

# Specialization of the Macrophage Plasma Membrane at Sites of Interaction with Opsonized Erythrocytes

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**ABSTRACT** We incubated mouse peritoneal macrophages for 3–8 min at 37°C with antibody-coated sheep erythrocytes and examined regions of close interaction between the two cell types by electron microscopy. At sites of focal macrophage-erythrocyte contact we observed a distinctive specialization of the macrophage plasma membrane consisting of a prominent subplasmalemmal band of electron-dense material, ~25–35 nm in thickness. In many instances, this band showed a periodic substructure similar to that seen in clathrin coats. Moreover, many slender erythrocyte processes penetrated into invaginations of the macrophage surface which were bristle-coated at their blind extremity. As previously shown for clathrin-coated pits, the segments of the macrophage plasma membrane beneath which the dense material was found were selectively resistant to the membrane-perturbing effect of the antibiotic, filipin. This structural specialization of the macrophage plasma membrane at sites of ligand-receptor interaction during immune phagocytosis of antibody-coated erythrocytes may represent the morphological counterpart of the zipper mechanism of phagocytosis previously demonstrated by functional studies.

Phagocytosis is the uptake of foreign particles within intracellular plasma membrane-derived vacuoles (1–3). In mammals, this function is primarily assumed by macrophages and polymorphonuclear leukocytes, which are considered as “professional” phagocytes (4) playing a crucial role in host defense against invading microorganisms (1, 2, 5, 6).

Although a wide variety of foreign bodies are ingested by phagocytosis, the uptake of antibody-coated (opsonized) particles has received particular attention, since it involves a specific interaction between the Fc-portion of particle-bound antibodies and Fc receptors on the surface of the phagocytic cell (2, 7–10). Griffin and co-workers (11–13) have demonstrated that the initial interaction of particle-bound ligands with their corresponding macrophage receptors generate a signal leading to the extension of pseudopods in the area of contact between the macrophage and the particle to be ingested. This brings additional receptors into apposition with ligands on the particle’s surface, resulting in the generation of additional signals and further pseudopod extension. The process continues in stepwise fashion until the plasma membranes of the advancing pseudopods meet and fuse with one another around the particle, enclosing the latter within a phagocytic vacuole. Thus, according to the above outlined “zipper model” (11–13), phagocytosis requires the repeated, circumferential

apposition of receptor-carrying macrophage plasma membrane segments with particle-bound ligands.

Whether the membrane segments involved in phagocyte-particle interaction have a specific organization at the ultrastructural level is still unknown. To investigate this point, we studied by electron microscopy the early steps of phagocytosis of antibody-coated erythrocytes by peritoneal macrophages. Our results indicate that regions of the macrophage plasma membrane which establish close contacts with the opsonized erythrocytes are underlined on their cytoplasmic side by a dense coat resembling that of clathrin-coated pits and vesicles (14–16). We suggest that these coated membrane segments represent the morphological counterpart of the “zipper.”

## MATERIALS AND METHODS

**Collection and Cultivation of Macrophages:** Stimulated macrophages were obtained from peritoneal washing of T6T6/C57 or T6T6/BALB mice injected intraperitoneally 3 d previously with 2 ml of 3% thioglycollate broth (Difco Laboratories, Detroit, MI). They were plated on 16-mm culture wells in Dulbecco’s modified Eagle’s medium (DME) supplemented with 5% fetal calf serum. After 2 h, nonadherent cells were removed by repeated washing with DME.

**Preparation of Opsonized Erythrocytes:** Sheep erythrocytes in Alsever’s solution were washed three times in phosphate-buffered (50 mM, pH

