

# Initial Events during Phagocytosis by Macrophages Viewed from Outside and Inside the Cell: Membrane-Particle Interactions and Clathrin

JUDITH AGGELER and ZENA WERB

*Department of Anatomy and Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143*

**ABSTRACT** The initial events during phagocytosis of latex beads by mouse peritoneal macrophages were visualized by high-resolution electron microscopy of platinum replicas of freeze-dried cells and by conventional thin-section electron microscopy of macrophages postfixed with 1% tannic acid. On the external surface of phagocytosing macrophages, all stages of particle uptake were seen, from early attachment to complete engulfment. Wherever the plasma membrane approached the bead surface, there was a 20-nm-wide gap bridged by narrow strands of material 12.4 nm in diameter. These strands were also seen in thin sections and in replicas of critical-point-dried and freeze-fractured macrophages. When cells were broken open and the plasma membrane was viewed from the inside, many nascent phagosomes had relatively smooth cytoplasmic surfaces with few associated cytoskeletal filaments. However, up to one-half of the phagosomes that were still close to the cell surface after a short phagocytic pulse (2–5 min) had large flat or spherical areas of clathrin basketwork on their membranes, and both smooth and clathrin-coated vesicles were seen fusing with or budding off from them. Clathrin-coated pits and vesicles were also abundant elsewhere on the plasma membranes of phagocytosing and control macrophages, but large flat clathrin patches similar to those on nascent phagosomes were observed only on the attached basal plasma membrane surfaces. These results suggest that phagocytosis shares features not only with cell attachment and spreading but also with receptor-mediated pinocytosis.

Phagocytosis, the ingestion of foreign particles by cells, has been the subject of active investigation since Metchnikoff (1) first described the protective role of the “wandering cells” of starfish larvae over a century ago. In mammals, the major wandering phagocytes are macrophages and polymorphonuclear granulocytic leukocytes, and their roles in defense against microorganisms and in inflammation are well documented (2–5). Macrophages are long-lived cells specialized in ingesting and sequestering a wide variety of foreign bodies and cell debris, but the exact cellular mechanism by which they engulf such particles is incompletely understood.

During the initial steps of phagocytosis, particles first bind to the plasma membrane surface and then enter the cell, enclosed within a plasma membrane-derived phagosomal membrane. At least three specific receptors for the Fc portion of various classes of IgG have been identified, and their roles in the uptake of IgG-coated erythrocytes have been described

(6, 7). Receptors recognizing the C3b and C3d fragments of complement can also mediate phagocytosis of erythrocytes by activated macrophages (8). A receptor that recognizes mannose residues of glycoconjugates may be responsible for ingestion of yeast cell walls (9, 10), and so-called nonspecific receptors that mediate the uptake of latex beads and other particles are also known (5). The overall process of phagocytosis requires metabolic energy (5) and is temperature-sensitive, with a threshold at 18°C (11). Divalent cations are not required for cultured macrophages to bind particles (12), but Ca<sup>2+</sup> and especially Mg<sup>2+</sup> probably play a role in subsequent ingestion of particles (12, 13). Opsonization of bacteria by factors in serum may facilitate uptake (13, 14); macrophages cultured in serum-free medium ingest particles, but at a reduced rate (12, 13).

Phagocytosis of particles by macrophages has been likened to cell spreading on a substratum, because a dense network of actin filaments appears beneath the plasma membrane in both

cases (15). This has led to the suggestion that actin is directly involved in the plasma membrane movement that accompanies the phagocytic process (16). Hartwig et al. (17) have presented a model of phagocytosis in which an actin-binding protein interacts with cytoskeletal elements, and subsequent reorganization of subplasmalemmal actin filaments forces the plasma membrane up over the particle, forming the phagosome. Their evidence indicates that similar actin-binding proteins may function during both phagocytosis and cell spreading (18). Recently, Boyles and Bainton (19) studied the formation of focal attachment sites for cytoskeletal filaments on the plasma membrane of spreading polymorphonuclear leukocytes and found that similar plaques are formed on the cytoplasmic surface of phagosomes when these cells ingest yeast (20). Such foci also appear to act as attachment sites in spreading macrophages (21).

We have examined the plasma membranes of macrophages fixed during ingestion of latex beads using the high-resolution platinum replica techniques devised by Heuser (22, 23) to visualize the initial events of phagocytosis from both outside and inside the cell. On the external surface of phagocytosing macrophages, narrow strands of material were observed between the plasma membrane and the bead surface. On the cytoplasmic faces of nascent phagosomes, large areas of clathrin basketwork were observed. Preliminary reports of this work have been presented in abstract form (24–26).

## MATERIALS AND METHODS

### *Cell Culture and Phagocytosis*

Macrophages were harvested from CD-1 mice (Flow Laboratories, Inc., Rockville, MD) by peritoneal lavage using Dulbecco's phosphate-buffered saline (PBS) with 100 U/ml sodium heparin. Cells were plated onto (5-mm)<sup>2</sup> acid-cleaned glass cover slips in 16-mm culture wells ( $5 \times 10^5$  cells/well) and incubated overnight in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum. In some experiments, the J774.2 macrophage cell line (6) was used. Before each experiment, cells were washed free of serum and cultured briefly in modified Eagle's medium (Earle's salts) (MEM), containing 0.2% lactalbumin hydrolysate (LH), and buffered with 25 mM HEPES. For electron microscopy, latex beads (polystyrene 0.45  $\mu$ m diameter; Polysciences, Inc., Warrington, PA) were washed once in Ca<sup>2+</sup>-free MEM-LH-HEPES, sonicated briefly, and added to cells at a concentration of 0.5 mg/ml. In one experiment, 0.1- $\mu$ m diameter beads (Polysciences, Inc.) were used without prior washing. Decreasing the Ca<sup>2+</sup> concentration before breaking open cells helped to reduce small vesicle formation from plasma membrane fragments; the kinetics and extent of bead uptake in Ca<sup>2+</sup>-free MEM-LH-HEPES did not differ from that in medium containing Ca<sup>2+</sup> when measured by a turbidimetric assay (OD<sub>600 nm</sub>) (27) (data not shown).

### *Fixation, Freezing, and Replica Preparation*

After the cells were exposed to latex beads for 1–5 min at 37°C, the cover slips were washed to remove excess beads. Some cells were immediately fixed for 1 h at 22°C in 1% glutaraldehyde and 1% paraformaldehyde (pH 7.2) in medium E, composed of 155 mM NaCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 20 mM HEPES buffer, pH 7.2, which is isotonic with extracellular fluid. In other experiments, cells were transferred into medium I, composed of 100 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM EGTA, and 20 mM HEPES buffer, pH 7.0, which is isotonic with intracellular fluid, and were rapidly broken open by placing a poly-L-lysine-coated cover slip over them and pulling it away, or by gently scraping away cell tops with a loop of fine platinum wire. Broken-open cells were immediately fixed for 1 h in 1% glutaraldehyde and 1% paraformaldehyde at 22°C in medium I. After fixation, cells were rinsed briefly in distilled H<sub>2</sub>O and then 15% methanol before being rapidly frozen against a copper block that had been cooled to liquid helium temperature (28). Frozen samples were freeze-dried in a Balzers freeze-fracture apparatus (Balzers, Hudson, NH) by warming them to –90°C at a vacuum of  $2 \times 10^{-6}$  torr or better and then rotary replicated with platinum-carbon (22, 23). Replicas were removed from the glass cover slips with 50% hydrofluoric acid, and cellular material was digested away in undiluted household bleach (1 h). In most experiments, replicas were also treated with 1,4-dioxane (diethylene dioxide) to dissolve the latex beads, which otherwise adhered to the replica and obscured much of the interesting detail. This was done by floating

the replicas onto Formvar-coated copper grids, then immersing grid and replica in a small beaker of dioxane and agitating gently for 15–20 s. Cleaned replicas were examined at 100 kV in a JEOL 100B or 100C electron microscope. All micrographs of platinum replicas are printed as negatives to give an appearance comparable to that of scanning electron micrographs.

