag.) 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.) 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 speed 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 the 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. Spherical RBCs are rigid and are not able to squeeze through the narrow spaces; they become trapped against the endothelial cells and basement membrane that form the sieve. In this situation, the RBC is like a fleeing thief who is trapped against a fence at the end of an alley. There, it is readily ingested by macrophages that patrol along the sinusoidal lining (Figure 8-12). The mechanisms that signal macrophages to ingest senescent cells are currently under investigation. It is highly likely that there is no single mechanism, but rather that macrophages recognize several signals. Whether a single signal is sufficient to elicit ingestion or whether a cell must express more than one signal is uncertain. Those mechanisms receiving the greatest amount of research interest include binding of autologous immunoglobulin G (IgG) to band 3 clusters,41,42 exposure of phosphatidylserine on the exterior (plasma side) of the membrane,43 and inability to maintain cation balance.44 The macrophage is able to recognize these three and other characteristics of senescent cells that differentiate them from younger cells and thus targets the older cells 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 22. Mechanical Hemolysis (Intravascular Hemolysis) Although most natural RBC deaths occur in the spleen, a small portion of RBCs rupture, normally intravascularly (within the lumen of blood vessels). An RBC's trip around the vascular system can be traumatic, 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 trap and rupture (i.e., lyse) cells. A few cells lyse in the blood vessels from purely mechanical or traumatic causes; this is called 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 hemo globin so that its iron is not lost. Hemolysis and the functions of haptoglobin and hemopexin are discussed in Chapter 22. 100 PART II Hematopoiesis 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, polychromatic normoblast, orthochromic normoblast, and reticulocyte. • As erythroid precursors age, the nucleus becomes condensed and ultimately is ejected from the cell, which produces the reticulocyte stage. The cytoplasm changes color from blue, reflecting numerous ribosomes, to 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 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 cells of the kidney, which then produce EPO. • EPO, the primary hormone that stimulates the production of erythrocytes, is able to (1) rescue the CFU-E from apoptosis, (2) shorten the time between mitoses of precursors, (3) release reticulocytes from the marrow early, and (4) reduce the number of mitoses of precursors. • Apoptosis is the mechanism by which excessive production 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 younger cells. • EPO rescues cells from apoptosis by stimulating the production of antiapoptotic molecules that counteract the effects of Fas and FasL. • Survival of RBC precursors in the bone marrow depends on cytokines and adhesive molecules, such as fibronectin, which are elaborated by macrophages. RBCs are found in erythroid islands, where erythroblasts at various stages of maturation surround a macrophage. • As RBC precursors mature, they lose fibronectin receptors and can leave the bone marrow. Egress occurs between 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, membrane lipid changes, and cation balance changes. • Intravascular hemolysis results when mechanical factors breach 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 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. Polychromatic normoblast 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. Polychromatic normoblast 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 CHAPTER 8 Erythrocyte Production and Destruction 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 101 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 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. 9. Intravascular hemolysis is the result of trauma to RBCs while in the circulation. a. True b. False REFERENCES 1. Dessypris EN, Sawyer ST: Erythropoiesis. In Greer JP, Foerster J, Rodgers GM, et al, editors: Wintrobe's clinical hematology, ed 12, Philadelphia, 2009, Wolters Kluwer Health/Lippincott Williams & Wilkins, pp 106-125. 2. Shumacher HR, Erslev AJ: Bone marrow kinetics. 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Bookchin RM, Etzion Z, Sorette M, et al: Identification and characterization of a newly recognized population of highNa+, low-K+, low-density sickle and normal red cells. Proc Natl Acad Sci U S A 97:8045-8050, 2000. Energy Metabolism and Membrane Physiology of the Erythrocyte 9 Kathryn Doig and George A. Fritsma* OUTLINE OBJECTIVES Energy Production— Anaerobic Glycolysis Glycolysis Diversion Pathways (Shunts) After completion of this chapter, the reader will be able to: Hexose Monophosphate Pathway Methemoglobin Reductase Pathway Rapaport-Luebering Pathway RBC Membrane RBC Deformability RBC Membrane Lipids RBC Membrane Proteins Osmotic Balance and Permeability 1. List red blood cell (RBC) processes that require energy. 2. Discuss the Embden-Meyerhof anaerobic glycolytic pathway (EMP) in the erythrocyte, with attention to adenosine triphosphate generation and consumption. 3. Name three diversion pathways from the EMP within RBCs and state the function or purpose of each. 4. Describe the role of 2,3-bisphosphoglycerate in erythrocyte metabolism and the sacrifice made to produce it. 5. Name the components related to the hexosemonophosphate pathway that act to detoxify peroxide and the type of chemical process that accomplishes the detoxification. 6. Describe the methemoglobin reductase pathway that diverts from glycolysis. 7. Explain the importance of semipermeability of biologic membranes. 8. Discuss the arrangement of lipids in the RBC membrane. 9. Discuss the function of glycolipids in the RBC membrane. 10. Explain the concept of lipid exchange between the RBC membrane and the plasma, including factors that affect the exchange. 11. Define, locate, and provide examples of transmembranous versus skeletal membrane proteins of RBCs. 12. Discuss the general structure of spectrin and its function and arrangement in the membrane. 13. Discuss how ankyrin, protein 4.1, actin, adducin, tropomodulin, dematin, and band 3 interact with α- and β-spectrin and the lipid bilayer. 14. Describe the functions of transmembranous proteins as they affect RBC function. 15. Cite the relative concentrations of sodium and potassium inside RBCs and name the structure that maintains those concentrations. 16. Name conditions that develop from abnormalities of RBC membrane constituents. CASE STUDY After studying the material in this chapter, the reader should be able to respond to the following case study: Cyanosis is blue skin coloration, most obvious in whites, 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 and/or is distri buted 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.1 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 authors extend appreciation to Dr. John Griep, whose coverage of energy metabolism in the prior edition provided the foundation for that content in this chapter. 103 104 PART II Hematopoiesis T he red blood cell (RBC, erythrocyte) transports oxygen from the lungs to tissues and organs and helps to trans port carbon dioxide to the lungs to be exhaled. The mammalian RBC has evolved to do this quite efficiently but has sacrificed greatly for this purpose by losing its nucleus.2,3 The resulting RBC shape facilitates oxygen acquisition and release. However, the cell is left without the ability to synthe size replacement proteins for vital pathways of energy produc tion and membrane integrity, among others. As a result, its life span is limited to about 120 days because these processes inevitably fail. Mechanisms have evolved to maximize oxygen exchange during the life span of the cell and to counter the natural chemical processes that oxidize heme iron, which would leave it nonfunctional for oxygen transport. This chapter examines the energy production pathways of the RBC, dis cusses mechanisms for maintaining reduced heme iron and facilitating oxygen delivery, and explores the features of the RBC membrane that contribute to effective oxygen delivery for RBCs. ENERGY PRODUCTION— ANAEROBIC GLYCOLYSIS At the time the RBC ejects its nucleus, the cellular organelles are also packaged and ejected from the cell. Without mitochon dria, the RBC is left to rely on glycolysis for energy production. Fortunately, oxygen exchange and oxygen binding to heme iron do not require energy.4 Other RBC metabolic processes do require energy, however, and they are listed in Box 9-1. Loss of energy leads to RBC death. In fact, a hereditary deficiency of nearly every enzyme involved in glycolysis has been identified. A common result of these deficiencies is shortened RBC sur vival, known as hereditary nonspherocytic hemolytic anemia. Transmembranous cation gradient concentration proteins, called cation pumps, provide a particular example of how energy failure, even in otherwise normal cells, leads to cell death. The pumps maintain high concentrations of intracellular potas sium (K+), low concentrations of sodium (Na+), and very low concentrations of calcium (Ca2+) in contrast to extracellular concentrations of low K+ and high levels of Na+ and Ca2+. The pumps consume approximately 15% of RBC adenosine tri phosphate (ATP) production as they prevent adverse accumu lations of intracellular sodium and calcium. As energy-producing enzymes degrade over time, the pumps fail, Na+, Ca2+, and BOX 9-1 Erythrocyte Metabolic Processes Requiring Energy Maintenance of intracellular cationic electrochemical gradients Maintenance of membrane phospholipid Maintenance of skeletal protein plasticity Maintenance of functional ferrous hemoglobin Protection of cell proteins from oxidative denaturation Initiation and maintenance of glycolysis Synthesis of glutathione Mediation of nucleotide salvage reactions water flow in, and the RBC swells and is destroyed. This process is accelerated in hereditary nonspherocytic anemias in which the cells begin their life in the circulation with diminished enzyme concentrations. Plasma glucose enters the RBC through a facilitated mem brane transport system.5 Anaerobic glycolysis, the EmbdenMeyerhof pathway (EMP; Figure 9-1), requires glucose to generate ATP, a high-energy phosphate source that is the greatest reservoir of energy in the RBC. Energy reserves like glycogen are not available in RBCs, so the RBCs rely mostly on external glucose for glycolysis-generated ATP. Via the EMP, glucose is catabolized to pyruvate, 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 this information into three phases for better comprehension. The first phase of glucose catabolism involves glucose phosphorylation, isomerization, and diphosphorylation to yield fructose 1,6-bisphosphate (F-1,6-P). F-1,6-P serves as the substrate for TABLE 9-1 Glucose Catabolism: First Phase Substrates Enzyme Products Glucose, ATP G6P F6P, ATP F-1,6-P Hexokinase Glucose phosphate isomerase Phosphofructokinase Fructodiphosphate adolase G6P, ADP F6P F-1,6-P; ADP DHAP, G3P ADP, Adenosine diphosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; F-1,6-P, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; G3P, glucose 3-phosphate; G6P, glucose 6-phosphate. TABLE 9-2 Glucose Catabolism: Second Phase Substrates Enzyme Product G3P Glyceraldehyde-3-phosphate dehydrogenase Phosphoglycerate kinase Bisphosphoglyceromutase Bisphosphoglycerate phosphatase 1,3-BPG 1,3-BPG, ADP 1,3-BPG 2,3-BPG 3-PG, ATP 2,3-BPG 3-PG 1,3-BPG, 1,3 Bisphosphoglycerate; 2,3-BPG, 2,3 bisphosphoglycerate; 3-PG, 3-phosphoglycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; G3P, glucose 3-phosphate. TABLE 9-3 Glucose Catabolism: Third Phase Substrates Enzyme Product 3-PG 2-PG PEP, ADP Monophosphoglyceromutase Phosphopyruvate hydratase (enolase) Pyruvate kinase 2-PG PEP Pyruvate, ATP 2-PG, 2-Phosphoglycerate; 3-PG, 3-phosphoglycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate. CHAPTER 9 Energy Metabolism and Membrane Physiology of the Erythrocyte Embden-Meyerhof Pathway (Anaerobic Pathway of Glucose Metabolism) H2O2 H 2O 105 Hexose Monophosphate Pathway Glutathione peroxidase GSH Glucose Glutathione reductase ATP Hexokinase GSSG (–1 ATP) ADP NADP Glucose 6-phosphate NADPH Glucose-6-phosphate dehydrogenase 6-phospho-gluconate Glucose phosphate isomerase Pentose phosphate Fructose 6-bisphosphate ATP Phosphofructokinase (–1 ATP) ADP Fructose 1,6-bisphosphate aldolase Dihydroxyacetone Glyceraldehyde phosphate 3-phosphate Triose phosphate isomerase Glyceraldehyde 3-phosphate dehydrogenase NAD NADH 1,3-Bisphosphoglycerate Bisphosphoglycerate mutase Rapaport-Luebering Pathway ADP Phosphoglycerate kinase 2,3-Bisphosphoglycerate (2,3-BPG) Bisphosphoglycerate phosphatase ATP Methemoglobin reductase +H Methemoglobin reductase Methemoglobin Hemoglobin Methemoglobin Reductase Pathway (+2 ATP) 3-Phosphoglycerate Phosphoglyceromutase 2-Phosphoglycerate Enolase Phosphoenolpyruvate ADP Pyruvate kinase ATP (+2 ATP) Pyruvate NADH NAD Lactate Figure 9-1 Glucose metabolism in the erythrocyte. ADP, adenosine diphosphate; ATP, adenosine triphosphate; G6PD, glucose-6-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); NADP, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form). 106 PART II Hematopoiesis aldolase cleavage for the final product of phase 1 glycolysis: glyceraldehyde 3-phosphate (G3P; see Figure 9-1 and Table 9-1). Hexokinase and phosphofructokinase are rate-limiting in steady-state anaerobic glycolysis, even though hexokinase has the lowest activity of all the glycolytic enzymes. 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 summa rized in Table 9-2. During the first reaction step, G3P is phos phorylated with a high-energy phosphate and oxidized to 1,3-bisphosphoglycerate (1,3-BPG) through the action of glyceraldehyde-3-phosphate dehydrogenase (G3PD). 