H. Löffler
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Atlas of Clinical Hematology

6th Edition

Springer

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Atlas of Clinical Hematology

Initiated by L. Heilmeyer
and H. Begemann

Sixth Revised Edition

With 199 Figures, in 1056 separate Illustrations,
Mostly in Color, and 17 Tables

Springer

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Preface to the Sixth Edition

Soon after the 5th edition of this volume appeared, the WHO published details on the pathology and genetics of the hematopoietic and lymphatic tissues. Work in progress found in short journal articles had already been integrated into the last edition. Now it was possible to incorporate the new proposals for classification and diagnosis and to include figures of new types of leukemia and lymphoma. These include leukemias of dendritic cells, intravascular large B-cell lymphoma, the liver-spleen T-cell lymphoma as well as persistent polyclonal B-cell lymphocytosis, which is placed between benign and malignant.

The present volume completes and extends the cytogenetic and molecular-genetic characterization of the different diseases and incorporates new figures. At this point we would like to thank PD Dr. Claudia Schoch, Munich, for her valuable help and for graciously providing new zytogenetic and FISH figures. In addition, several figures and tables were replaced, and a schematic drawing of the topography of lymphoma infiltration in bone marrow (courtesy of Prof. Dr. H.E. Schaefer, Freiburg) was added to the lymphoma chapter.

Even in 2004, diagnosis in hematology and lymphomas starts, as a rule, with the morphological examination of blood, bone marrow or lymphatic tissues. It can direct the subsequent use of immunophenotyping, cytogenetics and molecular genetics, in this way demonstrating ways of saving money and avoiding unnecessary investigations.

Gene expression profiling and, in the future, proteomics still represent very expensive methods that must find their place in diagnosis and prognostic evaluation. Gene profiling studies have already confirmed morphological subtypes in AML, e.g., M3 and M3V, which cannot be distinguished into strictly separate groups by cytogenetic and molecular-genetic methods. New therapeutic measures (especially immunotherapy) have brought interesting progress into the MDS group. For example, the biological entity 5q minus syndrome, which is well defined by morphology and cytogenetics, responds very well to treatment with the thalidomide derivative CC 5013. The fusion gene BCR-ABL, which was originally detected by cytogenesis and is today routinely detected by FISH or PCR in CML, was the first example of a specifically tailored molecular therapy in a tumor; certainly other examples will follow. Cases of ALL involving t(9;22), t(4;11) and t(8;14) have also been established as separate prognostic groups with special therapeutic problems.

All of these examples demonstrate that a comprehensive arsenal of diagnostic methods has to be used today for diagnostic and prognostic decisions and individualized therapeutic planning.

We are again grateful to Prof. Dr. R. Disko of Munich who agreed to revise and update the chapter on the principal causative agents of tropical diseases. Finally we wish to thank Mrs. Stephanie Benko and the entire staff of Springer-Verlag in Heidelberg as well as Ms. Marina Litterer at ProEdit GmbH for their thoughtful and effective support.

Preface to the Fifth Edition

The first edition of the Atlas of Clinical Hematology was published over 40 years ago. The first four editions were coauthored by Herbert Begemann, who died unexpectedly in April of 1994. We wish to dedicate the fifth edition as a memorial to this dedicated physician and hematologist.

Since the fourth edition was published in 1987, hematology has undergone profound changes. New methods such as cytochemistry and immunophenotyping have been joined by cytogenetics and, more recently, molecular genetic techniques, which have assumed a major role in routine diagnostic procedures. This has been due in part to significant advances in methodology and new tools in molecular biology. When used in standardized protocols, these tools can furnish swift results that are relevant to patient care. Since the advent of cytogenetics and molecular genetics, we have formulated new definitions for clinical and biological entities. An example is promyelocytic leukemia with its two variants (M3 and M3v), the (15;17) translocation, and the PML/RARA fusion gene, which has been successfully treated for the first time with differentiation therapy. Another example is acute myelomonocytic leukemia with abnormal eosinophiles (M4Eo), inversion 16, and the MYH/11/CBFB fusion gene, which has a very good prognosis. The transmission of morphologic findings by electronic data transfer is also gaining importance in hematology, as it permits the immediate review of difficult findings by specialists. Several colleagues seated at their own desks and microscopes can communicate with one another instantaneously by computer monitor. These advances do not alter the fact that hematologists must still have a sound grasp of morphologic principles. Diagnostic problems often arise when modern counting devices and cell sorters, with their impressive capabilities, are used without regard for cellular morphology. There is no question that classical morphology has gained much from its competition and comparison with the new techniques, leading to significant diagnostic and prognostic advances.

While retaining the basic concept of the previous editions, we found it necessary to eliminate several chapters. Now that many hematologic centers and laboratories are equipped with fluorescence-activated cell sorters (FACS) for immunotyping, and given the availability of reliable commercial kits and precise staining instructions for immunocytochemistry, the chapter by B. R. Kranz has been omitted from the present edition. We have also dropped the methodology section and most of the electron micrographs supplied by Prof. D. Huhn. Both colleagues merit our sincere thanks. Ever since the first edition, Prof. W. Mohr of Hamburg has authored the chapter on blood parasites as the principal causative agents of tropical diseases, and we gratefully acknowledge his contribution. Following the death of Prof. Mohr, we have chosen to include this chapter owing to the special importance of tropical diseases in the modern world. We are grateful to Prof. R. Disko of Munich, who agreed to revise and update the chapter.

The chapters on chronic myeloproliferative diseases, and especially those dealing with myelodysplasias, acute leukemias, malignant lymphomas, and malignant mastocytoses, had to be extensively revised or rewritten. We have added new sections and illustrations on therapy-induced bone marrow changes, cytologic changes in the cerebrospinal fluid due to leukemic or lym-

phomatous meningeal involvement, and NK cell neoplasias. We have also endeavored to give due attention to issues in pediatric hematology.

In compiling this revised fifth edition, in which over 90 % of the illustrations are new, we benefited greatly from our two decades of central morphological diagnostics for the ALL and AML studies in adults and the morphological consulting of the BFM treatment study on AML in children (H. L.). We thank the directors of these studies, Professors D. Hoelzer, T. Büchner, U. Creutzig, and J. Ritter, for their consistently fine cooperation. We also thank the Institute of Pathology of the University of Kiel, headed by Prof. Karl Lennert, and the current head of the Department of Hematologic Pathology, Prof. Reza Parwaresch, for preparing histologic sections of the tissue cores that we submitted.

Acknowledgements

We are indebted to Prof. Brigitte Schlegelberger, Prof. Werner Grote (director of the Institute of Human Genetics, University of Kiel), Dr. Harder, and Mr. Blohm for providing the cytogenetic findings and schematic drawings. We limited our attention to important findings that have bearing on the diagnosis or confirmation of a particular entity.

A work of this magnitude cannot be completed without assistance. My secretary of many years, Mrs. Ute Rosburg, often freed me from distracting tasks so that I could gain essential time. Mrs. Margot Ulrich efficiently organized the processing of the photographic materials, while Mrs. Ramm-Petersen, Mrs. Meder, and Mrs. Tetzlaff were meticulous in their performance of cytologic, cytochemical, and immunocytochemical methodologies. My senior staff members in Kiel, Prof. Winfried Gassmann and Dr. Torsten Haferlach, helped with the examination and evaluation of many of the specimens pictured in the Atlas. My colleague Dr. Haferlach collaborated with the study group of Prof. Schlegelberger to introduce the FISH technique into routine clinical use. Finally, we thank Mrs. Monika Schrimpf and the entire staff at Springer-Verlag in Heidelberg as well as Ms. Judith Diemer at PRO EDIT GmbH for their thoughtful and effective support.

St. Peter and Munich
Summer 1999

Helmut Löffler · Johann Rastetter

Preface to the First Edition

So far the diagnostic advances of smear cytology have found only limited applications in medical practice. This is due largely to the fact that available illustrative materials have been too stylized to give the novice a realistic introduction to the field. In the present atlas we attempt to correct this situation by portraying the great morphologic variety that can exist in individual cells and in pathologic conditions. In so doing, we rely mainly on artist's depictions rather than photographs. On the one hand the "objectivity" of color photos, though much praised, is inherently questionable and is further degraded by the process of chemographic reproduction. An even greater drawback of photomicrographs is their inability to depict more than one plane of section in sharp detail. By contrast, a person looking through a microscope will tend to make continual fine adjustments to focus through multiple planes and thus gain an impression of depth. A drawing can recreate this impression much better than a photograph and so more closely approximates the subjective observation. We have avoided depicting cells in black and white; while there is merit in the recommendation of histologists that students' attention be directed toward structure rather than color, this is rarely practicable in the cytologic examination of smears. The staining methods adopted from hematology still form the basis for staining in smear cytology. For this reason most of the preparations shown in this atlas were stained with Pappenheim's panoptic stain. Where necessary, various special stains were additionally used. For clarity we have placed positional drawings alongside plates that illustrate many different cell types, and we have used arrows to point out particular cells in films that are more cytologically uniform.

We were most fortunate to have our color plates drawn by an artist, Hans Dettelbacher, in whom the faculties of scientific observation, technical precision, and artistic grasp are combined in brilliant fashion. We express our thanks to him and to his equally talented daughter Thea, who assisted her father in his work. Without their contribution it is doubtful that the atlas could have been created.

We are also grateful to a number of researchers for providing scientific help and specimens, especially Prof. Dr. Henning and Dr. Witte of Erlangen, Dr. Langreder of Mainz, Prof. Dr. Mohr of the Tropical Institute of Hamburg, Dr. Moeschlin of Zurich, Dr. Undritz of Basel, and Dr. Kuhn of our Freiburg Clinic. We also thank our translators, specifically Dr. Henry Wilde of our Freiburg Clinic for the English text, Dr. Rene Prevot of Mulhouse for the French text, and Dr. Eva Felner-Kraus of Santiago de Chile for the Spanish text. We must not fail to acknowledge the help provided by the scientific and technical colleagues at our hematology laboratory, especially Mrs. Hildegard Trappe and Mrs. Waltraud Wolf-Loffler. Finally, we express our appreciation to Springer Verlag, who first proposed that this atlas be created and took the steps necessary to ensure its technical excellence.

Freiburg, Spring 1955 Ludwig Heilmayer · Herbert Begemann

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Methodology

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Blood Smear

Differentiation of the peripheral blood is still an important procedure in the diagnosis of hematologic disorders. The requisite blood smears are usually prepared from venous blood anticoagulated with EDTA (several brands of collecting tube containing EDTA are available commercially). However, many special tests require that the blood be drawn from the fingertip or earlobe and smeared directly onto a glass slide with no chemicals added. The slide must be absolutely clean to avoid introducing artifacts. Slides are cleaned most effectively by degreasing in alcohol for 24 h, drying with a lint-free cloth, and final wiping with a chamois cloth (as a shortcut, the slide may be scrubbed with 96 % alcohol and wiped dry).