### *Thin-section Transmission Electron Microscopy*

Peritoneal macrophages were cultured in 60-mm plastic dishes, exposed to latex beds at 37°C, and washed as described above. Cells were then fixed at 22°C in 1% glutaraldehyde in 0.02–0.1 M sodium cacodylate buffer (pH 7.4) for 1 h, followed by 1 h each in 1% OsO<sub>4</sub> in acetate-veronal buffer, 1% tannic acid (Mallinckrodt Inc., Science Products Div., St. Louis, MO) (29) in 0.05 M sodium cacodylate buffer, and 1% uranyl acetate in acetate-veronal buffer. Cells were dehydrated through graded alcohols and detached from the plastic dish by adding propylene oxide. The resulting suspension was pelleted in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) and embedded in Epon before sectioning on a Porter-Blum MTB-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT).

### *Size Measurements*

The diameter and spacing of the surface strands observed between beads and cells were determined by selecting three typical electron microscope negatives made at  $\times 60,000$  and measuring all of the strands and spaces around two beads in each micrograph. These measurements were made directly on the negatives on a standard light box using a magnifying micrometer eyepiece and recorded to the nearest 0.1 mm. Strand spacing was measured from center to center as nearly as possible. 95 strands and 94 interstrand spaces were measured. Results are expressed as mean and standard deviation and the range is noted.

The number of clathrin-coated areas on the plasma membrane (excluding phagosome membranes) was quantified in two ways: (a) Micrographs of broken-open cells taken at  $\geq \times 40,000$  were projected onto blank paper, and exposed membrane and clathrin patches, pits, and vesicles were traced. Micrographs taken at  $< \times 36,000$  could not be used because preliminary results indicated that some coated areas were not visible in such views. At  $\times 40,000$ , each micrograph represents 3.8  $\mu$ m<sup>2</sup> of plasma membrane. The tracings were then cut out and individually weighed. The results are expressed as percentage of plasma membrane surface covered with clathrin basketwork (mean and standard error). (b) The profiles of latex bead phagosomes and associated clathrin coats were measured on thin-section electron micrographs printed at  $\times 50,000$ . For approximately one-third of these beads (17 out of 51) the presence of clathrin could not be unambiguously determined because of dense cytoplasmic background or unclear sectioning of the plasma membrane. Results are expressed as percentage of phagosomes positive for clathrin coating.

## RESULTS

### *Phagocytosis Viewed from Outside the Cell*

Replicas of the outside surface of macrophages viewed by transmission electron microscopy provided high-resolution images of the plasma membrane similar to those described previously for fibroblasts (23). These surfaces were covered with many small bumps, slightly larger than intramembranous particles, that were distributed randomly against a homogeneous background of granular platinum (Fig. 1). The surface of 0.45- $\mu$ m diameter beads either on cells (Figs. 1–3) or on the cover slip away from cells (Fig. 4a) had a rough, cobbled appearance with occasional larger bumps. Low magnification views of phagocytosing macrophages showed that beads were most abundant on the rounded perinuclear portion of ingesting cells, as previously described (30), although beads were also found on the thinner, spreading lamellipodia of these cells (not shown). There was a marked tendency for small beads to be found at the base of microvilli and surface ruffles. We could not use particles  $>1.0$ - $\mu$ m diameter in these studies because replicas of structures taller than  $\sim 0.5$   $\mu$ m collapse on themselves and produce confusing images.

All stages of bead uptake, from early attachment to late engulfment, were observed when replicas of phagocytosing macrophages were examined at high magnification (Figs. 1–3).

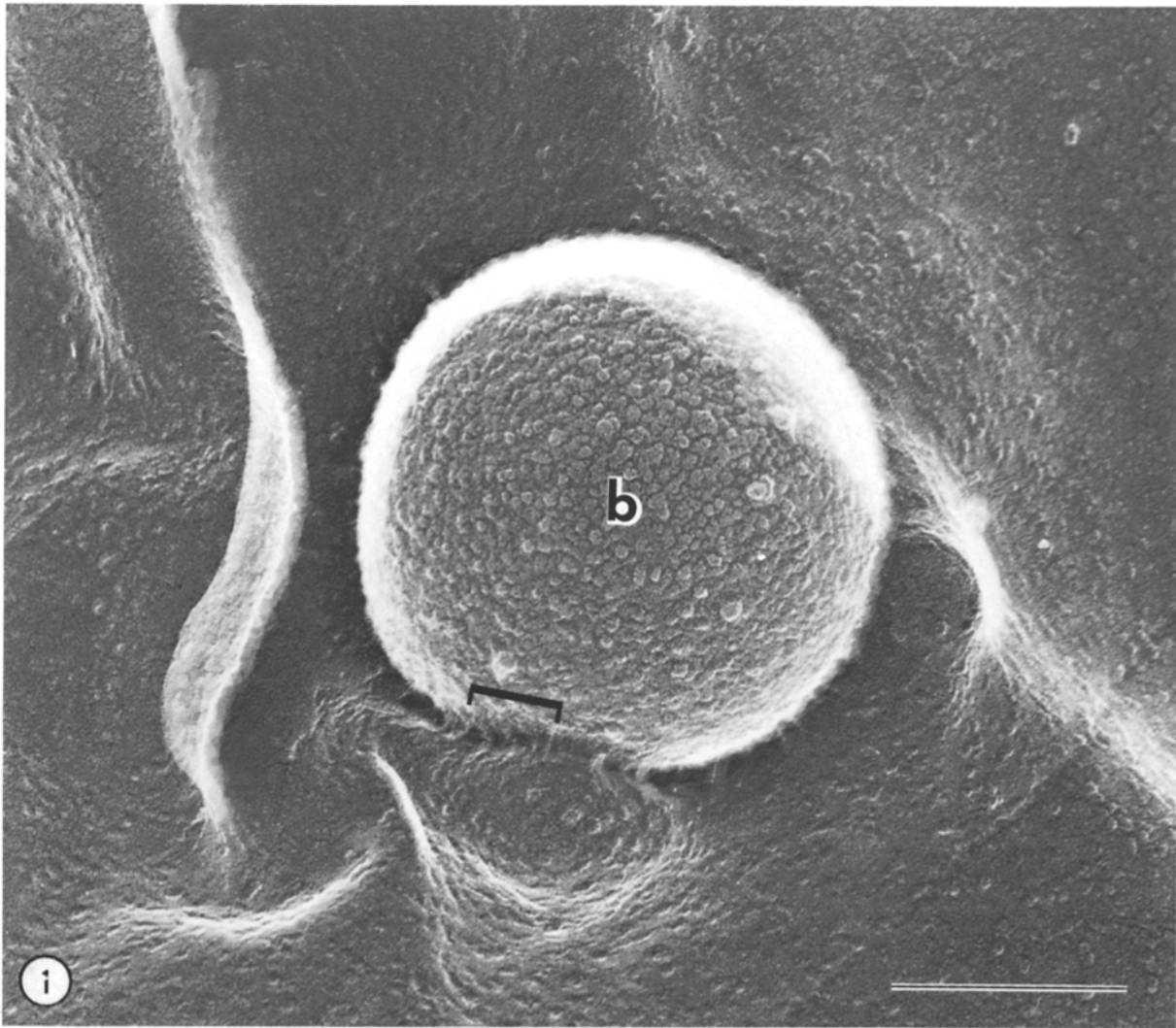


FIGURE 1 Rotary-deposited platinum replica of the outside surface of a freeze-dried, fixed macrophage ingesting a 0.45- $\mu\text{m}$  latex bead (*b*). Some of the rough material on the bead surface may be adsorbed protein. Where the cell membrane closely approaches the bead surface, a 20-nm gap is present, spanned by 12.4-nm strands (bracket). Bar, 0.2  $\mu\text{m}$ .  $\times 160,000$ .

