The localization of peroxidase in the organelles of mononuclear phagocytes has been extensively studied by means of the cytochemical technique of Graham and Karnovsky (1). In the bone marrow of some species, such as man (2) and the rat (3), peroxidase is synthesized early in monocyte development and can be seen in promonocyte rough endoplasmic reticulum, Golgi cisternae, and storage granules. As differentiation progresses to the monocyte stage, peroxidase can no longer be detected within the RER and Golgi complex, indicating that its synthesis has ceased, and a second population of granules, which are peroxidase-negative, is formed. Circulating blood monocytes are similar to bone marrow monocytes in that all of their organelles are unreactive for peroxidase, with the exception of those granules that were formed early in differentiation. Rabbit promonocytes (4) differ significantly from those of the human and rat because no peroxidase has been demonstrated in them during maturation in the bone marrow; therefore, all organelles of the rabbit circulating blood monocyte, including its granules, are unreactive for this enzyme.

Certain tissue macrophages also contain peroxidase, cytochemically identifiable in the RER and perinuclear cisterna. This peroxidase reactivity in the RER is characteristic of resident peritoneal macrophages of the rat (5) and guinea pig (6), Kupffer cells (7) and medullary lymph-node macrophages (8) of the rat, and resident, but not exudate, peritoneal macrophages of the rabbit (9). Such reactivity has not been described in the tissue macrophages of man (10). Although considerable evidence (11) supports the view that circulating monocytes evolve into tissue macrophages, including peritoneal macrophages and Kupffer cells, the different distribution of peroxidase in monocytes compared to its localization in these tissue mononuclear phagocytes has been cited as evidence that the blood monocyte is not a direct precursor (12, 13).

During studies of blood monocyte differentiation in vitro, we observed that peroxidase activity rapidly appears in the RER after the cells adhere to a...
surface. This finding, confirmed with monocytes from three species—rabbit, rat, and human—indicates that blood monocytes have the capacity to develop this enzymatic reaction typical of certain tissue macrophages. Moreover, its emergence may be a consequence of plasma membrane:external surface interaction.

Materials and Methods

Preparation and Incubation of Monocytes. Sterile conditions were maintained throughout these procedures. Blood was drawn by venipuncture (human) or cardiac puncture (adult male rats and rabbits) into heparinized syringes (10 U/ml, final concentration). Buffy coat cells were separated by centrifugation in Kaplow tubes (Virtis Co., Gardiner, N. Y.) or, alternately, we obtained mononuclear cells using a modification of the Ficoll-Hypaque technique (14). The methods were as described previously for processing human blood (15), except that centrifugation of rabbit blood was performed at 600 g for 50 min. Leukocytes were washed once in Hanks' balanced salt solution (HBSS) with heparin (10 U/ml, final concentration), then suspended in medium 199 with glutamine and Hanks' salts, 50 U/ml penicillin, 50 μg/ml streptomycin (Grand Island Biological Co., Grand Island, N. Y.), and 20% heat-inactivated homologous (rat or rabbit) or autologous (human) serum. Subsequently, one or more aliquots were removed for fixation (0 time). For incubation, 3-ml aliquota, each containing about 2 × 10⁵ cells/ml, were pipetted into 60 × 15-mm polystyrene tissue culture dishes (no. 3002, Falcon Plastics, Oxnard, Calif.).

Cells were incubated at 37°C in 5% CO₂-air for 2-96 h. In some experiments, we used fibrin-coated dishes with bovine fibrinogen (90% clottable, Pentex), prepared by the methods of Unkeless et al. (16). In other experiments, 5-ml aliquota of the leukocyte suspension were shaken for 2 h under a 5% CO₂ atmosphere in sealed beakers in a Dubnoff shaking incubator, with or without heat-killed *Staphylococcus albus* (15). Peritoneal macrophages were obtained from normal rabbits by lavage with 100 ml heparinized HBSS after sacrificing the animals.

