Enzyme Histochemistry on Bone Marrow Biopsies: Reactions Useful in the Differential Diagnosis of Leukemia and Lymphoma Applied to 2-Micron Plastic Sections

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Attempts to encourage widespread use of plastic embedding to improve morphological detail in human bone marrow biopsy specimens have failed because the procedures were too time-consuming and laborious to replace the simpler and more expeditious procedures for paraffin embedding. We have systematically investigated a variety of fixation and embedding procedures and arrived at a method that allows a plastic section to be produced as rapidly as a routine paraffin section. This method permits the use of histochemical procedures that are rapidly becoming mandatory in hematologic diagnosis. Because of the scarcity of normal human marrow samples, rat bone marrow was used. We established the method's applicability to human marrow by using it extensively with human buffy coat cells and in preliminary trials with human marrow samples. Biopsy specimens were fixed in a mixture of paraformaldehyde, glutaraldehyde, and acrolein, and embedded in a mixture of glycol and methyl methacrylate. Sections 2 microns thick were cut; incubated for chloroacetate esterase, α-Naphthol butyrate esterase, acid phosphatase (with and without tartrate), or alkaline phosphatase; and examined by light microscopy. Specimens could be prepared for examination within 48 hours. Tissues fixed with this basic fixation procedure can subsequently be used for electron microscopic study if desired. This approach, which provides histochemical markers of various hematopoietic cell lines in intact tissues and excellent ultrastructural preservation, promises to be useful in the diagnosis of neoplasms and inflammatory lesions, particularly when applied to bone marrow, spleen, and lymph nodes.

The bone marrow biopsy has become a standard diagnostic procedure, performed concomitantly with aspiration of marrow content, since the introduction of appropriate needles around 1960 and especially since the improvement of the needles in the early 1970s. The main value of the biopsy is that it permits a more accurate quantitation of marrow cellularity than does examination of aspirated material. In addition, it greatly facilitates the detection of focal lesions, such as granulomas, lymphomas, and metastatic tumors. Its utility has remained limited, however, because precise cytologic identification of many nucleated cells is difficult in sections. This difficulty is due largely to shrinkage and loss of cytoplasmic detail caused by decalcification of bone trabeculae and paraffin embedding. Furthermore, with the exception of the Leder procedure for demonstrating chloroacetate esterase in the neutrophilic series, recent histochemical techniques valuable in the differential diagnosis of leukemic cell lines cannot be applied to paraffin-embedded material. Clearly, the biopsy specimen would be more informative if cytologic detail were better preserved and if advances in histochemistry and electron microscopy could be applied directly to it. The additional information would increase the biopsy's value as an adjunct to the smears of aspirates and would be particularly important when aspirated material is unobtainable.

The newer embedding materials, such as methacrylates, have several advantages over paraffin—notably, they cause less shrinkage of tissue and permit the cutting of thinner sections. Burkhardt in Europe and Block in the United States have vigorously championed the value of plastic embedding in the diagnosis of hematologic disorders. Unfortunately, its use has not become widespread. We believe that this is due largely to its cost, its complexity, and especially its time requirements.

Traditional staining methods have been widely adapted to plastic embedding, and several investigators have demonstrated that certain histochemical procedures can be applied to plastic sections. Indeed, Westen and Bainton of this laboratory recently demonstrated the presence of alkaline-phosphatase-positive reticulum cells in plastic sections of rodent bone marrow.

We have therefore systematically investigated fixation and plastic embedding procedures. We used rat bone marrow because of the scarcity of normal human marrow specimens and the possibility that the variation in patient specimens—even in those classified as normal—might introduce differences that might be erroneously ascribed to particular fixation or embedding procedures. We used human buffy coat to check possible differences between human and rat blood cells. We modified the procedures to maximize the survival of diagnostically useful enzymes (chloroacetate esterase [CAE], α-naphthol butyrate esterase [αNBE], acid phosphatase [AcPase], and alkaline
phosphatase (AlkPase) without significant loss of morphological detail. The attainment of this goal was compatible with good preservation of organelles at the ultrastructural level. We have sought to simplify all of the procedures so that maximal information could be obtained from a single specimen within 2 days. Preliminary trials indicate that the procedure is suitable for use with human marrow.

