

# DISTRIBUTION OF ACTIN-BINDING PROTEIN AND MYOSIN IN MACROPHAGES DURING SPREADING AND PHAGOCYTOSIS

OLLE I. STENDAHL, JOHN H. HARTWIG, ERICA A. BROTSCHI, and THOMAS P. STOSSEL

From the Hematology-Oncology Unit, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

## ABSTRACT

Actin-binding protein (ABP) and myosin are proteins that influence the rigidity and movement, respectively, of actin filaments *in vitro*. We examined the distribution of ABP and myosin molecules in acetone-fixed rabbit lung macrophages by means of immunofluorescence. The staining for both of these proteins in unspread cells was quite uniform, but was reduced in the nucleus and concentrated slightly in the periphery. The peripheral accumulation of staining attenuated in uniformly spread cells, although filopodia and hyaline veils definitely stained. In cells fixed during ingestion of yeast particles, the brightest staining correlated with the disposition of organelle-excluding pseudopodia initially surrounding the yeast. After phagocytosis was complete and the yeasts resided in intracellular vacuoles, no concentration of staining around the ingested yeasts was detectable. We conclude that ABP and myosin molecules are components of the structural unit of the cell responsible for spreading and phagocytosis, the hyaline cortex, a region known to be rich in actin filaments. The findings are consistent with the theory that these molecules control the rigidity and movement of filaments in the periphery of the living macrophage.

**KEY WORDS** actin-binding protein · immunofluorescence · macrophages · myosin · phagocytosis · spreading

Spreading and phagocytosis by macrophages are activities that involve movements of an organelle-excluding region of peripheral cytoplasm, the hyaline cortex (16). Actin filaments are the principal subcellular structures visible in thin-section electron micrographs of the cortical cytoplasm, including the extensions of cortex, which forms the pseudopodia that spread on surfaces or that surround objects during phagocytosis (20). Therefore, actin filaments seem to have a function in these motor activities. Other macrophage proteins interact with actin *in vitro* to cause consistency changes and

movement. Macrophage actin-binding protein (ABP) cross-links actin filaments *in vitro* to form a solid gel (3, 23). Macrophage myosin contracts the actin-ABP gel in the presence of  $Mg^{2+}$ -ATP and an incompletely characterized myosin-activating factor (11, 22). If ABP and myosin are important in the consistency changes and movement of cortical cytoplasm perceived to occur during spreading and phagocytosis by living macrophages, they should associate with actin in the cortical cytoplasm and in pseudopodia. However, these proteins comprise a relatively small fraction of the total cytoplasmic protein (11) and hence are not readily identifiable in thin-section electron micrographs. In this paper, we report the demonstration by means of immunofluorescence that

ABP and myosin molecules reside in the cortical cytoplasm of resting macrophages and especially in accumulations of cortex engaged in spreading and phagocytosis. An abstract of this research has been published (4).

## MATERIALS AND METHODS

### *Preparations of Cells and Antigens*

Lung macrophages were obtained by tracheal lavage (18) from rabbits previously treated with an intravenous injection of complete Freund's adjuvant (17). The harvested cells were washed twice in 0.15 M NaCl and suspended in Krebs-Ringer's phosphate buffer, pH 7.4 (1 ml packed cells/100 ml of buffer). Macrophage myosin and ABP were extracted and purified as previously described (11, 13).

### *Preparations and Conjugation of Antibodies*

Purified myosin or ABP (1 mg/ml) were emulsified in equal volumes of complete Freund's adjuvant, and 0.2-ml aliquots were injected into multiple intradermal sites of male pygmy goats. The antisera used were collected 7 wk after the first immunization. The specificity of the antisera was assessed by immunoelectrophoresis in agar gel set in 40 mM sodium pyrophosphate, pH 9.0. A soluble extract of macrophages was prepared for reaction against the antisera as previously described (11), except that the homogenizing medium contained 40 mM sodium pyrophosphate buffer, pH 8.6, instead of imidazole-HCl, pH 7.0. IgG was isolated from serum by precipitation with 37% ammonium sulfate in 1 mM EDTA, pH 7.0, dialyzed against 0.01 M sodium phosphate buffer, pH 7.6, and chromatographed on a diethylaminoethyl cellulose column with the same buffer. The IgG fractions were pooled, concentrated to 10 mg/ml with a dialysis membrane and negative pressure, and dialyzed against 0.13 M NaCl, 20 mM sodium phosphate buffer (phosphate-buffered NaCl) pH 7.4, containing sodium azide, 100 µg/ml. For direct immunofluorescence, the IgG was conjugated with tetramethyl rhodamine isothiocyanate by the procedure of Cebra and Goldstein (5). Rhodamine-conjugated F(ab')<sub>2</sub> fragments were prepared to eliminate interaction between the Fc moiety of IgG and the macrophage surface. Conjugated IgG was digested with pepsin (19), and the F(ab')<sub>2</sub> was isolated by gel filtration on a G-100 Sephadex column. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (7, 15) was performed to identify the F(ab')<sub>2</sub> and IgG.

