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PHAGOCYTIC RELEASE OF LYSOSOMAL ENZYMES FROM GUINEA PIG NEUTROPHILS—REGULATION BY CORTICOSTEROIDS, AUTONOMIC NEUROHORMONES AND CYCLIC NUCLEOTIDES*

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Abstract—The effects of various pharmacologic agents on the capacity of guinea pig neutrophils to phagocytize serum-treated zymosan particles and release lysosomal enzymes were determined. Neutrophils (10^7) and zymosan were incubated in Krebs–Ringer phosphate (KRP) medium containing 7.5 mM glucose, pH 7.4, at 37° in the absence and presence of corticosteroids, cyclic nucleotides, and adrenomimetic and cholinomimetic agents. Methylprednisolone hemisuccinate, triamcinolone acetonide, dexamethasone acetate, paramethasone acetate and hydrocortisone hemisuccinate reduced particle uptake by and discharge of lysosomal enzymes from guinea pig neutrophils. Aldosterone hemisuccinate and deoxycorticosterone acetate were inactive. Adrenomimetic (e.g. epinephrine) agents inhibited particle uptake by and lysosomal enzyme secretion from neutrophils, and cholinomimetic (e.g. acetylcholine) agents accelerated lysosomal enzyme release but had no effect on phagocytosis. Cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and one of its analogs inhibited particle ingestion by and lysosomal enzyme release from neutrophils; and this inhibition was potentiated by theophylline. Cyclic 3′,5′-guanosine monophosphate (cyclic GMP), in contrast to the actions of corticosteroids, adrenomimetic agents and cyclic AMP, accelerated lysosomal enzyme secretion but had no effect on particle uptake. Cyclic GMP did not affect release of cytoplasmic lactate dehydrogenase, thus indicating maintenance of cell viability during release of lysosomal enzymes. Cytochalasin B, an agent which blocked phagocytic uptake of zymosan, did not interfere with inhibition of lysosomal enzyme secretion by corticosteroids, adrenomimetic agents and cyclic AMP or acceleration of this event by cholinomimetic agents or cyclic GMP. These studies indicate that guinea pig neutrophils are capable of releasing lysosomal enzymes during phagocytosis of zymosan and that certain agents can modulate lysosomal enzyme secretion and/or phagocytosis.

The pathogenesis of many inflammatory reactions is characterized by the accumulation of polymorphonuclear (PMN) leukocytes and the phagocytosis of immune complexes by these cells [1–5]. Certain phagocytic cells such as the PMN have the capacity to selectively discharge lysosome granule-associated enzymes during phagocytosis of particulate material [6–11]. Quite often, tissue injury occurs in regions where phagocytic leukocytes are secreting lysosomal hydrolases [12, 13]. The role of lysosomal enzymes as mediators of acute [14–16] and chronic [17, 18] inflammation is well documented. Thomas [19] observed that local injection of granules obtained from rabbit PMN produced a Shwartzman-like reaction and enhanced the Arthus reaction.

Corticosteroids have been employed for many years in the treatment of various inflammatory conditions. However, aside from their metabolic activities, there are many possible mechanisms by which these agents might exert their anti-inflammatory effects. Drugs such as hydrocortisone have been demonstrated to inhibit the release of hydrolases from isolated lysosomes by stabilizing lysosomal membranes [20–23]. Quite recently Smith *et al.* [24] demonstrated the capacity of such anti-inflammatory corticosteroids as dexamethasone and paramethasone to inhibit directly the activity of the lysosome granule-associated enzyme, aryl sulfatase.

Several models of inflammation have been employed to demonstrate the anti-inflammatory activity of certain adrenomimetic agents [25–28]. In addition, certain autonomic neurohormones have been reported to inhibit the immunological discharge of mediators of inflammation from various cells [29, 30]. The actions of these neurohormones are thought to be mediated by cyclic AMP. Furthermore, agents such as theophylline, cyclic AMP and epinephrine have been reported to block the release of enzymes from isolated lysosomes [31, 32].

The objective of this study was to evaluate the effects of anti-inflammatory corticosteroids as well as adrenomimetic and cholinomimetic agents on phagocytosis by and lysosomal enzyme secretion from guinea pig neutrophils. In addition, cyclic AMP and cyclic GMP, which are thought to mediate the cellular responses to adrenomimetic and cholinomimetic agents [29, 30, 33–37], respectively, were investigated for their effects on phagocytosis by and release of lysosomal enzymes from neutrophils.

METHODS

Isolation of guinea pig neutrophils. Male Hartley albino guinea pigs (400–650 g) received one intraperi-

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toneal injection (20 ml) of 12% (w/v) sodium caseinate in 0.9% NaCl. Eighteen hr later neutrophils were harvested according to the method of Michell *et al.* [38]. This procedure consistently yielded cell suspensions containing a minimum of 95% neutrophils. Viability of the neutrophils was always greater than 98 per cent as determined by trypan blue exclusion.

Preparation of zymosan particles. Zymosan particles (Sigma Chemical Co.) measuring 0.5 to 3 μm in diameter were opsonized with normal guinea pig serum according to the procedure of Ignarro *et al.* [39] Each ml of final serum-treated zymosan suspension contained approximately 4×10^8 particles.

