The Immunologic Phenotyping of Bone Marrow Biopsies and Aspirates: Frozen Section Techniques

By Gary S. Wood and Roger A. Warnke

Techniques were developed for the preparation of frozen sections from undecalcified bone marrow biopsy cores and bone marrow aspirates. Selected indirect antibody and biotin-avidin detection systems, employing immunoperoxidase or immunofluorescent labels, were studied to determine which were best suited for bone marrow frozen section immunopathology. Methods employing murine hybridoma monoclonal antibodies illustrate the immunophenotyping of representative lymphoid neoplasms involving bone marrow. The results of immunohistologic staining were comparable to those of fluorescence-activated cell sorter (FACS) analysis of cell suspensions. The advantages and limitations of immunohistologic techniques are discussed as they relate to immunophenotypic studies previously feasible only with bone marrow cell suspensions. Frozen section immunohistologic techniques serve as useful adjuncts to the conventional evaluation of bone marrow aspirates and biopsies.

IMMUNOHISTOLOGIC techniques applied to lymphoid tissues have contributed to our understanding of normal lymphoid organ structure and function and to our knowledge of lymphomas. Immunologic phenotyping has aided in the diagnosis of difficult lymphoid lesions and may prove to be of prognostic significance in certain lymphoproliferative disorders. Immunologic phenotyping is also a useful adjunct in the diagnosis of acute leukemia, especially in adults where 10%–25% of the cases cannot be subclassified with confidence. Heteroantisera, and more recently monoclonal antibodies, directed against cell surface, cytoplasmic, and nuclear antigens have been utilized to divide the acute leukemias into several immunologic subgroups. There is immediate clinical relevance to the immunophenotyping of leukemias since therapeutic regimens and/or prognoses differ depending not only on the conventional diagnosis but also on the immunologic phenotype.

The immunophenotyping of bone marrow lesions has previously been based principally on cell suspension studies. Major disadvantages of cell suspension immunophenotyping include the time and effort involved in specimen processing, the potential artificial alteration of relative cell populations, and the total loss of architectural features. Architectural features are helpful in the recognition of bone marrow involvement by non-Hodgkin’s lymphomas and in the evaluation of lymphoid nodules. In addition, architectural preservation would aid in the study of a host response associated with tumor cells and manifested by infiltrates of B and T lymphocyte subpopulations, as well as other inflammatory cells.

Furthermore, the immunophenotyping of hairy cell leukemia by cell suspension techniques is often not practical, since bone marrow aspiration commonly yields a “dry tap” and since the peripheral circulation may not contain sufficient numbers of hairy cells for harvesting. Immunophenotypic analysis of various other leukemias may likewise be difficult or impossible during an aleukemic phase without bone marrow immunophenotyping techniques that utilize intact tissue fragments rather than cell suspensions. Such techniques may also prove useful in distinguishing early relapse from normal regenerating marrow or in the early detection of a change in immunologic phenotype, such as that occurring in the blast crisis of chronic myelogenous leukemia which is also commonly associated with fibrosis and a “dry tap.”

Immunohistologic studies based on bone marrow tissue sections have been confined largely to disorders involving plasma cells and plasmacytoid cells that contain sufficient quantities of antigen (in this case immunoglobulin) to generally be detectable after fixation and embedding. Such antigens appear less able to survive decalcification. B-lymphocyte subpopulations cannot be demonstrated as reliably following routine fixation and embedding, although newer techniques may yield better results. Similarly, T-lymphocyte surface antigens could not be demonstrated in tissues fixed in 10% formalin or B-5.

Frozen section techniques offer excellent preservation of both intracytoplasmic and cell surface antigens. Tissues can be processed quickly, stored for extended periods of time, and studied when convenient. The main disadvantage of frozen section preparations is the relative lack of cytologic detail; however, as discussed below, several important cytologic features can be assessed in bone marrow frozen sections. In
addition, histochemical data can readily be obtained from frozen section or aspirate smear material, and this, combined with the cytologic information obtained from aspirate smears or paraffin sections, complements the immunophenotypic data.

The purpose of this study was to develop techniques for the preparation of frozen sections from bone marrow aspirates and undecalcified bone marrow biopsy cores. Selected staining systems were examined to determine which were best suited for bone marrow immunohistologic staining, and a study was made of the correlation between tumor immunophenotyping by frozen section and cell suspension techniques.

