

ANALOGOUS ULTRASTRUCTURE AND SURFACE PROPERTIES DURING CAPPING AND PHAGOCYTOSIS IN LEUKOCYTES

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ABSTRACT

Ultrastructural analyses have revealed striking similarities between Concanavalin A capping and phagocytosis in leukocytes. Both processes involve extensive membrane movement to form a protuberance or pseudopods; a dense network of microfilaments is recruited into both the protuberance and the pseudopods; microtubules are disassembled either generally (capping) or in the local region of the pseudopods (phagocytosis); and cells generally depleted of microtubules by colchicine show polarized phagocytosis via the microfilament-rich protuberance rather than uniform peripheral ingestion of particles via individual pseudopods. Cap formation can thus be viewed as occurring as an exaggeration of the same ultrastructural events that mediate phagocytosis.

Similar changes in cell surface topography also accompany capping and phagocytosis. Thus, in nonfixed cells, Concanavalin A-receptor complexes aggregate into the region of the protuberance in colchicine-treated leukocytes (conventional capping) or into the region of pseudopod formation in phagocytizing leukocytes. In the latter case, the movement of lectin-receptor complexes occurs from membrane overlying peripheral microtubules into filament-rich pseudopods that exclude microtubules. These data provide evidence against a role for microtubules as "anchors" for lectin receptors. Rather, they indicate a preferential movement of cell surface Concanavalin A-receptor complexes towards areas of extensive (the protuberance) or localized (pseudopods) microfilament concentration.

In conventional capping, Concanavalin A must be added to the colchicine-treated cells before fixation in order to demonstrate movement of receptors from a diffuse distribution into the protuberance. However, Concanavalin A receptors are enriched in the membrane associated with phagocytic particles as compared to the remaining membrane. This particle-induced redistribution of receptors is particularly prominent in colchicine-treated cells that phagocytize and are then fixed and Concanavalin A labeled; both lectin receptors and beads are concentrated over the protuberance. Thus, the final analogy between conventionally capped and phagocytic cells is that in both cases the properties of the plasma membrane in regions of microfilament concentration are modified by Concanavalin A itself (capping) or by the phagocytized particle, to limit locally the diffusion of Concanavalin A receptors.

KEY WORDS phagocytosis · microtubules · microfilaments · Concanavalin A · lysosomes

The concept that microtubules influence cell surface function in polymorphonuclear leukocytes (PMN) as well as monocytes, macrophages, and lymphocytes has arisen from three types of phenomena: First, the capping of surface-bound ligands such as concanavalin A (Con A) which is greatly enhanced by exposure of all these cells to colchicine and a variety of other agents that inhibit microtubule assembly (7, 19, 20); second, the specific preservation of membrane transport (29, 30), loss of membrane lectin receptors (21), and decrease in membrane microviscosity (4) that normally occur during phagocytosis in PMN and are inhibited by colchicine; and third, the reported inhibition by colchicine of fusion between lysosomes and phagocytic vacuoles and/or the plasmalemma in PMN (17, 33). These same processes are also believed to be regulated in part by microfilaments. Thus, cytochalasin B, a putative inhibitor of microfilament function, prevents capping and phagocytosis and enhances lysosomal fusion with the plasmalemma. Neither the precise roles of microtubules and microfilaments in these three processes nor the functional and structural interrelationships of the two cytoskeletal elements has been clear.

We recently described in detail the ultrastructure of Con A capping in microtubule-depleted human blood PMN, monocytes, and lymphocytes (1). Microtubule disassembly alone leads to a marked redistribution of microfilaments from a relatively uniform submembranous distribution into a protuberance. When Con A is present under these conditions, it is accumulated over the protuberance in a "cap." Phagocytosis is also associated with a concentration of microfilaments, in this case to form pseudopods that engulf particles attached to the cell surface (2, 15, 28).

An analysis of cytoskeleton and Con A distribution with phagocytosis is presented here. The ultrastructural and immunofluorescence studies of neutrophils and macrophages described show that pseudopods involved in the ingestion of particles are highly enriched for microfilaments and actin while devoid of microtubules. The absence of microtubules is localized to the pseudopod since we show (and have established previously (6, 23)) that centriole-associated microtubules are extensively assembled during phagocytosis. The changes in microtubule and microfilament distri-

bution are concluded to be analogous in pattern to those seen in capping but localized to regions in proximity to the particle(s) being phagocytized. On the basis of the analogy, predictions are made of the behavior of membrane-bound ligands during phagocytosis. The behavior of bound Con A is shown consistent with the analogy. In addition, evidence is obtained for reorganization of Con A receptors by phagocytic particles in the absence of exogenous ligand. The relationship of the distribution of microfilaments to that of lysosomes is also examined. Our observations suggest a novel interpretation of the colchicine sensitivity of lysosome-phagosome fusion.

MATERIALS AND METHODS

Cells

Suspensions of human leukocytes containing approx. 80% PMN and 20% mononuclear cells were obtained from buffy coat of freshly drawn heparinized blood as previously described (19). Suspensions of peritoneal macrophages (approx. 65% pure) were obtained by the method of Becker (3) from rabbits injected 28-36 h previously with sterile glycogen in saline. Peritoneal PMN had been harvested from the same rabbits at 12-16 h after stimulation. These cells were diluted to 10^6 macrophages/ml in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (DMEM-FCS) and plated in 35-mm plastic culture dishes containing three sterile 15-mm glass cover slips. After 1 h at 37°C in the CO₂ incubator, the medium containing exudate fluid and nonadherent cells was replaced with 2 ml of fresh DMEM-FCS.

Phagocytosis

Human leukocytes were exposed to the following phagocytic stimuli: zymosan (Sigma Chemical Co., St. Louis, Mo.) previously opsonized by incubation for 30 min at 37°C with fresh human serum as before (6); lipopolysaccharide (Difco Laboratories, Detroit, Mich.) or bovine serum albumin (Sigma) coated paraffin oil prepared and opsonized by the method of Stossel (27); and carboxylated polystyrene beads (0.785 μ m diameter: Polysciences, Inc., Warrington, Pa.). Rabbit peritoneal macrophages were incubated with sheep erythrocytes (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) opsonized with sheep anti-rabbit immunoglobulin (IgG, Cappel) using the procedure of Griffin et al. (12).

Antibodies

Human serum containing anti-actin antibody was a generous gift of Dr. A. Fagraeus (Karolinska Institute, Stockholm, Sweden). Fagraeus et al. (8) and Norberg et

al. (18) have established by immunodiffusion that the serum reacts specifically with purified actin and have employed the serum at dilutions up to 1:200 to label microfilaments in a range of cells and tissues (8, 9, 18). We previously confirmed the specificity of the serum for actin and used it to label microfilaments in fibroblasts and polymorphonuclear leukocytes (22). Antibody against bovine brain tubulin was raised in goats in collaboration with Drs. E. L. Becker and H. J. Showell (Dept. of Pathology, University of Connecticut Health Center, Farmington, Conn.). The tubulin was isolated as before (20) and subjected to phosphocellulose column chromatography to remove high molecular weight components and retain only 6S tubulin according to Weingarten et al. (31). The resulting serum gave an immunoprecipitation reaction with both crude and 6S tubulin but not with other proteins including actin. Microtubules are labeled in a range of tissue culture cells as well as in leukocytes at serum dilutions up to 1:500. Labeling is prevented by exposure of cells to colchicine (10^{-6} M; 30 min) or by prior adsorption of the immune serum with purified 6S tubulin.

