EVIDENCE FOR CONTRACTILE PROTEIN TRANSLOCATION IN MACROPHAGE SPREADING, PHAGOCYTOSIS, AND PHAGOLYSOSOME FORMATION

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ABSTRACT

Macrophage pseudopodia that surround objects during phagocytosis contain a meshwork of actin filaments and exclude organelles. Between these pseudopodia at the base of developing phagosomes, the organelle exclusion ceases, and lysosomes enter the cell periphery to fuse with the phagosomes. Macrophages also extend hyaline pseudopodia on the surface of nylon wool fibers and secrete lysosomal enzymes into the extracellular medium instead of into phagosomes. To analyze biochemically these concurrent alterations in cytoplasmic architecture, we allowed rabbit lung macrophages to spread on nylon wool fibers and then subjected the adherent cells to shear. This procedure caused the selective release of β-glucuronidase into the extracellular medium and yielded two fractions, cell bodies and isolated pseudopod blebs resembling podosomes, which are plasmalemma-bounded sacs of cortical cytoplasm. Cytoplasmic extracts of the cell bodies eluted from nylon fibers contained two-thirds less actin-binding protein and myosin, and ~20% less actin than extracts of cells sheared in the absence of nylon wool fibers. Nearly all of the actin and two-thirds of the other two proteins were accounted for in podosomes. The alterations in protein composition correlated with assays of myosin-associated EDTA-activated adenosine triphosphatase activity, and with a diminution in the capacity of extracts of nylon wool fiber-treated cell bodies to gel, a property dependent on the interaction between actin-binding protein and F-actin. However, the capacity of the remaining actin in cell bodies to polymerize did not change.

We propose that actin-binding protein and myosin are concentrated in the cell cortex and particularly in pseudopodia where prominent gelation and syneresis of actin occur. Actin in the regions from which actin-binding protein and myosin are displaced disaggregates without depolymerizing, permitting lysosomes to gain access to the plasmalemma. Translocation of contractile proteins could therefore account for the concomitant differences in organelle exclusion that characterize phagocytosis.

KEY WORDS macrophages · contractile proteins · spreading · secretion · phagocytosis · cytoplasmic gels

A cortical margin of hyaline cytoplasm separates the lysosomes of mammalian phagocytic leukocytes from their plasma membranes. During phagocytosis, the cells surround particulate objects with pleats of plasma membrane containing this hyaline ectoplasm. These folds ultimately fuse, thereby encasing the objects within phagocytic vacuoles, and the lysosomes merge with the surfaces of these developing vacuoles (6, 12, 24, 25, 30). Evidence is accumulating that an actin filament gel constitutes the peripheral hyaline ectoplasm and regulates both the movement of pseudopodia and the traffic of lysosomes that occur during phagocytosis: (*a*) cytoplasmic extracts of diverse cells, including phagocytic leukocytes, form gels of actin filaments, and the ultrastructure of these gels resembles that of cortical cytoplasm of intact cells (3, 14, 19, 23, 26); some of these gels synerese, indicating a capacity to undergo movement (3, 19, 23); (*b*) cytochalasin B, a substance which dissolves cytoplasmic actin gels (11, 28), inhibits phagocytosis (1, 11) yet enhances the extracellular secretion of lysosomal enzymes by phagocytic cells (13, 29, 31); and (*c*) human polymorphonuclear leukocytes that were defective in phagocytosis, yet secreted excessive amounts of lysosomal enzymes into phagocytic vacuoles and into the extracellular medium, contained actin that was markedly defective in its capacity to polymerize (2). Taken together, these facts suggest that actin gelation is necessary for the creation of pseudopodia required for phagocytic vacuole formation, whereas lysosomal secretion depends on a local dissolution of the gel which permits the lysosomes to gain access to the plasma membrane. Because the events occur simultaneously, different influences on the state of actin must exist in different regions of the developing phagocytic vacuole; factors promoting actin gelation predominate in the advancing pseudopodia, and gel dissolution occurs at the base of the vacuole where lysosomes fuse.

Two factors which we believe control the state of actin in phagocytic leukocytes are the high molecular weight proteins, myosin and actin-binding protein, both of which can cross-link actin filaments (22, 23). Assuming that these (and possibly other molecules) regulate the state of actin, two mechanisms are possible to explain how these accessory proteins might control the actin state during phagocytosis. First, these regulatory proteins could be uniformly distributed around the cell and become "activated"[1] as a consequence of contact of an ingestible particle with the plasma membrane. Activation of these proteins could propagate with the sequential spreading of the pseudopodia around an object. The control proteins residing at the original site of membrane:particle contact might become inactivated, thereby returning actin to the sol state. A second alternative is that the regulatory proteins are in asymmetrical distribution throughout the cell and actually advance with the pseudopodia, abandoning the central region that becomes the site of lysosomal secretion. The location of these proteins therefore determines the presence or absence of an actin gel state. Both mechanisms more or less imply that actin-binding protein and myosin are located in the cell periphery at least near the plasmalemma, but proof of this point is necessary. In addition to deciding between these mechanisms, the question as to whether the depolymerization of actin filaments accompanies the solvation of actin gels requires resolution.

To approach these problems, we have taken advantage of the similarity between cell spreading and phagocytosis (18). Electron micrographs of cells spreading on flat surfaces show filament-rich regions of organelle exclusion to be most prominent in lateral pseudopodia (4, 5, 15, 20). Phagocytes recognize nylon wool fibers and spread pseudopodia on them in an apparent attempt at ingestion, and lysosomes approach the plasmalemma at the nylon wool fiber contact site. Because no true phagocytic vacuole can form on the flat nylon wool fiber surface, the lysosomal enzymes are secreted to the extracellular medium (15). Removal of the spread cells from the nylon wool surface results in the separation of the spread pseudopodia from the cell bodies, and these pseudopodia are isolable as a pure population. We have compared the consistency of extracts from cell bodies of spread and unspread rabbit lung macrophages and correlated the findings with the protein composition of cell bodies and of pseudopodia. A preliminary report of this work has been published (7).

