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J Clin Invest. 1974;54(6):1337-1343. <https://doi.org/10.1172/JCI107880>.

Research Article

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Effects of Corticosteroids on Human Monocyte Function

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ABSTRACT This report examined the effect of corticosteroids in vitro on human peripheral blood monocytes, essential cells in both immune and nonimmune cellular defense mechanisms. Monocyte chemotaxis in response to sera, *Escherichia coli* filtrate, and lymphokine chemotactic factor was markedly reduced ($P < 0.01$) by hydrocortisone succinate (HCS) at 16 $\mu\text{g}/\text{ml}$. Methylprednisolone succinate and unesterified hydrocortisone produced similar impairment of monocyte chemotaxis while two drugs which unmodified do not enter cells, hydrocortisone phosphate (HCP) and cortisone acetate, had no effect on chemotaxis. HCS also significantly impaired monocyte random migration at 16 $\mu\text{g}/\text{ml}$. Monocyte bactericidal activity was reduced by HCS at 16 $\mu\text{g}/\text{ml}$ ($P < 0.01$) but was not affected by HCP even at 120 $\mu\text{g}/\text{ml}$. In comparison, HCS did not alter granulocyte chemotaxis even at 500 $\mu\text{g}/\text{ml}$, and bactericidal activity was reduced at 16 $\mu\text{g}/\text{ml}$ ($P < 0.01$). Monocyte phagocytosis of cryptococci was reduced only 20% ($P < 0.05$) at 120 $\mu\text{g}/\text{ml}$. HCS at 120 $\mu\text{g}/\text{ml}$ did not alter monocyte base-line or postphagocytic hexosemonophosphate shunt activity, viability by trypan blue exclusion, adherence to tissue culture flasks, or surface binding of IgG globulin. These corticosteroid-induced defects in monocyte function may contribute to reduced cellular defense during corticosteroid therapy.

INTRODUCTION

Corticosteroid therapy is thought to interfere with cellular immunity in man since recipients have an increased incidence of granulomatous infections (1, 2) and reduced delayed hypersensitivity reactions (3). The cellular immune response involves the participation of two

This work has in part been published in abstract form. 1973. *Clin. Res.* 21: 878.

Dr. Sagone is a Leukemia Society of America Scholar, Dr. LoBuglio is an American Cancer Society Professor, and Dr. Rinehart is a National Institutes of Health supported research fellow.

Received for publication 8 April 1974 and in revised form 30 July 1974.

major cell types, the lymphocyte and the monocyte-macrophage. Although a number of studies indicate that corticosteroids induce a deficit in human lymphocyte number and function (4-6), no studies have examined the effects of corticosteroids on monocyte function in man.

Studies in experimental animals suggest that the function of the monocyte-macrophage system may be seriously impaired by corticosteroids. Weston, Mandel, Yeckley, Kruegers, and Claman found that lymphocytes from corticosteroid-treated guinea pigs immunized with tuberculin had a normal capacity to passively transfer tuberculin reactivity to normal guinea pigs. In contrast, corticosteroids impaired skin reactivity to tuberculin in guinea pigs receiving lymphocytes from donors with strong tuberculin immunity (7). In vitro experiments suggest that one aspect of this defective macrophage function may be related to interference with normal macrophage response to lymphocyte products thought to mediate the cellular immune reaction (lymphokines). These studies demonstrated that corticosteroids interfered with the effects of migration inhibition factor and macrophage aggregating factor on guinea pig macrophages (8, 9). Further, North has shown that corticosteroids can inhibit macrophage but not granulocyte exudation in rabbits (10).

These observations suggest that monocyte-macrophage defects may contribute to the adverse effects of corticosteroids on cellular defenses. This report describes the in vitro effects of corticosteroids on a variety of assays of human monocyte function.