Electron Microscopy

Cell suspensions (approx. 10^7 human leukocytes/ml) in phosphate-buffered saline (PBS) containing 2 mM CaCl_2 , 2 mM MgCl_2 and 5 mM glucose were exposed to phagocytic particles at 30°C (for zymosan) or 37°C (for oil emulsion and latex) for 0.5–10 min. The cells were then collected by centrifugation (3 s at 12,000 rpm in an Eppendorf microcentrifuge), the supernatant fluid was aspirated, and the cells were fixed at room temperature for 30 min in 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. The cell pellets were washed in buffer, postfixed in 1% aqueous osmium tetroxide, dehydrated through a graded series of ethanols and embedded in Spurr resin (6). Thin (silver) sections were collected on 300-mesh grids (Formvar coated for zymosan samples), stained with aqueous uranyl acetate and lead salts as previously described (1), and examined in a Philips 300 electron microscope.

Fluorescein-Con A Labeling

Fluorescein isothiocyanate-conjugated Con A (fluorescein-Con A) was prepared as before (19). Suspensions of human leukocytes (10^6 cells/ml) either were exposed for 5 min to carboxylated polystyrene beads simultaneously with fluorescein-Con A (10 $\mu\text{g}/\text{ml}$) or were treated with beads, fixed with 2% paraformaldehyde in PBS, and subsequently incubated with fluorescein-Con A. The cells were washed, mounted in 50% glycerol and observed by phase and fluorescence microscopy. Phagocytosis was not inhibited by the low concentration of Con A employed. Carboxylated polystyrene beads were chosen because, unlike nonderivatized beads, they do not bind fluorescein-Con A.

Immunofluorescence Staining of Actin and Tubulin

The labeling methods followed the general outline of Brinkley et al. (5) and Lazarides and Weber (16) and have been described before in studies with polymorphonuclear leukocytes (22). Briefly, monolayers of peritoneal macrophages were exposed to opsonized erythrocytes for 10–20 min at 37°C. Nonadherent erythrocytes were removed by rinsing the cover slips through four changes of PBS. For antitubulin staining, the cells were fixed for 30 min at 37°C with 3% formaldehyde followed by rinsing in PBS, 5-min fixation in acetone at -10°C , and further rinsing in PBS. The monolayers were then incubated sequentially for 30 min at room temperature with goat antitubulin antibody (1:200 dilution in PBS) and rabbit anti-goat fluorescein isothiocyanate-conjugated IgG (fluorescein-IgG) (1:20 dilution in PBS; Meloy Laboratories, Inc., Springfield, Va.). Control cells were exposed to a 1:200 dilution of preimmune serum from the same goats, followed by fluorescein-IgG. The cover slips were rinsed and mounted on slides in 50% glycerol-PBS. For antiactin staining, the procedure was identical except that paraformaldehyde fixation was omitted, and labeling of acetone-fixed cell monolayers was done with human antiactin antibody (1:100) and sheep anti-human fluorescein-IgG (Cappel). Control monolayers were exposed to a 1:100 dilution of normal human serum (from donors other than those used for the immune serum) followed by fluorescein-IgG. The cells were observed by combined phase and fluorescence microscopy in a Zeiss Photomicroscope III. Fluorescence associated with antibody-labeled monolayers was photographed with Kodak Tri-X-Pan film. Control monolayers were insufficiently fluorescent to permit photographic recording. This absence of nonspecific labeling of macrophages by fluorescein-IgG facilitated the evaluation of labeling patterns.

RESULTS

Microfilament Distribution during Phagocytosis

Resting leukocytes show a thin, uniform band of microfilaments underlying the whole membrane and occupying numerous small folds and projections in the cell surface (illustrated in reference 1). This relatively simple architecture is rapidly altered during phagocytosis. Fig. 1 shows a human neutrophil within 30-s contact with opsonized zymosan. Several particles have been enveloped by pseudopods packed with microfilaments which exclude subcellular organelles. This same rapid concentration of microfilaments in pseudopods is characteristic of phagocytizing human blood mon-

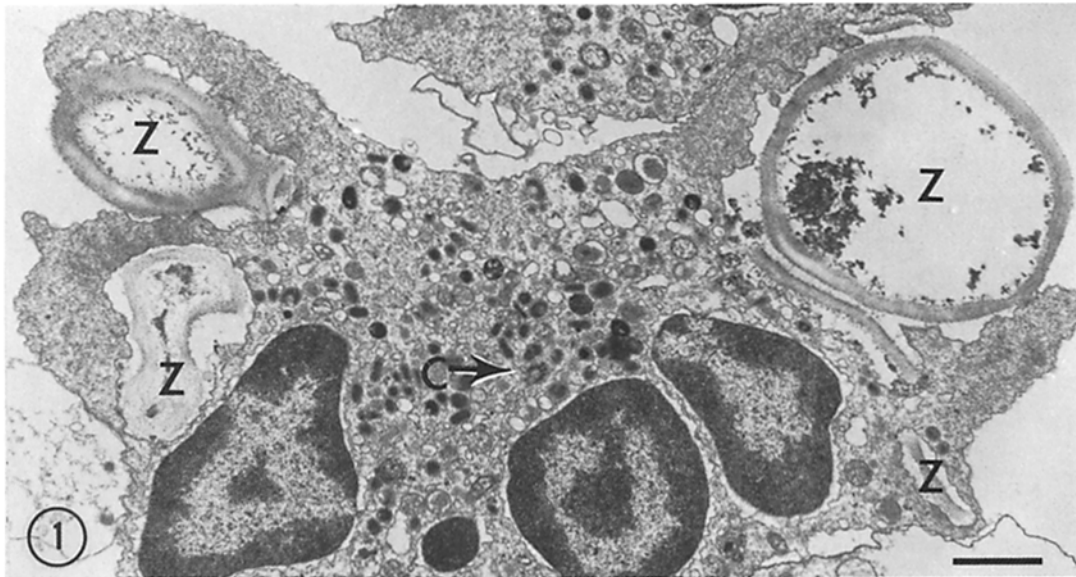


FIGURE 1 Human neutrophil ingesting zymosan. The cell was exposed to opsonized zymosan (Z) at 30°C for 0.5 min before fixation. Prominent microfilament-rich pseudopods that exclude granules encircle the particles. The base of these pseudopods is depleted of microfilaments and contains granules in process of discharge towards the zymosan particles. A centriole (C) with associated microtubules occupies a central position in the cell. Bar, 1 μ m. \times 11,600.

ocytes and rabbit peritoneal macrophages (shown subsequently). Regions of the cell not involved in phagocytosis retain their original thin band of peripheral microfilaments. Fig. 2 is a higher magnification of a pseudopod (upper left) of the cell in Fig. 1. The pseudopod is seen to contain a mat of fine filaments. It appears adherent at discrete points of attachment to the zymosan. These points suggest the morphological equivalent of the "teeth" of the zipper postulated by Griffin et al. (11) as the mechanism by which pseudopods progressively envelop particles. The attachment to particles such as polystyrene beads (Fig. 3) or mineral oil emulsion (28) seems relatively continuous, and suggests that the intermittent zymosan attachment is not the result of artifactual particle shrinkage. The base of the large zymosan particle is essentially clear of filaments, and granule fusion is apparently occurring there. However, with smaller particles (Fig. 3) the particle is internalized within a cage of microfilaments and granule fusion is correlated with the disappearance of the microfilaments. A similar sequence has been observed by others (2, 15) and will become clearer from analysis of capped cells (below).

