

MECHANISMS OF LYSOSOMAL ENZYME RELEASE FROM HUMAN LEUKOCYTES

I. Effect of Cyclic Nucleotides and Colchicine

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ABSTRACT

In order to study mechanisms underlying selective enzyme release from human leukocytes during phagocytosis, the effects were studied of compounds which affect microtubule integrity or the accumulation of cyclic nucleotides. Human leukocytes selectively extrude lysosomal enzymes (β -glucuronidase) from viable cells during phagocytosis of zymosan or immune complexes, or upon encounter with immune complexes dispersed along a non-phagocytosable surface such as a millipore filter. In each circumstance, lysosomal enzyme release was reduced by previous treatment of cells with pharmacological doses of drugs which disrupt microtubules (e.g. 10^{-8} - 10^{-5} M colchicine) or with agents which affect accumulation of adenosine 3'5'-monophosphate (cAMP) (e.g. 10^{-8} M cyclic nucleotides and 2.8×10^{-4} - 2.8×10^{-6} M prostaglandin E (PGE) and A (PGA) compounds). Preincubation of cells with 5 μ g/ml cytochalasin B resulted in complete inhibition of zymosan ingestion, but not of adherence of zymosan particles to plasma membranes or selective enzyme release. In this system, in which enzyme release was independent of particle uptake, preincubation of cells with colchicine, vinblastine, dibutyryl cAMP, or PGE₁ also reduced extrusion of lysosomal enzymes. When cell suspensions were incubated with membrane-lytic crystals of monosodium urate (MSU), cytoplasmic as well as lysosomal enzymes were released with subsequent death of the cells. However, enzyme release followed phagocytosis of crystals (as measured by enhanced C-1 oxidation of glucose) and was due to "perforation from within" of the lysosomal membrane, rather than lysis by crystals of the plasma membrane. Enzyme release after MSU ingestion was also reduced when cells were treated with pharmacological doses of the test agents. When cells were killed by Triton X-100, acting on the plasma membrane, C-1 oxidation of glucose was abolished and enzyme release could *not* be inhibited pharmacologically. These observations suggest that lysosomal enzyme release from human phagocytes can be an active process which accompanies plasma membrane stimulation, is independent of cell death, and may be controlled by cyclic nucleotides and agents which affect microtubules.

Although a series of studies has indicated that extracts of leukocyte lysosomes can provoke acute and chronic inflammation in experimental animals (1), a clear definition of discrete mecha-

nisms which account for release from inflammatory cells of such materials is just beginning to emerge. Four separate circumstances are recognized under which substances ordinarily se-

questered within lysosomes may gain access to the exterior of cells.

One mechanism has been termed "regurgitation during feeding" (2), and may be important to the propagation of joint inflammation in rheumatoid arthritis. When cells engage in phagocytosis (e.g. leukocytes which engulf immune complexes in the synovial fluid of patients with rheumatoid arthritis) they release a portion of their lysosomal hydrolases into the surrounding medium. This effect appears due to extrusion of lysosomal materials from incompletely closed phagosomes open at their external border to tissue space while joined at their internal border with granules discharging acid hydrolases into the vacuole (phagolysosome). Under such circumstances lysosomal enzymes are selectively released to the outside of the cell without necessarily causing cytoplasmic damage. Electron-microscope images consistent with such a mechanism have in fact been published (3, 4).

A second mechanism has been called "reverse endocytosis" (5), and may be pertinent to the pathogenesis of tissue injury in nephritis, vasculitis, and rheumatoid arthritis. When leukocytes encounter immune complexes which have been dispersed along a nonphagocytosable surface such as a Millipore filter (Millipore Corp., Bedford, Mass.) or collagen membrane there is similar, selective release of lysosomal enzymes directly to the outside of the cell (6, 7). Enzyme release may occur when leukocytes are in apposition to immune complexes in a blood vessel wall, in the glomerular basement membrane, or when pannus encounters articular cartilage.

Another mechanism for enzyme release ("perforation from within" [8]) occurs when certain materials gain access to the vacuolar system wherein they interact with, and finally rupture, lysosomal membranes. A wave of membrane damage results with release of cytoplasmic and lysosomal enzymes followed by cell and tissue death. The inflammatory episodes of acute gout appear due to this type of encounter between leukocytes and crystals of monosodium urate (MSU) (9).

Finally, inflammatory substances may leak from cells simply as a result of cell death due to plasma membrane injury. A number of animal, bacterial, and chemical toxins, as well as synthetic detergents may cause such lysis of the outer cell membrane (10).

Since enzyme release under each circumstance may be crucial to the perpetuation of tissue injury, it may be that *reduction* of such enzyme release would prove beneficial. The effect on these four mechanisms of enzyme release was therefore studied of agents which regulate the secretion of stored proteins in tissues such as pancreas, salivary gland, and thyroid. Two types of compounds were studied: those which affect the function of microtubules and microfilaments, and those which influence the accumulation within cells of cyclic nucleotides. The results suggest that selective enzyme release from human phagocytes is independent of cell death and that pharmacologic agents can reduce lysosomal enzyme release in three of four experimental challenges. When cells were killed by means of a lethal injury to the plasma membrane (Triton X-100), enzyme release could not be inhibited by pharmacologic means.

MATERIALS AND METHODS

Separation of Leukocytes

Leukocytes were obtained from the venous blood of healthy young men. Blood (40 cm³) was drawn into plastic syringes (60 cm³; Sherwood Medical Industries, Inc., Deland, Fla.) previously moistened with 0.4 ml of heparin (1000 U/ml; Liquaemin Sodium®, Organon Inc., West Orange, N. J.) and containing 8 ml of a 6% dextran solution (average molecular weight of dextran; 248,000; Pharmachem, Bethlehem, Pa.). After gentle mixing, sedimentation was allowed to proceed in the same syringe for 10–20 min at room temperature. The cell-rich supernatant was carefully transferred to centrifuge tubes and the cells were sedimented at 800 rpm (100 g) for 8 min in an International (Model UV) centrifuge at room temperature. Supernatant fluid was removed, the erythrocytes were removed by hypotonic lysis (8 cm³ 0.85% NaCl, add 24 cm³ distilled water for 30 s, add 8 cm³ 2.6% NaCl), and the leukocytes were washed once in 0.15 M NaCl and resuspended in the buffered medium.

Medium

Calcium and magnesium, at final concentrations of 0.6 and 1 mM respectively, were added to phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.) to produce "PiCM", pH 7.3.

