INHIBITION OF PHAGOCYTOSIS AND PLASMA MEMBRANE MOBILITY OF THE CULTIVATED MACROPHAGE BY CYTOCHALASIN B

Role of Subplasmalemmal Microfilaments

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ABSTRACT

Functional and morphologic effects of cytochalasin B on the cultivated macrophage were examined to determine the basis for plasma membrane movements of the type required for endocytosis and/or spreading on a substratum. Inhibition of phagocytosis and changes in cell shape by cytochalasin B exhibited nearly identical dose-response curves requiring 2–5 × 10⁻⁶ M and 1–2 × 10⁻⁵ M cytochalasin B to inhibit these functions by 50% and 100%, respectively. In contrast, hexose transport was ten times more sensitive to the drug requiring 2–3 × 10⁻⁷ M cytochalasin B to achieve 50% inhibition of 2-deoxyglucose uptake. Inhibition of phagocytosis and changes in cell shape could not be explained solely by drug effects on hexose transport. Analysis of serial thin sections showed that cytochalasin B doses inhibitory for hexose transport had no effect on distribution or organization of either of the two subplasmalemmal microfilament types. However, cytochalasin B concentrations (2.0 × 10⁻⁵ M) that inhibited phagocytosis and altered cell shape disorganized and/or disrupted oriented bundles of 40–50-Å subplasmalemmal microfilaments, but had no effect on the microfilamentous network. Comparative dose-response studies showing positive correlations among cytochalasin B effects on phagocytosis, changes in cell shape, and alterations in oriented subplasmalemmal microfilament bundles provide additional support for the hypothesis that microfilamentous structures play a role in translocation of plasma membrane required for endocytosis and cell motility.

INTRODUCTION

The diverse cell functions of endocytosis, attachment and spreading of cells on a substratum, maintenance of cell shape, and cell movement all share a requirement for extensive translocation of plasma membrane. Although the precise mechanisms are incompletely understood, recent studies have suggested that microfilaments may participate in the movement of plasma membrane (1–6). A recent report from our laboratory, based on ultrastructural analysis of the cultivated macrophage, has provided additional support for this hypothesis (7). The capacity of the macrophage to...
readily attach and spread on a substratum and its special ability to engage in extensive phagocytosis and pinocytosis make this cell type particularly well suited for such studies. An organized system of 40-50-A microfilaments was found in specific association with the subplasmalemmal region of newly forming phagocytic vacuoles (7). Morphologically similar microfilamentous structures were also found subjacent to the substrate attached surface of the cell (7).

Further characterization of the relationship of microfilaments to plasma membrane movement based on studies employing cytochalasin B form the subject of the current report. Since cytochalasin B can paralyze the plasma membrane microfilaments to plasma membrane movement also found subjacent to the substrate attached logically similar microfilamentous structures were associated from other actions of the drug. To answer these questions comparative studies of dose-response relationships for cytochalasin B inhibition of phagocytosis, changes in cell shape, and inhibition of hexose transport as well as ultrastructural analysis of cytochalasin B effects on subplasmalemmal microfilaments were performed.

MATERIALS AND METHODS

Cultivation of Mononuclear Phagocytes

Cells from the peritoneal cavity of unstimulated male mice (25-30 g) of the NCS/PA strain (pathogen free) were harvested by techniques previously described (7). Monolayers of macrophages free of lymphocytes were prepared and maintained in medium no. 199 containing 20% heat-inactivated newborn calf serum (NBCS) (Grand Island Biological Co., Santa Clara, Calif.) for 24-48 h before use. For experiments requiring glucose-free media, cells were maintained in carbohydrate-free medium no. 199 containing 20% dialyzed NBCS (glucose concentration <5 x 10^{-4} M).

