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A contractile activity that closes phagosomes in macrophages

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SUMMARY

Studies of Fc-mediated phagocytosis by mouse macrophages identified a contractile activity at the distal margins of forming phagosomes. Time-lapse video microscopic analysis of macrophages containing rhodamine-labeled actin and fluorescein dextran showed that actin was concentrated at the distal margins of closing phagosomes. Phagocytosis-related contractile activities were observed when one IgG-opsonized erythrocyte was engaged by two macrophages. Both cells extended pseudopodia until they met midway around the erythrocyte. It was then constricted and pulled into two phagosomes, which remained interconnected by a string of erythrocyte membrane. Butanedione monoxime, an uncompetitive inhibitor of class II and perhaps other myosins, and wortmannin and LY294002, inhibitors of phosphoinositide 3-kinase, prevented the constrictions without inhibiting the initial pseudopod extension. Immunofluorescence microscopy showed the presence of myosins IC, II, V and IXb in phagosomes. Of these, only myosin IC was concentrated around the strings connecting shared erythrocytes, suggesting that myosin IC mediates the purse-string-like contraction that closes phagosomes. The sequential processes of pseudopod extension and contraction can explain how macropinosomes and spacious phagosomes form without guidance from a particle surface.

Key words: Phagosome, Macrophage, Myosin, Contraction

INTRODUCTION

Phagocytosis is a complicated rearrangement of the actin cytoskeleton that delivers extracellular particles into intracellular vacuoles called phagosomes. In macrophages, a particle opsonized with IgG can stimulate Fc receptor-mediated phagocytosis, in which pseudopodial extensions of the cell surface cover the particle and enclose it. Although it is established that these pseudopodia contain actin and many actin-associated proteins (Allen and Aderem, 1995), and that a functional actin cytoskeleton is necessary for phagocytosis (Allison et al., 1971), it is not yet known how the actin cytoskeleton is regulated to achieve this apparently coordinated combination of extension and closure.

No single extant model explains all kinds of phagocytosis. According to the zipper model for phagocytosis, pseudopod advance over a particle is guided by interactions between opsonins and cognate receptors in the macrophage plasma membrane. Such zippering of a membrane along a particle surface, together with a membrane fusion process, has been considered sufficient to enclose particles completely (Greenberg and Silverstein, 1993; Swanson and Baer, 1995). However, in the analogous process of macropinocytosis, actin-rich pseudopodia extend and close into intracellular vesicles without any particle surface to guide them (Swanson and Watts, 1995). Moreover, some bacteria enter macrophages by a process resembling macropinocytosis (Alpuche-Aranda et al., 1994). Thus, without an additional mechanism for closing phagosomes, the zipper model cannot explain how pseudopodia close to form macropinosomes or spacious phagosomes.

Recent studies indicate that phagocytosis requires two component activities of the actin cytoskeleton. Inhibitors of phosphoinositide 3-kinase (PI3-kinase) allow pseudopodia to extend onto an opsonized particle, but prevent them from closing into phagosomes, indicating that PI3-kinase regulates phagosome closure (Araki et al., 1996). Phagosome closure could occur by regulated actin polymerization, reorganized actin gel networks, altered membrane curvature or a localized contractile activity. Although myosins localize to phagosomes (Allen and Aderem, 1995; Stendahl et al., 1980) and evidently participate in the process (Ostap and Pollard, 1996), their contribution to phagocytosis remains undefined. Evans et al. (1993), measured forces generated by leukocytes during phagocytosis of yeast particles, and identified a contraction of the entire cell that followed pseudopod extension over a particle. This indicated that some contractile activity accompanies phagocytosis. Here we describe a localized

contraction that constricts the margins of forming phagosomes and allows extended pseudopodia to close into intracellular phagosomes. To begin to look for a motor responsible for this process, we probed bone marrow-derived macrophages for class I, II, V, VI, VII and IX myosins. Myosin-IC, myosin IIa, myosin Va and myosin IXb were present in these cells and all localized to phagosomes. The localization and timing of appearance of myosin IC suggest that it plays a role in the constriction.

MATERIALS AND METHODS

Cells

Bone marrow-derived macrophages were obtained from C3H-HeJ mice and cultured for 6 days as previously described (Swanson, 1989). Erythrocytes were labeled with biotin and fluorescein-streptavidin, and opsonized with IgG. 5×10^8 washed, sheep erythrocytes (ICN Biomedical, Costa Mesa, CA) were labeled with 1 mM NHS-x-biotin (Calbiochem, La Jolla, CA; prepared from a 50 mM stock in dimethyl formamide) in 150 mM carbonate-bicarbonate buffer, pH 9.5. After incubation for 20 minutes at 4°C, cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.4), opsonized for 60 minutes with rabbit anti-sheep erythrocyte IgG (ICN Biomedical), and washed again with PBS. To label erythrocyte surfaces, fluorescein-streptavidin (6 μg/ml final concentration) was added to erythrocytes when they were mixed with the macrophages; adding at this time reduced particle clumping. For some studies, as indicated, fluorescein-streptavidin was added to macrophages after first allowing 30 minutes for phagocytosis. Macrophages were chilled after phagocytosis and incubated with fluorescein-streptavidin (20 minutes at 4°C), then fixed. In other studies, erythrocytes were opsonized with IgG without additional labeling with NHS-x-biotin or fluorescein-streptavidin.

Phagocytosis

Macrophages were incubated in Ringer's buffer (RB: 155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM Hepes, pH 7.2, 10 mM glucose) for 15 minutes, then in RB with or without inhibitors (100 nM wortmannin, 50 μM LY294002 or 15 mM BDM) for 15 minutes, all at 4°C. Opsonized erythrocytes were added (5×10^6/well), together with fluorescein streptavidin, then dishes were centrifuged at 500 rpm for 1 minute. Warm solutions of RB ± inhibitors were added to initiate phagocytosis. Dishes were incubated for 20 or 30 minutes at 37°C, then fixative was added (3.7% formaldehyde in 6.8% sucrose, 40 mM Hepes, pH 7.3; 30 minutes at 37°C). To measure the reversibility of inhibition, the first incubation for phagocytosis was followed by 40 minutes in fresh solutions of RB ± inhibitors, then cells were fixed. Fixed cells were washed with PBS, then incubated with 10 units/ml rhodamine-phalloidin (Molecular Probes, Eugene, OR) in PBS plus 0.25% Triton X-100 for 30 minutes at room temperature. Cells were washed again with PBS and prepared for observation by fluorescence microscopy.

