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Mechanisms of Lysosomal Enzyme Release from Human Leukocytes: Microtubule Assembly and Membrane Fusion Induced by a Component of Complement

(C5a/chemotaxis/cytochalasin B/cAMP: cGMP antagonism)

IRA GOLDSTEIN*, SYLVIA HOFFSTEIN*, JOHN GALLIN†, AND GERALD WEISSMANN*

* Department of Medicine, New York University School of Medicine, New York, N.Y., 10016; and † Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **A low-molecular-weight component of complement, similar to or identical with human C5a, interacts with human polymorphonuclear leukocytes treated with cytochalasin B and provokes extracellular release of lysosomal enzymes from these cells. Enzyme release occurs in the absence of particles and is selective in that it is not accompanied by release of cytoplasmic enzymes. Cell viability is not altered. Pharmacologic agents that regulate secretion of other inflammatory mediators influenced complement-dependent enzyme release: cAMP and theophylline, prostaglandin E_1 and colchicine *inhibited*, whereas cGMP *enhanced* release of enzymes. Ultrastructural histochemistry of cells exposed to this component of complement revealed degranulation, fusion of lysosomal with plasma membranes, and transient assembly of microtubules associated with the release of endogenous myeloperoxidase. Our findings suggest that these intracellular events are common to two important responses of polymorphonuclear leukocytes in inflammation and tissue injury: (*a*) release of lysosomal hydrolases and (*b*) chemotaxis.**

Enzymes and mediators of inflammation ordinarily sequestered within lysosomes are released from viable human polymorphonuclear leukocytes (PMNs) during phagocytosis by "regurgitation during feeding" (1–3) or when cells adhere to an immunologically prepared, nonphagocytosable surface by "reverse endocytosis" (3, 4). These two mechanisms of lysosomal enzyme release have been studied in our laboratory and depend upon the integrity of cytoplasmic microtubules (3,5). Enzyme extrusion is also enhanced or inhibited by agents that affect the levels within cells of adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) (1–3, 5).

Recently, these mechanisms of enzyme extrusion have become amenable to more detailed analysis in cytochalasin B-treated PMNs (5, 6). Cytochalasin B interferes with the function of cytoplasmic microfilaments and inhibits membrane transport of sugars and nucleosides in cultured cells (7–12). Cytochalasin B-treated human PMNs are unable to ingest particles, but nevertheless selectively extrude lysosomal but not cytoplasmic enzymes when particles come into contact with their surfaces (6, 13, 14). Ultrastructural and biochemical studies of cytochalasin B-treated PMNs have demonstrated fusion of lysosomal granules with each other and the plasma membrane as the morphological basis of enzyme release (6). Consequently, it has become possible to monitor, *extracellularly* (after fusion of lysosomes with plasma membranes) processes that ordinarily occur *intracellularly* (fusion of lysosomes with phagocytic vacuoles).

We have recently demonstrated that stimulation of the alternate pathway of complement activation in fresh human serum generates a factor, lysosomal enzyme-releasing factor (LRF), which mediates selective lysosomal enzyme release from cytochalasin B-treated human PMNs in the absence of particles (15). Studies of the mechanism of action upon human leukocytes of LRF, its isolation, and its identification as a low-molecular-weight product of C5, are the subject of this report.

Abbreviations: PMN, polymorphonuclear leukocyte; LRF, lysosomal enzyme-releasing factor.

MATERIALS AND METHODS

Assay for LRF-Mediated Lysosomal Enzyme Release. The methods used have been described (15). Briefly, serum and leukocyte suspensions containing about 85% PMNs in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) were prepared from human venous blood. Aliquots of leukocyte suspensions containing 2×10^6 PMNs were preincubated with cytochalasin B (5 μg/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) at 37° for 15 min before addition of appropriate compounds in buffer and serum.

LRF was generated in untreated serum, or in serum containing 0.25 M ε-aminocaproic acid, by adding zymosan (1 mg/ml) (Nutritional Biochemicals Corp., Cleveland, Ohio) or lipopolysaccharide B from *E. coli* 026:B6 (50 μg/ml) (Difco Laboratories, Detroit, Mich.). After 15 min of incubation, the serum was rendered free of particles by filtration through a Millipore filter of 0.45-μm pore diameter (Millipore Corp., Bedford, Mass.).