Incubation conditions. Neutrophils (10^7) in 1.0 ml of Krebs–Ringer phosphate (KRP) medium containing 7.5 mM glucose were incubated at 37° in a Dubnoff metabolic shaker set at 120 excursions/min. Untreated or serum-treated zymosan particle suspensions (0.1 ml) were added to the neutrophil suspensions. In those experiments involving drugs, cells and drug(s) were preincubated for 10 or 30 min (as indicated in the table legends) at 37° followed by the addition of zymosan particles and then further incubated at 37° for 60 min. After incubation the samples were centrifuged at 150 *g* for 10 min and the supernatants were assayed for β-glucuronidase and lactate dehydrogenase activities.

Enzyme assays. β-Glucuronidase activity was determined by a modification of the procedure of Gianetto and DeDuve [40]. Aliquots (0.5 ml) of supernatants, obtained as described above, were incubated in 2.5 ml of 0.1 M sodium citrate, pH 4.8, containing 5.0 mg of phenolphthalein glucuronic acid as substrate, at 37° for 18 hr. Enzyme reactions were terminated by adding 0.4 ml alkaline glycine and absorbancy (540 *E*) was determined. Data are expressed as μg phenolphthalein liberated/18 hr of incubation/10^7 cells.

Lactate dehydrogenase activity was measured according to the method of Bergmeyer *et al.* [41]. Data were calculated as absorbancy units/min/10^7 cells.

Measurement of phagocytosis. Uptake of zymosan particles by neutrophils was quantitated by enumeration of ingested zymosan particles by oil immersion light microscopy. The data are expressed as the average number of zymosan particles ingested/100 neutrophils.

Drug solutions and sources. Solutions of the catecholamines contained 0.01% (w/v) 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 20 min. The following agents were purchased from Sigma Chemical Co: acetylcholine chloride, acetyl-β-methylcholine chloride, carbamycholine chloride, l-epinephrine bitartrate, adenosine-5′-monophosphoric acid, adenosine-3′:5′-cyclic monophosphoric acid, N^6,$O^{2'}$-dibutyryl adenosine-3′:5′-cyclic monophosphoric acid, guanosine-5′-monophosphoric acid, guanosine-3′:5′-cyclic monophosphoric acid, N^2,$O^{2'}$-dibutyryl guanosine-3′:5′-cyclic monophosphoric acid, sodium metabisulfite, *d*-aldosterone-21-hemisuccinate, deoxycorticosterone acetate, and atropine sulfate. The l-isomer of isoproterenol was obtained from Pfaltz & Bauer, Inc.; theophylline (Schwarz/Mann); propranolol hydrochloride (Ayerst Laboratories, Inc.); methylprednisolone hemisuccinate and hydrocortisone hemisuccinate were obtained from The UpJohn Co.; paramethasone acetate was obtained from Syntex Laboratories, Inc.; triamcinolone acetonide (E. R. Squibb & Sons, Inc.); and dexamethasone acetate (Schering Corp.). Cytochalasin B was the generous gift of Dr. Philip Davies.

All agents tested were dissolved in aqueous buffer

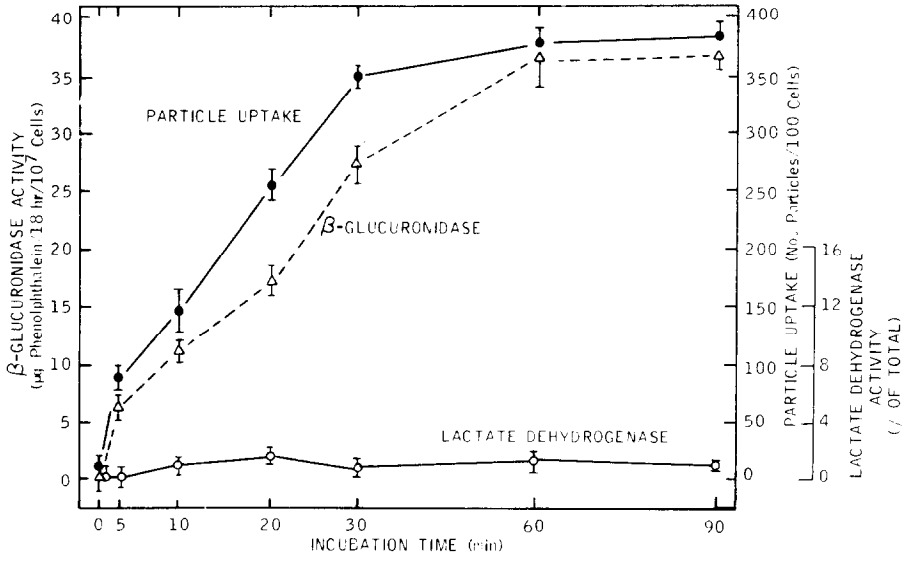

Fig. 1. Secretion of β-glucuronidase and lactate dehydrogenase from and particle uptake by guinea pig neutrophils in the presence of zymosan. Neutrophils (10^7) were incubated in 1.0 ml KRP containing 4×10^8 zymosan particles treated with normal serum. Values for total cell enzyme activities, determined after incubating cells in 0.2% Triton X-100–0.04 M Tris acetate, pH 7.4, for 15 min, were: 161.3 ± 12.7 μg phenolphthalein/18 hr/10^7 cells for β-glucuronidase and 195.3 ± 13.7 absorbancy units/min/10^7 cells for lactate dehydrogenase. Data represent the mean ± S.E.M. of five separate experiments.

except the corticosteroids and cytochalasin B, which were dissolved in 0.1% dimethylsulfoxide (DMSO). All the drugs were soluble under the defined incubation conditions and they produced no appreciable alteration of the pH of the incubation media. The small amounts of DMSO employed as a vehicle did not alter cell viability or enzyme release.