Cytochemical Procedures. Cells were fixed for 10 min at 4°C in 1.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.), washed in 0.1 M sodium cacodylate buffer, and reacted for peroxidase using the glucose oxidase technique (17) in which H₂O₂ is generated during the incubation. Incubation media consisted of 0.05 M Tris buffer (pH 7.6), 0.5 mg/ml 3,3'-diaminobenzidine (DAB), 2 mM glucose, and 25 μg/ml glucose oxidase (A-grade, fungal, lyophilized, Calbiochem, La Jolla, Calif.). Incubations were continued for 2 h at 22°C. In some experiments, the peroxidase method of Graham and Karnovsky (1) (pH 7.6) was used, with a final concentration of 0.01% H₂O₂. We selected the glucose oxidase technique as the preferred method because it appeared to be more sensitive for demonstrating reaction product in RER. All the results reported here are based on the use of this method.

After incubation for enzyme reaction, the cells were washed, postfixed in OsO₄, processed for electron microscopy as previously described (18), and embedded in Epon. Uranyl acetate was always omitted, both in en-bloc and on-grid staining, since it may extract or mask peroxidase reaction product. Cells adhering to tissue culture dishes were fixed, washed, reacted for the enzyme, and processed as above, still in the dishes, until dehydration in absolute alcohol was complete. Then propylene oxide (4 ml) was added to each dish (19), and the surfaces were rinsed vigorously with a Pasteur pipette. As the cell layer detached, it was transferred to polypropylene tubes (Falcon Plastics), then concentrated into small pellets and embedded in Epon.

Control preparations consisted of cells from incubations in which (a) glucose oxidase was omitted, or (b) 0.02 M 3-amino-1,2,4-triazole or 0.01 M KCN was added to preincubation and incubation media. Cells were also incubated for 1 h in 0.05 M Tris-HCl buffer containing 0.1% catalase and 0.05% DAB. After three washes, they were transferred to 10⁻³ M potassium ferricyanide for ½ h. The localization of catalase was assessed by the method of Roels et al. (20).

All data reported from examining thin sections were derived from two to five experiments, in each of which a minimum of 50 monocytes or macrophages was studied.

Results

Our initial experiments were undertaken to document the localization of peroxidase in the blood monocytes and resident peritoneal macrophages of normal rabbits. As previously reported (4), no peroxidase activity could be
demonstrated in rabbit blood monocytes (Fig. 1). In contrast, peroxidase activity was prominent in 90–95% of the resident macrophages. Reaction product was localized in the entire RER and perinuclear cisterna, but was not seen in Golgi cisternae, cytoplasmic vesicles, or in the larger inclusions of these cells (Fig. 2).

Next, we cultured blood monocytes to see if they were capable of developing peroxidase reactivity during differentiation in vitro. Leukocytes were separated from the blood by both the Ficoll-Hypaque and Kaplow buffy-coat methods, cultured for varying intervals (2–96 h), and tested for the enzyme. Within 2 h, the majority (60%) of monocytes adhering to the plastic culture dishes developed peroxidase reactivity in the RER and perinuclear cisterna. The reaction remained strongly positive for 5–8 h, began to diminish at 18 h, and by 3–4 days most cells were peroxidase-negative. Some culture dishes were then coated with fibrin to obtain a surface more nearly resembling that which might be encountered in vivo. When the cells were subsequently tested for peroxidase, enzyme reactivity in monocytes was even more pronounced (Fig. 3) at 2 h than in the previous experiment, persisting in most cells for at least 18 h. Thus these experiments indicated that after blood monocytes adhere to surfaces, peroxidase reactivity rapidly appears in RER, a localization identical to that found in resident macrophages.

Because adherence to a surface seemed to be important to the development of reactivity, aliquots of these same blood monocytes were incubated on a shaking incubator to prevent adherence. After 2 h, they had developed no peroxidase reactivity. Similarly, cells shaken in suspension with staphylococci remained peroxidase-negative after 2 or 18 h of incubation without surface adherence, although the bacteria became phagocytized. These results indicate that peroxidase reactivity in monocyte RER is not stimulated by brief attachment of bacteria to the cell membrane, nor by the process of phagocytosis.