Leukocyte Suspensions

Cell pellets were resuspended in PiCM to a concentration of 4×10^6 leukocytes per ml ($83 \pm 5\%$

polymorphonuclear leukocytes. $N = 10$). The platelet:leukocyte ratio was 5:1. Even in 20-fold excess platelets contained negligible proportions of total β -glucuronidase activity (11). The remaining cells were lymphocytes and monocytes which when separated on Hypaque-Ficoll gradients (12) proved to contain only 1.6% of the total β -glucuronidase activity and 9.4% of the lactate dehydrogenase (LDH) activity of the lymphocyte/monocyte contaminated polymorphonuclear leukocyte preparation. Portions of cell suspensions (1.0 ml) were dispensed into 10 \times 75 mm plastic test tubes. The cells were incubated at 37°C with appropriate drugs, autologous serum was added to a concentration of 10%, then cells were exposed to particles. At the end of experiments, tubes were centrifuged at 2500 rpm (755 g) at 4°C in a Sorvall centrifuge (Sorvall Superspeed, Rotor No. SS-1, Ivan Sorvall, Inc., Newtown, Conn.). The cell-free supernatants were removed for enzyme determination. Cells were fixed for electron microscopy as previously described (5). Cells were also stained for peroxidase by the method of Graham and Karnovsky (13).

Viability of Cells

The integrity of leukocytes in the pellets at the end of experiments was assayed by several techniques. (a) Exclusion of eosin Y: a cell pellet was diluted in 0.5% eosin Y in saline, mixed, and cells were counted in a hemocytometer. Nonviable cells lost their ability to exclude eosin Y and stained pink. In no instance did the proportion of nonviable cells exceed 3% (except in studies with MSU and Triton X-100). (b) Phagocytosis: the percent of cells capable of phagocytosis (>85%) when zymosan was added after a 5-h incubation period was no less than when cells were incubated immediately with zymosan. (c) LDH determinations: extracellular escape of cytoplasmic enzymes was taken to indicate cell death. (d) A normal metabolic response (C-1 oxidation of glucose) to phagocytosis was determined (14).

Particles

Zymosan particles (Nutritional Biochemicals Corporation, Cleveland, Ohio), measuring 3–5 μ m in diameter, were suspended in saline, boiled, washed twice, and resuspended (5 mg/ml) in PiCM. Particle counts were performed with a standard hemocytometer.

MSU Crystals

Microcrystalline urate was prepared as described by McCarty and Faires (15). Samples of crystals were ground to produce particles 0.5–30 μ m in length.

Immune Complexes

Aggregated human γ -globulin was prepared from Cohn Fraction II (courtesy of Dr. Edward C. Franklin) by heating at 63°C for 20 min. The antigen, present in excess (1.0 ml of a 1% solution in 0.15 M NaCl), was added to 5 ml of a high titer (1:5120) rheumatoid serum (antibody) and reacted at 4°C for 24 h. The precipitate was washed three times in 0.15 M NaCl and resuspended in PiCM. The preparation is referred to as rheumatoid factor complex (RF- algG), or algG/IgM . Protein concentration of the immune precipitates was adjusted to 100 μ g/0.1 ml.

Enzyme Assays

β -glucuronidase was determined at 18 h of incubation with phenolphthalein glucuronidate as substrate, as previously described (16). Readings were obtained in a Beckman-DB Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 540 nm. LDH was determined by the method of Wacker et al. (17), measuring reduction of nicotinamide adenine dinucleotide phosphate (NADP). Readings were obtained every minute for 3 min at 340 nm in a Beckman-DU Spectrophotometer.

Enzyme Recovery

Appropriate control experiments performed as previously described (11) indicated that there was no preferential degradation of enzyme activity (β -glucuronidase or LDH) in resting cells, excluded the possibility that particles and test compounds interfered with enzyme assays, and tested for selective adsorption of enzymes to cells or particles after their release into the medium. Recovery of enzymes was unimpaired.

Compounds

Prostaglandins were kindly furnished by Dr. John Pike, Upjohn Co., Kalamazoo, Mich.; 2-chloroadenosine (2-CA) compliments of Dr. Colin Bayley, Cyclo Chemical Corp., Los Angeles, Calif.; theophylline: Mann Research Labs, Inc., New York; 3'5'-cAMP and dibutyryl 3'5'-cAMP; Sigma Chemical Co., St. Louis, Mo.; cytochalasin B: ICI Research Laboratories, Alderley Park, Cheshire, England. Cytochalasin B was dissolved in 0.1% dimethylsulfoxide (DMSO). This concentration of DMSO did not alter cell viability or morphology.

C-1 Oxidation of Glucose

Stimulation of leukocyte hexose monophosphate shunt activity in response to phagocytosis of particles was measured utilizing minor modifications of a pre-

viously described method (14). 1-ml portions of leukocyte suspensions containing $2.4\text{--}4.8 \times 10^6$ cells/ml in PICM buffer were added to 25-ml Erlenmeyer flasks containing 0.25 ml of autologous serum, 0.25 μCi D-[1- ^{14}C]glucose in 0.5 ml buffer, and either buffer alone or particles suspended in buffer, to a final volume of 2.5 ml. The evolution of $^{14}\text{CO}_2$ measured as counts per minute, by leukocytes incubated with particles compared to $^{14}\text{CO}_2$ evolution from cells incubated without particles, has been shown to reflect the degree of phagocytic activity (14). The increment of $^{14}\text{CO}_2$ evolution from cells exposed to MSU was linear over a dose range of 0.1 mg/ml–10 mg/ml.

Nonphagocytosable Surface

Millipore filters (Millipore Corp.; pore diameter 0.22 μm) were soaked overnight in phosphate-buffered saline (PBS), then rinsed. Appropriate concentrations of the aIgG/IgM complex were layered on the filter surface and allowed to dry. Filters were washed with PBS before use. Experiments were performed in vessels described by Gerber and Schubert (18). Two Plexiglas chambers were separated by the Millipore filter. The cell suspensions were preincubated in tubes as in the bulk phase experiments, then transferred to the chamber. The cells were allowed to settle onto the filter. The two chambers were clamped tightly together so that none of the cell suspension leaked around the filter. The bottom chamber re-

mained dry, indicating that medium did not penetrate the filter.

RESULTS

Cells Exposed to Zymosan and to Immune Complexes in the Bulk Phase

Exposure of human leukocytes to zymosan or to aIgG/IgM complexes results in the selective release of lysosomal enzymes with maintenance of cell viability (11). The selective nature of the enzyme release, the kinetics of enzyme release, and the influence on enzyme release of cell: particle ratio are all demonstrated in Fig. 1. Release of enzyme was maximum after 120 min of incubation with a particle:cell ratio of 30:1. Although in this experimental system nearly all phagocytosis occurs in the first 30 min of incubation, further phagocytosis was observed during a subsequent 30-min period. Release of enzyme therefore appears to be very closely related to phagocytosis and not to an event which is significantly delayed. However, when cells are exposed to smaller numbers of particles (particle: cell ratio of 3:1) phagocytosis is completed by 30 min but there is a substantial increment of *selective* enzyme release during the following 90 min. Thus, ingestion of large numbers of par-