MATERIALS AND METHODS

Materials

The experimental animals were Sprague-Dawley rats, body weight 150–400 g. Immediately after the animals were killed by ether anesthesia, the femurs were exposed and the proximal ends broken. A Pasteur pipette was then inserted into the marrow cavity, and the distal end of the femur was broken below the tip of the pipette. The pipette was then withdrawn, and the marrow specimen was immediately blown into fixative.

Normal human blood leukocytes were obtained by collecting human peripheral blood in a syringe containing 10 U heparin/ml of blood. The anticoagulated blood was centrifuged for 10 min in Kaplow tubes (VIRTUS CO, Gardner, N.Y.). The plasma was then removed and replaced with fixative. After 1 hr, the buffy coat, which solidifies as it fixes, was separated from the red cells as a disc of cells and placed in wash buffer. The discs could be handled like tissue specimens.

Fixatives

Several fixatives were tested initially: freshly prepared paraformaldehyde in various concentrations, buffered with 0.1 M sodium cacodylate (NaCa) at pH 7.4; acrolein and glutaraldehyde in various concentrations, in the same buffer; aldehyde fixatives in various combinations; Zenker-formal;21 Bouin’s solution;21 Burkhardt’s formalin-methanol;4 and periodeate-lysine-paraformaldehyde.22

For the best morphological preservation, we recommend fixation in 2% paraformaldehyde, 1% glutaraldehyde, and 1% acrolein in 0.1 M NaCa buffer at pH 7.4 for 2 hr at 22°C. For the best enzyme survival, we recommend fixation in 1% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% acrolein in the same buffer for 1 hr at 4°C. We will subsequently refer to the former fixative as PGA (2%, 1%, 1%) and to the latter as PGA. For the best results, these fixatives should be used within 1 wk of their preparation.

Embedding

After fixation, tissues were washed for 1 hr at 4°C in 0.1 M NaCa buffered to pH 7.4 with 3% sucrose. They were then dehydrated at –20°C for 10 min in 50% aceton (reagent grade) with 50% 0.1 M NaCa buffer containing 3% sucrose, followed by 10 min in 75% acetone and 10 min in 100% acetone. The final dehydration was performed under vacuum of 15–20 in of mercury (vacuum desiccator, Pelco, Box 510, Tustin, Calif. 92680). The dehydrated tissues were transferred to components of JB-4 (Polysciences, Inc., Warrington, Pa. 18976). First, the tissue was placed in solution A (glycol-methacrylate monomer) for 2–12 hr at –20°C under vacuum. After infiltration, it was transferred to the complete embedding mixture (18 ml solution: A + 0.09 g benzol peroxide + 0.5 ml solution B + 2 ml methyl methacrylate) at –20°C and placed in embedding molds (Sorvall, Norwalk, Conn.) under vacuum. The vacuum chamber was moved to 4°C after 2 hr at –20°C, and the specimens were allowed to polymerize overnight.

The next morning, the blocks were allowed to warm to room temperature. Sections 2 μ thick were then cut with glass knives on a Sorvall JB-4 microtome, floated on water, placed on coverslips, and allowed to air-dry at room temperature.

Staining

For routine morphology, we used a modified Maximow’s stain: Gill’s double-strength hematoxylin for 2 min; rinse in H2O; 1% aqueous eosin Y for 15 min; rinse in H2O; 1% azure II for 1 min; rinse in H2O; clear with ethylene glycol monomethyl ether for 5 sec; rinse in H2O. The periodic acid-Schiff23 and Sudan Black23 stains were also applied.

Enzyme Histochemistry

The following techniques, modified by extending the incubation period to 90 min at 37°C, were used to demonstrate enzymatic activity on the plastic sections: AlkPase,24 AcPase with and without tartaric acid,25,26 αNBE with and without NaF,27 CAE1 and β-glucuronidase.28 Peroxidase29 was incubated at 22°C for 20 min.