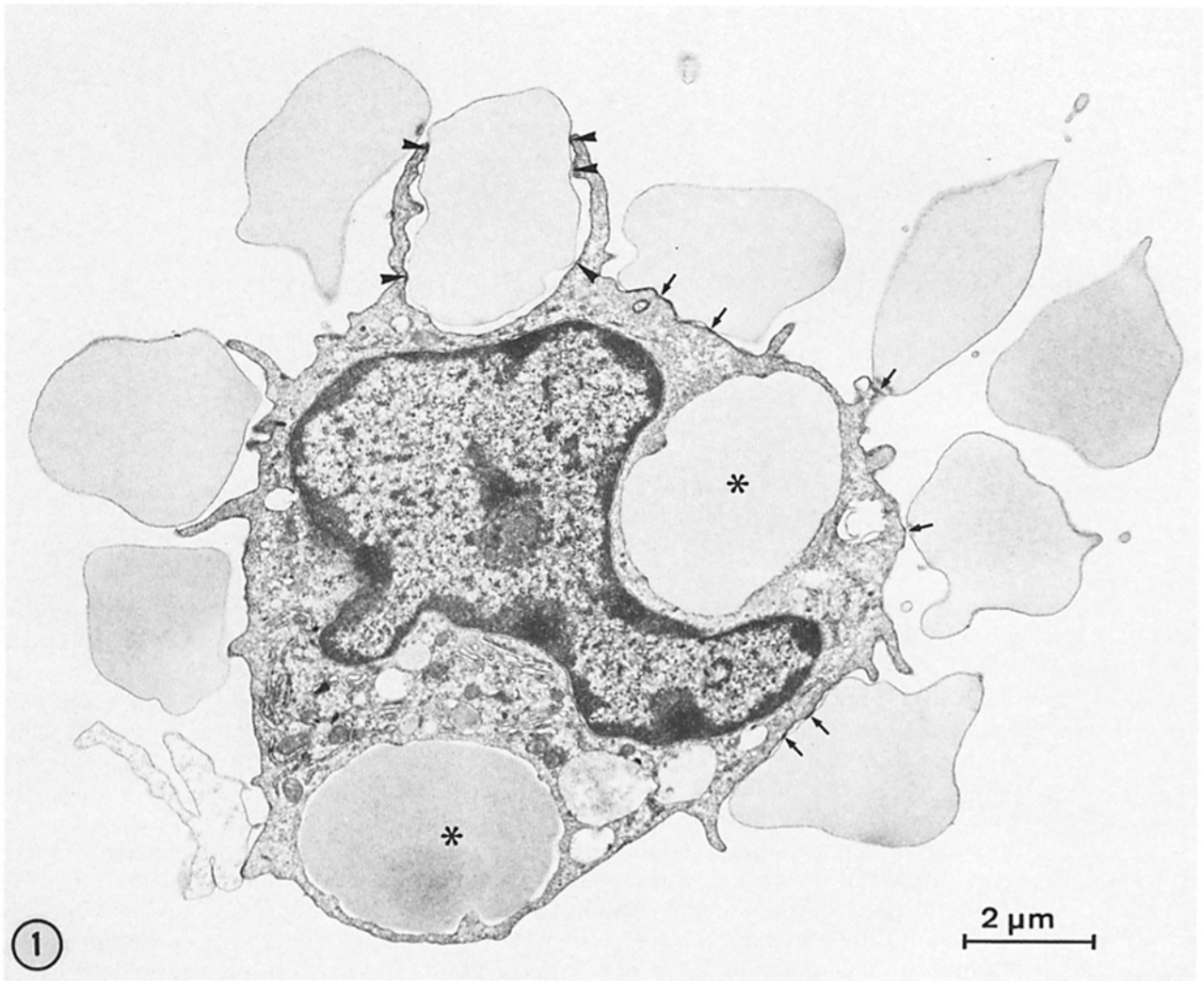


FIGURE 1 Thin section of a peritoneal macrophage incubated for 5 min at 37°C with antibody-coated sheep erythrocytes. The macrophage is surrounded by a "rosette" (19-21) of erythrocytes at various stages of attachment and internalization. Some erythrocytes are bound to the macrophage plasma membrane at one or several points of attachment (arrows). In the upper part of the picture, an erythrocyte is partially surrounded by macrophage pseudopods. The latter are not continuously and uniformly apposed to the erythrocyte surface, but focal areas of close contact (arrowheads) alternate with regions of wider separation of the two plasma membranes. Two erythrocytes (asterisks) are apparently enclosed within phagocytic vacuoles.  $\times 9,000$ .

