Enzyme Histochemistry and Immunohistochemistry on Biopsy Specimens of Pathologic Human Bone Marrow

By Jay H. Beckstead, Paula S. Halverson, Curt A. Ries, and Dorothy F. Bainton

We have systematically investigated a variety of fixation and plastic embedding procedures and arrived at a method that allows processing of ~2-μm sections of bone marrow biopsies for examination by light microscopy. More importantly, this method permits the use of enzyme histochemical and immunohistochemical procedures that are rapidly becoming mandatory in the diagnosis of hematologic malignancies. Over 200 full-length bone marrow biopsy specimens were fixed in a mixture of paraformaldehyde, glutaraldehyde, and acrolein, dehydrated in acetone, and embedded in a mixture of methyl and glycolmethacrylate. All procedures were carried out at 4°C. Decalcification was unnecessary. Sections 2-μm thick were cut and incubated for peroxidase, naphthol AS-D chloroacetate esterase, α-naphthyl butyrate esterase, acid phosphatase (with and without tartrate), or alkaline phosphatase and then examined by light microscopy. Specimens could be prepared for examination within 48 hr. This approach, which provides definitive markers for various hematopoietic cell lines in intact tissues, is invaluable when aspirated material is unavailable. It is also useful in the analysis of focal lesions of bone marrow due to inflammation or neoplasia and shows potential as an investigative tool. For example, we have discovered that early myelofibrosis is accompanied by a marked increase in the number of alkaline-phosphatase-positive reticulum cells.

The paraffin-embedded bone marrow biopsy specimen is very valuable in the evaluation of hematologic disorders because it provides a means of assessing intact tissue for cellularity and of detecting focal lesions. It has not achieved its full diagnostic potential, however, because of the difficulty in precisely identifying certain individual cells. Furthermore, recent advances in the use of enzyme histochemistry in diagnosing hematopoietic malignancies have been largely applied to aspirated material or to touch preparations of biopsy specimens. We felt that the biopsy specimen could provide much more information if advances in fixation and embedding methods, enzyme and immunohistochemistry could be applied directly to it. We hoped that the additional information obtained would increase the biopsy specimen’s value as an adjunct and would make it invaluable when aspirated material is unavailable.

Various proponents of plastic embedding for bone marrow biopsy specimens, notably Block and Burkhardt, have noted that thin sections of plastic-embedded material provide improved cytologic detail. Others have noted some enzyme survival in such sections. Unfortunately, many of the recommended procedures for plastic embedding have been considered too expensive and time-consuming for routine use. We have recently shown in animals that a relatively simple, rapid procedure can provide superior sections with excellent survival of a wide variety of the histologic enzymes used in diagnosing and classifying hematologic malignancies. We have applied this technique to more than 200 abnormal human bone marrow biopsies. In this article we illustrate its usefulness in diagnosing hematologic abnormalities, particularly the leukemias. The technique shows potential as an investigative tool as well.

MATERIALS AND METHODS

Human bone marrow specimens were obtained by Jamshidi needle biopsy (11-gauge) of the posterior iliac crest. Three normal volunteers were also examined. The specimens were immediately placed in cold (4°C) paraformaldehyde, glutaraldehyde, acrolein fixative (PGA). Two hours later, they were transferred to wash buffer. They were held in the wash buffer for 1–48 hr. and then were dehydrated in graded acetones and infiltrated with glycol methacrylate monomer. They were then transferred to small cups containing the complete embedding mixture and placed under vacuum for 12 hr. The entire procedure from fixation through embedding was carried out at 4°C. The hardened blocks were sectioned at 1–3μm with a Sorvall JB-4 microtome, stained with hematoxylin-eosin-azure (HEA) for morphology, and tested for the following enzymes: α-naphthyl butyrate esterase (αNBE), peroxidase, naphthol AS-D chloroacetate esterase (CAE), alkaline phosphatase (AcPase), acid phosphatase (AcPase), and lysozyme (see Appendix for further details).

RESULTS

Jamshidi marrow cores up to 3.5 cm in length proved easy to embed by the technique outlined under Materials and Methods. Decalcification is unnecessary and was not carried out because it is time-consuming and may cause enzyme inactivation. Most sectioning problems were the result of inadequate dehydration or infiltration time. Since fat and clotted blood are difficult to infiltrate, care was taken to remove clotted blood surrounding biopsy specimens.
<table>
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</table>

0, Negative; 0/+, some patients with occasional weakly positive cells; +, weakly positive; ++, moderately positive; ++++, most cells strongly positive.

