Differentiation of Macrophages from Normal Human Bone Marrow in Liquid Culture

ELECTRON MICROSCOPY AND CYTOCHEMISTRY

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ABSTRACT To study the various stages of human mononuclear phagocyte maturation, we cultivated bone marrow in an in vitro diffusion chamber with the cells growing in suspension and upon a dialysis membrane. At 2, 7, and 14 days, the cultured cells were examined by electron microscopy and cytochemical techniques for peroxidase and for more limited analysis of acid phosphatase and arylsulfatase. Peroxidase was being synthesized in promonocytes of 2- and 7-day cultures, as evidenced by reaction product in the rough-surfaced endoplasmic reticulum, Golgi complex, and storage granules. Peroxidase synthesis had ceased in monocytes and the enzyme appeared only in some granules. By 7 days, large macrophages predominated, containing numerous peroxidase-positive storage granules, and heterophagy of dying cells was evident. By 14 days, the most prevalent cell type was the large peroxidase-negative macrophage. Thus, peroxidase is present in high concentrations in immature cells but absent at later stages, presumably a result of degranulation of peroxidase-positive storage granules. Clusters of peroxidase-negative macrophages with indistinct borders (epithelioid cells), as well as obvious multinucleated giant cells, were noted. Frequently, the interdigitating plasma membranes of neighboring macrophages showed a modification resembling a septate junction—to our knowledge, representing the first documentation of this specialized cell contact between normal macrophages. We suggest that such junctions may serve as zones of adhesion between epithelioid cells.

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INTRODUCTION

Recent studies have convincingly demonstrated that macrophages of inflammatory exudates originate from precursor cells in the bone marrow, are transported as blood monocytes via the peripheral circulation, and differentiate further in tissues and cavities to become large macrophages (1–3), epithelioid cells, and multinucleate giant cells (4–7). Seeking to identify the precursor cells of mononuclear phagocytes in human bone marrow, Nichols et al. (8) and Nichols and Bainton (9, 10) found that electron microscopy and peroxidase cytochemistry afford several advantages over light microscopy and routine electron microscopy in the definitive identification of the various developmental stages of immature leukocytes. In addition, the stages of monocyte maturation could be more explicitly recognized.

In normal human marrow, peroxidase is synthesized early, during the promonocyte stage, and is localized in all granules and organelles of the secretory apparatus—i.e., cisternae of the rough endoplasmic reticulum (RER)[1] and Golgi complex. It has been determined that the peroxidase-positive granules also contain the enzymes arylsulfatase and acid phosphatase and hence are modified primary lysosomes. In the monocyte stage, peroxidase production ceases and is no longer visible in the RER or Golgi complex, and a second population of granules is produced. Formation of these peroxidase-negative granules continues while monocytes are being transported in the blood, but their content is unknown (9, 10).

[1] *Abbreviation used in this paper*: RER, rough endoplasmic reticulum.

Relatively little research has been devoted to the cytochemical characteristics of the human mononuclear phagocyte system in vivo after the blood monocyte stage. However, the cultivation of normal bone marrow cells in a liquid medium in a Marbrook in vitro diffusion chamber has recently been achieved, and provides a convenient means for studying the differentiation of this cell line in short-term cultures (11, 12). This system is advantageous because cells can be cultivated in the absence of exogenous stimulatory substances and are easily retrieved for analysis of function, cytogenetics, and cytochemistry. Using this method of culture, Golde and Cline (11) were able to identify the various maturational stages of normal human mononuclear phagocytes by morphological and cytochemical procedures using light microscopy, and also demonstrated that macrophages maturing in vitro possess surface receptors for IgG and are capable of actively phagocytizing microorganisms (11, 12).

The purpose of the present study was to characterize these immature and mature human mononuclear phagocytes by electron microscopy combined with peroxidase and lysosomal enzyme localization. The previous investigations by Nichols and Bainton (9, 10), using normal human bone marrow and blood, afford a basis for comparing in vivo cellular differentiation with that observed in vitro after, 2, 7, or 14 days of culture. In this report, we describe the fine structural and enzymatic alterations which occur during the in vitro maturation of promonocytes to large macrophages and giant cells.

METHODS

Materials. Bone marrow was obtained from the posterior iliac crests of five healthy, adult volunteers.

The histochemical reagents, Grade I β-glycerophosphate and *p*-nitro-catechol sulfate, were obtained from Sigma Chemical Co., St. Louis, Mo., and 3,3′-diaminobenzidine tetrahydrochloride was supplied by either Sigma Chemical Co. or ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio. Glucose oxidase (A-grade) was purchased from Calbiochem, San Diego, Calif.

Collection of tissues. Techniques for obtaining suspensions of bone marrow cells and culturing them in a liquid medium have been described (11). Specimens were harvested at 2, 7, and 14 days.

Fixation. Cells processed for enzyme localization were fixed in 1.5% distilled glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) containing 1% sucrose at 4°C for 10–30 min.

Enzyme procedures. After fixation, cells were washed three times in sodium cacodylate-HCl buffer (pH 7.4) with 7% sucrose. They were then incubated at 22°C in each of the following enzyme media containing 5% sucrose: (*a*) Graham and Karnovsky's medium for peroxidase (13), pH 7.4, for 1 h (Figs. 1–3); (*b*) Tice's medium for peroxidase (14), using the glucose oxidase method for the generation of H_2O_2, for 2 h at 22°C (Figs. 4–8); (*c*) modified Gomori's medium for acid phosphatase (15), pH 5.0, for 90 min (Figs. 9 and 10A); and (*d*) Goldfischer's medium for arylsulfatase (16), pH 5.5, for 90 min (Fig. 10B), followed by treatment with 2% $(NH_4)_2S$ (17).

Subsequent processing. Enzyme preparations were postfixed for 1 h at 4°C in 1% OsO_4 in acetate-Veronal (Winthrop Laboratories, Evanston, Ill.) buffer, pH 7.6, with or without staining en bloc in uranyl acetate for 60 min, and then processed and examined as described (8, 9).

RESULTS

The number and differential counts of normal bone marrow cells grown in a liquid medium have already been reported (11). In brief, at 2 days (and up to 4 or 5 days), the predominant cell line is the neutrophilic granulocyte; thereafter, the proportion of macrophages progressively increases until by 7–14 days, they are preponderant. These macrophages stain intensely for α-naphthyl butyrate esterase, but peroxidase activity has been noted to decrease with maturation (12).

Localization of peroxidase in 2-, 7-, and 14-day cultures

Promonocytes. The promonocytes (Fig. 1) can be identified without difficulty at 2 and 7 days, but were rare by 14 days (Table I). These immature cells appeared similar to those observed in freshly obtained normal human bone marrow (8–10), except that the plasma membrane finger-like extensions were not seen in vivo. Peroxidase was being synthesized during the initial stages of maturation, since reactivity was visible as a flocculent density in all cisternae of the RER, all Golgi cisternae, and in both immature and mature cytoplasmic granules (≅300 nm).

Monocytes. The monocyte differed from the promonocyte in several aspects: (*a*) The nucleus became more indented and often horseshoe shaped, and the chromatin was moderately condensed. (*b*) Peroxidase reaction product disappeared from the RER and Golgi cisternae, indicating that synthesis of the enzyme had ceased (Table II). (*c*) Two populations of granules were usually present, one peroxidase-positive and the other peroxidase-negative. These cultured monocytes (Fig. 2) differed from typical blood monocytes in that they contained fewer peroxidase-negative granules. In addition, secondary lysosomes filled with peroxidase reaction product were observed (arrow, Fig. 2), reflecting partial degranulation of the peroxidase-positive storage granules. In other monocytes (Fig. 3), we commonly encountered heterophagy of cells such as neutrophils and eosinophils.