To determine whether the appearance of peroxidase reactivity in blood monocytes occurs in other species, we next conducted preliminary experiments with human and rat blood monocytes, both before and after adherence. At 0 time, enzyme activity was present only in some storage granules, as reported previously in both human (2) and rat (3) monocytes. After 2–18 h of incubation, however, RER peroxidase reactivity, identical to that observed in rabbit cells, developed in ~30% of the human monocytes (Fig. 4) and in a smaller percentage of the rat monocytes. Under the conditions of our experiments, these latter cells adhered slowly, and spreading was delayed and less marked than in the cells of the other species.

**Control Cells.** The cytochemical reaction was inhibited in 5-h adherent monocytes or resident peritoneal macrophages of the rabbit when aminotriazole was added before and during incubation. When glucose oxidase was omitted from the reaction mixture, no reaction product could be found in 90% of the cells, but ~10% of the cells were weakly reactive in the RER, presumably because of a low level of endogenous H$_2$O$_2$ production.

A negative cytochemical reaction followed the incubation of tissue in the presence of DAB and catalase, with consecutive incubation in the presence of potassium ferricyanide. This ruled out the possibility that a positive reaction might result from nonspecifically bound DAB subsequently oxidized by OsO$_4$. 
Fig. 1. Rabbit monocyte reacted for peroxidase immediately after isolation from the blood. The eccentrically situated nucleus (N) and cluster of azurophil granules (g) near the Golgi complex (G) are characteristic. No peroxidase reaction product can be seen in the RER cisternae (rer), Golgi complex (G), or azurophil granules (g). However, the adjacent reticulocyte (R) appears dense due to the reactivity of its constituent heme protein, hemoglobin. m, mitochondrion; ce, centriole. The cells were separated from buffy coat, fixed for 10 min in glutaraldehyde at 4°C, then washed and incubated for 2 h at 22°C in a medium (pH 7.6) containing 0.5 mg/ml DAB plus H$_2$O$_2$ generated by the glucose oxidase method. Specimens were postfixed in OsO$_4$, dehydrated in ethanol, and embedded in Epon. Thin sections were stained on-grid with Reynolds’ lead citrate. Magnification × 20,000.
Fig. 2. Resident macrophage from the rabbit peritoneal cavity, tested for peroxidase. The extensive RER (rer), including the perinuclear cisterna (pn), is densely reactive for the enzyme. In contrast, the Golgi cisternae (Gc) and cytoplasmic vesicles (v) are unreactive. Specimens were obtained by lavage with HBSS, fixed, and processed as outlined in Fig. 1. Magnification × 15,000.

The reaction product was completely inhibited by KCN in 90% of the cells; however, weakly-to-moderately positive cells were observed in 5-10% of the monocyte population. In resident macrophages, inhibition was virtually absolute.

To ascertain whether the reaction might be due to catalase rather than peroxidase, we reacted both cell types for the former enzyme, using the method of Roels et al. (20). No catalase reaction product appeared in the RER or perinuclear cisterna, but it was noted in small, membrane-bound organelles—microperoxisomes (21)—as well as in occasional large inclusions.
Discussion

Our results demonstrate that rabbit blood monocytes, which initially contain no cytochemically detectable peroxidase, develop such reactivity in the RER after 2 h of incubation on a serum- or fibrin-coated surface. No reactivity
FIG. 4. Human blood monocyte, which has adhered to a serum layer (arrows) on a plastic surface for 7 h, tested for peroxidase. In this species, both circulating blood monocytes and adherent cells in vitro contain peroxidase-positive granules (g). None of the peroxidase-negative granules normally present in vivo can be seen in this micrograph. The major change which has occurred in the adherent cell is the appearance of reaction product in the perinuclear cisterna (pn) and less prominently in the RER (rer). m, mitochondria; gl, glycogen. Specimen preparation as in Fig. 3. Magnification × 21,000.
appears during incubation without surface adherence or after phagocytosis of bacteria by nonadherent cells. Peroxidase reactivity in monocytes is identical in localization and characteristics to that of the enzyme normally found in rabbit resident peritoneal macrophages. Moreover, since human and rat monocytes develop similar RER reactivity after adherence, the phenomenon is not peculiar to the rabbit.

