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# CYTOCHALASIN B REVERSIBLY INHIBITS PHAGOCYTOSIS: FUNCTIONAL, METABOLIC, AND ULTRASTRUCTURAL EFFECTS IN HUMAN BLOOD LEUKOCYTES AND RABBIT ALVEOLAR MACROPHAGES‡

Phagocytosis involves several specific sequential and overlapping activities, which include contact between particle and cell, ingestion, increased metabolic activity, fusion of lysosomal structures with the vacuoles containing ingested material, and intracellular killing and digestion. The techniques for separating these activities may distinguish those that are causally related from others that are merely associated, and may reveal the specific pathways involved.

In human polymorphonuclear leukocytes (PMN), for example, colchicine and vinblastine are known to cause the disruption of normal microtubules.[3-5] These agents also interfere with lysosomal degranulation and the formation of digestive vacuoles in PMN, without altering particle ingestion and intracellular killing. Thus, microtubules may facilitate the association of lysosomes with vacuoles containing the ingested material.[6-10]

The recently introduced mold metabolite, cytochalasin B, may provide an opportunity to dissect the phagocytic process further. This agent also affects certain types of motility, of cells and within cells,[11] and may specifically interfere with the function of contractile systems of microfilaments, as distinguished from microtubules.[12-14] In order to study the possible role of microfilaments in phagocytosis, we examined the effects of cytochalasin B on phagocytosis of bacteria by human blood leukocytes and rabbit alveolar macrophages.

## MATERIALS AND METHODS

Human blood leukocytes. As described previously,[6] heparinized venous blood was sedimented in 2 volumes of 3 percent dextran, and the leukocyte-rich supernatant was sedimented and washed in modified Krebs-Ringer phosphate buffer, pH 7.4. The cells were osmotically shocked (to lyse red cells), restored to isotonicity, washed once more in buffer, counted, and adjusted to about $3 \times 10^7$ white blood cells (WBC) per ml. Differential counts were performed.

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*Rabbit alveolar macrophages.* As described previously,[15] alveolar macrophages were harvested in Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose. The average yield was $1 \times 10^8$ cells per rabbit.

*Bacteria. Staphylococcus aureus* strain 502A[16] was shaken overnight in beef heart infusion broth at 37°C, centrifuged, and washed once in normal saline, and the bacteria were suspended in albumin (10 mg/100 ml) buffer. The concentration of staphylococci was estimated from a constructed curve of transmittance at 600m$\mu$ and confirmed by quantitative plating in agar. For metabolic studies heat-killed staphylococci[6,15] were used.

*Drugs.* Cytochalasin B (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, England; mol. wt. 479) was dissolved in dimethylsulfoxide (DMSO spectranalyzed, Fisher Scientific Co., Pittsburgh, Pa.) at a concentration of 3 mg/ml. Aliquots were stored at minus 70°C until use, at which time they were diluted in aqueous media. In most experiments, DMSO was used as an additional control in at least the concentration at which it was present with cytochalasin B (and sometimes two-to-four times that concentration).

*Phagocytosis.* Leukocytes in 12 percent autologous serum-heparinized buffer, with or without cytochalasin B and DMSO, were shaken in an Eberbach Incubator Model 6250 (Eberbach Corp., Ann Arbor, Michigan), 100 reciprocations per minute, at 37°C for 10 minutes. Then bacteria were added (0 time), and the incubation continued. Duplicate samples were taken at intervals of 20 and 40 minutes, and the cells were separated from the media by centrifugation and washing. Both the bacteria remaining in the supernatants and the cell-associated live bacteria were enumerated to determine, respectively, uptake and killing of bacteria by leukocytes. Bacteria were also enumerated after incubation in serum-buffer without white cells. The detailed procedures were as previously described.[9,6] Experiments were done in 25-ml Erlenmeyer flasks, with total volumes of 2.8, 4.2, or 5.6 ml, and at various ratios of staphylococci to polymorphonuclear leukocytes.

In experiments designed to test the reversibility of effects of cytochalasin B, aliquots of cells, one with cytochalasin B, 2 $\mu$g/ml, were shaken in 35 ml centrifuge tubes at 37°C for 10 minutes. Then all were diluted, washed, resuspended in flasks, and again incubated, one (other) with cytochalasin B, 2 $\mu$g/ml, and then all with bacteria, and samples taken as usual.

After a series of preliminary experiments to establish good conditions for incubation of alveolar macrophages, studies were done like those designed to detect reversibility with human leukocytes, except that (a) the concentration of cytochalasin B was increased from 2 $\mu$g/ml to 5 $\mu$g/ml, (b) the time of each preincubation with drug was increased from 10 minutes to 20 minutes, (c) the times of (duplicate) sampling after addition of bacteria were changed to 30 and 60 minutes, and (d) there were five flasks containing cells: two control, two that received the drug after the wash, and one that received it before the wash.

*Metabolic studies.* In leukocytes, the oxidation of glucose-1-$^{14}$C was measured as described previously.[9] In macrophages, the oxidations of glucose-1-$^{14}$C, glucose-6-$^{14}$C, pyruvate-1-$^{14}$C, acetate-1-$^{14}$C, and succinate-1-$^{14}$C, were all measured as described previously.[15]

*Enzyme studies.* Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6 PGD) were measured by the methods of Strauss *et al.*[17] Units of either enzyme represent m$\mu$ moles NADP reduced/minute/$10^6$ cells.

*Morphology.* Samples of either cell type at various intervals were centrifuged directly on to glass (Shandon Cytocentrifuge, Shandon Scientific Co., Inc., Sewickley, Penna.) and stained with Wright's stain. Other samples were added to an equal volume of 2 percent buffered glutaraldehyde solution, pH 7.3, centrifuged and overlayed with more fixative. Later they were rinsed briefly in buffered, isotonic sucrose solution, post-fixed in $OsO_4$, dehydrated and embedded in Maraglas.[18-20] Ultrathin sections, having been stained with uranyl and lead salts,[21] were studied in an Elmiskop 101 electron microscope. One-micron-thick sections of the plastic-embedded tissue were stained with metachromatic dyes and examined by conventional and phase microscopy.

*Statistics.* The t test for paired variates was used throughout.

## RESULTS

*The effects of cytochalasin B on the uptake and killing of staphylococci by human blood leukocytes.*

Compared to controls treated with DMSO, cytochalasin B (2 or 5 μg/ml) inhibited the disappearance of bacteria from the supernatant after both 20 and 40 minutes of incubation (Fig. 1; mean values are depicted, ± SEM). These findings were consistent over a range of ratios of bacteria to PMN that varied from 4:1 to 39:1 (mean 17:1, median 18:1, mode 18:1). Since cytochalasin B did not affect the recovery of bacteria from control flasks incubated without leukocytes, the observed difference represents a decreased uptake of bacteria by cells. Although the drug-treated cells took up fewer bacteria than their controls, 40-minute samples of cells treated with 5 μg/

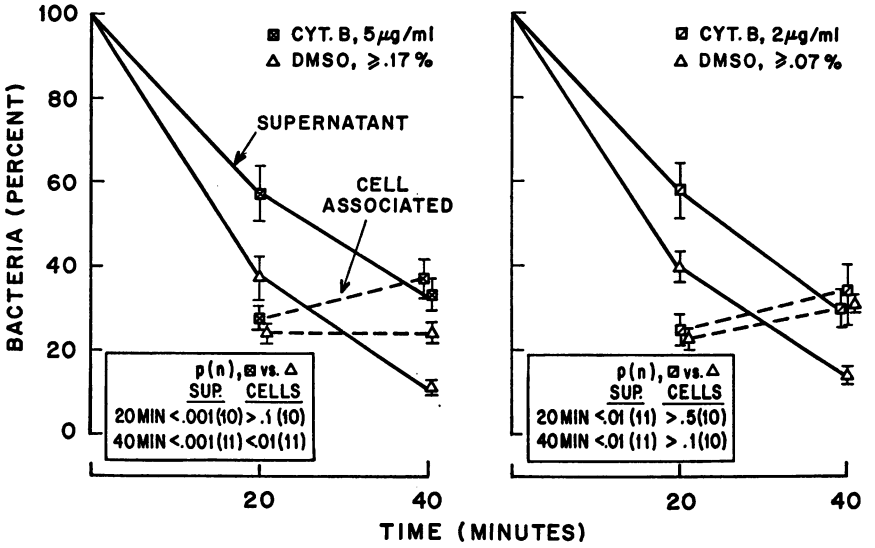


FIG. 1. Cytochalasin B inhibits the uptake and killing of staphylococci by human blood leukocytes.

ml cytochalasin B yielded significantly more live bacteria than their controls (Fig. 1). This finding indicates either that bacteria were adherent to treated cells but not ingested, or were ingested but not normally killed, or both.

