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INITIAL EVENTS DURING PHAGOCYTOSIS BY MACROPHAGES VIEWED FROM OUTSIDE AND INSIDE THE CELL: RECEPTOR-LIGAND INTERACTIONS, CYTOSKELETON, AND CLATHRIN

by

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DISSERTATION

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ABSTRACT

The initial events during phagocytosis of latex beads by mouse peritoneal macrophages cultured on glass coverslips were visualized by high-resolution electron microscopy of platinum replicas of quick-frozen freeze-dried cells or of critical-point-dried cells, by conventional thin-section electron microscopy of macrophages postfixed with 1% tannic acid, and by indirect immunofluorescence microscopy. 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 27 nm-wide gap bridged by narrow strands of material 12.4 nm in diameter. These strands were also seen surrounding phagosomes in thin sections and in replicas of critical point-dried and freeze-fractured macrophages. Similar strands were observed between the plasma membrane and the coverslip at the edges of spreading cells. When cells were broken open and the plasma membrane was viewed from the inside, small foci of cytoskeletal attachment were scattered over the upper plasma membrane surface, and both focal attachments and linear arrays of filaments covered much of the adherent basal plasma membrane. Many of the 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 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 basal surfaces. Clathrin basketworks covered 1-2 % of the free upper surfaces and 10-20 % of the adherent lower surfaces of macrophages spread onto glass or IgG-coated substrata. Clathrin was identified in radiolabeled, isolated phagosomes by co-migration with bovine brain coated vesicle proteins on SDS-polyacrylamide gels. Clathrin constituted 1% of the total ^{35}S-methionine labeled protein of macrophages, and 5% of the protein associated with 5 min phagosomes. These results suggest that phagocytosis shares features not only with cell attachment and spreading, but also with receptor-mediated pinocytosis.

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CONTENTS

" There is grandeur in this view of life, with its several powers, having been originally breathed by the Creator into a few forms or into one, and that...from so simple a beginning endless forms most beautiful and most wonderful have been and are being evolved."

Charles Darwin, 1859

"The whole secret of the study of nature lies in learning how to use one's eyes."

George Sand, 1869

INTRODUCTION

Historical

Phagocytosis, the ingestion of foreign particles by cells, has been the subject of active investigation since Metchnikoff first described the protective role of the "wandering cells" of starfish larvae over a century ago (Karnovsky, 1981). 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 (Metchnikoff, 1893; Gordon and Cohn, 1973; Silverstein, et al, 1978; Stossel, 1974; Karnovsky, et al, 1975). Those long-lived cells specialized in ingesting and sequestering a wide variety of foreign bodies and cell debris were named 'macrophages' by Metchnikoff in 1887.[1] In 1893, he described phagocytosis as

> "a phenomenon of considerable complexity. When it is exhibited by leucocytes, these cells are in the first place affected by various substances which possess an attraction for them. They proceed towards these substances by means of their amoeboid movements and englobe them. Intracellular digestion may afterwards occur. Here then we have phenomena of sensibility, contraction, ingestion, and production of digestive fluids."

The exact cellular mechanism by which macrophages 'englobe' particles is still incompletely understood.

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1. J'adopte par contre le nom de macrophages pour les cellules fixes du tissu conjonctif, les cellules épithéliales des alvéoles pulmonaires, en général, toutes les sortes d'éléments capables d'englober des corps solides... E. Metchnikoff. 1887. Inst. Pasteur Annal. 1:324.

Surface binding and receptors

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 (Unkeless, 1977; Diamond and Yelton, 1981). Receptors recognizing the C3b fragment of complement can also mediate phagocytosis of erythrocytes by activated macrophages (Ehlenberger and Nussenzweig, 1977). A receptor that recognizes mannose residues of glycoconjugates may be responsible for ingestion of yeast cell walls (Danley and Hilger, 1981; Sung, et al, 1981), and so-called nonspecific receptors that mediate the uptake of latex beads and glutaraldehyde-treated erythrocytes are also known (Karnovsky, et al, 1975). The uptake of latex is inhibited by treating cells with human or bovine albumin (Deierkauf, et al, 1977) or with glutaraldehyde-treated BSA (Benoliel, et al, 1980), although this effect may be mediated by electrostatic interactions rather than by competition for a specific receptor. Opsonization of bacteria by factors in serum, including complement, immunoglobulins, and fibronectin, facilitates their uptake (Stossel, 1973; Gudewicz, et al, 1980; Villiger, et al, 1981); macrophages cultured in serum-free medium ingest particles, but at a reduced rate (Rabinovitch, 1967; Stossel, 1973).

Metabolic requirements

The overall process of phagocytosis requires metabolic energy (Karnovsky, et al, 1975; Vray, et al, 1981) and is temperature sensitive,

with a threshold at 18°C (Silverstein, et al, 1977). 2-Deoxyglucose selectively inhibits Fc- and C3-mediated phagocytosis, but does not affect uptake of latex or zymosan; this effect is not mediated by cAMP (Michl, et al, 1976). Divalent cations are not required for cultured macrophages to bind particles (Rabinovitch, 1967), but Ca^{2+} and, especially, Mg^{2+} probably play a role in subsequent ingestion of particles (Rabinovitch, 1967; Stossel, 1973). Phagocytic vesicles have a Mg^{2+}-ATP-dependent Ca^{2+} pump that is sensitive to trifluoperazine (Lew and Stossel, 1980), and this calmodulin-inhibiting drug has been shown to reduce particle uptake by macrophages, suggesting that intracellular Ca^{2+}-calmodulin may play a role in phagocytosis (Horwitz, et al, 1981). Phagocytosis of latex particles causes rapid (30 sec) membrane hyperpolarization in macrophages by a mechanism involving K^{+} and Ca^{2+} (Kouri, et al, 1980).

Microbicidal function

There are several metabolic consequences of particle binding and ingestion by macrophages that are of special importance for their microbicidal function. Particle binding and ingestion stimulate a respiratory burst in phagocytes that leads to the production of various reactive oxygen metabolites (Johnston, et al, 1976; Babior, 1978). In addition, phagocytosis stimulates the secretion of various lysosomal hydrolases (Gordon, et al, 1974), as well as other neutral proteinases (Werb and Gordon, 1975a,b), that may aid in the destruction of extracellular particulates.

Phagolysosome formation

After ingested particles enter the cytoplasm, they fuse with primary lysosomes, and are eventually found within secondary lysosomes in the perinuclear area. Phagolysosomal fusion probably takes place soon after particle engulfment (Pesanti and Axline, 1975; Geisow, et al, 1981) and is complete within an hour (Kielian and Cohn, 1980). Fusion itself is not sensitive to colchicine (Pesanti and Axline, 1975), but movement of phagolysosomes to the perinuclear cytoplasm is inhibited by this drug, indicating that microtubules may play a role in cytoplasmic translocation (Allison, et al, 1971).

Membrane recycling

As is the case with pinocytosis (Steinman, et al, 1976) particle phagocytosis results in large amounts of plasma membrane being internalized. Current evidence indicates that macrophages can rapidly recycle this endocytosed membrane, so that 80-90 % of marker proteins reappear on the plasma membrane surface within 10-15 min of ingestion (Muller, et al, 1980a,b; Storrie, et al, 1981).

Cell spreading and cytoskeleton

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 (Reaven and Axline, 1973). This has led to the suggestion that actin is directly involved in the plasma membrane movement that accompanies the phagocytic process, especially because phagocytosis of some particles may be reduced by treating cells with cytochalasin B, an agent that causes disorganization

of cellular actin filaments (Allison, et al, 1971;Axline and Reaven, 1974;Allison and Davies, 1974). Hartwig, et al, (1980) 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 (Stendahl, et al, 1980). Recently, Boyles and Bainton (1979) 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 (Boyles and Bainton, 1981). Such foci also appear to act as attachment sites in spreading macrophages (Trotter, 1981), but they have not been demonstrated during phagocytosis by this cell type.

Aims

I have used the high resolution electron microscopic techniques developed by Heuser (1980,1981) to investigate the sequence of events that accompanies particle binding and phagosome formation in macrophages. This study was first undertaken with two problems in mind. First, it had been found using scanning electron microscopy that phagosomes formed around IgG-coated erythrocytes by extension and cupping of the plasma membrane, while C3-coated erythrocytes and latex beads appeared to sink into the cytoplasm without active extension of the plasma membrane (Kaplan, 1977). It seemed that the high-resolution, platinum-replica technique might provide a clearer understanding of

these two modes of phagosome formation. Second, specific cytoskeletal filament attachments had been described on yeast phagosomes in polymorphonuclear leukocytes, again using scanning electron microscopy, (Boyles and Bainton, 1981), so the power of the high-resolution replica techniques to display cytoskeletal filaments (Heuser and Kirschner, 1980) promised to yield information about the role of these contractile proteins in phagocytosis. These original aims have been only partially fulfilled by the present study. In answer to the first question, I found that latex beads were engulfed initially by migration of small lamellipodia up over the particle, followed by the particle sinking into the cytoplasm, so that both mechanisms may function during phagocytosis of these beads. In answer to the second question, I obtained an essentially negative result. Surprisingly few cytoskeletal filaments were attached to phagosomes in my samples, and I cannot draw any definite conclusions about their role in phagocytosis from my studies. On the other hand, the experiments that I describe here have produced an unexpected finding of great potential interest; the hexagonal clathrin basketworks typical of micropinocytic vesicles during receptor-mediated endocytosis of soluble ligands are also present on nascent phagosomes and may play a similar role in both pinocytosis and phagocytosis. The original observations using high resolution electron microscopy of platinum replicas have been confirmed and expanded by studies using thin section transmission electron microscopy, indirect immunofluorescence microscopy, and biochemical analyses.

MATERIALS AND METHODS

Cell Culture and Phagocytosis

Resident macrophages were harvested from CD-1 mice (Flow Laboratories, Inc., Rockville, Md.) by peritoneal lavage using Dulbecco's phosphate-buffered saline (PBS) with 100 units/ml sodium heparin. For some experiments, macrophages were elicited by intraperitoneal injection of thioglycollate broth 5 days before collection. Cells were plated onto (5 mm)^2 acid-cleaned glass coverslips in 16-mm culture wells (5 x 10 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 (Unkeless, 1977) 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 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). For electron microscopy, polystyrene (latex) beads (0.45 μm diam., Polysciences, Inc., Warrington, Pa.) were washed once in Ca^{2+}-free MEM-LH-HEPES, sonicated briefly, and added to cells at a concentration of 0.5 mg/ml. In one experiment, 0.1 μm diam. beads (Polysciences, Inc.) were used without prior washing because these particles were too small to pellet in a Beckman microfuge (8700 x g). Decreasing the Ca^{2+} concentration before breaking open cells helped to reduce small vesicle formation from plasma membrane fragments; the kinetics and extent of bead uptake in Ca^{2+}-free MEM-LH-HEPES did not differ from that in medium containing Ca^{2+} (Table 1).

Table 1

Effect of Ca^{2+} on Latex Bead Ingestion by Macrophages

Medium	$OD_{500\ nm}$	$\mu g/10^6$ cells
DME-LH	0.319	15.3
Ca^{2+}-free MEM	0.325	15.5
Ca^{2+}-free overnight	0.221	10.7

Thioglycollate-elicited peritoneal macrophages were plated at 1.5 x 10^5 cells/well in 24-well culture plates in DME-FCS and incubated for several hours to allow cell attachment. Washed cells were then incubated overnight in DME-FCS or Ca^{2+}-free-MEM-FCS before phagocytosis. To measure latex bead ingestion, 0.45 µm beads were washed, resuspended in DME-LH or Ca^{2+}-free-MEM-LH at 2.0 mg/ml and added to cells for 30 min at 37°C.

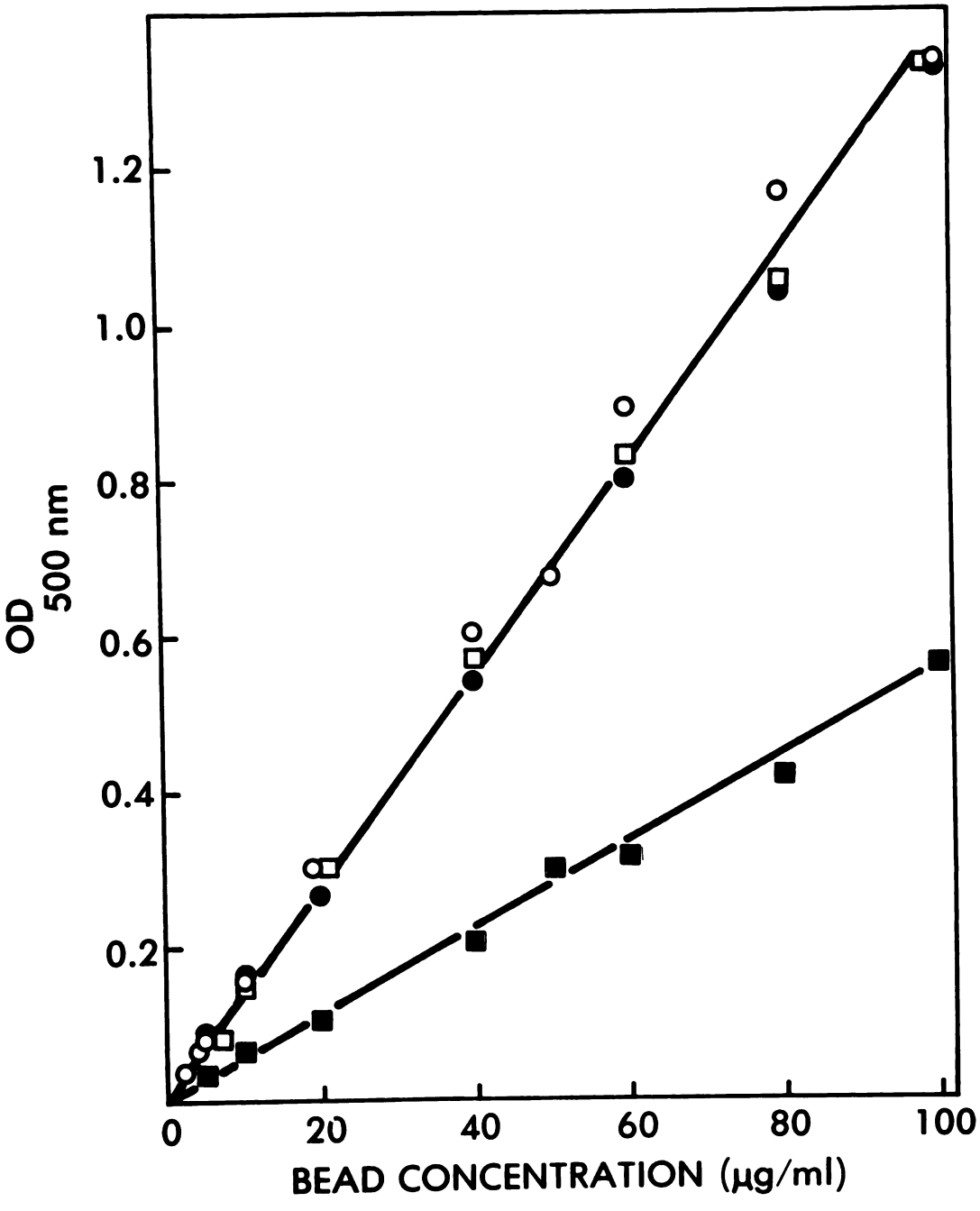
Quantification of Latex Bead Ingestion

Uptake of latex beads was measured by the turbimetric assay of Muller, et al (1980). Mouse peritoneal macrophages (resident or thioglycollate-elicited) or J774.2 macrophage cells were plated at 8 x 10^5 cells/16 mm well (confluent) in 24-well culture plates (Costar) in DME-FCS and cultured overnight at 37°C. For phagocytosis experiments, cells were washed with HBSS and cultured briefly in MEM-LH-HEPES or Ca^{2+}-free MEM-LH-HEPES as indicated. Latex beads of various diameters (0.45 μm, Polysciences, Inc.; 0.86 μm, 1.01 μm, Dow Chemical Co., 2.02 μm, polyvinyltoluene, Dow Chemical Co.) were washed and added to cells as indicated. To stop phagocytosis and float away uningested beads, cells were washed several times with ice-cold 15% sucrose, lysed with 0.5% Triton-X-100, and scraped off of the plate with a rubber policeman. The $OD_{500\ nm}$ of the cell suspension was measured using a Gilford Spectrophotometer, and latex bead uptake (in μg) was determined by comparison with a standard curve. Fig. 1 shows that the OD_{500nm} of latex bead suspensions increased linearly with increasing bead concentration. Polystyrene beads (0.45 μm, 0.86 μm and 1.01 μm) produced a single standard curve, while the 2.02 μm polyvinyltoluene beads exhibited a characteristically different curve.

Fixation, Freezing, and Replica Preparation

After exposing the cells to latex beads for 1-5 min at 37°C, the coverslips were washed to remove excess beads. Some cells were immediately fixed for 1 h at 22°C in 1% glutaraldehyde and 1% paraformaldehyde in buffer E, composed of 155 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, and 20 mM HEPES buffer, pH 7.2, which is isotonic with

Figure 1. Calibration curve showing concentration-dependent turbidity ($OD_{500\ nm}$) of suspensions of latex beads. Polystyrene latex beads, 0.45 μm dia. (O), 0.86 μm dia. (●); 1.01 μm dia. (□). Polyvinyltoluene beads, 2.02 μm dia. (■).


1.2
1.0
0.8
0.6
0.4
0.2
OD 500 nm
0
20
40
60
80
100
BEAD CONCENTRATION (μg/ml)

①

extracellular fluid. In other experiments cells were transferred into buffer I, composed of 100 mM KCl, 5 mM $MgCl_2$, 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 coverslip over them and pulling it away, or by gently scraping away cell tops with a loop of fine platinum wire. Broken cells were immediately fixed for 1 h in 1% glutaraldehyde and 1% paraformaldehyde at 22°C in buffer I. After fixation cells were rinsed briefly in distilled H_2O and then 15% methanol before being rapidly frozen against a copper block that had been cooled to liquid helium temperature (Heuser, et al, 1979;Heuser, 1980,1981). Frozen samples were stored in liquid N_2 before being loaded into a Balzers freeze-fracture apparatus, where they were freeze-dried by warming them to -90°C at a vacuum of 2×10^{-6} torr or better. Finally, they were rotary-replicated with platinum-carbon. Replicas were removed from the glass coverslips with 50% hydrofluoric acid and washed in distilled water before 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, being careful not to dislodge the replica. If residual latex remained, this treatment was repeated. 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.

Preparation of Antibody-Coated Latex Beads

Antibody-coated latex beads were prepared by coupling BSA to carboxylated latex beads (Polysciences, Inc.) with the water soluble carbodiimide, EDAC, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (Fiddler and Gray, 1978). Carboxylated latex beads (0.46 μm dia.) were washed in normal saline and resuspended in 10 mg/ml BSA (with a small amount of ^{125}I-BSA added as tracer). EDAC was added dropwise to the beads to a final concentration of 5 mg/ml and the suspension was incubated for 24 h at room temperature on a shaking platform. After incorporation, the BSA-coated beads were washed with saline and then incubated at 37°C for 30 min with excess rabbit anti-BSA. Washed, antibody-coated beads were used for microscopy as described above. Approximately 1.6 mg of BSA was coupled to 50 mg of latex beads by this method.

Critical Point Drying

In some experiments, fixed cells were carefully dehydrated through graded alcohols and critical point dried from CO_2 in a Bomar critical point-drying apparatus instead of quick-freezing and deep-etching. Critical point dried samples were immediately placed in the Balzers apparatus and rotary-replicated with Pt-C as described above. To study cytoskeletal elements in one experiment, cultured rabbit fibroblasts were extracted with 0.5% Triton-X-100 in a microtubule-stabilizing buffer containing 0.1% HEPES, at pH 6.9, 1 mM EGTA, and 4% glycerol (M. Lopata, personal communication), fixed, dehydrated, critical point dried and replicated as indicated above. Care was taken never to allow these specimens to air dry, as this instantly and totally destroyed all

ultrastructural detail.

Thin-Section Transmission Electron Microscopy

Peritoneal macrophages were cultured in 60-mm plastic dishes, exposed to latex beads 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_4 in acetate-veronal buffer, 1% tannic acid (Mallinckrodt) (Begg, et al, 1978) 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 and embedded in Epon before sectioning on a Porter Blum MTB-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.).