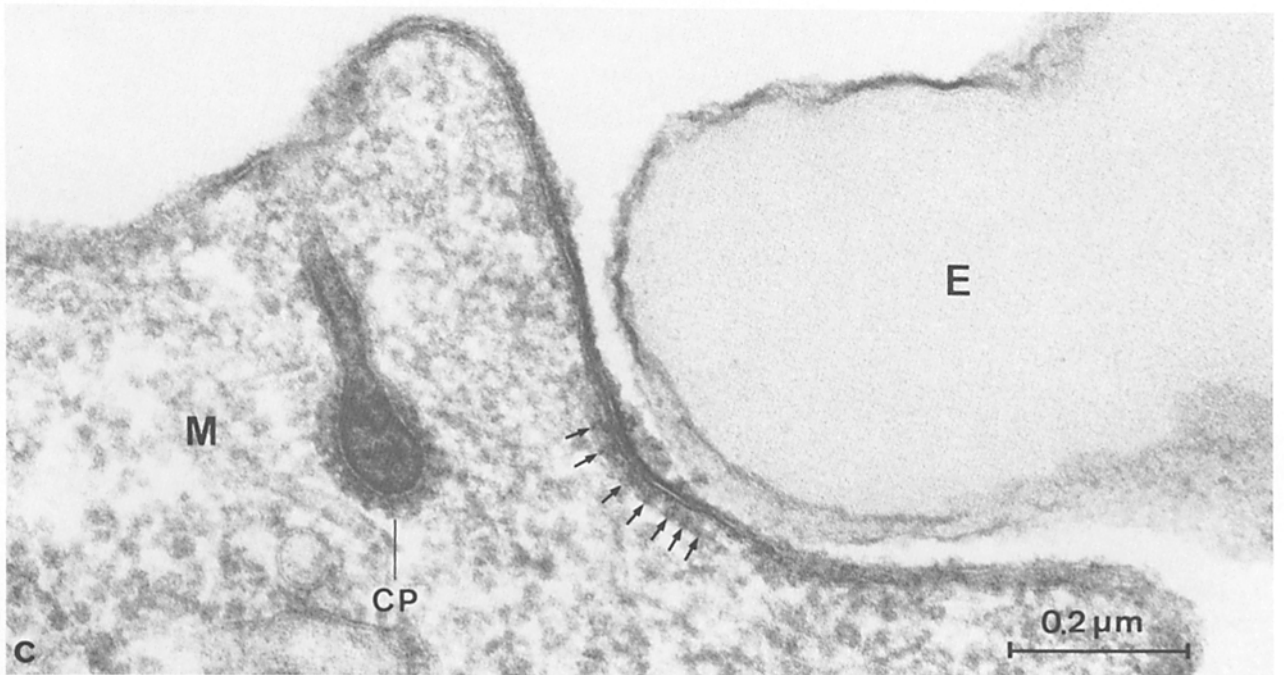
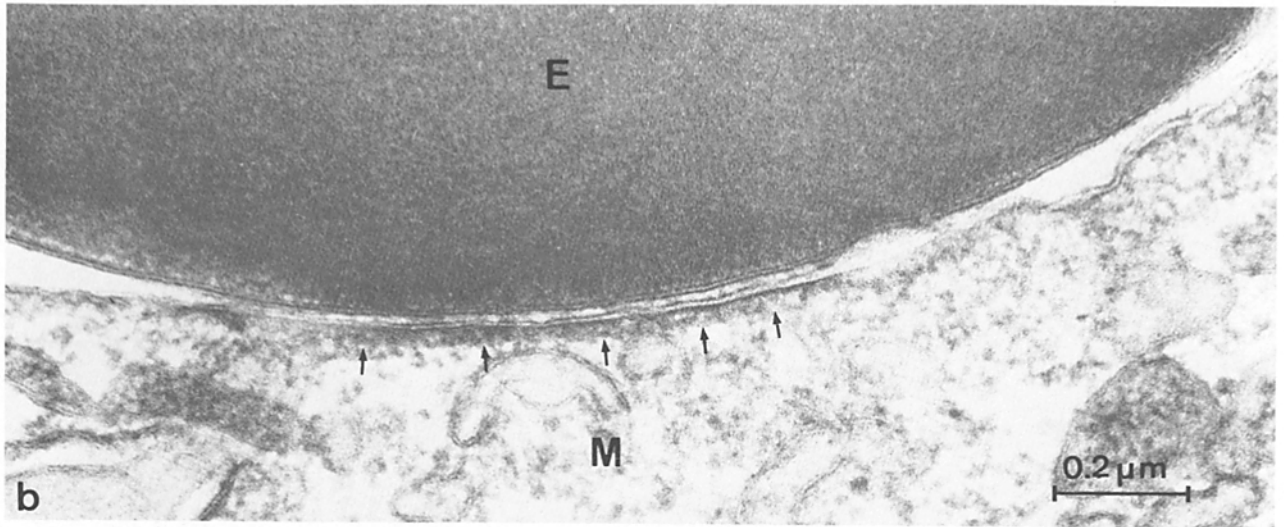
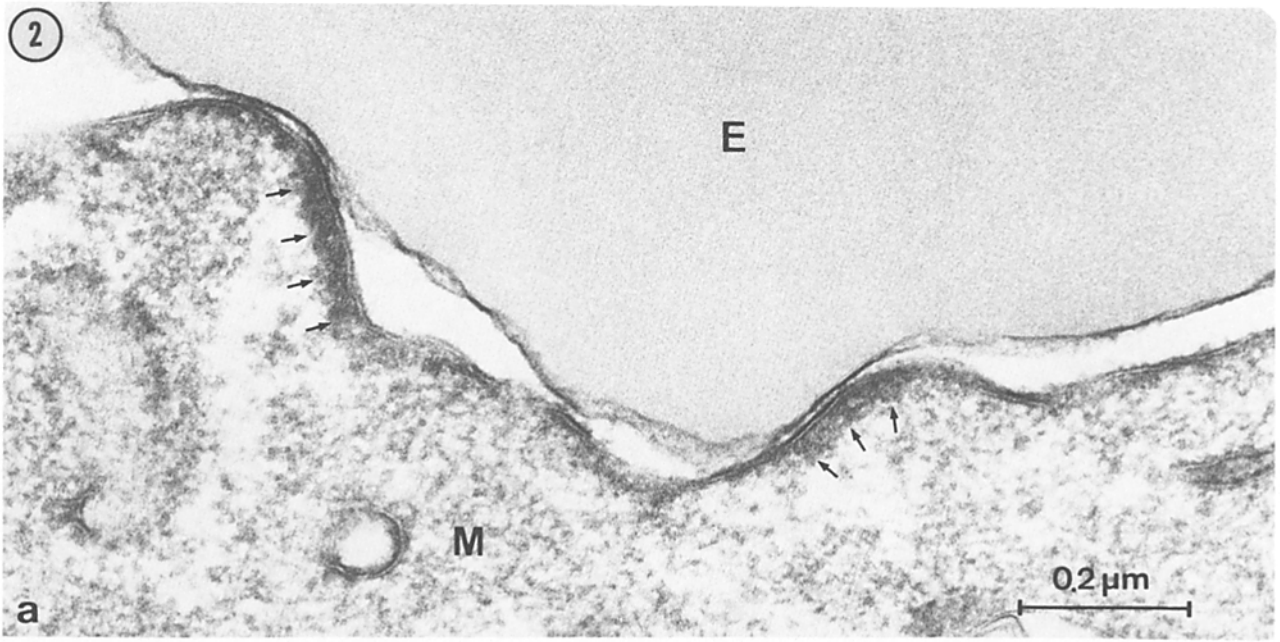
7.4) isotonic saline (PBS), resuspended as a 1% (vol/vol) suspension in bovine serum albumin (BSA) in PBS and mixed with rabbit antiserum to sheep erythrocytes diluted 1:1,000 in 1% BSA in PBS. The mixture was incubated for 30 min at room temperature, washed twice in PBS and resuspended as a 50% suspension in PBS.

