Laboratory techniques

Glycol methacrylate (GMA) embedding for light microscopy. II Immunohistochemical analysis of semithin sections of undecalcified marrow cores

A ISLAM,* E ARCHIMBAUD,† E S HENDERSON,* T HAN* *Department of Medical Oncology, Roswell Park Memorial Institute, Buffalo, New York, United States of America, and †Service d'Hematologie, Hôpital Edouard Herriot, Lyon, France

SUMMARY A routine method allows bone marrow biopsy specimens to be embedded in glycol methacrylate (GMA), a water miscible plastic, and to benefit from the advantages of good morphology with immunoperoxidase detection of a wide range of cellular antigens useful in diagnosing and classifying various haematopoietic disorders. Marrow cores were fixed in cold Bouin's solution, rinsed in cold phosphate buffer, dehydrated in cold methanol, infiltrated and embedded in cold GMA, then polymerised at 4°C. Sections were cut at 2 μm thickness with a Tungsten carbide knife in a Jung's high performance microtome (Autocut). Antigenicity was preserved when drying slides at room temperature but pronase digestion was necessary to re-expose the antigens in bone marrow biopsy sections embedded in GMA. Histostik, a new adhesive, was used to coat the glass slides to prevent section loss during enzyme digestion and immunostaining procedures.

This method of adapting plastic embedding to undecalcified marrow cores preserves marrow architecture and cellular details and it can serve as a useful adjunct to analyse the bone marrow from patients with myeloproliferative and lymphoproliferative disorders. This technique may also be applicable in non-haematological malignant conditions which affect the marrow.

We recently reported that embedding bone marrow trephine biopsy specimens in glycol methacrylate (GMA), a water miscible plastic, permits more precise histochemical localisation of enzyme activity than frozen or paraffin wax sections.1 The superior histological GMA embedding technique has been used successfully for enzyme histochemical analysis, but its use in immunohistochemistry has been restricted because GMA embedding inhibited the antigen-antibody reaction.2 We have found that by using embedding procedures carefully, however, bone marrow biopsy sections embedded in GMA can be used for light microscopical demonstration of antigen-antibody reaction by an enzyme labelled (immunoperoxidase) antibody method, without removing the embedding medium.

In this paper we describe the labelling of a wide range of cellular antigens in bone marrow biopsy sections embedded in GMA using monoclonal and polyclonal antibodies. With this approach, it is possible to combine optimal morphological studies with enzyme and immunohistological studies, and to carry them out simultaneously on one biopsy specimen. In this way both the morphology and immunoenzymatic composition of marrow cells can be visualised in the same biopsy specimen and in their own natural environment. In bone marrow biopsy sections embedded in GMA, not only can the cell morphology be seen at its best, but the marrow architecture is well preserved. Thus proportions and architectural features of phenotypically distinct bone marrow subpopulations can be determined in the same relative proportions in vitro as well as in vivo. This would be helpful in recognising to what extent bone marrow is affected in non-Hodgkin's lymphoma and in evaluating lymphoid nodules4 and focal bone marrow lesions.5

Material and methods

Islam needles4 were used to obtain posterior iliac crest marrow biopsy specimens from patients with various malignant haematological disorders. The biopsy samples were placed immediately into cold Bouin's solution and fixed for three hours at 4°C on a rotor mixer.
GMA embedding for light microscopy

After washing overnight in buffer the samples were dehydrated in graded ethanol and embedded in GMA according to the procedures described previously. A Tungsten carbide knife in a Jung's high performance microtome (Autocut) was used to obtain semithin sections from the undecalcified marrow cores. Sections were floated on distilled water and mounted on glass slides coated with Histostik (Accurate Chemical, Westbury, New York).

ANTISERA
Antiser to human IgG, IgA, IgM, κ, λ, and polyclonal rabbit antisera to factor VIII related antigen were obtained from Dako (Dako Corporation, Santa Barbara, California). Monoclonal antibodies to Leu series (Leu-M1 (CD 15), Leu-M3, Leu-M5, Leu-12 (CD 19), Leu-14 (CD 22), anti-CALLA (CD 10)) were obtained from Becton Dickinson (Mountain View, California). The anti-mouse monoclonal antibody L12-2 was the generous gift of Dr G Rovera (the Wistar Institute of Anatomy and Biology, Philadelphia).

IMMUNOSTAINING OF PLASTIC-EMBEDDED BONE MARROW BIOPSY SECTIONS
The sections were dipped in a 0.3% hydrogen peroxide solution in absolute methanol for 20 minutes to inhibit endogenous peroxidase activity. Then the sections were washed three times for 10 minutes in Tris-buffered saline (TBS). After washing, the excess liquid was wiped from the slide carefully, but the sections were never allowed to dry. The sections were digested with pronase (0.2 mg/ml in TBS) for 10 minutes at 37°C in a moist chamber. The sections were then washed in TBS as described above.