Indirect Immunofluorescence

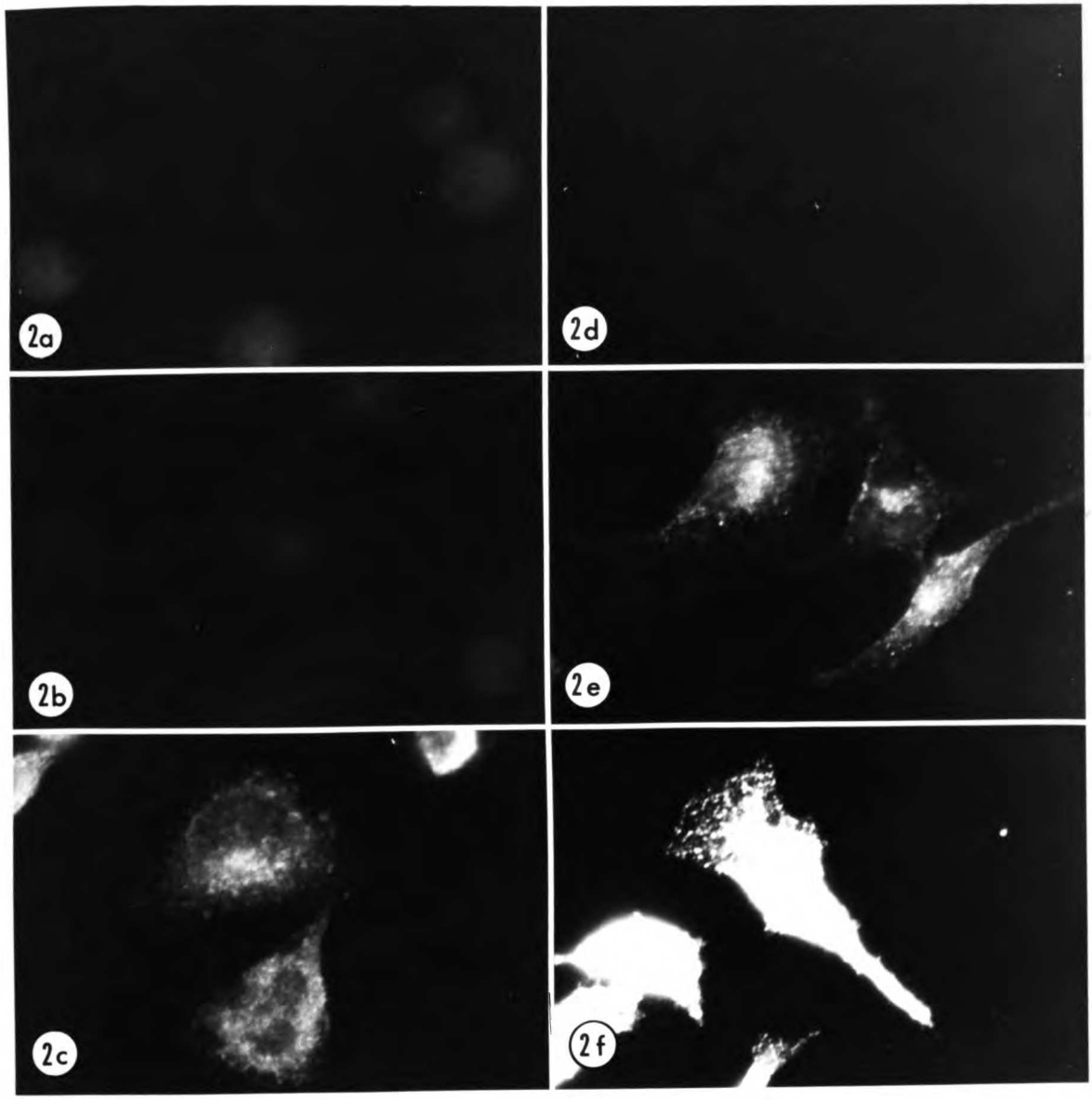
Peritoneal macrophages were cultured on 12-mm glass coverslips and exposed to latex beads (5.7 μm diam., Dow Chemical Co., Indianapolis, Ind.) at 37°C for various times. After rinsing in saline to remove excess beads, cells were fixed with 2% formaldehyde in PBS (10 min), rinsed twice with 3% horse serum in PBS (2 h), and permeabilized with 0.1% Triton-X-100 (10 min). For some experiments, control or phagocytosing cells were broken open in buffer I using poly-L-lysine-coated coverslips as described above and fixed without subsequent detergent permeabilization. Cells were then incubated (45 min) with 10 μg/coverslip of the IgG fraction of an antiserum raised in rabbits against bovine brain coated vesicle proteins and kindly provided by Dr.

R. Anderson, University of Texas. These antibodies produce a characteristic punctate staining pattern in fibroblasts that is believed to indicate the scattered distribution of coated pits and vesicles in these cells (Anderson, et al, 1978). After overnight rinsing in PBS with horse serum, specific antibody binding was visualized using rhodamine-labeled second antibodies. Initially, rhodamine-conjugated $F(ab')_2$ fragments of goat (anti-rabbit IgG) (Cappel Labs, Cochranville, Pa.) were employed, but this probe adsorbed nonspecifically to the macrophage surface, even after it was absorbed first against a crude homogenate of mouse liver, kidney, and spleen, and then against a suspension of whole fixed J774.2 macrophages (Fig. 2a). In later experiments, a rhodamine-conjugated sheep anti-(rabbit-IgG) was used that showed no background fluorescence in control experiments (Fig. 2d). After incubation with labeled second antibody, coverslips were washed extensively with 3% horse serum in PBS and mounted on microscope slides with 1% polyvinyl alcohol (Gelvatol, Monsanto Corp., Santa Clara, Calif.) (Lockwood,1978). Immunofluorescence was observed and recorded with a Zeiss Photomicroscope using epi-illumination.

Coated Vesicle Preparation

Coated vesicles were isolated from beef brains according to the method of Pearse (1975), as modified by Blitz, et al. (1977). Brain (675 g) was homogenized in extraction buffer (MES buffer:0.1 M 2-[N-morpholino]-ethanesulfonic acid; 1 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetate (EGTA); 0.5 mM $MgCl_2$; 0.02% sodium azide, pH 6.5) and the homogenate centrifuged at 20,000 g for 30 min. (These and all subsequent steps were carried out at $4^{o}C$.) The supernatant was

Figure 2. Controls showing specificity of indirect immunofluorescent staining with antibodies to bovine coated vesicle proteins. (a-c) Fluorescent second antibody was a rhodamine-conjugated $F(ab')_2$ fragment of goat (anti-rabbit IgG); (d-f) second antibody was a rhodamine-conjugated whole IgG of sheep (anti-rabbit IgG). Staining with rhodamine-labeled second antibodies only is shown in (a & d); staining with both first antibody directed against coated vesicles and fluorescent second antibody probes without permeabilization by Triton is shown in (b & e); staining of Triton-permeabilized macrophages with both first and second antibodies is shown in (c & f). x 1,230.


2a
2d
2b
2e
2c
2f

then centrifuged at 85,300 g for 1 h and the resulting pellet resuspended in 21.0 ml MES buffer with the aid of a Dounce homogenizer. This suspension was layered on each of six 5-60% sucrose step gradients and centrifuged for 2 h at 50,000 g. (All sucrose gradients were prepared in MES buffer.) The broad middle bands of all six gradients were collected and pooled (~ 100 ml), leaving the reddish soluble protein and the dense white material at the bottom, and 300 ml cold MES buffer was added before centrifuging the suspension for 1 h at 100,000 g. The pellets were resuspended in MES buffer, layered on each of six 20-60% sucrose step gradients and centrifuged for 16 h at 50,000 g. The opalescent band at the 50-55% interface was collected, diluted with MES buffer, and centrifuged at 100,000 g for 1 h. The resulting pellets were resuspended in MES buffer, layered on two 5-30% sucrose step gradients, and centrifuged for 45 min at 50,000 g. The opalescent bands at the 10-20% and 20-30% interfaces and at the top of the gradients were collected, diluted and centrifuged at 100,000 g for 1 h. These final pellets were each resuspended in 200 µl of MES buffer and stored at -20°C for further use. Clathrin (M.W. 180,000) was the major protein in all three fractions, as determined by polyacrylamide gel electrophoresis. Total protein in these fractions was 0.5 mg (10-20% interface), 2.4 mg (20-30% interface), and 1.0 mg (top of gradient) as determined by the method of Lowry, et al. (1951).

Phagosome Isolation

J774.2 macrophages (10^7 cells/100 mm culture dish) were radiolabeled by incubating them overnight at 37°C with 20 µCi/ml [^{35}S]-labeled methionine in methionine-poor medium (1 part DME:8 parts

methionine-free DME:1 part fetal calf serum). Prelabeled cells were washed and cooled to 4°C in Ca^{2+}-free MEM-LH. Latex beads (1.01 μm dia., Dow Chemical Co.) were added to the chilled cells at 5 mg/ml in Ca^{2+}-free MEM-LH and allowed to bind at 4°C for 30 min. Unbound beads were then washed off and cells were either immediately scraped into a small volume (~ 1 ml) of 10% sucrose in buffer I (see Fixation, Freezing, and Replica Preparation) or allowed to ingest the bound beads by warming at 37°C in Ca^{2+}-free MEM for various times before scraping into cold 10% sucrose. (This and all subsequent steps were carried out at 4°C.) Harvested cells were homogenized by 50 to 60 strokes in a tight-fitting Dounce homogenizer, brought to 30% sucrose, and aliquots (sample in 30% sucrose, 1 ml; 20% sucrose, 2.5 ml; 10% sucrose, 1.2 ml) placed at the bottom of 10-30% sucrose step gradients according to the method of Werb and Cohn (1972). After centrifugation for 45 min at 60,000 rpm in a SW 50 Ti rotor, the latex beads and associated membranes were found floating at the 10-20% interface. These isolated phagosomes were collected, mixed with 2x Laemmli sample buffer with β-mercaptoethanol (Laemmli, 1970), boiled for 3 min, and run on 5-15% SDS-polyacrylamide slab gels. In some experiments, aliquots of both the original homogenate and the isolated bead fractions were concentrated by precipitating with 5% TCA before SDS-PAGE analysis.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) as described by Werb and Chin (1981). For most experiments 5-15% continuous gradient reducing gels were used with 3% stacking gels. Either fifteen or twenty samples were run on each gel

using up to 50 µl (30,000 cpm) of each sample so that each lane contained approximately the same number of counts. Latex beads were not removed from the samples even though their presence sometimes caused streaking of the polypeptide bands, because it was found that much of the labeled protein remained associated with the beads even after boiling in sample buffer and was released only upon electrophoresis. Gels were run at 20 mA per gel. Usually gels were stained for 1 h using Coomassie Brilliant Blue R, and then destained overnight before drying. Dried gels were fluorographed using Kodak X-Omat AR X-ray film preflashed before exposure. In some cases, the fluorographs were scanned using a Canalco Scanning Densitometer and the resulting tracings were cut out and weighed on an analytical balance to determine the percentage of counts incorporated into specific polypeptide bands.

Radioiodination

Isolated coated vesicles in which clathrin was the major protein, as determined by Coomassie Blue staining of SDS-PAGE gels, were iodinated using Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) according to the method of Fraker and Speck (1978) to provide a labeled standard for comparison with phagosome-associated proteins. Coated vesicles (37 µg) were added to 20 µCi of Na[^{125}I] in 50 µl PBS and placed in an Iodogen-coated glass test tube for 10 min at 22°C. The iodinated sample was then removed and mixed immediately with 50 µl of 2x Laemmli sample buffer containing 10% β-mercaptoethanol. This was boiled for 3 min and used directly on SDS-PAGE gels. Unincorporated ^{125}I migrated off the gels under these conditions. To determine the extent of ^{125}I incorporation into coated vesicle proteins, an aliquot of the

iodinated sample was precipitated with 5% TCA in the presence of excess carrier BSA, washed free of unincorporated ^{125}I by centrifugation in a Beckman microfuge, and counted in a Packard Scintillation counter. Total incorporation was 980,000 cpm or 2650 cpm/μg protein. Clathrin was the major radiolabeled polypeptide in these samples along with a band of M_r 52,000 that is probably tubulin (Fig. 20, Lane 2)(Pfeffer and Kelly, 1981).

Immunoprecipitation

Clathrin was immunoprecipitated from J774.2 macrophages by the method of Mishell and Shiigi (1980) using formalin-fixed Staphylococcus aureus (Staph A) as a carrier. The antibodies used were an IgG fraction of an antiserum raised in rabbits against bovine brain coated vesicles (Anderson, et al, 1978). These antibodies are ~80% against clathrin with the remaining fraction directed against the triskelion light chains, as shown by Burridge gel analysis (A. Kalov, personal communication). Briefly, Staph A bacteria (Zysorbin brand, Zymed Labs, Burlingame, CA) were washed several times in a buffer containing 20 mM KPO_4, pH 8.1, 150 mM NaCl, 0.5% NP40, and 2 mM methionine (SAC buffer). The coated vesicles were preabsorbed with washed Staph A and preimmune antiserum for 15 min at 4^oC, and then the bacteria were removed by centrifugation in a Beckman microfuge. Preabsorbed vesicles were reacted with antibodies to clathrin for 30 min at room temperature, freshly washed Staph A was added as carrier, and the suspension was centrifuged. The Staph A pellet was then washed several times before being resuspended in 50-100 μl of Laemmli sample buffer, boiled and run on SDS-polyacrylamide gels. Although some streaking resulted, the entire

sample, including Staph A was loaded on the gels. This was necessary because most of the proteins appeared to be trapped with the Staph A pellet when only the boiled supernatant was run (see above, re phagosomes). These samples showed a number of protein bands that were adsorbed non-specifically to the Staph A. To reduce this non-specific adsorption, the immunoprecipitation was repeated, but the final Staph A was preincubated with excess unlabeled sample, and the radiolabeled samples were preincubated with Staph A before incubation in the presence of antibody to coated vesicles. Although this procedure reduced non-specific adsorption somewhat, it did not eliminate it entirely, and the gels presented in Fig. 21 still display a number of extraneous bands.

Size Measurements

The length, diameter, and spacing of the surface strands observed between beads and cells and at the edges of cells were determined by measuring all of the strands and spaces around 10 beads in 7 typical electron microscopic negatives taken at a magnification of X 60,000. 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. For thin-section electron micrographs, measurements were made on photographic enlargements at X 50,000 magnification. Results are expressed as mean and standard deviation and the range noted. In estimating strand width from micrographs of platinum replicas, it should be noted that the replicas themselves are approximately 5-10 Å thick, so that the true strand widths are at least 1-2 nm less than the measured widths reported in Table 2. The density of the

strands on the plasma membrane surface was estimated by assuming a hexagonal distribution with each side of the hexagons 25 nm long. This array was plotted on graph paper and the number of strands (apices)/μm^2 was counted.

The number and extent of clathrin-coated areas on the plasma membrane was quantified in three ways. 1) Micrographs of broken-open cells taken at a magnification $\geq$ X 40,000 were scored for clathrin patches, pits, or vesicles. Micrographs taken at magnification < 36,000 were not used because preliminary results indicated that some coated areas were not visible in such views. At a magnification of X 40,000 each micrograph represents 3.8 μm^2 of plasma membrane. These scores were converted to percentages and a cumulative step function was constructed. The cumulative percentage scores were tested for significant differences using the nonparametric Kolmogorov-Smirnov two-sample test (Daniel, 1974). The tabulated results are expressed as number of clathrin basketworks per μm^2 of plasma membrane. 2) Micrographs of broken-open cells taken at a magnification $\geq$ X 40,000 were projected onto blank paper, and exposed membrane and clathrin patches, pits and vesicles were traced. The tracings were then cut out and individually weighed. Rounded vesicles were counted twice, but no attempt was made to compensate for the dome shape of coated pits. The results are expressed as percentage of plasma membrane surface covered with clathrin basketwork (mean and standard error). 3) The profiles of latex bead phagosomes and associated clathrin coats were measured on thin-section electron micrographs printed at a magnification of X 50,000. For approximately one third of these beads (17/51) 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.

"To see a world in a grain of sand
And a Heaven in a Wild Flower,
Hold Infinity in the palm of your hand
And Eternity in an hour."

William Blake, 1805

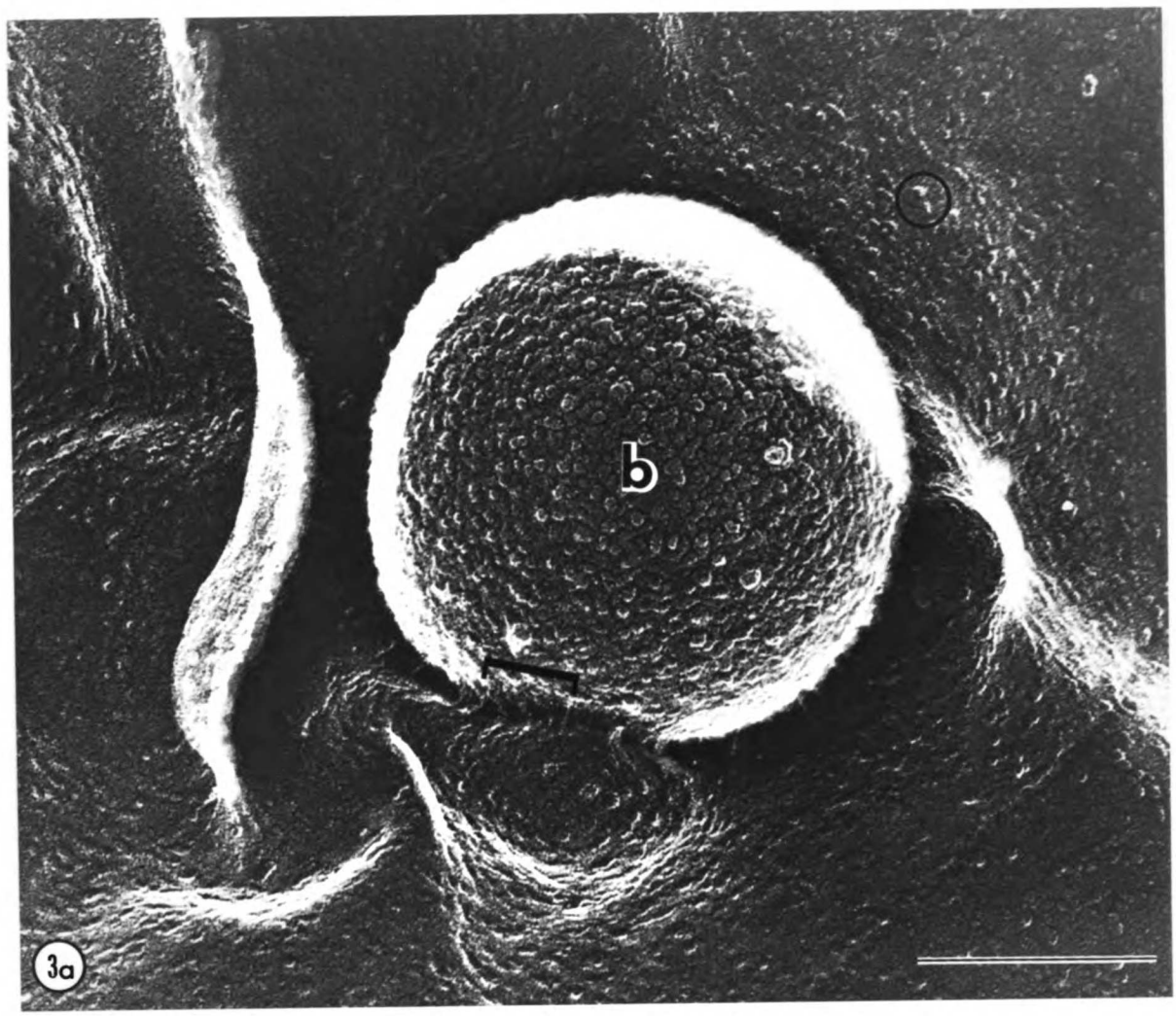
PHAGOCYTOSIS VIEWED FROM OUTSIDE THE CELL

Experimental Observations

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 (Heuser,1980). These surfaces were covered with many small bumps, slightly larger than intramembranous particles, that were distributed randomly against a homogeneous background of granular platinum (Figs. 3 and 6). The surface of 0.45 μm diam. beads either on cells (Figs. 3,4) or on the coverslip away from cells (Fig. 5a) 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 (Walter, et al, 1980), 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. It was not practical to use particles larger than 1.0 μm diam. in these studies because replicas of structures much taller than 0.5 μm 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. 3,4). At the earlier stages of

Figure 3. Rotary-deposited platinum replicas of the outside surface of freeze-dried, fixed macrophages ingesting 0.45-μm latex beads. (a) Some of the rough material on the bead surface (b) may be adsorbed protein. Numerous globular islands of material approximately the size of intramembranous paticles (circle) are visible on the cell surface, distinct from the fine, granular background of the platinum replica. X 160,000. Bar = 0.2 μm. (b) Replica of the outside surface of a freeze-dried macrophage ingesting a 0.45-μm latex bead shown in stereo to illustrate more clearly a small pseudopodium advancing over the bead surface. X 72,000. Bar = 0.2 μm.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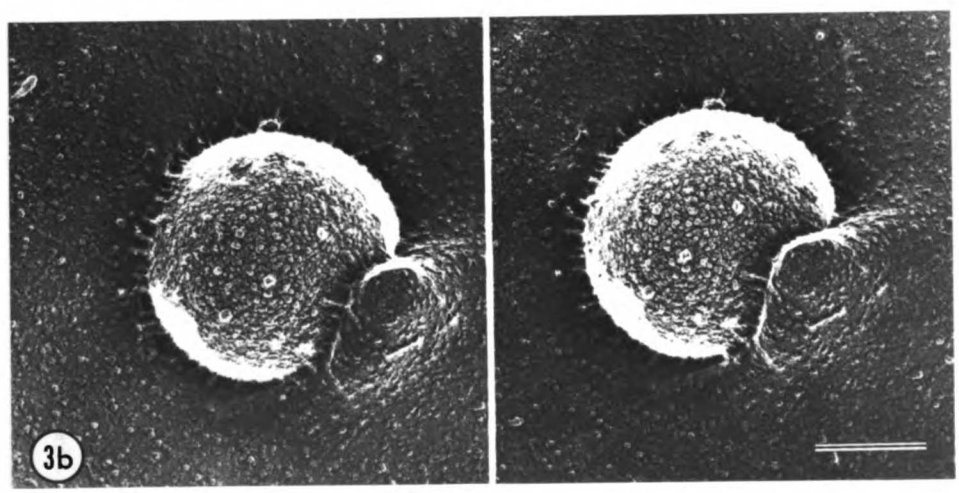

3b

Figure 4. Replicas of the outside surface of freeze-dried macrophages showing later stages of latex bead ingestion. Where the cell membrane closely approaches the bead surface, a 27-nm gap is present, spanned by 12.4-nm strands (bracket). (a,b) Intermediate stages in bead ingestion showing small surface ruffles engulfing beads. X 160,000. (c,d) Late stages in ingestion showing beads sinking below the cell surface. X 90,000. Bars = 0.2 μm.

