The Regional Association of Actin and Myosin With Sites of Particle Phagocytosis

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Contractile proteins are thought to play a causative role in motile processes such as phagocytosis. In order to investigate their role in phagocytosis further, simultaneous immunofluorescence localization of F-actin and myosin was carried out in resident mouse peritoneal macrophages after phagocytosis of opsonized zymosan particles. Both actin and myosin appeared to concentrate rapidly at sites of particle phagocytosis. The observed concentration of both proteins at such sites preceded ultimate particle engulfment. Cytochalasin B, a drug which was shown to block pseudopod extensions around the particle, did not prevent the concentration of the two contractile proteins at cell-particle binding sites. This result ruled out path-length effects as an explanation for the observed concentration of actin and myosin at phagocytic sites. Kinetic analysis showed that actin rapidly concentrates at particle-cell binding sites within minutes (or less) of contact with cell surface. The two proteins are present throughout the engulfment phase until and after ingestion is complete. Finally, at later times the particles become clustered over the cell nucleus and the particle-associated actin-myosin seen earlier is no longer evident.

Key words: phagocytosis, actin, myosin, macrophages, immunofluorescence

Contractile proteins are known to play a role in cell movement along substrate surfaces (see Korn [1] for a review). Several lines of evidence exist that implicate both actin and myosin and perhaps other contractile proteins in cell movement along surfaces. However, gross cell movement along infinite surfaces necessarily involves intimate contact of a large portion of the cell surface with the substratum, making interpretation of morphologic data obtained by immunofluorescence or electron microscope techniques difficult at best.

Phagocytosis of particles by contrast is a motile process possessing several unique features which simplify the analysis of immunofluorescence data particularly with respect to the localization of the contractile apparatus (for a review see Stossel [2] and Silverstein

et al [3]). For example, Silverstein and his colleagues have shown that pseudopod extension around a particle is receptor-dependent and involves only those regions of the membrane in close opposition to the particle being ingested [4, 5]. This strict regional responsiveness allows sites of particle-membrane interaction to be compared with uninvolved regions of the cell.

Actin has been detected in pseudopod extensions by both electron microscope and immunocytochemical methods [6, 7]. Myosin, which would be expected to participate in any postulated work-producing contraction, has not thus far been reported at sites of phagocytosis.

For the above reasons, we have determined the intracellular distributions of both actin and myosin by simultaneous immunofluorescence techniques. Our data show that myosin and actin are present at particle-membrane binding sites *prior* to pseudopod extension and ultimate particle ingestion.

MATERIALS AND METHODS

Preparation of F-Actin-Specific Fluorescent Reagents

Biotinyl-heavy meromyosin (B-HMM) and fluorescein-labeled egg white avidin (Fl-Av) were prepared and characterized as described by Heggeness and Ash [8]. The preparations used in these studies had 10–12 moles biotin/mole HMM and 2–3 moles fluorescein/mole avidin.

Preparation of Uterine Myosin and Rabbit Antimyosin Antibodies

Human uterine myosin was prepared from surgical specimens, within 3–4 h of removal, essentially as described by Pollard et al [9]. The protein was further purified by DEAE-Sephadex A-50 chromatography as described by Wang [10].

Rabbit antibodies were prepared with this material by subcutaneous injection of 100 μg of the protein homogenized in an equal volume of Freund's complete adjuvant into the footpads. Animals were boosted again at monthly intervals with 100 μg of immunogen emulsified in Freund's incomplete adjuvant. Bleedings were taken 10–14 days after the last boost and the IgG fraction was prepared by DEAE-cellulose chromatography.

F(ab')$_2$ fragments of the IgG fraction of immune or preimmune IgG were prepared by limited pepsin digestion as described by Nisonoff et al [11].

Preparation of Affinity-Purified Antiuterine Myosin

Affinity-purified antibodies were prepared by affinity chromatography of 20 mg of immune IgG on a human uterine myosin-Sepharose 4B column that contained a total of 2.5 mg myosin per 2.5 ml of packed gel bed which was prepared with CNBr-activated Sepharose 4B (Pharmacia, Piscataway, New Jersey) in accordance with the instructions of manufacturer. The column was exhaustively washed with 0.5 M NaCl and the bound antibodies were eluted with 0.1 M acetic acid. The eluted antibodies were neutralized immediately with 1/10 volume of 1 M Na acetate, dialyzed versus 0.14 M NaCl/0.02 M Na phosphate (pH 7.3) and ultracentrifuged for 30 min (100,000g) to remove denatured protein aggregates.

For some experiments the affinity-purified antibody was radioiodinated with ^{125}I by the chloramine-T method of McConahey and Dixon [12].

Preparation of Mouse Peritoneal Macrophage Monolayers and Phagocytosis of Zymosan Particles

Peritoneal exudate cells were isolated from Balb/cSt mice as described by Russell et al [13] and allowed to adhere to 16-mm-diameter glass coverslips by culturing of the washed cells for 3–4 h at 37°C in HEPES-buffered Eagles' minimum essential medium (MEM) containing 10% fetal calf serum (Gibco, Inc., Santa Clara, California) followed by vigorous rinsing with Tyrode's balanced salt solution containing 0.1% bovine serum albumin (Tyrode's-BSA) prior to use as described below.

When stimulation of particle phagocytosis was desired, cells were covered with 200 μl of zymosan particles (2 $\times$ 10^7 per milliliter) which had been previously opsonized with fresh human serum. Uptake was allowed to proceed at ambient temperature for up to 90 min. The reaction was terminated at specified time intervals by immersion of the coverslips in Tyrode's-BSA for a few seconds followed by fixation with 2% formaldehyde solution buffered with 0.1 M Na phosphate buffer (pH 7.4) for 20 min. Duplicate coverslips were either immunofluorescently stained for actin and myosin or assayed for degree of particle engulfment as described below.

Drug Inhibition Experiments

When drugs were used as inhibitors of phagocytosis, the cells were pretreated with the indicated concentration of drug for 15 min prior to zymosan challenge. During the 30-min particle incubation period, drug was included in the medium. In order to determine if inhibitory drug effects were reversible, parallel coverslips were washed free of excess zymosan and drug and were incubated in drug-free medium for an additional 30 min.