[1] Neither the receptor nor signal mechanisms of this "activation" are known or presupposed by the alternatives being considered and tested.

MATERIALS AND METHODS

Preparation and Incubation of Cells

Lung macrophages were obtained from rabbits by tracheal lavage (17) as previously described (23), washed twice with ice-cold 0.15 M NaCl solution by centrifugation at 270 *g* for 10 min, and suspended in 10 vol (per packed milliliter of cells) of modified Krebs' Ringer phosphate medium (24) containing 10 μg/ml of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and adjusted to pH 7.4 with 1 M NaOH (hereafter identified as Medium). This suspension contained an average of 8 mg of cell protein per milliliter of Medium.

Macrophage suspensions in Medium were warmed to 37°C for 5 min in a 50-ml polypropylene conical centrifuge tube placed in a water bath. 10 ml of the suspension was then added to prewarmed 15-ml plastic syringes, some of which contained 500 mg of nylon wool fibers (Baxter Laboratories, Inc., Fenwal Div., Deerfield, Ill.) previously washed with Medium. The syringes were sealed with a plastic cap over the nozzle. After addition of the cells, the plunger was withdrawn to ~ the 12-ml mark, utilizing a thin metal spatula to create an air vent during sealing. The syringes were placed in a 37°C water bath. After selected intervals, the syringes were removed from the water bath and tapped gently on a table top for 30 s. The plastic caps were then removed and the plungers were compressed. These maneuvers were required to dislodge cells from the nylon wool fibers. The effluent was collected in 50-ml graduated polypropylene conical centrifuge tubes. The plastic cap was removed and 5–10 ml of ice-cold Medium containing 0.2 vol of acid-citrate dextrose anticoagulant (NIH Formula A) was aspirated into the syringe. The cap was replaced and the syringe again tapped for 30 s, followed by elution of the syringe contents, which were pooled with the initial eluate. The entire tapping and elution process is hereafter referred to as "shear."

Fractionation Procedure

The pooled eluates were centrifuged at 2°C for 270 *g* for 10 min, which pelleted whole cells or cells (designated residual cell bodies) sheared on nylon wool which had lost hyaline blebs. The supernatant fluid was aspirated with Pasteur pipettes and centrifuged in 15-ml glass (Corex, Corning Glass Works, Corning, N. Y.) tubes at 11,000 *g* for 10 min at 2°C. The pellet derived from this step contained hyaline blebs when examined in the phase-contrast microscope, and is designated the bleb or podosome (8) fraction. The supernatant fluid of this step was aspirated, its volume recorded, and analyzed as the extracellular "medium" fraction.

Cytoplasmic extracts were prepared from cells and residual cell bodies. The cell pellets were washed once in ice-cold 0.15 M NaCl solution with centrifugation at 270 *g* for 10 min at 2°C, and once with ice-cold distilled water to lyse any erythrocytes present. The final pellet was suspended in 2 vol of ice-cold 0.34 M sucrose homogenizing solution containing 10 mM dithiothreitol, 1 mM adenosine triphosphate (ATP), 2 mM EDTA, 10 mM imidazole HCl, pH 7.0. Cell disruption was monitored by phase-contrast microscopy during homogenization at 0°C in a glass Dounce shearing device (Kontes Co., Vineland, N. J.) with a tight-fitting pestle. The homogenates were centrifuged at 100,000 *g* for 1 h at 2°C. The supernatant fluids obtained are referred to as cytoplasmic extracts.

Measurements of Cytoplasmic Extract Consistency

All extracts were made 0.1 M in KCl by the addition of 0.03 vol of 3 M KCl solution, and then warmed in 15-ml glass (Corex) tubes, without agitation, at room temperature for 60 min. During warming, some extracts gelled. As we and others have reported (14, 19, 23, 26), the gel strength of these extracts can be crudely estimated in various ways. However, these estimates are subjective and poorly reproducible, because the gels are easily disrupted, and movement of the solution during warming weakens the final gel formed. In this study we refer to a 4+ gel as one that permits the tube containing it to be turned upside down without the gel falling out. A ± gel is a viscous solution that gives the impression of having chunks of gelatinous material within it.

We have found an indirect but more satisfactory way of assaying "gel" state to be the measurement of extract protein sedimentable at relatively low *g* forces (9, 11). This approach is based on the premise that the Stoke's radius of a gel lattice is greater than that of individual fibrous strands. In the present studies, samples of warmed extracts were centrifuged for 10 min at 10,000 *g* at 25°C. To obtain a measurement of the amount of protein present in extracts as polymers or ungelled aggregates, samples were also centrifuged at 100,000 *g* for 90 min at 25°C. The protein concentrations of the supernatant fluids were assayed before and after sedimentation.

To determine the viscosity of extracts, extracts warmed to 25°C for 60 min were cooled at ice bath temperature for 30 min and shaken to disrupt any of the gel not dissolved by cooling. Viscometry was then performed with a Cannon-Manning semimicro instrument (Cannon Instrument Co, State College, Pa.) with a charge volume of 0.27 ml and an outflow time for water of 3.6 s. All measurements were done at 4°C. Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (21).

Measurement of Contractile Proteins

Quantitative densitometry of Coomassie Blue stained 5% polyacrylamide gels was utilized as previously described (23) to measure the concentration of actin-binding protein, myosin heavy chain, and actin monomer

after electrophoresis of intact macrophages, podosomes, or cytoplasmic extracts in 0.1% sodium dodecyl sulfate. Myosin was also quantified by assay of its EDTA-activated ATPase activity in 0.6 M KCl as done before (10). In some experiments, rabbit skeletal muscle heavy meromyosin was added to cytoplasmic extracts, and its EDTA-activated ATPase activity was determined.

Other Assays

Lactate dehydrogenase activity was assayed by the method of Wacker et al. (27). The rate of hydrolysis of *p*-nitrophenyl-β-glucuronide at pH 5.0 in the presence of 0.1% Triton X-100 (β-glucuronidase activity) was measured as previously described (21). Protein was determined by the Lowry procedure with bovine serum albumin as a standard (16). Corrections were made for interference by dithiothreitol present in some solutions by means of standard curves. Fluoride-stimulated adenylate cyclase activity was assayed as previously described (8).

