Technical methods

A simple method for histological examination of bone marrow particles using hydroxyethyl methacrylate embedding

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Paraffin sections prepared from particles of bone marrow obtained by needle aspiration of the sternum, iliac crest, or tibia (or any other suitable site) may be useful in the diagnosis of blood diseases or in the general study of this tissue. The cells seen in these sections differ markedly from those seen in smears from the same material. The paraffin method causes considerable shrinkage and distortion and thin ($1-2\mu$) sections cannot be easily obtained. In addition, the Romanowsky type of stain, which allows beautiful differentiation of the cell types in smears, is rarely successful with paraffin sections. The procedure described in this paper provides sections of suitable thickness after using an embedding medium based on hydroxyethyl methacrylate and the same apparatus that is commonly used in preparing paraffin sections. The cells in these sections are clearly defined and relatively undistorted and, with suitable staining, the various types may be identified and results approaching those seen in a Romanowsky-stained smear obtained.

The procedure is based on the work of Ramon (1955) who showed that marrow particles could be separated from blood and precipitated protein by using a mixture of ethanol and formalin as a fixative and that of Ruddell (1967a and 1967b) who devised an embedding medium based on hydroxyethyl methacrylate which could be easily sectioned at about 1μ using the microtomes and steel knives commonly used for paraffin work.

Materials

FIXATIVE

Mix together equal volumes of absolute isopropanol and 15% formalin (15 ml of commercially available 40% formaldehyde solution

mixed with 85 ml distilled water). When this is mixed a fine, often nearly invisible, precipitate is formed and it is a wise precaution to remove this by filtration using Whatman no. 42 filter paper held in a Buchner funnel attached to a suitable side-arm flask and vacuum line. For use the fixative is held in wide-mouth jars of about 400 ml capacity which are threequarters filled.

EMBEDDING MEDIUM: SOLUTION A

To 80 ml hydroxyethyl methacrylate add 16 ml 2-butoxyethanol. Mix well and add about 0.3 g benzoyl peroxide. Stir to dissolve using gentle heat ($40-50^{\circ}\text{C}$). A magnetic stirrer-hotplate is useful.

EMBEDDING MEDIUM: SOLUTION B

To 15 parts of polyethylene glycol 200 (or 400) add 1 part N,N-dimethyl aniline and mix thoroughly.

The constitution of these media follows Ruddell (1967b).

Methods

COLLECTION AND FIXATION OF MARROW PARTICLES

Immediately after aspiration the marrow and blood obtained are ejected from the syringe into 200-400 ml of fixative. A small portion may be placed on a glass slide and smears made from this for haematological study. If it has not clotted that remaining on the slide may also be added to the fixative. The syringe is also flushed several times with fixative. When the blood and marrow are added to the fixative the blood proteins are coagulated and if much blood is present the coagulum is bulky. This flocculent material tends to remain floating for a longer time than the marrow particles which usually sink rapidly to the bottom of the jar. The supernatant fluid with the suspended protein floccules can then be carefully sucked off using a tube attached to a Venturi water pump. It may be necessary to leave a good deal of fluid in the jar at the first attempt to avoid removing the marrow. More fixative is added and the process repeated until all the flocculated protein has been removed and only particles of marrow remain in the bottom of the jar. If the particles are rather fatty, or small, and slow to sink, 20-50 ml of absolute isopropanol may be added. After mixing and standing for a short time the particles will sink quite rapidly and may be more readily sepa-

¹ The hydroxyethyl methacrylate was obtained from Imperial Chemical Industries Ltd. The other reagents were supplied by British Drug Houses.

rated from the protein. Fixation is allowed to continue until the next day. A shorter time of around three hours may be satisfactory in urgent cases. Fixation for longer than 24 hours tends to impair staining and should be avoided. If embedding cannot be continued after 24 hours it is best to store the marrow in 70% ethanol.