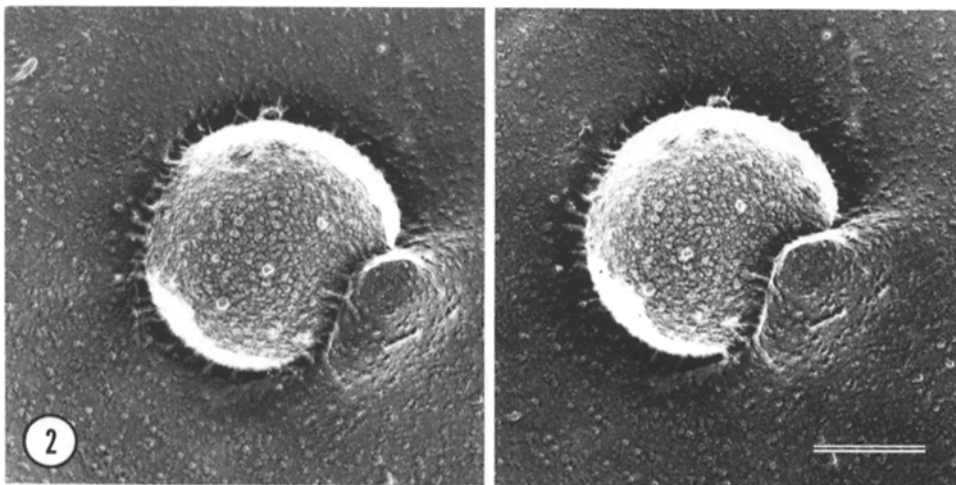


FIGURE 2 Replica of the outside surface of a freeze-dried macrophage ingesting a 0.45- $\mu\text{m}$  latex bead shown in stereo to illustrate more clearly a small pseudopodium advancing over the bead surface. Bar, 0.2  $\mu\text{m}$ .  $\times 72,000$ .

At the earlier stages of attachment, small lamellipodia and surface ruffles were often observed creeping up the sides of the beads (Figs. 1 and 2), whereas at later stages the beads usually appeared to be sinking into the cytoplasm below the plasma

membrane surface (Fig. 3 *c* and *d*). Invariably, there was a gap of  $\sim 20$  nm between the bead and the adjacent plasma membrane. This gap was bridged by many narrow strands of material ( $12.4 \pm 3.5$ -nm diameter, mean  $\pm$  SD), which were

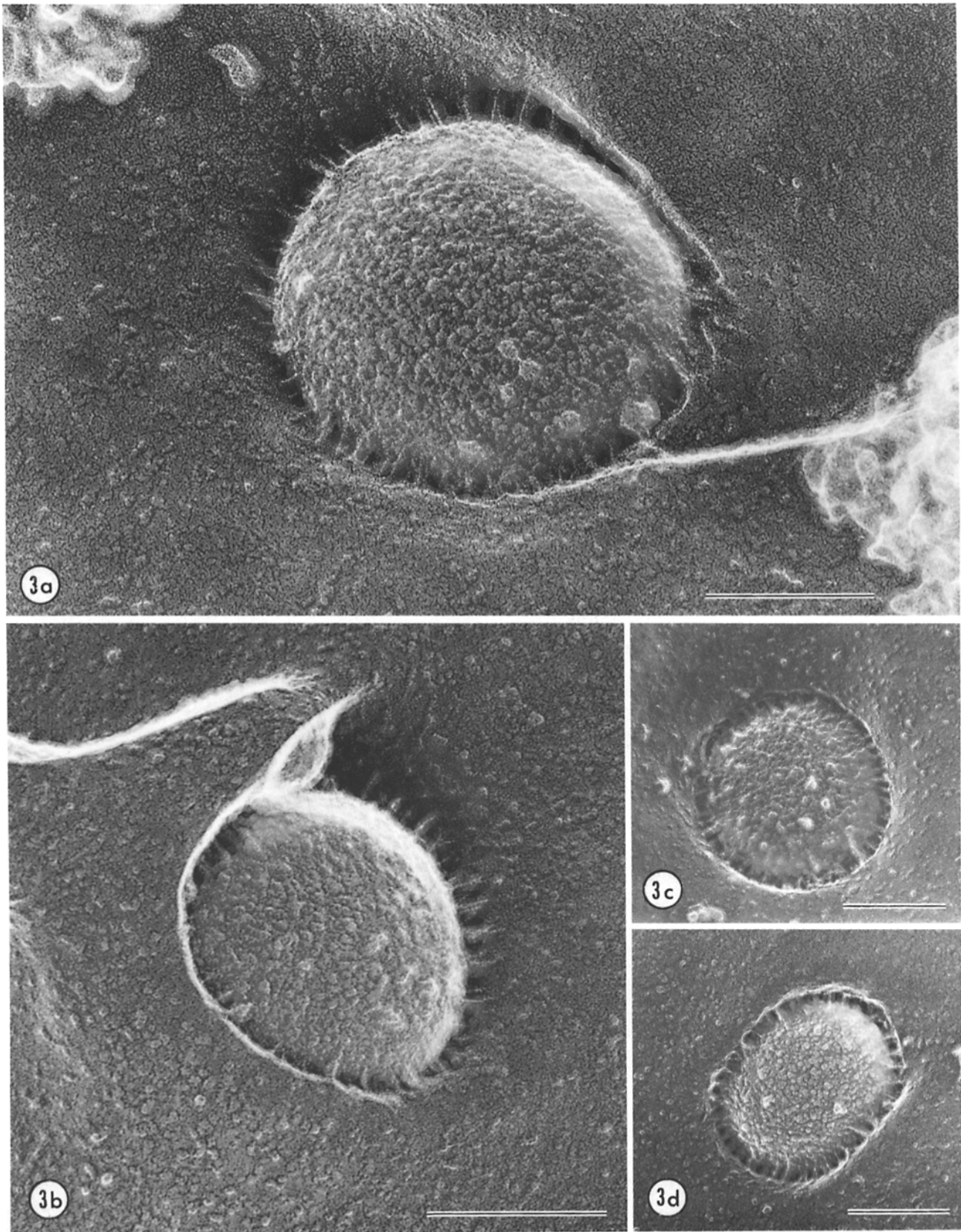


FIGURE 3 Replicas of the outside surface of freeze-dried macrophages showing later stages of latex bead ingestion. (a and b) Intermediate stages in bead ingestion showing small surface ruffles engulfing beads.  $\times 160,000$ . (c and d) Late stages in ingestion showing beads sinking below the cell surface. Bars,  $0.2 \mu\text{m}$ .  $\times 90,000$ .

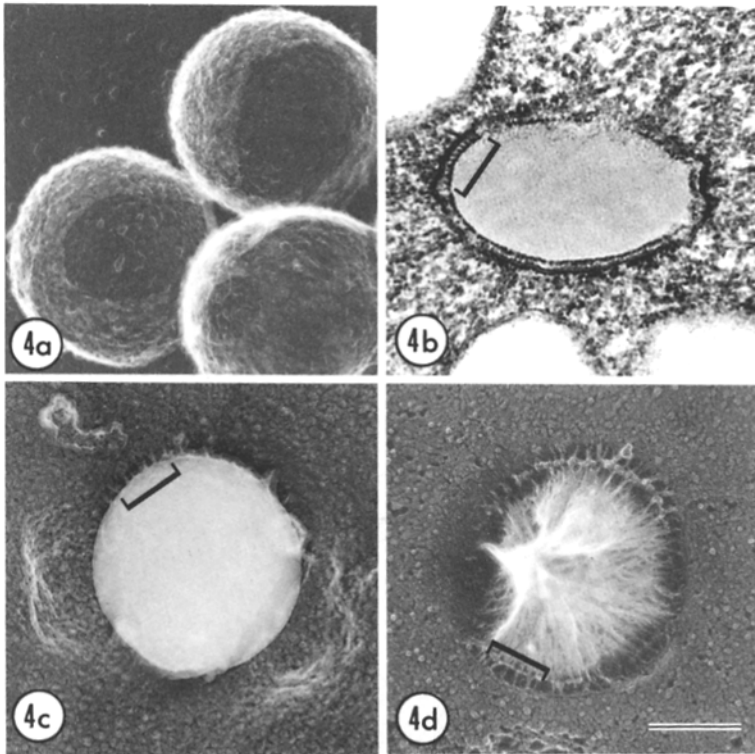


FIGURE 4 Demonstration that 12.4-nm strands are present only during ingestion of beads by cells. (a) 0.45- $\mu\text{m}$  beads isolated on the surface of the glass cover slip with no strands. The presence of 12.4-nm strands (brackets) in replicas of freeze-dried macrophages is not unique to quick-frozen samples because similar structures are also observed surrounding beads in conventional thin-section electron micrograph (b), in replica of a cell prepared by ethanol dehydration and critical point drying (c), and in replica of a freeze-fractured cell (d). In c and d, the electron-dense beads have not been dissolved with dioxane and still adhere to the platinum replica. Bar, 0.2  $\mu\text{m}$ .  $\times 60,000$ .