RESULTS

Release of enzymes from and particle uptake by guinea pig neutrophils in the presence of zymosan. In the presence of serum-treated zymosan, neutrophils demonstrated particle uptake after 5 min of incubation, and phagocytosis increased linearly through the next 25 min, after which time particle ingestion began to reach a maximum (Fig. 1). The release of β-glucuronidase from neutrophils started almost immediately and increased significantly during the following 60 min of incubation. Enzyme release reached a maximum by 60–90 min. Thus a strong temporal relationship exists between particle uptake by and β-glucuronidase release from neutrophils. Therefore, release of β-glucuronidase occurs during, rather than subsequent to, phagocytosis. There was no significant release of cytoplasmic lactate dehydrogenase during 90 min of incubation, which is indicative of selective lysosomal enzyme release during phagocytosis. Serum which was not treated with zymosan did not stimulate β-glucuronidase secretion from neutrophils in the absence of zymosan particles.

Effect of methylprednisolone hemisuccinate on the time course of β-glucuronidase secretion from and phagocytosis by guinea pig neutrophils. The data in Table 1 indicate that methylprednisolone hemisuccinate inhibited significantly the secretion of β-glucuronidase from and phagocytosis by guinea pig neutrophils when the cells were in contact with the serum-treated zymosan particles for 30 min. The inhibitory influence of methylprednisolone increased through 30 and 45 min of incubation and appeared to reach a maximum between 45 and 60 min. Methylprednisolone was inactive at the 90 min-time interval.

Effect of corticosteroids on particle uptake by and β-glucuronidase secretion from guinea pig neutrophils. Methylprednisolone hemisuccinate, triamcinolone acetonide, dexamethasone acetate, paramethasone acetate and hydrocortisone hemisuccinate inhibited the release of β-glucuronidase from and particle uptake by neutrophils in the presence of zymosan particles treated with normal serum (Table 2). The relative effects of the corticosteroids indicate that methylprednisolone > triamcinolone acetonide > dexamethasone > paramethasone > hydrocortisone. Aldosterone hemisuccinate and deoxycorticosterone acetate were inactive.

Effect of adrenomimetic agents and cyclic AMP on particle uptake by and β-glucuronidase secretion from guinea pig neutrophils. Epinephrine and isoproterenol inhibited the release of β-glucuronidase from and particle uptake by guinea pig neutrophils in the presence of zymosan particles treated with normal serum (Table 3). Propranolol, a β-adrenergic receptor antagonist, while having no effect by itself, blocked the inhibitory action of epinephrine and isoproterenol on particle ingestion and enzyme secretion. Theophylline, a potent inhibitor of phosphodiesterase which degrades cyclic AMP, while having no effect by itself, did potentiate the effects of epinephrine and isoproterenol on enzyme release and phagocytosis. It is important to note that catecholamines were most effective when preincubated with neutrophils for 10 min prior to exposure to zymosan.

Cyclic AMP, at high concentrations, inhibited β-glucuronidase secretion and particle uptake (Table 3). Theophylline, while having no effect itself, did potentiate the effects of cyclic AMP on enzyme release and particle uptake. The dibutyryl analog of cyclic AMP was more active than cyclic AMP whereas adenosine-5′-monophosphate was inactive.

Effect of cholinomimetic agents and cyclic GMP on secretion of β-glucuronidase from and particle uptake by guinea pig neutrophils. Acetylcholine, acetyl-β-methylcholine and carbamylcholine stimulated β-glucuronidase secretion from but not particle ingestion by guinea pig neutrophils in the presence of zymosan

Table 1. Effect of methylprednisolone hemisuccinate (MPH) on the kinetics of phagocytosis by and β-glucuronidase secretion from guinea pig neutrophils

Incubation time* (min)	Per cent of total β-glucuronidase activity released†		Particle uptake (No. Particles/100 cells)	
	without MPH	with MPH	without MPH	with MPH
0	2.1 ± 0.1‡	2.6 ± 0.3	8 ± 0.7	13 ± 1.4
5	4.3 ± 0.3	3.9 ± 0.2	43 ± 3.8	37 ± 3.5
15	12.4 ± 0.7	10.7 ± 1.3	124 ± 10.2	110 ± 12.6
30	19.5 ± 0.2	13.7 ± 0.8§	263 ± 19.8	185 ± 14.5§
45	24.6 ± 0.1	13.4 ± 0.6‖	337 ± 27.4	203 ± 16.3‖
60	27.8 ± 0.2	9.3 ± 0.3‖	384 ± 36.5	169 ± 11.9‖
90	21.3 ± 0.3	17.5 ± 0.9	375 ± 29.3	346 ± 27.8

* Neutrophils (10^7) were preincubated with methylprednisolone hemisuccinate (10^{-5} M) in 1.0 ml of Krebs–Ringer phosphate medium, pH 7.4, for 30 min followed by incubation with serum-treated zymosan (4×10^8 particles) for the time periods indicated.

† Total cell β-glucuronidase activity was 142 ± 9.6 μg phenolphthalein/18 hr/10^7 cells.

‡ Data represent the mean ± S. E. M. of three separate experiments.

§ Significant at $P < 0.05$ vs control.

‖ Significant at $P < 0.01$ vs control.

