INTERACTIONS OF ACTIN, MYOSIN, AND AN ACTIN-BINDING PROTEIN OF RABBIT PULMONARY MACROPHAGES

III. Effects of Cytochalasin B

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As part of our endeavor to understand the mechanism by which mammalian phagocytes ingest particulate objects, we have been studying the contractile proteins of rabbit pulmonary macrophages. We reconstituted a macrophage contractile system comprised of actin, a high-molecular-weight actin-binding protein, myosin, and a protein cofactor (6, 16, 17). The actin-binding protein promotes the assembly and gelation of low concentrations of purified actin or of actin in extracts of pulmonary macrophages. Myosin and the cofactor effect the Mg\(^{2+}\)-ATP-dependent contraction of the gels composed of actin and actin-binding protein. More actin-binding protein is extracted from macrophages which have previously phagocytized particles than from resting cells. We speculate that contact of ingestible particles with the macrophage plasma membrane alters the state of the actin-binding protein, that activation of the actin-binding protein promotes the assembly and gelation of actin in the cell periphery, and that myosin and cofactor then compress the gel to form narrow pseudopods. This series of events would explain the apparent sol-to-gel transformation observed by microscopists many years ago in the peripheral cytoplasm of macrophages ingesting particles (9).

The fungal metabolite, cytochalasin B, inhibits phagocytosis by macrophages at concentrations greater than 10\(^{-6}\) M, and the inhibition is maximal at a concentration of 10\(^{-5}\) M (1, 2, 8, 11). Logical inference and interpretation of electron micrographs have led to the idea that cytochalasin B inhibits phagocytosis and influences a vast number of other cellular functions by acting on cytoplasmic microfilaments believed to be actin polymers (see reference 18 for a review). However, investigators have found that cytochalasin B influences the behavior of muscle and cytoplasmic actins only at concentrations much higher than those generating maximal effects on phagocytosis or not at all (4, 10, 13, 14).

We report here that cytochalasin B prevents the temperature-dependent assembly and gelation of actin in macrophage extracts, and that it inhibits the gelation of purified actin by actin-binding protein. It dissolves gels formed by warming macrophage cytoplasmic extracts or mixtures of actin and actin-binding protein. These effects of cytochalasin B are essentially maximal at concentrations of 10\(^{-5}\) M. A preliminary account of this work has been published (5).

MATERIALS AND METHODS

The procurement of rabbit pulmonary macrophages, the preparation of extracts of these cells, and the purification of macrophage contractile proteins have been described in our earlier publications (6, 16, 17). Rabbit skeletal muscle actin was made by the method of Spudich and Watt (15). Analytical procedures including assays of the rate of phagocytosis, Mg\(^{2+}\)-ATPase activity, polyacrylamide gel electrophoresis with sodium dodecyl sulfate, quantitative densitometry of polyacrylamide gels, analysis of total protein, and electron microscopy were performed as described previously (6, 16, 17). The viscosity of protein solutions was determined with a Wells-Brookfield cone-plate microviscometer (Brookfield Engineering Laboratory, Inc., Stoughton, Mass.) utilizing a 1.565° cone angle. Cytochalasin B was purchased from the Aldrich Chemical Company (Milwaukee, Wis.).
RESULTS

Cytochalasin B Reversibly Prevents the Assembly of Actin in Sucrose Extracts of Macrophages

