ROLE OF MICROTUBULE ASSEMBLY IN LYSOSOMAL ENZYME SECRETION FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

A Reevaluation

SYLVIA HOFFSTEIN, IRA M. GOLDSTEIN, and GERALD WEISSMANN

From the Division of Rheumatology of the Department of Medicine, New York University Medical Center, New York 10016

ABSTRACT

The dose-related inhibition by colchicine of both lysosomal enzyme release and microtubule assembly was studied in human polymorphonuclear leukocytes (PMN) exposed to the nonphagocytic stimulus, zymosan-treated serum (ZTS). Cells were pretreated with colchicine (60 min, 37°C) with or without cytochalasin B (5 μg/ml, 10 min) and then stimulated with ZTS (10%). Microtubule numbers in both cytochalasin B-treated and untreated PMN were increased by stimulation and depressed below resting levels in a dose-response fashion by colchicine concentrations above 10^{-7} M. These concentrations also inhibited enzyme release in a dose-response fashion although the inhibition of microtubule assembly was proportionately greater than the inhibition of enzyme release. Other aspects of PMN morphology were affected by colchicine. Cytochalasin B-treated PMN were rounded, and in thin sections the retracted plasma membrane appeared as invaginations oriented toward centrally located centrioles. Membrane invaginations were restricted to the cell periphery in cells treated with inhibitory concentrations of colchicine, and the centrioles and Golgi apparatus were displaced from their usual position. After stimulation and subsequent degranulation, the size and number of membrane invaginations greatly increased. They remained peripheral in cells pretreated with > 10^{-7} M colchicine but were numerous in the pericentriolar region in cells treated with < 10^{-7} M. Similarly, untreated PMN that were permitted to phagocytose immune precipitates had many phagosomes adjacent to the centriole. After colchicine treatment, phagosomes were distributed randomly, without any preferential association with the centrioles. These data suggest that microtubules are involved in maintaining the internal organization of cells and the topologic relationships between organelles and the plasma membrane.

Secretion of previously "packaged" molecules from a variety of cell types appears to be dependent upon the normal function of cytoplasmic microtubules since the process is inhibitable by colchicine and other microtubule-active agents (3, 4, 12, 21, 24, 35, 36). The precise role of microtu-

bules in the secretory process has, however, been difficult to elucidate, since morphologic studies have demonstrated relatively few microtubules in secretory cells, and those microtubules that are seen do not appear to be oriented with respect to the organelles whose mobility they putatively control. Recent evidence (1) indicates that only 35–40% of the tubulin pool in secretory cells is in the assembled form. The remainder probably assembles into functional microtubules in response to secretory stimuli. Thus, most of those microtubules seen in secretory cells under resting conditions may not represent the population involved in secretory events.

Colchicine, the drug used in most studies of microtubule function, binds to depolymerized tubulin and prevents its subsequent polymerization into functional microtubules (34). Only a fraction of the theoretical number of tubulin molecules need be complexed with colchicine to prevent polymerization (17). Although colchicine binds to membrane proteins (1, 26, 27), the high affinity binding sites appear to be membrane-associated tubulin (1, 27). However, in order to ascribe the effects of colchicine on cell function to its effect on microtubule polymerization, it is necessary to combine biochemical studies of cell function with ultrastructural studies of microtubule assembly.

Secretion of lysosomal enzymes by human polymorphonuclear leukocytes (PMN)[1] is similar to secretion of stored proteins by other cell types (6, 8, 9, 31, 32, 38). Enzyme secretion occurs in response to a number of phagocytic and nonphagocytic stimuli and is associated with enhanced microtubule assembly (6, 11, 33). Secretion can be partially inhibited by colchicine (13, 18, 39) and by agents which increase cellular levels of cyclic $3',5'$-adenosine monophosphate (41, 42), and can be enhanced by agents which increase cyclic $3',5'$-guanosine monophosphate (41, 42). We have previously reported positive correlations between pharmacological modulation of in vivo microtubule assembly and enzyme release (33) and consequently have designed the experiments described below to test whether colchicine concentrations that inhibited biochemically measured enzyme release from stimulated PMN also inhibited microtubule assembly.

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[1] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte(s); ZTS, zymosan-treated serum; LDH, lactate dehydrogenase.

In these experiments microtubule assembly was stimulated in both cytochalasin B-treated and untreated PMN by serum in which complement was activated via the alternate pathway by zymosan-treated serum (ZTS). Such a fluid phase stimulus provides a population of uniformly stimulated cells which are amenable to electron microscope morphometry of microtubule assembly in vivo. Furthermore, cytochalasin B-treated PMN release lysosomal enzymes to the extracellular milieu upon stimulation by ZTS. Thus, microtubule assembly and lysosomal enzyme release can be studied in the same system. The data reported here provide morphologic confirmation that colchicine concentrations that inhibit enzyme release from PMN also inhibit microtubule assembly in a dose-related fashion. They also suggest that the process affected by the absence of functional microtubules is the ability to translocate phagocytic vacuoles from the cell periphery to the granule-rich, pericentriolar cytoplasm.

MATERIALS AND METHODS

Preparation of PMN

Venous blood (32 ml) was drawn from healthy adult volunteers and mixed with 8 ml of acid citrate dextrose (ACD) anticoagulant (National Institutes of Health formula A) plus 20 ml of 6% dextran in normal saline. The erythrocytes were allowed to sediment 30–45 minutes. Portions (15 ml) of the leukocyte-rich supernates were then mixed with 35 ml of 0.87% ammonium chloride and centrifuged for 10 min at $160\,g$. The cell pellets were resuspended in normal saline, combined in a volume of 50 ml, and centrifuged at $160\,g$ for 10 min. The resulting pellet was suspended in buffer (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.6 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4). Aliquots containing 2×10^6 PMN were preincubated with varying concentrations of colchicine (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 1 h. Cytochalasin B (5 μg/ml) (ICI Research Laboratories, Alderly Park, Cheshire, Eng.) in 0.1% dimethyl sulfoxide (Matheson, Coleman and Bell, East Rutherford, N. J.) was added for the last 10 min before addition of ZTS. This concentration of dimethyl sulfoxide did not influence cell morphology, enzyme release or enzyme assays.

Activation of Complement

Complement was activated in fresh autologous serum containing 250 mM epsilon-aminocaproic acid (Sigma Chemical Co.) by adding zymosan (1.0 mg/ml). After 15 min of incubation at 37°C, the serum was rendered free of particles by centrifugation (3,000 g). Zymosan-treated serum containing epsilon-aminocaproic acid

(ZTS) was added to the reaction mixtures in a final concentration of 10% (vol/vol), and enzyme release was measured after 5 min, by which time maximal enzyme release has been achieved in this system. The bulk of evidence regarding the identity of the lysosomal enzyme-releasing activity obtained by these methods indicates that it is a low molecular weight ($\sim$ 17,000 daltons) product of C5, probably C5a (6). Chromatography on Sephadex G-75 of zymosan-treated serum, endotoxin-treated serum, and trypsinized human C5 yield similar low molecular weight fractions containing enzyme-releasing activity that is inhibitable by antibodies to human C5 but not by those to human C3. Furthermore, the activity is resistant to heat (56°C for 30 min), is obtained in enhanced yields from serum containing epsilon-aminocaproic acid, and is chemotactic for human PMN (6). These properties are identical to those of C5a.

