Morphologic and Functional Characteristics of Bone Marrow Macrophages From Imferon-treated Mice

By Martha E. Fedorko

The iron storage macrophage has been isolated from the marrow of Imferon-treated mice and studied in vitro by morphologic, histochemical, and functional tests and isotope labeling techniques. These macrophages on stained preparations are large, many times binucleate cells (up to 150 μ), and show Prussian blue reactivity. In Epon-embedded, stained thick sections they contain elongated narrow basophilic inclusions. These macrophages are actively phagocytic and pinocytic; histochemical studies show that these cells are rich in acid phosphatase, nonspecific esterase, and PAS diastase-resistant activity. Iron storage macrophages do not incorporate the 3H-thymidine. The electron microscopic appearance of this macrophage shows that the cell has ferritin free in the cytoplasm and several types of cytoplasmic granules: those with large quantities of electron-dense ferritin and/or hemosiderin (type A), elongated granules (type B) with moderately electron dense homogeneous matrix and some ferritin at the periphery, and granules with heterogeneous content (type C). The above findings demonstrate that the iron storage cell is a mature macrophage which contains hydrolases, ferritin, and a unique population of cytoplasmic granules which are lysosomal in nature. There is some evidence to suggest that the unusual lysosome (type B granule) occurs after macrophages have ingested erythrocytes.

The macrophages of the mononuclear phagocyte system in spleen, liver, and bone marrow play an important role in erythropoiesis, for they have been demonstrated to engulf aged erythrocytes and use the iron liberated from them for new hemoglobin production or storage. Morphologic studies on stained smears of marrow give very little information on the characteristics of the macrophage, and staining for Prussian blue reactivity is most often performed mainly to determine the presence or absence of iron. The amount of cell disruption on such smears can be considerable. Studies on bone marrow tissue in block, whether at the light or ultrastructural level, are somewhat limited because the cell cytoplasm covers large surface area, extends between many cell types, and only narrow areas of cytoplasm can be identified in any one section. Initially, methods for isolating bone marrow monocyte precursors were used to try to study the macrophages from this organ, but these methods were found to be inadequate because the macrophages are easily disrupted during the isolation.

Studies by others at the ultrastructural level have concentrated on some morphologic characteristics of marrow macrophages and their relation in tissue block to red cell precursors. In the present report, a method is described for isolating suitable numbers of these bone marrow macrophages, maintaining...
them in vitro, together with illustrating their structural and functional characteristics.

**MATERIALS AND METHODS**

**Preparation of Mouse Bone Marrow**

NCS mice (Rockefeller Colony) received 0.25 cc Imferon (Lakeside Laboratories, Milwaukee, Wis.) intraperitoneally 2-4 wk before use. Tibias and femurs from six treated mice were placed in a container lined on the bottom with filter paper moistened with saline. The epiphyseal ends of the bones were cut off, and the bone marrow was flushed out with a syringe fitted with a 19-gauge needle and filled with Hanks' medium adjusted to pH 7.4. The marrow pieces were teased apart into minute fragments and the suspension then transferred to a 25-ml sterile graduated flat-bottomed cylinder. The marrow particles were then allowed to sediment for 15-20 min at room temperature. The supernatant 17 cc (fraction 1, Table 1), which contained single cells was then aspirated off. The remainder which contained bone marrow particles in approximately a 2-cc volume was mixed with 0.05% collagenase, 383 U/mg (Worthington Biochemical Corp., Freehold, N.J.), in 5 ml of Hanks' medium, pH adjusted to 7.4, at 37°C for 30 min with gentle agitation. The collagenase-containing cell and particle suspension was next diluted to 20 cc with Hanks' medium, filtered through a wire mesh sieve (25 square openings/sq mm), placed back into the cylinder, and sedimented at 1 g for 20 min. The upper 15 cc (fraction 2) which contained predominantly single cells was separated off from the lower 5 cc (fraction 3) which contained small particles of marrow. Both the collagenase-treated cell suspension and marrow particles were washed in medium and each suspended in a 5-ml volume of 10% heat-inactivated fetal calf serum in RPMI medium (Grand Island Biological Co., New York, N.Y.). Fractions 1 and 2 were plated at a concentration of $3.6 \times 10^6$/cu cm. Fraction 3 was plated at the same concentration of single cells, but the number of explants was $3.6 \times 10^3$/cu cm. The specimens were either allowed to settle for 4 or 18 hr onto glass cover slips, after which time cells from fractions 2 and 3 were exposed to trypsin (Grand Island Biological Company, New York, N.Y.), 0.06% final concentration in saline, to remove cells on the macrophage surface. Specimens from fraction 1 did not require exposure to trypsin.