Western blotting

Macrophage lysates were analyzed for the presence of myosins by western blotting. Cells in Petri dishes were rinsed with PBS, lysed in 1 ml 5% trichloroacetic acid (4°C), and scraped from the dishes with a rubber policeman. The cell suspension was homogenized in a Dounce homogenizer. The protein precipitate was pelleted by centrifugation, then resuspended in 1 ml SDS-PAGE sample buffer containing protease inhibitors (complete inhibitor tablets, Boehringer-Mannheim, 1 tablet/7 ml water). Immediately after resolubilization of proteins, 40 μg protein per sample was separated by 5% SDS-PAGE, transferred to PVDF membranes, then processed with affinity-purified rabbit antibodies raised against myosin IC (J. Skowron, W. Bement and M. Mooseker, unpublished), human platelet myosin II (Biomedical Technologies, Stoughton, MA), chick brain myosin V (Espreafico et al., 1992), porcine myosin VI (Hasson and Mooseker, 1994), human myosin VIIa (Hasson et al., 1995), human myosin IXb (Wirth et al., 1996) and horseradish peroxidase-labeled secondary antibodies.

Immunofluorescence

Macrophages on coverslips were allowed to phagocytose sheep erythrocytes opsonized with mouse anti-sheep erythrocyte IgG (Harlan Bioproducts, Indianapolis, IN). Cells were fixed 15 minutes at 37°C in buffer A (30 mM Hepes, pH 7.4, 10 mM EGTA, 0.5 mM EDTA, 5 mM $MgSO_4$, 33 mM potassium acetate, 5% polyethylene glycol 400, 0.02% sodium azide) containing 4% paraformaldehyde; then fixed and permeabilized for 15 minutes in buffer A containing 4% paraformaldehyde and 1% Triton X-100. Cells were then washed 3×5 minutes in buffer B (buffer A plus 2% heat-inactivated goat serum), then incubated with anti-myosin antibodies in buffer B, 60 minutes at 37°C. Cells were washed again and incubated for 60 minutes at 37°C in buffer B containing 10 units/ml Texas Red-phalloidin, plus Oregon Green-labeled goat anti-rabbit IgG (1:5000) and aminomethylcoumarin-labeled goat anti-mouse IgG (dil 1:500) (all from Molecular Probes, Eugene, OR). Preparations were washed again with buffer B, fixed again with buffer A plus 4% paraformaldehyde, washed again with buffer B, and finally mounted in glycerol containing 1 mg/ml phenylenediamine. Cells were observed using a Zeiss Axiophot 2 fluorescence microscope.

Fluorescent analog cytochemistry

Macrophages were labeled by scrape-loading (McNeil et al., 1984) with fluorescein dextran (f-dextran, average MW 10,000, Molecular Probes, Eugene OR) and rhodamine-labeled actin (a gift from Dr Geneva Omann, University of Michigan). To prevent actin polymerization during the loading procedure, cells were scrape-loaded in solutions with cytochalasin B (Sigma Chemical Co., St Louis, MO). 10^6 cells on a Petri dish were rinsed with PBS at 37°C, then 40 μl of 500 μg/ml f-dextran, 50 μg/ml rhodamine-actin and 5 μM cytochalasin B in PBS were added and cells were scraped off the dish with a rubber policeman. The scraped cells were quickly combined with 2 ml DM10F and plated onto 25 mm diameter, circular coverslips. After 10 minutes, medium was replaced with DM10F to remove extracellular fluorophores, and cells were incubated for another hour (in 5% CO_2, 37°C) before observation by fluorescence microscopy.

Imaging

For video microscopy, macrophages were plated onto 25 mm circular coverslips, which were assembled into Leiden chambers on a heated stage (Medical Systems Corp.) of a Zeiss IM35 inverted microscope. Cells in RB were viewed by phase-constrast optics using a 100× objective lens. Images were collected via a nuvicon video camera, digitized using Metamorph software (Universal Imaging Co., West Chester, PA) and stored on optical discs.

For fluorescence microscopy of living cells, scrape-loaded macrophages on coverslips were prepared as described for video microscopy. Cells were observed using a 60× planapo objective lens. Metamorph software controlled a shutter and filter wheel that obtained pairs of fluorescein and rhodamine fluorescence signals, collected every 10 seconds using a digital cooled CCD camera (Quantix, Photometrics). Images were processed to obtain ratio images, dividing the rh-actin fluorescence by the f-dextran fluorescence.

For fluorescence confocal microscopy, macrophages fixed and stained with fluorescein streptavidin and rhodamine phalloidin were observed using a Zeiss LSM 410, as described by Araki et al. (1996).

RESULTS

Movements of actin during phagocytosis

During Fc receptor-mediated phagocytosis, actin-rich pseudopodia extend around a particle and enclose it. We reasoned that a contractile activity associated with phagosome closure should increase the concentration of actin at the distal margin of the closing phagosome. Actin concentrations were monitored directly by observing phagocytosis of IgG-opsonized sheep erythrocytes in macrophages scrape-loaded with rh-actin and f-dextran (Fig. 1). The f-dextran served as an indicator of the distribution of cytoplasm; it was accessible to the nucleus as well as the phagocytic cups, and reported the movements of cytoplasm during phagocytosis. Because rh-actin was larger than f-dextran, it was excluded from the nucleus. Ratiometric images were obtained by dividing the pixels for rh-actin by the corresponding pixels for f-dextran; and pseudocolor applied to the ratiometric image showed high concentrations of rh-actin at the cell margin and forming phagosomes as yellow and orange, and the low concentrations in the nucleus, relative to f-dextran, as purple. The time series indicated that actin was concentrated at the distal margins of closing phagosomes (Fig. 1, at 1.5, 3.5 and 4 minutes). In one time series, two labeled macrophages engaged a cluster of erythrocytes (Fig. 2). The cells appeared to bite individual erythrocytes from the cluster, and showed an increase in actin concentration coincident with phagosome closure (Fig. 2, at 2 and 3 minutes). Similar increases in actin concentration were observed in four separate observations; in no instance did phagocytosis occur without an increase in actin concentration. This increase in actin concentration indicated that closure required more than the continued advance of the pseudopod around the particle, and that perhaps a contractile activity was mediating the constriction that closed the phagosome.

