HORMONAL CONTROL OF LYSOSOMAL ENZYME RELEASE FROM HUMAN NEUTROPHILS

Effects of Autonomic Agents on Enzyme Release, Phagocytosis, and Cyclic Nucleotide Levels*

By L. J. IGNARRO, T. F. LINT, and W. J. GEORGE

(*From The Departments of Pharmacology and Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112*)

Release of lysosomal enzymes from phagocytic leukocytes in the presence of various immune reactants is well documented (1–6). The tissue injury that results from the accumulation of, and phagocytosis by, granulocytes is probably a direct consequence of the extracellular release of granule constituents (7, 8). Indeed, lysosome granules proteins are capable of mediating inflammation (9, 10) and cartilage degradation (11–13).

The anti-inflammatory action of certain catecholamines in various animal models of acute (14, 15) and chronic inflammation (16, 17) has been reported. In fact, catecholamines have been thought to play a regulatory role in the inflammatory process (18). More recent studies have uncovered specific actions of catecholamines at the cellular and organelle level. For example, certain sympathomimetic amines were reported to inhibit the allergic release of histamine from sensitized human leukocytes (19) and lung fragments (20). In both of these studies it was suggested that the actions of the catecholamines were mediated by cyclic AMP. Release of enzymes from isolated lysosomes was also inhibited by epinephrine, norepinephrine, isoproterenol, theophylline, and cyclic AMP (21–24).

In order to understand more completely the anti-inflammatory effects of catecholamines at the cellular level, several adrenergic and cholinergic amines and cyclic nucleotides were tested for their capacity to modify the release of β-glucuronidase, a lysosomal marker enzyme, from and particle ingestion by human neutrophils during phagocytic uptake of serum-treated zymosan particles. In addition, the effects of autonomic neurohormones on neutrophil levels of cyclic AMP and cyclic GMP were determined in order to assess the possible involvement of cyclic nucleotides in mediating the actions of the neurohormones on phagocytic enzyme release. Preliminary reports of some of the present findings appeared earlier (25, 26).

Materials and Methods

Isolation of Human Neutrophils and Mixed Leukocytes.—Human neutrophils were isolated from fresh heparinized venous blood drawn from healthy volunteers by a modification of the glass bead column purification procedure described by Rabinowitz (27). Briefly, leukocyte-rich plasma was obtained from heparinized blood after sedimentation of erythrocytes in 0.5% (wt/vol) Dextran 200 C (Sigma Chemical Co., St. Louis, Mo.) in saline. The leukocyte-rich plasma was placed on the siliconized glass bead column and incubated at 37°C for 90 min. Lymphocytes, erythrocytes, and platelets were eluted with 50% (vol/vol) Eagle's minimum essential medium in autologous plasma. Neutrophils were then eluted from the column with 0.1 M sodium phosphate buffer, pH 7.3, containing 0.1% (wt/vol) glucose. EDTA was not employed in any step. The final cell suspension was prepared in Hanks' balanced salt solution containing 0.1% (wt/vol) glucose (HBSS)[1] and diluted to 5×10^6 neutrophils per ml. This procedure yielded consistently a cell suspension containing at least 97% neutrophils. Mononuclear cells were present to the extent of only 1–3%, and erythrocytes and platelets were absent. Viability of the neutrophils was always greater than 99% (trypan blue or eosin Y exclusion) and recoveries of neutrophils from leukocyte-rich plasma was 25–45%. Human mixed leukocytes were obtained from heparinized venous blood after sedimentation of erythrocytes in a suspension consisting of five parts of blood mixed with one part of 6% (wt/vol) Dextran 70. After centrifuging the upper leukocyte layer (150 g for 10 min), cell sediments were washed with and then resuspended in HBSS. The final cell concentration was adjusted to 5×10^6 mixed leukocytes per ml. Microscopic examination revealed 60–75% neutrophils, and over 99% of the granulocytes excluded trypan blue.

Preparation of Zymosan Particles.—Zymosan particles (Sigma), measuring 0.5–3 μ in diameter, were suspended and boiled for 10 min in HBSS (10 mg/ml). The supernate was discarded and the zymosan particle sediment was completely resuspended in either normal or rheumatoid arthritic serum to the extent of 25 mg/ml. High titer (1:10,240–1:20,480 by rheumatoid factor latex agglutination) rheumatoid arthritic (RA) serum was obtained from individuals with active rheumatoid arthritis. Zymosan suspensions were then incubated at 37°C for 30 min, while shaking adequately to maintain the particles in suspension, and the particles were allowed to settle. Supernates were discarded, sediments were washed extensively with cold saline, and resuspended thoroughly in HBSS (10 mg/ml) with the aid of a glass tissue grinder equipped with a teflon pestle. All particle suspensions were completely homogeneous and absence of particle aggregation was confirmed by phase contrast microscopy. Each ml of final zymosan suspension contained approximately 4×10^8 particles. In some experiments the particle concentration was varied from 0.25×10^8 to 4×10^8 particles per ml.

Preparation of Zymosan-Treated Serum.—Zymosan particles (0.5–3 μ in diameter) were suspended and boiled for 10 min in saline (10 mg/ml). The sediment was resuspended in 0.01 M sodium phosphate-buffered (pH 7.4) saline, containing 0.001 M magnesium chloride. The zymosan suspension (10 mg/ml) was then diluted fivefold with fresh human serum and incubated at 37°C for 20 min while shaking gently. Incubations were followed by rapid filtration through a 0.22 μ (pore diameter) Millipore filter. Epsilon aminocaproic acid was dissolved in the zymosan-treated serum, to a final concentration of 0.2 M, in order to retard the possible loss of lysosomal enzyme releasing activity from the activated serum by a serum carboxypeptidase-like enzyme(s) (28). The above procedure is similar to that reported recently by Goldstein et al. (29).

Incubation Conditions.—Neutrophils (5×10^6 in 1.0 ml of HBSS) were incubated at 37°C

---

[1] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution containing 0.1% wt/vol glucose; RA, rheumatoid arthritic.

in a Dubnoff metabolic shaker while agitating at 120 excursions per min. Zymosan particles, either untreated or treated with normal or rheumatoid arthritic serum, were added to the extent of 0.1 ml of particle suspension to 1.0 ml of cell suspension. In those experiments where drugs were tested, cells and appropriate drug(s) were preincubated at 37°C for 5 min before the addition of zymosan particles and then further incubated at 37°C for 15 min. Following incubation the samples were centrifuged at 350 g for 10 min at 4°C and the supernates were assayed for β-glucuronidase and lactate dehydrogenase activities.

Enzyme Assays.—β-glucuronidase activity was measured by a modification of the procedure reported by Gianetto and DeDuve (30). Aliquots (0.2 ml) of supernates, obtained as described above, were incubated in 4.0 ml of 0.1 M sodium citrate, pH 4.8, containing 5.0 mg of phenolphthalein glucuronide as substrate, at 37°C for 18 h. Enzyme reactions were terminated by adding 0.4 ml of alkaline glycine-maleate and absorbancy (540 nm) was determined. Data are expressed as μg of phenolphthalein liberated per 18 h of incubation. Lactate dehydrogenase activity was measured according to the method of Bergmeyer et al. (31). Data were calculated as the change (Δ) in absorbancy at 366 nm per min. Determinations of total neutrophil enzyme activities were made after cell lysis by eight freeze-thaw cycles, employing a dry ice-acetone mixture, and running cool tap water. Broken cell preparations were incubated and centrifuged, as with intact cell preparations, and enzyme activities were then determined.