Preparation of the Smear. The first drop of blood is wiped away, and the next drop is picked up on one end of a clean glass slide, which is held by the edges. (When EDTA-anticoagulated venous blood is used, a drop of the specimen is transferred to the slide with a small glass rod.) Next the slide is placed on a flat surface, and a clean coverslip with smooth edges held at about a 45 tilt is used to spread out the drop to create a uniform film. We do this by drawing the coverslip slowly to the right to make contact with the blood drop and allowing the blood to spread along the edge of the coverslip. Then the spreader, held at the same angle, is moved over the specimen slide from right to left (or from left to right if the operator is left-handed), taking care that no portion of the smear touches the edge of the slide. The larger the angle between the coverslip and slide, the thicker the smear; a smaller angle results in a thinner smear.

Once prepared, the blood smear should be dried as quickly as possible. This is done most simply by waving the slide briefly in the air (holding it by the edges and avoiding artificial warming). The predried slide may be set down in a slanted position on its narrow edge with the film side down. For storage, we slant the slide with the film side up, placing it inside a drawer to protect it from dust and insects.

The best staining results are achieved when the smear is completely air-dried before the stain is applied (usually 4 – 5 h or preferably 12 – 24 h after preparation of the smear). In urgent cases the smear may be stained immediately after air drying.

Bone Marrow

Percutaneous aspiration of the posterior iliac spine is the current method of choice for obtaining a bone marrow sample. It is a relatively safe procedure, and with some practice it can be done more easily and with less pain than sternal aspiration. Marrow aspirate and a core sample can be obtained in one sitting with a single biopsy needle (e.g., a Yamshidi needle). When proper technique is used, the procedure is not contraindicated by weakened host defenses or thrombocytopenia. However, there is a significant risk of postprocedural hemorrhage in patients with severe plasmatic coagulation disorders (e.g., hemophilia), in patients on platelet aggregation inhibitors, and in some pronounced cases of thrombocytosis. In all cases the biopsy site should be compressed immediately after the needle is withdrawn, and the patient should be observed. The procedure should be taught by hands-on training in the clinical setting.

Aspiration is usually performed after a core biopsy has been obtained. The needle is introduced through the same skin incision and should enter the bone approximately 1 cm from the biopsy site. A sternal aspiration needle may be used with the guard removed, or a Yamshidi needle can be used after removal of the stylet.

The operator rechecks the position of the spine and positions the middle and index fingers of the left hand on either side of the spine. The sternal aspiration needle, with adjustable guard removed, is then inserted until bony resistance is felt and the needle tip has entered the periosteum. This is confirmed by noting that the tip can no longer be moved from side to side. The needle should be positioned at the center of the spine and should be perpendicular to the plane of the bone surface. At this point a steady, gradually increasing pressure is applied to the needle, perhaps combined with a slight rotary motion, to advance the needle through the bone cortex. This may require considerable pressure in some patients. A definite give will be felt as the needle penetrates the cortex and enters the marrow cavity. The needle is attached to a 20-mL glass syringe, the aspiration is performed, and specimens are prepared from the aspirated material.

After the needle is withdrawn, the site is covered with an adhesive bandage and the patient instructed to avoid tub bathing for 24 h.

The usual practice in infants is to aspirate bone marrow from the *tibia*, which is still active hematopoietically.

We prefer to use the needle described by Klima and Rosegger, although various other designs are suitable (Rohr, Henning, Korte, etc.). Basically it

does not matter what type of needle is used, as long as it has a bore diameter no greater than 2–3 mm, a well-fitting stylet, and an adjustable depth guard. All bone marrow aspirations can be performed in the ambulatory setting.

Sternal aspiration is reserved for special indications (prior radiation to the pelvic region, severe obesity). It should be practiced only by experienced hematologists. It is usually performed on the sternal midline at approximately the level of the second or third intercostal space. The skin around the puncture site is aseptically prepared, and the skin and underlying periosteum are desensitized with several milliliters of 1 % mepivacaine or other anesthetic solution. After the anesthetic has taken effect, a marrow aspiration needle with stylet and guard is inserted vertically at the designated site. When the needle is in contact with the periosteum, the guard is set to a depth of about 4–5 mm, and the needle is pushed through the cortex with a slight rotating motion. A definite give or pop will be felt as the needle enters the marrow cavity. Considerable force may have to be exerted if the cortex is thick or hard. When the needle has entered the marrow cavity, the stylet is removed, and a 10- or 20-mL syringe is attached. The connection must be airtight so that an effective aspiration can be performed. The plunger is withdrawn until 0.5 to 1 mL of marrow is obtained. Most patients will experience pain when the suction is applied; this is unavoidable but fortunately is of very brief duration. If no marrow is obtained, a small amount of physiologic saline may be injected into the marrow cavity and the aspiration reattempted. If necessary, the needle may be advanced slightly deeper into the marrow cavity. The procedure is safe when performed carefully and with proper technique. Complications are rare and result mainly from the use of needles without guards or from careless technique. The procedure should be used with caution in patients with plasmacytoma, osteoporosis, or other processes that are associated with bone destruction (e.g., metastases, thalassemia major). Bone marrow aspirations can be performed in the outpatient setting.

For preparation of the smears, we expel a small drop of the aspirated marrow onto each of several glass slides (previously cleaned as described on p. 3) and spread it out with a coverslip as described for the peripheral blood. We also place some of the aspirate into a watch glass and mix it with several drops of 3.6 % sodium citrate. This enables us to obtain marrow particles and prepare smears in a leisurely fashion following the aspiration. If the aspirate is not left in the citrate solution for too long, the anticoagulant will not introduce cell changes that could interfere with standard investigations. We vary our smear preparation technique according to the nature of the inquiry and the desired tests. Spreading the marrow particles onto the slide in a meandering pattern will cause individual cells to separate from the marrow while leaving the more firmly adherent cells, especially stromal cells, at the end of the track. In every bone marrow aspiration an attempt should be made to incorporate solid marrow particles into the smear in addition to marrow fluid in order to avoid errors caused by the admixture of peripheral blood. We see no advantage in the two-coverslip method of smear preparation that some authors recommend. We find that simple squeeze preparations often yield excellent results: Several marrow particles or a drop of marrow fluid are expelled from the syringe directly onto a clean glass slide. A second slide is placed over the sample, the slides are pressed gently together, and then they are pulled apart in opposite directions. This technique permits a quantitative estimation of cell content. All marrow smears are air dried and stained as in the procedure for blood smears. Thicker smears will require a somewhat longer staining time with Giemsa solution. Various special stains may also be used, depending on the nature of the study.

If cytologic examination does not provide sufficient information, the *histologic examination of a marrow biopsy specimen* is indicated. This is especially useful for the differentiation of processes that obliterate the bone marrow, including osteomyelosclerosis or -fibrosis in neoplastic diseases and abnormalities of osteogenesis, the blood vessels, and the marrow reticulum. In recent years the Yamshidi needle has become increasingly popular for bone marrow biopsies.

Fine-Needle Aspiration of Lymph Nodes and Tumors

The fine-needle aspiration of lymph nodes and tumors is easily performed in the outpatient setting. The diagnostic value of the aspirate varies in different pathologic conditions. An accurate histologic classification is usually essential for sound treatment planning and prognostic evaluation, and so the histologic examination has become a standard tool in primary diagnosis. The unquestioned value of the cytologic examination of aspirates is based on the capacity for rapid orientation and frequent follow-ups, adding an extra dimension to the static impression furnished by histologic sections.

The technique of lymph node aspiration is very simple: Using a 1 or 2 gauge (or smaller) hypoder-

mic needle with a 10- or 20-mL syringe attached, we fixate the lymph node between two fingers of the free hand, insert the needle into the node, and apply forceful suction to aspirate a small amount of material. A thinner needle should be used for tissues that contain much blood, and some authors routinely use needles of gauge 12, 14, or 16 (outer diameter 0.6 – 0.9 mm). Special equipment is available that permits one-handed aspiration (e.g., the Cameco pistol grip syringe holder) and even the use of one-way syringes.

The tissue fragments on the needle tip and inside the needle are carefully expelled onto a glass slide, and a smear is prepared. It is rare for tissue to be drawn into the syringe, but if this material is present it may be utilized for bacteriologic study. The smears are stained like a blood film, and special stains may be used as needed. The aspiration is almost painless and does not require anesthesia. If the lymph node is hard or if histologic examination of the aspirate is desired, we use a somewhat larger gauge needle (approximately 1 – 2 mm in diameter) that has a stylet and a sharp front edge. The stylet is withdrawn before the node is punctured. Of course, the use of a larger needle requires preliminary anesthesia of the skin and lymph node capsule. All *tumors* that are accessible to a percutaneous needle can be aspirated in similar fashion.

Splenic Aspiration

Splenic aspiration is rarely practiced nowadays and is always performed under some form of radiologic guidance. Today it is indicated only in certain forms of hypersplenism or unexplained splenic enlargement. We consider the Moeschlin technique to be the safest. Splenic aspiration is contraindicated in patients with hemorrhagic diathesis, septic splenomegaly, splenic cysts, or painful splenomegaly due to excessive capsular tension or infarction. The procedure should be used with caution in patients with hypertension of the portal or splenic vein (Banti syndrome, splenic vein thrombosis, splenomegalic cirrhosis). It should be withheld from dazed patients who are unable to cooperate. Moeschlin recommends that splenic aspiration be performed only when definite splenic enlargement is noted and only under stringent aseptic conditions. The procedure is safest when performed under ultrasound guidance, as this will demonstrate not only the size and position of the spleen but also any pathologic changes (e.g., splenic cysts) that would contraindicate the procedure.

Concentrating Leukocytes from Peripheral Blood in Leukocytopenia

Principle. White blood cells are centrifuged after sedimentation of the erythrocytes to concentrate the nucleated cells and make it easier to detect abnormal cell forms.

Reagents

1. Gelatin, 3 %, in 0.9 % NaCI (or plasma gel infusion solution; B. Braun, Melsungen)
2. Heparin (cresol-free)

Method. Place 3 – 5 mL of venous blood or EDTA-treated blood into a narrow tube, add 1/4 volume gel to the sample and carefully mix by tilting. Let stand at 37 for 14 min, 7 min at a 45 slant, and 7 min upright. Pipet off the leukocyte-rich layer and centrifuge lightly at 2000 rpm. Decant the supernatant, gently shake out the sediment, and prepare the smears.

Demonstration of Sickle Cells

Method. Place 1 drop of blood onto a slide and cover with a coverslip.

Place 1 drop of 2 % sodium thiosulfate ($Na_2S_2O_4$) along one edge of the coverslip and hold a blotter against the opposite edge, the object being to draw the Na thiosulfate beneath the coverslip so that it mixes with the blood. (If this is unsuccessful, it may be necessary to raise the coverslip slightly or even add the Na thiosulfate directly to the blood before covering. However, it is best to mix the thiosulfate and blood in the absence of air, as described above!)

Create an airtight seal around the coverslip with paraffin, and let stand for 30 min at room temperature. Examine the unstained slide under the microscope.

Light Microscopic Procedures

II

1. Staining Methods for the Morphologic and Cytochemical Differentiation of Cells

1.1 Pappenheim's Stain (Panoptic Stain)

The hematologic stain that we use most frequently, and which was used in most of the plates pictured in this atlas, is Pappenheim's panoptic stain. It is based on a combination of the Jenner-May-Grünwald stain and Giemsa stain.