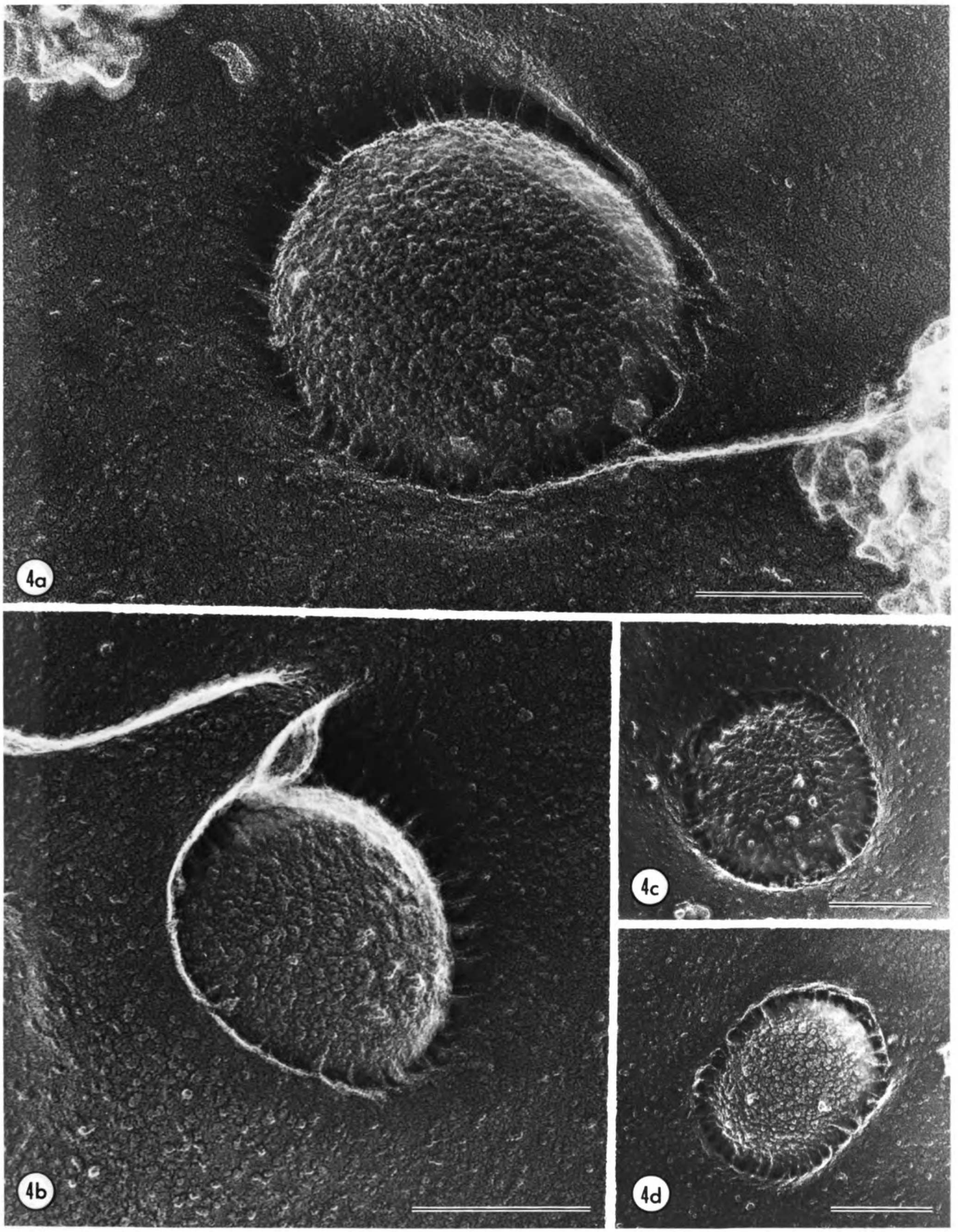
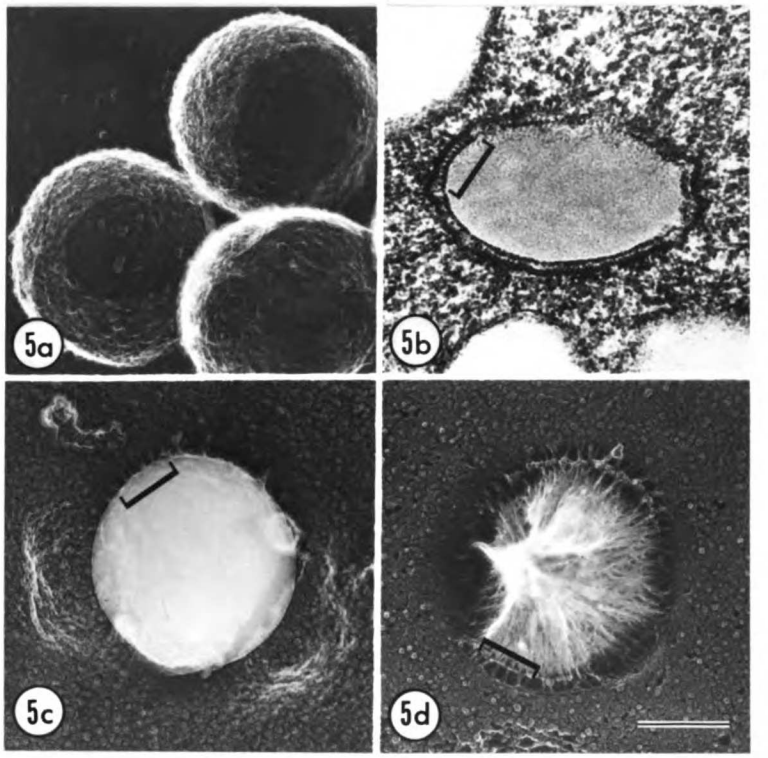

4a
4b
4c
4d

Figure 5. Demonstration that 12.4-nm strands are present only during ingestion of beads by cells. (a) 0.45-μm beads isolated on the surface of the glass coverslip 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 regular structures are also observed surrounding beads in (b) conventional thin-section electron micrograph, in (c) replica of a cell prepared by ethanol dehydration and critical point drying, and in (d) replica of a freeze-fractured cell. In (c) and (d), the electron-dense beads have not been dissolved with dioxane and still adhere to the platinum replica. X 60,000. Bar = 0.2 μm.


5a
5b
5c
5d

attachment, small lamellipodia and surface ruffles were often observed creeping up the sides of the beads (Fig. 3), whereas at later stages the beads usually appeared to be sinking into the cytoplasm below the plasma membrane surface (Fig. 4c,d). Invariably, there was a gap of 27 nm between the bead and the adjacent plasma membrane. This gap was bridged by many narrow strands of material (12.4 ± 3.5 nm diam., mean ± S.D.)(Table 2) which were spaced about 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 coverslip (Fig. 5a). Once these structures had been recognized in replicas of freeze-dried cells, they were also found in conventional thin-section electron micrographs and in replicas of critical-point-dried or freeze-fractured cells (Fig. 5 b-d)(Table 2). Similar strands were often observed at irregular intervals along the edges of spread cells, extending from the plasma membrane to the surface of the culture dish (Fig. 6)(Table 2). These strands were approximately twice as long as those around phagosomes (57 nm) and slightly narrower (9.1 nm). Such strands have also been observed extending between HeLa cells and the underlying substratum in thin-section electron micrographs (T. Pollard, personal communication).

Choice of Particle

The small (0.45 μm diam.) latex beads that I used in the present study have provided a number of advantages over other particles and served as a model for phagocytosis of debris by macrophages. These beads were small enough that their delicate platinum replicas were relatively stable. (Replicas of structures > 1 μ tall tended to collapse

Table 2

Dimensions of Macrophage Surface Strands

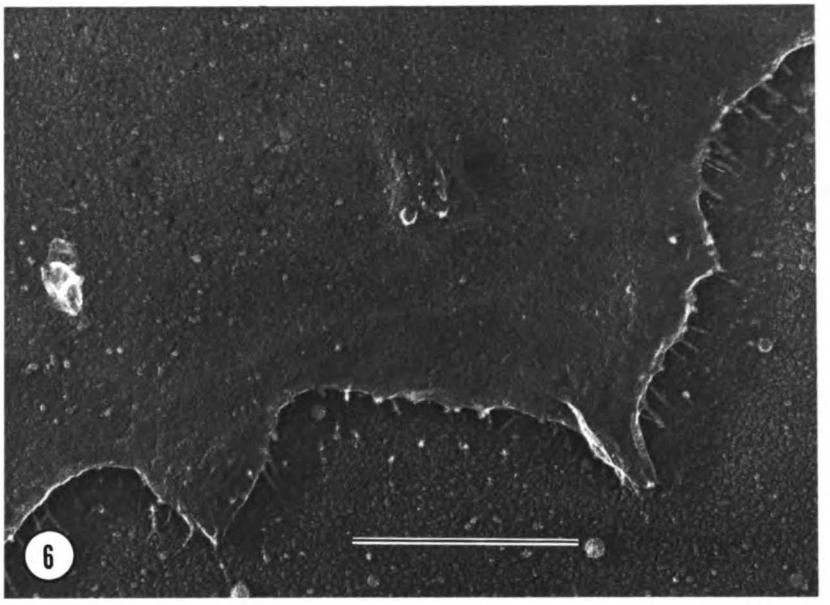
Location	Width (nm)	Length (nm)
Phagosome (Surface replica)	12.4 ± 3.5 (95)	26.4 ± 9.3 (40)
(Freeze fracture)	n.d.	27.8 ± 4.8 (27)
(Thin section)	6.1 ± 2.1 (44)	17.2 ± 7.0 (51)
Cell Edge (Surface replica)	9.1 ± 2.7 (41)	57.2 ± 18.3 (43)

Width and length of surface strands were measured directly on electron microscopic negatives of replicas using a magnifying micrometer. For thin section micrographs, strands were measured on photographic prints magnified to X 50,000.

*Indicates number of strands measured.

Figure 6. Replica of spread edge of a cultured macrophage showing extensions (bracket) between the plasma membrane and the underlying coverslip. x 60,000. Bar = 0.5 μm.

Figure 7. Replica of the surface of a macrophage ingesting IgG-coated latex beads. BSA/anti-BSA complexes (stars) appear as small mounds scattered over the bead surface with areas of uncoated bead visible between (arrowheads). 12.4 nm strands (bracket) can be seen connecting antibody-coated beads and the cell surface in some areas, but are not obvious between individual beads in this clump. x 60,000. Bar = 0.5 μm.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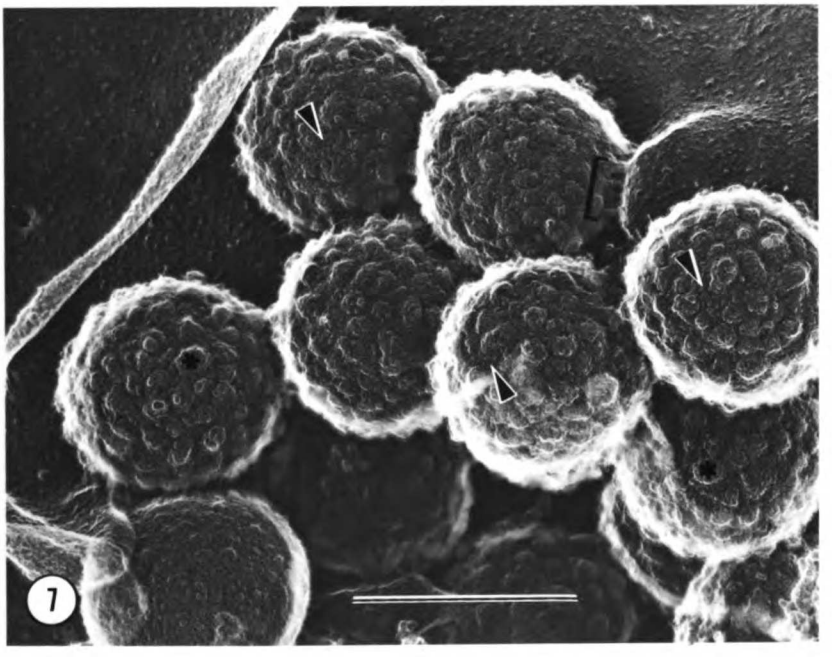
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and produce confusing images.) In addition, these beads were of a non-biological size and shape, so that phagosomes could be unambiguously identified within cells even after the beads were dissolved with dioxane (see Fig. 15d,e). The fact that these particles were not deformed during phagocytosis and replica preparation also aided in their identification, especially within phagosomes.

Nature of Nonspecific Receptor

One disadvantage of studying latex beads is that the biochemical nature of the nonspecific receptor that mediates their uptake is not yet known. Phagocytosis of a variety of particles by macrophages is known to be mediated by specific receptors on the cell surface (Karnovsky, et al, 1975; Unkeless, 1977; Diamond and Yelton, 1981; Ehlenberger and Nussenzweig, 1977; Gudewicz, 1980). In the case of latex beads, this interaction may involve a cellular receptor that recognizes areas of negative charge density on the bead surface, but binding to secreted macrophage proteins that adhere to the beads is also a possibility. The latex receptor does not seem to be the same as the polyanion receptor described by Brown, et al, (1980), however, because uptake of latex beads was undiminished by treatment with heparin or polyinosinic acid, two potent inhibitors of the uptake of acetylated low-density lipoprotein mediated by this receptor (Table 3). The physiological function of this receptor may be to mediate the uptake of denatured proteins, and it is of interest in this regard, that excess glutaraldehyde-treated BSA can inhibit phagocytosis of latex particles by macrophages (Benoliel, et al, 1980).

Table 3

Effect of Polyanion Inhibitors on Latex Bead Ingestion

Drug		$OD_{500\ nm}$	$\mu g/10^6$ cells
DME-LH		0.166	6.0
Polyinosinic acid	(2.5 µg/ml)	0.197	7.0
	(25 µg/ml)	0.189	7.0
Heparin	(250 U/ml)	0.155	5.7

Thioglycollate-elicited peritoneal macrophages were plated at 2 x 10^6 cells/well in a 24-well culture plate in DME-FCS and incubated overnight at 37°C before phagocytosis. To measure latex bead ingestion, 0.45 µm beads were washed, resuspended at 0.5 mg/ml in DME-LH and added to cells for 30 min at 37°C in the presence or absence of polyanion inhibitors as indicated.

Discussion

Particle binding in phagocytosis--an external view of cell surface receptors

The elegant experiments of Griffin, et al, (1975,1976) 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 have called this process "zippering". I suggest that the narrow strands 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, which suggests that they are not primarily lipid. Nor is it clear whether they are extensions of some intrinsic membrane molecule (receptor) or part of an extrinsic cellular glycocalyx, although they appear, especially in thin section, to be outside the bilayer of the plasma membrane. In this regard, it is interesting to note that small, regular bumps of tannic-acid-stained material of similar dimensions were often observed on the outer surface of plasma membrane of macrophages viewed in thin section (see Fig. 18a). The dimensions of the strands observed both around phagosomes and at cell edges (Table 2) are consistent with known pericellular molecules; for example, fibronectin molecules are 65 nm in length and 2 nm in diameter (Erickson, et al, 1981). Although these strands have not been specifically noted in previous morphologic studies of phagocytosis, such structures can be seen in thin-section electron micrographs (Fig. 5b)

and are probably present during phagocytosis of latex particles by Kupffer cells (Wisse, 1977), and of E. coli by PMNs (Moore, et al, 1978). Kaplan, et al, (1975), using scanning electron microscopy, described finger-like connections between the macrophage surface and latex beads, but their beads were badly extracted by dehydration with amyl acetate and the connections appear to be much coarser and more widely spaced than the strands observed by means of the platinum replica technique.

The original description of zippering involved Fc-receptor-mediated phagocytosis of IgG-coated erythrocytes, so it was of some interest to know whether similar strands were present during ingestion of these particles. Because the replicas collapsed, I was not able to identify similar bridges in macrophages fixed during phagocytosis of IgG-coated erythrocytes. I attempted to look at phagocytosis of IgG-coated latex beads with equivocal results. Unfortunately, the immunoglobulin complexes did not coat the beads evenly, but appeared as irregular bumps on the surface with latex visible between. Strands were present, but their relationship to IgG complexes could not be seen clearly (Fig. 7). These strands had the same dimensions as those observed on uncoated latex beads (Table 2). Previous thin-section electron microscopic studies of Fc-receptor-mediated phagocytosis of erythrocytes have not shown such strands to be a regular feature (Silverstein, et al, 1978; Munte-Kaas and Kaplan, 1980; Griffin, et al, 1976), although I have observed occasional strands in small patches where macrophages were in close contact with erythrocytes (not shown). One reason for this discrepancy may be that, although Fc-receptor-mediated phagocytosis requires even distribution of IgG molecules over the erythrocyte surface (Griffin, et al,

1975), as few as 600 IgG molecules per erythrocyte, or a density of only 25 IgG molecules per μm^2 of erythrocyte surface, are sufficient for macrophage phagocytosis to proceed (Ehlenberger and Nussenzweig, 1977). In contrast, I estimate that the strands observed on latex bead surfaces had a density of 1400 per μm^2--more than 50 times as frequent as the IgG molecules. The relatively small number of Fc-receptor interactions necessary for ingestion may explain why similar structures have not been observed during Fc-mediated erythrocyte phagocytosis. It is interesting to note that the average spacing between strands around latex bead phagosomes (25 nm) corresponds closely to the length of the sides of the polygons that make up the basketwork of clathrin-coated pits (Crowther and Pearse, 1981). It is, therefore, possible, that each strand is in register across the plasma membrane with the apex of a clathrin triskelion bound to the cytoplasmic membrane surface.

"Human subtlety...will never devise an invention more beautiful, more simple or more direct than does nature."
Leonardo da Vinci, 1510

CYTOSKELETON

Experimental Observations

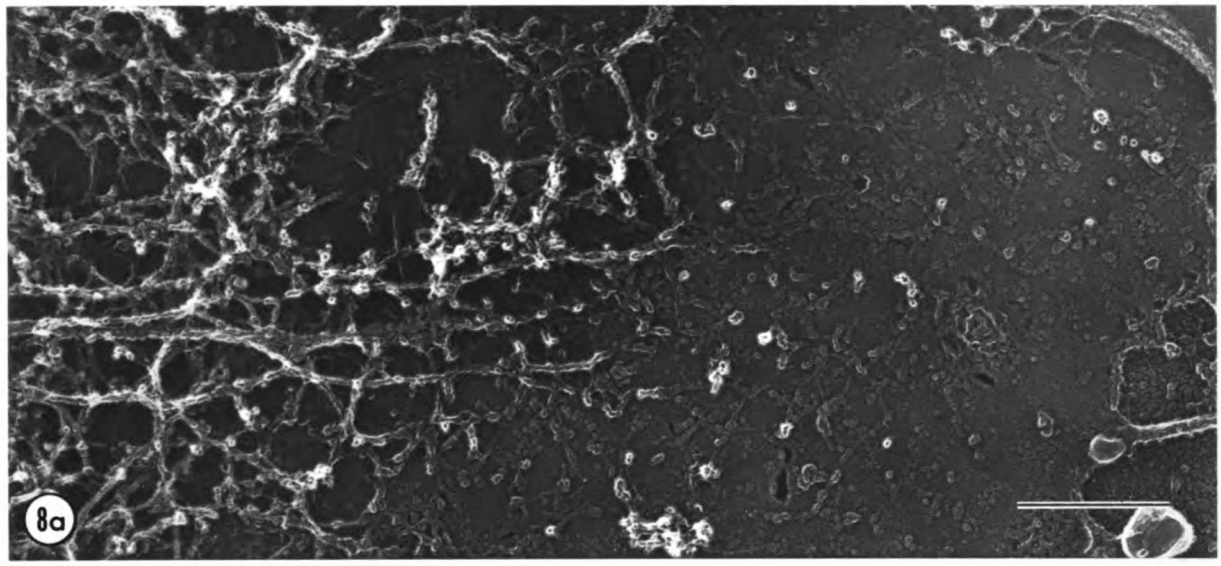
Cytoskeletal attachment to the plasma membrane

The focal attachment of cytoskeletal filaments to the cytoplasmic surface of the plasma membrane has been described in spreading polymorphonuclear leukocytes (Boyles and Bainton, 1979), in macrophages (Trotter, 1981), and in fibroblasts (Heuser and Kirschner, 1980). In the present study, scattered foci of cytoskeletal filaments were widely distributed on the inner surface of the plasma membranes of both control and phagocytosing macrophages (Figs. 8,9). On the adherent bottom plasma membranes of macrophages cultured for 24 hr, in addition to focal attachments, a dense cytoskeletal meshwork was often observed radiating out from the central perinuclear area to attach near the spread margin of the cell (Fig. 8a) (Trotter, 1981). In cells still in the process of spreading, large well-defined, starlike foci of filament attachment were observed (not shown) (Boyles and Bainton, 1979;Trotter, 1981). Most of the filaments in these foci were actin, judged by their size and morphology (Heuser and Kirschner, 1980) and by their conversion to distinctive rope-like helices after treatment with the S_1 fragment of myosin (not shown)(Heuser and Kirschner, 1980; Trotter, 1981).