**Phagocytosis:** Oponised erythrocytes were added to monolayers of macrophages and incubated for 3-8 min at 37°C. In some experiments the macrophages were first incubated with the erythrocytes at 4°C for 15 min, then warmed to 37°C for 5 min. After the incubation, they were washed with PBS and fixed for electron microscopy.

**Processing for Electron Microscopy:** Cells were fixed in a mixture of 2.5% glutaraldehyde and 1% tannic acid (Mallinckrodt Inc., Science Products Div., St. Louis, MO) in 0.1 M sodium cacodylate buffer. The monolayers

were then postfixated for 20 min in 1% osmium tetroxide in Veronal acetate buffer, stained en bloc with 0.5% uranyl acetate in Veronal for 20 min, dehydrated in graded ethanols and embedded in situ in Epon 812. For filipin labeling (17) macrophages were fixed in 2.5% glutaraldehyde in cacodylate buffer, then incubated overnight in the same buffer containing 100  $\mu\text{g/ml}$  filipin (The Upjohn Co., Kalamazoo, MI). The filipin solution was prepared by dissolving filipin in a small volume of dimethyl sulfoxide (DMSO) which was then added to the buffer to obtain the desired concentration. The final solution contained 1% DMSO. These cultures were postfixated and dehydrated as described above, with the exception that tannic acid treatment (0.5% tannic acid in 50 mM cacodylate buffer, pH 7.0, for 1 min) was carried out between osmification and uranyl acetate en bloc staining (18). Thin sections were cut either parallel or perpendicular to the plane of the culture, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope.

FIGURE 2 Higher magnification of regions of macrophage-erythrocyte interaction. A band of electron-dense material (arrows) is present underneath those segments of the macrophage plasma membrane establishing a close apposition with erythrocytes. In *a* and *b*, the subplasmalemmal densifications have a rather amorphous appearance and lack a distinct substructure, whereas the densification in *c* has periodically spaced "bristles" (arrows) similar to those seen in the clathrin coat of an adjacent coated pit (CP). The heavy tannic acid staining of the content of the coated pit is an indication that the latter is still in continuity with the cell surface. *E*, erythrocyte. *M*, macrophage. (*a*)  $\times 112,000$ . (*b*) 88,000. (*c*)  $\times 115,000$ .



