MECHANISMS OF LYSOSOMAL ENZYME RELEASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

Effects of Phorbol Myristate Acetate

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Human peripheral blood polymorphonuclear leukocytes (PMN's) selectively release, or secrete, granule-associated (lysosomal) enzymes when exposed to appropriate phagocytic and nonphagocytic stimuli (6, 7, 19–21). Secretion is dependent upon the translocation of granules to the cell periphery and membrane fusion between granule membranes and the plasma membrane (exocytosis). These phenomena have been demonstrated to be influenced in reciprocal fashion by the cyclic nucleotides, cyclic 3′,5′-guanosine monophosphate (cGMP) and cyclic 3′,5′-adenosine monophosphate (cAMP), and by pharmacologic agents which are known to promote either assembly or disassembly of cytoplasmic microtubules (6, 11, 19–21).

Phorbol myristate acetate (PMA), a potent tumor-promoter and proinflammatory agent (8, 12), has recently been found capable of raising the levels of cGMP in a variety of cell types (5). Consequently, we have investigated the effects of this compound upon microtubule assembly in, and lysosomal enzyme release from, cytochalasin B-treated human PMN's exposed to two stimuli, zymosan particles and the complement component, C5a. Enzyme release from stimulated cytochalasin B-treated cells occurs in the absence of chemotaxis and phagocytosis (6, 20). Therefore, any influence that PMA may have upon these processes (3) may be disregarded, and its effects upon enzyme release per se can be studied more exclusively. Furthermore, cytochalasin B-treated cells exposed to C5a provide the advantage of constituting a model experimental system in which relationships between microtubule morphology and enzyme release may be analyzed quantitatively (6).

MATERIALS AND METHODS

Preparation of Cells

Leukocyte suspensions containing approximately 85% PMN's were prepared from heparinized venous blood obtained from healthy adult donors by employing standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes (7). The cells were suspended in phosphate buffer (10 mM) in 140 mM NaCl, pH 7.4, containing 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Aliquots ($2–4 \times 10^6$ PMN's) were incubated with cytochalasin B (5 μg/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (Matheson, Coleman & Bell, East Rutherford, N. J.) at 37°C for 10 min. This concentration of dimethyl sulfoxide did not influence cell morphology, enzyme release or enzyme assays.

Stimuli for Lysosomal Enzyme Release

After preincubation with cytochalasin B, either zymosan (0.5 mg/ml) (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) plus fresh autologous serum (10% vol/vol), or C5a (see below), was added and the reaction mixtures were allowed to incubate for an additional 15 min or 5 min, respectively. Zymosan was suspended in 150 mM NaCl, boiled, washed twice, and resuspended in phosphate-buffered saline. The final particle-to-cell ratio in the reaction mixtures was 30:1. C5a was generated in fresh autologous serum containing 250 mM epsilon-amino-caproic acid (EACA) (Sigma Chemical Co., St. Louis, Mo.) by adding zymosan (1.0 mg/ml) (6, 15). After 15 min of incubation at 37°C, the serum was rendered free of particles by centrifugation ($3,000 \times g$). Previous studies

have established that the bulk of the lysosomal enzyme releasing activity in zymosan-treated serum containing EACA (EACA-ZTS) can be attributed to its content of C5a (6).

Enzyme Assays

Following incubation, the reaction mixtures were centrifuged in the cold (1,000 × 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 (18). Crystalline egg-white lysozyme (Worthington Biochemical Corp.) was used as a standard. Lactate dehydrogenase (LDH) was measured by the method of Wacker et al. (16). Total enzyme activity was measured in simultaneously run duplicate reaction mixtures to which had been added 0.2% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.).

Cell Viability

The integrity of PMN's in suspension and in cell pellets at the conclusion of experiments was assayed by two techniques: (*a*) exclusion of eosin Y; and (*b*) release of cytoplasmic LDH, an indicator of cell death (19).