Association of cytoskeletal filaments with phagosomes

Because many reports have suggested that microfilaments are

Figure 8. Demonstration of cytoskeletal filament association with macrophage membranes. (a) Long arrays of cytoskeletal filaments extend from the central part of the cell to attach to the membrane near the lower cell edge of a spread cell. The narrow band of membrane at the cell margin free of attachments is commonly observed in these specimens. x 40,000. Bar = 0.5 μm. (b) Replica of the cytoplasmic surface of the nonadherent upper cell membrane of a macrophage showing a small attachment site for cytoskeletal filaments. X 90,000. Bar = 0.2 μm.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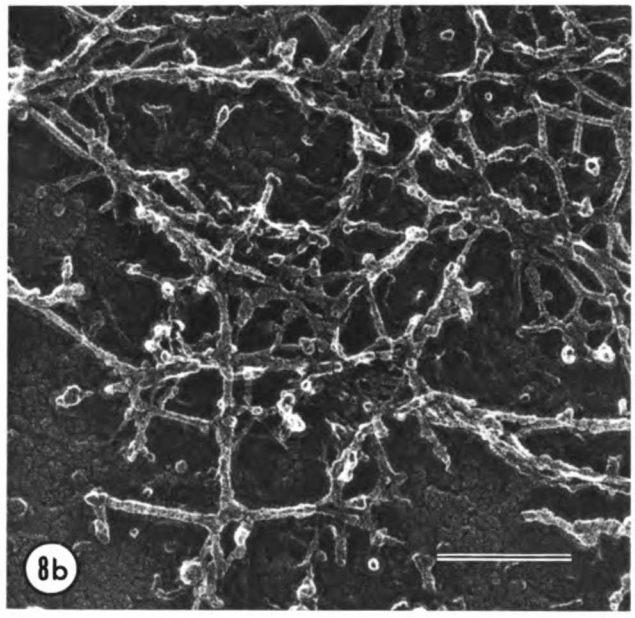
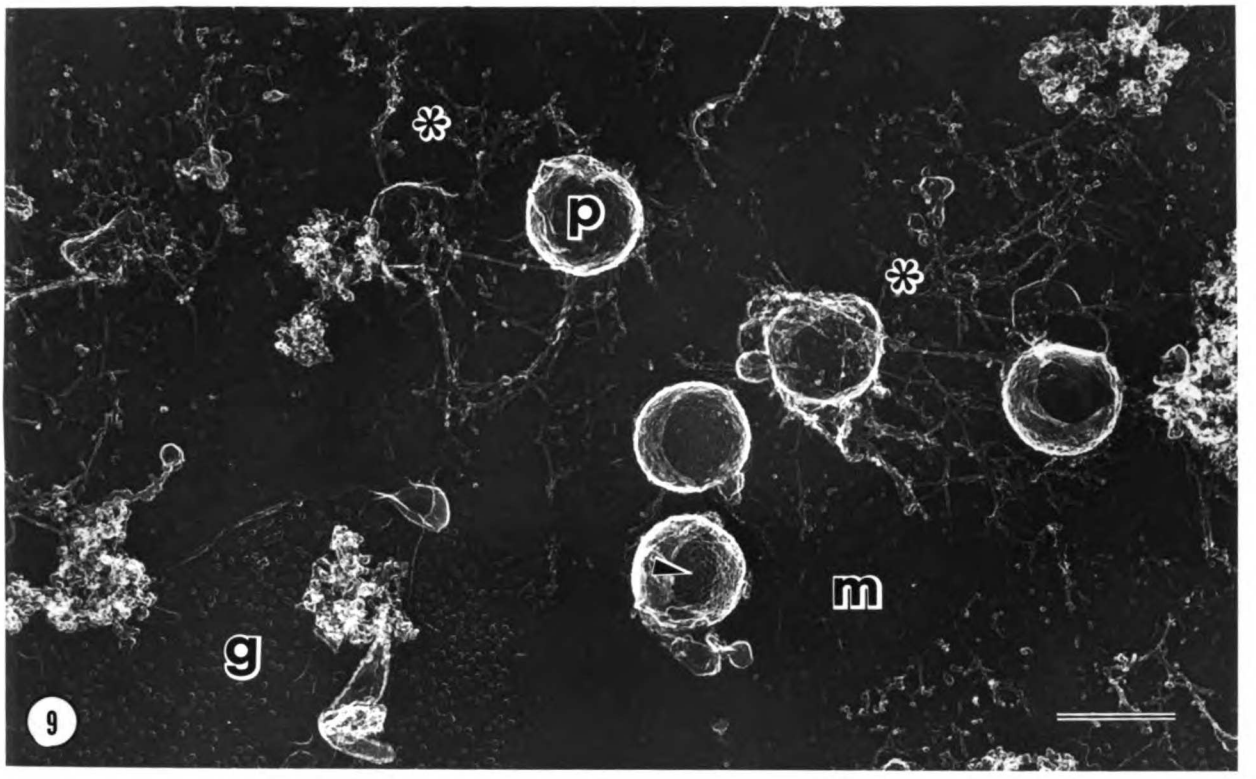
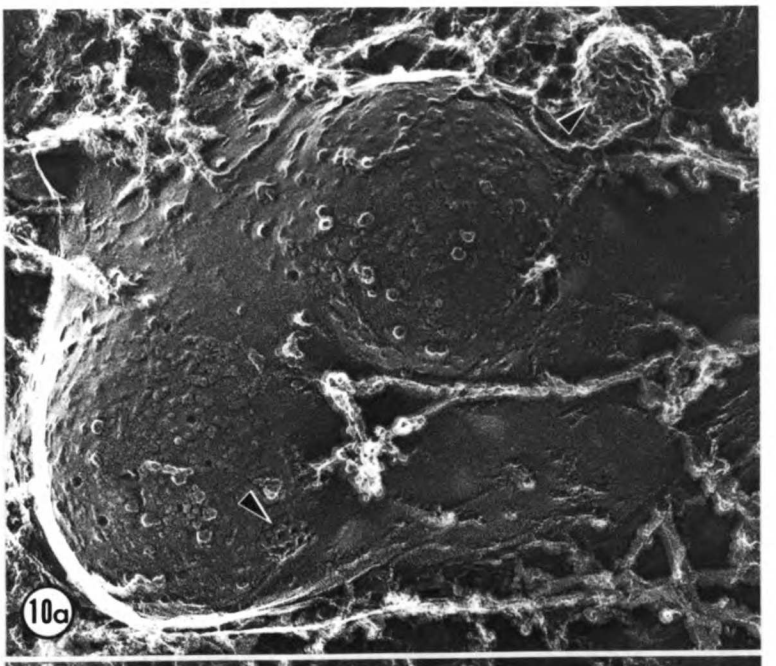

8b

Figure 9. Low magnification view of a replica of the cytoplasmic surface of upper cell membrane (m) spread on a poly-L-lysine-coated glass coverslip (g), showing several nascent phagosomes (0.45 μm) (p) and scattered foci of cytoskeletal attachment (asterisks). Large attachment foci are noticeably missing from the cytoplasmic surfaces of the phagosomes, although a few filaments are distributed in collar-like fashion around the closing lips of 4 out of the 5 phagosomes in this view. The pebbled appearance of the coverslip 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. X 30,000. Bar = 0.5 μm.


p
m
g
9

involved in the phagocytic process (Reaven and Axline, 1973;Painter and McIntosh, 1979;Boyles and Bainton, 1981;Hartwig, et al, 1980;Nagpal and Brown,1980;Storrie and Chadwick, 1980), I attempted to visualize specific associations between filamentous cytoskeletal elements and nascent phagosome membranes in these replicas of the cytoplasmic faces of broken-open macrophage membranes. In samples of the upper surfaces of macrophages prepared by adhesion to poly-L-lysine-coated coverslips, irregular areas of smooth cytoplasmic membrane were readily distinguished from the adjacent pebbled-appearing coverslip, and individual bead phagosomes still attached to these membranes were easily identified (Fig. 9). Surprisingly, there were few clear examples of specific microfilament attachments to the cytoplasmic surfaces of nascent phagosomes, and many phagosomes appeared quite smooth (Figs. 9,10a,15a). There was often a loose mesh of filaments that formed a collar around phagosomes, radiating out from the area of the advancing phagosome lip (Figs. 9 and 10a), although most of the cytoplasmic faces of such phagosomes appeared quite clear. When phagocytosis of 0.45-μm beads was observed by thin-section electron microscopy, an organized filamentous meshwork was seen radiating out from nascent phagosomes in the area of organelle exclusion (Fig. 10c), as described by others for larger particles (Reaven and Axline, 1973) and as observed on the basal surfaces of attached cells (Allison, et al, 1971). It is not clear at present why cytoskeletal filaments appear densely arrayed about phagosomes in thin section, but very sparce in replicas. Phagosomes that were located within the cytoplasm away from the plasma membrane were often observed to be enmeshed in the filamentous cytoskeleton in replicas of broken open cells (Fig. 10b,15b,c).

Figure 10. Demonstration of different amounts of cytoskeletal filament attachment to phagosomes. (a) Two smooth phagosomes forming after 2 min exposure to latex beads. Some filaments remain attached in collar-like fashion, but the cytoplasmic surfaces are free of filaments. Note coated vesicles and clathrin basketwork on phagosome (arrowheads). (b) A fully-formed phagosome that is completely within the cytoplasm after 5 min of phagocytosis and is enmeshed in cytoskeletal filaments. (c) Thin-section transmission electron micrograph showing the radial organization around a bead phagosome observed in the cortical cytoplasm. X 90,000. Bar = 0.2 μm.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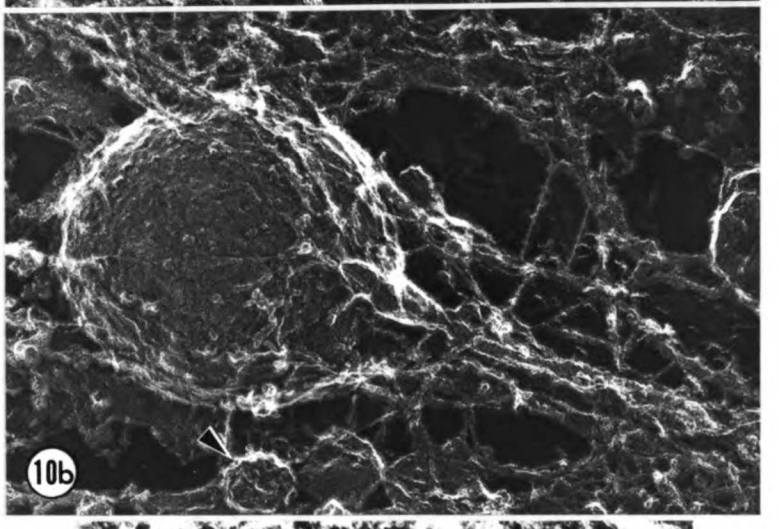
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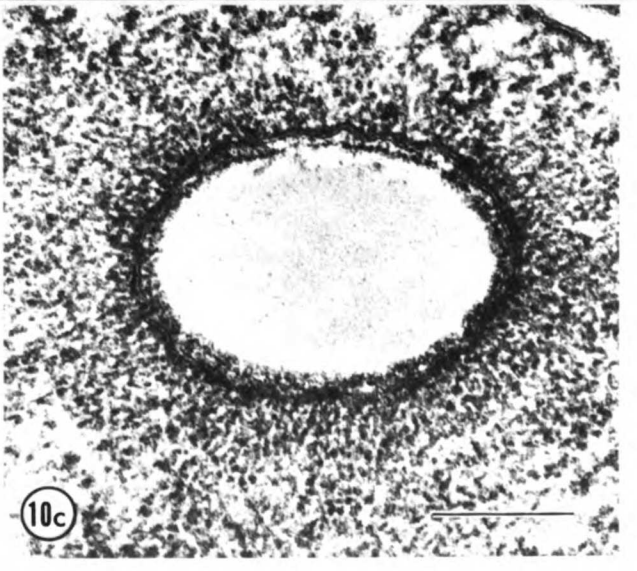

10c

Effects of cytochalasin B on phagocytosis

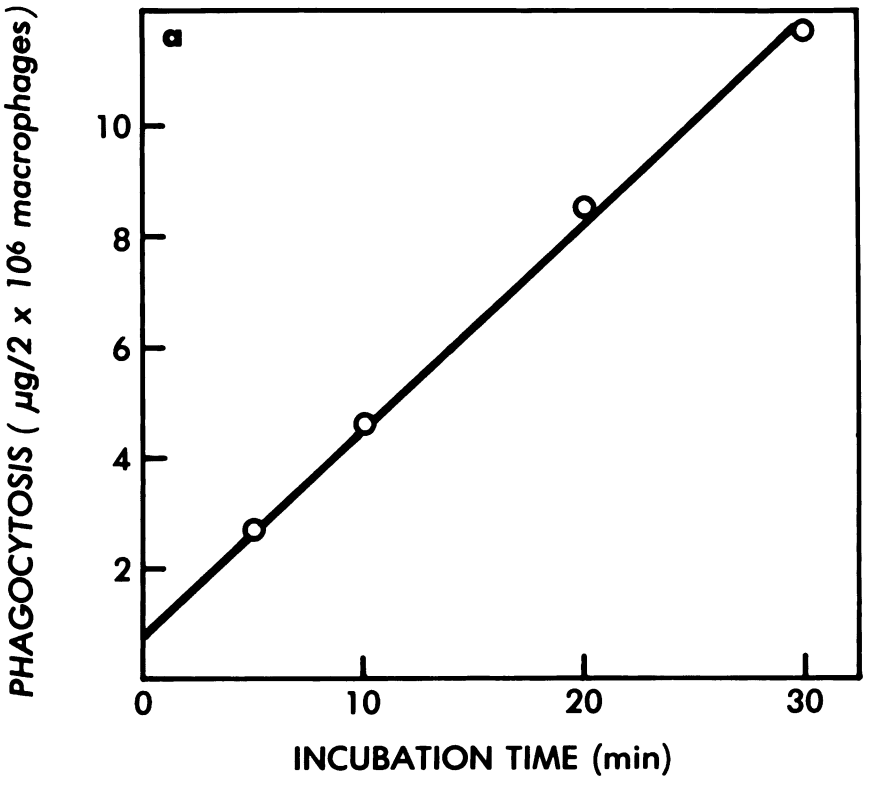
Because so few microfilament attachments were observed on nascent phagosomes, especially on the cytoplasmic faces where they might function to pull particles into the cell, I attempted to repeat earlier studies which showed that the microfilament-disrupting cytochalasins inhibit phagocytosis of a variety of particles (Table 4). I tested the ability of cytochalasin B to inhibit uptake of the small latex beads used in the morphologic studies. Figure 11 shows that latex ingestion after 30 min was reduced only 35 % even by 50 μg/ml cytochalasin B, a concentration >10-fold in excess of that necessary to disrupt microfilament associations. In addition, pretreating cells for 1 hr with 5 μg/ml cytochalasin B, so that they were already rounded when latex beads were added, had no effect on subsequent uptake (Table 4).

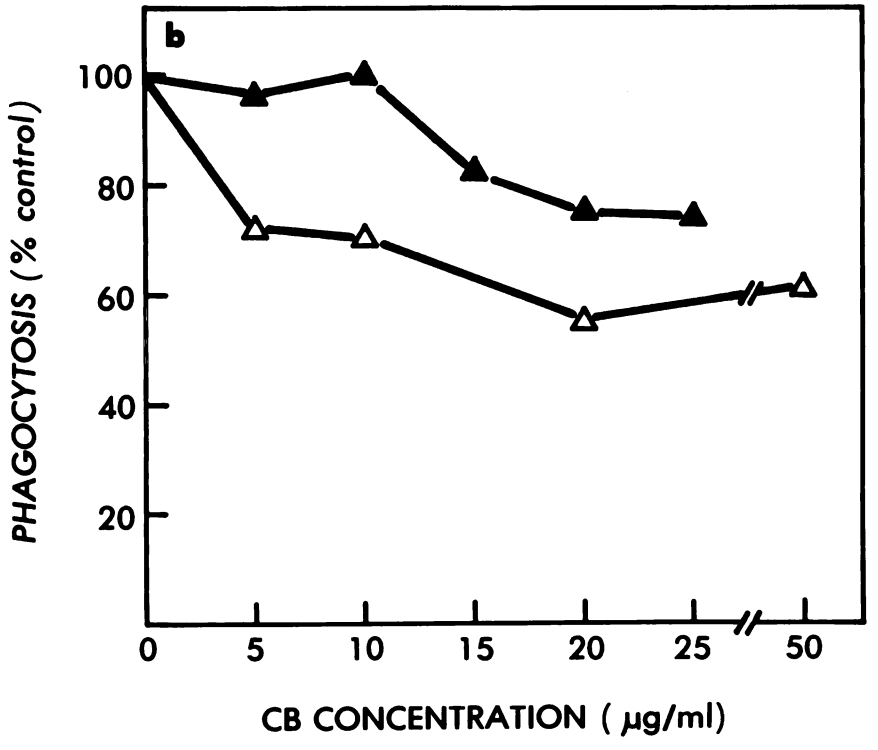
Discussion

Are microfilaments attached to phagosomes?

The many reports indicating active involvement of cytoskeletal microfilaments in phagocytosis and cell spreading originally led me to look for specific filament associations in replicas of broken-open cells during phagocytosis of small (0.45 μm diam.) latex beads. I found many focal attachments of cytoskeletal filaments to macrophage plasma membranes that closely resembled those previously described in polymorphonuclear leukocytes (Boyles and Bainton, 1979), macrophages (Trotter, 1981), and fibroblasts (Heuser and Kirschner, 1980), but I did not find similar structures attached to bead phagosome membranes. It is possible

Figure 11. Phagocytosis of latex beads by thioglycollate-elicited or resident peritoneal macrophages treated with cytochalasin B. Cells were harvested and cultured as described in Materials and Methods before being exposed to latex beads (0.5mg/ml; 0.45 μmdia.) in serum-free DME. (a) Uptake of latex beads by thioglycollate or resident macrophages (37°C). Results are the averages of three experiments. (b) Dose-response curve showing that high concentrations of cytochalasin B only partially inhibit latex bead uptake by thioglycollate (Δ) or resident (▲) macrophages after 30 min at 37°C. For the two experiments shown, untreated thioglycollate cells (10^6 cells) ingested 13.8 μg of latex and resident cells (10^6 cells) ingested 10.8 μg.


a
PHAGOCYTOSIS (μg/2 x 10⁶ macrophages)
10
8
6
4
2
0
10
20
30
INCUBATION TIME (min)


b
PHAGOCYTOSIS (% control)
100
80
60
40
20
0
5
10
15
20
25
50
CB CONCENTRATION (μg/ml)

(11)

Table 4

Effect of the Cytochalasins on Phagocytosis

Cell type	Particle	Time (min)	Drug conc. (μg/ml)	% Control Uptake	References
macrophages	SRBC*	90	10	5	Klaus (1973)
macrophages	IgGSRBC	60	0.5	30	Kaplan (1977)
	complement-coated RBC	60	0.5	80	
monocytes	IgGRBC	960	1	5	Fleer, et al (1978)
$P388D_1$ macrophages	SRBC	60	5	16	Goodell, et al (1978)
macrophages	SRBC	30	10	10-20	Lukehart & Miller (1978)
macrophages	OxRBC	90	0.5	20	Uher, et al (1981)
macrophages	zymosan	45	10	2	Painter & McIntosh (1979)
PMN+	zymosan	60	10	35	Zigmond & Hirsch (1972)
PMN	zymosan	15	5	0	Zurier, et al (1973)
macrophages	latex (2 μm)	240	10	0	Axline & Reaven (1974)
macrophages	latex (2 μm)	120	10	0	Chang (1978)
macrophages	latex (0.5 μm)	30	50	60-75	Aggeler, present study
		90	5	00	
PMN	latex (1 μm)	60	10	50	Zigmond & Hirsch (1972)
PMN	latex (2 μm)	30	48	40	Howard, et al (1981)
PMN	starch (0.5-1.5 μm)	40	5	0	Cannarozzi & Malawista (1973)
macrophages	oil red O	45	5	25	Mimura & Asano (1976)

+Polymorphonuclear leukocyte
*Sheep red blood cells

Table 4a

Effect of the Cytochalasins on Phagocytosis

Cell type	Particle	Time (min)	Drug conc. (μg/ml)	% Control Uptake	References
macrophages	opsonized bacteria	'prolonged'	10	0	Allison, et al (1971)
macrophages	E. coli	150	25	30	Gregory, et al (1979)
	C. psittaci	150	25	100	
macrophages	T. pallidum	30	10	20-30	Lukehart & Miller (1978)
PMN	opsonized E. coli	2	10	1	Davis, et al (1971)
PMN	E. coli	60	10	10	Zigmond & Hirsch (1972)
PMN	E. coli	60	10	20	Davies, et al (1973)
PMN	E. coli	35	5	0	Nakagawara, et al (1976)
macrophages	S. aureus	60	5	10-20	Malawista, et al (1971)
PMN	S. aureus	2	10	0	Davis, et al (1971)
PMN	S. aureus	60	10	90	Zigmond & Hirsch (1972)
PMN	S. aureus	40	5	55	Cannarozzi & Malawista (1973)

that such attachments are normally present in intact macrophages and are simply torn away during the breaking-open procedure used in these experiments; the appearance of filaments radiating out from latex bead phagosomes in thin-section micrographs (Fig. 10c) argues that this may be the case. Alternatively, the apparent lack of filament attachments to these small phagosomes may be real and physiologically significant, as the inability of cytochalasin B to inhibit ingestion of these small beads may indicate.

Small particles have few associated microfilaments

There are several ways to reconcile these different images of filament association with phagosomes. One possibility is that the same spreading mechanism is active during endocytosis of all particles and ligands, regardless of size, but that cytoskeletal attachments to small phagosomes or pinosomes are correspondingly small. Typical foci of microfilament attachment on the plasma membrane are 0.5 μm across or greater and, therefore, already larger than the latex bead phagosomes I studied (Fig. 8b). In addition, careful examination of bead phagosome surfaces often reveals small knobs of material that may be remnants of broken filament attachments (Fig. 15). According to this view, few filament attachments would be expected on an individual phagosome. To reconcile this interpretation with the apparently dense filament network observed in the subplasmalemmal area of organelle exclusion, I suggest that most thin-section images actually contain relatively few filaments attached to the plasma membrane, but that these filaments organize the surrounding cytoplasm into the observed linear patterns. Some support for such an interpretation comes from experiments using myosin subfrag-

ments to identify actin filaments (Heuser and Kirschner, 1980). Specifically decorated microfilaments attached to plasma membrane structures can be identified in this way, but they are infrequent and do not correspond to the apparent density of the cortical meshwork, as seen in thin section (Heuser and Kirschner, 1980; Salisbury, et al, 1980; Wang, et al, 1981). Thus, there may be few cytoskeletal filament attachments directly involved in membrane interactions even for large surfaces, and microfilaments may not be absolutely required for internalization of small vesicles.