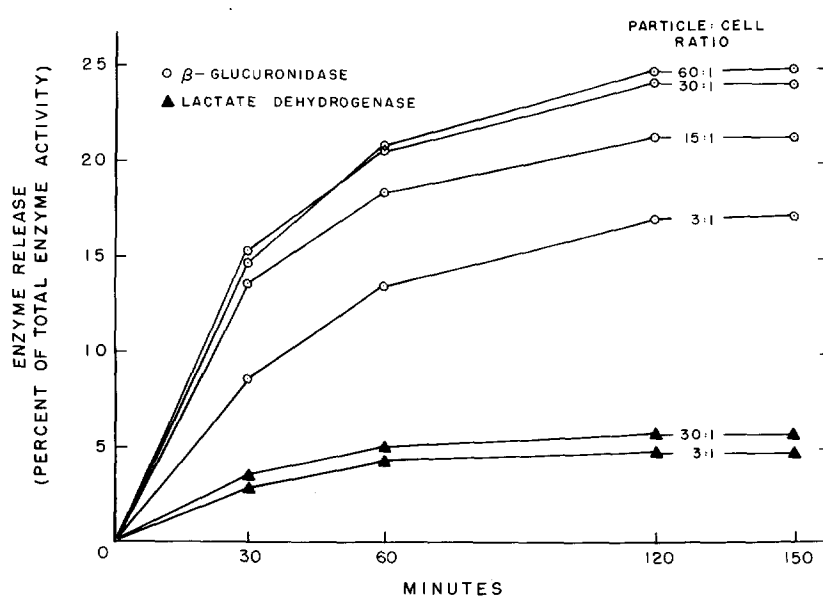


FIGURE 1 Selective enzyme release from human leukocytes. Kinetics of release and effect of cell: particle (zymosan) ratio.

ticles induces greater enzyme release earlier and abolishes the delay in enzyme release observed with the smaller particle:cell ratio. The kinetics of enzyme release suggest two possibilities: (a) formation within cells of phagocytic vacuoles may stimulate movement of granules to the cell periphery, or (b) phagocytic vacuoles remain open to the outside during the entire 120-min period.

When cells were incubated with compounds which increase cellular levels of cAMP or which interfere with microtubule integrity before their exposure to zymosan or immune complex, reduction in lysosomal enzyme release was observed (Tables I and II). cAMP itself had no

effect on extrusion of lysosomal enzymes. However, in combination with theophylline or 2-CA, both of which inhibit phosphodiesterase, cAMP caused a considerable reduction in enzyme release. Prostaglandin E₁ (PGE₁) also blocked selective hydrolase release. When theophylline or 2-CA was added during preincubation with PGE₁ it acted further to reduce hydrolase release. Theophylline and 2-CA alone produced a dose-dependent reduction in lysosomal enzyme release. Colchicine at a concentration of 10⁻³ M, but not at lower concentrations, also reduced enzyme release (Tables I and II).

PGE and PGA compounds stimulate the accu-

TABLE I
*Inhibition of Enzyme Release from Human Leukocytes Exposed to Zymosan**

Compound	Concentration	No.	Percent of control enzyme release	
			β -Glucuronidase	Lactate dehydrogenase
<i>M</i>				
None		10	100.0	100.0
3'5'-cAMP	10 ⁻³	5	94.9 \pm 2.0	99.4 \pm 1.1
Theophylline	10 ⁻³	5	77.8 \pm 2.5†	98.3 \pm 0.6
2-Chloroadenosine	10 ⁻³	5	73.4 \pm 4.5†	98.4 \pm 0.9
3'5'-cAMP	10 ⁻³			
+	+	5	39.3 \pm 8.4†	100.6 \pm 1.7
theophylline	10 ⁻³			
3'5'-cAMP	10 ⁻³			
+	+	5	47.2 \pm 9.1†	100.9 \pm 1.4
2-chloroadenosine	10 ⁻³			
Dibutyl cAMP	10 ⁻³	5	63.1 \pm 4.7†	98.9 \pm 1.0
PGE ₁	2.8 \times 10 ⁻⁴	5	67.4 \pm 7.6†	101.2 \pm 0.8
PGE ₁	2.8 \times 10 ⁻⁴			
+	+	5	46.1 \pm 3.8†	100.8 \pm 1.4
theophylline	10 ⁻³			
PGE ₁	2.8 \times 10 ⁻⁴			
+	+	5	44.3 \pm 4.2†	101.6 \pm 0.9
2-chloroadenosine	10 ⁻³			
Colchicine	10 ⁻³	6	68.2 \pm 4.1†	98.8 \pm 1.1
Percent of total enzyme release§				
			β -Glucuronidase	Lactate dehydrogenase
Resting cells		10	4.7 \pm 3.0	1.8 \pm 1.5
Cells + zymosan		10	22.4 \pm 2.9	2.1 \pm 1.3

* Leukocytes (4×10^6) incubated with drug for 60 min at 37°C, then exposed to zymosan particles for 60 min.

† Significant at $P < 0.01$ vs. control of 100%.

§ Enzyme activity expressed as a percentage of total activity released by 0.2% Triton X-100. Total activity (100%) of β -glucuronidase was $19.7 \pm 2.7 \mu\text{g}$ phenolphthalein/ 4×10^6 leukocyte/h; lactate dehydrogenase: 1260 ± 81 absorbancy units/ 4×10^6 leukocyte.

TABLE II
*Inhibition of Enzyme Release from Human Leukocytes Exposed to Immune Complex in Bulk Phase**

Compound	Concentration	No.	Percent of control enzyme release	
			β -Glucuronidase	Lactate dehydrogenase
<i>M</i>				
None	Control	10	100.0	100.0
3'5'-cAMP	10^{-3}	3	93.8 \pm 1.7	
Theophylline	10^{-3}	8	55.4 \pm 5.3 \ddagger	100.4 \pm 0.9
2-Chloroadenosine	10^{-3}	4	58.7 \pm 4.6 \ddagger	101.4 \pm 1.0
3'5'-cAMP	10^{-3}			
+	+	3	39.4 \pm 2.9 \ddagger	99.6 \pm 0.8
theophylline	10^{-3}			
Dibutyl cAMP	10^{-3}	7	54.2 \pm 4.4 \ddagger	98.7 \pm 1.1
PGE ₁	2.8×10^{-4}	7	57.8 \pm 8.6 \ddagger	99.8 \pm 0.6
PGE ₁	2.8×10^{-4}			
+	+	5	38.3 \pm 2.7 \ddagger	100.3 \pm 0.9
theophylline	10^{-3}			
PGE ₁	2.8×10^{-4}			
+	+	4	35.8 \pm 3.4 \ddagger	100.9 \pm 1.4
2-chloroadenosine	10^{-3}			
Colchicine	10^{-3}	6	60.1 \pm 4.9 \ddagger	101.0 \pm 1.6
Percent of total enzyme release \S				
			β -Glucuronidase	Lactate dehydrogenase
Resting cells		10	3.1 \pm 1.2	2.0 \pm 0.9
Cells + aIgG/IgM		10	14.4 \pm 1.6	2.9 \pm 1.0

* Leukocytes (4×10^6) incubated with drug for 60 min at 37°C, then exposed to aIgG/IgM for 120 min.