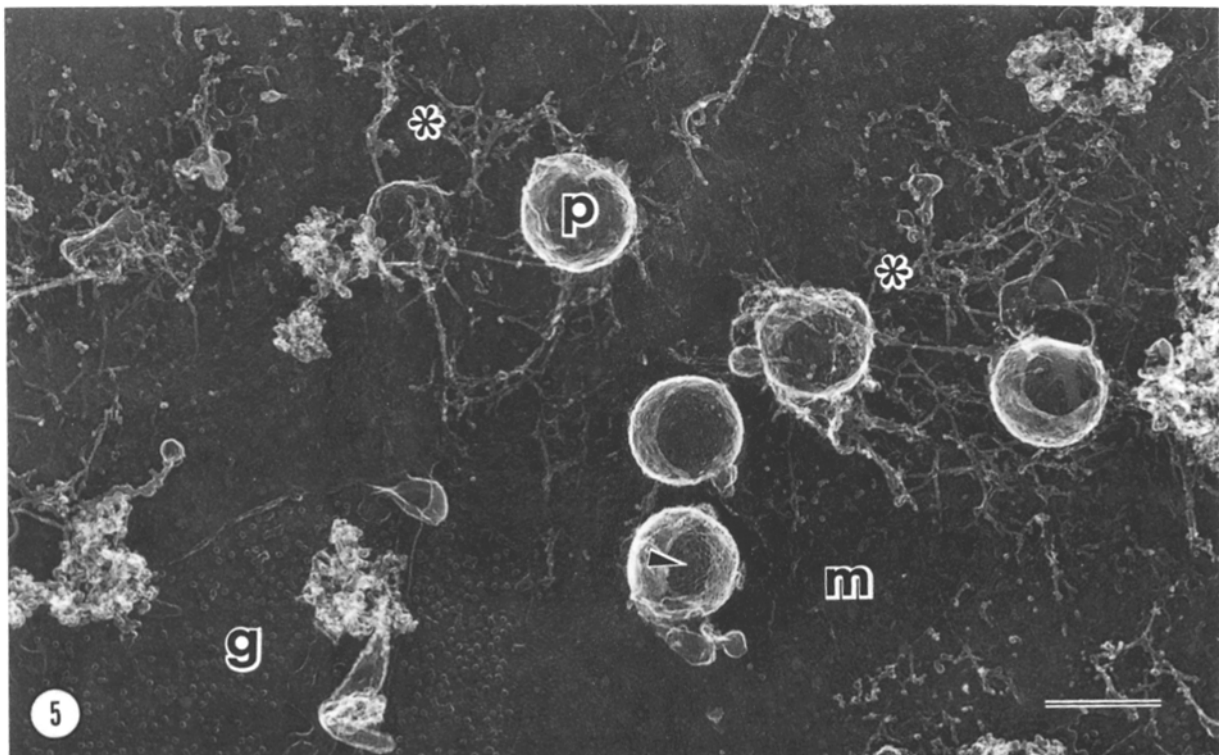


FIGURE 5 Low magnification view of a replica of the cytoplasmic surface of upper cell membrane (m) spread on a poly-L-lysine-coated glass cover slip (g), showing several nascent phagosomes (0.45  $\mu\text{m}$ ) (p) and scattered foci of cytoskeletal filament convergence (asterisks). A few cytoskeletal filaments can be seen adhering to the phagosomes. The pebbled appearance of the cover slip is clearly distinct from the area of smooth open membrane displayed in this replica. A large patch of clathrin basketwork (arrowhead) is present on one phagosome. Bar, 0.5  $\mu\text{m}$ .  $\times 30,000$ .

spaced  $\sim 25$  nm apart (range, 12–67 nm). These strands were always visible at the edge of the forming phagosome but were not observed between adjacent beads or between beads and the cover slip (Fig. 4a). Once these structures had been recognized

in replicas of freeze-dried cells, we also found similar strands in conventional thin-section electron micrographs and in replicas of critical-point-dried or freeze-fractured cells (Fig. 4 b–d).

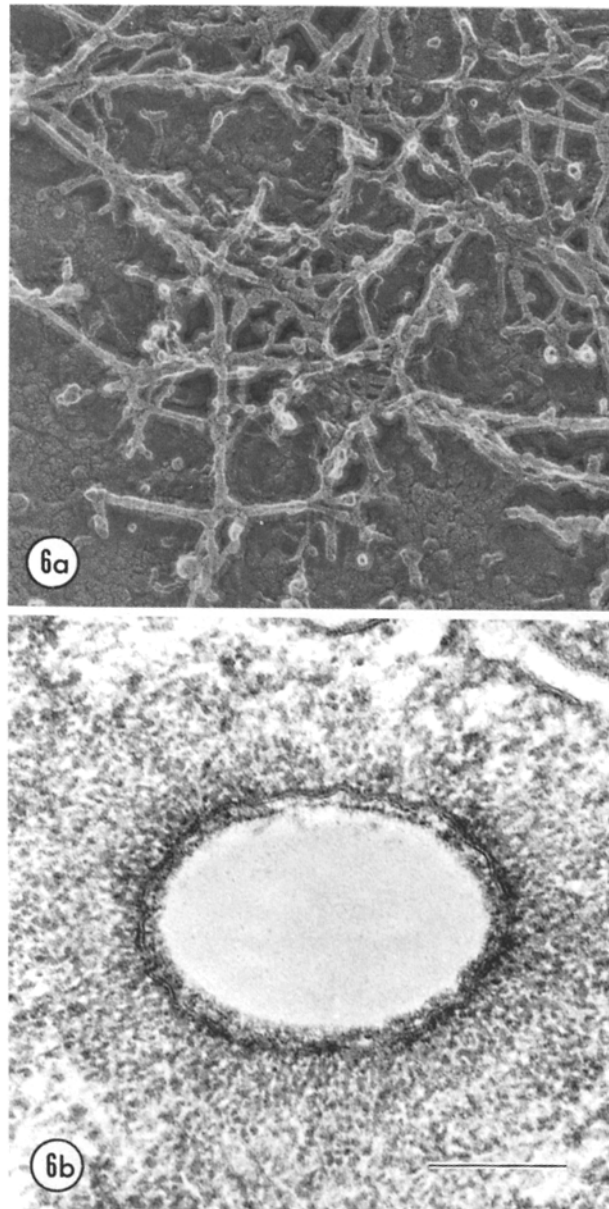


FIGURE 6 Demonstration of cytoskeletal filament association with macrophage membranes. (a) Replica of the cytoplasmic surface of the nonadherent upper cell membrane of a phagocytosing macrophage showing a starlike focus of cytoskeletal filament convergence. (b) Thin-section transmission electron micrograph of a phagocytosing macrophage showing cytoskeletal meshwork radiating out from a bead phagosome. Bar, 0.2  $\mu\text{m}$ .  $\times 90,000$ .