Quantitation of Phagocytosis

Polyvinyl toluene particles (PVT) of 2.0 μm diameter (Particle Information Service, Los Altos, Calif.) were added to media of Leighton tube cultures at a final concentration of 7.0 x 10^{5}/ml and phagocytosis was permitted to proceed at 37°C. Each Leighton tube cover slip preparation (10.5 x 35 mm) contained approximately 5 x 10^{5} macrophages. At selected time intervals, the monolayers were saline-rinsed to remove unattached particles and fixed in 1.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at 4°C. The uptake of particles by 100 or more cells per slide was evaluated by direct count of intracellular particles by oil immersion phase-contrast microscopy. The attachment and interiorization stages of phagocytosis could be differentiated by evaluation of the particle position relative to the macrophage surface. Intracellular particles were in the same focal plane as structural elements of the cytoplasm—most often in the centrosphere region—and appeared less refractile than extracellular particles. Particles exhibiting typical “rosette” attachment to the macrophage were scored as extracellular in location (11). Application of these criteria provided reproducible results with a standard error of <10%.

Cytochalasin B (Imperial Chemical Industries, Ltd., Alderley Park, Cheshire, England), prepared fresh as a stock solution of 1.0 x 10^{-5} M in dimethylsulfoxide (DMSO), was added to tissue culture medium to a final concentration of 1.0 x 10^{-4}-2.1 x 10^{-4} M. DMSO was added as necessary to a final concentration of 0.2%.

Measurement of 2-Deoxyglucose Transport

The uptake of [2-3H]deoxyglucose by macrophage monolayers was performed by the method of Mizel and Wilson (12). Macrophages were cultivated for 24 h in sterile liquid scintillation vials in 20% NBCS in medium 199 before use. The tissue culture medium was aspirated, and monolayers were rinsed twice with glucose-free Earle’s balanced salt solution containing 15 mM Tricine (Sigma Chemical Co., St. Louis, Mo.) and 5 mM NaHCO₃ at 37°C. 1 ml of assay buffer, of the same composition as the rinse buffer but containing 1.3 x 10^{-6} M [2-3H]deoxyglucose (1 μCi/ml) (New England Nuclear, Boston, Mass.) and desired concentration of cytochalasin B, was added to each vial. After a 10-min incubation of the vials in a shaking water bath at 37°C, the assay buffer was aspirated and the monolayers were rapidly rinsed three times with balanced salt solution at 0°C. Vials were prepared for liquid scintillation counting by incubation with 0.5 ml NaOH (0.5 N) for 18 h before the addition of the liquid scintillation fluid containing 40 ml Liquifluor (New England Nuclear, Boston, Mass.) and 5 mM NaHCO₃ at 37°C. Each vial was aspirated and the monolayers were harvested by techniques previously described (7). Monolayers were saline-rinsed to remove unattached particles and fixed in 1.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at 4°C. The uptake of particles by 100 or more cells per slide was evaluated by direct count of intracellular particles by oil immersion phase-contrast microscopy. The attachment and interiorization stages of phagocytosis could be differentiated by evaluation of the particle position relative to the macrophage surface. Intracellular particles were in the same focal plane as structural elements of the cytoplasm—most often in the centrosphere region—and appeared less refractile than extracellular particles. Particles exhibiting typical “rosette” attachment to the macrophage were scored as extracellular in location (11). Application of these criteria provided reproducible results with a standard error of <10%.

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Time-Lapse Phase-Contrast Photomicroscopy

Time-lapse phase photomicroscopy was performed using macrophage monolayers cultivated on circular glass cover slips (25-mm diam) and mounted in Sykes-Moore chambers (14). Culture chambers were attached to a continuous perfusion apparatus, placed on the stage of an inverted phase microscope, model M40 (Wild Heerbrugg, Ltd., Heerbrugg, Switzerland), and maintained at 37°C with a thermostatically controlled heater (Sage Instruments Div., Orion Research, Inc., White Plains, N. Y.). Photographs were taken at intervals.
varying from 1 to 10 s on Tri-X film (Eastman Kodak Co., Rochester, N. Y.) with a modified Beaulieu Model 2008S camera back (Hervic Corp., Sherman Oaks, Calif.).