Macrophages with peroxidase-positive granules. By 7 days in culture, the more typical features of macrophages emerged as follows: (*a*) there was a marked increase in cellular size which was mainly attributable to the augmented cytoplasm; (*b*) an increase in the number of mitochondria, expansion of

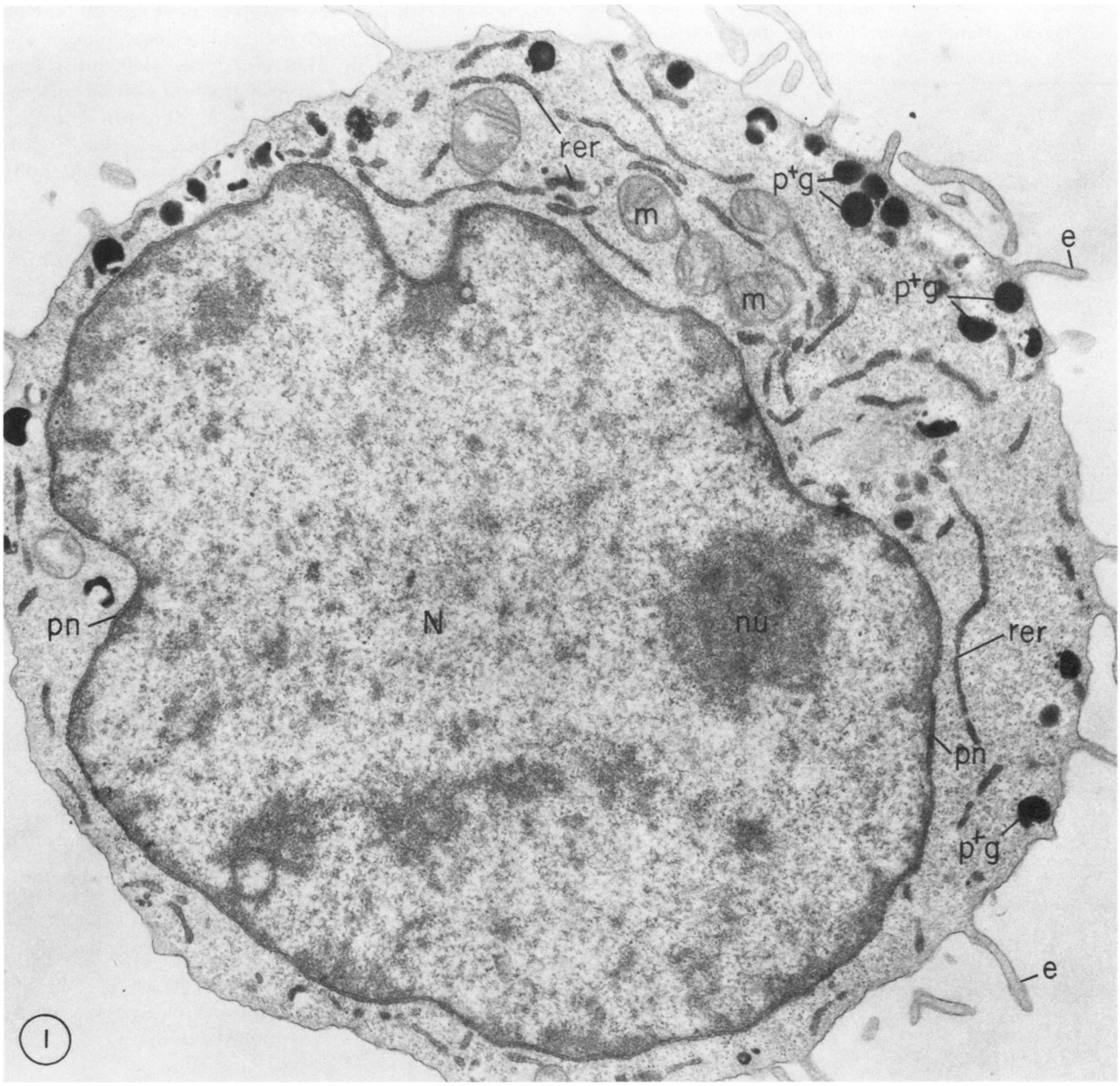

FIGURE 1 Promonocyte from a 2-day culture reacted for peroxidase, which can be seen as a fluocculent density in the RER (rer), perinuclear cisterna (pn), and all granules (p^+g). The large nucleus (N) contains a large nucleolus (nu) and heterochromatin is sparse. A few mitochondria (m) are visible, and several cytoplasmic extensions (e) extrude from the cell surface. (×14,000.)

the RER, an enlarged Golgi region with numerous vesicles, and microfilaments (100 Å) were also apparent (Fig. 4); (*c*) characteristically, the plasma membrane had many narrow extensions. The only peroxidase-positive organelles were the granules formed earlier, during the promonocyte stage, and secondary lysosomes. Although they were less prominent at 14 days, macrophages with peroxidase-containing granules persisted and sometimes developed into enormous cells with a host of storage granules. Some contained relatively few digestive vacuoles, whereas, in others, these secondary lysosomes were plentiful (Fig. 4, inset).

Macrophages lacking peroxidase—single macrophages. By 14 days, the dominant cell type was the large macrophage without endogenous peroxidase (Fig. 5 [Figs. 5–8 illustrate macrophages from 7- and 14-day cultures lacking peroxidase.]). Whereas some

TABLE I
Stages of Mononuclear Phagocyte Differentiation in Liquid Culture at Various Times

	Days in culture		
	2	7	14
Promonocyte	++	+	rare
Monocyte	++	+	rare
Macrophage			
(*a*) Peroxidase-positive	rare	++	+
(*b*) Peroxidase-negative	rare	+	++
Epithelioid cells	0	+	++
Giant cells	0	0	+

macrophages were free in the liquid culture (Fig. 5), many formed clumps (Fig. 10B) or had become adherent to the membrane (Fig. 10A).

Epithelioid cells. These tightly grouped cells had developed elaborate cytoplasmic projections (Fig. 6A), similar to those described in immature epithelioid cells (7, 18). Occasionally, the interdigitating processes of two adjacent macrophages had made intimate contact, and a modification of the two plasma membranes and cell surfaces was evident (Figs. 6B and 7). This contact resembled the septate-like zone of adhesion described in other cell types (19), but it has not been observed before between normal macrophages. In addition to clusters of lipid-filled macrophages with indistinct cell membranes (Fig. 8), we encountered conspicuous multinucleated giant cells (not illustrated).

Localization of lysosomal enzymes in 7- and 14-day cultures

Marrow from two subjects was cultured for 7 and 14 days, and the cells in liquid suspension were aspirated, fixed, and processed separately from the cells either adherent to or loosely settled upon the surface of the membrane. Acid phosphatase distribution followed two different patterns: in both suspended and adherent cells, we located ≅25% that contained reaction product within the entire RER and in digestive vacuoles (Fig. 9 [Figs. 9 and 10 depict 7- and 14-day cultured bone marrow cells which have been reacted for lysosomal enzymes.]). These cells were not ordinarily in close association with other macrophages.