Table 2. Effect of corticosteroids on secretion of β-glucuronidase from and particle uptake by guinea pig neutrophils*

Steroid	Per cent inhibition of β-glucuronidase secretion 10^{-5} M	10^{-6} M	10^{-7} M	Particle uptake in 10^{-5} M agent (No. particles/100 cells)
Control (no steroid)				346 ± 39.6
Methylprednisolone hemisuccinate	62 ± 5.2†	35 ± 4.2†	18 ± 2.1	146 ± 18.4†
Triamcinolone acetonide	55 ± 4.3†	30 ± 1.1‡	17 ± 0.3	173 ± 12.5†
Dexamethasone acetate	42 ± 6.4†	25 ± 2.2‡	11 ± 0.2	215 ± 30.8‡
Paramethasone acetate	49 ± 3.1†	30 ± 3.5‡	9 ± 0.3	197 ± 26.6†
Hydrocortisone hemisuccinate	33 ± 3.4‡	18 ± 2.3	6 ± 1.3	256 ± 20.3‡
Deoxycorticosterone acetate	4 ± 0.1	2 ± 0.05	0	338 ± 22.4
Aldosterone hemisuccinate	3 ± 0.07	0	0	329 ± 19.7

* Incubations of neutrophils (10^7) were conducted at 37° for 30 min with the respective steroid and for an additional 60 min in the presence of zymosan treated with normal serum. Control incubations yielded a value of 33.4 ± 3.1 μg phenolphthalein/18 hr/10^7 cells (21.6 per cent of total cell activity) for release of β-glucuronidase. Data represent the mean ± S. E. M. of five separate experiments.

† Significant at $P < 0.01$ vs control.

‡ Significant at $P < 0.05$ vs control.

particles treated with normal serum (Table 4). The action of acetylcholine, acetyl-β-methylcholine and carbamylcholine was blocked by atropine, an antagonist of muscarinic receptors. Atropine alone was inactive. As was the case with the catecholamines, maximum effects with the cholinomimetic agents were observed after a 10-min preincubation period.

Cyclic GMP and its dibutyryl analog accelerated the release of β-glucuronidase from, but not particle uptake by, neutrophils. Guanosine-5′-monophosphate was inactive (Table 4).

Effect of cholinomimetic agents and cyclic GMP on viability of guinea pig neutrophils during particle uptake. Acetylcholine, acetyl-β-methylcholine, carbamylcholine, cyclic GMP, and to a greater extent its dibutyryl analog, stimulated enzyme release from neutrophils that were actively phagocytizing zymosan particles. However, cell viability was maintained throughout the incubation period, as indicated by the failure of these agents to accelerate release of cytoplasmic lactate dehydrogenase from these cells and the continual exclusion of trypan blue by these neutrophils.

Table 3. Effect of adrenomimetic agents and cyclic AMP on secretion of β-glucuronidase from and particle uptake by guinea pig neutrophils

Agent(s) tested	Per cent inhibition of β-glucuronidase secretion 10^{-4} M	10^{-5} M	10^{-6} M	Particle uptake in 10^{-4} M agent (No. particles/100 cells)
Control (no agent)	0			354 ± 31.5
Epinephrine*	44 ± 3.1†	31 ± 2.4†	22 ± 1.6‡	205 ± 22.3†
Isoproterenol	47 ± 2.8†	28 ± 1.9†	20 ± 1.3‡	191 ± 17.1†
Epinephrine + propranolol (10^{-4} M)	9 ± 0.4	3 ± 0.2	0	339 ± 23.6
Isoproterenol + propranolol (10^{-4} M)	7 ± 0.3	0	0	319 ± 28.4
Propranolol (10^{-4} M)	0	0	0	347 ± 33.3
Epinephrine + theophylline (10^{-4} M)	62 ± 4.7†	45 ± 3.3†	34 ± 2.2†	121 ± 21.7†
Isoproterenol + theophylline (10^{-4} M)	67 ± 5.2†	39 ± 2.5†	29 ± 1.5†	118 ± 22.8†
Theophylline (10^{-4} M)	4.5 ± 0.42	2.4 ± 0.29	0	358 ± 32.7
Control (no agent)				384 ± 34.3
Cyclic AMP§	18 ± 1.2‡	11 ± 0.7	8 ± 1.1	285 ± 24.4‡
Theophylline	7 ± 0.4	4 ± 0.2	2 ± 0.07	346 ± 36.2
Cyclic AMP + theophylline (10^{-4} M)	41 ± 3.2†	28 ± 2.1‡	17 ± 0.9‡	224 ± 19.5†
Dibutyryl cyclic AMP	56 ± 4.6†	32 ± 1.8†	19 ± 1.7‡	207 ± 21.3†
AMP	0	0	0	380 ± 33.8

* Incubations of neutrophils (10^7) were conducted at 37° for 10 min with the respective agent(s) and for an additional 60 min in the presence of zymosan treated with normal serum. Control incubations yielded a value of 32.2 ± 2.3 μg phenolphthalein/18 hr/10^7 cells (21.4 per cent of total cell activity) for release of β-glucuronidase. Data represent the mean ± S. E. M. of three to four separate experiments.

† Significant at $P < 0.01$ vs control.

‡ Significant at $P < 0.05$ vs control.