The constriction of shared erythrocytes

Contractile activities could be observed directly when two macrophages attempted to phagocytose a single erythrocyte. Phase-contrast video microscopy showed that erythrocytes landing directly onto individual macrophages were quickly ingested by phagocytosis, as expected, but those landing between two macrophages were engaged by both cells. In such instances, pseudopodia extended around the erythrocyte from both macrophages until they met each other. Both phagocytes then constricted the middle of the erythrocyte, and pulled it into two phagosomes. These phagosomes remained connected (Fig. 3).

The geometry of the shared erythrocytes was visualized by fluorescence microscopy. Macrophages were fed IgG-opsonized erythrocytes labeled on their surfaces with fluorescein-streptavidin, then were fixed and observed by fluorescence microscopy. Although most erythrocytes were completely intracellular, some were clearly shared by two macrophages. Typically, the shared erythrocytes contained two bulbous ends, each enclosed in a macrophage vacuole and connected to the other by a long string of fluorescent membrane (Fig. 4a,b). Pseudopodia from each macrophage extended along the string, giving the appearance that nearly all of the erythrocyte was enclosed by one macrophage or the other. We expected that if the erythrocyte remained intact, and bridged phagosomes of two macrophages, then some portion

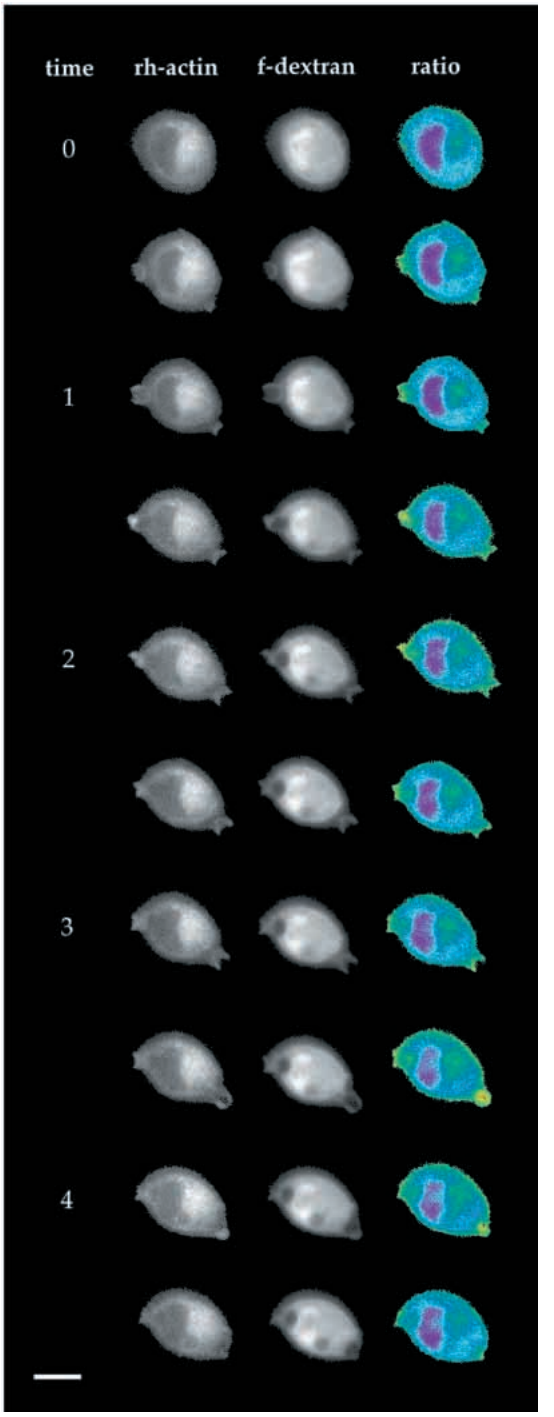

Fig. 1. Fluorescent actin inside macrophages. Macrophages were scrape-loaded with f-dextran and rh-actin, and fed IgG-opsonized erythrocytes. Digital images of the two fluorophores are shown as a time series from top to bottom. The rightmost column shows the ratio images of rh-actin divided by f-dextran, with pseudocolor applied to show high ratio values as yellow and green, intermediate ratios as blue, and low ratios as purple. Rh-actin was excluded from the nucleus and f-dextran was not; hence the ratio values in the nuclear region were low. Frames are separated by 30-second intervals. Phagocytic cups form first on the left side of the cell, then at the lower right side of the cell. Actin concentrations were highest at the distal margins of closing phagosomes (at 1.5 minutes, 3.5 minutes and 4 minutes). Bar, 10 μm.

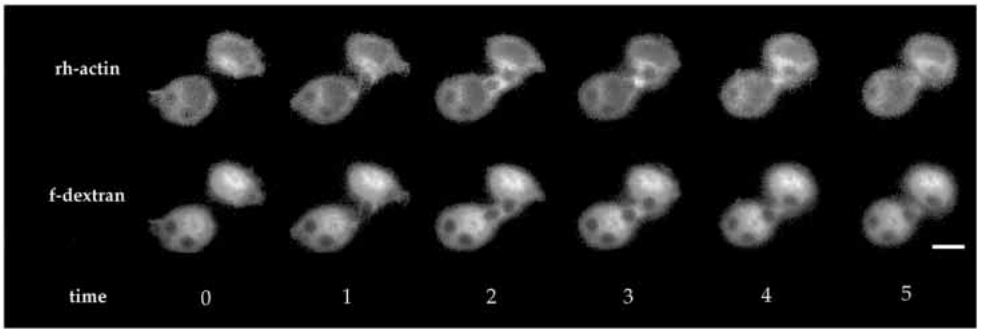

Fig. 2. Fluorescent actin is concentrated at the distal margin of the phagosome as phagocytosis is completed. Two macrophages scrape-loaded with rh-actin and f-dextran are shown during phagocytosis of IgG-opsonized erythrocytes. Frames are separated by 1-minute intervals. At time 0, the lower cell had already ingested two erythrocytes. During the next 5 minutes, the two macrophages each ingested another erythrocyte from a cluster of erythrocytes between them. As phagosomes closed (times 2 and 3 minutes), rh-actin was concentrated at the distal margins of the phagosomes. Bar, 10 μm.