TABLE II
Ultrastructural Characteristics of Various Stages of Mononuclear Phagocyte Differentiation in Culture

Cell type	Cell size	Peroxidase localization				Nuclear/ cytoplasmic ratio	Nucleus
		RER	Golgi complex	Granules	Secondary lysosomes		
	μm						
Promonocyte (Fig. 1)	10–15	+	+	+	–	<1	(*a*) Round; indentations (*b*) Minimally condensed chromatin; large nucleoli
Monocyte (Figs. 2 and 3)	8–10	–	–	+	+	1 to >1	(*a*) Variable shape (*b*) Heterochromatic, occasional nucleoli
Macrophage							
(*a*) Peroxidase-positive (Fig. 4)	10–20	–	–	+	+	>1	(*a*) Usually eccentric; oval or indented (*b*) Chromatin variable; may be very condensed or euchromatic
(*b*) Peroxidase-negative (Fig. 5)	10–20	–	–	–	–	>1	(*a*) Eccentric (*b*) Euchromatic, with large nucleoli
Epithelioid cell							
(*a*) Immature (Fig. 6)	15–20	–	–	–	–	>1	(*a*) Eccentric; indented
(*b*) Mature (Fig. 8)	15–20	–	–	–	–	>1	(*b*) Euchromatic, with nucleoli
Giant cell	>25	–	–	–	–	>1	(*a*) Multiple; randomly dispersed (*b*) Euchromatic, with large nucleoli

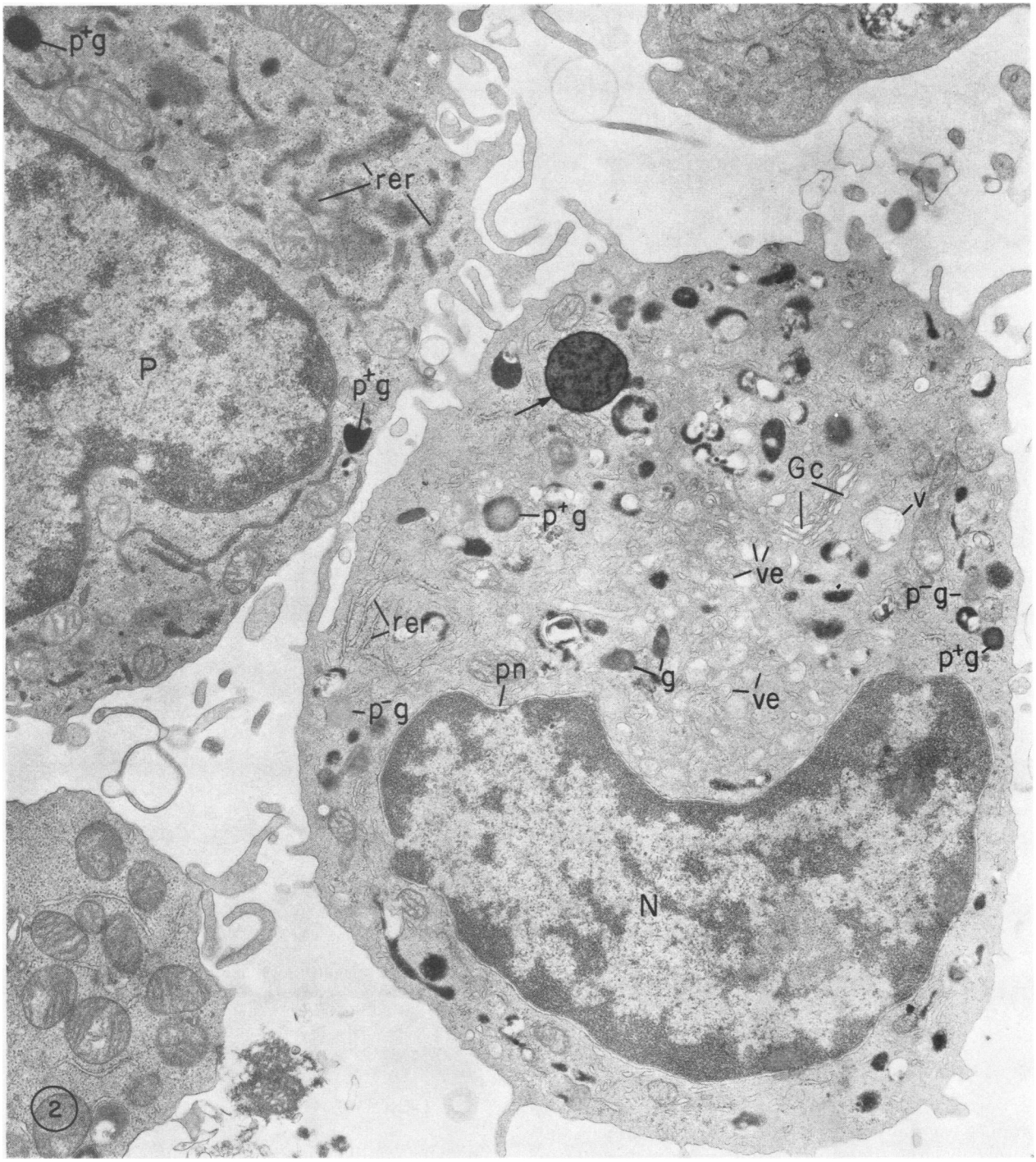

FIGURE 2 Monocyte from a 7-day culture. Peroxidase is present only in cytoplasmic granules (p^+g) and in one large vacuole (arrow)—presumably a secondary lysosome—in which degranulation has occurred. The many other vacuoles (v) and vesicles (ve) contain no peroxidase. Two peroxidase-negative granules can also be discerned (p^-g). In the upper left-hand corner, note the portion of a promonocyte (P) with peroxidase in the RER (rer) and granules (p^+g). (×15,000.)