Preparation of Ferritin- Anti-Ferritin

Ferritin (0.5 mg) was added to 1 ml of rabbit anti-horse ferritin (Cappel Laboratories Inc., Downington, Pa.) to form a visible precipitate. The precipitate was allowed to settle, washed 2 × in phosphate-buffered saline and suspended in 0.5 ml of buffered saline. The suspension was added to the reaction mixtures in a final concentration of 10% (vol/vol), and the cells were fixed after 5 and 15 min.

Enzyme Assays

Following incubation, the reaction mixtures were centrifuged in the cold (755 g for 10 min) and cell-free supernates removed for enzyme assays. Beta-glucuronidase was determined after 18 h of incubation with phenolphthalein glucuronidate as substrate (2). Lysozyme was determined by the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N. J.) measured by decrease in absorbancy at 450 nm (37). Crystalline hen egg-white lysozyme (Worthington Biochemical Corp.) was used as a standard. The cytoplasmic enzyme, lactate dehydrogenase (LDH), was measured by the method of Wacker et al. (29) and used as an indicator of cell viability. Total enzyme activities were determined in simultaneously run, duplicate reaction mixtures to which had been added the detergent, Triton X-100 (0.2%) (Rohm and Haas Co., Philadelphia, Pa.).

Electron Microscopy

Replicate cell suspensions were fixed at room temperature, 1 min after the addition of ZTS as described in (40), and embedded in Spurr's low viscosity epoxy.

Morphometry

All centrioles visible on six sections cut from each of three experiments were photographed at × 17,000 and printed at × 50,000 on high contrast paper. Pericentriolar microtubules were counted, as described (6), from all

of the centrioles photographed. To test the possibility that microtubules in the cell periphery were more or less sensitive to colchicine than pericentriolar microtubules, we took micrographs of the right hand side of 9 consecutive cells per treatment from each of four separate experiments. To minimize observer bias, we selected the cytoplasmic areas at a magnification too low to distinguish microtubules, and subsequently focused at higher magnification. Negatives were printed to a final magnification of × 50,000 at high contrast. Each print was examined carefully and microtubules were counted, measured, and circled in ink. Profiles were considered to be microtubules if they had straight, parallel sides, 240–280 Å long, and were more electron dense than the ground cytoplasm. The volume density of microtubules was then estimated by superimposing a coherent double lattice test system (30). The coarse points, 2.5 cm apart, were used to measure cytoplasmic areas, and the fine points, 0.25 cm apart, to measure microtubule density. A minimum of 100,000 points or 250 μ^2 of cytoplasm per treatment were examined for microtubules.

RESULTS

Effects of Cytochalasin B and Colchicine on PMN Ultrastructure

Untreated PMN, prepared as described and examined in thin sections, have irregular profiles with an occasional agranular pseudopod. The centrioles and Golgi apparatus are usually centrally located, with nuclear lobes behind and to either side. The bulk of the granule population is in the pericentriolar cytoplasm and around the nuclear lobes. The granules are always at least 0.1 μm from the plasma membrane.

Cytochalasin B-treated PMN cannot maintain the agranular pseudopods of untreated cells and appear rounded with small projections from the cell surface. The centrioles and Golgi apparatus remain centrally located, with nuclear lobes behind and to either side. The granules are evenly distributed throughout the cytoplasm but do not approach the plasma membrane more closely than do those in untreated cells. Portions of the plasmalemma are retracted and appear as electron-transparent vacuoles which extend inward toward the pericentriolar cytoplasm (Fig. 1a). Previous transmission electron microscope studies with thorotrast (10) as well as scanning electron microscopy (33) have established that these apparent vacuoles are continuous with the extracellular space. Microtubules, radiating from the centrioles, were sometimes observed adjacent to and parallel with
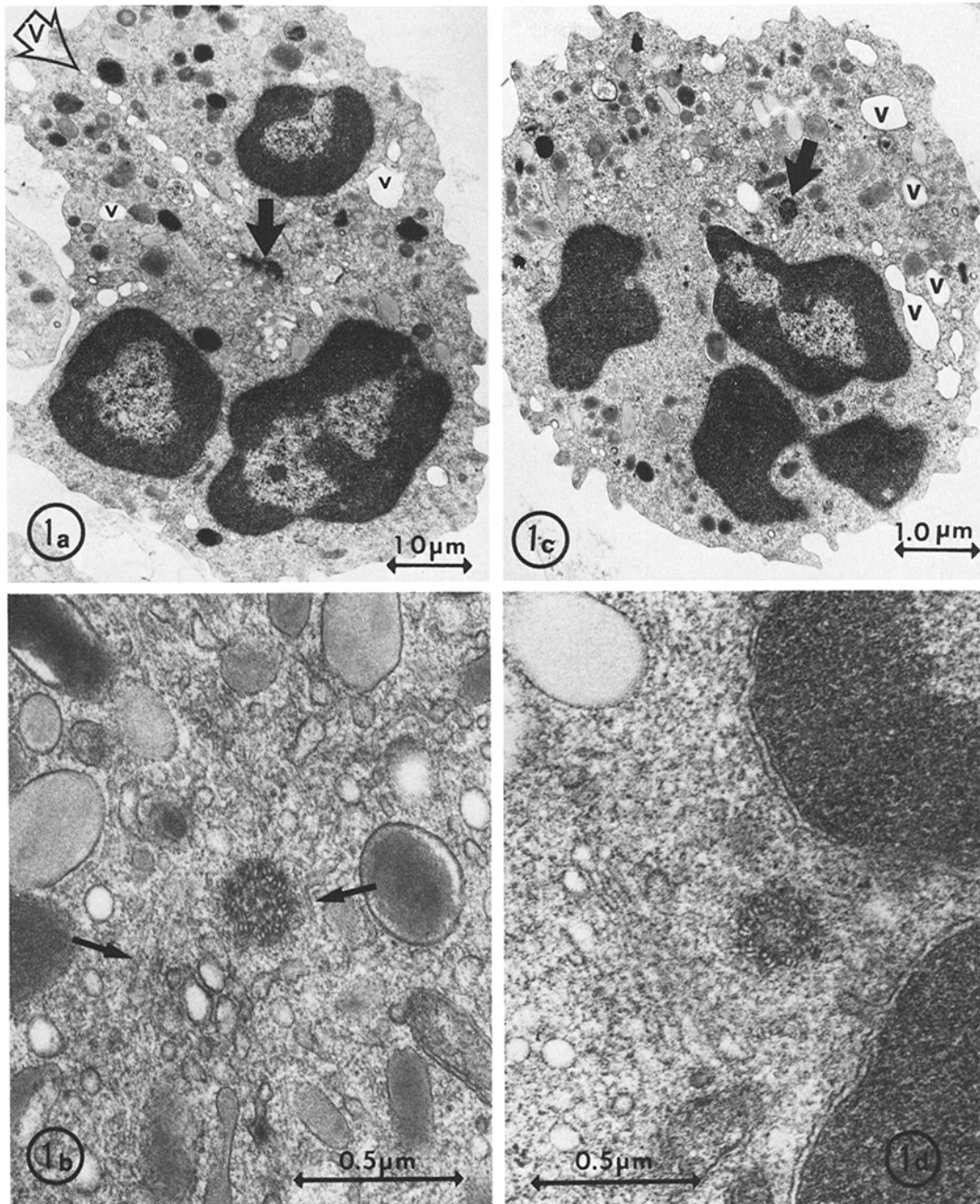
FIGURE 1 *a* An unstimulated, cytochalasin B-treated PMN. The centrioles (arrow) are between the nuclear lobes, and a row of vacuoles (*v*) can be seen extending from the cell periphery to the centriolar region. × 11,000. 1*b* A typical centriole from another unstimulated, cytochalasin B-treated PMN. Several microtubules (arrows) can be seen radiating from the centriole. × 50,000. 1*c* A PMN treated with both cytochalasin B and 10^{-5} M colchicine. The centriole (arrow) is now located to one side of the cell rather than between the nuclear lobes, and most of the vacuoles (*v*) are in the peripheral cytoplasm. × 11,000. 1*d* A centriole from an unstimulated, colchicine-treated (10^{-5} M) PMN sectioned in the same plane as the centriole in Fig. 1*b*. No microtubules are visible in the adjacent cytoplasm. × 50,000.