METHODS

Cells. Mononuclear cells were isolated by Ficoll-Hypaque separation of blood from normal volunteers as previously described (11). These preparations contained 20-30% monocytes and 70-80% lymphocytes. Blood leukocytes were obtained by dextran sedimentation (12) and contained 70-80% granulocytes. Immediately before functional assays, corticosteroids were added to cell suspensions. Cell viability was determined by trypan blue exclusion.

Chemotaxis. The chemotactic responsiveness of whole blood leukocytes or mononuclear cells was determined by the method of Snyderman, Altman, Hausman, and Mergenhagen (13). Mononuclear preparations were suspended to 5×10^6 cells/ml in Gey's balanced salt solution (GBSS)¹ (pH 7.1) containing various concentrations of corticosteroids. 1 ml of cell suspension was allowed to migrate across 5- μ m Nucleopore filters (Nucleopore Corp., Pleasanton, Calif.) at 37°C for 90 min. Granulocyte chemotaxis was performed under identical conditions except that 3.5×10^6 cells/ml of whole blood leukocytes were used. Various chemotactic stimuli were used including heat-inactivated sera (14), *Escherichia coli* filtrate (15), and the supernate of antigen (tuberculin)-stimulated lymphocyte cultures containing chemotactic factor (13). For assay of random movement, no chemotactic stimulus was used and chambers were incubated 4 h. Results were expressed as mean number of cells/high-power field (hpf) for heat-inactivated sera, and *E. coli* filtrate. Because it represents a relatively weak stimulus, results obtained by using chemotactic factor were expressed as mean number of cells/5 hpf. The data in some experiments were also expressed as percent inhibition of chemotaxis as compared to a non-corticosteroid-treated control.

Bactericidal assay. Bactericidal activity was determined by the method of Root, Rosenthal, and Balestra (16) by utilizing whole leukocyte preparations containing 2.5×10^6 granulocytes or mononuclear preparations containing 2.5×10^6 monocytes incubated with 12.5×10^6 *Staphylococcus aureus* organisms for 60 min at 37°C. Number of viable bacteria was determined by triplicate colony counts at 0 and 60 min of incubation, and results were expressed as number of bacteria killed in 60 min. Incubation of bacteria with no leukocytes, 2.5×10^6 lymphocytes, or iodoacetate-inhibited mononuclear cells produced no change in viable number of bacteria present at 60 min.

Glucose metabolism. Platelet-free monocyte monolayers of 95% purity were formed as previously described (17) in glass metabolic flasks. ¹⁴CO₂ production from [1-¹⁴C]glucose was monitored continuously in an ionization chamber-electrometer apparatus (18). Base-line ¹⁴CO₂ production of the monolayers was quantitated and the effect of phagocytosis of latex particles determined by addition of 0.25 ml of Lytron 612 (Monsanto Company, Cincinnati, Ohio) particles at a concentration of 60 mg/ml. Monocyte number was determined by DNA content of the monolayer. Results were expressed as nanomoles of ¹⁴CO₂ produced/hour/10⁷ monocytes.

Cryptococcal phagocytosis. Viable *Cryptococcus neoformans* were ⁵¹Cr-labeled by incubation with Na⁵¹CrO₄ for 1 h and then washed three times and suspended to 10⁷ organisms/ml. Monocyte monolayers (>95% pure) were prepared in Falcon tissue culture dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) (17) and incubated with 10⁷ cryptococci for 2 h at 37°C in the presence of 10% fresh AB sera and washed rigorously to remove noningested organisms. The monolayers were then lysed by freeze thawing five times, and the lysate was assayed for radioactivity in a Packard counter (Packard Instrument Co., Inc., Downers Grove, Ill.) to determine number of cryptococci present and DNA content (19) to determine

¹ Abbreviations used in this paper: GBSS, Gey's balanced salt solution; HBSS, Hanks' buffered salt solution; HCP, hydrocortisone phosphate; HCS, hydrocortisone succinate; HMP, hexosemonophosphate; hpf, high-power field; RBC, red blood cell.

number of monocytes present. The DNA content due to the number of cryptococci ingested was <5% of the DNA content of the monocyte monolayers. Phagocytosis was expressed as number of cryptococci ingested/microgram monocyte DNA. Morphologic examination of washed monolayers demonstrated that >90% of bound cryptococci was intracellular and that heat inactivation of sera abolished phagocytosis.