Compounds

PMA was the generous gift of Drs. Walter Troll and Andrew Sivak of the Department of Environmental Medicine, New York University School of Medicine. This compound, in dimethyl sulfoxide, was dissolved in 150 mM NaCl to desired concentrations. The final concentration of dimethyl sulfoxide never exceeded 0.1%.

Electron Microscopy

Cells were fixed at room temperature by a modification of the simultaneous fixation method of Hirsch and Fedorko (9). Lead citrate-stained sections were viewed in a Zeiss EM 9S. To eliminate observer bias, *all* centrioles visible on at least six sections from each of two specimens were photographed at 17,000 × and printed at 90,000 × on No. 6 paper (6) for ease in microtubule counting. Microtubules were counted from all of the centrioles photographed. Criteria for microtubule counting are described in Table II.

RESULTS

Effect of PMA on Beta-Glucuronidase Release

Cytochalasin B-treated human PMN's exposed to zymosan particles in the presence of autologous serum for 15 min released 22.3 ± 1.2% of total beta-glucuronidase. The release of this lysosomal enzyme was not accompanied by any enhancement of the release of cytoplasmic LDH or change in the percentage of cells which excluded eosin Y. When the cells were preincubated with PMA (20 ng/ml) before exposure to zymosan, there was either no change or there was enhancement of beta-glucuronidase release depending upon the duration of preincubation (Table I). Significant enhancement (+ 36.8%) of enzyme release was observed if PMA was added 1 min before zymosan, but maximal enhancement (+ 62.1%) occurred when the two were added simultaneously (0 min preincubation). The increment in beta-glucuronidase release was not accompanied by an increment in LDH release or change in eosin Y exclusion. Appropriate experiments revealed no evidence that PMA or dimethyl sulfoxide influenced the enzyme assays. Furthermore, recovery of enzymes (supernate plus pellet) was complete and there was no evidence that any of the reactants influenced the total values measured in reaction mixtures containing Triton X-100.

Cytochalasin B-treated PMN's exposed to EACA-ZTS (C5a) for 5 min released 21.7 ± 0.6% of total beta-glucuronidase. Lysosomal enzyme release in this system was also enhanced with the addition of PMA. The degree of enhancement varied with the concentration of PMA and the duration of preincubation (Table I). As was the case with zymosan, the greatest enhancement of enzyme release was observed following very brief exposure of cells to PMA.

PMA-Induced PMN Lysozyme Release

In the absence of external stimuli (either zymosan or C5a), beta-glucuronidase was not released in significant amounts from cytochalasin B-treated PMN's exposed to PMA (Table I). Lysozyme, however, was released from these cells (Fig. 1). In human PMN's lysozyme-containing granules (specifics) appear to be distinct from those granules (azurophils) which contain acid hydrolases and which more closely resemble lysosomes of tissues such as liver and kidney (14). These results are quite similar to those previously reported from studies of normal PMN's (4, 17).

Ultrastructure

Cytochalasin B-treated cells, exposed to PMA (20 ng/ml) for 30 s, in the presence of 10% fresh autologous serum, showed an increase, relative to

TABLE I

Enhancement by PMA of Lysosomal Enzyme Release from Cytochalasin B-Treated Human PMN