*Some lymphs focally positive.
†Moderately positive if paraformaldehyde fixed.
‡Pseudoperoxidase, dependent on hemoglobin concentration.
§Moderately positive in the zone of maturing cartilage.
| Basophil insufficiently sampled.

and those suspected of being extremely fatty were allowed extra dehydration or infiltration time.

The cytologic detail in our specimens was markedly better than that in conventional paraffin-embedded specimens because of improved fixation and the minimal shrinkage that occurs with plastic embedding. At times, a zonal variation in fixation, due to the different rates of diffusion of the components of PGA is noted, but this does not usually cause interpretive difficulties. When the specimens were thinly sectioned and stained with a polychrome stain, HEA, they were readily interpretable by those familiar with Romanovsky-type stains of aspirated material. We examined two sections on each specimen, a "thick" section (2.5μm) for pattern and cytoplasmic detail and a "thin" section (1.5μm) for nuclear detail. Figure 1* illustrates the results in a normal human bone marrow specimen. The easy identification of azurophilic granules greatly facilitated the identification of early myeloid cells. This advantage, coupled with the improved cytologic detail, made it possible to perform a differential count on the sections with much greater confidence than would be possible on paraffin-embedded material. Figure 1 (inset) shows a blast from a patient with acute myelogenous leukemia (AML). Auer rods can be confidently identified, a nearly impossible task in paraffin-embedded material.

We found that a wide variety of useful enzymes could be reliably identified in the plastic sections. Inconsistencies in enzyme survival could be generally traced to overfixation, temperature variations, or other alterations in processing, though some individual variation between patients may also have been important. The incubation conditions (see Appendix) were carefully monitored, since we found that prolonged incubation can increase the incidence of nonspecific cross-reactions. Specifically, the 90-min, 37°C incubation with αNBE that we originally used gave weakly positive reactions in the promyelocytes of some individuals. For this reason, we decreased the temperature of incubation to 30°C. In general, increasing the temperature and the length of incubation increased the strength of the reactions, but at the risk of introducing some nonspecificity. Increasing the thickness of the sections also increased the strength of the reactions. The enzymes were slowly labile in the embedded tissues; though some strong reactions persisted for up to 1 yr of storage, others diminished over the course of 2–3 mo. The expected results with various enzymes are shown in Table 1. Although we have been success-

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*Figures 1–9 are 2-μm sections of normal or pathologic human bone marrow biopsies that have been stained for the demonstration of various enzymes. All materials were fixed 1–2 hr at 4°C in PGA (2% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% acrolein) buffered with PO4 at pH 7.4. The following abbreviations are used: B, basophil; E, eosinophil; EB, erythroblast; P, promyelocyte; Au, Auer rod; M, macrophage; Sin, sinus; Al-RC, alkaline phosphatase-positive reticulum cell; R, reticulin.
Figs. 1–9. See legends facing page.
ful in using immunohistochemical techniques to demonstrate a number of proteins (lysozyme, lactoferrin), we have not yet reliably demonstrated any surface or cytoplasmic lymphoid markers.

We found many of the enzyme reactions helpful in the diagnosis and classification of malignancy, particularly in cases where aspirates were scanty or inadequate. Peroxidase-positive granules (Fig. 2) were readily identifiable in leukemic cells of most patients with AML, and Auer rods were well demonstrated when present. The CAE reaction* was also useful in identifying myeloid leukemias. This reaction in a rib mass specimen from a patient with chronic myelogenous leukemia (CML) (Fig. 3) readily identified the mass as a granulocytic sarcoma.

The α-naphthyl butyrate esterase reaction is a valuable marker of the monocyte macrophage or mononuclear phagocyte system (MPS) in hematopoietic tissues. The small number of monocytes and their precursors normally present in the bone marrow can be recognized by positive staining with αNBE. In a pathologic marrow specimen, such as the one from a patient with well differentiated monocytic leukemia illustrated in Fig. 4, the reaction can help to classify the cell lineage of the malignant process. In addition to the reaction in the cells of the MPS, some lymphocytes show a discrete focal reaction using the same substrate. This reaction has been correlated with T-cell markers by some authors (not illustrated).