In the preparation containing small marrow explants (fraction 3), the yield of iron storage macrophages from Imferon-treated mice varied from 10% to 30% of the cells present after trypsinization. Most of the studies were performed on Imferon-treated mice so that there could be a large quantity of iron stored in the cells. The yield of bone marrow macrophages from normal

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<tr>
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<td>2.</td>
<td>Tibias and femurs from six treated mice were placed in a container.</td>
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<td>3.</td>
<td>The bone marrow was flushed out with Hanks' medium.</td>
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<td>4.</td>
<td>The marrow pieces were teased apart.</td>
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<td>5.</td>
<td>The suspension was transferred to a cylinder.</td>
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<td>6.</td>
<td>The supernatant was aspirated off.</td>
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<td>7.</td>
<td>The remainder was mixed with collagenase and Hanks' medium.</td>
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<td>8.</td>
<td>The suspension was diluted and filtered.</td>
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<td>9.</td>
<td>The supernatant was separated from the sedimented particles.</td>
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<td>10.</td>
<td>The specimens were plated at different concentrations.</td>
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<td>11.</td>
<td>Some specimens were exposed to trypsin.</td>
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**Table 1. Procedure for Processing Mouse Bone Marrow**

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<th>Step</th>
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<tr>
<td>1.</td>
<td>20 cc marrow suspension</td>
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<td>2.</td>
<td>Sedimented (1 g)</td>
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<td>3.</td>
<td>17 cc Supernatant (fraction 1) abundant in monocyte precursors and monocytes</td>
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<td>4.</td>
<td>3 cc sedimented particles</td>
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<td>5.</td>
<td>Collagenase digestion</td>
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<td>6.</td>
<td>Dilution to 20 cc, passage through sieve</td>
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<td>7.</td>
<td>Sedimentation (1 g)</td>
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<td>8.</td>
<td>Cells (15 cc) (fraction 2) monocyte precursors and monocytes</td>
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<td>9.</td>
<td>Particles (5 cc) (fraction 3) abundant in iron storage macrophages</td>
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mice was reduced, but morphologic characteristics were similar. Monocytes and their precursors described here were those obtained from suspensions of marrow (fraction 1).

**Light-microscopic Methods**

Phase microscopic studies were performed on glass cover slips to which macrophages were attached. The cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15 min and studied with Zeiss phase optics.

The Prussian blue stain was used after glutaraldehyde fixation as described above by adding an equal volume of 2% HCl and 2% potassium ferrocyanide mixture for 30 min. The specimens were mounted in water and studied with both phase and light microscopy. Some specimens were air dried and counterstained with neutral red in sucrose, 0.1 mg/ml.

Wright-Giemsa-stained specimens were air dried, stained first with Wright's stain (Harleco Co., Philadelphia, Pa.), then buffered at pH 7.2-7.4, and stained with Giemsa (Gravellon Laboratories, St. Louis, Mo.) diluted 1 drop/ml.

Radioautographs were prepared on dried cover slips of specimens which were fixed in glutaraldehyde (see above), rinsed, and dried. The slides were then dipped in Ilford L-4 Nuclear Research emulsion prepared by diluting 0.7 g/cc at 45°C followed by 30 min at room temperature before coating. The emulsion-coated preparations were allowed to stand in the dark 2-4 wk. The slides were developed in D19 (Kodak) and then stained in citrate-buffered methanol-Giemsa, pH 5.75, as cited in van Furth et al.

