Glycol methacrylate embedding for light microscopy I enzyme histochemistry on semithin sections of undecalcified marrow cores

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SUMMARY A simple, routine procedure for water miscible glycol methacrylate (GMA) embedding of undecalcified bone marrow cores, which preserves the activity of enzymes useful in diagnosing various haematopoietic disorders, is described. The GMA used in this study has a low acid content that eliminates background staining, and the modified May-Grünwald-Giemsa stain provides good definition and excellent colour differentiation of various haematopoietic cells in the bone marrow, thereby providing optimal conditions for the study of the morphology and enzyme activity of bone marrow cells in the same preparation. The method is simple, reproducible, requires no expensive equipment, and is suitable for routine processing of small bone marrow cores in any histopathology or haematology laboratory.

Enzyme markers are useful in characterising leukaemic cells, particularly in the acute types.^{1,2} The use of enzyme markers to diagnose haematological malignancies, however, has been restricted to bone marrow aspirates or to touch preparations. Cytochemical reactions performed on bone marrow biopsy specimens could be very informative, because in histological preparations tissue organisation is maintained and the sampling errors inherent in bone marrow aspiration are avoided. These methods could be particularly important in analysing focal lesions of bone marrow, or when aspirated material is unobtainable.

Unfortunately, the superior histological technique of methyl-methacrylate embedding³⁻⁵ has been unsuccessful when used with enzyme histochemistry methods. Water miscible glycol-methacrylate (GMA) embedding, however, which was introduced by Rudell,⁶ provides good morphological detail and yields excellent histochemical results.^{7,8} It also has good stretching and mounting properties and, when applied to small size marrow cores, can provide sections with excellent preservation of a wide variety of enzymes used to diagnose and classify haematological malignancies.^{9,10}

The value of GMA embedding in diagnosing haem-

mopoietic disorders has been recognised for some years; its use, however, has been restricted, because the technique entails procedures that are not practical for histology laboratories in most general hospitals.

This paper describes a simple GMA embedding technique that requires no expensive microtome or elaborate apparatus, and can be easily adapted for routine use in a histopathology or haematology laboratory. It provides more precise histochemical localisation of enzyme activity than frozen or paraffin sections. In combination with the May-Grünwald-Giemsa (MGG) stain, haematopoietic tissue cells can be differentiated almost as well as in Romanowsky's stained smears of aspirated marrow. The MGG staining procedure is more rapid and less complicated than the usual Giemsa or haematoxylin and eosin procedures, though the results are similar to the routinely used Romanowsky's stain. Moreover, the staining procedure does not stain the GMA embedding material, thus avoiding any background colour.

Material and methods

Pieces of bone marrow cores (8-10 mm) were obtained from patients using a Jamshidi¹¹ or Islam¹² needle, or one of the disposable needles, and were fixed immediately in PGA (1% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% acrolein in 0.1 M

sodium cacodylate buffer at pH 7.4)⁹ for one hour at 4°C on a rotor mixer. Long (20–22 mm or over) marrow cores were not only difficult to process in GMA but were also difficult to section. Thus when long marrow cores were obtained they were divided into two smaller pieces and then fixed. After fixation marrow cores were washed for one hour at 4°C in 0.1 M sodium cacodylate buffer at pH 7.4 with 3% sucrose. They were then dehydrated at 4°C for 10 minutes in 50% acetone with 50% 0.1 M sodium cacodylate buffer containing 3% sucrose, followed by 10 minutes in 75% acetone and 10 minutes in 100% acetone.

The dehydrated tissues were placed in solution A (hydroxyethyl-methacrylate 100 ml, 2-butoxyethanol 15 ml, and benzoyl peroxide 1.0 g) at 4°C for two hours under vacuum. After two hours solution A was renewed and the specimens were left in fresh solution A for 24 hours under vacuum. After this infiltration the specimens were transferred to complete embedding mixture—42 parts solution A and one part solution B (polyethylene glycol 400, 10 ml, and NN dimethylaniline 1 ml)—placed in embedding moulds (Sorvall), and left to polymerise overnight at 4°C under vacuum. The polymerisation reaction is strongly exothermic and if allowed to proceed rapidly without adequate cooling, the boiling point of the monomer may be exceeded with resulting formation of gas bubbles which can be trapped in the plastic and seriously impair the properties of the block. This excess rise of temperature not only damages the tissue form overheating but also destroys the activity of the enzymes. The presence of even a single gas bubble in the block must be taken as a sign that temperatures have exceeded the boiling point of the monomer. The polymerisation reaction is also inhibited by low temperatures and oxygen. Thus polymerisation at 4°C under vacuum provides an effective cooling system and permits slow but uniform and adequate polymerisation.

