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INFLUENCE OF CORTICOSTEROIDS ON HUMAN POLYMORPHONUCLEAR LEUKOCYTE FUNCTION IN VITRO

Reduction of Lysosomal Enzyme Release and Superoxide Production[1]

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Abstract—In an experimental system in which phagocytosis or adherence of cells to surfaces were excluded as variables, we have investigated the possibility that corticosteroids may inhibit release of lysosomal constituents from viable human polymorphonuclear leukocytes into the extracellular environment. Release of β-glucuronidase and lysozyme from cytochalasin B–treated cells exposed to serum-treated zymosan, heat-aggregated human IgG, and the complement component C5a was significantly reduced by pretreatment with hydrocortisone sodium succinate and methylprednisolone sodium succinate (5×10^{-4} M). Both steroids also reduced superoxide production by these cells. These in vitro studies provide evidence that corticosteroids influence membrane-dependent responses of intact, viable polymorphonuclear leukocytes to immune reactants. Inhibition of these responses (lysosomal enzyme release, superoxide production) may explain, in part, both the antiinflammatory actions of steroids and their deleterious effects on host defenses.

INTRODUCTION

The influence of adrenal corticosteroids on polymorphonuclear leukocyte function has been extensively studied with respect both to their antiinflammatory actions and to their deleterious effects on host defenses. Since corticosteroids have previously been demonstrated to be capable of "stabilizing" lysosomal membranes (1,2), and because polymorphonuclear leukocyte

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lysosomes undoubtedly play important roles in the mediation of inflammation (3) and the killing and digestion of microorganisms (4), attention has naturally been drawn to the effects that corticosteroids have on these organelles. A recent study, however, demonstrated that corticosteroids had *no* effect on the structural integrity of lysosomes isolated from human peripheral blood leukocytes (5). This finding has cast some doubt on the often-quoted hypothesis that lysosomal stabilization is an important mechanism of corticosteroid action. Nevertheless, several investigators have presented evidence that corticosteroids do, indeed, influence release of lysosomal enzymes from whole cells (6–8). It is now appreciated that constituents of polymorphonuclear leukocyte lysosomes may be extruded from intact, viable cells by mechanisms that do not involve rupture of lysosomal membranes. Such release, or secretion, may occur as a consequence either of phagocytosis of particles (6,7) or of adherence of cells to immunologically prepared, nonphagocytosable surfaces (7,8). Release of lysosomal enzymes is dependent on fusion between the membranes of lysosomes and those of phagocytic vacuoles or the cell itself.

In an experimental system in which phagocytosis or adherence of cells to surfaces were excluded as variables, we have investigated the possibility that corticosteroids may influence membrane fusion in intact cells and consequently inhibit transport of lysosomal constituents either into phagocytic vacuoles or into the extracellular environment. We have examined the effects of two clinically useful steroid preparations—which possess the advantage of being soluble in aqueous media—on selective lysosomal enzyme release from cytochalasin B–treated human polymorphonuclear leukocytes exposed to three distinct stimuli: serum-treated zymosan, heat-aggregated human IgG, and the low-molecular-weight, soluble complement component C5a. Each of these immune reactants acts at the cell surface to launch events that lead to membrane fusion between lysosomes and the plasma membrane (9). We have also examined the effects of these compounds on another consequence of polymorphonuclear leukocyte cell surface stimulation by these immune reactants: the generation of superoxide anion, a highly reactive molecule involved in bacterial killing (9).

MATERIALS AND METHODS

Suspensions containing approximately 85% polymorphonuclear leukocytes were prepared from heparinized venous blood obtained from healthy adult donors by employing standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes (10). The cells were suspended in 10 mM phosphate-buffered 140 mM NaCl containing 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$, pH 7.4. This buffer was used throughout.

Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was boiled and washed with 140 mM NaCl and then incubated with fresh serum at a concentration of 10 mg/ml for

30 min at 37°C. After centrifugation and washing twice, this preparation of serum-treated zymosan was suspended in buffer. Serum-treated zymosan (but not untreated zymosan) was readily agglutinated by rabbit antibody to human C3 (Behring Diagnostics, Somerville, N.J.), confirming that fragments of C3 (presumably C3b) were bound to the zymosan particles.