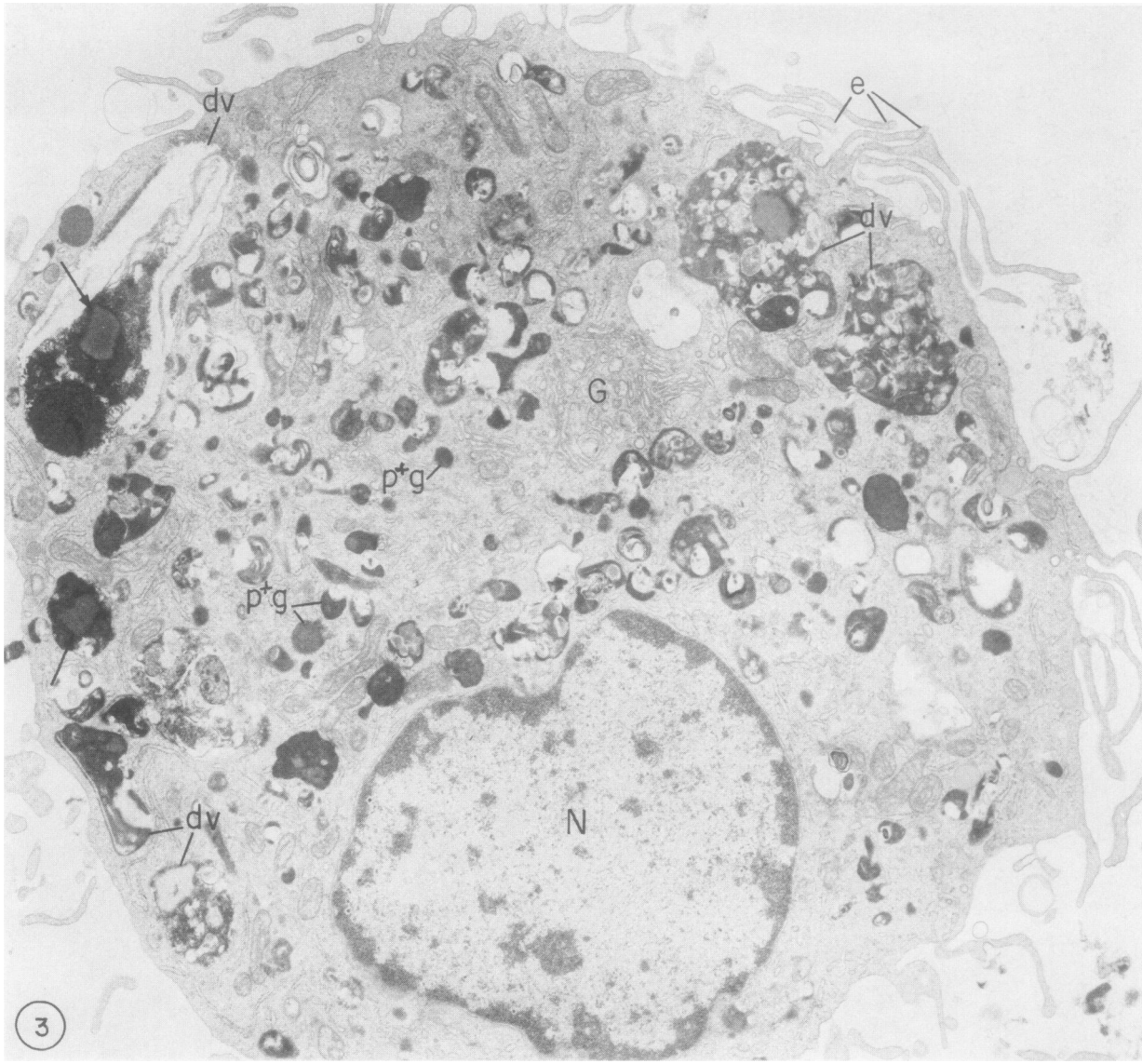

FIGURE 3 Monocyte (or early macrophage) from a 7-day culture, demonstration heterophagy of other leukocytes. Note the many digestive vacuoles (dv) or secondary lysosomes filled with cellular debris. Obvious eosinophil crystals (arrows) can be identified within several of these vacuoles. Some smaller peroxidase-positive granules (p^+g) are still present. Cytoplasmic extensions (e) on this cell outnumber those of the more typical monocyte depicted in Fig. 2. (×13,000.)

In the second pattern of distribution, weak reaction product was only seen in some Golgi cisternae, and mainly in large digestive vacuoles. As is apparent in Fig. 10A, some of these macrophages adhered to the dialysis membrane in compact masses. Cells found in clumps in the liquid suspension medium also followed this latter distribution. Reaction product for arylsulfatase was less extensive than that for acid phosphatase in that it was rarely seen in the RER or Golgi complex. Rather, it was observed most often in small vesicles and large secondary lysosomes (Fig. 10B).

DISCUSSION

Monocytes, unlike neutrophilic leukocytes, are not fully differentiated when released from the bone marrow. After circulating in the blood they enter the tissues, where they mature into large, long-lived macrophages (1–3). The maturation of rodent macrophages has been extensively investigated both in vitro and in vivo (22–25). Cohn and his co-workers (26–28) have shown that small, immature macrophages develop into very large phagocytes with sizable nuclei, plenti-

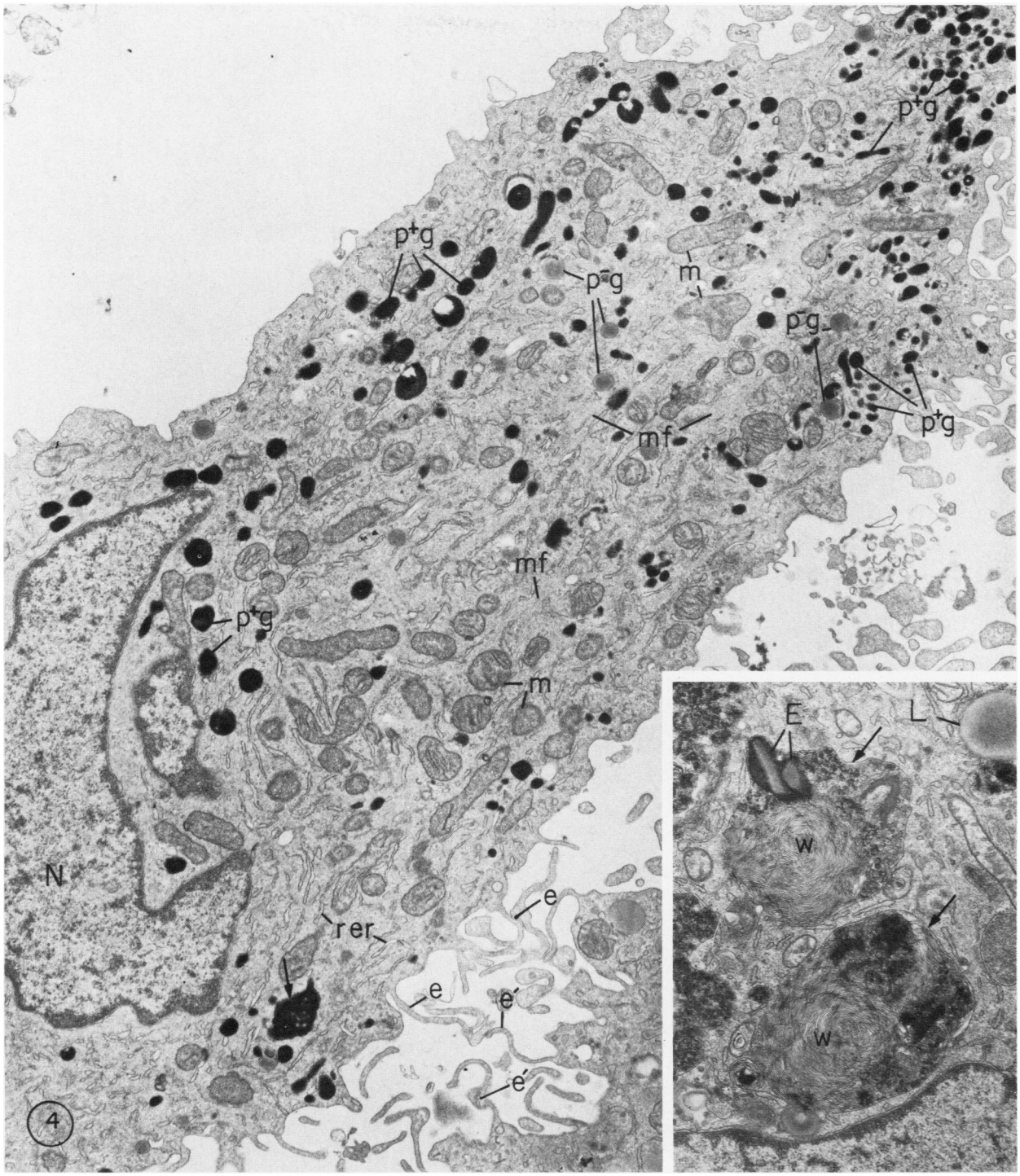

FIGURE 4 Large macrophage from a 14-day culture containing many peroxidase-positive granules (p^+g). m, mitochondrion; mf, microfilament; rer, scattered RER. A large peroxidase-positive inclusion is designated by an arrow. Note the slender cytoplasmic extensions (é) of an adjacent macrophage which partially interdigitate with the extensions (e) of the other cell. The inset shows two secondary lysosomes (arrows) from another macrophage, which contains whorls of membrane (w), two eosinophil granules (E), and other debris. A lipid droplet is also present. (×12,000; inset ×14,000.)