Additions to PMN's*	Enzyme Release‡	
	Beta-glucuronidase	LDH
	(μg phenolphthalein/18 h per 2 × 10⁶ PMN's)	*(Absorbance units per 2 × 10⁶ PMN's)*
Fresh serum (Control)	10.9 ± 0.5	28.2 ± 1.7
Fresh serum + PMA (20 ng/ml)§	10.4 ± 0.7	31.7 ± 3.5
Zymosan (Z)	56.5 ± 3.1	35.2 ± 3.5
PMA (20 ng/ml) (5 min) + Z	58.7 ± 1.4	33.5 ± 2.6
PMA (20 ng/ml) (1 min) + Z	73.3 ± 2.3 (+36.8%)‖	30.9 ± 4.4
PMA (20 ng/ml) (0 min) + Z	84.8 ± 2.9 (+62.1%)‖	35.3 ± 5.2
EACA-ZTS (C5a)	55.1 ± 1.4	30.8 ± 3.1
PMA (10 ng/ml) (5 min) +C5a	63.2 ± 1.6	31.1 ± 2.8
PMA (10 ng/ml) (1 min) +C5a	80.1 ± 1.9 (+56.6%)‖	29.7 ± 2.2
PMA (20 ng/ml) (5 min) +C5a	73.6 ± 1.8 (+41.9%)‖	33.2 ± 3.5
PMA (20 ng/ml) (1 min) +C5a	89.6 ± 2.3 (+78.1%)‖	32.8 ± 4.1

* PMN's were preincubated with cytochalasin B (5 μg/ml) for 10 min. at 37°C before additions.

‡ Total activity released by 0.2% Triton X-100: Beta-glucuronidase = 253 ± 20 μg phenolphthalein/18 h/2 × 10^6 PMN's; LDH = 883 ± 71 A.U./2 × 10^6 PMN's. Mean ± SEM, N = 6.

§ Cells were incubated with fresh autologous serum (10% vol/vol) ± PMA for 15 min, or with PMA for durations indicated before exposure to zymosan (0.5 mg/ml) plus fresh autologous serum (10% vol/vol) for 15 min or EACA-ZTS (10% vol/vol) for 5 min.

‖ Percent augmentation of enzyme release provoked by zymosan and C5a alone (minus control). *P* vs. zymosan (or C5a) alone <0.001.

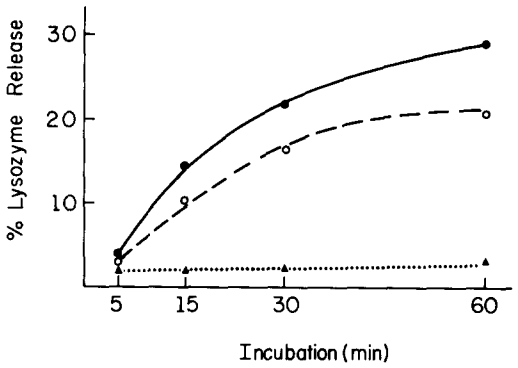

FIGURE 1 PMA-induced lysozyme release from cytochalasin B-treated human PMN's. After preincubation with cytochalasin B (5 μg/ml for 10 min.), cells were allowed to incubate for durations indicated with or without the addition of PMA. (▲···▲, Control; O---O, 10 ng/ml PMA; ●—●, 20 ng/ml PMA). Lysozyme release is expressed as the percent of total activity released by 0.2% Triton X-100.

control cells (in 10% serum), in the number of microtubules visible in the centriolar region (Fig. 2 and Table II). The number of visible microtubules was not significantly different from that observed in cells exposed to EACA-ZTS (C5a) alone for 30 s (6). Prominent vacuole formation, previously described by White and Estensen (17), was not observed when PMN's were exposed to PMA (20 ng/ml) for 30 s.

Although there is no proof that centriolar microtubules are representative of cytoplasmic microtubules, observations of numerous sections of PMN's fixed after exposure to PMA (and other stimuli for exocytosis) (10) indicate that microtubules in all portions of the cytoplasm are more frequently seen in these cells than in resting cells. However, larger numbers of microtubules are seen in the centriolar region, and differences due to experimental manipulation are most readily quantitated in this region. Furthermore, cytoplasmic microtubules that are not seen to be attached to an organizing center are usually oriented radially with respect to the centriolar region, suggesting that they originated there.