The C5a was generated in fresh human serum containing 250 mM epsilon aminocaproic acid (Sigma Chemical Co., St. Louis, Mo.) by adding zymosan (1.0 mg/ml) as previously described (11). The activated serum was chromatographed on Sephadex G-75, and those fractions with peak lysosomal enzyme-releasing activity were employed in the experiments described below. The bulk of evidence regarding the identity of the lysosomal enzyme-releasing activity obtained by these methods indicates that it is a low-molecular-weight (approximately 17,000 daltons) product of C5, probably C5a (11). The protein content of the fractions was determined by the Folin method (12).

The IgG was isolated from fresh human serum after precipitation with 37% ammonium sulfate, desalting on a column of Sephadex G-25, and treatment with DEAE-Sephadex A50 (13). This preparation of IgG (at 3.0 mg/ml), when reacted with either rabbit antibody to whole human serum or antibody to human IgG (Behring Diagnostics), yielded single precipitin bands in immunoelectrophoresis. The IgG was aggregated by heating at 62°C for 10–30 min (14) and suspended in buffer.

Duplicate reaction mixtures containing approximately $2–4 \times 10^6$ polymorphonuclear leukocytes, preincubated with cytochalasin B (5.0 μg/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (Matheson, Coleman and Bell, East Rutherford, N.J.) at 37°C for 10 min, were incubated with appropriate compounds and stimuli either in the presence or in the absence of 75 μM horse heart ferricytochrome *c* (Type III, Sigma Chemical Co.). Incubations were terminated by placing the tubes in ice, following which they were centrifuged at 4°C for 10 min at 755*g*. Cell-free supernatants (without cytochrome *c*) were assayed for the granule-associated (lysosomal) enzymes β-glucuronidase (15) and lysozyme (16) as previously described. The cytoplasmic enzyme lactate dehydrogenase (LDH) was measured by the method of Wacker et al. (17) and used as an indicator of cell viability. Under no circumstances was there significant extracellular release of this enzyme in the experiments described below. Total enzyme activity released by 0.2% Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa.) was measured in supernatants of simultaneously run duplicate reaction mixtures.

Cytochrome *c* reduction (an indication of superoxide generation) was measured in the remaining cell-free supernatants (9). With the aid of ferricyanide and dithionite, the amount of cytochrome *c* that was reduced and the total amount of cytochrome *c* present were calculated, using an absorbance coefficient of 21.1 $mM^{-1}cm^{-1}$ at 550 nm (reduced–oxidized) (18). Specificity of cytochrome *c* reduction was checked by assaying supernatants from reaction mixtures containing 10 μg/ml superoxide dismutase (Truett Laboratories, Dallas, Texas) in addition to appropriate compounds and stimuli. Cytochrome *c* reduction in reaction mixtures without cells was also measured and found not to be significantly influenced by any of the other reagents. Superoxide generation is expressed as nanomoles of cytochrome *c* reduced per 10^6 polymorphonuclear leukocytes.

Hydrocortisone sodium succinate and methylprednisolone sodium succinate were generously supplied by the Upjohn Co., Kalamazoo, Mich. Xanthine oxidase and xanthine were from Sigma Chemical Co.

RESULTS

Cytochalasin B–treated polymorphonuclear leukocytes selectively

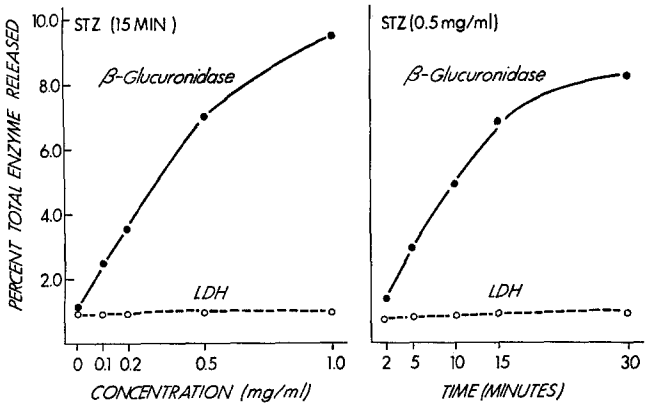

FIG. 1. Enzyme release from cytochalasin B–treated human polymorphonuclear leukocytes exposed to serum-treated zymosan (STZ) versus concentration and time. LDH: lactate dehydrogenase.