Phagocytosis of large particles requires active cytoskeletal interaction - Cytochalasin B effects

The possibility that active membrane extension and the dynamic formation of focal cytoskeletal attachments that accompanies this spreading process (Boyles and Bainton, 1979; Trotter, 1980) may be considerably more important for ingestion of some particles, such as IgG-coated erythrocytes or yeast, than it is for small latex beads, is also suggested by experiments using the microfilament-disrupting agent cytochalasin B (Fig. 11, Table 4). A review of the literature (Table 4) indicates that uptake of large particles, such as IgG-coated erythrocytes or zymosan (yeast cell walls), is almost completely inhibited by concentrations of cytochalasin up to 10 μg/ml. In contrast, latex bead ingestion (especially beads $\leq$ 1 μm dia.) is reduced $\leq$ 50% even by 50 μg/ml cytochalasin B. Adsorptive micropinocytosis of soluble ligands is not inhibited by cytochalasin treatment of cells, even for long periods of time (Table 5). (Such pinocytosis usually takes place via clathrin-coated pits and vesicles, but may occur through non-coated vesicles and

Table 5

Effect of the Cytochalasins on Pinocytosis

Cell type	Tracer	Time (hr)	Drug conc. (μg/ml)	% Control uptake	References
macrophages	bulk phase medium*	'prolonged'	10	0	Allison, et al (1971)
macrophages	colloidal gold, ferritin	4	50	110	Wills, et al (1972)
macrophages	$DNP_{40}BSA$, hemocyanin	1.5	10	95	Klaus (1973)
macrophages	colloidal gold	50	10	67	Pratten and Lloyd (1979)
	polyvinylpyrrolidine+	50	10	64	
macrophages	rat liver cytosol proteins	96	10	90	Dean (1979)
macrophages	horseradish peroxidase	2	10	100	Schubert, et al (1980)
Kupffer cells	colloidal gold	2.5	10	85	Munthe-Kaas (1977)
lymphocytes	transferrin	0.5	96	50	Galbraith and Galbraith (1980)
B lymphocytes	horseradish peroxidase	0.25	10	97	Salisbury, et al (1980)
liver (Chang cells)	sucrose+	2	20	0	Wagner, et al (1971)
hepatocytes	asialoglycoprotein	1	48	100	Kolset, et al (1979)
		1	96	70	
fibroblasts	α-N-acetylfucosamine	8	100	85	van Figura and Kresse (1974)
	β-N-acetylhexosaminidase	48	100	0	
fibroblasts	low density lipoprotein	4	20	45	Miller and Yin (1978)
yolk sac	polyvinylpyrrolidone+	6	10	50	Duncan and Lloyd (1978)

*These authors observed that the phase-dense macropinocytic vesicles believed to be formed by surface fusion of membrane ruffles ceased to appear in the presence of cytochalasin B.

+Uptake is probably by macropinocytosis rather than micropinocytosis.

channels in cytochalasin-treated cells (Nilsson, 1977; Salisbury, et al, 1980)). Thus, macrophages may be able to engulf small particles by a mechanism that involves only membrane flow and surface adhesion, but may require active extension of lamellipodia over the particle surface and well-formed focal attachment of cytoskeletal filaments for ingestion of large particles.

In addition to particle size, specific membrane receptor interactions may also modulate the importance of microfilament attachment in the ingestion of certain particles. Thus, the uptake of opsonized E. coli is completely inhibited by low doses of cytochalasin B, even though these particles are relatively small (Table 4a). This may indicate that Fc-mediated phagocytosis is especially dependent upon cytoskeletal filament interactions. Kaplan (1977) observed that Fc-mediated phagocytosis of erythrocytes was cytochalasin-sensitive, while C3b-mediated uptake was not. In this regard, the inhibition of zymosan uptake by cytochalasin is also of interest (Table 4). Phagocytosis of zymosan may be mediated via the macrophage mannose receptor (Danley and Hilger, 1981; Sung, et al, 1981), and uptake of glycoconjugates via this receptor in fibroblasts is the only type of adsorptive micropinocytosis that has been reported to be inhibited by cytochalasin (Table 5) (von Figura and Kesse, 1974).

Mechanism of action of cytochalasin B

The mechanisms whereby the cytochalasins inhibit some types of phagocytosis are not clear at present. It has been proposed that actin filaments are in dynamic equilibrium in cells, continuously polymerizing at one end and depolymerizing at the other (Kirschner, 1980).

Cytochalasins are known to bind specifically to the 'growing' end of microfilaments (MacLean-Fletcher and Pollard, 1980); they do not cause net depolymerization of these filaments in cells (Morris and Tannenbaum, 1980; Fox and Phillips, 1981). It has been reported that where microfilaments attach to or insert into the plasma membrane, they do so 'growing' end first (Salisbury, et al, 1980; Trotter, 1981; Weeds, 1982). Taken together, these results suggest that cytochalasin may interfere with cell spreading and phagocytosis by binding to microfilaments at the site of their attachment to the plasma membrane, thereby disrupting and/or preventing the normal dynamic interaction of the cytoskeleton with the membrane. In a specific example, Hartwig and Stossel (1976) have proposed that cytochalasin B inhibits phagocytosis by preventing the interaction of actin and an actin binding protein, thus causing solation of the cytoplasmic filament meshwork or gel. According to this view, the normal role of cytoskeletal filaments is not to pull endocytic vesicles (pinosomes or phagosomes) into the cell, but rather to push the plasma membrane out around entering particles (Hartwig, et al, 1979). The appearance of a collar of microfilaments radiating out from the closing lip of latex bead phagosomes is consistent with this model (Figs. 8a,9).

Summary

In summary, there are at least two possible reasons why phagosomes may appear enmeshed in cytoskeletal filaments when viewed in thin section, but relatively naked when viewed in replicas of broken-open cells. First, all microfilament attachments to the plasma membrane (including phagosomes) may be very labile, reflecting the dynamic interaction of

the cytoskeleton with the membrane. This would be especially true for actively motile cells, such as macrophages (Allison, et al, 1971; Trotter, 1981) and polymorphonuclear leukocytes (Boyles and Bainton, 1979), compared to more static cell types, such as fibroblasts. According to this view, these loose attachments are easily torn away by the mechanical stress of breaking cells open, leaving the plasma membrane and phagosomes relatively bare, but are preserved when viewed in thin section. Second, it is possible that the number of microfilaments attached to the membrane is actually much smaller than conventional thin-section views have suggested, but that the attached filaments orient the adjacent cytoplasm to produce an image of multiple radiating filaments.

In either case, the role of cytochalasin B *in vivo* is to prevent the normal interaction of filaments with the plasma membrane. Even in the presence of this drug, small plasma membrane vesicles or channels can flow into the cell by 'sinking' into the cytoplasm, but the dynamic interactions of cytoskeleton and membrane that produce ruffling and cell motility are paralyzed. In as much as phagocytosis of large particles depends on active extension of the plasma membrane over the particle surface by a mechanism that includes formation of lamellipodia and ruffles, cytochalasin B reduces or inhibits uptake of most large particles.

"What we experience of nature is in models, and all of nature's models are so beautiful."

R. Buckminster Fuller, 1966

CLATHRIN

Morphological Observations

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 (Heuser, 1980). Coated vesicles have been noted in macrophages studied in thin sections, especially in the Golgi area (Roth and Porter, 1962; Fawcett, 1965; Nichols, et al, 1971; Wisse, 1977; Munte-Kaas and Kaplan, 1980), but endocytic coated vesicles have been relatively difficult to see against the dense cortical cytoplasm of these cells. In replicas of broken-open macrophages, however, it was apparent that clathrin patches and vesicles were remarkably abundant (Figs. 12a,13), involving 1-4% of the total plasma membrane surface in control cells (Table 6). By using a hypotonic fixation buffer (0.02 M sodium cacodylate) and a postfixation protocol that included 1% tannic acid, I was also able to see numerous coated pits and vesicles in thin-section electron micrographs (Fig. 12b,c). Both the area (Table 6) and number (Table 7) of clathrin basketworks present on the adherent lower surfaces of macrophages were significantly greater than on the free upper plasma membrane surfaces. This was especially true for large flat patches of hexagonal basketwork, which were never observed on upper surfaces (Fig. 13a). After a phagocytic stimulus (2-5 min), the number and area of coated patches on the adherent cell bottoms was increased 2-fold

Figure 12. 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 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. X 90,000. Bar = 0.2 μm.

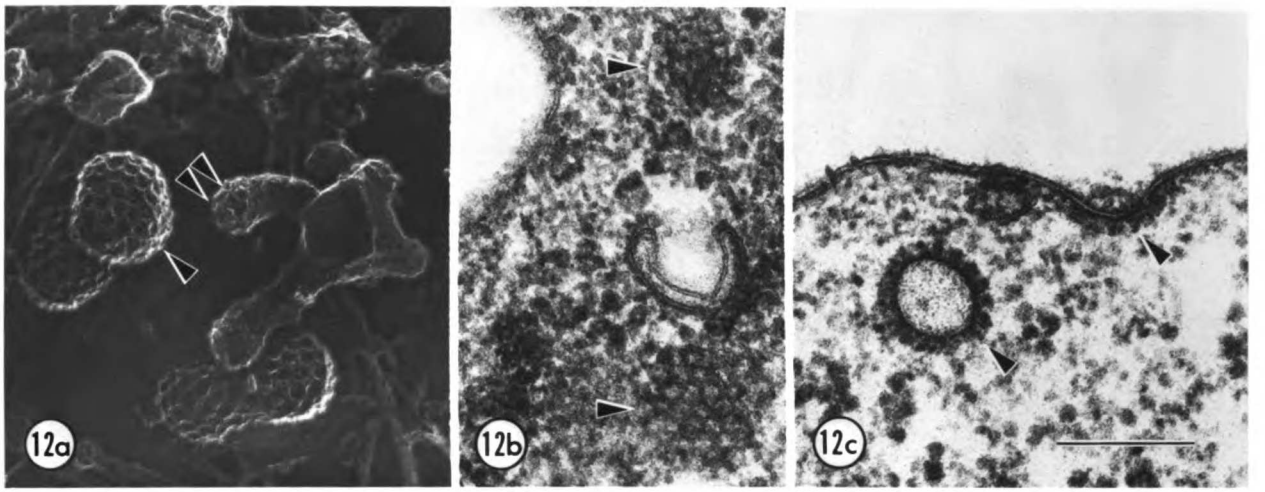

12a
12b
12c

Figure 13. 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 surfaces appears as flat patches, as in (a). Possible sites of cytoplasmic filament attachment to clathrin basketwork are indicated by circles. X 60,000. Bar = 0.2 μm.

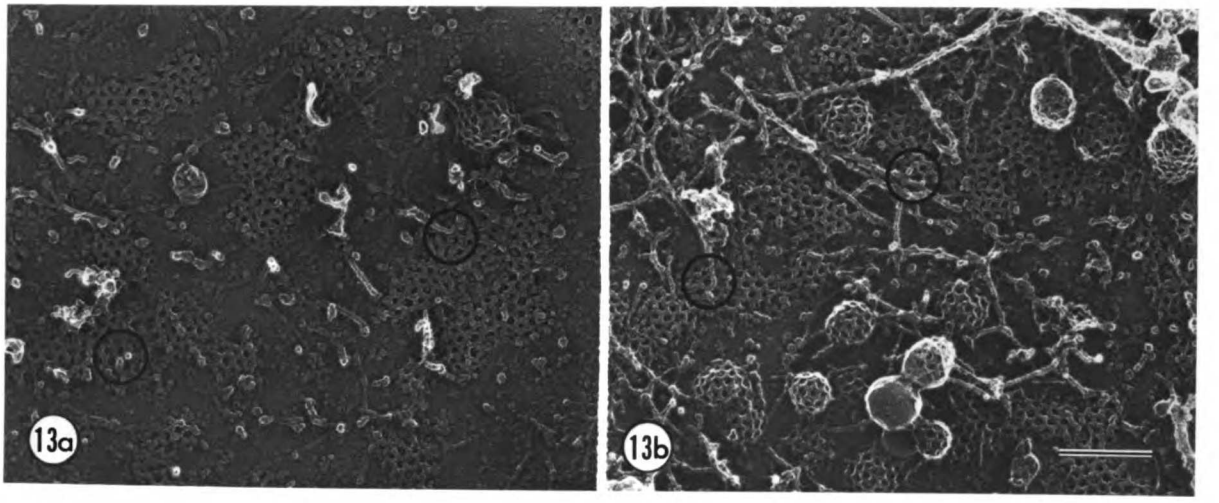

13a
13b

Table 6

Extent of Clathrin Basketwork on Macrophage Plasma Membranes

Cells	Surface area covered by clathrin basketworks (%) Upper plasma membrane	Lower plasma membrane	p^+
Control	1.3 ± 0.3 (24)	3.5 ± 1.0 (14)	0.001
Phagocytosing (2-5 min)	1.6 ± 0.3 (40)	9.7 ± 1.8 (31)	<0.001
p^+	>0.2	0.02	

Control or phagocytosing macrophages were broken open by the poly-L-lysine-coated coverslip 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 (Method (2)). Values tabulated represent mean ± standard error and numbers in parentheses indicate number of micrographs scored.

+Probability (p) was determined by the unpaired two-sample t-test.

Table 7

Frequency of Clathrin Basketworks on Macrophage Plasma Membrane

Cells	Number of clathrin basketworks/μm^2	
	Upper plasma membrane	Lower plasma membrane
Control	0.8 (28)	1.8 (16)
Phagocytosing (1 min)	0.9 (31)	2.8 (2)
(2 min)	0.7 (23)	3.9 (12)
(5 min)	0.7 (17)	4.1 (22)

Control or phagocytosing macrophages were broken open by the poly-L-lysine-coated coverslip 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 (Method (1)). Clathrin patches on the lower plasma membranes of macrophages after 2-5 min of phagocytosis were significantly increased compared to the upper plasma membranes of these cells ($P < 0.01$). The lower plasma membranes of control and phagocytosing (2-5 min) cells differed at the 10% level.

*Number in parentheses indicates micrographs scored.

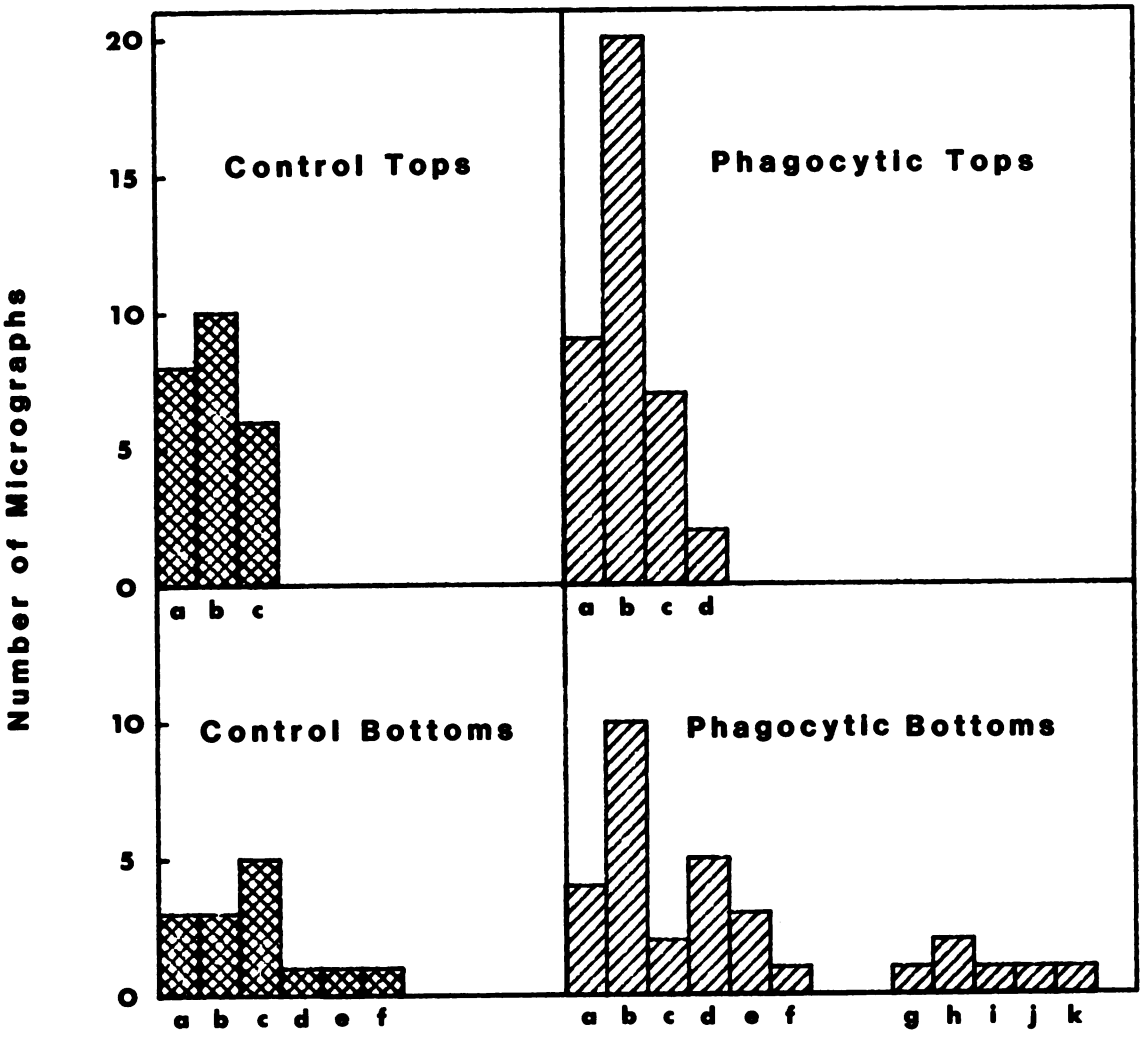
over controls (Tables 6,7), primarily because of the appearance of areas of membrane displaying a very high density of basketwork (Fig. 14). Because of the way that cells were torn open by the poly-L-lysine-coated coverslips, plasma membrane at the periphery of the cell was sampled more often than other areas of the ventral surface (see Fig. 8a). Nevertheless, areas of centrally located plasma membrane were observed, and the distribution of clathrin on them was similar to the peripheral areas.

Association of clathrin with phagosomes

Although the cytoplasmic surfaces of phagosomes were often quite smooth (Figs. 9,10a,15a), 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 8). These areas were often quite large, in several cases covering the entire observable surface of the phagosome, an area equivalent to 2 to 4 coated vesicles (Fig. 15c). Deeper coated pits and vesicles were also found on phagosome membranes (Figs. 15b,16). In Fig. 16 clusters of coated vesicles are seen budding off the cytoplasmic face of bead phagosomes observed in both replicas and thin sections. In one experiment I examined phagocytosis of very small beads (0.1 μm diam.). Most of these beads were surrounded by smooth, closely adherent phagosomes (Fig. 15d), but uptake within coated vesicles was occasionally observed (Fig. 15e).

Because the technique used to break 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

Figure 14. Distribution of clathrin basketwork on the plasma membranes of macrophages. Bars indicate the number of micrographs scored in which a given percentage of the visible plasma membrane was covered with baskets. (a) 0%; (b) 0.1-2.5% (c) 2.6-5.0%; (d) 5.1-7.5%; (e) 7.6-10.0% (f) 10.1-12.5%; (g) 17.6-20.0%; (h) 20.1-22.5%; (i) 22.6-25.0%; (j) 25.1-27.5%; (k) 27.6-30.0%.


Control Tops
Phagocytic Tops
Control Bottoms
Phagocytic Bottoms
Number of Micrographs
20
15
10
5
0
10
5
0
a b c
a b c d
a b c d e f
a b c d e f g h i j k
Surface Area With Baskets

(14)

Figure 15. Replicas of the cytoplasmic surfaces of bead phagosomes viewed from inside macrophages broken-open after 5 min of phagocytosis. In (a-c), 0.45- μm dia. latex beads were used; in (d,e), 0.1- μm dia. beads are shown. Most of the phagosomes visualized in this way have a relatively smooth surface (a,d), but large patches of clathrin basketwork can be seen on some (b,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,e), the replicas of 0.1-μm bead phagosomes 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. Asterisk in (d) indicates focal attachment of cytoskeleton to plasma membrane. X 90,000. Bar = 0.2 μm.