### *Macrophage-Particle Interaction*

To glass slides was added 50 µl (5 × 10<sup>6</sup> cells/ml) of macrophages suspended in Krebs-Ringer's phosphate buffer with 30% fresh rabbit serum. A coverslip was added over the cell suspension, and the slides were incubated in a petri dish containing drops of water for moisture and placed in a 37°C incubator for 15–30 min. The slides were then washed in phosphate-buffered NaCl to float the coverslip and rinse off nonadhering cells. Yeast cells (*Saccharomyces cerevisiae*), 10<sup>9</sup>/ml in 0.1 M carbonate buffer, pH 9.5, were incubated with fluorescein isothiocyanate, 1 mg/ml, for 1 h at 20°C, and washed four times in phosphate-buffered NaCl. Fluorescein-labeled yeast cells were added, air dried, and briefly heat-fixed in a Bunsen burner before the addition of macrophages. In some experiments, 0.2 vol of a suspension of sodium diisododecylphthalate oil droplets coated with *Escherichia coli* lipopolysaccharide and opsonized with fresh

rabbit serum (21) were incubated with the macrophage suspension for 30 min at 37°C. After washing, the cell suspension was smeared on the coverslip and dried gently with an air jet. Ingested oil particles appeared as lucent vacuoles within acetone-fixed macrophages, which permitted evaluation of the staining pattern in cells with unequivocally internalized particles.

### *Fixation and staining*

After washing in phosphate-buffered NaCl, the slides were immersed in cold acetone (–10° to –20°C) for 7 min and thereafter briefly in phosphate-buffered NaCl solution. In the indirect immunofluorescence technique, 50 µl of antimyosin (500 µg/ml) or anti-ABP IgG (200 µg/ml) was added to the slides, which were then covered with a coverslip. After incubation at 37°C for 30 min, the slides were washed in phosphate-buffered NaCl solution for 45 min, and then incubated with 50 µl of rhodamine-labeled rabbit anti-goat IgG (100 µg protein/ml; N. L. Cappel Laboratories Inc., Cochranville, Pa.) for 30 min at 37°C. The IgG and anti-IgG preparations were centrifuged at 15,000 g for 10 min to remove aggregates before use. The slides were finally washed in phosphate-buffered NaCl for 120 min, mounted with 90% glycerol-10% phosphate-buffered NaCl, and sealed with Permount (Fisher Scientific Co., Pittsburgh, Pa.). For direct immunofluorescence, 50 µl of rhodamine-conjugated antimyosin IgG and F(ab')<sub>2</sub> (340 µg/ml) was added to the slides and incubated for 45 min at 37°C. The slides were washed and mounted as described above, except without the addition of rabbit anti-goat IgG. The slides were examined in a Zeiss Universal fluorescence microscope, using appropriate filters for viewing rhodamine and fluorescein fluorescence. Phase and fluorescence photographs were taken with Kodak Tri-X Pan film, using an exposure time of 1/60–1/4 s and 15–30 s, respectively.

### *Controls*

Fixed macrophages were reacted with: (a) preimmune goat serum followed by rhodamine-conjugated rabbit anti-goat IgG; (b) rhodamine-conjugated preimmune IgG; (c) rhodamine-conjugated anti-myosin F(ab')<sub>2</sub> with and without prior absorption with myosin as described below; (d) antimyosin and anti-ABP IgG absorbed with purified macrophage myosin and ABP, respectively. For absorption, equal volumes of IgG (400 µg/ml for antimyosin and 200 µg/ml for anti-ABP) and respective antigens (1 mg/ml) were mixed and incubated at room temperature for 60 min and then at 4°C overnight in phosphate-buffered NaCl solution. The mixtures were then centrifuged at 15,000 g for 30 min, and the supernatant fluids were used for staining. Controls for dilution were done by omitting the antigen proteins from the solution. Monolayers of human rat skin fibroblasts (provided by courtesy of Dr. P. Erlich) were fixed and stained with antimyosin IgG and rhodamine-conjugated anti-IgG as described above.

## RESULTS

Fig. 1 shows the purity of the ABP and myosin preparations used as antigens as assessed by electrophoresis on polyacrylamide gels in sodium dodecyl sulfate. Fig. 1 also shows the reactions of anti-ABP and antimyosin antisera against their respective antigens after electrophoresis in agar gel. Both antisera gave single precipitin arcs. The myosin antiserum also yielded a single arc when reacted with a macrophage extract after electro-

phoresis in 40 mM pyrophosphate (Fig. 1A). Anti-ABP serum gave single lines of identity against ABP and macrophage extract in agarose immune diffusion (not shown) and no reaction with macrophage myosin (Fig. 1B).

When macrophages were suspended in fresh rabbit serum and added to glass slides coated with yeast particles, the cells spread on the surface and phagocytosed the fluorescent yeast. This process could easily be followed in the microscope, permitting fixation and staining at various steps. Before they spread, cells were spherical in shape. In such cells antimyosin IgG gave a pattern of diffuse cytoplasmic fluorescence with brightest staining in the periphery (Fig. 2A). The nuclear region appeared less brightly stained. Anti-ABP IgG exhibited a similar pattern, although the peripheral staining was relatively more pronounced (Fig. 3A). With both antibodies, the regions of brightest staining were away from the area of greatest cross-sectional thickness, the central cell body. In the cells which had spread, the staining intensity with both antimyosin (Fig. 2B) and anti-ABP (Fig. 3B) distributed more evenly over the cytoplasm. The nucleus persisted in showing very weak staining. Peripheral projections and veils stained (Figs. 2D and 3B).