Electron Microscopy

After initial fixation as described above, a small portion of the tissue was transferred to the stronger PGA (2%, 1%, 1%) for 3 hr of additional fixation at 4°C. It was then transferred to a buffered 0.1 M EDTA solution containing glutaraldehyde26 for decalcification. (Decalcification was not necessary for rat bone marrow, but was included because it will be needed for human biopsy specimens containing trabeculae of bone.) The decalcified tissue was stored in 0.1 M NaCa buffer with 3% sucrose. When an ultrastructural examination was desired, the tissue was postfixed for 1 hr in O.S O4 with actate-veranol buffer containing 5% sucrose, stained en bloc for 1 hr in 0.5% uranyl acetate at room temperature, dehydrated through graded alcohols, infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections were cut with diamond knives on a Sorvall MT-2 microtome and examined with a Siemens 101 microscope at 80KV.

RESULTS

Light-Microscopic Studies

Our goal was to devise a procedure that would allow certain diagnostically valuable histochemical reactions to be applied to plastic-embedded sections of bone marrow without destroying the improvements in morphological preservation made possible by plastic embedding. Using the procedure described in Materials and Methods in sections of rat bone marrow and human buffy coat, we have obtained consistent preservation of four of the enzymes used in the histochemical classification of leukemias14 and lymphomas.31,32

1. CAE. CAE is a good marker for neutrophilic series (pro-myelocyte → PMN). It is remarkably resilient and is known to survive in a variety of routine fixatives with subsequent paraffin4 and plastic23 embedding. We had no difficulty in demonstrating its presence in plastic sections. Mature polymorphonuclear cells (PMNs) and the neutrophilic precursors showed specific diffuse reddish staining (Figs. 1 and 2). Mast cells (not illustrated) stain strongly, some bone marrow macrophages and sinus endothelial cells show staining of vacuoles (secondary lysosomes), and rare monocytes stained very weakly; the other nucleated cells of the peripheral blood and bone marrow were negative.

2. αNBE. Since αNBE is fairly specific for the mononuclear phagocyte system in hematopoietic tissues, it is a very useful marker for these cells.27,29,43 In the bone marrow, the macrophages, which are often associated with erythroid islands, stain prominently (Fig. 3). Scattered monocytes and their precursors can also be identified in most marrows and in the peripheral blood. Human monocytes
The following abbreviations are used for Figs. 1–6: M, monocyte; N, neutrophil, L, lymphocyte; Mac, macrophage; P, neutrophil precursor; Meg, megakaryocyte. All materials were fixed at 4°C for 1 hr in PGA (1% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% acrolein), and buffered with 0.1 M NaCa at pH 7.4. The figures on the left (1, 3, and 5) are photomicrographs of 2-micron sections of plastic-embedded rat bone marrow. The figures on the right (2, 4, and 6) are photomicrographs of similar preparations of cells from human buffy coat.
stain specifically; PMNs are negative (Fig. 4). The reaction in these cells is specifically and completely inhibited by NaF, but some marrow macrophages are only partially inhibited by NaF. As reported by others, a subpopulation of human lymphocytes show a punctate esterase reaction that resists NaF inhibition (inset, Fig. 4). Some investigators have drawn attention to the correlation of this reaction with T lymphocytes. The cytoplasmic rim of occasional marrow fat cells also showed moderate diffuse staining and occasional sinus endothelial cells showed staining of small vacuoles (not illustrated).

(3) AlkPase. A prominent granular reaction is seen in myelocytes and later cells of the neutrophilic series in rat bone marrow (Fig. 5). In this species, eosinophils are also positive. Reticulum cells and small arterioles and capillaries also stain with this enzyme. In human buffy coat (not illustrated), reaction product is restricted to some neutrophils.

(4) AcPase. The activity of the ubiquitous enzyme AcPase was easily demonstrated in macrophages and osteoclasts in the rat marrow, but less consistently demonstrated in other cell types (particularly PMNs) from rat and in cells from human buffy coat. In Fig. 6, coarse granular staining can be seen in the mature PMNs and light staining in the platelets. Neutrophilic precursors in the rat marrow stained as well. Monocytes and macrophages showed considerable variation in their staining reactions, with both irregular blobs of reaction and some granular staining. A subpopulation of lymphocytes shows one or two distinct punctate blobs of reaction product, a reaction said to correlate well with T-cell markers. Both lymphocytic and monocytic patterns of reaction are illustrated in Fig. 6. Preincubation with Tartraric acid eliminates or markedly inhibits the enzyme reactions in all these cells, except for bone marrow osteoclasts and occasional macrophages.