RESULTS

Removal of Macrophages from Nylon Wool Fibers by Shear Releases β-Glucuronidase Activity and Peripheral Cytoplasmic Blebs (Podosomes)

Macrophages incubated at 37°C in the presence of Krebs' Ringer phosphate medium containing bovine serum albumin rapidly and firmly adhere to nylon wool fibers. Visualization of the adherence process is possible by focusing up and down with the phase-contrast microscope. Macrophages rapidly extend hyaline pseudopodia over the nylon wool.

To characterize the spreading and secretion mechanism, we analyzed the effect of nylon wool fibers on cell structure. To make this analysis, cells had to be removed from the fibers. Because the removal itself had effects on secretion, these effects had to be quantified. Removal of macrophages from nylon wool fibers required the addition of a divalent cation chelating agent and mechanical shear. These procedures eluted 56–75 of the adherent cell protein. The eluates consisted of apparently intact cells (designated cell bodies) and hyaline blebs resembling "podosomes," structures released by brief sonication of intact macrophages (8).

The specific adenylate cyclase and lactate dehydrogenase activities of blebs eluted after 5 min of attachment to nylon wool fibers were similar to those of podosomes removed from cells by sonication (Table I). The ultrastructure of blebs made by the different procedures was also similar, although blebs from nylon wool fiber-adherent cells appeared to be larger in diameter on average, and fewer cylindrical structures with dense filaments arrays were seen (Fig. 1). Because of their general similarity, both types of blebs are referred to as podosomes. Elution of cells immediately after contact with nylon wool fibers yielded podosomes, but the amount of cell protein recoverable in the podosome fraction was maximal after 5 min of incubation (not shown). Nonsedimentable β-glucuronidase activity rapidly appeared in the intracellular medium, and podosomes eluted from nylon wool fibers (Table II).

Control cells incubated without nylon wool fibers, but exposed to the eluting solution and mechanical shear, released small but finite quantities of soluble β-glucuronidase activity and podosomes (Tables II and III). Neither control nor nylon wool-attached cells released detectable lactate dehydrogenase (LDH) activity into the extracellular medium in response to shear and elution.

In summary, mechanical shearing of warmed macrophages rapidly causes the liberation of podosomes and the release of β-glucuronidase activity, but not of LDH activity.

Macrophage Cell Bodies Removed from Nylon Wool Fiber Are Deficient in Gelation

F-actin is the major component of cytoplasmic gels (3, 14, 19, 23, 26). Because one of the aims of this study was to relate functional changes occurring in the cytoplasm to the state of actin,

TABLE I

Adenylate Cyclase and Lactate Dehydrogenase Activities of Macrophage Podosomes Prepared by Sonication and by Shear of Cells Adherent to Nylon Wool

	Adenylate cyclase	Lactate dehydrogenase
	pmol/min/mg protein	*nmol/min/mg protein*
Podosomes prepared by:		
Sonication	379 ± 67 (8)	800 ± 110 (4)
Nylon wool	450 ± 132 (3)	1,100 ± 300 (3)

Values represent means ± standard errors and the number of determinations in parentheses.