the long axis of these vacuoles. The impression gained from viewing numerous such cells was that the centrioles constituted a focus of symmetry and that microtubules formed the architectural framework (Fig. 1b). PMN exposed to colchicine (10^{-5}-10^{-6} M) appeared superficially like untreated cells. Colchicine did not alter the distribution of granules or the ability of the cells to form agranular pseudopods. Closer examination showed that the centrioles and Golgi apparatus were not in their usual position but were most frequently found between a nuclear lobe and the plasma membrane. Few or no microtubules were seen in the cytoplasm of these cells. Cytochalasin B-treated cells exposed to colchicine also showed the displacement of the centrioles and Golgi apparatus and the absence of the normal complement of microtubules. In addition, the vacuoles formed by the retracted plasma membrane were limited to the cell periphery (Figs. 1c and d).

Effects of ZTS on Ultrastructure of Untreated and of Cytochalasin B-Treated PMN

Untreated PMN stimulated by ZTS had very irregular profiles with more pseudopodial extensions than resting cells. More microtubules were also present in the cytoplasm of stimulated cells, but they were never seen to extend into the pseudopods. No morphological evidence of degranulation was seen and no enzyme release was detected biochemically. Cytochalasin B-treated PMN exposed to ZTS also had increased numbers of microtubules compared to similarly treated resting cells, and the radial symmetry around the centrioles was striking (Figs. 2 and 3). Massive degranulation occurred, and the vacuoles increased in size and number and approached the centrioles in linear arrays. Continuities between the internalized and external plasma membrane were seen frequently. So much membrane appeared to be added by granule fusion that degranulating cells, sectioned through a centriole, sometimes resembled flowers with numerous petals. Long microtubules were commonly seen running parallel with a row of vacuoles, extending from a centriole to the cell perimeter. Short, dense filaments, up to 250 nm long by 10-20 nm wide, were present in all cytochalasin B-treated PMN after stimulation by ZTS but not before, and were oriented tangential to plasmalemmal or vacuolar membranes. The dense filaments were always observed within less

dense aggregates of 40-60 Å filaments, and had the appearance of PMN myosin aggregates prepared in vitro (28).

Effect of ZTS on the Ultrastructure of PMN Pretreated with Both Colchicine and Cytochalasin B

PMN treated with high concentraitons of colchicine before stimulation by ZTS had few or no visible microtubules. The vacuoles were irregular in outline, generally were more peripherally located, and their position bore no relationship to the position of the centriole which was also frequently located in the cell periphery (Figs. 4 and 5). With 5×10^{-7} M colchicine, more microtubules were visible and the centrioles retained their central position, but many vacuoles remained in the cell periphery. Only a few vacuoles approached the pericentriolar cytoplasm. Cells exposed to 10^{-7} M or 10^{-8} M colchicine were indistinguishable from controls without colchicine. High concentrations of colchicine did not alter the appearance of dense filaments (Figs. 6a and b).

Effect of Colchicine on Translocation of Phagocytic Vacuoles

PMN exposed to immune complexes ingested them with great avidity and formed many small phagocytic vacuoles. When sections that included a centriole were examined, the vacuoles were seen to have a nonrandom distribution. Most of the vacuoles were on the same side of the cell as the centriole and approached very close to it (Fig. 7a). They were frequently seen to be closely associated with microtubules.

Colchicine-treated PMN (10^{-5} M) also ingested immune complexes, but the vacuoles had a random distribution and were not preferentially located on one side or the other of the cell. Vacuoles were not commonly seen near the centriole (Fig. 7b), and, when they were, that centriole had a few persistent microtubules.