Immunofluorescence Staining Procedures

Simultaneous immunofluorescence staining for actin and myosin was carried out on coverslips essentially as described by Heggeness and Ash [8] and Heggeness et al [14], except that F(ab')$_2$ fragments (200 μg/ml) of the IgG fraction of antimyosin or preimmune sera were substituted for intact IgG. Briefly, formaldehyde fixed cells were made permeable with 0.1% Triton X-100 (no detectable staining was seen if this step was omitted), rinsed with buffer and incubated with B-HMM (100 μg/ml) and anti-myosin F(ab')$_2$ fragments (200 μg/ml) for 20 min. After extensive washing with buffer the cells were stained with Fl-Av (50 μg/ml) and rhodamine-labeled sheep anti-rabbit IgG (100 μg/ml). After washing, the coverslips were mounted on slides and viewed with a Zeiss microscope equipped with FL-IV epi-illumination optics with filter combinations that allowed for selective viewing of fluorescein and rhodamine fluorescence. Micrographs were recorded on Kodak Tri-X film.

Assay of Particle Engulfment

Zymosan particles that had been completely engulfed by cells were distinguished from surface-bound particles by incubation of formaldehyde-fixed but intact cells with rhodamine-labeled Con A (Rh-Con A) at a concentration of 100 μg/ml for 5 min. After washing, zymosan particles assessible to the extracellular millieu were brightly labeled while those which were completely engulfed were unstained. The percentage engulfment was obtained by calculating the ratio of unstained to total particles times 100. Partially stained particles were not considered to be engulfed. At least 200 particles were scored in each case. All data are the average of three separate experiments.

In experiments designed to relate actin distribution to the degree of particle engulfment, cells which had been first stained with Rh-Con A in an intact state as described above were made permeable with 0.1% Triton X-100 and then stained for F-actin as described above.

Electron Microscopy

Cells were prepared for electron microscopy by immersion of coverslips in Ca^{2+}-free Karnovsky's formaldehyde-glutaraldehyde fixative for 30 min at room temperature. The fixed cells were embedded in Epon in situ by the procedures of Maupin-Szamier and Pollard [15]. After curing, the coverslips were removed and the Epon blocks were remounted for ultrathin sectioning. Silver-colored thin sections were cut on an LKB Ultramicrotome 3 Model 8800 in a plane that was perpendicular to the original plane of cell growth. The sections were mounted on grids and stained with uranyl acetate and Pb citrate. Specimens were examined in a Hitachi 12A electron microscope.

Analytical Procedures

Polyacrylamide electrophoresis in sodium dodecyl sulfate (SDS PAGE) was performed as described by Laemmli [16] in 1.5-mm-thick slabs of 7% acrylamide with a Bio Rad Model #220 slab gel electrophoresis apparatus (Bio Rad, Richmond, California). Samples for SDS PAGE were prepared as described by Wang [10].

Direct staining of Coomassie Blue-stained SDS polyacrylamide gels strips with ^{125}I-labeled anti-human myosin was performed exactly as described by Burridge [17] as modified by Wallach et al [18]. The specific activity of the labeled antibody was 1.22×10^6 cpm/μg protein.

Ouchterlony analysis was carried out in 0.5% agarose gels containing 20 mM Na PP_i/ 0.5 M KCl (pH 7.0).

Reagents and Chemicals

Paraformaldehyde was obtained from Polysciences (Warrington, Pennsylvania) and fresh formaldehyde solutions were prepared immediately prior to use. Cytochalasin B was obtained from Sigma (St. Louis). Biotinyl-N-hydroxysuccinimide ester was prepared as described by Heitzmann and Richards [19]. All other reagents were reagent grade or better.

RESULTS

Characterization of Antimyosin Antibodies

The myosin antigen used to elicit the antibodies used in these studies was prepared from human uterine muscle as described by Pollard et al [9] and further purified by DEAE Sephadex A-50 chromatography. SDS-PAGE analysis (Fig. 1C) of the final purified protein (DM) shows the characteristic heavy-chain band with the light chains running in the dye front in this system. Actin, which was present at earlier stages of the purification, was not detected in this preparation. Traces of proteolytic degradation products are apparent just below the major heavy-chain band. These trace components are clearly proteolytic products, since they slowly increased during prolonged storage of the purified protein.

Anti-human uterine myosin was prepared by immunizing rabbits with the DEAE-Sephadex-purified protein. This antibody preparation was analyzed by Ouchterlony gel diffusion in 0.5% agarose gels containing 20 mM sodium pyrophosphate/0.5 M KCl (pH 7.0). In Figure 1A, a crude 0.6 M KCl/20 mM $NaPP_i$-1 mM dithiothreitol (DTT)

extract (EX) of human uterine tissue is compared with the DEAE-Sephadex A-50-purified myosin (DM) preparation. A single fused precipitin line is seen between the crude extract and the purified protein. When the antibody preparation was preabsorbed with myosin-Sepharose 4B, no detectable reaction was seen in either case (Fig. 1B).

To further verify the specificity of the antimyosin preparation, the Coomassie Blue-stained gels shown in Figure 1C were cut out as strips, neutralized with PBS, and directly stained with affinity-purified ^{125}I-anti-human myosin by the direct labeling technique of Burridge [17] as modified by Wallach et al [18]. Figure 2 shows an autoradiograph of the dried gels obtained after this procedure. Only one major band of radioactivity was seen in the crude uterine extract (EX); it has a mobility that is identical to that of the heavy chain of Sepharose 4B-purified myosin (SM). The lower-molecular-weight bands seen with the purified protein presumably represent antibody reaction with proteolytic fragments of the heavy chain, since they are not detected in the original extract. Thus, by a number of sensitive immunochemical criteria the anti-human uterine myosin used in these studies is monospecific for myosin.

Immunofluorescent Distribution of Actin and Myosin in Resting Macrophages

Figure 3 shows the immunofluorescence staining patterns observed in the same normal resting macrophage for F-actin (3A) and myosin (3B). The spread cell shows a fine network-like distribution of actin (3A) throughout the cell cytoplasm in addition to some areas of punctate staining. The so-called "stress" fibers seen in other adherent cells such as 3T3 fibroblasts were rarely seen in such macrophage preparations even after 24 h of culture.