Enzymes were measured in supernatants of reaction mixtures after 1–60 min of incubation at 37°. β-Glucuronidase (EC 3.2.1.31) was determined after 18 hr of incubation with phenolphthalein glucuronidate as substrate (16). Myeloperoxidase (EC 1.11.1.7) was measured by the Worthington method with orthodianisidine as substrate (17). Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Wacker *et al.* (18).

Compounds and Antisera. Prostaglandin E_1 was kindly furnished by Dr. John Pike, Upjohn Co., Kalamazoo, Mich. This compound was dissolved in 0.2 M sodium phosphate buffer (pH 7.4). Theophylline was from Mann Research Laboratories, New York, N.Y.; ε-aminocaproic acid, colchicine, cAMP, and cGMP were from Sigma Chemical Co., St. Louis, Mo.

Rabbit antiserum to human C3 was purchased from Custom Reagent Laboratory, San Diego, Calif. Goat antiserum to human C5 was the generous gift of Dr. Fred Rosen. This antiserum was prepared with highly purified human C5 (19). It did not react with human C3 and yielded a single precipitin band when reacted against whole human serum in immunoelectrophoresis. Antisera were heated at 56° for 30 min before use.

Molecular Sieve Chromatography was done in a 2.6 × 35-mm column of Sephadex G-75. Phosphate-buffered (10 mM) saline (pH 7.4) was the eluant. Filtration was done at 4° at 15 ml/hr, and fractions of 2.0 ml were collected.

Trypsin-Treated Human C5. Crystalline trypsin, free of carboxypeptidase activity, and soybean trypsin inhibitor (Worthington Biochemicals, Freehold, N.J.) were dissolved in Tris·HCl-buffered 0.15 M NaCl (pH 7.3). Commercial lyophilized human C5 (Cordis Laboratories, Miami, Fla.), prepared by the method of Vroon *et al.* (20), was reconstituted with distilled water (1000 CH_{50} units in 1.0 ml) and incubated with 5 μg of trypsin for 10 min at 37° before addition of 10 μg of trypsin inhibitor. The mixture was cooled before addition of sucrose (final concentration 10%) in preparation for chromatography. The human C5 preparation was characterized as functionally pure on the basis of three criteria: high reactivity (at least 1000 hemolytic units per ml); high specificity (other components, if present, occur at concentrations of less than 10 hemolytic units per ml); and low protein content (absorbance at 280 nm of 0.06 or less). Furthermore, it did not yield a precipitin band on immunodiffusion when reacted with antiserum to human C3.

PMN Chemotaxis was evaluated by a radioassay (21) that uses ^{51}Cr-labeled PMNs and a chemotaxis chamber containing two micropore filters separating the upper cell compartment from the lower compartment containing the chemotactic stimulus.

Electron Microscopy. Cell pellets were prepared for electron microscopy as described (6). Myeloperoxidase was cytochemically localized by the method of Graham and Karnovsky (22).

TABLE 1. *Enzyme release from human PMNs exposed to zymosan-treated serum**

	Enzyme activity released into supernatant†		
	β-Glucuronidase	Myeloperoxidase	Lactate dehydrogenase
Fresh serum	6.5 ± 0.2	3.1 ± 0.5	4.8 ± 0.2
Zymosan-treated serum	17.9 ± 0.6‡	16.3 ± 0.8‡	4.7 ± 0.2

* 1 mg/ml of zymosan incubated at 37° for 15 min before filtration, cells preincubated 15 min with 5 μg/ml of cytochalasin B.

† Expressed as percent of total activity released by 0.2% Triton X-100: β-glucuronidase, 13.5 ± 0.82 μg of phenolphthalein per 2 × 10^6 PMNs per hr; lactate dehydrogenase, 958 ± 64 absorbance units per 2 × 10^6 PMNs; or by sonication: myeloperoxidase, 248 ± 41 absorbance units per 2 × 10^6 PMNs Mean ± SEM. $n = 6$.

‡ Significantly different, $P < 0.001$, from the values for untreated serum.

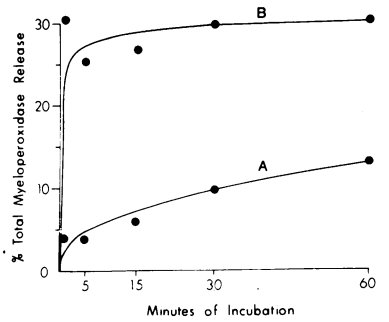

FIG. 1. Kinetics of enzyme release. Human PMNs were preincubated with 5 μg/ml of cytochalasin B for 15 min at 37°, then exposed to 10% zymosan-treated serum (*A*) or 10% zymosan-treated serum containing 0.25 M ε-aminocaproic acid (*B*). Myeloperoxidase in supernatants of reaction mixtures is expressed as percentage of total activity released by sonication.