§ Incubations of neutrophils (10^7) were conducted at 37° for 30 min with the respective agent(s) and for an additional 60 min in the presence of zymosan treated with normal serum. Control incubations yielded a value of 35.6 ± 2.7 μg phenolphthalein/18 hr/10^7 cells (22.3 per cent of total cell activity) for release of β-glucuronidase. Data represent the mean ± S. E. M. of three to four separate experiments.

Table 4. Effect of cholinomimetic agents and cyclic GMP on secretion of β-glucuronidase from and particle uptake by guinea pig neutrophils

Agent(s) tested	Per cent increase of β-glucuronidase secretion			Particle uptake in 10^{-4} M agent (No. particles/ 100 cells)
	10^{-4} M	10^{-5} M	10^{-6} M	
Control (no agent)				343 ± 27.4
Acetylcholine*	56 ± 4.1†	40 ± 2.6†	23 ± 1.4‡	351 ± 32.3
Acetyl-β-methylcholine	67 ± 5.5†	49 ± 4.3†	29 ± 0.8†	348 ± 30.7
Carbamylcholine	43 ± 2.2†	30 ± 3.1†	18 ± 1.3‡	357 ± 26.1
Acetylcholine + atropine (10^{-4} M)	21 ± 0.7	11 ± 1.7	2 ± 0.6	342 ± 34.5
Acetyl-β-methylcholine + atropine (10^{-4} M)	13 ± 1.2	9 ± 0.3	3 ± 1.1	338 ± 37.6
Carbamylcholine + atropine (10^{-4} M)	10 ± 0.5	18 ± 0.2	7 ± 0.4	345 ± 24.4
Atropine (10^{-4} M)	0	0	0	332 ± 35.3
Control (no agent)				357 ± 63.6
Cyclic GMP§	67 ± 5.6†	48 ± 3.7†	26 ± 1.7‡	352 ± 30.5
Dibutyryl cyclic GMP	88 ± 7.4†	62 ± 6.5†	38 ± 2.6†	368 ± 43.4
GMP	0	0	0	362 ± 37.3

* Incubations of neutrophils (10^7) were conducted at 37° for 10 min with the respective agent(s) and for an additional 60 min in the presence of zymosan treated with normal serum. Control incubations yielded a value of 29.4 ± 2.1 μg phenolphthalein/18 hr/10^7 cells (18.8 per cent of total activity) for release of β-glucuronidase. Data represent the mean ± S. E. M. of four separate experiments.

† Significant at $P < 0.01$ vs control.

‡ Significant at $P < 0.05$ vs control.

§ Incubations of neutrophils (10^7) were conducted at 37° for 30 min with the respective agent(s) and for an additional 60 min in the presence of zymosan treated with normal serum. Control incubations yielded a value of 31.7 ± 1.8 μg phenolphthalein/18 hr/10^7 cells (19.3 per cent of total activity) for release of β-glucuronidase. Data represent the mean ± S. E. M. of four separate experiments.

Recovery of enzyme activities from guinea pig neutrophils. Complete recovery of β-glucuronidase and lactate dehydrogenase activities (latent and nonlatent) was obtained from neutrophils and incubation media in the presence of pharmacologic agents. Therefore, the presence of serum-treated zymosan particles and test agents does not alter total enzyme activities significantly.

Effect of corticosteroids on enzyme release from and particle uptake by cytochalasin β-treated guinea pig neutrophils. In the presence of cytochalasin B, an agent which inhibits particle uptake, methylprednisolone hemisuccinate, triamcinolone acetonide, dexamethasone acetate, paramethasone acetate and hydrocortisone hemisuccinate inhibited β-glucuronidase secretion from neutrophils in the presence of serum-

Table 5. Effect of corticosteroids on enzyme secretion from and particle uptake by cytochalasin B-treated guinea pig neutrophils

Experimental condition*	Per cent of total enzyme activity released†		Particle uptake (No. particles/ 100 cells
	β-glucuronidase	Lactate dehydrogenase	
No treatment	3.4 ± 0.4‡	1.3 ± 0.07	
Cytochalasin B (CB)	4.8 ± 0.6	2.3 ± 0.15	
Serum-treated zymosan (STZ)	21.6 ± 1.9	1.8 ± 0.09	319 ± 28.7
CB + STZ (control)	30.8 ± 2.1§	1.5 ± 0.14	9 ± 0.3
Methylprednisolone hemisuccinate (10^{-5} M) + CB + STZ	12.3 ± 0.9‖	2.2 ± 0.21	5 ± 0.1
Triamcinolone acetonide (10^{-5} M) + CB + STZ	15.1 ± 1.3‖	1.1 ± 0.06	6 ± 0.5
Dexamethasone acetate (10^{-5} M) + CB + STZ	17.7 ± 1.5‖	2.1 ± 0.17	8 ± 0.9
Paramethasone acetate (10^{-5} M) + CB + STZ	16.2 ± 0.8‖	1.7 ± 0.12	11 ± 0.13
Hydrocortisone hemisuccinate (10^{-5} M) + CB + STZ	19.4 ± 1.7‖	1.4 ± 0.11	6 ± 0.4

* Neutrophils (10^7) were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°. The cells were then incubated with the respective agent(s) for 30 min followed by a 60-min incubation with zymosan treated with normal serum.

† Total cell enzyme activities were: β-glucuronidase, 172 ± 9.6 μg phenolphthalein/18 hr/10^7 cells; and lactate dehydrogenase, 199 ± 11.4 absorbancy units/min/10^7 cells.

‡ Data represent the mean ± S. E. M. of four separate experiments.