Semithin sections were then cut from the undecalcified bone marrow cores with a Tungsten-carbide knife in a Jung's high performance microtome (Autocut), floated on water, placed on glass slides, and allowed to air dry at room temperature. The sections were stained for myeloperoxidase activity using 3.3'-diaminobenzidine tetra hydrochloride (DAB) as the substrate¹³ and counterstained with Cole's haematoxylin. The slides were then washed in distilled water, allowed to dry, and mounted with diatex. The sections were also stained for acid phosphatase by the Goldberg and Barka method¹⁴ and for acid α -naphthyl acetate esterase by the method of Yam *et al.*¹⁵

For MGG staining, the sections were placed in May-Grünwald solution (MG stain 2 ml + 48 ml Sorenson's phosphate buffer pH 6.8) and incubated in

a dry oven at 45°C for 45 minutes. The sections were then transferred directly into Giemsa solution (Giemsa stain 1 ml + 49 ml of Sorenson's phosphate buffer pH 6.8) and incubated at 45°C for 45 minutes. The slides were washed briefly in distilled water, gently blotted dry, cleared in xylene, and mounted with diatex.

The MG and G solutions and the mounting medium diatex were obtained from Raymond A Lamb, London, and the Sorenson's phosphate buffer from Fisher Scientific, Springfield, New Jersey. In addition to MGG staining, most other conventional stains used on paraffin embedded tissue section can be used with good results.

Results

This method of infiltration and GMA embedding of bone marrow cores is relatively simple, does not contain holes or air bubbles (fig 1), is easy to section, and does not require longer time than standard methods. This technique provides consistently good quality histological sections of 2–3 μ m in thickness from undecalcified bone marrow cores. One micrometre thick sections were also possible, but they were too thin to handle and did not bind sufficient dye for easy interpretation.

Using GMA, the methods of Leduce and Bernhard¹⁶ and Spaur and Moriarty¹⁷ were tried.

Fig 1 Surface view of bone marrow biopsy core embedded in GMA. Note centrally placed marrow core and absence of any holes or air bubbles in this plastic block.

Fig 2 Detail of bone marrow biopsy preparation from patient with non-Hodgkin's lymphoma: (a) after decalcification and paraffin embedding; (b) after embedding in GMA without decalcification. Note cellular detail and excellent tissue preservation in this preparation. Plasma cells (arrows) can be easily recognised. (a) Haetoxylin and eosin; (b) May-Grünwald-Giemsa stain.

These authors advocated prepolymer formation from the final embedding mixture to reduce the swelling artefacts in the tissue during polymerisation, but consistent uniform prepolymer formation by their techniques was difficult. The heat released during prepolymer formation often drove the mixture into spontaneous polymerisation, and the Leduce and Bernhard¹⁶ technique of prepolymer formation over a Bunsen burner in a capped Erlenmeyer flask was too dangerous. The methods of Sims¹⁸ and Lee¹⁹ were much more satisfactory, but this resin failed to

polymerise uniformly, even after air was excluded and the temperature raised. Attempts to apply the technique to larger moulds to process the whole bone marrow cores were unsuccessful. Beckstead and Bainton⁹ and Westen *et al*²⁰ have combined methylmethacrylate with GMA to produce an embedding medium suitable for enzyme histochemistry, but it is not clear why both media are required when similar or better results can be obtained with GMA embedding alone; and our experience with the technique of Beckstead and Bainton for embedding and

sectioning of undecalcified marrow cores has been less satisfactory. According to our tests the GMA embedding technique described by Germain was the most satisfactory.²¹ Bone shattering was minimal and the cytological details of haemopoietic tissue were well preserved. The consistency of the final block, however, was too hard and brittle for adequate sectioning in the Autocut microtome.