The myosin distribution (Fig. 3B) is more or less uniformly punctate and shows some areas with an apparent periodicity.

Specificity of the Immunofluorescence Staining Reactions

As seen in Figure 4B, the reaction of B-HMM with the cell is completely inhibited by its specific inhibitor, sodium pyrophosphate. Although not shown, the staining of cells with B-HMM and F1-Av was also blocked by free biotin, a potent competitive inhibitor of avidin binding to B-HMM. Neither inhibitor had any apparent effect on the antimyosin-myosin staining reaction.

The cytoplasm-associated rhodamine staining reaction was not seen if preimmune F(ab')$_2$ fragments were substituted for specific antimyosin F(ab')$_2$ fragments (Fig. 4C). In addition, no reaction was observed if the antibodies were preabsorbed with myosin-Sepharose 4B (not shown).

Actin and Myosin Distributions in Macrophages Phagocytosing Zymosan

Figure 5B,C show the actin and myosin distributions, respectively, in a cell that had been exposed to serum-treated zymosan (Z) for 30 min. A thin ring of bright actin-associated staining is observed which is uniformly and intimately associated with the engulfed zymosan particle (Fig. 5B). The myosin (Fig. 5C) staining pattern in the same cell is also closely associated with the engulfed particle but, unlike the actin pattern, shows a discontinuous or "patchy" distribution. It should be noted that the apparent lack of staining of other cell cytoplasmic regions is due to the fact that the cell body is below the focal plane of the particles. When the cell body was brought into focus, the staining patterns in regions removed from particle engulfment sites were similar to those seen in resting cells.

Fig. 1. Characterization of antimyosin antibodies and antigens. A: DEAE-Sephadex A-50-purified human myosin (DM; 6 μg) and 0.6 M KCl/20 mM NaPP$_i$/1 mM DTT extract of human uterine muscle (EX; 126 μg) were placed in Ouchterlony wells as indicated and tested against 1.3 mg rabbit antimyosin immunoglobulin G (trough). Single Coomassie Blue staining lines of immunoprecipitation between the purified myosin and the crude extract which show apparent immunochemical identity are seen. B: As in A except that the antimyosin immune globulin was preabsorbed with myosin-Sepharose 4B (50 μg myosin). No detectable reaction is revealed with Coomassie Blue. C: SDS PAGE electrophoresis of the antigens used in A and B; 50 μg of human uterine extract (EX), 4 μg of human uterine myosin after Sepharose 4 B chromatography (SM), and 4 μg of final DEAE-Sephadex A-50-purified protein (DM) were electrophoresed on 7% SDS PAGE gels in the presence of 1% β-mercaptoethanol and 0.1% SDS. Stained with Coomassie Brilliant Blue.

Fig. 2. Direct staining of SDS gels with ^{125}I-anti-human myosin antibodies. The gels labeled EX and SM are the same as those shown in Figure 1C and were cut out as strips and neutralized by incubation in PBS (pH 8.0). After treatment of the gels with preimmune IgG (1 mg/ml) and BSA (1 mg/ml) for 2 h, affinity-purified ^{125}I-antihuman myosin (2.2 × 10^6 cpm/μg) was added at 1 μg/ml and incubated for 18 h at room temperature. Unbound antibody was removed by exhaustive washing as described by Wallach et al [18] prior to autoradiography of the dried gel strips.

Effect of Cytochalasin B on the Actin-Myosin Distributions in Particle-Treated Macrophages

It is conceivable that the bright actin and myosin staining results from a path-length effect due to viewing long pseudopod extensions which extend out around the particle along their vertical axis. To evaluate this possibility, cells were pretreated with cytochalasin B, a drug known to block particle ingestion and pseudopod extension, and then were
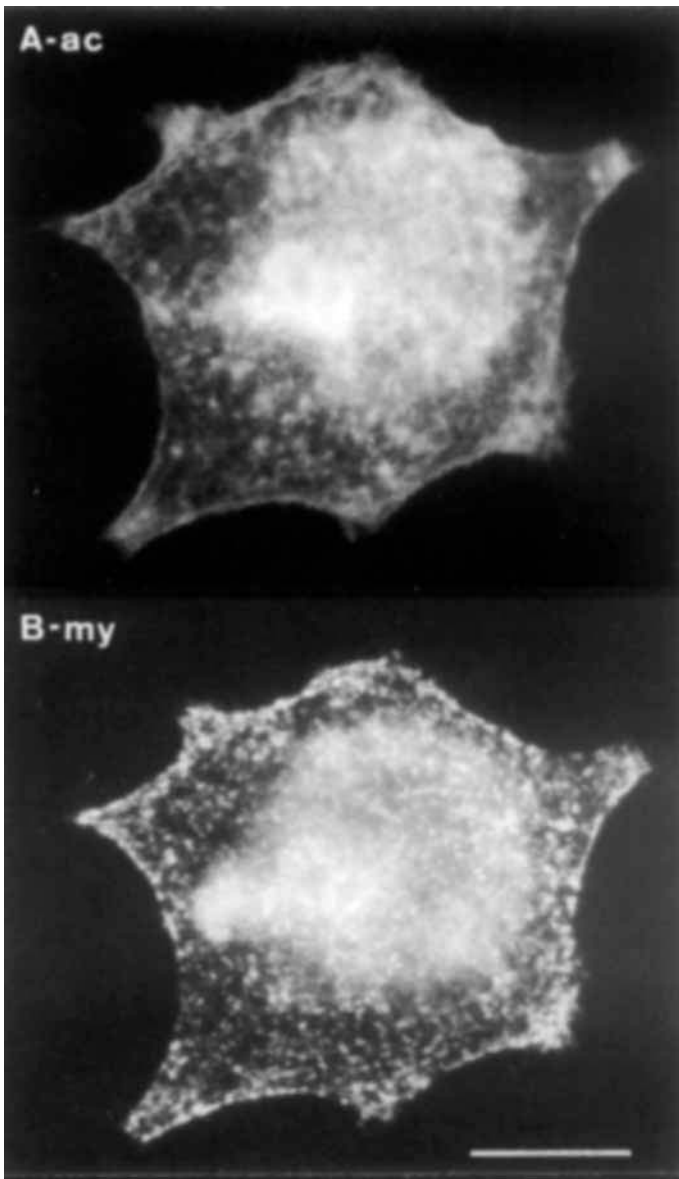
Fig. 3. Simultaneous immunofluorescence localization of actin (ac) and myosin (my) in resting mouse peritoneal macrophages. A: Actin distribution; B: myosin distribution. × 2,400; bar equals 10 μm.

challenged with particles in the presence of the drug. As shown in Figure 5D–F, after staining for actin (Fig. 5E) and myosin (Fig. 5F) both proteins are found in a focal plane which appears to underly the peripheral region of the zymosan particle (Z). As in the case of non-drug-treated cells the myosin distribution is "patchy" or punctate. Occasionally, particles are lost from such sites*, one of which is also shown in Figure 5D–F (arrowhead). A corresponding "oval-shaped" area of phase density (Fig. 5D) is also seen at such sites, which is consistent with the accumulation of protein at these regions of the cell.