Thick sections (1500 Å-2800 Å) of Epon-embedded material were transferred to glass slides and stained with 0.2% azure A in 1% sodium borate.

**Histochemistry.** Acid phosphatase reactivity was demonstrated on cover slip preparations fixed in glutaraldehyde as above and exposed Gomori substrate as described previously. Control preparations without β-glycerophosphate were also prepared.

The nonspecific esterase reaction was demonstrated on preparations fixed in phosphate-buffered 3% formalin, pH 7.4, followed by a water rinse and then incubation in substrate containing 0.5 mg naphthol-AS-acetate (Sigma Co., St. Louis, Mo.), 0.5 ml acetone, 15 ml glycol, and either 40 mg fast blue RR (Sigma) or Garnet GBC (Sigma) in 50 ml 0.02 M phosphate buffer, pH 6.5. Control specimens were incubated in 3 x 10^-3 M sodium fluoride for 30 min after fixation and before exposure to substrate.

The periodic Schiff reaction was carried out (1) on cover slip preparations fixed in 3.4% formalin buffered to pH 7.4 and processed by Longley's method. Some preparations were digested with malt diastase, 1 mg/ml, for 30 min at 37°C before periodic acid oxidation. There were also control preparations without the oxidation step: (2) on polyacrylamide gels prepared by the method of Ornstein, Davis to which 1 cc deironized Imferon was added before preparation (LakeSide Laboratories, Milwaukee, Wis.). After polymerization the gels were removed from the tube and exposed to the periodic acid Schiff reaction. These gels showed reactivity. Imferon was deironized by anaerobic incubation of 2 ml Imferon with dithionite in 5 g/250 cc of 1 M acetate buffer, pH 4.5, until dialysate became colorless.

**Phagocytosis of Organisms by Macrophages**

For assessment of phagocytic activity, heat-killed streptococci and zymosan (yeast cell walls obtained from Standard Brands, Inc., N.Y., Lot OB298, in saline, boiled 1 hr, washed three times) were added to the culture in serum-containing medium in the approximate ratio 10:1 (organisms: cells).

**In Vitro Nuclear Labeling of Monocytes and Macrophages**

Labeling experiments were performed with methyl 3H-thymidine, specific activity, 17.6 Ci/mM (New England Nuclear Corp., Boston, Mass.). In vitro labeling was performed as cited in van Furth et al. in medium which contained between 0.1 and 2 μCi/ml thymidine-3H for 12-18 hr. The preparations were then washed extensively in five changes of medium, fixed in glutaraldehyde, and air dried.
Red Cell Adherence to Bone Marrow Macrophages

A 5% solution of sheep erythrocytes in medium 199 was incubated in rabbit anti-red-cell antibody (kindly supplied by Dr. S. Gordon) at a final concentration of 1:4000 for 30 min at 37°C and washed. Macrophage cultures were incubated with a 0.1% solution of antibody-coated erythrocytes for 15 min in medium without serum. The cover slips were then washed and fixed in glutaraldehyde.

In Vitro Uptake of Collagenase-treated Erythrocytes

Mouse peritoneal macrophages were placed in tissue culture by the method of Cohn and Benson. Mouse erythrocytes were collected, washed, and 0.1 cc packed erythrocyte volume was diluted with 2 cc of 0.05% collagenase, 383 U/mg (Worthington Biochemical Corp., Freehold, N.J.), in Hanks' medium, pH 7.4. At the end of the incubation at 37°C for 20 min, the packed erythrocyte volume was washed, suspended in medium 199 with a final concentration of 1% erythrocytes and 10% heat-inactivated fetal calf serum, and added to the tissue culture for 1 hr. The cultures were then washed and replenished in medium for 2 days. Control preparations did not receive collagenase-treated erythrocytes. Both types of specimens were fixed for electron microscopy.