1,3-BPG is dephosphorylated by phosphoglycerate kinase, which gener ates ATP and 3-PG. The third phase of anaerobic glucose catabolism converts 3-PG to pyruvate with the generation of ATP. Substrates, enzymes, and products are listed in Table 9-3. The product 3-PG is isomerized by phosphoglyceromutase to 2-phosphoglycerate (2-PG). Enolase then converts 2-PG to phosphoenolpyruvate (PEP). Pyruvate kinase (PK) splits off the phosphates, forming ATP and pyruvate. PK activity is allosterically modulated by increased concentrations of F-1,6-P, which enhances the affinity of PK for PEP.5 Thus when the F-1,6-P is plentiful, increased activity of PK favors pyruvate production. Pyruvic acid may diffuse from the erythrocyte or may become a substrate for lactate dehydrogenase with regeneration of the oxidized form of nicotinamide adenine dinucleotide (NAD+). The ratio of NAD+ to the reduced form (NADH) may modify the activity of this enzyme. GLYCOLYSIS DIVERSION PATHWAYS (SHUNTS) Without organelles, RBCs take advantage of diversions or shunts from the glycolytic pathway to generate products that maximize oxygen delivery from several vantage points. The three diversions are the hexose monophosphate pathway (HMP) or aerobic glycolysis, the methemoglobin reductase pathway, and the Rapoport-Luebering pathway. Hexose Monophosphate Pathway Aerobic or oxidative glycolysis occurs through a diversion of glucose catabolism into the HMP, also known as the pentose phosphate shunt (see Figure 9-1). The HMP detoxifies accumu lated peroxide, an agent that oxidizes heme iron, proteins, and lipids, especially those containing thiol groups. Peroxide arises spontaneously from the reduction of oxygen in the cell’s aqueous environment.5 By detoxifying peroxide, the HMP extends the functional life span of the RBC. The HMP diverts glucose 6-phosphate (G6P) to pentose phosphate by the action of glucose-6-phosphate dehydrogenase (G6PD). In the process, oxidized nicotinamide adenine dinu cleotide phosphate (NADP+) is converted to the reduced form (NADPH). NADPH is then available to reduce the oxidized form of glutathione (GSSG) to its reduced form (GSH) using glutathione reductase. As the “thio” indicates, glutathione is a sulfur-containing compound and is, in fact, a tripeptide TABLE 9-4 Glucose Catabolism: Hexose Monophosphate Pathway Substrates Enzyme Product G6P 6-PG Glucose-6-phosphate dehydrogenase Phosphogluconolactonase Phosphogluconate dehydrogenase 6-PG R5P 6-PG, 6-Phosphogluconate; G6P, glucose 6-phosphate; R5P, ribulose 5-phosphate. containing cysteine. The designation GSH highlights the sulfur moiety. Once glutathione is reduced, it is available to reduce 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, the activity of the HMP may increase twentyfold to thirtyfold.6 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.7 Normal G6PD activity is able to detoxify oxidative compounds and safeguard hemoglobin, sulfhydryl-containing enzymes, and membrane thiols, allowing normally functioning RBCs to carry enormous quantities of oxygen safely. However, G6PD defi ciency is the most common human RBC enzyme deficiency worldwide resulting in hereditary nonspherocytic anemia (see Chapter 23). Methemoglobin Reductase Pathway Heme iron is constantly exposed to oxygen, an oxidizing agent.8 In addition, the accumulation of peroxide oxidizes heme iron from the ferrous to the ferric state, forming methe moglobin. Although the HMP is able to prevent some hemo globin 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 far more efficient in the presence of methemoglobin reductase, more properly called cytochrome b5 reductase (cytob5r). Using H+ from NADH formed when G3P is converted to 1,3-BPG, soluble cytochrome b5 reductase acts as an intermediate elec tron carrier, swiftly returning the oxidized iron to its ferrous, oxygen-carrying state. Cytochrome b5 reductase accounts for more than 65% of the methemoglobin-reducing capacity within the RBC.8 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 essen tially competing with oxygen for hemoglobin. When 2,3-BPG binds, 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 CHAPTER 9 Energy Metabolism and Membrane Physiology of the Erythrocyte immediately sacrifices the production of two ATP molecules via glycolysis. However, there is the loss of another two molecules of ATP 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 in ATP deficit by this diversion. The cell maintains a delicate balance between ATP generation to support the energy requirements of cell metabolism and the need to maintain the appropriate oxygenation/deoxygenation status of hemoglobin. Acidic pH and low concentrations of 3-PG and 2-PG inhibit the activity of bisphosphoglyceromutase, thus inhibiting 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 mainstream of glycolysis. In summary, these conditions favor generation of ATP by causing the conversion of more 1,3-BPG directly to 3-PG and also returning some 2,3 BPG to 3-PG for ATP generation downstream by PK. RBC MEMBRANE RBC Deformability RBCs are biconcave and average 90 fL in volume.9 Their average surface area is 140 µm2, 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 diam eter as they pass through narrow capillaries and through splenic pores 2 µm in diameter, a property called RBC deformability.10 The RBC plasma membrane, which is 5 µm thick, is 100 times more elastic than a comparable latex membrane, yet 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 oxygen from lungs to body tissue and carbon dioxide 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% (see Chapter 14 and inside front cover), and as hemoglobin concentration rises, viscosity rises.11 Hemoglobin concentrations above 36% compromise deformability and shorten the RBC life span, because the more viscous cells cannot accommodate to narrow capillaries or splenic pores. As RBCs age, they lose membrane surface area while retaining hemoglobin. The hemoglobin becomes more and more con centrated, and eventually the RBC, unable to pass through the splenic pores, is destroyed by splenic macrophages. Refer to Chapter 8 for a more complete discussion of RBC senescence theories. RBC Membrane Lipids Membrane elasticity (pliancy) is the third property that contrib utes to deformability. The RBC membrane consists of approxi mately 8% carbohydrates, 52% proteins, and 40% lipids.12 The lipid portion, equal parts of cholesterol and phospholipids, forms a bilayer universal to all animal cells (see Figure 13-9). Four main phospholipids form an impenetrable fluid barrier as their hydrophilic polar head groups are arrayed upon the 107 membrane’s surfaces, oriented toward both the aqueous plasma and the cytoplasm, respectively.13 Their hydrophobic nonpolar acyl tails arrange themselves to form a central layer dynamically sequestered from the aqueous plasma and cyto plasm. The membrane maintains extreme differences in osmotic pressure, cation concentrations, and gas concentra tions between the plasma external to the cell and the internal cytoplasm.14 Phospholipids reseal rapidly when the membrane is torn. Cholesterol, esterified and largely hydrophobic, resides paral lel to the acyl tails of the phospholipids, equally distributed between the outer and inner layers, and evenly dispersed within each layer, approximately one molecule per phospho lipid molecule. Cholesterol’s β hydroxyl group, the only hydro philic portion of the molecule, anchors within the polar head groups, while the rest of the molecule intercalates among and parallel to the acyl tails. Cholesterol confers tensile strength to the lipid bilayer.15 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. Defi ciencies in these enzymes are associated with membrane abnormalities such as acanthocytosis as the membrane loses tensile strength. 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 the phospholipids is energy dependent, relying on a number of membrane-associated enzymes, called flippases, floppases, and scramblases, for their position.16 When phospholipid distribution is disrupted, as in sickle cell anemia and thalassemia or in RBCs that have reached the end of 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 RBCs. Refer to Chapter 8 for a complete dis cussion of RBC senescence theories. Membrane phospholipids and cholesterol may also redistribute laterally so that the RBC membrane may respond to stresses and deform within 100 milliseconds of being chal-lenged 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 concentra tion also affects cholesterol exchange.17 In liver disease with low bile salt concentration, membrane cholesterol concentra tion becomes reduced. As a result, the more elastic cell mem brane shows a “target cell” appearance when the RBCs are spread on a slide (see Figure 18-1). Glycolipids (sugar-bearing lipids) make up 5% of the exter nal half of the RBC membrane.18 They associate in clumps or rafts and support carbohydrate side chains that extend into the aqueous plasma to help form the glycocalyx. The glycocalyx 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 108 PART II Hematopoiesis endothelium. Glycolipids may bear a few copies of carbohydratebased blood group antigens, for example, antigens of the ABH and the Lewis blood group systems. RBC Membrane Proteins Although cholesterol and phospholipids constitute the princi pal RBC membrane structure, transmembranous (integral) and skeletal (cytoskeletal, 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 trans membranous proteins. Of the purported 300 membrane pro teins, about 50 have been characterized and named, some with a few hundred copies per cell, others with over a million copies per cell.20 Transmembranous Proteins The transmembranous proteins serve a number of RBC func tions, 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 each other and to the vessel walls, promoting fragmentation (vesicu lation), reducing membrane flexibility, and shortening the RBC life span. Signaling receptors bind plasma ligands and trigger energy activation of submembranous G proteins that then initiate various energy-dependent cellular activities, a process called signal transduction. The transmembranous proteins assemble into one of two macromolecular complexes named by their respective skeletal anchorages, ankyrin or protein 4.1 (Figure 9-2). These com plexes and their anchorages provide RBC membrane structural integrity, because the membrane relies on the cytoplasmic skeletal proteins positioned immediately within (underneath) the lipid bilayer for its ability to retain (and return to) its biconcave shape despite deformability. The transmembranous proteins provide vertical membrane structure. Transmembranous 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 transmembranous proteins. Certain trans membranous proteins provide peptide epitopes. For instance, glycophorin A carries the peptide-defined M and N determi nants, and glycophorin B carries the Ss determinants, which together comprise the MNSs system.25,26 The Rh system employs two multipass transmembranous lipoproteins and a multipass glycoprotein that each cross the membrane 12 times.27-30 The two lipoproteins present the TABLE 9-5 Names and Properties of Selected Transmembranous RBC Proteins Transmembranous Protein Band Molecular Weight (D) Copies per Cell (×103) % of Total Protein 3 90,000-102,000 1200 27% 4.5 PAS-1 35,000-43,000 45,000-75,000 36,000 500-1000 5% 85% of glycophorins Glycophorin B PAS-4 20,000 100-300 10% of glycophorins Glycophorin C PAS-2 14,000-32,000 50-100 4% of glycophorins Aquaporin 1 Band 3 (anion exchanger 1) Ca2+ ATPase Duffy Glut-1 Glycophorin A ICAM-4 K+-Cl– cotransporter Kell Kidd Na+,K+-ATPase Na+-K+-2Cl– cotransporter Na+-Cl– cotransporter Na+-K+ cotransporter Rh RhAG Function Water transporter Anion transporter, supports ABH antigens Ca2+ transporter G protein–coupled receptor, supports Duffy antigens Glucose transporter, supports ABH antigens Transports negatively charged sialic acid, supports MN determinants Transports negatively charged sialic acid, supports Ss determinants Transport negatively charged sialic acid, supports Gerbich system determinants Integrin adhesion 93,000 46,000-50,000 Zn2+-binding endopeptidase, Kell antigens Urea transporter 30,000-45,500 50,000 D and CcEe antigens Necessary for expression of D and CcEe antigens; gas transporter, probably of CO2 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. CHAPTER 9 Energy Metabolism and Membrane Physiology of the Erythrocyte 109 Ankyrin complex Rh RhAG CD47 Band 3 Rh Glut 1 Dematin Adducin 4.1R Tropomyosin GPA LW Duffy Band 3 Band 3 GPA 4.1R complex Kell XK GPC -spectrin -spectrin p55 Protein 4.2 Ankyrin Tropomodulin Actin protofilament Spectrin–ankyrin 3 interaction Membrane surface Phospholipids Fatty acid chains Carbohydrates Ankyrin Integral protein Band 3 Actin Glycophorin β α Spectrin Peripheral protein Spectrin–actin 4.1 interaction Spectrin dimer–dimer association Band 4.1 Membrane cytoskeleton Lipid bilayer Figure 9-2 Representation of the human red blood cell membrane. The transmembranous proteins assemble in one of two complexes defined by their anchorage to skeletal proteins ankyrin and 4.1. Band 3 is the most abundant transmembranous 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 transmembranous 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. 110 PART II Hematopoiesis D and CcEe epitopes, respectively, but expression of the D and CcEe antigens requires the separately inherited glycopro tein RhAG, which localizes near the Rh lipoproteins in the ankyrin complex. Loss of the RhAG glycoprotein prevents expression of both the D and CcEd antigens (Rh-null) and is associated with morphologic RBC abnormalities. Additional blood group antigens localize to the 4.1 complex or specialized proteins.28-30 A few copies of phosphatidylinositol (PI), not mentioned in the RBC Membrane Lipids section, reside in the outer, plasmaside portion of the lipid bilayer. These serve as anchors for two surface proteins, 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 PI through a glycan core consisting of multiple sugars. CD55 and CD59 are linked to PI by phosphatidylinositol glycan class A (PIG-A). In paroxysmal nocturnal hemoglobin uria (see Chapter 23), PIG-A acquires a mutation, CD55 and CD59 become deficient, and the cell is susceptible to complement-mediated hemolysis. Numerical naming—for instance, band 3, protein 4.1, and protein 4.2—derives from historical (preproteomics) protein identification techniques that distinguished 15 membrane pro teins 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 molecu lar 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 mem brane to the skeletal 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 1 2 2.1 α-spectrin β-spectrin 2.2 Ankyrin 2.3 Adducin 3 Band 3 (anion exchanger) 4.1 4.2 Protein 4.1 Protein 4.2 4.9 5 Dematin, p55 Actin 6 G3PD 7 Stomatin, tropomyosin A B Globin Figure 9-3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of RBC membrane proteins stained with Coomassie blue bye. 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.) Skeletal Proteins The principal skeletal 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 lateral bonds.39 Antiparallel means that the carboxyl (COOH) end of one strand associates with the amino (NH3) end of the other. The spectrins form a hexagonal lattice (Figure 9-4) that is immediately adja cent to the cytoplasmic membrane lipid layer and provides lateral membrane stability.40 Because the skeletal proteins do not penetrate the bilayer, they are also called peripheral proteins. 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 (see Figure 9-4).35 A single helix at the amino terminus of α-spectrin consistently binds a pair of helices at the carboxy 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 Spectrin dimer bonds that appear along the length of the molecules disassociate and reassociate (open and close) during RBC deformation.44 Likewise, the 20 α-spectrin and 16 β-spectrin repeated helices unfold and refold. These flexible interactions plus the spectrin–actin–protein 4.1 junctions are key regulators of membrane elasticity and mechanical stability, and abnormalities in any of these proteins result in deformationinduced membrane fragmentation. For instance, autosomal dominant mutations that affect the integrity of band 3, RhAG, ankyrin, protein 4.1, or spectrin are associated with hereditary spherocytosis (see Chapter 23).45,46 In these cases there are too few vertical anchorages to maintain membrane stability. The lipid membrane peels off in small blebs called vesicles, whereas CHAPTER 9 Energy Metabolism and Membrane Physiology of the Erythrocyte 111 TABLE 9-6 Names and Properties of Selected Skeletal RBC Proteins Skeletal Protein α-spectrin β-spectrin Adducin Ankyrin Dematin F-actin G3PD Protein 4.1 Protein 4.2 (protein kinase) Tropomodulin Tropomyosin Band Molecular Weight (D) 1 2 2.9 2.1 4.9 5 6 4.1 4.2 5 7 240,000-280,000 220,000-246,000 80,000-103,000 206,000-210,000 43,000-52,000 42,000-43,000 35,000-37,000 66,000-80,000 72,000-77,000 41,000-43,000 27,000-38,000 Copies per Cell (×103) 240 240 60 120 40 400-500 500 200 200 30 80 % of Total Protein 16% 14% 4% 4.5% 1% 5.5% 3.5% 5% 5% — 1% Function Filamentous antiparallel heterodimer, primary cell skeleton Caps actin filament Anchors band 3 and protein 4.2 Actin bundling protein Binds β-spectrin Anchors 4.1R complex Anchors ankyrin complex Caps actin filament Regulates actin polymerization G3PD, Glucose-3-phosphate dehydrogenase glyceraldehyde; RBC, red blood cell. Spectrin dimer Actin Actin Band 3 Adducin Band 4.1 Band 4.1 A B Tropomyosin Glycophorin Junctional complex Spectrin dimer Ankyrin 100 nm Figure 9-4 Spectrin-based cytoskeleton on the cytoplasmic side of the human red blood cell membrane. A, Junctional complex composed of actin filaments containing 14 to 16 actin monomers, band 4.1, adducin, and tropomyosin, which probably determines the length of the actin filaments. B, Spectrin dimers form a lattice that binds band 3 and protein 4.2 (not shown) via ankyrin and band 3, Glut-1 and Duffy (not shown), and glycophorin via protein 4.1. the cytoplasmic volume remains intact. This generates sphero cytes with a reduced membrane-to-cytoplasm ratio. Conversely, hereditary elliptocytosis (ovalocytosis) arises from one of several autosomal dominant mutations affecting spectrin dimer-todimer lateral bonds or the spectrin–ankyrin–protein 4.1 junc tion.47 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.48 Osmotic Balance and Permeability The RBC membrane is impermeable to 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.49 Aquaporin 1 is a transmembranous protein that forms pores or channels whose surface charges create inward flow of water in response to internal osmotic changes. Glucose is transported without energy expenditure by the transmem branous protein Glut-1. Adenosine triphosphatase (ATPase)–dependent (energydependent) cation pumps (see Table 9-5) regulate the concentra tions of Na+ and K+, maintaining intracellular-to-extracellular ratios of 1:12 and 25:1, respectively.50,51 These enzyme-based pump mechanisms, in addition to aquaporin, maintain osmotic balance. Pump mechanism damage permits the influx of Na+, with water following osmotically. The cell swells, becomes spheroid, and eventually ruptures, spilling hemoglo bin. This phenomenon is called colloid osmotic hemolysis. 