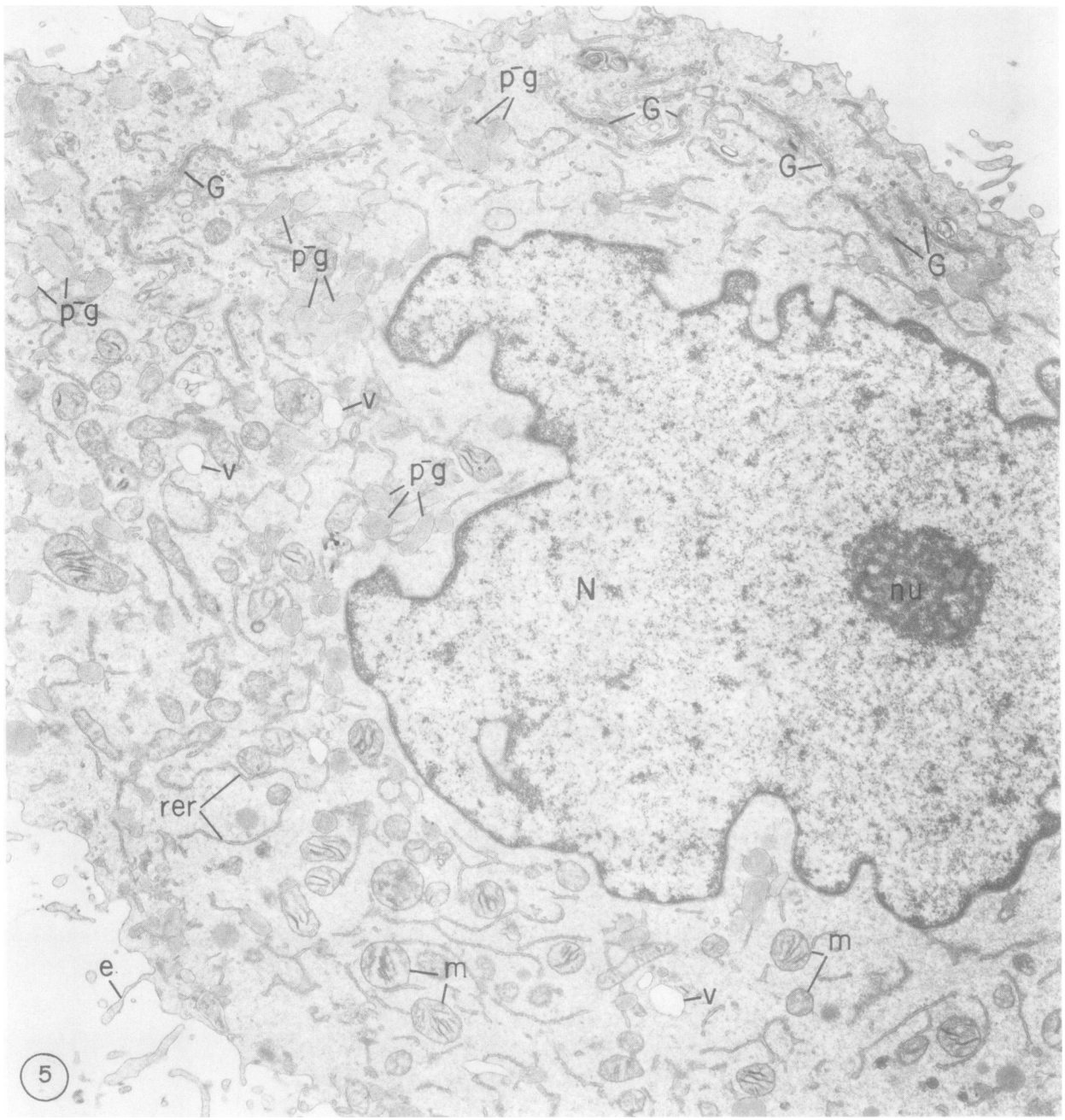

FIGURE 5 Peroxidase-negative macrophage from a 14-day culture. Although this type of macrophage is also observed at 7 days, it is the predominant type in the 14-day culture. It has an eccentric nucleus (N) with a distinct nucleolus (nu) and cytoplasm filled with many of the organelles described before—i.e. mitochondria (m); RER (rer); a large Golgi complex (G) is near the plasma membrane; and numerous small vesicles (≅600 Å). Note that no peroxidase reaction product can be detected at this late stage of maturation. In addition to occasional clear vacuoles (v), many inclusions with moderately dense matrices are evident. Their content is unknown; we are presently designating them as peroxidase-negative granules (p⁻g). Also note the paucity of 1,000 Å vesicles, which are usually present in great numbers in peritoneal macrophages fixed in vivo (8). (×19,000.)

