DIFFERENTIATION OF MONOCYTES

Origin, Nature, and Fate of Their Azurophil Granules

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ABSTRACT

The origin, content, and fate of azurophil granules of blood monocytes were investigated in several species (rabbit, guinea pig, human) by electron microscopy and cytochemistry. The life cycle of monocytes consists of maturation in bone marrow, transit in blood, and migration into tissues where they function as macrophages. Cells were examined from all three phases. It was found that: azurophil granules originate in the Golgi complex of the developing monocyte of bone marrow and blood, and ultimately fuse with phagosomes during phagocytosis upon arrival of monocytes in the tissues. They contain lysosomal enzymes in all species studied and peroxidase in the guinea pig and human. These enzymes are produced by the same pathway as other secretory products (i.e., they are segregated in the rough ER and packaged into granules in the Golgi complex). The findings demonstrate that the azurophil granules of monocytes are primary lysosomes or storage granules comparable to the azurophils of polymorphonuclear leukocytes and the specific granules of eosinophils. Macrophages from peritoneal exudates (72–96 hr after endotoxin injection) contain large quantities of lysosomal enzymes throughout the secretory apparatus (rough ER and Golgi complex), in digestive vacuoles, and in numerous coated vesicles; however, they lack forming or mature azurophil granules. Hence it appears that the monocyte produces two types of primary lysosomes during different phases of its life cycle—azurophil granules made by developing monocytes in bone marrow or blood, and coated vesicles made by macrophages in tissues and body cavities.

INTRODUCTION

The monocyte constitutes about 5% of the leukocytes of blood in most mammalian species and is differentiated from other white cells in Romanovsky-stained smears by its large size, kidney-shaped nucleus, and abundant pale blue cytoplasm with scattered small azurophil granules.

Recently it has been established by radioautography (1–3) and by chromosome marker techniques (4) that blood monocytes of rats and mice originate from a rapidly proliferating pool of precursor cells in the bone marrow. After a developmental period of 1–3 days (2, 3), they circulate briefly (~1½ days) in the blood (2) and subsequently migrate into the tissues to assume their ultimate role as macrophages (1, 2, 4). Although the morphologic and functional properties of tissue macrophages have been extensively studied (5, 6), less attention has been paid to earlier stages in the life cycle of this cell line, i.e., the developing monocytes in the bone marrow and blood. In particular,

little is known about the differentiation of monocytes or the nature of their azurophil granules.

The objective of the present investigation was to analyze by morphologic and cytochemical methods the stages in differentiation of monocytes—from marrow to blood to tissue—in an attempt to establish the origin, composition and fate of their azurophil granules.[1]

MATERIALS AND METHODS

Materials

Bone marrow and blood were examined from New Zealand albino rabbits, hematologically normal humans, and guinea pigs. In addition, peritoneal exudates from rabbits and guinea pigs were studied. *Escherichia coli* K12, used in the experiments on phagocytosis, was grown overnight in Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.) and washed three times in saline before use.

The following cytochemical substrates were obtained from Sigma Chemical Co., St. Louis, Mo.: grade I β-glycerophosphate, *p*-nitrocatechol sulfate, and 3,3′-diaminobenzidine (DAB) tetrahydrochloride. Endotoxin (lipopolysaccharide β, *S. typhosa*) was obtained from Difco Laboratories, Inc., Detroit, Mich.; *E. coli* K12 was obtained from American Type Culture Collection, Rockville, Md., pentobarbital (Beuthanasia Special) from H. C. Burns Pharmaceuticals, Oakland, Calif., and Desicote (used to coat all glassware) from Beckman Instruments, Inc., Fullerton, Calif.

Methods

PRODUCTION OF EXUDATES: Animals were given either a single injection or multiple intraperitoneal injections (at weekly intervals) of endotoxin in saline. The dosages used were 1 μg and 0.5 μg for the rabbit and guinea pig, respectively. Exudate was collected at 4 or 96 hr after endotoxin injection.

EXPERIMENTS ON PHAGOCYTOSIS: 2 ml of *E. coli* K12 suspension containing approximately 5×10^{10} bacteria/ml saline were injected into the peritoneal cavities of animals given endotoxin 4–20 hr previously. Exudate cells were collected and fixed 15 and 30 min after the injection.

COLLECTION OF TISSUES: Methods for collecting and handling bone marrow cells in suspension were described previously (8). Blood was collected from rabbits by cardiac puncture and from humans from the antecubital vein using plastic syringes containing heparin (20 units/ml blood); white cells were concentrated either by the buffy coat disc technique (9) or in buffy coat preparatory tubes (10). For collection of exudates, animals were given lethal intracardial injections of pentobarbital, the fixative was introduced immediately into the open peritoneum, and the exudate was collected with wide-bore pipettes.

FIXATION: Cells: were fixed either in (*a*) 1.5% distilled glutaraldehyde in 0.1 M sodium cacodylate–HCl buffer (pH 7.4) with 1% sucrose, at 4° and 25°C for 10 min–16 hr; or (*b*) the same fixative plus 2% acrolein, overnight; or, (*c*) 1% formaldehyde–3% glutaraldehyde with $CaCl_2$ (11), at 25°C for 10 min–4 hr. The suspension of fixed cells was either processed directly for morphologic studies, or washed three times in cacodylate-HCl buffer with 7% sucrose and incubated in the enzyme media described below. For cytochemical staining, the best results were obtained with fixation procedure (*a*) above. For morphologic studies procedures (*b*) and (*c*) were preferable and gave comparable results.

ENZYME PROCEDURES: Cells were incubated in the following media containing 5% sucrose: (*a*) modified Gomori's medium (12), pH 5.0, for acid phosphatase; (*b*) Goldfischer's medium (13), pH 5.5, for aryl sulfatase, followed by treatment with 2% $(NH_4)_2S$ (14); (*c*) Graham's and Karnovsky's medium (15), pH 7.6, for peroxidase. Details concerning incubation conditions and controls of cytochemical tests, as well as subsequent processing and microscopy were given previously (8).

RESULTS

Background Information

The source of circulating monocytes has remained controversial until relatively recently when it was established beyond reasonable doubt (see references in Introduction) that monocytes originate from precursor cells in the bone marrow and ultimately migrate into the tissues to become macrophages. Hence it is now possible to study the life cycle of this cell line, beginning with the early stages of its differentiation in the bone marrow.

Investigation of monocyte precursors is rendered difficult for two reasons: (*a*) the sample of monocytes in the bone marrow is small, as they constitute only 1–2% of the nucleated cell population (16), and (*b*) identification of immature monocytes is difficult, since they are not easily distinguished from developing granulocytes in some species. To minimize the latter problem, we initially selected the rabbit as the experimental animal, since immature monocytes can be readily identified in this

[1] A preliminary report of the findings was published previously (7).

species because their cytoplasmic granules differ from those of developing granulocytes in size and in the organization of their content (17, 18). After working out the developmental sequence of monocytes in the rabbit, we carried out similar though more limited studies on man and the guinea pig. The basic features of granule formation proved to be similar in all species (except for some differences to be described in granule contents). Hence the observations pertain to all three species investigated unless otherwise indicated.

In beginning these studies, criteria for identifying monocytes and their cytoplasmic granules were established by examining circulating monocytes from the blood. By utilizing the granules as a marker, we were then able to identify the early developing stages of the monocytic cell line in the bone marrow. The developmental sequence of monocytes will be described in some detail, since up to now only incidental observations on immature monocytes have appeared in the literature (19–22).[2]

Morphologic Observations on Mononuclear Phagocytes

Bone Marrow and Blood

Developing monocytes seen in Wright's-stained smears have been classically grouped into three subdivisions (23) based primarily on nuclear characteristics. However, since intermediate stages are difficult to classify precisely from thin sections (as opposed to the whole cells present in smears), we have elected to classify monocytes into two groups—promonocytes and monocytes—the characteristics of which are given below.

PROMONOCYTE: The early promonocyte (Fig. 1) varies in over-all size (7–15 μ) and normally is found only in bone marrow. Besides its content of azurophil granules, its distinguishing features are its large oval or round nucleus with two to five nucleolar profiles and abundant cytoplasm which contains numerous free polysomes, together with a conspicuous Golgi complex but relatively few cisternae of rough endoplasmic reticulum (ER). In addition, many small granules, presumably immature azurophils, are seen in the Golgi region, and some of the Golgi cisternae have a finely granular, moderately dense content similar to that of the immature granules (Fig. 2). These latter observations constitute presumptive evidence (along with the cytochemical evidence to be presented) that the azurophil granules represent secretory granules whose contents are concentrated and packaged in the Golgi complex, as are secretory granules in other systems.