\ddagger Significant at $P < 0.01$ vs. control of 100%.

\S Enzyme activity expressed as a percentage of total activity released by 0.2% Triton X-100. Total activity (100%) of β -glucuronidase was $20.8 \pm 1.7 \mu\text{g}$ phenolphthalein/ 4×10^6 leukocyte/h; lactate dehydrogenase: 1320 ± 102 absorbancy units/ 4×10^6 leukocyte.

mulation of cAMP in mixed populations of human leukocytes whereas prostaglandin F compounds have little or no effect on cAMP levels (19, 20). For example, whereas the concentration of cAMP in resting cells was 6 pmol/ 10^7 cells, incubation of cells with 10^{-2} M theophylline and 10^{-5} M PGE₁ raised the level to 18 and 66 pmol, respectively and the two compounds together caused an accumulation of 220 pmol/ 10^7 cells (21). Preincubation of leukocytes with prostaglandin E and A compounds consistently reduced selective extrusion of lysosomal enzymes from cells exposed to zymosan. However, pretreatment of cells with the same concentrations of prostaglandin F_{1 β} had little effect on enzyme release. Prostaglandin F_{2 α} enhanced enzyme

release by virtue of causing cell injury (Table III). Similar results were obtained when cells were exposed to the immune complex (Table IV).

The concentrations of prostaglandins employed were quite large. Although optimal results were obtained with 2.8×10^{-4} M concentrations, PGE₁ and PGA₂ retarded hydrolase release from phagocytes at concentrations as low as 2.8×10^{-6} M (1 $\mu\text{g}/\text{ml}$) (Table V).

In initial experiments PGE₂ did not reduce enzyme release. It has been demonstrated that PGE₂ reduces the deformability of red blood cells and it has been suggested that erythrocytes may be one of the primary receptors for PGE₂ (22). When contaminating erythrocytes were

removed from cell suspensions by means of hypotonic lysis, the effect of PGE₂ was comparable to that of PGE₁. The influence of erythrocytes on the action of PGE₂ is documented in Table VI. This effect of red blood cells was not observed when cells were incubated with other prostaglandin compounds. In all experiments reported in this paper, erythrocytes were removed by hypotonic lysis.

At the concentration used (10⁻³ M), colchicine significantly retarded particle uptake by phagocytic cells whereas the cyclic nucleotides and prostaglandin E and A compounds also depressed C-1 glucose oxidation, a metabolic concomitant of phagocytosis (Table VII). This suggested that reduced enzyme release might simply be a consequence of reduced particle uptake. Nonetheless, the experiments did not exclude additional effects

TABLE III
Effect of 2.8×10^{-4} M Prostaglandin and 10^{-3} M Theophylline on Enzyme Release from Human Leukocytes Exposed to Zymosan*

Compound	No.	Percent of control enzyme release	
		β -Glucuronidase	Lactate dehydrogenase
None (control)	10	100.0	100.0
Theophylline	10	68.6 \pm 3.0 \ddagger	98.4 \pm 0.4
PGE ₁	4	67.4 \pm 7.6 \ddagger	97.8 \pm 1.1
PGE ₁ + theophylline	4	46.1 \pm 3.8 \ddagger	96.9 \pm 1.0
PGE ₂	4	59.9 \pm 4.5 \ddagger	99.5 \pm 1.4
PGE ₂ + theophylline	4	48.4 \pm 5.2 \ddagger	98.7 \pm 3.0
PGA ₂	4	43.2 \pm 6.1 \ddagger	100.4 \pm 0.9
PGA ₂ + theophylline	4	39.0 \pm 3.1 \ddagger	101.8 \pm 2.1
PGF _{1β}	4	88.6 \pm 1.9	100.1 \pm 1.4
PGF _{1β} + theophylline	4	64.7 \pm 2.4 \ddagger	99.7 \pm 0.5
PGF _{2α}	4	135.8 \pm 5.2 \ddagger	140.4 \pm 6.2
PGF _{2α} + theophylline	4	125.1 \pm 4.8 \ddagger	131.2 \pm 4.8

* Leukocytes (4×10^6) incubated with drug for 60 min at 37°C, then exposed to zymosan for 60 min.

\ddagger Significant at $P < 0.01$ vs. control of 100%. (Control release = 22.4 \pm 2.9% of total β -glucuronidase activity. See cells + zymosan Table I.)

TABLE IV
Effect of 2.8×10^{-4} M Prostaglandin and 10^{-3} M Theophylline on Enzyme Release from Human Leukocytes Exposed to Immune Complex*

Compound	No.	Percent of control enzyme release	
		β -Glucuronidase	Lactate dehydrogenase
None (control)	7	100.0	100.0
Theophylline	7	54.9 \pm 2.1 \ddagger	98.3 \pm 2.0
PGE ₁	7	56.3 \pm 3.4 \ddagger	97.4 \pm 1.7
PGE ₁ + theophylline	7	36.2 \pm 2.9 \ddagger	97.2 \pm 1.5
PGA ₂	3	41.4 \pm 4.9 \ddagger	101.6 \pm 3.7
PGA ₂ + theophylline	3	32.8 \pm 5.0 \ddagger	101.1 \pm 0.7
PGF _{1β}	3	87.8 \pm 3.1	99.4 \pm 1.0
PGF _{1β} + theophylline	3	54.0 \pm 3.9 \ddagger	100.7 \pm 0.8
PGF _{2α}	3	127.4 \pm 4.7 \ddagger	139.6 \pm 6.8
PGF _{2α} + theophylline	3	119.9 \pm 4.2 \ddagger	122.9 \pm 5.1

* Leukocytes (4×10^6) incubated with drug for 60 min at 37°C, then exposed to aIgG/IgM for 120 min.

\ddagger Significant at $P < 0.01$ vs. control of 100%. (Control release = 14.4 \pm 1.6% of total β -glucuronidase activity. See cells + aIgG/IgM Table II.)

TABLE V
Inhibition of Enzyme Release from Human Leukocytes*

Compound	Concentration	β -Glucuronidase	
		Percent of total release	Percent of control release
<i>M</i>			
None	(Resting)	1.9 \pm 0.7	8.1
None	(Control)	23.6 \pm 2.7	—
PGE ₁	2.8 \times 10 ⁻⁴	15.5 \pm 0.4	65.9†
PGE ₁	2.8 \times 10 ⁻⁵	18.7 \pm 0.9	77.5†
PGE ₁	2.8 \times 10 ⁻⁶	20.4 \pm 1.9	83.5§
PGA ₂	2.8 \times 10 ⁻⁴	9.7 \pm 1.0	41.0†
PGA ₂	2.8 \times 10 ⁻⁵	17.7 \pm 0.7	73.2†
PGA ₂	2.8 \times 10 ⁻⁶	19.5 \pm 2.0	80.8†

* 1 h incubation with drug followed by 1 h incubation with zymosan particles.