Although we refer to this enzyme as a peroxidase, its identity is unknown. The cytochemical methods we used are not specific for peroxidases, for many heme-containing molecules, including cytochromes, hemoglobin, and catalase (22), also oxidize DAB in the presence of H$_2$O$_2$ to form the visible reaction product, oxidized DAB (1). The reactivity of different heme proteins with H$_2$O$_2$ and DAB varies, depending upon such factors as fixation time, pH, and H$_2$O$_2$ concentration. We do not believe that the RER activity described here is catalase, since when tested by an appropriate method, reaction product in adherent monocytes and resident macrophages has an entirely different distribution. No biochemical data are available on the peroxidase content of blood monocytes or resident peritoneal macrophages of the rabbit, although Romeo et al. reported that small amounts are present in rabbit exudate peritoneal macrophages elicited by casein (23).

Certain cell types other than macrophages also demonstrate similar RER peroxidase reactivity. These include lacrimal, submaxillary, and parotid gland cells (22, 24); thyroid acinar cells (25); and uterine epithelial cells, in which enzyme activity is induced by estrogen treatment (26). The peroxidase in thyroid cells presumably participates in the synthesis of thyroid hormone, but the function of the other peroxidases, like that of the macrophage enzyme, remains unknown. Reportedly, RER peroxidase in peritoneal macrophages of the guinea pig (27) and in rat Kupffer cells (12) is not delivered to phagocytic vacuoles, so that it is questionable whether these enzymes are involved in the killing of bacteria.

The reason for the appearance of peroxidase activity after adherence is unknown, but may relate to altered metabolic events resulting from surface stimulation. Both the morphology and growth characteristics of many cells change when they attach to surfaces (28, 29); increases in protein synthesis have been described (30, 31). Adherence of neuroblastoma cells apparently stimulates differentiation, including induction of acetylcholine esterase (31). When monocytes adhere to aggregated IgG, superoxide formation is stimulated briefly (32), but later metabolic changes have not been studied. In our experiments, it is not clear why the monocyte RER enzyme activity persists for many hours, but disappears after 1–2 days in tissue culture even though the cells remain adherent. To study these questions further, we are currently examining changes in peroxidase reactivity of resident and exudate peritoneal macrophages cultured under similar conditions. Studies of metabolic inhibitors are also underway in an attempt to distinguish whether the appearance of this enzyme is due to activation or induction.

Although most investigators agree that exudate macrophages are derived from blood monocytes, the origin of resident macrophages and Kupffer cells is controversial. Both cytochemical (12, 13) and kinetic (33) data have been used to
challenge the current view (11) that blood monocytes are the precursors of all tissue macrophages. The enzyme that appears in adherent monocytes is identical in localization to that of certain tissue macrophages, such as resident peritoneal and Kupffer cells (see introductory section). There is the possibility that different enzymes are being observed in the various cell types, although we have no evidence of this. Nevertheless, the fact that monocytes can rapidly acquire this feature typical of certain macrophages, i.e. resident macrophages and Kupffer cells, supports the theory that these cells derive from blood monocytes.

Summary

Rabbit blood monocytes, which contain no cytochemically demonstrable peroxidase, develop peroxidatic activity in the RER and perinuclear cisternae within 2 h after adherence to serum- or fibrin-coated surfaces. A similar reactivity appears in surface-adherent human and rat blood monocytes. In both localization and characteristics, this enzyme reactivity in monocytes resembles that normally seen in the resident peritoneal macrophages of the rabbit, as well as in several types of tissue macrophages in other species. Thus this observation supports the concept, presently based on the kinetic data of other investigators, that blood monocytes are the precursors of such cells. Moreover, the appearance of new enzyme activity after adherence may reflect alterations in cellular metabolism resulting from plasma membrane:surface interactions.

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