*This was rigorously proved by examination of identical fields of cell before and after immunofluorescence staining procedures.
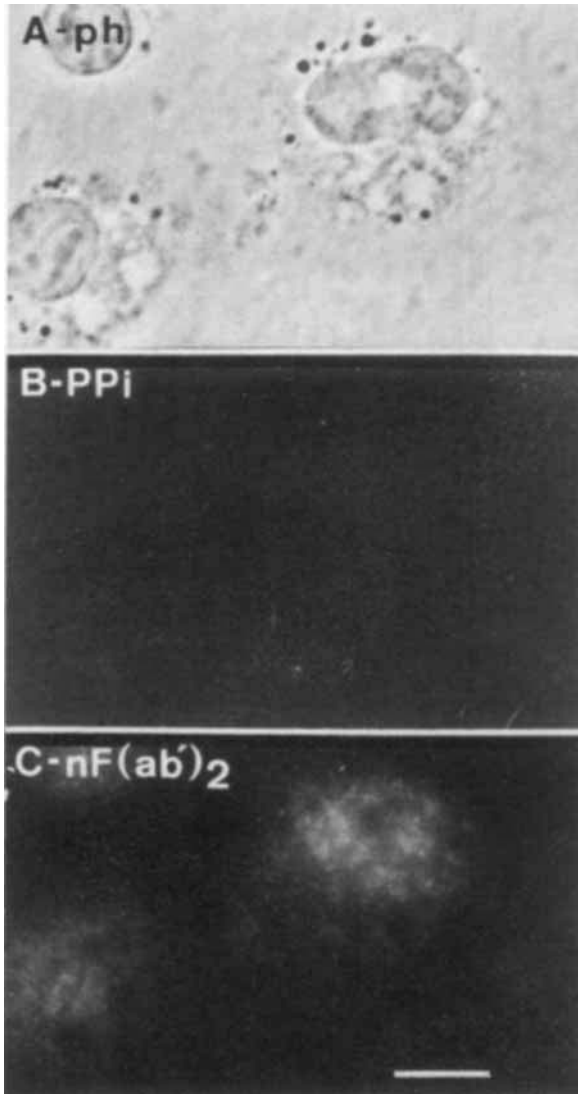
Fig. 4. Immunofluorescence specificity controls. A: Phase contrast (ph); fluorescein staining obtained with B-HMM and F1-Av with B-HMM in the presence of 10 mM NaPP$_i$/2 mM MgCl$_2$ in the same cells; C: staining of the same cells with F(ab')$_2$ fragment of preimmune IgG (200 μg/ml) in the place of F(ab')$_2$ fragments of antimyosin IgG followed by Rh-sheep anti-rabbit IgG. No detectable cytoplasmic staining is seen in either case. × 1,250; bar = 10 μm.

An electron micrograph of CB-treated cells which had been subsequently challenged with particles is shown in Figure 6. Such cells show no evidence of pseudopod extension around the particle surface (inset), a result which rules out path-length considerations as a viable explanation of the immunofluorescent results. Furthermore, in the region of the cell underlying the particle periphery, a dense zone of microfilaments (MF) is clearly evident (arrow).
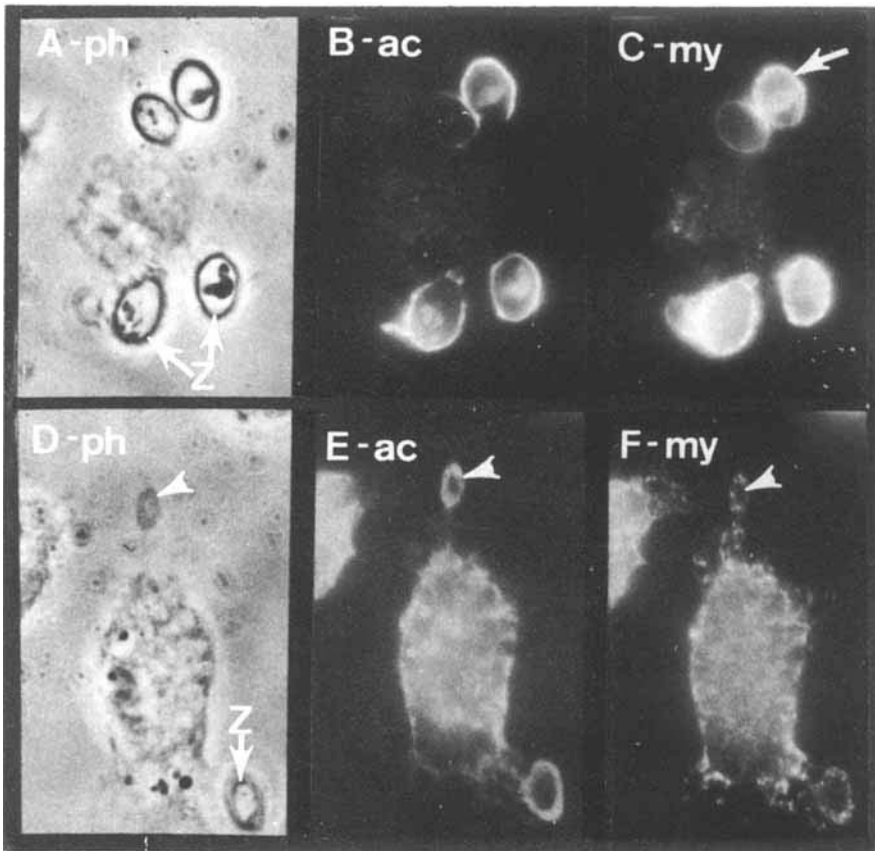
Fig. 5. Simultaneous immunofluorescence localization of actin (ac) and myosin (my) in mouse peritoneal macrophages 30 min after exposure to zymosan (Z) particles in the absence (A–C) and presence (D–F) of CB. A: Phase contrast (ph) micrograph showing macrophages and zymosan (Z); B: actin distribution in same cell as A; C: myosin distribution in same cell as A. Note the patchy distribution of the myosin-associated staining in comparison with the more uniform actin pattern. D: Phase contrast micrograph of a CB-treated cell which has been subsequently exposed to zymosan for 30 min. The cell was pretreated with drug (10 μg/ml) for 15 min, followed by addition of zymosan for an additional 30 min. The cells were then fixed and stained for actin and myosin as before. E: Actin distribution in same cell as D; F: Myosin distribution in same cell as D. The fluorescence associated with the particle core is a nonspecific autofluorescence. A–F × 1,250.