The presence of lysozyme is another marker of the MPS and myeloid differentiation. Lysozyme can be detected with an immunoperoxidase procedure employing an antilysozyme antibody. Diffuse brownish cytoplasmic staining was seen in cells from a patient with well differentiated monocytic leukemia (Fig. 5), in contrast to the normal granular pattern of reaction in myeloid cells (insert, Fig. 5).

Acid phosphatase was detectable in many cells of hematopoietic origin (Table 1). Cells of the MPS showed a variety of reactions, ranging from an intense positivity in osteoclasts and some macrophages to a moderate granular cytoplasmic reaction in monocytes. Some lymphocytes showed a focal positivity, which may also correlate with some T-cell malignancies. Neutrophils sometimes showed small granules, but were usually unreactive, presumably due to overfixation, since in the rat, neutrophils were reactive. The malignant cells in hairy cell leukemia were also positive and resisted tartrate inhibition (Fig. 6).

The alkaline phosphatase reaction is seen in a limited number of cell types in the human bone marrow. Certain fibroblast-like reticulum cells show a distinct plasma-membrane-associated AlkPase (Fig. 7). We have observed a striking increase in these alkaline-phosphatase-positive reticulum cells (Al-RC)

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*We have also found the CAE reaction valuable as a means of dramatically emphasizing, by the absence of staining, small focal lesions that might otherwise go unnoticed. This has been particularly helpful in evaluating patients with lymphomas. In addition, it provides a means of rapidly and accurately assessing granulocyte reserves and the myeloid:erythroid ratio.

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**Fig. 1.** Human bone marrow, stained with hematoxylin, eosin, and azure II to demonstrate the improved morphological preservation as compared to biopsies embedded in paraffin. Azurophilic granules, which stain pinkish-red, are visible in immature myeloid cells (arrows). Basophils (B), as well as mast cells, stain metachromatically. Larger, pink granules are present in portions of the cytoplasm of eosinophils (E); nucleated erythroblasts (EB) are also present (x 1000). Inset demonstrates that Auer rods (Au) can also be seen in a myeloblast of a patient with AML (x 1600).

**Fig. 2.** Bone marrow from a patient with AML (M) reacted for peroxidase. Peroxidase-positive azurophilic granules and Auer rods (Au) are clearly visible in promyelocytes (P). A fragment of an eosinophil (E) shows larger granules. A large Auer rod (Au) is seen in the inset (x 1600).

**Fig. 3.** Portion of a rib from a patient with CML that documents the presence of a granulocytic sarcoma. Most cells show strong red granular staining with naphth AS-D chloroacetate esterase reaction (CAE) and can therefore easily be identified as neutrophilic promyelocytes (P) (x 1600).

**Fig. 4.** Bone marrow biopsy from a patient with acute monocytic leukemia (M5), incubated for the α-naphthyl butyrate esterase reaction. A diffuse reddish blush is seen in all of the monoblasts. The irregular nuclei are very easily seen in these thin sections (x 1600).

**Fig. 5.** Same patient as in Fig. 4 reacted with the antilysozyme antibody using an immunoperoxidase procedure. The blast cells show a brownish cytoplasmic reaction, indicating the presence of cytoplasmic lysozyme. The inset shows the granular reaction in neutrophils (x 1600).

**Fig. 6.** Bone marrow biopsy from a patient with hairy cell leukemia. The tissue has been reacted for acid phosphatase in the presence of tartaric acid (TRAP). The leukemic population infiltrates the marrow, and most of the cells stain red. Most macrophages (M) lack tartrate resistant acid phosphatase (x 640). Some preparation at higher magnification (inset), to illustrate a TRAP-positive hairy cell (x 1600).

**Fig. 7.** Normal human bone marrow reacted for alkaline phosphatase to demonstrate the relatively sparse population of alkaline-phosphatase-positive (blue) reticulum cells (Al-RC). The elongated blue-staining cytoplasmic processes of the Al-RC extend throughout the hematopoietic parenchyma and near sinus (Sin) walls (x 640).

**Fig. 8.** Bone marrow biopsy from a patient with acute myeloblastosis reacted for alkaline phosphatase. A striking increase in the network of blue-staining cytoplasmic processes is obvious. The nuclei of these Al-RC are occasionally difficult to identify (x 800).