release, or secrete, lysosomal but not cytoplasmic enzymes on exposure to either serum-treated zymosan, aggregated IgG, or C5a (9). Enzyme release is both time- and concentration-dependent, and occurs independently of phagocytosis or cellular adherence to surfaces. Figure 1 shows the response of cytochalasin B–treated cells to serum-treated zymosan. Serum-treated zymosan presumably binds to these cells via receptors for the opsonic fragment of the third component of complement, which coats these particles, but is not ingested by them. Surface stimulation by this immune reactant was sufficient to provoke release of the lysosomal marker enzyme β-glucuronidase in a concentration- and time-dependent fashion. The granule-associated enzyme lysozyme was similarly released (not shown), but there was no significant leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating that cell viability was maintained in these experiments. Results similar to these were obtained with aggregated IgG and with the soluble stimulus C5a.

When cytochalasin B–treated polymorphonuclear leukocytes were preincubated with hydrocortisone sodium succinate or methylprednisolone sodium succinate before exposure to the three stimuli, the subsequent release of lysosomal enzymes was reduced (Table 1). The magnitude of the reduction varied with the concentration of steroid (negligible below 10^{-5} M) and with the stimulus for enzyme release. At all effective steroid concentrations, the greatest reduction of enzyme release occurred when cells were exposed to C5a. Significant reduction of enzyme release provoked by all three stimuli was observed with steroid concentrations of 5×10^{-4} M. Appropriate experiments revealed no evidence that these compounds either influenced the enzyme

TABLE 1

Influence of Hydrocortisone Sodium Succinate and Methylprednisolone Sodium Succinate on Enzyme Release from Cytochalasin B–Treated Human Polymorphonuclear Leukocytes

Additions[a]	Enzyme release[b]	
	β-Glucuronidase	Lysozyme
Serum-treated zymosan (0.5 mg/ml)	40.4±3.2	2.6±0.3
+ hydrocortisone (5×10^{-4} M)	30.4±2.4 (−24.8%)[c]	1.8±0.2 (−29.2%)[c]
+ methylprednisolone (5×10^{-4} M)	28.9±2.3 (−29.5%)[c]	1.7±0.2 (−31.3%)[c]
Aggregated IgG (0.3 mg/ml)	49.4±3.8	3.4±0.4
+ hydrocortisone (5×10^{-4} M)	28.2±2.4 (−42.9%)[d]	2.1±0.3 (−38.9%)[d]
+ methylprednisolone (5×10^{-4} M)	30.0±2.6 (−39.3%)[d]	2.0±0.2 (−42.6%)[d]
C5a (8.0 μg protein/ml)	40.2±3.6	2.7±0.3
+ hydrocortisone (5×10^{-4} M)	19.4±2.2 (−51.8%)[d]	1.3±0.2 (−52.3%)[d]
+ methylprednisolone (5×10^{-4} M)	21.1±3.0 (−47.5%)[d]	1.4±0.2 (−50.0%)[d]

[a] Leukocytes were incubated with steroids for 30 min at 37°C, then with cytochalasin B for 10 min and with additions for 15 min.

[b] Total enzyme activity released by 0.2% Triton X-100: β-Glucuronidase = 524±41 μg phenolphthalein/ 18 h per 4×10^6 leukocytes; lysozyme = 15.8±3.6 μg/4×10^6 leukocytes. Mean±SE, $n = 9$.

[c] Percent inhibition by steroid, $P<0.05$ (Student's *t-test*).

[d] Percent inhibition by steroid, $P<0.01$ (Student's *t-test*).

assays or affected the recovery of enzyme activity from these cells (supernatant activity plus activity in cell pellets was constant). Furthermore, experiments in which the effects of sodium succinate were examined revealed no evidence that this compound was capable of influencing enzyme release.

Whereas these findings are consistent with either direct or indirect effects of these corticosteroids on membrane fusion as the mechanism whereby they inhibited lysosomal enzyme release, they do not rule out the possibility that these agents acted merely by influencing the manner in which the various immune reactants interacted with the cell surface to generate the signals that provoke exocytosis. Therefore, we examined their effects on another consequence of cell surface stimulation.