RESULTS

Cytochalasin B-treated PMNs incubated with fresh serum for 60 min released minimal amounts of the lysosomal enzymes, β-glucuronidase and myeloperoxidase. Marked enhancement of enzyme release was observed when such cells were incubated with activated serum. Results with zymosan-treated serum are summarized in Table 1. Lysosomal enzyme release was not accompanied by release of the cytoplasmic enzyme, lactate dehydrogenase. PMN viability, as measured by eosin Y exclusion after 60 min of incubation (1), exceeded 98%. The possibility that zymosan particles that had escaped filtration accounted for the data was excluded by the observation that sera treated with this material retained enzyme-releasing activity after centrifugation at 7000 × *g* or refiltration through a filter of 0.22-μm pore diameter. Buffer treated with zymosan yielded no activity when added to fresh serum. Enhancement of lysosomal enzyme release from cytochalasin B-treated PMNs by zymosan-treated serum varied with the duration of incubation; significant amounts of myeloperoxidase activity were released into supernatants as early as 1 min after incubation started. Maximum enzyme release occurred, however, during the first minute of exposure to serum that had been pretreated with 0.25 M ε-aminocaproic acid before addition of zymosan (Fig. 1). This enhanced, rapid release of lysosomal enzymes was not associated with increased release of lactate dehydrogenase and was not accompanied by changes in pH or alterations of the osmolarity of the reaction mixtures sufficient in themselves to induce enzyme release.

In order to determine whether cyclic nucleotides and microtubule agents, which modify enzyme release in other systems (1, 3, 5), had similar effects upon LRF-mediated release, these were added to PMN suspensions before exposure to zymosan-treated serum for 60 min. Enzyme release was significantly diminished when cytochalasin B-treated PMN suspensions were preincubated for 40 min with cAMP (100 μM) and theophylline (100 μM), or prostaglandin E_1 (280 μM), or colchicine (10 μM), whereas preincubation for 5 min with cGMP (5 μM) enhanced the effect of treated serum (Table 2).

When serum that had been treated with zymosan or bacterial lipopolysaccharide was chromatographed on Sephadex G-75, enzyme-releasing activity was detected in two peaks. One peak was detected at or about the void volume and the other in the molecular weight range of 15,000–20,000 (Fig. 2). Heated serum exposed to zymosan yielded only the

TABLE 2. *Modification of LRF-mediated enzyme release from cytochalasin B-treated human neutrophils**

Compounds added before exposure to LRF	n	Enzyme activity released into supernatant† β-Glucuronidase	Lactate dehydrogenase
None (control)	6	17.9 ± 0.6	4.7 ± 0.2
cAMP (100 μM) + theophylline (100 μM)‡	4	11.9 ± 0.6§	4.3 ± 0.3
Prostaglandin E_1 (280 μM)‡	4	13.2 ± 0.5§	4.6 ± 0.2
Colchicine (10 μM)‡	4	15.0 ± 0.6§	4.7 ± 0.2
cGMP (5 μM)¶	4	21.2 ± 0.5§	4.3 ± 0.3

* Neutrophils preincubated with compounds for durations indicated below and with cytochalasin B for 15 min at 37° before exposure to zymosan-treated serum for 60 min.

† Expressed as percent of total activity released by 0.2% Triton X-100. Mean ± SEM.

‡ 40-min preincubation before exposure to zymosan-treated serum for 60 min.

§ Probability that the value differs from that of the control, $P < 0.01$.

¶ 5-min preincubation.

high-molecular-weight peak of activity, as did fresh, untreated serum. Enzyme-releasing activity in the low-molecular-weight fractions was resistant to heat (56° for 30 min) and treatment with antiserum to human C3, but was completely inhibited with antiserum to human C5. These antisera had variable effects on the activity in the high-molecular-weight fractions. Chromatography of trypsin-treated human C5 yielded similar low-molecular-weight fractions containing enzyme-releasing activity (Fig. 3).

PMN chemotactic activity in fractions obtained by chromatography of zymosan-treated serum is shown in Fig. 4. The peak of chemotactic activity corresponded to the peak of enzyme-releasing activity.