DMSO in these concentrations ($\geqq .17\%$) had only a small effect on the uptake of bacteria by cells. When DMSO-treated flasks were compared to no-drug controls (not depicted in Fig. 1), supernatants from 20-minute samples contained a small but significant increase in the mean percentage of the inoculum remaining ($38 \pm 4.5$ [SEM] vs. $33 \pm 4.0$; $n = 12$, $p < .05$); differences at 40 minutes were no longer significant ($11 \pm 1.7$ vs. $10 \pm 1.4$; $n = 12, p > .1$).

*Reversibility.* In four experiments (see Methods) the effect of cytochalasin B on the uptake of bacteria by leukocytes appeared to be reversible. The mean percentages of the inoculum remaining in the supernatant, $\pm$ SEM, from flasks given the drug after the wash, versus no-drug controls, were $65 \pm 12.3$ vs. $42 \pm 6.3$ ($p < .05$) at 20 minutes, and $32 \pm 9.6$ vs. $15 \pm 7.7$ ($p < .01$) at 40 minutes. In contrast, from flasks where the drug was applied before the wash, values were similar to controls: $43 \pm 6.8$ at 20 minutes, and $18 \pm 8.5$ at 40 minutes. Ratios of bacteria: PMN varied from 16 to 21:1.

*The effects of cytochalasin B on the uptake and killing of staphylococci by rabbit alveolar macrophages.*

In each of four studies (see Methods), the results were consistent; mean values, $\pm$ SEM, from the eight paired flasks (control vs. drug-after-wash) are depicted in Fig. 2. (The 60-minute supernatants were inadvertantly lost in one study; thus $n = 6$ at that point). At each interval there is both diminished uptake of bacteria from the supernatant, and diminished killing by cells that were treated with drug after the wash. Ratios of bacteria: macrophage varied from 11 to 17:1.

*Reversibility.* Cells treated with cytochalasin B before being washed, subsequently behaved similar to control cells (Fig. 2).

*The effect of cytochalasin B on the production of $^{14}CO_2$ from labelled glucose and from other labelled substrates.*

The increase in production of $^{14}CO_2$ from glucose-1-$^{14}$C via the pentose shunt pathway, that follows the addition of bacteria to control human leukocytes, was diminished in cells treated with cytochalasin B (Table 1). In two experiments in which cytochalasin B, 5 $\mu$g/ml, was used, values for treated cells were less than 5% those of controls (Experiments 1 and 2, Stimulated Cells). This inhibition was dosage-dependent, and was significant with as little as 0.5 $\mu$g/ml cytochalasin B (Experiments 3-6, Stimulated Cells). The inhibition was similar when the period of incubation was extended from 20

TABLE 1. CYTOCHALASIN B INHIBITS THE PRODUCTION OF $^{14}CO_2$ FROM GLUCOSE-1-$^{14}$C BY HUMAN BLOOD LEUKOCYTES

| | *Stimulated Cells*△ | | | | | | | *Resting Cells*△ | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Cytochalasin B, μg/ml* | | | | | | *DMSO** | *Cytochalasin B, μg/ml* | | | | | *DMSO** | *Ratio* |
| | *5* | *2* | *1* | *0.5* | *0.2* | *0* | *0.17%* | *5* | *2* | *0.5* | *0.2* | *0* | *0.17%* | *Bact:PMN* |
| (1)+ | 17 | 40 | | | | 352 | 328 | 4 | 6 | | | 11 | 13 | 18:1 |
| (2) | 1.5 | | 9 | | | 41 | 40 | 0.6 | | | | 1.6 | 1.9 | 23:1 |
| (3) | | | 9 | 15 | 25 | 27 | | | | 0.9 | 1.2 | 1.2 | | 30:1 |
| (4) | | | 13 | 21 | 28 | 35 | | | | 0.9 | 1.0 | 1.3 | | 21:1 |
| (5)□ | | | 16 | 24 | 24 | 34 | | | | 1.4‡ | 1.9 | 2.0 | | 22:1 |
| (6) | | | | 55 | 73 | 71 | | | | 3.4 | 3.9 | 4.5 | | 31:1 |
| Mean (3-6) ± SEM | | | | 29±9 | 38±12 | 42±10 | | | | 1.7±.6 | 2.0±.7 | 2.3±.8 | | |
| Mean % of Control | | | | 66% | 87% | | | | | 72% | 90% | | | |
| Mean (2-5) ± SEM | | | 12±2 | | | 34±3 | | | | | | | | |
| Mean % of Control | | | 35% | | | | | | | | | | | |
| p (n = 4) | | | <.01 | <.01 | >.2 | | | | | <.05 | >.1 | | | |

△ cpm ($\times 10^3$)/3 $\times$ $10^7$ cells/20 min., average counts from duplicate flasks.
* Cyt. B, 5 μg/ml, contained 0.17% DMSO.
+ Each flask received 1 μCi glucose-1-$^{14}$C, instead of the usual 0.2 μCi.
□ Time of incubation 40 min., instead of the usual 20 min.
‡ Single flask.

to 40 min. (Experiment 5). DMSO had no appreciable inhibitory effect (Experiments 1 and 2).

A similar though less pronounced effect of cytochalasin B to that in stimulated cells could be demonstrated in leukocytes incubated without bacteria (Table 1, Resting Cells).

Because the alveolar macrophage contains a complement of metabolic machinery some of which is only rudimentary in the mature PMN,[22,15] we tested various substrates in macrophages—resting and stimulated—treated with cytochalasin B. For the oxidation of glucose-1-$^{14}C$, there was once again significantly less $^{14}CO_2$ from both stimulated and resting cells treated