Thus, immunofluorescence and electron microscope examination of phagocytosing cells indicate that both F-actin and myosin concentrate at particle-binding sites even in CB-treated cells which have not extended pseudopods or engulfed the particle.

The Relationship of the Actin Response to the Degree of Particle Engulfment

As seen in Figure 5B, the actin staining regions do not always completely surround the zymosan particle. At early times after particle challenge especially, crescent-shaped profiles of actin staining are commonly seen, indicating that the contractile response occurs before complete ingestion of the particle.
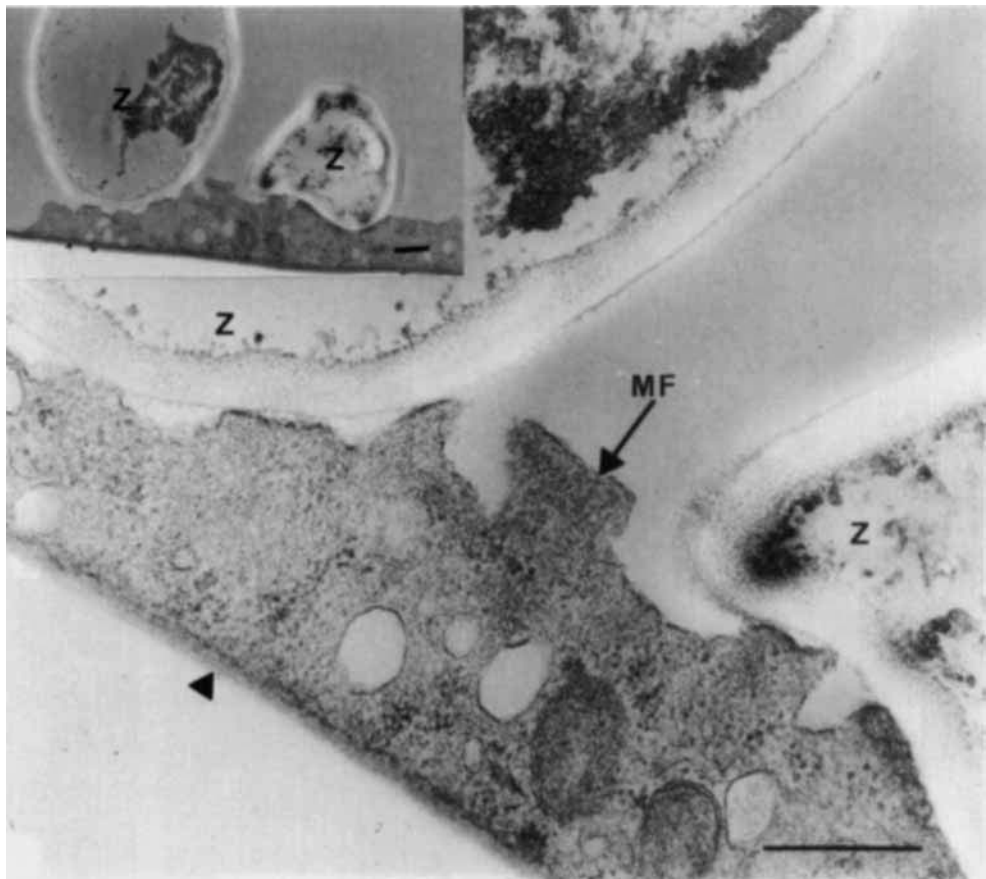
Fig. 6. Transmission electron micrograph of a thin section of Epon-embedded CB-treated macrophage exposed to zymosan (Z) for 30 min. Note the lack of long pseudopod extensions (inset) around the particles. At higher magnification, dense zones of microfilaments (MF) are apparent which lie in areas that are in close proximity to the particle-binding sites and that clearly underly the plasma membrane. The arrowhead indicates the substratum surface. × 55,000; inset × 12,000; bar = 0.2 μm.

In order to investigate the relationship between the degree of ingestion and the actin staining pattern, we took advantage of the fact that those areas of the particle in direct contact with the macrophage membrane did not appear to stain with Rh-Con A in *intact* cells. Thus, particles that are 50% ingested by the cell show a crescent-like staining profile when treated with Rh-Con A (Fig. 7B, dashed arrow).