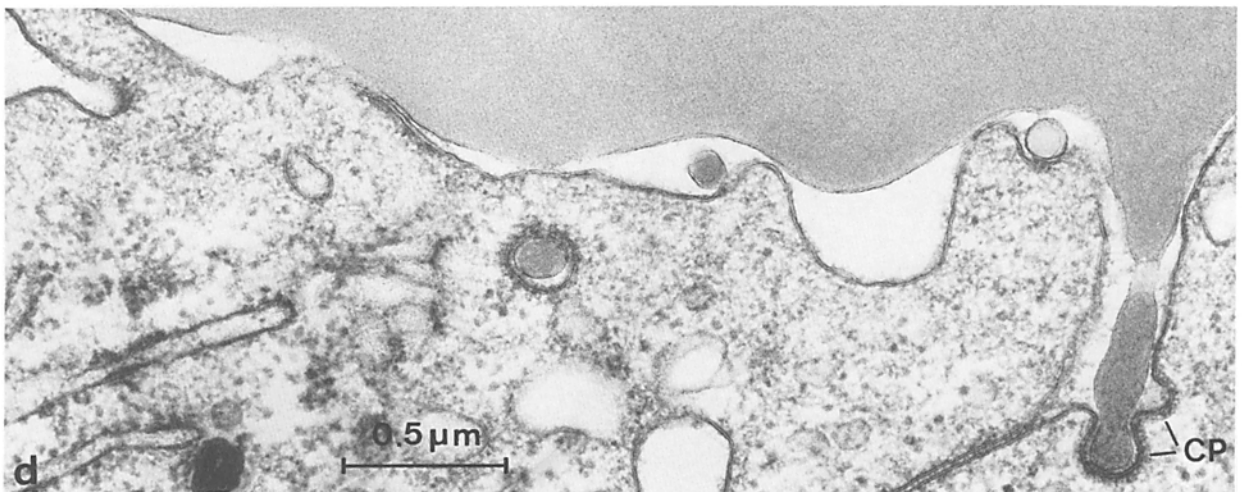
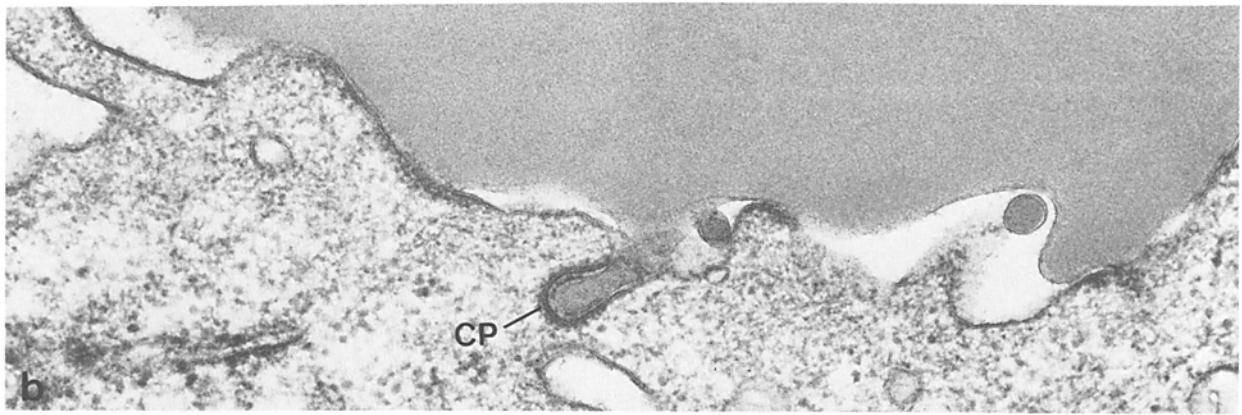
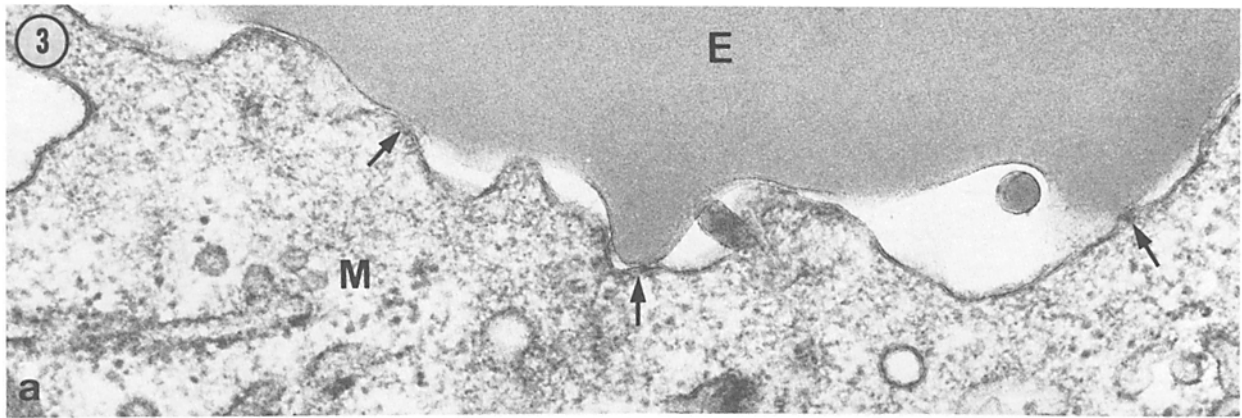


TABLE I  
Quantitative Evaluation of the Frequency of Coated and Noncoated Macrophage-Erythrocyte Contacts \*

	Number of contacts evaluated	Coated contacts	Noncoated contacts
Experiment 1	98	63 (64%)	35 (36%)
Experiment 2	74	43 (58%)	31 (42%)

\* "Contacts" were defined as zones of focal close apposition between macrophage plasma membrane and erythrocytes. Macrophage-erythrocyte contacts were screened for the presence of a dense coating on the cytoplasmic side of the macrophage plasma membrane by examining pictures taken at an appropriate magnification from two separate experiments. Only contacts in which the plasma membrane was clearly resolved were evaluated; oblique or tangential sections resulting in fuzzy images were not considered.

## RESULTS

When mouse peritoneal macrophages were incubated at 37°C for 3–8 min with antibody-coated sheep erythrocytes, various stages of attachment and ingestion of erythrocytes could be observed. A variable number of erythrocytes were bound to the macrophages in a "rosette" pattern (19–21) (Fig. 1). Erythrocyte/macrophage attachments appeared to involve one or several discrete segments of the respective plasma membranes (Fig. 1). Likewise, the apposition of macrophage pseudopods to erythrocytes was not uniform and continuous, but occurred at focal points of close contact alternating with areas of wider separation of the two plasma membranes (Fig. 1). At the time points studied, a number of erythrocytes appeared to be completely enclosed within phagocytic vacuoles (Fig. 1).