† $P < 0.01$ vs. control of 100%.

§ $P < 0.05$ vs. control of 100%.

of these compounds on intracellular events subsequent to particle ingestion. Therefore, experiments were designed so that enzyme release might be studied without concern for the engulfment phase of phagocytosis. Henson has demonstrated that the encounter between neutrophils and immune complexes dispersed along a non-phagocytosable surface results in selective release of lysosomal enzymes (4). The technique was adapted to our studies.

Cells Exposed to Nonphagocytosable Stimuli

Human leukocytes were allowed to settle onto Millipore filters (see Materials and Methods) and incubated at 37°C for 2 h. Under these circumstances, in the absence of phagocytosis, there was selective release of lysosomal enzymes. The kinetics of enzyme release are demonstrated in Fig. 2. Most enzyme release, as was the case in

TABLE VI
Influence of Erythrocytes on Inhibition of Enzyme Release by PGE₂*

Compound	β -Glucuronidase release			
	+ RBC		- RBC	
	Percent of total	Percent of control	Percent of total	Percent of control
None (resting)	1.7 \pm 1.0	7.5	2.1 \pm 0.7	9.7
None (control)	22.8 \pm 2.1	—	21.7 \pm 1.9	—
PGE ₂ (2.8 \times 10 ⁻⁴ M)	20.5 \pm 0.9	89.8	13.0 \pm 1.4	59.9

* 1 h incubation with PGE₂ followed by 1 h incubation with PGE₂ and zymosan. Values are mean (\pm SEM) of three experiments.

TABLE VII
Glucose Oxidation in Human Polymorphs during Particle Uptake

Compound added	Concentration	Exposure of cells to aIgG/IgM complex	
		Difference resting vs. phagocytic	Percent inhibition
None	<i>M</i> (Control)	96	00.0
PGE ₁	2.8 \times 10 ⁻⁴	48	50.0
Theophylline	10 ⁻³	61	36.5
PGE ₁ + theophylline	2.8 \times 10 ⁻⁴ + 10 ⁻³	24	75.0
Colchicine	10 ⁻³	28	70.6

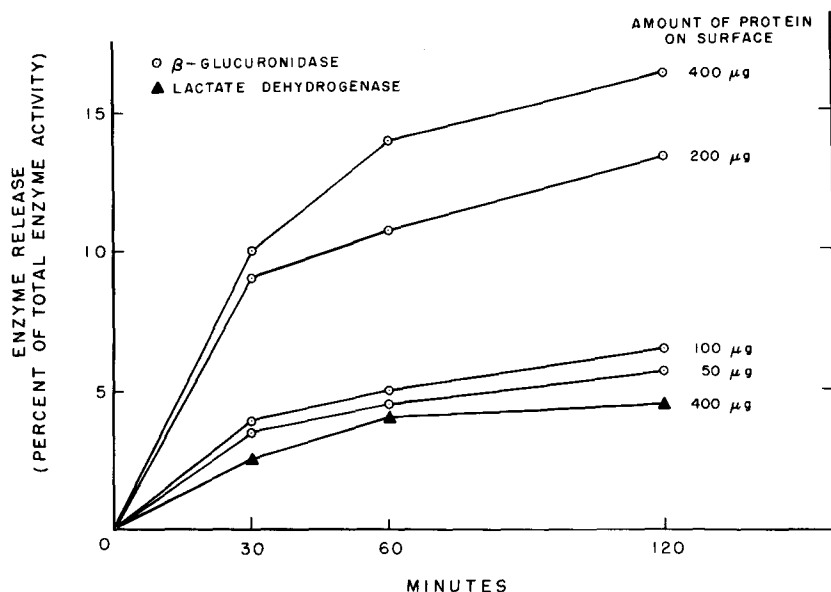


FIGURE 2 Selective enzyme release from human leukocytes. Kinetics of release and effect of immune complex quantity.

TABLE VIII
Adherence of Immune Complex to Millipore Filters*

Complex added to filter		Complex recovered	Percent recovery
μg	cpm	cpm	
25	118 (100-136)	127 (101-153)	107.5
100	508 (484-532)	497 (412-582)	97.7
200	1346 (1280-1412)	1259 (1199-1319)	93.6
400	1921 (1804-2038)	1788 (1704-1872)	92.8

Numbers represent mean (and range) of two experiments.

* Immune complex (^{125}I -labeled) applied to filters. Filters incubated with leukocytes at $37^\circ\text{C} \times 3$ h. Filters washed. Counts made in Nuclear-Chicago Corp. Model 4216 Automatic Gamma Sample Counting System.

bulk phase experiments, occurred during the first 60 min.

EVIDENCE THAT ENZYME RELEASE FROM CELLS ON MILLIPORE FILTER WAS NOT DUE TO UPTAKE OF COMPLEXES IN THE BULK PHASE: It was important to determine that immune complexes coated onto filters in fact remained there, and that leukocytes added to the chamber settled onto the filter. Recovery experiments utilizing ^{125}I -labeled γ -globulin indicated that only small amounts of the deposited complex were removed from the filter during the course of the experiment (Table VIII). Generally, 6×10^6 cells were added to the chamber. At the end of the experiment usually 2×10^5 cells

were recovered in the supernatant. Fluorescein staining of the leukocytes failed to demonstrate immune complexes within cells, indicating that those cells which did remain in the bulk phase had not engaged in phagocytosis. In addition, amounts of immune complexes (e.g. 200 μg) which, when dispersed along the filter, caused significant release of β -glucuronidase, did not induce significant enzyme release when added to cells in the bulk phase (Table IX). When cells were incubated with the dibutyryl analogue of cAMP, with prostaglandin E and A compounds, and with 10^{-6} M colchicine before being exposed to immune complex on filters, there was reduction

of induced enzyme release (Table X). $\text{PGF}_{1\beta}$ again failed to retard enzyme release.

EFFECT OF SERUM INACTIVATION: It has been shown that heat-inactivated serum sup-

ports particle uptake, C-1 oxidation, and enzyme release almost as well as fresh serum when cells are exposed to immune complexes or MSU crystals (11). However, when cells were challenged with zymosan, modest but significant increments in phagocytosis and its metabolic concomitants were observed with fresh serum. The effect on enzyme release of heat inactivation was also studied. The presence of complement components (fresh serum) augmented only modestly the release of lysosomal enzymes from cells which had encountered immune complexes on the Millipore filter (Table XI). Despite the evidence that phagocytosis of complexes does not occur in this system, it cannot be inferred that phagocytic vacuoles were not formed in neutrophils adherent to the Millipore filter. Hawkins has utilized ferritin-anti-ferritin complexes on the filters, and in electron micrographs has observed phagosomes which contain clusters of ferritin (23).

