EFFECT OF CYTOCHALASIN B ON THE ADHESION OF MOUSE PERITONEAL MACROPHAGES

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ABSTRACT

The adhesion of normal mouse macrophages to glass surfaces was reduced by nontoxic levels (1–50 μg/ml) of cytochalasin B in combination with a centrifugal force (1,000–8,000 *g*). Macrophages nonspecifically activated by *Corynebacterium acnes* were also detached by this treatment, but less effectively. The effects of cytochalasin B treatment on these cells were shown to be reversible. After detachment, the cells reattached to glass, appeared morphologically normal, and behaved like untreated cells as judged by adhesion, acid phosphatase levels, and phagocytosis. The effect of cytochalasin B on several parameters of phagocytosis by normal macrophages was also examined. The results demonstrate that cytochalasin B can be used to detach macrophages from surfaces and suggest a functional relationship between phagocytosis and macrophage adhesion to surfaces. Furthermore, the effect of cytochalasin B on adhesion of phagocytic cells provides a probe for further investigation of the adhesion of cells to surfaces.

Interest in the adhesion of nonphagocytic cells to substratum has received attention recently because of the evidence that normal and transformed cells have different adhesive properties (21). Electron microscope studies (3, 5, 7) as well as the reported capacity of agents such as proteases and divalent metal chelating agents (22), dibutyryl cyclic AMP (9), and Colcemid (21) to affect adhesion have prompted theories concerning the adhesion of nonphagocytic cells. It has been suggested that microtubule-microfilament arrays associated with attachment devices are located in patches at the cell substratum interface (3, 5, 6, 21) and attach to the substratum by a process which is sensitive to both ethylenediaminetetraacetic acid (EDTA) and proteases.

Adhesion of macrophages and other phagocytic cells has been more difficult to investigate, partially due to the lack of practical methods for detaching these cells; the only techniques currently available involve mechanical manipulation, i.e., scraping or the use of high shear forces, or treatment with toxic agents. Some of the first insights into this system were provided by North (17) who invoked morphological evidence to theorize that adhesion to and spreading of phagocytic cells on surfaces reflected an attempt by the cell to phagocytize that surface as a foreign body. Subsequently, Reaven and Axline (20), using electron microscopy, observed arrays of microfilaments, microtubules, and microchannels at the cell-substratum interface and at the free surface when in contact with a phagocytic stimulus. These arrays were shown in later studies to be disorganized by cytochalasin B and were thought to be involved in both adhesion and phagocytosis (2).

While attempting to enucleate macrophages by utilizing cytochalasin B and centrifugal force (8,

16), we observed that cells were detached from glass cover slips. This prompted an investigation into the possible effects of cytochalasin B on the adhesion of these cells. The cytochalasins are mold metabolites that have been shown to exert a wide variety of effects on cells (4, 15, 16) including the capacity to inhibit phagocytosis (1, 14), cell mobility and ruffled membrane formation (7), plasma membrane mobility (2), and protein synthesis (12). Many of these effects appear to be related to the cytochalasin's abilities either to alter microfilament function (1) or inhibit hexose transport across cell membranes (17), both of which are reversible upon removal of the drug.

In the experiments presented in this report, phagocytic cells (murine peritoneal macrophages) were detached from the substratum by relatively low centrifugal forces combined with cytochalasin B treatment under conditions which failed to affect the adhesion of nonphagocytic cells (fetal lamb kidney, mouse L929). These studies were undertaken to investigate this detachment process and the mechanism of macrophage adhesion to surfaces.

MATERIALS AND METHODS

Cells

Seiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) were injected intraperitoneally with 2 ml of 2.4% thioglycollate broth. 4 days later the macrophages were harvested by lavage of the peritoneal cavity with phosphate-buffered slaine (PBS). Cells were allowed to attach to circular glass cover slips, 22 mm in diameter (Van Water and Rogers, Salt Lake City, Utah), at a concentration of 1×10^6 macrophages per cover slip, and incubated 24 h before further use. Cell cultures were maintained in Eagle's minimal essential medium (MEM) supplemented with decomplemented fetal calf serum (10%), penicillin (100 μg/ml), streptomycin (μg/ml), and tylosine (24 μg/ml). Cells were incubated in an environment of 5% CO_2 in humidified air at 37°C.