Promonocytes resemble erythrocyte precursors in their large complement of free polysomes but can be readily identified by the presence of secretory granules and an active Golgi complex. As already mentioned, monocytes can be easily distinguished from granulocytes on the basis of the size and appearance of their granules. In the rabbit, the immature granules of both eosinophilic (18) and neutrophilic (PMN) (17) progranulocytes are easily recognized, since they appear as characteristic dense-cored vacuoles with variable numbers of cores. The mature eosinophil granules and mature azurophil granules of neutrophilic leukocytes (which measure up to 1500 and 800 mμ, respectively) are much larger than monocyte granules.

Most of the more mature monocyte azurophil granules have a homogeneous dense content, are usually round or oval in shape, but sometimes are pleomorphic (see Fig. 7). In the three species studied, the granules are similar in morphology and size (varying from 100–500 mμ) (Figs. 2, 4, 6). After formaldehyde-glutaraldehyde fixation, however, an occasional granule in a rabbit monocyte shows a denser center with an internal whorled, lamellated structure resembling a fingerprint. Such internal structure was not seen in azurophil granules of other species or in monocytes from rabbit blood. Occasionally promonocytes are seen in mitosis in the bone marrow.

MONOCYTE: The more fully differentiated, i.e. more nearly mature, monocyte found in the bone marrow or blood (Fig. 7) differs from the promonocyte in that (*a*) its over-all size (9–11 μ) is smaller; (*b*) its nucleus is smaller and there is a decreased nuclear/cytoplasmic ratio; (*c*) there are fewer free polysomes; and (*d*) azurophil granules are more numerous. Up to 50 granules can be counted in a single cell section in the rabbit monocyte and up to 120 in the human.[3] Moreover, more

[2] Recently, however, some work has been published describing the morphologic, functional, and kinetic properties of promonocytes and monocytes isolated from mouse bone marrow (46, 47, 49, 50.)

[3] Counts carried out on blood monocytes indicate that an average of 24 granules is present per cell profile in the rabbit and 59 in the human.

of the granules are mature; their average size is larger and their content is more uniformly dense and homogeneous.

Monocytes varying in maturity are present in both bone marrow and blood, but the early promonocytes of the bone marrow such as that shown in Fig. 1 are not seen in the blood under normal conditions. Approximately half of the sections of monocytes from normal blood contained nucleoli. This observation is contrary to previous reports by other investigators using light microscope techniques (23), who failed to find nucleoli in circulating monocytes. It is of interest that the over-all appearance of the Golgi region (presence of condensing secretory material and numerous immature granules) suggests that granule formation continues in circulating cells. Digestive vacuoles are observed only rarely in monocytes of normal bone marrow or blood, indicating that in the normal animal little or no phagocytosis occurs in these sites.

Peritoneal Exudate

It is well known that induction of an inflammatory response in the peritoneum with agents such as endotoxin produces an exudate composed primarily of leukocytes derived from the blood. Initially (up to 4 hr), the population of cells in the exudate consists mostly (95–99%, in the rabbit) of neutrophilic leukocytes, with only a few (1–5%) mononuclear cells plus occasional lymphocytes and eosinophils. With increasing time, the exudate gradually changes in composition until (after 72–96 hr) it consists predominantly of mononuclear cells.

4 HR EXUDATE: The few mononuclear cells present in the exudate at 4 hr resemble the monocytes of the blood in their general morphology and content of azurophil granules. Immature granules are frequently seen in the Golgi region (Fig. 13), and images suggesting continuing granule production are sometimes present, as in the blood. In other words, the cells present in the 4 hr exudate which have recently migrated from the blood (2) appear little changed from their state in the circulation.

96 HR EXUDATE: The cells obtained after 96 hr of stimulation consist primarily of mononuclear phagocytes which appear quite different from blood monocytes (Figs. 8, 9, and 12). These cells, most of which have spent many hours in the peritoneum, differ from circulating monocytes as follows (*a*) they are larger (11–14 μ) (Fig. 9), (*b*) they lack azurophil granules, but (*c*) they contain large numbers of digestive vacuoles of varying size with a heterogeneous content (Fig. 8), (*d*) they contain increased amounts of rough ER and mitochondria (Figs. 8 and 9), and (*e*) their Golgi complexes are larger and contain increased numbers of small ($\sim$ 600 A) vesicles, especially coated vesicles (Fig. 8), but no forming granules. Moreover, coated vesicles are frequently observed in continuity with Golgi cisternae (Figs. 8 and 10). The absence of either immature or mature azurophil granules suggests that mononuclear cells are depleted of their granules soon after arrival in the peritoneum and that in the new environment no new azurophil granules are being formed.

Enzyme Localization in Mononuclear Phagocytes

Since cells of the mononuclear line ultimately function as phagocytes (during the macrophage portion of their life cycle), it seemed reasonable to assume that their granules, like the azurophil granules of neutrophilic leukocytes (8, 24), may be lysosomal in nature. To test this hypothesis, we carried out cytochemical tests for lysosomal enzymes. Since monocytes of some species are also known to contain peroxidase (25–27), we also carried out tests for peroxidase.

Bone Marrow and Blood

ARYL SULFATASE AND ACID PHOSPHATASE: Reaction product for both enzymes was observed in monocytes of the bone marrow and blood[4] in the following organelles (*a*) in some segments of the ER cisternae (Figs. 3 and 5), (*b*) in some cisternae of the Golgi complex and associated vesicles (Figs. 4 and 5, inset), (*c*) in immature granules (Figs. 4 and 5), and, (*d*) in the case of aryl sulfatase, in most of the mature granules (Fig. 5). As is the case in neutrophils (8) and eosinophils (18, 28) with the Gomori method, reaction product for acid phosphatase could not usually be visualized in mature granules (Fig. 4). There was some variation in the frequency and intensity of the ER reaction from one species to

[4] Observations on lysosomal enzymes in blood monocytes were limited to the rabbit. Work is in progress to determine the localization of these enzymes in monocytes from human and guinea pig blood.