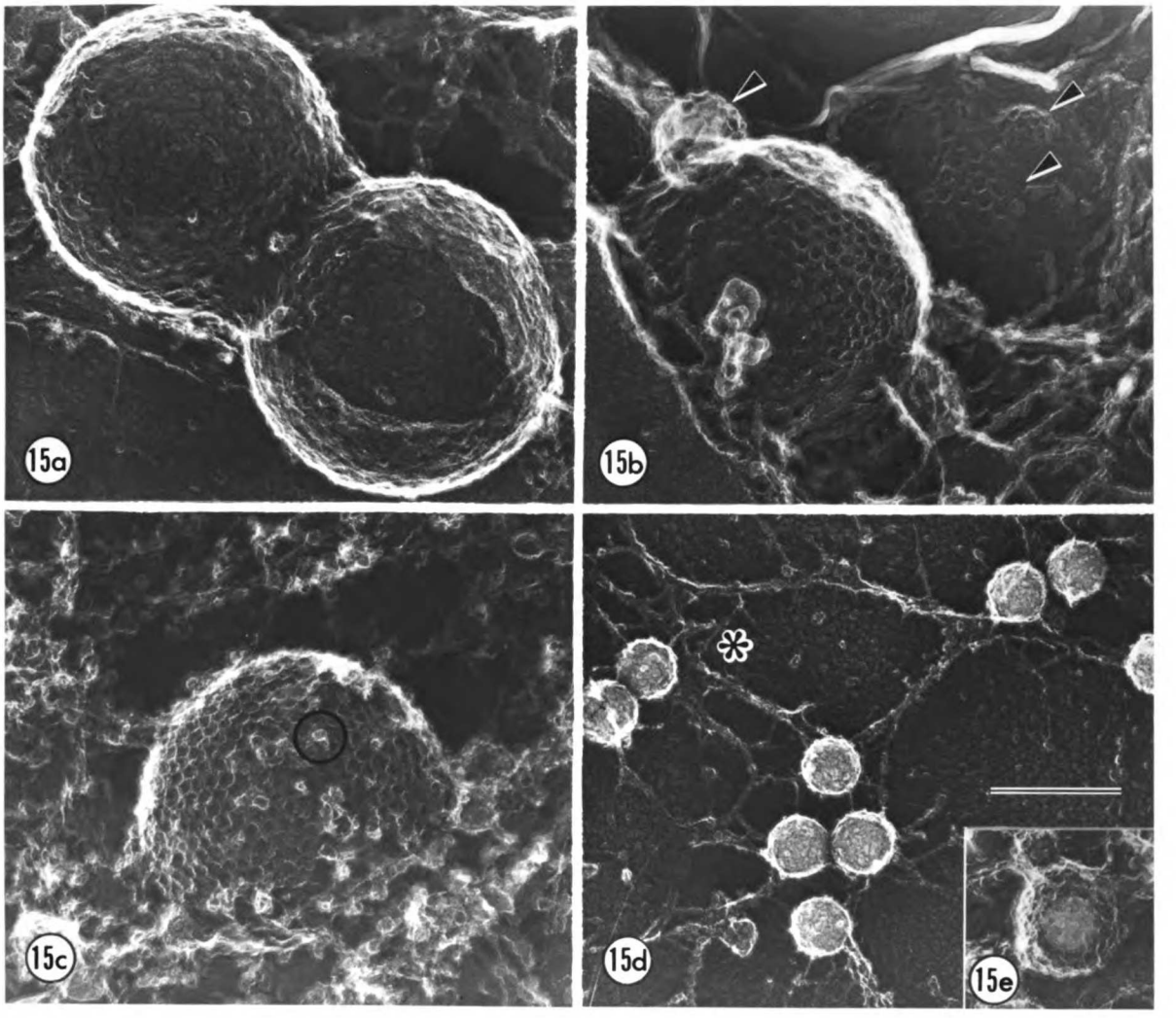

15a
15b
15c
15d
15e

Table 8

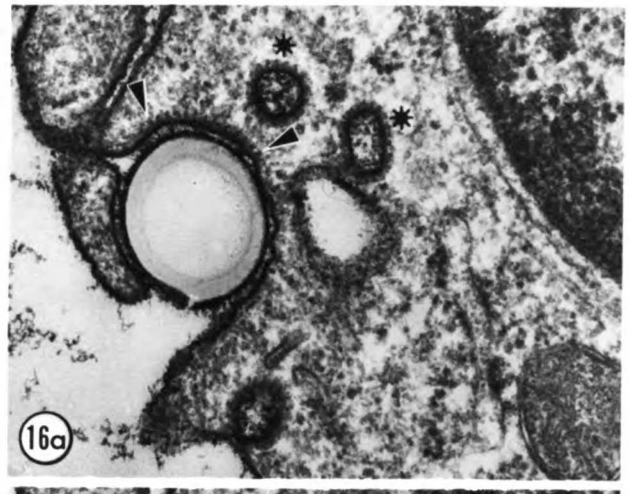
Frequency of Clathrin Basketwork on Phagosome Membranes

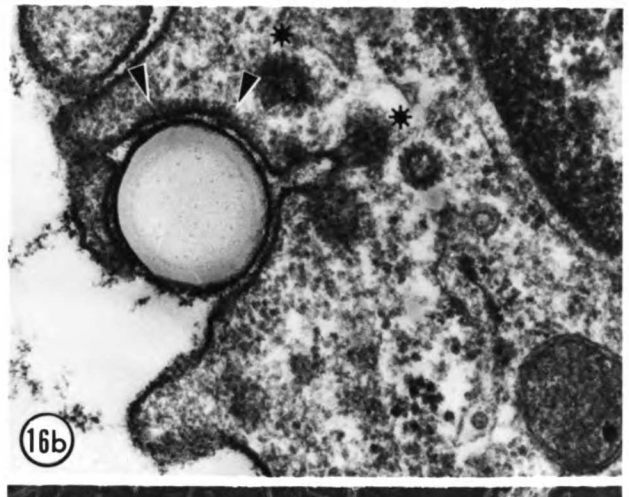
Method	Time of incubation	Phagosomes scored (#)	Phagosomes with clathrin (%)
Platinum replica	1-min pulse	19	37
	2-min pulse	36	36
	5-min pulse	67	37
Thin section	5-min pulse	34 (51)*	53
	5-min pulse followed by 30-min chase	19 (32)*	0

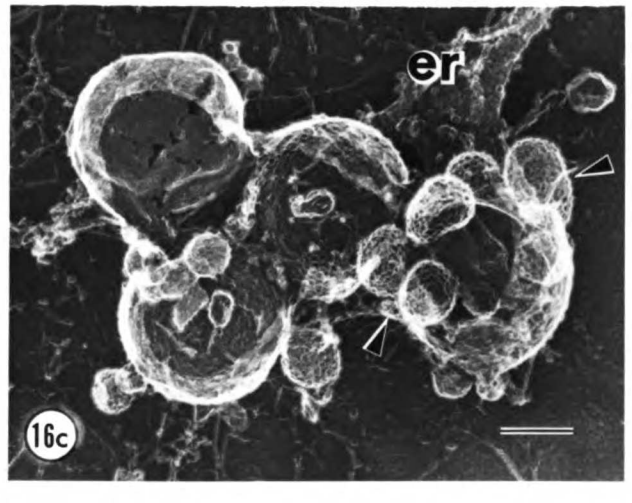
Electron micrographs of phagocytosing macrophages processed as described in Materials and Methods were scored for the presence of clathrin coats on phagosome membranes. In thin section micrographs, phagosomes scored after 5 min were within 1 μm of the plasma membrane. After a 30-min chase, all phagosomes scored were in a perinuclear location and many were associated with secondary lysosomes. Phagosomes were not observed in replicas of cells broken open after a 30-min chase, presumably because they were no longer connected to the plasma membrane and were lost when the cells were broken open.

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

Figure 16. Association of multiple clathrin-coated vesicles with bead phagosomes. (a,b) Two sections through the same bead phagosome, which has a cluster of several coated vesicles attached to it (stars). 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 the one on the right (arrowheads) and several flat patches of clathrin on the center one. A small piece of rough endoplasmic reticulum (er), studded with ribosomes, is visible at the upper right. X 48,000. Bar = 0.2 μm.


16a


16b


er
16c

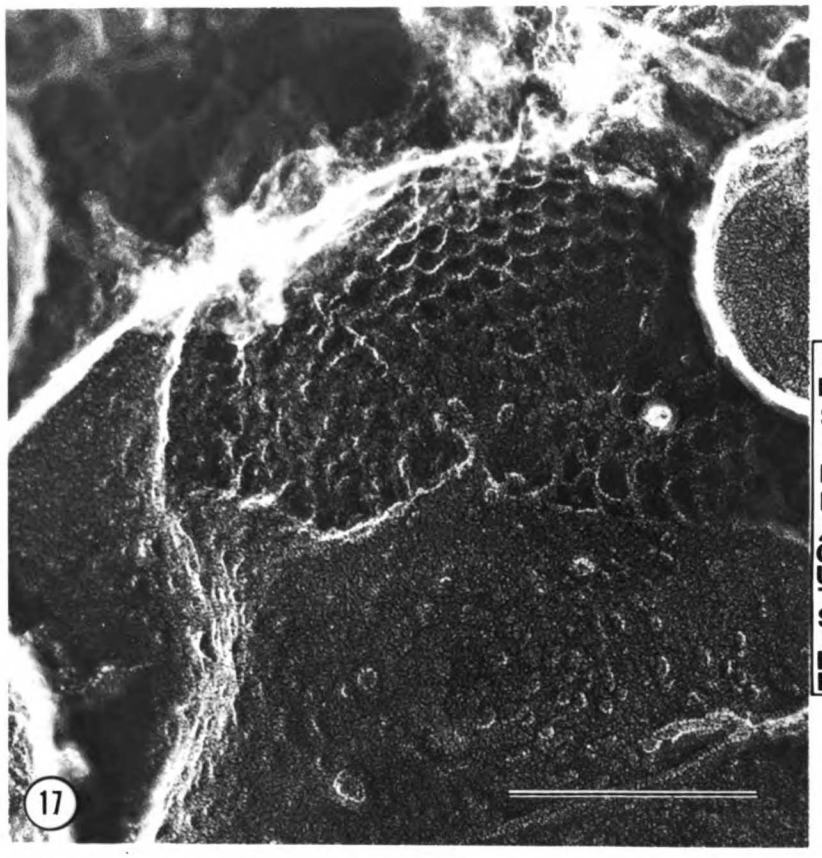
cases a fortuitous break preserved the relationship of the ingested bead to the plasma membrane, and a large clathrin patch could be seen just under the plasma membrane adjacent to the advancing lip of the forming phagosome (Fig. 17; see also Fig. 15b). This proximity of large clathrin patches to the advancing phagosome lip was often observed by thin-section transmission electron microscopy, as well (Figs. 16a,b,18a). The association of clathrin with 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. 18b).

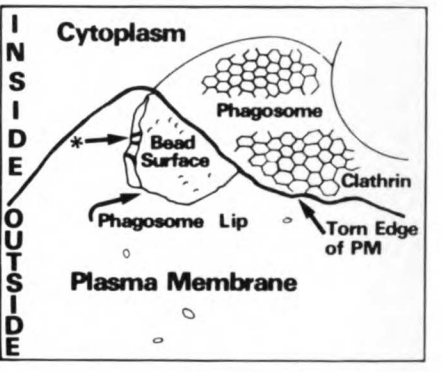
Immunofluorescence studies of clathrin in macrophages

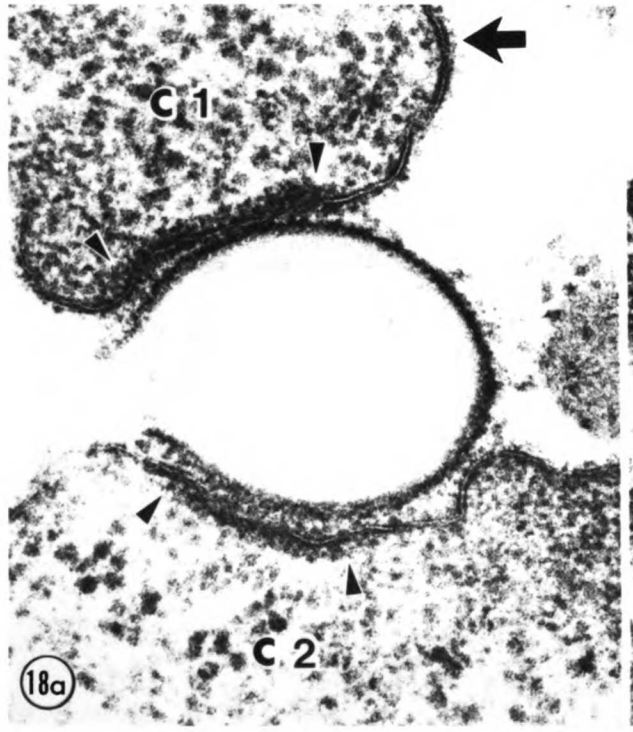
The distribution of clathrin in whole control and phagocytosing macrophages was further examined by indirect immunofluorescence using antibodies raised against bovine brain coated vesicle proteins. This antiserum was 80% against clathrin and 20% against triskelion light chains, as determined by Burridge gel analysis (A. Kalov, personal communication). In control macrophages it produced a pattern of punctate staining similar to that observed in fibroblasts (Anderson, et al, 1978), especially over the rounded perinuclear part of the cell (Figs. 2c,f,19b,d). When cells were exposed to 0.45 μm diam. beads, the pattern of clathrin staining appeared similar to that of controls, but these beads were too small for adequate resolution by the light microscope. I therefore examined the pattern of fluorescence after exposing macrophages to large (5.7 μm diam.) latex beads. These beads were themselves negative for fluorescence, but while they were being ingested by cells, a bright rim of fluorescence appeared around some beads (Fig.

Figure 17. (a) 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. (*) indicates 12.4-nm strands that can be seen bridging the gap at the phagosome lip. X 160,000. Bar = 0.2 μm.

Figure 18. Thin-section transmission electron micrographs 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. Small bumps of tannic-acid-stained material distributed at regular intervals along the cell surface are indicated by an arrow. (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. X 90,000. Bar = 0.2 μm.


17


INSIDE
OUTSIDE
Cytoplasm
Phagosome
Bead Surface
Clathrin
Phagosome Lip
Torn Edge of PM
Plasma Membrane


C 1
C 2
18a

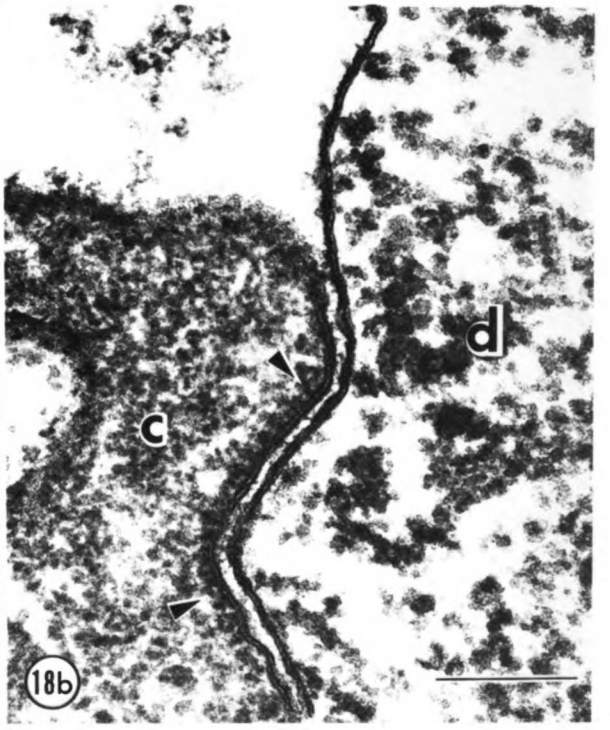
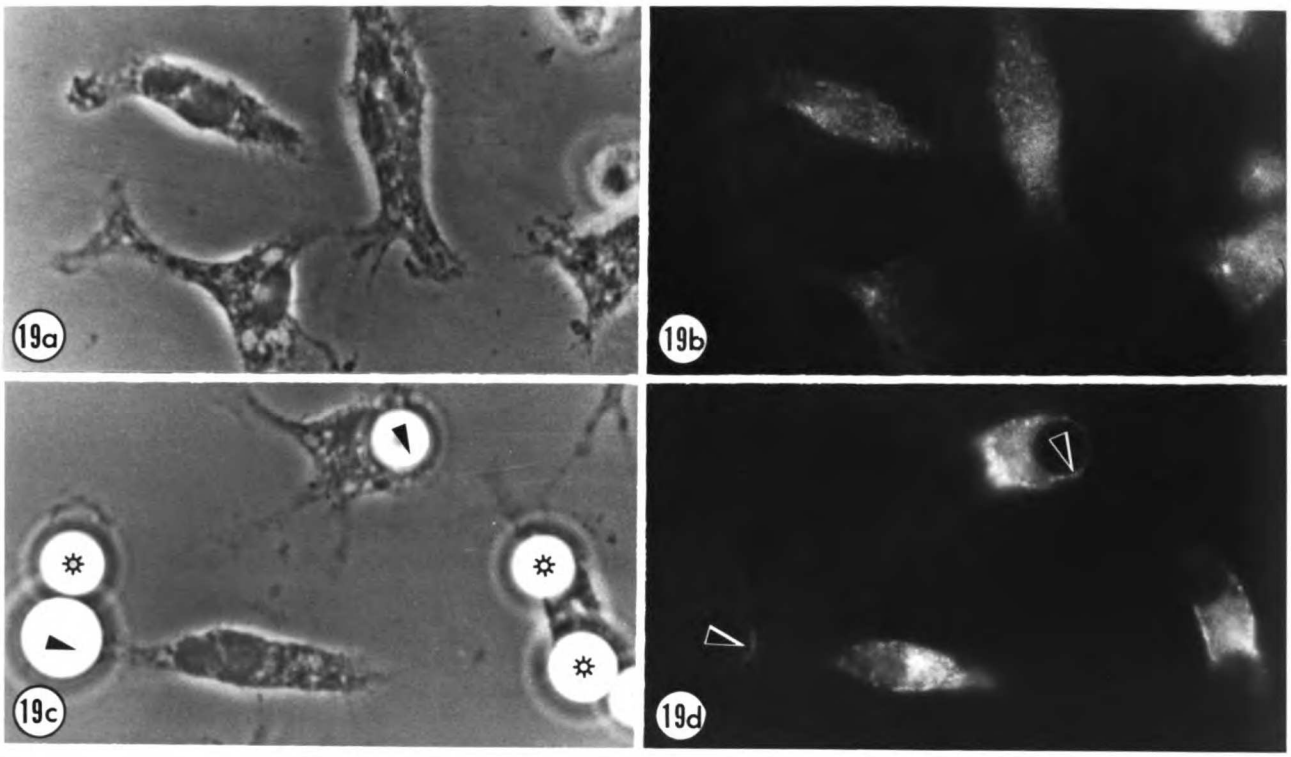

C
d
18b

Figure 19. Demonstration of coated vesicle formation in control and phagocytosing macrophages by indirect immunofluorescence. (a) Phase-contrast and (b) immunofluorescence micrographs of control macrophages showing punctate clathrin staining. (c) Phase-contrast and (d) immunofluorescence micrographs of macrophages exposed to 5.7-μm latex beads for 15 min showing punctate cytoplasmic staining. Some beads are entirely without fluorescence (*) but others show bright patches of clathrin staining around them (arrowheads). X 1,230.


19a
19b
19c
19d

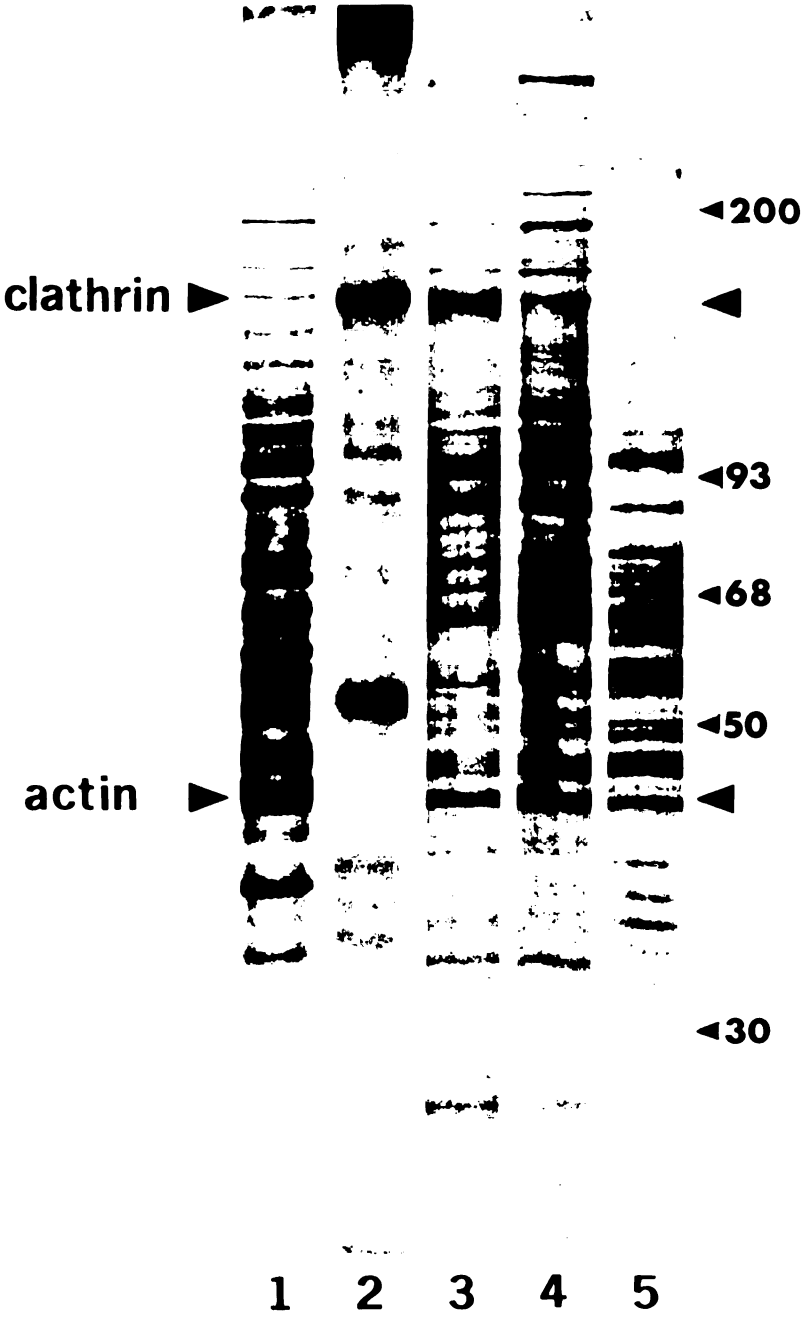
19d). In some cases this rim appeared punctate and, in others, almost continuous.