The non-specific reactivity to the secondary antibody was then blocked by applying a 2% solution of normal serum, from the species in which the secondary antibody was produced to the sections in TBS supplemented with 5% bovine serum albumin, for 20 minutes at 37°C. Then the blocking solution was poured off the slides and appropriate dilutions of the primary antibodies in TBS were applied to the sections and incubated at 37°C for 60 minutes. The primary antibody was washed off with TBS, as described above, and an appropriate horseradish peroxidase conjugated secondary antibody was applied for 20 minutes at 37°C. The slides were then washed in TBS as described before and after washing in TBS, peroxidase activity was shown using the methods of Graham and Kanovsky, and Graham, Lundholm, and Kanovsky, with either 3,3′-diamino-benzidine (DAB) or 3-amino-9-ethylcarbazole (AEC). The sections were counterstained with Mayer's haematoxylin and counterstained with glycergel (AEC) or Permound (DAB) for observation.

Results
Bone marrow biopsy sections, embedded in GMA and cut at 2 μm, facilitated specific localisation of immunoglobulins and various myeloid and lymphoid differentiating antigens in cells within the marrow cavity. This advantage, coupled with improved cytological detail attained by plastic embedding, made it possible to define the composition and to localise cellular disease with much greater confidence and sensitivity.

Figs 1 and 2 show GMA-embedded bone marrow biopsy sections from a patient with IgA-κ gammapathy—a lymphoproliferative disorder. The GMA embedded bone marrow biopsy section of this patient stained with May Grünwald and Giemsa stain showed an infiltrate of cells that were a mixture of mature looking lymphocytes and plasma cells (fig 1). When the sections were stained for immunoglobulins, the plasma cell cytoplasm stained heavily for κ light chains and IgA heavy chains (fig 2), indicating the clonal nature of plasma cell proliferation. A bone marrow biopsy specimen obtained at the same time from this

Fig 1 Details of bone marrow biopsy preparation from patient with lymphoproliferative disorder showing diffuse infiltration of marrow with well differentiated, mature looking lymphocytes and plasma cells. Biopsy specimen was processed into GMA and the semithin section stained with May-Grunwald and Giemsa stains.
patient but processed in paraffin showed infiltration of the marrow with mononuclear lymphoid cells alone; plasma cells were not noticed and diffuse well differentiated lymphocytic lymphoma was diagnosed.

Fig 3 shows a bone marrow biopsy section embedded in GMA from a patient with hairy cell leukaemia (HCL). Bone marrow aspiration in this patient was difficult and yielded a dry tap but a marrow core processed in GMA showed an infiltrate of loosely arranged mononuclear lymphoid cells (hairy cells) which were strongly positive for Leu M5 (CD11c) and Leu 14 (CD22) (fig 3). Positivity with the monoclonal antibody Leu M5 and Leu 14 is considered to be diagnostic of HCL.10

Fig 4 shows a bone marrow biopsy section embedded in GMA from a case of chronic granulocytic leukaemia in megakaryoblastic transformation. The section was stained for factor VIII related antigen, which showed the strong positive reaction on megakaryocytes in different stages of maturation. Because of

**Fig 2** Bone marrow biopsy section from same case as in fig 1, showing large number of plasma cells that are strongly positive for immunoglobulin IgA. Section was stained for immunoglobulin IgA by an indirect immunoperoxidase method and counterstained with Mayer's haematoxylin. Reaction product was shown by using DAB as substrate.

**Fig 3** Bone marrow biopsy preparation from patient with HCL showing strong positive reaction for Leu 14 among hairy cells. Section was stained with monoclonal antibody Leu 14, with an indirect immunoperoxidase method and counterstained with Mayer's haematoxylin. Reaction product was shown by using AEC as substrate.

**Fig 4** Bone marrow biopsy preparation from patient with chronic granulocytic leukaemia in megakaryoblastic transformation. Section was stained for factor VIII related antigen with an indirect immunoperoxidase method and counterstained with Mayer's haematoxylin. Reaction product was shown by using AEC as substrate. Note strong positive reaction throughout cytoplasm of megakaryocytes in different stages of maturation including megakaryoblasts.

**Fig 5** Bone marrow biopsy preparation from patient with chronic granulocytic leukaemia in the chronic phase. Section was stained with monoclonal antibody L12–2 with an indirect immunoperoxidase method and counterstained with Mayer's haematoxylin. Reaction product was shown by using AEC as substrate. Note strong positive reaction for L12–2 among all mature granuloid precursor cells.
increase in marrow fibre content, this patient’s marrow was difficult to aspirate and the above staining procedure helped in making an accurate diagnosis.

Fig 5 shows the bone marrow biopsy section embedded in GMA from a patient with chronic granulocytic leukaemia in the chronic phase. The section was stained with monoclonal antibody L12-2, which showed a strong positive reaction (fig 5) among all of the mature granuloid precursor cells.