**Fig. 9.** Same patient as in Fig. 8 stained for reticulin (x 800). The black linear deposits (R) between the cells illustrate the marked increase in reticulin fibers.
in patients with myelofibrosis (Fig. 8). The presence of reaction product appears to correlate with active reticulin deposition (Fig. 9), but tends to diminish markedly as mature collagen appears. With this enzymatic marker, increased numbers of stromal reticulum cells are easily recognized. We are now applying this method to the investigation of the evolution of myeloproliferative and lymphoproliferative disorders.* Osteoblasts, some chondrocytes, and the endothelial cells of small arterioles are also reactive with this enzyme. The expected positivity in neutrophils is very weak to negative in the PGA fixed material. If one is particularly interested in this reaction in neutrophils it can easily be demonstrated in material fixed in 4% paraformaldehyde.

In addition to the enzyme histochemical and immunohistochemical reactions noted above, most of the other special stains used in histology can be adapted to the plastic-embedded sections.31 We have been successful with periodic acid-Schiff (PAS) reactions, reticulin stains, Tri-chrome stains, and iron stains. In at least one instance, the Perl's reaction for hemosiderin, plastic embedding permitted superior staining. The problem of iron loss during decalcification is well known and forms the basis for the preference of thick smears as a means of evaluating iron stores.24 This problem is eliminated in plastic sections that are processed without decalcification. The resulting iron stains have the advantages of greater sampling and better counterstaining than thick smear preparations. The cytologic detail was sufficient to permit ready identification of ringed sideroblasts. As noted in our previous paper,12 we have been unsuccessful in trying to apply the Sudan black B stain.

DISCUSSION

In recent years, it has become apparent that conventional morphological observations alone do not permit completely accurate, reproducible classification of the leukemias. Many investigators are searching for more objective tests using enzyme histochemistry and immunohistochemistry, as well as surface markers. The widely used French-American-British (FAB) classification25 recommends the use of enzyme histochemistry and immunohistochemistry in conjunction with morphological observations. We have demonstrated that these histochemical procedures can be readily applied to sections of plastic-embedded human bone marrow. This makes it possible to use histochemistry as an aid in the diagnosis of hematopoietic disorders in any patient on whom a biopsy is performed. For the leukemias, in particular, this permits histochemical criteria to be utilized in the classification of each case. This method can be especially helpful in cases where the aspirate is inadequate or where the lesions are focal. Many different histochemical criteria have been put forth.22-27 We have utilized a relatively straightforward histochemical classification that is outlined below with suggestions for the extension of these criteria to elucidate ambiguous cases.

The rationale for using enzyme and immunologic markers in the classification of malignancies is an extension of the much older morphological concept that malignancies bear some resemblance to the normal cells from which they are derived. During differentiation, each cell line normally synthesizes particular chemical substances that enable the cells to perform their unique functional role when they are mature and fully differentiated. Many of these substances are unique to one cell line, and furthermore, may appear at a specific stage of maturation. If leukemic cells can be shown to contain such substances, they can be considered by analogy with normal cells to be of a particular cell lineage.

This basic rationale has been helpful in subdividing the acute leukemias. Peroxidase, for example, is formed very early in normal cells committed to nonlymphoid maturation28 and has never been reported in lymphoid cells. These features make it a nearly ideal enzyme for differentiating between lymphoid and nonlymphoid leukemias. It is not a good means for subclassifying the nonlymphoid leukemias, since it is found in cell lines29,30 developing into neutrophils, eosinophils, basophils, and monocytes. Fortunately, the two cell lines of paramount clinical importance, neutrophils and monocytes, develop esterases with differing specificities at the promyelocyte/promonocyte stage. The neutrophilic promyelocyte develops an enzyme that is extremely resistant to denaturation and hydrolyzes naphthol-AS-D chloroacetate esters.31 The monocyte has an estoenzyme32 that readily hydrolyzes p-naphthyl butyrate esters at acid pH, but is remarkably sensitive to sodium fluoride (NaF) inhibition.13,33 Since these enzymes are formed early in maturation, they can be very useful in subclassifying leukemic populations.