Fixation for Electron Microscopy

Specimens from fractions 2 and 3 were trypsinized in 0.06% trypsin in saline for 30 min at 37°C and then rinsed in saline before being fixed with glutaraldehyde, 2.5% in 0.1 M cacodylate buffer, pH 7.4, for 5 min at 37°C and then 10 min at 4°C. The cells were next exposed to a mixture of glutaraldehyde and osmium, 1% in 0.1 M cacodylate buffer, pH 7.4, in a ratio of 2:1 (v:v). The specimens were rinsed twice in cold saline and then postfixed in 0.25% uranyl acetate in 0.1 M acetate buffer, pH 6.3. After the fixation and two cold saline rinses, the cell monolayers on the dishes were dehydrated in graded alcohols, and during a subsequent brief exposure to propylene oxide the monolayer detached and floated off. This monolayer was centrifuged into a narrow tipped conical centrifuge tube after suspension in 2% Noble agar, then separated off and embedded in Epon. Thin sections were placed on Formvar carbon-coated grids, stained in most instances with uranyl acetate and lead citrate, and studied with the Siemens Elmiskop I A.

Electron Microscope Histochemistry

To demonstrate acid phosphatase reactivity, the glutaraldehyde fixation and substrate was the same as for phase microscopy; postfixation was in 1% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, followed by suspension in agar and processing as described above. Peroxidase reactivity was demonstrated by LeDuc's modification of 3,3'-diaminobenzidine reaction after fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 10 min. The specimens were postfixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.3, for 1 hr, dehydrated, exposed to propylene oxide, and embedded in Epon.

Evaluation of Pinocytosis

Explant cultures of bone marrow macrophages were trypsinized and placed in medium containing 40% heat-inactivated fetal calf serum and Thorotrast (Testgar, Kanakee, Wis.) in a dilution of 1:40 for 1 hr. The specimens were washed and processed for electron microscopy.

RESULTS

Light Microscopic Studies on the Marrow Macrophage

The bone marrow macrophage which stores iron characteristically is a large cell (~150 μ); after Giemsa and Prussian blue staining it may contain one to two nuclei and a heterogeneous array of cytoplasmic inclusions. The appearance of the cells harvested at 4 and 18 hr is essentially similar except that at 4 hr the cytoplasm is less extensive and contains fewer inclusions. Prussian blue staining shows abundant characteristic blue-green positivity which appears to be mainly
granular. Giemsa stain shows diffuse cytoplasmic basophilia, an occasional basophilic nucleus from ingested cells, ingested erythrocytes, brown granules, and clear vacuoles of various sizes.

The fibroblast is another cell type present after 18 hr in culture which can reach the same size as macrophages but on stained preparations has distinguishing morphologic characteristics, namely a diffuse cytoplasmic basophilia and only a few cytoplasmic inclusions and vacuoles. Prussian blue positivity is minimal or absent.

The phase microscopic appearance of the bone marrow macrophage reveals the large size of the cell and more accurately the extent of the cytoplasm which often shows a rim free of organelles (Fig. 1A) and has prominent membrane ruffling (Fig. 1B). The nucleus is round or oval and can be binucleate with prominent phase-dense nucleoli. The cytoplasm shows many organelles including lucent vacuoles, very refractile lipid droplets, phase-dense cytoplasmic granules, and phase-dense elongated narrow structures ~ 3 x 1 μ. When these preparations are viewed without phase optics, the elongated cytoplasmic inclusion appears yellow in color. There are demonstrated the comparative smaller size and fewer cytoplasmic organelles of the monocyte in contrast to the macrophage (see Fig. 2A insert).

Bone marrow macrophage cultures are phagocytic for zymosan and streptococci (Fig. 1D and E). Red cell adherence performed on occasional hypermature macrophages found in fraction 1 is found to be marked.