The cells with Rh-Con A-labeled particles shown in Figure 7 were subsequently stained for actin after being made permeable to the B-HMM/F1-Av reagents with detergent. This staining protocol allows a direct comparison of the extent of engulfment as judged by the Rh-Con A pattern with the intracellular actin distribution. Particles in every phase of engulfment are seen even within the same cell, ranging from those which are completely inaccessible to Rh-Con A (Fig. 7B, solid arrow), intermediate forms (Fig. 7B, broken arrow) and particles which are totally accessible to reagent (Fig. 7E, solid arrows).
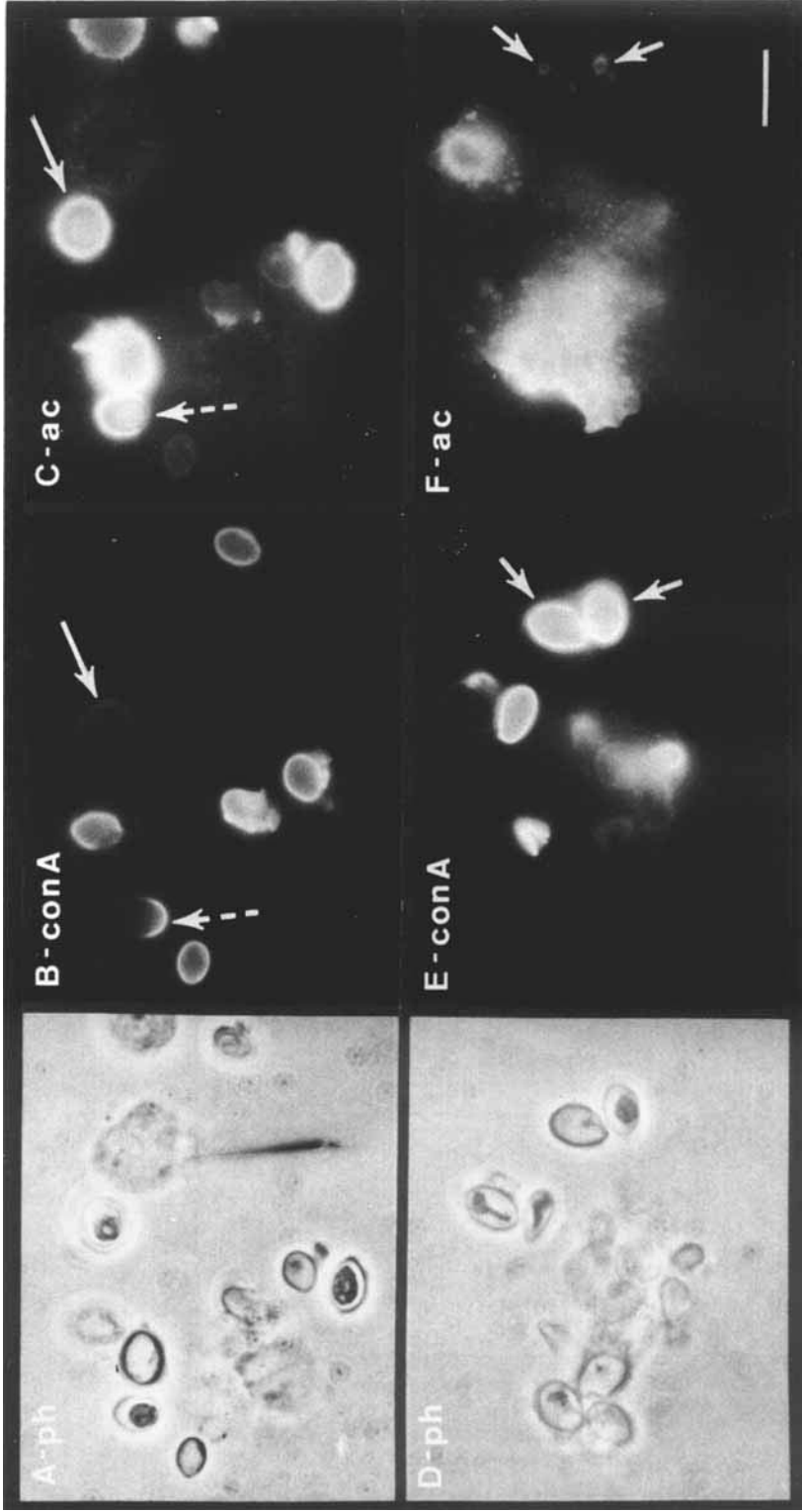
Fig. 7. Relationship of degree of particle phagocytosis to the cytoplasmic actin distribution. Macrophages which had been exposed to zymosan for 15 (D–F) or 30 (A–C) min were fixed with formaldehyde and stained with Rh-Con A immediately after rinsing. After rinsing, the cells were then rendered permeable with 0.1% Triton X-100 and stained for F-actin as described in Materials and Methods. Particles in various stages of engulfment are seen, ranging from initial surface contact (E, F, solid arrows) to 50% ingested (B, C, broken arrows), and finally to completely ingested (B, C, broken arrows).

The actin patterns in order of increasing degree of ingestion range from small rings of fluorescence that lie in a focal plane underlying the particle, and are the first recognizable response of the contractile system to the particle's presence at the cell surface, to the rings seen at later stages of ingestion (Fig. 7C, broken arrow), which lie within the focal plane of the particle and are in all probability pseudopod extensions that have advanced around the particle periphery. Finally, there are particles that are totally inaccessible to Rh-Con A and show actin "rings" that completely envelope the particle, indicating that the particle-associated actin staining persists for some time after the ingestion phase is completed (Fig. 7C, solid arrow).

Kinetics of Engulfment of Zymosan Particles by Macrophages

Through the accessibility of zymosan particles to Rh-Con A labeling in formaldehyde-fixed but intact macrophages, the kinetics of engulfment were followed over a 90-min incubation period at ambient temperature. As seen in Table I, the percentage of cell-associated particles that were *totally inaccessible* to Rh-Con A (an example of which is shown in Figure 7B, solid arrow), and were therefore completely engulfed by the cell, increased with time, plateauing approximately 60–90 min after particle challenge at levels of about 40% engulfment of total cell-bound zymosan. When these cells were rendered permeable with Triton X-100 *prior* to staining with Rh-Con A, >99% of cell-associated particles were stained, confirming that totally engulfed particles were capable of being stained by Rh-Con A.

As expected, CB was found to completely and reversibly block engulfment as measured by this assay (Table I). In addition, no partial engulfment profiles like those noted in Figure 7 were found.

Kinetics of the Actin-Myosin Response

Figure 8 shows a kinetic analysis of the particle-associated actin and myosin response to the presence of particles in phagocytosing macrophages. These data were obtained by immunofluorescently staining cells for actin and myosin at the indicated time points after particle challenge. In each case 200 or more cell-associated particles were microscopically scored for either 1) actin staining alone (o——o), 2) myosin alone (△——△), 3) both actin and myosin (●——●), or 4) no detectable associated staining (▲----▲).

