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IMPROVED HISTOCHEMICAL METHODS FOR THE EXAMINATION OF PLASTIC-EMBEDDED HUMAN MARROW

by

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ABSTRACT

Improved methods for processing, sectioning, and staining plastic (glycol methacrylate) embedded human marrow biopsies are described. Marrow biopsies processed by this technique were compared with biopsies processed by the conventional paraffin embedding method. The plastic embedded marrows provide better morphology enhancing diagnostic accuracy, permit assessment of bone as well as marrow and allow histochemical analysis of biopsy specimen.

Special stains including naphtol AS-D chloroacetate esterase, periodic acid Schiff (PAS), reticulin and iron have been modified so that they are suitable for undecalcified, two microns thick, plastic embedded human marrow biopsies.

Key Words:

Bone marrow, plastic embedment, Glycol methacrylate embedment, Histochemistry, naphtol AS-D chloroacetate esterase, periodic acid Schiff, reticulin and iron.

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INTRODUCTION

Paraffin-embedded marrow biopsies are widely used for diagnostic purposes. Such specimens are useful for diagnosis of granulomas, lymphomas, metastatic tumors and myeloproliferative disorders, although often because of their thickness the sections do not permit identification of a significant proportion of the hematopoietic cells. Moreover, the trabecular bone, vascular sinuses, osteoblasts, and osteoclasts are difficult to evaluate. Decalcification of the marrow for paraffin embedding produces shrinkage, interferes with evaluation of the bone, reduces the quality of staining of hematopoietic cells, and interferes with the ability to apply special stains to the marrow cells.

Plastic embedded sections can be cut thinly (1-2 μm) whereas paraffin sections are 4 to 8 μm thick, usually. Cell shrinkage is less in plastic embedded sections enhancing morphologic identification. Moreover, by preventing shrinkage in marrow, comparison of cells in marrow to those in soft tissues is greatly facilitated.

A serious limitation which has prevented widespread use of the plastic embedded tissue section has been its poor affinity for hematoxylin and eosin stain (H and E) or special histochemical stains, especially useful in hematologic diagnosis. The following report describes modification of staining techniques which has permitted use of H and E and certain special stains, including chloracetate esterase, periodic-acid Schiff, Prussian blue, silver stain for reticulin meshwork. In these studies the bone is not decalcified and can be evaluated as to its normalcy. Thus, the hematopoietic cells, marrow blood vessels, sinuses, osteoblasts, osteoclasts and metastatic cells can be evaluated.

MATERIALS AND METHODS

Jamshidi needle marrow biopsies were placed in Karnovsky's fixative. The fixative is prepared freshly each morning according to the procedure of Millinog.

The biopsy is left in fixative overnight and thereafter washed in phosphate buffer for one hour and dehydrated through a graded series of alcohols in the cold. The dehydration sequence based on the method of Rudell,[12] calls for 40 minutes in each of 40, 60, 80, and 95% ethyl alcohol. Following dehydration with 95% ethyl alcohol, a mixture of 1:1 100% ethyl alcohol to catalyzed plastic is added to the biopsy. The plastic used is JB-4 Plastic Embedding Kit from Polysciences, Inc. The plastic itself is glycol methacrylate catalyzed by benzoyl peroxide. The hardener for the plastic is polyethylene glycol 400 N,N dimethylaniline. The biopsy is left in the alcohol-plastic mixture for the rest of the working day (4-6 hours) and then is transferred to 100% plastic and is left overnight in the refrigerator.

The biopsy is embedded by placing it in the bottom of a small round mold (Sorvall) and covering it with 2 cc. of plastic to which hardener has been added. An aluminum block holder (Sorvall) is placed in the mold and the edges sealed with hot paraffin or dental wax. This seals out atmospheric oxygen which inhibits polymerization. Polymerization of the plastic will occur at room temperature in one to four hours, depending on the ratio of hardener to plastic. After polymerization, the blocks are peeled from the mold and excess plastic is removed using a dental drill.

The blocks were sectioned on a Sorvall JB-4 microtome using thick glass knives. Sections of two microns thickness were removed with fine forceps and placed in a room temperature water bath where they expand and are easily transferred to glass slides. These are air-dried or placed on a hot plate for better adherence of sections to the slide.

For hematoxylin and eosin (H and E) staining, Gill hematoxylin and Erythrosin B gave the most satisfactory results (See Appendix).