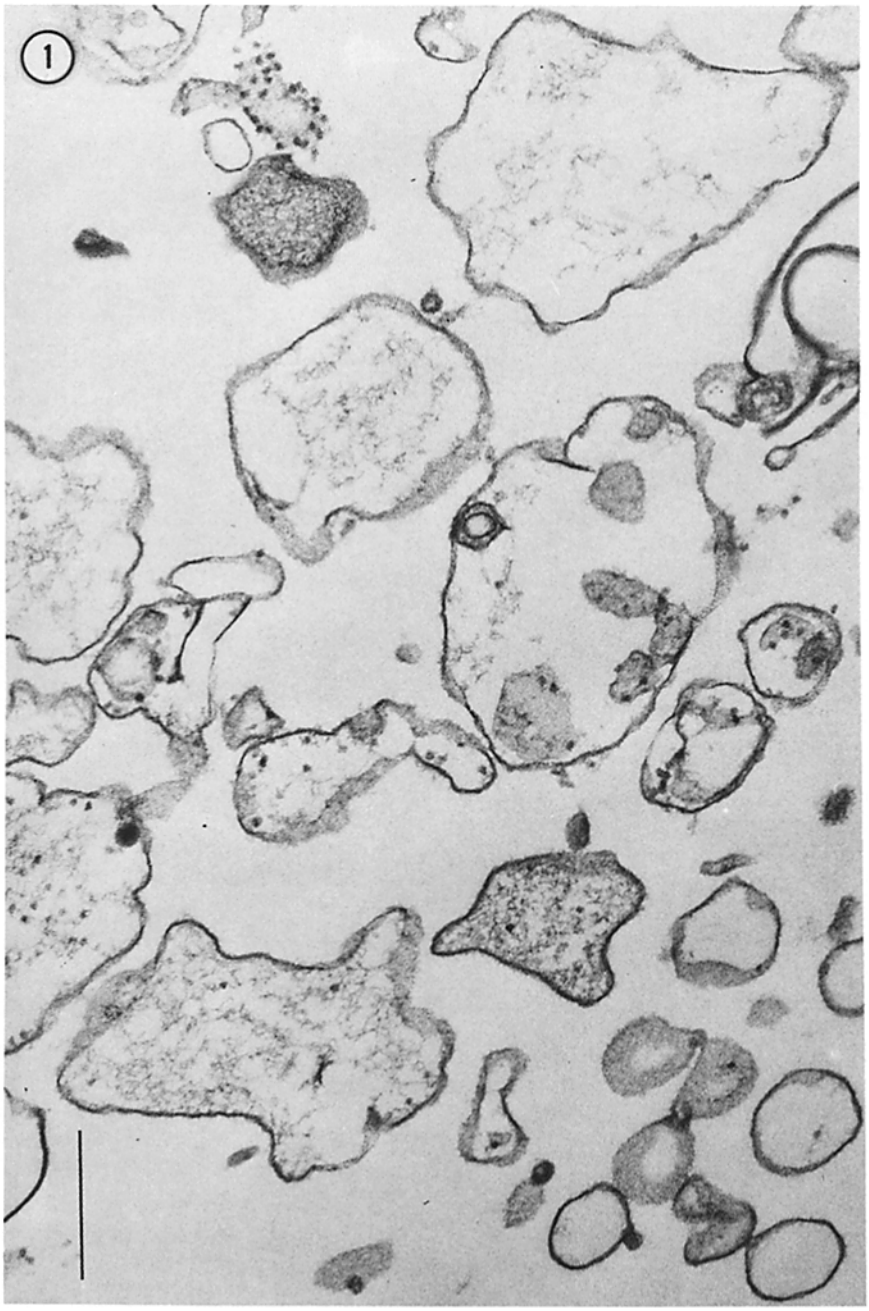

FIGURE 1 Appearance of podosomes released by shear from macrophages adherent to nylon wool fibers for 5 min. Electron micrograph of a thin section. Bar, 0.5 μm.

we first examined the effect of extract concentration and of actin addition to extracts on the various measurements of consistency.

Fig. 2*a* shows the effect of diluting extracts in homogenizing solution containing 0.1 M KCl on the sedimentability of protein at low and high speeds and in extract viscosity. The rate of fall in these properties with increasing dilution is not

uniform, viscosity declining more rapidly than protein sedimentability. The relationship between the change in viscosity and protein sedimentability at high speed is exponential (Fig. 2*a*, inset). Because dilution affects other extract contents as well as actin, the effect of adding purified rabbit skeletal muscle actin to macrophage extracts was examined. Fig. 2*b* indicates that the relationship between the consistency measurements in minimally diluted extracts holds as the actin concentration of extracts increases. The findings suggest that the assays do measure the state of actin assembly under these conditions and provide a basis for comparing changes in the different measurements obtained in extracts from cells subjected to various experimental conditoins.

The macromolecular state of actin in control and nylon wool-exposed cell bodies, both being treated with eluting solution and shear, differed. Sucrose extracts of control cell bodies made 0.1 M in KCl and warmed to room temperature, formed gels. However, identically treated extracts of nylon wool fiber-exposed cell bodies became viscous, but did not gel. This apparent lack of gelation was reflected by the relatively low quantity of extract protein sedimentable at low speed from warmed extracts of nylon wool-treated cell bodies (Table IV). The viscosity of warmed extracts of nylon wool fiber-treated cells was also lower than that of extracts of control cells, but the reduction in viscosity was proportionately less than the decrease in sedimentability of protein at low speed. The relatively smaller diminution in viscosity correlated with a minor reduction in the amount of protein sedimentable at high speed from the nylon wool fiber extracts (Table IV). The results suggested that the gel state of actin in nylon wool fiber cell bodies was considerably diminished, but that the actin present was not appreciably depolymerized. These findings were then correlated with concentrations of contractile proteins in the various fractions.

TABLE II

Effect of Nylon Wool Fibers on the Release of β-Glucuronidase Activity and Lactate Dehydrogenase Activity by Macrophages Subjected to Shear

Nylon treatment	β-glucuronidase activity	Lactate dehydrogenase activity
	nmol/min	
−	6.8 ± 1.4	Not measurable
+	24.8 ± 11.8	Not measurable

Macrophages (80 mg cell protein) were incubated at 37°C for 5 min and sheared in the presence or absence of nylon wool fibers. The extracellular medium, after removal of cells and podosomes by centrifugation, was assayed for enzymatic activities. The total activities released are expressed as mean ± SEM where $n = 4$.

TABLE III

Effect of Nylon Wool Fibers on the β-Glucuronidase Activity of Podosomes

Nylon wool fibers	β-Glucuronidase activity	
	Specific	Total
	nmol/min/mg protein	*nmol/min*
−	1.2 ± 0.1	1.2 ± 0.5
+	2.6 ± 0.3	6.9 ± 3.8

Macrophages (80 mg cell protein) were incubated at 37°C for 5 min in the presence or absence of 500 mg nylon wool fibers. The incubation was terminated by shearing the cells, and the podosomes and cell bodies were separated from each other by differential centrifugation. Activities are expressed as mean ± SEM where $n = 4$. Total podosome protein recovered was 0.9 ± 0.4 mg in the absence of nylon wool fibers, and 2.6 ± 1.4 mg in the presence of nylon wool fibers per 80 mg cell protein.

Attachment to and Removal of Macrophages from Nylon Wool Fibers Redistributes Contractile Proteins from Cell Bodies to Podosomes

Fig. 3*a* shows densitometric scans of dodecyl sulfate polyacrylamide gels after electrophoresis and staining of extracts prepared from cell bodies sheared with or without exposure to nylon wool fibers. Such scans were used to quantify the concentration of contractile proteins in these extracts. There are a number of differences in the polypeptide composition of the extracts, but we have restricted our analysis to those bands which correspond to previously purified and characterized macrophage proteins. The differences in the relative concentrations of actin-binding protein and myosin heavy chain evident in this scan are consistent and representative of several experiments (Table V). A densitometric scan of a stained dodecyl sulfate polyacrylamide gel after electrophoresis of solubilized podosomes isolated from nylon-contacted cell bodies removed from nylon wool is also shown for comparison (Fig.