During the interaction of macrophages and yeast particles, several patterns emerged: when the particles appeared attached to the cell surface, no change in staining pattern was noticeable compared to nonphagocytosing cells. However, during ingestion when pseudopodia partially surrounded the yeast, the brightest antimyosin and ABP fluorescence was in these pseudopodia (Figs. 2C, and 3C-E). Cells which had completed phagocytosis were indistinguishable from those of resting cells, except that these cells contained the fluoresceinated yeast particles. These particles did not have the distinct rim of rhodamine staining observed before completion of phagocytosis. When a rim of fluorescence was visible (Fig. 2C and 3D), the phagocytic vacuole was not completely closed, judged by viewing at different planes of focus. In cells which had ingested opsonized lipopolysaccharide-coated oil droplets, phagosomes were visible as vacuoles within the diffusely stained cell body and did not have a distinct antimyosin fluorescent encirclement (Fig. 2E). The statistical validity of this observation was verified by counting vacuoles with or without a stained rim (Table I).

Several sets of experiments were carried out to

establish the specificity of the antimyosin and anti-ABP staining. Direct staining of macrophages with rhodamine-conjugated antimyosin IgG and F(ab')<sub>2</sub> fragments gave identical but weaker staining patterns compared to IgG, indicating that the Fc-binding entities on the cell surface of the fixed macrophages did not interfere with the specific antimyosin staining (Fig. 4). Indirect immunofluorescence staining showed brighter staining but with a pattern indistinguishable from that of the direct technique (Figs. 2 and 4). Therefore, the indirect immunofluorescence technique was subsequently used. To evaluate the specificity of the antimyosin and anti-ABP antibody, the preparations were absorbed with purified macrophage myosin and ABP. The fluorescence was markedly reduced and indistinguishable from that of preimmune IgG and rhodamine-conjugated anti-goat IgG (Fig. 5). When fibroblasts were stained with antimacrophage myosin IgG, the brightest staining was restricted to stress fibers (Fig. 6). These stress fibers when viewed at high magnification showed typical periodic staining observed by others (8). Several staining procedures were also evaluated to rule out artifactual staining patterns. Neither formalin (8) nor ethyldimethylaminopropyl carbodiimide/glutaraldehyde-saponin (25) treatment changed or improved the staining pattern observed with acetone treatment. Cold acetone was the most suitable agent for rapid fixing of cell at different stages of phagocytosis.

## DISCUSSION

The cortical cytoplasm that extends from the cell during spreading and as pseudopodia surrounding objects during phagocytosis is characteristically devoid of phase-dense organelles. This hyaline cortex, which is rich in actin filaments, moves actively during spreading and phagocytosis and seems to be a rather plastic but distinct structural and functional motor unit of the cell. Therefore, we inquired whether it also contains the actin-associated proteins myosin and ABP. Our immunofluorescence staining experiments indicate that these proteins are in the cortex of macrophages. The reliability of the immunofluorescence findings depends on the specificity of the antibodies and avoidance of staining artifacts. Our evidence that the antibodies used were specific for myosin and ABP in macrophages is the lack of cell staining by preimmune serum and by reagents absorbed with antigens. Furthermore, the antigens utilized to