Effect of Colchicine on Enzyme Release

Cytochalasin B-treated PMN exposed to ZTS for 5 min released $22.2 \pm 1.7\%$ of total beta-glucuronidase and $35.3 \pm 3.1\%$ lysozyme. This release was not influenced by colchicine concentrations of 10^{-7} M or less. Colchicine concentrations above 10^{-7} inhibited enzyme release in a
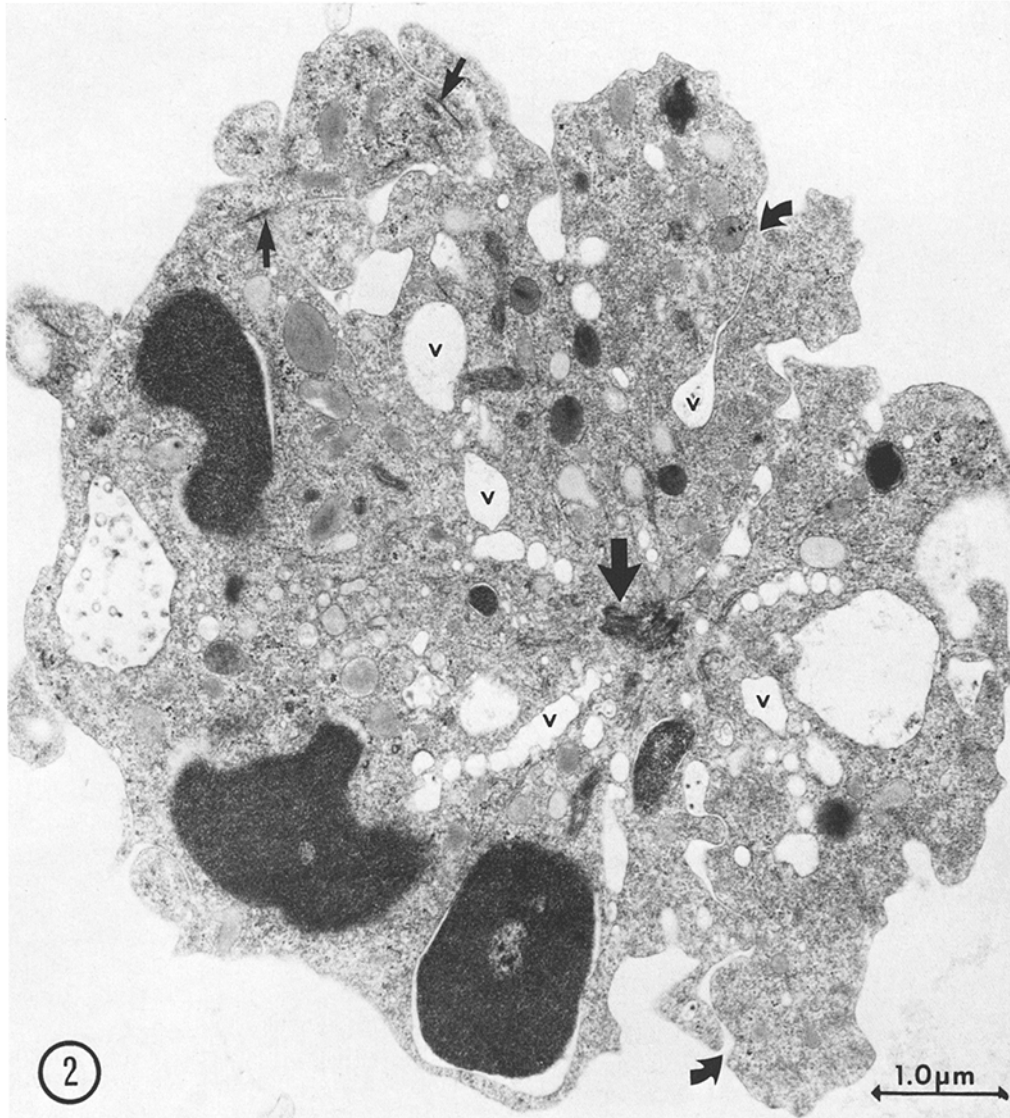
FIGURE 2   A cytochalasin B-treated PMN exposed to ZTS for one minute. The vacuoles (v) are more numerous and extend from the centriole (large arrow) to the cell periphery. Continuities between vacuoles and between vacuoles and plasma membrane are common (curved arrows). Dense filaments (small arrows) can be seen in the upper left of the figure. × 18,000.

dose-related fashion (see Table I). LDH release did not vary significantly from controls.