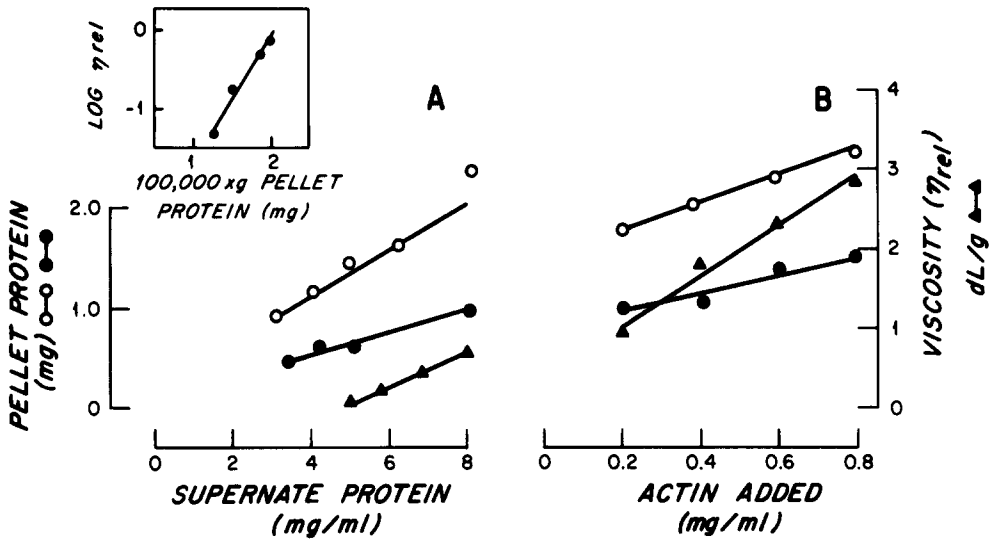

FIGURE 2 (A) Effect of protein concentration on protein sedimentation from warmed macrophage extracts and on extract viscosity. The amount of protein sedimented from a 0.4-ml vol of extract, made 0.1 M with KCl and warmed from 4 to 25°C for 1 h, was measured after centrifugation of the extract at 10,000 *g* for 10 min (●) or after 100,000 *g* for 90 min (○) at 25°C as described in Materials and Methods. The viscosity (◀) of the extract was measured at 4°C. The extract was warmed to 25°C for 1 h and then returned to 4°C for 30 min. In the inset, the amount of protein sedimented by centrifugation at 100,000 *g* for 90 min is plotted against the log of the extract viscosity at each concentration. (B) Effect of adding rabbit skeletal muscle actin on protein sedimented from a macrophage cytoplasmic extract and on the extract viscosity. Actin was added to the extract, making the final concentrations indicated. The extract volume was adjusted to 0.4 ml with sucrose homogenizing solution such that the final concentration of macrophage extract protein was 6.3 mg/ml.

TABLE IV

Gelation and Aggregation of Actin in Macrophage Cytoplasmic Extracts Prepared from Control Cells or Residual Bodies of Cells Previously Attached to Nylon

	Gelation		Aggregation	
	Visual	Protein sedimented at $10^4 g$, 10 min	Reduced viscosity	Protein sedimented at $10^5 g$, 60 min
		%	*d liter/g*	%
Control	4 + gel	17.2 ± 0.2	1.0 ± 0.2	30.0 ± 0.2
Nylon wool	± gel	7.5 ± 2.0	0.6 ± 0.3	25.7 ± 3.3

Macrophage extracts were prepared from cells sheared in the presence or absence of nylon wool fibers after 5 min incubation at 37°C. The extracts were made 0.1 M with KCl and warmed to 25°C for 1 h, and then assayed for protein sedimentability at the indicated centrifuge speeds and for viscosity. The data are expressed as mean ± SEM where $n = 4$. An impression as to gel strength was recorded visually as described in the text.

3*b*). Table V summarizes the contractile protein composition of extracts and podosomes isolated from cells sheared in the presence and absence of nylon wool fibers. Relative to control extracts, the average actin-binding protein and myosin concentrations were reduced 67 and 65%, respectively, in extracts prepared from nylon-contacted residual cell bodies. The actin concentration was only slightly diminished, by 19%, in these extracts compared to controls. Based on the correlations shown in Fig. 2, this much loss of F-actin from a cytoplasmic extract would be expected to be associated with a 37% fall in extract viscosity if the remaining actin in the extract were proportionally polymerized. This change is close to that actually measured (Table IV).

In separate experiments, myosin concentrations of cytoplasmic extracts of control and of nylon wool-contacted cell bodies were also compared by means of ATPase measurements as well as by electrophoresis and densitometry. The two determinations correlated well (Table VI). Nylon wool

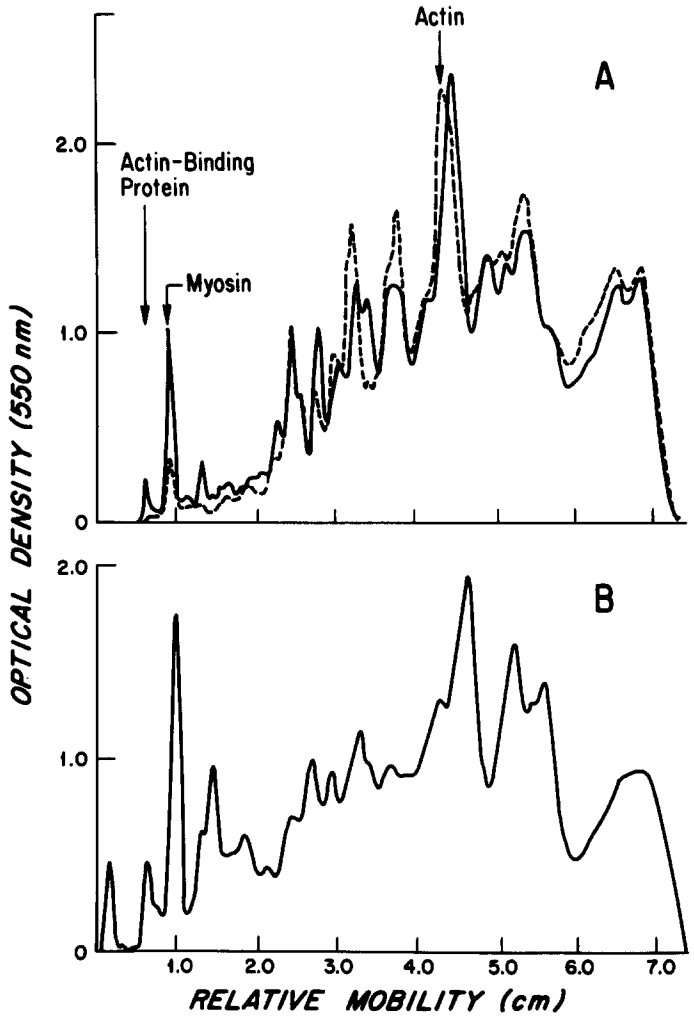

FIGURE 3 Densitometric scans of Coomassie Blue stained 5% polyacrylamide gels after electrophoresis in dodecyl sulfate of (A) sucrose extracts prepared from cells not exposed to nylon wool fibers but exposed to shear (——), or residual cell bodies removed from nylon by shear (- - -), and (B) podosomes isolated from cells attached to nylon wool fiber.