The myeloid leukemias are thus defined by the presence of peroxidase (light or ultrastructural level) and/or CAE and the absence of αNBE. The degree of

*It has also been reported that B lymphocytes from the mantle zone of normal lymph nodes show a distinct membrane-associated AlkPase and that certain lymphomas thought to derive from these cells also express this enzyme.32 We have made similar observations using lymph nodes embedded in plastic. This marker should be readily demonstrable in the bone marrows of patients with metastatic lymphomas.
morphological differentiation, including the presence of hypergranular promyelocytes (M1, M2, M3), should also be noted.25 The monocytic leukemias (M4) are defined by the presence of diffuse αNBE and the absence of CAE; peroxidase may or may not be present.34 Again, the degree of maturation (M4a, M4b) should be specified morphologically.25 It should also be noted that an occasional myeloid leukemia may show CAE in the absence of peroxidase, or vice versa.35,36 The presence of cytoplasmic lysozyme, as demonstrated by immunohistochemistry, is an additional characteristic of the nonlymphoid leukemias that may be useful as a marker. The term myelomonocytic leukemia (M5) should be restricted to leukemias that show clear-cut evidence of two leukemic populations, one showing monocytic differentiation and one showing myeloid differentiation.25 This form should be distinguished from a myeloid leukemia with a reactive monocytosis, an occasionally difficult task. Rare leukemias with cells containing both αNBE and CAE should also be designated as myelomonocytic.26,37,38

Erythroleukemia (M6) is identified only where a prominent abnormal erythropoiesis occupies more than 50% of a clearly leukemic bone marrow.25 The enzymes noted above should be negative in these cells, though weak to moderate nonspecific esterase positivity, which is resistant to NaF, has occasionally been noted,39-41 and a diffuse pseudoperoxidase reaction may be seen in cells showing hemoglobinization. In our experience, these leukemias are extremely rare when strictly defined.

Megakaryocytic leukemia is rarely recognizable at the light microscopic level, since the majority of these leukemias are quite undifferentiated. Megakaryocytic leukemia is best defined by testing for a fixation-sensitive platelet peroxidase at the ultrastructural level.30 These leukemias could be added to the FAB system as M7a and M7b, undifferentiated and differentiated, respectively. The enzymes αNBE, CAE, and peroxidase should all be negative at the light microscopic level, but AcPase and α-naphthyl acetate esterase (αNAE) may show some reactivity.13 This population of cells should constitute more than 50% of a clearly leukemic bone marrow.

The acute lymphoid leukemias are usually defined by the absence of the enzymes used in defining the acute nonlymphoid leukemias: peroxidase, diffuse NaF-sensitive αNBE, CAE, and lysozyme. Unfortunately, any definition based on the absence of specific features is certain to include some cases inappropriately. There has been much progress in recent years toward defining immunologic subgroups among the lymphoid leukemias.41-47 The widespread application of these markers would eliminate the criticism noted above by replacing a negative definition with a positive one. That is, lymphoid leukemias would be defined not only by the absence of the features of the nonlymphoid leukemias but by the presence of lymphoid markers. The clinical relevance of the application of T-cell (E-rosettes) and B-cell (surface immunoglobulin) markers to the subclassification of lymphoid leukemias is widely accepted.48,49 Wider application of differentiation markers such as human thymic antigens, la antigens, intracytoplasmic immunoglobulins, the common ALL antigen (Greave's), and terminal deoxynucleotidyl transferase may indicate additional useful subclasses. This positive approach would, of course, leave a small number of unclassified leukemias, which could be further reduced by the application of peroxidase at the ultrastructural level.30 Those leukemias that remained unclassified could then be designated as undifferentiated (M0).50

As an ideal approach, the acute leukemias should all be initially examined at the light microscopy level for the presence of peroxidase, CAE, and diffuse αNBE. Those leukemias not clearly identified by their histochemistry should be subjected to lymphoid marker studies. The remaining unclassified cases should be examined ultrastructurally for the presence of peroxidase and fixation-sensitive platelet peroxidase. The usefulness of induced differentiation in culture51 remains unsettled at this time, but may further clarify this group in the future. Focal reactions with AcPase and αNBE and lysozyme are helpful reactions, but are not considered primary in the classification scheme outlined above. This approach is summarized in Table 2.

