A Histochemical Study of the Reticuloendothelial System of Human Marrow—Its Possible Transport Role

By S. Trubowitz and B. Masek

Technical problems, confusing morphologic criteria and a troublesome anatomical location have made the study of the nonhematopoietic elements of the bone marrow most difficult. The anatomical and functional relationships of these cells, namely the capillary endothelial cells, the reticular cells and fat cells, to each other and to the developing hematopoietic cells are indeed blurred. Aschoff\(^1\) saw a functional relationship between the endothelial cells and reticular cells based on their common capacity for phagocytosis of colloidal dyes. He pointed out, however, that physiologic integration did not imply anatomical identity. Drinkar et al.\(^2\) reviewed the early studies on the extent of the marrow endothelium and after a thorough investigation of their own, concluded that the endothelial structures are closed structures not in communication with the parenchyma. Duan,\(^3\) utilizing similar injection technics of carbon granules and colloidal dyes, was able to identify a most extensive capillary system between and about the fat cells. He identified the reticular cells by their characteristic shapes and staining reaction, but indicated no distinct relationship between the reticular and endothelial cells. Duan was not completely satisfied with the resolution provided by his technics and found it unfortunate that a specific stain for the identification of the endothelial cell in tissue had not yet been developed. The histochemical demonstration of the phosphatases (the adenosine tri-, di- and mono-phosphatases as well as the alkaline phospho-mono-esterase) in the capillary endothelial cell is now fairly well established\(^4\) and provided the stimulus for the reexamination of some of the nonhematopoietic elements of human bone marrow. The present report deals with observations made on the endothelial, reticular and adipose cells of the human marrow visualized by a variety of histochemical methods.

Material and Methods

The observations were made on the marrows of some 70 patients. Most of the subjects had a wide variety of hematologic abnormalities, but a sufficient number of essentially normal marrow aspirates were available to provide a basis for comparison. The samples of marrow were obtained from the corpus sterni immediately below the sternal angle by the commonly employed aspiration technic. The initial 0.5 ml of marrow aspirate was placed in a Cooch crucible lined with Filtrator Coffee\(^*\) paper and washed with

\(^*\)Obtained from Filtrator Coffee Apparatus Co., New York, N. Y.
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cold physiologic saline to remove the contaminating blood and loose cells. The paper containing the individual marrow particles was immediately transferred to a small petri dish containing ice cold 6.25 per cent glutaraldehyde made with 0.2 M. phosphate buffer at pH 7.6. The total time required from moment of aspiration to fixation was less than one minute. Some particles were transferred to a vial containing fresh fixative and subsequently post fixed in 1 per cent buffered O2O4 and embedded in Epon for use in another study. After brief fixation in glutaraldehyde, the remaining particles were washed and stored in cold distilled water until a variety of histochemical procedures could be carried out on the intact marrow particles and on spreads prepared from the particles on glass slides. The entire operation was completed on the day of the marrow aspiration. For this study the following histochemical procedures were employed: alkaline phosphatase, acid phosphatase, adenosine triphosphatase, adenosine diphosphatase, adenosine monophosphatase, nonspecific esterase, toluidine blue, periodic acid Schiff reaction for neutral polysaccharides, iron, reticulum and Oil red O staining. The tissue spreads were prepared by gentle compression on clean glass slides just prior to incubation in the phosphatase medium.

Again, in this investigation, the reticular cells are defined as a system of widely disseminated cells which lie in close apposition to the vessels, the hematopoietic cells and fat cells of the marrow. They are recognized by their ovoid nuclei and abundant, pale-staining (as with the usual Romanowsky stains), ill-defined, stellate-cytoplasmic mass. The endothelial cells are characterized by their long narrow nuclei arranged either as a linear aggregate of single cells or as bundles of such aggregates.

Alkaline Phosphatase

Both marrow spreads and marrow particles were incubated for 15 minutes at room temperature in the medium described by Kaplow.8 The whole particles were washed in water and mounted in water on single concavity slides. The spreads were washed in water, counterstained in 3 per cent neutral red, dehydrated, cleared and mounted in Permount.9 Preparations mounted in glycerol jelly show superior intensity of stain.

