An Electron Microscopic Study of Erythrophagocytosis*

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ABSTRACT

The present study describes a submicroscopic surface fragmentation of erythrocytes which occurs in the ascitic fluid of rats bearing the Novikoff ascites hepatoma. The resulting fragments attach to the surface of macrophages and are phagocytized by pseudopod formation. Plasma membrane in the region of these phagocytosis vacuoles appears to condense into electron-opaque material, suggesting an alteration in its physicochemical state. Stages in intracellular digestion of intact erythrocytes or small fragments within the phagocytosis vacuoles are illustrated; no particles resembling ferritin are observed. The phagocytosis vacuoles possess high levels of acid phosphatase activity. They may be called phagosomes, a type of lysosome. There is no indication of a connection between phagosomes and other formed cytoplasmic organelles. Small vacuoles of the order of 80 μ in diameter, which may represent pinocytosis vacuoles, are present in the cytoplasm and some appear to be in contact with the phagosome membrane, reminiscent of observations of Rose with HeLa cells.

INTRODUCTION

Although the phenomenon of erythrophagocytosis has been recognized and investigated for almost a century (1), the essential features of this process still remain obscure. Recent cytological studies have added little to the earlier descriptions, due largely to limitations in the resolving power of the light microscope. With the concept of the reticuloendothelial system formulated by Aschoff (2), physiologists have generally confined themselves to measuring the rates at which erythrocytes are cleared from the circulating blood, leaving unanswered direct questions concerning the fundamental nature of this important process.

While it is generally agreed that only "aged," "worn," or otherwise "altered" erythrocytes are candidates for phagocytosis (3), opinion is divided on whether this represents the major pathway for their destruction. Recent studies (3) appear to support the earlier contention of Rous (4) that erythrocytes are eliminated primarily through fragmentation, but whether the resultant particles are subsequently phagocytized is not always clear.

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Other workers have not observed erythrocyte fragmentation (5). In any case, the sequential steps in the transport of erythrocytes or of their fragments across the surface membrane of the macrophage are not well known, nor is it clear what events occur in the intracellular digestion of this material.

With the currently available procedures of electron microscopy, phagocytosis and other mechanisms operating at the cell surface can be revealed in much greater detail. This should make it possible to clarify the interrelatedness of such processes as phagocytosis, pinocytosis (6, 7), and cytopempsis (8). Cytochemical staining procedures can supplement electron microscopy in delineating the enzymatic machinery of the cytoplasmic structures involved in the ingestion and digestion of materials.

The present work is concerned with erythrophagocytosis as it occurs in the hemorrhagic fluid within the peritoneal cavity of rats bearing the Novikoff ascites hepatoma (9). It describes surface fragmentation of the erythrocyte. The resulting fragments are engulfed by formation of pseudopods of the macrophage with the result that a vacuole is formed limited by the plasma membrane of the macrophage. By staining procedures, acid phosphatase activity can be demonstrated within these
phagocytosis vacuoles ("phagosomes" (10, 11)). From the work of de Duve (12) on lysosomes, it is reasonable to assume that other hydrolytic enzymes are localized there as well. Presumably, these play a role in the digestion of the erythrocyte and its fragments.

**Material and Methods**

The material for the present report was obtained from a Sprague-Dawley rat bearing an eighth generation generation of the Novikoff hepatoma in ascites form. The results were confirmed by examination of similar material taken from later transfers of this tumor.

Five days after transplantation, a few drops of the hemorrhagic ascites fluid, withdrawn by syringe from the peritoneal cavity, were added to 5 ml of cold buffered 1 per cent OsO_4 at pH 7.2 (13). The cells were fixed in suspension for 15 to 20 minutes and occasionally stirred to prevent settling. After several washings in veronal-acetate buffer, the suspension was centrifuged into a firm pellet. With a flat wooden splint, the pellet was gently lifted free from the lusteroid tube, floated in a Petri dish, and diced with a razor blade into appropriately sized pieces. The tissue samples were kept overnight in 1.6 per cent formaldehyde-0.28 M veronal-0.28 M acetate buffer and then dehydrated in the usual manner. The specimens were embedded in a 1:5 mixture of methyl and n-butyl methacrylate containing 1 per cent luperco CDB as catalyst. To minimize polymerization damage, 75 mg of uranyl nitrate/100 ml were added to each of the three methacrylate mixtures (14). Polymerization took place overnight at 60 °C. Sections with osmium-fixed preparations.