**Scanning Electron Microscopy**

Cover slip preparations of macrophages were fixed at room temperature in 1.25% glutaraldehyde in PBS (pH 7.4), thrice rinsed in distilled water, rapidly frozen by immersion in isopentane cooled to −150°C with liquid nitrogen, and freeze dried 18 h. Glass cover slips with dehydrated macrophages were attached to metal disks with silver paint, coated in vacuo with gold, and examined with a model S4-10 Cambridge stereoscan electron microscope (Cambridge Instruments, Ltd., London, England).

**Transmission Electron Microscopy**

48-h cultivated macrophages were incubated for time periods up to 60 min in medium to which cytochalasin B (in 0.2% DMSO) had been added in final concentrations of 0.06−2.0 × 10⁻⁷ M. Control cells were incubated for identical time periods with 0.2% DMSO. Cells were rinsed briefly with warm PBS, fixed, and embedded in Epon directly on glass cover slips using techniques previously described (7). Epon disks, trimmed and re-mounted on plastic cylinders, were sectioned parallel to the plane of cell attachment. Serial thin sections of oriented macrophages were placed on Formvar-coated slot grids, stained with uranyl acetate and Reynolds’ lead citrate, coated with a thin film of carbon, and examined with a JEM 100B electron microscope.

**RESULTS**

Previous studies employing other cell types have shown that cytochalasin B inhibits hexose transport (12, 15−17), as well as phagocytosis (5, 6, 18, 19) and various types of cell movement (4, 8, 10). However, it is not known if effects of the drug on hexose transport are sufficient to account for functional changes. Therefore, comparative dose-response studies were performed to investigate the relationship among inhibition of phagocytosis, changes in cell shape, and inhibition of hexose transport produced by cytochalasin B.

**Inhibition of Phagocytosis by Cytochalasin B**

In studies of the kinetics of phagocytosis the importance of examining maximal linear rate of particle uptake has been emphasized (20). Initial efforts, therefore, were directed toward the development of a reproducible system for the assay of particle ingestion by monolayers that would meet this requirement.

The kinetics of phagocytic uptake of PVT particles by macrophage monolayers are shown in Fig. 1. After an initial lag the rate of particle uptake by control macrophages was linear from 30 to 120 min after the addition of PVT. A concentration of 4.0 × 10⁻⁷ PVT particles/ml of medium was required to achieve the maximal rate of particle uptake. Cytochalasin B at a concentration of 1.0 × 10⁻⁴ M (0.2% for DMSO) inhibited phagocytosis by 95−99%. In contrast to the marked inhibition of particle uptake, cytochalasin B had no apparent effects on the attachment phase of phagocytosis. DMSO alone at concentrations up to 1% had no effect on particle uptake. The inhibition of phagocytosis by cytochalasin B was fully and rapidly reversible. The maximal linear rate of uptake of particles added 15 min after removal of cytochalasin B from the culture media was identical to that of control values.

The dose-response curve for cytochalasin B inhibition of phagocytosis is shown in Fig. 2. Less than 10% inhibition of particle uptake was produced by 5.0 × 10⁻⁷ M cytochalasin B and maximal inhibition required concentrations of 2.0 × 10⁻⁵ M cytochalasin B. Phagocytic uptake was inhibited 50% by a drug concentration of 2.0 × 10⁻⁶ M. The inhibition of particle uptake was fully and rapidly reversible over the entire range of concentrations at which cytochalasin B was effective.

**Changes in Cell Shape Produced by Cytochalasin B**

Cytochalasin B has been shown to produce marked changes in cell shape (8, 21). It was of interest, therefore, to examine the morphologic effects of cytochalasin B on the macrophage.

A control cell showing the typical phase-contrast appearance of a well-differentiated macrophage is shown in Fig. 3 a for reference. Treatment with 1.0 × 10⁻⁵ M cytochalasin B resulted in cells with the appearance seen in Fig. 3 b. The characteristic features were a thin, highly branched, arborized appearance of peripheral cytoplasm and a markedly thickened centrosphere region of the cell. At concentrations up to 2.1 × 10⁻⁵ M there was no detachment of cells from the glass substrate.