Measurement of Particle Uptake.—Uptake of zymosan particles by neutrophils, present in either the purified neutrophil or mixed leukocyte suspensions, was quantitated by enumeration of ingested zymosan particles by oil immersion phase contrast microscopy. The data are expressed as the average number of zymosan particles ingested per 100 neutrophils (phagocytic index), and was calculated from 200–400 cell counts on successive microscopic fields. This method can be employed to measure both the rate and the extent of phagocytosis of zymosan particles. In addition, in those circumstances where phagocytosis was either just beginning or proceeding at a reduced capacity, the percent of neutrophils which contained one or more zymosan particles was determined.

Measurement of Neutrophil Levels of Cyclic AMP and Cyclic GMP.—Cyclic AMP levels in neutrophils were measured by the protein-binding procedure described by Gilman (32). Levels of cyclic GMP were measured essentially according to the radioimmunoassay of Steiner et al. (33). Neutrophil samples were processed by rapid freezing of individual incubation mixtures containing cells, acidification, and ether extraction as described previously (34). Cyclic nucleotide levels in entire incubation mixtures were determined and the data are expressed as pmoles of cyclic nucleotide per 10^6 neutrophils.

Drug Solutions and Sources.—Solutions of the catecholamines contained 0.01% (wt/vol) sodium metabisulfite to prevent spontaneous oxidation and were utilized within 10 min of preparation. All other solutions of test agents were prepared fresh and used within 15 min. The complete names of all the chemical agents and drugs, including their salts, used in this study are indicated below. The following agents were purchased from Sigma Chemical: *l*-epinephrine bitartrate, *l*-phenylephrine hydrochloride, acetylcholine chloride, acetyl β-methylcholine chloride, carbamylcholine chloride, pilocarpine hydrochloride, choline chloride, atropine sulfate, hexamethonium bromide, *d*-tubocurarine chloride, theophylline, cyclic 3′,5′-adenosine monophosphate sodium, $N^6,O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate sodium, adenosine 5′-monophosphate sodium, cyclic 3′,5′-guanosine monophosphate sodium, $N^2,O^{2'}$-dibutyryl cyclic 3′,5′-guanosine monophosphate sodium, guanosine 5′-monophosphate sodium. The *l*-isomer of isoproterenol hydrochloride was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). 8-azido cyclic 3′,5′-adenosine monophosphate and 8-bromo cyclic 3′,5′-guanosine monophosphate sodium, respectively, were purchased from Plenum Scientific Research, Inc. (Hackensack, N. J.) and ICN. Corp. (Irvine, Calif.). CIBA-GEIGY Corp. supplied phentolamine methanesulfonate and Ayerst provided *dl*-propranolol hydrochloride.

RESULTS

Release of Enzymes and Particle Uptake by Human Neutrophils in the Presence of Zymosan.—The data in Table I illustrate that both human neutrophils and mixed leukocytes release β-glucuronidase into the extracellular medium during uptake or phagocytosis of serum-treated zymosan particles. More β-glucuronidase release from, and particle uptake by, cells occurs in the presence of zymosan particles treated with serum from RA than from normal individuals. Very little release of β-glucuronidase and uptake of particles are evident in the presence of serum-free zymosan. Even less enzyme release occurs in the absence of zymosan particles. In addition to the absence of significant release of the cytoplasmic marker enzyme lactate dehydrogenase (Table I), 99% of the neutrophils and granulocytes in the mixed leukocyte preparations exclude trypan blue after 15–30 min of incubation. Therefore, release of β-glucuronidase represents a selective process without concomitant loss of cell viability.

TABLE I

Release of Enzymes from and Particle Uptake by Human Neutrophils in the Presence of Zymosan*

Experimental condition‡	Release of enzymes§		Particle uptake
	β-glucuronidase	Lactate dehydrogenase	
			no. particles/100 neutrophils
Neutrophils			
Cells alone	4.1 ± 0.2 (1.4)	0.0029 ± 0.0003 (2.2)	—
Cells + zymosan	8.7 ± 0.3 (3.0)	0.0018 ± 0.0002 (1.4)	27 ± 5
Cells + zymosan-normal serum	37.2 ± 1.9 (12.7)	0.0022 ± 0.0003 (1.7)	325 ± 38
Cells + zymosan-RA serum	59.5 ± 3.7 (20.2)	0.0017 ± 0.0001 (1.3)	504 ± 62
Mixed leukocytes			
Cells alone	2.8 ± 0.4 (1.4)	0.0026 ± 0.0004 (2.4)	—
Cells + zymosan	4.1 ± 0.4 (2.0)	0.0031 ± 0.0004 (2.8)	20 ± 4
Cells + zymosan-normal serum	16.9 ± 1.4 (8.2)	0.0034 ± 0.0003 (3.1)	312 ± 41
Cells + zymosan-Ra serum	27.4 ± 1.9 (13.3)	0.0028 ± 0.0003 (2.6)	506 ± 66

* Data represent the Mean ± SEM of three to five separate experiments.

‡ Cells (5×10^6) were incubated at 37°C for 15 min as indicated.

§ β-glucuronidase activity is expressed as μg phenolphthalein/18 h/5×10^6 total cells; values for total cell activity were 294 ± 12 (neutrophils) and 206 ± 14 (mixed leukocytes). Lactate dehydrogenase activity is expressed as Δabsorbancy (366 nm)/min/5×10^6 total cells; values for total cell activity were 0.131 ± 0.008 (neutrophils) and 0.109 ± 0.010 (mixed leukocytes). Numbers in parentheses signify percent of total cell enzyme activity.

Neutrophils and mixed leukocytes respond to zymosan particles in a qualitatively similar manner with regard to particle uptake and enzyme release.

In the presence of zymosan particles treated with RA serum, release of β-glucuronidase from neutrophils is detectable after 5 min of incubation and increases markedly during the following 10 min (Fig. 1). Enzyme release appears to taper off by 20–30 min, although a small increment of enzyme release continues during incubation of cells for an additional 3 h (data not illustrated). Particle uptake starts almost immediately and increases linearly through the first 10 min of incubation, after which time particle ingestion begins to attain a maximum (Fig. 1). Over 80% of the neutrophils take up one or more particles at each time interval except 2 min where approximately 45% of the cells ingest particles. These data illustrate a close temporal relationship between discharge of β-glucuronidase into and ingestion of zymosan particles from the extracellular environment. Thus, release of β-glucuronidase occurs during phagocytosis and not subsequent to attainment of maximum particle uptake. These findings are similar to those reported previously which indicated a correlation between the extracellular appearance of granule enzyme and the time of phagocytosis (35, 36). Lactate dehydrogenase is not released to any significant extent during 30 min of incubation. In fact, no significant release of lactate dehydrogenase from neutrophils occurs until 6–8 h of incubation at 37°C, at which time a significant number of cells begin to take up trypan blue. It is important to indicate that neither normal serum nor RA serum, undiluted or
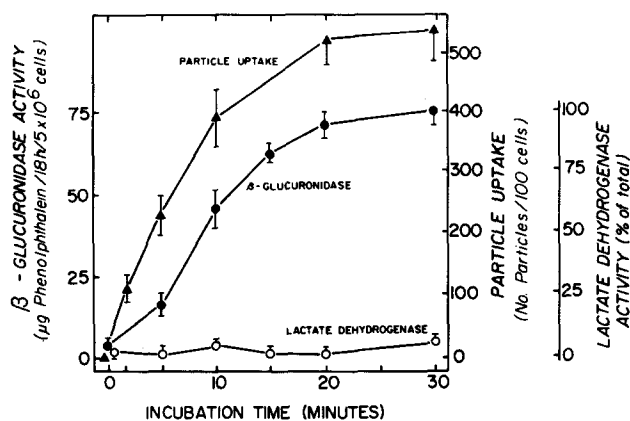