Activated macrophages were obtained from mice injected 10 days previously with 6-mg wet weight of heat-killed *Corynebacterium acnes* and 4 days previously with 2 ml of 2.4% thioglycollate broth. These cells were then harvested and treated in the same way as the normal macrophages.

Detachment Assays

In experiments to evaluate the effect of cytochalasin B on adhesion, cells were allowed to attach to glass cover slips and incubated for 24 h. Culture medium was then removed, and the cells were treated for 30 min at 37°C with cytochalasin B dissolved in MEM. The cover slips were then inverted (cell-side down) in 50-ml centrifuge tubes containing 5 ml of MEM containing the drug and centrifuged for 1 h at 37°C at various forces in a Beckman J-21 centrifuge with a type JS-13 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) prewarmed to 37°C. The cover slips were then removed and placed cell-side up in plates with fresh media lacking the drug. The amount of detachment was determined by examining five different portions of the cover slips and counting the number of remaining attached cells. The number of attached cells from the treated group was compared to control cells treated in an analogous manner except for the absence of the drug in the media. The efficiency of detachment was expressed as a percentage of the number of attached cells in the test group compared to the number of remaining attached cells in the control group.

Reattachment of Macrophages

For the studies on the effect of detachment on cell function, cells were allowed to attach to cover slips at a density of 1×10^6 per cover slip and incubated for 24 h before treatment with cytochalasin B (10 μg/ml) for 30 min. After centrifugation for 1 h at 5,860 *g* in an inverted position, the cover slips were then removed from the tubes, and the supernate was decanted. The detached cell pellet was resuspended in PBS and washed twice by centrifugation (250 *g* for 10 min). After the last wash, the cells were resuspended in new medium without cytochalasin B and allowed to reattach to clean cover slips at a density of 1×10^6 cells per cover slip. The cells were then incubated for 24 h before further testing.

Acid Phosphatase Assay

For acid phosphatase determination, 3×10^6 cells were scraped from cover slips, washed once in PBS, and resuspended in 0.5 ml of distilled water. The cells were then freeze-thawed twice, and the enzyme level was assayed using Sigma phosphatase kit no. 104 (Sigma Chemical Co., St. Louis, Mo.). The lysate was added to a buffered solution of the substrate, *p*-nitrophenyl phosphate, and incubated at 37°C. *p*-Nitrophenyl phosphate is a colorless compound until hydrolyzed by acid phosphatase to *p*-nitrophenol which is yellow at alkaline conditions. After 30 min, the reaction was stopped by the addition of NaOH, and the adsorbance at 410 nm was measured and converted to units of enzyme activity as detailed in the kit bulletin. The results of the test on macrophage lysates were expressed in units of enzyme activity per 1×10^6 cells.

Phagocytosis

For assay of phagocytic activity, cover slips with 1×10^6 cells were exposed to approx. 1.0×10^7 heat-killed yeast and incubated for 20 min at 37°C. The cover slips

were then removed, fixed in methanol, and stained with Wright's stain. Between 100 and 200 macrophages were examined, and the results were expressed with respect to three different parameters of yeast-cell interaction: (*a*) the fraction of cells that were actively phagocytic was calculated by dividing the number of cells with attached or ingested yeast by the total number of cells examined; (*b*) the average number of yeast phagocytized per cell was calculated by dividing the total number of attached or ingested yeast by the total number of observed cells; and (*c*) the percentage of phagocytized yeast actually ingested was calculated by dividing the number of ingested yeast observed by the total number of yeast observed attached to or ingested by cells.

Materials

Cytochalasin B (Calbiochem, San Diego, Calif.) was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and used at final concentrations from 1 to 50 μg/ml in MEM. Control medium consisted of MEM plus DMSO alone. Trypsin (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) was suspended at 0.2% in a citrate-buffered salt solution (pH 7.2). Disodium EDTA (Fisher Scientific Co., Fair Lawn, N. J.), was used at a concentration of 0.02% in a phosphate-buffered salt solution (pH 7.2).