Histochemistry is useful in the evaluation of several other malignancies that may involve the bone marrow. The presence of tartrate-resistant acid phosphatase (TRAP) is certainly a valuable aid in the diagnosis of hairy cell leukemia,52 and several other enzymes, such as αNBE,53 have recently been identified in these cells. The presence of αNBE activity or lysozyme in the cells of malignant histiocytosis and histiocytosis X, may be very useful in the diagnosis of these disorders. Though it has not yet been firmly established, it appears likely that some lymphomas25 may also show characteristic histochemical patterns. Our preliminary observations indicate that some caution is necessary in the interpretation of αNBE positivity in tissues other than bone marrow, since this enzyme is present in some cells of nonmonocyte origin.54

The improvement in morphology with plastic embedding has been noted by many others, but careful attention to fixation is essential to realize the full potential of this procedure. In fact, problems with fixation may be more obvious and distracting in plas-
tic-embedded materials than in conventionally prepared specimens. We must stress that fresh, carefully prepared fixative is essential for optimal results (see Appendix). The enzymes we have used vary markedly prepared specimens. We must stress that fresh, carefully attentive to the effects of fixation and processing. The acid and alkaline phosphatases in certain cell types, mainly neutrophils, can be very sensitive to fixation. We have selected our conditions to maximize the largest range of enzymes compatible with excellent morphology. It is also important to use standard conditions of incubation for the demonstration of these enzymes, since the specificity of some reactions may be lost with prolonged incubation or elevation of temperature.

At present, the cost of embedding a marrow biopsy specimen in plastic is about 2.5 times the cost of conventional embedding. The expense reflects the need for a great deal of hand processing and for somewhat more expensive materials. We are changing our procedures to decrease the cost of plastic embedding. We have substituted a simple, inexpensive phosphate buffer (see Appendix) for the cacodylate buffer originally recommended. We have found no difference in the results. We are automating the procedure up to the embedding step; when combined with an expected increase in utilization, this should allow us to decrease the costs to approximately twice the cost of conventional embedding, and possibly less. The histochemical tests cost no more on plastic-embedded specimens than they do on smear preparations.

In addition to the clinical usefulness of our plastic embedding procedure, it provides an opportunity to make new observations, since histochemistry has always been difficult on bony materials. For example, we have noted a striking increase in the alkaline-phosphatase-positive reticulum cells of the bone marrow early in acute myelofibrosis, but the enzyme is not present on the fibroblasts seen later in the disease when true collagen fibrosis occurs. A more extensive characterization of these cells in mouse and rat bone marrow has been the subject of a previous paper. An enzymatic marker like this may serve as a tool for understanding the pathogenesis of this disorder and might be of value in diagnosing early or acute myelofibrosis. We are presently attempting to isolate these stromal cells in order to better characterize and study them in vitro.

ACKNOWLEDGMENT

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Table 2. Classification of Acute Leukemias Using Cytochemical and Lymphoid Markers

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+ Positive in 3% or more of leukemic cells; - negative; +/- some cases positive, others negative.
* Peroxidase may only be identifiable at the ultrastructural level.
† Hemoglobin stains as a pseudoperoxidase.
‡ Peroxidase is negative but can be demonstrated at the ultrastructural level (see Discussion).
§ NaF-resistant esterase may be positive.
|| Focal NaF-resistant esterase in some T-cell ALL.
| As noted in the text, the best combination of positive markers for the lymphoid cells remains to be determined.
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APPENDIX

I. Fixative: 3% paraformaldehyde–0.2% glutaraldehyde–0.2% acrolein in 0.1 M phosphate buffer

Preparation of stock buffer:
0.2 M phosphate buffer stock, pH 7.4
A. monobasic sodium phosphate, NaH₂PO₄ 27.8 g in 1000 ml H₂O
B. dibasic sodium phosphate, Na₂HPO₄ 28.4 g in 1000 ml H₂O

Method
Mix 95 ml of A with 405 ml of B and adjust pH to 7.4. (The wash buffer is prepared by diluting the 0.2 M PO₄ stock with an equal part of distilled water and adding 2% sucrose.)

Preparation of Fixative:
(a) paraformaldehyde 9.0 g dissolved in distilled H₂O at 60°C (see method below)
(b) 25% glutaraldehyde stock, EM grade 2.4 ml
10% acrolein stock, EM grade 6.0 ml

Method
The solution of paraformaldehyde in H₂O is prepared in a water bath at 60°C with intermittent agitation. After 30 min, 1 M NaOH is introduced drop by drop; this clears the solution in seconds with only a slight shift of the pH. Prepare fixative fresh weekly and store in a dark bottle at 4°C. Shake well before using.