Monocyte membrane binding of IgG globulin. Group O, Rh positive red blood cells (RBC) were ⁵¹Cr-labeled (20) and coated with IgG globulin by incubation with a high titer anti-D sera as previously described (21). The RBC were then washed and suspended to 2×10^8 cells/ml in Hanks' buffered salt solution (HBSS). Monocyte monolayers were formed in Falcon tissue culture dishes (17), and 1 ml of IgG-coated RBC was incubated with each of triplicate dishes in the presence of 0 and 120 μ g of hydrocortisone succinate (HCS) for 2 h at room temperature. The monolayers were then washed five times with HBSS to remove unbound RBC. Monolayers were lysed by freeze thawing and lysates assayed for radioactivity in a Packard gamma counter to determine the number of RBC present. RBC binding was expressed as number of RBC bound/monolayer.

Monocyte adherence. Mononuclear cells suspended in HBSS with 10% sera containing 0-120 μ g/ml HCS were added to plastic Falcon dishes and incubated for 2 h at 37°C. Monolayers were washed five times and number of adherent cells measured by DNA assay (19).

Drugs. HCS (Solucortef, Upjohn Co., Kalamazoo, Mich.), methylprednisolone succinate (Solumedrol, Upjohn Co.), hydrocortisone (Sigma Chemical Co., St. Louis, Mo.), cortisone acetate (Merck Sharp & Dohme, Rahway, N.J.), and cortisone phosphate (Merck Sharp & Dohme) were dissolved in preservative-free buffered salt solutions and used within 1 h of preparation.

Statistical analysis. In all experiments, the effect of corticosteroids was analyzed by Student's *t* test.

RESULTS

Effect of corticosteroids on chemotaxis. The normal response of mononuclear cell preparations to chemotactic stimuli in our laboratory was 75-100 cells/hpf with *E. coli* filtrate, 50-75 cells/hpf with sera, and 70-75 cells/5 hpf with chemotactic factor preparations. In the absence of chemotactic stimuli migration was <4 cells/hpf. When the mononuclear preparation was used, the cells which migrated across the membrane were all monocytes by morphologic criteria. The effect of HCS on monocyte chemotactic response to chemotactic factor preparations is illustrated in Fig. 1. At concentrations of 16 μ g/ml, significant reduction ($P < 0.01$) in migration occurred while concentrations of 120 and 500 μ g/ml virtually abolished migration to levels seen in the absence of chemotactic stimuli. HCS also adversely affected the monocyte response to *E. coli* filtrate and sera (Fig. 2). At concentrations of 16 μ g/ml, HCS produced significant reduction ($P < 0.01$) in cells/hpf in response to these two stimuli with even greater inhibition at higher doses. Concentrations of HCS less than 16 μ g/ml produced an inconsistent suppression of chemotaxis.

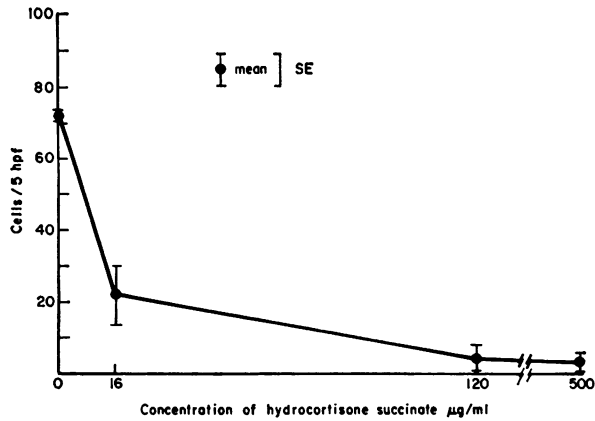


FIGURE 1 Effect of HCS on monocyte chemotactic response to lymphokine chemotactic factor. The results are expressed as mean cell migration from three experiments. A significant reduction in cell migration occurred at all HCS concentrations ($P < 0.01$).