THE EFFECT OF INHIBITING PHAGOCYTOSIS

TABLE IX
*Enzyme Release Induced by Immune Complex:
Bulk Phase versus Nonphagocytosable Surface**

Protein concentration μg	Percent of total β -glucuronidase release	
	Bulk phase	Nonphagocytosable surface
0 (cells only)	2.8 \pm 0.6	3.2 \pm 0.8
50	3.0 \pm 0.9	5.7 \pm 1.4
100	3.9 \pm 0.8	6.6 \pm 1.2
200	6.1 \pm 1.5	13.3 \pm 1.4
400	14.4 \pm 1.6	16.5 \pm 1.7

* Leukocytes exposed to immune complex for 2 h at 37°C.

Values are mean (\pm SEM) of three experiments.

TABLE X
*Inhibition of Enzyme Release from Human Leukocytes Exposed to Immune Complex on
Nonphagocytosable Surface**

Compound	No.	Percent of control enzyme release	
		β -Glucuronidase	LDH
None (control)	7	100.0	100.0
Dibutyl cAMP 10^{-3} M	4	75.7 \pm 2.6 \ddagger	99.4 \pm 1.1
PGE_1 2.8×10^{-4} M	4	81.3 \pm 1.9 \ddagger	97.8 \pm 0.9
Theophylline 10^{-3} M	4	83.1 \pm 2.4	101.4 \pm 1.7
2-Chloroadenosine 10^{-3} M	4	73.0 \pm 1.9 \ddagger	97.8 \pm 2.4
PGE_1 + theophylline	4	55.9 \pm 3.3 \ddagger	96.6 \pm 2.9
PGE_1 + 2-chloroadenosine	4	62.9 \pm 2.7 \ddagger	99.0 \pm 1.0
PGA_1 2.8×10^{-4} M	4	64.2 \pm 3.1 \ddagger	99.1 \pm 0.9
$\text{PGF}_{1\beta}$ 2.8×10^{-4} M	4	90.0 \pm 1.8	98.9 \pm 0.7
Colchicine 10^{-5} M	4	73.3 \pm 2.9 \ddagger	102.5 \pm 1.9

	No.	Percent of total enzyme release \S	
		β -Glucuronidase	LDH
Cells + filter (resting)	7	3.2 \pm 0.8	4.0 \pm 4.4
Cells + filter + complex (control)	7	16.5 \pm 1.7	4.8 \pm 3.7

* Human leukocytes (6×10^6) exposed to aIgG/IgM complex (400 μg) for 2 h. at 37°C. Pretreatment with drugs: 1 h, 37°C.

\ddagger $P < 0.01$ vs. control of 100%.

\S Enzyme activity expressed as a percentage of total activity released by 0.2% Triton X-100. Total activity (100%) of β -glucuronidase was $24.6 \pm 1.8 \mu\text{g}$ phenolphthalein/ 4×10^6 leukocyte/h; lactate dehydrogenase (LDH): 1640 ± 107 absorbancy units/ 4×10^6 leukocyte.

TABLE XI
Effect of Serum Inactivation on Enzyme Release
from Nonphagocytosable Surface*

	Percent of total β -glucuronidase release	
	Fresh serum	Heated serum
Surface + cells	8.4 \pm 0.7	7.7 \pm 1.0
Surface \pm aIgG/ RF + cells	17.7 \pm 1.6	13.1 \pm 1.2

* Leukocytes exposed to surface for 2 h at 37°C. Values represent mean of three experiments.

BY MEANS OF CYTOCHALASIN B: We therefore studied the morphological and biochemical consequences of exposing leukocytes to nonphagocytosable particles. This was accomplished by using cytochalasin B, a fungal metabolite known to disrupt microfilaments and inhibit ingestion of particles by human leukocytes (24-26). Incubation of cells with 5 μ g/ml cytochalasin B before exposure of cells to zymosan resulted in complete inhibition of zymosan uptake. Zymosan particles adhered to the plasma membrane and induced enzyme release but were not ingested (Fig. 3). Phagocytic vacuoles within neutrophils were not seen on any of many electron microscope grids observed; enzyme appeared to be released directly to the outside of the cell and, since treatment of cells with cytochalasin B did not enhance enzyme release (Table XII), this system converts the regurgitation during feeding model into the reverse endocytosis model in which release of lysosomal enzyme is independent of particle uptake and phagosome formation. It was therefore of interest to study the effect on enzyme release of colchicine and of agents which increase cAMP concentration. Incubation of cells with 10^{-5} M colchicine, 5×10^{-5} M vinblastine, or with dibutyryl cAMP and PGE₁ before treatment of cells with cytochalasin B and exposure to zymosan reduced extrusion of lysosomal enzymes (Table XII). As was the case for PGE₁, the effect of dibutyryl cAMP was dose dependent. Colchicine and vinblastine were not inhibitory at lower concentrations. There is apparently a threshold concentration at which colchicine becomes maximally effective, as greater concentrations in this system produced no further inhibitory effect and 10^{-3} M colchicine was needed to reduce enzyme release in the bulk phase (Tables I and II).

Cells Exposed to Membrane-Lytic Agents

Cell suspensions incubated with MSU crystals release cytoplasmic as well as lysosomal enzymes, with subsequent death of the cells (11). However, the kinetics of enzyme release from cells exposed to the membrane-lytic detergent Triton X-100 differed from those observed after cells ingested MSU crystals (Fig. 4). In the first instance there was rapid release of large proportions of total enzyme whereas enzyme release was delayed in the latter. In addition, incubation of cells with MSU crystals resulted in normal increments of C-1 oxidation (a metabolic consequence of phagocytosis) before enzyme release, whereas the addition of Triton X-100 prevented C-1 oxidation, and led to immediate release of enzymes (Table XIII). It may therefore be inferred that enzyme release follows phagocytosis of crystals and is due to "perforation from within" rather than lysis of plasma membrane by crystals in the media.

There is no evidence that agents which affect the intracellular level of cyclic nucleotides prevent rupture of lysosomal membranes by MSU. Were, however, merger of granules with phagocytic vacuoles to be impeded, or were phagocytosis inhibited, reduced release from cells of acid hydrolase would be expected. Leukocytes were therefore incubated with PGE₁, PGF_{1 β} , and dibutyryl cAMP before challenge by MSU crystals. Dibutyryl cAMP and PGE₁ (but not PGF_{1 β}) did in fact reduce release of lysosomal enzymes (Table XIV), whereas such agents had no effect on enzyme release from cells incubated with Triton X-100 (Table XV).

DISCUSSION

The release of enzymes from human leukocytes is reduced when cells are pretreated with pharmacologic doses of agents which increase cellular levels of cAMP or with compounds which disrupt microtubule integrity under three experimental circumstances: (a) phagocytosis of particles from the bulk phase, (b) encounter with nonphagocytosable particles, (c) exposure to crystals of MSU. In contrast, enzyme release from cells incubated with the detergent Triton X-100 was not prevented when cells were preincubated with such agents. These observations indicate that such compounds affect an intracellular event subsequent to particle encounter and/or ingestion.