The extent of microfilament assembly and its relationship to the particle inferred from thin

sections is substantiated by immunocytochemical staining for actin within spread macrophages ingesting opsonized erythrocytes (Fig. 4). Figs. 4a and b are, respectively, phase- and antiactin-stained spread rabbit peritoneal macrophages. Typically, long filaments that are somewhat more numerous but less ordered than the filaments described in fibroblasts by Lazarides and Weber (16) are present in these leukocytes. In addition, intensely fluorescent spots that may correspond to sites of attachment to the substratum are present in macrophages. Similar spots were observed before in PMN (22) and lymphoid cells (8, 9). Figs. 4c-f are similar phase fluorescence pairs of cells that are phagocytizing opsonized sheep erythrocytes. The phase-contrast images reveal erythrocytes both attached to the macrophage membrane and inside the cells. Pseudopods enclosing the attached particles are difficult to discern by direct observation. However, fluorescence due to antiactin labeling extends under and part way around these particles. This concentration of fluorescence that cups the erythrocytes corresponds to the microfilament-rich pseudopods observed above by electron microscopy. In contrast with the surface-associated particles, most erythrocytes within the cell are no longer surrounded by dense antiactin

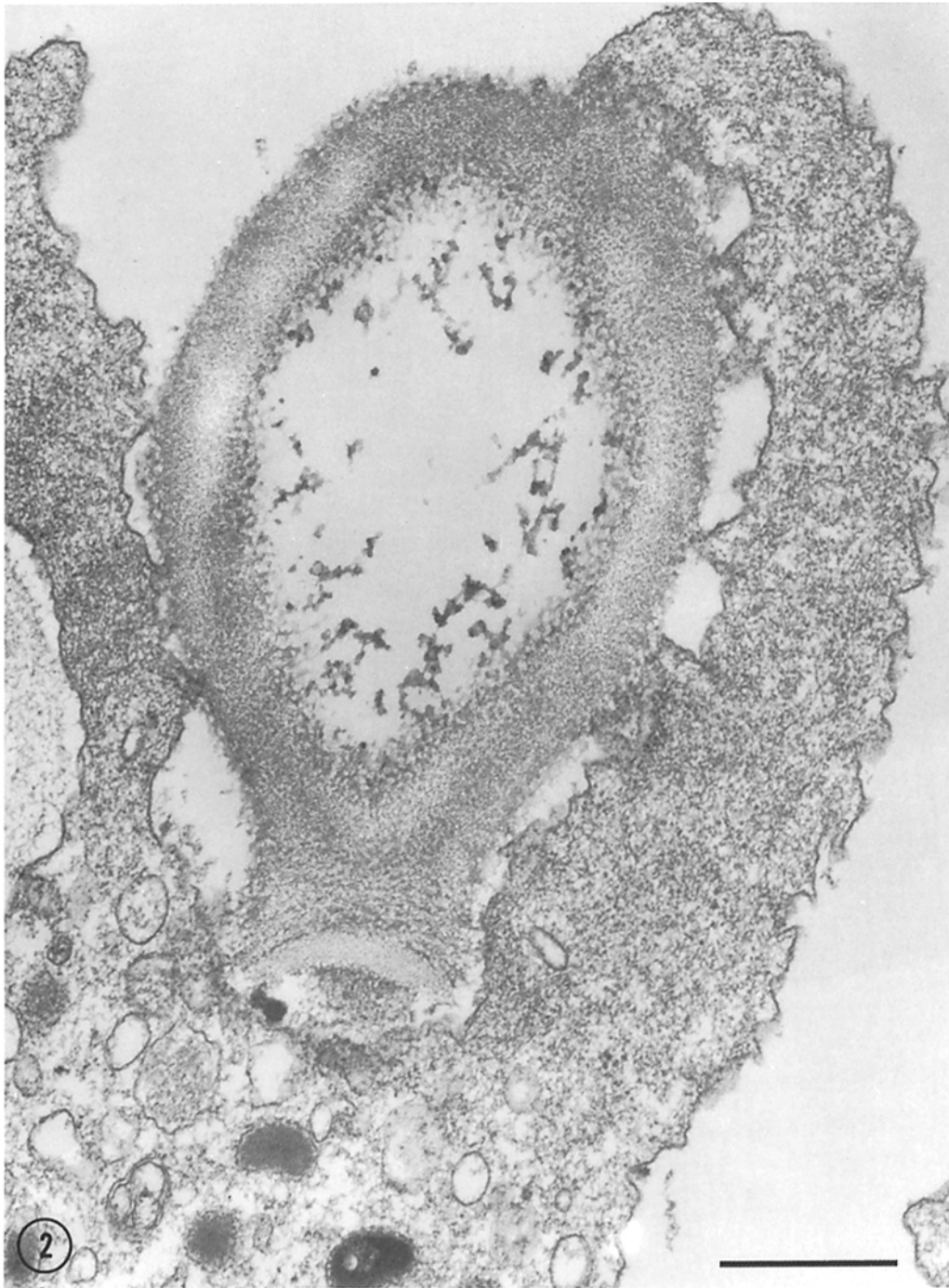


FIGURE 2 High magnification view of a pseudopod enclosing a zymosan particle (from Fig. 1). The dense mat of microfilaments within the pseudopods is readily apparent. Bar, $0.5 \mu\text{m}$. $\times 51,900$.

stain. The filamentous pattern of antiactin distribution is considerably less marked after internalization, perhaps reflecting a mobilization of fila-

ments to the cell periphery and/or the progressive change in cell shape from flattened to round as phagocytosis proceeds.

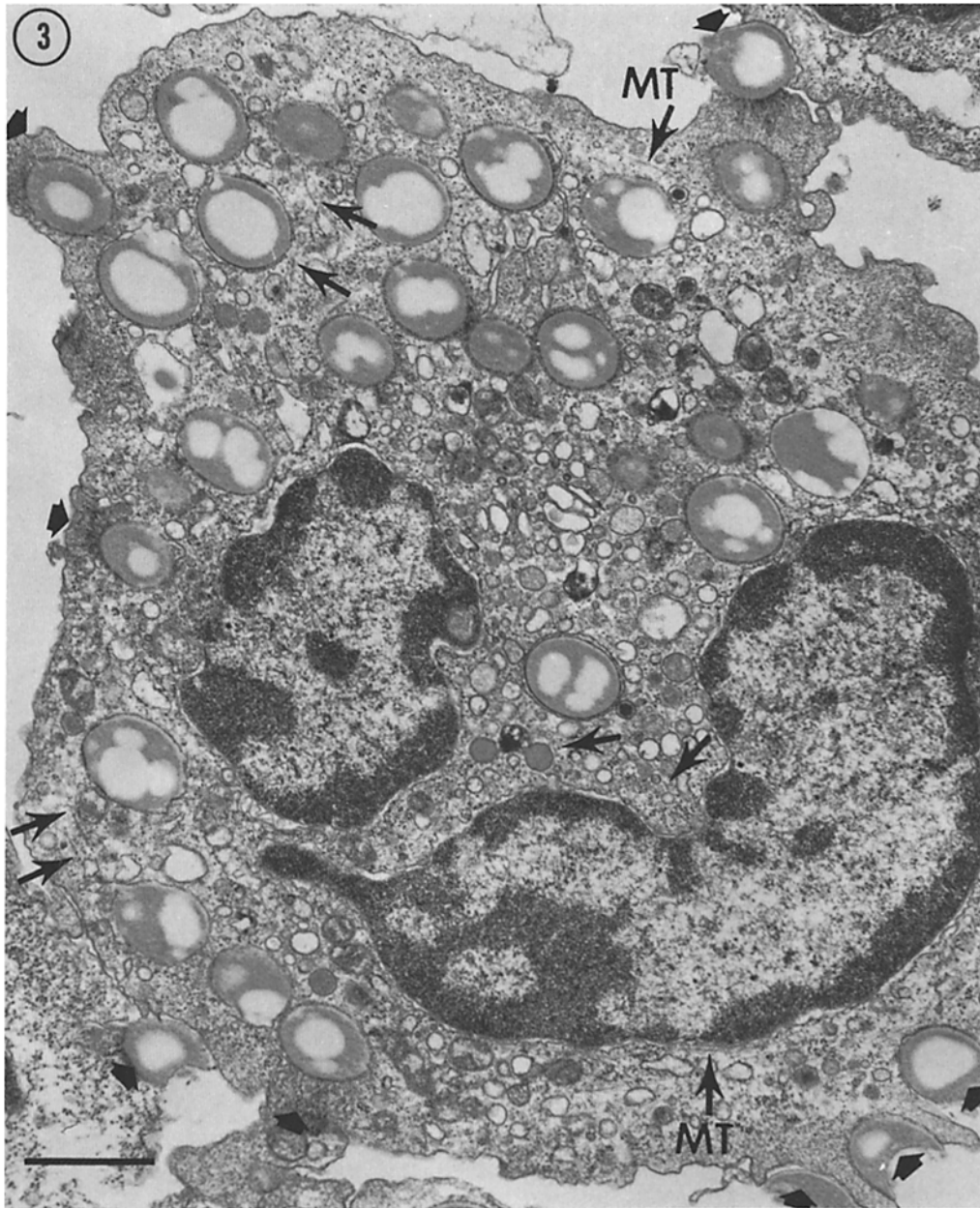


FIGURE 3 Human blood monocyte ingesting carboxylated polystyrene beads. The cell was exposed to polystyrene for 5 min at 37°C before fixation. Phagocytosis proceeds from all regions of the cell (short arrows), and ingested particles move progressively towards the cell center. The most peripheral particles are completely enclosed in a microfilament network that excludes cytoplasmic granules. Particles farther inside the cell are free of microfilaments and show close association and fusion with granules. Microtubules (arrows, MT) are present towards the cell center and also at the cell periphery. Note that the peripheral microtubules, although close to the membrane, are excluded from the microfilament-rich areas. Bar, 1 μm . $\times 17,800$.