Cytochemical studies were made of PMNs fixed within 1 min of exposure to treated serum and then incubated with 3,3′-diaminobenzidine to demonstrate endogenous peroxidase. These studies demonstrated that extensive degranulation had already occurred before fixation so that compared to control

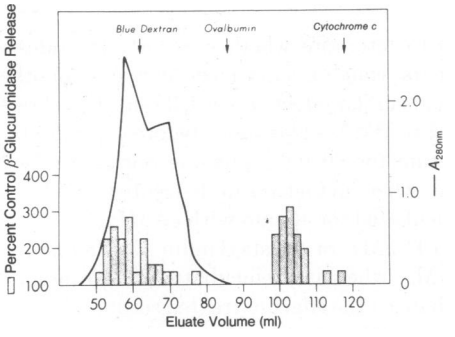

FIG. 2. Behavior of enzyme-releasing activity in zymosan-treated serum during Sephadex G-75 chromatography. Aliquots (0.5 ml) of fractions were incubated with cytochalasin B-treated PMNs for 60 min. Release of β-glucuronidase is expressed as percent of enzyme released from cells incubated with buffer alone. *Arrows* indicate elution volumes of marker molecules.

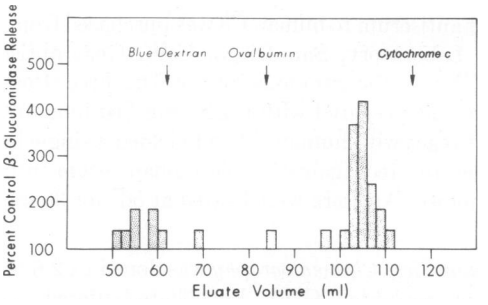

FIG. 3. Sephadex G-75 chromatography of enzyme-releasing activity in trypsin-treated human C5. Symbols and axes as in Fig. 2.

cells exposed to fresh serum (Fig. 5*a*), very few peroxidase-positive lysosomes remained intact in cells exposed to treated serum (Fig. 5*b*). Even more striking than the virtual absence of lysosomes was the presence of deep, membrane-bound, clefts which gave the cells the appearance of responding to several directional signals at the same time as if in a state of cognitive dissonance.

In order to determine what changes in subcellular architecture were associated with this massive change in cell form and content, similar preparations were fixed by a mixed fixative procedure that yields better ultrastructural preservation than the method used to demonstrate myeloperoxidase. Cells fixed rapidly in this way were seen to contain packets of released lysosomal contents within the clefts suggesting that extensive fusion of lysosomes with each other and with the plasma membrane might have contributed to their formation. Numerous microtubules were also apparent, radiating from the centriolar region, some of which were just under and parallel to the plasma membrane of the clefts (Fig. 6*b*). Microtubules were less numerous in PMNs exposed only to fresh serum (Fig. 6*a*) and in cells that had been exposed to treated serum for 5 min. To quantitate this phenomenon, microtubule profiles were counted in a 4-μm^2 area centered upon a visible centriole. The mean number of microtubules (Table 3) visible in this area was significantly larger in PMNs exposed to zymosan-treated serum for 1 min than in cells exposed to fresh serum or to zymosan-treated serum for 5 min. Treated serum appeared to stimulate microtubule assembly immediately upon contact with leukocytes, an effect which was quite reversible and which, after 5 min left the cells degranulated but otherwise ultrastructurally intact.

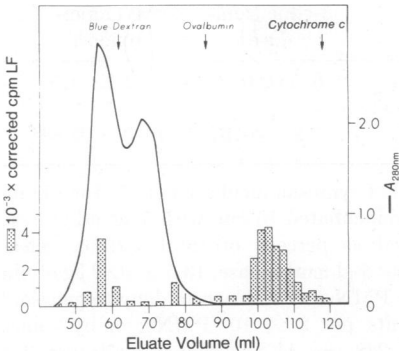

FIG. 4. Sephadex G-75 chromatography of chemotactic activity in zymosan-treated serum. Activity is expressed as corrected counts per minute on the lower filter (*corrected cmp LF*).