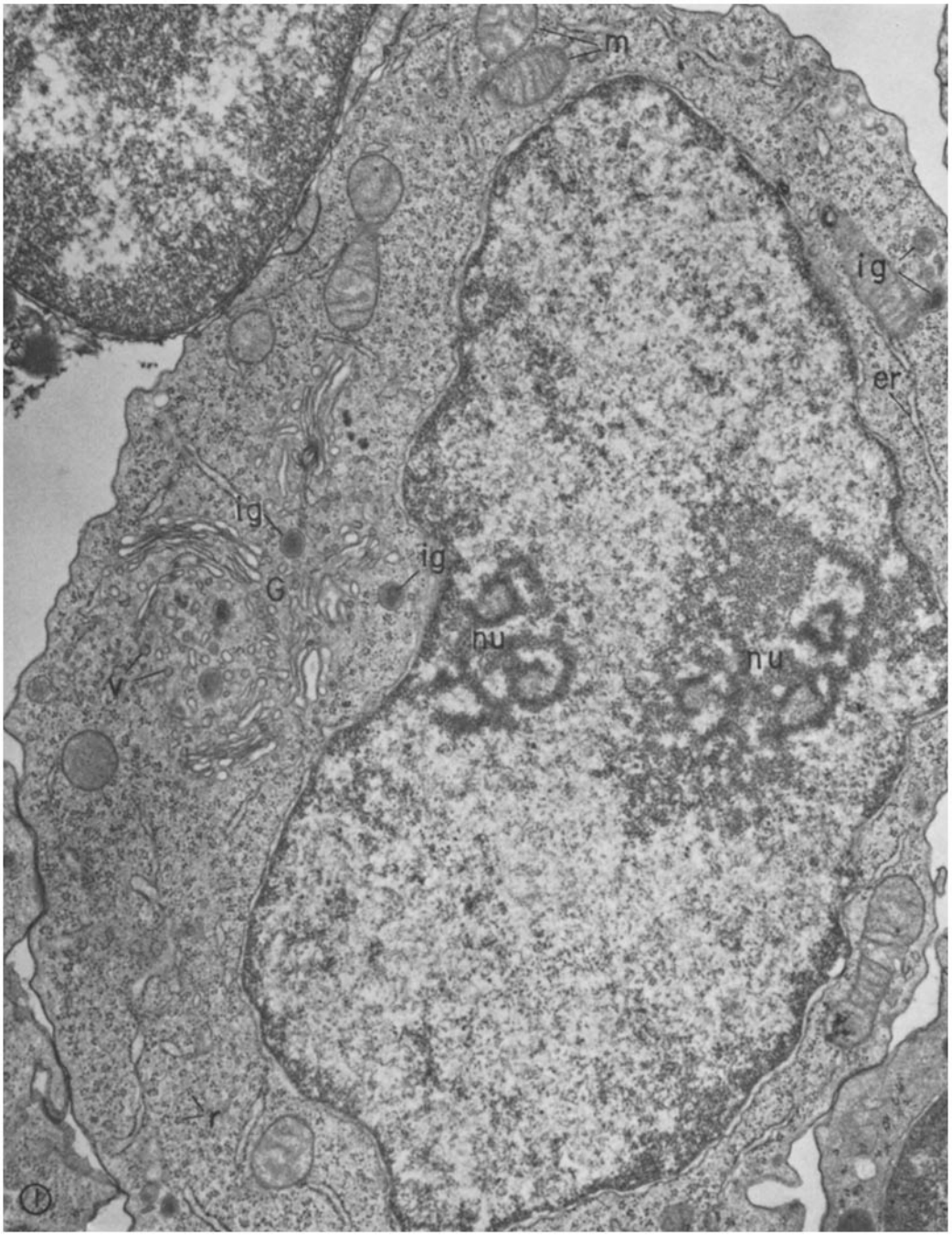

FIGURE 1 Early promonocyte from rabbit bone marrow. The large oval nucleus, which contains two large nucleoli (*nu*) and sparse heterochromatin, almost fills the cell. In the cytoplasm, there are numerous free polysomes (*r*), a few flattened cisternae of rough-surfaced ER (*er*), mitochondria (*m*), and a conspicuous Golgi complex (*G*). The latter consists of several stacks of four to six cisternae and small vesicles (*v*) with a content of low density. In the Golgi region there are several immature granules (*ig*), $\sim$ 150 mμ in diameter, characterized by their slightly irregular outline and a content that is typically denser in the center than at the periphery. No fully condensed or mature granules are present in this very young cell. The specimen was fixed in glutaraldehyde-acrolein overnight at 4°C. $\times$ 23,000.

another. With acid phosphatase, only a few ER cisternae contained reaction product in the rabbit, whereas in the human and guinea pig (Fig. 3), all cisternae, including that of the nuclear envelope, contained deposits. Reaction product for aryl sulfatase was seen in only occasional cisternae of the ER and Golgi complex in the rabbit and guinea pig (Fig. 5, inset), and was not seen in ER or Golgi components in man.

PEROXIDASE: Rabbit monocytes of bone marrow and blood were not reactive for peroxidase. In many promonocytes from human marrow, reaction product was seen in all cisternae of the rough ER, including the perinuclear cisterna (Fig. 6 and inset *a*), all cisternae of the Golgi complex (Fig. 6, inset *b*), and all immature and mature granules. In some monocytes from human marrow, the ER and Golgi complex were not reactive, in such cells, which appear to be more mature, some of the granules were strongly reactive, and others were negative. In monocytes from guinea pig bone marrow, reaction product was not found in the ER or Golgi complex. As in the cells of man, some of the granules were reactive and others were negative.

In the blood of both man and the guinea pig, reaction product was restricted to the granules, of which some were reactive and others were not. The actual and relative numbers of peroxidase-positive and peroxidase-negative granules varied considerably from cell to cell, but granule counts carried out on representative samples (25 cells) indicated that approximately one-half of the granules of human monocytes and about 1/3 to 1/4 of those of the guinea pig were reactive for this enzyme.

The fact that reaction product for acid phosphatase (all species) and aryl sulfatase (rabbit and guinea pig) and peroxidase (human) was found throughout the secretory apparatus (rough ER, Golgi cisternae, immature granules) during the period when monocyte azurophil granules are being formed indicates that these enzymes are probably synthesized by developing monocytes, segregated in the rough ER, and concentrated and packaged by the Golgi complex into azurophil granules. In the rabbit, the demonstration of reaction product for lysosomal enzymes in the ER and Golgi complex of circulating monocytes substantiates the morphologic observations indicating that synthesis and packaging of granule contents continue during the transit of monocytes in the blood. The presence of the lysosomal enzymes mentioned above in azurophil granules and the secretory apparatus together with the lack of evidence of endocytosis indicate that these granules are primary lysosomes.

Peritoneal Exudate

ARYL SULFATASE AND ACID PHOSPHATASE: The distribution and amount of reaction product found in mononuclear phagocytes from the early (4 hr) exudate were the same as in the

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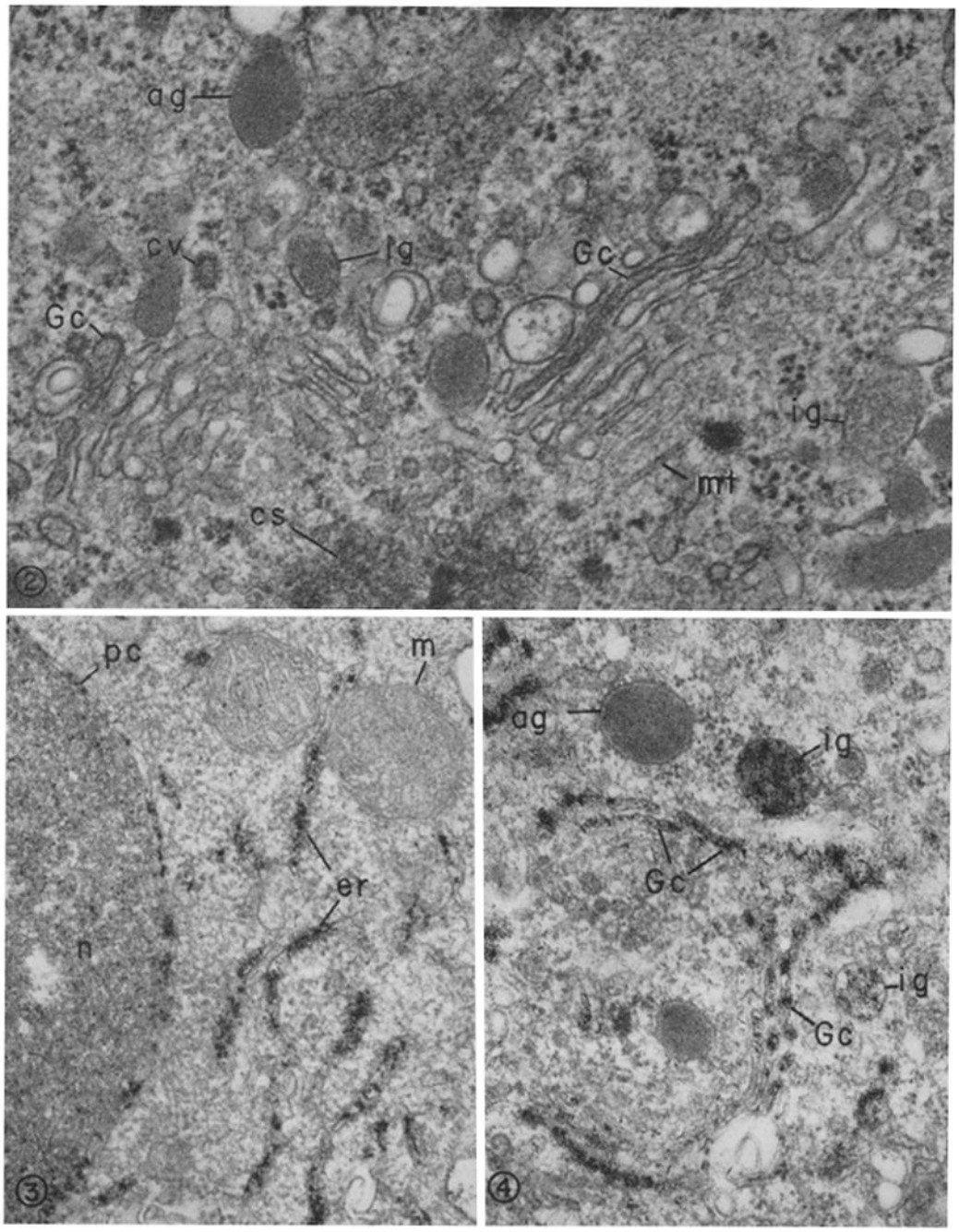
Key to symbols
G, Golgi complex
Gc, Golgi cisterna(e)
P, neutrophilic (PMN) leukocyte
ag, azurophil granule
b, bacterium
c, centriole
cs, centriolar satellite
cv, coated vesicle
dv, digestive vacuole
er, rough ER
f, filaments
ig, immature azurophil granule
m, mitochondrion
n, nucleus
mt, microtubule
nu, nucleolus
pc, perinuclear cisterna
pv, phagocytic vacuole
ps, pseudopodia
r, ribosome
v, vesicle

Figs. 1–7 are electron micrographs of developing monocytes in bone marrow, and Figs. 8–15 are macrophages from peritoneal exudate. After fixation as described in the figure legends, specimens were postfixed in OsO_4, and all except that shown in Fig. 7 were subsequently stained in block in buffered uranyl acetate. Sections examined for morphology were doubly stained in aqueous 5% uranyl acetate and alkaline lead enzyme preparations were lightly stained with lead citrate.