To determine if this impairment of chemotaxis was a direct effect of HCS on monocytes or mediated through the interaction of HCS and lymphocytes, the following two experiments were done. 5×10^6 lymphocytes/ml were incubated with 0, 16, and 120 µg HCS/ml in GBSS for 90 min at 37°C. The supernates were dialysed against distilled water, lyophilized, and re-suspended at a 10-fold concentration in GBSS. In two separate experiments, the concentrated supernates of lymphocytes incubated with either dose of HCS did not have chemotactic activity for monocytes. Further, the addition of these supernates to mononuclear preparations in the monocyte chemotaxis assay produced no impairment of monocyte response to *E. coli* filtrate. Thus, we could not identify a nondialysable inhibitor

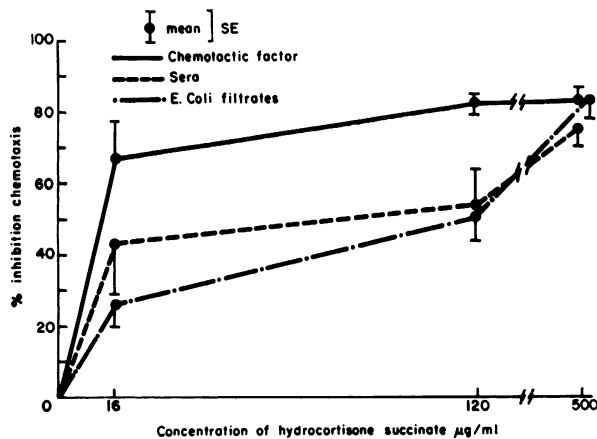


FIGURE 2 Comparison of HCS inhibition of monocyte chemotaxis to three stimuli. The results are expressed as mean percent inhibition of chemotaxis from three experiments. Inhibition is significant at all drug concentrations ($P < 0.01$).

TABLE I
Effect of HCS on Monocyte Chemotaxis with HCS Mixed with Chemotactic Stimulus, *E. coli* Filtrate

Hydrocortisone concentration µg/ml	Cells/hpf*	
	Exp. 1	Exp. 2
0	86±4	76±5
16	91±2	79±3
120	79±5	67±4

* Results expressed as mean and SE of 10 hpf.

of chemotaxis released from lymphocytes by HCS under the experimental conditions present in our chemotactic assay.

More importantly, the chemotactic assay system was modified to allow examination of the effect of HCS on monocyte chemotaxis in the absence of lymphocytes. For these studies, 5×10^6 mononuclear cells were placed in the upper portion of the chemotactic chamber without any material in the lower chamber. After 2 h incubation at 37°C, the upper chamber was washed rigorously with GBSS to remove nonadherent lymphocytes leaving a monocyte monolayer on the Nuclepore filter (> 95% monocytes by morphologic criteria). 1 ml of GBSS containing 0, 16, or 120 µg was preincubated with the monocyte monolayer for 30 min, and then the chemotactic stimuli (*E. coli* filtrate) was placed in the lower chamber and the assay completed in the usual fashion. The mean and standard error for the inhibition of chemotaxis in three experiments by 16 µg and 120 µg HCS was 62 ± 16 and 95 ± 2 , respectively. Thus, the inhibition of monocyte chemotaxis by HCS is not dependent on the presence of lymphocytes.