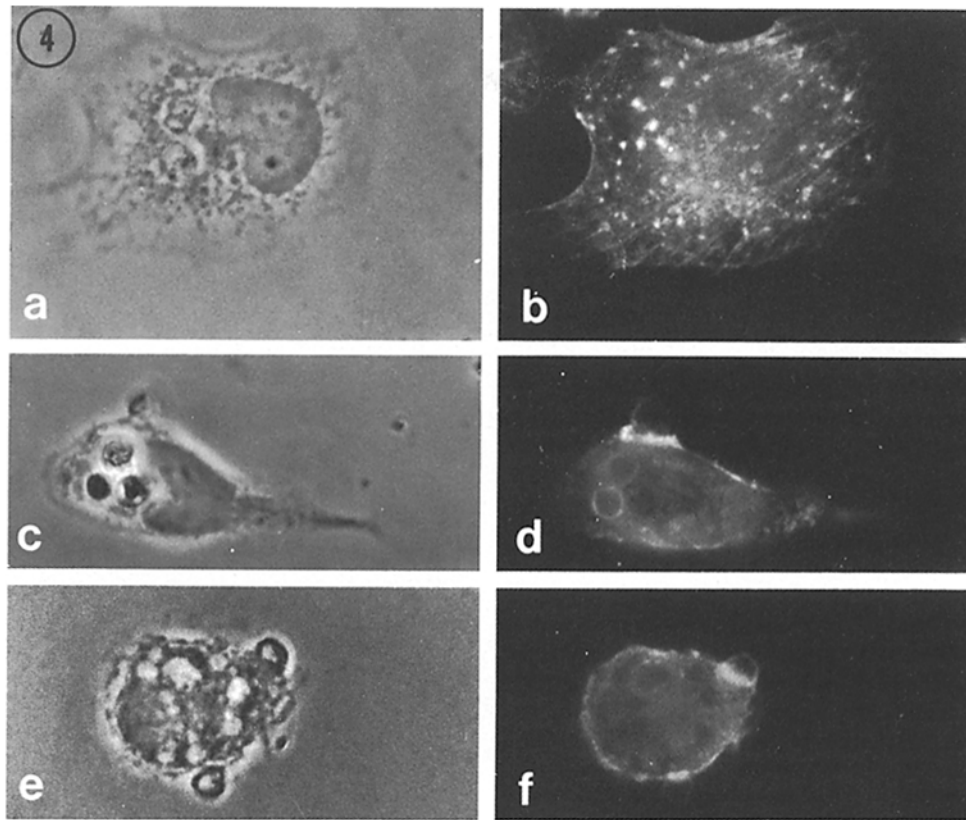


FIGURE 4 The distribution of actin during phagocytosis in rabbit peritoneal macrophages. Macrophages were incubated with or without opsonized erythrocytes at 37°C for 20 min before acetone fixation and labeling with human antiactin antibody and antihuman fluorescein-IgG. Cells were photographed on Kodak Tri-x-Pan film using both phase and fluorescence optics. Control cells (*a* and *b*) are spread and show a relatively organized system of oriented fibers as well as a series of fluorescent patches that may correspond to sites of actin attachment to the substratum. In contrast, phagocytizing cell pairs (*c-f*) show a marked concentration of fluorescence at sites of attachment of erythrocytes to the plasmalemma. Cytoplasmic fibers are not prominent in the phagocytizing cells. Initial magnification, $\times 1,250$.

Microtubule Distribution during Phagocytosis

We have shown previously that human neutrophils maintained in suspension have few assembled microtubules (20). The extent of microtubule assembly in PMN as determined from direct counts of centriole-associated microtubules is rapidly increased by binding of surface ligands such as Con A, zymosan, oil emulsion, and polystyrene latex particles (6, 13, 20, 22). Similarly, Reaven and Axline (25) have shown increased densities of microtubules in macrophages after phagocytosis or spreading of cells on a surface. This stimulation is very rapid. Thus, the number of centriole-associated microtubules in PMN phagocytizing zymo-

san and oil was found to be increased three- to fivefold in cells fixed within 0.5–1.0 min of addition of zymosan and oil (reference 6, and unpublished results). Similarly, microtubule numbers were doubled within 2 min after addition of Con A (the earliest time studied in our laboratory), and Hoffstein et al. (13) have reported even earlier increases induced by Con A.

The question here is the distribution of microtubule assembly in relationship to the ingested particle. Reexamination of micrographs of neutrophils exposed to zymosan revealed that, in contrast to the increased numbers of centriole-associated microtubules, no microtubules are observed in the microfilament-enriched processes that surround the zymosan particle (Fig. 2). A similar

situation holds for the phagocytic ingestion of polystyrene latex. As shown in Fig. 3 (arrows), microtubules are observed at the cell periphery but always beneath the pseudopod and not within it. This distribution is also suggested in the micrographs of adherent mouse macrophages obtained by Reaven and Axline (25). Since occasional microtubule profiles in the processes could be missed in thin sections, it is particularly important to confirm this apparent distribution of microtubules by immunofluorescence.

Fig. 5*a* and *b* are paired phase-fluorescence micrographs of rabbit peritoneal macrophages that have spread on glass cover slips. Microtubule assembly has been induced after adherence, and microtubules are present in a delicate array. Many appear to originate from the centriolar region. Fig. 5*c-f* are macrophages phagocytizing erythrocytes as in Fig. 4, but now stained with antitubulin. With ingestion of erythrocytes, the macrophages tend to round up. However, the microtubule array within the cell remains largely unchanged, and antitubulin staining is present up to the margins of the ingested erythrocytes. Most importantly, there is no concentration of fluorescence in the areas of erythrocyte attachment and enclosure by pseudopods (see areas marked by arrows in Fig. 5*d* and *e*) in macrophages. This stands in strong contrast to the concentration of fluorescence into pseudopods that enclose the attached particles in antiactin-stained cells.

The concept of a reciprocal relationship between the distribution of microfilaments and microtubules in the pseudopods surrounding the particle is reinforced by two pairs of phase-fluorescence micrographs (Fig. 6) which show macrophages that are engulfing the same attached erythrocyte. The upper pair indicates clearly the intense concentration of actin in processes that surround the particle. In the lower pair, tubulin is clearly absent from the equivalent processes, and in fact, the intensity of cytoplasmic microtubule labeling appears reduced at the attachment sites.

Membrane Events during Phagocytosis and Capping

The described combination of membrane folding, extensive microfilament organization, and the absence of assembled microtubules at regions of particle engulfment is precisely analogous to the ultrastructure of the leukocyte cap (1). Capping is necessarily associated with microtubule disassem-

bly and leads to the massive aggregation of microfilaments at one pole of the cell beneath a region of extensive membrane folding. The aggregation of microfilaments in the analogous phagocytic event is localized to the region of the cell immediately adjacent to the particle which is being enveloped and internalized. Thus, capping is a more global structural response that under physiological conditions is contained by a particle-oriented process.