of the erythrocyte membrane should remain extracellular. To identify extracellular portions of shared erythrocytes, fluorescent label was added after phagocytosis. Macrophages were first fed opsonized, biotinylated erythrocytes, then fluorescein-streptavidin was added to label exposed erythrocyte membrane. Most erythrocytes were intracellular, inaccessible to the probe and therefore dark; but the shared erythrocytes were labeled along a short stretch of the string connecting the two macrophages. Even when the phagosomes were separated by many μm, only 1-2 μm of erythrocyte membrane was accessible to fluorescent probe (Fig. 4c,d), indicating that macrophage pseudopodia extended along the length of the elongated erythrocytes.

Inhibition of phagosome constriction

Several studies have indicated a role for PI3-kinase in Fc receptor-mediated phagocytosis. Ligation of Fc receptors increased PI3-kinase activity and led to the generation of phosphatidylinositol 3,4,5-trisphosphate (Ninomiya et al., 1994). Moreover, the PI3-kinase inhibitors wortmannin and LY294002 inhibited macrophage phagocytosis late in the process (Araki et al., 1996). To examine the role of PI3-kinase in the constriction of shared erythrocytes, macrophages were incubated with the PI3-kinase inhibitors wortmannin or LY294002 before and during phagocytosis of fluorescent erythrocytes. Under such conditions, constricted erythrocytes were absent. Instead, many erythrocytes were wedged between two macrophages, without any evidence of constriction (Fig. 4e,f). Fluorescent labeling of erythrocyte surfaces after phagocytosis showed two non-fluorescent ends of the erythrocyte and an equatorial band of exposed erythrocyte membrane, indicating that pseudopodia extended from each neighboring macrophage along the particle surface (Fig. 4g,h). This was consistent with earlier observations that wortmannin did not inhibit pseudopod extension along opsonized erythrocyte surfaces (Araki et al., 1996). Thus, inhibition by wortmannin and LY294002 indicated a role for PI3-kinase in the purse-string-like constriction at the outer margin of the pseudopod.

To analyze the contribution of F-actin to this string formation in shared erythrocytes, F-actin of macrophages fixed during phagocytosis was labeled with rhodamine-phalloidin. In intermediate stages of the constriction process, visualized by confocal microscopy, the outer margins of the pseudopodia contained dense accumulations of F-actin (Fig. 5a,b). F-actin was also present in the extensions of macrophage surface that surrounded the connecting string of erythrocyte membrane

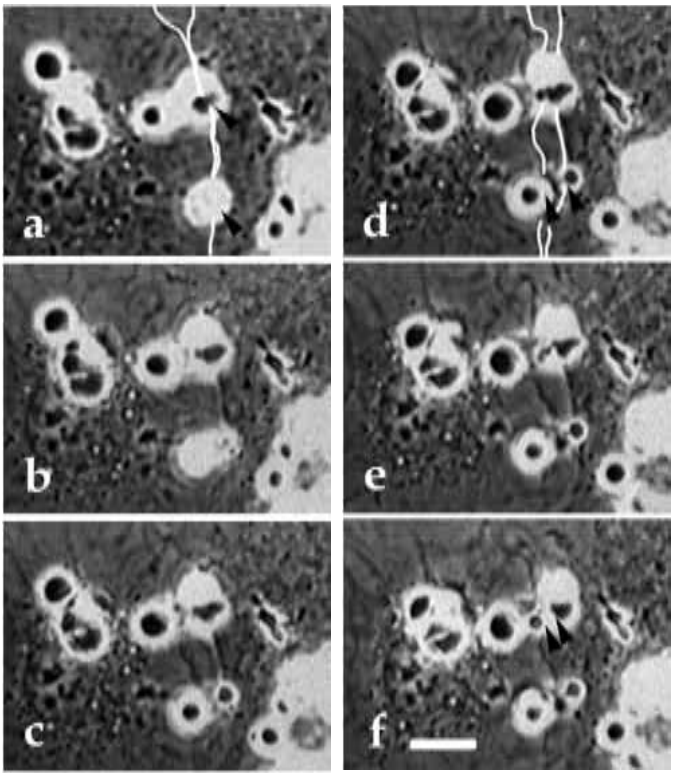

Fig. 3. Video sequence showing the constriction of opsonized erythrocytes shared by two macrophages. Two phase-bright erythrocytes landing at the border between adjacent macrophages are indicated by arrowheads in (a). Boundaries of adjacent macrophages are indicated by white lines in (a) and (d). Constriction by the macrophages followed first for the lower erythrocyte (b and c), then for the upper erythrocyte (b-f). Arrowheads indicate the distances between the phagosomes that share an erythrocyte. Frames are separated by 1 minute intervals. Bar, 10 μm.