To determine if HCS reduced chemotaxis by binding or inactivating chemotactic substances, HCS was added to the lower chamber with *E. coli* filtrates rather than to the upper chamber with the mononuclear suspension. No significant reduction in chemotaxis was observed at 16 or 120 µg/ml HCS (Table I). The lack of direct

TABLE II
Effect of HCS on Monocyte Random Movement

Hydrocortisone concentration µg/ml	Cells/5 hpf*	
	Exp. 1	Exp. 2
0	35±6	36±5
16	18±4‡	22±2‡
120	10±0.3§	7±1§

* Expressed as mean and SE of triplicate chambers.

‡ Significantly reduced as compared to control, $P < 0.05$.

§ Significantly reduced as compared to control, $P < 0.01$

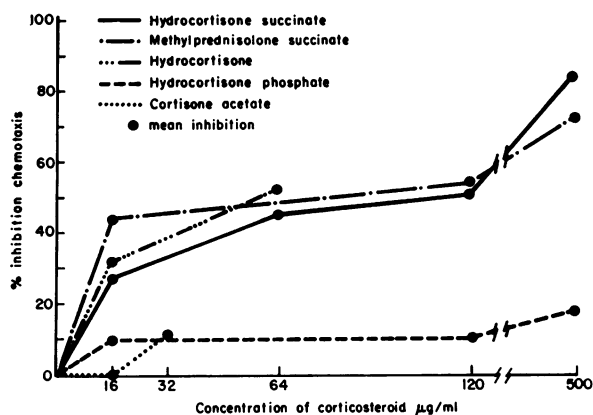


FIGURE 3 Inhibition of monocyte chemotaxis by various corticosteroid preparations. The results are expressed as mean percent inhibition of chemotaxis from three experiments. Hydrocortisone, methylprednisolone succinate, and HCS reduced chemotaxis significantly at concentration $\geq 16 \mu\text{g/ml}$ ($P < 0.01$).

effect of HCS on monocytes in this experiment is probably due to the poor diffusion of HCS across the filter in this nonagitated, short incubation (90 min) system. Random movement of monocytes was significantly reduced by HCS at $16 \mu\text{g/ml}$ ($P < 0.05$) and $120 \mu\text{g/ml}$ ($P < 0.01$) in duplicate experiments (Table II). HCS was not itself chemotactic at either 16 or $120 \mu\text{g/ml}$.

To determine if the chemical structure of the corticosteroid modified this adverse effect, the chemotactic response of *E. coli* filtrate was studied with a variety of cortisone preparations (Fig. 3). Hydrocortisone phosphate and cortisone acetate, which unmodified are unable to cross cell membranes (22), had no significant effect on monocyte chemotactic response while methylprednisolone succinate and unesterified hydrocortisone produced effects comparable to HCS. Significant re-

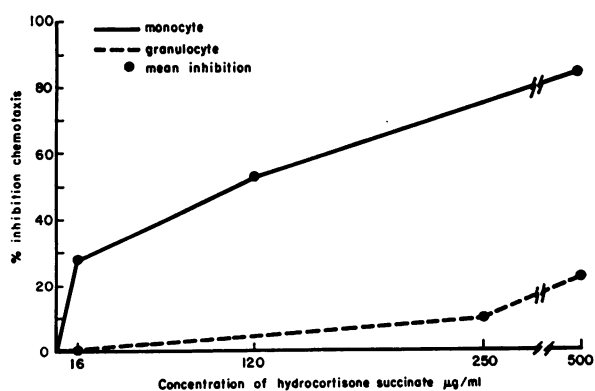


FIGURE 4 Comparative inhibition of monocyte and granulocyte chemotaxis to *E. coli* filtrates by HCS. The results are expressed as mean inhibition found in three experiments.