(5) Other Stains. The distribution of staining of β-glucuronidase is not illustrated, but moderate staining could be seen in macrophages and some lymphocytes, and weaker staining in neutrophils and platelets, in both human and rat preparations. We have not been able to demonstrate Sudan Black B staining, and peroxidase staining has been limited to eosinophils and RBC.

Electron-Microscopic Studies

Throughout the experiments, materials were also embedded in Epon and examined with the electron microscope. The aldehyde fixatives, in general, gave satisfactory fine structural morphology, but the precipitant fixatives (Zenker’s, Burkhhardt’s, Bouin’s) caused severe distortion of organelle detail that made fine structural examination unprofitable. The combination of precipitant fixatives, particularly the PGA, gave pleasing preservation of most ultrastructural organelles, with good penetration of the biopsy cores. Figures 7 and 8 illustrate the fine structural morphology of cells from rat bone marrow cores fixed by immersion in PGA for 1 hr at 4°C and subsequently in PGA (2%/1%/1%) for 3 hr at 22°C. We recommend this fixation procedure because it yields material suitable for both light-level enzyme histochemistry and ultrastructural analysis.

Technical Considerations

We devised our processing procedure by examining the influences of various fixation and embedding procedures on morphological detail and enzyme survival. We initially concentrated on obtaining good morphology, but we always tested enzymatic reactions and eliminated fixatives that strongly inhibited them. We began by fixing rat bone marrow for 1 hr at 4°C in 4% paraformaldehyde in NaCa buffer, 4% paraformaldehyde/1.4% glutaraldehyde with NaCa buffer, Bouin’s solution, Zenker’s solution, Burkhhardt’s fixative, or periodate-lysine-paraformaldehyde. Differences in morphological preservation with these fixatives initially appeared to be minor, but examination at higher magnifications suggested that Burkhhardt’s, Zenker’s, and Bouin’s solutions caused extreme precipitation of proteins—a finding later confirmed by electron microscopic examination. In addition, the Zenker’s and Bouin’s fixatives almost entirely abolished enzymatic reactivity.

At the conclusion of these preliminary experiments, we decided to concentrate on the aldehyde fixatives in a standard 0.1 M NaCa buffer at pH 7.4. We tested various concentrations and combinations and found that a mixture of 2% paraformaldehyde, 1% glutaraldehyde, and 1% acrolein gave the best morphological detail. We retested this mixture against the original fixatives and again found that it gave superior morphology.

Despite intermittently encouraging results, obtain-
Figures 7 and 8, electron micrographs of rat bone marrow, illustrate the preservation of organelles and relationships among cells in specimens fixed in PGA (1%, .25%, .25%) for 1 hr at 4°C and then in the stronger PGA (2%, 1%, 1%) for 3 hr at 22°C.

Fig. 7. A large bone marrow macrophage (Mac) can be identified by the prominent secondary lysosomes (sI) in its cytoplasm. Portions of its cytoplasm are elongated and are in close contact with a basophilic erythroblast (BE), orthochromatic erythroblasts (OE), and a neutrophilic promyelocyte (P). Note the complexity of interdigitations (arrow) with the basophilic erythroblast (BE), an observation emphasized previously by La Pushin and Trentin. Several erythroblasts have slits in their perinuclear cisternae (s), presumably an artifact of fixation. × 11,500.

The results with these enzymes were much more consistent, however, when fixation and embedding were carried out at 4°C. AcPase and aNBE were much more difficult to preserve. Varying the enzyme procedures and increasing the incubation period produced little improvement. We therefore examined the effect of each of the solutions used in processing.