DISCUSSION

Our previous studies (15) and results of experiments described in this report support the conclusion that a factor (LRF) capable of stimulating selective lysosomal enzyme release from PMNs is generated by activation of the alternate complement pathway. The bulk of evidence regarding the identity of LRF indicates that it is a low-molecular-weight product of C5, probably C5a. Chromatography on Sephadex G-75 of zymosan-treated serum, endotoxin-treated serum, and trypsinized human C5 yielded similar low-molecular-weight fractions containing enzyme-releasing activity that was inhibited by antibodies to human C5 but not by those to human C3. These fractions possessed chemotactic activity for PMNs, a characteristic property of human C5a (23).

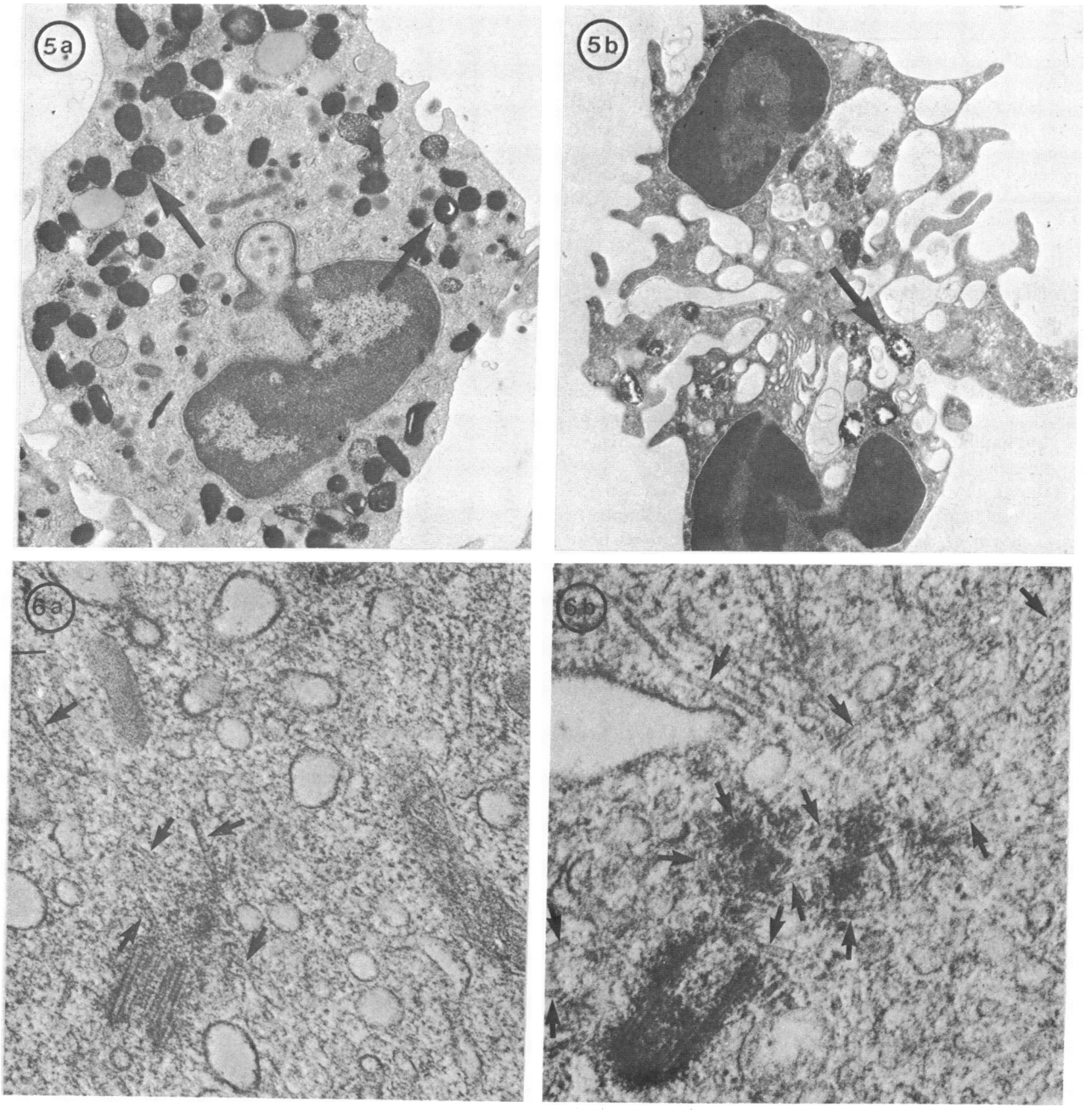

FIG. 5. (*a*) A human peripheral blood neutrophil pretreated with cytochalasin B (5 μg/ml, 10 min) then exposed to 10% fresh serum containing 0.25 M ε-aminocaproic acid for 1 min. It was then fixed and incubated for cytochemical localization of myeloperoxidase. Many peroxidase-positive (*arrows*) as well as peroxidase-negative lysosomes are visible scattered throughout the cytoplasm. ×9,280. (*b*) A human peripheral blood neutrophil prepared exactly as in (*a*) except that zymosan-treated ε-aminocaproic acid serum was used instead of fresh. Clefts can now be seen extending into the interior of the cell and very few intact lysosomes remain (*arrows*). ×9280.