§ Significant at $P < 0.05$ vs STZ.

‖ Significant at $P < 0.01$ vs CB + STZ.

Table 6. Effect of adrenomimetic and cholinomimetic agents on enzyme secretion from and particle uptake by cytochalasin B-treated guinea pig neutrophils

Experimental condition*	Per cent of total enzyme activity released†		Particle uptake (No. particles/100 cells)
	β-glucuronidase	Lactate dehydrogenase	
No treatment	2.5 ± 0.3‡	2.4 ± 0.21	
Cytochalasin B (CB)	3.7 ± 0.2	3.5 ± 0.37	
Serum-treated zymosan (STZ)	23.6 ± 1.3	2.9 ± 0.16	327 ± 22.3
CB + STZ (control)	32.2 ± 2.7§	3.8 ± 0.41	6 ± 2.2
Epinephrine (10^{-4} M) + CB + STZ	18.6 ± 0.6‖	1.9 ± 0.17	4 ± 0.8
Epinephrine (10^{-4} M) + theophylline (10^{-4} M) + CB + STZ	11.3 ± 0.8‖	2.6 ± 0.24	7 ± 0.4
Isoproterenol (10^{-4} M) + CB + STZ	16.9 ± 1.5‖	3.1 ± 0.30	3 ± 0.2
Isoproterenol (10^{-4} M) + theophylline (10^{-4} M) + CB + STZ	9.3 ± 0.4	2.3 ± 0.11	4 ± 0.3
Theophylline (10^{-4} M) + CB + STZ	30.4 ± 1.6	3.6 ± 0.25	6 ± 0.4
Acetylcholine (10^{-4} M) + CB + STZ	49.6 ± 3.3	1.8 ± 0.05	5 ± 0.1
Acetyl-β-methylcholine (10^{-4} M) + CB + STZ	53.1 ± 4.2‖	3.3 ± 0.18	7 ± 0.3
Carbamylcholine (10^{-4} M) + CB + STZ	45.4 ± 3.7‖	2.2 ± 0.23	9 ± 0.7

* Neutrophils (10^7) were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°. The cells were then incubated with the respective agent(s) for 30 min followed by a 60-min incubation with zymosan treated with normal serum.

† Total cell enzyme activities were: β-glucuronidase, 158 ± 10.7 μg phenolphthalein/18 hr/10^7 cells; and lactate dehydrogenase, 192 ± 13.3 absorbancy units/min/10^7 cells.

‡ Data represent the mean ± S. E. M. of three separate experiments.

§ Significant at $P < 0.05$ vs STZ.

‖ Significant at $P < 0.01$ vs CB + STZ.

treated zymosan particles (Table 5). Although the cytochalasin B inhibited particle ingestion, and thus prevented any drug effect on this neutrophilic function, the serum-opsonized particles did adhere to the surface of the plasma membrane of the neutrophils. Cytochalasin B alone did not induce lysosomal enzyme release from neutrophils. However, when cells were incubated with serum-treated zymosan after cytochalasin B, an enhancement of β-glucuronidase secretion was observed (Table 5). In the presence of cytochalasin B, no significant amount of lactate dehydrogenase was released from neutrophils. The capacity of cytochalasin B to augment lysosomal enzyme release from human and rabbit neutrophils in the presence of zymosan or various immune complexes has been reported previously [42, 43].

Effect of adrenomimetic and cholinomimetic agents on enzyme release from and particle uptake by cytochalasin B-treated guinea pig neutrophils. Epinephrine and isoproterenol inhibited the release of β-glucuronidase from cytochalasin B-treated guinea pig neutrophils in the presence of serum-treated zymosan particles but in the absence of particle uptake (Table 6). Cytochalasin B inhibition of phagocytosis disallowed any drug effect on particle uptake. Theophylline potentiated the actions of these agents in the absence of phagocytosis.

Acetylcholine, acetyl-β-methylcholine and carbamylcholine accelerated lysosomal enzyme secretion from guinea pig neutrophils treated with cytochalasin B.

Effect of cyclic nucleotides on enzyme release from and particle uptake by cytochalasin B-treated guinea pig neutrophils. Cyclic AMP and its dibutyryl analog inhibited β-glucuronidase secretion from cytochalasin B-treated neutrophils (Table 7). In addition, the effect of cyclic AMP on enzyme release was potentiated by theophylline in the absence of particle ingestion. Cyclic GMP and its dibutyryl analog stimulated β-glucuronidase secretion from cytochalasin B-treated neutrophils. There was no effect of these agents on particle uptake due to the blockade of phagocytosis by cytochalasin B. Cytochalasin B, while enhancing the release of β-glucuronidase in the presence of serum-treated zymosan, had no effect on lactate dehydrogenase release from neutrophils.

Complete recovery of enzyme activities (latent and nonlatent) was obtained from neutrophils and incubation media in experiments where cytochalasin B was employed.

DISCUSSION

Guinea pig neutrophils actively phagocytized serum-treated zymosan particles and secreted lysosome granule-associated β-glucuronidase but not cytoplasmic lactate dehydrogenase. This selective release of a lysosomal enzyme by phagocytic cells occurred without a loss of cell viability. Particle uptake by these cells occurred at approximately the same rate as discharge of β-glucuronidase under conditions where the incubation time was varied; and these results agree with data reported by other investigators employing human neutrophils [6, 39, 44].