Consistent enzyme survival was our largest problem. AlkPase and CAE are present in large amounts in rat leukocytes and were fairly easy to demonstrate.
Fig. 8. This field contains mostly promyelocytes (P), early neutrophil precursors. Note the presence of slender cellular processes and of a granular and fibrillar matrix between the cells (arrows). The cell at the top is an eosinophilic myelocyte (EM). x 11,500.

The tissue on AcPase and αNBE. To do this, we incubated air-dried smears of rat bone marrow individually in various concentrations of fixatives, ethanol, acetone, and embedding materials (glycol methacrylate and methyl methacrylate) and then examined the smears for survival of AcPase and αNBE. We identified a number of factors that decreased enzyme survival—notably, increasing the concentration of fixatives and increasing the period of fixation. This observation held true for the individual aldehydes as well as the combinations. We also noted that glutaraldehyde and acrolein were somewhat more inhibitory than paraformaldehyde alone. Ethanol markedly inhibited αNBE in rat macrophages, whereas acetone caused little inhibition. Unfortunately, the complete infiltrating solution (monomer and benzol peroxide) almost totally inhibited the enzymes. Decreasing the concentration of benzol peroxide resulted in some improvement of enzyme survival, especially in the methyl methacrylate monomer and mixtures of the two.
monomers. Even a prolonged period (up to 24 hr) in the monomer alone produced little apparent change in enzyme activity.

We repeated these experiments on smears prepared from human buffy coat. The results were largely similar to those in the rat, though there were several apparent species differences, e.g., AlkPase was much more sensitive to inhibition in man. Bearing these factors in mind, we modified our procedures in an attempt to obtain a balance between enzyme survival and the superior morphology that we had focused on in earlier experiments. We found that good penetration of the fixative into a biopsy specimen obtained with a Jamshidi needle required at least 1 hr of fixation, but that the fixative concentration could be dropped to 1%, 0.25%, or 0.25% PGA with little effect on the morphology. We also decreased the amount of benzol peroxide in the embedding mixture and found that 0.09 g 20 ml gave blocks of adequate hardness, but that further decreases gave inconsistent polymerization. Infiltation took place without benzol peroxide. Glycol methacrylate was easier to work with than methyl methacrylate and produced better sections, but methyl methacrylate appeared to improve enzyme survival slightly. A mixture of the two monomers retained the advantages of both. During the course of these experiments, we evolved a procedure that involved processing in part at −20°C. We have subsequently found that processing at 4°C throughout was adequate for enzyme preservation. Once the specimens were embedded and stored at room temperature, enzymes were demonstrated to be stable for 6 mo, the longest period tested thus far.

**DISCUSSION**

We have shown that morphological improvements at least as good as, and possibly superior to, those demonstrated by earlier proponents of plastic embedding are possible with simplified techniques. More important, we have shown that with a few simple modifications in tissue processing, a number of histochemical procedures can be applied to 2-μ-thick sections of plastic-embedded tissue. Furthermore, the sensitivity of these procedures seems to closely parallel that of the procedures used with the traditional air-dried smears. (It needs emphasizing, however, that with a slice of cell only 2 μ thick, focal staining may be missed, and smaller amounts of enzyme are available for reaction than with smears of whole cells.) Finally, with our techniques, specimens can be prepared for light microscopic examination within 36–48 hr and are preserved well enough to be subsequently processed for electron microscopy if further characterization at the ultrastructural level is desired.

The morphological improvement at the light-microscopic level should promote better correlations between findings from aspirated material, histologic sections, and ultrastructural studies; and when aspirated material is unavailable, it should improve diagnostic accuracy by improving cytologic detail. Histochemistry is increasingly used in the diagnosis and classification of hematologic disorders, particularly malignancies, because it has been shown to contribute to the reproducibility of classifications of the acute leukemias in laboratories throughout the world. The most recent classification schemes are based on a combination of morphologic and histochemical features. Specifically, since CAE appears early in maturation, at the neutrophilic promyelocyte stage, and is present at all later stages, including the mature PMN, it serves as a useful marker for the myeloid series in hemopoietic malignancies. The Leder procedure has already proven useful in detecting chloromas, the tumor masses composed of early neutrophilic precursors, in paraffin-embedded material. We would like to emphasize the usefulness of CAE in determining whether extramedullary granulopoiesis is present.