FIG. 1. Release of β-glucuronidase and lactate dehydrogenase from and particle uptake by human neutrophils in the presence of zymosan. Neutrophils (5×10^6) were incubated at 37°C in 1.0 ml of HBSS containing 4×10^8 zymosan particles treated with rheumatoid arthritic serum (particle/cell ratio was 80). Values for total cell enzyme activities, determined after lysis of cells by freezing and thawing, were 288 ± 15 μg phenolphthalein/18 h/5×10^6 neutrophils for β-glucuronidase and 0.139 ± 0.011 Δabsorbancy (366 nm)/min/5×10^6 neutrophils for lactate dehydrogenase. Data represent the Mean $\pm$ SEM of three separate experiments.

diluted 10-fold, provoked release of β-glucuronidase from neutrophils or mixed leukocytes in the absence of zymosan particles.

The close relationship between β-glucuronidase release and zymosan particle uptake, under conditions where the particle per cell ratio was varied, is illustrated in Fig. 2. Over 85% of the neutrophils ingest one or more particle at each particle/cell ratio except five where 60-80% of the cells take up particles. Lactate dehydrogenase is not discharged regardless of the numbers of particles associated with neutrophils.

In order to assess the possible role of complement components in provoking the sera-treated zymosan-induced release of β-glucuronidase from, and particle uptake by, human neutrophils sera were heated at 53°C for 30 min before incubation with zymosan particles. Particle uptake and release of β-glucuronidase are reduced by about 45-60% when neutrophils are incubated with zymosan particles treated with heated normal serum (Table II). However, zymosan treated with heated RA serum provokes the same degree of particle uptake and enzyme release as does zymosan treated with unheated RA serum. Therefore, RA serum appears to be heat-resistant with regard to its capacity to promote zymosan-induced phagocytosis by, and lysosomal enzyme release from, human neutrophils.

Effect of Catecholamines and Cyclic AMP on Release of β-Glucuronidase from and Particle Uptake by Human Neutrophils.—Epinephrine and isoproterenol inhibit release of β-glucuronidase from, and particle uptake by, neutrophils in the presence of zymosan treated with RA serum (Table III). Phenylephrine, a
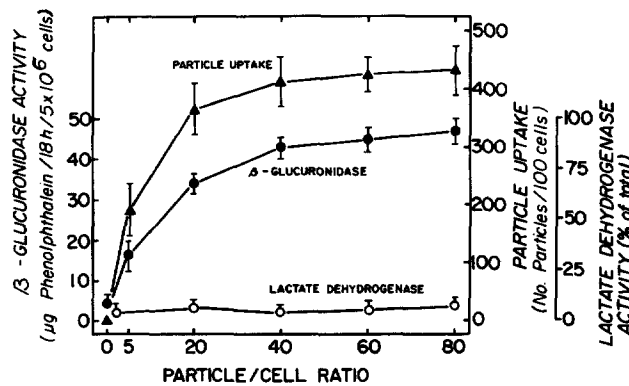

Fig. 2. Effect of particle/cell ratio on release of β-glucuronidase and lactate dehydrogenase from and particle uptake by human neutrophils in the presence of zymosan. Neutrophils (5 × 10^6) were incubated at 37°C for 10 min in 1.0 ml of HBSS containing various numbers of zymosan particles treated with rheumatoid arthritic serum. Values for total cell enzyme activities, determined after lysis of cells by freezing and thawing, were 308 ± 19 μg phenolphthalein/18 h/5 × 10^6 neutrophils for β-glucuronidase and 0.125 ± 0.009 Δabsorbancy (366 nm)/min/5 × 10^6 neutrophils for lactate dehydrogenase. Data represent the Mean ± SEM of four separate experiments.

TABLE II

*Effect of Heated and Cobra Venom Factor-Treated Sera on Particle Uptake by and β-Glucuronidase Release from Human Neutrophils**

Experimental condition‡	Particle uptake	β-glucuronidase release
	no. particles/100 neutrophils	*μg/18 h/5 × 10⁶ cells*
5 × 10⁶ neutrophils (N)	—	3.6 ± 0.8
N + zymosan (Z)	21 ± 5	7.3 ± 0.6
N + normal serum-treated Z (NSZ)	334 ± 34	40.2 ± 2.3
N + heated NSZ	140 ± 21§	24.3 ± 3.2§
N + cobra venom factor-treated NSZ	112 ± 16§	14.8 ± 2.1§
N + rheumatoid arthritic serum-treated Z (RASZ)	528 ± 60	55.6 ± 4.1
N + heated RASZ	506 ± 44	49.9 ± 3.7
N + cobra venom factor-treated RASZ	468 ± 49	47.2 ± 4.6

* Data represent the Mean ± SEM of four separate determinations. Incubations were conducted at 37°C for 15 min as indicated.

‡ Heated sera were prepared by heating fresh sera at 56°C for 30 min. Zymosan particles were treated with heated sera in the same manner as for unheated sera. 10 U of cobra venom factor (0.1 ml of stock 100 U per ml) were mixed with each ml of the sera and incubated at 30°C for 60 min. Zymosan particles were treated with cobra venom factor-treated sera in the same manner as for untreated sera.

§ Significantly different ($P < 0.001$) from corresponding controls (N + normal serum-treated Z).

related amine but lacking β-adrenergic receptor stimulating activity, affects neither enzyme release nor particle uptake. Propranolol, a β-adrenergic receptor antagonist, but not phentolamine, an α-adrenergic receptor antagonist, blocks the inhibitory action of epinephrine on enzyme release and particle uptake. Theophylline, which is well known to inhibit the phosphodiesterase-catalyzed degradation of endogenous cyclic AMP and thereby elevate tissue levels of this cyclic nucleotide, enhances the effect of epinephrine on both enzyme release and particle uptake. In fact, in the presence of theophylline, cyclic AMP inhibits β-glucuronidase release and particle uptake. The dibutyryl and 8-azido analogs of cyclic AMP are more active than cyclic AMP whereas adenosine 5'-monophosphate is inactive. Thus, certain catecholamines and cyclic AMP possess the capacities to inhibit β-glucuronidase release and phagocytosis or uptake of zymosan particles by neutrophils.