Effects of Colchicine on Microtubule Assembly

The mean number of microtubules visible in the centriolar region of cytochalasin B-treated cells preincubated with a high-dose (10^{-5} M) of colchi- cine was 3.2 ± 0.6, compared to 21.5 ± 1.7 in control cells. This was not significantly different from the mean number seen in cells without cyto-chalasin B. Those microtubules visible after col-chicine treatment were morphologically similar to those in control PMN. Stimulation with ZTS in-creased the number of microtubules visible in un-treated cells and in cells treated with less than 10^{-7}
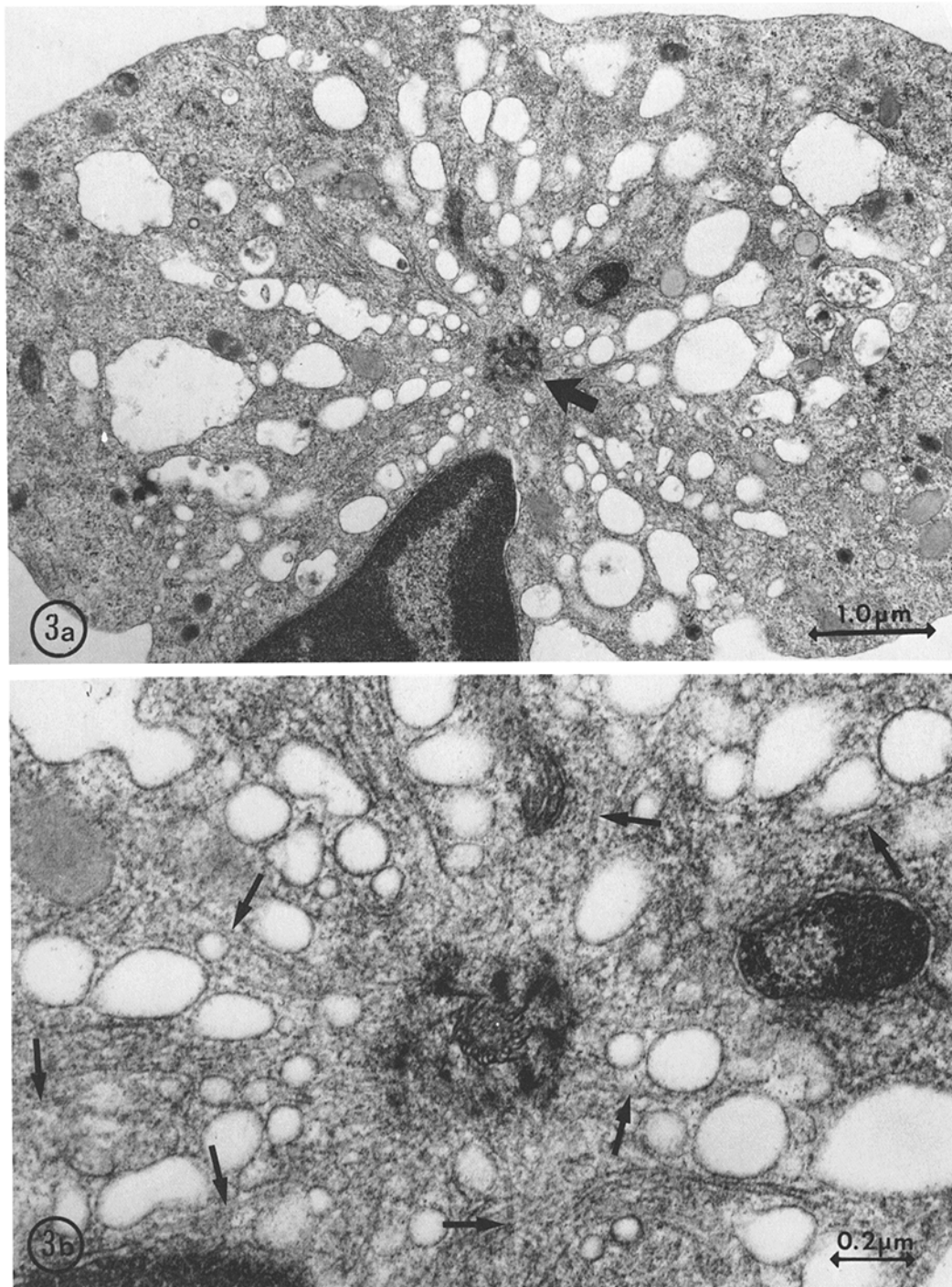
FIGURE 3 *a* A cell similar to that in Fig. 1. Degranulation is almost complete and the radial symmetry around the centriole is striking. × 16,000. 3*b* A higher magnification view of the centriolar region of the stimulated cell in Fig. 3*a*. Many microtubules (arrows) are visible radiating from centriole-associated organizing sites. It can be seen in this micrograph that the radial arrangement of vacuoles and organelles around the centriole is paralleled by a similar radial arrangement of microtubules. × 50,000.
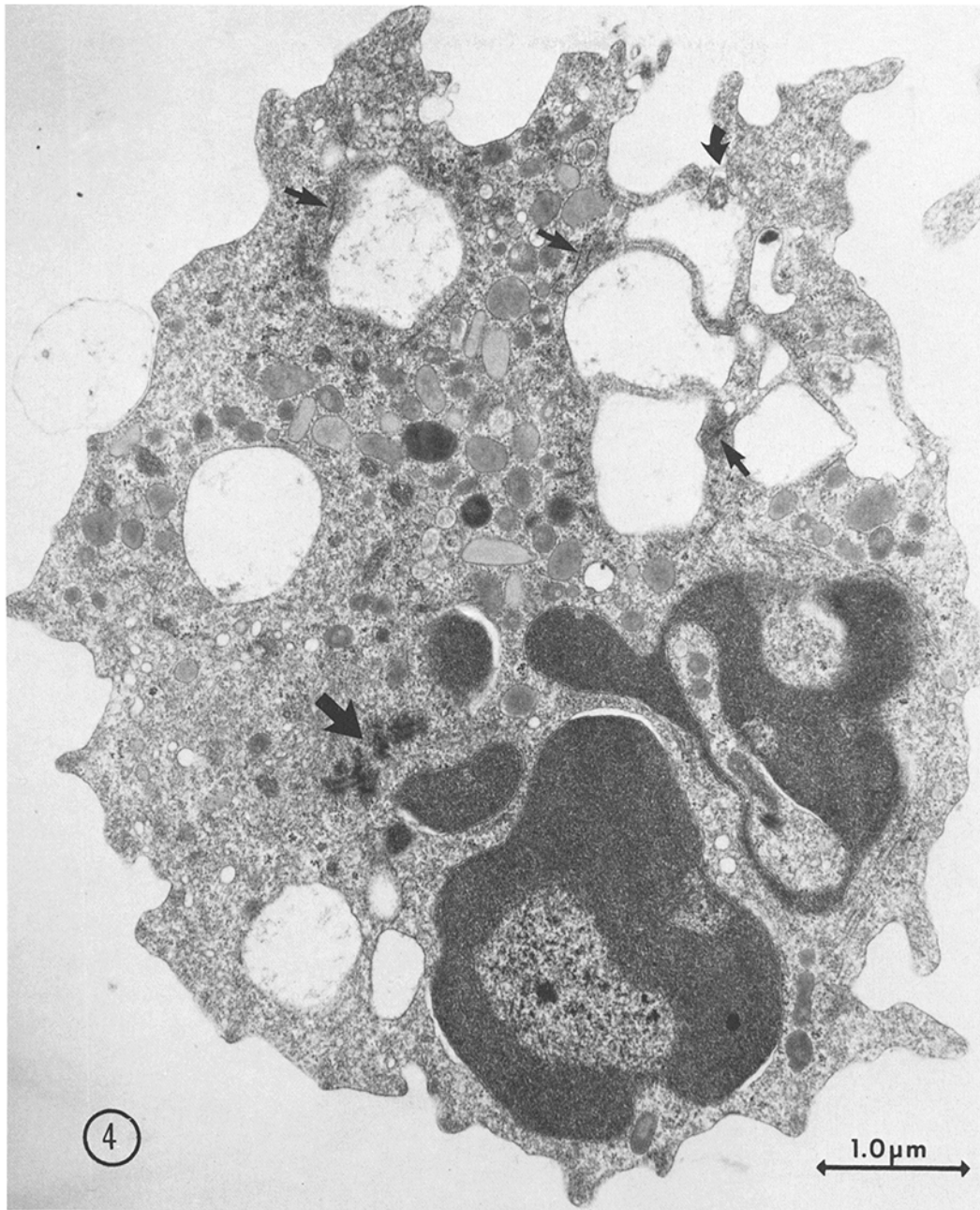
FIGURE 4 A cytochalasin B-treated PMN preincubated with 10^{-5} M colchicine for one hour and exposed to ZTS for one minute. The centrioles (large arrow) can be seen in the lower left of the figure, and large vacuoles are scattered around the cell periphery. Continuity between a vacuole and the plasma membrane can be seen near the top of the figure (curved arrow). Regions of cytoplasm can be seen that still contain many granules, and dense filaments (small arrows) are present adjacent to vacuoles in the upper portion of the figure. × 18,000.
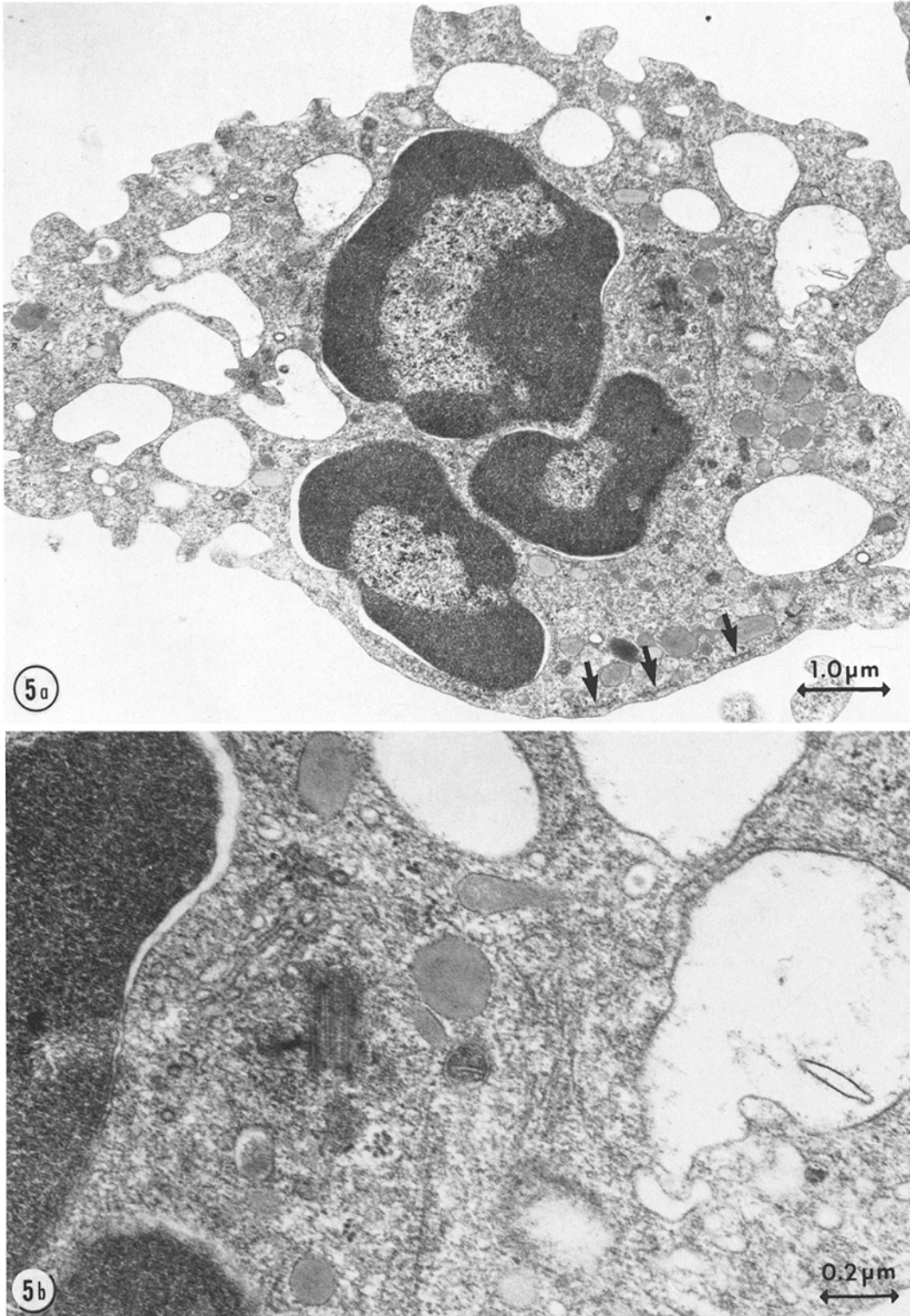
FIGURE 5 *a* Another cell treated as in Fig. 4. The vacuoles are large and without orientation with respect to the centriole (arrow). Dense filaments are present adjacent to the plasma membrane in the lower right of the figure (small arrows), and regions of cytoplasm without vacuoles contain numerous granules. × 16,000. 5*b* A higher magnification view of the centriolar region of the colchicine-treated, stimulated cell shown in Fig. 5*a*. No microtubules are visible adjacent to the centriole-associated microtubule organizing site. × 50,000.
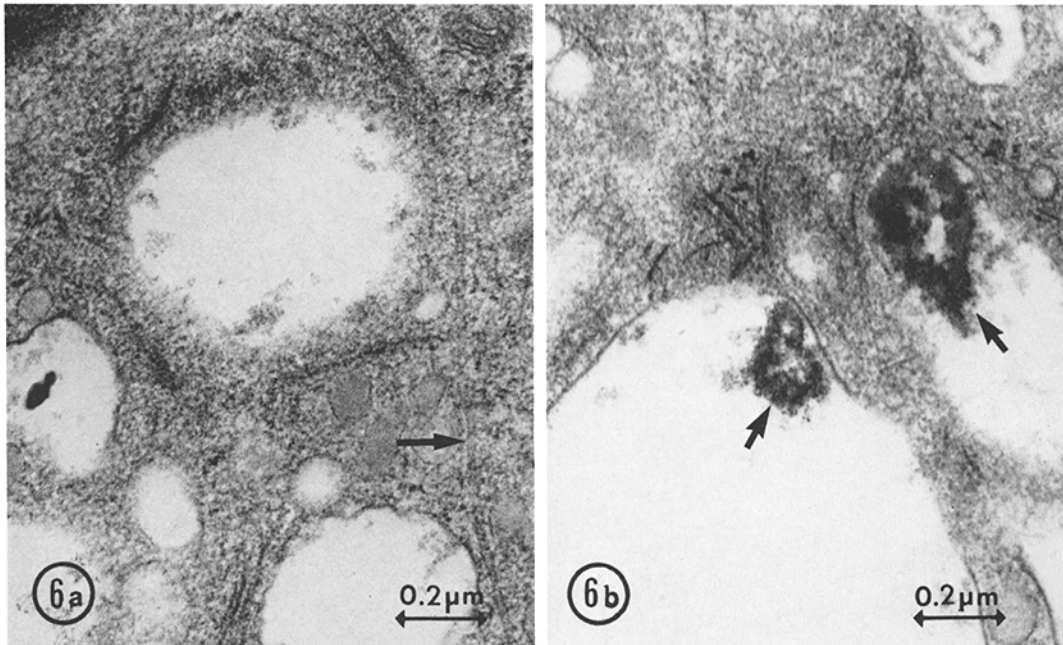
FIGURE 6 *a* A higher magnification view of a vacuole from a cytochalasin B-treated, ZTS-stimulated cell showing a vacuole with tangentially arranged, dense filaments and an adjacent microtubule (arrow). × 50,000. 6*b* A portion of a cell treated with both cytochalasin B and colchicine (10^{-5} M), then stimulated with ZTS. Many dense filaments can be seen, but no microtubules. Discharge of granule contents into vacuoles (arrows) is visible near the filaments. × 50,000.