### Phagocytosis Viewed from Inside the Cell

In samples of the upper surfaces of macrophages prepared by adhesion to poly-L-lysine-coated cover slips, irregular areas of smooth cytoplasmic membrane were readily distinguished from the adjacent pebbled-appearing cover slip, and individual bead phagosomes still attached to these membranes were easily identified (Fig. 5). Scattered foci of cytoskeletal filament convergence were widely distributed over the plasma membrane surface in such samples (Figs. 5 and 6a). As reported for spreading polymorphonuclear leukocytes (19) and macrophages (21), these dense foci were also abundant on the adherent bottom plasma membranes of these macrophages (not shown).

**ASSOCIATION OF CYTOSKELETAL FILAMENTS WITH PHAGOSOMES:** Because many reports have suggested that microfilaments are involved in the phagocytic process (15, 17, 31), we attempted to identify specific associations between filamentous cytoskeletal elements and nascent phagosome membranes in broken-open cells. Surprisingly, there were few clear examples of filament attachments to the cytoplasmic surfaces of nascent phagosomes in these preparations, although an organized meshwork of cytoskeleton was seen radiating out from nascent phagosomes in the area of organelle exclusion in macrophages observed by standard thin-section electron microscopy (Fig. 6b), as described by others for larger particles (15) and for the basal surfaces of attached cells (16). The reason for the small number of filament attachments to phagosomes observed in replicas of broken-open cells is not known at present, but this lack may be largely an artifact of our procedures for breaking cells open.

**DISTRIBUTION OF COATED VESICLES IN MACROPHAGES:** One cytoplasmic structure that can be dramatically visualized by the deep-etch replica technique is the clathrin basketwork surrounding coated vesicles (23). Coated vesicles have been noted in macrophages studied in thin sections (32, 33), but they have been relatively difficult to see against the dense cytoplasm of these cells. In replicas of broken-open macrophages, however, it was apparent that clathrin patches and vesicles were remarkably abundant (Fig. 7a), involving 1%–4% of the total plasma membrane surface (Table I). By using a hypotonic fixation buffer (0.02 M sodium cacodylate) and a postfixation protocol that included 1% tannic acid, we were also able to see numerous coated pits and vesicles in thin-section electron micrographs (Fig. 7b and c). Both the number and area of clathrin basketworks present on the adherent lower surfaces of macrophages were significantly greater than on the free upper plasma membrane surfaces (Table I). This was especially true for large flat patches of hexagonal basketwork, which were never observed on upper surfaces (Fig. 8). After a phagocytic stimulus (2–5 min), the number and area of coated patches on the adherent cell bottoms was increased twofold over controls (Table I).

**ASSOCIATION OF CLATHRIN WITH PHAGOSOMES:** Although the cytoplasmic surfaces of phagosomes were often quite smooth (Figs. 5 and 9a), examination of both replicas and thin-section micrographs indicated that up to one half of the phagosomes observed after a short (2–5 min) phagocytic pulse had areas of clathrin basketwork associated with them

TABLE I  
Extent of Clathrin Basketwork on Macrophage Plasma Membrane

Cells	Surface area covered by clathrin basketworks (%)		P*
	Upper plasma membrane	Lower plasma membrane	
Control	1.3 $\pm$ 0.3 (24)	3.5 $\pm$ 1.0 (14)	0.001
Phagocytosing (2–5 min)	1.6 $\pm$ 0.3 (40)	9.7 $\pm$ 1.8 (31)	<0.001
P*	>0.2	0.02	

Control or phagocytosing macrophages were broken open by the poly-L-lysine-coated cover slip method and replicas of the cytoplasmic surfaces of both the upper and lower plasma membranes were observed by transmission electron microscopy. Micrographs were scored as described in Materials and Methods. Values tabulated represent mean  $\pm$  SE and numbers in parentheses indicate number of micrographs scored.

\* P was determined by the unpaired two-sample Student's *t* test.

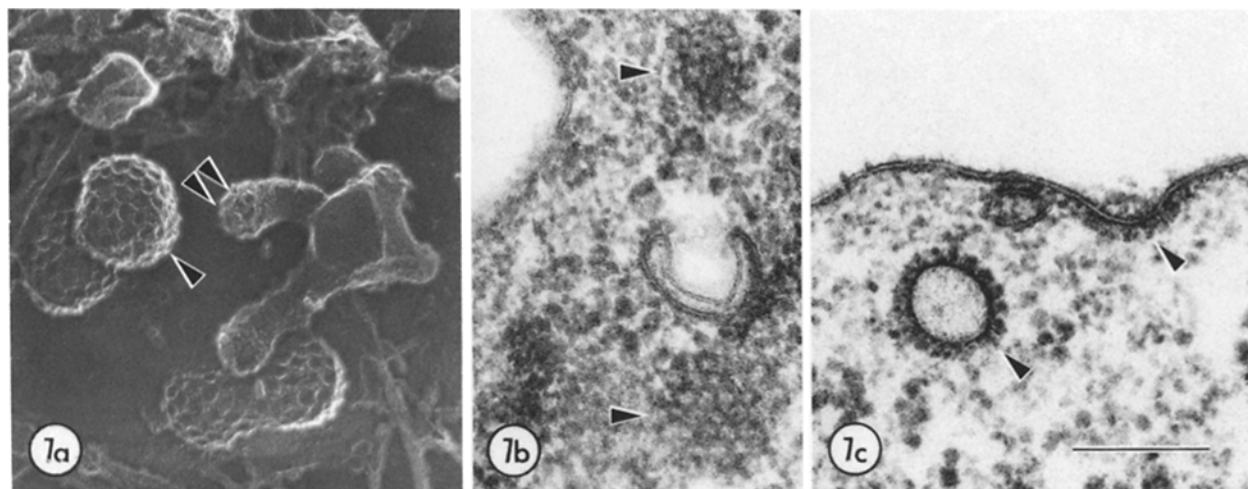


FIGURE 7 Demonstration of coated pits and vesicles in macrophages. (a) Replica of the cytoplasmic surface of the adherent bottom of a phagocytosing macrophage showing several forming coated pits and vesicles (arrowhead), including a small vesicle (double arrowhead) budding off from what appears to be a remnant of smooth endoplasmic reticulum or Golgi apparatus. (b) Thin-section electron micrograph showing two en face views of coated vesicles (arrowheads). (c) Thin-section transmission electron micrograph of two coated pits (arrowheads) in a cell of the mouse macrophage line J774.2. The granular tannic acid-labeled content of these coated pits indicates that both are still in continuity with the cell surface. Bar, 0.2  $\mu\text{m}$ .  $\times 90,000$ .

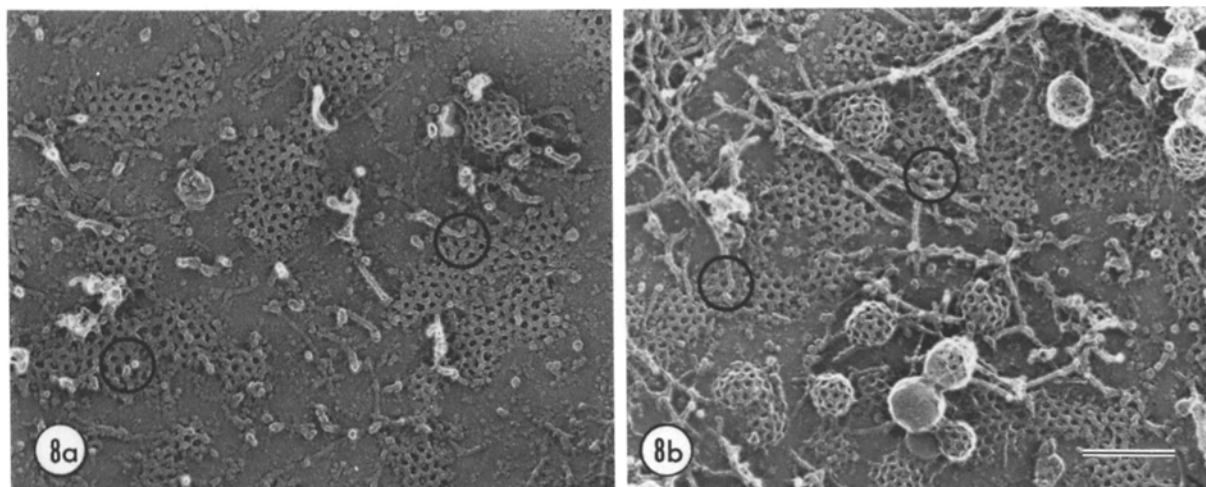


FIGURE 8 Replicas of the cytoplasmic surface of adherent bottoms of mouse peritoneal macrophages after 5 min of phagocytosis, showing areas where the density of clathrin basketwork is very high. In a, 19% of the plasma membrane is clathrin-coated; in b, 28% is coated. It is noteworthy that much of the basketwork observed on these lower plasma membrane surfaces appears as flat patches, as in a. Possible remnants of cytoplasmic filament attachment to clathrin basketwork are indicated by circles. Bar, 0.2  $\mu\text{m}$ .  $\times 60,000$ .