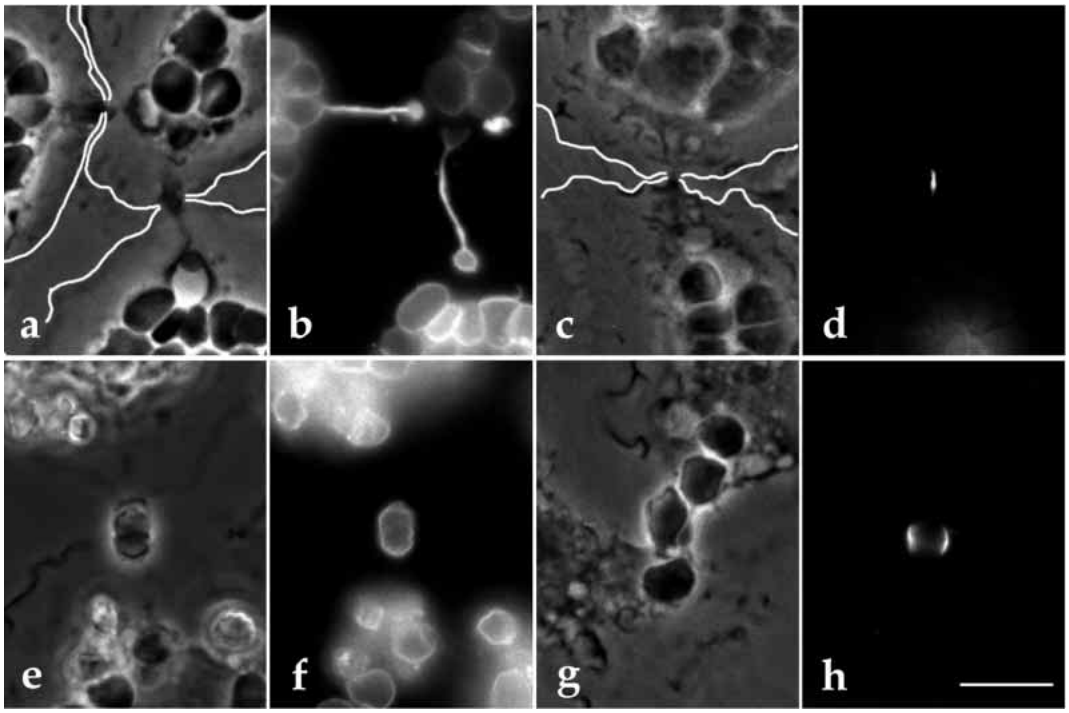

Fig. 4. Phase contrast (a,c,e,g) and fluorescence (b,d,f,h) images of shared erythrocytes, showing the threads produced by the contractile activity at the distal margins of macrophages. Sheep erythrocytes were surface-labeled with biotin, then opsonized with IgG. Fluorescein-streptavidin was added either during phagocytosis, to label the entire erythrocyte surface (a,b,e,f), or after phagocytosis was completed, to label only exposed erythrocyte membrane (c,d,g,h). Macrophages were incubated in buffer alone (a-d) or with 100 nM wortmannin (e-h), which inhibited the contractile activity. White lines in a and c indicate macrophage borders; profiles are interupted at regions containing shared erythrocytes. Bar, 10 μm.

(Fig. 5c). When phagosome constriction was blocked by wortmannin, the phagocytic cups surrounding erythrocytes contained a uniform distribution of F-actin (Fig. 5d), indicating that the PI3-kinase inhibitor had not prevented actin assembly or recruitment, but had prevented its concentration at the margins of contractile pseudopodia.

Myosins I and II have been localized to phagosomes (Allen and Aderem, 1995; Stendahl et al., 1980), and may contribute to the contractile activity described here. To examine the role of myosin, macrophages were incubated in 2,3-butanedione monoxime (BDM), an uncompetitive inhibitor of myosin II and perhaps other myosins (Herrmann et al., 1992; Cramer and Mitchison, 1995; McKillop et al., 1994). BDM inhibited phagocytosis and the constriction of shared erythrocytes. Like wortmannin and LY294002, BDM did not prevent the formation of actin-rich phagocytic cups around the erythrocytes: rhodamine-phalloidin staining of macrophages in BDM produced images of shared erythrocytes essentially identical to Fig. 3d. Thus, in wortmannin-, LY294002- and BDM-treated cells, string formation was completely inhibited; instead, many erythrocytes bound to macrophage surfaces were nested in cup-shaped phagosomes that labeled brightly with rhodamine-phalloidin.

An assay was devised to quantify the contractile activity, counting the number of fluorescent erythrocyte membrane strings per 100 macrophages on a coverslip. Dose-response curves for string formation in wortmannin and LY294002 were similar to those published for inhibition of PI3-kinase (Vlahos et al., 1994; Yano et al., 1995), and for the inhibition of phagocytosis in macrophages (Araki et al., 1996), further indicating that PI3-kinase was involved in the contractile activity (data not shown). In control preparations, the number of strings measured after 60 minutes was less than after 20 minutes, because the macrophages were apparently capable of degrading or breaking the strings and completing phagocytosis (Fig. 6). Complete inhibition of string formation was observed for wortmannin, LY294002 and BDM during 20- and 60-minute incubations, indicating that the inhibitors were not simply slowing the contractile activity. Inhibition of phagocytosis and thread formation by LY294002 and BDM were reversible, indicating that the drugs were not exerting some general cytotoxicity. The inhibition by wortmannin was not reversible.

Localization of myosins in phagosomes

Western staining of macrophage lysates showed immunoreactivity for myosins IC, II, V and IXb (Fig. 7), but not for myosins VI or VII (not shown). Immunofluorescent staining of macrophages engaged in phagocytosis yielded positive labeling of phagosomes with myosins IC, II, V and IXb (Fig. 8). Particles were recognized by phase-contrast optics and by fluorescent labeling of particle surfaces with aminomethylcoumarin-labeled secondary antibodies (not shown); phagocytic cups were recognizable by labeling F-actin with Texas Red-phalloidin. Although all four myosins could be localized in phagocytic cups, no one class of myosin labeled

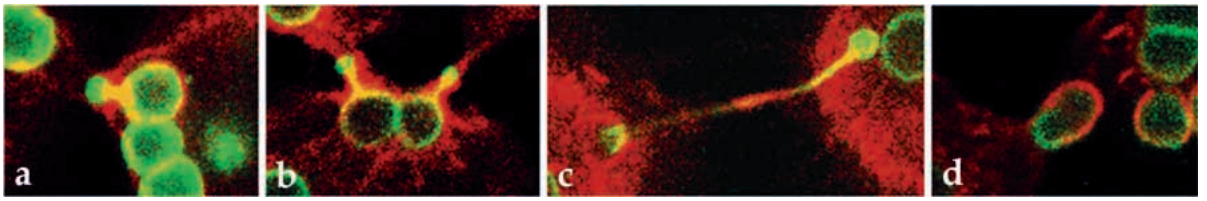

Fig. 5. Fluorescence confocal microscopy of macrophages constricting erythrocytes. Green shows the fluorescent profile of the fluorescein-streptavidin-labeled erythrocyte membrane, red shows the distribution of F-actin, visualized with rhodamine-phalloidin, yellow shows regions with both signals. (a-c) Control preparations. Note rhodamine-phalloidin staining that extends along the erythrocyte thread in (c). (d) Macrophages incubated with 100 nM wortmannin, with an unconstricted erythrocyte shared by both cells.

all phagosomes. Rather, it appeared that myosins II and IXb labeled ruffles and early phagocytic cups, myosin IC labeled a later stage of phagosome closure, and myosin V labeled fully internalized phagosomes.