Examination at higher magnification of the regions of focal apposition between macrophages and erythrocytes after fixation in glutaraldehyde-tannic acid revealed a distinctive specialization of the macrophage plasma membrane. The membrane segments which were closely apposed to opsonized erythrocytes were underlined on their cytoplasmic side by a prominent band of electron-dense material, ~25–35 nm thick (Fig. 2). The appearance of these subplasmalemmal densities was somewhat variable. Some consisted of a highly osmiophilic, compact material with barely recognizable substructure (Fig. 2*a* and *b*), while others had regularly spaced "bristles" as characteristically found in clathrin coats (14, 16, 22, 23) (Figs. 2*c* and 3*c*). In addition, fingerlike processes extending from the erythrocyte surface (cf. references 19, 21, 24) were seen to project into deep invaginations of the macrophage plasma membrane, which were always surrounded by a typical bristle-coat, at least at their distal end (Fig. 3*b* and *d*). It is not clear at present whether these invaginated contacts and the "flat" regions of contact are separate phenomena or are different time points of the same process. Dense subplasmalemmal coats were not observed outside regions of close apposition with erythro-

cytes (with the exception of coated pits). A quantitative evaluation showed that ~40% of the macrophage-erythrocyte contacts were devoid of cytoplasmic coat (Table I). However, in serial sections, contacts that were apparently uncoated in one section had discrete patches of coat material in adjacent sections (Fig. 3). Likewise, apparently unspecialized appositions often turned out to be superficial regions of invaginated contacts between erythrocyte processes and macrophage coated pits (Fig. 3). When serial sections are examined, therefore, virtually all macrophage-erythrocyte interactions are seen to involve a coated segment of the macrophage plasma membrane.

Since clathrin-coated pits in macrophages and other cells are selectively resistant to the membrane-perturbing effect of the polyene antibiotic, filipin (25, 26), we examined the ultrastructural appearance of coated macrophage-erythrocyte contacts following incubation with filipin. As previously described (26), exposure of prefixed macrophages to filipin resulted in a marked alteration of cholesterol-containing membranes with loss of their characteristic trilaminar appearance. Fig. 4 shows an area of interaction between an erythrocyte and a macrophage in a preparation treated with filipin. The trilaminar appearance of the macrophage plasma membrane is disrupted by filipin except at the level of its coated segments, which appear, therefore, resistant to the effects of the antibiotic. The erythrocyte membrane was less severely affected by filipin than the macrophage plasma membrane (concerning the limited reactivity of the erythrocyte plasma membrane to filipin, see reference 27).

The presence of a dense mat of microfilaments in the regions of cytoplasm involved in particle binding and ingestion is well known (12, 28–34) and was confirmed in this study. In addition, however, we frequently observed distinct condensations of the filamentous network just beneath segments of pseudopodal membrane which were in contact with erythrocytes (Fig. 5). The mass of condensed filaments often tapered away from the region of contact, assuming a triangular shape, with the base on the subplasmalemmal coat (Fig. 5).

## DISCUSSION

During phagocytosis of antibody-coated erythrocytes by peritoneal macrophages, the plasma membrane of the advancing pseudopods establishes focal contacts with the erythrocyte membrane. At these sites, a specific interaction occurs between the Fc-portion of erythrocyte-bound antibodies and Fc-receptors at the surface of the macrophage. According to the zipper model of phagocytosis (11–13), repeated particle-phagocyte interactions are essential for the progression of pseudopods over the entire surface of the particle to be ingested. Our results indicate that macrophage-erythrocyte interactions, whether they involve relatively flat segments of the respective plasma membranes or invaginated contacts, are characterized by the presence of an electron-dense coat beneath the inner, cytoplas-

FIGURE 3 Consecutive serial sections through a region of macrophage-erythrocyte interaction. The arrows in *a* indicate three focal areas of close apposition between the macrophage (*M*) and the erythrocyte (*E*). No distinct coat is visible underneath the macrophage plasma membrane in these areas. However, examination of the successive sections (*b–d*) clearly demonstrates that all three regions of contact are associated with a coated segment of the macrophage plasma membrane. The contact to the left in *a* becomes underlined by a fuzzy material in *b* and by a distinct coat (bracket) in *c*. The apparently uncoated appositions in the middle and on the right of the picture in *a* (arrows) turn out to represent specialized invaginated contacts between fingerlike erythrocyte processes and macrophage bristle-coated pits (*CP*) as revealed by the sections in *b* and *d*, respectively.  $\times 42,000$ .