Effect of Cholinergic Agents and Cyclic GMP on Release of β-Glucuronidase from and Particle Uptake by Human Neutrophils.—Acetylcholine, acetyl β-methylcholine, carbamylcholine, and pilocarpine markedly accelerate release of β-glucuronidase from, but not particle uptake by, human neutrophils in the presence of zymosan treated with RA serum (Table IV). Choline, a precursor in the biosynthesis of acetylcholine, is completely without effect on enzyme

TABLE III

*Effect of Catecholamines and Cyclic AMP on Release of β-Glucuronidase from and Particle Uptake by Human Neutrophils**

Agent(s) tested	% inhibition of β-glucuronidase release			Particle uptake in $10^{-6}M$ agent
	Concn. of agent: $10^{-6}M$	$10^{-7}M$	$10^{-8}M$	
				no. particles/100 neutrophils
Control (no agent)	—	—	—	505 ± 53
Epinephrine (E)	56 ± 4.1	35 ± 2.2	19 ± 0.9	386 ± 35‡
Isoproterenol	55 ± 3.8	30 ± 1.7	12 ± 0.5	380 ± 39‡
Phenylephrine	0	0	0	512 ± 66
E + propranolol ($10^{-6}M$)	12 ± 0.7	0	0	478 ± 52
E + phentolamine ($10^{-6}M$)	54 ± 2.1	36 ± 2.7	23 ± 1.1	—
E + theophylline ($10^{-6}M$)	71 ± 6.5	52 ± 4.3	32 ± 1.6	309 ± 43‡
Cyclic AMP + theophylline ($10^{-6}M$)	37 ± 2.1	22 ± 1.8	9 ± 0.5	380 ± 34‡
Dibutyryl cyclic AMP	43 ± 2.3	28 ± 1.4	15 ± 0.8	312 ± 41‡
8-azido cyclic AMP	62 ± 4.7	48 ± 3.7	26 ± 1.6	—
AMP	0	0	0	516 ± 47

* Data represent the Mean ± SEM of three to four separate experiments. Incubations of 5 × 10⁶ neutrophils were conducted at 37°C for 15 min in the presence of zymosan treated with rheumatoid arthritic serum. Control incubations (no agents added) yielded a value of 56.4 ± 2.7 μg phenolphthalein/18 h/5 × 10⁶ neutrophils (19.8% of total cell activity) for release of β-glucuronidase. Theophylline ($10^{-6}M$), propranolol ($10^{-6}M$), or phentolamine ($10^{-6}M$), when tested alone, did not affect significantly the release of β-glucuronidase.

‡ Significantly different ($P < 0.05$) from control.

release. Atropine, a muscarinic receptor antagonist, blocks the action of acetylcholine on enzyme release. Neither hexamethonium (ganglionic blocking agent) nor d-tubocurarine (neuromuscular blocking agent) affects the accelerating action of acetylcholine on enzyme release. Cyclic GMP and its dibutyryl and 8-bromo analogs, but not guanosine 5'-monophosphate, also accelerate the release of β-glucuronidase from, but not particle uptake by, neutrophils (Table IV).

Although cholinergic agents and cyclic GMP stimulate enzyme release from cells that are actively engaged in phagocytic uptake of zymosan particles, these agents do not accelerate enzyme release from cells in the absence of normal or RA serum-treated zymosan particles. In addition, the extent of phagocytic uptake of RA serum-treated zymosan particles during a given 15-min incubation at 37°C is not altered by either cholinergic agents or cyclic GMP (Table IV). Cell viability is maintained throughout the course of incubation with these agents, as indicated by failure of such agents to accelerate release

TABLE IV

Effect of Cholinergic Agents and Cyclic GMP on Release of β-Glucuronidase from and Particle Uptake by Human Neutrophils[*]

Agent(s) tested	% increase in β-glucuronidase release			Particle uptake in 10^{-6}M agent
	Concn. of agent: 10^{-6}M	10^{-7}M	10^{-8}M	
				no. particles/100 neutrophils
Control (no agent)	—	—	—	494 ± 44
Acetylcholine (A)	121 ± 4.1	66 ± 3.8	30 ± 0.9	538 ± 56
Acetyl β-methylcholine	168 ± 7.2	89 ± 4.3	37 ± 1.6	520 ± 48
Carbamylcholine	149 ± 7.1	76 ± 5.2	28 ± 1.0	490 ± 52
Pilocarpine	154 ± 6.0	73 ± 4.8	22 ± 1.2	—
Choline	0	0	0	509 ± 50
A + atropine (10^{-6}M)	28 ± 0.8	3 ± 0.1	0	—
A + hexamethonium (10^{-6}M)	129 ± 5.7	70 ± 3.4	27 ± 1.1	—
A + d-tubocurarine (10^{-6}M)	117 ± 3.9	66 ± 4.6	32 ± 1.3	—
Cyclic GMP	128 ± 7.4	75 ± 2.8	24 ± 1.7	512 ± 42
Dibutyryl cyclic GMP	167 ± 9.5	98 ± 4.9	37 ± 2.2	—
8-bromo cyclic GMP	208 ± 11	134 ± 8.4	82 ± 5.6	536 ± 40
GMP	0	0	0	516 ± 38

[*] Data represent the Mean ± SEM of three to four separate experiments. Incubations of 5×10^6 neutrophils were conducted at 37°C for 15 min in the presence of zymosan treated with rheumatoid arthritic serum. Control incubations (no agents added) yielded a value of 61.5 ± 3.8 μg phenolphthalein/18 h/5 × 10^6 neutrophils (20.4% of total cell activity) for release of β-glucuronidase. Atropine (10^{-6}M), hexamethonium (10^{-6}M), or d-tubocurarine (10^{-6}M), when tested alone, did not affect significantly the release of β-glucuronidase.

of lactate dehydrogenase from cells and by the continual exclusion of eosin Y by cells (Table V).

The data illustrated in Table VI indicate that complete recovery of β-glucuronidase and lactate dehydrogenase activities from neutrophils and incubation media, in the presence of various test agents, is obtained. Therefore, all enzyme activities are accounted for after all incubations and the presence of serum-treated zymosan particles and test agents do not alter enzyme activities appreciably.

Effect of Autonomic Agents and Cyclic Nucleotides on Release of β-Glucuronidase from Human Neutrophils in the Presence of Zymosan-Treated Serum.— Incubation of neutrophils with particle-free zymosan-treated fresh human serum results in the discharge of β-glucuronidase but not of lactate dehydrogenase (Table VII). Treatment of human serum with zymosan probably activates complement, via the alternate pathway, and thereby generates one or more components (i.e., activated fifth component) that are capable of selectively

TABLE V

Effect of Cholinergic Agents and Cyclic GMP on Viability of Human Neutrophils During Particle Uptake*

Experimental condition‡	Release of lactate dehydrogenase§	% of neutrophils with eosin Y uptake
5 × 10⁶ neutrophils (N)	0.0034 ± 0.0002	0.2 ± 0.01
N + zymosan-RA serum (Z)	0.0039 ± 0.0004	1.1 ± 0.2
N + Z + 10⁻⁶M acetylcholine	0.0037 ± 0.0003	1.6 ± 0.3
N + Z + 10⁻⁶M carbamylcholine	0.0030 ± 0.0004	1.2 ± 0.2
N + Z + 10⁻⁶M cyclic GMP	0.0034 ± 0.0003	1.9 ± 0.5
N + Z + 10⁻⁶M 8-bromo cyclic GMP	0.0038 ± 0.0005	1.4 ± 0.1

* Data represent the Mean ± SEM of four separate determinations.

‡ Incubations were conducted at 37°C for 15 min as indicated.

§ Expressed as Δabsorbancy (366 nm)/min/5 × 10⁶ neutrophils; value for total cell enzyme activity was 0.126 ± 0.008.