RESULTS

Comparison of Paraffin and Plastic Embedded Marrow: Three marrow biopsies, which were previously found to be normocellular were chosen to compare. One was a two micron-thick undecalcified plastic-embedded section of an ordinary quality, the two others were five microns thick decalcified, paraffin-embedded sections. One was of good quality and the other was of poor quality. Five hundred cells were counted under 1000x and under 400x magnification for each specimen. The result is summarized in Table 1 which demonstrates that about 16% of cells in an excellent paraffin embedded section cannot be identified. In a poor section, about 40% of the cells were unclassifiable. In the plastic section all cells were identifiable. Also, this table indicates that in a decreasing order the lymphocytes, normoblasts, and myelocytes are missidentified in paraffin sections. Examination of the biopsy at 1000x magnification can help in identification of the paraffin embedded sections but this is not necessary for plastic embedded marrows. These findings are representative of our experience with, first paraffin embedded and subsequently plastic embedded, sections.

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In a case of multiple myeloma and of aplastic anemia marrow biopsies that had been decalcified, embedded in paraffin and from which five microns thick sections, stained with conventional hematoxylin and eosin were prepared, were compared with a second biopsy that had been embedded in plastic and from which two microns thick sections stained with modified hematoxylin and eosin were made.

The paraffin embedded section of multiple myeloma was markedly hypercellular and the marrow was infiltrated by malignant mononuclear cells with marked anisonucleosis and hypochromatosis (Fig. 1A). Although occasional cells could be identified as plasma cells, the main tumor mass seemed to be in syncytial aggregates, without obvious cellular borders; therefore, the diagnosis of multiple myeloma could not be easily made on the bases of the biopsy, and it was read as a poorly differentiated malignant neoplasm. A definitive diagnosis could be made only after examining the Wright's stained smear of the marrow aspirate. The plastic embedded biopsy (Fig. 1B) was also hypercellular and was infiltrated by cells which were clearly recognized as malignant plasma cells. The characteristic clock wheel chromatin arrangement, intranuclear Dutcher bodies as well as "flaming" plasma cells with multiple cytoplasmic projections were identified, readily.

The paraffin embedded bone marrow sections of the case of aplastic anemia were markedly hypocellular and consisted of occasional fibrocytes and plasma cells, and some mononuclear cells. The other mononuclear cells were not identifiable (Fig. 2A). These cells were recognizable as histiocytes in the plastic embedded sections (Fig. 2B). Other cells such as plasma cells and fibrocytes were also recognized

more easily. Another interesting observation was the presence of a homogeneous, pink intercellular substance in the cellular compartment of the marrow, which was absent in the paraffin embedded sections.

Hematopoietic Cell Morphology

Erythroid Cells: With plastic embedment and 2 microns-thick sections marked improvement in morphological details of the marrow was evident compared to paraffin sections (Fig. 3). Middle and late stage erythroid cells were easily identified by their pinkish-red cytoplasm, round hyperchromatic nuclei with abundant coarse chromatin and small amount of parachromatin. Nuceloli were not prominent. Erythroid precursors were in aggregates (islets) often. The nuclear size and the nucleocytoplasmic ratio were a function of stage of maturity: the early erythroblasts had large nuclei with a rim of pink cytoplasm; the more mature nucelated red cells had small, condensed and eccentric nuclei and more abundant cytoplasm.

Granulocytes, Monocytes, Macrophages: Myeloblasts had round nuclei with abundant parachromatin and small amounts of finely granular chromatin. The nuclei contains one or two round, eosinophic nucleoli. The cytoplasm was scant and grayish-pink. Neutrophilic myelocytes contained orange cytoplasmic granules. Eosinophilic myelocytes contained bright red granules and basophiles contained dark blue granules. Specific differential counts could be done with ease, therefore.

Monocytes had oval or kidney-shape nuclei with finely granular chromatin and one or two chromocenters. The cytoplasm was amphophilic, finely granular and usually finely vaculated.

Macrophages had small round, oval or kidney-shape nuclei, finely granular chromatin and one chromocenter. The eosinophilic cytoplasm was abundant, vaculated and usually contained nuclear debris and/or iron granules which were stained dark brown. Majority of macrophages were located at the periphery of the erythroid islets.

Megakaryocytes: The megakaryocytes had their characteristic appearance: abundant grayish-pink cytoplasm which was reticulated, irregular, multilobated nuclei containing abundant parachromatin and a small amount of finely granular chromatin and multiple chromocenters (Fig. 3). Platelets formation could be observed at the periphery of some of the megakaryocytes.