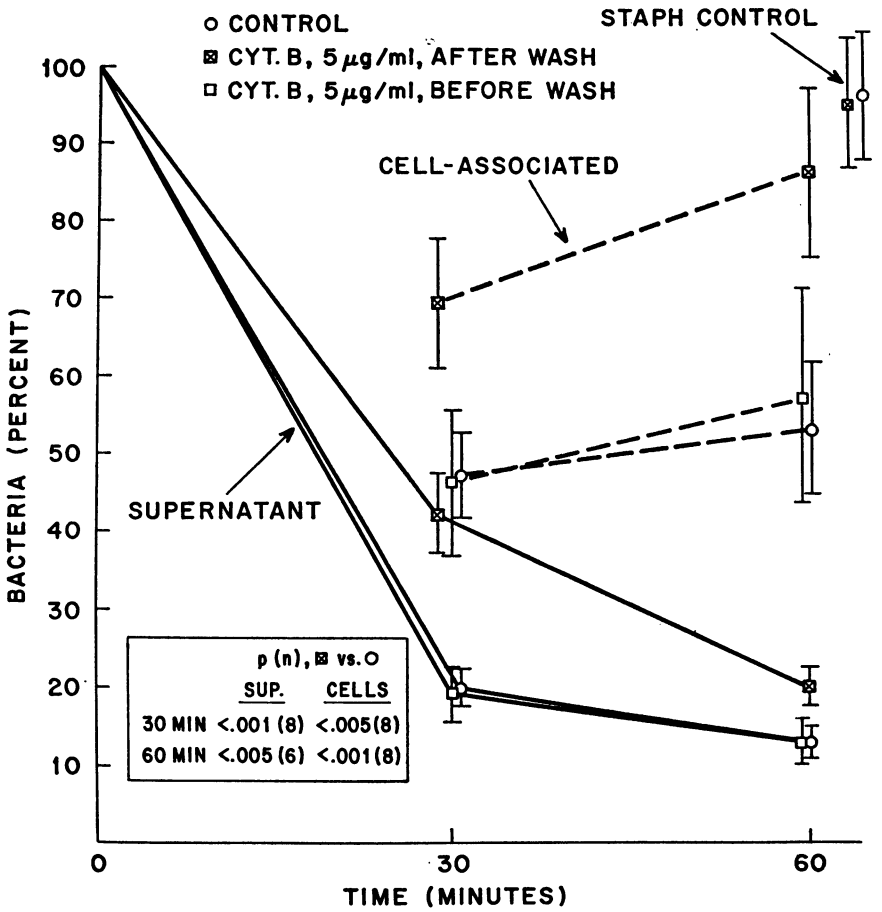


FIG. 2. Cytochalasin B inhibits the uptake and killing of staphylococci by rabbit alveolar macrophages.

with cytochalasin B (2 $\mu$g/ml) than from their respective controls (Fig. 3). Effects on the oxidation of glucose-6-$^{14}$C were similar (Fig. 3) but significantly less marked. Thus, in stimulated cells treated with cytochalasin B, the release of $^{14}CO_2$ compared to controls was 30% from glucose-1-$^{14}$C and 65% from glucose-6-$^{14}$C ($p < 0.025$).

Cytochalasin B, 2 $\mu$g/ml, had no significant inhibitory effect on the release of $^{14}CO_2$ from pyruvate-1-$^{14}$C, acetate-1-$^{14}$C, or succinate-1-$^{14}$C, in either resting or stimulated cells (Table 2).

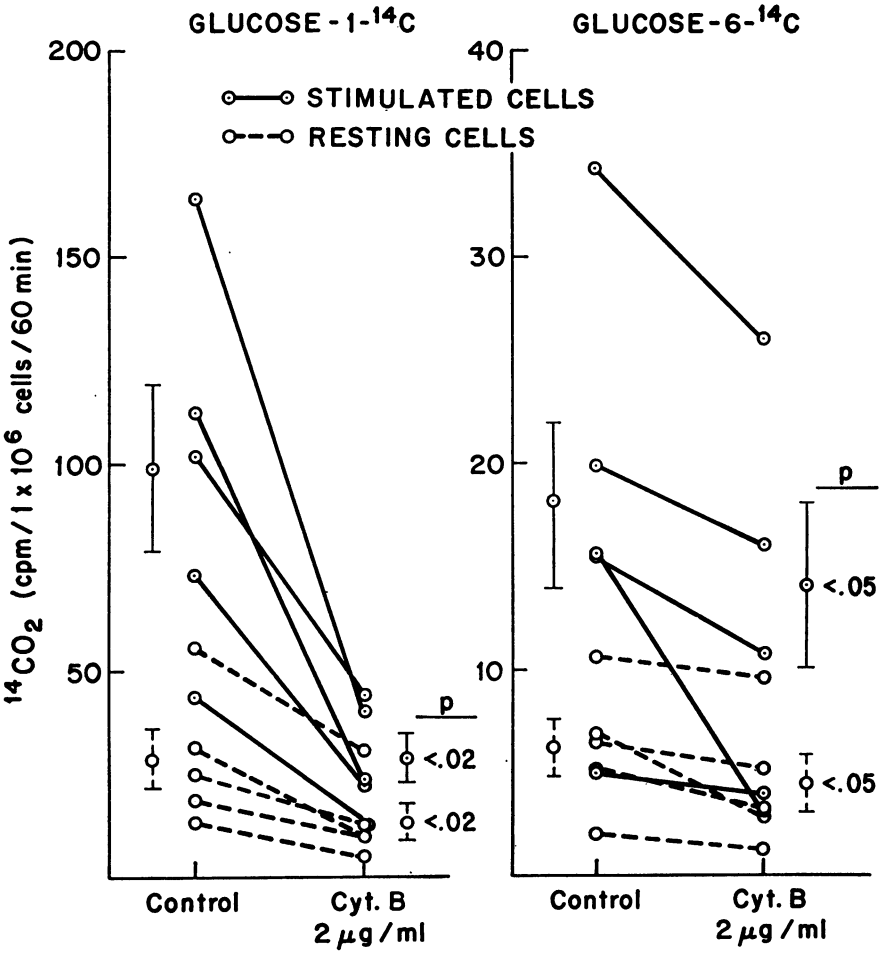


FIG. 3. Cytochalasin B inhibits the production of $^{14}CO_2$ from glucose-$^{14}$C by rabbit alveolar macrophages.

*Reversibility of the effect of cytochalasin B on the production of $^{14}CO_2$ from glucose-1-$^{14}C$.* When bacteria were presented to cells, either leukocytes or macrophages, previously incubated with cytochalasin B and then washed, the production of $^{14}CO_2$ from glucose-1-$^{14}C$ was similar to that from controls that had not received cytochalasin B (Table 3, Before Wash). Stimulated cells that received cytochalasin B after the wash, released only 13% to 23% as much $^{14}CO_2$ as did controls. Thus this metabolic effect of cytochalasin B appears to be reversible in both leukocytes and macrophages.

*The lack of inhibition by cytochalasin B of the activities of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6 PGD).* In view of the inhibition of pentose shunt activity in the leukocyte and the preferential reduction in $^{14}CO_2$ production from glucose-1-$^{14}C$

TABLE 2. CYTOCHALASIN B DOES NOT INHIBIT THE PRODUCTION OF $^{14}CO_2$ FROM LABELLED PYRUVATE, ACETATE AND SUCCINATE

| | | *Resting Cells*△ | | *Stimulated Cells*△ | |
|---|---|---|---|---|---|
| *Cyt. B, 2 μg/ml* | | − | + | − | + |
| Pyruvate-1-$^{14}C$ | (1) | 23 ± 2.1 | 33 ± 3.1 | 34 ± 3.7 | 48 ± 5.1 |
| | (2) | 20 ± 3.1 | 30 ± 2.9 | 30 ± 3.7 | 43 ± 3.9 |
| Acetate-1-$^{14}C$ | (1) | 38* ± 3.1 | 43 ± 5.1 | 36 ± 5.2 | 45 ± 7.3 |
| | (2) | 18 ± 3.2 | 29 ± 3.1 | 33 ± 4.1 | 34 ± 5.2 |
| Succinate-1-$^{14}C$ | (1) | 11 ± 3.7 | 13 ± 2.1 | 14 ± 2.1 | 15 ± 2.2 |
| | (2) | 27 ± 3.1 | 23 ± 3.0 | 25 ± 2.7 | 24 ± 1.8 |

△ cpm/$1\times10^6$ cells/60 min.; average counts from triplicate flasks, ± SEM.
* Duplicate flasks.