Method. Place the air-dried slide with the film side up in prepared May-Grünwald eosin-methylene blue solution for 3 min. Dilute with water or buffer solution (phosphate buffer pH 7.3, see below) for an additional 3 min. Pour off this solution and apply Giemsa stain immediately, without intermediate rinsing. The stock Giemsa stain is diluted with neutral distilled water by adding 10 mL water per 10 drops of Giemsa solution. Stain the specimen for 15 to 20 min. The dilution ratio and Giemsa staining time should be individually adjusted to allow for inevitable variations in the composition of the solution. After Giemsa staining, wash the slide with neutral water and tilt to air-dry. Fixation is effected by the methyl alcohol already contained in the May-Grünwald solution. The quality of the stain depends greatly on the pH of the water that is used. The smear will be too red if the water is too acidic and too blue if the water is too alkaline. Standard pH strips can be used to test the water for proper acidity. Water left standing in the laboratory can easily become too acidic through exposure to acid fumes, especially from carbon dioxide. The latter problem is solved by preboiling. A more accurate way to ensure correct acidity for staining is to use a pH 7.3 buffer solution (22.3 mL of 1/15 mol/L KH_2PO_4 + 77.7 mL of 1/15 mol/L Na_2HPO_4) instead of water.

1.2 Undritz Toluidine Blue Stain for Basophils

Reagent. Saturated toluidine blue-methanol: dissolve 1 g toluidine blue in 100 mL methanol. The solution will keep indefinitely.

Method. Fix and stain the air-dried smears on the staining rack by covering with the toluidine blue-methanol for 5 min. Wash in tap water, air dry.

Interpretation. The granulations in basophils and mast cells stain a red-violet color owing to the strong metachromatic effect of the sulfate present in the heparin. As a result, these cells are easily identified even at moderate magnification. By contrast, azurophilic granules (even in severe "toxic" granulation) and the coarse granules in leukocytes affected by Adler anomaly show very little violet transformation of their blue color.

1.3 Mayer's Acid Hemalum Nuclear Stain

This is used for the blue contrast staining of nuclei in assays of cytoplasmic cell constituents (glycogen, enzymes; pp. 9 ff.) and in immunocytochemistry.

Reagents. Dissolve 1 g hematoxylin (Merck) in 1 L distilled water and add 0.2 g sodium iodate ($NaIO_3$) and 50 g aluminum potassium sulfate ($KAl(SO_4)_2 \cdot 12H_2O$). After these salts are dissolved, add 50 g chloral hydrate and 1 g crystallized citric acid. The hemalum will keep for at least 6 months at 20 °C with no change in staining properties. The solution can also be purchased in ready-to-use form.

Method. The necessary staining time in the hemalum bath varies with the method of specimen preparation and must be determined by progressive staining. After staining, wash the slide for at least 15 min in several changes of *tap* water (acid residues may reduce the intensity of the stain).

1.4 Heilmeyer's Reticulocyte Stain

Draw a 1 % brilliant cresyl blue solution in physiologic saline to the 0.5 mark of a white cell counting pipet, and draw up the blood to the 1.0 mark. Expel the mixture carefully, without forming air bubbles, into a paraffinated watchglass dish, mix carefully with a paraffinated glass rod, and place in a moist chamber for 15 – 20 min. Then remix carefully with a paraffinated glass rod. With the rod, transfer 1 or 2 drops of the mixture to a microscope slide and smear in standard fashion using a ground coverslip. Examine the air-dried slides under oil-immersion magnification, and count the number of reticulocytes per 1000 red cells at multiple sites in the smear. Very high-quality films can be obtained by Giemsa counterstaining.

1.5 Heinz Body Test of Beutler[1]

This test is used to detect defects of red cell metabolism that do not allow glutathione to be

[1] After Huber H, Löffler H, Faber V (1994) Methoden der diagnostischen Hämatologie. Springer, Berlin Heidelberg New York Tokyo.

maintained in a reduced state. The defect may result from a glucose-6-phosphate dehydrogenase deficiency, a glutathione reductase deficiency, diseases with "unstable hemoglobin," or an "idiopathic" Heinz body anemia. The test involves the oxidative denaturation of hemoglobin to intraerythrocytic "Heinz bodies" following incubation of the red cells with acetylphenylhydrazine.

Reagents

1. Sörensen phosphate buffer, pH 7.6, 0.67 M:
   1/15 M KH_2PO_4 13 parts.
   1/15 M Na_2HPO_4 87 parts.
2. Glucose phosphate buffer: dissolve 0.2 g glucose in 100 mL phosphate buffer. The solution may be stored frozen or at 4 °C (watch for clouding!).
3. Acetylphenylhydrazine solution: dissolve 20 mg acetylphenylhydrazine in 20 mL glucose phosphate buffer at room temperature. This solution is prepared fresh and should be used within 1 h.
4. (a) Dissolve saturated alcohol solution of brilliant cresyl blue; or (b) 0.5 g methyl violet in 100 mL of 0.9 % NaCI, and filter; blood: heparinized, defibrinated, or treated with EDTA.

Method. Centrifuge the blood lightly for 5 min. Pipet 0.05 mL of test erythrocytes into 2 mL of the acetylphenylhydrazine solution. Suspend normal erythrocytes in an identical solution to serve as a control. Aerate the suspensions by drawing them up into the pipet and carefully blowing them out with a small quantity of air; repeat several times. Incubate for 2 h at 37 °C, aerate again, and incubate 2 h more.

To stain with brilliant cresyl blue: spread a small drop of stain solution 4(a) onto a clean, degreased slide and dry the thin stain film rapidly in air. Place a small drop of the incubated erythrocyte suspension on a coverglass and invert the glass onto the stain; examine with the microscope.

To stain with methyl violet: mix a small drop of the erythrocyte suspension with 2 or 3 drops of stain solution 4b on the slide and cover with a coverslip. Let the mixture stand for 5–10 min and examine with the microscope.

Interpretation. The percentage of erythrocytes that contain more than four Heinz bodies is determined. Normal values range from 0 % to 30 %. The number of Heinz bodies is elevated in the diseases listed above.

1.6 Nile Blue Sulfate Stain

This stain is used for the visualization of Heinz inclusion bodies. A 0.5 % Nile blue sulfate solution in absolute alcohol is transferred to the end of a slide with a glass rod until about 1/3 of the slide is covered. The slide is dried by blowing on it, and the stain film is spread out evenly with a cotton swab. Slides prepared in this way are placed face-to-face and wrapped in paper for storage. Staining is performed by dropping 2 or 3 large drops of blood onto the prepared part of the slide and covering with the prepared part of the second slide. The slides, held by their unstained outer ends, are separated and placed back together several times to thoroughly mix the blood with the stain. Finally the slides are left together for 3 to 5 min, separated, and a ground coverslip is used to collect the blood from each slide and smear it onto another slide, which is allowed to dry. The Heinz bodies appear as small, dark blue bodies situated at the margin of the yellow to bluish erythrocytes.

1.7 Kleihauer-Betke Stain for Demonstrating Fetal Hemoglobin in Red Blood Cells

Principle. Normal adult hemoglobin (HbA) is dissolved out of the red cells by incubating air-dried and fixed blood smears in citric acid phosphate buffer (of McIlvain), pH 3.3, at 37 °C. Fetal hemoglobin (HbF) is left undissolved in the red cells and can be made visible by staining.

Reagents

- Ethyl alcohol, 80 %
- McIlvaine citric acid-phosphate buffer, pH 3.3
- Stock solution A:
  Sörensen citric acid, 21.008 g in 1 L water = 0.1 M
- Stock solution B:
  Disodium hydrogen phosphate $Na_2HPO_4 2H_2O$, 27.602 g in 1 L water = 0.2 M
- For pH 3.3:
  266 mL of solution B + 734 mL of solution A,
  Ehrlich hematoxylin, 0.1 % erythrosin solution

Method. Prepare thin blood smears, air dry, and fix in 80 % ethyl alcohol for 5 min. Wash in water and dry. If further processing is delayed, the slides may be stored in a refrigerator for 4–5 days. For elution, place the slides upright in a beaker containing the buffer in a 37 °C water bath for 3 min, moving the slides up and down after 1 and 2 min to keep the buffer mixed. Then wash in running water.

Staining. Stain in Ehrlich hematoxylin for 3 min, then poststain in 0.1 % aqueous erythrosin solution for 3 min. Examine at 40× using dry or oil-immersion magnification.

Interpretation. Erythrocytes that contain HbA appear as unstained "shadows," while cells that contain HbF will stain a bright red color.

The method can be used for the diagnosis of thalassemia major and for the detection of fetal erythrocytes that have entered the maternal circulation.

1.8 Kleihauer-Betke Stain for Demonstrating Methemoglobin-Containing Cells in Blood Smears

Principle. Methemoglobin combines with KCN to form cyanmethemoglobin, while oxyhemoglobin does not react with cyanides. Oxyhemoglobin functions as a peroxidase, whereas cyanmethemoglobin has very low perixodase activity.

Method. Add 1/50 vol of a 0.4 M KCN solution to blood anticoagulated with heparin or sodium citrate. Prepare thin smears from this mixture, dry, and immerse in the following mixture at room temperature: 80 mL of 96 % ethyl alcohol + 16 mL of 0.2 M citric acid + 5 mL of 30 % H_2O_2. Move the smears rapidly in the solution for about 1 min, then leave them in the solution for 2 min. Wash the smears first in methyl alcohol, then in distilled water, and stain with hematoxylin and erythrosin (see stain for HbF). Examine at 40× using dry or oil-immersion magnification.

Interpretation. Oxy-Hb-containing cells stain a bright red. Cells that contain met-Hb (converted to cyanmet-Hb) are eluted and appear as shadows.

The same staining procedure can be used to differentiate erythrocytes with a glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency by combining it with the Brewer test (method of Betke, Kleihauer and Knotek). This test is based on the principle that hemoglobin converted to met-Hb by the addition of nitrite reduces to oxy-Hb in the presence of methylene blue and glucose. Red blood cells with a G-6-PDH deficit cannot undergo this reduction. Even after several hours, when all the methemoglobin in normal erythrocytes has converted back to oxy-Hb, cells with a G-6-PDH deficiency retain all or most of their met-Hb. This causes the deficient cells to appear "blank" with appropriate staining (see top of this section).

1.9 Berlin Blue Iron Stain

Principle

The Berlin blue reaction is used for the histochemical demonstration of trivalent iron. Iron in protein compounds can also be demonstrated by the addition of dilute hydrochloric acid. Iron in hemoglobin is not detected.