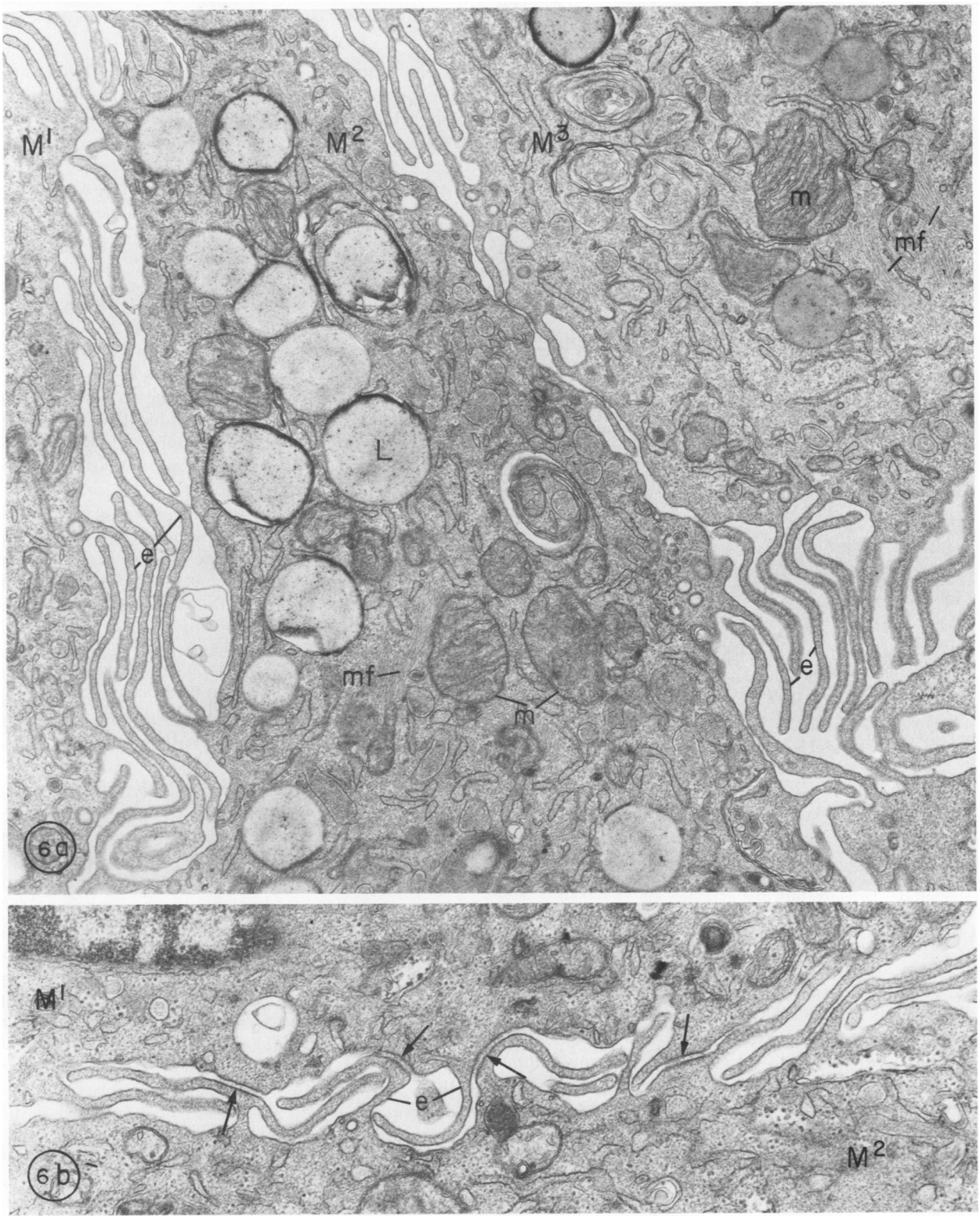

FIGURE 6 (A and B). High magnifications of adjacent macrophages, presumably epithelioid cells, cultured for 14 days and reacted for peroxidase, illustrating the numerous finger-like extensions and cell contacts. (A) Depicts portions of cytoplasm from three macrophages (M^1, M^2, and M^3); and (B) cytoplasm from two macrophages (M^1 and M^2). At four sites (arrows), the plasma membranes of the two cells in (B) are in close contact and manifest a structural modification which is better seen in Fig. 7—a higher magnification of two adjacent membranes. These macrophages represent early or immature epithelioid cells, containing many lipid droplets (L) which nonspecifically bind diaminobenzidine, thus accounting for their dark rims. Multiple mitochondria (m) and 100 Å microfilaments (mf) are present. ((A) ×21,000; (B) ×28,500.)

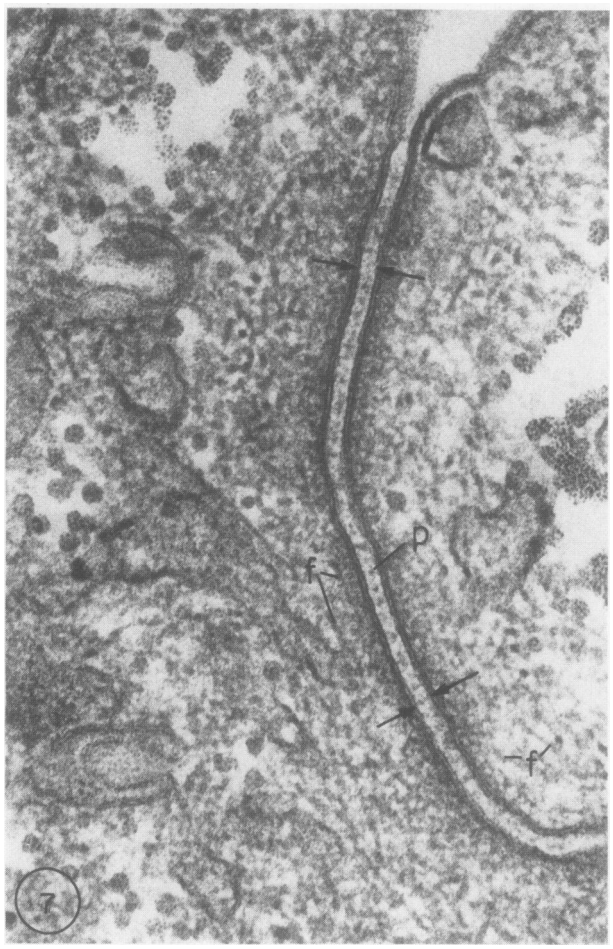

FIGURE 7 A septate-like zone of adhesion with characteristic spacing between two macrophages. The space between the two plasma membranes (arrows) measures more than 200 Å and contains irregularly spaced extracellular particles (p). The intracellular face exhibits fine filaments (f). (×100,000.)

ful cytoplasm, numerous Golgi complexes, lysosomes, stacks of smooth and rough endoplasmic reticulum, and many mitochondria. These structural evolutionary changes are associated with increases in the cellular content of protein, RNA, oxidative enzymes, and lysosomal hydrolases. More limited electron microscopic and cyto-chemical studies have been conducted with human mononuclear phagocytes obtained from bone marrow (29, 30) and blood (31, 32), with soft agar (29, 31, 32) or methylcellulose (30).

In this study, we used a liquid culture system and electron microscopy and cytochemistry to facilitate the detailed characterization of cellular organelles and enzyme contents of human bone marrow-derived mononuclear phagocytes. The peroxidase technique is useful for identifying promonocytes, because they are reactive for this enzyme and thus, can be distinguished from peroxidase-negative cells such as erythroblasts, lymphoblasts, and plasma cells. Although the granulocytic promyelocytes (neutrophilic, eosinophilic, and basophilic) are also peroxidase positive, their storage granules differ sufficiently in fine structure to make them distinguishable from promonocytes (9, 33, 34). Hence, with van Furth and Fedorko (22), we support the conclusion that mononuclear phagocytes do not develop from immature granulocytes but represent a separate line of differentiation. In this in vitro system, we demonstrated that peroxidase is synthesized early, in the promonocyte stage, and packaged into storage granules by the same pathway (RER → Golgi complex via vesicles → granules) which has been defined for other secretory proteins (35). Monocytes did not synthesize the enzyme but contained it in their storage granules.[2] With the endocytosis of dying cells, these granules degranulated; and, within 7 to 14 days, the cells became peroxidase-negative macrophages.

Phagocytosis seems to be a major cause of the loss of storage granules in the liquid culture system. However, some cells retain such peroxidase-positive granules in great quantities, even after 14 days of in vitro culture. Nevertheless, the vast number of macrophages are peroxidase-negative by 14 days, and epithelioid and giant cells, also all negative for peroxidase, are frequently observed. The lipid-filled clusters of epithelioid cells seen in Fig. 8 may correspond to the "giant fat" cells recently described by Dexter et al. (37) in the adherent cells cultivated from mouse bone marrow.

Macrophages tended to form clusters, with extensive interdigitation between cells. Such groups have previously been noted in animals (5, 7, 18, 38) and man (39–41) and referred to as "immature epithelioid cells" (7, 18). We found in this study that some of the cytoplasmic extensions had modified membrane contacts resembling septate junctions. This membrane modification of the mononuclear phagocyte was similar to that described earlier by Friend and Gilula (19) in the rat adrenal cortex and in other steroid hormone-secreting cells. Along this distinctive cell contact (19), the membranes of apposing cells are separated by 210–300 Å and bisected by irregularly spaced 100–150-Å extracellular particles. In freeze-fracture replicas, the cell membranes in the area of the septate contact is no different from nonjunctional areas of membrane (19). Somewhat similar membrane modifications have been described by Sanel and Serpick (42) in leukemic monocytes, by Daniel and Flandrin (43) in hairy cells undergoing

[2] During studies on blood monocytes in vitro, Bodel et al. (36) observed that peroxidase activity rapidly appeared in the RER and perinuclear cisterna within 2 h after monocyte adherence to a fibrin-coated surface but disappeared after the cells had been cultured for 1–2 days.