Radioautographic preparations of bone marrow given a pulse of thymidine-3H in culture show a labeling index of 20%–50% for immature cells (from fraction 1) but an index of 0.5% for iron storage macrophages (400 cells counted in four preparations from fraction 3). Sometimes ingested cell nuclei within the large macrophage's cytoplasm are covered with radioautographic grains, but these are distinguished from the nucleus of the macrophage by their very basophilic staining characteristics. One example of a macrophage which shows no nuclear label is shown in Fig. 1F. A corresponding positive promonocyte is shown in the insert, same figure.

The large iron-containing macrophages show marked PAS reactivity (Fig. 1G) which is partially resistant to diastase digestion. Reactivity appears to be localized both in cytoplasmic granules and in the cytoplasmic matrix. Monocyte precursors show positive reaction in the nuclear hof region (Fig. 1G insert).

The iron storage macrophages show marked acid phosphatase reactivity which is localized predominantly in large and small granules scattered throughout the cytoplasm (Fig. 1H) and in the perinuclear hof area. By comparison there are fewer acid phosphatase-positive granules in the cytoplasm of monocytes and their precursor cells (insert, Fig. 1H). Fibroblasts show minimal acid phosphatase reactivity.
Fig. 1. Bone marrow mononuclear phagocytes and fibroblasts from Imferon-treated mice after 18 hr in culture and trypsinization (see Materials and Methods). Monocytes and their precursors were obtained from fraction 1 and not trypsinized. (A) Phase microscopic appearance of bone marrow macrophage fixed in glutaraldehyde and mounted in water. Shown is its large cell size, binucleate features, prominent nucleoli, cytoplasm filled with vacuoles of varying sizes, phase-dense granules, elongated narrow phase-dense inclusions (one indicated at the arrow), and highly refractile lipid droplets. Inset shows the typical size, uniform nucleus of the macrophage precursor in the bone marrow. Demonstrated is the quantity of cytoplasmic organelles and amount of membrane ruffling. × 2500. (B) Phase microscopic appearance of a bone marrow macrophage similar to that described in (A). One nucleus is visible, but the plane of focus shows very prominent membrane ruffling of the peripheral cytoplasm. Intra Ryder cytoplasmic organelles appear to be concentrated in the central areas of the cell. × 2500. (C) Phase microscopic appearance of bone marrow fibroblast. Shown is the somewhat smaller size of the cell as compared to the macrophage in (B). The nucleus is oval with prominent nucleoli. The cytoplasm shows long narrow pseudopods which are relatively free of organelles. A few elongated mitochondria, small vacuoles, and phase-dense granules are located around the nucleus. × 2500. (D) Two bone marrow macrophages which were exposed to medium containing zymosan for 4 hr before washing and fixation. There are many relatively large phase-dense organisms within the plane of focus of the cell. Many other morphologic features of the cells are obscured by these phagocytosed organisms. × 2500. (E) Wright-Giemsa-stained preparation of mouse marrow macrophage. The cell is binucleate (arrows), and within the cytoplasm are present many heat-killed streptococci to which the culture was exposed before fixation and washing. × 2500. (F) Radioautographic preparation of mouse marrow macrophage, after an in vitro pulse of 3H-thymidine. Shown is the nucleus with prominent nucleolus. No radioautographic grains are present over the nucleus. The cytoplasm contains very
Nonspecific esterase activity is prominent within these cells and appears localized primarily in cytoplasmic granules (Fig. 1!). There is partial inhibition of the reaction with fluoride ($3 \times 10^{-2} M$). Monocyte precursors and monocytes show few positive cytoplasmic granules (insert, Fig. 11). Fibroblasts are negative.

**Electron Microscopy on Bone Marrow Macrophages**

At the ultrastructural level, bone marrow macrophages exhibit characteristics common to cells of the macrophage series and certain unique features. The method of tissue culture which uses bone marrow explants enables macrophages to settle out on glass, and the surrounding cells remain above (Fig. 2). The nucleus shows features common to those macrophages from other sites, namely, few nucleoli and moderate margination of nuclear chromatin. The Golgi complex is multifocal and contains the usual components: cisternae, vesicles, and vacuoles. There are few lipid droplets in the cytoplasm and a moderate amount of rough endoplasmic reticulum and mitochondria. Phagocytosis of erythrocytes in all stages of digestion is also evident.