RESULTS

Cytochalasin B Effect on Adhesion

The effect of cytochalasin B on the adhesion of normal macrophages was evaluated by treating the cells with different concentrations of the drug (1–50 μg/ml) and then subjecting them to varying centrifugal forces (1,000–8,000 *g*) in a direction perpendicular to their attached surfaces. Cells not treated with cytochalasin B were not detached by applied forces up to 8,000 *g*, nor were cells detached without centrifugation when treated with cytochalasin B at concentrations up to 50 μg/ml. However, significant detachment was observed when cytochalasin B treatment was combined with a centrifugal force. Fig. 1 shows the percentage of cells detached in relation to the applied centrifugal force for each concentration of cytochalasin B tested. As little as 1 μg/ml of cytochalasin B was able to cause detectable detachment when forces greater than 4,000 *g* were used. As the concentration of drug was increased forces were required to produce similar results. For example, 5 μg/ml combined with 6,000 *g* was able to effect detachment of approx. 90% of the cells, while increasing the concentration of drug to 10 μg/ml decreased the force required to detach 90% of the cells to 2,400 *g*. Increasing the cytochalasin B concentration above 10 μg/ml resulted in little change in the kinetics of detachment, and concentrations of 25 and 50 μg/ml were indistinguishable in their effect on adhesion. However, increasing concentrations of cytochalasin B appeared to be somewhat toxic to the remaining attached cells as evidenced by their fragmentation and loss of internal organization.

It has been reported that macrophages stimulated by various agents such as BCG or endotoxin spread more rapidly and adhere more firmly to surfaces than normal macrophages (13). It was of interest to determine if we could detect, by our assay, differences in the adhesive properties of these two different cell types. The effectiveness of combined cytochalasin B treatment and centrifugation to detach macrophages activated nonspecifically by exposure of the donor animal to *Corynebacterium acnes* was evaulated, and the results are

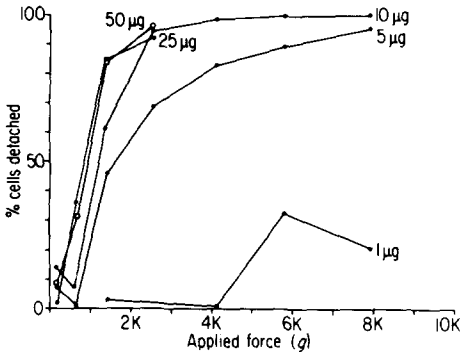

FIGURE 1 Effect of combined cytochalasin B treatment and applied centrifugal force on adhesion of normal macrophages. Cells were allowed to attach to glass cover slips and incubated for 24 h. Cells were then treated with different concentrations of cytochalasin B for 30 min at 37°C. The coverslips were then inverted in 50-ml centrifuge tubes containing 5 ml of drug suspension and were centrifuged for 1 h at 37°C in a Beckman J-21 centrifuge with a type JS-13 rotor which had been prewarmed. The cover slips were then removed and inverted in plates with fresh media without drug. The amount of detachment was determined by examining five different areas on the cover slips and counting the number of remaining, attached cells. The number of attached cells in the drug-tested group was expressed as a percentage of cells treated in an analogous manner but without the drug. Each line represents one concentration of cytochalasin B with applied centrifugal force plotted on the abscissa and the percentage of cells detached by this procedure plotted as the ordinate.

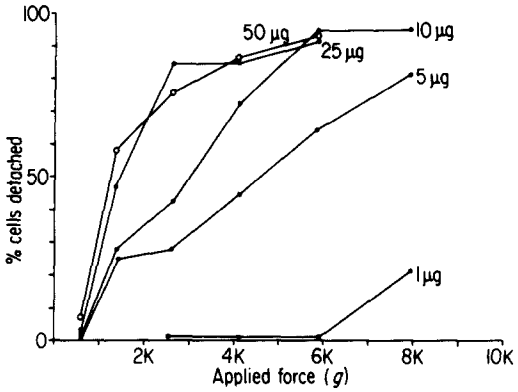

FIGURE 2 Effect of combined cytochalasin B treatment and applied centrifugal force on adhesion of activated macrophages. Activated macrophages were treated and evaluated as in Fig. 1.