Extracts of pulmonary macrophages containing 0.34 M sucrose, 10 mM dithiothreitol, 2 mM EDTA, 1 mM ATP, and 20 mM Tris-maleate buffer, pH 7.0, gel when warmed from 0°C to room temperature (Fig. 1). Gelation is associated with the assembly of cytoplasmic actin, because the gels consist of interdigitating actin filaments (Fig. 2 A) (17), and because actin (together with other proteins) sediments when the warmed extract is spun in the ultracentrifuge (Fig. 3). None of these changes occurs in macrophage extracts kept at ice-bath temperature. In the presence of 10⁻⁶ M cytochalasin B, macrophage extracts warmed from 0°C to room temperature do not increase their viscosity, do not gel (Fig. 1) and do not acquire filaments visible in the electron microscope (Fig. 2 B). Macrophage sucrose extracts made 0.1 M in KCl at ice-bath temperature also gel when warmed to room temperature. A final concentration of 10⁻⁶ M cytochalasin B prevents the gelation of this salt-treated extract. However, even in the presence of 10⁻⁶ M cytochalasin B, a salt-treated extract increases its viscosity after 60 min at room temperature (Table I), and filaments are visible in the electron microscope (not shown). Therefore, cytochalasin B inhibits extract gelation either in the presence or in the absence of KCl, but does not prevent totally the salt-induced assembly of extract actin. As shown in Fig. 3, decreasing amounts of extract protein are sedimentable in the ultracentrifuge when the concentration of cytochalasin B is raised above 2 × 10⁻⁶ M. The decrease in protein sedimentability is maximal at cytochalasin B concentrations above 2 × 10⁻⁶ M. The relative distribution of the major polypeptides in the pellets of which actin constituted 25% of the total pellet protein formed was not altered by various cytochalasin B concentrations. Dimethylsulfoxide, the vehicle for dissolving cytochalasin B, has no effect on macrophage extract consistency or protein sedimentability (Fig. 3). Fig. 3 also shows the effect of different cytochalasin B concentrations on the initial rate of phagocytosis by rabbit pulmonary macrophages. When extracts containing 10⁻⁶ M cytochalasin B are chromato-

![Figure 1](image_url)

**Figure 1** Test tubes containing a macrophage extract (supernatant fraction of pulmonary macrophages homogenized in 0.34 M sucrose, 10 mM dithiothreitol, 2 mM EDTA, 1 mM ATP, 20 mM Tris-maleate buffer, pH 7.0 at 0°C and centrifuged at 80,000 g for 1 h at 2°C) warmed from 0°C to 25°C in the presence of 0.15% dimethylsulfoxide (left) or 10⁻⁶ M cytochalasin B, 0.15% dimethylsulfoxide (right). The total protein concentration of the extract was 7 mg/ml.
graphed at 4°C on Sephadex G-15 equilibrated with 0.34 M sucrose, 5 mM dithiothreitol, 2 mM EDTA, 1 mM ATP, and 20 mM Tris-maleate, the eluting protein gels and forms filaments when warmed to room temperature. Therefore, removal of cytochalasin B by chromatography reverses the inhibition of actin assembly and gelatin in macrophage extracts.

**Cytochalasin B Dissolves Macrophage Extracts Actin Gels but does not Depolymerize Actin Filaments**

Addition of cytochalasin B to macrophage extract gels dissolves them within 10 min (Fig. 4), and the sols have the viscosity of solutions containing F-actin concentrations equivalent to those found in macrophage extracts. Filaments persist in the sols and are visible in the electron microscope (Fig. 2C). The sedimentability of protein in the gels dissolved by cytochalasin B is the same as that of control extracts permitted to gel in the absence of cytochalasin B. Dimethylsulfoxide alone has no effect on the consistency of extract gels.

**Cytochalasin B Inhibits and Reverses the Gelation of Actin by Actin-Binding Protein but does not Inhibit the Polymerization of Actin by Salt**

As shown in Table II, a low concentration of G actin warmed in 0.1 M KCl solution becomes viscous but does not gel after 1 h. Therefore, it sediments at high speed but not at low speed. Actin-binding protein causes the same concentration of actin to gel. Both proteins sediment together at low and high speeds. Cytochalasin B, 10⁻⁶ M, prevents the gelation of actin by actin-binding protein, and neither actin nor actin-binding protein sediments at low speed. Actin still sediments at high speed, but the pellet contains less actin-binding protein than the high-speed pellet formed in the absence of cytochalasin B. Therefore, cytochalasin B prevents completely the gelation of actin in the presence of actin-binding protein but has less effect on the polymerization of actin either in the presence or in the absence of actin-binding protein after 1 h.