(Table II). These areas were often quite large, in several cases covering the entire observable surface of the phagosome, an area equivalent to two to four coated vesicles (Fig. 9c). Deeper coated pits and vesicles were also found on phagosome membranes (Figs. 9b and 10). In Fig. 10, clusters of coated vesicles are seen budding off from the cytoplasmic face of bead phagosomes observed in both replicas and thin-sections. In one experiment, we examined phagocytosis of very small beads (0.1- $\mu\text{m}$  diameter). Most of these beads were surrounded by smooth, closely adherent phagosomes (Fig. 9d), but uptake within coated vesicles was occasionally observed (Fig. 9e).

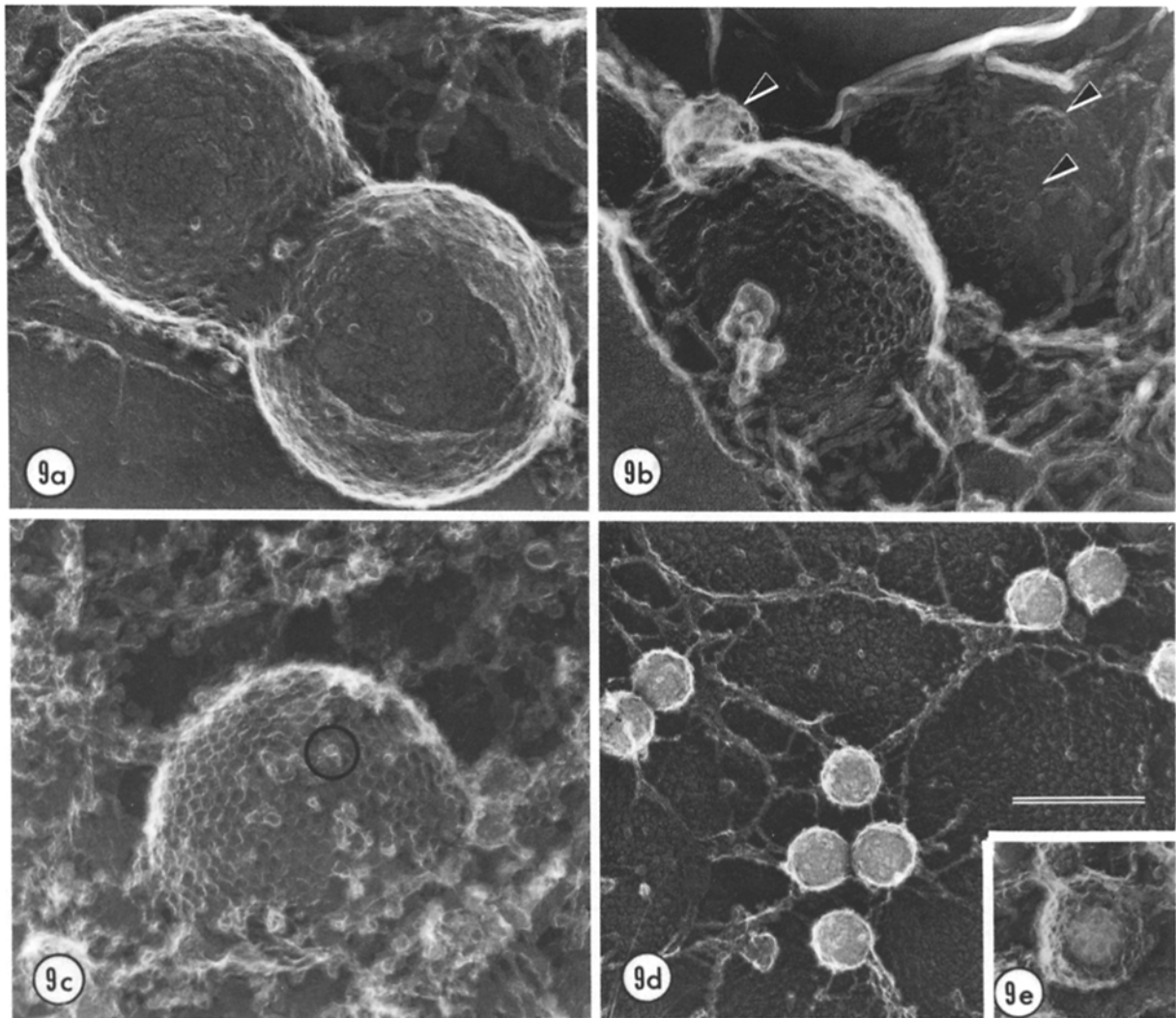
Because our techniques for breaking open cells disrupted many cytoplasmic structures, it was often difficult to ascertain the distance of a phagosome from the cell surface in replicas. However, in a number of cases a fortuitous break preserved the relationship between the ingested bead and the plasma

membrane, and a large clathrin patch could be seen just under the plasma membrane adjacent to the advancing lip of the forming phagosome (Fig. 11; see also Fig. 9b). This proximity of large clathrin patches to the advancing phagosome lip was often observed by thin-section transmission electron microscopy as well (Figs. 10a and b, 12a, and Table II). The association between clathrin and phagosomes was not limited to ingestion of latex beads of various sizes, as shown by the large clathrin patch visible on the advancing plasma membrane of a macrophage ingesting a dead cell (Fig. 12b).

## DISCUSSION

### *Particle Binding in Phagocytosis: An External View of Cell Surface Interactions*

Phagocytosis of a variety of particles is known to be mediated



**FIGURE 9** Replicas of the cytoplasmic surfaces of bead phagosomes viewed from inside broken-open macrophages. Many of the phagosomes visualized in this way have a relatively smooth surface (a), but large patches of clathrin basketwork can be seen on some (b and c). In b two adjacent phagosomes with clathrin-coated patches, pits, and vesicles (arrowheads) are shown just beneath the torn edge of the plasma membrane (lower left). (c) A phagosome entirely covered with clathrin basketwork. Possible remnant of a cytoskeletal filament attachment to this phagosome is indicated by a circle. In d and e, replicas of macrophages that had ingested 0.1- $\mu\text{m}$  latex beads are shown; these replicas were not treated with dioxane so the electron-dense latex beads are still present. Most of these small beads were taken up in individual, smooth phagosomes (d), but e shows a bead that appears to have been trapped and taken up within a coated vesicle. Bar, 0.2  $\mu\text{m}$ .  $\times 90,000$ .

by specific receptors on the macrophage surface (5–8, 14), although the biochemical nature of the so-called nonspecific receptor that mediates uptake of latex beads is not yet known (5). This interaction may involve recognition of areas of negative charge density on the bead surface, but binding to secreted macrophage proteins that adhere to the beads is also a possibility. Phagocytosis of latex beads does not seem to be mediated by the polyanion receptor described by Brown et al. (34), however, because ingestion was undiminished by treating cells with heparin or polyinosinic acid, two potent inhibitors of the uptake of acetylated low density lipoprotein mediated by this receptor (J. Aggeler, unpublished observations).