Osteoclasts, Osteoblasts, and Bone Trabeculae

Osteoclasts were identified as large multinucleated cells with abundant amphophilic cytoplasm (Fig. 4). The cell membrane was irregular and the cytoplasm contained abundant red granules unevenly distributed throughout the cytoplasm. The nuclei were individual and tend to aggregate in the center of the cell. A prominent nuclear membrane, abundant parachromatin, and one or more small eosinophilic nucleoli was characteristic.

Osteoblasts had an oval to pyramidal shape with an even cytoplasmic membrane. The cytoplasm contains a wide rim of amphophilic, finely granular cytoplasm and a paracentral light pink zone. The single nucleus was oval to round and usually eccentrically located. The nuclear membrane was not prominent, and the chromatin was finely granular and evenly distributed. One or two small eosinophilic nucleoli were present.

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The plastic embedded marrow sections were suitable for evaluation of metabolic bone diseases. Osteoid stained light pink and the calcified bone stained darkly amphophilic. The morphologic details of the Haversian Systems, osteocytes, and laminated bone could be evaluated easily.

Metastatic Tumors

Foci of metastatic tumors were easily recognizable in the thin sections. Breast carcinoma cells that had metastasized to marrow are shown in Figure 5. The tumor cells are clustered in a variable sized aggregates in the marrow space. In this case, the tumor cells are larger than the hematopoietic cells. The cellular and nuclear pleomorphism, hyperchromatism, irregularity of the nuclear membrane and irregularity and multiplicity of the nucleoli were more evident than they were in the paraffin embedded sections. A marked increase in osteoclasts was present in this marrow biopsy.

Vascular Structures

Marrow sinuses could be identified as irregular channels. They are lined by endothelial cells which have scanty elongated pink cytoplasm and an oval nucleus. The nuclear membrane is prominent, the parachromatin is abundant and a chromocenter is present in the nucleus (Fig. 6). The basement membrane can be visualized by PAS stain. Egress of the differentiated hematopoietic cells through the sinusoidal wall also could be identified.

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Arterioles, venules, and nerve trunks can be identified in these sections. Although their basic morphology is identical to the paraffin embedded sections, their structure is much better preserved, and differentiation of arterioles from venules is more precise.

Histochemistry on Plastic Embedded Marrow

Differentiation of some cases of acute myelogenous leukemias from acute lymphocytic leukemias may be difficult in decalcified, paraffin-embedded marrow biopsies. Histochemical stains such as the naphthol AS-D chloroacetate and the periodic acid Schiff are helpful in such a differentiation. Napthol AS-D chloroacetate reactivity is absent in a decalcified marrow. Also, the tissue section should be thin enough for examination of individual marrow cells. These requirements are present in undecalcified, plastic-embedded marrow biopsies.

Naphtol AS-D chloroacetate esterase stains the cytoplasm of the myelomonocytic cells and the bone marrow mast cells bright red and granular (Fig. 7). The mast cells are elongated cells while the myelomonocytic cells are round to oval. The lymphoid cells, normoblasts and megakaryocytes were not stained. The nuclei of all cell types are stained purple gray.

PAS stains the cytoplasmic granules of late myelocytes bright red while myeloblasts are PAS negative (Fig. 8A). The large lymphocytes and lymphoblasts contain occasional large perinuclear granules (Fig. 8B), while the late granulocytes contain more uniform and smaller PAS-positive granules. Megakaryocytes are also slightly PAS positive.

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Reticulin stain demonstrates the amount of marrow fibrosis by the number of branching, black reticulum fibers (Fig. 9). The nuclei stain red and the collagen if present will stain blue. Reticulin fibers are increased in myeloproliferative disorders, lymphocytic and myelomonocytic leukemias and lymphomas.

The presence of storage iron in marrow could be evaluated by Gomori's method. Also, marrow identification of ring sideroblasts with perinuclear arrangement of the iron granules is possible in plastic embedded sections. These cells are shown in Figure 10 from a case of sideroblastic anemia.

DISCUSSION

Smears prepared from aspirated marrow are easy to prepare, provides good morphology and hematologists are used to their morphological characteristics.[3,14] However, in some cases aspiration of marrow is not possible or provides inadequate material. The cellularity of the marrow is difficult to quantify as is the frequency of megakaryocytes. Granulomas and marrow fibrosis cannot be studied in aspirated marrow smears. Metastatic tumor cells are more easily identified in biopsy sections. Although biopsies obviate these problems, paraffin embedded biopsy sections have not been a satisfactory substitute for the aspirate. The cytologic features of the specimen are often inadequate for diagnosis.