Time-lapse photomicroscopy was performed to examine the time course for morphologic changes. Cells were fully arborized within 10 min of application of the drug with early changes visible by 3 min. After the initial period of centrosphere rounding and arborization of peripheral pseudopodia,
the cells remained fixed in position and shape. The characteristic intense undulating movement of plasma membrane was markedly reduced within 10 min of addition of cytochalasin B to the media. All of the morphologic changes were fully and rapidly reversible. Within 1 min of removal of cytochalasin B, the retracted cells began to spread and by 10 min the cells were normal in appearance and undulating membrane activity was fully restored.

Dose-response studies of the cytochalasin B effect on macrophage morphology were performed. The results of such a study using centrosphere rounding and cytoplasmic retraction as...
Figure 3 Phase-contrast appearance of untreated control macrophage (3a) and cell treated with cytochalasin B (1.0 x 10^{-5} M) (3b). × 2,400. (3a) Control macrophage shown from reference. Centrosphere region contains abundant, large, phase-dense secondary lysosomes which are sharply demarcated from more peripheral cell regions. Note absence of perinuclear halo. (3b) Macrophage showing perinuclear halo resulting from marked rounding of the centrosphere region. Thin, tortuous cytoplasmic extensions remain in cell periphery at sites of attachment to glass.

indices of the cytochalasin B effect are shown in Fig. 2. It can be seen that the dose-response relationship describing the cytochalasin B-induced changes in cell shape is nearly the same as that for inhibition of phagocytosis. Less than 10% of the cells exhibited the morphologic changes by 5 x 10^{-7} M cytochalasin B and 98% of the cells were affected by 2.1 x 10^{-5} M cytochalasin B. A concentration of 5.0 x 10^{-4} M cytochalasin B was required to produce changes in 50% of the cells.

Inhibition of Hexose Transport by Cytochalasin B

The 2-deoxy analogue of glucose, a hexose which is transported and phosphorylated but not metabolized, was selected for the hexose transport studies. Cytochalasin B at a concentration of 1.0 x 10^{-5} M resulted in complete inhibition of [2-^4H]deoxyglucose transport (Fig. 4). Hexose uptake was reduced to 50% of control value by 2 x 10^{-7} M cytochalasin B. DMSO (0.2%) had no effect on hexose transport. Preincubation of the cells with cytochalasin B was not required to achieve the full effect (results not shown).

The finding that cytochalasin B-induced changes in cell shape and inhibition of phagocytosis required tenfold greater concentrations of the drug than were necessary to inhibit 2-deoxyglucose uptake provided evidence against, but did not exclude, the possibility that these effects could be explained solely by cytochalasin B inhibition of hexose transport. To examine this possibility the effects of glucose deprivation on macrophage morphology and phagocytic activity were examined. The phase-contrast appearance of cells cultivated for time periods up to 24 h in hexose-free medium was identical to that for controls. There was no evidence of toxicity, no decrease in cell number, and the arborization and cell rounding typical of cytochalasin B-treated cells were not observed. In addition it was found that the phagocytic uptake of PVT particles by macrophages preincubated in hexose-free media for 2 h was identical to that for control cells.
Ultrastructural Studies of Cytochalasin B Effects

Scanning electron microscopy was performed to obtain a more detailed view of the gross morphologic changes produced by cytochalasin B. A control macrophage with its low profile, thin veil of peripheral cytoplasm, and relatively smooth surface is shown in Fig. 5 a. After 10 min of exposure to cytochalasin B (1.0 × 10⁻⁵ M) (Fig. 5 b) the peripheral cytoplasm of the cells was markedly retracted, producing the rounded and raised central portion of the cell. It was the zone of abrupt change in cell thickness that produced the perinuclear halo seen in the phase micrographs. Extensive infolding of plasma membrane resulted in the wrinkled appearance of the cytochalasin B-treated cell.

Longitudinal sections through cytochalasin B-treated cells confirmed the fact that unique configurational changes are induced by the drug. Fig. 6 illustrates the severe rounding of the central portion of the cell but also shows that certain portions of the “base” of the cell (arrows) maintained tight contact with substratum despite cytochalasin B treatment.