illustrated in Fig. 2. Activated macrophages, like normal macrophages, were not detached by either drug treatment or centrifugation alone, and detachment could be seen only when the two were combined. On a qualitative basis, the response to the drug was similar to that exhibited by normal macrophages, but activated macrophages seemed to be less sensitive. For example, 10 µg/ml of cytochalasin B combined with 2,400 *g* was able to detach 95% of normal macrophages, but only 43% of activated macrophages. To detach 95% of the activated macrophages using the same drug concentration, it was necessary to increase the force to 6,000 *g*. It is not known whether activation stimulates all macrophages in the peritoneal cavity or merely a subpopulation, and in fact it is not clear from our data whether we are measuring the increased adhesiveness of activated macrophages as a whole or just an altered subpopulation of normal macrophages.

Effect of Detachment on Macrophage Function

The next series of experiments was designed to determine whether cytochalasin B actually affects the adhesion process of the cell or whether simply detachment results from a general toxic effect of the drug. For these experiments normal macrophages, which had been attached for 24 h, were detached by using cytochalasin B at 10 µg/ml combined with an applied force of 6,000 *g*. The detached cell pellet was resuspended and washed twice in PBS. Approx. 95% of the macrophages initially seeded onto the cover slip could be recovered with a viability greater than 90% as evaluated by trypan blue exclusion. There was also no evidence of enucleation in the detached cells when stained with Wright's stain and examined by light microscopy.

The detached and washed cells were allowed to reattach to clean cover slips at a density of 1×10^6 cells per cover slip and incubated for 24 h at 37°C. At this time, they were firmly attached to the glass surface and could not be detached by an applied force of up to 8,000 *g* unless combined with cytochalasin B treatment. Their sensitivity to cytochalasin B was not lost after the initial detachment, and cells could be detached and allowed to reattach several times without any obvious loss of effectiveness of the drug. Normal macrophages are also resistant, relative to nonphagocytic cells, to detachment by treatment with trypsin or EDTA. Reattached macrophages were completely resistant to treatment with 0.2% trypsin or 0.02% EDTA without centrifugation, while these compounds at these concentrations can detach most nonphagocytic cells.

Reattached cells were also examined with the scanning electron microscope. Samples were washed, fixed in 2% glutaraldehyde, and then observed at various magnifications. Fig. 3 A shows a normal macrophage which has not been treated with cytochalasin B. It has attached and spread out across the surface, exhibiting the pseudopodia characteristic of these cells when not in direct contact with other cells. Fig. 3 B shows a normal macrophage which has been treated with 10 µg/ml of cytochalasin B for 90 min. While the outline of the cell appears to be fairly normal, most of the internal material of the cell has retracted towards the center, causing it to "round up." If this cell is now subjected to centrifugation, detached, and allowed to reattach, it takes on the appearance of the cells in Fig. 3 C. These cells have been reattached for 20 min and have not yet begun to spread out onto the surface. If these cells are then incubated further, they begin to spread out on the glass and assume an appearance indistinguishable from untreated cells. Fig. 3 D shows macrophages detached by cytochalasin B, allowed to reattach, and incubated for 48 h. They appear similar to normal cells in size and shape.

The intracellular enzyme level was also examined in reattached cells as another parameter of normal macrophage function. Acid phosphatase was selected as a representative enzyme and the intracellular content was measured as described in