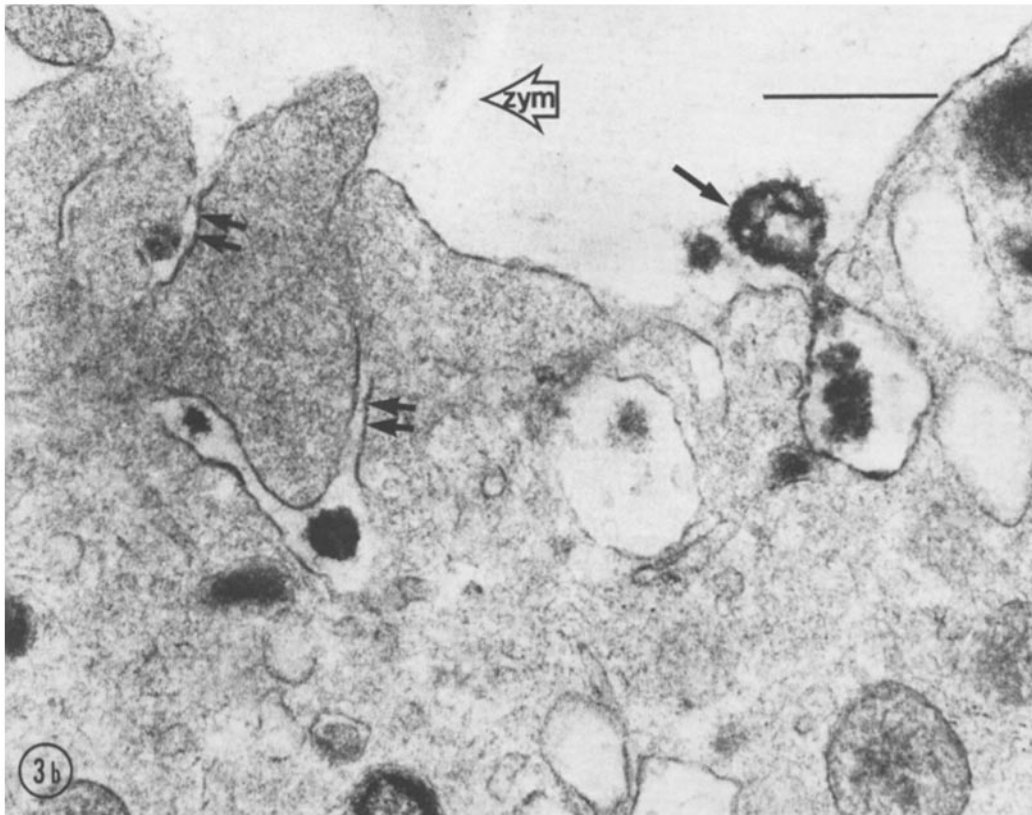
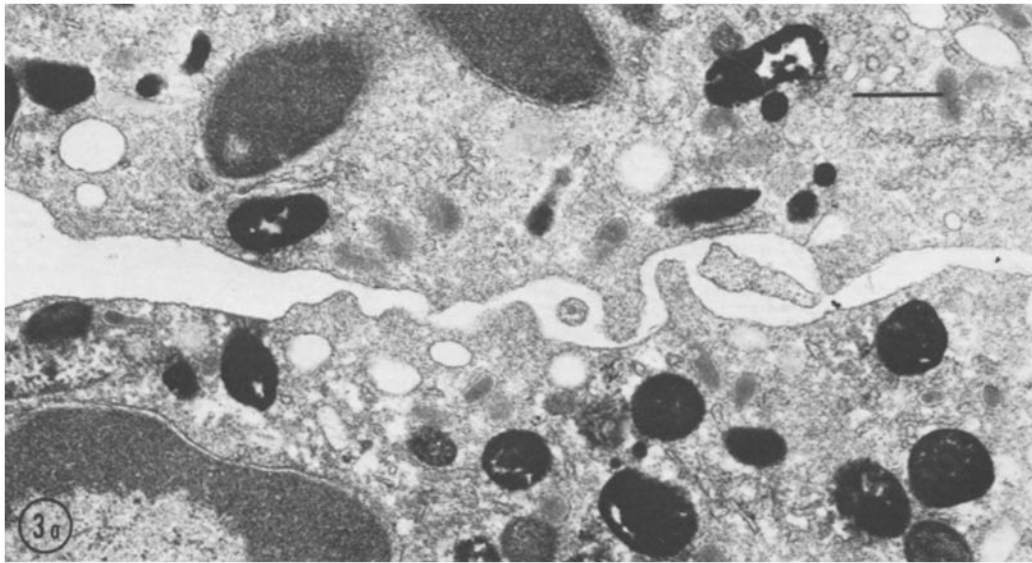


FIGURE 3 a Portions of two human peripheral blood neutrophils treated with cytochalasin B and then fixed and stained for peroxidase by the method of Graham and Karnovsky (13). Many peroxidase-positive lysosomes are seen at the cell periphery. Line indicates $0.5 \mu\text{m}$. $\times 23,000$.

FIGURE 3 b A portion of a human peripheral blood neutrophil treated with cytochalasin B and then exposed to zymosan (*zym*) (60 min). Peroxidase-positive material is seen emerging from a lysosome that has fused with the cell membrane (arrow). At the left are other peroxidase-positive deposits in lysosomes that are open to the outside of the cell (double arrows). Line indicates $0.5 \mu\text{m}$. $\times 46,000$.

TABLE XII
Enzyme Release from Human Leukocytes Treated with Cytochalasin B*

Compounds added	Enzyme activity released into supernatant†		
	Percent β -glucuronidase	Percent lactate dehydrogenase	Percent of control β -glucuronidase release
None (resting)	5.8 \pm 2.4	3.7 \pm 0.9	—
Cytochalasin B (CB) 5 μ g/ml	6.4 \pm 2.9	4.5 \pm 1.0	—
CB + zymosan (control)	32.6 \pm 3.4	5.1 \pm 1.2	100.0
Dibutyl cAMP 10^{-3} M + CB + zymosan	19.7 \pm 2.6	5.0 \pm 0.9	60.4 \pm 3.7§
PGE ₁ 2.8×10^{-4} M + theophylline 10^{-3} + CB + zymosan	17.8 \pm 2.0	4.0 \pm 1.0	54.3 \pm 4.1§
Colchicine 10^{-5} M + CB + zymosan	22.8 \pm 3.1	4.6 \pm 1.3	70.0 \pm 6.7§
Vinblastine 5×10^{-5} M + CB + zymosan	20.9 \pm 2.7	5.2 \pm 2.9	64.2 \pm 5.8§

* Leukocytes (4×10^6) incubated with drug for 60 min at 37°C, then with CB for 15 min, then exposed to zymosan for 60 min.