10⁻⁶ M cytochalasin B dissolves actin gels formed by addition of actin-binding protein to either G- or F-actin. Filaments are visible in both intact and dissolved gels (Fig. 5).

**Table I**

| Viscosity | Cytochalasin B | 0.1 M KCl |
|-----------|---------------|-----------|
| gel       | -             | +         |
| cP/min    | 1.0           | 8.1       |

1-ml samples of macrophage extracts in 0.34 M sucrose, 5 mM dithiothreitol, 2 mM EDTA, 1 mM ATP, 10 mM Tris-maleate, pH 7.0, were warmed from ice-bath temperature to room temperature for 60 min. 10⁻⁷ M cytochalasin B, 0.1 M KCl, or both (final concentrations) were added as indicated. All samples contained 0.15% dimethylsulfoxide.

**Cytochalasin B does not Inhibit the Mg²⁺-ATPase Activity of Cofactor-Activated Macrophage Actomyosin**

Cytochalasin B at concentrations ranging from 10⁻⁶ to 10⁻⁵ M has no effect on the Mg²⁺-ATPase activity of macrophage myosin in the presence of macrophage actin and cofactor. In these experiments, the Mg²⁺-ATPase activity was 0.215 μmole Pi released/mg myosin protein/min at 37°C with or without cytochalasin B.

**DISCUSSION**

Cytoplasmic gelation, long thought to be relevant to cell movement (9), can now be demonstrated in vitro (7, 12, 17). Protein gels are cross-linked fiber networks (3). Therefore, macrophage actin-binding protein promotes the gelation of actin either by creating stable cross-links for the actin polymers or else by increasing the rigidity of the cross-linked actin strands. In either case, cytochalasin B impairs the interaction between macrophage actin-binding protein and actin. It prevents the gelation of actin by actin-binding protein and it dissolves gels of the same material. Cytochalasin B affects the gelation reaction in crude extracts of actin and actin-binding protein whether or not added salt is present. We reported previously that actin-binding protein appears to promote the temperature-dependent assembly as well as gelation of actin in sucrose extracts of macrophages in the absence of added salt (17). The inhibition of actin assembly by cytochalasin B...
Figure 2 Morphology of a macrophage extract treated in different ways, negatively stained, and photographed in the electron microscope. (a) Shows the extract warmed from 0°C to 25°C in the presence of 0.15% dimethylsulfoxide in which the extract gelled. (b) Shows the extract warmed in the presence of 10^{-6} M cytochalasin B, 0.15% dimethylsulfoxide. The extract did not gel, and its viscosity was 1.5 cP/min. (c) Shows the extract allowed to gel by warming from 0°C to 25°C for 1 h and then dissolved by the addition of 10^{-6} M cytochalasin B, 0.15% dimethylsulfoxide (final concentrations). The viscosity of the extract after dissolution of the gel was 7 cP/min. × 82,000.
TABLE II

Effect of Cytochalasin B on the Viscosity and Sedimentability of Actin and Actin-binding Protein

| Protein sedimentability | (130,000 g-min) | (730,000 g-min) |
|-------------------------|----------------|-----------------|
|                         | Pellet         | Supernate       |
| Actin                   | 7.0            | 0               |
| Actin + cytochalasin B  | 6.7            | 0               |
| Actin + actin-binding   | 228            | 32              |
| Actin + actin-binding   | 7.5            | 0               |
| protein gel             |                | 539             |
| Actin + actin-binding   |                | 11              |
| protein + cytochalasin B|                | 210             |

0.6 mg of rabbit skeletal muscle G-actin was dissolved in 1 ml of ice-cold 0.1 M KCl, 1 mM ATP, 5 mM Tris-maleate buffer, pH 7.0, containing 0.15% dimethylsulfoxide in the presence or absence of 10^{-4} M cytochalasin B or 0.05 mg of purified macrophage actin-binding protein and warmed to 25°C for 1 h. The solutions were assayed for viscosity and then centrifuged as indicated at 25°C. The total protein contents of the pellet and supernatant fractions were measured, and the distribution of protein in the pellet and supernatant fractions was determined by quantitative densitometry of stained polyacrylamide gel electrophoreograms.

in sucrose extracts of macrophages without added salts supports this idea. Kinetic studies will be required to determine whether cytochalasin B alters the rate of actin polymerization in the presence of salt and actin-binding protein. However, under relatively physiological conditions (0.1 M KCl) the major qualitative effect of cytochalasin B appears to be on the gel state of actin.