TABLE VI

Recovery of Enzyme Activities from Human Neutrophils

Experimental condition	Percent recovery*	
	β-glucuronidase	Lactate dehydrogenase
5 × 10⁶ neutrophils (N)	98.4 ± 6.7	102.4 ± 2.2
N + zymosan	103.2 ± 8.1	98.8 ± 2.7
N + zymosan-normal serum	94.1 ± 7.0	96.9 ± 2.8
N + zymosan-RA serum (Z)	97.8 ± 4.8	101.5 ± 3.1
N + Z + 10⁻⁶M epinephrine	106.8 ± 9.4	105.4 ± 4.4
N + Z + 10⁻⁶M cyclic AMP	96.0 ± 3.9	100.8 ± 2.5
N + Z + 10⁻⁶M acetylcholine	99.6 ± 5.4	95.8 ± 3.8
N + Z + 10⁻⁶M cyclic GMP	94.2 ± 8.6	104.4 ± 4.3

* Data represent the Mean ± SEM of three separate determinations. Incubations were conducted at 37°C for 15 min as indicated. Samples were then frozen-thawed, centrifuged, and the clear supernates were assayed for enzyme activities. Controls consisted of neutrophils that were not incubated, and recoveries of enzyme activities were calculated on the basis of control values of total cell activities. Values for control total cell enzyme activities were: β-glucuronidase, 251 ± 8.9 μg phenolphthalein/18 h/5 × 10⁶ neutrophils; lactate dehydrogenase, 0.138 ± 0.009 Δabsorbancy (366 nm)/min/5 × 10⁶ neutrophils.

releasing lysosomal enzymes from polymorphonuclear leukocytes in the absence of particles (37). Epinephrine, but not phenylephrine, inhibits the release of β-glucuronidase provoked by zymosan-treated serum and this effect is blocked by propranolol (Table VII). Dibutyryl cyclic AMP, but not AMP, also inhibits enzyme release. Conversely, carbamylcholine, but not choline, enhances β-glucuronidase release and this effect is blocked by atropine. Dibutyryl cyclic GMP, but not GMP, also enhances the discharge of β-glucuroni-

TABLE VII

Effects of Autonomic Agents and Cyclic Neucleotides on Release of β-Glucuronidase and Lactate Dehydrogenase from Human Neutrophils in the Presence of Zymosan-Treated Serum*

Agent(s) tested	β-glucuronidase release	Lactate dehydrogenase release
	$\mu g/18 \ h/5 \times 10^6$ cells	$\Delta \ absorb./min/5 \times 10^6$ cells
Control (no agent)	42.2 ± 3.8	0.0041 ± 0.0005
Epinephrine 10^{-6}M (E)	24.1 ± 1.7‡	0.0036 ± 0.0003
Phenylephrine 10^{-6}M	44.0 ± 3.4	0.0032 ± 0.0004
E + propranolol 10^{-6}M	38.5 ± 2.9	0.0038 ± 0.0005
Dibutyryl cyclic AMP 10^{-6}M	27.0 ± 1.9‡	0.0035 ± 0.0002
AMP 10^{-6}M	43.6 ± 3.2	0.0040 ± 0.0005
Carbamylcholine 10^{-6}M (C)	96.5 ± 8.8‡	0.0039 ± 0.0005
Choline 10^{-6}M	40.4 ± 3.8	0.0033 ± 0.0002
C + atropine 10^{-6}M	53.8 ± 6.2	0.0042 ± 0.0004
Dibutyryl cyclic GMP 10^{-6}M	126.1 ± 11.4‡	0.0046 ± 0.0005
GMP	47.0 ± 4.2	0.0036 ± 0.0004

* Data represent the Mean ± SEM of four separate experiments. Incubations of 5 × 10^6 neutrophils were conducted at 37°C for 15 min in 1.0 ml of HBSS containing 10% of zymosan-treated serum (containing 0.2 M ε-aminocaproic acid). Values for total cell enzyme activities were 322 ± 16 (β-glucuronidase) and 0.128 ± 0.016 (lactate dehydrogenase).

‡ Significantly different ($P < 0.05$) from control.

dase. Lactate dehydrogenase is not released appreciably nor is the release of this cytoplasmic enzyme altered by any of the agents tested.

Effect of Zymosan and Epinephrine on Human Neutrophil Levels of Adenosine 3',5'-Monophosphate.—Basal levels of cyclic AMP in normal human neutrophils range from 8–10 pmol per 10^6 purified neutrophils. Incubation of neutrophils with or without RA serum-treated zymosan particles at 37°C for up to 15 min does not affect the basal levels of cyclic AMP (Table VIII). Epinephrine, in the presence of zymosan particles, inhibits release of β-glucuronidase and, at the same time, elevates neutrophilic levels of cyclic AMP. In the absence of the zymosan, epinephrine does not affect enzyme release and produces only a slight increase in intracellular cyclic AMP levels. Propranolol, an antagonist of β-adrenergic receptors, reduces markedly the actions of epinephrine on both enzyme release and cyclic AMP levels (Table X). Phentolamine, an alpha-receptor antagonist, does not reduce and, in fact, enhances both actions of epinephrine on neutrophils.

Effect of Zymosan and Acetylcholine on Human Neutrophil Levels of Guanosine 3',5'-Monophosphate.—Basal levels of cyclic GMP in normal human neutrophils are in the range of 1–2 pmol per 10^6 purified neutrophils. Incubation of neutrophils with RA serum-treated zymosan particles at 37°C for 2 and 15 min results in elevation of intracellular cyclic GMP levels and extracellular release of β-glucuronidase (Table IX). Acetylcholine stimulates further both

the elevation of cyclic GMP levels (at 2 min of incubation) and the release of β-glucuronidase in the presence of RA serum-treated zymosan particles. In the absence of zymosan acetylcholine elicits little or no effect on cyclic GMP levels and enzyme release. Atropine, a muscarinic receptor antagonist, greatly

TABLE VIII

Effect of Human Neutrophil Interaction with Zymosan and Epinephrine on Intracellular Concentrations of Cyclic AMP*

Experimental condition	Cyclic AMP concentration			β-glucuronidase release		
	Incubation: 0 min	5 min	15 min	Incubation: 0 min	5 min	15 min
	pmol/10^6 cells			*μg/18 h/5 × 10^6 cells*		
5 × 10^6 neutrophils (N)	8.9	7.8	9.4	3.7	4.0	3.8
	±0.5	±0.4	±0.7	±0.1	±0.1	±0.1
N + zymosan-RA serum (Z)	7.8	9.8	8.8	4.3	18.7	51.2
	±0.4	±0.7	±0.3	±0.3	±0.8‡	±4.1‡
N + 10⁻⁶M epinephrine (E)	10.4	20.7	11.4	3.5	4.1	4.6
	±0.6	±1.2‡	±0.7	±0.3	±0.2	±0.2
N + Z + E	9.9	71.3	46.1	3.7	5.3	24.6
	±0.5	±4.2§	±3.0§	±0.2	±0.3§	±2.3§

* Data represent the Mean ± SEM of four to six separate determinations. Incubations were conducted at 37°C as indicated.

‡ Significantly different ($P < 0.01$) from corresponding controls (5 × 10^6 neutrophils).

§ Significantly different ($P < 0.05$) from corresponding controls (N + zymosan-RA serum).