Reagents

- Methanol
- Potassium ferrocyanide (potassium hexacyanoferrate), 2 %
- HCl, 37 %
- Pararosaniline solution in methanol, 1 % (alternative: nuclear red stain)

Method

- Fix the air-dried smears in formalin vapor for 30 min (alternative: fix in methanol for 10 – 15 min).
- Wash in distilled water for 2 min and air dry.
- Place the specimens in a cuvet that contains equal parts of a 2 % solution of potassium ferrocyanide and a dilute HCl solution (1 part 37 % HCl mixed with 50 parts distilled water) for 1 h.
- Wash in distilled water.
- Nuclear stain in pararosaniline solution: 300 µL of 1 % pararosaniline solution in methanol diluted with 50 mL distilled water.
  Alternative. Stain nuclei with nuclear true red solution (which yields a fainter nuclear stain).

All materials should be iron-free, and metal forceps should not be introduced into the solution. Pappenheim- or Giemsa-stained smears can subsequently be used for iron staining. They are first prepared by destaining them for 12 – 24 h in pure methanol. These smears do not need to be fixed prior to staining.

Interpretation

Iron is stained blue, appearing either as diffusely scattered granules or as clumps in the cytoplasm. There are two applications for iron staining in hematology:

(a) demonstrating sideroblasts and siderocytes, and
(b) demonstrating iron stored in macrophages and endothelial cells.

Regarding (a): sideroblasts and siderocytes are, respectively, erythroblasts and erythrocytes that contain cytochemically detectable iron. This iron can be demonstrated in the form of small granules that may be irregularly scattered

throughout the cytoplasm or may encircle the nucleus of erythroblasts like a ring. Normally the granules are very fine and can be identified in erythroblasts only by closely examining the smears with oil-immersion microscopy in a darkened room. Generally 1 to 4 fine granules will be seen, rarely more. When iron deficiency is present, the percentage of sideroblasts is reduced to less than 15 %. Sideroblasts containing coarse iron granules that form a partial or complete ring around the nucleus (ringed sideroblasts) are definitely abnormal. The detection of siderocytes has little practical relevance: they are increased in the same diseases as sideroblasts, and they are elevated in the peripheral blood following splenectomy, as the spleen normally removes iron from intact red blood cells.

Regarding (b): the content of stored iron is assessed by examining bone marrow fragments in smears or sections. Iron stored in macrophages may occur in a diffusely scattered form, a finely granular form, or in the form of larger granules or clumps that may cover part of the nucleus. Iron can also be demonstrated in plasma cells as a result of alcohol poisoning or sideroblastic anemia and hemochromatosis.

The differential diagnosis afforded by iron stain is summarized in **Table 1.**

1.10 Cytochemical Determination of Glycogen in Blood Cells by the Periodic Acid Schiff Reaction and Diastase Test (PAS Reaction)

Principle

This method is based on the oxidation of α-glycols in carbohydrates and carbohydrate-containing compounds. The resulting polyaldehydes are demonstrated with the Schiff reagent (leukofuchsin).

Reagents

- Formalin.
- Periodic acid solution, 1 %, in distilled water.
- Sulfite water: add tap water to 10 mL of a 10 % sodium metabilsulfite solution ($Na_2S_2O_5$) and 10 mL of 1 mol/L HCL to make a volume of 200 mL. The stock solutions can be stored in the refrigerator; the mixture should always be freshly prepared.
- Prepare Schiff reagent (commercially available) as follows: completely dissolve 0.5 g pararosaniline in 15 mL of 1 mol/L HCl by shaking (no heating) and add a solution of 0.5 g potassium metabisulfite ($K_2S_2O_5$) in 85 mL distilled water. The clear, bright red solution will gradually lighten to a yellowish color. After 24 h, shake with 300 mg activated charcoal (powdered) for 2 min and then filter. The colorless filtrate is ready to use and, when stored in a dark stoppered bottle in a cool place, will keep for several months. Schiff reagent that has turned red should no longer be used!

Method

- Fix the smears for 10 min in a mixture of 10 mL 40 % formalin and 90 mL ethanol (alternative: fix for 5 min in formalin vapor).
- Wash for 5 min in several changes of tap water.
- Place the smears in 1 % periodic acid for 10 min (prepared fresh for each use).
- Wash in at least two changes of distilled water and dry.
- Place in Schiff reagent for 30 min (in the dark at room temperature).
- Rinse in sulfite water (changed once) for 2 – 3 min.
- Wash in several changes of distilled water for 5 min.
- Nuclear stain with hemalum for approx. 10 min, then blue in tap water for approx. 15 – 20 min, and air dry.

Even older slides that have been stained with Giemsa or Pappenheim can be reused for the PAS reaction. Specimens that have been treated several times with oil or xylene should not be used for PAS staining. The smears can be placed unfixed in periodic acid after washing in distilled water (Step 3) to remove the color.

Interpretation

PAS-positive material in the cytoplasm may produce a diffuse red stain or may appear as pink to burgundy-red granules, flakes, or clumps of varying size that may occupy large areas of the cytoplasm. The distribution of PAS-positive material in normal leukocytes is summarized in the **Table.** Some plasma cells, macrophages, and osteoblasts may also show a positive PAS reaction, and megakaryocytes are strongly positive.

Table 1. Differential diagnosis by iron stain in the bone marrow

	Sideroblasts	Iron-storing reticulum cells, sideromacrophages	Special features
Normal bone marrow	~ 20 – 60 % finely granular, 1 – 4 granules	Isolated, mostly finely granular deposits	Siderocytes in peripheral blood 0 – 0.3 ‰
Hypochromic anemias			
– Iron deficiency	< 15 % finely granular	None	Serum Fe ↓
– Infection, tumor	< 15 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	Serum Fe ↓
– Sideroachrestic anemias (RARS)	> 90 % coarsely granular; ringed sideroblasts (> 15 %)	Greatly increased, many diffuse or coarsely granular deposits	Serum Fe ↑, siderocytes may be increased
– Lead poisoning	> 90 % coarsely granular; ringed sideroblasts	Greatly increased, many diffuse or coarsely granular deposits	Serum Fe ↑, siderocytes may be increased
– Thalassemia	> 90 % coarsely granular; ringed sideroblasts	Greatly increased, many diffuse or coarsely granular deposits	Serum Fe ↑, siderocytes may be increased
Hemolytic anemias	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Secondary sideroachrestic anemias	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Vitamin B_6 deficiency	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Megaloblastic anemias	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Aplastic anemias	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Myeloproliferative disorders	≤ 80 % finely granular	Increased finely granular or (rarely) coarsely granular deposits	
Hemochromatosis	≤ 80 % finely granular	Increased Plasma cells contain iron	Bone marrow is not useful for diagnosis except positive plasma cells
Postsplenectomy state	≤ 80 % finely granular	Somewhat increased	Siderocytes greatly increased

PAS reaction in normal leukocytes

Cell type	PAS reaction
Myeloblast	∅
Promyelocyte	(+)
Myelocyte	+
Metamyelocyte	++
Band and segmented cells	+++
Eosinophils	+ (intergranular reaction)
Basophils	+ (granular!)
Monocytes	(+) to +
Lymphocytes	∅ to + (granular)

Reaction: ∅ = negative; (+) = weakly positive; + = positive; ++ = markedly positive; +++ = strongly positive

1.11 Sudan Black B Stain

Principle

Sudan black B is a fat-soluble dye that becomes highly concentrated in lipids. The sudanophilia, which occurs even after degreasing, is based on an oxidative coupling of Sudan black derivatives with phenols. It is peroxidase-dependent and thus corresponds to the peroxidase reaction. It is hardly used anymore.

1.12 Cytochemical Determination of Peroxidase

Principle

Benzidine or diaminobenzidine (more often used) is converted, in the presence of peroxide, from the leuko form into a high-polymer form that is detectable by cytochemical staining.

Reagents.

- Fixative: methanol + 37 % formalin (10 : 1).
- DAB solution: 5 mg diaminobenzidine tetrahydrochloride in 20 mL of 0.05 mol/L tris-HCl buffer (pH 7.6) with 50 µL of 1 % H_2O_2 added
- Tris-HCl: 50 mL of solution A (121.14 g trishydroxymethylaminomethane dissolved in 1 L distilled water) + 40 mL of solution B (1 mol/L HCl) + 960 mL distilled water
- Mayer's hemalum:

Method

- Fix the air-dried smears for 15 s at 4 °C (30 s for thicker bone marrow smears).
- Wash 3 times in tap water.
- Air dry.
- Incubate in DAB solution for 10 min.
- Wash briefly in tap water.
- Incubate in Mayer's hemalum for 3 min.
- Wash in tap water for 3 min.
- Air dry.

Interpretation

From the promyelocytic stage on, neutrophils and eosinophilic granulocytes show a yellowish green to brownish granular stain. Monocytes may show a positive reaction, which is weaker than that of granulocytes.

1.13 Hydrolases

Principle

The principle is the same for all hydrolases and may be summarized as follows: Today only the azo dye method is still in routine clinical use. It is based on the hydrolytic splitting of an aryl ester by the enzyme and the immediate coupling of the liberated phenol derivative to a dye substance, usually a diazonium salt or hexazotized pararosaniline.

1.13.1 Cytochemical Determination of Leukocyte Alkaline Phosphatase (LAP) in Blood Smears

Reagents

- Fixative: 10 % formalin in absolute methanol (one part 37 % formalin, 9 parts 100 % methanol)
- Staining solution: dissolve 35 mg sodium-α-naphthyl phosphate in 70 mL of 2 % veronal sodium solution, pH 9.4; add 70 mg concentrated variamine blue salt B, and stir. Immediate filter the solution and use.
- Mayer's hemalum.

Method

- Fix the air-dried smears at 4 °C for 30 s.
- Wash 3 times thoroughly in tap water.
- Incubate in refrigerator at 4 – 7 °C for 2 h.
- Wash thoroughly in tap water.
- Nuclear stain in Mayer's hemalum for 5 – 8 min.
- Air dry the smears and mount in glycerine gelatin or Aquatex.

Interpretation

Neutrophilic granulocytes (a few band cells, mostly segmented forms) are the only types of blood cell that show enzymatic activity. The intensity of the phosphatase reaction is usually scored on a four-point scale. The activity score,

or index, is based on groups of 100 cells and is calculated from the sum of the cells assigned to the different reaction grades, which is multiplied by a corresponding factor (1–4). The index ranges from 0 to 400. Cells in the bone marrow that have phosphatase activity are neutrophilic granulocytes, vascular endothelial cells, and osteoblasts. The location of structures in bone marrow smears, lymph node touch preparations, and sections can be determined more accurately by using methods that employ the substrates naphthol-AS-BI phosphate or -MX phosphate.

1.13.2 Cytochemical Determination of Acid Phosphatase

Reagents

- Fixative: see Appendix
- Staining solution: mix together 0.8 mL hexazotized pararosaniline (mix equal parts 4 % sodium nitrite and 4 % pararosaniline in HCl, see Appendix) + 30 mL Michaelis buffer pH 7.4 (58 mL of 0.1 mol/L sodium barbital + 41.9 mL of 0.1 mol/L HCl) + 10 mg naphthol-AS-BI phosphate, dissolved in 1 mL dimethylformamide. Adjust the solution to pH 4.9–5.1 and filter before use.
- Mayer's hemalum

Method

- Fix the air-dried smears at 4 °C for 30 s.
- Wash 3 times in tap water.
- Air dry.
- Incubate in stain solution for 3 h at room temperature.
- Wash briefly in tap water.
- Place in Mayer's hemalum for 3 min.
- Blue in tap water for 3 min.
- Air dry.