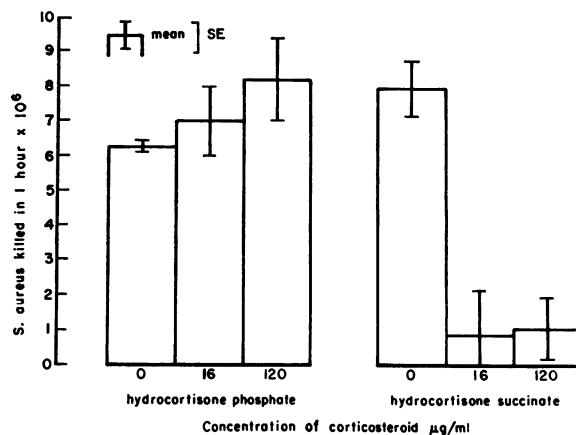


FIGURE 5 Effect of hydrocortisone on monocyte bactericidal activity. The results are expressed as mean number of *S. aureus* $\times 10^6$ killed by 2.5×10^6 monocytes in 1 h (three experiments). Significant inhibition ($P < 0.01$) occurred with both doses of HCS but not with HCP.

duction of migration occurred with both of these preparations at concentrations of $16 \mu\text{g/ml}$ or greater ($P < 0.01$). Because of limited solubility, cortisone acetate and unesterified hydrocortisone could not be studied in the high-dose range.

In contrast to the monocyte, granulocytes appeared to be resistant to the adverse effect of corticosteroids. In four experiments, the mean and SE of granulocyte chemotactic response to *E. coli* filtrate was 58 ± 5 cells/hpf and in the presence of HCS at a concentration of $500 \mu\text{g/ml}$ was 43 ± 7 (not significantly different). The contrast in effect of HCS on monocytes and granulocytes is illustrated in Fig. 4.

Effect of corticosteroids on bactericidal activity. HCS produced significant depression of monocyte bactericidal activity at $16 \mu\text{g/ml}$ or greater (Fig. 5). Concentrations

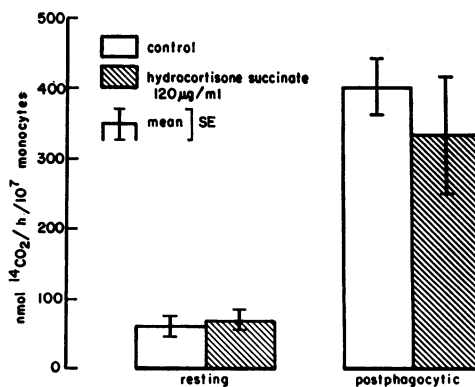


FIGURE 6 Effect of HCS on monocyte HMP shunt activity. Results are expressed as mean $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ glucose by monocyte monolayers (three experiments). The presence of $120 \mu\text{g/ml}$ HCS had no significant effect on resting or postphagocytic $^{14}\text{CO}_2$ production.

less than 16 $\mu\text{g/ml}$ produced variable effects in this relatively brief incubation period (60 min). Similar to the chemotaxis experiments HCP, which unmodified enters cells poorly, had no adverse effect on monocyte bactericidal function. HCS also impaired the *S. aureus* killing ability of granulocytes. The whole leukocyte preparation killed $9.3 \pm 1.1 \times 10^6$ bacteria in 1 h; when 16 $\mu\text{g/ml}$ of HCS was present, killing was reduced to $3.6 \pm 0.8 \times 10^6$ bacteria (three experiments, $P < 0.01$).

Effect of corticosteroids on glucose metabolism. Since intracellular bactericidal activity appears to be dependent upon several metabolic events including peroxide generation and stimulation of the hexosemonophosphate (HMP) shunt in phagocytic cells, we examined the effect of HCS on HMP shunt activity. We previously have shown that monocyte monolayers respond to ingestion of latex particles with a 6–10-fold increase in $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]\text{glucose}$ and little or no change in $^{14}\text{CO}_2$ production from $[6-^{14}\text{C}]\text{glucose}$ (Krebs' cycle) (23). As depicted in Fig. 6, 120 $\mu\text{g/ml}$ of HCS did not impair either base-line or postphagocytic $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]\text{glucose}$, reflecting an intact HMP shunt response to this maximal stimulus.