colchicine, but assembly was inhibited at higher concentrations and few microtubules were visible at 10^{-5} M or 10^{-6} M (Fig. 4 and Table I). Colchicine inhibition of ZTS-stimulated microtubule assembly was also observed in cells without cytochalasin B.

Since the enzyme release studies indicated that inhibition of beta-glucuronidase release was significantly greater at 10^{-5} M than 10^{-6} M, a morphometric study of microtubules in the peripheral cytoplasm was undertaken to determine whether there was a corresponding difference at the two dose levels. Whereas there was a marked increment in the number of microtubules per square micron in untreated cells after ZTS stimulation (0.23 ± 0.06 to 0.42 ± 0.05), pretreatment with colchicine prevented this increase. Instead, the frequency of visible microtubules was below that in resting control cells. After 10^{-5} colchicine the frequency was 0.05 ± 0.02, and after 10^{-6} M the frequency was 0.14 ± 0.03 (Table II). Point counting estimates of microtubule volume density confirmed the results obtained by counting. The ratio of points on microtubules to points on cytoplasm increased in untreated cells after stimulation

but was below resting levels in high-dose colchicine-treated cells. Again, the volume density of microtubules appeared to be somewhat higher in 10^{-6} as compared to 10^{-5} M colchicine-treated cells (see Table III).