Biochemical Observations

Proteins associated with isolated phagosomes

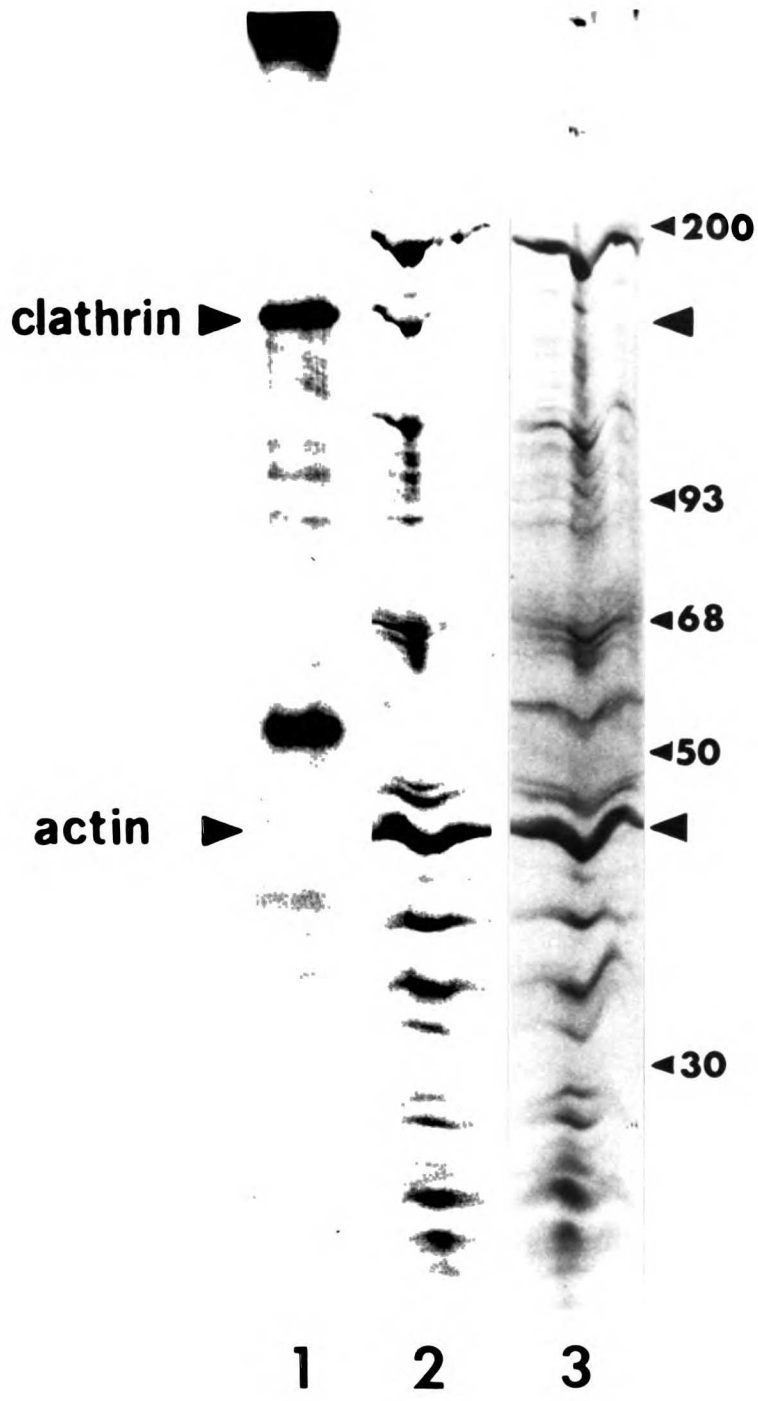
Because of their buoyant density, latex bead phagosomes can be purified from cell homogenates by flotation on sucrose density gradients (Nachman, et al, 1971; Werb and Cohn, 1972). When the ^{35}S-methionine labeled proteins of such isolated phagosomes were analyzed by SDS-polyacrylamide gel electrophoresis, phagosomes were observed to contain a distinct subset of the polypeptides present in whole cell homogenates (Fig. 20, Lane 1). Proteins of M_r 41,000, 98,000, 104,000, 110,000, 119,000, 127,000, 172,000, and 269,000 were particularly prominent in phagosomes isolated after 5 min of ingestion (Fig. 20, Lane 3). The 41,000 dalton polypeptide is probably actin and was present in varying amounts in different phagosome preparations. Polypeptides of M_r 71,000, 135,000, 141,000, 180,000 and 195,000 were also present, but were reduced in intensity on 5 min phagosomes compared to beads isolated after binding at 0 ^{o} C (Fig. 20, Lanes 3,4). I have identified the 172,000 polypeptide as clathrin on the basis of the following observations: 1) This band comigrated with radiolabeled brain coated vesicle clathrin on SDS-PAGE (Fig. 20, Lane 2). 2) Immunoprecipitation with antibodies to brain coated vesicle proteins precipitated a band of 172,000 which was not present after immunoprecipitation with a non-specific antiserum (Fig. 21, Lanes 2,3). As noted in Materials and Methods, the immunoprecipitation procedure resulted in non-specific adsorption to the Staph A of a number of bands in addition to clathrin.

Figure 20. Polyacrylamide gel patterns of proteins associated with isolated phagosomes. Mouse J774.2 macrophages were labeled with ^{35}S-methionine and latex bead phagosomes were isolated as described in Materials and Methods. Lane (1), total cell protein; lane (2), ^{125}I-labeled bovine brain coated vesicles (the major iodinated bands are clathrin and tubulin); lane (3), latex bead phagosomes isolated after incubation for 5 min at 37° C; lane (4), latex beads isolated after binding to cells for 30 min at 4° C (no phagocytosis occurs at this temperature); lane (5), latex beads added to cell homogenates after disruption. The positions of clathrin and actin are indicated by large arrows. Molecular weight standards (as K daltons) are indicated by small arrows: myosin (200 Kd), phosphorylase B (93 Kd), bovine serum albumin (69 Kd), immunoglobulin (heavy chain)(50 Kd), and carbonic anhydrase (30 Kd).



20

Figure 21. Immunoprecipitation of macrophage clathrin. Mouse J774.2 macrophages were labeled with ^{35}S methionine and allowed to ingest latex beads. Clathrin was immunoprecipitated from cell homogenates using antibodies to bovine brain coated vesicles, as described in Materials and Methods. Lane (1), ^{125}I-labeled clathrin prepared from brain coated vesicles; lane (2), immunoprecipitation with antibodies to clathrin; lane (3), immunoprecipitation with preimmune rabbit serum. The positions of clathrin and actin are marked by large arrowheads and the molecular weight standards are indicated.



(21)

Nevertheless, it is clear that the 172,000 dalton clathrin band was present in these samples only after specific immunoprecipitation (Fig. 21, Lane 2) and was missing from the sample incubated with a preimmune antiserum (Fig. 21, Lane 3).

When fluorograms of these gels were traced by densitometer, clathrin was found to constitute 5% of the total protein associated with phagosomes after 5 min of ingestion (Table 9). The clathrin band accounted for approximately 1% of the total labeled cellular protein in macrophage homogenates and 2.4% of that associated with latex beads bound to the cell surface at $0^{\circ}C$ (Table 9). When latex beads were added to cell homogenates after disruption, no clathrin was detected on beads isolated from the mixture (Fig. 20, Lane 5). Actin accounted for 2-5% of the total cell- and bead-associated protein in these samples.

Discussion

Distribution of clathrin on macrophage plasma membranes

Endocytic coated pits were first described in detail by Roth and Porter (1964) in the mosquito oocyte and similar structures were subsequently identified in a wide variety of cell types (Fawcett, 1965;Friend and Farquhar, 1967). Using negative staining, Kanaseki and Kadota (1969) were able to demonstrate the hexagonal lattice structure of the coat, and Pearse (1975) later purified the major structural protein of this lattice and named it "clathrin" from the Greek word for basket. Infrequent coated vesicles, especially of the smaller, Golgi-related variety, have been noted previously in macrophages (Roth and Porter, 1962; Fawcett, 1965; Nichols, et al, 1971;Wisse, 1977;Munte-Kaas and

Table 9

Cell- and Phagosome-Associated Proteins

Source	Protein	
	Clathrin (%)	Actin (%)
Phagosomes (5 min)	4.9	2.6
Surface-bound beads (0°C)	2.4	3.9
Cell homogenate (control)	0.8	3.2
(0°C binding)	1.4	4.9
(5 min phagocytosis)	1.3	4.7

Mouse J774.2 macrophages were prelabeled with ^{35}S-methionine, incubated with latex beads, homogenized, and phagosomes isolated as described in Materials and Methods. Labeled proteins were run on SDS-polyacrylamide gels, fixed, dried and fluorographed. The fluorograms were scanned and the densitometric tracings were cut out and weighed to determine the % protein in chosen polypeptide bands.

Kaplan, 1980), and the present observations using platinum replicas, thin-section electron microscopy, and indirect immunofluorescence indicate that 150-200 nm plasma membrane-associated clathrin-coated vesicles are at least as abundant in these actively endocytic cells as they are in normal fibroblasts (Heuser, 1980;Pfeiffer, et al, 1980; Salisbury, et al, 1980;Wall and Hubbard, 1981). As shown in Table 6, clathrin basketworks occupied about 1.5% of the free upper plasma membrane surface of macrophages and up to 10% of the adherent lower surfaces. I also observed all stages of coated vesicle formation in these samples, from small flat patches to spherical forms 150-200 nm in diameter (Figs. 12a,13), as has been described by Heuser (1980) in cultured mouse fibroblasts. Addition of a tannic acid post-fixation step during processing for thin-section electron microscopy allowed easy visualization of coated membranes, both associated with the plasma membrane (Fig. 12b,c) and in the perinuclear cytoplasm. Even though our method of rupturing cells by sticking upper cell surfaces to a poly-L-lysine-coated coverslip produced a somewhat uneven pattern of opened cells, the sample of upper and lower plasma membrane surfaces that I observed appeared to be reasonably random. 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 (Tables 6 and 7). This increase was especially true for large flat patches (Fig. 13a), which were never observed on the upper cell membranes (except associated with phagosomes). Similar flat clathrin carpets have been reported recently on the bottom surfaces of cultured HeLa cells (Maupin and Pollard, 1981), where they may play a role in adhesion of cells to the coverslip. 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 (Reaven and Axline, 1973). A two-fold increase of clathrin-coated pits on the ventral surface of migrating chick embryo fibroblasts was noted by Abercrombie, et al, in their original description of adhesion plaques (1971). Preliminary experiments in which macrophages were allowed to spread onto IgG-coated surfaces have indicated that very large areas of basketwork were assembled in response to this specific ligand-receptor (Fc) interaction (Aggeler, et al, 1982). These findings suggest that extracellular ligand binding to cell surface receptors, followed by conformational changes in the complex, may signal clathrin binding at the cytoplasmic surface of the interacting membranes.

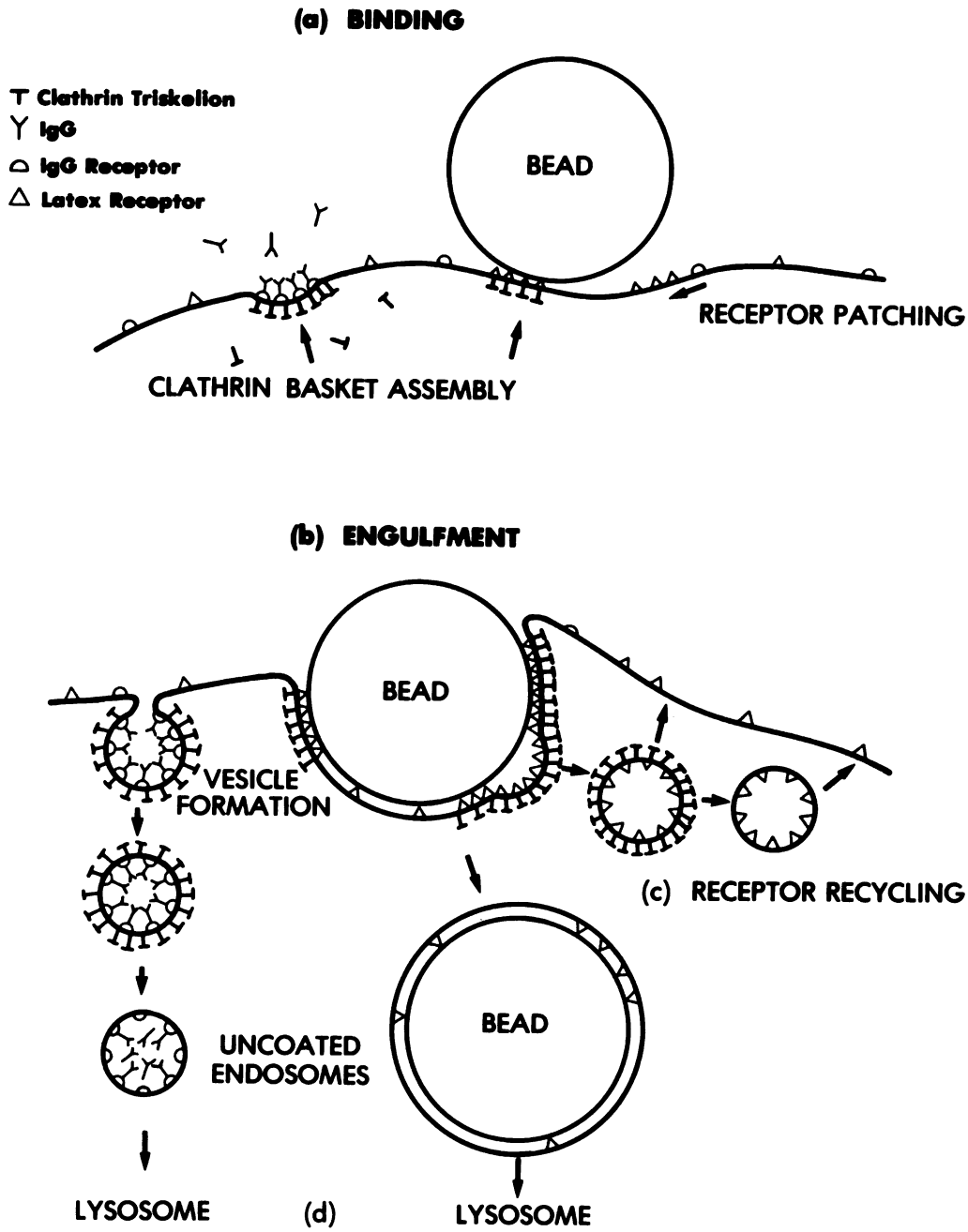
The role of clathrin in phagocytosis

Receptor-ligand interactions

One view of phagocytosis is that it represents the successful attempt of a cell to ingest a very large receptor-ligand complex and is, thus, similar mechanistically to receptor-mediated pinocytosis of soluble ligands (Fig. 22). There are several reasons for thinking that these processes may share similar mechanisms. Foremost, each requires specific membrane-ligand interactions. In the case of phagocytosis, both receptor and ligand must be evenly distributed over the interacting surfaces or ingestion of bound particles cannot proceed (Griffin, et al, 1975,1976). Particles that allow free movement of ligands on their surface are more readily bound by macrophages than non-fluid ones (Lewis, et al, 1980), suggesting that some clustering of ligands facilitates

Figure 22. Model for the assembly of clathrin during various stages of endocytosis by macrophages. (a) Triskelions bind to the cytoplasmic face of the plasma membrane at sites of binding to cell surface receptors during both phagocytosis of particles and pinocytosis of soluble ligands. (b) After particle or ligand engulfment, coated vesicles may function to sort various membrane components (i.e. receptors) from vesicle contents for ultimate transport either back to the cell surface (c) or into lysosomes (d).

POSSIBLE ROLES FOR CLATHRIN IN ENDOCYTOSIS



binding. Receptors for soluble ligands may be either diffusely distributed over the cell surface or preclustered within coated pits (Goldstein, et al, 1979;Pearse and Bretscher, 1981). In either case, the first step in internalization appears to be binding to a specific cell surface receptor. For latex beads, the biochemical nature of the receptor/ligand interaction is not yet known (see previous chapter), but specific membrane/particle recognition is assumed to take place (Karnovsky, et al, 1975).

Clathrin basket assembly

Many different soluble ligands have now been found to enter cells while bound to receptors clustered in clathrin-coated pits. This may be a general mechanism for receptor-mediated endocytosis (Anderson, et al, 1978; Gorden, et al, 1978; Youngdahl-Turner, et al, 1978;Goldstein, et al, 1979;Kaplan, 1981; Matlin, et al, 1981; Pearse and Bretscher, 1981), although exceptions have been noted (Bretscher, et al, 1980;Huet, et al, 1980; Kaplan, 1981;Montesano, et al, 1982;Nature and Orci, 1982). The signals for clathrin basket formation are unknown at present, and it is not clear whether clathrin is actively involved in receptor patching, or only in stabilizing already-patched receptors within coated pits. The basic subunit of the clathrin basket is a triskelion made up of three clathrin molecules with their ends associated at hexagon vertices (Ungewickell and Branton, 1981;Crowther and Pearse, 1981). In addition to the clathrin trimer, each triskelion contains three low molecular weight proteins (~35,000) (Kirchhausen and Harrison, 1981) and a protein of M_r 110,000 that probably mediates binding to the plasma membrane (Ungewickell and Branton, 1981;Pfeffer and Kelly, 1981). Blitz, et al,

(1977) have reported that the 110,000 dalton protein associated with brain coated vesicles is phosphorylated, and the recent finding of Patzer, et al, (1982) of ATP-dependent release of clathrin from isolated coated vesicles suggests that phosphorylation may be an important step in clathrin binding to membranes. The initial rate of clathrin basket formation in vitro increases with temperature between 10 and 30°C, but the extent of basket formation is actually decreased at higher temperatures, suggesting that baskets are labile under physiologic conditions (Jaarsveld, et al, 1981).

It is probable that coats grow by addition of triskelions at their periphery (Heuser, 1980), but the size and location of any cytoplasmic pool of clathrin is presently unknown. My estimate of the amount of clathrin in a macrophage is approximately 6.3×10^6 molecules or 2.1×10^6 triskelions per cell based on gel scans indicating that ~1.2% of total protein is clathrin (Table 9). This is enough clathrin to cover ~ 800 μm^2 of membrane with basketwork, well over 50% of the surface area of a macrophage (Steinman et al, 1976; Phaire-Washington, et al, 1980; Petty, et al, 1980). This estimate, therefore, suggests that a sizeable pool of cytoplasmic clathrin must exist in these cells. This pool need not be soluble, but may be present in the form of small clathrin-coated vesicles, although Heuser (1980) has argued that growth of patches by such 'quantal' addition of baskets does not occur in fibroblasts. Evidence has been presented that coated patches never leave the plasma membrane of 3T3 cells and that no cytoplasmic pool of clathrin exists in these cells (Wehland, et al, 1981; Willingham, et al, 1981). In contrast, both the work of Heuser (1980) showing gradual growth of coated pits by peripheral addition of hexagons and my estimates of the total

clathrin content of macrophages indicate that these basketworks form by assembly from a cytoplasmic pool of triskelions.

Association of clathrin with phagosomes

Although it is generally believed that phagocytosis is the only type of adsorptive endocytosis in which coated vesicles do not play a major role (Pearse and Bretscher, 1981), the early appearance of clathrin patches and vesicles on the surface of nascent phagosomes, as reported here (Figs. 15-18, Table 8), shows that clathrin-coated structures probably function during phagocytosis as well. The presence of clathrin patches near the advancing lip of phagosomes that were not yet completely closed (Figs. 16-18) indicates that association of clathrin with phagosome membrane can be a very early event. After a 1-min phagocytic pulse, 37% of phagosomes had detectable clathrin patches on their surface (Table 8), suggesting that such an early transient association of basketwork may occur during the formation of most phagosomes. The marked increase of clathrin associated with isolated phagosomes after 5 min of ingestion also supports this idea (Table 9).

Endosome uncoating

During receptor-mediated uptake of soluble ligands, clathrin coats disappear from pinocytic vesicles within 2-5 min (Brown, et al, 1980; Wall, et al, 1980; Wall and Hubbard, 1981). The signals for and timing of small vesicle uncoating are unknown, but similar mechanisms probably act during uncoating of coated phagosomes, as well. Local changes in Ca^{2+} concentration may play a role in this process. Both coated vesicles (Blitz, et al, 1977) and phagosomes (Lew and Stossel, 1980) can

sequester Ca^{2+}, and localized reduction of divalent cation concentrations near these structures may contribute to clathrin disassembly. In addition, it has recently been reported that vesicles become uncoated by an ATP-dependent mechanism (Patzer, et al, 1982), and increased pH is also known to cause basket dissociation (Jaarsveld, et al, 1981). The fact that 40-50 % of phagosomes remain coated after a 5-min phagocytic pulse (Table 8) may reflect the longer time necessary for phagosome closure. By 30 min after a phagocytic pulse, all of the phagosomes were located deep within the cytoplasm and none were positive for clathrin (Table 8) (Orci,et al, 1982). When macrophages were allowed to spread on IgG-coated coverslips ("frustrated phagocytosis"), large numbers of clathrin-coated areas were observed, and these were evident even after 20 min at 37°C (Aggeler, et al, 1982). This stabilization of basketworks on these immobilized membranes may be further indication that vesicle closure contributes to uncoating. In addition, some phagosomes may become uncoated by virtue of budding of coated vesicles off of them as frequently observed in this study (see Figs. 15b,16).

Membrane recycling

The function of clathrin during endocytosis is not clear at present, but one possibility is that clathrin-coated vesicles may form to mediate recycling of internalized membrane back to the plasma membrane in response to a phagocytic or pinocytic stimulus. The involvement of clathrin in membrane shuttling was first described by Heuser and Reese (1973) at the presynaptic membrane of the frog neuromuscular junction, in this case mediating retrieval of newly fused synaptic vesicle membrane. A number of recent reports have documented the massive and

rapid recycling of membrane in the opposite direction, from pinocytic and phagocytic vesicles back to the plasma membrane. Macrophages can turn over their entire plasma membrane every 30 minutes by pinocytosis alone (Steinman, et al, 1976). Membrane internalized in pinosomes reappears at the plasma membrane surface within 10 minutes in hamster fibroblasts (Storrie, et al, 1981), and labeled phagosome membrane proteins also return rapidly to the plasma membrane (Muller, et al, 1980a and b). In addition, a number of specific receptors return so rapidly to the plasma membrane surface that it is difficult to lower their effective number (Stahl,et al,1980; Wall and Hubbard,1981). It is possible that some of the clathrin baskets observed on phagosomes formed to mediate such return. Shuttling of membrane components and vesicle contents by clathrin-coated vesicles during protein synthesis (Farquhar,1978), transport of IgG across the newborn rat intestine (Abrahamson and Rodewald, 1981), and infection of cells by vesicular stomatitis virus (Rothman and Fine, 1980) and by influenza virus (Matlin,et al,1981) has been suggested, although the cellular mechanisms for identification and sorting of these vesicles are not known. The presence of clathrin patches near the advancing lip of phagosomes that were not yet completely closed (Figs. 16-18) indicates that association of clathrin with phagosome membrane can be a very early event, easily within the time span necessary for rapid membrane recycling (5 to 10 minutes).