TABLE IX

Effect of Human Neutrophil Interaction with Zymosan and Acetylcholine on Intracellular Concentrations of Cyclic GMP*

Experimental condition	Cyclic GMP concentration			β-glucuronidase release		
	Incubation: 0 min	2 min	10 min	Incubation: 0 min	2 min	10 min
	pmol/10^6 cells			*μg/18 h/5 × 10^6 cells*		
5 × 10^6 neutrophils (N)	1.67	1.08	1.44	3.2	3.9	3.8
	±0.36	±0.11	±0.35	±0.1	±0.2	±0.2
N + zymosan-RA serum	1.48	4.23	11.47	4.4	11.8	43.4
(Z)	±0.28	±1.46‡	±0.57‡	±0.3	±0.7‡	±3.2‡
N + 10⁻⁶M acetylcholine	1.71	1.54	1.55	4.3	7.1	6.8
(A)	±0.44	±0.40	±0.52	±0.2	±0.7	±0.8
N + Z + A	1.18	9.88	11.67	4.4	50.3	94.6
	±0.11	±1.68§	±0.63§	±0.3	±8.8§	±7.9§

* Data represent the Mean ± SEM of four to six separate determinations. Incubations were conducted at 37°C as indicated.

‡ Significantly different ($P < 0.05$) from corresponding controls (5 × 10^6 neutrophils).

§ Significantly different ($P < 0.001$) from corresponding controls (N + zymosan-RA serum).

reduces the effects of acetylcholine on both cyclic GMP levels and enzyme release (Table X). Hexamethonium, a nicotinic receptor antagonist, does not reduce either effect of acetylcholine on neutrophils.

DISCUSSION

The model system employed for study was the interaction of purified human neutrophils with RA serum-treated zymosan particles. Under the defined experimental conditions the cells ingested the particles and extruded β-glucuronidase but not lactate dehydrogenase activity. This selective discharge of granule contents from phagocytic cells without loss of cell viability has been reported by other laboratories. Lysosomal enzyme release during phagocytosis has been termed "regurgitation during feeding" (38), as distinguished from "reverse endocytosis" (39), where phagocytes are in contact with immune reactants that have been rendered nonphagocytizable by immobilization to solid surfaces (2, 3, 5, 40). The data in the present study exemplify the former mechanism of enzyme release. Extrusion of β-glucuronidase from neutrophils paralleled closely the phagocytosis of zymosan particles under various experimental conditions including those in which incubation time, particle/cell ratio and type of treatment of particles were varied.

Zymosan particles treated with seropositive RA serum were more effective then particles treated with seronegative normal serum with regard to the extent of both particle uptake and lysosomal enzyme release. The reason for this difference is presently unknown, but it may derive from the knowledge that fresh seropositive RA serum contains immune complexes (rheumatoid factors, altered IgG, and complexes of these abnormal proteins) and activated comple-

TABLE X

Effect of Specific Receptor Antagonists on Alterations in β-Glucuronidase Release and Cyclic Nucleotide Levels in Human Neutrophils by Autonomic Neurohormones*

Experimental condition‡	Cyclic nucleotide concentration (% of control)§		Release of β-glucuronidase (% of control)§
	Cyclic AMP	Cyclic GMP	
Epinephrine, 10^{-6}M (E)	321 ± 15	—	45 ± 3.1
E + propranolol, 10^{-6}M	138 ± 17‖	—	97 ± 4.4‖
E + phentolamine, 10^{-6}M	546 ± 37‖	—	33 ± 2.1‖
Acetylcholine, 10^{-6}M (A)	—	202 ± 18	251 ± 19
A + atropine, 10^{-6}M	—	89 ± 11¶	109 ± 8¶
A + hexamethonium, 10^{-6}M	—	182 ± 16	242 ± 21

* Data represent the Mean ± SEM of three to five separate determinations.

‡ Incubations with epinephrine and acetylcholine, respectively, were conducted at 37°C for 10 min and 5 min.

§ Control signifies incubation of 5×10^6 neutrophils and the zymosan particles in 1.0 ml of HBSS at 37°C for 5 or 10 min.

‖ Significantly different ($P < 0.01$) from the group with epinephrine alone.

¶ Significantly different ($P < 0.001$) from the group with acetylcholine alone.

ment components (although total complement levels might be lower than normal). Zymosan particles treated with either heated (56°C for 30 min) or cobra venom factor-treated normal serum possessed a much lower capacity to provoke particle uptake and enzyme release. These data suggest that zymosan-mediated particle uptake and enzyme release require the presence of discrete complement components, since heating serum destroys the conventional sequence of complement activation (41) and cobra venom factor inactivates the third component of complement in fresh serum (42, 43), thereby inhibiting the alternate pathway of complement activation (43). Subjection of RA serum to either heat or cobra venom factor before treatment of zymosan did not reduce the actions of RA serum-treated zymosan on neutrophil function. Thus, it appears from these data that complement components, or the activation of complement components, are not required for RA serum-treated zymosan particles to trigger particle ingestion and enzyme discharge. One possible explanation for the differences between normal and RA serum is that adsorption onto zymosan of complex macromolecules such as rheumatoid factors and/or complexes of rheumatoid factors and altered IgG provides the particles with the capacity to trigger phagocytosis and enzyme release. This view is supported by the findings that phagocytosis of IgG aggregates by leukocytes is enhanced by rheumatoid factors (44), and this phagocytosis and concomitant lysosomal enzyme release do not require the presence of complement components (1, 6, 40).

The inhibition of lysosomal enzyme discharge and particle uptake by epinephrine and isoproterenol, both of which are β-adrenergic receptor agonists, but not by phenylephrine, which is a specific α-adrenergic receptor agonist, suggests that interaction with β-adrenergic receptors associated with the surface of the neutrophil accounts for the inhibitory actions of the catecholamines on neutrophil function. This interpretation is supported by the findings that propranolol, a β-receptor antagonist, but not phentolamine, an α-receptor antagonist, blocked the inhibitory action of epinephrine on phagocytosis and enzyme release. Enhancement of the inhibitory actions of epinephrine by theophylline suggested that cyclic AMP in neutrophils might be associated with these actions of the catecholamine. Indeed, cyclic AMP levels in neutrophils were elevated markedly by epinephrine during phagocytosis. The close temporal relationship between inhibition of β-glucuronidase discharge and elevation of cyclic AMP levels by epinephrine during contact of human neutrophils with phagocytizable particles further suggests that the inhibitory action of the catecholamine is mediated by intracellular cyclic AMP. Additional evidence in support of this hypothesis derives from the findings that β-adrenergic receptor blockade reduced markedly the capacity of epinephrine to elevate cyclic AMP levels in neutrophils. Finally, the addition of either cyclic AMP or analogs of this nucleotide to incubation mixtures of neutrophils and zymosan particles resulted in inhibition of β-glucuronidase release and particle uptake.

Cyclic AMP levels associated with the neutrophils were not altered signifi-
cantly during particle uptake and release of granule enzymes in the absence of
catecholamine. These data are in accordance with those of Manganiello et al.
(45) and Seyberth et al. (46) which indicate that cyclic AMP levels in phago-
cytic cells do not increase during particle ingestion.

Our findings that cyclic AMP and related analogs reduced particle uptake by
neutrophils are in agreement with those of other investigators (47–49). These
data could imply that inhibition of lysosomal enzyme discharge from phago-
cytes by cyclic AMP is attributed simply to inhibition of phagocytosis and,
therefore, to the reduction of enzyme discharge from phagocytic vacuoles par-
tially opened to the exterior of the cell. However, such a clear-cut mechanism
may not be the only operational one as ample evidence exists that lysosomal
enzyme release from leukocytes is inhibited by cyclic AMP, catecholamines
and related agents in the absence of particle uptake (40). Further, dibutyryl
cyclic AMP and prostaglandin E_1 reduced granule enzyme release from cyto-
chalasin B-treated human leukocytes, under which condition particle ingestion
is completely inhibited and, therefore, independent of enzyme release (50). Simi-
larly, in the present study epinephrine and dibutyryl cyclic AMP inhibited
the selective discharge of β-glucuronidase that was provoked by particle-free,
zymosan-treated serum. These data indicate that several independent but
perhaps complementary neutrophil functions are sensitive to the inhibitory
actions of cyclic AMP.