The concentrations of cytochalasin B required to inhibit actin gel formation by actin-binding protein or to dissolve actin gels are an order of magnitude lower than those which begin to inhibit the rate of phagocytosis by macrophages (Fig. 2 and references 1, 2, 8, 11). Therefore, it is reasonable to suggest that cytochalasin B may inhibit macrophage phagocytosis by its interference with the
FIGURE 3 Effect of cytochalasin B concentrations on the initial rate of phagocytosis (●) by intact pulmonary macrophages and on the sedimentability of protein in macrophage extracts (○△). Macrophage extracts containing 2 mg of total protein in 0.5 ml were warmed from 0°C to 25°C for 1 h in the presence or absence of cytochalasin B as indicated. All extracts contained 0.15% dimethylsulfoxide. The extracts were then centrifuged at 80,000 g for 3 h at 25°C. The total protein content of the pellets formed was assayed and their polypeptide composition analyzed by electrophoresis on 5% polyacrylamide gels with 1% sodium dodecyl sulfate. The gels shown are, from left to right, of the pellets sedimented from the control and four extracts containing increasing quantities of cytochalasin B. The arrows indicate, from top to bottom, the polypeptides of actin-binding protein, myosin heavy chain, and actin. (△) Indicates the sedimentable protein of an extract gelled by warming from 0°C to 25°C and then dissolved by the addition of 10^-6 M cytochalasin B, 0.15% dimethylsulfoxide. Its final viscosity was 7 cP/min. F actin, 1 mg/ml in 0.34 M sucrose solution, had a viscosity of 10.5 cP/min. The sixth polyacrylamide gel from the left shows the high-speed pellet of the extract gel dissolved with cytochalasin B.

FIGURE 4 Photograph of a macrophage sucrose extract gelled by warming from 0°C to 25°C and shown supporting its weight on a surface (left). The macrophage extract was gelled in a glass cylinder that was removed after gelation. The addition of cytochalasin B (final concentration 10^-6 M, 0.15% dimethylsulfoxide) caused the gel to collapse (right).
Figure 5. Morphology of a gel (left) composed of rabbit muscle actin (0.5 mg/ml) and macrophage actin-binding protein (0.1 mg/ml) in 0.1 M KCl and of the same mixture after the gel was dissolved by the addition of 10^{-6} M cytochalasin B (right).
interaction between macrophage actin-binding protein and actin. Our findings confirm earlier studies which showed that these low concentrations of cytochalasin B do not markedly influence the viscosity, morphology, or function of polymerized muscle or cytoplasmic actins (4, 10, 13, 14). If our findings can be confirmed in other cytoplasmic contractile systems, then the "microfilament-disruptive" action of cytochalasin B is ascribable to its dissolution of actin filament gels and is now amenable to molecular analysis.

SUMMARY
Low concentrations (≥10−7 M) of cytochalasin B reversibly inhibit the temperature-dependent gelation of actin by an actin-binding protein. The cytochalasin B concentrations which maximally inhibit actin gel formation are 10-fold lower than the concentrations which maximally impair phagocytosis by intact macrophages. Cytochalasin B also prevents the polymerization of monomeric actin in sucrose extracts of macrophages in the absence but not the presence of 0.1 M KCl. 10−6 M cytochalasin B dissolves macrophage extract gels and gels comprised of purified actin and actin-binding protein by dissociating actin-binding protein from actin filaments. This concentration of cytochalasin B, however, does not depolymerize the actin filaments.

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