Concanavalin A (Con A) has been shown to bind to cell surface glycoproteins of polymorphonuclear leukocytes (PMN's). The reported sequelae of Con A binding to PMN are capping, in which the lectin and bound membrane glycoproteins form a dense collection in one area of the cell surface, and internalization of plasma membrane bearing both bound lectin and glycoproteins (14, 15). These phenomena may depend upon the state of assembly of microtubules since the effects of Con A can be modified by either the addition of colchicine (5 μM) or incubation at 4°C (11, 14), treatments which move the microtubule = tubulin monomer equilibrium to the right (13). Furthermore, leukocytes of the beige mouse (a homologue of Chediak Higashi) behaved like colchicine-treated cells with respect to Con A-induced capping but resembled normal cells if pretreated with agents that raise intracellular cGMP (12) and enhance microtubule assembly (18). These observations suggest a relationship between Con A-influenced cell surface phenomena and microtubule assembly.

This report presents evidence that Con A does indeed influence the dynamics of microtubule assembly or stability as shown by the presence of greater numbers of microtubules in Con A-treated human PMN's than in resting cells. In addition, Con A-treated PMN's selectively discharge specific granules without the concomitant discharge of azurophilic granules.

**MATERIALS AND METHODS**

Leukocyte suspensions containing approximately 85% PMN's were obtained from human peripheral blood (22), and suspended in 5 mM HEPES (Grand Island Biological Co., Grand Island, N.Y.), and 150 mM NaCl, adjusted to pH 7.4.

**Enzyme Release**

Leukocyte suspensions (1 ml, 2 × 10⁶ cells) were incubated at 37°C with or without Con A (Sigma Chemical Co., St. Louis, Mo.) and/or cytochalasin B for the durations indicated. The tubes were then centrifuged (800 g, 10 min) and the cell-free supernates assayed for
lysozyme (20), β-glucuronidase (2), and lactate dehydrogenase (LDH) (16). Enzyme activities are expressed as percentage of total activity released from duplicate reaction mixtures by 0.2% Triton X-100.

Ultrastructural Studies

Leukocyte suspensions were incubated with ferritin-Con A (Cappel Laboratories, Downington, Pa.) or with 100 μg/ml Con A at 37º C for varying periods of time, then fixed in suspension by a rapid procedure which gives relatively good preservation of microtubule organization (21). In a few experiments, cell pellets from enzyme release studies were fixed and reacted for myeloperoxidase (5) and then used for morphometric studies of granule volume.

Morphometry

All centrioles visible on silver sections cut from two blocks from each of two specimens were photographed at × 17,000 and printed at × 50,000 on high-contrast paper. Microtubules were counted from all of the centrioles photographed. Profiles were considered to be microtubules if they had straight parallel sides, 240–280 Å apart, were at least 550 Å long and were more electron dense than the ground cytoplasm. Only those microtubules were counted that were within a 2 μm × 2 μm square centered upon a centriole. To test the possibility that increases in mean numbers of pericentriolar microtubules reflect redistribution rather than an increase in cytoplasmic microtubules, counts were also made in four randomly selected 1 μm × 1 μm areas of peripheral cytoplasm from electron micrographs of individual control and Con A-treated cells.

Granule volume as a percentage of cytoplasmic volume was determined by point counting on neutrophil profiles from cell pellets of two replicate enzyme release experiments. Micrographs (10–15 per treatment) were printed to a final magnification of 16,000 and a coherent square lattice with 1-μm spacing was superimposed. The number of intersections that fell on granules (Ng) over the number of total cytoplasmic intersections exclusive of vacuoles or nuclear lobes (Nc + Ng) gave the granule to total cytoplasm ratio (Ng/Nc + Ng) or fractional granule volume (17). The entire cytoplasmic area in each micrograph was sampled and at least 1,000 points were counted per treatment. The significance of the differences between means was determined by Student’s t test.