FIGURES 2–4 Fields from developing promonocytes in bone marrow, illustrating immature or forming azurophil granules. Fig. 2 shows that in the rabbit an occasional Golgi cisterna (*Gc*) contains homogeneous material of moderate density similar to the content of immature granules (*ig*). It is believed that as granule formation proceeds, small vesicles pinch off the Golgi cisternae and subsequently several coalesce and the content condenses to form the mature azurophil granule (*ag*). Note that several coated vesicles (*cv*) are also present in the Golgi region, and microtubules (*mt*) are observed converging on centriolar satellites (*cs*). Figs. 3 and 4 are preparations of developing promonocytes collected from the guinea pig and rabbit, respectively, and reacted for acid phosphatase. Reaction product is seen in the rough ER (*er*) and perinuclear cisterna (*pc*), and within the Golgi cisternae (*Gc*) and immature granules (*ig*). Note that all the ER cisternae and all the cisternae (three to four) in the Golgi stack contain reaction product. No reaction product is present in the mature granule (*ag*). The specimen in Fig. 2 was fixed in formaldehyde-glutaraldehyde for 4 hr at 23°C. Those in Figs. 3 and 4 were fixed for 1 hr at 4°C in glutaraldehyde and incubated for 2 hr in a modified Gomori medium (pH 5.0). Fig. 2, × 54,000, Fig. 3, × 46,000; Fig. 4, × 47,000.

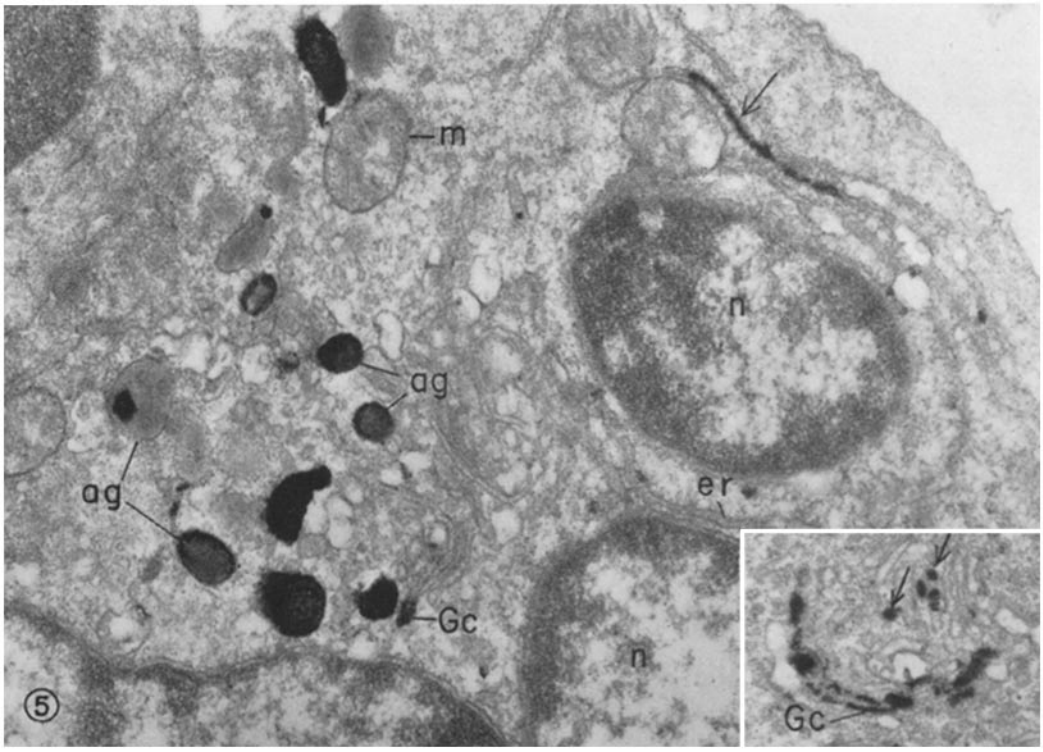


FIGURE 5 Promonocyte from rabbit bone marrow incubated for aryl sulfatase. Reaction product is present in one segment of a cisterna of the rough ER (arrow). The other ER cisternae (*er*) are not reactive. Most of the azurophil granules (*ag*) contain reaction product. A small amount of reaction product is seen at the tip of a Golgi cisterna (*Gc*). Note that in some granules the reaction product is uniformly distributed throughout, but in others it forms a peripheral rim or focal spot. The *inset* depicts the Golgi complex of a similar cell from guinea pig bone marrow. Reaction product is localized within two Golgi cisternae (*Gc*) and in adjacent vesicles (arrows). Specimens were fixed for 1 hr at 4°C in glutaraldehyde, incubated for 90 min (Fig. 5) or 4 hr (*inset*) in Goldfischer's aryl sulfatase medium (pH 5.5), and treated with $(NH_4)_2S$. Fig. 5, × 28,000; *inset*, × 28,000.

marrow and blood. In the macrophage of the 96 hr exudate, however, over-all reaction for both enzymes was noticeably greater in that (*a*) the ER and Golgi reaction in a given cell was more extensive (Figs. 9 and 12, inset), (*b*) digestive vacuoles were reactive in all cells (Figs. 9, 11 and 12), and (*c*) numerous small vesicles closely associated with the cisternae were reactive in many cells (Figs. 9, inset, and 10). Some of these vesicles were of the coated variety. Occasionally such a coated vesicle containing enzyme reaction product was found in continuity with one of the Golgi cisternae (Fig. 10), and vesicles of similar size were observed in continuity with digestive vacuoles (Fig. 11). These observations suggest that in macrophages, as in other systems (see reference 29), some of the coated vesicles represent primary lysosomes which serve to shuttle hydrolytic enzymes from Golgi complex to secondary lysosomes. The localization of enzymes was similar in exudates examined after single or multiple injections of endotoxin.

PEROXIDASE: The macrophages of the rabbit are not reactive for peroxidase. Those of the guinea pig and human were not examined for the presence of this enzyme.

Controls

ACID PHOSPHATASE: No reaction product was seen in monocytes obtained from any source (bone marrow, blood, or peritoneal exudate) and incubated without substrate.