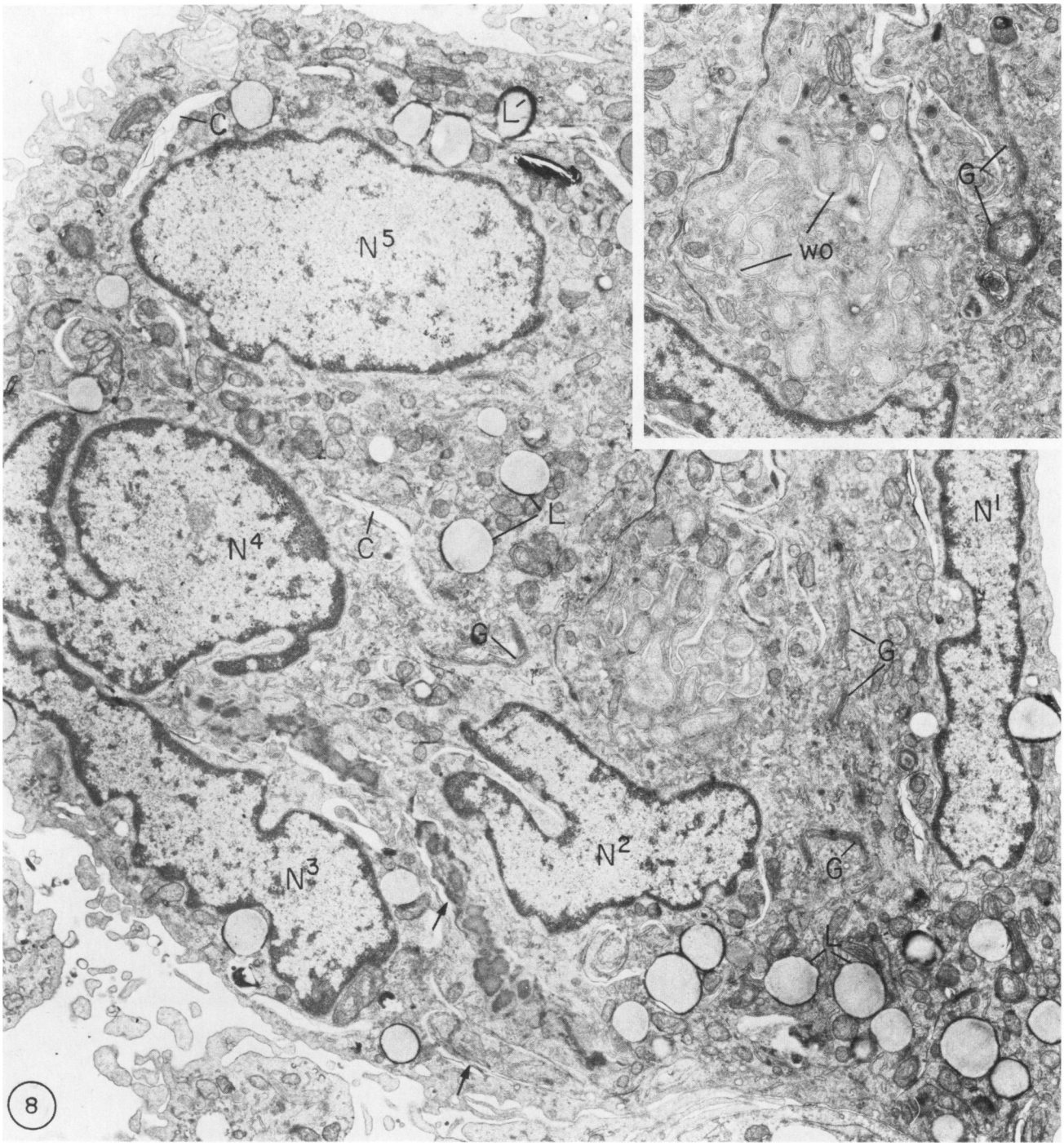

FIGURE 8 A group of large peroxidase-negative macrophages from a 14-day culture, with a view of the nuclei of five cells (N^1–N^5). It is difficult to distinguish the cell borders (arrows)—a situation which has been noted previously in regard to mature "epithelioid" macrophages (18). Numerous lipid droplets (L), some with very dense borders (L'), as well as mitochondria, many vesicles, clefts (C) (20), and several Golgi complexes (G) are present. Here, the inset reveals intracellular membranes, including a Golgi region (G), at a higher magnification. The worm-like structures (wo) have been demonstrated in guinea pig macrophages (21) and are thought to represent plasma membrane modifications. (×10,000; inset ×11,000.)

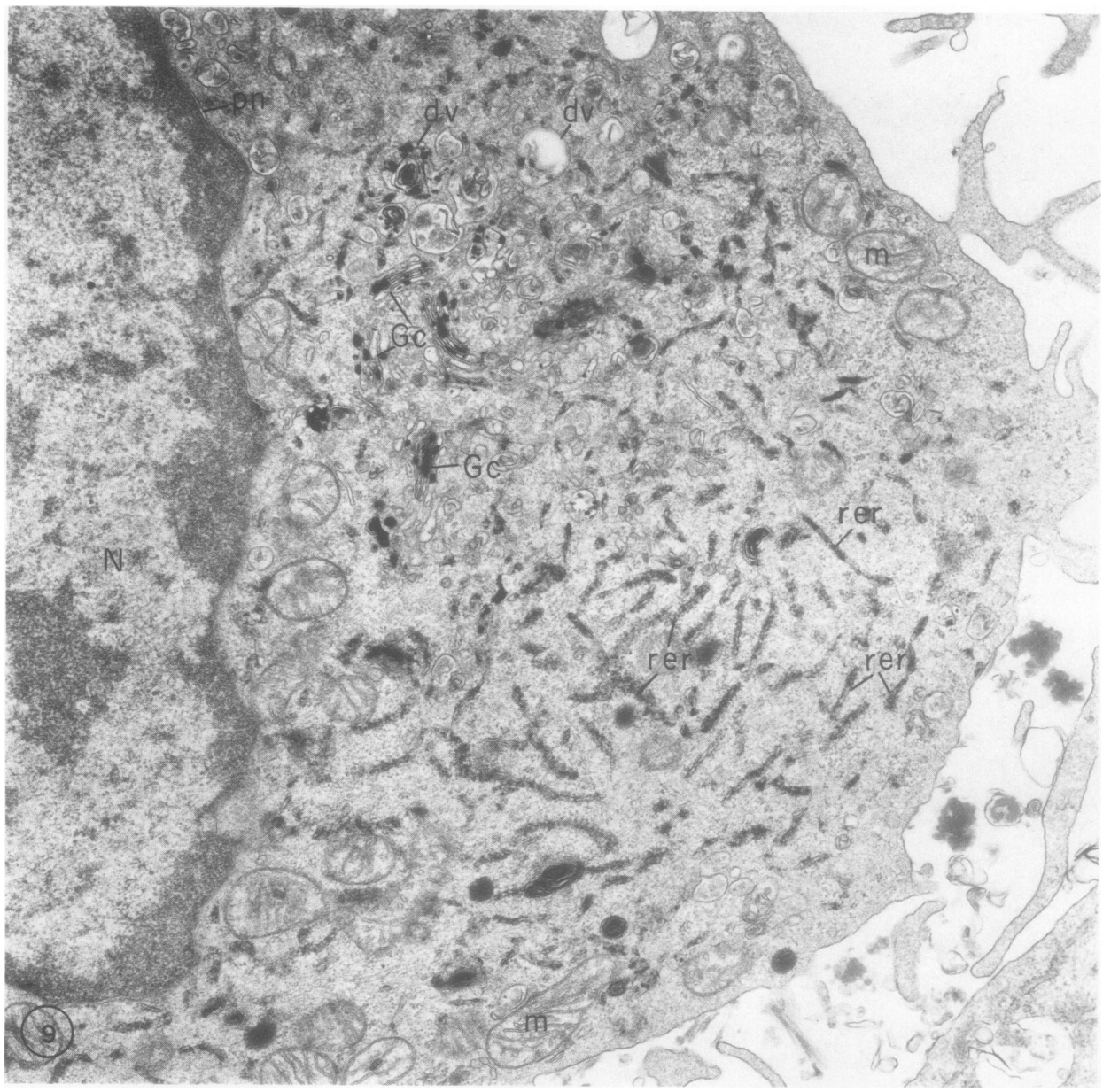

FIGURE 9 A macrophage growing in suspension in a 7-day culture and reacted for acid phosphatase. The entire RER (rer), excluding the perinuclear cisterna (pn), contains moderate amounts of lead phosphate. In addition, this lysosomal enzyme appears in most Golgi cisternae (Gc) as well as in some digestive vacuoles (dv). (×23,000.)