112 PART II Hematopoiesis Ca2+ ATPase extrudes calcium, maintaining exceptionally low intracellular levels of 5 to 10 µmol/L. Calmodulin, a cytoplasmic Ca2+-binding protein, controls the function of Ca2+ ATPase.52 Sickle cell disease provides an example of increased cation permeability. When crystallized sickle hemoglobin deforms the cells, internal levels of Na+, K+, and especially Ca2+ rise, which results in hemolysis.53 SUMMARY • Glucose enters the erythrocyte by facilitated membrane transport. • Glycolysis in the EMP occurs aerobically and anaerobically. • The primary reservoir of energy is ATP, which is generated by glycolysis. • Through anaerobic glycolysis in the EMP, glucose is metabolized to pyruvate, using two molecules of ATP per molecule of glucose and providing four molecules of ATP per molecule of glucose, for a net gain of two molecules of ATP. • By means of the Rapaport-Luebering pathway (a shunt off the EMP), 2,3-BPG is generated. It is necessary for the facilitation of oxygen delivery to the tissues. • The methemoglobin reductase pathway (also a bypass from the EMP) is able to convert oxidized heme iron (methemoglobin) to its reduced state, returning the molecule to its oxygen-carrying function. • Aerobic glycolysis occurs in the HMP, which converts glucose into pentose with the generation of NADPH. NADPH reduces glutathione, which then reduces peroxide to water and oxygen. Proteins, lipids, and heme iron are then protected from oxidation by peroxide. • The RBC membrane is a typical lipid bilayer with hydrophobic components oriented away from the water-rich plasma and cytoplasm. • The two lipid layers of the membrane differ in the composition of phospholipids. • The membrane provides a semipermeable barrier separating the plasma constituents from the cytoplasm, which allows the cell to maintain necessary intracellular differences. • Membrane cholesterol is restored from the plasma by an enzymeenhanced process of lipid exchange. • Abnormalities in membrane lipids lead to abnormal cell shapes, such as the “target cells” that develop with liver disease, which result from the changes in plasma bile salts. • The membrane proteins include the transmembranous (integral) proteins. These include ion channels and various receptors. • The proteins that localize to the interior surfaces of the membrane are called skeletal (peripheral) proteins. • Membrane proteins are extracted using sodium dodecyl sulfate, separated using polyacrylamide gel electrophoresis, and stained with Coomassie blue for visualization. They are numbered from the point of application; lower numbers correlate to high protein molecular weight and lower net charge. • PAS staining is used to localize electrophoresed proteins with high carbohydrate composition, which are designated as PAS-1 through PAS-4. • The shape and flexibility of the RBC, which are essential to its function, are related to the cytoskeleton. The cytoskeleton is derived from a group of peripheral proteins on the interior side of the lipid membrane. The major structural proteins are α- and β-spectrin, which are bound together and to the lipid membrane by ankyrin, actin, protein 4.1, adducin, tropomodulin, dematin, and band 3. • Abnormalities of the peripheral structural proteins such as spectrin and actin lead to abnormal RBC shapes. Hereditary spherocytosis and hereditary elliptocytosis are examples of diseases associated with abnormalities and deficiencies of structural proteins. • The concentration of potassium inside the RBC is higher than that in the plasma, whereas the sodium concentration is lower. This disequilibrium is maintained by the Na+-K+ pump in the membrane. Failure of the pump leads to an influx of sodium, with water following by osmosis, which results in cell swelling and possible lysis. Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented. R E V I E W Q UESTIONS 1. An RBC process that does not require energy is: a. Maintenance of intracellular cationic electrochemical gradients b. Oxygen transport c. Maintenance of skeletal protein plasticity d. Initiation and maintenance of glycolysis 2. ATP is generated by the: a. Embden-Meyerhof pathway b. Hexose monophosphate pathway c. Luebering-Rapaport pathway d. Aerobic glycolytic pathway CHAPTER 9 Energy Metabolism and Membrane Physiology of the Erythrocyte 3. Which of the following is true concerning 2,3-BPG? a. It enhances the release of oxygen from hemoglobin. b. It provides a source of glucose for the RBC. c. It is unnecessary for RBC survival. d. It is the least abundant of all organophosphates in the RBC. 113 9. The numbering system for membrane proteins gives the lowest number to proteins that are: a. Smallest b. Most negatively charged c. Largest d. Most positively charged 4. The activity of the hexose monophosphate pathway increases the RBC source of: a. Glucose and lactic acid b. 2,3-BPG and methemoglobin c. NADPH and reduced glutathione d. ATP and other purine metabolites 10. Which of the following is an example of an integral mem brane protein? a. Spectrin b. Glycophorin A c. Actin d. Ankyrin 5. The layer of the erythrocyte membrane that is largely responsible for the shape, structure, and deformability of the cell is the: a. Integral protein layer b. Exterior lipid layer c. Peripheral protein layer d. Interior lipid layer 11. Which of the following statements about spectrin structure is correct? a. Spectrin forms homodimers that twist into ropelike strands. b. Spectrin dimers associate into tetramers that are tightly bound to each other throughout their length. c. Seven spectrin tetramers join at their ends to create a lattice. d. The spectrin strands are connected to the lipid bilayer by ankyrin. 6. The glycolipids of the RBC membrane: a. Provide flexibility to the membrane b. Constitute ion channels c. Carry RBC antigens d. Attach the cytoskeleton to the lipid layer 7. The lipids of the membrane are arranged: a. In chains beneath a protein exoskeleton b. So that hydrophobic portions are facing the plasma c. In a hexagonal lattice d. In two layers that are not symmetric in composition 8. Lipid exchange between the RBC membrane and the plasma occurs: a. To replace lost lipids in the membrane b. To provide a mechanism for excretion of lipid-soluble RBC waste products c. To ensure symmetry between the composition of the interior and exterior lipid layers d. To provide lipid-soluble nutrients to the RBC 12. All of the following are functions of band 3 except: a. Providing a channel for anion movement through the membrane b. Contributing to membrane flexibility c. Attaching spectrin to the lipid bilayer d. Identifying senescent RBCs 13. Na+,K+-ATPase maintains a concentration of ions in which: a. Potassium is in higher concentration inside the RBC than in the plasma, and sodium is in lower concentration in the RBC than in the plasma b. Sodium and potassium are in higher concentration inside the RBC than in the plasma c. Sodium is in higher concentration inside the RBC than in the plasma, and potassium is in equilibrium with the plasma d. Potassium and sodium are in lower concentration inside the RBC than in the plasma REFERENCES 1. Percy MJ, Lappin TR: Recessive congenital methaemoglobi naemia: cytochrome b5 reductase deficiency. Brit J Haem 141:298-308, 2008. 2. Bull B: Morphology of the erythron. In Lichtman MA, Beutler E, Kipps TJ, et al, editors: Williams hematology, ed 7, New York, 2006, McGraw-Hill, pp 369–385. 3. Thelen MJ: The mature erythrocyte. In Greer JP, Foerster J, Rodgers GM, et al, editors: Wintrobe’s clinical hematology, ed 12, Philadelphia, 2009, Wolters Kluwer Health/Lippincott Williams & Wilkins, pp 126-155. 4. Beutler E: Disorders of red cells resulting from enzyme abnor malities. In Lichtman MA, Beutler E, Kipps TJ, et al, editors: 5. 6. 7. 8. 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Hemoglobin Metabolism 10 Mary Coleman OUTLINE OBJECTIVES Hemoglobin Structure After completion of this chapter, the reader will be able to: Heme Structure Globin Structure Complete Hemoglobin Molecule Hemoglobin Biosynthesis Heme Biosynthesis Globin Biosynthesis Hemoglobin Assembly Hemoglobin Ontogeny Hemoglobin Production Regulation Heme Regulation Globin Regulation Hemoglobin Regulation Hemoglobin Function Variant Hemoglobins Methemoglobin Sulfhemoglobin Carboxyhemoglobin Hemoglobin Measurement 1. Describe the primary structure of the globin chains found in hemoglobin. 2. Describe the quaternary structure of hemoglobin. 3. Describe the biosynthesis of heme and globin. 4. Differentiate steps in heme synthesis that occur in the mitochondria and the cytoplasm. 5. Identify the ontogeny of hemoglobin with emphasis on the hemoglobin of newborns and adults. 6. Identify the three types of normal hemoglobin in adults and their reference intervals. 7. Describe the regulatory effects of hemoglobin metabolism. 8. Identify the important role that hemoglobin plays in maintaining body functions. 9. Describe the mechanism by which hemoglobin carries oxygen to the tissue. 10. Describe the Bohr effect. 11. Explain the significance of the sigmoid shape of the oxygen dissociation curve. 12. Correlate right and left shifts in the oxygen dissociation curve with conditions that can cause shifts in the curve. 13. Identify the P50 value (amount of oxygen needed to saturate 50% of hemoglobin) as it pertains to a normal oxygen dissociation curve. 14. Differentiate T and R forms of hemoglobin. 15. Identify the source of production of 2,3- bisphosphoglycerate and describe its impact on hemoglobin oxygenation. 16. Identify the oxygen affinity of fetal hemoglobin. 17. Compare and contrast the composition of the chemically modified hemoglobins—methemoglobin, carboxyhemoglobin, and sulfhemoglobin—and their affinity for oxygen. 18. Compare and contrast oxygenated, deoxygenated, and oxidized hemoglobin and ferric versus ferrous iron. 19. Describe how hemoglobin is routinely measured in the laboratory. 20. Identify how different kinds of hemoglobins are identified by laboratory tests. 21. Identify the gene locations of the globins that make up the hemoglobin molecule, including the number of genes for each globin chain (for Hb A, A2, and F) and their general arrangement on chromosomes. CASE STUDY After studying the material in this chapter, the reader should be able to respond to the following case study: Hemoglobin and hemoglobin electrophoresis testing were performed on a mother and her newborn infant, both pre sumed to be healthy. The assays were part of a screening program to establish reference values. The mother’s hemo globin value was 14 g/dL (140 g/L), and the infant’s was 20 g/dL (200 g/L). The mother’s hemoglobin electrophoresis results were 97% Hb A, 2% Hb A2, and 1% Hb F. 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 results so different? 3. What is the difference between a hemoglobin test and the hemoglobin electrophoresis test? 4. Why were the mother’s and newborn’s hemoglobin electrophoresis results so different? HEMOGLOBIN STRUCTURE groups and two heterogenous pairs of polypeptide chains (Figure 10-1). Hemoglobin is the main cytoplasmic component of eryth rocytes (red blood cells, or RBCs). Free (non-RBC) hemoglo bin, generated from RBCs through hemolysis, has a short Hemoglobin (Hb) is the first protein whose structure was described using x-ray crystallography.1-4 The hemoglobin mole cule is a conjugated globular protein consisting of four heme 115 116 PART II Hematopoiesis 1 2 2 1 Heme Heme Heme 1 2 bonds and 2 1 bonds are located between the dimers, 1 1 bonds are in the front, 2 2 bonds are behind. 1 Heme 2 2 2 2 1 1 1 Figure 10-1 Hemoglobin: a tetramer of four heme molecules, each attached to a globin polypeptide chain. TABLE 10-1 Globin Chains = CH2 CH CH3 H C CH=CH2 CH3 N N CH Fe2+ HC N N CH3 CH3 C H CH2 HOOC • CH2 CH2 CH2 • COOH Figure 10-2 Heme is protoporphyrin IX that carries a central ferrous ion (Fe2+). half-life, is rapidly salvaged, and is catabolized or excreted renally. The concentration of hemoglobin within RBCs is approximately 34 g/dL, and its molecular weight is 64,000 D (Daltons). Hemoglobin’s main function is to transport oxygen from the lungs to tissues. Hemoglobin also modulates vascular dilation by transporting nitric oxide (NO) and transports carbon dioxide from the tissues to the lungs for exhalation. Heme Structure Heme consists of a ring of carbon, hydrogen, and nitrogen atoms called protoporphyrin IX with an atom of divalent ferrous iron (Fe2+) attached (ferroprotoporphyrin, Figure 10-2). Each of the four heme groups is positioned in a pocket of the poly peptide chain near the surface of the hemoglobin molecule. Each heme molecule combines reversibly with one oxygen molecule. Owing to its double bonds, heme renders blood red. 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 Symbol Name No. of Amino Acids α β γA γG δ ε ζ θ Alpha Beta Gamma A Gamma G Delta Epsilon Zeta Theta 141 146 146 (position 136: alanine) 146 (position 136: glycine) 146 Unknown 141 Unknown give rise to different types of polypeptide chains. Each chain is designated by a Greek letter (Table 10-1).5 Each globin chain is divided into eight helices and 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. The 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, sec ondary, 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. 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 asso ciation with the distal histidine residue in E7. The distal histi dine appears to swing in and out of position to permit the CHAPTER 10 Hemoglobin Metabolism C – Terminus TYR HIS 117 ALA PRO PRO LYS ALA GLN ALA HIS ALA GLN THR ALA GLY VAL ALA LYS VAL VAL TYR PHE VAL LEU 130 140 H GLU ASN 120 LYS G ASN GLU ARG LEU 100 PRO ASN PHE ASP VAL VAL N – Terminus LEU LEU THR PRO VAL ASP LEU VAL ASP HIS E GLY ALA ASP ASN 110 LYS ASP 70 Close spatial contact ALA LEU LEU VAL GLY LEU VAL GLU TYR GLU GLY ARG TRP VAL PRO B THR E7 (Distal histidine residue) ALA PHE SER GLY LEU GLY PHE VAL LEU HIS F8 (Proximal histidine) LEU 20 GLY HIS CYS GLU THR SER PHE GLY THR LEU HIS LYS LEU GLY LYS F ALA 90 80 ASN GLU LYS 10 SER ALA A VAL THR ALA TRP LEU LEU LEU LEU ALA HIS GLU HIS GLY VAL CYS VAL VAL LYS LYS GLY HIS ALA LYS C GLN PHE ARG PHE 40 GLU LYS VAL 60 PRO ASN GLY D VAL MET ALA ASP 50 THR PRO SER PHE GLY ASP LEU SER Figure 10-3 A β-globin chain: a polypeptide with helical and nonhelical segments. (From Huisman TH, Schroder WA: New aspects of the structure, function, and synthesis of hemoglobins, Boca Raton, Fla, 1971, CRC Press; modified from Stamatoyannopoulos G: The molecular basis of blood diseases, ed 2, Philadelphia, 1994, Saunders.) passage of oxygen into and out of the hemoglobin molecule. 