Various plastic embedding techniques have been used by the investigators since 1949, when Newman, et al.[10] introduced methyl methacrylate for this purpose. Rosenberg, et al.[11] in 1960 suggested the use of a water-soluble acrylic ester, glycol methacrylate also known as GMA as

the embedment of choice for light and electron microscopy. This technique was studied and further developed by Ledna and Bernhard[9] in 1967, by Cope[5] in 1968, by Ashford, et al.[1] in 1972 and by Hoshina and Kobayashi[7,8] in 1971, 1972. The value of this embedment for light microscopy was emphasized by Feder and O'Brian[6] in 1968. In 1974, Sims and Cole[4,13] used this embedding media for routine experimental work. In 1976, Bennett, et al.[2] anticipated that plastic embedment will replace the paraffin for routine purposes.

The lack of a good quality hematoxylin and eosin stain, the inability to perform special stains, the small-sized biopsies, the length of time required for processing and sectioning the biopsies and the cost have been among the major criticisms of the use of plastic embedded marrows for diagnosis. The preposed method solves the above problems and demonstrates an easier processing, sectioning, and staining method, which provides a superior morphology in a short time. Plastic embedded marrow cores are ready for histological evaluation within two days from the time the biopsies are obtained. This is shorter than the time needed for preparation of decalcified, paraffin embedded bone marrow samples. The procedure is not costly either, especially if the technique is adapted for other specimens such as kidney, liver, and gastrointestinal biopsies. After a short period of training the sections can be cut easily by histology technologists.

APPENDIX

Staining method for hematoxylin and eosin: The sections were exposed to Gill hematoxylin for one hour at room temperature and rinsed in deionized water three times. The sections were blued in 0.2% ammonia water for about two minutes and rinsed with deionized water five times. The sections were exposed to erythrosin B for ten minutes and rinsed with deionized water three times. They were dipped quickly in 95% alcohol three times and then in 100% alcohol. They were exposed to xylene for a few minutes and then mounted.

Staining method for naphthol AS-D chloroacetate esterase: One drop of 4% p-rosanilin* in 2N hydrochloric acid and one drop of 4% sodium nitrite were mixed in a 50 ml. flask and were left to stand for one minute. Thirty-five ml. of 0.1 M phosphate buffer, pH 6.5 was added to this and was mixed. This solution was then added to 10 mg naphthol AS-D chloroacetate in 1 ml N,N-dimethylformamide was mixed well, and then filtered. The sections were immediately placed in this solution and stained for 30 minutes. They were then washed in running tap water for five minutes and were counterstained with Harris' hematoxylin acidified with acetic acid for one minute. The sections were washed in running tap water for five minutes and rinsed in distilled water. They were air dried and then cleared in xylene and mounted with Permount.

Staining method for reticulin: The sections were oxidized with 0.3% potassium permanganate in 0.3% sulfuric acid for three minutes, rinsed with three changes of distilled water and were reduced with 2%

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*NOTE: p-rosanilin, acridine-free may be obtained from the Roboz Surgical Instrument Co., 810-18th Street, N. W., Washington, D. C. 20006

potassium metabisulfite for one minute. They were washed with running tap water for three minutes and rinsed with four changes of distilled water. Ammoniacal silver solution was prepared by addition of 10 ml of 10% silver nitrate to 2.5 ml of 10% potassium hydroxide. Concentrated ammonium hydroxide was added drop by drop with constant shaking, until the precipitate just dissolved. The solution was made up to 45 ml with distilled water. The sections were exposed to ammoniacal silver solution for two minutes, rinsed with three changes of distilled water, reduced in 20% formalin for two minutes, washed with running tap water for three minutes, and rinsed with two changes of distilled water. They were then toned in 0.2% gold chloride for 30 seconds, rinsed with two changes of distilled water, and fixed in 2% sodium thiosulfate for one minute. The sections were washed well with tap water and then rinsed with two changes of distilled water. The slides were counterstained with 0.1% nuclear fast red for five minutes, rinsed with three changes of distilled water, and dehydrated through graded alcohols. The sections were cleared in xylene four times and mounted with Permount.

Staining methods for PAS: The sections were placed in 0.5% periodic acid for 15 minutes, rinsed with distilled water four times and were exposed to the Lillie's Schiff solution for 30 minutes. They were then rinsed in distilled water twice and washed in running tap water for ten minutes. The sections were again rinsed in distilled water twice, and were left in the acidified Harris' hematoxylin for ten minutes. They were then washed in running tap water for five minutes, rinsed with distilled water twice and were dehydrated through graded alcohols. The sections were cleared in xylene three times and were mounted with Permount.