The cytoplasm of the marrow macrophage contains three types of membrane-bound granules (Figs. 3, 6). The dark, very electron-dense deposit within cytoplasmic granules (type A) corresponds to one type of lysosome containing ferritin. Another morphologic type of cytoplasmic granule (type B) is a membrane-bound long narrow structure with moderately electron-dense matrix which can show some ferritin deposition in the peripheral zone beneath the limiting membrane; few to many of these granules may be present. Cells which contain large numbers of type B granules do not usually spread out on glass or show the ruffled peripheral cell membrane. A third type of cytoplasmic granule (type C) contains a heterogeneous mixture of components (Fig. 3).

Unstained specimens are useful to determine the extent of ferritin deposition and show that ferritin is located not only in membrane-bound granules, but dispersed as well throughout the cytoplasmic matrix.

The macrophage can be distinguished from fibroblasts (Fig. 4) which contain a few membrane-bound cytoplasmic granules with very electron-dense content.
Fig. 2. Electron microscopic appearance of bone marrow explant adherent to the plastic surface of the tissue culture dish 18 hr after plating. The explant culture was not trypsinized. The edge of the cell occupying the left part of the electron micrograph is a portion of a bone marrow macrophage (1) which was attached to the surface of the tissue culture dish. The entire length of the cell is not shown, and the nucleus is not visible. Representative areas include: portion of the multifocal Golgi complex (Go) with its associated lamellae, vesicles, and vacuoles; the midzone of the cytoplasm filled with a variety of cytoplasmic organelles including very electron-dense ferritin-laden lysosomes (A) representative of type A lysosome; elongated narrow, moderately electron-dense type of lysosome—type B—filled with moderately electron-dense cytoplasmic matrix (B); mitochondria (m); lipid droplets (L); a variety of vacuoles, some of which are pinocytic (V₁) whereas others apparently contain content of ingested cell debris and remnants of type B lysosomal matrix (V₂). Certain other vacuoles contain ferritin-laden lysosomes which still retain apparent remnants of their granule membrane (V₃). The most peripheral areas of the cell show stacks of rough endoplasmic reticulum (RER). Surrounding the bone marrow macrophage are degenerating forms of myelocytes (2) and monocyte precursors which have also ingested apparent cell debris (3). × 8400.
Fig. 3. Electron micrograph of mouse bone marrow macrophage after 18 hr in culture. Shown are the different types of lysosomes: one type which contains predominantly ferritin (A) shows on its margin only part of the membrane which surrounds it; the most prominent type of lysosome visible is the long narrow elongated one (B) which shows its limiting membrane and moderately electron-dense matrix; other lysosomal structures (C) contain a mixture of components of varying size, shape, and electron density. Scattered strips of partially rough and partially smooth endoplasmic reticulum and mitochondria are present. x23,400.

but do contain large numbers of polyribosomes and long strips of rough endoplasmic reticulum.

**Pinocytic Function of Marrow Macrophages**

The large marrow macrophages actively engage in pinocytosis. The uptake of Thorotrast is seen in peripheral small vesicles and larger vacuoles (Fig. 6). Cytoplasmic granules fuse with pinocytic vacuoles and vesicles, but their contents do not appear to be readily solubilized within the time interval after initial exposure to the pinocytic marker (Fig. 5). In some instances after Thorotrast administration there appears to be a degree of autophagy present, i.e., inclusion of mitochondria, lipid, and some ferritin-laden granules showing peripheral membrane within pinocytic vacuoles.