Effect of corticosteroids on phagocytic function. Although the normal metabolic response of monocytes after latex ingestion would suggest an intact phagocytic capacity in the presence of 120 $\mu\text{g/ml}$ HCS, this assay is done in a vast excess of latex particles and is not a sensitive measure of phagocytic activity. We, therefore, examined the effect of HCS on monocyte cryptococcal phagocytosis. In three experiments, HCS at 16 $\mu\text{g/ml}$ had no adverse effect while at 120 $\mu\text{g/ml}$ a modest 20% reduction in phagocytosis was noted. This represented a decrease in cryptococci ingested/ μg monocyte DNA from $21.4 \pm 1.0 \times 10^4$ to $14.9 \pm 1.5 \times 10^4$ ($P < 0.05$).

Effect of corticosteroids on monocyte membrane function. We have previously noted that monocyte binding of IgG-coated red cells is not dependent on intracellular metabolic activity but is adversely effected by chemical modification of the cell membrane (21). HCS did not alter monocyte binding of IgG globulin-coated RBC in two experiments. RBC bound/monolayer were $12.6 \pm 1.5 \times 10^8$ and $16.5 \pm 3.7 \times 10^8$ in the absence of HCS and $17.1 \pm 1.6 \times 10^8$ and $17.0 \pm 1.3 \times 10^8$ in the presence of 120 $\mu\text{g/ml}$ HCS.

We also examined the effects of HCS on monocyte adherence to plastic tissue culture dishes. In three experiments, HCS in concentrations of 16 $\mu\text{g/ml}$ and 120 $\mu\text{g/ml}$ did not alter the number of monocytes able to adhere to the tissue culture dishes in a 2-h period. Also, HCS in concentrations as high as 1,000 $\mu\text{g/ml}$ did not alter monocyte viability as judged by trypan blue exclusion.

DISCUSSION

These studies indicate that blood monocytes have a unique in vitro sensitivity to corticosteroids. They have impaired random movement, chemotaxis, and bactericidal activity after only brief periods (60–90 min) of contact with concentrations of HCS as low as 16 $\mu\text{g/ml}$. Similar concentrations have been reached in vivo with administration of 100–500 mg of HCS and methylprednisolone succinate (24, 25). In contrast, granulocyte migration and chemotaxis was not effected by HCS concentrations as high as 500 $\mu\text{g/ml}$ as previously reported (26). Granulocyte bactericidal activity, however, was impaired by low doses of HCS in the present study, while Mandell, Rubin, and Hook (27) showed consistent impairment only at high concentrations (1,200 $\mu\text{g/ml}$) of HCS. The explanation for this lack of agreement of the effect of HCS on granulocyte bactericidal activity is unclear although a number of differences exist in the methodology utilized in these studies.

These observations suggest that defective monocyte function may contribute to impaired cellular immune resistance in patients receiving corticosteroids. The blood monocyte represents the circulating cell which responds to chemotactic stimuli and matures to a tissue macrophage at sites of infection or inflammation (28). The chemotactic stimuli may represent products of immune lymphocytes (lymphokines) or products of the infectious agent themselves. The observation in this study that HCS interferes with the monocyte response to both of these types of chemotactic stimuli suggests that this effect on chemotaxis may account at least in part for impaired macrophage exudation (10) and poor delayed hypersensitivity skin test reactions (3, 7) observed in vivo. Once at the site of reaction, macrophages are called upon to ingest and kill intracellular organisms (28) and may exert tumoricidal activity (29). Our studies indicate interference by HCS of the bactericidal process, and a study by Hibbs has demonstrated interference of macrophage tumoricidal activity in vitro with similar concentrations of HCS (30). Thus, corticosteroid interference of monocyte-macrophage killing activities may further contribute to in vivo impairment of cellular defense mechanisms. These observations on chemotaxis and bactericidal activity of monocytes represent effects of corticosteroids in vitro and may not be operational in vivo. Preliminary studies in our laboratory, however, indicate that corticosteroids can also produce defects in monocyte function in vivo (31).