MATERIALS AND METHODS

Tissue Preparation

Bone marrow biopsy cores were obtained by conventional needle biopsy techniques. The biopsies were placed in normal saline and kept on ice. Portions of the cores, free of cortical bone, were transferred into airtight plastic capsules (John L. Beem, size 00, Pelo, Tustin, Calif.) filled with embedding medium (OCT compound, Lab Tek II; Lab Tek Products, Division Miles Laboratories, Inc., Naperville, Ill.). The capsules were frozen in a mixture of dry ice and isopentane, and stored at −70°C. Before immunoperoxidase staining, sections were fixed in acetone for 10 min at 4°C, since similar cutting was confined to a narrow segment of the knife. Biopsies oriented vertically dulled the knife more quickly, since cutting was confined to a narrow segment of the knife. The resulting Ficoll-Hypaque/plasma interface layer was layered over the cells. The specimens were then transferred into airtight plastic capsules (John L. Beem, size 00, Pelo, Tustin, Calif.), some of this last reagent was biotinylated as previously described.3 Avidin-TRITC conjugates were dialyzed against two washes of PBS, pH 7.2-7.4, to remove sinusoidal blood cells. All the techniques involved in the generation and screening of these antibodies have been described elsewhere.42

F(ab)2 fragments of purified goat anti-human kappa or lambda light chain antibodies, TRITC-conjugated F(ab)2 fragments of purified rabbit anti-goat antibody, TRITC-conjugated F(ab)2 fragments of purified goat anti-mouse antibody, TRITC-conjugated purified goat anti-mouse antibody, and HRP-conjugated purified rabbit anti-goat antibody were prepared as described elsewhere.42 HRP-conjugated purified swine anti-goat IgG (heavy and light chains) was obtained from Tago, Inc., Burlingame, Calif. The following avidin and biotin conjugates were used: avidin conjugated with horseradish peroxidase (avidin-HRP); avidin conjugated with tetramethylrhodamine isothiocyanate (avidin-TRITC) (Vector Laboratories, Inc., Burlingame, Calif.); avidin-TRITC (E.Y. Laboratories, Inc., San Mateo, Calif.); purified goat anti-mouse IgG (heavy and light chains) (Tago, Inc., Burlingame, Calif.). Some of this last reagent was biotinylated as previously described.42 Avidin-TRITC conjugates were dialyzed against two

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<tr>
<th>Antibody Clone/ (Antigen Recognized)</th>
<th>Lymphoid and Hematopoietic Cell Reactivity</th>
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<tr>
<td>L203/Ia (Ia)</td>
<td>B cells, macrophages, thymic epithelium, Langerhans and related dendritic cells, early myeloid cells, some activated T cells, B-cell neoplasms, most non-T/non-B ALL, occasional T-cell neoplasms, some AML</td>
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<tr>
<td>Anti-light and heavy human Ig chains</td>
<td>B cells, Ig (+) B-cell neoplasms</td>
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<tr>
<td>L3B12</td>
<td>Lymphocytes, monocytes, macrophages, Ia+ dendritic cells, lymphomas, lymphocytic leukemias, monocytic leukemias</td>
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<tr>
<td>L12E7</td>
<td>Thymocytes (cortical &gt; medullary), T-ALL, some non-T/non-B ALL, lymphoblastic lymphoma</td>
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<td>L17F12/Leu-1</td>
<td>Thymocytes, peripheral T cells, T-cell neoplasms, B-CLL (Leu-1)</td>
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<td>SK7/Leu-4</td>
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<td>ATM3.2/Leu-5</td>
<td>Helper peripheral T cells, some T-cell neoplasms, some thymocytes</td>
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| SK3/Leu-3a                          | Help 
changes of PBS for 24 hr to remove unconjugated fluorochrome. Fetal calf serum or bovine serum albumin was added to a final concentration of 2% to some aliquots of these conjugates in an effort to minimize nonspecific background staining.

**Immunohistologic Staining Procedures**

Two biotin-avidin staining systems and five indirect antibody staining systems were employed in this study (see section, Comment on Immunohistologic Techniques). Various controls were employed, including cross-comparisons between the staining patterns of various monoclonal antibodies, staining with one or more stages deleted, and the substitution of first stage reagents with other monoclonal antibodies devoid of specificity for the target antigens.