FIG. 6. (*a*) The centriolar region of neutrophil treated with 10% fresh serum (0.25 M ε-aminocaproic acid) and then fixed rapidly by a mixed fixative procedure. Microtubules (*arrows*) were preserved by this method although relatively few can be seen. ×43,200. (*b*) The centriolar region of a neutrophil exposed to zymosan-treated ε-aminocaproic acid serum and then fixed rapidly as above. 24 Microtubules (*arrows*) can be seen in the area shown, compared to nine in the corresponding area shown in Fig. 6*a* (see Table 3). ×43,200.

TABLE 3. *Mean number of microtubule profiles adjacent to centrioles**

Treatment (time)	*n*	Mean SEM	*P* against control
Fresh serum + EACA†	27	11.9 ± 0.8	
Zymosan-treated EACA serum (1min)	10	27.3 ± 1.8	<0.001
Zymosan-treated EACA serum (5 min)	5	14.6 ± 2.4	Not significant

* Counted from electron photomicrographs at ×54,000 magnification. 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 by 2 μm square centered upon a centriole. All cells were pretreated with cytochalasin B at 5 μg/ml (10 min).

† 0.25 M ε-aminocaproic acid (*EACA*).

Enhancement of activity by ε-aminocaproic acid in serum is also a characteristic of this component, which is sensitive to degradation by the C5a inactivator, a carboxypeptidase-like serum enzyme (24). In view of the great similarity, if not identity, of LRF to C5a, we would expect that activation of the classical complement pathway would also generate this factor; preliminary experiments in our laboratory suggest this.

Lysosomal enzyme release induced by LRF (C5a) is modified by those pharmacologic agents that either enhance or diminish enzyme release from normal cells exposed to particles (1, 3, 5) and cytochalasin B-treated PMNs exposed to particles that they cannot engulf (5, 6). Inhibition of enzyme release by colchicine, cAMP and theophylline, and by prostaglandin E_1 has been considered to be due to the direct or indirect effects of these agents on cytoplasmic microtubules (disassembly) (3, 5, 6). It is in their aggregated state that microtubules regulate intracellular flow of granules to phagocytic vacuoles in the cell periphery. In contrast, stimulation of enzyme release by cGMP, as in other systems (25, 26), is mimicked by D_2O, an agent that promotes assembly of microtubules (27, 28). The concordance of our observations on LRF-induced enzyme release in cytochalasin B-treated leukocytes with observations of others with these same agents on antigenic release of histamine and slow-reacting substance of anaphylaxis from sensitized leukocytes (27, 28) and lung tissue (25, 29) leads us to suggest that various mediators of inflammation are subject to pharmacologic control at the level of cyclic nucleotide/tubulin interaction. Proof of this must await precise determinations of the intracellular levels of cyclic nucleotides and of the state of assembly of tubulin. However, actual determinations of cyclic nucleotides in Hypaque-Ficoll preparations of PMNs are rendered especially difficult because of both dialyzable and nondialyzable inhibitors of the assay in extracts (30).

The precise mechanisms whereby C5a stimulates intracellular membrane fusion and transient assembly of microtubules in cytochalasin B-treated PMNs is unknown. The almost instantaneous change in cell profile observed after an encounter with zymosan-treated ε-aminocaproic-acid serum (and its prompt reversal) does, however, suggest that this protein affects processes common to chemotaxis and membrane fusion. An attractive hypothesis is that C5a acts through a plasma-membrane receptor linked to a system capable of regulating intracellular levels of cGMP, thereby regulating microtubular assembly and the traffic of cytoplasmic organelles. Whatever the exact mechanism, these studies provide evidence for a new function for C5a. This component of complement provokes secretion of lysosomal hydrolases either into phagocytic vacuoles (as in normal cells) or into extracellular spaces (as in normal, and especially cytochalasin B-treated, cells).

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