In view of the suggestions that microfilaments may participate in the movement of plasmalemma required for cell spreading and phagocytosis, it was of interest to examine the effects of cytochalasin B on the microfilamentous structures of the macrophage. The ultrastructural characteristics and intracellular distribution of the microfilamentous system of normal-cultivated mouse peritoneal macrophages have been described in a previous publication from our laboratory (7). The microfilament system is normally composed of two components: (a) a randomly oriented microfilament network, composed of short segments of 40–50-Å microfilaments, which is present as a 400–600-Å layer immediately subjacent to the plasmalemma of all regions of the cell and (b) an organized system of bundles of 40–50-Å microfilaments found immediately subjacent to the microfilament network on the glass-attached surface of macrophages or in association with newly forming phagocytic vacuoles on the free surface of macrophages. Fig. 7 is a typical section through the substrate-associated subplasmalemmal region of a normal macrophage in which portions of the microfilament network (A) and organized microfilaments (B) are represented.

Exposure of the cells to cytochalasin B at a concentration that inhibits hexose transport by 70% (6 × 10⁻⁷ M), but does not appreciably interfere with phagocytosis, has no discernible effect on either the microfilamentous network or the organized bundles of subplasmalemmal microfilaments normally present in these cells. However, exposure of cells to 1–2 × 10⁻⁵ M cytochalasin B, concentrations which normally inhibit phagocytosis by 95–99%, produced marked morphologic changes in the region of the organized bundles of microfilaments. Within 10 min of
FIGURE 5 a Scanning electron micrograph of control macrophage showing well spread peripheral cytoplasm and gently curving convex cell surface. × 7,500.

FIGURE 5 b Macrophage treated with cytochalasin B (1.0 × 10⁻⁴ M) for 10 min. Note marked centrosphere rounding, retraction of peripheral cytoplasm leaving slender cytoplasmic extensions, and marked infolding of plasma membrane. × 7,500.
addition of cytochalasin B to the media, the organized, long-strand 40-50-Å microfilaments of this layer were no longer visible. Instead the region appeared filled with short segments of microfilaments which were not oriented in any particular manner and which were morphologically similar to components of the microfilamentous network that normally occupies only the most superficial 400-600-Å subplasmalemmal layer of these cells.

These ultrastructural changes were best appreciated by analysis of serial thin sections of the subplasmalemmal region of cells cut parallel to the plane of glass attachment. Fig. 8 is a low power view of a slice taken through the bulbous tip of a substrate-attached cytoplasmic extension of such a cytochalasin B-treated cell. The encircled area represents the region from which higher magnification electron micrographs (Fig. 9 a-d) were obtained from 4 adjacent sections. These sections extended from a region of the cell which included plasma membrane (9 a) through the subplasmalemmal area (9 b and 9 c) into the deeper regions of the cell characterized by ribosomes, lysosomes, and other cell organelles (9 d). The

FIGURE 6 Longitudinal section of 48-h cultivated macrophage treated with cytochalasin B (2.0 × 10⁻⁵ M) for 60 min. Note severe rounding of the central portion of the cell. Projections such as (A) may remain in contact with the substrate surface (arrows) for long distances. The numerous vacuoles (V) suggest that the section was taken from a cell with a convoluted surface. O₂,OsO₄-glutaraldehyde fixation. × 27,600.
important feature of this series of sections is a lack of the well-defined layer of organized microfilaments always seen in control cells.

DISCUSSION

Dose-response relationships for cytochalasin B inhibition of phagocytosis and alteration of cell shape in the cultivated macrophage have been examined to determine if a correlation exists between these processes requiring plasma membrane translocation. Our findings suggest that inhibition of phagocytosis produced by cytochalasin B cannot be dissociated from effects of the drug on plasma membrane movement. Dose-response curves for the inhibition of phagocytosis and changes in cell shape were virtually identical requiring $2-5 \times 10^{-4}$ M and $1-2 \times 10^{-6}$ M cytochalasin B to produce inhibition of these functions by 50% and 100%, respectively. The time course required to produce the effects as well as that required to reverse them after removal of cytochalasin B were similar.