Acid Phosphatase

Bone marrow spreads and bone marrow particles were incubated for 15 minutes at room temperature in a medium using hexazonium pararosanilin as a coupler according to recommendations described by Barka.9 Following incubation, the whole particles were washed in distilled water and mounted in water in single concavity slides. The spreads were washed in water and mounted in glycerine jelly.

Adenosine Tri- and Diphosphatase and 5' Nucleotidase

Marrow spreads and particles were incubated for 60 minutes at 37 C. in a mixture of 10.0 ml. of a 125 mg./100 ml. solution of the respective substrate (ATP, ADP and AMP); 10.0 ml. of a 0.2M tris-maleate buffer pH 7.2, 1.5 ml. of 2 per cent lead nitrate, 2.5 ml. of M/10 magnesium sulfate and 1.0 ml. distilled water.10 The spreads and particles were then washed in a weak aqueous solution of ammonium sulfide and washed well in distilled water. The spreads were mounted in glycerine jelly and the whole particles in water on single concavity slides.

Nonspecific Esterase

Esterase activity was demonstrated by the use of naphthol AS-D chloroacetate substrate as described by Moloney.11 Incubation was maintained for 30 minutes at room temperature. The spreads were counterstained with hematoxylin. The specimen were washed and mounted as described above.

*Obtained from Fisher Scientific Co., New York, N. Y.
Fig. 1.—Marrow particle stained with toluidine blue. The large adipose cells appear as bright spheres. The stromal and hematopoietic cells grow out over the fat cell surfaces. (250×)

Toluidine Blue

Both spreads and particles were stained in 0.03 per cent aqueous toluidine blue at room temperature for 5 minutes. Spreads were washed, dehydrated, cleared and mounted in Permount. The particles were washed and mounted in single concavity slides in water.

Periodic Acid Schiff (PAS)

Particles and spreads were stained by a modification of the PAS reaction introduced by McManus.12 The spreads were counterstained with hematoxylin, washed in water, dehydrated in alcohol, cleared in xylol and mounted in Permount. The particles were mounted in water on single concavity slides.
Iron

A modification of Perl’s Prussian Blue method was used to identify the presence of nonheme iron in the specimens. The tissues were handled with bone-tipped forceps and incubated in medium consisting of 1 part 2 per cent aqueous potassium ferrocyanide and 3 parts 1 per cent aqueous hydrochloric acid. Incubation was carried out at 60 C. for 20 minutes. After thorough rinsing in distilled water, the spreads were counterstained with 3 per cent neutral red, dehydrated, cleared and mounted in Permount. The whole particles were mounted in water in single concavity slides.

Reticulum

A gold chloride solution as described by Soule was used to demonstrate reticular fibers in the marrow spreads and particles. Again, the whole particles were mounted in water on single concavity slides and the spreads dehydrated, cleared and mounted in Permount.

Oil Red O

Marrow particles were stained in 0.5 per cent solution of Oil Red O in 60 per cent
Fig. 3.—Endothelial cells, reticular cell and mast cell. Toluidine blue. (1000×)

Fig. 4.—Marrow capillaries. Alkaline phosphatase stain. (400×)
triethyl phosphate for 5-10 minutes after which they were washed thoroughly in water and mounted in single concavity slides.

The mounts of intact marrow particles were examined at about 40 diameters magnification under the stereo microscope, and by transmitted light at 125 and 250 diameters magnification and where possible at 400 diameters. The spreads were sufficiently thin to allow examination at 1000 diameters and showed surprisingly little disturbance of cellular detail.