We have recently reported that cell surface stimulation was sufficient to provoke superoxide generation by human polymorphonuclear leukocytes exposed to appropriate particulate and soluble stimuli (9). Figure 2 shows the responses of cytochalasin B–treated cells to serum-treated zymosan, C5a, and aggregated IgG. Superoxide generation by these cells, as measured by superoxide dismutase–inhibitable cytochrome *c* reduction, varied with the concentration of immune reactants (as demonstrated here), even though

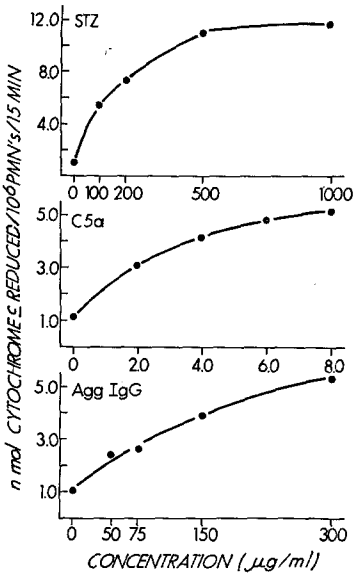

FIG. 2. Superoxide generation by cytochalasin B–treated human polymorphonuclear leukocytes (PMN's) exposed to serum-treated zymosan (STZ), C5a, and heat-aggregated human IgG (Agg IgG) for 15 min, versus concentration of immune reactants

TABLE 2

Influence of Hydrocortisone Sodium Succinate and Methylprednisolone Sodium Succinate on Superoxide Production by Cytochalasin B–Treated Human Polymorphonuclear Leukocytes

Additions[a]	Superoxide production[b]
Serum-treated zymosan (0.5 mg/ml)	11.0±0.4
+ hydrocortisone (5×10^{-4} M)	9.6±0.6 (−12.7%)[c]
+ methylprednisolone (5×10^{-4} M)	7.5±0.5 (−31.8%)[c]
Aggregated IgG (0.3 mg/ml)	3.9±0.3
+ hydrocortisone (5×10^{-4} M)	2.1±0.2 (−46.2%)[d]
+ methylprednisolone (5×10^{-4} M)	2.0±0.2 (−48.7%)[d]
C5a (8.0 μg protein/ml)	4.2±0.4
+ hydrocortisone (5×10^{-4} M)	2.3±0.3 (−45.2%)[d]
+ methylprednisolone (5×10^{-4} M)	2.1±0.2 (−50.0%)[d]

[a] Leukocytes were incubated with steroids for 30 min at 37°C, then with cytochalasin B for 10 min and with additions (plus cytochrome *c*) for 15 min.

[b] Cytochrome *c* reduction inhibitable by superoxide dismutase (10 μg/ml) = nmol cytochrome *c* reduced/10^6 leukocytes per 15 min. Mean ± SE, $n = 9$.

[c] Percent inhibition by steroid, $P<0.05$ (Student's *t-test*).

[d] Percent inhibition by steroid, $P<0.01$ (Student's *t-test*).

phagocytosis was inhibited by means of cytochalasin B. It also increased with respect to time of incubation with constant amounts of reactants (not shown). The influence of hydrocortisone and methylprednisolone on this metabolic response to cell surface stimulation is shown in Table 2. Hydrocortisone sodium succinate (5×10^{-4} M) significantly inhibited superoxide generation by cytochalasin B–treated polymorphonuclear leukocytes exposed to C5a and aggregated IgG approximately to the same extent that it reduced lysosomal enzyme release. With serum-treated zymosan, however, the degree of inhibition was less as compared to the inhibition of enzyme release observed under identical experimental conditions. Methylprednisolone sodium succinate (5×10^{-4} M), on the other hand, reduced superoxide generation by these cells in response to all three immune reactants to an extent comparable to its inhibition of enzyme release. Neither hydrocortisone nor methylprednisolone influenced the measurement of superoxide generated in an experimental system employing xanthine and xanthine oxidase (19). Thus, the observed effects of these compounds on polymorphonuclear leukocyte superoxide generation were not due simply to their ability to interfere with the assay system.

These findings indicate that in the case of cell surface stimulation by serum-treated zymosan, which presumably involves the receptor for C3b, both hydrocortisone and methylprednisolone are capable of inhibiting lysosomal enzyme release, but have more variable effects on one of the early biochemical events signaled by stimulus–membrane interaction: superoxide generation. These findings also suggest that corticosteroids may influence, in a more general fashion, the manner in which all three immune reactants interact with membranes.