TABLE 3. REVERSIBILITY OF THE EFFECT OF CYTOCHALASIN B ON THE PRODUCTION OF $^{14}CO_2$ FROM GLUCOSE-1-$^{14}C$

| | *Stimulated Cells*△ | | | | |
|---|---|---|---|---|---|
| | *Human Blood Leukocytes** | | | *Rabbit Alveolar Macrophages*+ | |
| | (1) | (2) | (3) | (1) | (2) |
| Control (No Cyt. B) | 77 | 62 | 85 | 95 | 110 |
| Cyt. B, 2 μg/ml, Before wash | 87 | 56 | 87‡ | 84 | 99 |
| Cyt. B, 2 μg/ml, After wash | 9 | 10 | 18‡ | 22 | 23 |
| Ratio, Bacteria: Cell | 20:1 | 12:1 | 12:1 | 150:1 | 150:1 |

△ Aliquots of cells were incubated, some with cytochalasin B, then diluted, washed, resuspended, and again incubated, some with cytochalasin, and then all with glucose-1-$^{14}C$ and heat-killed staphylococci (see Methods).
* cpm ($\times10^3$)/$3\times10^7$ cells/20 min.; average counts from triplicate flasks.
+ cpm/$1\times10^6$ cells/60 min.; average counts from triplicate flasks.
‡ Duplicate flasks only.

over that from glucose-6-$^{14}$C in the macrophage, the effects of cytochalasin B on two important enzymes in the pentose shunt were examined. In two experiments with homogenates of macrophages, mean values for G6PD without and with added cytochalasin B (5 $\mu$g/ml) were 3.62 and 3.80 units, respectively. In five experiments, the mean values for 6 PGD were 1.01 and 1.16 units respectively. DMSO in corresponding dilution had no effect on activities of either enzyme.

*Structural and ultrastructural observations*

The diminished uptake of bacteria by cells treated with cytochalasin B was frequently confirmed by examining samples centrifuged on to glass slides and stained (Figs. 4 and 5). In addition to having fewer cell-associated bacteria than controls or DMSO-treated cells, drug-treated PMN could be identified by the tendency of their cytoplasm to spread more upon the glass than that of untreated cells, and of the lobes of their nuclei to be less mutually constrained, producing a rosette effect when viewed at low power (Fig. 3). Drug-treated macrophages tended also to spread out more than their controls, and to lose their normal three-dimensional appearance; whatever cell-associated bacteria were present appeared to be primarily in one plane (Fig. 5).

In one-micron-thick sections, diminished numbers of intracellular bacteria could be counted in drug-treated cells compared to controls. For example, in one experiment the mean number of bacteria per control PMN slice ($n = 20$ slices) after 40 minutes of incubation was $7.4 \pm 0.6$ (SEM), versus $1.2 \pm 0.3$ per slice from drug-treated cells (Figs. 6 and 7). In another, there were $7.0 \pm 0.5$ bacteria per macrophage slice ($n = 20$, 60 minutes of incubation) versus $5.0 \pm 0.4$ from drug-treated cells (5 $\mu$g/ml, 20-minute preincubation).

PMN treated with cytochalasin B frequently showed partial engulfment of bacteria as depicted in Fig. 8, an observation that was rarely made on control cells. Control cells, on the other hand, contained proportionally more and larger digestive vacuoles (often containing partly digested micro-organisms) than did drug-treated cells. However, in treated cells, fusion of leukocyte granules with phagocytic vacuoles (phagosomes) was on occasion definitely observable.

The hyaloplasm of control leukocytes contained their usual, scant complement of centrioles, microtubules and skeins of microfilaments (Fig. 9). Filaments were not seen in PMN treated with cytochalasin B (Fig. 8). Similarly, there was a pronounced decrease in the incidence of microfilaments in drug-treated macrophages compared to controls (Figs. 10 and 11); however, microtubules remained prominent in drug-treated cells (Fig. 11).

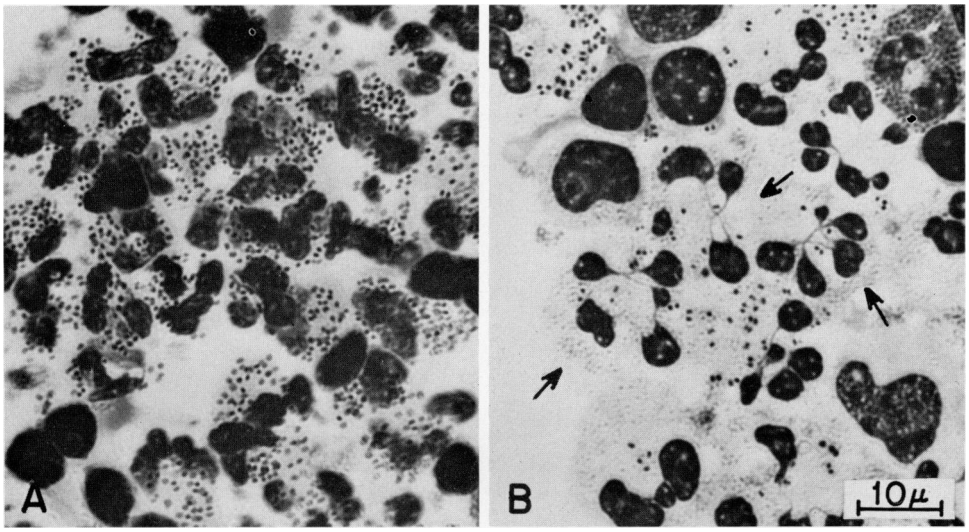


FIG. 4. The effect of cytochalasin B on human blood leukocytes inoculated with bacteria for 20 minutes is seen in cells centrifuged on to glass slides. (A) No drug. Many bacteria per cell are seen. (B) Cytochalasin B, 5 μg/ml, 10 minute preincubation. Fewer bacteria per cell are seen, cells appear more spread out than in (A), and lobes of PMN nuclei show the rosette effect (arrows). Wright's stain. (Taken from ref. 1).

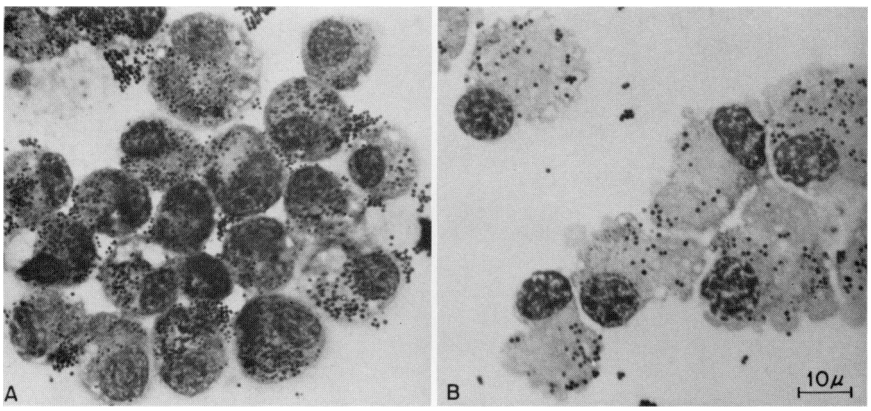


FIG. 5. The effect of cytochalasin B on rabbit alveolar macrophages incubated with bacteria for 60 minutes is seen in cells centrifuged on to glass slides. (A) No drug. Many bacteria per cell are seen. The cells have depth; bacteria are in different planes. (B) Cytochalasin B, 5 μg/ml, 10 minute preincubation. Fewer bacteria per cell are seen, and the cells appear flatter and more spread out than in (A). Wright's stain.