TABLE I. Engulfment of Zymosan (Z) by Macrophages as Assayed by Accessibility of Zymosan to Rh-con A in Intact Cells*

Treatment	Time after Z addition (min)	Total Z/cell[a] (SEM)	% Engulfment[a] (SEM)
No drug	30	5.7 (0.9)	26 (6)
	60	5.4 (0.8)	38 (3)
	90	7.8 (0.6)	40 (5)
CB (10 µg/ml)	30	6.0 (0.4)	0.4 (0.07)
30 min after CB removal	60	ND	23 (4)

*Peritoneal macrophage monolayers on glass coverslips were pretreated with the indicated concentration of drug in Tyrode's-BSA medium 15 min prior to zymosan addition. Drug was included during the zymosan exposure period. To test for drug reversibility cells were rinsed in drug-free medium and incubated an additional 30 min. At the indicated time, cells were rinsed and fixed, and surface-accessible zymosan was stained with Rh-Con A as described in Materials and Methods. ND – not determined.
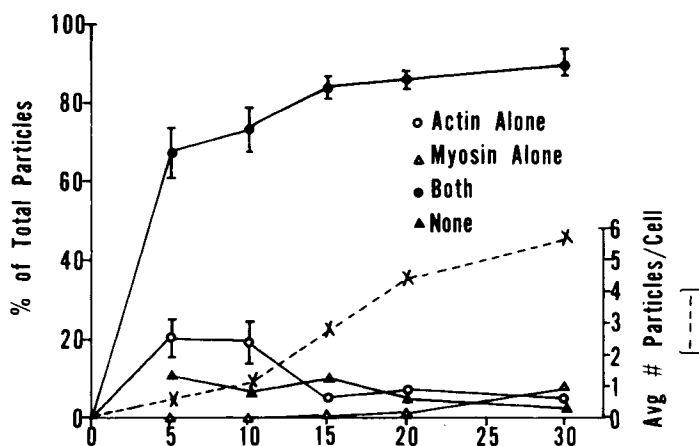[a]Mean of three separate experiments.

Fig. 8. Kinetic analysis of the actin-myosin response to zymosan challenge in mouse macrophage monolayers. Adherent cells on glass coverslips were challenged with serum-treated zymosan at time zero. At indicated times, cells were fixed with 2% formaldehyde for 20 min and immunofluorescently stained for actin and myosin. At least 200 particles were microscopically scored for a dectable response for actin alone (o———o); myosin alone (△———△); both actin and myosin (●———●); or no detectable staining (▲———▲). The average number of cell-associated particles was likewise determined (– – –). Average of three separate experiments ± SEM.

The data indicate that the two contractile proteins accumulated rapidly at regions associated with adherent particles at a time prior to any significant engulfment by the cell (see Table I). In addition, the data suggest that actin may associate slightly faster than myosin, since there is a significant and reproducible percentage (20%) of particles at 10–15 min which have only actin associated with them.* At later times this value diminished nearly to zero with a corresponding increase in the percentage of particles with both proteins associated. Particles with associated myosin but no detectable actin (△———△) were only rarely seen.

At later times after particle addition (> 30 min) cells contained too many particles for accurate quantitation. However, at these later time points, clusters of particles were seen (generally over the cell nucleus) which no longer stained for actin or myosin (Fig. 9).

DISCUSSION

The data presented here indicate that two major macrophage contractile proteins, F-actin and myosin, accumulate rapidly at phagocytic sites. Detailed kinetic analysis and morphologic evidence further suggest that the observed regional contractile protein response clearly precedes the ultimate engulfment of membrane-associated particles (Figs. 7 and 8).

The specificity of the reagents used in these studies is worthy of comment. First, the BHMM/F1-Av technique has been shown to be highly specific for actin [8]. In addition, unlike antiactin antibodies which have been used by others [7, 20] to localize actin, the B-HMM reagent is specific for F-actin but not G-actin [21].

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*Such a result could also be explained by a difference in the sensitivities of the two fluorescent reagents for their respective target proteins.
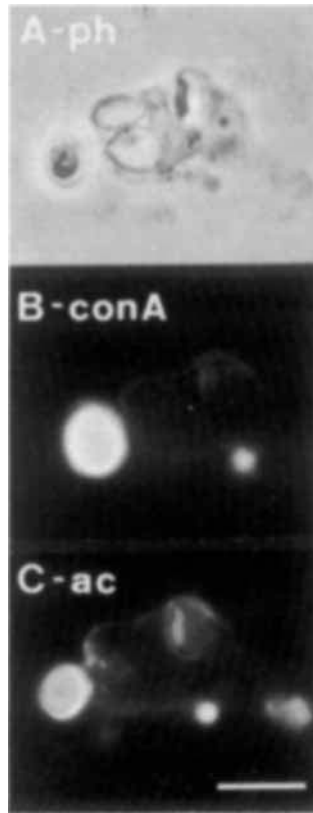
Fig. 9. Con A (B) and actin (C) distributions in a macrophage containing a cluster of zymosan. (A, phase contrast.) This cell was fixed and stained 1 h after particle challenge. Note that the adjacent particle at the cell periphery is stained for actin while those within the cluster are completely unstained. The fluorescence associated with the particle core is a nonspecific autofluorescence. × 1,250; bar = 10 μm.

    The use of antibodies for the localization of myosin by immunofluorescence has led to conflicting results in some cases. For example, myosin has been detected on the surface of platelets [22] and fibroblasts [23] by some investigators but not by others using different antibody preparations [24, 25]. The latter result was also found to be the case in these studies. Since these differences could be explained by the presence of antibodies to trace impurities, such as glycolipids, we have extensively characterized the antibody preparation used in these studies. The evidence for monospecificity includes the demonstration of a single line of identity between the highly purified antigen and the crude extract used for preparation of the antigen (Fig. 1A). This reacting antibody species is removed by pre-absorbsion of the antibody with myosin-Sepharose 4B (Fig. 1B). Furthermore, direct staining of SDS gels of the crude extract with ^{125}I-antimyosin labeled only one major protein species which comigrated with myosin heavy chain (Fig. 2). Since this technique is probably an order of magnitude more sensitive than immunofluorescence we conclude that myosin is the major fluorescent species detected in our studies.
    The actin and myosin distributions seen in mouse peritoneal macrophages is similar to those noted by others in cultured fibroblasts [8, 20]. The only striking difference that we have noted is the absence of actin-containing "stress fibers" seen in many other cultured

cell types. The reason for this is unclear but could conceivably be related to the higher motility rate of this cell type compared with fibroblasts.