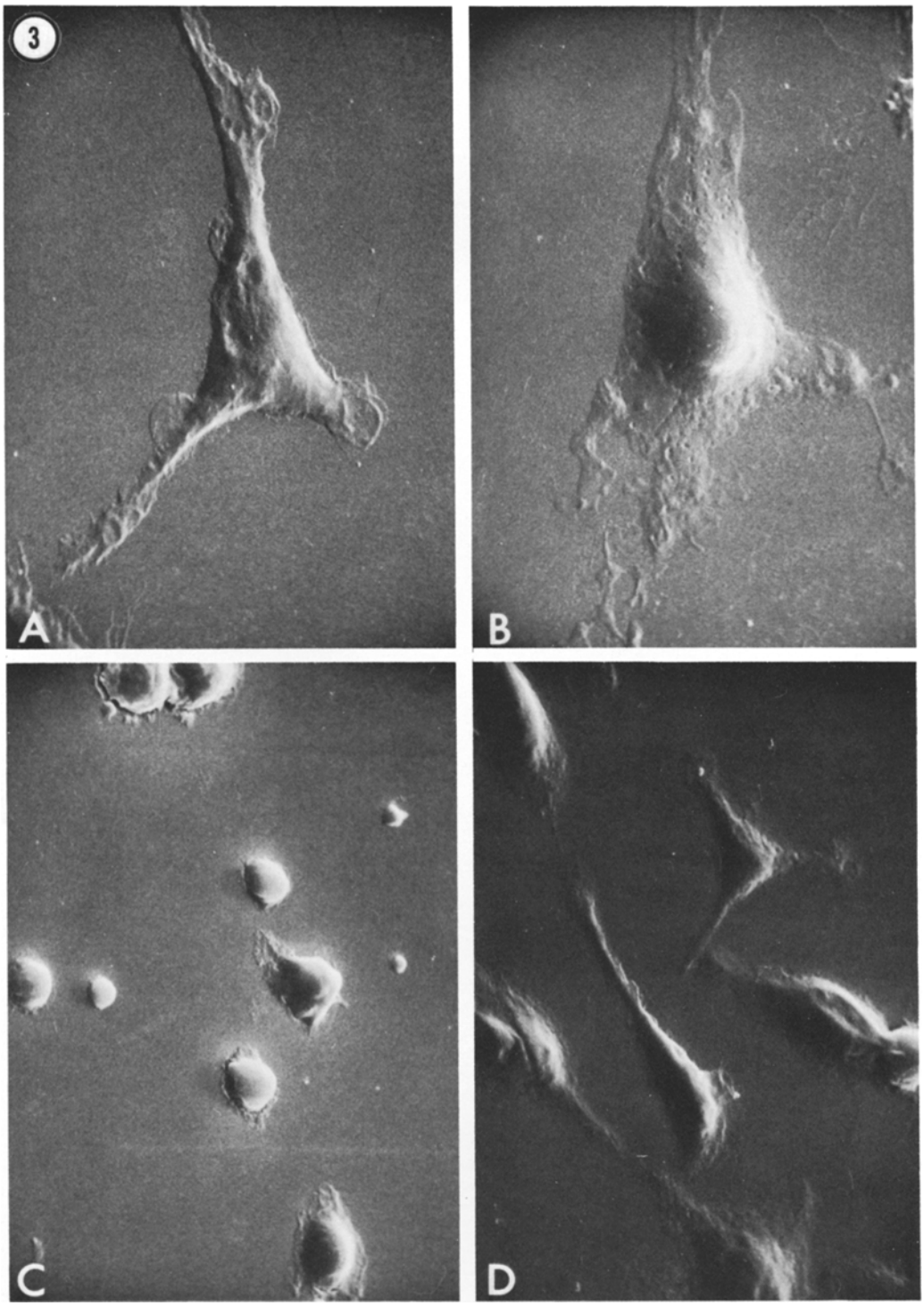

FIGURE 3 Scanning electron micrographs of macrophage treated and detached with cytochalasin B. (A) Normal, untreated macrophage. × 2,000. (B) Normal macrophages treated with 10 μg per ml of cytochalasin B for 90 min at 37°C. × 2,000. (C) Normal macrophages treated with cytochalasin B at 10 μg per ml, detached by centrifugation at 6,000 *g*, and allowed to reattach for 20 min. × 1,000. (D) Normal macrophages treated with cytochalasin B at 10 μg per ml, detached by centrifugation at 6,000 *g*, and allowed to reattach for 48 h. × 1,000.