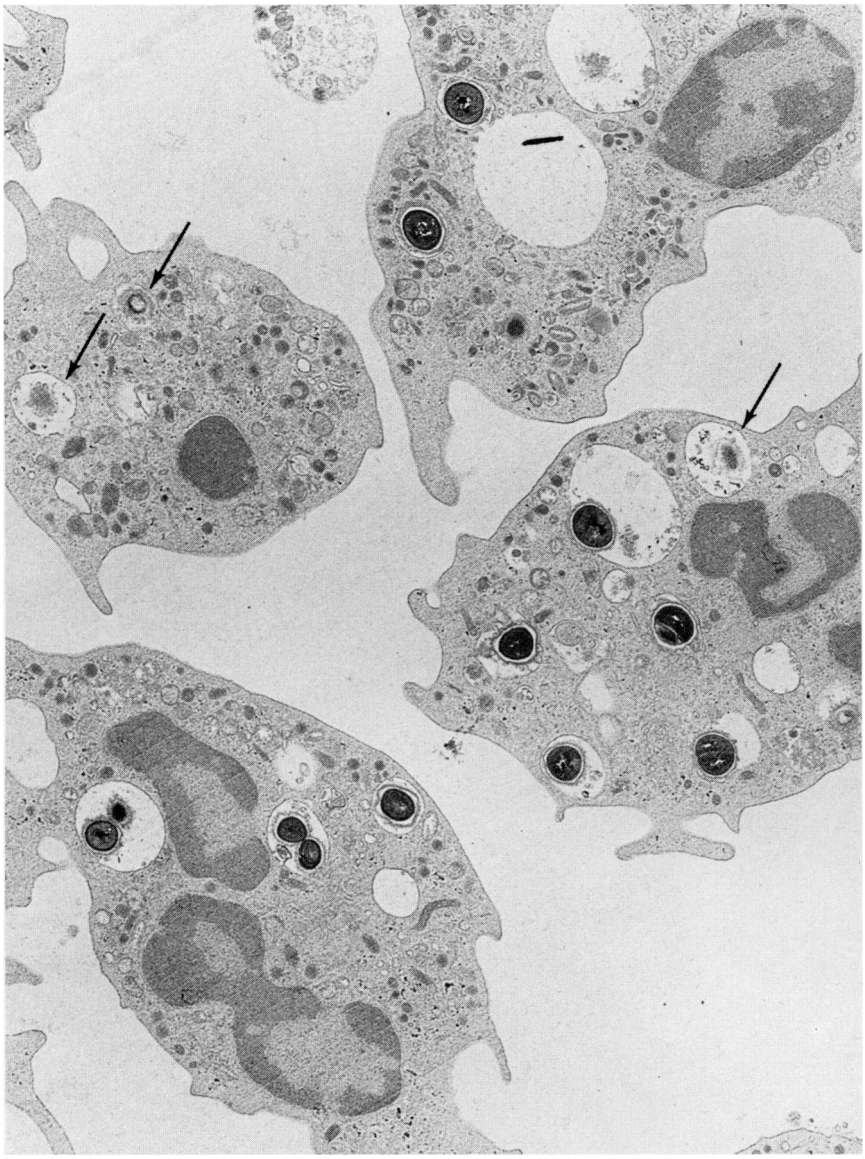

FIG. 6. Control leukocytes (no drug) incubated with bacteria for 40 minutes contain numerous ingested bacteria in varying stages of digestion. The arrows point at vacuoles with partly hydrolzed micro-organisms; the large, "empty" digestive vacuoles most likely represent final-stage digestive vacuoles in which bacteria have been completely hydrolzed. Mag: 9,000 ×.

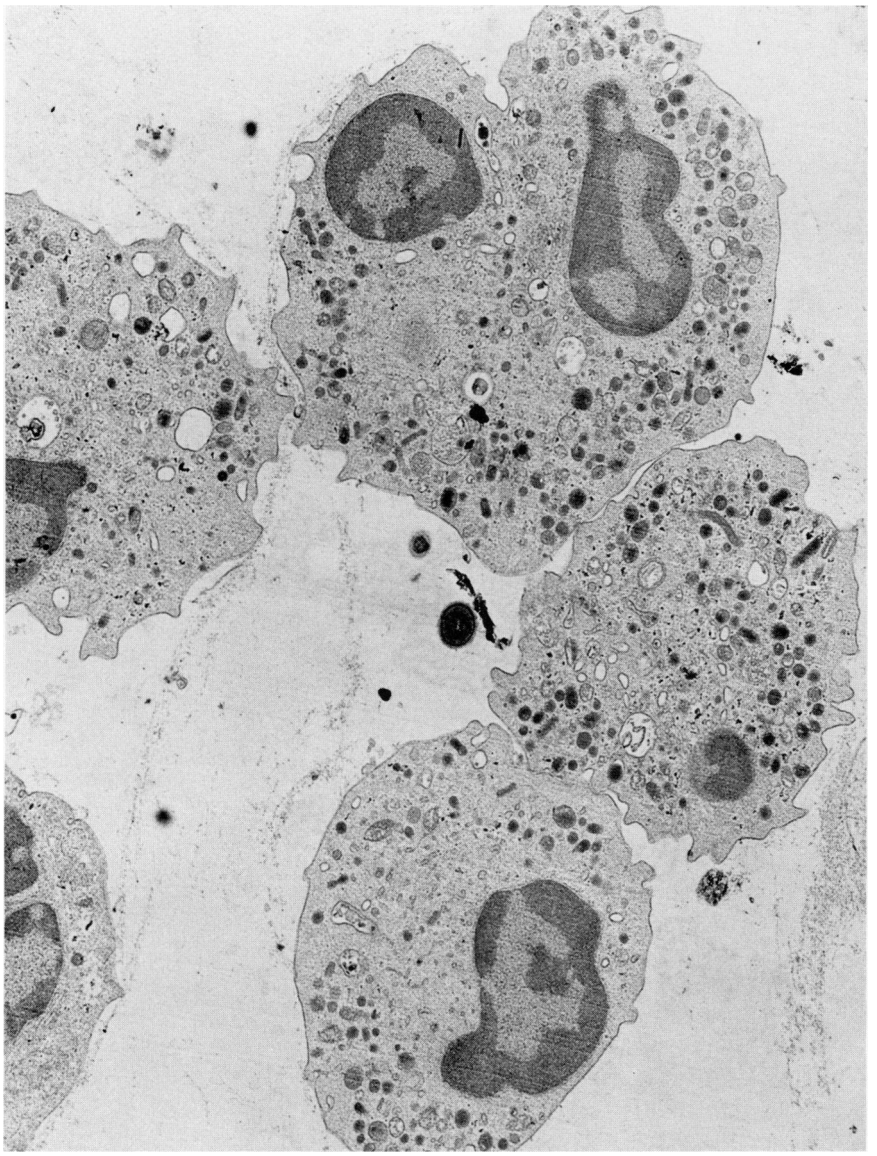

FIG. 7. Leukocytes exposed to 5 $\mu$g/ml cytochalasin B (10 minute preincubation) and incubated with bacteria for 40 minutes, typically show fewer intracellular bacteria. Note also their full complement of leukocyte granules. Mag: 9,000 $\times$.