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 oxygen molecules) or deoxygenated (not carrying oxygen molecules). The quaternary structure of hemoglobin, also called a tetra meric molecule, describes the complete hemoglobin molecule. The complete hemoglobin molecule is spherical, has four heme groups attached to four polypeptide chains, and may carry four molecules of oxygen. It is composed of two α globin chains and two non-α globin chains. Each globin chain has a heme group attached. Each heme molecule is capable of carry ing one molecule of oxygen. Strong α1,– non-α1 and α2,–non-α2 dimeric bonds hold the molecule in a stable form. Tetrameric α1–non-α2 and α2,–non-α1 bonds also contribute to the stabil ity of the structure (Figure 10-4).6,7 118 PART II Hematopoiesis Heme C α1 Heme F D F C B E E G α2 H G H B A D A D A H F B E A G G C E H B C Heme Heme F non−α2 D non−α1 Figure 10-4 Hemoglobin molecule illustrating tertiary folding and quaternary assembly. Heme is suspended between the E and F helices of the polypeptide chain. Pink represents α1 (left) and α2 (right); yellow represents non-α2 (left) and non-α1 (right). HEMOGLOBIN BIOSYNTHESIS Heme Biosynthesis Heme biosynthesis occurs in the mitochondria and cytoplasm of bone marrow RBC precursors, beginning with the pronor-moblast (also known as proerythroblast) through the circu lating polychromatic (also known as polychromatophilic) erythrocyte (see Chapter 8). As they lose their mitochondria and the citric/tricarboxylic acid cycle, mature RBCs can no longer make hemoglobin. Heme biosynthesis begins with the condensation of glycine and succinyl coenzyme A (CoA) catalyzed by aminolevulinate syn thase (ALAS) to form aminolevulinic acid (ALA). ALA dehydratase (also known as ALA dehydrase, porphobilinogen synthase) in the presence of ALA catalyzes the formation of porphobilinogen. Porphobilinogen deaminase, also known as hydroxymethylbilane synthase, in the presence of porphobilinogen catalyzes the formation of hydroxyl methylbilane. This pathway continues until, in the final step of production of heme, Fe2+ combines with protoporphyrin IX in the presence of ferrochelatase/heme synthase to make heme (Figure 10-5).6 Transferrin, a plasma protein, carries iron in the ferric (Fe3+) form to developing RBCs. Iron passes through the RBC mem brane to the mitochondria 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 control the synthesis of the six globin chains. The α and ζ genes are on chromosome 16; the γ, β, δ, and ε genes are linked on chromosome 11. In the human genome, there is one copy of each globin gene per chromatid for a total of two genes per person with the exception of α and γ. There are two copies of the α and γ genes per chromatid for a total of four genes per person. The production of globin chains takes place in RBC pre cursors from the pronormoblast through the circulating poly-chromatic (polychromatophilic) erythrocyte, but not in the mature RBC.8,9 Globin proteins arise via transcription of the genetic code to messenger ribonucleic acid (mRNA) and translation of mRNA to the globin polypeptide chain. A slight excess of α-globin mRNA is present in pronormoblasts (pro erythroblasts); however, β-globin mRNA is translated more efficiently than α-globin mRNA. This results in synthesis of sets of chains in approximately equal amounts. When syn thesized, the chains are released from the ribosomes in the cytoplasm.7 119 CHAPTER 10 Hemoglobin Metabolism MITOCHONDRION Glycine ALA synthase Succinyl CoA CYTOSOL ALA ALA dehydratase Porphobilinogen (PBG) PBG deaminase Hydroxymethylbilane Uroporphyrinogen III synthase Uroporphyrinogen III Uroporphyrinogen decarboxylase Protoporphyrinogen IX Coproporphyrinogen oxidase Coproporphyrinogen III Protoporphyrinogen oxidase Protoporphyrin IX Ferrochelatase Fe2 Heme Ribosomes Heme polypeptide chain Heme Non- polypeptide chain Non α Ribosomes Heme , non- dimer α Heme Non α Heme Heme α Non α Heme α Non α α 2, non-2 tetramer Heme Heme Heme Figure 10-5 Hemoglobin assembly begins with glycine and succinyl coenzyme A (CoA), which assemble in the mitochondria catalyzed by aminolevulinic acid (ALA) synthase to form ALA. In the cytoplasm, ALA undergoes several transformations to form coproporphyrin III, which, catalyzed by coproporphyrinogen oxidase, becomes protoporphyrinogen IX. In the mitochondria protoporphyrinogen IX is converted to protoporphyrin IX by protoporphyrinogen oxidase. Ferrous (Fe2+) ion is added, catalyzed by ferrochetolase to form heme. In the cytoplasm, heme assembles with α chain and non-α chain globins to form dimers and ultimately hemoglobin tetramers. Hemoglobin Assembly After their release from ribosomes, each globin chain binds to a heme molecule and they pair off. An α chain and a non-α chain combine to form heterodimers. Two heterodimers spon taneously combine to form tetramers. The tetrameric α1β2 and α2β1 bonds also contribute to the stability of the structure. This completes the hemoglobin molecule (see Figure 10-5).6,7 The combination of two α and two β chains along with the four heme molecules forms Hb A. This is the predominant hemoglobin in postnatal life. Hb A2 contains two α and two δ chains. The δ chains are inefficiently expressed; only small amounts of Hb A2 are found in the RBCs. Hb F contains two α and two γ chains. In adults, Hb F is distributed unevenly among RBCs; it is present in a few RBCs called F cells.7 The various globin chains affect the net negative charge of the hemoglobin molecule. In hemoglobin electrophoresis, hemoglobins under the influence of an electrical field exhibit varying mobilities, which allows differentiation of hemoglobin 120 PART II Hematopoiesis Birth Months Site of blood cell production 1 2 3 Yolk sac 4 5 6 7 8 2 Liver 4 6 8 10 Bone marrow Spleen A 50 40 Globin chain 30 synthesis % of total 20 α γ ζ ε β 10 δ Weeks 0 10 20 Intrauterine life 30 0 10 20 Birth 30 40 Infant B Figure 10-6 Timeline of globin chain production from intrauterine life to adulthood. See also Table 10-2. types. Various support media, buffer, and pH are employed for definitive identification (see Chapter 26). TABLE 10-2 Normal Hemoglobins Stage Globin Chain Hemoglobin ζ2 + ε2 α2 + ε2 ζ2 + γ2 α2 + γ2 Gower-1 Gower-2 Portland F α2 + γ2 α2 + β2 F, 60-90% A, 10-40% α2 + γ2 α2 + δ2 α2 + β2 F, 1-2% A2, 95% Intrauterine HEMOGLOBIN ONTOGENY Hemoglobin composition differs with prenatal gestation time and postnatal age. Hemoglobin changes reflect changes in the activation and inactivation or switching of the globin genes, progressing from the ζ to the α gene on chromosome 16 and from the ε to the γ, δ, or β genes on chromosome 11. The ζ and ε genes normally appear only during the first 3 months of embryonic development. These two chains in addition to the α and γ chains are constituents of embryonic hemoglobins (Figure 10-6). At birth, Hb F is the predominant hemoglobin. Normal adult hemoglobin is predominately Hb A (α2β2) with small amounts of Hb A2 (α2δ2) and Hb F (α2γ2).7 Table 10-2 presents adult reference intervals. A small percentage of Hb A is glycated. Glycation is a posttranslational modification formed by nonenzymatic binding of various sugars with globin chain amino groups. The most glycated hemoglobin is Hb A1c, in which glucose attaches to the N-terminal valine of the β chain. Normally, about 4% to 6% of Hb A circulates in the A1c form. In uncontrolled diabetes mellitus, the amount of A1c is increased. Other posttranslational modifications seem to be of little importance.7 Early embryogenesis (product of yolk sac erythroblasts) Begins in early embryogenesis; peaks during midgestation and declines rapidly just before birth Birth Adulthood HEMOGLOBIN PRODUCTION REGULATION Heme Regulation The key rate-limiting step in heme synthesis is the initial re action of glycine and succinyl CoA to form ALA, catalyzed by ALAS. Transcription of the ALAS gene is inhibited by heme, CHAPTER 10 Hemoglobin Metabolism Globin Regulation Globin production is regulated by the rate at which the deoxy ribonucleic acid (DNA) is transcribed to mRNA. The amount of the specific globins synthesized is proportional in general to the content of their individual globin mRNAs. Hemin—the Fe3+ oxidation product of heme—is important in controlling the rate of globin synthesis in intact polychromatic erythrocytes and various cell-free systems, and in its absence, an inhibitor of globin synthesis accumulates. Balanced globin chain and heme synthesis is important, because excess components of hemoglobin—unpaired chains, protoporphyrin, and iron— reduce RBC survival. Normal mature RBCs contain only com plete hemoglobin molecules; pools of free heme or globin chains are minute.7,9 Hemoglobin Regulation Hemoglobin synthesis is normally stimulated by tissue hypoxia. Hypoxia causes the kidneys to produce increased erythropoi etin, which stimulates the production of hemoglobin and RBCs. Although each laboratory must establish its own refer-ence intervals based on their instrumentation, methodology, and patient population, in general, reference intervals for hemoglobin are as follows: Men: Women: Newborns: 14 to 18 g/dL (140 to 180 g/L) 12 to 15 g/dL (120 to 150 g/L) 16.5 to 21.5 g/dL (165 to 215 g/L) 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 The function of hemoglobin is to readily bind oxygen mole cules in the lung, which requires high oxygen affinity; to trans port oxygen; and to efficiently unload oxygen to the tissues, which requires low oxygen affinity. During oxygenation, each of the four heme iron ions in a hemoglobin molecule can reversibly bind one oxygen molecule. Approximately 1.34 mL of oxygen is bound by each gram of hemoglobin. The affinity of hemoglobin for oxygen depends on the partial pressure of oxygen (PO2), often defined in terms of the amount of oxygen needed to saturate 50% of hemoglobin, 7.4 pH 40 pH B 60 7.2 pH My 80 7.6 og lob in 100 % O2 saturation which leads to a decrease in heme production (a negative feedback mechanism). Other enzymes in the heme pathway inhibited by heme are ALA dehydrase and porphobilinogen deaminase/hydroxymethylbilane synthase. An increased demand for heme induces an increased synthesis of ALAS.9 Ferrochelatase (also known as heme synthase) also plays a regulatory role in heme biosynthesis. A negative feedback mechanism by heme or substrate inhibition by protoporphyrin IX is believed to inhibit the ferrochelatase/heme synthase enzyme.9 121 P50 C A Hemoglobin 20 0 20 40 60 PO2 (mm Hg) 80 100 Figure 10-7 Oxygen dissociation curve. A, Normal dissociation curve. B, Left-shifted curve with reduced P50 caused by a decrease in 2,3-bisphosphoglycerate (2,3-BPG), partial pressure of carbon dioxide (PCO2), temperature, and H+ ions (raised pH). A left-shifted curve is also seen with hemoglobin variants that have increased oxygen affinity. C, Right-shifted curve with increased P50 caused by an elevation in 2,3-BPG, PCO2, temperature, and H+ ions (lowered pH). A right-shifted curve is also seen with hemoglobin variants that have decreased oxygen affinity. 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. Heme units do not undergo simultane ous oxygenation or deoxygenation; rather, the state of each heme unit in comparison with the other units influences further binding. That is, hemoglobin that is completely deoxygenated has little affinity for oxygen, but with each oxygen molecule that is bound, the avidity increases, and the hemoglobin mole cule quickly becomes fully oxygenated.7 Shifts of the curve to the left or right occur if there are changes in the pH of the blood. This shift in the curve as a result of pH is termed the Bohr effect. 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 left, 50% oxygen saturation of hemoglobin occurs at a PO2 of less than 27 mm Hg. If there is a shift of the curve to the right, 50% oxygen saturation of hemoglobin occurs at a PO2 higher than 27 mm Hg. The reference interval for arterial oxygen saturation is 96% to 100%. If the oxygen dissociation curve shifts to the left, a patient with arterial and venous PO2 concentrations in the refer ence intervals (80 to 100 mm Hg arterial and 30 to 50 mm Hg venous) will have a higher percent oxygen saturation and a higher affinity for oxygen than a patient for whom the curve is normal. With a shift to the right, a lower oxygen affinity is seen. Hemoglobin absorbs less oxygen in the lungs but delivers more oxygen to the tissues than it normally would, as in the presence of Hb S (see Chapter 26). 122 PART II Hematopoiesis 2 1 2 2 15° Heme O 2 2,3-BPG 1 O2 Heme 2 2 Hem e 1 Hem Heme 1 Heme e 1 O2 Heme O 2 2 1 1 Deoxygenated (tense, "T") state 2 1 2 Oxygenated (relaxed, "R") state Figure 10-8 Tense (T) and relaxed (R) forms of hemoglobin. The tense form incorporates one 2,3-bisphosphoglycerate (2,3-BPG) molecule and salt bridges and is unable to transport oxygen. The relaxed form develops as 2,3-BPG is released, the salt bridges are broken, and oxygen molecules are incorporated. The effect of 2,3-bisphosphoglycerate (2,3-BPG, formerly 2,3-diphosphoglycerate), on oxygen affinity is complex. The hemoglobin molecule is allosteric; that is, its function and structure are influenced by other molecules. In the presence of large amounts of 2,3-BPG, the hemoglobin molecule changes from a relaxed (R) oxygenated molecule to a tense (T) deoxy genated molecule. The T structure is stabilized by salt bridges, which become broken as the molecule switches to the R struc ture. When the salt bridges are broken, the hemoglobin mole cule is able to bind to oxygen (Figure 10-8). Carbon dioxide, hydrogen ions, and chloride ions all decrease the affinity of hemoglobin for oxygen by strengthening the salt bridges that lock the molecule into its T conformation. Some abnormal hemoglobins with a high oxygen affinity and low P50 occur as a result of amino acid substitutions that lead to loss of bonds that would have stabilized the tetramer in the T conformation. Without these binding sites, the hemoglobin molecule holds on more tightly to oxygen—hence a higher oxygen affinity.7 Clinical conditions that produce a shift 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 abnormal hemoglobins, carboxyhemoglobin, or some other hemoglobin variants. Conditions producing a shift of the curve to the right include increased body temperature, increased 2,3-BPG concentrations, increased H+ concentration (decreased pH), and abnormal hemoglobins with a low affinity for oxygen. Clinical conditions that produce a right shift include a high fever, acidosis, and circumstances that produce hypoxia, such as high altitude, pulmonary insufficiency, congestive heart failure, severe anemia, and cardiac right-to-left shunt.7 The sigmoidal oxygen curve generated by normal hemoglobin is in contrast with myoglobin's hyperbolic curve (see Figure 10-7). Myoglobin, present in cardiac and skeletal muscle, is a 17,000-D 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 that it is not as effective as hemoglobin in releasing oxygen to the tissues at physiologic 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 through the kidney, but levels may become elevated in renal failure. Testing for serum myoglobin aids in detecting myocardial infarction in patients who have no underlying trauma, rhabdomyolysis, or renal failure. An elevated myoglo bin level generates a positive result on the urine hemoglobin dipstick test that must be differentiated from a response caused by hemoglobin. In contrast to myoglobinuria, hemoglobinuria is seen in intravascular hemolytic disorders.7 Hb F (fetal hemoglobin, the primary hemoglobin in new borns) has a P50 of 19 to 21 mm Hg, which results in a left shift of the oxygen dissociation curve and increased affinity relative to that of Hb A. Consequently, fetal circulation extracts oxygen from maternal blood. If Hb F delivers oxygen less readily to tissues, however. To achieve adequate tissue oxygenation, fetal hemoglobin concentration is high. The concentration approaches adult values by 6 months as Hb F production is reduced. A second crucial function of hemoglobin is the transport of carbon dioxide. In the blood, carbon dioxide undergoes a pair of reactions in which it combines with water to form car bonic acid. Carbonic acid then disassociates to release H+ and bicarbonate: CO2 + H2O → H2CO3 H2CO3 → H+ + HCO3− The first of these reactions