phagocytosis, and by Breton-Gorius et al. (44) in abnormal erythroblasts. To our knowledge, this is the first report of septate-like zones of attachment in normal macrophages, and we believe that they may serve as adhesive elements in these cells. "Close junctions" have been observed in cultured murine macrophages (45) and as so-called "junctional formations" in mouse-spleen cultures (46), but the morphology of these membrane contacts is quite dissimilar from that illustrated here. Moreover, this membrane modification differed considerably from the cell coats of adjacent macrophages depicted by Brederoo and Daems (21), who described a spacing of 630 Å, much thicker than our 300 Å contact.

Another finding of this research was the observation that adherent macrophages can pierce the dialysis membrane, as demonstrated in Fig. 10A. This may be an important discovery, because some investigators are designing experiments and interpreting data based on the

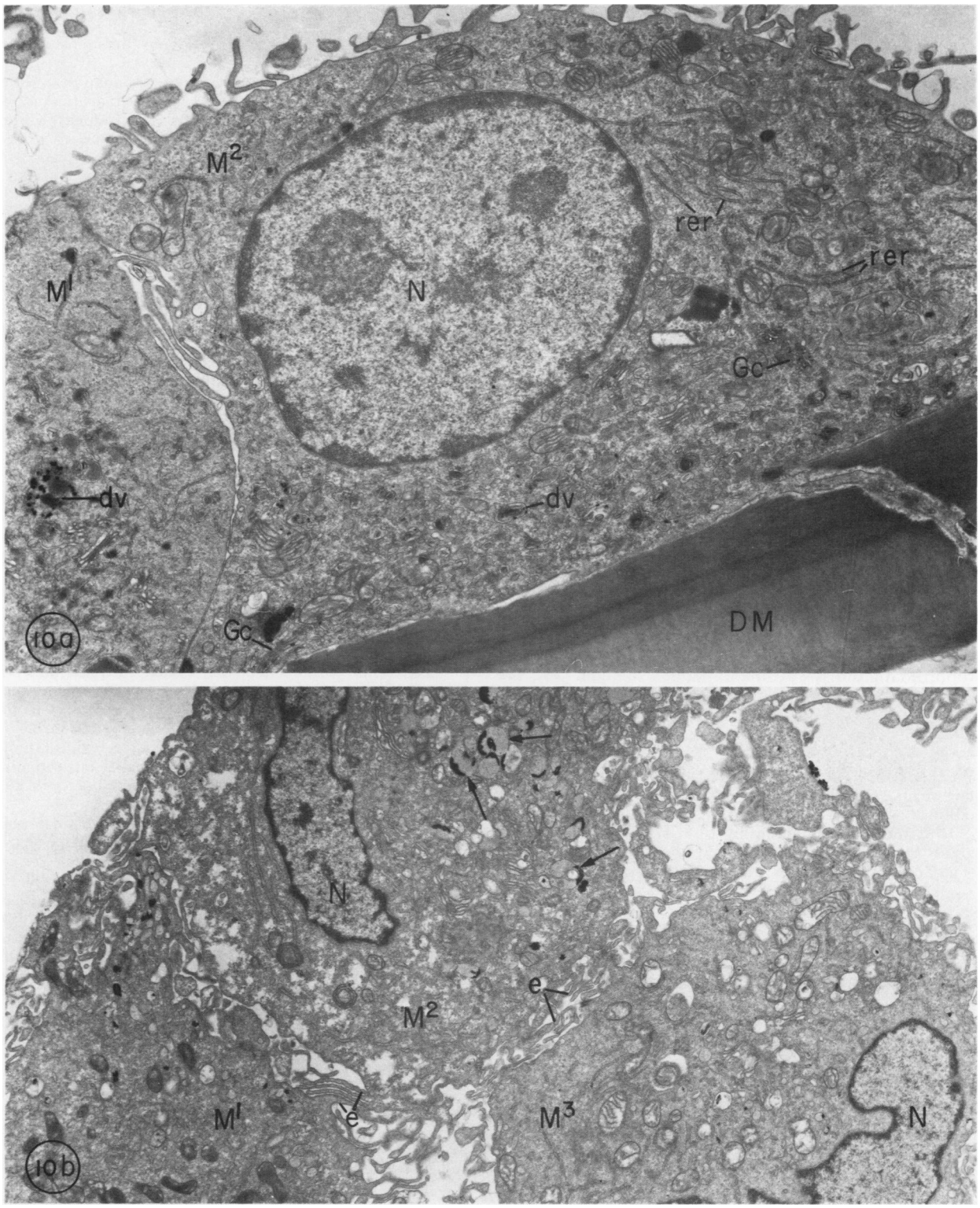

FIGURE 10 (A) Adherent macrophages (M^1–M^2) growing on the membrane in a 7-day culture and reacted for acid phosphatase. Only scant amounts of lead phosphate are demonstrable in some Golgi cisternae (Gc) and a few digestive vacuoles (dv). Note the close cell-to-cell contact. One process has actually pierced the dialysis membrane (DM) (right corner). N, nucleus; rer, RER; m, mitochondrion. B: Three closely adjoining macrophages (M^1–M^3) from the liquid medium of a 14-day culture which were reacted for another lysosomal enzyme—arylsulfatase. Only the cytoplasmic vacuoles (arrows) contain reaction product. Note the many plasma membrane extensions (e); such cells have been called immature epithelioid cells because of their dense packing and numerous extensions. N, nucleus. ([A] ×13,000; [B] ×9,500.)

assumption that only small dialyzable molecules can penetrate such membranes.

A further pertinent point is the unresolved question of why some macrophages, usually single cells, are actively synthesizing acid phosphatase, whereas macrophages in clusters seem to contain low levels of the enzyme. Perhaps the individual cells are better phagocytes, and, having ingested a "meal," are induced to synthesize lysosomal enzymes, as documented by Axline and Cohn (47). Alternatively, the phagocytic capacity of cellular aggregates may be less than that of relatively free macrophages. In this connection, Spector and Mariano (48) found that epithelioid cells phagocytize fewer latex beads and bacteria than do free macrophages.

In conclusion, it should be re-emphasized that these observations on the localization of peroxidase were derived from macrophages in culture. As far as we know, similar electron microscopic and cytochemical studies of human macrophages in various tissues have not been attempted. In animal experiments, certain tissue macrophages have been found to contain peroxidase, cytochemically identifiable in the RER and perinuclear cisterna. This enzyme reactivity in the RER is characteristic of resident peritoneal macrophages of the rat (49), guinea pig (50, 51), rabbit (36), and mouse (52), as well as Kupffer cells (53) and medullary lymph node macrophages (54) of the rat. Such reactivity has not yet been reported in the tissue macrophages of man (55), but this point should be further explored.

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