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An observation of interest is the increased opacity to electrons of the membranes in the immediate area of the erythrocyte fragments where such fragments make contact with the surface of the phagocyte (Figs. 3, 5). This may represent an alteration in the physicochemical properties of the surface layer (cf. Novikoff (11) who observed in *Amoeba proteus* an increased acid phosphatase activity in the region of the plasma membrane to which food particles are attached, prior to engulfment). Only in rare instances do fragments appear to gain access to the cytoplasm by formation of pits or indentations in the surface membrane. An example of this is seen in Fig. 6. Here, an erythrocyte bud appears to be indenting the plasma membrane of the phagocyte without formation of pseudopodia.

Neither newly formed phagocytosis vacuoles at their sites beneath the surface membrane nor internal vacuoles show any relationship to the endoplasmic reticulum, the free ribonucleoprotein particles which form the bulk of the visible cytoplasmic
ground substance, the prominent Golgi apparatus, or any other cell organelle.

The erythrophagocytes contain but little endoplasmic reticulum even during obvious periods of intense phagocytic activity. These macrophages thus differ from those of mouse lung, in which Kar rer (18) found a general correspondence between the amount of endoplasmic reticulum and the degree of phagocytic activity.

Apparently intact erythrocytes are phagocytized in the same fashion as the red cell fragments. Generally, however, they are in the process of fragmenting when engulfed (Fig. 7) and fragments continue to be detached into the cavity between the erythrocyte membrane and phagosome membrane. This cavity enlarges (Figs. 7, 9, 10, 12) and usually contains within it an amorphous material of low density (Figs. 8, 10, 11). Stages in the actual digestive process, as evidenced by successive losses in opacity, were not frequently observed. Most phagocytized erythrocytes were either intact or had suffered a marked loss of material to a point where only the "ghost" of the cell together with a small amount of residual material remained. The fragments may persist in the phagosome after the erythrocyte is broken down, but in time they, too, disappear (Fig. 11).

At no time in the process are electron-opaque particles of the size order of ferritin observed. This is in contrast to the situation in reticulocytes of the bone marrow in man where Bessis (19) has described the accumulation of ferritin following digestion of phagocytized red cells. It is possible that the erythrocyte degradation in the peritoneal macrophages that we have studied does not involve conservation of iron for subsequent resynthesis of hemoglobin.

The importance of macrophages in the elimination of erythrocytes is emphasized by recent studies of hemolysis in a number of pathological conditions. Whether this process involves whole cells or cell fragments has never adequately been settled. Rous (4) contended that phagocytosis per se could not account for the total elimination of red cells from the circulating blood and that therefore fragmentation must occur. This view is supported by more recent studies employing microcinematography (see Miescher (3) for references). Whether the resultant fragments are subsequently phagocytized is, however, not clear. The present study describes a type of fragmentation which occurs in erythrocytes of the ascitic fluid in which Novikoff hepatoma cells are growing and there is no doubt that these fragments are rapidly phagocytized by peritoneal macrophages. To what extent similar processes occur in other body sites and under other conditions requires further investigation with both light and electron microscopy.

In the phagocyte here described the erythrocyte fragments are generally phagocytized by pseudopod formation and apparently only rarely by indentation of the plasma membrane. The region of the plasma membrane to which the fragments adhere appears to condense into electron-opaque material, as do similar regions of the phagosome membrane. The underlying physicochemical alteration is probably analogous to that which appears to occur in amoebae where the first step in pinocytosis may involve protein-binding and alteration of the physical state of the plasmalemma (6). The same phagocytes which produce pseudopodia to engulf erythrocyte fragments appear also to form small pinocytosis vacuoles. The linear arrangement of these vacuoles (Figs. 6, 9, 10, 5) suggests a mode of formation similar to that in amoebae (20). Recent work (see Holter, (7)) indicates that one of the decisive factors determining the manner of entry into an amoeba is the nature of the material making contact with the cell. This may have its counterpart in the specificity of bacteriophage attachment to and penetration into bacteria (see Weidel, (21) for a review of recent work on receptor sites of bacteria). In any case, in the cell we have studied, erythrocytes or fragments induce pseudopod formation leading to phagosome formation; perhaps certain substances in solution induce formation of the pinocytosis vacuoles.

There is no indication in our studies of any connection between the phagosomes or their contents and any other cell organelle. We have encountered nothing comparable to the connection of fat-containing droplets to the endoplasmic reticulum as seen in intestinal epithelial cells by Palay and Karlin (22). The erythrocyte fragments are usually surrounded by a smooth surfaced membrane (Fig. 5).