DEHYDRATION, INFILTRATION, AND POLYMERIZATION

The particles of marrow are gathered together and, using a Pasteur pipette, transferred to a suitable small embedding vessel. The 2.5 ml polystyrene flat-bottomed cups with polythene caps which are supplied for use with an AutoAnalyzer (Technicon Instruments Ltd) are suitable. The fixative overlying the marrow particles is replaced by 70% ethanol which in turn is replaced with three to five changes of absolute ethanol, each change lasting for about 30 minutes. Thorough dehydration is essential and this part of the process may be conveniently completed between 9 am and 2 pm. The ethanol is then replaced by solution A of the embedding medium. The marrow particles float to the surface at first but they soon sink. When this occurs, after 15 to 20 minutes, the overlying fluid is removed and replaced by fresh solution A. Four or five more changes follow, each lasting 20-30 minutes, before leaving the particles therein overnight.

The following day the block is formed by replacing the solution A with a freshly prepared mixture of 45 volumes solution A mixed thoroughly with 1 volume of solution B. The marrow particles and any solution A remaining around them must be thoroughly mixed with this before filling the cup to the brim and capping with a coverslip to exclude all air. The cup is then placed in a vessel of cold water to help remove any heat formed during polymerization. This should be complete in about 30 minutes. A little contraction occurs and a few small bubbles form beneath the coverslip.

TRIMMING BLOCKS AND CUTTING SECTIONS

The block is prepared for sectioning by first using a fine toothed saw to cut off the coverslip and 3-4 mm of underlying plastic. Two vertical cuts are made on opposite sides of the cup, which can be easily broken and peeled from the block. The block is trimmed to 3-4 mm in height but left with a circular cutting face. It is moderately hard, but can be indented slightly with a thumb nail.

The block is fixed to a carrier by sticking with Ester wax (Steedman, 1960). The carrier should be made from a rigid material. Tufnol or metal are preferable but reasonable results are obtained with hardwood blocks. We have also used a modified carrier on a base sledge microtome

Fig. 1 Base sledge microtome with special holder for cutting sections of bone marrow particles embedded in hydroxyethyl methacrylate. This may make sectioning easier but is not essential.

which was devised by G. W. Fynn at the Royal Radar Establishment, Malvern, with some success (Fig. 1). For the best results the microtome used should be rigid and in good mechanical order. A well sharpened knife is also essential otherwise juddering and the alternate cutting of thick and thin sections will occur. We have used the MSE base sledge microtome with the thickness setting at 1 or 1.5 μ . Independent checking of the instrument showed these to be accurate. The knives were sharpened either by the lapping plate method (Bell, 1958) or using wood blocks armed with diamond abrasive. Doubtless other methods would be satisfactory. A defect-free edge is essential.

MOUNTING SECTIONS

The sections are taken from the microtome knife with fine forceps and floated onto the surface of distilled water which is at room temperature. The sections immediately flatten and float so that they can be picked up with a microscope slide in the same way as paraffin sections. After standing vertically to drain until apparently dry at room temperature for about 30 minutes they are subjected to thorough drying at 55° to 60°C for two to four hours. Initial slow drying at room temperature is essential to avoid microscopic crazing and cracking while further drying at a higher temperature ensures that the sections

are firmly attached to the slide. (We use a slide drying fan heater.)

STAINING THE SECTIONS

In all the staining procedures the hydroxyethyl methacrylate remains around and throughout the tissue section. No suitable solvent has been found. Ruddell (1967a and b) reported success with various methods, including periodic acid-Schiff, Alcian blue, cresyl fast violet, alizarin staining for calcium, Perls's reaction for iron and methyl green differentiated with a mixture of 2-ethoxy-ethanol and acetone for desoxyribonucleic acid. For the study of bone marrow cells a differential stain is useful but initial trials with Romanowsky type stains were not fully satisfactory. The only useful results obtained were by staining with Giemsa's stain diluted approximately 1:25 with 0.2M acetate buffer (Walpole) at pH 5.5 but the colours were rather pale. So far the best results have come from Dominici's method (as quoted by Gray, 1954) which with some modification is given below.

- 1 Place the slides directly into a solution containing 0.5 g eosin yellowish and 0.5 g orange G dissolved in 100 ml of distilled water and leave for 10 to 15 minutes.

- 2 Rinse for three to four seconds in distilled water.

- 3 Place in a solution of 0.5 g toluidine blue dissolved in 100 ml distilled water for three to four minutes.

- 4 Rinse for three to four seconds in distilled water and blot dry. Allow to dry in air at room temperature for five to 10 minutes, clear briefly in xylol, and attach a coverslip with DPX or other suitable mountant.