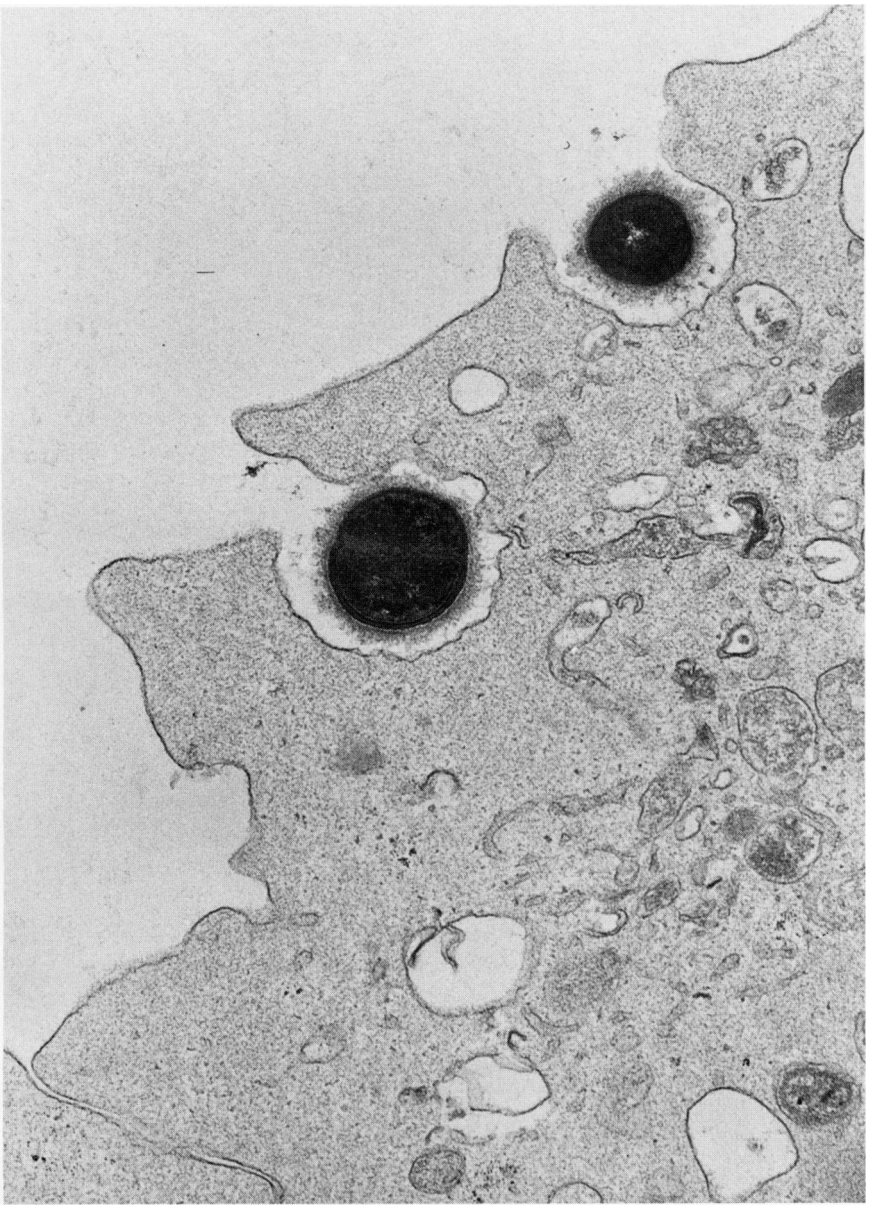

FIG. 8. Part of a leukocyte exposed to cytochalasin B as in Fig. 7. The depicted partial or nearly complete engulfment of bacteria was observed with many of the treated cells. Note the finely granular cytoplasm devoid of microfilaments. Mag: 33,000 ×.

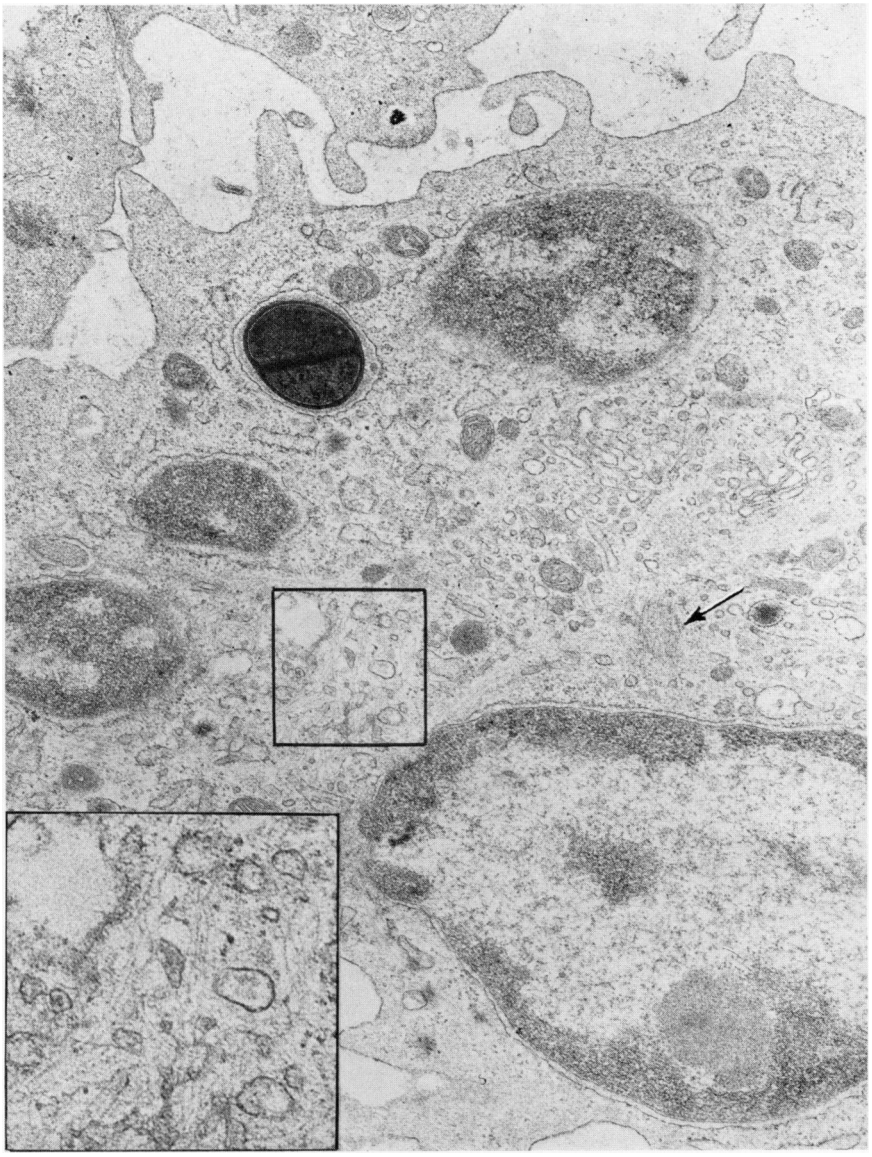

FIG. 9. In contrast to Fig. 8, part of a control leukocyte contains, aside from a bacterium, skeins of microfilaments (insert). A centriole (arrow) and microtubules are also visible. Mag: 22,000 ×. Insert mag: 50,000 ×.