The release of deleterious constituents (e.g. lysosomal enzymes) from neutrophils during phagocytosis of immune complexes may explain the tissue injury that accompanies the release of these inflammatory agents [12, 13]. The inhibition of lysosomal enzyme release would most certainly alleviate the tissue destruction that accompanies many inflammatory conditions.

Methylprednisolone hemisuccinate, triamcinolone acetonide, paramethasone acetate, dexamethasone

Table 7. Effect of cyclic nucleotides on enzyme secretion from and particle uptake by cytochalasin B-treated guinea pig neutrophils

Experimental condition*	Per cent of total enzyme activity released†		Particle uptake (No. particles/100 cells)
	β-glucuronidase	Lactate dehydrogenase	
No treatment	3.4 ± 0.4‡	1.3 ± 0.07	
Cytochalasin B (CB)	4.8 ± 0.6	2.3 ± 0.15	
Serum-treated zymosan (STZ)	21.6 ± 1.9	1.8 ± 0.09	319 ± 28.7
CB + STZ (control)	30.8 ± 2.1§	1.5 ± 0.14	9 ± 0.3
Cyclic AMP (10^{-4} M) + CB + STZ	24.5 ± 2.2‖	1.9 ± 0.18	10 ± 0.4
Theophylline (10^{-4} M) + CB + STZ	28.3 ± 2.3	2.3 ± 0.20	8 ± 0.6
Cyclic AMP (10^{-4} M) + theophylline (10^{-4} M) + CB + STZ	16.5 ± 1.1¶	2.4 ± 0.16	10 ± 0.9
Dibutyryl cyclic AMP (10^{-4} M) + CB + STZ	11.4 ± 0.8¶	1.2 ± 0.05	7 ± 0.3
Cyclic GMP (10^{-4} M) + CB + STZ	58.8 ± 4.9¶	1.6 ± 0.15	12 ± 1.4
Dibutyryl GMP (10^{-4} M) + CB + STZ	75.5 ± 3.4¶	2.5 ± 0.23	6 ± 0.8

* Neutrophils (10^7) were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°. The cells were then incubated with the respective agent(s) for 30 min followed by a 60-min incubation with zymosan treated with normal serum.

† Total cell enzyme activities were: β-glucuronidase 172 ± 9.6 μg phenolphthalein/18 hr/10^7 cells; and lactate dehydrogenase, 199 ± 11.4 absorbancy units/min/10^7 cells.

‡ Data represent the mean ± S. E. M. of four separate experiments.

§ Significant at $P < 0.05$ vs STZ.

‖ Significant at $P < 0.05$ vs CB + STZ.

¶ Significant at $P < 0.01$ vs CB + STZ.

acetate and hydrocortisone hemisuccinate inhibit both phagocytic uptake of serum-treated zymosan particles by and secretion of β-glucuronidase from guinea pig neutrophils. Extracellular release of lactate dehydrogenase is not affected by these steroids. These data are supported by reports showing corticosteroids to suppress the phagocytic release of lysosomal enzymes from human neutrophils [45]. Furthermore, the relative effectiveness of these corticosteroids (methylprednisolone > triamcinolone acetonide > dexamethasone > paramethasone > hydrocortisone) in inhibiting the phagocytic secretion of lysosomal enzymes from guinea pig neutrophils approximates the rank order of anti-inflammatory activity of these agents in human and laboratory animals. Therefore, the effects of these corticosteroids on an inflammatory cell such as the neutrophil may be related to the anti-inflammatory activity of these drugs. It is interesting to note that aldosterone hemisuccinate and deoxycorticosterone acetate, which are mineralocorticoids and do not possess anti-inflammatory activity, had no effect on the phagocytic discharge of β-glucuronidase from neutrophils. The observed actions of steroids on neutrophils appear to be specific for those corticosteroids possessing anti-inflammatory activity. It is possible that the demonstration of the inhibition of two important physiological functions of the neutrophil (phagocytosis and enzyme release) by certain antiarthritic corticosteroids may serve to elucidate the mechanism(s) of actions of these agents in various pathological conditions.

We have shown adrenomimetic agents such as epinephrine and isoproterenol to inhibit phagocytosis of zymosan particles by and secretion of β-glucuronidase from guinea pig neutrophils. Both of these agents are β-agonists and the observation that the β-receptor antagonist, propranolol, blocked the action of these agents on phagocytosis and enzyme release, is evidence for the presence of β-receptors on the surface of the guinea pig neutrophil. The finding that theophylline enhanced the action of epinephrine and isoproterenol indicates that the effects of these adrenomimetic agents on the phagocytic discharge of lysosomal enzymes from guinea pig neutrophils may be mediated by intracellular cyclic AMP. We report here that cyclic AMP and its dibutyryl analog inhibited both particle uptake by and enzyme release from guinea pig neutrophils. These effects are potentiated by theophylline. These results are in agreement with reports indicating that exogenous adrenomimetic agents and cyclic AMP inhibited these cellular activities *in vitro* [32, 46, 47] in human neutrophils.

Whereas adrenomimetic agents have been demonstrated to exert an inhibitory effect on phagocytosis and enzyme release, the data in this report indicate that the cholinomimetic agents, acetylcholine, acetyl-β-methylcholine and carbamylcholine accelerate the secretion of β-glucuronidase from neutrophils but have no effect on particle uptake. The observation that atropine, a muscarinic receptor antagonist, blocked the action of these agents suggests that there are muscarinic receptors associated with the neutrophil surface.