is facilitated by the RBC enzyme carbonic anhydrase. The H+ from the second reaction binds deoxygenated hemoglobin. The bicarbonate diffuses across the RBC membrane, and a portion is exchanged with plasma Cl−; this is called the chloride shift. The plasma bicarbonate travels to the lungs, where it is expired. About 70% to 85% of tissue carbon dioxide is processed in this manner.10 Approximately 10% to 20% of carbon dioxide binds to the N-terminal amino group of each globin chain as carbaminohemoglobin. Carb aminohemoglobin has a lower affinity for oxygen than does hemoglobin in the absence of carbon dioxide.11 CHAPTER 10 Hemoglobin Metabolism VARIANT HEMOGLOBINS The variant hemoglobins methemoglobin, sulfhemoglobin, and carboxyhemoglobin are hemoglobin variants whose structure has been modified by drugs or environmental chemicals. Methemoglobin Methemoglobin is a form of hemoglobin that contains iron in the oxidized or ferric state (Fe3+). Methemoglobin is contin ously being formed by spontaneous oxidation, but it fails to accumulate because several reducing enzyme systems restrict its concentration to less than 1% of total hemoglobin (see Chapter 9). Methemoglobin is brownish to bluish and does not revert to red upon oxygen exposure. Ferric iron cannot bind oxygen, but when one or more ferric ions are present the conformation of the molecule changes, and the oxygen affinity of the remain ing heme groups increases.12,13 Increased methemoglobin pro duces a shift to the left in the oxygen dissociation curve, so that oxygen is not delivered efficiently to the tissues. If metheмо globin comprises more than 30% of total hemoglobin, patients present with hypoxia and cyanosis.5,14 Elevated levels of methemoglobin are seen when oxidants such as nitrites are present or when there is decreased activity of methemoglobin reductase, a genetic deficiency. It is also seen in patients who inherit Hb M disease, which is caused by an abnormality in the structure of the globin portion of the hemoglobin molecule (see Chapter 26).15 Methemoglobin is assayed by spectral absorption analysis instruments such as the CO-oximeter. Methemoglobin shows a peak in the range of 620 to 640 nm at a pH of 7.1. Acquired methemoglobinemia may be treated by removal of the offending substance or administration of ascorbic acid or methylene blue.15 Sulfhemoglobin Sulfhemoglobin is formed by the irreversible oxidation of hemo globin by sulfonamides, phenacetin, acetanilide, or phenazo pyridine. It is created in vitro by the addition of hydrogen sulfide to hemoglobin and has a greenish pigment. Sulfhemoglobin is ineffective for oxygen transport, and patients with elevated levels present with cyanosis. Sulfhemoglobin cannot be converted to normal adult hemoglobin; it persists for the life of the cell. Treatment consists of prevention by avoidance of the offending agent. Sulfhemoglobin, like methemoglobin, shows a peak at 620 nm on a spectral absorption instrument. The sulfhemoglobin 123 spectral curve does not shift when cyanide is added, a feature that is used to distinguish it from methemoglobin.13 Carboxyhemoglobin Carboxyhemoglobin results from the combination of carbon mon oxide with heme iron. Although carbon monoxide binds hemo globin more slowly than oxygen, its affinity is 240 times that of oxygen and its release is 10,000 times slower. Carbon monoxide has been termed the silent killer because it is an odorless and colorless gas, and victims may quickly become hypoxic.5,13 Some carboxyhemoglobin is produced endogenously. The reference interval is 0.2% to 0.8%. Exogenous carbon monox ide is derived from the exhaust of automobiles and from indus trial pollutants, such as coal, gas, charcoal burning, and tobacco smoke. In smokers, levels may vary from 4% to 20%.5,13 Expo sure 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.16 Even when heating systems in the home are properly maintained, accidental poisoning with carbon monoxide may occur.17 Toxic effects, such as headaches and dizziness, are experienced at levels of 10% to 15%. Levels of more than 50% may cause coma and convulsions. Carboxyhemoglobin may be detected by spectral absorption instruments at 541 nm. It gives blood a cherry-red color, which is also imparted to the skin of victims. Hyperbaric oxygen therapy has been used for treatment. HEMOGLOBIN MEASUREMENT The cyanmethemoglobin method is the reference method for hemoglobin assay.18 A lysing agent present in the cyanmethemoglobin reagent, which converts the ferrous 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 concen tration, is measured at 540 nm spectrophotometrically and compared with a standard (see 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 (see Chapter 39). Hemoglobin electrophoresis is used to separate the different types of hemoglobins such as Hb A, A2, and F (see Chapter 26). SUMMARY • The hemoglobin molecule is composed of four heme groups (protoporphyrin IX + Fe2+) and two pairs of unlike polypeptide chains. Each heme molecule combines with one polypeptide chain. • Hemoglobin, contained in RBCs, carries oxygen to the tissue bound to the ferrous iron in heme. • Hemoglobin biosynthesis is regulated by hormones, oxygen tension in the kidneys, and enzymes in the heme synthesis pathway. • 2,3-BPG produced by the glycolytic pathway facilitates the delivery of oxygen from hemoglobin to the tissues. 124 PART II Hematopoiesis • The oxygen dissociation curve of hemoglobin is sigmoid owing to cooperativity among the hemoglobin subunits. • The Bohr effect is the influence of pH on the hemoglobin oxygen release mechanism. • The three hemoglobins found in normal adults are Hb A, Hb A2, and Hb F. Hb A, composed of two αβ heterodimers, is the predominant hemoglobin of adults. • Hemoglobin ontogeny describes which hemoglobins are produced by the body from the fetal period through birth to adulthood. • Chemically modified hemoglobins do not transport oxygen to the tissues well, which results in cyanosis. Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented. R E V I E W QUESTIONS 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. Transferrin mRNA synthesis c. Iron oxidase d. Protoporphyrin IX reductase 4. Which of the following forms of hemoglobin molecule has the lowest affinity for oxygen? a. Tense b. Relaxed c. Arterial d. Venous 5. Using the normal hemoglobin-oxygen dissociation curve in Figure 10-7 for reference, predict the position of the oxygen saturation curve for methemoglobin: a. Normal b. Shifted to the right of normal c. No change d. Shifted to the left of normal 6. The predominant hemoglobin found in a normal newborn is: a. Gower-1 b. Gower-2 c. A d. F 7. What is the distribution of normal hemoglobins in adults? a. 80% to 90% Hb A, 5% to 10% Hb A2, 1% to 5% Hb F b. 80% to 90% Hb A2, 5% to 10% Hb A, 1% to 5% Hb F c. >95% Hb A, 90% Hb A, 5% Hb F, 1% Hb A2 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 the term "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 in each cell 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 CHAPTER 10 Hemoglobin Metabolism 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 125 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 1. Perutz MF: Molecular anatomy, physiology and pathology of hemoglobin. In Stamatoyannopoulos G, Nienhuis AW, Leder P, editors: The molecular basis of blood diseases, Philadelphia, 1987, Saunders, pp 127-173. 2. Perutz MF, Rossman MG, Cullis AF, et al: Structure of hemoglobin: a three dimensional Fourier synthesis at 5.5A resolution obtained by x-ray analysis. Nature 185:416-422, 1960. 3. Perutz MF, Kendrew JC, Watson HC: Structure and function of haemoglobin: II. Some relations between polypeptide chain con?guration and amino acid sequence. J Mol Biol 13:669-678, 1965. 4. Perutz MF, Muirhead H, Cox JM, et al: Three dimensional Fourier synthesis of horse oxyhaemoglobin at 2.8 Å resolution: the atomic model. Nature 219:131-139, 1968. 5. 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In Lichtman MA, Beutler E, Kipps TJ, et al, editors: Williams hematology, ed 7, New York, 2006, McGraw-Hill Professional, pp 11-12. 11 Iron Metabolism Mary Coleman OUTLINE OBJECTIVES Dietary Iron Iron Absorption and Excretion Iron Cycle and Transport Iron Storage Laboratory Assessment of Iron Metabolism After completion of this chapter, the reader will be able to: 1. Discuss the role of iron as an essential nutrient for human survival. 2. List the sites in which iron is distributed in the body and state the approximate amount at each site. 3. State the minimum daily intake of iron required at various ages for men, women, and children. 4. Describe the mechanism of iron absorption and distinguish between the absorption of heme and nonheme iron. 5. State the site of iron absorption. 6. Describe transferrin, transferrin receptor, hepcidin, hemosiderin, and ferritin, including function and regulation. 7. Diagram the transport of iron from ingestion to incorporation into heme. 8. Define sideroblast, siderocyte, and siderosome. 9. Discuss regulation, excretion, transport, and storage of iron in the body. 10. Identify and discuss the laboratory tests currently used to assess iron status in the body. 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. The measured Hct, Hb, serum ferritin, and transferrin saturation in samples from the donors, who gave an average of 15 units (approximately 485 mL) of blood over 3 1 2 years. The investigators collected comparable data from nondonors. Of the donors, 10 women and 6 men took a dietary supplement providing approxi mately 20 mg of iron per day. In addition, mean iron dietary intake was 16.4 mg/day for the women and 19.9 for the men. Over the period of the study, mean iron stores in women decreased from 12.53 to 1.14 mg/kg of body weight. Mean iron stores in men declined from 12.45 to 1.92 mg/kg. Nondonors' iron stores remained unchanged. Based on Hb and Hct results, no donors became anemic. There was no I ron in its cationic bivalent ferrous (Fe2+) and trivalent ferric (Fe3+) states is essential for the life of all organisms, plant and animal.1 In humans, 70% of total body iron is trans ported in blood noncovalently bound in the ferrous state to the heme portion of hemoglobin, where it binds, transports, and releases oxygen (O2, Table 11-1).2 In mitochondria, ferrous iron is transferred to protoporphyrin IX (see Figure 10-5) to form heme. Four heme molecules become bound, one each, to four 126 statistically significant difference in iron stores between the men who took supplements and those who did not, although a difference was seen for the women. Total iron losses over 80 days, the average interval between donations, were calcu lated to be 4.32 mg/kg for the women and 3.93 mg/kg for the men. As iron stores decreased, the calculated iron absorp tion rose to 3.55 mg/day for the women and 4.10 mg/day for the men. 1. Why did the donors' iron stores decrease? 2. What is the average daily iron absorption rate? 3. Why did the donors' absorption rate rise? 4. List the laboratory tests performed on serum that are used to evaluate iron stores. 5. What are the reference intervals for these iron study tests? 6. What does each test result indicate about iron stores? globin chains to produce tetrameric hemoglobin. Myoglobin, present in muscle cells, is a single-chain heme protein. Iron likewise binds to myoglobin, the O2 transport molecule in muscle. The primary and secondary structures of myoglobin resemble hemoglobin, however, myoglobin is monomeric, and myoglobin oxygen binding is irreversible. Approximately 5% of total human body iron is bound to myoglobin. Ferritin and hemosiderin are the storage forms of iron, con taining 25% of human body iron, and are distributed to the CHAPTER 11 Iron Metabolism TABLE 11-1 Iron Compartments in Normal Humans Compartment Hemoglobin iron Storage iron: hemosiderin, ferritin Myoglobin iron Other compartments: Peroxidase, catalase, cytochromes, riboflavin enzymes Percent of Total Body Iron ~65% ~25% ~5% ~0.1% ~3.6% Iron Content (mg/kg Body Weight) 2.6 14.2 1.0 0.003 0.140 liver and bone marrow in hepatocytes and macrophages. Plasma ferritin protein concentration assays are employed clinically to assess the adequacy of iron stores. Less than 1% of iron is transported through plasma in the ferric state bound to transferrin. Plasma iron and transferrin saturation assays may be used clinically to diagnose iron deficiency. Finally, heme-bound iron is essential to the mitochondrial cytochrome P-450 (CYP 450) system in all animals. Also known as cytochrome oxidase, this enzymatic system is bound to mitochondrial membranes and supports oxidation-reduction reactions such as the hydroxylation of organic molecules from free oxygen. Cytochrome oxidase iron cycles between the ferrous and ferric state as it facilitates electron transfer. Less than 1% of human body iron is located in the CYP 450 system. Iron exists only transiently as a free cation; it is normally bound by or incorporated into various proteins. Because of its catalytic properties in single-electron oxidation-reduction reactions, free iron forms oxygen radicals that can damage cellular proteins and lipids. The concentration of storage and transport iron is con trolled by dietary intake and iron loss through bleeding. Iron deficiency occurs when there is inadequate intake or excessive blood loss, causing anemia. Iron overload results from increased absorption owing to genetic predisposition or repeated blood transfusions and pro duces potentially fatal heart and liver disease. Evolution has given humans a mechanism for absorbing dietary iron effi ciently but not for eliminating excess iron effectively. Disorders of iron metabolism are discussed in Chapter 19. DIETARY IRON Although many foods have high iron content, the iron may be minimally bioavailable. The bioavailability of iron depends on its chemical form and the presence of non-iron foods that promote or inhibit absorption. An average American diet may provide 10 to 20 mg of iron/day, but only 1 to 2 mg/day is absorbed. Iron is absorbed in two forms: heme and nonheme. Heme-bound iron, mainly from meat, is absorbed more efficiently than nonheme, inorganic iron and in a different manner.3 Heme iron is present in hemoglobin, myoglobin, 127 and heme-containing enzymes. Approximately 5% to 35% of heme iron is absorbed as hemin (iron-containing porphyrin). Nonheme iron, found in nonmeat sources such as legumes and leafy vegetables, accounts for approximately 90% of dietary iron, but only 2% to 20% of it is absorbed, depending on the iron status of the individual and the ratio of dietary enhancers and inhibitors.1 Ascorbate, citrate, and other organic acids and amino acids enhance absorption of nonheme iron by the formation of soluble chelates. Cooking in iron pots increases the amount of iron consumed. Substances that interfere with nonheme iron absorption include phytates, polyphenols, phosphates, oxalates, and calcium.4,5 Dietary iron may be supplemented with tablets or multi vitamins that supply ferrous sulfate. Since the 1940s, infant formula and some cereals have been fortified with iron in the United States. Iron supplementation should be targeted to populations that are at risk for iron deficiency, because the potential for iron overload exists in individuals with adequate iron status. IRON ABSORPTION AND EXCRETION The duodenum and upper jejunum are sites of maximal absorp tion of iron. For transport of oxygen in Hb, iron must be in the ferrous form (Fe2+). To be absorbed from food, iron must be in the form of heme iron (Fe2+) or converted from ferric nonheme iron to the soluble ferrous form by a duodenumspecific cytochrome b–like protein, DCYTB.6 Uptake of heme iron occurs on heme carrier protein 1, located on the apical membrane of the duodenal enterocyte.7 Heme iron binds to the enterocyte in the mucosal epithelium and is internalized (Figure 11-1). Here the enzyme heme oxygenase degrades heme to produce ferrous iron, carbon monoxide, and bilirubin-IXa. Ferrous iron is transported across the duodenal epithelium bound to the apical divalent metal transporter 1 (DMT1). The ferrous iron is carried to the basolateral membrane (base and sides of the enterocyte membrane), from which it is exported to the portal circulation, a process mediated by ferroportin, a basolateral transport protein. Ferroportin works in conjunction with a copper-containing iron oxidase known as hephaestin. Hephaestin may facilitate iron egress by reoxida tion of ferrous to ferric iron.8 The ferric iron becomes bound to transferrin for transport through the circula tion. Some iron remains in the enterocytes as ferritin and is released to the circulation over a few hours. Enterocytestored ferritin iron is excreted when the cells are exfoliated in the stool. Hepcidin, an antimicrobial peptide produced in the liver, seems to act as a negative regulator of intestinal iron absorption and iron release from macrophages. Hepcidin binds to the ferroportin receptor, causing degradation of fer roportin and trapping iron in the intestinal cells. Hepcidin synthesis rises when transferrin is carrying its maximum capac ity of iron (transferrin saturation of more than 50% in females or 60% in males), and diminishes when iron is unavailable.7-9 The role of hepcidin in the anemia of chronic inflammation is discussed in Chapter 19. 