DISCUSSION

Numerous studies from this and other laboratories have shown that release of lysosomal enzymes to the outside of PMN during phagocytosis is affected by agents that influence the state of assembly of microtubules (18, 32, 39, 41, 42), and have interpreted these data as applying directly to fusion of lysosomes with phagosomes (14, 15, 32, 33, 41, 42). In these earlier studies, in which selective enzyme release from stimulated PMN was measured, agents known to promote disassembly of microtubules, i.e., colchicine and vinblastine, decreased enzyme release. In contrast, D_2O, which increases microtubule stability (16, 25), increased enzyme release (42). This interpretation was supported by data which showed inhibition of phagocytic degranulation by colchicine (10, 39). Moreover, cyclic nucleotide-modulated increments and decrements in enzyme release (cGMP
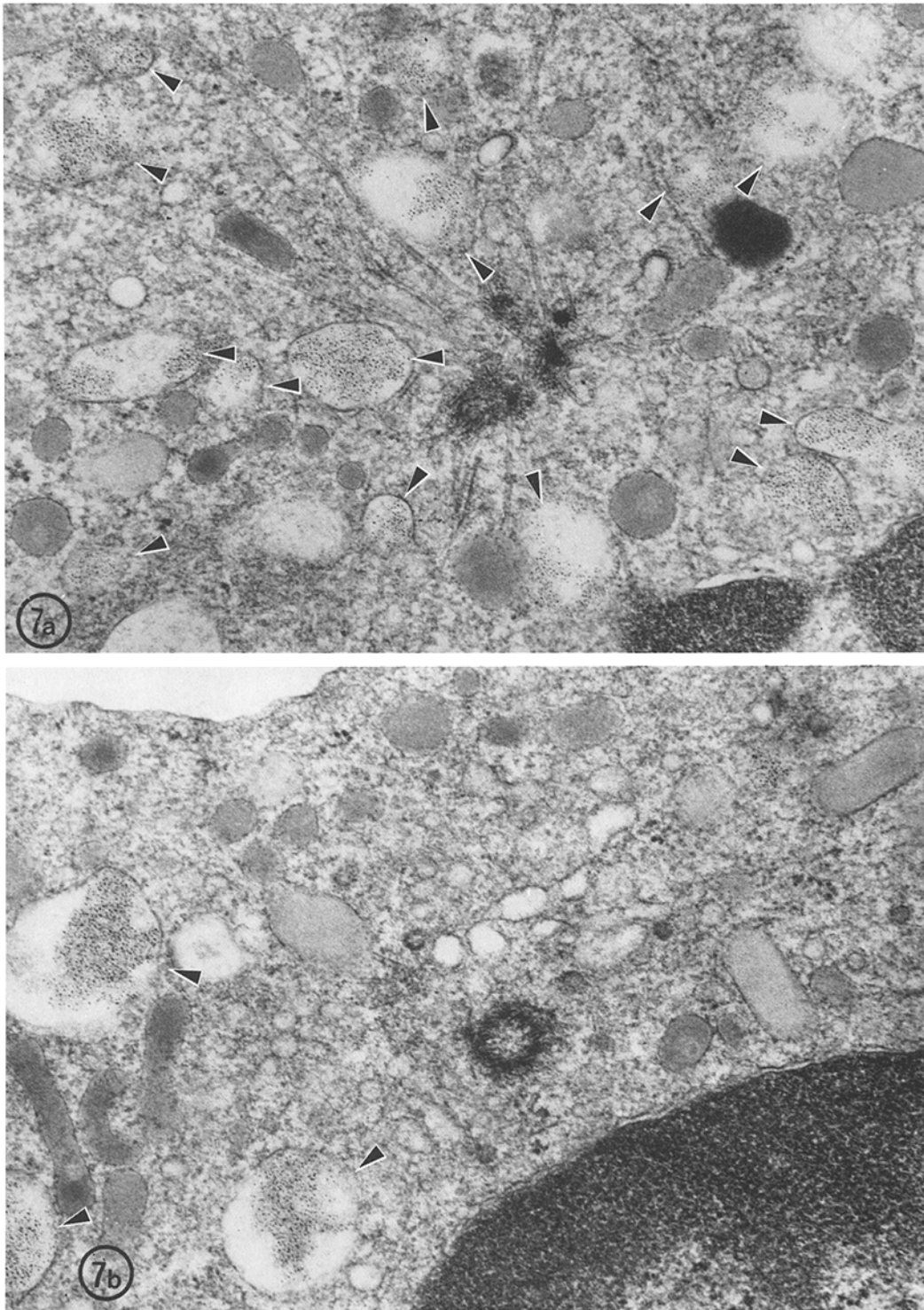
FIGURE 7 *a* The pericentriolar cytoplasm of an untreated PMN exposed for 5 min to ferritin- anti-ferritin immune complexes. Many microtubules radiate from centriole-associated microtubule organizing sites. Fourteen ferritin-containing phagocytic vacuoles (arrowheads) are visible in this small portion of the cytoplasm. × 25,000. 7*b* A corresponding portion of a colchicine-treated PMN (10^{-5} M) similarly exposed to ferritin- anti-ferritin. In contrast to the cell in Fig. 7*a*, only a few microtubules and three phagocytic vacuoles are visible. × 25,000.

↑ cAMP ↓) correlated closely with increments and decrements in microtubule numbers (33). Finally, increased numbers of microtubules were consistently seen in stimulated as compared to resting cells. The data presented here show that, in both cytochalasin B-treated and untreated PMN, colchicine causes a dose-related inhibition of microtubule assembly. Colchicine concentrations which affect microtubule assembly also inhibit lysosomal enzyme release in a dose-related fashion, but concentraitons which cause the virtual

disappearance of microtubules inhibit enzyme release by no more than 40% (31, 41). Thus, lysosomal enzyme release from PMN is influenced by the state of assembly of microtubules but not in a simple direct way.

Lysosomal enzyme secretion to the external medium in response to a given stimulus reflects varying rates of: (a) cell motility, or chemotaxis; (b) phagocytosis; (c) translocation of phagosomes to the granular cytoplasm; and (d) fusion of granules with complete or incomplete (open) phagosomes. In order to understand the role of microtubules in enzyme release from phagocytes, we must clearly distinguish the steps involved. Pesanti and Axline (19, 20) have shown that doses of colchicine sufficient to inhibit microtubule assembly in resting macrophages have no effect on fusion as measured by transfer of acid phosphatase to phagocytic vacuoles or degradation of ingested material, although phagocytosis *per se* is reduced. They suggest that fusion of organelles may be modulated by contractile elements (microfilament system) but not by means of microtubules.

In the present combined morphologic and biochemical study of the role of microtubules in enzyme release, we have used ZTS, a soluble stimulus for enzyme release. Its active component (C5a) is a low molecular weight protein which makes contact with all areas of the plasma membrane simultaneously and equally, and all enzymes released by fusion of lysosomal granules with the plasma membrane appear in the surrounding medium. Thus, cell motility and phagocytosis are eliminated as possible variables. Therefore, the only variables remaining to be influenced by mi-

TABLE I

Inhibition of Microtubule Assembly in, and Enzyme Release from, Cytochalasin B-Treated Human PMN Stimulated by ZTS*

Colchicine	Mean microtubule numbers‡	Enzyme release (%)§	
		Beta-Glucuronidase	Lysozyme
[M]			
0	35.6 ± 2.0	100	100
10^{-8}	32.2 ± 2.1	101.8 ± 6.5	95.6 ± 1.1
10^{-7}	36.0 ± 2.5	91.4 ± 5.2	99.7 ± 5.3
5×10^{-7}	11.6 ± 1.3‖	78.5 ± 3.9‖	86.7 ± 4.2‖
10^{-6}	4.0 ± 0.4‖	74.1 ± 3.4‖	82.7 ± 3.4‖
10^{-5}	2.8 ± 0.4‖	59.8 ± 3.9‖	61.2 ± 4.2‖

* Cells were preincubated with cytochalasin B (5.0 μg/ml) for 10 min and exposed to ZTS (10%, vol/vol) for 5 min at 37°C.