Novikoff (11, 23) has recently proposed that the Gomori staining procedure for acid phosphatase activity, performed on formal-calcium fixed cells not embedded in paraffin, may be used to identify lysosomes in liver and other tissues. It is, therefore, of considerable interest that the phagosomes are intensely stained in the acid phosphatase procedure (Figs. 13, 14). If they resemble liver and kidney
lysosomes, the phagosomes contain a host of important hydrolytic enzymes of which acid phosphatase is but one (12). Presumably erythrocyte digestion results from the activity of these hydrolyses.

It is not known where in the cell the enzymes are manufactured and how they gain access to the phagosomes. In a special strain of HeLa cells displaying marked pinocytosis activity, Rose (24) has suggested that lysosomal enzymes are brought to the pinocytosis vacuole by small "microkinetosomes." It is possible that in the erythrophagocytosis this is accomplished by the small vacuoles frequently seen in linear array (Fig. 9).

We should comment on the use of the word phagosome for the phagocytosis vacuoles. The term was recently coined by Straus (10) to describe the lysosomes or "droplets" of proximal convoluted tubule cells, because protein injected into the rat is concentrated in these "droplets." Novikoff (11) has raised the question whether these droplets may not be thought of as pinocytosis vacuoles ("pino-

somes") rather than phagocytosis vacuoles (phago-
somes), restricting the term phagosome to vacuoles containing solid material. Novikoff (11, 23) has found acid phosphatase activity in the phagosomes of all macrophages tested (liver, spleen, uterus, lung, etc.), irrespective of whether, as in our study, the phagosomes are electron-lucid except for the ingested material or whether they contain numerous electron-opaque particles (e.g., in Kupffer's cells, (25)).

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**BIBLIOGRAPHY**


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EXPLANATION OF PLATES

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FIG. 1. Fragmentation of an erythrocyte* in the ascitic fluid of an ascites tumor-bearing rat, and attachment of fragments to the phagocyte surface. Fragment A appears to be in the process of detaching from the surface; C may be attaching to the macrophage while D is attached. Note increased electron-opacity of the plasma membrane at D. The erythrocyte membrane is evident on fragment B. \( \times \) 20,000.

FIG. 2. Erythrocyte fragmentation. Arrows point to two fragments about to be cast off from the erythrocyte surface. Four fragments appear to be free in the ascitic fluid. Fragment A is attached to or is being ingested by a macrophage of which only a part lies in this plane of section. In the cell at the bottom note two small vacuoles \( (P) \) (pinocytosis?) below the plasma membrane. \( \times \) 20,000.

FIG. 3. Phagocytosis of erythrocyte fragments. Part of an erythrocyte (upper left) and four small fragments lie, apparently free in the ascitic fluid. At the arrow, one (or two) small fragments are attached to the surface of the macrophage. A slender pseudopod \( (PS) \) is seen over the relatively large erythrocyte fragment being phagocytized. \( \times \) 36,000.

FIG. 4. Phagocytosis of erythrocyte fragments. Three fragments are seen at the cell surface. Note increased opacity of the plasma membrane in that area. A slender pseudopod \( (PS) \) is apparently forming a phagocytosis vacuole (phagosome) in which at least four erythrocyte fragments are seen. Apparently free ribonucleoprotein granules \( (R) \) are scattered through the cytoplasm of the phagocyte. \( \times \) 48,000.

* In these thin sections intact erythrocytes cannot readily be distinguished from large fragments.
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Fig. 5. Phagocytosis of erythrocyte fragments. At the upper left is a portion of an erythrocyte which may be forming a bud. There are two fragments attached to the phagocyte surface, and seven fragments in vacuoles beneath the plasma membrane. Note the increased opacity of the plasma membrane in the region of the erythrocyte fragments, within the phagocytosis vacuoles (A), and on the surface (B). Mitochondria (M) appear dense in this relatively thick section but their typical structure may be seen. Endoplasmic reticulum is sparse or absent but apparently free ribonucleoprotein granules (R) are numerous. To the lower left is an erythrocyte particle (arrow) without an enveloping membrane. The structure marked E, probably represents the remains of a digested erythrocyte. Within the cell small vacuoles (pinocytosis ?) may be seen, comparable to those in Figs. 2 and 6; at P, they are seen in linear array. The body U, is too dense to be analyzed. X 28,000.
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Fig. 6. Phagocytosis of erythrocyte fragments by direct indentation of the cell surface. The process was not frequently observed in this material. A single bud from an erythrocyte (E) is seen indenting the plasma membrane; note increased opacity of plasma membrane (arrow). Presumably it would have entered the cell without formation of a pseudopod. Within the cell note the small vacuoles (pinocytosis ?) in linear arrays (P). X 42,000.