Interpretation

A bright red homogeneous or granular precipitate forms in the cytoplasm of cells with acid phosphatase activity. In the case of plasmacytomas, the abnormal plasma cells tend to show stronger activity than normal plasma cells or plasma cells affected by reactive changes. A dotlike staining pattern is seen in T-lymphocytes, while the blasts of T-ALL usually show a circumscribed (focal) paranuclear acid phosphatase reaction.

Acid Phosphatase Reaction with Inhibition by Tartrate

Method

Add 60 mg of L-tartaric acid to 30 mL of the staining solution, then analyze as described for acid phosphatase. Fast garnet GBC can be used as a coupling salt instead of the pararosaniline solution. This requires the following modifications in the staining solution: Dissolve 10 mg naphthol-AS-BI phosphate in 0.5 mL dimethylformamide, and add 0.1 mol/L acetate buffer pH 5.0 to make 10 mL. Dissolve 10–15 mg of fast garnet GBC in 20 mL of 0.1 mol/L acetate buffer solution. Mix both solutions well. Filtering is not required. Incubate the smears at 37 °C for 60–90 min.

Interpretation

Most of the cells of hairy cell leukemia are positive even after tartrate inhibition, and macrophages and osteoclasts do not show significant inhibition. Today immunophenotyping, especially with CD 103, is more important.

1.13.3 Detection of Esterases with Naphthyl Acetate or Naphthyl Butyrate ("Neutral Esterases")

Reagents

- Solution a: mix 1 drop (0.05 mL) sodium nitrite solution (4 %) + 1 drop (0.05 mL) pararosaniline solution (4 % in 2 mol/L HCl) for about 1 min (yields a pale yellow solution), then dissolve in 5 mL of 0.2 mol/L phosphate buffer, pH 7.0–7.1 (250 mL Na_2HPO_4+130 mL NaH_2 PO_4).
- Solution b: dissolve 10 mg α-naphthyl acetate in 0.2–0.3 mL chemically pure acetone; add 20 mL of 0.2 mol/L phosphate buffer pH 7.0–7.1 while stirring vigorously.
- Mix solutions a and b and filter into small cuvets.

Method

- Fix the thin, air-dried smears (will keep up to 3 days when sheltered from dust, longer at 4–8 °C) in formalin vapor for 4 min or in the fixative solution for 30 s (see Appendix).
- Wash in tap water.
- Incubate for 60 min.
- Wash in tap water.
- Stain in Mayer's hemalum for approx. 8 min.
- Blue in tap water for approx. 15 min.
- Mount smears with glycerine gelatin or Aquatex (Merck).
- Air-dried smears may be mounted with Eukitt.

Interpretation

Positive cells stain with a brown to reddish-brown diffuse or granular pattern. The α-naphthyl butyrate stain yields a dark red color. The result is very similar to the α-naphthyl acetate stain, so the slightly different method used with α-naphthyl butyrate will not be described in detail.

Monocytes in the peripheral blood are strongly positive for α-naphthyl acetate stain, while neutrophilic and eosinophilic granulocytes are negative. Some lymphocytes stain with a circumscribed, dotlike pattern. The strongest activity in bone marrow cells is found in monocytes, macrophages, and megakaryocytes.

Acid α-Naphthyl Acetate Esterase (ANAE)

Reagents

- Fixative: see Appendix.
- Staining solution: dissolve 50 mg α-naphthyl acetate in 2.5 mL ethyleneglycolmonomethyl ether + 44.5 mL of 0.1 mol/L phosphate buffer pH 7.6 + 3.0 mL hexazotized pararosaniline (1.5 mL 4 % pararosaniline in 2 mol/L HCl + 1.5 mL 4 % sodium nitrite solution). Adjust the solution to pH 6.1 – 6.3 with 1 mol/L HCl and filter before use. The solution must be clear.
- Mayer's hemalum.

Method

- Fix air-dried smears in fixative solution at 4 °C for 30 s.
- Wash 3 times in tap water.
- Air dry for 10 – 30 min.
- Incubate in staining solution at room temperature for 45 min.
- Rinse briefly in tap water.
- Place in Mayer's hemalum for 3 min.
- Blue in tap water for 3 min.
- Air dry.

Interpretation

The reaction product appears as a reddish-brown homogeneous or granular precipitate. Acid esterase is used to identify T-lymphocytes. The method is reliable only for more mature forms, however, and inconsistent results are obtained in acute lymphocytic leukemias with T characteristics.

1.13.4 Naphthol AS-D Chloroacetate Esterase (CE)

Reagents

- Methanol-formalin solution, 9 : 1 (v/v).
- 0.1 mmol/L Michaelis buffer, pH 7.0.
- Naphthol AS-D chloroacetate.
- Dimethylformamide.
- Sodium nitrite solution, 4 %.
- Pararosaniline solution, 4 %, in 2 mol/L HCl.
- Staining solution A: mix 0.1 mL sodium nitrite solution and 0.1 mL pararosaniline solution with 30 mL Michaelis buffer.
- Staining solution B: dissolve 10 mg naphthol AS-D chloroacetate in 1 mL dimethylformamide.
- Staining solution C: mix solutions A) and B), adjust to pH 6.3 with 2 mol/L HCl, and filter into a cuvet. Use immediately.

Method

- Fix smears in methanol-formalin for 30 s at room temperature, wash thoroughly in tap water without delay.
- Place smears in staining solution for 60 min, then wash thoroughly in tap water.
- Nuclear stain with hemalum for 5 – 10 min, wash thoroughly with tap water, and blue for approx. 10 min.
- After air drying, the smears may be directly examined or mounted with Eukitt.

Interpretation

A bright red reaction product forms at sites of enzymatic activity in the cytoplasm. Neutrophilic granulocytes normally display a positive reaction from the promyelocytic stage on, the late promyelocyte to myelocyte stages showing the strongest reaction. A slightly weaker reaction is seen in band and segmented forms. Monocytes may also show a weak chloroacetate esterase reaction. Besides neutrophils, tissue mast cells display very strong activity. In acute myelomonocytic leukemia, which is associated with an anomaly of chromosome 16, some of the abnormal eosinophils show a positive chloroacetate esterase reaction. Normal eosinophils are negative.

1.14 Appendix

Fixation (Suitable for Esterase, Acid Phosphatase, DAP IV)

The fixative solution is composed of:

- 30 mL buffer solution (20 mg disodium hydrogen phosphate · $12H_2O$ and 100 mg potassium dihydrogen phosphate dissolved in 30 mL distilled water; pH should be 6.6)
- +45 mL analytical grade acetone
- +25 mL formalin (37 %)

Fix air-dried smears in this solution for 30 s at 4 – 10 °C, wash in three changes of distilled water, and dry at room temperature for 10 – 30 min.

Schaefer universal fixative. Mix 0.5 mL of 25 % glutardialdehyde solution and 60 mL analytical grade acetone in distilled water to make 100 mL. Air-dried smears are incubated in this fixative solution at room temperature: 1 min for peroxidase, 10 min for chloroacetate esterase, 5 min for detecting esterase with naphthyl acetate or naphthyl butyrate, 1 min for acid phosphatase, 1 min for alkaline phosphatase, 10 min for detecting iron, and 10 min for the PAS reaction.

Sodium Nitrite Solution 4 %

Dissolve 4 g sodium nitrite in distilled water to make 100 mL.

Pararosaniline Solution 4 %

Dissolve 2 g Graumann pararosaniline (Merck) in 50 mL of 2 mol/L HCl by gentle heating. Cool and filter the solution.

The sodium nitrite and pararosaniline solutions will keep for several months when stored in a dark bottle under refrigeration. Most of the reagents and even commercial staining kits can be ordered from pharmaceutical houses (Merck, Serva, Sigma, etc.). Before kits are used for routine tests, they should be compared against solutions prepared by the methods indicated.

The cytochemical features of blood cells and bone marrow cells are reviewed in **Table 2.**

Table 2. Cytochemistry of blood and bone marrow cells

	Peroxidase	PAS	Esterase		Phosphatases		Remarks
			α-Naphthyl-acetate-, Naphthol-AS-acetate-	Naphthol-AS-D-chloracetate-	alkaline	acid	
Reticulum cells	∅	∅ – +	++	∅	∅ (1)	++	(1) vascular endothelia +++
Plasma cells	∅	∅	(+)	∅	∅	+	Acid phosphatasc is strongly positive in multiple myeloma
Myeloblast	∅	∅ – (+)	∅ – (+)	∅	∅	∅	
Promyelocyte	++	(+)	∅ – (+)	+++	∅	+	
Myelocyte	++	+	∅ – (+)	+++	∅	+	
Metamyelocyte	++	++	∅ – (+)	+++	∅ – (+)	(+)	
Band form	++	+++	∅ – (+)	+++	∅ – (+)	(+)	
Segmented form	+++	+++	∅ – (+)	+++	∅ – +++	(+)	
Eosinophils	++	+	∅ – (+)	∅	∅	(+) – +	
Basophils Blood Tissue	∅ – +	+	∅ – (+)	∅ ++	∅	∅ ++	
Monocytes	∅ – +	(+) – +	+++	(+)	∅	∅	
Lymphocytes	∅	∅ – +	+	∅	∅	∅	Hairy cells are acid-phosphatase positive
Erythroblasts	∅	∅	++	∅	∅	∅	Positive PAS reaction in erythremias and erythroleukemias and some MDS
Erythrocytes	∅	∅	(+)	∅	∅	∅	
Megakaryocytes and platelets	∅	+	+++	∅	∅	++	PAS reaction may be decreased in Werlhof's disease
Osteoblasts	∅	∅	+	∅	+++	+	
Osteoclasts	∅	∅ – (+)	++	∅	∅	+++	

Reaction: ∅ = negative; (+) = weakly positive; + = positive; ++ = markedly positive; +++ = strongly positive

2. Immunocytochemical Detection of Cell-Surface and Intracellular Antigens

Today the immunologic characterization of cells is based on the use of monoclonal antibodies. This may involve the immunocytologic staining of smears or analysis by flow cytometry, in which a number of different fluorochrome-labeled antibodies are used for studies of cell suspensions. We refer the reader to commercial kits, which come with detailed instructions, and to the information that has become available in recent textbooks on immunocytology and diagnostic hematology. (Hrušák O, Porwit-MacDonald A (2002) Antigen expression patterns reflecting genotype of acute leukemias. Leukemia 16: 1233 – 1258)

3. Staining Methods for the Detection of Blood Parasites[1]

3.1 "Thick Smear" Method

One drop of blood is placed on a slide and spread with the edge of a second slide to cover an area the size of a dime. The film should not be too thick, or it will flake off during drying or displace during staining (it should be thin enough that printed text can still be read through it). The film is air dried and may be stained after it is completely dry.