Consequently, various combinations of the components of embedding medium were tested²² and, after repeated trials, a mixture was achieved that produces blocks free of air bubbles and was of a consistency that could be cut at any thickness from 30 µm to 1 µm with a Tungsten-carbide knife in a Reichert Jung Microtome (Autocut). Sections as thick as 20–30 µm were necessary to trim the block to reach an adequate depth before the final sections of 2–3 µm were cut.

Early in this study several fixatives—paraformaldehyde, glutaraldehyde, phosphate buffered formalin, Schaffer's solution,²³ Bouin's solution and PGA—were tested²² as fixative for tissue embedded in GMA to find the best fixative for routine and special stains for enzyme histochemistry. The Schaffer's solution produced the best nuclear and cytoplasmic detail which facilitated recognition of cell types in haematopoietic tissue in the sections of bone marrow cores stained by MGG, but tissues fixed in PGA or Bouin's solution at 4°C for one hour were the most satisfactory for enzyme histochemistry.

Unlike many other plastics, GMA cannot be dissolved out of the section by any technique available at the present time, and the unremoved resin stains heavily with most basic dyes, thus obscuring the sharpness of cellular details. It has been suggested that this background staining can be removed completely from the matrix by differentiating the section in either 2-butoxyethanol or absolute ethyl alcohol, or in mixtures of these two solvents.⁶ In our hands, however, such treatment often produced uneven staining and caused detachment of sections from the slides. Among the various GMA's tested,²² the one obtained from Hartung Associates was the most satisfactory, and its low acid content produced little or no background staining. Thus our combination of Hartung's GMA and the modified MGG staining technique described herein produced the best Romanowsky's staining for sections of undecalcified marrow cores embedded in GMA.

Fig 2 compares the details of bone marrow biopsy sections from a patient with non-Hodgkin's lymphoma after GMA and conventional paraffin embedding techniques. One half of the biopsy specimen was fixed in formaldehyde solution, decalcified, and processed into paraffin (fig 2a); the other half was fixed in PGA and processed into GMA without

decalcification (fig 2b). The superiority of GMA over paraffin in preserving morphological detail is evident. The plasmal cell infiltration, which was missed in the haematoxylin and eosin stained paraffin-embedded sections, was clearly shown in sections stained with MGG and embedded in GMA. The MGG stain provided good definition and excellent colour differentiation of various haematopoietic cells for easy and accurate identification.

The enzyme myeloperoxidase (MPO) is a good marker for the granulocytic series. By light microscopy, the Harker method, using 3,3'-diaminobenzidine (DAB) at pH 7.6, seemed to be most sensitive.¹³ It was not difficult to show myeloperoxidase activity in GMA embedded plastic sections. The reaction product was shown in all the cells of granulopoietic series, including myeloblasts (fig 3) and mature polymorphonuclear neutrophils, after only 10 minutes of incubation. The lymphoblasts were negative; the enzyme was also very active in mast cells, basophilic granulocytes, and eosinophilic granulocytes. Peroxidase rich, larger granules of these cells exhibited more intense brownish coloration (fig 5). Enzyme activity was also shown in cells of monocytic lineage, but the reaction products were more finely granular, with less intense coloration.

Enzyme acid phosphatase activity was also easily shown in the sections of GMA embedded bone marrow cores, and the reaction product was especially heavy in osteoclasts (fig 6) and macrophages. Monocytes showed considerable variation in staining reactions, and megakaryocytes were mainly unreactive. Tartrate resistant acid phosphatase was also shown in GMA embedded sections of marrow cores from patients with hairy cell leukaemia (fig 7). Enzyme acid alphanaphthyl acetate esterase (ANAE) activity mainly followed the distribution pattern of acid phosphatase. The reaction product was particularly heavy in monocytes and macrophages (fig 8). A diffuse positive reaction was also observed in megakaryocytic cells, but the reaction product was much weaker.

Discussion

Improved plastic embedding techniques that produce reliable, semithin sections of high histological quality from undecalcified bone marrow cores for light microscopic study provide for better evaluation of the morphological and structural detail of the bone marrow in various haematopoietic disorders. For light microscopic examination of bone and bone marrow specimens, embedding in methyl-methacrylate is the method of choice, as decalcification is avoided and semithin sectioning of large specimens is possible.⁵ Unfortunately, this technique is not successful for

Fig 3 Bone marrow biopsy section from case of acute myeloid leukaemia showing myeloperoxidase positive blast cells. Bone marrow specimen was processed into GMA and stained for MPO by Hanker's method.