128 PART II Hematopoiesis Body Location Lumen of duodenum Enterocyte mucosal membrane Function Type of iron: Heme or Non-heme Iron from food Hemoglobin or myoglobin Non-heme iron (Fe3+) + chelator (e.g., ascorbate) Conversion to absorptive form Heme DCYTB Receptor Membrane transporter Unknown (Fe2+) Divalent metal transporter Heme Heme oxygenase Enterocyte cytoplasm Enterocyte basolateral membrane Iron stored as ferritin or transported to plasma Membrane transporter Oxidizer Fe2+ + bilirubin + CO Fe2+ Fe2+ Ferroportin Hephaestin Fe3+ (aPo) Transferrin Plasma of portal blood vessels Carried in plasma to hepatocytes, macrophages, developing RBCs, etc Divalent transferrin Figure 11-1 Simplified version of the mechanism for absorption of iron in the small intestine. aPo, (Apo) transferrin; CO, carbon monoxide; DCYTB, duodenum-specific cytochrome b–like protein; RBC, red blood cell. Transferrin transports ferric iron (Fe3+) to hematopoietic and other tissues, where it is bound by cell membrane transferrin receptors (Figure 11-2). Transferrin receptors are expressed in larger amounts on normoblasts and rapidly dividing cells, whether normal or malignant, but not on highly differentiated cells such as reticulocytes.10 Transferrin is taken into the cell by endocytosis. An endosome forms containing the iron-loaded transferrin and its receptor. Iron is released from transferrin by acidi fication of the endosome to a pH of 5.5. Iron is transported across the endosomal membrane by DMT1 and used in the synthesis of iron-containing proteins. Excess iron is stored as ferritin or hemosiderin (see section on iron storage).6 Humans have no effective means to excrete iron. Instead we regulate iron by controlling absorption. The amount of iron absorbed is inversely proportional to iron stores and the rate of erythropoiesis. As stated previously, normal absorption of iron is 1 to 2 mg/day. With decreased iron stores, iron absorp tion may increase to 3 to 4 mg/day. In iron overload, only 0.5 mg/day is absorbed. The body conserves iron extremely well, losing only about one one-thousandth of the total body iron content. This amount is replaced if dietary sources are adequate. Normal iron losses occur mainly by way of the feces and amount to about 1 mg/day. Perspiration and exfoliation of the skin and dermal appendages result in minimal losses. Lactation and menstruation result in an additional loss of about 1 mg/d.11 IRON CYCLE AND TRANSPORT Iron is absorbed from the gastrointestinal tract and transported via the circulation to the bone marrow, where it is inserted into protoporphyrin IX in the mitochondria of the erythroid CHAPTER 11 Iron Metabolism Location Form of Iron Transferrin + Fe3+ Plasma Transferrin receptor (TfR) on cell membrane of marrow erythroblasts Transferrin + Fe3+ at pH ~ 5.5 Iron released and used to synthesize iron-containing protein such as Hb Hb production (normoblast Circulation reticulocytes) Older reticulocytes and mature RBCs Macrophages 120-day-old RBCs Iron + protoporphyrin IX + globin Plasma Transferrin + Fe3+ Figure 11-2 Cycling of an iron molecule through the body after it has been absorbed in the plasma. Iron is incorporated into marrow red blood cell (RBC) precursors, released into the circulation for the life of the RBC, and sent back to the plasma, bound to transferrin. Hb, Hemoglobin. precursors to make heme (refer to Figures 10-2 and 10-5; see also Figure 11-2). Hb synthesis is completed in the reticulocyte stage. Iron circulates in red blood cells (RBCs) in the ferrous form noncovalently bound to the heme molecule. Iron from senescent RBCs is turned over to macrophages and reused.6 Ferrokinetics involve transferrin, transferrin receptor, and ferritin. These are regulated by iron-responsive protein (IRP). The genes for these three principal proteins have been located and sequenced.12 Researchers may someday be able to modify these genes to treat hereditary iron disorders. Most plasma transferrin is produced by hepatocytes. The major function of transferrin is to transport iron from the enterocytes of the duodenum to transferrin receptors on marrow erythroblasts. Transferrin has a half-life of 8 days and migrates to the β fraction in serum electrophoresis. The amino terminus and carboxy terminus each independently bind a ferric ion. A bicarbonate ion "locks" the iron in place within transferrin by serving as a bridging ligand between the protein and iron. The transferrin molecule can exist as a single-chain glycoprotein with no iron attached, called apotransferrin, or in a monoferric or diferric form.13 The transferrin gene is located on the long arm of chromosome 3, at 3q21-qter (Figure 11-3).12 The transferrin receptor is a glycoprotein dimer, and it is located on virtually all cells except mature RBCs. It provides transferrin-bound iron access into the normoblast; it also plays a crucial role in the release of iron from transferrin within the cell. It is present in large numbers on normoblasts, in the placenta, and in the liver. The transferrin recep tor can bind two molecules of transferrin. The transferrin recep tor's affinity for transferrin depends on the iron content of transferrin and the pH. At high levels of transport iron and a pH of 7.4, the transferrin receptor selectively binds diferric transferrin in preference to monoferric transferrin or apotransferrin. The transferrin receptor gene is located near the transferrin gene at chromosome band 3q26.2-qter (see Figure 11-3).12 Control of transferrin receptor biosynthesis is a major mechanism for regulation of iron absorption in normoblasts. When transferrin is fully saturated, iron is deposited in the liver. When transferrin is congenitally absent, iron is absorbed by the intes tine and deposited in the liver, pancreas, spleen, and other tissues.14 When it is absent, iron stores are severe hypochromic microcytic anemia results.12 13 130 PART II Hematopoiesis iron (see Chapter 10).6,11 There is evidence that, in addition to regulation at the mRNA level, transcriptional regulation of iron metabolism also occurs.11 IRON STORAGE p q 26.1 Transferrin receptor gene 3q26.2-qter 26.2 26.3 27 28 29 Transferrin gene 3q21-qter Chromosome 3 Figure 11-3 Location of the transferrin and transferrin receptor genes on chromosome 3. Cellular uptake of iron is mediated largely by interaction of the transferrin receptor and the transferrin molecule. The ferric iron–transferrin receptor complex is endocytosed, iron is released into the cell, and the receptor-transferrin complex is returned to the cell surface, where the transferrin is released for reuse. Iron enters a "chelatable" soluble pool in the cell, where it is used for synthesis of cellular constituents or for deposition as ferritin, a nontoxic storage form of iron. IRPs are messenger ribonucleic acid (mRNA)-binding pro teins that coordinate the intracellular expression of transferrin receptor, ferritin, and other proteins important for iron meta bolism. IRPs bind to iron-responsive elements (IREs), which are stem-loop mRNA structures. IRPs bind IREs when iron supply is decreased and release them when iron supply is increased. When there is little intracellular iron, IRPs regulate the increase of the translation and stability of the mRNA for transferrin receptor and aminolevulinic acid synthase and decrease the translation of apoferritin mRNA. This ensures that the number of molecules of transferrin receptor on the cell membrane, while decreasing the ferritin trapping of iron that enters the cell. More Hb can be formed if there is more iron uptake by the cell. At the same time, an increase in production of aminolevulinic acid synthase ensures that enough protopor phyrin can be made to accommodate the expected increase in Iron is stored in accessible reserve form as ferritin or as the partially degraded or precipitated form of ferritin called hemosiderin. Apoferritin, the protein component of the ferritin molecule without the iron, is an empty sphere 12 nm in dia meter and 1 nm thick and is composed of 24 light (L) and heavy (H) subunits. Within the apoferritin shell, ferric ions, hydroxyl ions, and oxygen are bound to form ferritin. The liver and spleen, which have major iron storage deposits, have a large amount of L subunits. Tissues such as heart tissue that do not normally store ferritin have a higher proportion of H sub units. Genes for the H and L chains belong to multigene families with members on several chromosomes. The gene for two types of L chains is on chromosome 19; the gene for heavy chains is on chromosome 11.12 Hemosiderin is a degradation product of ferritin produced by the partial digestion of protein and release of iron micelles, which form insoluble ferritin aggregates. Most stored iron is soluble ferritin, but as iron stores increase, so does the propor tion of hemosiderin in relation to ferritin. Ferritin and hemosiderin stores are found in the liver, bone marrow, spleen, and muscle, but most in the liver. When iron is needed from ferritin stores, it is returned to transferrin to be used by the cells that need the iron for metabolism. LABORATORY ASSESSMENT OF IRON METABOLISM Reference intervals for laboratory tests of iron status are pro vided but are meant to be a guide only, because values may differ depending on the assessment method used (Table 11-2). A single assay may not provide sufficient iron status informa tion. An understanding of what the tests measure and what variations can occur because of diurnal variation and clinical status is helpful in interpreting the significance of the test result. Serum iron concentration is a measure of Fe3+ bound to serum transferrin and does not include free serum Hb iron. Serum iron concentration is measured using chromogen spectropho tometry. Iron exhibits diurnal variation, being at its highest concentration in the morning and at its lowest in the evening. Serum iron concentration is decreased not only in iron deficiency but also in inflammatory disorders, in acute infection, after certain immunizations, and after myocardial infarction. Only about one third of transferrin iron-binding sites are normally occupied by Fe3+; two thirds of the iron-binding sites do not carry iron, and these sites are referred to collectively as the serum unsaturated iron-binding capacity (UIBC). The total of available sites is referred to as the total iron-binding capacity (TIBC). UIBC is measured using chromogen spectrophotometry. TIBC can be measured indirectly by chemical means and CHAPTER 11 Iron Metabolism TABLE 11-2 Assessment of Body Iron Status Laboratory Assay Adult Reference (Intervals) Diagnostic Use Serum ferritin level Serum iron level Serum transferrin level (TIBC) Transferrin saturation Serum transferrin receptor (sTfR) sTfR-F index 12-300 mcg/L 10-30 μmol/L 47-70 μmol/L Indicator of iron stores Indicator of tissue iron supply Female: 16-50% Male 16-60% 1.15-2.75 mg/L Indicator of tissue iron supply RBC zinc protoporphyrin Bone marrow or liver biopsy Bone marrow sideroblast count 6000/mm3 1 : 200 in isotonic saline 5 mL Undiluted Dilute with saline to 100-200/mm3 nucleated cell count Dilute by nucleated cell count; if RBC count ≥ 1 million/mm3, make a push smear and differential cells that are pushed out on the end 3 RBC, WBC, blood cell; WBC, white blood cell. *Expected cell yield (WBC count for no. of cells recovered on slide): 0/mm3 for 0-70, 1-2/mm3 for 12-100, >3/mm3 for >100 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. The number of cells recovered is small, the area covered in 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 (see Figure 17-1). 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.6,7 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 a lubrication for the central nervous system. Figure 17-1 Wright-stained cytocentrifuge slide showing a concentrated button of cells within a marked circle. (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) to perform the differential. In this case, the differential count 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 5 drops of fluid (undiluted or diluted) is 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/mm3. 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 Gross Examination Normal CSF is nonviscous, clear, and colorless. A cloudy or hazy appearance may indicate the presence of WBCs (greater than 200/mm3), RBCs (greater than 400/mm3), or micro organisms.6,7 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 of the fluid. Tubes usually are numbered in order of collection. If blood is present because of a traumatic puncture, the first tube will contain the most 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 tap, whereas a yellowish or pinkish yellow tinge may indicate a subarachnoid hemorrhage. This yellowish color sometimes is referred to as xanthochromia, but this term also may be used because CHAPTER 17 Body Fluids in the Hematology Laboratory 229 CSF Gross appearance Cloudy Bloody Yellow Clear Report Spin and report Supernatant appearance Cell counts Clear Cloudy or bloody Count Extremely turbid Report Tube diluted Türk (1:2) or (1:20) Cytospin slide RBC (1:200) Report RBC Report WBC Figure 17-2 Flow chart for examination of cerebrospinal fluid (CSF). RBC, Red blood cell; WBC, white blood cell. (Modified from Kjeldsberg CR, Knight JA: Body fluids, ed 3, Chicago, 1993, ASCP Press; reprinted with permission.) TABLE 17-2 Characteristics of Cerebrospinal Fluid Traumatic Tap Pathologic Hemorrhage Clear supernatant Clearing from tube to tube Bone marrow contamination Cartilage cells Colored or hemolyzed supernatant Same appearance in all tubes Erythrophages Siderophages (may have bilirubin crystals) RBC, Red blood cell. 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 17-2 and Table 17-2).5,6 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. Normal cell counts in CSF are 0 to 5 WBCs/mm3 and 0 RBCs/mm3. If a high RBC count is obtained, one may determine whether the source of WBCs is peripheral blood contamination by using 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: WBCB × RBCCSF ÷ RBCB = WBCs added by traumatic puncture RBCB where WBCB is the WBC count for peripheral blood, RBCCSF is the RBC count for CSF, and RBCB is the RBC count for peri pheral blood. The corrected or true CSF WBC count (WBCCSF) is calculated as follows:5 True WBCCSF = CSF WBC hemacytometer count − 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 patients with infections such as meningitis. 