**Observations**

The toluidine blue-Oil Red O stained fragments of intact marrow reveal the intimate relationship between the fat cells, which make up the bulk of the marrow volume, and the stromal and blood elements. In the normal marrow the fat cells average about 85 micra in diameter in the glutaraldehyde-fixed state and are stacked as spheres in a three dimensional array. The hematopoietic cells and stained stromal components fill the spaces between the fat cells and spread out over the surfaces of the large adipose cells in a manner highly reminiscent of a monolayer growth in a tissue culture medium (Fig. 1). Mast cells are easily identified; appear singly and widely separated from each other. The alkaline-phosphatase and adenosine-triphosphatase stains of the marrow particle reveal an extensive capillary network coursing between and around the fat cells whose contours they follow very closely. It is important to point out that staining of the marrow fragments
Fig. 6.—Network of phosphatase active granules between and about individual blood cells. Note hexagonally arranged network surrounding the hematopoietic cells. Alkaline-phosphatase stain. (1000×)

takes place on the surface of the particles and penetrates but a short distance into the interior. The patchy and irregular staining of the particles in no way hinders an appreciation of the three dimensional relationship of some of the structures. In the same phosphatase stained preparations, large spindle-shaped or star-shaped cells, highly positive for phosphatase activity, are present, scattered between and about the fat cells, but separate and distinct from the capillaries. These cells can be readily distinguished from the mature granulocytic cells which are known to possess alkaline-phosphatase activity.

The intensity of the Perl stain for iron varies considerably. In the iron rich marrows the iron appears to occupy anatomical sites similar to those observed for the phosphatases. The blue-staining hemosiderin granules lie in a linear arrangement between and about the fat cells and as more diffuse homogeneous material in the spindle-shaped cells described above.

The reticulum stained particles are very dense and difficult to transillumi-
Fig. 7.—Fat cell with adjacent phosphatase rich capillaries. Phosphatase rich granules surround the blood cells on surface of the fat cell. Alkaline-phosphatase stain. (1000×)

The Oil Red O stains the adipose cells in the marrow particles most vividly, reveals their relationship to each other and makes them manifest in hyperplastic marrows where they are usually not visible in the unstained state. As anticipated, the fat cells in hypoplastic marrows are larger and more tightly packed than in the normal marrow and become smaller and widely spaced in the hyperplastic states.

The marrow particles provide a good three dimensional view of the overall organization of the marrow components—fat, hematopoietic and stromal.
Spreads of the marrow particles, however, offer a much better appreciation of intracellular and intercellular detail.

The toluidine-blue stained marrow spreads afford fairly ready recognition of most of the cellular elements. Stromal or reticular cells, characterized by their large oval shaped nuclei and abundant, diffuse cytoplasm, are found within and about the collections of nucleated red cells, granulocytic cells and megakaryocytes (Fig. 2 and 3). These cells are sometimes difficult to distinguish from the fat cells. Capillary fragments course through the cell mass: their elongated endothelial cells are easily recognized. Aggregates of brown granules are often present between the poles of these cells and prove to contain iron following Prussian-blue staining. Reticular cells are in close approxi-
Fig. 9.—Bands of intense alkaline phosphatase activity arising at intervals about surface of large capillary. Alkaline-phosphatase stain. (1000×)

mation to the vessels. A sprinkling of mast cells is often present in the immediate environs of the endothelial cells.

The alkaline-phosphatase and the adenosine-tri, di and monophosphatase show essentially identical distribution throughout the marrow. Since the alkaline-phosphatase stains are more intense than the others, this enzyme can serve as the prototype for the group. The capillaries (Fig. 4) and the reticular (Fig. 5) stain intensely in the marrow spreads prepared for alkaline phosphatase activity. The reticular cells are now readily distinguished from the collapsed fat cells which show no phosphatase activity. Alkaline-phosphatase activity is also present between and around the individual, differentiating
hematopoietic cells covering their surfaces as an hexagonally arranged network (Fig. 6). In Figure 7 intensely stained capillaries lie at the margin of a fat cell over whose surface a network of alkaline-phosphatase granules spread out to surround the individual blood cells. Figure 8 shows similar distribution of the alkaline-phosphatase staining granules along the course of the vessel and around the individual, neighboring cells. Figure 9 is a dramatic presentation of a larger vessel with bands of intense alkaline-phosphatase activity arranged at intervals about its surface and which extend to envelope the surrounding cells. Figure 10 and 11 are adenosine-triphosphatase stained preparations which show many of the features described for the alkaline phosphatase.