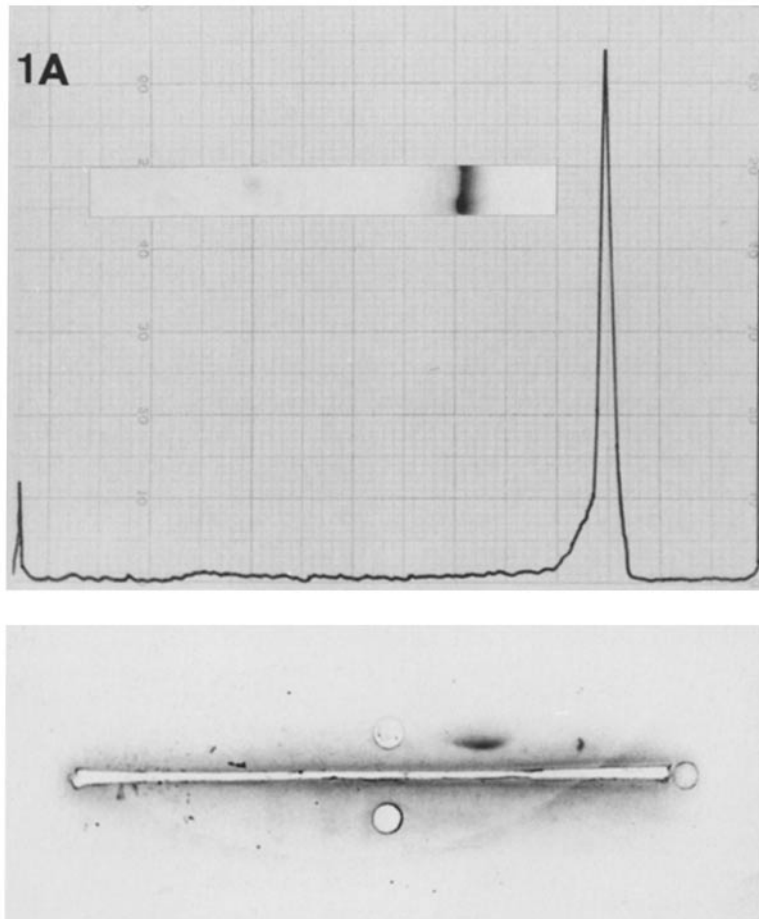
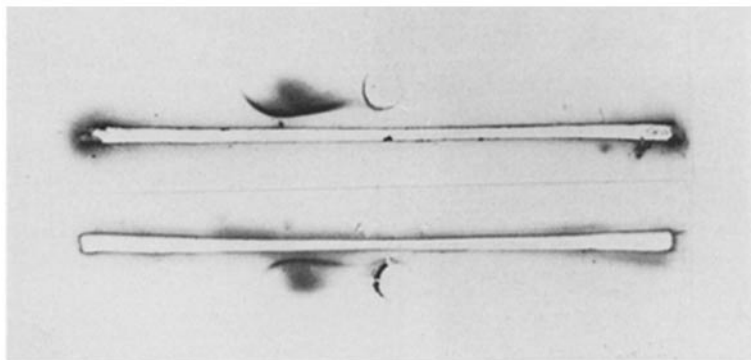
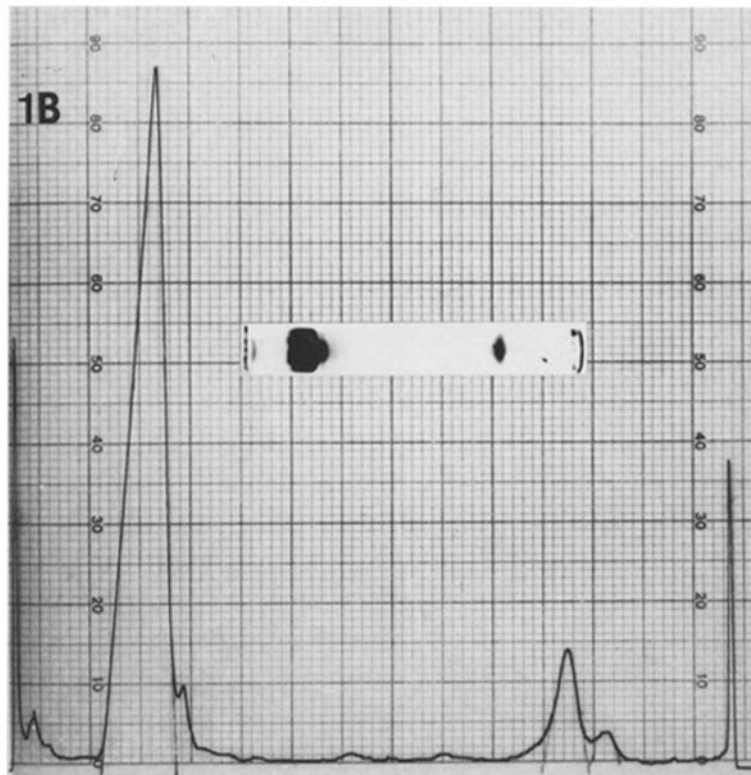


FIGURE 1 Polyacrylamide gels and densitometric scans showing (A) the subunit polypeptide of actin-binding protein (ABP) and (B) heavy and light subunits of myosin. The proteins were isolated from rabbit lung macrophages and used to immunize goats. The lower part of Fig. 1A shows the reaction of goat anti-rabbit macrophage ABP antiserum against purified macrophage ABP (top well) and lack of a reaction with macrophage myosin (bottom well). The lower part of Fig. 1B shows the reaction of goat anti-rabbit myosin antiserum against purified macrophage myosin (top well) and macrophage extract (bottom well) after electrophoresis in agar gel.

generate the antibodies were highly purified from the same cell type in which the localization exercise was done. Staining artifacts are harder to rule out. Other workers have previously used the fixation and immunofluorescent staining technique employed in this work to localize actin-associated proteins in various nonmuscle cells (e.g., references 2, 9, and 24). Fibroblasts and cultured epithelial cells spread on surface have phase and electron-dense fibers which are bundles of actin-containing filaments (10). Fluorochrome-conjugated antibodies against various proteins including myosin

brightly stain these fibers (24). Lung macrophages do not form such bundles, even after spreading, and the staining pattern in macrophages is more uniform. However, our antimyosin IgG yielded typical staining of fibers in spread fibroblasts fixed by the same procedure used to fix macrophages. Therefore, our technique gave results comparable to those of previous staining experiments by others. The strongest support for the veracity of the staining results is that they complement earlier cell fractionation studies, which revealed that cortical cytoplasmic fragments contained actin and were



enriched in ABP and myosin relative to the rest of the macrophage (6, 12).