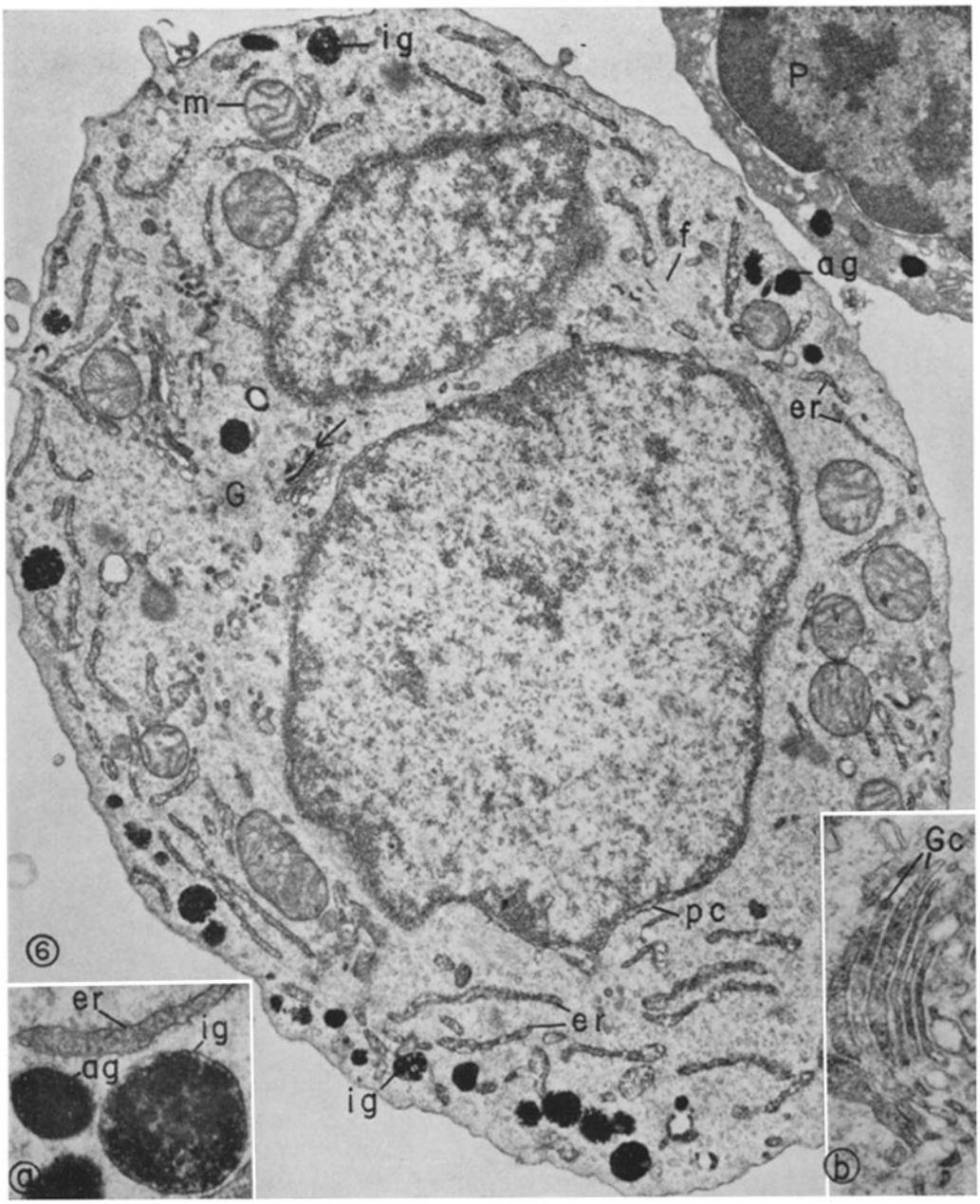


FIGURE 6 Promonocytes from human bone marrow reacted for peroxidase. Reaction product is distributed throughout the entire rough ER (*er*), including the perinuclear cisterna (*pc*), all Golgi cisternae (*Gc* and arrow), and all immature (*ig*) and mature granules (*ag*). Note that the reaction is more intense in the granules than in the ER and that one Golgi cisterna (arrow, Fig. 6) is more intensely reactive than the others, suggesting that a concentration gradient exists across the Golgi complex. In the human monocyte, clusters of fine filaments (*f*) are common. *Inset a* is a higher magnification view showing the dense globular reaction product at low concentration within the rough ER (*er*) and at higher concentration in immature (*ig*) and mature (*ag*) azurophil granules. In the immature granules, whose content is presumably still undergoing concentration, the aggregates of reaction product are less compact than in the mature granules. *Inset b* shows reaction product filling six or seven successive cisternae (*Gc*) of the Golgi complex of another cell. Specimens were fixed in glutaraldehyde for 10 min at 4°C and incubated for 1 hr in Graham and Karnovsky's medium at 23°C. Fig. 6, × 15,000; *Inset a*, × 47,000; *Inset b*, × 41,000.

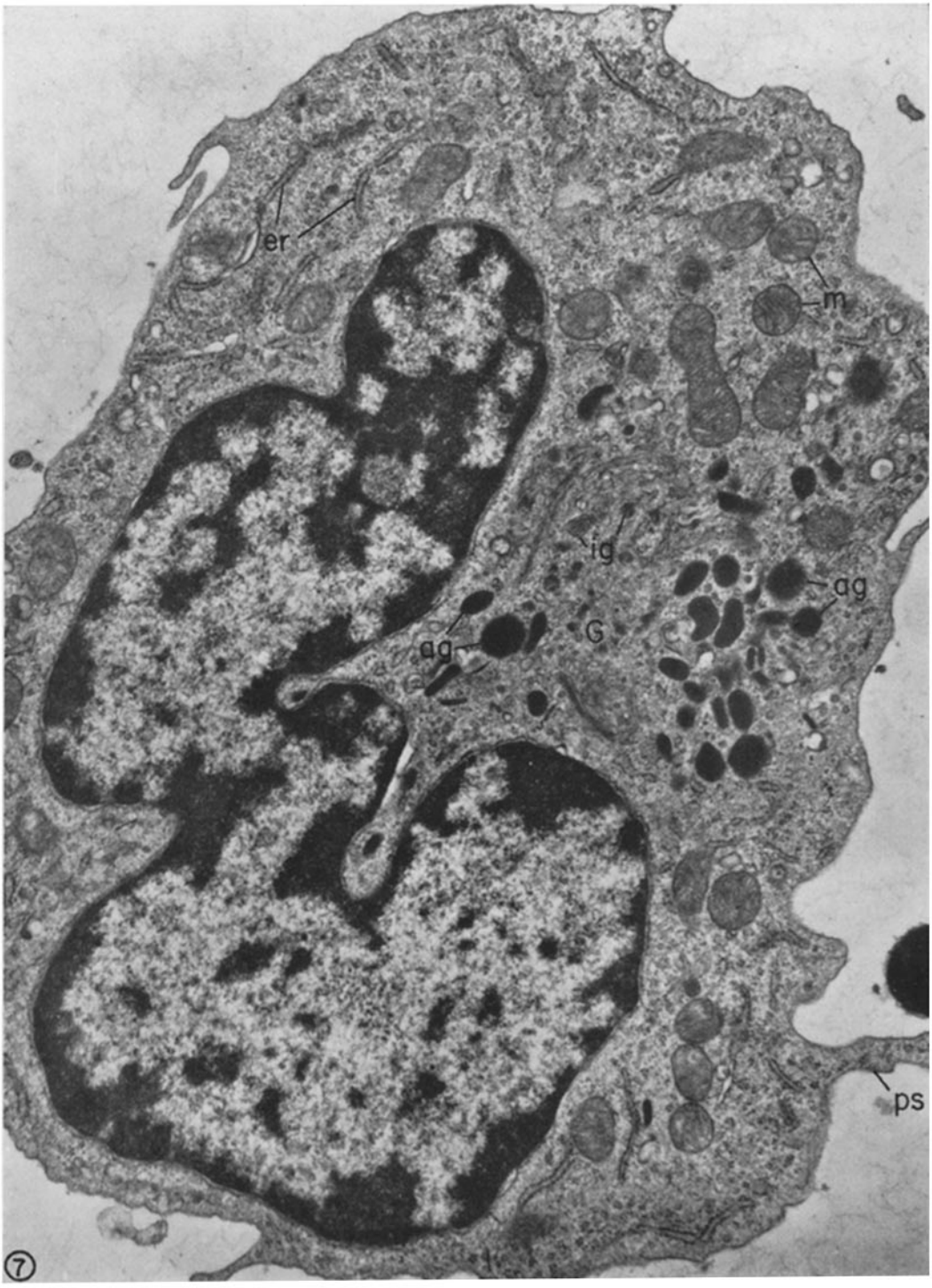


FIGURE 7 Mature monocyte from rabbit bone marrow, showing its characteristic features: the eccentric, kidney-shaped nucleus with moderately condensed chromatin, and the "rosette" of azurophil granules (*ag*) clustered near the Golgi complex (*G*) at the "hof" of the nucleus. About 30 mature, homogeneously dense azurophil granules (*ag*) which are variable in shape can be counted. In addition, numerous small immature granules (*ig*) are seen in the center of the Golgi region. A few ER cisternae (*er*) are present near the cell periphery, and scattered mitochondria (*m*) are seen. Note that a few pseudopodia (*ps*) extend from the surface. Specimen was fixed in glutaraldehyde at 4°C overnight. × 19,500.