Fig 4 Bone marrow biopsy section from a case of CML in lymphoblastic transformation shows a focus myeloperoxidase negative blast cell. Note peroxidase positive mature granulocytic precursor cells at periphery of lesion. This specimen was processed into GMA and stained for myeloperoxidase by Hanker's method.

Fig 5 Bone marrow biopsy section from case of chronic myeloid leukaemia in chronic phase showing intense peroxidase activity in eosinophilic granulocytes. Biopsy specimen was processed into GMA and stained for myeloperoxidase by Hanker's method.

Fig 6 Biopsy section reacted for enzyme acid phosphatase (AP). Note strong positive reaction in osteoclasts. BT = bony trabecula.

Fig 7 Bone marrow biopsy specimen from newly diagnosed case of hairy cell leukaemia showing tartrate resistant acid phosphatase positive "hairy" cells. Bone marrow biopsy specimen was processed into GMA and stained for AP in presence of tartaric acid.

Fig 8 Bone marrow biopsy section reacted for enzyme acid α-naphthyl acetate esterase (ANAE). Note strong ANAE reaction in macrophages.

enzyme histochemistry. GMA embedding is the most effective medium for preserving optimal morphological details and staining enzymes.

This cold GMA embedding technique for processing undecalcified marrow cores shows the presence of several enzymes useful in diagnosing various haematopoietic disorders using special stains and histochemical techniques; and the new MGG staining produces the best Romanowsky's staining for sections of undecalcified marrow cores embedded in GMA. Thus the morphology and enzymatic composition of marrow cells in their own natural environment can be studied, and on the sections from the same plastic embedded bone marrow biopsy specimens. In this way valuable information, which might otherwise go unnoticed, can be obtained.

Myeloperoxidase (MPO) is an enzyme found primarily in the cells of granulocytic series and has never been reported in lymphoid cells. This feature makes it useful for distinguishing between lymphoid and non-lymphoid leukaemias. This reaction, when performed on sections of bone marrow biopsy specimens, gives accurate information on the nature, localisation, homogeneity, and extent of leukaemic infiltrate in the marrow. With this enzyme marked on sections, it is possible to identify more precisely the spatial localisation of granuloid precursors in relation to bone, vascular, and fatty stroma at diagnosis and during haemopoietic regeneration following chemotherapy for acute myeloid leukaemia, which may provide some insight into the origin and migration of cells of granulocytic series within the marrow cavity.²⁴⁻²⁵ These findings may have important biological implications.

The enzyme acid phosphatase is a valuable marker for lymphoid malignancies and helps distinguish different types of acute and chronic lymphoproliferative disorders. It is important in characterising T and non-T acute lymphoblastic leukaemia.²⁶ The acid phosphatase reaction is positive in blast cells of most (about 90%)²⁷ of T derived acute lymphoblastic leukaemia with typical localisation in a small perinuclear area. In contrast, in most non-T-ALL (about 90%), this reaction is absent or weak, and present in only a small proportion of blast cells.²⁷ In chronic lymphocytic leukaemia (CLL) the reaction of the lymphocytes for acid phosphatase is negative or weak, while in hairy cell leukaemia (HCL), a disorder which can be confused with CLL, the cells' reaction to acid phosphatase is moderately to strongly positive and they are tartrate resistant. The enzyme ANAE gives similar results to acid phosphatase and has been correlated well with T cell markers by some,²⁸ but this reaction is particularly useful in characterising the monocytic component in a leukaemic marrow.

The adaptation of plastic embedding to

undecalcified marrow cores provides excellent preservation of the marrow architecture and cellular detail. Even in such improved histological section preparation, however, it is sometimes difficult to identify precisely the nature of cellular diseases in the marrow, and enzyme or immunohistological studies become essential to characterise the type of cells or sanctuary of cells participating in the malignant process. This can now be done by embedding undecalcified marrow cores in GMA. Although water miscibility and low temperature processing make GMA a suitable embedding medium for enzyme histochemistry, the nature and type of fixative used is vitally important. We tested various fixatives and the general conclusion is that PGA and Bouin's solution best preserve the enzyme activity of marrow cells in semithin sections of undecalcified marrow cores processed in glycol methacrylate.

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