The ability to visualize the nucleus in contrast to background cytoplasmic fluorescence implies that some myosin and ABP reside throughout the cytoplasm of the cell. The proteins could have some function there or could represent newly synthesized myosin and ABP in transit to the cell periphery.

The antimyosin and anti-ABP staining was

equally bright throughout macrophages spread relatively uniformly in all directions, as in resting cells, presumably reflecting a broad distribution of cortical cytoplasm. However, in phagocytosing cells, the fluorescent staining concentrated strikingly in the cortical pseudopodia initially surrounding yeasts undergoing phagocytosis. This redistribution of fluorescence correlated with the asymmetrical distribution of cortical cytoplasm into pseudopodia, visible in phase-contrast mi-

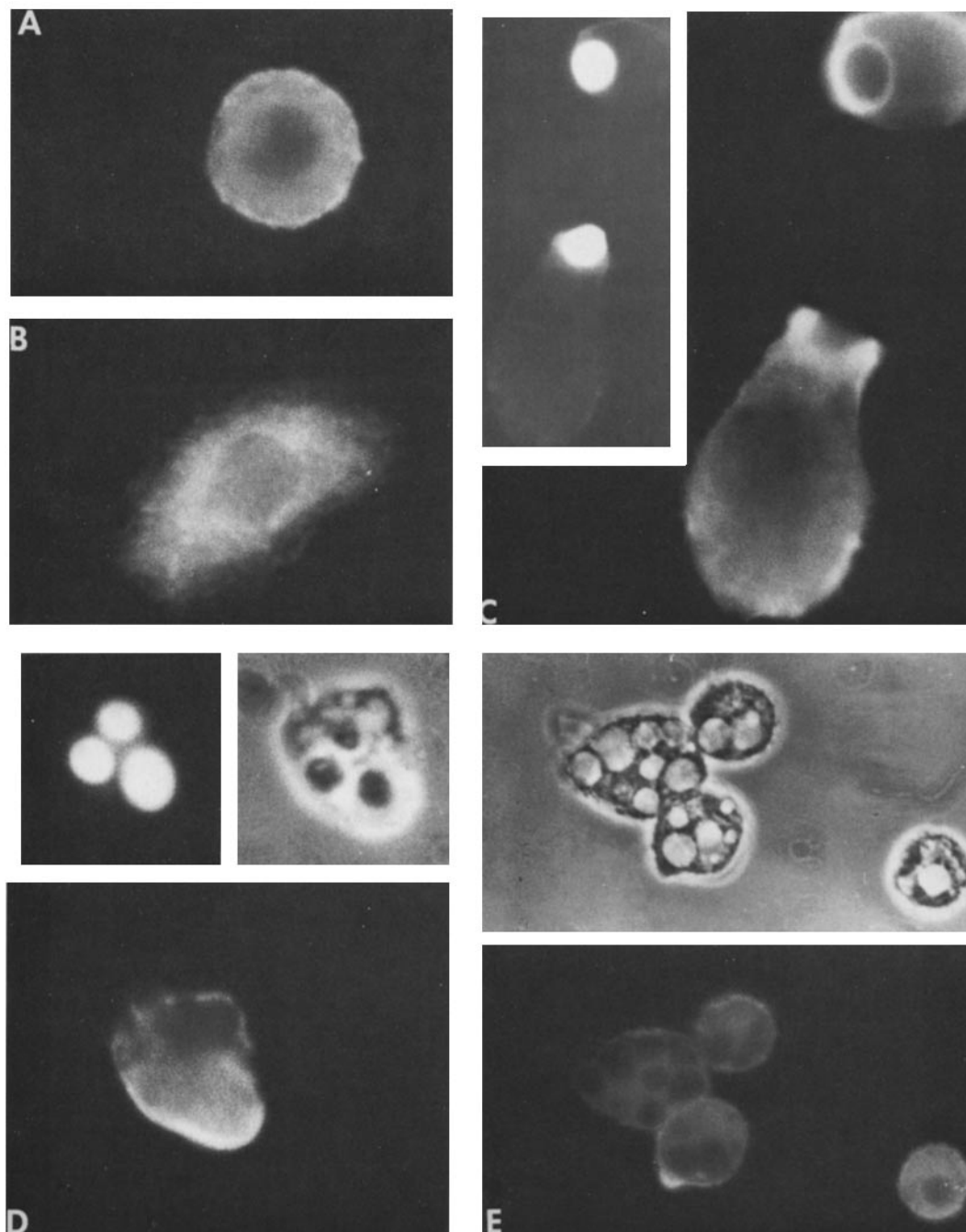


FIGURE 2 Rabbit lung macrophages fixed in acetone and subjected to indirect immunofluorescence staining with goat anti-myosin IgG and rhodamine-labeled rabbit anti-goat IgG. *A* shows a resting macrophage, *B* shows a spreading macrophage, *C* shows macrophages phagocytosing fluorescein-labeled yeast cells viewed for rhodamine fluorescence (*inset* shows the same macrophages and yeasts viewed for fluorescein fluorescence), *D* shows a macrophage with three completely ingested yeast cells viewed for rhodamine fluorescence (*inset* shows the same macrophage and yeasts viewed for fluorescein fluorescence and the phase-contrast photomicrograph of the same cell), and *E* shows macrophages having ingested oil droplets (phase-contrast photomicrograph (top) and rhodamine fluorescence after direct staining with rhodamine-labeled antimyosin  $F(ab')_2$  (bottom)).  $\times 1,000$ .