Results

Nuclei are blue with clear staining of the chromatin pattern. Basophilic cytoplasm stains varying shades of pale blue or greyish blue. Mast cell granules are basophilic and show metachromatic staining with the toluidine blue. Erythrocytes are pink. Eosinophil granules are bright red. The haemoglobinizing cytoplasm of red cell precursors are shades of pink. Differentiation of cell type is possible primarily on nuclear pattern and cell size and secondarily on cytoplasmic coloration. The cells are all well defined and their relationships to each other are clearly shown. The results are very much better than those obtained by the paraffin method. Examples are shown in Figures 2 to 4.

The hydroxyethyl methacrylate surrounding the marrow particles stains pale blue, the intensity increasing with section thickness, but this does not interfere with microscopic examination. It is on occasion possible to cut sections thinner

Fig. 2 Bone marrow from patient suffering from megaloblastic anaemia $\times 170$.

Fig. 3 Same case as in Fig. 2 $\times 660$.

Fig. 4 Bone marrow section from patient suffering from acquired haemolytic anaemia $\times 660$.

than 1μ but these are rather too thin for general study of marrow.

OTHER STAINING OR HISTOCHEMICAL PROCEDURES

The following have been used with success: haematoxylin and eosin, Perls's reaction for ferric iron, the periodic acid-Schiff reaction for carbohydrates, the alcian blue method for acid mucopolysaccharides, the Feulgen reaction for desoxyribonucleic acid, the periodic acid-hexamine silver method, Luxol fast blue for myelin, solochrome cyanine as a general stain, Heidenhain's iron haematoxylin method, and simple stains with basic dyes such as methylene blue and cresyl fast violet. Van Gieson's picrofuchsin and connective tissue stains of the Masson type do not work. Poor results were given by the methyl green-pyronin stain (Trevan and Shorrock) for nucleic acids. (Technical details for all these methods may be found in any current text of histological technique, for example, that of Drury and Wallington, 1967.)

TISSUES OTHER THAN BONE MARROW

The method has been applied with success to a number of tissues fixed in either glutaraldehyde or formalin, including spleen, lymph glands, kidney, liver, heart valves, tissues of the alimentary tract, and the retina. Blocks varying in size up to about 15×20 mm have been handled but it is advisable to keep the tissue slices 1-2 mm thick to ensure complete impregnation by the hydroxyethyl methacrylate.

Blocks of decalcified bone marrow have also been studied. Decalcification in a 5% solution of the di-potassium salt of diamino-ethane-tetra-acetic acid (EDTA), adjusted to pH 7 by the addition of 40% sodium hydroxide, gives good results. Decalcification may be hastened by warming to either 37° or 56°C without detriment to the tissue. Wash in running water overnight before embedding in hydroxyethyl methacrylate and stain with the Dominici procedure.

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Preparation of thin epoxy resin sections from thick sections of paraffin-embedded material

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The value of thin sections prepared from epoxy resin-embedded tissue for the routine histological examination of renal biopsies and other tissues was emphasized recently by Eastham and Essex (1969), thus confirming the use of thin sections for renal tissues as advocated earlier by Jones (1957a). However, most epoxy resin techniques, when compared with paraffin methods, possess several disadvantages. They include a slow embedding rate, considerable restriction in area and thickness of specimen that can be processed satisfactorily, orientation problems, irretrievability of specimen once embedded, and resistance of sections to routine histological or histochemical methods.

If, as seems likely, the thin section epoxy resin method is to be used as a routine diagnostic tool then it is vital that most of these disadvantages be overcome. It is hoped that the method described in this paper will help to meet this need.

Reagents

ARALDITE

10 ml epoxy resinCY 212 (Ciba)
 10 ml hardenerHY 964 (Ciba)
 0.5 ml acceleratorDY 064 (Ciba)
 1.0 ml di-N-butyl phthalate (British Drug Houses)

Prepared according to the method of Glauert and Glauert (1958):

The epoxy resin, hardener, and di-N-butyl phthalate were mixed together in a 100-ml conical flask for five minutes, heated at 60°C for a further five minutes, and then accelerator was added. The whole was then mixed thoroughly for 10 minutes and allowed to debubble for 20 minutes at 60°C before use. The mixture minus accelerator has a shelf life of one month at 4°C .

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