In the normal marrow acid phosphatase is present regularly in the myelocytic cells and is distributed fairly uniformly in the promyelocytes, myelocytes and polymorphonuclear leucocytes. Both the endothelial and reticular cells are devoid of acid-phosphatase activity. In the iron rich marrows, however, those reticular cells containing large granules of hemosiderin display

Fig. 10.—Adenosine-triphosphatase activity in marrow capillary and about individual hematopoietic cells. (1000 ×)
an intense acid-phosphatase stain. In the iron rich marrows stained for both alkaline and acid phosphatase, acid phosphatase but little alkaline phosphatase, is present in the hemosiderin laden reticular cells. However, no acid-phosphatase activity is visible in the rich alkaline-phosphatase active sites, namely the endothelial cells, other reticular cells and in the areas immediately about the proliferating hematopoietic cells. The adenosine tri-, di- and monophosphatases occupy the same topographic distribution but unlike the alkaline phosphatase are present in the hemosiderin loaded reticular cells.

The Prussian-blue stained spreads show dense blue-staining granules in the capillaries (Fig. 12). In addition, smudges of nongranular diffuse blue-
staining material spreads out between and around the blood cells, red cells, white cells and megakaryocytes, in a manner similar to that seen for the alkaline-phosphatase activity (Fig. 13 and 14). Iron is present in the large, stellate stromal cells, the reticular cells, described above.

The reticulum stain of the marrow spreads reveals presence of a red-staining material, presumably a mucopolysaccharide containing structure, which surrounds the individual cells. However, stromal cells containing this material, as seen for alkaline phosphatase and iron, cannot be identified. The reticulum unmasked by this stain is spread as a diffuse and fiber-like structure (Fig. 15). In contrast, the PAS reaction is limited to the cytoplasm of the myelocytic cells.

Focusing on the sites of primary interest in this study, we can summarize the findings as follows:
**Fig. 13.**—Iron granules in capillaries and diffuse iron staining material about the blood cells. Prussian blue stain. (1000 x)

<table>
<thead>
<tr>
<th></th>
<th>Endothelial Cell</th>
<th>Reticular Cell</th>
<th>Surrounding the hematopoietic cells</th>
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<tbody>
<tr>
<td>Alkaline Phosphatase</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Adenosine Triphosphatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Adenosine Diphosphatase</td>
<td>+</td>
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<tr>
<td>Adenosine Monophosphatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Iron</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>0</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Reticulum</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Toluidine Blue</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAS</td>
<td>0</td>
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<tr>
<td>Esterase</td>
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Fig. 14.—Iron containing material about megakaryocytes. Prussian blue stain. (1000×)

The intensity of the iron stain varies considerably, from complete absence in the iron-deficiency states to marked increase in the iron-overload states. The Prussian blue reaction unmasks the iron of hemosiderin but not of ferritin, hence the observations described for iron, probably concern storage rather than transport of iron with the presumption that the two processes are probably related. The distribution and intensity of the phosphatases are independent of the degree of iron deposition except in the very heavily laden reticular cells where the alkaline phosphatase is replaced by acid phosphatase activity.

DISCUSSION

Cytochemical investigations have demonstrated intense alkaline-phosphatase activity in the capillary endothelial cells and in the reticular cells of bone marrow. The observations presented in the present study confirm these findings and call attention to alkaline-phosphatase activity surround-
Fig. 15.—Bone marrow spread. Reticulum stain. (1000×)

ing the individual blood cells. Whether this activity outlines a complex structure of reticular cells which bind the hematopoietic cells or identifies an alkaline-phosphatase rich substance which is secreted into the intercellular space cannot be resolved by the techniques employed in this study. The additional demonstration of adenosine tri-, di- and monophosphatase activity in both the endothelial and reticular cells suggests a functional relationship between these two cell systems.