The film is *stained* without preliminary fixation. Owing to the concentrating effect of the thick smear method, a parasitic infection can be detected even when the organisms are present in small numbers. Staining without preliminary fixation induces a massive hemolysis that dislodges the parasites from the erythrocytes so that they can be identified.

The staining solution is prepared fresh for each use and consists of 1 drop of stock Giemsa stain distilled to 1.0 mL and buffered water (pH 7.2). This solution hemolyzes and stains simultaneously.

The stain is applied for 20 – 30 min, then the slide is carefully washed by dipping it in tap water. It is dried in an upright position.

Besides the thick smear preparation, a thin blood smear (fixed in methanol for 5 min) should also be prepared so that the parasites can be accurately identified if doubt exists. Often this is difficult to accomplish in thick smears.

Thick smear preparations for trypanosomes (T. *gambiense, T. rhodesiense, T. cruzi)* are stained in the same way as for malaria parasites. This method is also used to examine for *Borrelia recurrentis.*

3.2 Bartonellosis

Bartonella organisms are most readily detected by the examination of Pappenheim-, or Giemsa-stained blood smears.

3.3 Detection of Blood Parasites in Bone Marrow Smears

Blood parasites are best demonstrated in marrow smears by Giemsa staining (17 mm) after fixation in methanol (5 min) (see p. 7).

3.4 Toxoplasmosis

Giemsa staining of the touch preparation or other sample is also recommended for the detection of toxoplasmosis. Direct immunofluorescence and the peroxidase reaction can detect the organism with high sensitivity.

3.5 Microfiliariasis

1. Wet preparation (thick smear method): Examine a drop of fresh (anticoagulated) blood under a coverslip on a microscope slide (bearing in mind the periodicity in microfilarial activity, see p. 403). The highly motile organisms are clearly visible even at low magnification (250×).

2. Concentrating the sample: To 3 – 5 mL of drawn venous blood, add 10 – 15 mL of a mixture of 95 mL formalin (5 %), 5 mL acetic acid, and 2 mL of an alcoholic gentian violet solution (4 g per 100 mL 96 % alcohol). Centrifuge the mixture, and examine the sediment for stained microfilariae. (Membrane filtration methods provide a particularly good yield.)

3. Examination of a skin snip for microfilariae (*Onchocerca volvulus*). Place a large drop of physiologic saline solution onto a slide. Immerse in the saline a pinhead-size piece of skin excised with a Walser dermatome (if that is not available, use a razor blade). Cover with a coverslip, let stand 20 min, then examine with the microscope at low power (~300×). The organisms will pass from the skin into the saline medium and will move vigorously in the fluid.

3.6 Mycobacterium Species (*M. tuberculosis, M. leprae*)

One or two of the following reactions are used to examine a suspicious sample. The Kinyoun and auramine stains are usually combined and have largely replaced the Ziehl-Neelsen stain. The mycobacteria stain red with both the Kinyoun and Ziehl-Neelsen stains.

a. Kinyoun cold stain (alternative to Ziehl-Neelsen):

1. Fix the specimen (with heat or methanol).
2. Immerse in Kinyoun solution for 3 min.
3. Wash with water for 30 s.
4. Place in Gabett solution for 2 min.
5. Wash and dry.

[1] Revised by Prof. Dr. R. Disko, München.

b. Auramine stain:
1. Fix the specimen with heat.
2. Stain with Auramin solution for 3 min.
3. Decolorize with acid alcohol for 1 min.
4. Wash off acid alcohol with water.
5. Restain with blue-black ink solution for 1 min.
6. Rinse off ink solution with water and dry.

c. Ziehl-Neelsen stain:
1. Fix the specimen with heat.
2. Cover with 10 % carbolfuchsin and heat to steaming 3 times; stain for 3 min.
3. Decolorize in 3 changes of acid alcohol for 3 min.
4. Wash with water.
5. Counterstain with dilute methylene blue solution for 3 min.
6. Wash with water and dry between sheets of blotting paper.

Illustrations

Overview of Cells in the Blood, Bone Marrow, and Lymph Nodes

Figure 1 presents an overview of the various cells of hematopoiesis. The figure does not attempt to answer unresolved questions of cell origins and is intended only as an introductory scheme to help the beginner find some order in the bewildering variety of cells. The cells of hematopoiesis develop from CD 34 – positive stem cells, which resemble large lymphocytes or small, undifferentiated blasts (**Fig. 2**). When cultured, these cells form colonies that can sometimes be identified by their intrinsic color (**Fig. 3**).

Red and white cell precursors account for most of the cells found in normal bone marrow. In addition there are variable numbers of *reticulum cells, vascular and sinus endothelial cells, megakaryocytes, tissue mast cells, lymphocytic elements, plasma cells* and, very rarely, osteoblasts and osteoclasts (more common in children). The earliest precursors of the red and white blood cells have a basophilic cytoplasm and are very similar to one another. As hemoglobin synthesis increases, the *erythroblasts* lose their basophilic cytoplasm while their nuclei undergo a characteristic structural change. After losing their nuclei, the young erythrocytes still contain remnants of their former cytoplasmic organelles as evidence of their immaturity. They are reticulocytes and are released as such into the peripheral blood. The reticulocytes can be demonstrated by supravital staining (see p. 8).

The *myeloblasts*, which are the precursors of neutrophilic granulocytes and monocytes, develop into neutrophilic promyelocytes and promonocytes. The eosinophilic and basophilic granulocytes pursue their own lines of development and therefore have their own promyelocytes with specific granules.

Platelets (thrombocytes) develop from the cytoplasm of the megakaryocytes.

The common progenitor cell from which monocytes and neutrophilic granulocytes originate might be termed the myelomonoblast (CFU-GM).

The *reticulum cells* described and counted in cytologic preparations from bone marrow, lymph nodes, and spleen form a heterogeneous group. A large portion belong to the macrophage system and are derived from blood monocytes. They also include segregated vascular and sinus endothelial cells in addition to dendritic cells belonging to the stroma. The reticulum cells of the bone marrow constitute the reticular or spongy tissue of the bone marrow in which the actual hematopoietic cells reside. Apparently they perform important tasks relating to nutrition and differentiation of the blood cell precursors.

Two different types of reticulum cell are known to occur in the lymph nodes and spleen: the "dendritic reticulum cell," which occurs exclusively in germinal centers, primary follicles, and occasionally in the peripheral zones of follicles, and the "interdigitating reticulum cell," which is specific to the thymus-dependent region of the lymph node (see **Fig. 132** for details).

The "fibroblastic reticulum cell" described by Lennert and Müller-Hermelink can occur in all regions of the lymph node as well as in bone marrow, but as yet it has not been positively identified by light microscopy. The cells formerly described as small "lymphoid reticulum cells" are probably tissue lymphocytes.

In the *lymphatic system,* a basic distinction is drawn between B lymphocytes and T lymphocytes based on the development, differentiation, and function of the cells. Unfortunately, the differentiation of these two cell types cannot be accomplished with traditional staining methods and must rely on immunocytologic or flow cytometric analysis. Both lymphatic cell lines appear to arise from a common, committed stem cell that probably resides in the bone marrow. Thereafter the primary differentiation of the T cell line takes place in the thymus, while that of the B cells (in humans) takes place in the bone marrow, which today is viewed as the equivalent of the *fabrician bursa* in birds. Further development and proliferation of both cell lines take place in the lymph nodes.

The final maturation stage of B lymphocytes is the plasma cell, whose function is to produce immunoglobulins. Plasma cells occur ubiquitously. Apparently they can develop anywhere in the body but are most plentiful in lymph nodes, spleen, and bone marrow. A positive correlation exists between the amount of immunoglobulins present in the serum and the size of the plasma cell population.