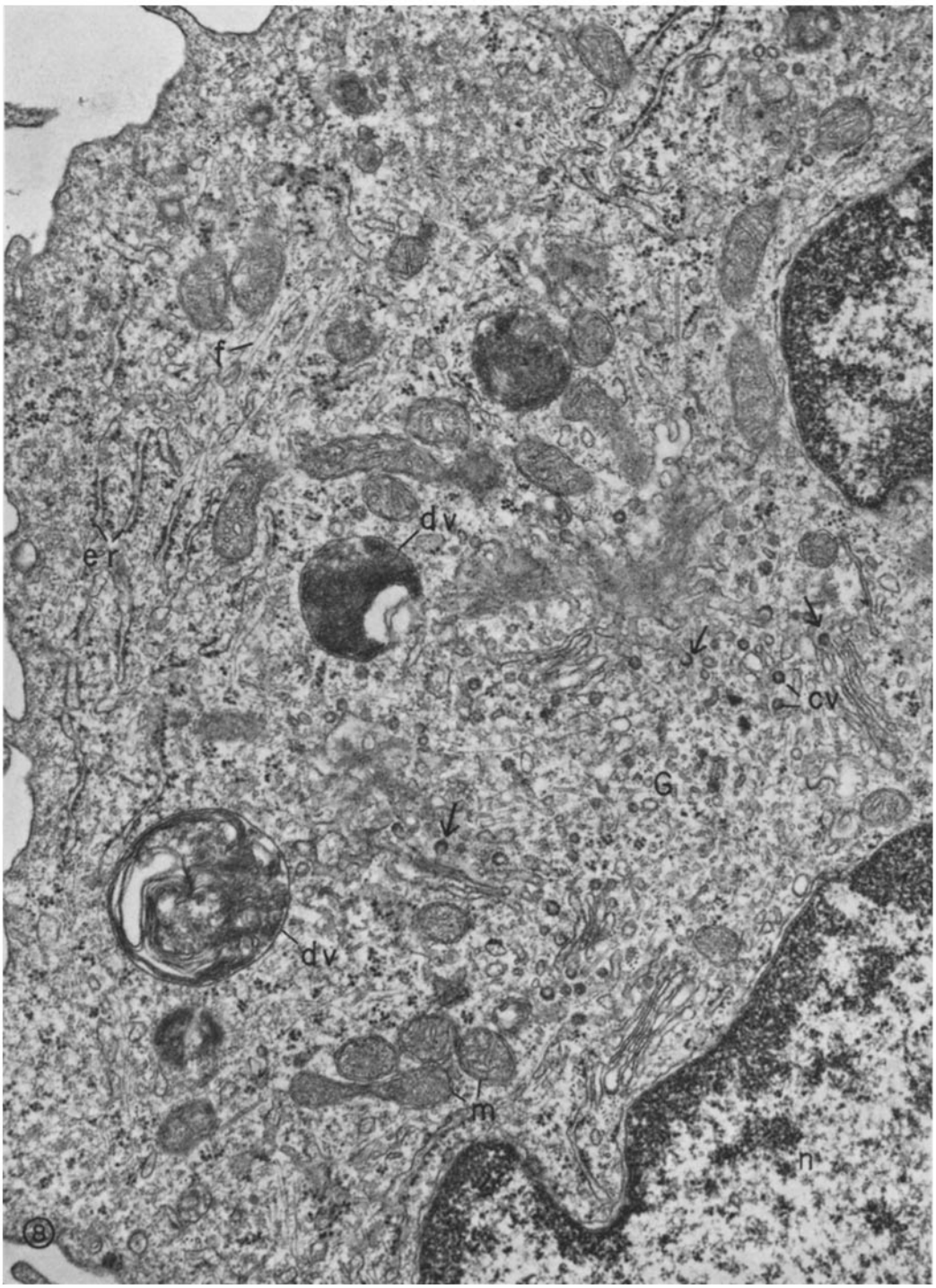


FIGURE 8 Portion of a macrophage (taken from a 96 hr peritoneal exudate produced in a rabbit) showing the Golgi region and surrounding cytoplasm. Numerous digestive vacuoles (*dv*) which vary in size and content are present but no azurophil granules are seen. The Golgi complex (*G*) is large and contains many vesicles, most of which are coated. Some of the coated vesicles (arrows) appear to be in direct continuity with Golgi cisternae. Rough ER (*er*) is moderately abundant, and mitochondria (*m*) are numerous. The cytoplasm is interlaced with fine (~100 A) filaments (*f*) which are sometimes oriented in bundles and sometimes scattered randomly. Specimen preparation was the same as for Fig. 7. × 23,000.

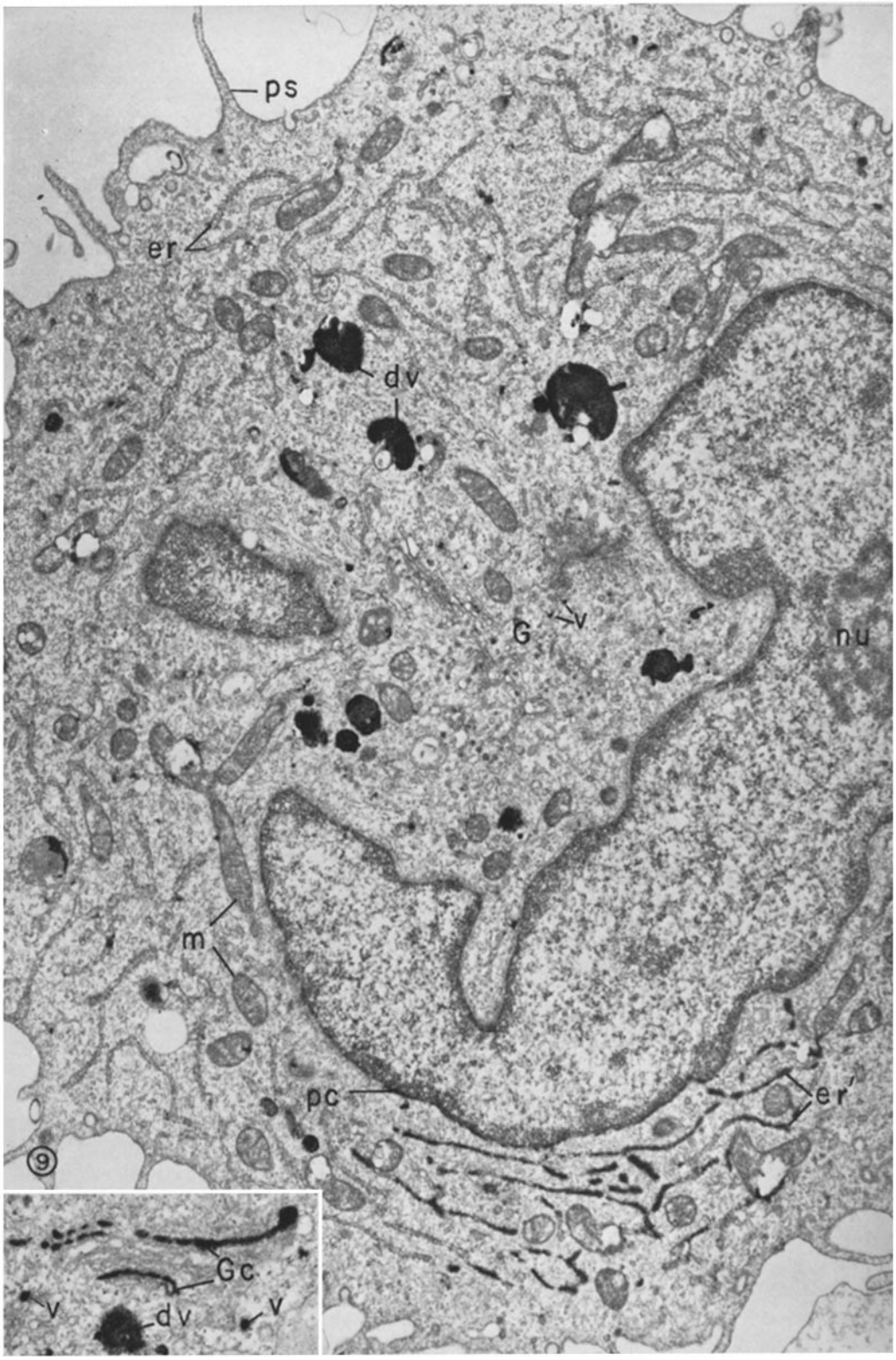
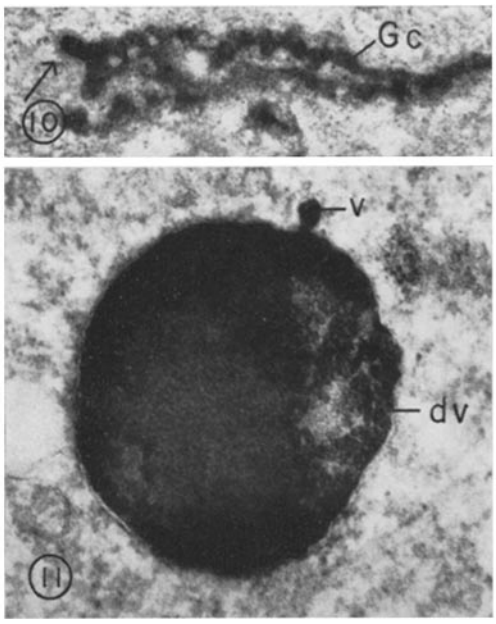


FIGURE 9 Macrophage from a 96 hr rabbit peritoneal exudate incubated for aryl sulfatase. The cell is characterized by its abundant cytoplasm, eccentric nucleus with nucleoli (*nu*), large Golgi region (*G*), numerous mitochondria (*m*), and moderately abundant ER (*er*). Lead sulfide reaction product is present in segments of the rough ER (*er'*) and perinuclear cisterna (*pc*). Reaction product is also found within numerous small vesicles (*v*) in the Golgi region (*G*) (seen to better advantage in the *inset*), and in digestive vacuoles (*dv*) of various sizes. Near the cell surface, note the presence of larger coated vesicles which do not contain reaction product and are probably pinocytic in origin (39). Numerous pseudopodia (*ps*), measuring ~ 50–70 mμ in width, extend from the cell surface. The *inset* depicts reaction product in several Golgi cisternae (*Gc*) and adjacent vesicles (*v*) of another cell. Specimen was prepared as for Fig. 5. Fig. 9, × 22,000; *inset*, × 37,000.



FIGURES 10 and 11 Small fields from macrophages obtained from a 96 hr rabbit peritoneal exudate reacted for aryl sulfatase. Fig. 10 shows fenestrated Golgi cisternae (*Gc*) containing lead sulfide reaction product. A coated vesicle is depicted in continuity with one of the Golgi cisternae (arrow). In Fig. 11, a vesicle (*v*) of similar size and content appears to be fusing with a digestive vacuole (*dv*). Specimen preparation was as for Fig. 5. Fig. 10, × 77,000; Fig. 11, × 93,000.