These structural considerations provide a useful framework within which one can examine the relationship of microfilaments and microtubules to the movement of membrane constituents. We note again that in the case of phagocytosis microtubule assembly outside the particle regions is actually increased whereas with cap formation microtubule assembly is abrogated everywhere. Thus, it will be of particular interest to compare the fate of membrane constituents during phagocytosis under conditions in which microtubule assembly has been prevented throughout the cell by antimicrotubule agents, with the physiological condition in which microtubule assembly is increased everywhere except the region associated with the particle.

Fate of Con A Receptors during Phagocytosis

These experiments were performed in two ways. In exp *A* below, fluorescein-Con A was added with the phagocytic particles; in exp *B*, fluorescein-Con A was added to fixed cells after they had phagocytized.

(A) FLUORESCHEIN-CON A AND BEADS GIVEN TOGETHER: We and others have shown that fluorescein-Con A is bound to the surface of the majority of normal neutrophils in a random or diffuse pattern (Fig. 7*b*) whereas after treatment with antimicrotubule agents such as colchicine, fluorescein-Con A is drawn into a protuberance to form a surface aggregate or cap on most cells (Fig. 7*d*). Typically in normal populations, 80-90% of cells are diffusely labeled, 5-10% are capped, and the remaining 5-10% show small aggregates or patches of fluorescence along the cell perimeter. Microtubule disruption induces capping on 75-90% of cells, with the remainder showing patchy (5-10% of cells) or diffuse (10-20%) fluorescence (i.e., references 19, 20).

On the basis of the structural analogy developed in the preceding sections, it might be predicted

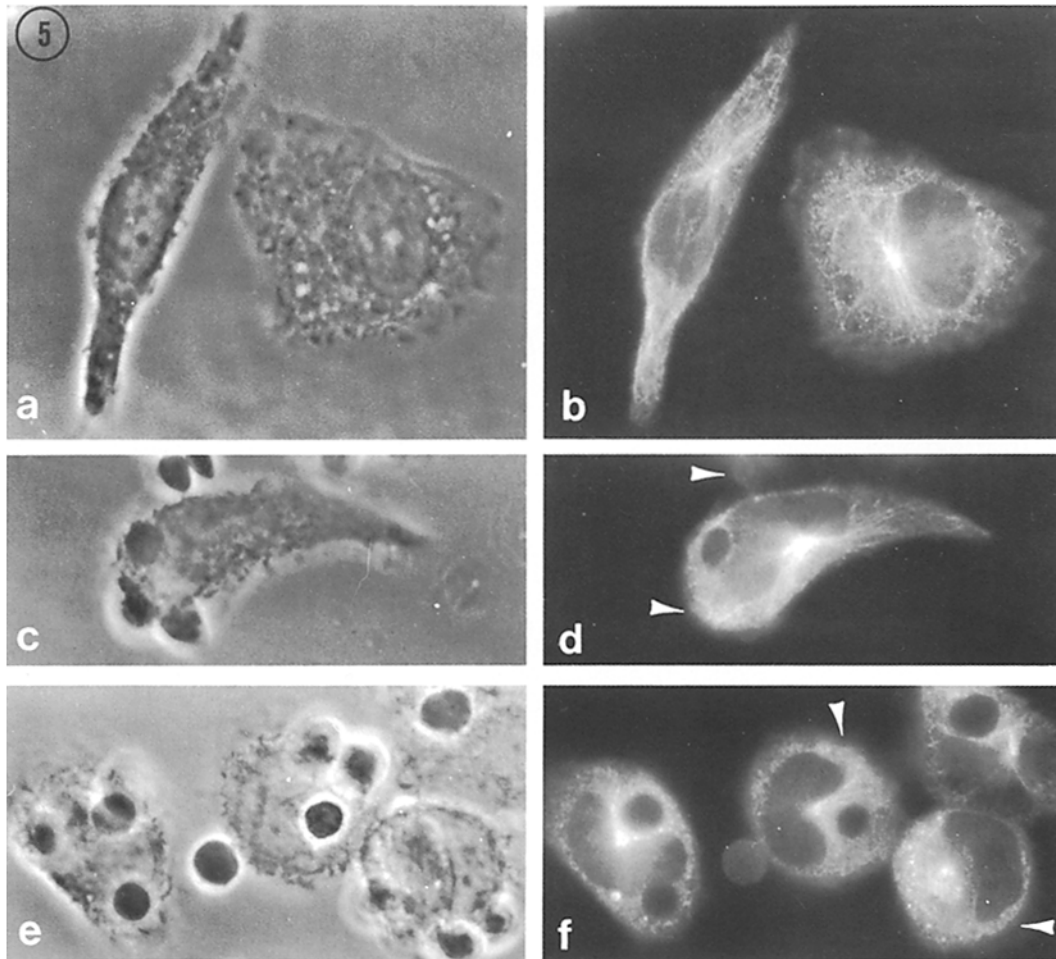


FIGURE 5 The distribution of microtubules during phagocytosis in rabbit peritoneal macrophages. Cells were treated as described in Fig. 4 and microtubules labeled by sequential incubation with goat antitubulin antibody and anti-goat fluorescein-IgG. Control cells are spread and show an extensive network of microtubules originating from the centriolar region adjacent to the cell nucleus and terminating near the membrane (*a* and *b*). Phagocytizing cells retain this network although individual microtubules are less readily resolved as phagocytosis and cell rounding progresses (cf. *5d* and *5f*). Regions of erythrocyte attachment to the surface of cells (visible by phase; marked with arrows by fluorescence) specifically lack associated microtubules (*c-f*). However, microtubules are apparent in regions immediately adjacent to intracellular erythrocytes. Initial magnification, $\times 1,250$.

that during phagocytosis fluorescein-Con A would move into the localized "cap" region surrounding the particle being phagocytized just as it migrates into the protuberance induced by antimicrotubule agents to form polar caps. This appears to be the case. Fig. 7*e* and *f* are matched phase-fluorescence micrographs of a cell that has been incubated for 5 min with carboxylated polystyrene beads and fluorescein-Con A. Even in this short interval, many beads are internalized (Fig. 3 illus-

trates an example). Bright areas of fluorescein-Con A are observed scattered through the cytoplasm of all phagocytizing cells. Thus, phagocytosis clearly induces an aggregation of fluorescein-Con A-receptor complexes into discrete regions. We emphasize that the fluorescein-Con A distribution in all cells during phagocytosis is distinct from the peripheral patching seen in a small percentage of nonphagocytizing cells. We caution, however, that the resolution of the fluorescence