The findings in this report indicate that cyclic GMP and its dibutyryl analog accelerate lysosomal enzyme secretion but have no effect on the ingestion of serum-treated zymosan particles. Because cholinomimetic agents also accelerate lysosomal enzyme release from guinea pig neutrophils, it is possible that cyclic GMP may mediate the actions of these agents. In this regard, Smith and Ignarro [48] showed that an increase in cellular cyclic GMP and the subsequent secretion of lysosomal enzymes from human neutrophils required calcium in the extracellular

medium. Indeed, acetylcholine failed to stimulate enzyme release from neutrophils in the absence of calcium [48, 49]. Calcium has been reported to stimulate guanylate cyclase activity [50] and it is reasonable to postulate that cholinomimetic agents, in the presence of an immune reactant (e.g. serum-treated zymosan), may facilitate calcium mobilization into neutrophils. This event would be followed by calcium activation of guanylate cyclase, elevation of cyclic GMP levels and secretion of lysosomal enzymes. Therefore, calcium, in a way not well understood, may serve as the link between the stimulus (immune reactant) and secretion in neutrophils by stimulating guanylate cyclase. At this time it is important to note that, unlike cyclic GMP levels in neutrophils which are elevated when the cells are in contact with immune reactants [39, 48], neutrophil cyclic AMP levels do not vary during particle ingestion and lysosomal enzyme secretion in the absence of adrenomimetic agents [39, 51, 52].

Corticosteroids, adrenomimetic agents and cyclic AMP were demonstrated to inhibit both particle uptake and lysosomal enzyme secretion. In order to ascertain whether inhibition of enzyme discharge occurred independently of inhibition of phagocytosis, we employed the fungal metabolite, cytochalasin B, which inhibits particle uptake by phagocytic cells such as neutrophils [53]. Neutrophils treated with cytochalasin B, and thus rendered incapable of phagocytizing particulate material, are still capable of secreting lysosomal enzymes but not lactate dehydrogenase. This has been referred to as "reverse endocytosis" [54]. In the studies reported here, when serum-treated zymosan particles are incubated with cytochalasin B-treated neutrophils, the particles adhere to the plasma membrane but are not internalized. Methylprednisolone hemisuccinate, triamcinolone acetonide, dexamethasone acetate, paramethasone acetate and hydrocortisone hemisuccinate inhibited β-glucuronidase release from cytochalasin B-treated guinea pig neutrophils. Epinephrine and isoproterenol inhibited β-glucuronidase secretion from cytochalasin B-treated neutrophils. Cyclic AMP also inhibits the discharge of β-glucuronidase from neutrophils treated with cytochalasin B. Thus, it appears that these agents exert independent effects on particle ingestion and enzyme release. However, their effect on phagocytosis probably contributes to the inhibition of lysosomal enzyme release. These data confirm a preliminary report issued from this laboratory [55]. The metabolism of glucose by neutrophils is markedly increased during phagocytosis [56, 57]; and corticosteroids have been reported to interfere with neutrophil metabolic enzymes [58] which could affect the phagocytic capabilities of these cells. Corticosteroids have been demonstrated to stabilize lysosomal membranes of isolated lysosomes from human neutrophils [59], an effect which may be responsible for the inhibition of enzyme release from neutrophils by these agents. Furthermore, adrenomimetic agents and cyclic AMP have been reported to inhibit and cholinomimetic agents and cyclic GMP to accelerate the release of hydrolases from lysosomes isolated from guinea pig neutrophils [32]. These findings might help to elucidate the mechanism(s) by which autonomic neurohormones and cyclic nucleotides influence the phagocytic release of lysosomal enzymes from guinea pig neutrophils. It is interesting to note that cholinomimetic agents and cyclic GMP, while accelerating enzyme release from neutrophils, had no effect on particle uptake. These data seem to indicate that phagocytosis activates a series of intracellular events which leads to lysosomal enzyme secretion, and that cyclic GMP may be one of the mediators of this secretory process.

The data presented in this report indicate that certain corticosteroids inhibit the phagocytosis of serum-treated zymosan particles by and the selective secretion of lysosomal enzymes from guinea pig neutrophils. Whereas adrenomimetic agents and cyclic AMP inhibit particle ingestion and enzyme release, cholinomimetic agents and cyclic GMP accelerate enzyme release and have no effect on phagocytosis. In the presence of cytochalasin B, which inhibits phagocytosis, the corticosteroids, adrenomimetic agents and cyclic AMP continue to inhibit lysosomal enzyme discharge from neutrophils. Cholinomimetic agents and cyclic GMP accelerate the release of β-glucuronidase from cytochalasin B-treated guinea pig neutrophils.

In conclusion, the data reported here show that certain antiarthritic corticosteroids may function to curtail the inflammatory process by modulating the release of lysosomal enzymes from neutrophils into the surrounding tissue where they have been reported to mediate cartilage damage and other forms of tissue injury. The fact that these agents inhibit the secretion of lysosomal enzymes in the absence of phagocytosis suggests that these mechanisms may be independent of one another and/or that certain corticosteroids may indeed exert separate effects on these cellular activities. Furthermore, the data show that autonomic neurohormones may function in inflammatory conditions to modulate the neutrophilic functions of phagocytosis and lysosomal enzyme release. In addition, it appears that cyclic AMP and cyclic GMP may play bioregulatory roles in mediating the actions of adrenomimetic and cholinomimetic agents on guinea pig neutrophils.

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