Additional studies, employing time-lapse phase-contrast photomicroscopy, confirmed the finding of Allison et al. (5) that cytochalasin B markedly reduced undulating movement of macrophage plasma membrane. Inhibition of both undulating plasmainema movement and phagocytosis required similar concentrations of cytochalasin B.

In contrast to the finding that cell functions requiring plasma membrane movement exhibited nearly identical sensitivity to cytochalasin B, we found that tenfold lower concentrations of cytochalasin B were required to achieve similar inhibition of hexose transport. Our finding that 50% inhibition of 2-deoxyglucose uptake by the macrophage required $2-3 \times 10^{-7}$ M cytochalasin B is in close agreement with results obtained by Mizel and Wilson (12) for other mammalian cell lines. In HeLa cells, for example, 50% inhibition of uptake of $1.3 \times 10^{-7}$ M and $1.0 \times 10^{-3}$ M 2-deoxyglucose...
was produced by $8 \times 10^{-8}$ M and $2.4 \times 10^{-7}$ M cytochalasin B, respectively.

We consider it unlikely that inhibition of phagocytosis and cell movement produced by cytochalasin B can be accounted for solely by inhibition of hexose transport. In addition to marked differences in dose-response relationships outlined above, our findings that macrophages maintained in hexose-free media showed neither a reduction in maximal linear rate of particle uptake nor exhibit-

FIGURE 8 Low-power electron micrograph taken through bulbous extension of macrophage treated as in Fig. 6. Section has been cut parallel to substrate-attached surface of cell. Encircled area denotes region of attached cytoplasm from which four serial sections (9 a–d) were photographed. O$_2$O$_2$-glutaraldehyde fixation. $\times 12,600$.

FIGURE 9a–9d Progressively deeper 500-Å thick serial sections through entire subplasmalemmal region of portion of macrophage shown in Fig. 8. The primary feature of all sections in this series is the uniformity of the microfilament network which comprises the background material of each slice. The most superficial slices (9 a, b) also contain numerous small vacuoles which have been formed by shallow indentations of the plasma membrane, whereas the deeper slices (9 c, d) show increasingly prominent clusters of polysomes and other structures characteristic of the central portion of cells. Section 9 c shows sparse arrays of organized microfilaments at arrow. Compare these with microfilaments from comparable slice of untreated cell (Fig. 7, site B). Asterisks suggest landmarks to be used in following the development of structures in adjacent sections. $\times 30,000$. 

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The cytochalasin B-sensitive microfilaments we described in the cultivated macrophage were not visualized. The authors did not examine the cytochalasin B sensitivity of the microfilaments. Malawista et al. (6) reported the existence of "skeins" of randomly oriented microfilaments near the periphery of rabbit alveolar macrophages that were less prominent after cytochalasin B treatment. In contrast, we could find no ultrastructural evidence that cytochalasin B, at doses comparable to those used by Malawista et al., altered the morphology of randomly oriented microfilaments. Although it is possible that mouse and rabbit macrophages differ in their microfilamentous structures, an alternate and more plausible explanation for the apparently conflicting findings in the two studies may lie in differences in technique used for maintenance and processing of cell preparations. We have found that microfilament bundles are seen rarely in preparations of randomly oriented cells but are readily visualized in monolayer preparations in which the original cell orientation has been preserved. It is thus possible that the microfilament "skeins" observed by Malawista et al. in rabbit macrophage suspensions are analogous to the class of microfilaments identified as oriented microfilament bundles in mouse macrophage monolayers.

Thus, specific organized subplasmalemmal microfilaments appear to be disaggregated by the same dose of cytochalasin B that inhibits macrophage plasma membrane functions relating to movement. It seems relevant that microfilaments of this class are not always present in the subplasmalemmal region of cells but appear to form only when specifically required (7). We believe that the unique response of organized subplasmalemmal microfilaments to the same dose of cytochalasin B which interfered with related plasma membrane movement strengthens the hypothesis (5-7) that microfilaments participate in these functions.

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