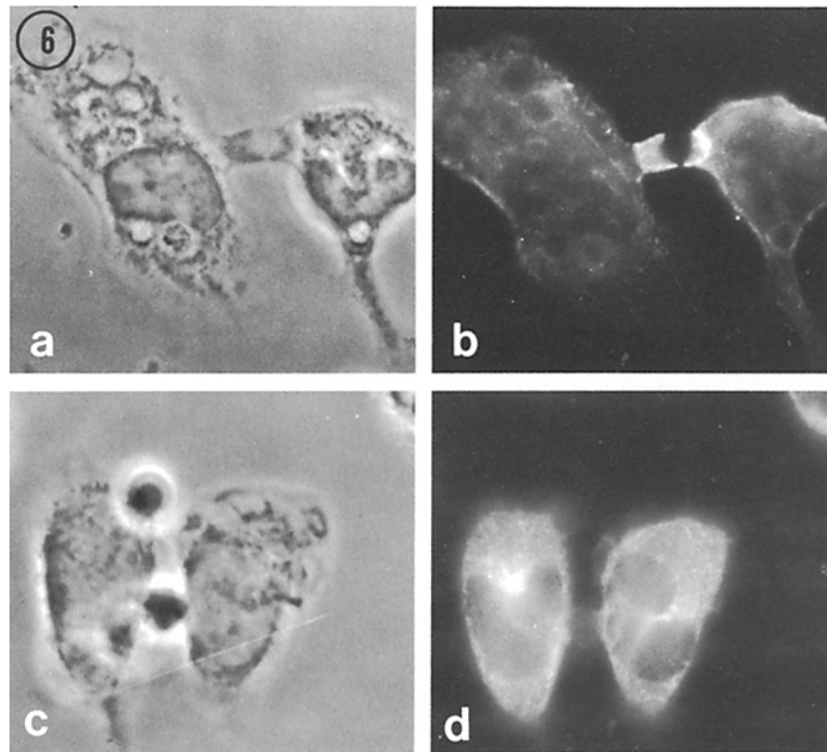


FIGURE 6 Immunofluorescence staining of phagocytizing peritoneal macrophages with antiactin and antitubulin. Two cells pairs are depicted, in which both cells of each pair are attempting to engulf a single erythrocyte (*a* and *c*). Antiactin staining is concentrated in the immediate region of the attached particle (*b*); antitubulin is excluded from the regions of particle attachment (*d*). Initial magnification, $\times 1,250$.

microscope is insufficient to prove that the fluorescein-Con A is exclusively associated with membrane that has enveloped the phagocytized bead. The precise relationship between fluorescein-Con A and the bead is being studied by recently developed fluorescence resonance energy transfer techniques (10).

When fluorescein-Con A and beads are given to cells that have been treated with colchicine or diamide, which induces characteristic large microfilament-filled protuberances, beads are associated preferentially with the protuberance (Fig. 7*g*) and fluorescein-Con A is drawn into a polar cap (Fig. 7*h*). This cap is very similar to the cap formed with fluorescein-Con A in nonphagocytizing cells (Fig. 7*d*).

The preferential localization of beads on the protuberance of colchicine-treated cells was further investigated by electron microscopy. Fig. 8*a* shows a section through a colchicine-treated cell undergoing phagocytosis (in this case without the addition of fluorescein-Con A). The characteristic

massive aggregation of microfilaments within the entire region of cell shape change is apparent and phagocytosis is polarized to this region. The absence of lectin in this experiment eliminates a role for Con A in the apparent affinity of beads with the region of the protuberance. Note that granules are excluded from the large microfilament-rich zone and that internalized beads are enmeshed in a filament bed several micrometers thick.

Lymphocytes which are treated in a similar fashion also show polystyrene beads preferentially associated with the microfilament-rich protuberance (Fig. 8*b*) even though they ingest the beads slowly if at all. This preference may signify a unique composition of the membrane of the protuberance that enables beads either to bind directly at this region or to move into the region after an initial association with dispersed binding sites.

(B) DISTRIBUTION OF FLUORESCHEIN-CON A ADDED TO CELLS THAT HAVE BEEN FIXED AFTER PHAGOCYTOSIS: As noted

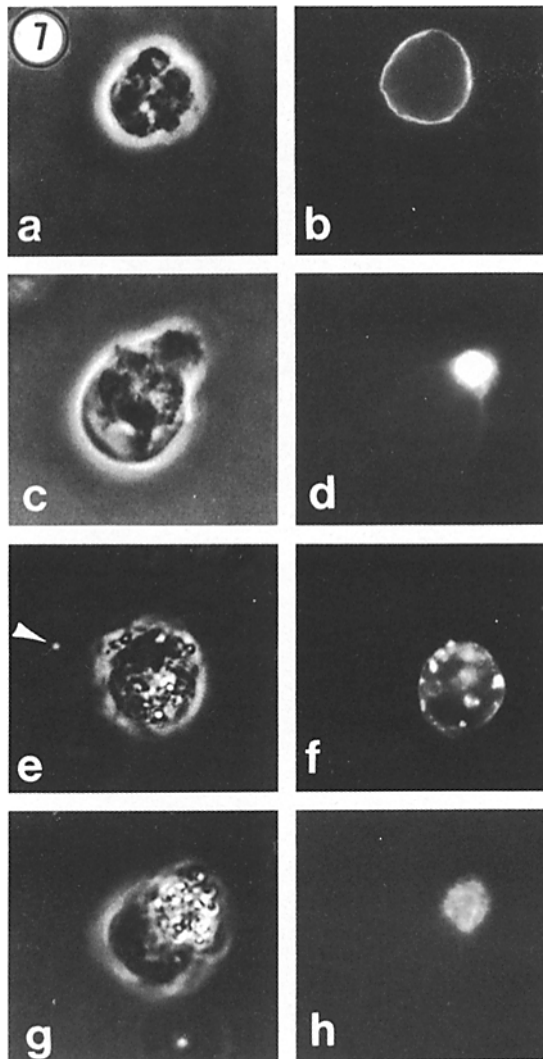


FIGURE 7 The distribution of polystyrene beads and fluorescein-Con A on human PMN. In Fig. 7*a-d*, cells were incubated at 37°C for 5 min with fluorescein-Con A (10 $\mu\text{g}/\text{ml}$) alone without (*a* and *b*) or with (*c* and *d*) previous exposure for 30 min to 10^{-6} M colchicine. In Fig. 7*e-h*, cells were incubated at 37°C for 5 min with carboxylated polystyrene beads plus fluorescein-Con A without (*e* and *f*) or with (*g* and *h*) prior exposure to colchicine. Note that free particles (in Fig. 7*e* and 7*g*) show no associated fluorescence. Initial magnification, $\times 1,250$.

before (1), the surface distribution of fluorescein-Con A on prefixed nonphagocytizing neutrophils, macrophages, or lymphocytes is random or diffuse (Fig. 9*a* and *b*). Similarly, when these cells are pretreated with antimicrotubule agents so that

they are induced to assume the "capped" structural configuration (Fig. 9*c*) in the absence of Con A, are fixed and then labeled with fluorescein-Con A (Fig. 9*d*), the distribution is also relatively diffuse. A small inhomogeneity in surface density of fluorescein-Con A cannot be ruled out, but a more likely explanation for the modest apparent concentration of fluorescence seen in Fig. 9*d* is that the membrane is extensively folded in cells with protuberances (illustrated in reference 1).

Neutrophils were allowed to phagocytize carboxylated polystyrene beads, were fixed, washed free of excess beads, and then labeled with fluorescein-Con A for 5-10 min at 37°C. For normal neutrophils, the distribution was patchy. The distribution of patches appeared to correspond to bound particles (Fig. 9*e* and *f*). However, the case for an inhomogeneous distribution of Con A receptors is more easily made when a similar experiment is performed with cells that have been treated with antimicrotubule agents. Fig. 9*g* shows the characteristic association of particles with the cell protuberance. The distribution of fluorescein-Con A on such cells is no longer uniform as in cells given colchicine alone. Rather, PMN exposed to colchicine and beads, then fixed and labeled with Con A, show a marked concentration of fluorescence in the protuberance (Fig. 9*h*). This "capping" of Con A receptors by beads is reminiscent of but probably not quite identical to capping induced by Con A itself; in particular, cells capped by Con A show essentially all of the associated fluorescence in the membrane (Fig. 7*d* and *h*) while some dispersed Con A receptors persist after capping caused by beads (Fig. 9*h*).

DISCUSSION

The central observations of this study are the structural similarities in the distributions of microtubules and microfilaments in capped and phagocytizing leukocytes. The cap may be viewed as an exaggeration or limit extension of a more localized physiological phenomenon that is centered on the phagocytized particle. The following observations form the basis of a structural and functional analogy: (*a*) Localized concentration of microfilaments or actin filaments beneath plasma membrane in the region of the cap and the phagocytic particle, (*b*) absence of assembled microtubules, either localized (phagocytosis) or generalized (capping), and (*c*) movement of fluorescein-Con A into the cap or membrane surrounding the particle when given to nonfixed cells.