CONCLUSION

Initial Events during Phagocytosis by Macrophages

What then are the initial events that take place during phagocytosis by macrophages? I would like to define the initial stages of phagocytosis as those events that begin with particle binding to the macrophage surface and end with membrane fusion, leaving the phagosome vesicle completely within the cytoplasm. Various aspects of particle binding to the cell surface have been described and studied in some depth (Gordon and Cohn, 1973; Stossel, 1974; Silverstein, et al, 1978), but a detailed understanding of the subsequent steps of engulfment is still incomplete. These early steps of phagocytosis are of interest not only because of their importance to normal immunological function by macrophages, but also as an example of several general principles of cell biology in action.

Structure and Function of the Plasma Membrane

In the ten years since Singer and Nicholson (1972) first proposed their fluid-mosaic model for the structure of the plasma membrane, our view of this organelle has changed dramatically. It is now clear that the plasma membrane is a highly dynamic and active structure capable of rapid changes in local composition and shape (Nicolson and Poste, 1976; Bretscher, et al, 1980). At the same time, cells can also maintain specific domains within the fluid bilayer of the membrane that are easily distinguished by their lipid and protein composition (Montesano, et al, 1981; Friend, 1982). In addition, it is evident that there is extensive interaction and cycling between various intracellular membrane

compartments and the plasma membrane and that cells are able to shift membrane from one compartment to another, probably by small vesicle shuttling, in order to maintain a homeostatic balance among these compartments.

The active participation of the plasma membrane in the initial stages of phagocytosis provides examples of several important aspects of plasma membrane function.

1) The requirement for particle binding to specific plasma membrane receptors is an example of the cell's ability to detect specific extracellular ligands by means of a wide variety of recognition molecules on the plasma membrane surface.

2) The requirement for circumferential binding during phagocytosis provides an excellent demonstration of protein movement within the plane of the membrane (Griffin, et al, 1975, 1976).

3) The assembly of clathrin basketworks on the cytoplasmic surface of nascent phagosomes may be an example of transmembrane signalling proceeding from cell surface receptor binding, to a conformational change in the integral membrane receptor, to triskelion binding.

The Cytoskeleton

Many studies during the past few years have produced a new image of the structure of the cytoplasmic ground substance. It is now clear that the cell is not a fluid-filled bag in which subcellular organelles float, but that the cytoplasm is highly structured by an elaborate network of filaments forming an intracellular scaffolding that organizes

the cellular contents (Porter and Tucker, 1981; Heuser and Kirschner, 1981). This cytoskeleton is not a static structure, however, especially in motile cells such as macrophages. Studies of phagocytes both in culture and in vivo demonstrate that these cells are highly motile and show a great deal of plasma membrane activity, such as ruffling and formation of lamellipodia. This high degree of motility is due in part to interactions between cytoskeletal filaments which probably undergo constant gel/sol transformations (Weeds, 1982), and in part to a pattern of dynamic interactions of these filaments with the plasma membrane (Reaven and Axline, 1974; Boyles and Bainton, 1979; Trotter, 1981; Aggeler and Werb, 1982). These data suggest that focal adhesions of microfilaments to the plasma membrane surface are constantly forming and reforming. Perhaps as a result of this process, small membrane ruffles and lamellipodia are thrown up. In addition, these cells possess the capacity of rapid migration over extracellular surfaces, and the ability to interact with and engulf large particles. Although the focal attachment of microfilaments to the plasma membrane has been amply demonstrated in macrophages, the molecular basis for this binding is unknown and specific membrane-associated actin-binding proteins remain to be identified in these cells. However, the effect of the fungal agent, cytochalasin B, on both spreading and phagocytosis by these cells (Allison, et al, 1971; Axline and Reaven, 1974) suggests that such specific binding proteins do exist. The cytochalasins are known to bind preferentially to the "growing" end of actin filaments (MacLean-Fletcher and Pollard, 1980), and a number of reports have indicated that it is this growing end that is inserted into the plasma membrane at sites of microfilament attachment (Salisbury, et al, 1980; Trotter, 1981; Weeds,

1982). I suggest that the cytochalasins act in cells by intercalating between microfilaments and the plasma membrane, thereby causing cytoskeletal detachment. Without the ability to reform focal attachments to the membrane, macrophages would be paralyzed, both in spreading (Allison, et al, 1971) and in phagocytic capacity (Table 4). Some uptake of soluble ligands can apparently still take place (Table 5), and it is interesting to note that this may occur via plasma membrane channels that "sink" deep into the cytoplasm in the presence of cytochalasin B (Nilsson, 1977). This observation implies that one normal function of the dynamic cytoskeletal interactions with the plasma membrane may actually be to keep that membrane at the cell surface.

Rapid Cellular Response

Most studies of phagocytosis in the past have emphasized the process as a whole, establishing such requirements as specific binding, metabolic energy, and cell contractility, but detailed descriptions of the steps necessary for the formation of a single phagosome are lacking. In this study, I have looked primarily at these earliest events, and from my results, it seems clear that formation of an individual phagosome (at least for small latex beads) is a very rapid event, taking less than 2 min to complete. During this time the plasma membrane forms a vesicle, plasma membrane receptors are bound, released, and recycled, clathrin basketworks assemble on the phagosome, and microfilament attachments to the plasma membrane are made and broken. This complicated process serves to emphasize the rapidity with which the cell can respond to its extracellular environment.

Clathrin Basket Assembly - An Example of Transmembrane Signalling

There are many biological systems in which extracellular signals, acting at the plasma membrane surface, produce profound metabolic changes in major cell functions. The polypeptide hormones, for example epidermal growth factor (EGF), are examples of this important phenomenon. In the case of EGF, binding of the circulating hormone to specific cellular receptors (Gorden, et al, 1978), followed by irreversible covalent linkage of hormone to receptor (Baker, et al, 1979;Linsley, et al, 1979), perhaps by proteolytic alteration of the receptor complex (Glenn and Cunningham, 1979), is sufficient to produce subsequent stimulation of DNA synthesis in responsive cells. It has been suggested that the signal for this response is transmembrane alteration of the receptor marked by specific phosphorylation of its cytoplasmic domain (Carpenter, et al, 1978; Maciag, 1982). The nature of the second messenger that actually reaches the nucleus is unknown in this case. After these initial signalling events, both hormone and receptor are internalized via coated pits, and ultimately degraded within lysosomes (Gorden, et al, 1978). Recent studies of the endocytosis of a number of different macromolecules, including EGF, via coated pits indicate that clathrin basket assembly at sites of surface ligand binding may be another example of specific transmembrane signalling.

In the case of clathrin basket assembly, a sequence of signalling events can be proposed:

1) Initially specific cell surface receptors interact with an extracellular site or molecule. These receptors, or other closely associated proteins, will be integral membrane proteins that possess a

cytoplasmic domain.

2) Upon specific ligand binding, a conformational change takes place in these membrane-spanning proteins leading to an alteration in their cytoplasmic domain.

3) This altered cytoplasmic end now recognizes and binds triskelions. Such an integral membrane binding protein that recognizes the 110K polypeptide associated with triskelions has been hypothesized, but not yet identified (Ungewickell and Branton, 1981).

4) Clathrin-coated patches grow at their periphery by addition of triskelions to the original small nucleus of hexagons (Heuser, 1980). It is not known whether such subsequent addition requires binding of triskelions to membrane proteins, or whether some attachment to free clathrin arms can take place, as well. The minimum number of triskelion/membrane interactions necessary to nucleate a patch is also unknown.

5) Rearrangement of the hexagonal lattice into pentagons and heptagons causes the originally flat patches to round and form vesicles (Kanaseki and Kadota, 1969; Heuser, 1980).

6) Closure of the endocytic vesicle activates a membrane Mg^{2+}-ATP-dependent Ca^{2+} pump (Lew and Stossel, 1980).

7) Local reduction of Ca^{2+} causes baskets to fall off the endosome membrane (Nandi, et al, 1981).

Variations of this sequence could also be invoked for formation and shuttling of the class of small coated vesicles usually associated with

the rough endoplasmic reticulum and the Golgi-GERL region.

A possible alternate sequence for some specific receptors is suggested by the proposal of Bretscher, et al, (1980) that coated vesicles act as molecular filters. These authors propose that clathrin baskets can act to immobilize some proteins within the plane of the plasma membrane, while allowing others to diffuse away freely. This concept has been invoked to explain how some receptors appear to mediate ligand uptake but never leave the plasma membrane surface (Stahl, et al, 1980; Wall and Hubbard, 1981). According to this idea, the sequence stated above might be altered in the following ways:

4) Several (> 2) adjacent receptors must bind triskelions in order to nucleate a coated patch. Patches grow at their periphery, as outlined above.

5) Rearrangement of the lattice causes rounding and formation of coated pits and vesicles.

6) At some short interval after receptor binding, the ligands fall off. This must occur within pits or channels that have limited access to the exterior in order for the ligand to be included within the vesicle contents.

7) Freed receptors now rapidly diffuse out of the coated pits and back onto the plasma membrane surface (Bretscher`s molecular filter).

8) Unbinding of ligand reverses conformation and coats fall off the cytoplasmic surface.

This alternate pathway raises a number of questions for which there is

very little answering data. The most controversial issue is whether endocytic coated vesicles ever exist (i.e. completely sealed off from the exterior but still clathrin-coated). The evidence rests on interpretation of thin-section micrographs, and high-quality work in this field is difficult to obtain. The current consensus appears to be that they do exist (D. Friend, personal communication), but they may be a very transient species. This alternative does, however, try to address the difficult problem of how cells may be able to internalize certain ligands without reducing their capacity (number of surface receptors) to take up more. Cells can clearly recognize and correctly route a large number of macromolecules to several different fates within the cell. This ability appears to rest with specific recognition events that take place at one membrane surface and are translated across this membrane to be interpreted within the cytoplasm.

Summary

In summary, according to the information currently available, the initial steps during phagocytosis are probably the following:

1) Initial binding of particle to cell surface receptor. This step probably requires binding to two or more adjacent receptors in order to be functionally irreversible (Lewis, et al, 1980).

2) Circumferential binding or 'zippering', as proposed by Griffin, et al, (1975, 1976).

3) Transmembrane signalling. At least four possible results of such signalling are known, i.e. clathrin basket assembly (Aggeler and Werb, 1982), activation of a Ca^{2+} pump (Lew and

Stossel, 1980; Horwitz, et al, 1981), protein phosphorylation (Blitz, et al, 1977; Patzer, et al, 1982), and activation of a proton (H^+) pump (D. Branton, personal communication).

4) Recycling of receptors (Bretscher, et al, 1980).

5) Ongoing attachment and detachment of cytoskeletal microfilaments. These interactions cause the plasma membrane to be thrown up into ruffles and lamellipodia.

6) Membrane fusion and true intracellular phagosome formation. Little is known about the mechanism whereby the plasma membrane leaflets fuse to close any endosome vesicle. This event in itself, at which time vesicle contents are no longer in contact with the extracellular milieu, may be an important signal for subsequent events (e.g. clathrin basket uncoating).

As I have emphasized above, all of these steps take place within a very short time span (30 sec to 2 min). Clearly much careful work will be necessary to establish the exact timing and the causal interrelationships of these events.

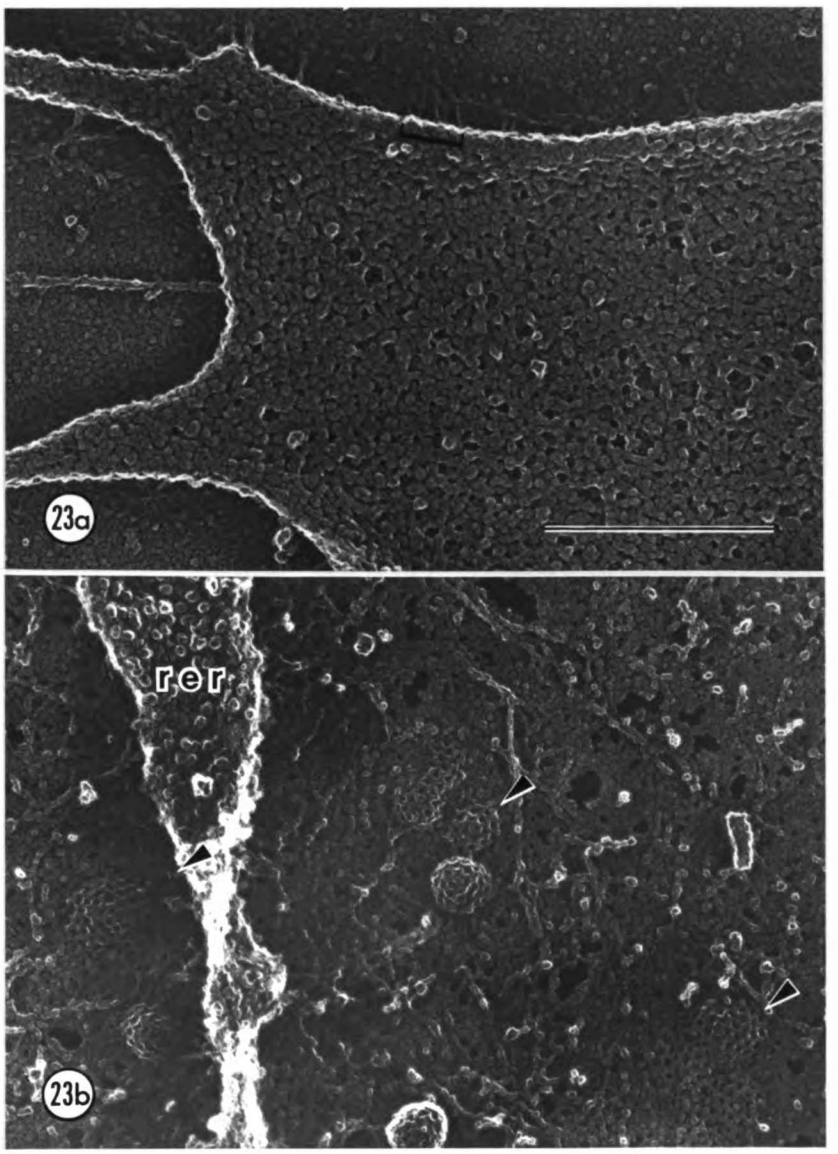
APPENDIX

Comparison of Critical Point Drying and Quick Freezing

An elaborate and increasingly detailed model of the three-dimensional arrangements of various contractile and other proteins into a microtrabecular cytoskeletal meshwork has been built up over the past few years, largely through the use of a variety of electron microscopic techniques (Porter and Tucker, 1981). The morphology and distribution of actin and myosin in non-muscle cells has been studied in whole mounts (Buckley and Raju, 1976; Temmink and Spiele, 1978) and detergent-extracted cytoskeletons (Webster, et al., 1978) of critical-point-dried cells and in platinum replicas of detergent-extracted, quick-frozen cells (Heuser and Kirschner, 1980). Small (1981) studied the influence of OsO_4 post-fixation and of dehydration (a necessary step before critical point drying) on the ultrastructural appearance of actin meshworks in cultured cells and concluded that dehydration altered actin filament organization compared to quick-frozen samples. On the other hand, Pinto da Silva (1980) has criticized quick freezing as a method for preserving membrane morphology. I examined rotary-deposited Pt-C replicas of critical-point-dried whole and broken-open macrophages and of detergent-extracted cytoskeletons to compare this method of sample preparation to the quick-freeze, deep-etch technique.

Figure 22 illustrates the appearance of replicas of critical point dried macrophages. In (a), the spreading edge of a cell showed obvious aggregation and probable extraction of plasma membrane components. (Compare this to the smooth appearance of the cell in Fig. 6). The small strands extending from the cell to the dish surface were still

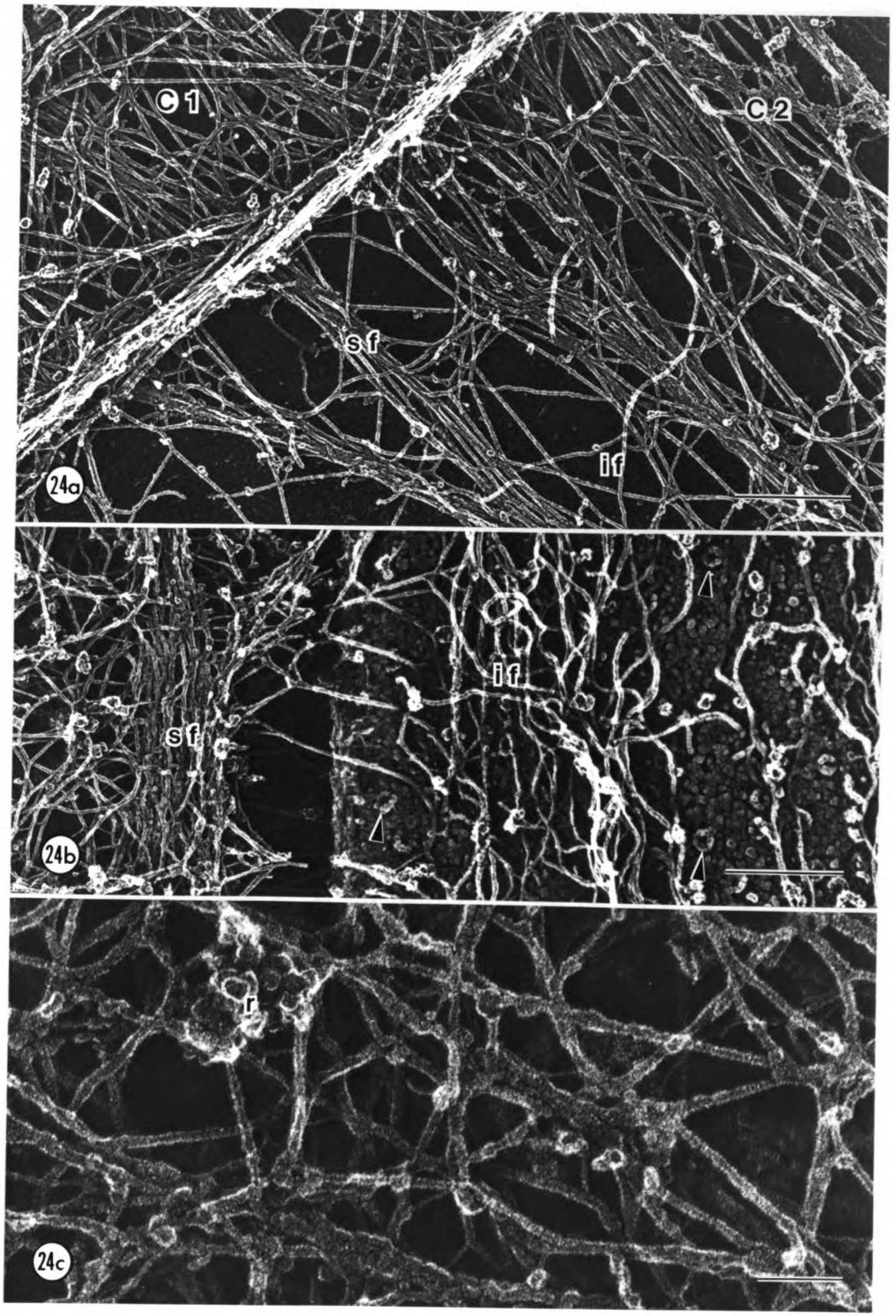
Figure 23. Replicas of critical point dried macrophages. (a) Thin spreading edge of a macrophage showing cobbled appearance of plasma membrane and presence of strands extending from cell surface to the culture dish (bracket). (b) Lower plasma membrane showing ribosome-studded rough endoplasmic reticulum (rer) and numerous clathrin-coated patches and pits (arrowheads). x 60,000. Bar = 0.5 μm.


23a
rer
23b

apparent. Cytoskeletal filaments and other intracellular structures, such as rough endoplasmic reticulum and clathrin basketwork, were reasonable well preserved in replicas of broken-open cells (Fig. 23b), although the underlying plasma membrane was aggregated and holey. In cells spread onto IgG-coated substrata, large areas of clathrin basketworks were observed, and these areas of membrane appeared to be protected from extraction somewhat (Aggeler, et al, 1982). When replicas of detergent-extracted, critical-point-dried fibroblasts were examined, the preservation of cytoskeletal filaments was excellent (Fig. 24). The structure and distribution of microfilaments and intermediate filaments appeared very similar to that in quick-frozen cytoskeletons, as reported by Heuser and Kirschner (1980). The nuclear envelope with nuclear pores was preserved (Fig. 24b), and polyribosomes attached to cytoskeletal filaments were observed (Fig. 24c).

From the above results, I suggest that careful critical point drying followed by rotary replication with Pt-C may be a more generally accessible method than quick-freezing for studying some details of cytoskeletal and plasma membrane structure. In fixed cells, the fine details of the plasma membrane were clearly lost, but some other structures, such as coated vesicles, were quite well preserved. In isolated cytoskeletons, the details of filament structure and distribution were comparable to quick-frozen specimens, and this would seem to be a fruitful experimental method to employ for future studies of cytoplasmic filaments.

Figure 24. Replicas of detergent-extracted rabbit fibroblast cytoskeletons. (a) Overlapping edges of two adjacent cells (C1 and C2) showing well-defined stress fibers (sf) with intermediate filaments (if) coursing between them. x 40,000 (b) Part of the nuclear envelope of an extracted cell enmeshed in a tangle of intermediate filaments. Outlines of nuclear pores (arrowheads) are clearly seen. x 40,000. Bar = 0.5 μm. (c) High magnification view showing cytoskeletal filaments and attached ribosomes (r). x 150,000. Bar = 0.1 μm.


C1
C2
sf
if
24a
sf
if
24b
r
24c

ABBREVIATIONS

Buffer E, extracellular buffer

Buffer I, intracellular buffer

BSA, bovine serum albumin

EDAC, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride

EGTA, ethyleneglycol-bis-(β-aminoethyl-ether)N,N,N',N'-tetraacetic acid

FCS, fetal calf serum

HBBS, Hank's buffered salt solution

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HS, horse serum

Iodogen, 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril

LH, lactalbumin hydrolysate

MEM, minimum essential medium

MES, 2-(N-morpholino)-ethanesulfonic acid

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline

SDS, sodium dodecyl sulfate

Staph A, Staphylococcus aureus

TCA, trichloroacetic acid

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