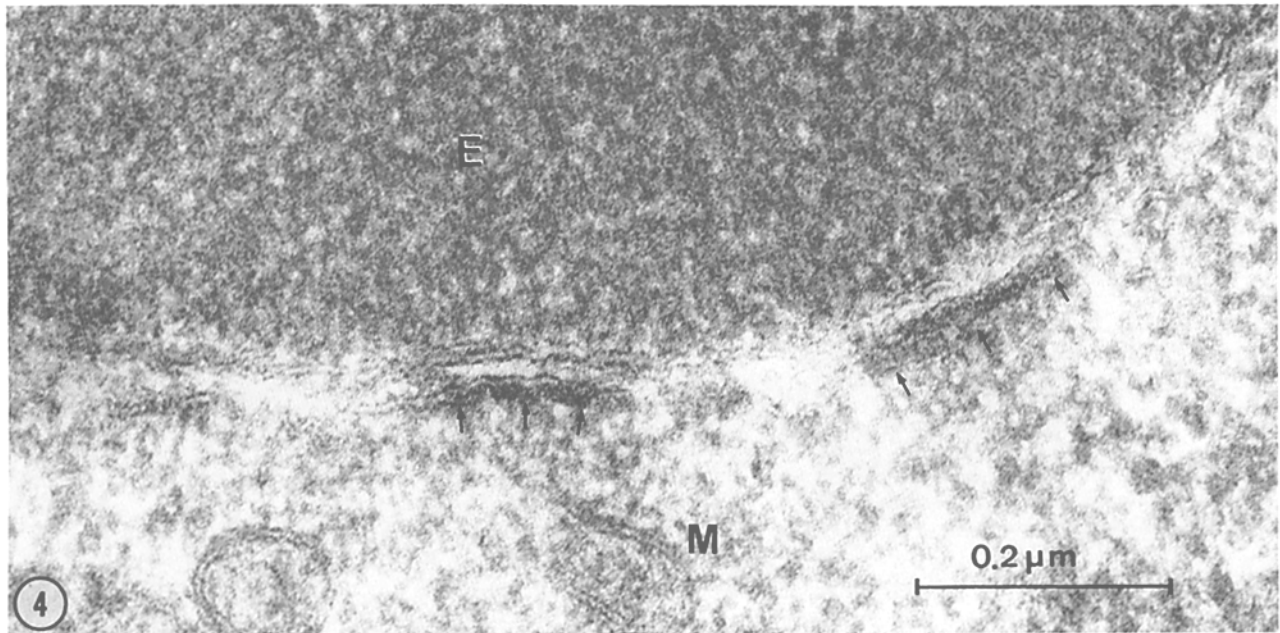


FIGURE 4 Region of macrophage-erythrocyte interaction after filipin treatment. The trilaminar appearance of the macrophage plasma membrane is lost due to the interaction with filipin, except at the level of two coated segments (arrows). The erythrocyte membrane is only slightly affected by filipin (see reference 27). *E*, erythrocyte. *M*, macrophage.  $\times 163,000$ .