Materials and Methods. At 24 h postreattachment, reattached cells contained more acid phosphatase (0.24 ± 0.02 U/10^6 cells) than untreated cells (0.18 ± 0.03 U/10^6 cells). By 48 h postreattachment, however, both untreated and reattached cells contained identical levels of acid phosphatase activity (0.29 ± 0.01 U/10^6 cells).

Phagocytosis of heat-killed yeast was also used to evaluate the function of reattached macrophages, and the results are summarized in Table I. Untreated macrophages were examined 24 and 48 h after attachment, and detached cells, 24 and 48 h after reattachment. In Table I, the second column represents the fraction of macrophages of the total population of cells which had yeast attached to them. The third column shows the average number of yeast attached per cell, and the last column shows the fraction of attached yeast which had actually been ingested by the macrophages. At 24 h postreattachment, all three parameters were depressed relative to the values found for untreated cells. By 48 h postreattachment, both the number of actively phagocytic cells and the percentage of attached yeast actually ingested were within the normal range. The average number of yeast per cell remained below the values found for untreated cells.

These data suggest that cytochalasin B does not exert its effect on macrophage adhesion through a permanently toxic mechanism. Although some cell functions are altered at 24 h after treatment, by 48 h postreattachment the treated cells are practically indistinguishable from untreated cells. Apparently, treatment for 90 min followed by removal of the drug does not permanently alter macrophage functions.

Cytochalasin B Effect on Phagocytosis

As stated previously, North has postulated that the attachment to and spreading upon a surface reflects an attempt by that macrophage to phagocytize the surface. It has also been previously demonstrated that cytochalasin B can reversibly inhibit phagocytosis (2, 10, 14). To further examine this relationship between adhesion and phagocytosis, we examined the effects of cytochalasin B on phagocytosis at the drug concentrations which affected adhesion.

Cells were treated with different concentrations of cytochalasin B for 70 min and then exposed to 10^7 heat-killed yeast. Incubation was continued for 20 min, and the cells were washed free of unattached yeast, fixed, and stained. Phagocytosis was evaluated as before, and the results are shown in Table II. All three parameters were seen to be affected by as little as 1 μg/ml. The number of actively phagocytic cells is reduced slightly by 1 μg/ml, and no further change is seen until 50 μg/ml. The average number of yeast per cell is reduced by 50% by as little as 1 μg/ml, but no further reduction is seen until the 50 μg/ml level is reached. This reduction may be due in part to the fact that the cells round up, which may reduce their effective surface area. Ingestion of yeast is also affected by increasing the drug concentration to 25 μg/ml. Increasing the drug concentration above this level does not change the effect upon ingestion.

TABLE I

Comparison of Phagocytosis by Normal and Reattached Macrophages

Cell type post-adsorption		Cells with yeast absorbed / total cells	Total yeast / total cells	Ingested yeast / total cells
	h			
Untreated	24	0.98 ± 0.01	6.38 ± 0.66	0.84 ± 0.03
Reattached	24	0.90 ± 0.01	5.58 ± 1.79	0.68 ± 0.02
Untreated	48	0.98 ± 0.02	6.29 ± 1.22	0.85 ± 0.06
Reattached	48	0.97 ± 0.01	4.38 ± 0.80	0.84 ± 0.02

For assay of phagocytic activity, 1 × 10^6 cells were incubated with 10 × 10^6 heat-killed yeast and incubated for 20 min at 37°C. The cells were washed, fixed in methanol, and stained with Wright's stain. Between 100 and 200 were examined with light microscopy. Results are expressed as the average of three experiments ± the standard deviation. The second column represents the fraction of the total cells which had yeast attached. Column 3 represents the average number of yeasts attached per cell. Column 4 represents the fraction of yeasts ingested of all attached yeasts.