fiber contact did not change the actin-binding protein, myosin, or actin concentrations of podosomes, although by engendering the production of considerably more of these structures, it resulted in greater total amounts of these proteins being associated with the podosome fraction. Therefore, the simplest interpretation of the fall in actin-binding protein and myosin concentrations of extracts of nylon wool-treated cells is a redistribution of these proteins from the cell body to the podosomes. The sums of total actin protein in cell extracts plus podosomes of nylon wool fiber-treated cells was 93% of the total actin protein in extracts and podosomes of an equivalent quantity of control cell protein, whereas the recoveries of total actin-binding protein and myosin in nylon wool fiber-treated cell fractions were 59 and 66%, respectively, of control cell fractions. Because the accounting for these proteins in the fractions was not 100%, we undertook experiments to determine whether or not the decreased myosin and actin-binding protein content of cytoplasmic extracts of nylon wool fiber-exposed cells was the result of increased proteolysis. First, the EDTA-activated ATPase activity of cytoplasmic extracts of control and nylon wool

TABLE V

Content of Actin-Binding Protein, Myosin, and Actin in Extracts and Podosomes of Macrophages Sheared after 5 min of Incubation With or Without Nylon Wool Fibers

	Actin-binding protein		Myosin		Actin	
	%*	μg‡	%	μg	%	μg
Control						
Extract	0.6 ± 0.1	207 ± 35	1.7 ± 0.2	588 ± 69	19.6 ± 0.5	6,761 ± 172
Podosomes	0.9 ± 0.1	11 ± 2	2.8 ± 0.1	34 ± 5	17.2 ± 0.7	207 ± 18
Total		218 ± 35		622 ± 69		6,968 ± 173
Nylon Wool						
Extract	0.2 ± 0.1	69 ± 7	0.6 ± 0.1	207 ± 36	15.8 ± 0.7	5,452 ± 241
Podosomes	1.0 ± 0.1	58 ± 5	3.4 ± 0.4	200 ± 23	18.0 ± 1.6	1,062 ± 94
Total		127 ± 9		407 ± 43		6,514 ± 259

Data is expressed as mean ± SEM where $n = 4$.

* The percentage of each protein in individual fractions was calculated by densitometry of Coomassie Blue stained 5% polyacrylamide disc gels.

‡ μg of protein, based on 100 mg of total cell protein, is the total mass of protein in each fraction multiplied by percentage content.

TABLE VI

Effect of Nylon Wool Fibers and Storage on the Myosin Content of Macrophage Cytoplasmic Extracts

Time of storage, *h*		0	1	24
Temperature of storage, °C		–	37	2
		Myosin EDTA ATPase activity, *nmol/min/mg protein*		
Nylon wool fibers				
	–	3.4 ± 0.2	3.5 ± 0.1	3.4 ± 0.2
	+	2.5 ± 0.1	2.5 ± 0.2	2.5 ± 0.2
		Myosin heavy chain, *% of extract protein*		
	–	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.2
	+	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1

Macrophage cytoplasmic extracts were stored for the times and at the temperatures indicated. For the ATPase assay, 0.1 ml of macrophage cytoplasmic extract was added to 0.4 ml of a solution containing 0.6 M KCl, 1 mM ATP, 1 mM EDTA, and 10 mM imidazole Cl, pH 7.0, and incubated at 37°C for 30 min. The quantity of myosin heavy chain in the extracts was determined by densitometry of Coomassie Blue stained 5% polyacrylamide disc gels. The values are expressed as mean ± SEM where $n = 4$.

cells was determined at various intervals after extract preparations (Table VI). Second, the EDTA-activated ATPase activity of rabbit skeletal-muscle heavy meromyosin added to control and nylon wool cell homogenates was assayed at various times (Table VII). In both instances, the stability of the activity was equivalent in control and nylon wool extracts. Another possibility to be considered was the idea that the 25–30% of cells not recovered from nylon wool fibers by shear contained higher concentrations of actin-binding protein and myosin than control cells. If such were the case, normal control cells could be a mixture of cells having high and low concentrations of these proteins. The high-concentration cells might stick most firmly to nylon wool fibers, whereas the low-concentration cells would be most easily removed by shear. This unlikely possibility was ruled out by examining the protein composition of cells attached to nylon wool fibers. However, there was no significant difference in the actin-binding protein or myosin concentrations of intact control cells and of cells attached to nylon wool fibers (Table VIII). The incomplete recoveries of actin-binding protein and myosin are not explained, but most likely reflect the difficulty in completely recovering proteins present in relatively small quantities in fractionation experiments.

DISCUSSION

Comparing the behavior of contractile proteins in

TABLE VII

Effect of Nylon Wool and Storage on the EDTA-Activated ATPase Activity of Heavy Meromyosin in Macrophage Extracts

Time of Assay, *h*	0	1	24
Temperature of storage, °C	–	37	2
	EDTA ATPase activity, *μmol P/min/mg heavy meromyosin protein*		
Control	1.09 ± .30	1.07 ± .60	1.07 ± .60
Nylon wool	1.10 ± .90	1.15 ± .11	1.07 ± .30

100 μg of rabbit skeletal muscle heavy meromyosin (HMM) was added to sucrose homogenizing solution to a final concentration of 50 μg HMM/ml. 2 ml of this solution was used to homogenize either control macrophages or macrophage residual cell bodies (20 mg cell protein/ml). The homogenates were centrifuged at 100,000 *g* for 1 h at 2°C, and the supernatant extracts collected. Some of these extracts were stored for the times and at temperatures indicated. The EDTA ATPase activity of 0.1 ml of each extract was assayed in a 0.5-ml system containing 0.6 M KCl, 1 mM ATP, 1 mM EDTA, and 10 mM imidazole-Cl, pH 7.0 for 15 min at 37°C. The amount of ATPase activity contributed by the added HMM was calculated by subtracting any background extract ATPase activity determined in extracts prepared with homogenizing solution minus HMM (<10% of the activity in the presence of HMM).