DISCUSSION

Corticosteroids have previously been shown to be capable of reducing selective lysosomal enzyme release from polymorphonuclear leukocytes exposed either to a variety of particles they were capable of ingesting (6,7) or to immune reactants deposited on nonphagocytosable surfaces (7,8). Since phagocytosis and surface adherence are absolute prerequisites for enzyme release in these experimental systems and may by themselves be influenced by corticosteroids (20), it has been difficult to distinguish between the effects of these compounds on these stimuli for secretion versus their effects on the secretory process per se (i.e., organelle translocation and membrane fusion). Consequently, we have elected to study the influence of corticosteroids on lysosomal enzyme release in an experimental system in which neither phagocytosis nor cellular adherence is a prerequisite for secretion. The fungal

metabolite cytochalasin B reversibly inhibits particle ingestion by polymorphonuclear leukocytes (21). These cells, nevertheless, selectively release lysosomal constituents when appropriate particulate and nonparticulate stimuli come into contact with their surfaces (22). Cytochalasin B, therefore, converts polymorphonuclear leukocytes from phagocytic cells into model secretory cells and allows one to measure an intracellular event, that is, membrane fusion, by monitoring the release of lysosomal enzymes.

Our data suggest that at least two clinically useful corticosteroid preparations can indeed retard the release of lysosomal constituents from intact, viable human polymorphonuclear leukocytes. They may do so either by directly inhibiting membrane fusion between lysosomes and their target membranes or by acting on other intracellular events that lead to this process. The data also suggest that these compounds may act primarily at the level of the surface membrane so as to modify or modulate stimulus–membrane interactions. Consequently, as suggested before (23), the stabilizing effects of antiinflammatory corticosteroids on isolated liver lysosomes in vitro simply represent a general capacity of these steroids to enter into, and influence the deformability of, lipid bilayers. That hydrocortisone, so inserted, can prevent membrane perturbation by immune reactants, such as proteins, is evidenced by recent experiments in our laboratory that have demonstrated that hydrocortisone can diminish the perturbation (measured by electron spin resonance spectroscopy) produced in artificial lipid membranes (liposomes) by aggregated IgG (24). The molecular mechanisms whereby corticosteroids stabilize biomembranes are unknown. It has been appreciated, however, that these compounds do interact with lipid bilayers in structurally specific fashion and become incorporated within both natural and artificial membranes (25)

The concentrations of steroids required to produce these in vitro effects are considerably above any conceivable pharmacological concentration in vivo. But this statement is based on two assumptions: (1) that the steroids remain undegraded (at concentrations above 0.5 mM) in the presence of live cells, and (2) that these water-soluble esters release free steroid at appropriate sites (i.e., the plasma membrane) at concentrations equivalent to their concentration in the bulk phase. Since free steroids preferentially enter lipid bilayers (23,24), since as little as 1 mol steroid/1000 mol other membrane lipid suffices to stabilize bilayers (23–25), and since we do not as yet know the actual amount of steroid released from esterified form, the relationship between in vitro and in vivo concentrations must remain conjectural.

Other possible modes of action of corticosteroids on intact cells cannot be excluded by these studies. For example, the agents we studied may have acted directly or indirectly on structural elements that are known to be important for exocytosis (e.g., microfilaments and microtubules) (11), or on

intracellular or ectoenzymes, or both (e.g., adenyl cyclase, NADH or NADPH oxidases) (26–28). Inhibition of pyridine nucleotide oxidation, for example, could certainly explain the reduction of superoxide generation by stimulated polymorphonuclear leukocytes (29). Whatever the precise mechanism may be, these in vitro studies provide evidence that corticosteroids influence two functions of intact, viable human polymorphonuclear leukocytes that may relate both to the antiinflammatory actions of these compounds and to their deleterious effects on host defenses. Retardation of fusion between polymorphonuclear leukocyte lysosomal and plasma membranes can limit the access of inflammatory and antimicrobial lysosomal constituents either to surrounding tissues or to ingested microorganisms. The former consequence could potentially limit inflammation and tissue injury, whereas the latter (in addition to diminished superoxide generation) could at least partially contribute to the defects in intracellular bacterial killing previously noted in corticosteroid-treated polymorphonuclear leukocytes (28). Diminished superoxide generation can also explain the reduced nitroblue tetrazolium dye reduction (30) and hexose monophosphate shunt activity (28,31) observed in these cells, since superoxide is very likely an intermediate in both of these reactions (29,32). These observations notwithstanding, it remains to be determined whether, indeed, the pharmacological concentrations of corticosteroids required to produce these in vitro effects on polymorphonuclear leukocyte function relate directly to their clinical efficacy.

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