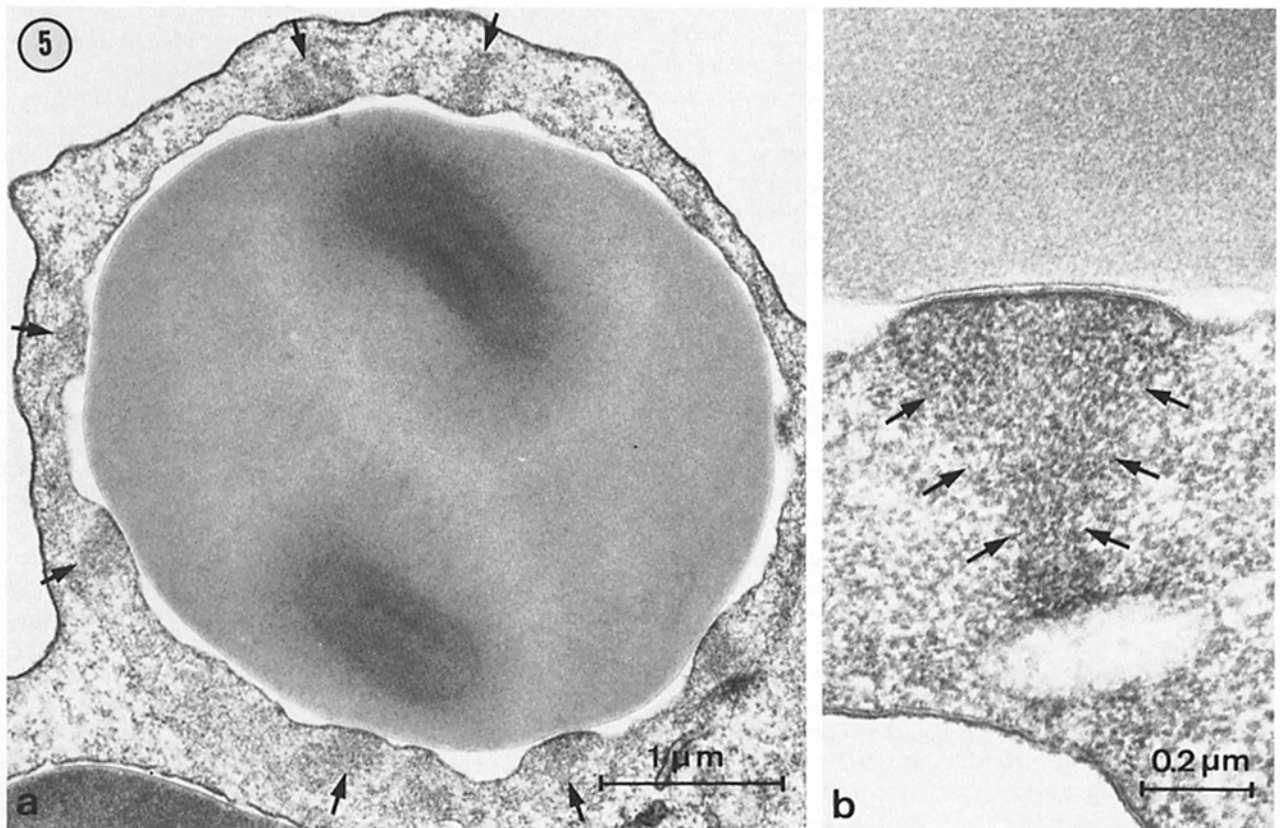


FIGURE 5 The picture in *a* shows the occurrence of several distinct condensations of the microfilament network in regions of macrophage pseudopods establishing focal contacts with an erythrocyte (arrows). The picture in *b* is a higher magnification showing the characteristic triangular shape (with the base facing the region of contact and resting on the subplasmalemmal coating) of the microfilament condensation (arrows). (*a*)  $\times 24,000$ . (*b*)  $71,000$ .

mic leaflet of the macrophage plasma membrane. In the case of invaginated contacts, the cytoplasmic coat surrounding the invaginations of the macrophage surface was morphologically indistinguishable from typical clathrin coats (14, 16, 22, 23).

However, in flat regions of contact periodically spaced bristles were not always visible and the coat sometimes had a more compact, amorphous appearance. Nevertheless, plasma membranes of both types of contact zone retained their trilaminar

structure when exposed to filipin and it is known that filipin does not disrupt coated pits (25, 26). Whether both contact zones do, indeed, involve clathrin coats will be determined by future immunocytochemical studies using anticlathrin antibodies. Concerning the functional significance of these coated membrane segments, one possibility is that they play a role in the recruitment of Fc- and possibly other receptors at sites of interaction with erythrocyte-bound ligands, and/or in maintaining their topographical distribution in these areas. A role in receptor clustering is well established for the clathrin cage surrounding coated pits (14–16, 35) so it is conceivable that clathrin coats might also be involved in receptor-mediated phagocytosis. However, clathrin is not necessarily the only candidate for this function, since subplasmalemmal dense material apparently unrelated to clathrin is also associated with receptor-rich membrane areas (36, 37), where it could help to maintain the spatial organization of the receptors within the plane of the membrane (37).

During this study we observed frequently, but not consistently, a marked condensation of the microfilament meshwork at regions of focal macrophage-erythrocyte contact. The zipper model of phagocytosis (11–13) predicts that ligand-receptor interaction triggers an assembly or recruitment of contractile proteins (30, 32–34), which would be in turn responsible for stepwise pseudopod extension during particle engulfment. That the observed condensations of microfilaments might reflect such an assembly or recruitment is an attractive possibility that should be explored further.

While this manuscript was in preparation, a paper appeared (38) reporting the occurrence of clathrin coats (as seen with the quick-freeze, deep-etch, rotary-shadowing technique) around nascent phagosomes in macrophages engaged in phagocytosis of latex beads. However, our experimental conditions differ from those of Aggeler and Werb (38), since the phagocytosis of opsonized erythrocytes is mediated by a specific Fc receptor-antibody interaction, whereas during the phagocytosis of latex beads, specific receptor-ligand interactions are not known to occur (2, 10, 39). Moreover, the differential sensitivity of immune (Fc-mediated) and nonimmune phagocytosis to 2-deoxyglucose inhibition (40) as well as the occurrence of other distinguishing properties (reviewed in references 39, 41) suggest that the two types of phagocytosis might not be mediated by the same mechanism. Nevertheless, results from these two different systems both point to the involvement of clathrin-coated membrane segments in phagocytosis.

In conclusion, our observations reveal for the first time the occurrence of a distinctive structural specialization of the macrophage plasma membrane at sites of ligand-receptor interaction during immune phagocytosis. We propose that this specialization represents the morphological counterpart of the zipper mechanism of phagocytosis demonstrated by functional studies (11–13).

We are grateful to Dr. J.-E. Ryser for his help in the initial experiments, to Dr. P.-F. Piguet for advice and to Dr. D. Brown for critical reading of the manuscript. We also thank J. Rial and M. Schramm for technical assistance and M. Sidler-Ansermet for excellent photographic work.

This study was supported by grants from the Swiss National Science Foundation (nr. 3.668.80, 3.324.78, and 3.059.81).

Received for publication 9 December 1982.

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