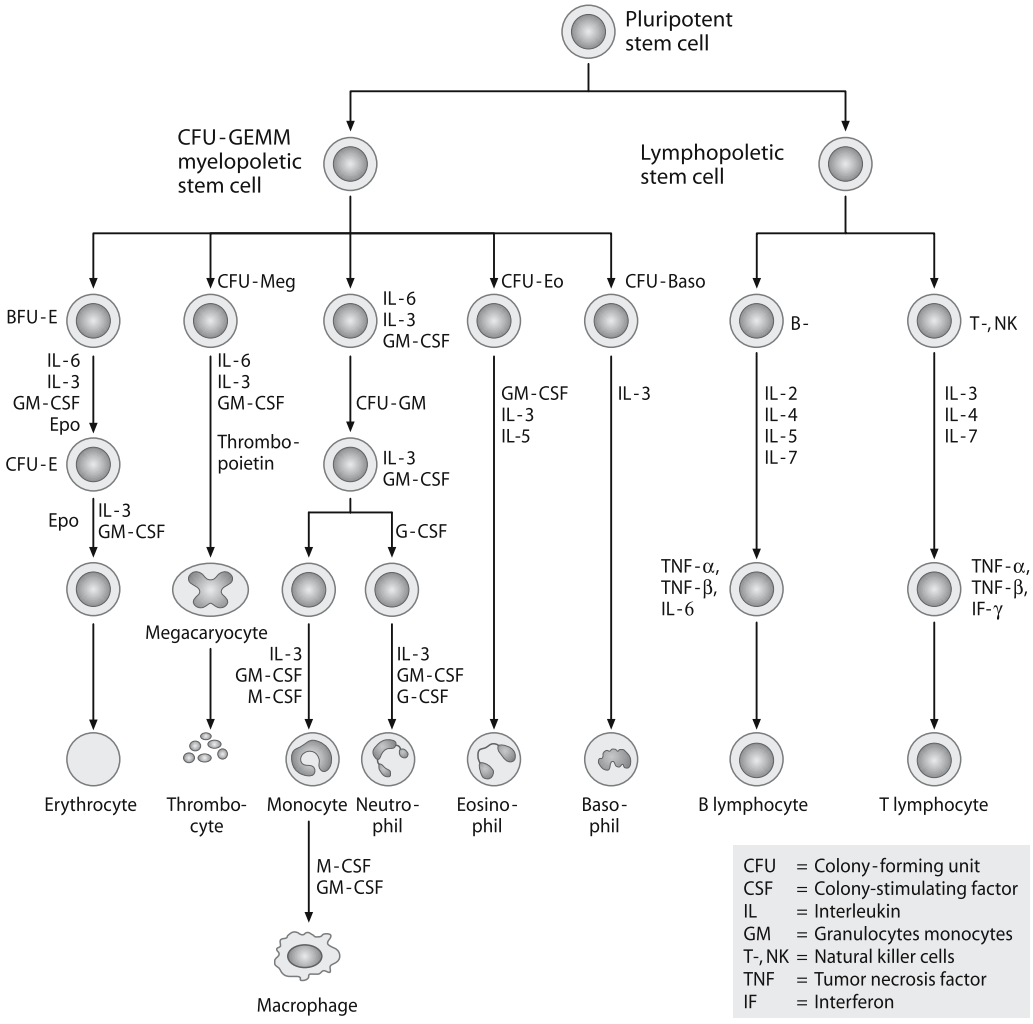


Fig. 1. The various cell lines of hematopoiesis

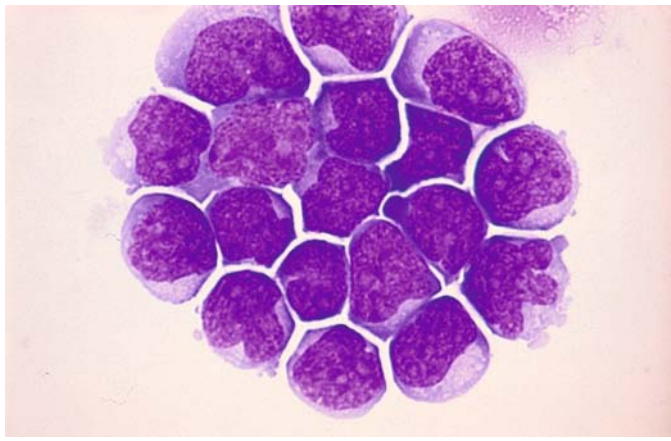


Fig. 2. CD 34 – positive stem cells

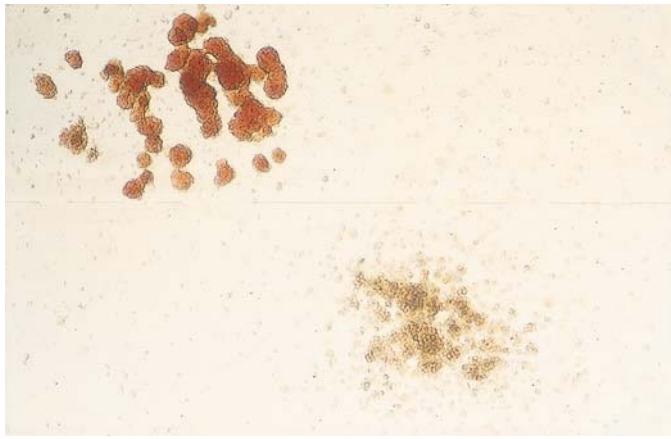


Fig. 3. Colonies of CD34-positive stem cells in cultures

Blood and Bone Marrow

4. Individual Cells

4.1 Light Microscopic Morphology and Cytochemistry

4.1.1 Cells of Erythropoiesis (Fig. 4 a – f)

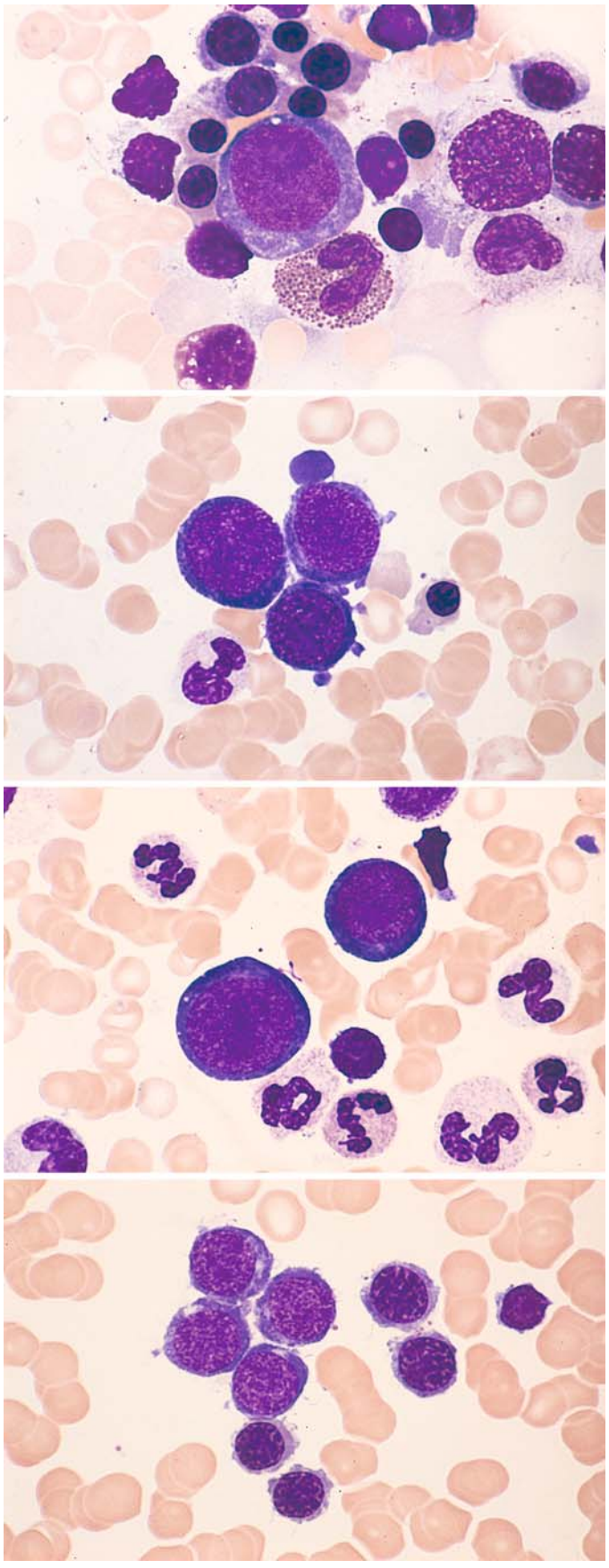
The **proerythroblasts,** called also **pronormoblasts** or **rubriblasts,** are the earliest precursors of erythropoiesis. They range from 15 to 22 µm in size and do not yet contain hemoglobin. They typically have a darkly basophilic, often shadowy cytoplasm that sometimes shows pseudopodia. The nucleus has a dense, finely honeycombed chromatin structure (**Fig. 4 a – c**). Most proerythroblasts have several (at most five) indistinct pale blue nucleoli, which disappear as the cell matures. Like all erythropoietic cells, proerythroblasts tend to produce multinucleated forms. Typically there is a perinuclear clear zone, which is found to contain minute granules on phase contrast examination. Hemoglobin first appears adjacent to the nucleus and produces a flaring of the perinuclear clear zone, later expanding to occupy the whole cell and heralding a transition to the polychromatic forms. Meanwhile the nucleus undergoes a characteristic structural change: the nucleoli disappear while the chromatin becomes coarser and acquires typical erythroblastic features.

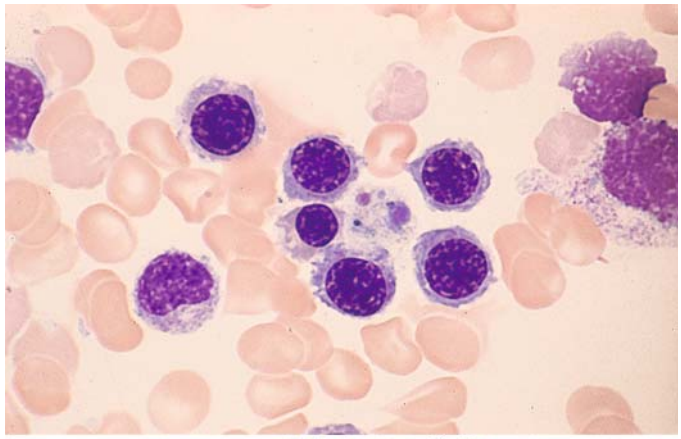
A continuum exists from the proerythroblasts to the **basophilic erythroblasts (macroblasts)** (**Fig. 4d**). These cells tend to be smaller than proerythroblasts (8 – 15 µm in diameter). The nuclear-cytoplasmic ratio is shifted in favor of the cytoplasm. The polychromatic erythroblasts show a coexistence of basophilic material with a greater abundance of hemoglobin. The nucleus appears coarse and smudgy, and there is partial clumping of the nuclear chromatin.

As development progresses, the cell loses more of its basophilic cytoplasm and further diminishes in size (7 – 10 µm in diameter), gradually entering the stage of the **orthochromatic normoblast** (**Fig. 4e**). The nuclear- cytoplasmic ratio is further shifted in favor of the cytoplasm, which acquires an increasingly red tinge ultimately matching that of the mature erythrocyte. Supravital staining of the youngest erythrocytes reveals a network of strands (see p. 8) called the "substantia reticulofilamentosa" of the reticulocytes. Staining with brilliant cresyl blue causes the aggregation or precipitation of ribonucleoproteins. It takes four days for the cells to pass through the four maturation stages. The clumplike erythroblastic nucleus then condenses to a streaklike, featureless, homogeneous mass. Some authors subdivide the normoblasts into basophilic, polychromatic, and orthochromatic forms according to their degree of maturity, while others use the terms *rubricyte* (basophilic normoblast) and *metarubricyte* (orthochromatic normoblast). Such fine distinctions are unnecessary for the routine evaluation of marrow smears, however. Normoblasts are incapable of dividing. The nucleus is expelled through the cell membrane.

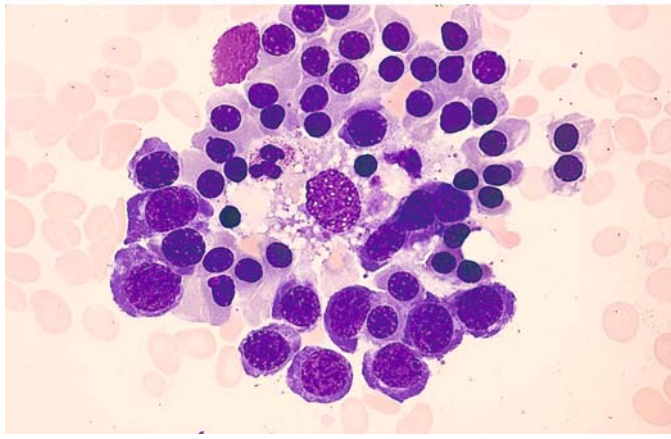
Particularly when erythropoiesis is increased, examination of the smear will reveal *nests or islands of erythroblasts* with central reticulum cells whose cytoplasm is in close contact (metabolic exchange) with the surrounding erythroblasts (**Fig. 4 f**).

Fig. 4 a–d





e



f

Fig. 4 e – f

Erythrocytes (Figs. 5 a–h, 6 a–i)

The morphologic evaluation of erythrocytes is based on the following criteria:
- Size
- Shape
- Hemoglobin: concentration, distribution
- Stainability
- Distribution in the smear
- Inclusions

Normal erythrocytes (Fig. 5 a) (diam. 7–8 µm).

Hypochromic erythrocytes (Fig. 5 b) in iron deficiency anemia. The cells, which have normal diameters, are conspicuous for their paucity of hemoglobin, which may form only a thin peripheral rim (anulocytes).

Poikilocytes (Fig. 5 c) are dysmorphic erythrocytes of variable shape that occur in the setting of severe anemias. Their presence indicates a severe insult to the bone marrow. Teardrops and pear shapes are particularly common and are not specific for osteomyelosclerosis or -fibrosis.