ARYL SULFATASE: In preparations incubated without substrate, no reaction product was seen over the cytoplasm of monocytes. However, a fine stippling of lead precipitate was often observed over the periphery of the nucleus in both experimental and control specimens (see references 14 and 18).

PEROXIDASE: Controls consisted of preparations from which H_2O_2 or DAB was omitted. No reaction product or enhanced density was observed in any of the controls.

PHAGOCYTOSIS EXPERIMENTS

The finding that azurophil granules were present in mononuclear phagocytes from blood and from early exudates (which represent cells recently mobilized from the blood) but were absent from those in late exudates suggested that mononuclear cells might degranulate during the initial phagocytic events that take place in the tissues. To test this hypothesis, cells of early exudates (4–20 hr) from guinea pigs and rabbits were challenged in vivo with *E. coli*, the exudate cells were collected and examined at selected intervals (15 and 30 min) thereafter, and phagocytosis was studied.

At 15 min after initiation of phagocytosis, the bacteria were surrounded by macrophage pseudopods and were present within phagocytic vacuoles (Fig. 13) in the cytoplasm of many cells. A single section of a macrophage was seen to contain as many as 20–25 bacteria. Occasionally images were encountered in which there was continuity between the azurophil granule membrane and that of the phagocytic vacuole (Figs. 13, inset, and 14), suggesting that these two organelles fuse, releasing the granule contents into the vacuole. Moreover, the results of cytochemical tests for lysosomal marker enzymes are in accord with this view, since reaction product was present in granules, in phagocytic vacuoles, and in the pockets created by the fusion of the two organelles (Figs. 14 and 15).

DISCUSSION

Origin and Nature of Monocyte Azurophil Granules

In this study, the monocyte has been examined in various phases of its life cycle, with attention focused primarily on the origin, content, and fate of its azurophil granules. Our morphological results indicate that azurophil granules are secretory granules, the formation of which begins in immature monocytes in the bone marrow and continues during subsequent monocyte maturation in the marrow and blood. However, no azurophil granules are formed by macrophages in the tissues after endocytosis begins. Our cytochemical results demonstrated that azurophils contain lysosomal enzymes (aryl sulfatase and acid phosphatase) which are segregated in the ER and packaged into granules in the Golgi complex, as are secretory products in many other cells (18, 30). We therefore conclude that azurophil granules of monocytes are primary lysosomes or storage granules similar to the azurophil granules of neutrophilic leukocytes (8, 24) and the specific granules of eosinophilic leukocytes (18) in that the granules contain stored hydrolytic enzymes but have not yet participated in digestion.

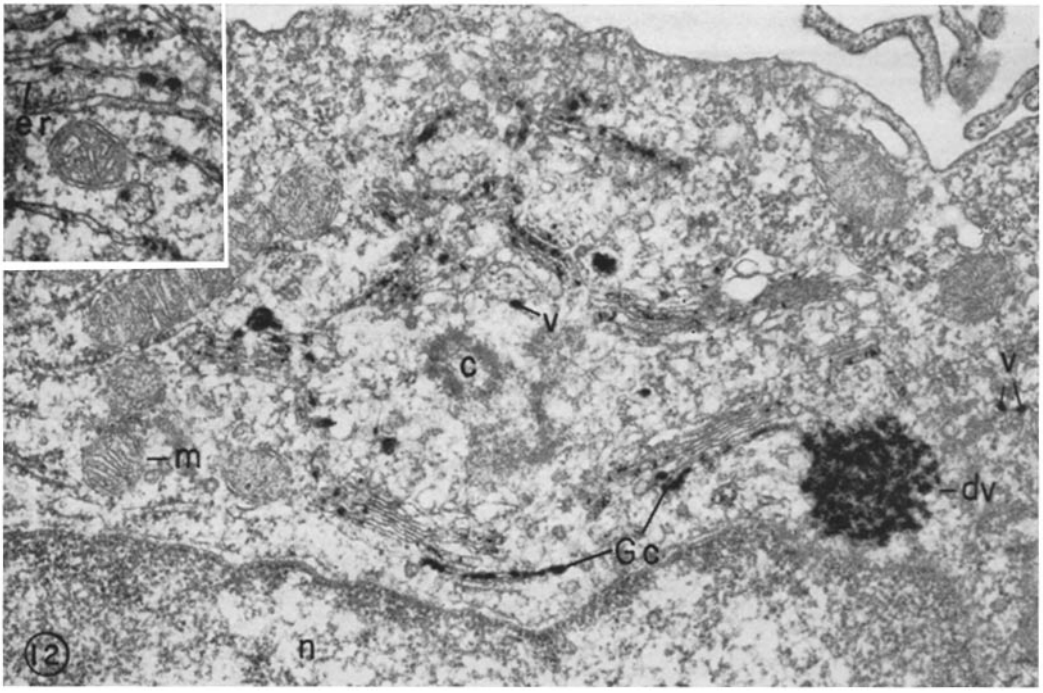


FIGURE 12 Golgi region of a macrophage in a 96 hr peritoneal exudate from the rabbit; specimen reacted for acid phosphatase. Enzyme reaction product is deposited in the majority of the cisternae of this large Golgi complex which is oriented around the centriole (*c*). Reaction product is also present in numerous small vesicles (*v*), in a large digestive vacuole (*dv*), and in segments of the rough ER (*er*) (see *inset*). Specimen preparation was the same as in Figs. 3 and 4. Fig. 12, × 39,000; *inset*, × 44,000.

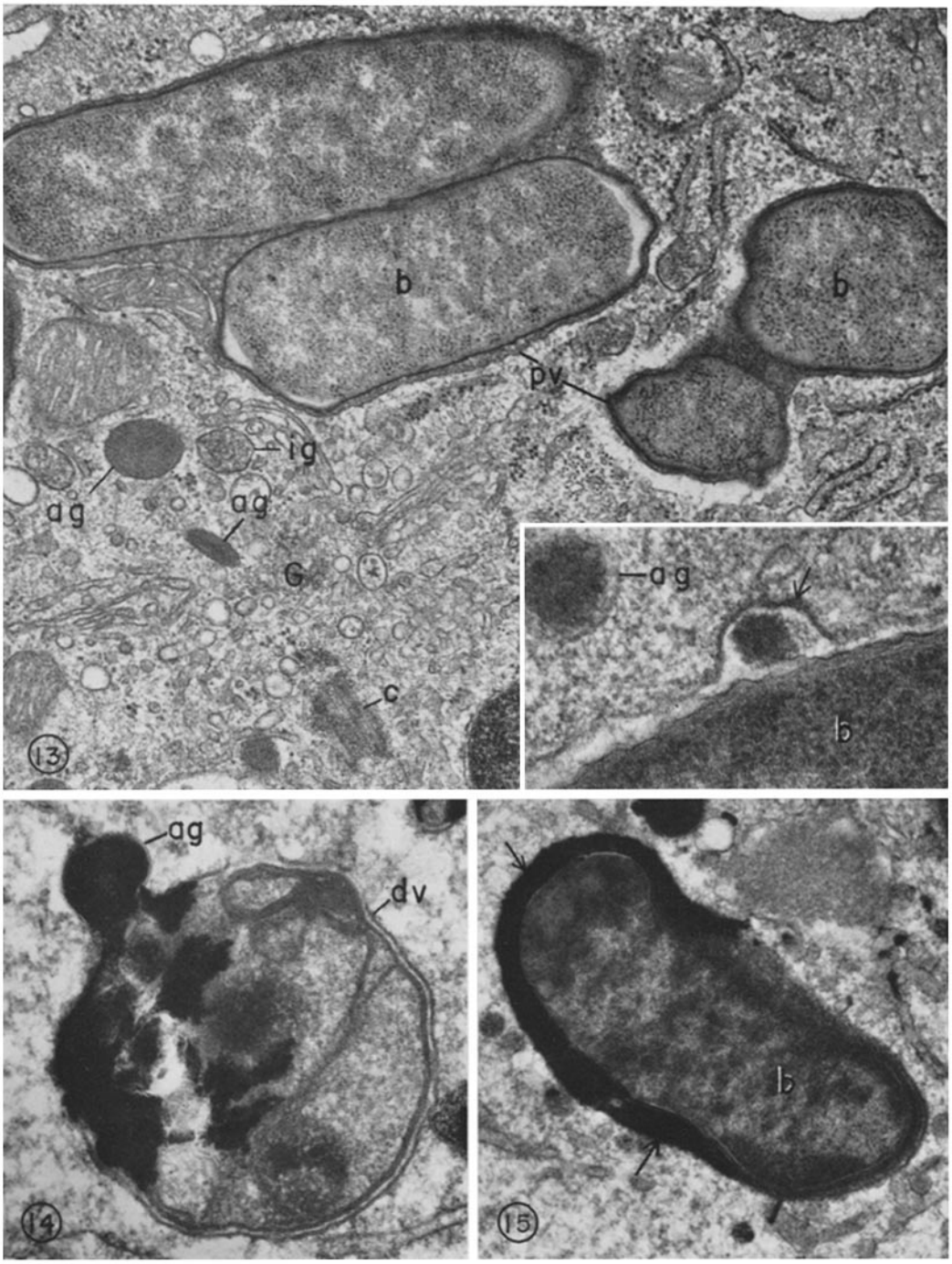
Fate of Azurophil Granules

Our observations on mononuclear phagocytes challenged with *E. coli* indicate that the fate of the monocyte azurophil granules—namely, fusion with phagocytic vacuoles—is similar to that of the aforementioned granules of neutrophils (31) and eosinophils (32). When *E. coli* is introduced into the peritoneal cavities of endotoxin-treated animals, mononuclear cells phagocytize the bacteria and degranulation takes place within minutes: the granule contents are emptied into adjacent phagosomes following fusion of the corresponding membranes, thereby releasing digestive enzymes into the vacuole.