The mechanisms involved in corticosteroid impairment of monocyte-macrophage function are unclear, but observations made in this report shed some light on this problem. The lack of adverse effect with corticosteroid preparations previously demonstrated to be unable to pass across cell membranes suggests that the inhibitory

action is an intracellular event and not a surface phenomena. Corticosteroids apparently are not toxic to monocytes since these drugs did not effect glass adherence, resting or stimulated HMP shunt activity, or trypan blue exclusion, all of which are parameters of cell viability.

Chemotaxis represents a complicated and only partially defined process of surface and intracellular events. A cell-surface receptor is thought to bind or interact with the chemotactic agent and trigger a series of intracellular activities in which alignment of microfilaments and microtubules allow directional cell migration (32-34). The corticosteroid inhibition of monocyte chemotaxis is not due to inactivation of the chemotactic materials since they retained chemotactic activity for granulocytes. Further, HCS did not effect monocyte response when it was directly mixed with the chemotactic substances rather than with the monocytes. Since chemotaxis can be inhibited by several metabolic inhibitors (33), corticosteroids may interfere with a necessary metabolic event. Against this possibility is the normal glucose metabolism of HCS-treated monocytes. A third alternative would be that HCS interferes with monocyte membrane interaction with chemotactic stimuli with subsequent lack of triggering of intracellular events. Corticosteroids, in fact, have been shown to interfere with the interaction between migration inhibition factor and guinea pig macrophages (8, 9). The observation that random migration is similarly inhibited, however, might indicate interference with a basic mechanism involved in cell migration. This could well be due to an abnormality in microfilament or microtubule function. Cytochalasin B which totally disrupts microfilament function is a potent inhibitor of chemotaxis (34). This gross distortion of microfilament function, however, also prevents cell adherence to cell surfaces and phagocytosis (35), effects which were not seen with corticosteroids. Corticosteroids might influence some other, as yet undefined, component in cell migration.

In vitro bactericidal activity represents a sequence of events including membrane binding of antibody-complement-coated bacteria, ingestion, and intracellular killing. The presence of normal monocyte binding of IgG-coated red cells and lack of inhibition of latex and cryptococcal phagocytosis at low doses of HCS suggest that membrane binding and ingestion are not seriously affected. Intracellular killing of bacteria requires lysosomal migration to and fusion with bacteria containing phagosomes (36). A sequence of metabolic events is associated with this event including the enzymatic generation of peroxide and stimulation of the HMP shunt (37). The observation that HCS did not prevent stimulation of HMP shunt activity of monocytes after ingestion of latex particles suggests that the metabolic pathways involved in peroxide generation and

HMP shunt response are not directly inhibited. The microfilament-microtubule systems in phagocytic cells are thought to play a role in lysosomal migration to phagosomes (38). Also, corticosteroids appear to "stabilize" lysosomes (39) which may prevent their fusion with the phagosome. Recent studies by Hibbs (30) have demonstrated that HCS in similar concentrations and under similar experimental conditions can interfere with lysosomal exocytosis in activated murine peritoneal macrophages. Whether the effect of corticosteroids in this study is due to impairment of lysosomal function or an effect on a crucial intracellular enzymatic or metabolic activity is unclear but represents a fertile area of future investigation.

ACKNOWLEDGMENTS

The authors wish to thank Miss Geraldine Bain and Mr. Jon Paine for excellent technical assistance and Miss Helen Ilc for manuscript preparation.

The work was supported by grants from the National Institutes of Health (1R01-CA12786 and 5R01-CA13381) and the American Cancer Society (CI-36A).

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