Palade\textsuperscript{18} observed vesicle formation in endothelial cells of capillaries and conceived of a transport system through the cytoplasm of endothelial cells based on transfer by means of micropinocytic activity. Phosphatase activity has been associated with, and in some manner may serve as the enzymatic mechanism of transport.\textsuperscript{19} Phosphatase activity has been demonstrated to reside in the pinocytotic vesicle of the endothelial cells.\textsuperscript{20}

Bessis and Breton-Gorius\textsuperscript{21} thought of the reticular cell and surrounding erythro- and normoblasts as a functioning unit—the erythroblastic island, in
which ferritin is transferred from the reticular cell to the differentiating erythroblasts by pinocytosis. Fawcett22 presented similar evidence for ferritin transfer to the erythroblast and directed special attention to selected sites on the erythroblast surface capable of binding the ferritin.

Hyman and Paldino23 assigned a protein transport role to the reticuloendothelial system and suggested that the reticulum cell may be capable of passing protein molecules to the extravascular side. They visualized the reticulum cell as a “window” built into the vascular wall to allow transfer of protein molecules from one side to the other. Some investigators24,25 attributed a lipid transport role to the reticuloendothelial system, while others26 saw a broader transport function involving many materials from blood to the extravascular spaces.

Shields27 speculated that the reticular cells acquire a transport function by giving rise to lymphoid cells which by shedding, form a plasma containing a variety of low and high molecular weight proteins.

The observations reported in this study, support the suggestion that the endothelial cells and reticular cells together form a transport system in the marrow. In this scheme the endothelial cells are the site of exchange of material with the blood in the capillary or sinusoidal lumen. The material is then transferred either directly to neighboring differentiating cells or into a complex of reticular cells which stretch from the vessels to surround the more distant, maturing blood cells. The reticular cells in turn transport these substances to the developing blood cells. Reticular cells also lie in close approximation to fat cells from which they may conceivably transfer lipid complexes to the growing cells. The reticulum which was found about the blood cells and not within the endothelial or reticular cells may represent mucopolysaccharides whose role may be similar to that of the mucopolysaccharides seen adjacent to endothelial cells in blood vessels. Anatomically the reticular cells form membrane-like structures which stretch from the external surface of the capillary or sinusoidal wall into and around the neighboring hematopoietic cells. This membranous complex of reticular cells may be thought of as a modified capillary without a lumen, capable of transporting a variety of materials from the endothelial cells to the blood cells.

The model of marrow structure derived from these histochemical studies is quite similar to the structure described by Weiss28,29 from his electron microscopic observations of rat marrow. The vascular sinus is visualized as a trilaminar wall consisting essentially of a double layer of reticular cells; one looking into the sinusoidal lumen, the other into the hematopoietic mass which it further subdivides by adventitial spurs.

The phosphatase activity of bone marrow tissue may reflect the transport requirements of this tissue and its intensity may therefore vary under a variety of physiologic and pathologic conditions. In this regard it is interesting that Hostettler and Ackerman30 found a striking relationship between alkaline-phosphatase activity and ensuing formation of lymphocytes in certain non-thymic lymphocytic organs of the embryonic and neonatal rabbit. The enzyme activity was localized in the reticular cells which condensed around the region...
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of the arterial branch. Pearse has suggested that increased levels of alkaline phosphatase activity in a blood vessel wall could be interpreted as a manifestation of increased transport function. Samorajski and McCloud were able to show an increase in endothelial alkaline phosphate activity of brain vessels with increase in the permeability of these structures.

It is also important to point out that the application of labeling technics to the stem cell question has thrown considerable doubt on the stem cell function of the reticular cells. The reticular cells may indeed be more closely related embryologically to the blood vessel cells than to the blood cells themselves.

SUMMARY

Intense alkaline adenosine tri-, di- and monophosphatase activity is present in the capillary endothelial cells and reticular cells of the bone marrow as well as about the individual marrow cells. The phosphatase activity of the granulocytic cells is weak by comparison and offers no difficulty in differentiation. Iron is confined to a similar topographic distribution. The described observations are discussed in terms of transport. It is suggested that the reticulo-endothelial system of the marrow is essentially a transport system for the differentiating hematopoietic cells.

REFERENCES


A Histochemical Study of the Reticuloendothelial System of Human Marrow—Its Possible Transport Role

S. TRUBOWITZ and B. MASEK