**Morphologic Changes in Mouse Peritoneal Macrophages After Ingestion of Erythrocytes**

Mouse peritoneal macrophages 2 days in culture after ingestion of collagenase-treated autologous erythrocytes show the presence of the type B lyso-
Fig. 4. Electron micrograph of fibroblast which may be present in the culture. Note the paucity of lysosomal elements. The amount of very electron-dense material which could represent biologic compounds containing iron is small. The cytoplasm is filled with clusters of ribosomal material (circled area), scattered strips of partially rough, partially smooth endoplasmic reticulum, occasional mitochondria and vacuoles. The lower cell margin (arrow) represents the surface which was attached to the plastic tissue culture dish. Note the bundle of microfilaments which is present beneath the cell membrane. x 7980.

Ultrastructural Histochemistry on Bone Marrow Macrophages

In granules showing minimal ferritin deposition (type B) and those with mixed composition (type C), acid phosphatase reactivity could be distinguished from ferritin deposition because of its larger size, amorphous shape, and irregular dense deposits of the lead reaction product. In type A granules which were heavily laden with ferritin, no lead reaction product could be distinguished. Phosphatase reactivity was localized in some elements of the Golgi complex, granules, and in a narrow rim at the periphery of the elongated narrow (type B) cytoplasmic granules (Fig. 8).

A positive diaminobenzidine reaction in cells could indicate the presence of heme-containing components. Type B cytoplasmic granules give a negative reaction, or at most, trace positivity (Fig. 9).

DISCUSSION

The focus of the work reported here was directed toward the mature bone marrow macrophage which stores iron with the view that this cell forms part of the monocyte-macrophage series and functions just as those in the spleen to
Fig. 5. An electron micrograph of mouse marrow macrophage which had received a pulse label with Thorotrast. In addition to labeling of the various vesicles and small vacuolar elements with Thorotrast, note the two vacuoles on the lower part of the micrograph (arrows); each contains the elongated lysosome whose membrane has apparently fused with that of the vacuole, but its contents remain intact within the vacuole. × 19,500.

Fig. 6. Electron micrograph of mouse marrow macrophage to illustrate that the cell may contain large numbers of both types of lysosomes after Imferon treatment of mice: those lysosomes which are predominantly ferritin laden (short arrow) and those which are long, narrow, and elongated (long arrow). × 14,000.

Fig. 7. Electron micrograph of mouse peritoneal macrophage in culture which had received collagenase-treated autologous erythrocytes 2 days earlier. Note the presence of the elongated structure (arrow) in the cytoplasm which resembles one type of lysosome shown in Fig. 6. × 15,600.

sequester defective erythrocytes and injected particles. Apart from the precursor cell types in the bone marrow giving rise to erythrocytes and leukocytes, the marrow stroma, as it were, can be considered to contain several cell types: endothelial cells, fibroblasts, adipose cells, and mature macrophages. The cells of the monocyte–macrophage series were the ones probably principally characterized with neutral red staining in the early studies initiated by Aschoff.
Fig. 8. Electron micrograph of acid phosphatase reaction on mouse marrow macrophage. Shown is the elongated narrow granule (type B, arrow), which shows reactivity to be irregular, amorphous in shape around the rim of the granule. Nonspecific precipitate is seen in the cytoplasm. Electron-dense deposits in the type A ferritin-laden granules seen in upper left and lower right corners of the micrograph differ from the reaction product in the histochemical reaction. × 27,300.

and others. Both quantitative and morphologic studies can give information on the cells responsible for sequestering particles. Some of the more recent morphologic studies have been reported previously. Others have attempted to define differences in endothelial, “reticular,” and adipose cells of human marrow by histochemical methods.

In the past, the one most emphasized aspect of bone marrow mature macrophages was their central location in erythroblastic islands and their function to act as “nurse cells” to developing erythroid elements. In phenylhydrazine-induced anemias, macrophages have been demonstrated to contain ingested erythrocyte nuclei. The exact mechanisms showing how the ingested iron of erythrocyte hemoglobin is transferred from mature macrophages to developing erythroid cells are not clear. Two possibilities have been suggested: (1) transferring ferritin directly to erythrocytes; (2) processing iron in ferritin to a form that can be bound by transferrin.