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 may also be obtained in patients with bacterial meningitis (in the thousands) or in patients with viral meningitis (in the hundreds).8 The predominant cell type may be obtained in patients with inflammatory processes and malignancies. Differential Cell Counts The cells normally seen in CSF are lymphocytes and monocytes (Figure 17-3). In adults, the predominant cells are lymphocytes; in newborns, the predominant cells are monocytes.6,7 Neutrophils are not found in normal CSF but may be seen in small 230 PART III Routine Laboratory Evaluation of Blood Cells Figure 17-3 Monocyte (left) and lymphocyte (right) seen in normal cerebrospinal fluid (×1000). Figure 17-5 Reactive (viral) lymphocytes in cerebrospinal fluid from a patient with viral meningitis (×1000). D A B Figure 17-4 Neutrophil with bacteria in cerebrospinal fluid from a patient with bacterial meningitis (×1000). (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) 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 in early bacterial meningitis (Figure 17-4). In viral meningitis, the predominant cells seen are lymphocytes, including reactive or viral lymphocytes and plasmacytoid lymphocytes (Figure 17-5). Eosinophils and basophils are not normally seen in CSF. Their presence has been linked to the presence of foreign materials such as shunts, in parasitic infections, or in allergic reactions (Figure 17-6).6,7 When nucleated RBCs are seen, they are most often the result of 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. If 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 large cells with abundant cytoplasm that stains lavender with Wright stain. They most often appear in clumps, and although they are not B C Figure 17-6 Eosinophil (A), lymphocytes (B), monocyte (C), and neutrophil (D) in cerebrospinal fluid from a patient with a shunt (×1000). Figure 17-7 Clump of ependymal cells in cerebrospinal fluid (×200). diagnostically significant, it is important not to confuse them with malignant cells (Figure 17-7). 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 17-8). CHAPTER 17 Body Fluids in the Hematology Laboratory 231 Figure 17-8 Cartilage cells in cerebrospinal fluid (×400). (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) Figure 17-9 Siderophage with bilirubin crystals (hematoidin) in cerebrospinal fluid (×400). Figure 17-11 Clump of breast tumor cells in cerebrospinal fluid (×400). Erythrophages 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 hemorrhage. Siderophages appear approximately 48 hours after hemorrhage and may persist for 2 to 8 weeks after the hemorrhage has occurred (Figure 17-9). A high percentage of patients with acute lymphoblastic leukemia or acute myelocytic leukemia have central nervous system involvement.6,7 It is always important to look carefully for 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 show central nervous system involvement. These blast cells have the characteristics of blast forms in the peripheral blood, including a high nucleus-tocytoplasm 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 17-10). 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 rather than 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.6,7 In children metastases to the central nervous system are related to Wilms tumor, Ewing sarcoma, neuroblastoma, and embryonal rhabdomyosarcoma.7 Malignant cells are usually large with a high nucleus-to-cytoplasm ratio and are often basophilic or hyperchromic. They often occur in clumps but may occur singly. Within clumps of malignant cells, there is dissimilarity between cells, and in multinucleated cells, there may be dissimilarity between the nuclei. The cells are usually large, often with abnormal distribution of chromatin, and they may have an indistinct or jagged border, or there may be "blebbing" at the border. Increased mitosis may be shown by the presence of several mitotic figures in the cell button. Malignant cells frequently have a bizarre appearance (Figure 17-11 and Table 17-3).9 232 PART III Routine Laboratory Evaluation of Blood Cells TABLE 17-3 Characteristics of Benign and Malignant Cells Benign Malignant Occasional large cells. Light to dark staining. Rare mitotic figures. Round to oval nucleus; nuclei are uniform in size with varying amounts of cytoplasm. Nuclear edge is smooth. Many cells may be very large. May be very basophilic. May have several mitotic figures. May have irregular or bizarre nuclear shape. Nucleus is intact. Nucleoli are small, if present. In multinuclear cells (mesothelial), all nuclei have similar appearance (size and shape). Moderate to small N : C ratio. Clumps of cells have similar appearance among cells, are in the same plane of focus, and have "windows" between cells. Edges of nucleus may be indistinct and irregular. Nucleus may be disintegrated at edges. Nucleoli may be large and prominent. Multinuclear cells have varying sizes and shapes of nuclei. May have high N : C ratio. 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. N : C, Nucleus-to-cytoplasm. SEROUS FLUID Serous fluids, including pleural, pericardial, and peritoneal fluids, are contained in a cavity that is lined by a membrane. The membranes consist of two layers: the parietal membrane, which lines the body wall, and the visceral membrane, which covers the organs. Normally serous fluid is present in very small quantities and serves as lubricant 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, in the space between the intestine and the peritoneal sac (Figure 17-12). An accumulation of fluid in a cavity is termed an effusion. When an effusion is in the peritoneal cavity, it also may be known as ascites or ascitic fluid.4 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 larger 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 17-4). TABLE 17-4 Serous Fluid: Transudates versus Exudates Characteristic Transudates Exudates Specific gravity Protein Lactate dehydrogenase White blood cells 0.6 Cloudy or yellow, amber, or grossly bloody Extremely large 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, monohistiocytes (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 17-13). Mesothelial cells are the lining cells of body cavities and are shed into these cavities constantly. These are large (12- to 30-μm) cells with a "fried egg" appearance with basophilic cytoplasm, oval nucleus with smooth nuclear borders, stippled nuclear chromatin pattern, and one to three nucleoli.6,7 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, they usually have "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.6 They tend to have a similar appearance to each other on a slide. Mesothelial cells are seen in almost all serous effusions, and their numbers are increased in sterile inflammations and decreased in tuberculous pleurisy and bacterial infections (Figure 17-14).6,7 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 17-15). 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. CHAPTER 17 Body Fluids in the Hematology Laboratory Lung 233 Parietal membrane (pleura) Pericardial cavity Visceral membrane (pericardium) Parietal membrane (pericardium) Body wall Parietal membrane (peritoneum) Liver Peritoneal cavity Stomach Visceral membrane (peritoneum) Intestine Figure 17-12 Parietal and visceral membranes of the pleural, pericardial, and peritoneal cavities. Parietal membranes line the body wall, and visceral membranes enclose organs. The two membranes are actually one continuous membrane. The space between the two opposing surfaces is identified as the body cavity (i.e., pleural, pericardial, and peritoneal cavities). (From Brunzel NA: Fundamentals of urine and body fluid analysis, ed 2, Philadelphia, 2004, Saunders.) TABLE 17-5 Gram-Stained Organisms Most Commonly Seen in Body Fluids Fluid Organism Cerebrospinal Gram-negative diplococci Gram-positive cocci Gram-positive cocci Gram-negative coccobacilli Yeast—stains gram-positive Cryptococcus—look for capsule Gram-positive cocci Yeast—stains gram-positive Gram-positive bacilli Gram-negative bacilli Gram-negative diplococci Gram-negative coccobacilli Serous (peritoneal, pleural, or pericardial) Figure 17-13 Hypersegmented neutrophil with prominent filaments. Normal appearance of neutrophils in body fluids (×1000). (From Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders.) 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 so that it can be used in rapid identification if bacteria are present. The most commonly seen organisms in the fluid are not listed above for that fluid. In each case the order of Gram stain is from left. Table 17-5 lists Gram stained organisms commonly seen in body fluids. Lupus erythematosus cells may be seen in patients with systemic lupus erythematosus, because all the Synovial (joint) fluids. NOTE: If the Gram-stained organisms seen in the fluid are not listed above for that fluid. Save the slide for review. factors necessary for the formation of these cells are present in 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 234 PART III Routine Laboratory Evaluation of Blood Cells A A B B Figure 17-14 A, Mesothelial cells in peritoneal fluid (×200). Note "fried egg" appearance. B, Mesothelial cell with 21 nuclei in pleural fluid (×400). Figure 17-15 A, Erythrophage in peritoneal fluid (×1000). B, Signet ring cell (arrow) in peritoneal fluid (×200). erythematosus cells can form in vivo and in vitro in serous and synovial fluids and should be reported (Figure 17-16). Malignant cells are seen in serous fluids from primary or metastatic tumors. They have the characteristic of malignant cells found in CSF (Figure 17-17). Figure 17-18 presents a flow chart for examination of serous fluid. SYNOVIAL FLUID Gross Examination Synovial fluid is 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. Normally this fluid is clear and colorless to pale yellow. Synovial fluid contains hyaluronic acid, which makes it very viscous. A small amount 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 Figure 17-16 Lupus erythematosus cell (arrow) in pleural fluid (×1000). the synovial cavity. They resemble mesothelial cells but are usually present in smaller numbers (Figure 17-19). Lupus erythematosus 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. A B C D Figure 17-17 A, Clump of tumor cells in pleural fluid (×200). B, Tumor cells and mitotic figure in pleural fluid (×400). C, Adenocarcinoma cells in pleural fluid (×200). D, Tumor cells in peritoneal fluid (×200). Note cannibalism. Serous fluids (Pleural, peritoneal, pericardial, ascitic) Gross appearance Cell count (clear, cloudy) Cell counts WBC RBC Türk (1:2) or (1:20) Undiluted or (1:20) dilution with saline Cytospin slide If dilution is necessary, base dilution on NCC. Dilute to 100-200/mm3 Normal Segmented leukocytes Tumor Lymphocytes Histiocytes/monocytes Mesothelial cells Look for bacteria Send to cytology laboratory Perform Gram stain on Cytocentrifuge slide Report Figure 17-18 Flow chart for examination of serous fluid. NCC, Nucleated cell count; RBC, red blood cell; WBC, white blood cell. 236 PART III Routine Laboratory Evaluation of Blood Cells B A A Figure 17-19 Synovial cells in synovial fluid (×400). Note similarity to mesothelial cells. B A B Figure 17-20 Intracellular calcium pyrophosphate crystals in synovial fluid (×1000). A, Wright stain. B, Polarized with red compensator. (B courtesy George Girgis, MT[ASCP], Indiana University Health, Indianapolis, Ind.) Many neutrophils are present in synovial fluid in acute inflammation of joints. As always, a careful search should be made for bacteria in such cases. Crystals Intracellular and extracellular crystals may be present in synovial fluid. Crystal examination may be performed by placing a drop of fluid on a slide and adding a coverslip or by examining Figure 17-21 Intracellular (A) and extracellular (B) monosodium urate crystals in synovial fluid (×1000). A, Wright stain. B, Polarized with red compensator. (A from Carr JH, Rodak BF: Clinical hematology atlas, ed 3, Philadelphia, 2009, Saunders. B courtesy George Girgis, MT[ASCP], Indiana University Health, Indianapolis, Ind.) a cytocentrifuge preparation. Since crystals should be fresh, without hyaluronidase added. All synovial fluids should be examined 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.10 They are seen in patients with chronic effusions, particularly patients with rheumatoid arthritis. Calcium pyrophosphate crystals are small intracellular crystals that are small rhomboid, platelike, or rodlike crystals.10 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 17-20).10 Monosodium urate crystals are seen in gout. They are large needlelike crystals that 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 17-21).10 Figure 17-22 presents a flow chart for synovial fluid analysis. CHAPTER 17 Body Fluids in the Hematology Laboratory 237 Synovial (joint) fluids Viscosity Crystals (polarize to confirm) Add hyaluronidase Cell counts RBC WBC Undiluted or (1:20) Türk (1:2) or (1:20) 1. Monosodium urate (needlelike) 2. Calcium pyrophosphate 3. Cholesterol (notched wedge) Cytospin slide If dilution is necessary, base dilution on NCC. Dilute to 100-200/mm3 Normal Lymphocytes Histiocytes/monocytes Synovial cells Segmented leukocytes Look for bacteria Look for LE cells Perform Gram stain on cytocentrifuge slide LE cells seen on cytocentrifuge slide Report results Figure 17-22 Flow chart for examination of synovial (joint) fluid. LE, Lupus erythematosus; NCC, nucleated cell count; RBC, red blood cell; WBC, white blood cell. BRONCHOALVEOLAR LAVAGE SPECIMENS Procedure and Precautions BAL specimens are not naturally occurring fluids; they are produced when the fluid is 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 be handled with caution. 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 are the lining cells of the alveoli, may be seen but are difficult to distinguish from macrophages. Malignant cells and 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. They occur singly or in clusters. The cells have a round nucleus at one end of the cell, elongated cytoplasm, and cilia at the opposite end of the cell from 238 PART III Routine Laboratory Evaluation of Body Cells Figure 17-23 Ciliated epithelial cells in bronchoalveolar lavage fluid (×100). Figure 17-24 Histiocytes with carbonaceous material in bronchoalveolar lavage fluid (×40). 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, and they can be propelled by their cilia (Figure 17-23). Histiocytes laden with carbonaceous material are seen in patients who smoke tobacco. These cells resemble side rophages in other fluids, but the carbonaceous material is black, brown, or blue-black and is more dropletlike (Figure 17-24). 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 17-25). Figure 17-25 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 but are rare in synovial fluid. • Malignant cells may be seen in any fluid but are rare in synovial fluid. • Synovial fluid should be examined for crystals using a 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. R E V I E W QUESTIONS 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. Six 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. CHAPTER 17 Body Fluids in the Hematology Laboratory 1. The nucleated cell count is: ___/mm3. a. 13 b. 263 c. 1050 d. 5830 3. Based on the cell counts from the RBC count. a. 3 b. 7 c. 13 d. 66 2. The RBC count is: ___/mm3. a. 131 b. 263 c. 1050 d. 5830 3. Based on the cell counts, the appearance of the fluid is: a. Clear b. Turbid c. Hemolyzed d. Bloody 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/ mm3 and 24 WBCs/mm3. On the cytocentrifuge preparation, several nucleated RBCs were seen. The differential was 5% 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/mm3. On the cytocentrifuge preparation, there were many cells that occurred in clumps and had nuclear "windows." These cell clumps appeared "three-dimensional" and contained some mitotic figures. What is the most likely identification of the cells in clusters? 239 a. Metastatic tumor cells c. Cartilage cells d. Pneumocytes 7. A serous fluid was obtained and sent to the laboratory. The fluid was straw colored and clear, with a specific gravity of 1.010, protein concentration of 1.5 g/dL, and fewer than 500 mononuclear cells/mm3 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 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 great toe. Fluid aspirated from the toe was straw-colored and cloudy. The WBC count was 2543/mm3. The cells included many neutrophils and monocytes/histiocytes. Intracellular crystals were seen on the cytocentrifuge preparation. The crystals were needle-shaped, and when polarized with 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 1. Clausen L: Cells in cerebrospinal fluid. Diagn Med 4:33-50, 1981. 