The ability to demonstrate αNBE on tissue sections is particularly valuable, since the cell line of the monocuclear phagocytes (promonocyte → monocyte → macrophage) can almost invariably be delineated by the intense αNBE staining. This enzyme can be profitable applied to tissues other than bone marrow. Indeed, the morphological term “histiocyte” might be replaced by a more precise description—the esterase-positive tissue mononuclear phagocyte (or macrophage). The cytochemical markers of the monocuclear phagocyte system can be used to evaluate malignant histiocytosis and to identify true histiocytic lymphomas as well as to identify this cell line in inflammatory reactions. It should be pointed out, however, that Schmalzl and Braunsteiner, using α-naphthol acetate as substrate, have shown that NaF-sensitive esterase activity is present in megakaryocytes, hepatocytes, sinus lining cells of the spleen, some kidney tubular cells, and some cells of the central nervous system. Ornstein et al. and Li et al. have concluded that α-naphthol butyrate is a more specific substrate, but to our knowledge, its body-wide distribution has not been similarly examined. We are currently using our new techniques to investigate the distribution of αNBE in body tissues of rat, mouse, and man.

Histochemistry may also play a role in defining some of the various types of lymphatic leukemias and lymphomas. The level of AcPase has been shown to be high in some neoplasms of T lymphocytes, but relatively low in lymphoid neoplasms of non-T lympho-
cytes. In addition, some investigators believe that the punctate density found in certain lymphocytes, which is positive for αNBE and which resists NaF inhibition, is a marker for certain T cells. Another possible histochemical feature of T cells was reported by Naeim et al., who demonstrated the presence of tartrate-resistant AcPase in T cells from patients with Sezary syndrome, a known malignancy of T cells. A high content of tartrate-resistant AcPase has also been helpful in establishing the diagnosis of hairy cell leukemia, although the cell origin of this malignancy remains obscure. Nanba et al. have shown that a distinct subgroup of B-cell lymphomas related to the mantle zone are characterized by a membrane AlkPase.

It is disappointing that peroxidase and Sudan Black, stains recommended for the differential diagnosis of leukocytic cell lines by the French-American-British classification system, are ineffective with plastic-embedded sections. This is probably because peroxide, an initiator of polymerization, inhibits peroxidase, and because the phospholipids ordinarily stained by Sudan Black are removed during dehydration of tissues. Since it is possible to demonstrate CAE in the neutrophilic series and αNBE in the mononuclear phagocytes, and since these stains are more specific than either peroxidase or Sudan Black, we feel that the disadvantage is small. Other useful stains, such as the reticulum stain and the periodic-acid-Schiff stain, can be successfully applied to plastic sections, as reported by others.

Preliminary trials of our procedure with human bone marrow biopsy specimens, conducted with Dr. Curt Ries, suggest that the bone present in human marrow need not be decalcified. Furthermore, the procedure is simple enough and fast enough to be used on clinical specimens with only a moderate increase in cost and attention. (This use of the procedure will be the subject of a future paper.) We have also had some success in applying immunohistochemical methods to these specimens. The ability to demonstrate intracytoplasmic or plasma-membrane-associated immunoglobulins as well as antilysozyme or antilactoferrin would, of course, be useful in the differential diagnosis of lymphocytic, mononuclear phagocytic, or neutrophilic cell lines in inflammatory and malignant states. Finally, because histochemical techniques can now be applied to intact tissue embedded in plastic as well as smears, touch preparations, and cryostat sections, the range of material available for histochemical evaluation can be extended to lymphoid tissues and other solid organs, both normal and pathologic.

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NOTE ADDED IN PROOF

Since the acceptance of this paper we have succeeded in staining for the enzyme peroxidase in plastic sections by making the following modifications during the incubation procedure as follows: (1) Presoak in 0.9% saline for 10 min, (2) Incubate in Graham and Karnovsky procedure for peroxidase for 20 min at 22°C; (3) If desired, the reaction product may be darkened by treating with Cu(NO₃)₂, as described by Hanks JS et al: Cancer Res 39:1635, 1979.

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