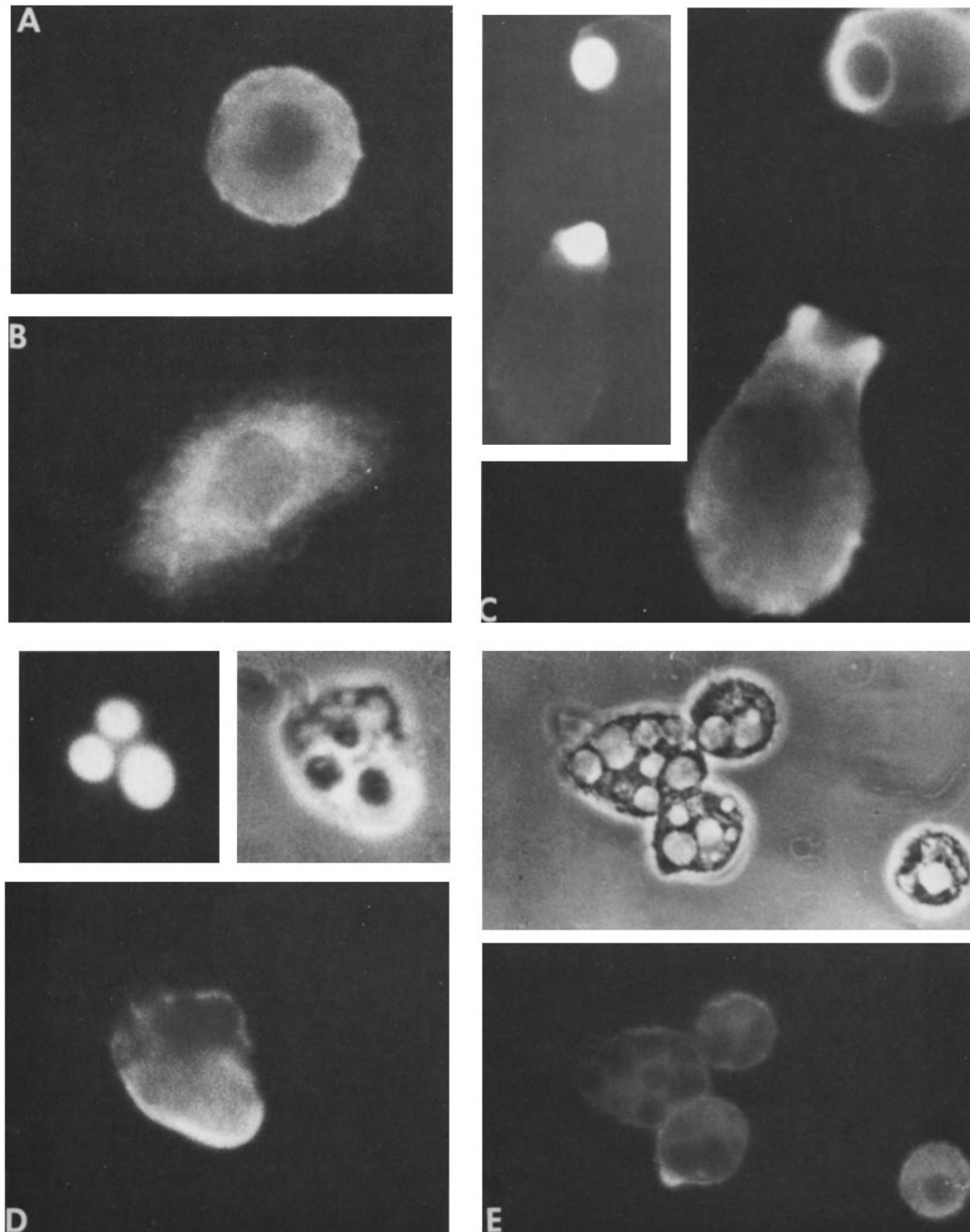


FIGURE 3 Rabbit lung macrophages fixed in acetone and subjected to indirect immunofluorescence staining with goat anti-rabbit macrophage ABP IgG and rabbit rhodamine-labeled anti-goat IgG. *A* shows resting macrophages, *B* shows a spreading macrophage, *C* shows a macrophage phagocytosing a fluorescein-labeled yeast cell viewed for rhodamine fluorescence (*inset* shows the same macrophage and yeast viewed for rhodamine fluorescence), *D* shows a macrophage phagocytosing two fluorescein-labeled yeast cells viewed for rhodamine fluorescence (*inset* shows the same macrophage and yeasts viewed for fluorescein fluorescence), and *E* shows a macrophage with three completely ingested and one partly ingested fluorescein-labeled yeast cells viewed for rhodamine fluorescence (*inset* shows the macrophage and the yeast cells viewed for fluorescein fluorescence).  $\times 1,000$ .