‡ Microtubules within 4 μm² centered on a centriole. Mean ± SEM (n = 17).

§ 100% = enzyme release from cells exposed to ZTS in the absence of colchicine: beta-glucuronidase = 70.7 ± 3.9 μg phenolphthalein per 2 × 10⁶ PMN; lysozyme = 2.8 ± 0.3 μg crystalline hen-white lysozyme equivalents per 2 × 10⁶ PMN. Enzyme release from resting cells (not exposed to ZTS) was 3.8 ± 0.7 and 1.8 ± 0.3, respectively. Release from unstimulated cells was not altered significantly by pretreatment with colchicine. Total activity released by 0.2% Triton X-100: beta-glucuronidase = 262 ± 20; lysozyme = 7.8 ± 1.2.

‖ Significant at $P < 0.005$ vs. control of 100%.

TABLE II

Effect of Colchicine on Microtubule Frequency* in Peripheral Cytoplasm of Cytochalasin B-Treated Human PMN Stimulated by ZTS‡

Experiment	Resting cells (No colchicine)	Stimulated cells (Colchicine concentrations)		
		None	$10^{-5} M$	$10^{-6} M$
1	0.23	0.60	0.11	0.15
2	0.33	0.43	0.06	0.09
3	0.21	0.49	0.07	0.19
4	0.14	0.33	0.12	0.15
M ± SEM	0.23 ± 0.04§	0.46 ± 0.06	0.09 ± 0.01§	0.15 ± 0.02§‖

* Mean number of microtubules per square micron of peripheral cytoplasm. Determined from 9 micrographs per treatment per experiment.

‡ Cells were preincubated with cytochalasin B (5.0 μg/ml) for 10 min and with colchicine for 60 min, then exposed to ZTS (10% vol/vol) for 1 min at 37°C.

§ $P < 0.002$ vs. stimulated cells prepared without colchicine.

‖ $P < 0.025$ vs. 10^{-5} M colchicine by matched pair t test.

TABLE III

Effect of Colchicine on Volume Density $(\times 10^{-4})*$ of Microtubules in Peripheral Cytoplasm of Cytochalasin B-Treated Human PMN Stimulated by ZTS‡

Experiment	Resting cells (No colchicine)	Stimulated cells (Colchicine concentrations)		
		None	10^{-5} M	10^{-6} M
1	12.5	25.7	5.6	5.8
2	8.3	11.6	1.4	3.5
3	8.1	19.0	1.7	3.1
4	5.9	14.3	3.0	5.1
M ± SEM	8.8 ± 1.4§	17.7 ± 3.1	2.9 ± 1.0§	4.4 ± 0.6§‖

* Ratio of test points on microtubule profiles to total points on cytoplasm. Determined from 9 micrographs per treatment per experiment.

‡ Cells were preincubated with cytochalasin B (5.0 μg/ml) for 10 min and with colchicine for 60 min, then exposed to ZTS (10% vol/vol) for 1 min at 37°C.

§ $P < 0.005$ vs. stimulated cells by matched pair t test.

‖ $P < 0.01$ vs. 10^{-5} M colchicine by matched pair t test.

crotubules are the translocation of stimulated plasma membrane (now a surrogate phagosome) into granule-rich areas within the cytoplasm and/or the fusion of lysosomal membranes with the plasma membrane: processes (c) and (d). The data suggest that it is the former rather than the latter process that is microtubule dependent.

The morphologic correlate of reduced microtubule assembly in unstimulated PMN is an apparent disorganization of the central region of the cell, with the centrioles and Golgi apparatus displaced from their usual position between the nuclear lobes. The agranular cytoplasm appears not to be adversely affected by colchicine and remains capable of forming pseudopods. Cytochalasin B prevents the formation of pseudopods, but the pericortical cytoplasm retains sufficient integrity to exclude granules.

Upon stimulation, microtubules were seen in greater numbers in both cytochalasin B-treated and untreated PMN than in resting cells. Increased microtubule volume density was also documented by point counting morphometry. Interestingly, a similar volume density was obtained for resting PMN as was obtained by Reaven et al. (23) for rat parathyroid glands. The increase in microtubule numbers was seen throughout the cytoplasm, but microtubules were most conspicuous extending in a radial pattern from centriole-associated organizing sites. Invaginations of the plasma membrane in cytochalasin B-treated, ZTS-stimulated PMN were prominent along these radiating microtubules in the pericentriolar cytoplasm of cells permitted to assemble microtubules. Cells that were prevented by colchicine from assembling microtubules had a more random pattern of invag-

inations. In the absence of microtubule assembly, there appeared to be no coordinated translocation of stimulated plasma membrane to the centriolar region, a finding in keeping with the observations of Freed and Lebowitz (5).

Unfortunately, although ZTS induces microtubule assembly in both cytochalasin B-treated and untreated PMN, enzyme release occurs only from stimulated cells pretreated with cytochalasin B (6). This may be because cytochalasin B inhibits the gelation of actin by interfering with actin-binding protein interactions, although it does not depolymerize actin filaments or inhibit the Mg^{++} ATPase activity of a cofactor-activated actinomyosin (7, 22). Phagocytosis is adversely affected by cytochalasin B-treatment but not stimulus-coupled secretion. To ensure that the phenomena observed were not due to cytochalasin B-treatment, we also examined the morphologic consequences of inhibition of microtubule assembly in cells without cytochalasin B which were permitted to phagocytose small particles of immune precipitates. These true phagosomes were also translocated to the centriole region when microtubule assembly was permitted and were closely associated with microtubules. When microtubule assembly was inhibited by colchicine, phagosomes were distributed randomly throughout the cytoplasm.

The data suggest that translocation of phagosomes within the cytoplasm is modulated by microtubule assembly rather than fusion itself and that correlations between tubule assembly and disassembly and enhanced or diminished enzyme release (33) probably reflect events earlier in the secretory process of PMN. Although our data do not show that fusion is unaffected by colchicine, it

is significant that inhibition of microtubule assembly inhibits enzyme release by no more than 40%. Microtubules, therefore, appear to be involved in maintaining the internal organization of PMN and the topologic relationships between their organelles and the plasma membrane. Assembly may enhance, or disassembly diminish, the chances for contact between PMN granules and stimulated areas of the plasma membrane. Other structures, perhaps contractile proteins, may play a more direct role in permitting fusion between granules and phagosomes or the membrane of the cell itself.

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