Discussion

The importance of immunological markers in characterising cellular phenotypes in leukaemias and lymphomas is well recognised, but using immunological markers to diagnose haematological malignancies has been restricted either to peripheral blood and bone marrow aspirates or to frozen and paraffin embedded tissue sections. Although the Romanowsky’s stained smears of peripheral blood and bone marrow aspirates normally provide excellent cellular details, they do not always reflect the cellular changes within the marrow accurately; and cells that are firmly anchored within the marrow and do not readily enter into suspension cannot be studied. Thus any immunological studies done on such samples have had the same drawbacks as those associated with the examinations of peripheral blood and bone marrow aspirates. On the other hand, a marrow biopsy overcomes all of the sampling problems associated with peripheral blood and bone marrow aspiration techniques, but when the marrow biopsy specimens are decalcified and processed into paraffin wax, they lose cytological details, and most antigens of diagnostic relevance detected with currently available monoclonal or polyclonal antibodies are also destroyed. Because of this, many laboratories have adopted immunohistological labelling of frozen tissue sections. Although immunolabelling of cryostat sections normally produces reliable and reproducible results, there are many disadvantages. First, making good quality frozen sections from undecalcified bone is difficult. Second, tissue morphology is usually poor, making it difficult to visualise the labelling reactions of neoplastic cells. Third, the cryostat sections often show artefactual staining reactions due to diffusion of antigens. When bone marrow biopsy specimens are embedded in water miscible plastic GMA, the problems associated with frozen sections are overcome. The adaptation of plastic (GMA) embedding for undecalcified marrow cores also provides excellent preservation of marrow architecture and cellular details. In many laboratories GMA is polymerised at room temperature and as the reaction is strongly exothermic substantially higher temperatures are achieved. This excess rise of temperature not only damages the tissue from overheating but also destroys the activity of various enzymes and different cellular antigens. The polymerisation reaction is inhibited by low temperatures and oxygen. Thus polymerisation at 4°C under vacuum provides an effective cooling system which not only permits slow, uniform, and adequate polymerisation but also preserves a wide range of cellular antigens by preventing heat damage due to excess rise of temperature during the polymerisation process. As a result, it is now possible to show various myeloid and lymphoid differentiating antigens on the marrow cells without sacrificing cytological or histological details. Thus with the immunoperoxidase detection of myeloid and lymphoid antigens, coupled with improved cytological details achieved by plastic embedding technique, it is now possible to identify and characterise the precise nature of cellular disease affecting the marrow. Moreover, this immunohistological method may provide the only means of studying bone marrow cell populations which are firmly anchored within special regions (paratrabecular) of the marrow, and which do not readily enter into suspension. In conditions such as hairy cell leukaemia and megakaryoblastic transformation of chronic granulocytic leukaemia where there is associated marrow fibrosis, bone marrow aspiration is often difficult and yields either a dry tap or an unsatisfactory sample. In such conditions the ability to perform immunological study on bone marrow biopsy sections is not only useful but may also provide an important means of making an accurate diagnosis as has been observed in two such cases reported here. In the patient with IgA-gammopathy where the conventional method of decalcification and paraffin wax embedding failed to show the presence of plasma cells, this method of plastic embedding proved very useful. Using bone marrow biopsy sections embedded in GMA it was not only possible to show the presence of large numbers of plasma cells in this patient’s marrow but with the immunoperoxidase method it was also possible to show the type of immunoglobulins (which were IgA heavy chain and κ light chain) present in those plasma cells indicating the clonal nature of plasma cell proliferation.

Although water miscibility and low temperature processing make GMA a suitable embedding medium for immunohistochemical analysis, the nature and type of fixative used can be of vital importance. We tested various fixatives and our general conclusion is that Bouin’s solution is superior in preserving the immunoreactivity of marrow cells in semithin sections of undecalcified marrow cores processed in GMA. We also observed that protease digestion was necessary to restore the antigenicity of bone marrow biopsy sections embedded in GMA. The mechanism of protease action is still debatable, but it is generally believed that when proteases are applied to GMA sections, they
may act by eliminating chemical bonds between GMA monomers and the free amino groups of the antigenic determinants, thus unmasking the determinants.2,21

While immunostaining, detachment of GMA sections from the glass slides can also be a real problem, especially when the sections are treated with methanolperoxide solution and digested with pronase. We have observed that pretreating the glass slides with alcin blue22 or Histostik leads to firm adherence of the GMA sections to glass slides and allows little or no section to be lost during the staining procedure.

In conclusion, it is suggested that this improved method of bone marrow biopsy processed by plastic embedding, coupled with immunoperoxidase detection of myeloid and lymphoid antigens in semithin sections of plastic embedded marrow cores, can serve as useful adjunctive diagnostic tools to analyse the bone marrow from patients with myeloproliferative and lymphoproliferative disorders. This technique may also be applicable to a variety of research studies, as well as being of value in non-haematological malignant conditions affecting the marrow.3

This work was supported by NIH grant CA 42683.

References


Requests for reprints to: Dr Anwarul Islam, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, New York 14263, USA.