† Enzyme activity expressed as a percentage of total activity (4×10^6 leukocytes) released by 0.2% Triton X-100. Total activity (100%) of β -glucuronidase was $20.6 \pm 2.8 \mu$ g phenolphthalein/ 4×10^6 leukocyte/h; lactate dehydrogenase: 1420 ± 77 absorbancy units/ 4×10^6 leukocyte.

§ Significant at $P < 0.01$ vs. control of 100% (No. = 4).

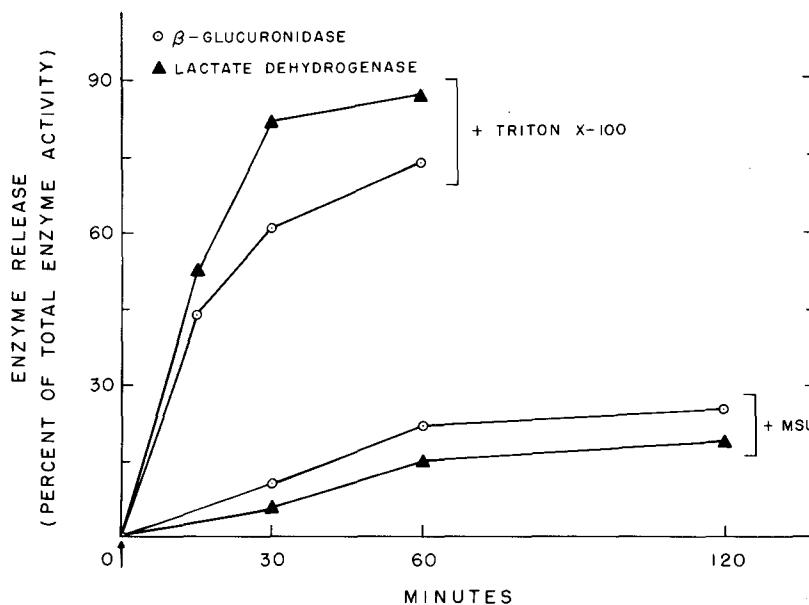


FIGURE 4 Enzyme release from leukocytes. Leukocytes challenged with MSU crystals (0.1 mg/ml) and Triton X-100 (0.5% vol/vol).

Intracellular movements of lysosomes, and of secretory granules in a variety of cell types, appear to be accomplished by an interaction between cyclic nucleotides, microtubules, and micro-

filaments. In order for degranulation and enzyme release to proceed in response to perturbation of the cell membrane, microtubules must remain under appropriate controls. Although micro-

TABLE XIII
Effect of Triton X-100 on Phagocytosis*

	no.	Cl-glucose oxidation <i>cpm</i>
Control (resting)	3	1387 ± 202
Cells + MSU	3	5711 ± 501
Cells + MSU + Triton X-100	3	1519 ± 317

* Human leukocytes (2×10^6) exposed to MSU or MSU + Triton X-100 for 60 min, 37°C.

tubules are in a dynamic state of assembly and disassembly, it is probably in their aggregated state that microtubules exert their influence on cell mechanics (27). cAMP and colchicine both favor disassembly of microtubules (28) and both reduce selective enzyme release. cAMP and compounds which increase its cellular concentration also reduce antigen-induced histamine release from sensitized leukocytes and lung tissue (29, 30). In contrast, deuterium oxide (D_2O) favors formation of microtubules (31) and aug-

TABLE XIV
Inhibition of Enzyme Release from Human Leukocytes Exposed to Monosodium Urate Crystals*

Compound with concentration	No.	Percent of control enzyme release	
		β -Glucuronidase	Lactate dehydrogenase
None (control)	6	100.0	100.0
Dibutyl cAMP 10^{-3} M	4	58.6 ± 4.7‡	67.1 ± 3.0‡
PGE ₁ 2.8×10^{-4} M	4	68.2 ± 5.4‡	70.9 ± 1.9‡
PGF _{1β} 2.8×10^{-4} M	4	97.0 ± 3.1	100.0 ± 2.6
Enzyme release (percent of total activity)§			
Resting cells	6	4.5 ± 1.1	3.1 ± 0.9
Cells + MSU (control)	6	21.0 ± 1.9	25.6 ± 2.3

* Leukocytes (4×10^6) incubated with drugs at 37°C for 1 h, then exposed to MSU (0.1 mg/ml) for 1 h.

‡ $P < 0.01$ vs. control of 100%.

§ Enzyme activity expressed as a percentage of total activity released by 0.2% Triton X-100. Total activity (100%) of β -glucuronidase was 18.1 ± 1.0 μ g phenolphthalein/ 4×10^6 leukocyte/h; lactate dehydrogenase: 1176 ± 78 absorbance units/ 4×10^6 leukocyte.

TABLE XV
Enzyme Release from Human Leukocytes Exposed to Triton X-100*

Compound and concentration	No.	Percent of control enzyme release	
		β -Glucuronidase	Lactate dehydrogenase
None (control)	3	100.0	100.0
PGE ₁ 2.8×10^{-4} M	3	105.1 ± 1.8	101.9 ± 0.7
Dibutyl cAMP 10^{-3} M	3	103.3 ± 2.4	105.2 ± 1.9
Colchicine 10^{-3} M	3	91.4 ± 4.1	97.4 ± 2.3
Enzyme release (percent of total activity)‡			
Resting cells	3	3.4 ± 1.6	3.3 ± 2.7
Cells + Triton X-100	3	33.9 ± 4.1	36.7 ± 3.2

* Leukocytes (4×10^6) incubated with drugs at 37°C for 1 h, then exposed to Triton X-100 (0.05% vol/vol) for 15 min.

‡ Enzyme activity expressed as a percentage of total activity released by 0.2% Triton X-100. Values are means of three experiments. Total activity (100%) of β -glucuronidase was 16.8 ± 1.7 μ g phenolphthalein/ 4×10^6 leukocyte/h, lactate dehydrogenase: 1060 ± 94 absorbance units/ 4×10^6 leukocyte.

ments histamine release from leukocytes (32). In addition, it has recently been reported that β -adrenergic agents (which increase cAMP levels) reduce, whereas cholinergic agonists enhance, lysosomal enzyme release from leukocytes (33), and immunologic release of histamine and SRS-A¹ from human lung fragments (34). Cholinergic stimulation of heart and brain preparations has produced an increase in levels of cyclic guanosine 3'5'-monophosphate (cGMP) (35), and the introduction of 8-bromo-cGMP to sensitized lung tissue (34) and of cGMP to leukocyte suspensions (33) was associated with enhancement of antigen-induced release of histamine and SRS-A in the first instance and enhancement of lysosomal enzyme release in the latter circumstance. Thus, it appears that in human leukocytes granule movement and acid hydrolase release may be modulated through changes in concentrations of at least two cyclic nucleotides, cAMP and cGMP. It is therefore possible that the release of inflammatory substances from phagocytes can be controlled by humoral means and that many of these may affect microtubule function.

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¹ SRS-A: slow-reacting substance of anaphylaxis.