Depending on particular needs, the proportions of paraformaldehyde, glutaraldehyde, and acrolein may be adjusted. In general, increasing the percent of acrolein and glutaraldehyde improves fixation, particularly at the EM level, but at the cost of enzyme reactivity. Alternatively, further decreases in the concentration of these elements or the use of paraformaldehyde alone may improve the survival of fixation-sensitive enzymes. The length of fixation may also be varied to achieve similar purposes.

II. Tissue Processing
The entire procedure is carried out at 4°C.

Method
(1) Fix 2 hr in PGA fixative.
(2) Wash in 0.1 M PO₄ buffer with 2% sucrose for 1 hr (may be held in buffer up to 48 hr).
(3) Dehydration—10 min 50% acetone (reagent grade) × 2
10 min 95% acetone × 2
10 min 100% acetone × 2
10 min 50% acetone/30% JB-4 monomer × 2
10 min JB-4 monomer
(4) Infiltrate in JB-4 monomer (Solution A, Polysciences Inc.) overnight.
(5) Preparation of embedding medium
JB-4 solution A 18 ml
methyl methacrylate monomer 2 ml
(#834 - Polysciences, Inc.) benzol peroxide 0.09 g
JB-4 solution B 0.5 ml

Embed tissue in this medium under vacuum (15–20 in of Hg) at 4°C in molds (Sorvall, Dupont Instruments, Newton, Conn. 06470) for 12 hr: then allow blocks to warm to room temperature.
(6) Cut sections 1–3 μ thick with glass knives on a Sorvall JB-4 microtome. The sections are transferred via water to coverslips and air dried at room temperature.
III. Morphology Stain (HEA)

Method

Stain coverslips in the following reagents:

1. Hematoxylin (Gill’s double strength) for 2 min; wash in H₂O.
2. Scott’s buffer (0.2% sodium bicarbonate, 1% magnesium sulfate in tap water) for 2 min; wash in H₂O.
3. Eosin Y, 1% aqueous, for 3 min; wash in H₂O.
4. Azure II, 0.1% aqueous, for 1 min; wash in H₂O.
5. Dry completely.
6. Decolor background in ethylene glycol monomethyl ether (#E-5378, Sigma Chemical Co., Box 14508, St. Louis, Mo., 63178) for 15 sec; wash in H₂O.
7. Dry and mount with Permount.

Times are approximate and should be varied to give the depth of color most desired. Intensity of color is also affected by the thickness of the section.

IV. Enzyme Reactions

A. Peroxidase procedure:

Method

1. Presoak sections in normal saline for 15 min.
2. Preincubate for 10 min at room temperature in 5 mg DAB (3,3-diaminobenzidine, Sigma Grade II) dissolved in 10 ml 0.05 M Tris buffer pH 7.6. Adjust pH (7.6), if needed, and filter.
3. Add 0.1 ml of 1% H₂O₂ to DAB-Tris solution and incubate for 20 min at room temperature (stock solution of H₂O₂ should be replaced frequently).
4. Rinse x 3 with 0.05 M Tris buffer.
5. Counterstain with hematoxylin.
6. Air dry and mount in Permount.

Note

If large numbers of coverslips are stained, substrate exhaustion may result in staining of eosinophils and red blood cells only. If this happens, repeat with small number of coverslips and careful checking of pH.

B. Chloroacetate esterase procedure:

Preparation of reagents:

1. Solution A. 10 mg naphthol AS-D chloroacetate (#N-0758, Sigma Grade III) dissolved in 1 ml N,N-dimethylformamide (#D-4254, Sigma).
2. Hexazotized pararosanilin:
   1. Prepare a 4% pararosanilin solution by dissolving 1 g EM grade pararosanilin hydrochloride (TAAB Lab, 52 Kidmore End Road, Emma Green, Reading, England) in 20 ml distilled H₂O and 5 ml 10% HCl; warm gently to increase solubility, then filter. May be stored at room temperature.
   2. Just before use, mix equal volumes of pararosanilin solution and fresh 4% sodium nitrite. Test with starch iodide paper. Paper should turn blue instantly; if not, add more nitrite.
3. Solution B. to 30 ml of 0.1 M veronal acetate buffer (pH 7.6), add 3 drops of freshly prepared hexazotized pararosanilin and bring pH to exactly 6.3 with 1 N HCl.