Fig. 7. Erythrophagocytosis. An erythrocyte is shown, presumably soon after being phagocytized. PS indicates the pseudopod which effected the engulfment and F the probable point of its fusion to the surface. Arrows point to fragments within the phagocytosis vacuole, probably the result of continued intracellular fragmentation of the erythrocyte. In the cytoplasm there is a phagocytosis vacuole with two particles (arrow) which may have detached from the phagocytized erythrocyte or have entered the cell directly. Also seen are ribonucleoprotein granules (R) and small vacuoles (P) (pinocytosis ?) near the phagosome membrane. X 28,000.
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Fig. 8. Intracellular digestion. Two phagocytes are shown side by side. In the cell on the left is a phagocytized erythrocyte showing no evidence of digestion. In the cell at the right, the erythrocyte appears to have been digested. Each of these phagocytosis vacuoles (phagosomes) contains an amorphous substance of low electron opacity. The phagosome at the left also contains four small erythrocyte fragments while the phagosome on the right contains two. At A, two erythrocyte fragments are attached to the cell surface. At B, two recently phagocytized fragments are seen in a small vacuole, under a bridge of cytoplasm. At C, two other fragments are seen wedged into the cleft between the two macrophages. A nucleus (N) and several mitochondria (M) are also evident. X 16,000.

Fig. 9. Higher magnification of a phagocytized erythrocyte. Numerous fragments have accumulated in the phagocytosis vacuole (phagosome). Note the increased opacity of the phagosome membrane where it is indented by the erythrocyte fragments. At arrow, four fragments are seen in a small vacuole which may have been detached (or is being detached?) from the larger phagosome. Within the cytoplasm small vacuoles (P) (pinocytosis?) may be seen. Some (arrow at P) may be in contact with the phagosome membrane. Others (P) are clearly arranged in linear fashion. Free ribonucleoprotein granules (R), mitochondria (M), and an unknown particle (U) are shown in the surrounding cytoplasm. X 25,000.
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Fig. 10. General view of erythrophagocyte. The erythrocyte to the right (E) has not yet undergone digestion; note the thin outer membrane (arrow) closely applied to its surface. To the left is a digested erythrocyte (E) still retaining an outer membrane. Within the same phagosome are many small fragments. Note that the phagosome membrane, originally derived from the plasma membrane, lies close to the erythrocyte “ghost” membrane over much of its surface. The isolated phagosomes (arrows) containing erythrocyte fragments, may have originated from the large phagosome. Small vacuoles (pinocytosis?) may be seen at P. Mitochondria (M), a lipid droplet (L), vacuoles (P) (pinocytosis?) near the surface, and unidentified structures (U) are also seen. X 19,000.
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Fig. 11. Intracellular digestion of erythrocyte fragments. Between the “ghost” of the digested erythrocyte (E) and the phagosome membrane are numerous small fragments derived from the erythrocyte. Some of the latter appear to be undergoing dissolution (area marked D). A small sac (S.A) appears to have pinched off from the main body of the phagosome, although it may have been separated from the plasma membrane. The nucleus (N) and Golgi area (G) are also seen. × 28,000.

Fig. 12. Erythrophagocyte in a smear of the ascites hepatoma, fixed in formal-calcium and stained with hematoxylin and eosin. N is the nucleus and E, a phagocytized erythrocyte. × 2000.

Fig. 13. Erythrophagocyte in a smear of the ascites hepatoma, fixed in formal-calcium and incubated for 30 minutes at 37°C. in the acid phosphatase medium (15), and counterstained with hematoxylin. The enzyme reaction product is restricted to one large (E) and numerous smaller (P) phagosomes. The nucleus, at N, is stained by hematoxylin but has no acid phosphatase reaction product. × 2000.

Fig. 14. Erythrophagocyte in a smear of the ascites hepatoma, fixed in formal-calcium and incubated for 30 minutes at 37°C. in the acid phosphatase medium (15) and counterstained with hematoxylin. The enzyme reaction product is restricted to the numerous small phagosomes (P). The nucleus is seen at N. × 2000.
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