The elegant experiments of Griffin and colleagues (35, 36) showed: that phagocytosis of particles by macrophages requires extensive interaction between specific receptors on the cell surface and recognition sites on the particle; and that engulfment stops if either receptors or recognition sites are not distributed continuously over the interacting surfaces. These

observers called this process “zippering.” We suggest that the narrow strands that we observed bridging the space between the plasma membrane and the latex beads are the physical counterparts of this “zipper.” The chemical composition of these strands is not known, although they are not destroyed by alcohol dehydration; nor is it clear whether they are extensions of some intrinsic membrane molecule (receptor) or part of an extrinsic cellular glycocalyx.

One reason why these strands have not been specifically noted in previous morphologic studies of phagocytosis (3, 33, 36) may be that as few as 600 IgG molecules per erythrocyte, or a density of only 25 IgG molecules/ $\mu\text{m}^2$  of erythrocyte surface, are sufficient for macrophage phagocytosis to proceed (8). In contrast, we estimate from the average spacing of the strands we observed between latex beads and the macrophage surface that cell/bead interactions had a density of 1,400/ $\mu\text{m}^2$ —more than 50 times as frequent as IgG/Fc receptor interactions necessary for erythrocyte ingestion. The relatively



TABLE II

## Frequency of Clathrin Basketwork on Phagosome Membranes

Method	Time of incubation	Micrographs scored	Phagosomes scored	Phagosomes with clathrin
		<i>n</i>	<i>n</i>	%
Platinum replica	2-min pulse	18	32	44
Thin section	5-min pulse	24	34 (51)*	53
	5-min pulse followed by 30-min chase	9	19 (32)*	0

Electron micrographs of phagocytosing macrophages processed as described in Materials and Methods were scored for the presence of clathrin coats on phagosome membranes. All phagosomes scored after 5 min were within 1  $\mu\text{m}$  of the plasma membrane. After a 30-min chase, all phagosomes scored were in a perinuclear location and many were associated with secondary lysosomes.

\* Numbers in parentheses indicate total phagosomes present in these micrographs, about one-third of which could not be scored.

small number of Fc-receptor interactions necessary for ingestion may explain why similar structures have not been observed during erythrocyte phagocytosis.

#### Distribution of Clathrin on Macrophage Plasma Membranes: Role in Phagocytosis

Many different soluble ligands have now been found to enter cells while bound to receptors clustered in clathrin-coated pits and vesicles, and this may be a general mechanism for receptor-mediated endocytosis (37–40). Infrequent coated vesicles have been noted previously in macrophages (32, 33), but our observations using platinum replicas and thin-section electron microscopy indicate that clathrin-coated vesicles are at least as abundant in these actively endocytic cells as they are in normal fibroblasts (23, 37–39). As shown in Table I, clathrin basketworks occupied  $\sim 1.5\%$  of the free upper plasma membrane surface of macrophages and up to 10% of the adherent lower surfaces. We also observed all stages of coated vesicle formation in these samples, from small flat patches to spherical forms 150–200 nm in diameter (Figs. 7a and 8), as has been described by Heuser (23) in cultured mouse fibroblasts. Addition of a tannic acid postfixation step during processing for thin-section electron microscopy allowed easy visualization of coated membranes, both associated with the plasma membrane (Fig. 7b and c) and in the perinuclear cytoplasm.

Although it is generally believed that phagocytosis is the only type of adsorptive endocytosis in which coated vesicles do not play a major role (40), the early appearance of clathrin patches and vesicles on the surface of nascent phagosomes as reported here (Figs. 9–13 and Table II) indicates that clathrin-coated structures may function during phagocytosis as well. The presence of clathrin patches near the advancing lip of phagosomes that were not yet completely closed (Figs. 10–12) indicates that association between clathrin and phagosome membrane can be a very early event. After a 2-min phagocytic pulse, 44% of phagosomes had detectable clathrin patches on their surface (Table II), suggesting that such an early transient association of basketwork may occur during the formation of most phagosomes. During receptor-mediated uptake of soluble ligands, clathrin coats disappear from pinocytic vesicles within 2–5 min (39). By 30 min after a phagocytic pulse, all of the

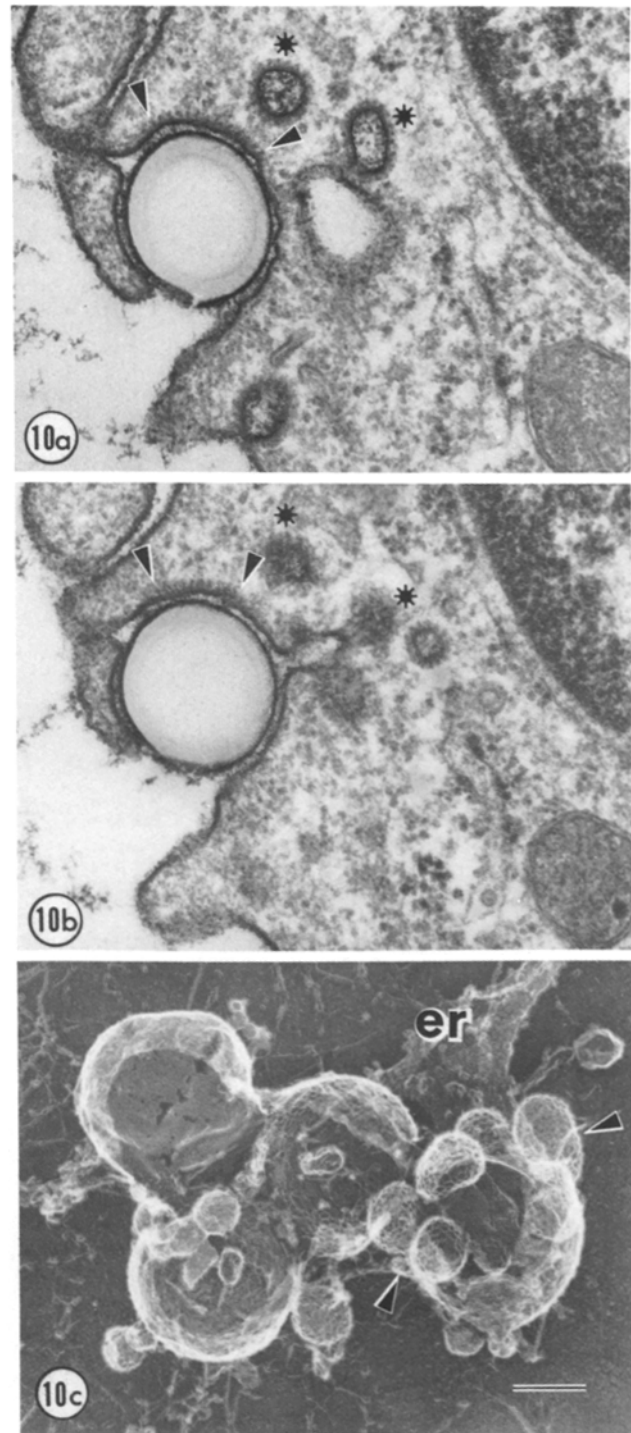


FIGURE 10 Association of multiple clathrin-coated vesicles with bead phagosomes. (a and b) Two sections through the same bead phagosome, which has a cluster of several coated vesicles attached to it (asterisks). This phagosome also shows a large flat clathrin patch adjacent to its advancing lip (arrowheads). (c) Parts of four bead phagosomes are visible with at least six well-formed coated vesicles budding off from the one on the right (arrowheads) and several flat patches of clathrin on the center one (arrowheads). A small piece of rough endoplasmic reticulum (er), studded with ribosomes, is visible at the upper right. Bar, 0.2  $\mu\text{m}$ .  $\times 48,000$ .

phagosomes were located deep within the cytoplasm and none were positive for clathrin (Table II). The function of clathrin during phagocytosis is not clear at present, but one possibility is that clathrin-coated vesicles may form to mediate recycling