scopy, that occurred during phagocytosis. However, after the pseudopodia had fused, the brighter fluorescence embracing yeasts or oil droplets disappeared and neither myosin nor ABP staining was visible around these totally internalized particles. These observations fit with the attenuation of organelle exclusion that begins at the base of the phagosomes, permits approach of lysosomes to the phagosomes, and facilitates their eventual fusion with the phagosome membranes. The simplest explanation for this attenuation is that cortical cytoplasm associated with the invaginated mem-

brane "peels off" of the phagosome and returns to the periphery. Boxer et al. (1) reported that an IgG fraction reactive against ABP stained phagolysosomes isolated from human granulocytes as well as the periphery of intact granulocytes. However, because only purified phagolysosomes derived from homogenized cells were examined in their study, the relative staining intensity in the phagosomes with respect to the rest of the cell was not compared. Furthermore, adsorption of ABP to the phagolysosomes during homogenization and fractionation was not ruled out.

We have argued that during phagocytosis and spreading of macrophages, actin, myosin, and ABP concentrate in the advancing pseudopodia (12) and that this concentration is part of the mechanism of pseudopod advancement (14). The findings of this work showing ABP and myosin in pseudopodia by immunofluorescence are consistent with these ideas.

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TABLE I  
Distribution of Antimyosin Staining in Macrophages after Phagocytosis

Exp	Phagocytic vacuoles counted	Vacuoles without stained rims	Vacuoles with stained rims
		%	
1	60	91	9
2	70	90	10
3	70	93	7

Macrophages were incubated with opsonized oil droplets for 30 min (exp 1 and 2) or 60 min (exp 3) and, after washing and fixation, were treated with rhodamine-conjugated anti-rabbit macrophage myosin F(ab')<sub>2</sub>.

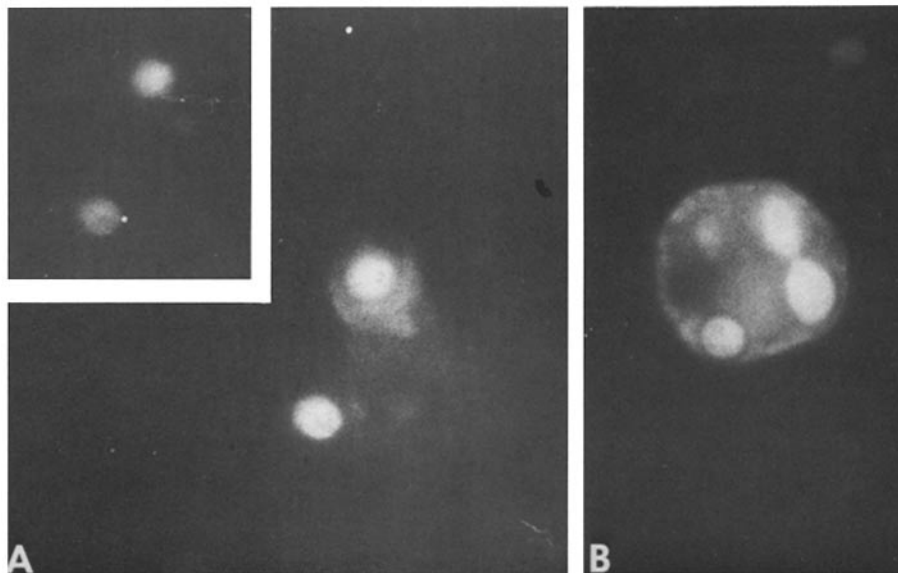


FIGURE 4 Rabbit lung macrophages fixed in acetone and stained directly with rhodamine-conjugated goat anti-rabbit macrophage myosin F(ab')<sub>2</sub>. A shows a macrophage ingesting a fluorescein-labeled yeast cell viewed for both rhodamine and fluorescein fluorescence (*inset* shows the macrophage and yeast cell viewed for fluorescein fluorescence), and B shows a macrophage which has ingested three fluorescein-labeled yeast cells viewed for both rhodamine and fluorescein fluorescence.  $\times 1,000$ .