Other Constituents of Azurophil Granules

Lysosomal enzymes were found in azurophil granules in all species examined in this study. Some variations in azurophil granule content apparently occur from species to species insofar as peroxidase is concerned, in that some azurophil granules of human and guinea pig monocytes contain peroxidase (as previously reported by other investigators (25–27), whereas those of the rabbit do not. Results obtained by histochemical methods for light microscopy (see reference 33) indicate that a number of enzymes are present in blood monocytes (e.g. a variety of esterases, dehydrogenases, and hydrolytic enzymes). However, the granules have not yet been isolated as a distinct fraction and analyzed biochemically, so that except for lysosomal enzymes (all species) and peroxidase (human and guinea pig)[5] demonstrated in this work, their composition remains unknown. In this regard, it should be noted that a "granule fraction" was prepared by Cohn and Wiener (34) from rabbit BCG-induced alveolar macrophages and found to contain numerous hydrolytic enzymes and lysozyme. However, the fact that the starting

[5] Recent findings by van Furth et al. (49) suggest that the azurophil granules of mouse monocytes also contain peroxidase.



FIGURES 13–15 These fields, from cells exposed in vivo to *E. coli*, illustrate the events that occur during phagocytosis of microorganisms by macrophages. Fig. 13 (and *inset*) are from a 4 hr exudate produced in the guinea pig and fixed 15 min after intraperitoneal injection of *E. coli*. A number of bacteria (*b*) have been taken up and segregated into phagocytic vacuoles (*pv*). Two mature azurophil granules (*ag*) as well as several immature granules (*ig*) are present in the Golgi region (*G*). The *inset* depicts a field in which fusion has occurred between an azurophil granule and a phagosome containing a bacterium (*b*). The content of the azurophil granule is visible in the membrane pocket (arrow). Note the high density of the content of the phagocytic vacuole. Another azurophil granule (*ag*) with a content of comparable texture and density is present in the same field. Figs. 14 and 15 are from a 20 hr rabbit exudate fixed 30 min after exposure to *E. coli* and reacted for aryl sulfatase. They illustrate stages which presumably occur shortly after fusion of a phagocytic vacuole with an azurophil granule. In Fig. 14, an azurophil granule (*ag*) containing dense reaction product is observed in continuity with a phagocytic vacuole (*dv*). Fig. 15 shows reaction product (arrows) around a bacterium (*b*) in a phagocytic vacuole. Specimens were fixed for 4 hr at 23°C (Fig. 13) or 10 min at 4°C (Figs. 14 and 15) in formaldehyde-glutaraldehyde. The cells in Figs. 14 and 15 were incubated in Goldfischer's aryl sulfatase medium (pH 5.5) at 23°C for 3 hr, and treated with $(NH_4)_2S$. Fig. 13, × 30,000; *inset*, × 69,000; Fig. 14, × 39,000; Fig. 15, × 32,000.

material in this work consisted of tissue macrophages and the fraction obtained was heterogeneous suggests that the structures isolated were primarily or exclusively digestive vacuoles rather than azurophil granules.

The Monocyte Compared with Granulocytes

In many aspects of its development and function, the monocyte is similar to the granulocytes, particularly the neutrophil; they both originate from a proliferating pool of immature cells in the bone marrow, where they produce a population of granules which are primary lysosomes (8, 24), both kinds of cells are distributed throughout the body via the blood, and both function as phagocytes in the tissues.

Monocytes differ from neutrophils, however, in several significant respects. First, kinetic data (1–3) indicate that their maturation time in the marrow is shorter than that of neutrophils. Monocytes are released randomly into the circulation after a developmental period of 1–3 days and continue their maturation in the blood, whereas neutrophils normally have a somewhat longer developmental period ($\sim$ 3 days), during which they complete their maturation in the bone marrow and are stored for an additional 3 or 4 days (35). Second, the monocyte makes smaller and fewer granules than the neutrophil of the same species. Moreover, the neutrophil makes two morphologically distinct types of granules with different contents whereas the monocyte appears to produce a single population of granules among which no distinctions can be made on morphologic grounds. However, it should be pointed out that heterogeneity of enzyme content is possible since only some of the granules in a given preparation are reactive for any enzyme. The third and most important distinction between the two cell lines is the fact that whereas the neutrophil is an "end cell" which does not divide or form granules after it leaves the marrow and probably degenerates after a day or two in the tissues (35), the monocyte is capable of continuing enzyme synthesis in the tissues (36) and may live for up to two months (2, 37).

Two Kinds of Primary Lysosomes in the Monocytic Cell Line

Cells of the monocyte line are unusual in that they make two different kinds of primary lysosomes, each of which is manufactured at a different time in the life cycle. The first is a storage granule or azurophil granule, made during cellular differentiation in the bone marrow and blood. These storage granules are utilized during the initial phases of phagocytosis in the tissues and additional granule formation has not been observed. Our cytochemical observations are in agreement with previous data which show that peritoneal exudate cells contain (38) and produce (36) large quantities of digestive enzymes. Moreover, they confirm the work of Cohn et al. (39) indicating that these cells package lysosomal enzymes into small "Golgi" vesicles, primarily coated vesicles, which therefore represent a second type of primary lysosome.

As information becomes available on the lysosomal or vacuolar system in various tissues (see reference 40), it is apparent that relatively few cells store lysosomal enzymes in morphologically distinct structures recognizable as granules. The most notable examples of those that do are other leukocytes, i.e. the neutrophilic (8, 24, 41) and eosinophilic (18, 42) granulocytes. In most other cells in which primary lysosomes have been identified, they occur as small vesicles—so-called Golgi vesicles (14, 40, 43)—which are sometimes recognized as coated (29). Such vesicles cannot be distinguished on morphologic grounds from other similar cytoplasmic vesicles (44, 45), and they have been identified so far only by cytochemical staining procedures. In summary, mononuclear phagocytes may well be unique in producing two varieties of primary lysosomes—one which is recognizable as a large granule, and another which is a vesicle distinguishable as a lysosome only by cytochemical techniques. The fact that the cell can shift from one mode to another in the packaging of the secretory product resembles the situation described in the pancreatic exocrine cell in which a partially similar shift occurs in vitro upon continuous stimulation (48).

Comments on the Distribution of Cytochemical Staining Reactions

In promonocytes as well as macrophages partial reactions of the ER and Golgi for acid phosphatase and aryl sulfatase were observed. The fact that not all of the compartments involved in the secretion of lysosomal enzymes are reactive in a given cell can be explained either on the basis of (*a*) an artifact of the cytochemical procedure, or (*b*) local

specialization, with certain cisternae producing some enzymes and certain cisternae producing other enzymes.

As in other leukocytes (8, 28), the azurophil granules are not uniformly reactive for acid phosphatase and aryl sulfatase. The situation differs from that encountered in neutrophils of the rabbit (8) in that some of the mature azurophil granules are reactive for aryl sulfatase. Moreover, in the case of the human and guinea pig monocyte only some of the azurophils are reactive for peroxidase. For the moment there is no clear explanation for these findings, but it could be due to differences in the permeability of the granule membrane to the incubation media, to differences in the packaging of the granule contents, or to real differences in the enzyme content of the granules. This situation requires further investigation.

We are grateful for the excellent technical assistance of Mrs. Jean Sarris, Miss Yvonne Jacques, Miss Irene Rudolf, and Miss Cassandra Lista. We also wish to thank Dr. Daniel S. Friend for his helpful suggestions during the course of this work.

This investigation was supported by grants Nos. AM 09090 and AM 10486 from the United States Public Health Service. The work was carried out while Dr. Nichols was the recipient of a National Institutes of Health Postdoctoral Fellowship (5 F02 HE 28917) and Dr. Farquhar of a Public Health Service Career Award (1-K3-GM-25,109).

Received for publication 30 October 1970, and in revised form 13 January 1971.

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