DISCUSSION

Previous studies in our laboratory have established a relationship between selective lysosomal enzyme (beta-glucuronidase) release from human PMN's and cyclic nucleotides (6, 19–21). Exogenous

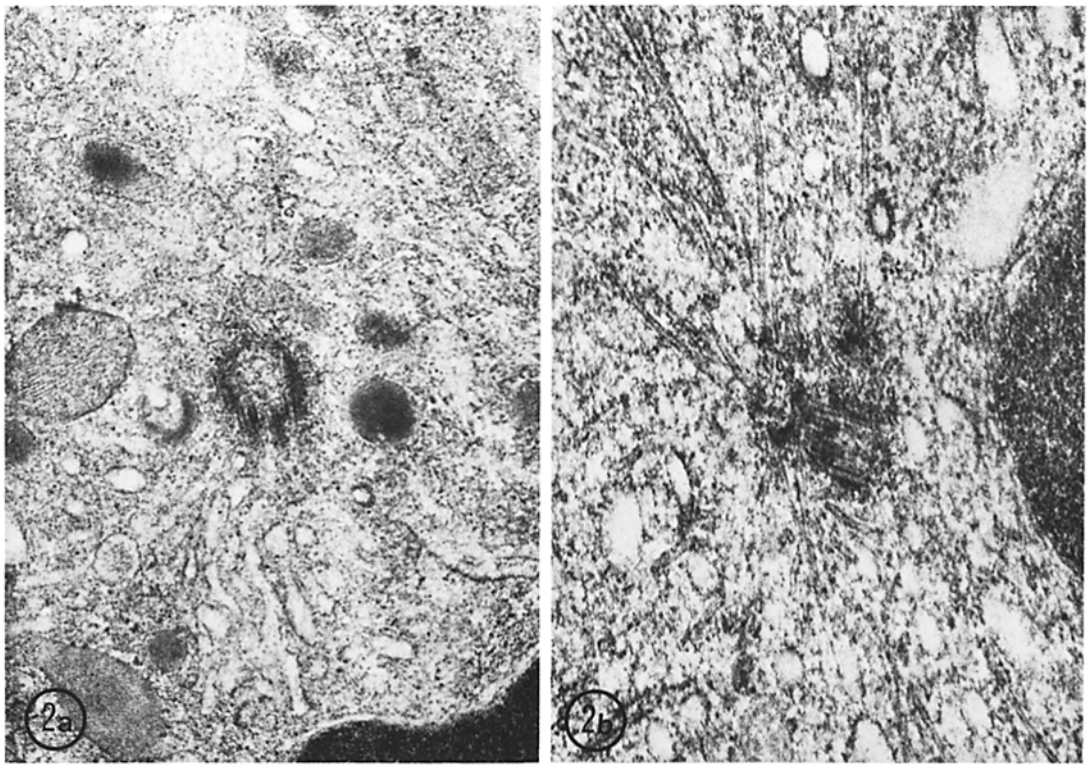

FIGURE 2 (*a*) Centriolar region of a cytochalasin B-treated human neutrophil incubated with buffer and 10% (vol/vol) fresh serum. × 50,000. (*b*) Centriolar region of a similar cell except that PMA (20 ng/ml) was added 30 s before fixation. Many more microtubules are visible. × 50,000.

cAMP (plus theophylline) and agents which elevate cellular levels of cAMP (e.g., prostaglandin E_1, isoproterenol) reduce, whereas exogenous cGMP and agents which elevate levels of cGMP (e.g., carbamylcholine) enhance, enzyme release from cytochalasin B-treated PMN's exposed to either zymosan particles or C5a (6, 21). We have also noted a relationship between enzyme release and the potential state of assembly of microtubules. Agents which promote disassembly (e.g., colchicine, vinblastine) reduce, whereas agents which promote microtubule assembly (e.g., deuterium oxide) enhance, lysosomal enzyme release (6, 21). Although it has been tempting to suggest that a relationship exists between cyclic nucleotides, microtubules, and enzyme release, there is, as yet, no firm evidence to support this. In fact, it is quite possible that the pharmacologic agents which we have studied exerted their influence upon enzyme release by mechanisms we have not recognized.