TABLE VIII

Contractile Protein Composition of Control Macrophages, Macrophages Attached to Nylon, and Cell Bodies Removed from Nylon Fibers by Shear

Treatment	Actin-binding protein	Myosin	Actin
	%	%	%
None*	0.6 ± 0.2	1.3 ± 0.1	12.2 ± 2.0
Residual cell bodies eluted from nylon wool fibers	0.1 ± 0.5	0.5 ± 0.1	8.5 ± 2.0
Cells remaining attached to nylon wool fibers	0.6 ± 0.1	1.1 ± 0.2	12.2 ± 0.8

The percent of total stainable protein was determined on Coomassie Blue stained 5% polyacrylamide disc gels.
* Addition of nylon wool fibers to macrophages solubilized in dodecyl sulfate electrophoresis buffer did not influence the electrophoretic pattern.

extracts of resting and phagocytizing cells is analogous to comparing contractile proteins from muscle in states of contraction and relaxation. The success of this approach depends on the occurrence of stable alterations in contractile protein structure or function during phagocytosis. The only such change hitherto detected was an increase in the extractibility of actin-binding protein from phagocytizing macrophages compared to resting cells (23). The failure to observe other striking alterations of contractile proteins in extracts of phagocytizing cells could mean that stable changes do not occur, and that activation results from local perturbations of the ionic or metabolic environment. These effects would most likely be neutralized by the extracting media. Another reason for such failure is the fact that analysis of whole cell extracts may not shed light on the localized and dynamic process of phagocytosis which involves reciprocal changes in the state of actin. The fractionation of spread macrophages into cell bodies and podosomes permits monitoring of alterations in contractile proteins occurring in different regions of the cell.

Nylon wool fiber contact diminished the capacity of cell body extracts to form a gel. We have shown that the gelation of cytoplasmic extracts correlates with the quantity of actin-binding protein present in the extracts, that purified actin-binding protein causes purified actins to gel, and that antiserum against actin-binding proteins prevents the gelation of cytoplasmic extracts (3, 9, 11, 23). The decrease in gelation capacity of nylon wool fiber-treated cell bodies correlated with an increased recovery of actin-binding protein in podosomes of nylon wool fiber-treated cells. Concomitant with the shift in actin-binding protein into podosomes was a transfer of myosin, documented by ATPase measurements as well as by electrophoretic quantitation. This redistribution of myosin indicates that the myosin-dependent syneresis of the macrophage actin gel

(23) would have been impaired in extracts of nylon wool fiber-treated cell bodies, although this point was not specifically examined. Only a small percentage of the total macrophage actin translocated from spread and sheared cells to podosomes, but the total amount of actin that shifted was substantial because of the large quantity of actin in these cells. This transfer of actin could account for the decrease in viscosity and quantity of protein sedimentable at high speed in extracts of nylon wool fiber-treated cell bodies. A corollary to this point is that the fraction of the total actin that was polymerizable was not different in extracts of control cells or of residual cell bodies. Therefore, the evidence favors that gelation, rather than polymerization changes, are the dominant variables affecting the state of actin in macrophages under these conditions.[2]

The findings of this and the accompanying study demonstrate that actin-binding protein and myosin are concentrated in the cell cortex (8), and that the "peeling" away of this cortex depletes the cell of these proteins. Our results and the documented morphology of spread cells (4, 5, 15, 20) show that a displacement of cortical cytoplasm accompanies spreading. Shearing of the spread cell with its cortex extended into pseudopodia results in formation of more podosomes than the shearing of unspread cells. An extended cell surface is probably a requirement for podosome formation (8). The shearing process which dislodges cells from nylon and podosomes from cells, accelerates the secretion of lysosomal enzymes. An explanation for these findings is that the effect of shear is to disrupt part of the cortical actin gel, thereby destabilizing the plasma membrane, fostering bleb formation (8), and also facilitating the entry of lysosomes into the cell periphery.

Because residual cell bodies retain an envelope of plasmalemma, the depletion of actin-binding protein and mysoin from them after spreading and shear strongly supports one of the alternative hypotheses under consideration in this study, viz. that displacement of activated myosin and actin-binding protein, rather than reversible activation of these proteins in fixed positions, as the mechanism of propagation of the contractile event. Therefore, we conclude that actin-binding protein, myosin, and some actin move with the tips of advancing pseudopodia during spreading. At present, we have insufficient information to explain the mechanism of this postulated movement of myosin and actin-binding protein, or how these movements relate to the extension of the pseudopodia. We infer that gelation and compression of actin brought about by these proteins serves to exclude organelles from the pseudopod. Actin filaments behind the advancing pseudopodia are actively disaggregated by the egress of myosin and actin-binding protein into the pseudopodia. This disaggregation permits lysosomes to enter the cell periphery, make contact with the plasmalemma, and undergo secretion. The cost of cell spreading may be an attenuation of the actin gel, even in the pseudopodia, which would explain the increase in lysosomal enzyme activity in the podosomes from cells subjected to nylon wool and shear, relative to those from cells treated by shear alone (Table III). This increase accompanies an increased release of lysosomal enzyme into the extracellular medium surrounding cells attached to nylon wool fibers, and may reflect lysosomal traffic through the cortical cytoplasm.

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[2] Depending on the solvent conditions, actin in extracts of macrophages undergoes temperature-dependent reversible polymerization and gelation (buffered sucrose solution without added salts) (3, 23), or else (in the presence of salt) reversibly gels, but remains polymerized (9, 11).