Myosin IC labeling of phagosomes indicated a role for that mechanoenzyme in phagosome closure. It formed a transient dense label around phagosomes coincident with closure. The label was internal to the phagosomal F-actin, such that the particle was closely wrapped with myosin Ic, with F-actin forming an outer layer (Fig. 8a,b). Erythrocytes that were shared between two macrophages contained myosin Ic around the connecting string (Fig. 9), whereas myosins II, V and IXb did not label those structures. This suggests that myosin IC participated in the contractions that formed strings and, by inference, in the contractions that closed phagosomes.

DISCUSSION

Contractile activities that close phagosomes

The experiments described here distinguish two component activities for ingestion by phagocytosis. The first is the extension of pseudopodia around a particle and the second is a purse-string-like constriction of the pseudopod margin (Fig. 10). Pseudopod extension probably occurs by localized polymerization of actin beneath the plasma membrane, as is the case for pseudopod advance during cell crawling (Mitchison and Cramer, 1996). The constrictions close the circular margin of the phagosome to a narrower aperture which, during normal phagocytosis, closes by membrane fusion into an intracellular vacuole or, when an erythrocyte is shared with another macrophage, remains plugged by a string of erythrocyte membrane. The contractile activity could be restricted to the margin of the phagosome, or it could occur throughout the phagocytic cup and culminate in the final closure. Consistent with this latter model, video microscopic studies of phagocytosis indicate that deformable particles, such as erythrocytes, are squeezed by phagosomes during the engulfment process (J. A. S., data not shown).

The contractile activity that closes phagosomes may be related to the contractions identified by Evans et al. (1993), who measured forces generated during phagocytosis in leukocytes. Although the contractions they described were not localized to the phagosomes, their timing was comparable to that observed for constriction of shared erythrocytes.

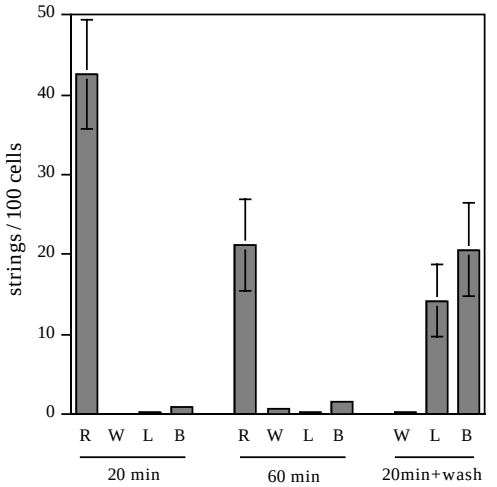

Fig. 6. Reversible inhibition of thread formation by BDM or LY294002. Macrophages were fed opsonized, fluorescent erythrocytes in RB containing 100 nM wortmannin (W), 50 μM LY294002 (L), 15 mM BDM (B) or no additions (R). They were fixed after 20 minutes, 60 minutes, or 20 plus a 40 minute incubation in RB (wash). For each coverslip, at least 100 cells were scored for the presence of fluorescent strings. Each bar represents a total of five preparations measured during three separate experiments. Values are mean ± s.e.m.

Mechanism

This contractility most likely involves actin and myosin. Actin is concentrated at the distal margins of closing phagosomes, and at the points of constriction of shared erythrocytes. Moreover, the myosin inhibitor BDM inhibits the constriction of shared phagosomes, consistent with the involvement of one

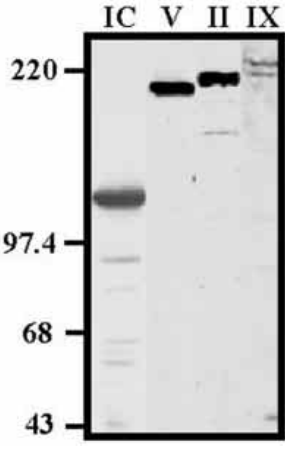

Fig. 7. Western blot of macrophage lysates, stained with affinity-purified rabbit anti-myosins IC, V, II and IXb. Note that each antibody recognizes a protein band of the correct molecular mass for each of these myosins: 127 kDa for myosin-IC, 190 kDa for myosin-V, 200 kDa for myosin-II, and a doublet at 230 kDa for myosin-IXb.

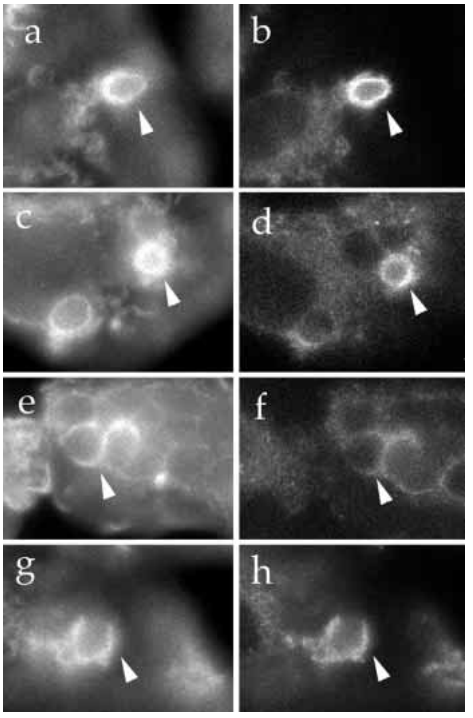

Fig. 8. Immunofluorescence showing phagosomes labeled with anti-myosin antibodies. Macrophages were fixed during phagocytosis of sheep erythrocytes opsonized with mouse anti-sheep erythrocyte IgG. Cells were stained with Texas Red-phalloidin (a,c,e,g) and rabbit anti-myosins IC (b), II (d), V (f) and IXb (h), followed by Oregon Green-labeled secondary antibodies. Arrowheads indicate phagosomes, which were recognized by phase contrast microscopy and by staining particles with AMCA-labeled goat anti-mouse IgG (not shown). All four myosins labeled some phagosomes. Anti-myosin IC sometimes produced a prominent labeling of phagosomes.