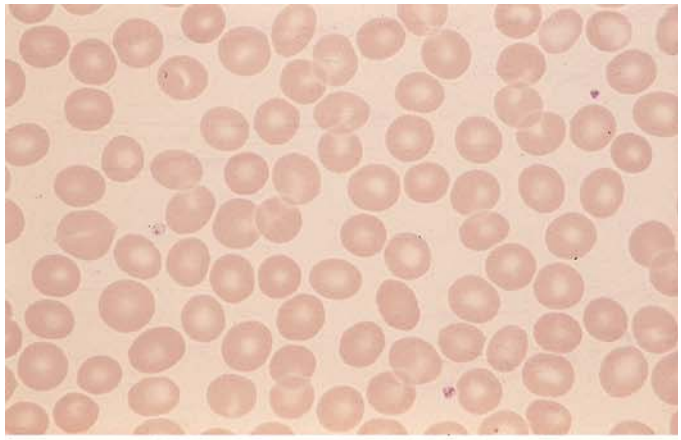
Microspherocytes (Fig. 5 d) are smaller than normal erythrocytes (diam. 3–7 µm) but are crammed with hemoglobin and have a greater thickness, giving them an approximately spherical shape. They are typical of congenital hemolytic jaundice (spherocytic anemia) but also occur in acquired hemolytic anemias.

Elliptocytes (ovalocytes) (Fig. 5 e) result from an inherited anomaly of erythrocyte shape that is usually innocuous but may be linked to a propensity for hemolytic anemia (elliptocytic anemia).

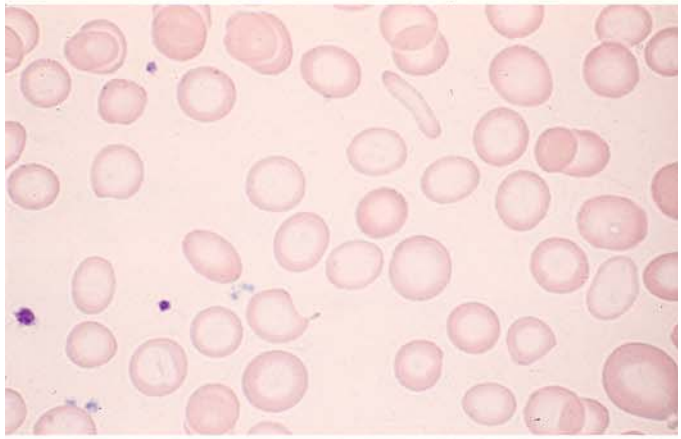
Basophilic stippling (Fig. 5 f) of erythrocytes is a sign of increased but abnormal regeneration. It is particularly common in lead poisoning. The normal prevalence of basophilic stippling is 0–4 erythrocytes per 10,000

Polychromatic erythrocytes (Fig. 5 g) (diam. 7–8 µm), **Cabot rings.** Polychromasia occurs when mature erythrocytes show increased staining with basic dyes (violet stain) in addition to hemoglobin staining. It is usually associated with reticulocytosis. Polychromasia occurs in red cells that still have a relatively high RNA content and in which hemoglobin synthesis is not yet complete. It is especially common in chronic hemolytic anemias. The variable staining of the erythrocytes is also termed *anisochromia.* Cabot rings are remnants of spindle fibers and are a product of abnormal regeneration (see also **Fig. 46c**).

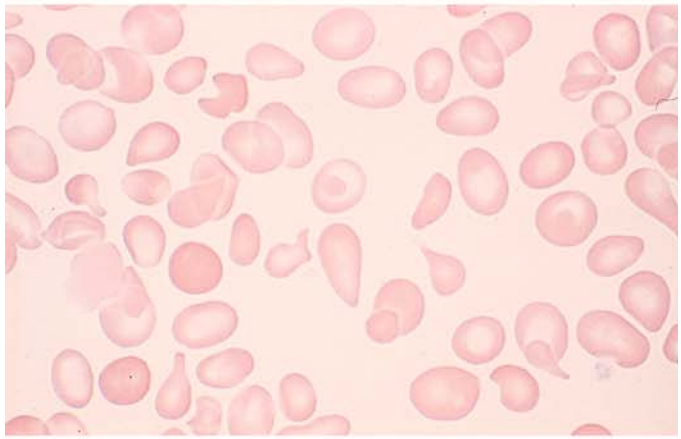
Megalocytes (Fig. 5 h) are very large, mostly oval erythrocytes that are packed with hemoglobin (> 8 µm in diameter). They occur predominantly in megaloblastic anemias (see sect. 5.2.3)



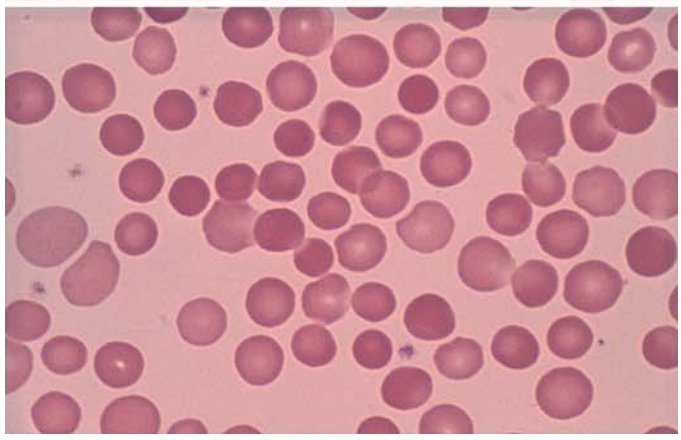
a



b



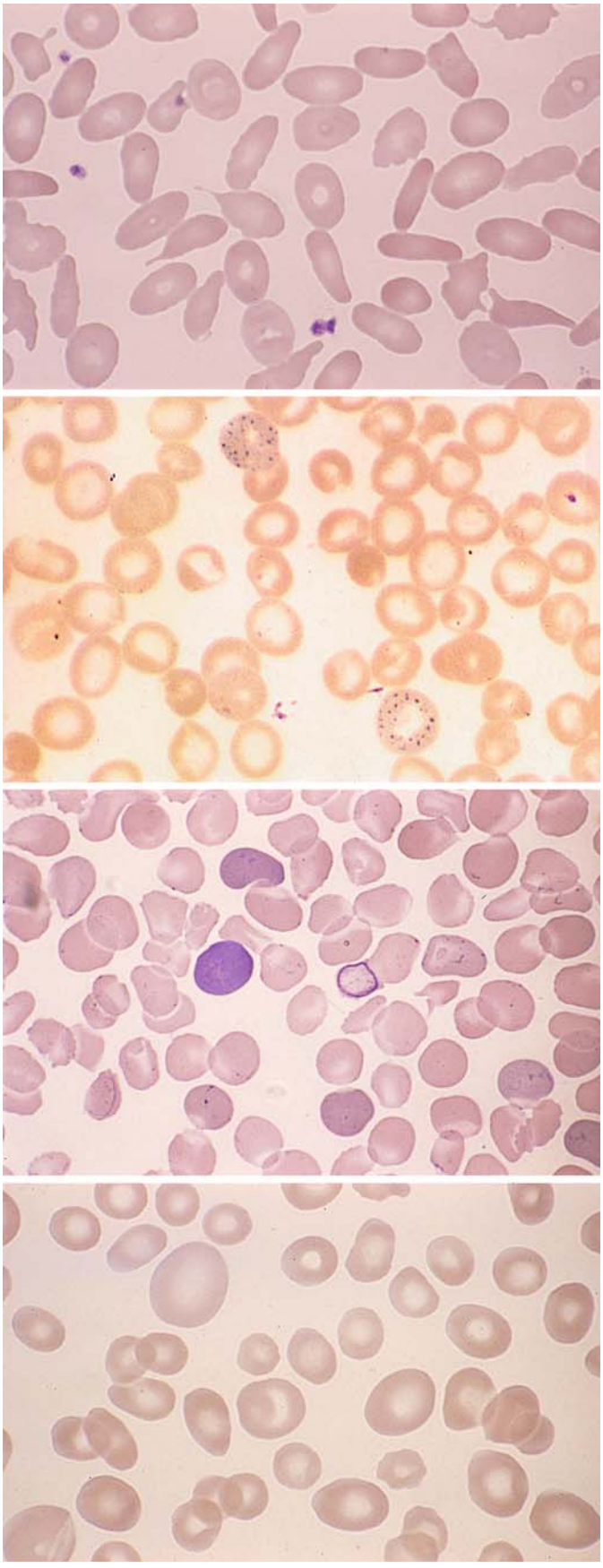
c



d

Fig. 5 a – d

Fig. 5 e – h



Erythrocytes (Fig. 6 a) containing *nuclear remnants* in the form of *Howell-Jolly bodies*, which are observed after splenectomy and in cases of splenic atrophy. Chromatin dust, like the Howell-Jolly bodies, consist of nuclear remnants.

Target cells (Mexican hat cells) (Fig. 6 b) are distinguished from anulocytes by the deeper staining of their central zone and peripheral rim. They are particularly common in hemoglobin abnormalities, occurring also in other hemolytic anemias, severe iron deficiency, and after splenectomy.

Acanthocytes or **"burr cells" (Fig. 6 c)** are distinguished by their jagged surface, which usually is deeply clefted. Acanthocytes are seen in a rare hereditary anomaly, A-β-lipoproteinemia. They are also a feature of uremia and hepatic coma, where large numbers of these cells are considered a poor prognostic sign. Acanthocyte formation has also been linked to the use of alcohol and certain drugs.

Sickle cells (drepanocytes) (Fig. 6 d). Sickle-shaped erythrocytes occasionally form spontaneously, but sickling is consistently induced by oxygen withdrawal in the sickle cell test (see p. 5). It signifies a common hemoglobinopathy, HbS disease (sickle cell anemia), which affects blacks almost exclusively. Red cell sickling also occurs in the less common HbC disease.

Knizocytes (triconcave erythrocytes) (Fig. 6 e) occur mainly in hemolytic anemias. The affected erythrocyte appears to have a "handle."

Stomatocytes (Fig. 6 f) have a slitlike central lucency. They are found in the very rare hereditary stomatocytosis and in other anemias.

Schizocytes (fragmentocytes) (Fig. 6 g) result from the fragmentation of erythrocytes, consisting either of a fragmented red cell or a fragment detached from such a cell. They resemble bits of broken egg shell. They may be caused by increased mechanical hemolysis (turbulence from artificial heart valves) or by increased intravascular coagulation (e.g., in hemolytic uremic syndrome) as fast-flowing red cells are sliced apart by fibrin filaments.

Siderocytes (Fig. 6 h) are erythrocytes that contain iron granules detectable with iron staining. They are a common feature of severe hemolytic anemias, lead poisoning, and pernicious anemia. Siderocytes containing coarse iron granules, which may encircle the nucleus (see **Fig. 60**), are pathognomonic for sideroachrestic anemias. Normal blood contains 0.5–1 siderocyte per 1000 red cells.

Left: At the center is a siderocyte containing several large iron granules and two sideroblasts also containing coarse iron granules, which normally are very small and difficult to see.

Right: At the center are three erythrocytes with numerous gray-violet granules that contain iron (Pappenheimer bodies). This is a clear-cut pathologic finding that is rarely observed.

Reticulocytes (Fig. 6 i) in various stages of maturity. The more filamentous reticula are characteristic of younger cells (brilliant cresyl blue stain, see p. 8).

Fig. 6 a – d