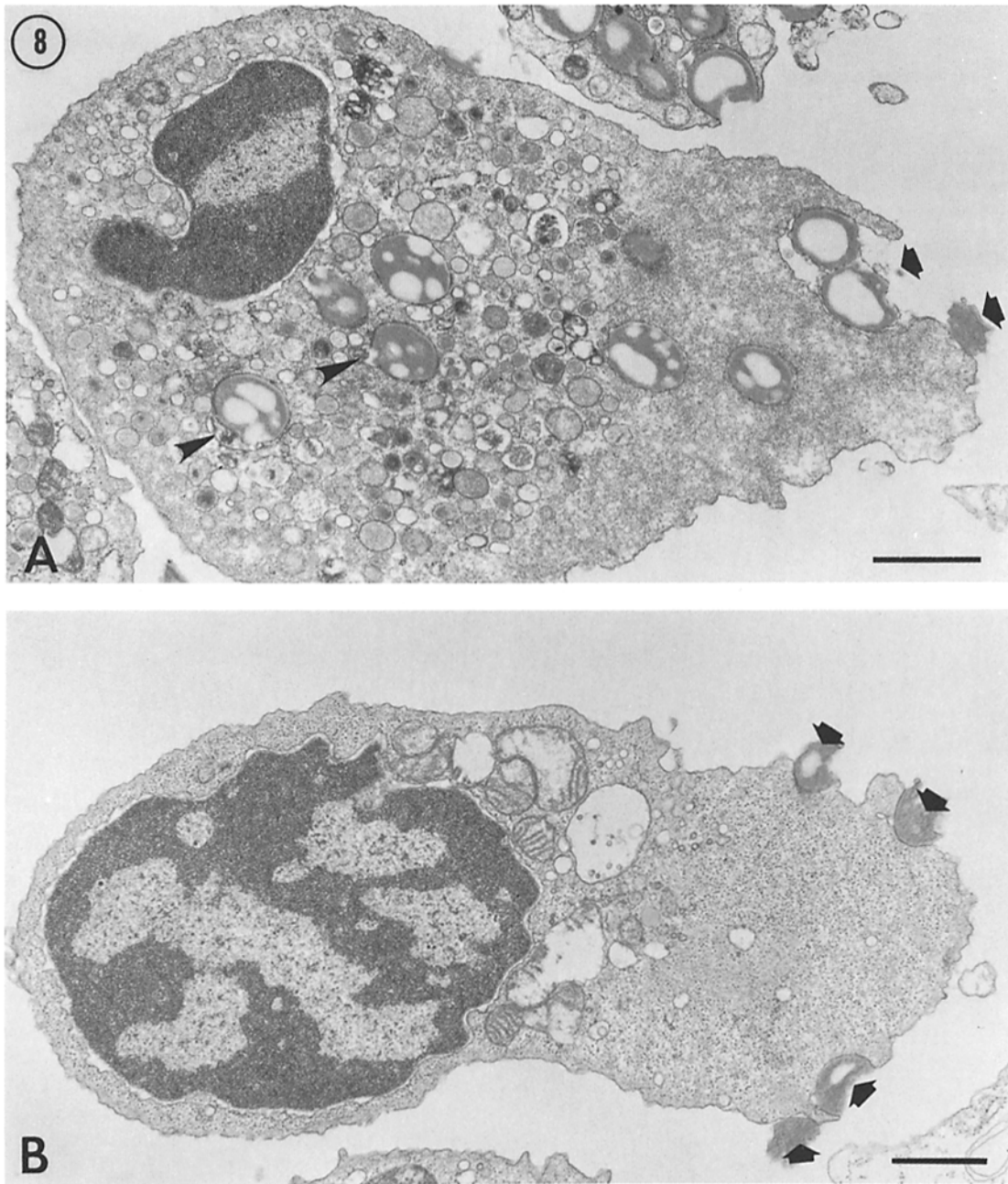


FIGURE 8 The ultrastructure of colchicine-treated leukocytes incubated with polystyrene beads. In Fig. 8A, a human blood monocyte exposed to colchicine (10^{-6} M; 30 min) and carboxylated polystyrene (5 min at 37°C) is shown in process of internalizing beads. Unlike the control monocyte in Fig. 3 which engulfed particles uniformly, this cell appears to be internalizing particles only from the region of the extensive microfilament-rich, granule-excluding protuberance (small arrows). Granule fusion with engulfed particles (arrowheads) is confined to regions of the cell far removed from the original site of phagocytosis. In Fig. 8B, a lymphocyte from a similar preparation is seen. This cell is not a phagocyte. Nevertheless, after colchicine, it develops a prominent microfilament-rich protuberance to which beads can become attached. The central core of intermediate (100 \AA) filaments in the protuberance is commonly observed in microtubule-depleted lymphocytes. Bars, $1 \mu\text{m}$. (A) $\times 15,300$; (B) $\times 17,800$.

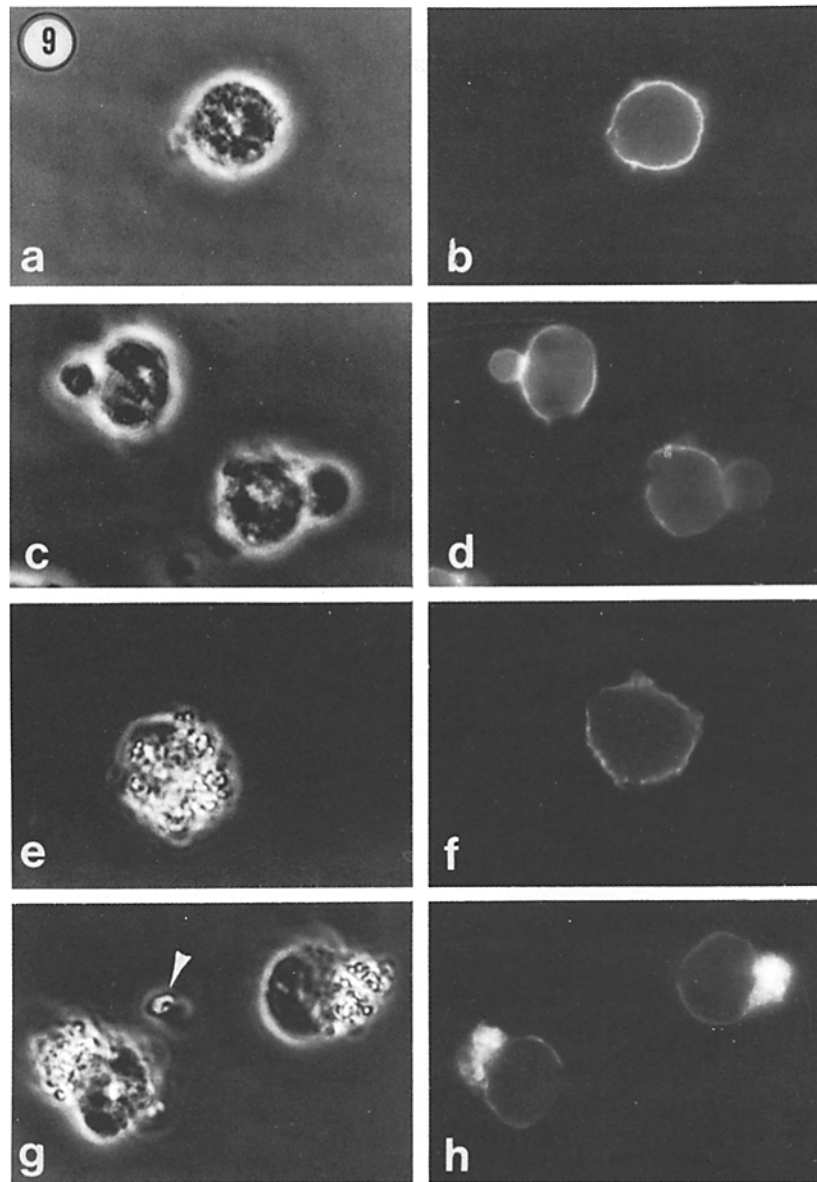


FIGURE 9 The distribution of fluorescein-Con A on leukocytes fixed after phagocytosis. In Fig. 9a-d, cells were fixed and labeled for 5 min with fluorescein-Con A ($10 \mu\text{g/ml}$) without (a and b) or with (c and d) prior disruption of microtubules with colchicine. In Fig. 9e-h, cells were exposed to carboxylated polystyrene beads for 5 min after a 5-min incubation without (e and f) or with (g and h) colchicine. The cells were then fixed and labeled for 5 min with fluorescein-Con A (1 ml). Note that no fluorescence is associated with the free particles (arrow) visible by phase in Fig. 9g. Initial magnification, $\times 1,250$.