Morphologic analyses of the various cell types have been attempted by several investigators, but in general have been limited by the methods used to study these aspects. A satisfactory method that was found to be adequate depended on migration of these cells intact out of a marrow explant. An early report of such a culture was given by Lewis and Lewis and has had many modifications. The explant method used here permitted the studies to be performed, but was limited by the fact that trypsinization of the culture altered cell

Fig. 9. Electron micrograph of diaminobenzidine reaction product in mouse marrow macrophage. Note that the elongated lysosomes (type B, short arrow) show negative reaction when compared to an extracellular erythrocyte (long arrow) which is positive. × 31,200.
surface characteristics so that immediately afterwards some studies could not be performed, i.e., red cell adherence.

Previous work has described the maturation phases of monocytes from their precursors. The cells in the maturation scheme can be classified as: immature (blast, promonocyte), intermediate (monocyte), mature (macrophage). In culture these extremely large cells form a separate class of macrophages apart from promonocytes and monocytes which acquire macrophage morphology. The maturation stages which precede the mature form have been described elsewhere. Several investigators have studied blood monocytes' maturation to macrophages. Table 2 summarizes some points of comparison that we illustrate between monocytes and mature marrow macrophages studied here. Some studies on normal marrow showed similar changes both in iron storage cells and their monocyte precursors.

Macrophages may show some features in common with those found in all areas of the organism and distinctive ones as a consequence of their special area of tissue localization. They may have some pleomorphic features and diverse functions based on localization of the macrophages. Most information has been obtained on macrophages from the peritoneal cavity, lung, and liver. In general, the outstanding features of these macrophages include their large size, abundant hydrolytic enzymes, ability to perform phagocytosis and pinocytosis, and the interphase nucleus in the normal homeostatic state. If macrophages from the lung are compared to those from the peritoneal cavity, it is evident that those from the lung contain more hydrolytic enzymes, are larger than the macrophage obtained from the peritoneal cavity, and may contain particles of carbon, or, in cases of intra-alveolar hemorrhage, products of erythrocyte digestion and/or ferritin. The energy for metabolic processes in alveolar macrophages depends more heavily on aerobic pathways in contrast, for example, to the processes in peritoneal cells.

The unique, narrow, elongated membrane-bound cytoplasmic organelle has been noted in thin sections of mouse bone marrow and rabbit spleen macrophages by others, but its function and etiology remained unclear. This or-

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<td>Nucleus</td>
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<td>Polyribosomes/unit area</td>
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<td>Ferritin in cytoplasmic granules—type A</td>
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<td>Cytoplasmic granules—type B (elongated, narrow)</td>
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ganelle has been observed in bone marrow macrophages from guinea pigs (Levine, unpublished) and from macrophages in the spleen after intravenous injection of homologous erythrocytes (Steinman, unpublished). In this report the lysosomal nature of the organelle has been described: its content of acid hydrolases, its capability of storing ferritin in situations of iron overloading, and its ability to fuse with pinocytic vesicles and vacuoles.

There is some evidence presented here that formation of the unusual lysosome in mouse peritoneal macrophages (which usually do not contain such a structure) can be produced in these cells after ingestion of collagenase-treated autologous erythrocytes. The possibility exists, therefore, that some undigested erythrocyte component or its digestion product, nonheme in nature, or bilirubin, is contained within the unusual lysosomes. Further information will await isolation and chemical characterization of this type of lysosome. If the presence of the structurally distinct lysosome indicates that the macrophage has undergone previous erythrocyte ingestion and partial digestion, it would then seem that the macrophages in the mouse and guinea pig marrow normally play a significant role in engulfing effete erythrocytes. The findings reported here may reflect either that the macrophage digests some unusual erythrocyte components or an unusual capacity of the macrophage to process derivatives of hemoglobin.

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