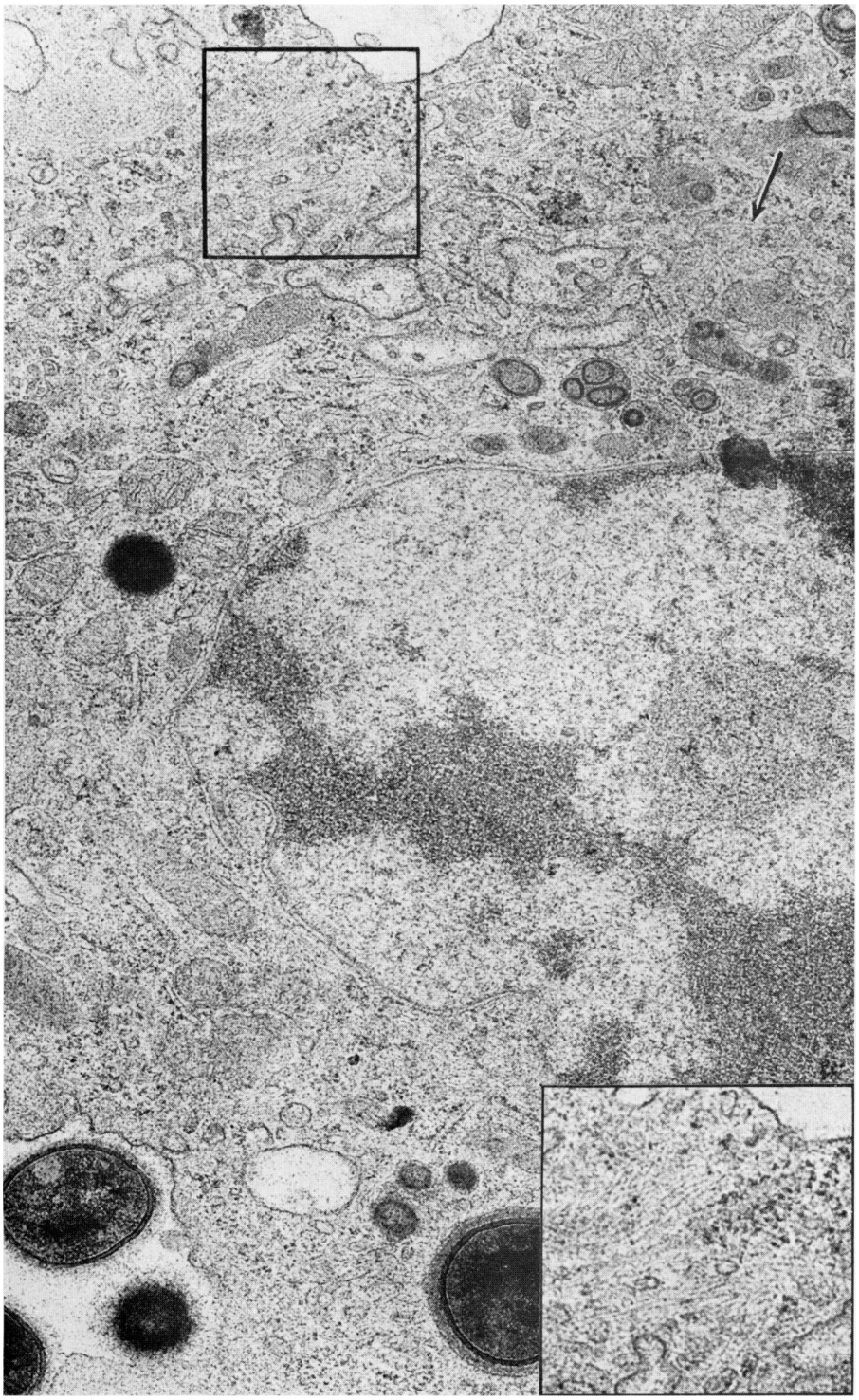

FIG. 10. Part of a control macrophage, incubated with bacteria for 60 minutes, shows ingested micro-organisms (lower margin of illustration). Note the abundant microtubules (arrow) and microfilaments (insert) which are particularly prominent in the upper one-third of the illustration. Mag: 30,000 ×. Insert mag: 50,000 ×.

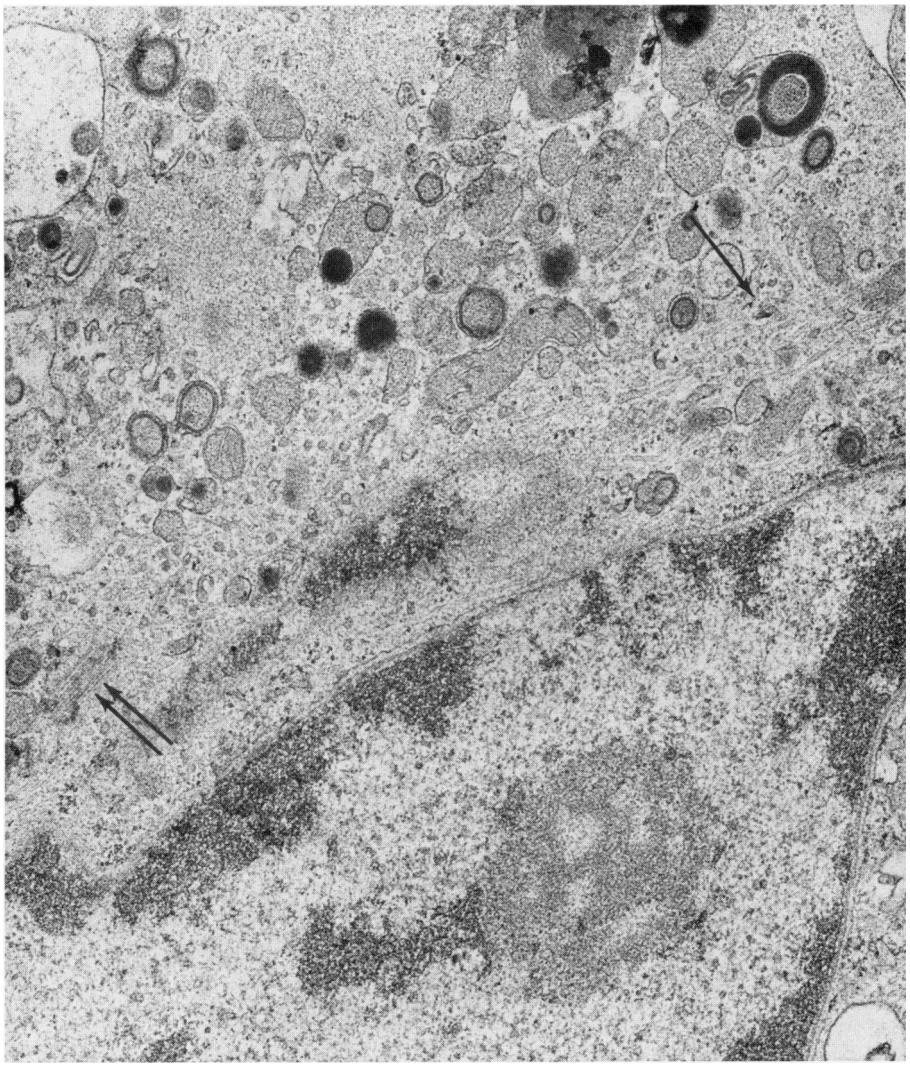

FIG. 11. Part of a macrophage exposed to 5 $\mu$g/ml cytochalasin B (20 minute pre-incubation) and incubated with bacteria for 60 minutes is seen. Microfilaments are not observable, but microtubules are prominent (arrow); a centrole is present on the left margin of the photograph (double arrow). Mag: 35,000 $\times$.

## DISCUSSION

*Uptake and killing of bacteria.* Cytochalasin B produced a decrease in the uptake of *Staphylococcus aureus* by both human blood leukocytes and rabbit alveolar macrophages (Figs. 1 and 2). Despite diminished uptake, however, the drug-treated cells showed more live cell-associated bacteria than their controls, indicating either that bacteria were adherent to treated cells but not ingested, or were ingested but not normally killed, or both. These effects with both cell types were reversible.

Recently, Davis *et al.* found similar results with human PMN using a higher concentration of cytochalasin B (10 $\mu$g/ml), a low ratio of bacteria: PMN (1:1), and measuring at intervals the survival of bacteria from samples of the entire suspension (supernatant + cells).[23] They too found no direct effect of cytochalasin B on the survival of staphylococci incubated without cells, or on the ability of serum to opsonize the bacteria. They also added penicillin and streptomycin to the medium; at low ratios of bacteria: PMN, this maneuver makes it possible to distinguish bacteria adherent to cells from those ingested and thus protected from the antibiotics by their intracellular location. They concluded that the effect of cytochalasin B appeared to be on the process of engulfment of bacteria rather than on intracellular killing.

Since we employed much higher ratios of bacteria: cell than did Davis *et al.*, it was possible to determine how many bacteria were actually ingested. Counts of bacteria in one-micron-thick sections indicated that both cell types ingested fewer bacteria when treated with cytochalasin B (also, Figs. 6 and 7). Furthermore, adherent bacteria could be found, some invaginated into the cell, but without the membrane fusion that would internalize them (Fig. 8). Allison *et al.* recently noted attachment of bacteria without engulfment under the light microscope, using drug-treated guinea-pig peritoneal macrophages adherent to a surface.[24]

Although our current findings indicate that the drug-induced increase in cell-associated live bacteria is due at least in part to adherent, non-ingested bacteria, they do not rule out the additional possibility of less intracellular killing. Moreover, such an additional effect seems likely from other evidence. Allison *et al.* found that cytochalasin B stopped all movements of ruffled membranes in macrophages and of the cells themselves, together with pinocytosis, and that pinosomes already formed remained immobile in what remained of the pseudopods.[24] If endosomes in general and lysosomes in addition were to cease their usual intracellular translocations,[25] the expected result would be a decrease in fusion of the two structures, possibly dramatic enough to effect a decrease in intracellular killing. Supporting this view is

the recent evidence that cytochalasin B interferes with the translocation of pigment granules induced in melanocytes by melanocyte-stimulating hormone.[26]

The failure of drug-treated cells to engulf bacteria completely may be due to a slowing of cytoplasmic flux about the adherent micro-organism, or to a defect in fusion of the plasmalemma after the bacterium has been surrounded.[28] Whatever the mechanism, the failure may be a special instance of the general effect of cytochalasin B on motility and the formation of a ruffled membrane, first described by Carter in L cells,[11] and confirmed by Allison *et al.* in macrophages[24] and by Harris and Ramsey in human blood PMN.[27] (The last workers found reversible effects, within seconds, with 0.5 $\mu$g/ml cytochalasin B). The considerable (though subnormal) uptake of bacteria onto the surfaces of presumably obtunded, drug-treated cells, can be attributed to the system employed: serum (for opsonin) and buffer, a high concentration of leukocytes, and a high ratio of bacteria:PMN, shaken vigorously to maximize the number of "hits" and minimize the necessity for locomotion or chemotaxis.