TABLE II

Cytochalasin B Inhibition of Phagocytosis

Drug	Cells with yeast absorbed / total cells	Total yeast / total cells	Ingested yeast / total cells
$\mu g/ml$			
0	0.98 ± 0.01	6.38 ± 0.66	0.84 ± 0.03
1	0.87 ± 0.05	3.52 ± 0.59	0.53 ± 0.01
5	0.91 ± 0.05	3.89 ± 0.39	0.34 ± 0.01
10	0.90 ± 0.01	3.67 ± 0.86	0.20 ± 0.02
25	0.87 ± 0.06	3.30 ± 0.36	0.17 ± 0.04
50	0.68 ± 0.01	1.83 ± 0.51	0.14 ± 0.01

1×10^6 cells were exposed to different concentrations of cytochalasin B for 70 min at 37°C. They were then incubated with 10×10^6 yeast for 20 min at 37°C while still in the presence of the drug. The results were evaluated and expressed as in Table I.

The effect of cytochalasin B on adhesion parallels most closely the inhibition of ingestion rather than attachment of the target to the cell.

DISCUSSION

The data from these experiments demonstrate that cytochalasin B inhibits the adhesion of mouse peritoneal macrophages to surfaces. The treatment of cells by cytochalasin B renders them susceptible to removal when a centrifugal force is applied. The effect of the drug appears to be completely reversible, as are many of its effects on cells. After detachment, macrophages will readhere to surfaces, remain protease and EDTA resistant, possess normal acid phosphatase level, and phagocytize yeast, all suggesting that they retain normal function after removal of the drug.

The mechanism of the cytochalasin B effect on macrophage adhesion is not entirely clear from these studies. The cytochalasins exhibit a variety of effects which might be responsible. For instance, it has been reported that cytochalasin B inhibits protein synthesis in HeLa cells (12). In preliminary results not reported here, we investigated this possibility by measuring protein synthesis in macrophages treated with cytochalasin B and could find no significant effect. Moreover, cycloheximide at 10 μg/ml, which inhibited protein synthesis over 90% in these cells, had no effect on adhesion as measured by our assay.

Another possible mechanism is the reported effect of the cytochalasins on cell microfilaments (8, 16). Dependent upon the cell type, the cytochalasins can cause either disruption or contraction of some of the cell's microfilament system. The importance of microfilaments in macrophage adhesion and phagocytosis has been emphasized by Reaven and Axline (20). Using electron microscopy, these authors examined serial sections of attached cells cut parallel to the adhering surface and found that organized bundles of microfilaments in the resting cells were found mainly at the cell surface adjacent to the substratum. In phagocytizing cells, these structures were also found in membrane areas in contact with attached targets. It was hypothesized that these bundles are organized in response to contact with a foreign object, either yeast or a glass surface, and assisted in the spreading of the cell around that object. This increases the surface area of the cell in contact with the target and hence increases the number of adhesion organs which can adhere to the target. In the model proposed by these authors, cytochalasin B-sensitive microfilaments may not represent the actual adhesion organs but may only facilitate their contact with the target. In our studies, cytochalasin B treatment by itself is never sufficient to detach cells completely. Some force must be applied to separate the cells from the surface; cytochalasin B only reduces that required force. In our hands, macrophages settle and attach quite naturally in the presence of cytochalasin B, but their spreading across the substratum is completely inhibited. Also, in our phagocytosis studies the attachment phase is inhibited to a much lesser degree than ingestion, a process thought to be analogous to cell spreading. Our data, therefore, seem to be consistent with the concept that cytochalasin B either reduces the actual number of adhesion organs in contact with a target or at least reduces their efficiency of operation. There are many other effects of cytochalasin B on cells,

however, and it is not possible to define the role of each of these activities, but the interference with microfilament function presents a plausible mechanism in light of other reports.

Although the method described in this report permits detachment of macrophages from surfaces, the actual mechanism or process of adhesion remains unclear. However, this technique does provide a method for removing macrophages from surfaces with little long-term effect on the cells or for possibly even allowing the selective separation of these cells from a mixed, adherent population. At 2,500 *g* with 10 μg/ml, the effect of cytochalasin B is minimal on nonphagocytic cells, and yet 90% of the macrophages can be detached. More importantly, a combination of cytochalasin B and centrifugation can aid in the study of the adhesion organelles, and inhibitors of other cell functions can be more efficiently screened for their effect on adhesion, possibly providing evidence concerning the function of the adhesion organelles themselves.

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