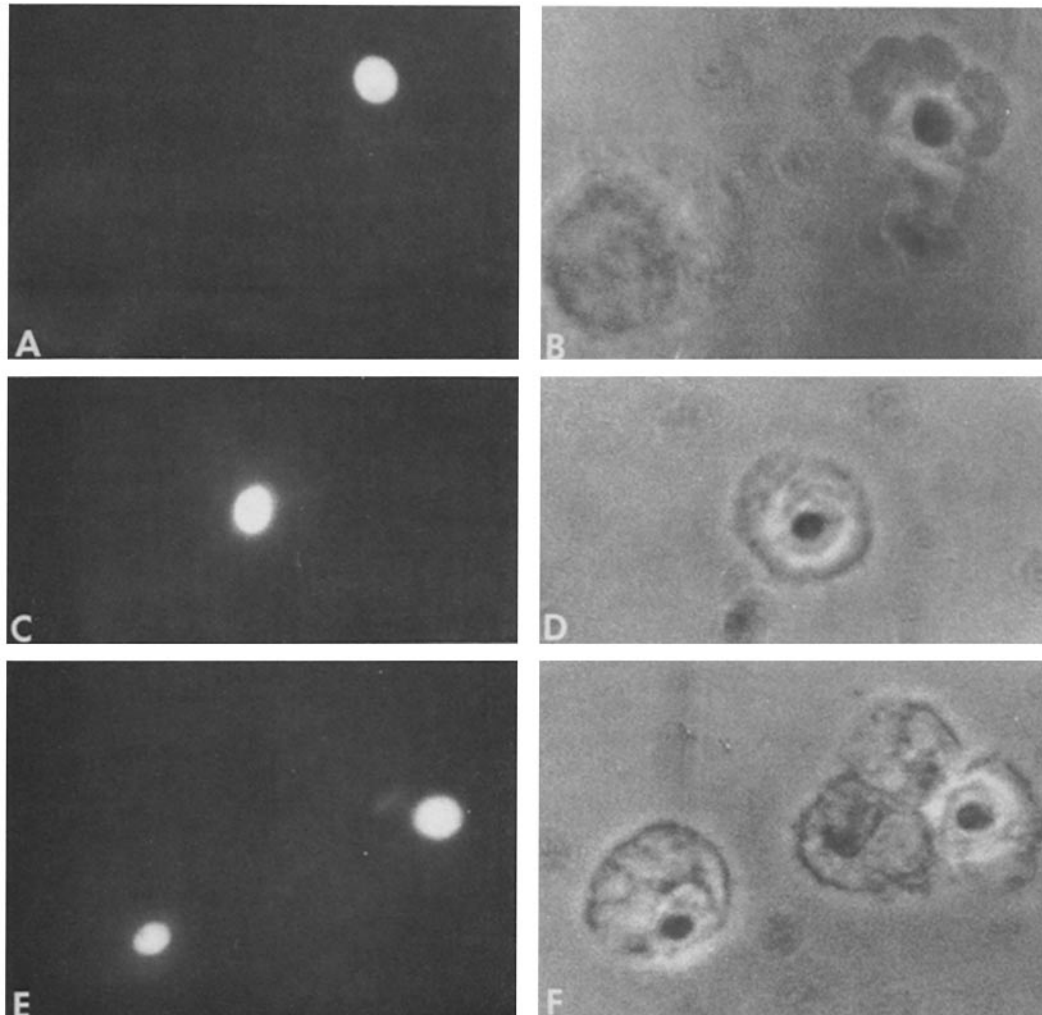


FIGURE 5 Rabbit lung macrophages fixed in acetone and treated with (A and B) preimmune goat serum, (C and D) goat anti-rabbit macrophage myosin IgG absorbed with purified macrophage myosin, and (E and F) goat anti-rabbit macrophage ABP IgG absorbed with purified macrophage ABP. The cells were then stained indirectly with rhodamine-labeled rabbit anti-goat IgG. The left part of the figure (A, C, and E) shows the macrophages with ingested fluorescein-labeled yeast cells viewed for both rhodamine and fluorescein fluorescence. The right part of the figure (B, D, and F) shows the phase-contrast photomicrographs of the corresponding cells.  $\times 1,000$ .

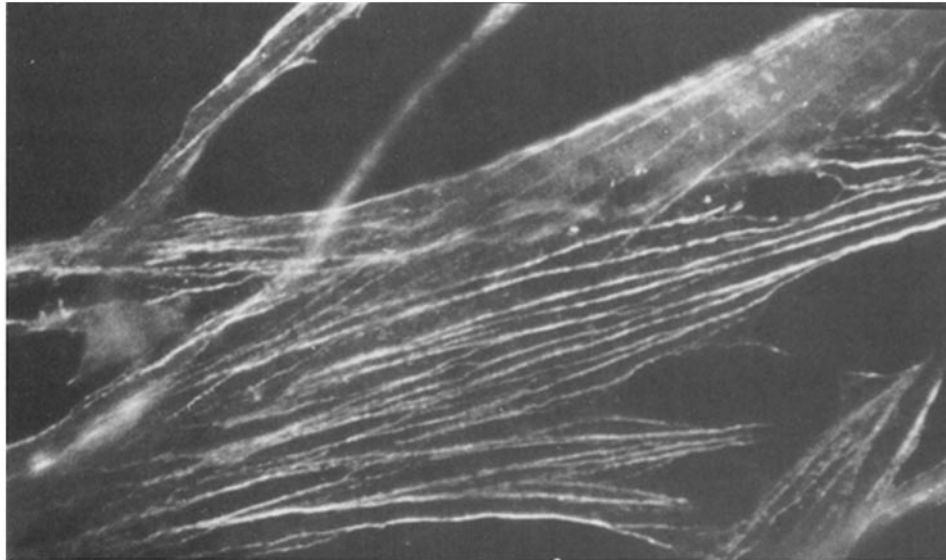


FIGURE 6 Monolayer of rat skin fibroblasts fixed in cold acetone and subjected to indirect immunofluorescence staining with goat anti-rabbit macrophage myosin IgG and rhodamine-conjugated rabbit anti-goat IgG.  $\times 1,000$ .

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