In contrast to the inhibitory action of catecholamines and cyclic AMP,
cholinergic agents and cyclic GMP accelerated the release of β-glucuronidase
from phagocytizing human neutrophils. Cyclic GMP may mediate the effect
of acetylcholine since neutrophil levels of cyclic GMP were elevated markedly
by acetylcholine at times when release of β-glucuronidase was also enhanced.
The relationship between enhancement of β-glucuronidase release and eleva-
tion of cyclic GMP levels in human neutrophils by acetylcholine is made even
more evident by the finding that atropine, but not hexamethonium, greatly
reduced both actions of acetylcholine. This suggests that specific muscarinic
receptors are associated with the neutrophil surface and are responsible for the
cholinergic stimulation of enzyme release. Neither cholinergic agents nor cyclic
GMP analogs altered phagocytosis at times when granule enzyme discharge
was enhanced. Moreover, these agents enhanced enzyme release from neutro-
phils in the presence of either particle-free, zymosan-treated serum or non-
phagocytizable particles (40). Thus, both control as well as cyclic GMP-
stimulated lysosomal enzyme release can proceed independently of particle
ingestion.

Release of β-glucuronidase from human neutrophils during cell contact with
immune reactant is accompanied by a concomitant elevation of cyclic GMP
levels. Elevation of cyclic GMP levels is associated with enhanced lysosomal
enzyme release whether the neutrophil is "triggered" by immune reactant or

immunologic release of enzyme is enhanced by acetylcholine. These data suggest that intracellular cyclic GMP may serve as a second messenger in mediating the extracellular release of neutrophilic granule contents. The concept of cyclic GMP acting as a second messenger is not a new one. Cardiac cyclic GMP has been implicated in mediating the cardiac actions of acetylcholine and perhaps certain other cholinergic agents (34, 51).

At this time the precise intracellular mechanism by which endogenous nucleotides modulate the immunologic discharge of lysosomal enzymes from neutrophils is unknown. Cyclic AMP and cyclic GMP have been shown to influence markedly the permeability of lysosomes to enzyme proteins (21–24). In view of the knowledge that cyclic AMP and cyclic GMP bind to and activate nucleotide-dependent protein kinases and, thereby, stimulate certain phosphorylation reactions, nucleotide-mediated phosphorylation of one or more subcellular components could play a role in regulating lysosomal enzyme discharge. For example, intracellular phosphorylation reactions could alter the physical and/or functional properties of lysosomes and interfere with specific functions of these organelles such as the translocation and subsequent fusion of granules with heterophagic vacuoles or the plasma membrane. In addition, or alternatively, nucleotides may alter the function of microtubule structures and modify the translocation of lysosome granules (1, 29, 50). Regardless of the mechanism, alterations in the immunologic discharge of lysosome granule contents from human neutrophils by cyclic nucleotides and autonomic neurohormones suggest that these endogenous chemical agents play a role in regulating the inflammatory process.

SUMMARY

The purpose of this investigation was to examine the effects of autonomic neurohormones, cyclic nucleotides, and related agents on the immunologic discharge of lysosomal enzymes from, and phagocytosis by, purified human neutrophils. In order to discern the possible intracellular mechanisms by which certain neurohormones influence neutrophil function, the concentrations of cyclic AMP and cyclic GMP in neutrophils were assessed during cell contact with phagocytizable particles and autonomic agents. The model system employed for study was the interaction of purified human neutrophils with rheumatoid arthritic (RA) serum-treated zymosan particles at 37°C in a neutral, balanced salt solution containing glucose. Neutrophils ingested the particles and discharged β-glucuronidase but not lactate dehydrogenase activity during 30 min of incubation. Treatment of zymosan particles with RA serum was more effective than treatment with normal serum with regard to the extent of both particle uptake and lysosomal enzyme release. During contact of neutrophils with RA serum-treated zymosan particles epinephrine, isoproterenol, and cyclic AMP inhibited both particle ingestion and β-glucuronidase discharge. These actions of epinephrine were associated with a concomitant elevation of cyclic AMP levels. In contrast to the actions of catecholamines and cyclic

AMP, acetylcholine and cyclic GMP accelerated lysosomal enzyme release without affecting particle uptake. The actions of acetylcholine were associated with a concomitant elevation of cyclic GMP levels. Increases in neutrophil levels of cyclic GMP but not of cyclic AMP were associated also with the discharge of β-glucuronidase provoked by particles in the absence of added cholinergic agents. The data suggest that the immunologic release of lysosomal enzymes from human neutrophils can be regulated by autonomic neurohormones, perhaps via the selective formation of appropriate nucleotides.

REFERENCES

1. Weissmann, G., R. B. Zurier, P. J. Spieler, and I. M. Goldstein. 1971. Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. *J. Exp. Med.* **134** (Suppl.):149.
2. Henson, P. M. 1971. The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *J. Immunol.* **107**:1535.
3. Hawkins, D. 1972. Neutrophilic leukocytes in immunologic reactions: evidence for the selective release of lysosomal constituents. *J. Immunol.* **108**:310.
4. Wright, D. G., and S. E. Malawista. 1972. The mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis. *J. Cell Biol.* **53**:788.
5. Oronsky, A. L., L. J. Ignarro, and R. J. Perper. 1973. Release of cartilage mucopolysaccharide-degrading neutral protease from human leukocytes. *J. Exp. Med.* **138**:461.
6. Ignarro, L. J. 1973. Neutral protease release from human leukocytes regulated by neurohormones and cyclic nucleotides. *Nat. New Biol.* **245**:151.
7. Cochrane, C. G., and B. S. Aikin. 1966. Polymorphonuclear leukocytes in immunologic reactions. The destruction of vascular basement membrane in vivo and in vitro. *J. Exp. Med.* **124**:733.
8. Hawkins, D., and C. G. Cochrane. 1968. Glomerular basement membrane damage in immunological glomerulonephritis. *Immunology.* **14**:665.
9. Weissmann, G. 1967. The role of lysosomes in inflammation and disease. *Ann. Rev. Med.* **18**:97.
10. Ignarro, L. J., and J. Slywka. 1972. Changes in liver lysosome fragility, erythrocyte membrane stability, and local systemic lysosomal enzyme levels in adjuvant-induced polyarthritis. *Biochem. Pharmacol.* **21**:875.
11. Ziff, M., H. J. Gribetz, and J. LoSpalluto. 1960. Effect of leukocyte and synovial membrane extracts on cartilage mucoprotein. *J. Clin. Invest.* **39**:405.
12. Ali, S. Y. 1964. The degradation of cartilage matrix by an intracellular protease. *Biochem. J.* **93**:611.
13. Ignarro, L. J., A. L. Oronsky, and R. J. Perper. 1973. Breakdown of noncollagenous chondromucoprotein matrix by leukocyte lysosome granule lysates from guinea pig, rabbit and human. *Clin. Immunol. Immunopath.* **2**:36.
14. Brown, J. H., H. K. Mackey, D. A. Riggilo, and N. L. Schwartz. 1968. Studies

on the acute inflammatory response. II. Influence of antihistaminics and cate-cholamines on formaldehyde-induced edema. *J. Pharmacol. Exp. Ther.* **160**:243.