RESULTS

The number of microtubules visible in Con A-treated leukocytes was enhanced above levels seen in resting cells within 2 min of adding Con A, and remained elevated for as long as the study was conducted (60 min). Microtubules were most numerous in the centriolar region of the cells (Fig. 1). They did not appear to arise from the centrioles themselves but radiated from adjacent, electron-dense centers (Figs. 1 and 2). Microtubules from more than one such center were usually visible in any single section through a centriole. Thus, there was relatively little variation, from section to section, in the number of microtubules visible in the centriolar region of a single cell, although their distribution varied with the plane of section. Many of these microtubules were very long, extending to the pericortical cytoplasm, where some were seen to curve and run for a short distance parallel to the plasma membrane. Many microtubules were also seen in some, but not all, of the lymphocytes present in these preparations. As in the neutrophils, they were most numerous in the centriolar region, although in some cells they were seen in parallel arrays just under the plasma membrane. Since microtubules were most abundant in the pericentriolar cytoplasm, most of the microtubule counts were made in this region. However, to test the possibility that Con A caused a redistribution of pre-existing microtubules from peripheral areas to the centriolar region, microtubules were also counted in equivalent areas of peripheral cytoplasm from control and Con A-treated cells. The mean number of microtubules visible in peripheral areas of resting cells was 1.8 ± 0.3 (n = 21, four specimens) as opposed to 5.9 ± 0.7 in cells exposed

FIGURE 1 (a) Centriole region of a control human neutrophil showing centriole-associated microtubule organizing sites but few microtubules (arrows). × 50,000. (b) A similar region from a human neutrophil exposed to Con A (100 μg/ml, 5 min, 37º C). Microtubules (arrows) are longer and more numerous than in control cells incubated without Con A. × 50,000.

FIGURE 2 Part of a neutrophil fixed after a 5-min exposure to ferritin-Con A. This section is tangential both to a centriole and to a portion of the plasma membrane bearing the ferritin label (arrowheads). It also includes a centriolar-associated microtubule organizing site (curved arrow). Many microtubules can be seen extending from the centriolar region to the labeled portion of the plasma membrane. In the lower left of this figure is a vacuole with a small amount of bound ferritin and adjacent microtubules. Material which may have been derived from granules can be seen in the vacuole. × 42,000.
to Con A for 15 min ($n = 15$, five specimens). The corresponding numbers in the pericentriolar region were $17.8 \pm 1.4$ (resting) and $43.3 \pm 2.3$ (Con A). Therefore, the increased number of microtubules found in the pericentriolar region probably is not due to redistribution of pre-existing, peripherally located tubules, but reflects a general assembly of tubules in all areas of cytoplasm.

Membrane internalizations bearing the ferritin marker were seen within neutrophils as early as 1 min after the addition of ferritin-Con A. Although most of these were peripherally located at this time, some invaginations extended down into the perinuclear region. Many microtubule profiles were seen in close proximity to these invaginations (Fig 2). By 15 min large ferritin-containing vacuoles were present in the perinuclear region of many cells. Microtubules were seen associated with these vacuoles throughout the 1-h course of the experiment. Some of the vacuoles may be closed to the outside since a far smaller proportion of bound Con A can be eluted from PMN's incubated with Con A at $37^\circ$ C than at $4^\circ$ C (11). Both the large perinuclear vacuoles and the elongated invaginations frequently contained a moderately electron-dense substance, and profiles of degranulation into these vacuoles were common. A few of the peripheral vacuoles were large and electron-lucent and appeared to result from active pseudopod formation. Occasionally, two PMN's were seen to be closely entwined with interdigitating pseudopods. In these instances, ferritin and the moderately electron-dense substance were seen between the cells. The images closely resembled those of cooperative endocytosis seen when PMN's were fixed while ingesting antigen-antibody complexes (10).

Since profiles suggestive of granule discharge were seen, cell supernates were assayed for lysozyme (present in both specific and azurophilic granules) and LDH to measure cell death. It was not possible to determine biochemically whether one or both types of granules were discharged since azurophilic enzymes (myeloperoxidase and $\beta$-glucuronidase) (1) sedimeted with Con A-treated cells and cell fragments. Adsorption of $\beta$-glucuronidase by Con A-treated PMN's may account for some of the apparent inhibition, by Con A, of $\beta$-glucuronidase release from PMN's exposed to phagocytic stimuli (9).

Con A induced a dose- and time-dependent release of lysozyme (60 min, $37^\circ$ C) (Figs. 3 and 4) over the range tested. LDH release was not significantly different from controls, indicating that Con A-induced release of lysozyme was not due to enhanced cell death. Release of lysozyme was reduced almost to control levels when 60 mM alpha-methyl-D-mannoside was added to the reaction mixture simultaneously with Con A. Pretreatment with cytochalasin B ($5 \mu g/ml$, 10 min), a dose which inhibits phagocytosis (3) and enhances lysosomal enzyme release (21), enhanced the specific release of lysozyme. Incubation of cytochalasin B-treated cells with 100 $\mu g/ml$ of Con A resulted in the release of 28.9 $\pm$ 5.7% of total lysozyme within 15 min and of 49.9 $\pm$ 3.4% in 60 min, whereas control cells with cytochalasin B but without Con A released 8.9$\% \pm 3.8$% of total lysozyme within 15 min, and this control release did not increase by 60 min.