2. Barnes PW, Eby CS, Shimer AG: An evaluation of the utility of performing body fluid counts on the Coulter LH 750. Lab Hematol 10:127-131, 2004. 3. 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PART IV Hematopathology: Erythrocyte Disorders Anemias: Red Blood Cell Morphology and Approach to Diagnosis 18 Rakesh P. Mehta* OUTLINE OBJECTIVES Definition of Anemia Clinical Findings Physiologic Adaptations Mechanisms of Anemia After completion of this chapter, the reader will be able to: Ineffective and Insufficient Erythropoiesis Acute Blood Loss and Hemolysis Laboratory Diagnosis of Anemia Complete Blood Cell Count with Red Blood Cell Indices Reticulocyte Count Peripheral Blood Film Examination Bone Marrow Examination Other Laboratory Tests Approach to Evaluating Anemias Clinical Importance of the Reticulocyte Count Red Blood Cell Distribution Width Hemolytic Anemias Physiologic Classification 1. Define anemia and recognize laboratory results consistent with anemia. 2. Discuss the importance of the history and the physical examination in the diagnosis of anemia. 3. Describe clinical signs and symptoms of anemia and recognize them in clinical scenarios. 4. List procedures that are commonly performed for the detection and diagnosis of anemia. 5. Distinguish among effective, ineffective, and insufficient erythropoiesis when given examples. 6. Discuss the importance of the reticulocyte count in the evaluation of anemia. 7. Characterize the three groups of anemias involving decreased production that are categorized on the basis of mean cell volume (MCV) and give one example of each. 8. Recognize the importance of reviewing the peri pheral blood film when assessing anemias and distinguish the important findings. 9. Describe the use of the red blood cell distribution width (RDW) in the diagnosis of anemias. 10. Briefly explain how the body adapts to anemia over time and the impact on the patient's experience of the anemia. 11. Use an algorithm incorporating the reticulocyte count, MCV, and RDW to narrow the differential diagnosis of anemia. 12. Classify given examples of variations in red blood cell morphology as inclusions, shape changes, volume changes, or color changes. 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 female phoned her physician and complained of fatigue, shortness of breath on exertion, and general malaise. She requested some "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. The hematocrit performed in the office was 20%. 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 MCV and reticulocyte count help determine the classification of the anemia? 3. Why is the examination of the peripheral blood film important in the work-up of an anemia? *The foundation for this chapter is the work of Ann Bell. The author would like to express his gratitude for the opportunity to continue to amend her fine endeavor. 241 242 PART IV Hematopathology: Erythrocyte Disorders R ed blood cells (RBCs) perform the vital physiologic function of oxygen delivery to the tissues. The hemoglobin within the erythrocyte has the remarkable capacity to bind oxygen in the lungs and then release it appropriately in the tissues.1 The term anemia is derived from the Greek word anaimia, meaning "without blood."2 A decrease in the number of RBCs, or the amount of hemoglobin in the RBCs, results in decreased oxygen delivery and subsequent tissue hypoxia. Anemia is a commonly encountered condition affecting an estimated 1.62 billion people worldwide.3 Anemia should not be thought of as a disease, but rather as a manifestation of other underlying disease processes.4,5 Therefore, the cause of all anemias should be thoroughly investigated. This chapter provides an overview of the diagnosis, mechanisms, 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 oxygencarrying capacity of the blood. It can arise if there is insufficient hemoglobin or the hemoglobin is nonfunctional. The former is the more frequent cause. Anemia is defined operationally as a reduction, from the baseline value, in the total number of RBCs, amount of circulating hemoglobin, and RBC mass for a particular patient. In practice, this definition is not applicable, because a patient's baseline value is rarely known.5,6 A more conventional definition is a decrease in RBCs, hemoglobin, and hematocrit below the reference range for healthy individuals of the same age, sex, and race, under similar environmental conditions.4-8 Problems with this conventional definition may occur for several reasons. The reference ranges are derived from large pools of "normal" individuals; however, the definition of normal is different for each of these data sets. This has led to the development of different reference ranges, depending on which pool of individuals was used. Furthermore, these pools of individuals lack the heterogeneity required to be universally applied to all the different populations.6 Examples of hematologic reference ranges 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 ranges 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. CLINICAL FINDINGS The history and physical examination are important components in making a clinical diagnosis of anemia. The classic symptoms associated with anemia are fatigue and shortness of breath. If oxygen delivery is decreased, then patients will not have enough energy to perform their daily functions. Obtaining a good history requires carefully questioning the patient, particularly with regard to diet, drug ingestion, exposure to chemicals, occupation, hobbies, travel, bleeding history, ethnic group, family history of disease, neurologic symptoms, previous medication, jaundice, and various underlying diseases that produce anemia.4,7-9 Although inquiry in these areas can reveal common conditions that can lead to anemia, there are numerous other possibilities as well. 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.10 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 pallor, jaundice, petechiae), eyes (for hemorrhage), and mouth (for mucosal bleeding). The examination should also look for sternal tenderness, lymphadenopathy, cardiac murmurs, splenomegaly, and hepatomegaly.4,7-9 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. Moderate anemias (hemoglobin concentration of 7 to 10 g/ dL) may not produce clinical signs or symptoms if the onset of anemia is slow.4 Depending on the patient's age and cardiovascular state, however, moderate anemias may be associated with pallor of conjunctivae and nail beds, dyspnea, vertigo, headache, muscle weakness, lethargy, and other symptoms.4,7-9 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. The severity of the anemia is gauged by the degree of reduction in RBC mass, cardiopulmonary adaptation, and the rapidity of progression of the anemia.4 PHYSIOLOGIC ADAPTATIONS Reduced delivery of oxygen to tissues caused by reduced hemoglobin causes 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 (see Chapter 8). With persistent anemia, the body implements physiologic adaptations to increase the oxygen-carrying capacity of a reduced amount of hemoglobin. Heart rate, respiratory rate, and cardiac output are increased for a more rapid delivery of oxygenated blood to tissues. In addition, the 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 (see Chapter 10).11 This is a significant mechanism in chronic CHAPTER 18 Anemias: Red Blood Cell Morphology and Approach to Diagnosis anemias that enables patients with low levels of hemoglobin to remain relatively asymptomatic. With persistent and severe anemia, however, the strain on the heart can ultimately lead to cardiac failure. 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 continuously produces RBCs to replace those lost. Hematopoietic stem cells develop into erythroid precursor cells, and the bone marrow appropriately releases reticulocytes that mature into RBCs in the peripheral circulation. Adequate RBC production requires several nutritional factors, such as iron, vitamin B12, and folate. Globin synthesis also must 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.4,7,8 Ineffective and Insufficient Erythropoiesis Erythropoiesis is the term used for marrow erythroid proliferative activity. Normal erythropoiesis occurs in the bone marrow (see Chapter 8).4 When erythropoiesis is effective, the bone marrow is able to produce functional RBCs that leave the marrow and supply the peripheral circulation with adequate numbers of cells. Ineffective erythropoiesis refers to the production of erythroid progenitor cells that are defective. These defective progenitors are often destroyed in the bone marrow before their matu ration and release into the peripheral circulation. Several conditions, such as megaloblastic anemia, thalassemia, and sideroblastic anemia, are characterized by ineffective erythropoiesis. In these anemias, the peripheral blood hemoglobin is low despite an increase in RBC precursors in the bone marrow. The effective production rate is considerably less than the total production rate, which results in a decreased number of normal circulating RBCs. Consequently, the patient becomes anemic.12 Insufficient erythropoiesis refers to a decrease in the number of erythroid precursors in the bone marrow, resulting in decreased RBC production and anemia. Several 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, the hormone that stimulates erythroid precursor proliferation and maturation (renal disease); loss of the erythroid precursors due to an autoimmune reaction (aplastic anemia, acquired pure red cell aplasia) or infection (parvovirus B19); or suppression of the erythroid precursors due to infiltration of the bone marrow with granulomas (sarcoidosis) or malignant cells (acute leukemia).12 Acute Blood Loss and Hemolysis Anemia can also develop as a result of acute blood loss (such as traumatic injury) or premature hemolysis resulting in a 243 shortened RBC life span. (Note that chronic blood loss leads to iron deficiency and is covered under insufficient erythro poiesis in the previous section.) With acute blood loss and excessive hemolysis, the bone marrow is able to increase production of RBCs, but the level of response is inadequate to compensate for the excessive RBC loss. 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.4,8,12 LABORATORY DIAGNOSIS OF ANEMIA Complete Blood Cell 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 (WBC) count, and platelet count. The RBC indices include the mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) (see Chapter 14).13 The most important of these indices is the MCV, a measure of the average RBC volume in femtoliters (fL). Reference ranges for these determinations are listed on the inside front cover of the text. Automated hematology analyzers also provide an RBC histogram and the red blood cell distribution width (RDW). A relative and absolute reticulocyte count, described subsequently, should be performed for every patient when anemia is found. Automated analyzers are available to perform reticulocyte counts with greater accuracy and precision than manual counting methods. The RBC histogram is an RBC volume frequency distribution curve with the relative number of cells plotted on the ordinate and RBC volume in femtoliters on the abscissa. With a normal population of RBCs, the distribution is approximately gaussian. Abnormalities include a shift in the curve to the left (microcytosis) or to the right (macrocytosis), and a widening of the curve caused by a greater variation of RBC volume about the mean or by the presence of two populations of RBCs with different volumes (anisocytosis). The histogram complements the peripheral blood film examination in identifying variant RBC populations.12 (A discussion of histograms with examples can be found in Chapter 39.) The RDW is the coefficient of variation of RBC volume expressed as a percentage.13 It indicates the variation in RBC volume within the population measured and 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 244 PART IV Hematopathology: Erythrocyte Disorders TABLE 18-1 Formulas for Reticulocyte Counts and Red Blood Cell Indices Test Formula 9 Absolute reticulocyte count (× 10 /L) Corrected reticulocyte count (%) Reticulocyte production index (RPI) Mean cell volume (MCV) (fL) Mean cell hemoglobin (MCH) (pg) Mean cell hemoglobin concentration (MCHC) (g/dL) Adult Reference Range = [reticulocytes (%)/100] × RBC count (× 10 /L) = reticulocytes (%) × patient's Hct (%)/45 = corrected reticulocyte count/maturation time = Hct (%) × 10/RBC count (× 1012/L) = Hb (g/dL) × 10/RBC count (× 1012/L) = Hb (g/dL) × 100/Hct (%) 12 25-75 × 109/L — In anemic patients, RPI should be >3 80-100 fL 26-32 pg 32-36 g/dL Hb, Hemoglobin; Hct, hematocrit; RBC, red blood cell. but still contain residual ribonucleic acid (RNA). Normally, they circulate peripherally for only 1 day while completing their development. The adult reference range for the reticulocyte count is 0.5% to 1.5% expressed as a percentage of the total number of RBCs.13 The newborn reference range is 1.5% to 5.8%, but these values change to approximately those of an adult within a few weeks after birth.7-9 An absolute reticulocyte count is determined by multiplying the percent reticulocytes by the RBC count. The reference range for the absolute reticulocyte count is 25 to 75 × 109/L, based on a normal adult RBC count.4 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 × 1012/L RBCs and 3% reticulocytes has an absolute reticulocyte count of 45 × 109/L. The percentage of reticulocytes is above the reference range, but the absolute reticulocyte count is within the reference range. For the degree of anemia, however, both of these results are inappropriately low. In other words, production of reticulocytes within the reference range is inadequate to compensate for an RBC count that is approximately one third of normal. The reticulocyte count may be corrected for 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). The RPI is a better indication of the rate of RBC production than is the corrected reticulocyte count (Table 18-1).4 Analysis of the reticulocyte count plays a crucial role in determining whether an anemia is due to an RBC production defect or to a premature destruction 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. This increased production of RBCs results in the release of more reticulocytes into the peripheral circulation and a higher reticulocyte count. Although an increased reticulocyte count can also be observed in acute blood loss, it is more commonly observed in the hemolytic anemias.4,13 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. An inappropriately low reticulocyte count results from decreased production of normal RBCs, due to either insufficient or ineffective erythropoiesis. Reticulocytes and related calculations are discussed in Chapter 14. 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 18-2 and 18-3). Examples of abnormal shapes and inclusions are provided in Figure 18-1. Finally, a review of the WBCs 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. (See Chapter 15 for a complete discussion of the peripheral blood film evaluation.) Additional 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.4,8 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 abnormal infiltration of the marrow. Important findings in the marrow that can point to the underlying cause of the anemia include abnormal cellularity of the marrow (e.g., hypocellularity in aplastic anemia); evidence of ineffective erythropoiesis and megaloblastic CHAPTER 18 Anemias: Red Blood Cell Morphology and Approach to Diagnosis 245 TABLE 18-2 Description of Red Blood Cell (RBC) Abnormalities and Commonly Associated Disease States RBC Abnormality Cell Description Commonly Associated Disease States Anisocytosis Macrocyte Abnormal variation in RBC volume or diameter Large RBC (>8 µm in diameter), MCV >100 fL Oval macrocyte Microcyte Large oval RBC Small RBC (

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