15. Tolone, G., Bonasera, and R. Bruno. 1971. The effects of adrenergic blocking agents on passive cutaneous anaphylaxis reactions in the rat. *Pathol. Microbiol.* **37**:194.

16. Henson, E. C., and J. G. Brunson. 1969. Prevention of experimental allergic encephalomyelitis by combination of epinephrine and a phenothiazine derivative, propiomazine. *Proc. Soc. Exp. Biol. Med.* **131**:752.

17. Henson, E. C., and J. G. Brunson. 1970. Studies of adjuvant-induced arthritis in the albino rat (CFN strain). *Ann. Rheum. Dis.* **29**:185.

18. Spector, W. G., and D. A. Willoughby. 1964. Vasoactive amines to acute inflammation. *Ann. N.Y. Acad. Sci.* **116**:839.

19. Lichtenstein, L. M., and S. Margolis. 1968. Histamine release in vitro: inhibition by catecholamines and methylxanthines. *Science (Wash. D. C.).* **161**:902.

20. Orange, R. P., W. G. Austen, and K. F. Austen. 1971. Immunological release of histamine and slow reacting substance of anaphylaxis from human lung. I. Modulation by agents influencing cellular levels of cyclic 3',5'- adenosine monophosphate. *J. Exp. Med.* **134** (Suppl.):136.

21. Ignarro, L. J., J. Slywka, and N. Krassikoff. 1971. Inhibition of lysosomal enzyme release by catecholamines: possible mediation by cyclic 3',5'-adenosine monophosphate, *Life Sci.,* Part I. **10**:1309.

22. Ignarro, L. J., N. Krassikoff, and J. Slywka. 1972. Inhibition of lysosomal enzyme release by adenosine 5'-triphosphate and $N^6,O^{2'}$-dibutyryl cyclic 3',5'-adenosine monophosphate. *Life Sci.,* Part I. **11**:317.

23. Ignarro, L. J., N. Krassikoff, and J. Slywka. 1973. Release of enzymes from a rat liver lysosome fraction: inhibition by catecholamines and cyclic 3',5'-adenosine monophosphate, stimulation by cholinergic agents and cyclic 3',5'-guanosine monophosphate. *J. Pharmacol. Exp. Ther.* **186**:86.

24. Ignarro, L. J., and C. Colombo. 1973. Enzyme release from polymorphonuclear leukocyte lysosomes: regulation by autonomic drugs and cyclic nucleotides. *Science (Wash. D. C.).* **180**:1181.

25. Ignarro, L. J. 1973. Phagocytic release of neutral protease from human leukocytes: acceleration by acetylcholine and cyclic 3',5'-guanosine monophosphate (cGMP), inhibition by norepinephrine and cyclic 3',5'-adenosine monophosphate (cAMP). *Pharmacologist.* **15**:156.

26. George, W. J., R. J. Paddock, and L. J. Ignarro. 1973. Cyclic 3',5'-guanosine monophosphate (cGMP) and cyclic 3',5'-adenosine monophosphate (cAMP) levels in human neutrophils during lysosomal enzyme release: elevation of cGMP by acetylcholine and of cAMP by epinephrine. *Pharmacologist.* **15**:157.

27. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood.* **23**:811.

28. Vallota, E. H., and H. J. Müller-Eberhard. 1973. Formation of C3a and C5a anaphylatoxins in whole human serum after inhibition of the anaphylatoxin inactivator. *J. Exp. Med.* **137**:1109.

29. 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.

30. Gianetto, R., and C. DeDuve. 1955. Tissue fractionation studies: IV. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat liver particles. *Biochem. J.* **59:**433.

31. Bergmeyer, H. U., E. Bernt, and B. Hess. 1965. Lactic dehydrogenase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York.

32. Gilman, A. G. 1970. A protein binding assay for adenosine 3′,5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.* **67:**305.

33. Steiner, A. L., C. W. Parker, and D. M. Kipnis. 1972. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* **247:**1106.

34. George, W. J., R. D. Wilkerson, and P. J. Kadowitz. 1973. Influence of acetylcholine on contractile force and cyclic nucleotide levels in the isolated perfused rat heart. *J. Pharmacol. Exp. Ther.* **184:**228.

35. Crowder, J. G., R. R. Martin, and A. White. 1969. Release of histamine and lysomal enzymes by human leukocytes during phagocytosis of staphylococci. *J. Lab. Clin. Med.* **74:**436.

36. Tew, J. G., W. M. Hess, and D. M. Donaldson. 1969. Lysozyme and β-lysin release stimulated by antigen antibody complexes and bacteria. *J. Immunol.* **102:**743.

37. 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.

38. Weissmann, G., P. Dukor, and R. B. Zurier. 1971. Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat. New Biol.* **231:**131.

39. Weissmann, G., R. B. Zurier, and S. Hoffstein. 1972. Leukocytic proteases and the immunologic release of lysosomal enzymes. *Amer. J. Path.* **68:**539.

40. Ignarro, L. J. 1974. Nonphagocytic release of neutral protease and β-glucuronidase from human neutrophils: Regulation by autonomic neurohormones and cyclic nucleotides. *Arthritis Rheum.* **17:**25.

41. Müller-Eberhard, H. J. 1969. Complement. *Ann. Rev. Biochem.* **38:**389.

42. Maillard, J. L., and R. M. Zarco. 1968. Decomplementation par un facteur extrait du venim de cobra. *Ann. Inst. Pasteur (Paris).* **114:**756.

43. Gotze, O., and H. J. Müller-Eberhard. 1971. The C3 activator system: an alternate pathway of complement activation. *J. Exp. Med.* **134:**90.

44. Hurd, E. R., J. LoSpalluto, and M. Ziff. 1970. Formation of leukocyte inclusions in normal polymorphonuclear cells incubated with synovial fluid. *Arthritis Rheum.* **13:**724.

45. Manganiello, V., W. H. Evans, T. P. Stossel, R. J. Mason, and M. Vaughan. 1971. The effect of polystyrene beads on cyclic 3′,5′-adenosine monophosphate concentration in leukocytes. *J. Clin. Invest.* **50:**2741.

46. Seyberth, H. W., H. Schmidt-Gayk, K. H. Jakobs, and E. Hackenthal. 1973. Cyclic adenosine monophosphate in phagocytizing granulocytes and alveolar macrophages. *J. Cell Biol.* **57:**567.

47. Bourne, H. R., R. I. Lehrer, M. J. Cline, and K. L. Melmon. 1971. Cyclic 3′,5′-

adenosine monophosphate in the human leukocyte: synthesis, degradation, and effects on neutrophil candidacidal activity. *J. Clin. Invest.* **50:**920.

48. Stossel, T. P., R. J. Mason, J. Hartwig, and M. Vaughan. 1972. Quantitative studies of phagocytosis by polymorphonuclear leukocytes: use of emulsions to measure the initial rate of phagocytosis. *J. Clin. Invest.* **51:**615.

49. Cox, J. P., and M. L. Karnovsky. 1973. The depression of phagocytosis by exogenous cyclic nucleotides, prostaglandins, and theophylline. *J. Cell Biol.* **59:**480.

50. 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.

51. George, W. J., J. B. Polson, A. G. O'Toole, and N. D. Goldberg. 1970. Elevation of guanosine 3',5'-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proc. Natl. Acad. Sci. U.S.A.* **66:**398.