The concept of a regional absence of microtubules during phagocytosis is particularly important. In previous studies, we have shown that phagocytosis is associated with the rapid assembly of microtubules most readily observed near centrioles (6, 23). Numerous microtubules are also

seen at the periphery in cells phagocytizing beads (e.g., Fig. 3). Importantly, they are absent from the microfilament-rich pseudopods. Establishment of this segregation of microtubules has allowed us to ask whether or not Con A-receptor complexes can move from a region that overlies assembled

microtubules to one that does not.

The data presented here indicate that receptors can indeed move out of microtubule-rich into microtubule-depleted regions of the cell surface. Thus, when cells are exposed to fluorescein-Con A alone, they show increased microtubule assembly within 2 min at 37°C. However, Con A alone induces no asymmetry in the distribution of submembranous microfilaments, and no asymmetry of lectin receptor distribution is evident. By contrast, cells exposed to fluorescein-Con A plus beads show microtubule assembly but also a marked polarization of microfilaments to regions of the membrane associated with beads. An apparent movement of Con A-receptor complexes into these microfilament-rich and microtubule-poor phagocytic regions then follows. We realize that this interpretation assumes that microtubule assembly in PMN precedes or occurs simultaneously with receptor movement. In fact, the relative timing of lectin movement and microtubule assembly is not known precisely. However, as described before (6), microtubule assembly occurs with equal or greater rapidity on exposure to phagocytizable particles (0.5 min or less) as on exposure to Con A (13). Yet, the assembly induced by Con A is associated with a homogeneous or diffuse distribution in nearly 90% of leukocytes.

Assuming that movement does indeed follow microtubule assembly, how can these observations be reconciled with the notion that microtubules anchor membrane receptors? The "anchorage" or "modulation" concept is based in part on the observation that Con A (above a certain minimal dose) binds uniformly to lymphocyte surface receptors and prevents capping of ligands such as IgG added subsequently. Both Con A and IgG move into caps after treatment with antimicrotubule agents. IgG capping is also prevented by locally bound Con A (as when Con A-coated platelets interact with lymphocytes). From this, it is inferred that movement into a cap is prevented by Con A-induced connections of membrane receptors to underlying microtubules or complexes of microfilaments and microtubules termed the SMA, "surface modulating assembly" (7, 32).

We as well as Hoffstein et al. (13) have confirmed that Con A induces a generalized microtubule assembly in leukocytes. However, we do not consider that the concomitant immobilization of Con A or IgG reflects their direct or indirect association with microtubules. Rather, immobili-

zation occurs because there is no local region depleted of microtubules and enriched in microfilaments into which ligand-receptor complexes can accumulate. Thus, the diffuse labeling in Con A-treated cells is not so much the result of microtubule assembly as it is the absence of regions of microtubule *dis*assembly associated with local microfilament recruitment.

The surface modulation hypothesis also suggests that the movement of receptors into caps after release of microtubule-imposed restraints is mediated by microfilaments (7). Indeed, it has now been shown that both Con A and IgG caps are underlaid by microfilaments (1, 26). However, it has also been established that microfilament redistribution can precede capping (1). Thus, the physical movement of receptors is probably not obligatorily dependent on connections with contractile filaments.

Why, then, do Con A receptors accumulate into a cap or phagocytic region? One explanation is that direct attachments develop between Con A-receptor complexes and microfilaments once the receptors have moved into the cap or phagocytic membrane. Alternatively, a molecular complex such as Con A itself or a bound particle may alter the physical properties of the cap or phagocytic membrane locally to limit the diffusion of the Con A receptor. We have suggested that such a local change could involve a regional phase separation (1, 4) but this remains to be proven. In favor of indirect receptor-microfilament interaction is the observation that there appears to be free mixing of Con A-receptor complexes within the capped region (1). Evidence that the influence of the bound particle on Con A receptor mobility extends beyond the membrane surrounding the particle itself comes from our studies of colchicine-treated cells that have phagocytized and been labeled after fixation (Fig. 9). It seems unlikely that the intense fluorescein-Con A labeling of the capped region is restricted to the membrane associated directly with the particles; the space between membrane and particle is too small to allow free penetration of the lectin into the space, and the ratio of total capped membrane to capped membrane associated with beads (Fig. 8a) is too high. Rather, the entire membrane region near the particle seems modified to allow the capture of Con A receptors.

The role of microtubules in determining surface topography thus emerges as one that controls the distribution and function of microfilaments. As-

sociated with the microfilament distribution may be localized changes in adjacent (perhaps connected) cytoplasmic membrane. We have shown previously that lectin receptors are selectively lost from the plasma membrane during phagocytosis but that this selectivity is diminished after colchicine treatment (21). The present experiments allow us to develop the interpretation of these events as follows: Normally, lectin receptors become associated with precisely that membrane which envelops the particle; after colchicine, particles are endocytized through the microfilament-rich protuberance whose entire membrane has been modified, perhaps by the proximity of attached particles, to have high affinity for the Con A receptors. Thus, the receptors are no longer entrained exclusively in the particle-associated membrane that will be endocytized but remain equally fixed in a membrane of similar properties but dissociated from the endocytic event. This function of microtubules does not of course rule out a role for membrane bound tubulin in affecting membrane structure.

We emphasize that the association of microfilaments with internalized membrane in normal cells is a transient one. The particle is enveloped by a layer of actin filaments that thins and disappears associated with lysosomal fusion. Similar observations were made previously in neutrophils by Allison and Davies (2) and in *Acanthamoeba* by Korn et al. (15). This orderly progression of microfilament aggregation and dispersal is lost or prolonged in the absence of microtubule assembly. Thus, in colchicine-treated cells, endocytosis occurs preferentially through the microfilament-rich protuberance (Fig. 8a), and particles remain for a prolonged interval enmeshed in a bed of microfilaments from which lysosomes are excluded.

On the basis of the latter observations, we suggest that in fact microtubules may play no direct role in membrane fusion leading to lysosomal discharge into phagocytic vesicles or incubation medium. Rather, the inhibition of fusion by colchicine first reported by Malawista and Bodel (17) could at least in part be an indirect effect dependent on the rearrangement of filaments induced by microtubule disassembly. The variability of colchicine inhibition of lysosomal fusion which has led to some controversy (e.g., reference 14, 24) could reflect the variability of the extent of microfilament polarization which may be diminished in cells adherent to a surface. This explana-

tion obviates the need to show direct microtubule-granule interactions in order to explain the colchicine sensitivity of lysosomal degranulation in leukocytes. Various other colchicine-inhibitable secretory processes (e.g., the discharge of secretory granules in pituitary or pancreas) could also reflect microfilament rearrangements after microtubule disassembly.

We thank Ms. C. B. Pearson and J. A. Krawiec for their excellent technical assistance. Rabbit peritoneal macrophages were obtained through the courtesy of Dr. E. L. Becker and Mr. H. J. Showell.

This paper was supported by grants ES-01106, CA15544, CA18564, and GM22621 from the National Institutes of Health and grant BC-179 from the American Cancer Society. J. M. Oliver is an American Cancer Society Faculty Research Awardee.

Received for publication 12 September 1977, and in revised form 23 January 1978.

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