In macrophages that are phagocytosing particles the data show that both myosin and actin are associated with and concentrate at phagocytic sites. Unlike the more uniform actin distribution around the phagosome vacuole, the myosin distribution seen is discontinuous or "patchy" (Fig. 5C). This pattern is somewhat reminiscent of myosin staining patterns seen directly underlying capped Ig receptors [26] in B lymphocytes, and it is consistent with the suggestion that phagocytosis and capping are related processes [7].

Our data indicate that actin concentrates at sites in close opposition to particle-cell interactions shortly after the particle has made receptor-mediated contact with the cell surface (Fig. 7F). This initial response of the contractile apparatus continues to propagate outward from the point of initial contact as more of the particle surface comes in contact with the cell surface. Pseudopods, rich in actin and myosin, begin to extend around the particle, becoming so firmly attached to membrane receptors that Rh-Con A is unable to penetrate such regions (Fig. 7B, broken arrow). The close association of the contractile apparatus at phagocytic sites from the point of first contact throughout all phases of the engulfment process is consistent with a major role for this cytoplasmic system in the engulfment phase of the process. Our data also suggest that, in addition to an involvement in the engulfment phase per se, the contractile apparatus may also function to move completely ingested particles within the cell cytoplasm since their association with the phagosome persists after ingestion is completed.

It could be argued that the apparent concentration of actin and myosin with phago-somal vacuoles is due to a path-length effect. Thus, a large particle surrounded by long vertical pseudopodal extensions which contain normal cytoplasmic concentrations of actin and myosin would be expected to show an increased fluorescence when compared to other thinner cell regions. This possibility is ruled out by several lines of evidence. First, cyto-chalasin B (a drug which prevents pseudopod extension but not particle adhesion to the cell surface [27]) does *not* block the concentration of actin or myosin at cell regions directly underlying the peripheral regions of particle-binding sites (Fig. 5D–F). Second, electron microscope studies of CB-treated cells with attached zymosan show little evidence of pseudopod extension around the particle surface and in fact contain dense regions of microfilaments which underly the vicinity of the particle-membrane binding site (Fig. 6). Finally, increased concentrations of actin are seen which underly particles well before significant pseudopod extension has occurred (Fig. 7F, arrow).

Thus, the observed concentration of these two contractile proteins at particle-binding sites which lack pseudopod extensions rules out gross path-length effects and indicates that particle binding is associated with a localized increase in actin and myosin which *precedes* pseudopod extension and ultimate ingestion.

At present, it is unclear whether the observed actin concentration at such sites represents assembly of F-actin filaments or simply a redistribution of filaments from other cell regions. Nevertheless, the observed highly regional response of the contractile system to a local membrane perturbation places severe restraints upon the nature of any transmembrane signal(s) which may exist to communicate the particle's presence to the contractile apparatus of the cell. Our current data are consistent with a recent suggestion advanced by Bourguignon and Singer [28] that aggregation of surface receptors by a multi-valent ligand (a large particle in this case) induces the association of an as yet unidentified actin-binding membrane component (X) with such receptor "patches" leading in turn to actin binding at that site. If their suggestions are correct, on the basis of our data, one would expect to find X in high concentration in isolated phagosomal membranes.

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REFERENCES

1. Korn ED: Proc Natl Acad Sci USA 75:588, 1978.
2. Stossel TP: J Reticuloendothel Soc 19:237, 1976.
3. Silverstein SC, Steinman RM, Cohn ZA: Annu Rev Biochem 46:669, 1977.
4. Griffin FM Jr, Silverstein SC: J Exp Med 139:323, 1974.
5. Griffin FM Jr, Bianco C, Silverstein SC: J Exp Med 141:1269, 1975.
6. Keyserlingk DG: Exp Cell Res 51:79, 1968.
7. Berlin RJ, Oliver JM: J Cell Biol 77:789, 1978.
8. Heggeness MH, Ash JF: J Cell Biol 73:783, 1977.
9. Pollard TD, Thomas SM, Niederman, R: Anal Biochem 60:258, 1974.
10. Wang K: Biochemistry 16:1857, 1977.
11. Nisonoff A, Wissler FC, Woernley DL: Biochem Biophys Res Commun 1:318, 1959.
12. McConahey P, Dixon FJ: Int Arch Allergy Appl Immunol 29:185, 1966.
13. Russell SW, Doe WF, Hoskins RG, Cochrane CG: Int J Cancer 18:322, 1976.
14. Heggeness MH, Wang K, Singer SJ: Proc Natl Acad Sci USA 74:3883, 1977.
15. Maupin-Szamier P, Pollard TD: J Cell Biol 77:837, 1978.
16. Laemmli UK: Nature 227:680, 1970.
17. Burridge K: Proc Natl Acad Sci USA 73:4157, 1976.
18. Wallach D, Davies PJA, Pastan I: J Biol Chem 253:3328, 1978.
19. Heitzmann H, Richards FM: Proc Natl Acad Sci USA 71:3537, 1974.
20. Pollack R, Osborn M, Weber K: Proc Natl Acad Sci USA 72:994, 1975.
21. Offer G, Baker H, Baker L: J Mol Biol 66:435, 1972.
22. Puszkin EG, Maldonado R, Spaet TH, Zucker MB: J Biol Chem 252:4371, 1977.
23. Willingham MC, Ostland RE, Pastan I: Proc Natl Acad Sci USA 71:4144, 1974.
24. Pollard TD, Fujiwara K, Handin R, Weiss G: Ann NY Acad Sci 283:218, 1977.
25. Painter RG, Sheetz M, Singer SJ: Proc Natl Acad Sci USA 72:1359, 1975.
26. Schreiner GF, Fujiwara K, Pollard TD, Unanue ER: J Exp Med 145:1393, 1977.
27. Axline SG, Reaven EP: J Cell Biol 62:647, 1974.
28. Bourguignon LY, Singer SJ: Proc Natl Acad Sci USA 74:5031, 1977.