Sections were initially incubated for 10 min at 24°C in modified phosphate-buffered saline (PBS) (0.12 M NaCl, 0.01 M NaH₂PO₄, pH 7.5, 0.04 M K₂HPO₄, 4.9 × 10⁻⁵ M thimerosal). Reagents were used at titers ranging from 5 to 50 μg/ml and were pipetted onto section in 20-μl aliquots. Each reagent was incubated for 15 min, followed by washing with PBS for 5 min. Purified heteroantibodies and fluorochrome conjugates were ultracentrifuged at 100,000 g for 15 min prior to use to remove aggregates that might bind via Fc receptors or otherwise nonspecifically adhere to tissue sites. After the last PBS wash, immunoperoxidase sections were incubated with 0.3% 3,3-diaminobenzidine (DAB) in 0.3% hydrogen peroxide in PBS for 5 min. The sections were then washed for 5 min in PBS, washed for 5 min in distilled water, incubated for 5 min in 0.5% CuSO₄ in 0.9% NaCl to darken the DAB reaction product, washed for 5 min in distilled water, counterstained, passed through alcohols and xylene, and mounted in synthetic mounting medium.

Various counterstains were utilized, including hematoxylsin and cosin, Wright's Giemsa, Giemsa, toludine blue, and methylene blue. In selected cases, endogenous peroxidase activity was suppressed with phenylhydrazine using a technique similar to that of Straus. Sections stained with fluorochrome conjugates were mounted in Tris-buffered glycerol, pH 9.2, following the final PBS wash. They were examined in a Zeiss microscope equipped with vertical illumination (Carl Zeiss, Inc., New York) with an HBO 50 mercury vapor lamp and exciter barrier filter combinations for fluorescein 440-490 nm excitation or rhodamine 546-561 nm excitation. Color slides were taken with a Zeiss 35-mm camera on high speed Ektachrome film (Eastman Kodak Company, Rochester, New York) with routine processing (ASA 400).

**Comment on Immunohistologic Techniques**

Three immunoperoxidase staining procedures were utilized: (1) monoclonal mouse anti-human antibody/biotinylated purified goat anti-mouse antibody/avidin-TRITC, (2) monoclonal mouse anti-human antibody/TRITC-conjugated purified goat anti-mouse antibody, (3) monoclonal mouse anti-human antibody/TRITC-conjugated F(ab')₂ fragments of purified goat anti-mouse antibody, (4) F(ab')₂ fragments of purified goat anti-human antibody/TRITC-conjugated F(ab')₂ fragments of purified rabbit anti-goat antibody.

Fluorescent background was most prominently localized to certain polymorphonuclear leukocytes (probably eosinophils), but there was also significant background scattered throughout the remainder of the bone marrow. In the biotin-avidin system, this background appeared to result from nonspecific adherence involving the third stage. In the indirect antibody systems, the background appeared largely due to Fc receptor binding of the second stage, since background was significantly reduced by using F(ab')₂ antibody fragments. The indirect antibody staining systems involving F(ab')₂ antibody fragments exclusively or monoclonal antibodies in conjunction with F(ab')₂ antibody fragments were therefore superior to the other immunofluorescent staining systems employed in this study.

In our laboratory, the three-stage monoclonal mouse anti-human antibody/biotinylated goat anti-mouse IgG/avidin-HRP technique has proven optimal for the immunohistologic study of bone marrow frozen sections. Whether biopsies or aspirates are employed, the time and expense involved are comparable to those required for similar studies of lymph node biopsies.

**Histochemistry**

The naphthol AS-D chloroacetate esterase (N-AS-D-CAE) and α-naphthyl butyrate esterase (α-NBE) reactions were performed on frozen sections as previously described. Sections were counterstained with hematoxylin.

**Immunofluorescence Staining of Cell Suspensions**

Ficoll-Hypaque/plasma interface preparations of bone marrow aspirates were stained and analyzed on the fluorescence-activated cell sorter (FACS) as previously described. Briefly, the two-stage indirect immunofluorescence staining technique employed monoclonal antibodies (Table 1) as the first stage reagents and a fluorescein conjugate of goat anti-mouse IgG (heavy and light chains) (Tago, Inc., Burlingame, Calif.) as the second stage reagent.

**RESULTS**

**Morphology of Cryostat Sections**

Bone marrow aspirate preparations sectioned easily and were similar to lymph nodes in most technical aspects. In most cases, frozen sections that contained multiple fields of intact tissue, including some paratrabeicular areas, could be cut from undecalcified biopsies and were more than adequate for immunophenotypic and histochemical evaluation (Fig. 1 A). Although the cytologic detail of frozen sections was less than that of conventional bone marrow preparations, several important features could still be assessed histologically (Fig. 1 B and C). These included the overall cellular-
Fig. 1. Normal bone marrow. All illustrations are of frozen sections of the same undecalcified bone marrow needle biopsy. (A) Low power appearance of a representative section to illustrate the retention of architectural integrity. (B and C) Oil immersion fields show preservation of many cytologic features, including nuclear morphology and cytoplasmic granules. The tinctorial characteristics of metachromatically stained sections are similar to those of comparably stained marrow aspirate smears. (A) Wright's-Giemsa, x 75. (B and C) Wright's-Giemsa, x 1200.