Perhaps the simplest interpretation of the results cited in this report is that PMA enhances the selective extrusion of beta-glucuronidase from cytochalasin B-treated human PMN's exposed to the two different stimuli by virtue of its ability to increase cellular levels of cGMP. The requirement for either a brief period of preincubation with, or simultaneous addition of, PMA to enhance enzyme release is reminiscent of our previous experience with carbamylcholine (21), and is in keeping with the observations of others regarding the kinetics of the cGMP response to PMA. For example, exposure of 3T3 fibroblasts in culture to PMA (100 μg/ml) resulted in a 50-fold increase of cGMP levels by 45 s with a decline toward control levels at 60 s (5). It is possible, of course, that PMA acts directly and independently to promote granule movement to the cell periphery and subsequent membrane fusion between lysosomal membranes and plasma membrane. Nor can these experiments resolve whether the increase in microtubule numbers observed in PMA-treated cells is the result of enhanced assembly (or stability)

TABLE II

*Mean Microtubule Numbers in PMA and Complement-Stimulated Human PMN**

Additions to PMN's	*n*	Mean ± SEM
Fresh serum (10% vol/vol) (Control)	(13)	10.7 ± 0.8
Fresh serum (10% vol/vol) + PMA (20 ng/ml)	(13)	27.9 ± 3.3‡

* Counted from electron micrographs at 90,000 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 (5 μg/ml) for 10 min, and with additions for 30 s before fixation.

‡ P vs. control <0.001

induced by PMA per se or is a secondary response to elevations of cGMP.

Enhancement of lysosomal enzyme (beta-glucuronidase) release from azurophil granules by PMA appears to be independent of the action of this compound on the release of specific granule enzymes (lysozyme). PMA does not consistently provoke the release of beta-glucuronidase from PMN's in the absence of other stimuli, whereas no additional such stimuli are necessary for provoking release of lysozyme. The facility with which specific granule constituents are extruded from PMN's in response to PMA is apparently not shared by primary, or azurophil granules (lysosomes). The nature of this difference is currently unclear, but may relate to differences between the membranes surrounding the two types of granules (1, 13), or to distinct requirements for microtubule assembly.

SUMMARY

PMA enhanced release of the azurophil granule enzyme, beta-glucuronidase, as well as lysozyme, from cytochalasin B-treated PMN's exposed to either zymosan particles or C5a. PMA was active at nanomolar concentrations, was not toxic to the cells, and was most effective when present for brief durations (0–1 min) before exposure of the cells to the stimuli. Beta-glucuronidase was not released in significant amounts from PMN's exposed to PMA alone, in the absence of stimuli such as zymosan or C5a. In contrast, only the specific granule enzyme, lysozyme, was released from unstimulated cells. Electron micrographs of cells exposed to PMA revealed an increase in the number of visible cytoplasmic microtubules as compared to control cells. Enhancement of lysosomal enzyme (beta-glucuronidase) release by PMA appears to be independent of effects on release of specific granule enzymes (lysozyme), but rather is likely due to PMA-induced elevations of cellular cGMP.

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REFERENCES

1. AVILA, J. L., and J. CONVIT. 1973. Studies on human polymorphonuclear leukocyte enzymes. II. Comparative studies of the physical properties of primary and specific granules. *Biochim. Biophys. Acta.* **293**:409–423.
2. BRITTINGER, G., R. HIRSCHHORN, S. D. DOUGLAS, and G. WEISSMANN. 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* **37**:394–411.
3. ESTENSEN, R. D., H. R. HILL, P. G. QUIE, N. HOGAN, and N. D. GOLDBERG. 1973. Cyclic GMP and cell movement. *Nature (Lond.).* **245**:458–460.
4. ESTENSEN, R. D., J. G. WHITE, and B. HOLMES. 1974. Specific degranulation of human polymorphonuclear leukocytes. *Nature (Lond.).* **248**:347–348.
5. GOLDBERG, N. D., M. K. HADDOX, R. ESTENSEN, J. G. WHITE, C. LOPEZ, and J. W. HADDEN. 1974. Evidence of a dualism between cyclic GMP and cyclic AMP in the regulation of cell proliferation and other cellular processes. *In* Cyclic AMP, Cell Growth, and the Immune Response. W. Braun, L. M. Lichtenstein, and C. W. Parker, editors. Springer-Verlag New York, Inc. 247–262.
6. GOLDSTEIN, I., S. HOFFSTEIN, J. GALLIN, and G. WEISSMANN. 1973. Mechanisms of lysosomal enzyme release from human leukocytes: microtubule assembly and membrane fusion induced by a component of complement. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2916–2920.
7. GOLDSTEIN, I. M., M. BRAI, A. G. OSLER, and G. WEISSMANN. 1973. Lysosomal enzyme release from