or more myosins in this process; although it is important to note that there is no reliable information on how this drug impacts myosins other than myosins-II. In this study, we probed bone marrow-derived macrophages for class I, II, V, VI, VII and IX myosins and detected class I, II, V and IX myosins present (Fig. 7).

These myosins have previously been implicated in signal transduction and membrane trafficking. Myosin-V has been implicated in membrane trafficking events (reviewed by Mermall et al., 1998). Recently, mutations in human myosin-Va have been identified as the basis for Griscelli disease (Pastural et al., 1997). Griscelli disease patients have partial albinism, neurological defects (similar to *dilute* mice), and immunodeficiencies, principally in delayed type hypersensitivity responses (Griscelli et al., 1978; Hurvitz et al., 1993; Klein et al., 1994). Human myosin-IXb is most highly expressed in peripheral blood leukocytes. Its protein expression is upregulated during differentiation of HL60 cells into macrophage-like cells and it redistributes from the actin-based cortex to the cytoplasm in the differentiated cells (Wirth et al., 1996). Class IX myosins have a GAP domain in their tails for the small GTPase rho (Post et al., 1998; Muller et al., 1997); Rho regulates the organization of the actin cytoskeleton in many kinds of cells, including leukocytes (reviewed by Dharmawardhane and Bokoch, 1997). Thus, this myosin has the potential to regulate its transducing element. Presently, roles for myosins II, V and IXb in phagosomes are simply suggested by their presence in phagosomes.

Human myosin-IC, a homolog of the ‘long-tailed’ amoeboid myosins-I, has a tail containing a putative membrane-binding domain (TH1), a putative ATP-independent actin-binding domain (TH2), and an SH3 domain (Bement et al., 1994). SH3 domains allow protein-protein interactions (Feller et al., 1994; Feng et al., 1994) and recently, an *Acanthamoeba* protein was

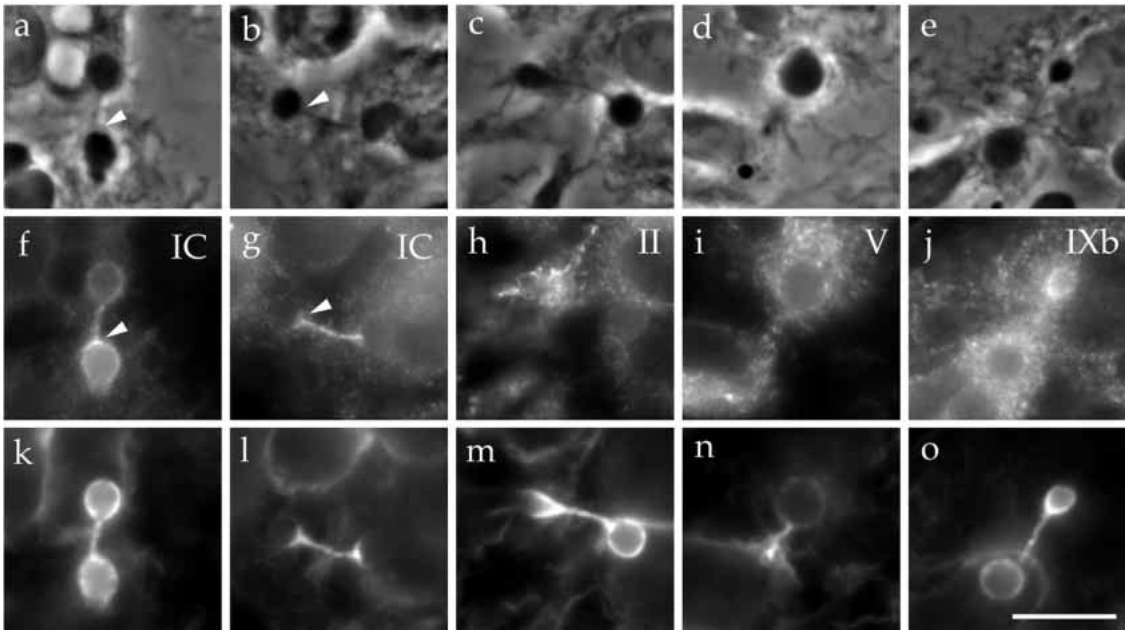

Fig. 9. Immunofluorescence showing myosins in shared phagosomes. Panels show phase-contrast images of shared erythrocytes (a-e), Oregon Green-labeled secondary antibody labeling of anti-myosin primary antibodies IC (f,g), II (h), V (I) and IXb (j); and Texas Red-phalloidin labeling of F-actin (k-o). Only anti-myosin IC labels connecting strings of erythrocyte membrane. Arrowheads indicate concentrations of myosin IC at points of constriction. Bar, 10 μm.