Immunohistologic Staining

Figure 2 illustrates frozen section immunologic staining of a filtered bone marrow aspirate obtained from a patient with a lymphoproliferative disorder with an IgM (lambda) gammopathy. The marrow contained an infiltrate of cells that stained for lambda light chains (Fig. 2 A and B) and mu heavy chains (Fig. 2C) but not for kappa light chains (Fig. 2D). The results were consistent with a mu/lambda neoplastic B-cell infiltrate.

Figure 3 illustrates frozen section immunologic staining of a Ficoll-Hypaque fractionated bone marrow aspirate obtained from a patient with a lymphoblastic lymphoma involving the bone marrow. The specimen contained an extensive infiltrate of cells that stained with pan-T-cell antibodies (Fig. 3A), as well as an antibody that recognizes immature T cells and some non-T/non-B ALL (Fig. 3B). Only occasional T cells...
Fig. 2. B-cell lymphoproliferative disorder with IgM (lambda) gammopathy. All illustrations are of frozen sections of the same filtered bone marrow aspirate. See text for a discussion of the staining results. (A) Sequential staining with monoclonal mouse anti-human lambda/goat antimouse IgG/HRP-conjugated swine anti-goat IgG. Endogenous peroxidase suppressed with phenylhydrazine. Lambda-positive B cells are distributed singly and in clusters throughout the marrow. Occasional eosinophils are identified by their dense granular staining. No counterstain (x190). (B) Sequential staining with F(ab')2 fragments of goat anti-human lambda and F(ab')2 fragments of TRITC-conjugated rabbit anti-goat antibody. The lambda-specific immunofluorescent staining pattern is similar to the lambda-specific immunoperoxidase pattern (A) (x300). (C) Staining similar to (A) with monoclonal mouse anti-human mu as the first stage. The pattern of specific staining parallels that seen in (A). No counter stain (x190). (D) Staining similar to (B) with F(ab')2 fragments of goat anti-human kappa as the first stage. Only occasional kappa-positive plasma cells (illustrated) and small B cells (not illustrated) were present indicating lambda light chain restriction of the B-cell infiltrate (x300).

Immunohistologic Staining Versus FACS Analysis

In order to determine if the results of frozen section immunohistologic staining were comparable to those of cell suspension staining as analyzed on the FACS, bone marrow preparations from five patients were stained for several antigens by both techniques (Table 2). This provided 29 separate comparisons between immunohistologic staining and cell suspension staining. In each instance, the results were similar.

DISCUSSION

The results demonstrate that the proportions and architectural features of phenotypically distinct bone marrow subpopulations can be determined through the immunohistologic study of frozen sections. As shown in Table 2, the immunohistologic results are comparable to those obtained by FACS analysis of cell suspen-
and 4C). Time and laboratory equipment. Tissues are easily

Fig. 3. T-cell lymphoblastic lymphoma with bone marrow involvement. All illustrations are of frozen sections of the same Ficoll-Hypaque fractionated bone marrow aspirate. See text for a discussion of the staining results. (A) Sequential staining with anti-Leu-4 (anti-T-cell)/biotinylated goat anti-mouse IgG/avidin-HRP. Most cells stained, indicating the T-cell nature of the lymphomatous infiltrate. Methylene blue counterstain (×300). (B) Staining similar to (A) with L12E7 as the first stage. Most cells stained strongly (a finding characteristic of immature T cells and their corresponding neoplasms). Mature peripheral T cells stain only weakly with L12E7 (×300). (C) Staining similar to (A) with anti-Leu-2a (anti-cytotoxic/suppressor T cell) as the first stage. Occasional T cells bearing the cytotoxic/suppressor immunophenotype are identified by their ring-like surface antigen staining pattern. Dense granular staining of eosinophils is also present (×300). (D) Staining similar to (A) with anti-Leu-3a (anti-helper T cell) as the first stage. Occasional T cells bearing the helper immunophenotype are identified by their ring-like surface antigen staining pattern (×300).

sions. In addition, it is possible to distinguish normal or reactive marrow elements when they are of a different immunophenotype than that of the neoplastic infiltrate. In the cases illustrated above, these elements included both B- and T-cell subsets (Figs. 2D, 3C, 3D, and 4C).