human leukocytes: mediation by the alternate pathway of complement activation. *J. Immunol.* **111**:33–37.

8. HECKER, E. 1968. Cocarcinogenic principles from the seed oil of *Croton tiglium* and other Euphorbiaceae. *Cancer Res.* **28**:2338–2349.
9. HIRSCH, J. G., and M. FEDORKO. 1968. Ulstastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. *J. Cell Biol.* **38**:615–627.
10. HOFFSTEIN, S., R. B. ZURIER, and G. WEISMANN. 1974. Mechanisms of lysosomal enzyme release from human leucocytes. III. Quantitative morphologic evidence for an effect of cyclic nucleotides and colchicine on degranulation. *Clin. Immunol. Immunopathol.* **3**:201–217.
11. IGNARRO, L. J., T. F. LINT, and W. J. GEORGE. 1974. Hormonal control of lysosomal enzyme release from human neutrophils. Effects of autonomic agents on enzyme release, phagocytosis, and cyclic nucleotide levels. *J. Exp. Med.* **139**:1395–1414.
12. JANOFF, A., A. KLASSEN, and W. TROLL. 1970. Local vascular changes induced by a cocarcinogen, phorbol myristate acetate. *Cancer Res.* **30**:2568–2571.
13. NACHMAN, R., J. G. HIRSCH, and M. BAGGIOLINI. 1972. Studies on isolated membranes of azurophil and specific granules from rabbit polymorphonuclear leukocytes. *J. Cell Biol.* **54**:133–140.
14. SPITZNAGEL, J. K., F. G. DALLDORF, M. S. LEFFELL, J. D. FOLDS, I. R. H. WELSH, M. H. COONEY, and L. E. MARTIN. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Lab. Invest.* **30**:774–785.
15. VALLOTA, E. H., and H. J. MULLER-EBERHARD. 1973. Formation of C3a and C5a anaphylatoxins in whole human serum after inhibition of the anaphylatoxin inactivator. *J. Exp. Med.* **137**:1109–1123.
16. WACKER, W. E. C., D. D. ULMER, and B. L. VALLEE. 1956. Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc concentration in serum. *N. Engl. J. Med.* **255**:449–456.
17. WHITE, J. G., and R. D. ESTENSEN. 1974. Selective labilization of specific granules in polymorphonuclear leukocytes by phorbol myristate acetate. *Am. J. Pathol.* **75**:45–52.
18. Worthington Enzyme Manual. 1972. Worthington Biochemical Corp., Freehold, N. J. 100–101.
19. ZURIER, R. B., S. HOFFSTEIN, and G. WEISSMANN. 1973. Mechanisms of lysosomal enzyme release from human leukocytes. I. Effect of cyclic nucleotides and colchicine. *J. Cell Biol.* **58**:27–41.
20. ZURIER, R. B., S. HOFFSTEIN, and G. WEISSMANN. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:844–848.
21. ZURIER, R. B., G. WEISSMANN, S. HOFFSTEIN, S. KAMMERMAN, and H. H. TAI. 1974. Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists, and agents which affect microtubule function. *J. Clin. Invest.* **53**:297–309.