Staining method for iron: The sections were placed in equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide solution for twenty minutes. They were then rinsed with distilled water five times and were left in the nuclear fast red solution for five minutes. They were rinsed with distilled water three times, and dehydrated through graded alcohols as usual. The sections were cleared in xylene four times and were mounted with Permount.

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TABLE

PERCENTAGE OF IDENTIFIABLE HEMATOPOIETIC CELLS IN PARAFFIN AND PLASTIC EMBEDDED MARROWS

Marrow Cells	EMBEDMENT					
	Paraffin Embedment (Good Specimen)		Paraffin Embedment (Poor Specimen)		Plastic Embedment	
	1000x	400x	1000x	400x	1000x	400x
Myeloblast	2.1	1.1	0.5	0.3	1.6	1.6
Myelocytes	39.7	41.5	32.0	30.0	46.9	46.9
Polymorphonuclear Cells	18.1	18.5	14.5	14.0	17.9	18.1
Nucleated Red Cells	18.2	16.7	11.5	10.8	20.3	20.4
Megakaryocytes	0.5	0.4	0.4	0.4	0.5	0.4
Monocytes and Histiocytes	2.1	1.1	1.0	0.9	2.0	2.0
Lymphocytes	3.1	2.2	1.1	0.7	10.2	10.0
Plasma Cells	1.0	1.0	0.8	0.8	0.6	0.6
Unclassified Cells	15.2	17.5	38.2	42.1	0	0

Legends To Figures

Figure 1A Paraffin embedded section of marrow involved with multiple myeloma, demonstrating atypical mononuclear cells with pleomorphic, hyperchromatic nuclei occasionally with plasmacytic features (arrow), diagnosed as poorly differentiated neoplasm suggestive of multiple myeloma.

B Plastic embedded section of the marrow in A, demonstrating malignant plasma cells with characteristic chromatin pattern and large nucleoli. Note the tissue preservation and lack of cellular shrinkage in this preparation. H and E stain. (x1250)

Figure 2A Paraffin embedded marrow from a case of aplastic anemia demonstrating a markedly hypocellular marrow containing fat cells. Unidentifiable mononuclear cells are present in small number.

B Plastic embedded section of the marrow in A, demontrating aplasia. In this preparation plasma cells (arrow) and several histiocytes one of which shows erythrophasocytosis (open arrow) and another of which contains hemosiderin granules (asterisk) can be identified. Note the homogeneous pink intercellular substance in the cellular compartment. H and E stain. (x1250)

Figure 3 Plastic embedded normal human marrow with developing erythroblasts in aggregates (arrow), granulocytes and megakaryocytes. H and E stain. (x625)

Figure 4 Plastic embedded marrow showing a binucleated osteoclast at the margin of a bone spicule. H and E stain. (x1250)

Figure 5 Plastic embedded marrow containing a cluster of metastatic mammary carcinoma cells. The prominant nucleoli, irregular nuclear membranes, and cellular pleomorphism characteristic of metastatic tumor to the marrow are identifiable. A group of pyknotic tumor cells are also present. H and E stain. (x1250)

Figure 6 Plastic embedded marrow from a case of acute undifferentiated leukemia showing a blood sinus containing leukemic blast cells. Note the oval nucleus of a sinus wall endothelial cell (arrow) PAS stain. (x1250)

Figure 7 Plastic embedded marrow from a case of chronic granulocytic leukemia demonstrating markedly naphthol AS-D chlroacetate esterase positive granules in myelocytes.

Figure 8A PAS stained plastic embedded marrow from a case of acute granulocytic leukemia showing bright red granules in eosinophilic myelocytes (arrow), homogeneous red stain of mature granulocytes (open arrow) and negative myelocytes.

B This is PAS stain of plastic embedded marrow from a case of acute lymphoblastic leukemia demonstrating perinuclear positive granules in the tumor cells (arrow). (x1250)

Figure 9 Reticulin stain of plastic embedded marrow of a case of idiopathic myelofibrosis showing marked marrow fibrosis. (x625)

Figure 10 Iron Stain of plastic embedded marrow from a case of sideroblastic anemia demonstrating several ring sideroblasts (insert) and increased iron in the histiocytes (asterisk). (x1250)