In order to determine the intracellular source of the released lysozyme, leukocytes, reacted for myeloperoxidase, were examined by electron microscopy. Numerous peroxidase-positive azurophilic and some peroxidase-negative specific granules remained visible in the cytoplasm after Con A treatment. Most of the perinuclear vacuoles were peroxidase negative. Peroxidase reaction product, when present, usually was seen to line the vacuole and frequently outlined peroxidase-negative material. The percent volume of peroxidase-negative granules (determined as percent of cytoplasm, exclusive of vacuoles) was significantly reduced with respect to control cells. The percent volume of
peroxidase-positive granules was not significantly affected by Con A (Table I). Cytochalasin B- treated cells exposed to Con A contained no peroxidase-positive or -negative vacuoles. Whereas peroxidase-positive granules were numerous, peroxidase-negative granules were scant and were not seen at all in some cell profiles. The percent volume of peroxidase-positive granules was not significantly different from controls, but the percent volume of peroxidase-negative granules was greatly reduced (Table I) corresponding to the enhancement of Con A-induced lysozyme release from cytochalasin B-treated neutrophils.

Because of the possibility that cytochalasin B may have induced a change in cytoplasm volume rather than cell shape (10) or that the loss of specific granules caused a significant decrease in the denominator (or total cytoplasmic volume), the raw data obtained by point counting were re-calculated as the ratio of granule volume to agranular cytoplasm volume. This re-calculation had no effect upon the relationships shown in Table I. Differences that were statistically significant remained significant, and those that were without significance remained without significance. Thus, the lysozyme released from Con A-treated PMN's appears to be derived mainly, if not exclusively, from specific granule discharge.

Con A therefore induces both microtubule assembly or stability and secretion of lysozyme from human neutrophils and adds another example to the literature of an association between enhanced microtubule numbers and secretion in human neutrophils (6, 7). These results also suggest that although microtubule assembly may be necessary, it may not by itself be sufficient to provoke discharge of both azurophil and specific granules from human neutrophils. They suggest further that the signal for discharge of specific granules differs either qualitatively or quantitatively from the signal for discharge of azurophilic granules. No

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\text{Table I}
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Granule Volume as Percentage of Cytoplasmic Volume* in Concanavalin A-treated Human Neutrophils (± Cytochalasin B)

| Treatment | Peroxidase-pos. granules | % | Peroxidase-neg. granules | % | Lysozyme release % of total |
|-----------|--------------------------|---|--------------------------|---|---------------------------|
| None (n = 14) | 16.7 ± 1.3 | NS | 11.6 ± 0.7 | <0.001 | 2.6 |
| Con A (n = 15) | 19.5 ± 1.8 | 6.0 ± 1.1 | 20.2 |
| Cytochalasin B§ (n = 10) | 16.2 ± 1.6 | NS | 8.9 ± 0.7 | 8.6 |
| Cytochalasin B + Con A (n = 11) | 14.3 ± 1.9 | NS | 1.2 ± 0.3 | <0.001 | 49.9 |

* Leukocyte suspensions were treated as above, then centrifuged. The pellets were fixed for myeloperoxidase cytochemistry, and the supernates assayed for released enzymes. Granule volume as a percentage of cytoplasmic volume was determined as described in Materials and Methods.

§ 100 µg/ml.

§ 5 µg/ml 10 min.
agents have as yet been reported which trigger release of the latter without, at the same time, inducing release of the former, although Ca supp and phorbol myristate acetate have been reported to induce release of specific but not azurophil granules.

**SUMMARY**

Human neutrophils stimulated by concanavalin A (Con A, 100 μg/ml) contained markedly enhanced numbers of microtubules and discharged peroxidase-negative (specific) but not peroxidase-positive (azurophil) granules. Release of lysozyme from specific granules was dose and time dependent, and could be inhibited by alpha-methyl-o-mannoside, and enhanced by cytochalasin B. Many microtubules were associated with internalized plasma membrane bearing Con A binding sites.

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