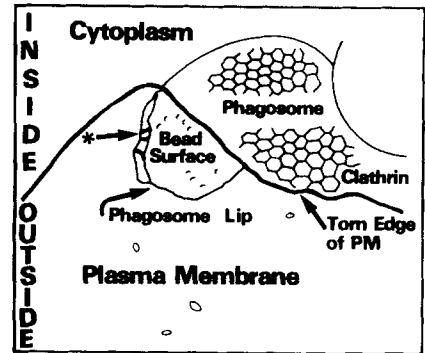
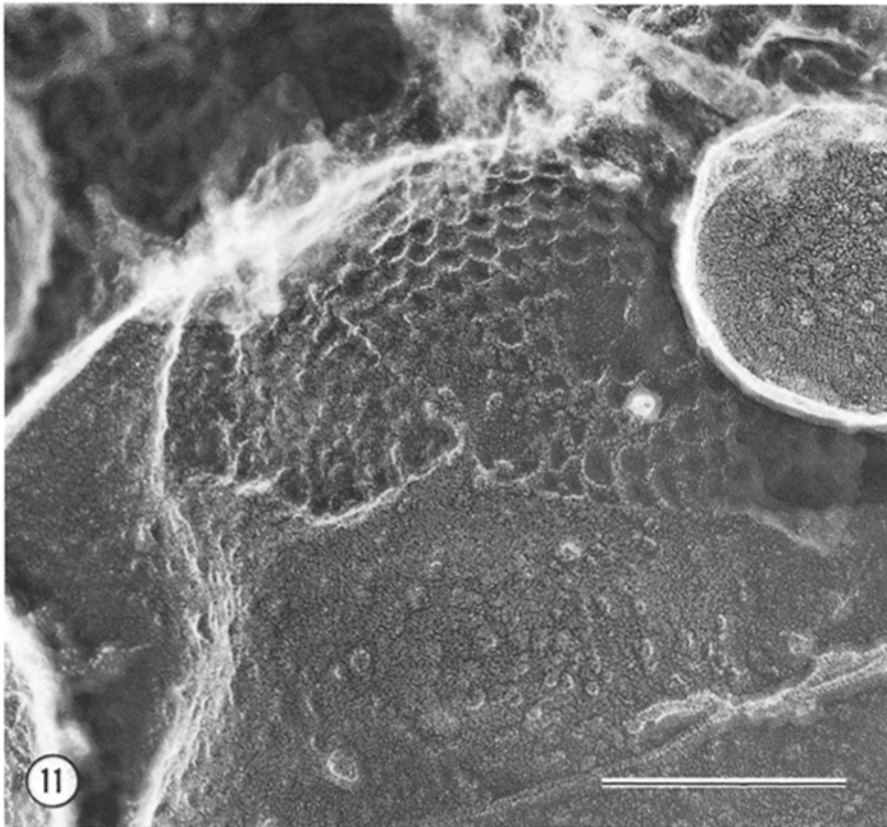


FIGURE 11 Replica of a phagocytosing macrophage that has been scraped open to reveal a large clathrin basketwork immediately below the advancing lip of a nascent phagosome. The accompanying diagram indicates the small area of bead surface visible where the phagosome is still open to the exterior, as well as the line along which the plasma membrane was torn open. Asterisk indicates 12.4-nm strands that can be seen bridging the gap at the phagosome lip. Bar, 0.2  $\mu\text{m}$ .  $\times 160,000$ .

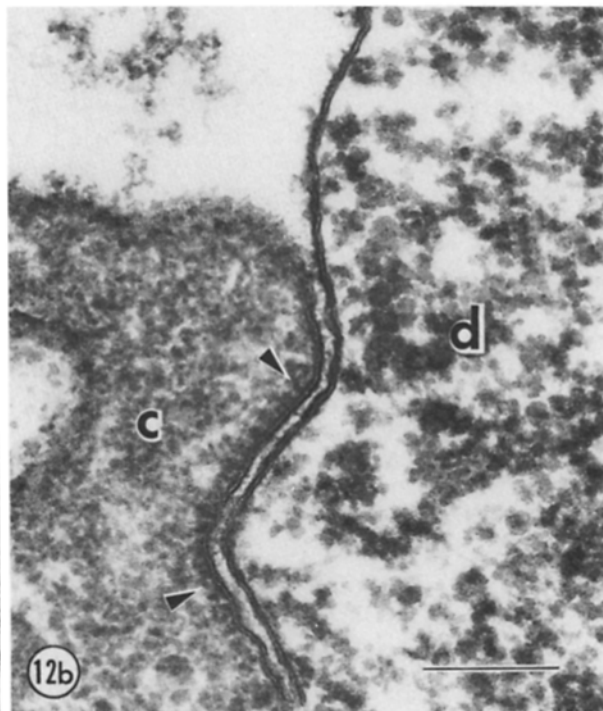
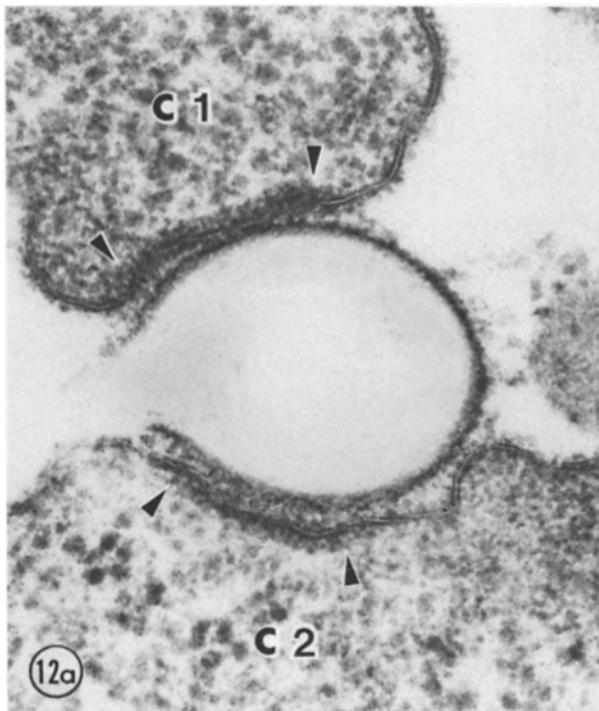


FIGURE 12 Thin-section transmission electron micrographs of phagocytosing macrophages showing large patches of clathrin (arrowheads) adjacent to the advancing lip of nascent phagosomes. (a) Large clathrin patches are seen in two J774.2 macrophages (c1 and c2) attempting to phagocytose a single latex bead. (b) A large clathrin patch can be seen near the advancing phagosome lip as a mouse peritoneal macrophage (c) begins to ingest the dead cell (d) at right. Bar, 0.2  $\mu\text{m}$ .  $\times 90,000$ .

of internalized membrane back to the plasma membrane in response to a phagocytic or pinocytic stimulus. Membrane internalized in pinosomes reappears at the plasma membrane surface within 10 min in hamster fibroblasts (41), and labeled

phagosome membrane proteins also return rapidly to the plasma membrane (27, 42).

Because our method of rupturing cells by sticking upper cell surfaces to a poly-L-lysine-coated cover slip produced an un-

even pattern of opened cells, we cannot be sure that we observed a completely random sample of upper and lower plasma membrane surfaces. Nevertheless, the number and extent of clathrin basketworks on the plasma membrane of adherent cell bottoms in a representative sample of micrographs was significantly greater than the number on upper plasma membranes (Table I). This increase was especially true for large flat patches (Fig. 8a), which were never observed on the upper cell membranes (except associated with phagosomes). The reason for the large clathrin patches observed in replicas of cell bottoms and of phagosomes is not clear, but similar flat clathrin carpets have been reported recently on the bottom surfaces of cultured HeLa cells (43), where they may play a role in adhesion of cells to the cover slip. In this regard, the large patches of clathrin basketwork found on latex bead phagosome membranes may also reflect an adhesion event and, thus, reinforce the analogy between particle phagocytosis and cell spreading on a substratum (15).

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