The immunohistologic techniques developed during this study offer certain advantages over alternatives such as those involving cell suspensions or cytocentrifuge preparations. The initial processing of the specimen is straightforward and requires a minimum of time and laboratory equipment. Tissues are easily
Fig. 4. Non-T/non-B acute lymphoblastic leukemia. All illustrations are of frozen sections of the same undecalcified bone marrow needle biopsy. The immunophenotypic diagnosis of non-T/non-B ALL was consistent with the diagnosis of ALL based on conventional marrow aspirate cytology and cytochemistry. See text for a discussion of the staining results. (A) Staining as in Fig. 3 with L3B12 (anti-lymphocyte/monocyte) as the first stage. Approximately 40% of the cells stained, indicating an abnormal increase in marrow lymphoid or monocytoid elements \((\times300)\). (B) Staining as in Fig. 3 with L203 (anti-la) as the first stage \((\times300)\). There is widespread staining since normal early myeloid elements as well as the neoplastic infiltrate express la antigen. (C) Staining as in Fig. 3 with anti-Leu-4 (anti-T-cell) as the first stage. Only scattered T cells were present. Similarly, the leukemic infiltrate did not stain for B-cell immunoglobulins (not illustrated) \((\times300)\).
experience in adults, comparative morphological studies have found a greater yield of lymphomatous involvement from the examination of aspirates.21,49 The principal disadvantage of frozen section techniques is diminished cytologic detail. In those instances that require cytologic detail combined with antigen characterization on a cell-by-cell basis, immunocytochemical, rather than immunohistochemical, techniques would probably be the methods of choice. Such techniques appear particularly useful for defining the cell specificity of new monoclonal antibody reagents and for detecting small numbers of leukemic cells.14 However, such applications constitute only a portion of the spectrum of research and diagnostic problems to which bone marrow immunophenotyping methods can potentially be applied.

There are relative advantages to the use of either biopsies or aspirates for bone marrow immunohistochemical studies. Although frozen sections are easier to cut from aspirates, the initial processing of the specimen is more involved and there is less architectural integrity. In contrast, biopsies are convenient to process and offer maximum architectural preservation. In those settings where biopsy rather than aspiration is the routine procedure or where the marrow cannot be aspirated, the use of biopsies is also of practical advantage to the clinician. In pediatric patients, the use of aspirates may be preferable to biopsies since, contrary to the general experience in adults, comparative morphological studies have found a greater yield of lymphomatous involvement from the examination of aspirates.21,49

The merits of alternative staining methods also warrant consideration. Sections stained with immuno-peroxidase offer permanent immunophenotypic records and do not require special immunofluorescence microscopy equipment for their examination. In addition, HRP-unlabeled, as well as HRP-labeled, cells can be visualized simultaneously with appropriate counterstains. Immunoperoxidase methods offer the potential for combined immunophenotypic and enzyme-histochemical staining. Simultaneous staining of tissue sections for more than one antigen is possible with either immunofluorescence or immunohistochemical techniques.7

Alternative enzyme labels can avoid endogenous peroxidase background, but generally possess other background, substrate, and/or localization problems. Glucose oxidase and alkaline β-galactosidase are two enzymes with nil background in human tissues. While reports concerning the use of glucose oxidase as an immunohistologic label are promising,5 we have been unable to localize alkaline β-galactosidase label to antigenic sites in immunohistologic studies of human tissues (unpublished data). Immunofluorescent methods avoid the endogenous enzyme background often inherent in immunohistochemical methods but can present background problems of their own.43

Frozen sections of either aspirates or biopsies offer adequate material for the histochemical differentiation of bone marrow cell types. As a marker for myeloid elements, the N-AS-D-CAE reaction allowed assessment of the relative neutrophil population ranging from promyelocytes to mature neutrophils.3,13,47,51,52 Leukemic myeloblasts have also been reported to be generally positive.13,42 Similarly, the α-NBE reaction served as a reasonably specific marker for monocyte precursors, monocytes, and macrophages,47,53,54 although punctate, NaF-resistant nonspecific esterase staining of some lymphocytes has been reported.47,52,55-57

The potential for long-term preservation of enzyme activity in frozen tissue is an advantage compared to
enzyme preservation in unstained smears or touch preparations. In addition, after frozen sections are cut for immunophenotyping, the remainder of the frozen specimen can be processed for plastic embedding. The resulting plastic sections can be studied histochemically and possess cytologic detail roughly comparable to that of paraffin sections (Beckstead, J., University of California at San Francisco, unpublished data).

Immunophenotypic and enzyme histochemical evaluation of frozen sections can serve as useful adjuncts to the conventional assessment of bone marrow biopsies and aspirates and afford an alternative to cell suspension techniques for the immunophenotyping of pathologic bone marrow infiltrates.

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The immunologic phenotyping of bone marrow biopsies and aspirates: frozen section techniques

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