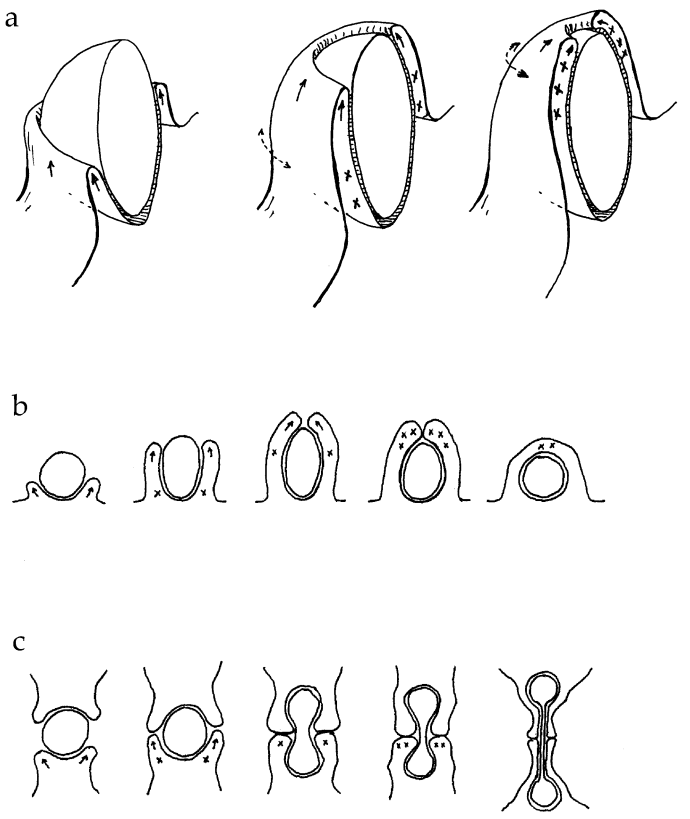

Fig. 10. A model for phagocytosis in macrophages. Ingestion entails two component activities, the extension of pseudopodia along the particle (arrows) and closure of the phagosome to form an intracellular vacuole (x). (a) The pseudopod advances by localized actin polymerization, and constricts by myosin-mediated contractions that follow. As the particle is completely enveloped, the contractile activity closes the phagosome. (b) Contractile activities that move along the forming phagosome should constrict or squeeze deformable particles. (c) When two macrophages engage a single erythrocyte, neither can enclose all of it, so the contractile activity constricts the erythrocyte.

found to associate with the SH3 domain of a class I myosin (Xu et al., 1995, 1997). Possibly myosin-Ic associates with membranes or a protein complex in the phagosome through its tail domain. Interestingly, the rat ortholog of human myosin-Ic (rat myr3) has been localized to elongate structures in regions of cell-cell contact in NRK cells (Stoffler et al., 1995). These structures are rich in filamentous actin and can be induced to form with the lectin Con A, suggesting a regulation by cell surface receptors (Stoffler et al., 1995). These structures in the rat kidney cells may be homologous to those seen here in the shared phagosomes.

Although the class of myosin that mediates closure remains undetermined, these studies indicate a significant role for myosin IC. Myosins IC, II, V and IXb localized to macrophage phagosomes, apparently at different times during the phagocytic process: myosins II and IXb were abundant in ruffles and unclosed phagosomes (M. Diakonova and J. Swanson, unpublished). Only myosin IC labeled the strings connecting shared erythrocytes, which indicates its presence during the constriction process. Previous studies of macrophages have shown phagosomal association of myosin I (Allen and Aderem, 1995) and myosin II (Stendahl et al., 1980).

Inhibition by wortmannin and LY294002 indicated that the contractions require PI3-kinase activity. Dose-response curves for string formation, using wortmannin and LY294002, showed inhibitions comparable to their effects on PI3-kinase (Vlahos et al., 1994; Yano et al., 1993) and on phagocytosis (Araki et al., 1996). Although wortmannin can inhibit myosin light chain kinase as well, it does so at higher concentrations than those used here (Yano et al., 1995). Similarly, 50 μM LY294002 does not significantly inhibit myosin light chain kinase (Yano et al., 1995). Therefore, although inhibition by BDM suggests a role for myosin in the phagocytic contractions, the effects of wortmannin and LY294002 are most likely not via direct inhibition of myosin light chain kinase but rather by their effects on PI3-kinase.

Regulation

To engulf a particle, pseudopod extension and contraction must be coordinated temporally and spatially. Pseudopod extension must be oriented such that the particle is enveloped and not simply pushed away. Contractions must be delayed long enough to allow pseudopodia to extend around the particle. To generate a purse-string-like contraction, contractions must also be oriented parallel to the pseudopod margin. Similar coordination is required for the formation of macropinosomes, which form from actin-rich cups that close into intracellular vesicles (Swanson and Watts, 1995). We propose that the signals that stimulate pseudopod extension also activate a slower or delayed contractile activity, producing a wave of constriction that follows pseudopod extension (Fig. 10). When that contraction reaches the end of the pseudopod, it constricts the distal end of the phagocytic cup to a narrow opening, that then closes by membrane fusion, creating an intracellular organelle. When two macrophages engage a single erythrocyte, the process is interrupted at the point of phagosome closure; the erythrocyte membrane that bridges the two phagosomes prevents membrane fusion and phagosome closure. The fact that myosin IC remains caught at this point of interuption indicates a role for that mechanoenzyme in the closure activity.

The molecules that regulate these two activities are not yet known, but they may be identifiable by their abilities to affect phagocytic cup formation and phagosome closure. Inhibition of molecules that regulate actin polymerization should prevent cup formation, whereas those that affect closure should allow cups to form but not close. PI3-kinase apparently falls into this second category, as does the tyrosine kinase syk (Cox et al., 1996). Fc receptor-mediated phagocytosis requires syk, which affects the activity of PI3-kinase. Macrophages from syk-deficient mice are capable of forming phagocytic cups, but fail to complete phagocytosis (Crowley et al., 1997). This indicates a role for syk in phagosome closure. In addition, the actin regulatory GTPases Rac, Rho and CDC42 are evidently present in macrophages (Allen et al., 1997), and may regulate the coordinated extensions and contractions of phagocytosis, particularly in conjunction with the myosin-IXb rho GAP. Phosphorylated phosphoinositides generated in the phagosome by the action of PI3-kinase may position the contractile apparatus, either directly by binding a myosin, or indirectly by

activating Rho (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996), or by organizing a complex of integral membrane proteins that can assemble a contractile apparatus. Alternatively, activation of the regulatory GTPases could activate p21-activated kinase-1 (PAK-1), which has been shown in other systems to localize to pinocytic vesicles (Dharmawardhane et al., 1997) and to activate myosin I (Sells and Chernoff, 1997).

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