*Metabolic studies.* Cytochalasin B inhibited the conversion of glucose to $CO_2$ in both leukocytes and macrophages (Table 1 and Fig. 3). Several possibilities should be considered. First, cytochalasin B may exert a nonspecific toxic effect on cell metabolism. However, few metabolic effects of cytochalasin B are known,[14] and in the present work there was no inhibition of the oxidation of pyruvate, succinate or acetate (Table 2), or of the activities of G6PD and 6 PGD. Furthermore, the effects of cytochalasin B were reversible (Table 3). Second, cytochalasin B may interfere with the active transport of glucose into these cells. This possibility is unlikely because the drug preferentially inhibited $CO_2$ production from glucose-1-$^{14}$C, at least in the macrophage (Fig. 3). Third, the inhibition of glucose oxidation may reflect the demonstrated inhibition of ingestion of bacteria. This alternative may partially explain the effect on cells stimulated by bacteria, but it does not explain the effect on resting cells (Table 1, Fig. 3). The latter effect could be related to interference with baseline cell motility (see above).

*Role of microfilaments.* We have identified skeins of microfilaments in ultrathin sections of both PMN and macrophages (Figs. 9 and 10). Treatment of either cell type with cytochalasin B interfered with our ability to distinguish these microfilaments. In contrast, microtubules were still present (Fig. 11). Schroeder first noted that cytochalasin B made microfilaments disappear in the cleavage furrow of dividing *Arbacia* eggs, and that furrowing ceased or regressed.[12,13] Mitosis was not affected; thus, microtubules presumably maintained their integrity. Since Carter's original observations

on functional effects of cytochalasin B,[11] a catalogue of effects related to motile functions, of or within cells, has arisen through the observations of many investigators, sometimes with documentation of ultrastructural effects on presumably-contractile microfilament systems.[14] De Petris[24] has found, in the peripheral or cortical cytoplasm of guinea pig peritoneal macrophages, a network of microfilaments which, in glycerol-extracted cells, bind heavy meromyosin, implying that they are "actin-like."[28]

In summary, if we postulate that cytochalasin B has a specific direct or indirect effect on the organization of systems of microfilaments, the current studies would support the following functions for microfilaments: production of the phagosome (i.e., the surrounding by the cell of an attached particle, or subsequent membrane closure, or both) and the provision of microstructural constraints which keep normal cells, centrifuged on to glass, from flattening and spreading (Figs. 4 and 5), and which maintain the spatial interrelationships of the lobes of PMN (Fig. 4). Other possible functions, based on observations of others, are those relating to cellular motility, external or internal; i.e., locomotion[24,27] and therefore chemotaxis (directed locomotion), pinocytosis[24] (or, more generally, endocytosis), exocytosis (by similar means), intracellular movement of endosomes[24] and lysosomes, and consequently intracellular killing and digestion. Glucose oxidation, in nonphagocyting cells, and especially glucose-1 oxidation, may be related to baseline motility.

*Relationship between microtubules and microfilaments.* It is useful to compare the suggested role of microtubules with that of microfilaments. Microtubules also appear to be involved in structure and movement in cells.[29] They have been thought possibly to perform mechanical work[30] and to contribute to the strength of protoplasmic gels.[31] It therefore seemed possible that in PMN, protoplasmic gels and microtubules might be involved in locomotion,[32] which requires frequent reversible cytoplasmic structural alterations. Indeed, colchicine, which causes the dissolution of microtubules in PMN,[3] does interfere with the motility of PMN crawling on glass,[33] but compared with cytochalasin, requires many minutes rather than seconds,[27] and molar concentrations 1000 times higher. However, at much lower concentrations, colchicine interferes with chemotaxis,[34] which involves direction as well as locomotion.

In addition to causing dissolution of normal microtubules,[3-5] both colchicine and vinblastine inhibit degranulation of PMN and the formation of digestive vacuoles during phagocytosis, without inhibiting ingestion or intracellular killing.[6,9] To correlate these activities, we turned to the work of Freed and associates, who consider microtubules to be involved in a basic intracellular transport system.[35] They studied long saltatory movements,

which are non-Brownian displacements of cytoplasmic particles, independent of flow of cytoplasm, and (like microtubules) generally radially directed. Because both lysosomal granules and ingested material underwent these movements in Freed's system, and because colchicine caused the movements to stop, we suggested that in PMN, microtubules may facilitate the association of lysosomes with phagosomes, to form digestive vacuoles.[6-10] Interference with the normal organization of microtubules in PMN might then be the basis for the observed effects of colchicine and vinblastine. However, it is not clear whether the microtubules provide a motive force for the fusion of lysosome and phagosome, or merely facilitate fusion by orienting these structures.

A working hypothesis for the relationship between microtubules and microfilaments, similar to one proposed by others,[24] is that a system of labile microtubules forms a series of oriented infrastructures against which a system of contractile microfilaments can perform mechanical work. This bone-and-muscle conception is surely oversimplified, but reasonably fits the data presently available. Endocytosis, studied here, would appear primarily to require the integrity of microfilaments, given a system in which easy contact is provided between particle and cell.

SUMMARY

Cytochalasin B, which affects certain types of motility, of cells and within cells, is currently thought to interfere rather specifically with the function of contractile microfilaments. To study the possible role of microfilaments in phagocytosis, we examined the effects of cytochalasin B (0.5-5 $\mu$g/ml) on phagocytosis of *Staphylococcus aureus* by human blood leukocytes and by rabbit alveolar macrophages.

Cytochalasin B diminished the uptake of bacteria by both cell types. Decreased numbers of intracellular bacteria were confirmed in one-micron-thick sections of cell pellets. However, *increased* numbers of live, cell-associated bacteria were recovered from drug-treated cells, as compared to controls, indicating that bacteria were adherent to cells but not ingested, or were ingested but not normally killed, or both. Effects on uptake and killing were reversible by diluting and washing the cells.

Cytochalasin B inhibited $^{14}CO_2$ from glucose-1-$^{14}$C both in resting cells and in cells stimulated by bacteria; this effect was also reversible. In macrophages, $^{14}CO_2$ from glucose-6-$^{14}$C was depressed less than that from glucose-1-$^{14}$C in both resting and stimulated cells; $^{14}CO_2$ from labelled pyruvate, acetate and succinate was not depressed.

Cytochalasin B caused cells centrifuged on to glass slides to become flatter and more spread out than normal, and the lobes of PMN nuclei to be less

mutually constrained (a rosette effect). In addition, microfilaments were no longer visible in drug-treated PMN, and their incidence was markedly decreased in drug-treated macrophages, while microtubules remained prominent.

These results suggest that contractile microfilaments may be important for endocytosis, for the provision of certain microstructural constraints, and possibly for other motile aspects of phagocytosis. Possible relationships between microfilaments and microtubules are discussed.

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