REFERENCES

1. ALLISON, A. C., P. DAVIES, and S. DEPETRIS. 1971. Subplasmalemmal microfilaments in macrophage movement and endocytosis. *Nat. New Biol.* **232**:153-155.
2. BOXER, L. A., E. T. HEDLEY-WHYTE, and T. P. STOSSEL. 1974. Neutrophil actin dysfunction and abnormal neutrophil behavior. *N. Engl. J. Med.* **291**:1093-1099.
3. BOXER, L. A., and T. P. STOSSEL. 1976. Interactions of actin, myosin and an actin-binding protein of chronic myelogenous leukemia leucocytes. *J. Clin. Invest.* **57**:964-976.
4. BRETON-GORIUS, J. 1968. Étude des leucocytes étalés par coupes sériées parallèles et perpendicu-

laires au plan d'étalement. *J. Microsc. (Paris).* **7**:95-106.

5. BRAGINA, E. E., JU M. VASILEV, and I. M. GELFAND. 1976. Formation of bundles of microfilaments during spreading of fibroblasts on the substrate. *Exp. Cell Res.* **97**:241-248.
6. COHN, Z. A. 1970. Endocytosis and intracellular digestion. *In* Mononuclear Phagocytes. R. van Furth, editor. Blackwell Scientific Publications Ltd., Oxford, England. 121-128.
7. DAVIES, W. A., J. H. HARTWIG, and T. P. STOSSEL. 1977. Translocation of contractile proteins in macrophage spreading and secretion. *Clin. Res.* **25**:474*a* (Abstr.).
8. DAVIES, W. A., and T. P. STOSSEL. Peripheral hyaline blebs (podosomes) of macrophages. *J. Cell Biol.* **75**:941-955.
9. HARTWIG, J. H., and T. P. STOSSEL. 1977. Actin-binding protein as an actin-gelling factor: quantitation and mechanism. *J. Cell Biol.* (Abstr.). In press.
10. HARTWIG, J. H., and T. P. STOSSEL. 1975. Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* **250**:5699-5705.
11. HARTWIG, J. H., and T. P. STOSSEL. 1976. Interactions of actin, myosin and actin-binding protein of rabbit alveolar macrophages III. Effects of cytochalasin B. *J. Cell Biol.* **71**:295-303.
12. HIRSCH, J. G. 1963. Cinemicrophotographic observations on granule lysis in polymorphonuclear leucocytes during phagocytosis. *J. Exp. Med.* **116**:827-833.
13. JOHNSTON, R. B., JR., J. E. LEHMEYER, and L. A. GUTHRIE. 1976. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin. *J. Exp. Med.* **143**:1551-1556.
14. KANE, R. E. 1975. Preparation and purification of polymerized actin from sea urchin egg extracts. *J. Cell Biol.* **66**:305-315.
15. KLOCK, J. C., and D. F. BAINTON. 1976. Degranulation and abnormal bactericidal function of granulocytes procured by reversible adhesion to nylon wool. *Blood.* **48**:149-161.
16. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein determination with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
17. MYRVIK, Q. N., E. S. LEAKE, and B. FARISS. 1961. Studies on pulmonary alveolar macrophages from the normal rabbit: a technique to procure them in a high state of purity. *J. Immunol.* **86**:128-132.
18. NORTH, R. J. 1970. Endocytosis. *Semin. Hematol.* **7**:161-171.
19. POLLARD, T. D. 1976. The role of actin in the temperature-dependent gelation and contraction of *Acanthamoeba* extracts. *J. Cell Biol.* **68**:579-601.
20. REAVEN, E. P., and S. G. AXLINE. 1973. Subplasmalemmal microfilaments and microtubules in resting and phagocytizing cultivated macrophages. *J. Cell Biol.* **59**:12-27.
21. SPUDICH, J. A., and S. WATT. 1971. The regulation of rabbit skeletal muscle contraction I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* **245**:4866-4871.
22. STOSSEL, T. P., and J. H. HARTWIG. 1975. Interactions between actin, myosin and a new actin-binding protein of rabbit alveolar macrophages. Macropahge myosin Mg^{++}-adenosine triphosphatase requries a cofactor for activation by actin. *J. Biol. Chem.* **250**:5708-5712.
23. STOSSEL, T. P., and J. H. HARTWIG. 1976. Interactions of actin, myosin and a new actin-binding protein of rabbit pulmonary macrophages II. Role in cytoplasmic movement and phagocytosis. *J. Cell Biol.* **68**:602-619.
24. STOSSEL, T. P., R. J. MASON, T. D. POLLARD, and M. VAUGHAN. 1972. Isolation and properties of phagocytic vesicles II. Alveolar macrophages. *J. Clin. Invest.* **51**:604-614.
25. STOSSEL, T. P., T. D. POLLARD, R. J. MASON, and M. VAUGHAN. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. *J. Clin. Invest.* **50**:1745-1757.
26. TAYLOR, D. L., J. A. RHODES, and S. A. HAMMOND. 1976. The contractile basis of amoeboid movement. II. Structure and contractility of motile extracts and plasmalemma-ectoplasm ghosts. *J. Cell Biol.* **70**:112-143.
27. WACKER, W. E. C., D. D. ULMER, and L. VALLEE. 1956. Metallo-enzymes and myocardial infarction II. Malic and lactic dehydrogenase activities and zinc concentration in serum. *N. Engl. J. Med.* **255**:449-456.
28. WEIHING, R. R. 1976. Cytochalasin B inhibits actin-related gelation of HeLa cell extracts. *J. Cell Biol.* **71**:303-307.
29. WELSCHER, H. D., and A. CRUCHAUD. 1976. The influence of various particles and 3',5' cyclic adenosine monophosphate on release of lysosomal enzymes by mouse macrophages. *J. Reticuloendothel. Soc.* **20**:405-420.
30. ZUCKER-FRANKLIN, D., and J. G. HIRSCH. 1964. Electron microscope studies on the degranulation of rabbit peritoneal leukocytes during phagocytosis. *J. Exp. Med.* **120**:569-575.
31. ZURIER, R. B., S. HOFFSTEIN, and G. WEISSMAN. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:844-848.