Method

1. Mix solution A and solution B together and filter. Add sodium fluoride (17 mg/10 ml) to obtain a 0.04 M final concentration.
2. Incubate sections for 1 hr at 30°C in the medium prepared above.
3. Rinse with H₂O.
4. Counterstain with hematoxylin.
5. Air dry and mount with Permount.

Note

Because of large amounts of nonspecific esterase remaining in cells processed this way, we have modified Dr. Leder’s procedure by the addition of sodium fluoride to inhibit cross-reactions.

C. Alkaline phosphatase procedure:

Preparation of reagent:

A stock solution is prepared of 30 mg naphthol AS phosphate dissolved in 0.5 ml N,N-dimethylformamide, to which is added 100 ml of 0.2 M Tris buffer (pH 9.1). This may be stored at −5°C for up to 6 mo.

Method

1. Dissolve 10 mg fast blue BB salt in 10 ml of the stock solution. Filter before use.
2. Incubate sections for 1.5 hr at 37°C.
3. Rinse with H₂O.
4. Counterstain with 0.1% aqueous neutral red.
5. Air dry and mount with Permount.
D. Acid phosphatase procedure:  
Preparation of reagents:
1. Michaelis veronal-acetate buffer stock solution.
   - sodium acetate-3 H₂O 9.714 g
   - sodium barbiturate 14.714 g
   - add distilled H₂O to make 500.00 ml
2. Incubation medium
   - naphthol AS-Bi phosphate 10 mg
   - dissolved in N,N-dimethylformamide 1 ml
   - Michaelis stock solution 5 ml
   - in distilled H₂O 1 2 ml
   - freshly prepared hexazotized pararosanilin (see above for preparation procedure) 1.6 ml
   - Adjust pH to 5.0 with 1N NaOH

Method
1. Incubate sections for 1.5 hr at 37°C.
2. Rinse with H₂O.
3. Counterstain with hematoxylin.
4. Air dry and mount with Permount.

Note
For tartrate-resistant acid phosphatase, add 75 mg of L+ tartaric acid to each 10 ml of incubation medium. The pH drops to 2 and must be readjusted to pH 5 with NaOH. The fast garnet method, may also be utilized; it is somewhat more sensitive, but dirtier. Sections should be air dried and mounted in Aqua-mount (Lerner Labs, Stanford, Conn. 06902).

E. α-Naphthyl butyrate esterase procedure:  
Preparation of incubation medium:
- α-Naphthyl butyrate dissolved in 10 mg
- ethylene glycol monomethyl ether 0.5 ml
- 0.15M phosphate buffer, pH 6.3 9.5 ml
- freshly prepared hexazotized pararosanilin
   (see above for preparation procedure) 0.05 ml
- Adjust pH to 6.3 and filter

Method
1. Incubate sections for 1.5 hr at 30°C in incubation medium.
2. Rinse in H₂O.
3. Counterstain in hematoxylin.
4. Air dry and mount with Permount.

Note
To show inhibition, add sodium fluoride (17 mg/10 ml) to the incubation medium, 0.04 M final concentration. The change from 37°C incubation helps diminish the small amount of cross-reacting esterase seen in neutrophilic promyelocytes in some individuals.

V. Lysozyme Procedure  
Method
1. Block endogenous peroxidase by exposing sections to 0.3% H₂O₂ in methanol for 30 min.
2. Wash with phosphate-buffered saline (PBS).
3. Incubate with rabbit anti-human lysozyme (Dako, Copenhagen, Denmark) for 36 hr at 4°C. The antibody is used at a dilution of 1:20 in PBS.
4. Wash with PBS.
5. React with Protein A peroxidase (Zymed Labs, Burlingame, Calif., 94010) for 2 hr at RT.
6. Wash with PBS.
7. React for peroxidase.

Note
All substrates used were obtained from Sigma Chemical, unless otherwise noted.

Note added in proof. Recently, in order to simplify the procedure, we have made two changes: (1) Freshly prepared 3% paraformaldehyde in phosphate buffer (pH 7.4) can be used as the primary fixative for 2 to 24 hr at 4°C. While enzyme survival is equivalent or better, morphological preservation is suboptimal but satisfactory.

(2) We have eliminated the methyl methacrylate monomer in preparation of the embedding medium and now use 20 ml of JB-4 solution A.
Enzyme histochemistry and immunohistochemistry on biopsy specimens of pathologic human bone marrow

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