

Effects of In Vivo Prednisone on In Vitro Eosinophil and Neutrophil Adherence and Chemotaxis

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The mechanisms by which corticosteroids affect circulating granulocytes are not completely understood. Since granulocyte adherence and chemotaxis are both prerequisite for an exudative response in inflammation, we studied these functions in vitro before and at 4, 24, and 48 hr after administration of 60 mg of prednisone to healthy human volunteers and patients with idiopathic hypereosinophilia. Adherence was determined by the number of eosinophils and neutrophils from heparinized whole blood that remained adherent to nylon-wool columns after a 15-min incubation. Prior to prednisone administration, $68 \pm 7\%$ neutrophils and $60 \pm 18\%$ eosinophils were adherent; 4 hr after prednisone administration, adherence of both cell types decreased by one-third ($p < 0.02$); at 24 hr adherence had partially recovered (neutrophils $63 \pm 10\%$; eosinophils $60 \pm 5\%$). Chemotaxis was measured with a morphologic Boyden-chamber assay using endotoxin-

activated serum as the chemoattractant. Twenty-four hours after prednisone administration eosinophil chemotaxis was reduced by more than 50%, and it had fully recovered by 48 hr. Neutrophil chemotaxis was never depressed, and at 48 hr it was significantly increased over the baseline value. Hence, these studies show that whereas corticosteroids have similar effects in decreasing both neutrophil and eosinophil adherence, they have differential effects on locomotion, in that eosinophil chemotaxis is significantly suppressed by drug administration while neutrophil chemotaxis is not suppressed. It should be emphasized that the eosinophils used in this study were taken from patients with the hypereosinophilic syndrome, and thus they may not truly represent normal eosinophils. However, these studies still suggest fundamental differences in the mechanisms of modulation of locomotion of different granulocyte populations involved in the inflammatory response.

FOR THE PAST 30 YR pharmacologic amounts of glucocorticoids have been widely used in many inflammatory and autoimmune disorders such as rheumatoid arthritis, chronic active hepatitis, ulcerative colitis, asthma, systemic lupus erythematosus, pemphigus vulgaris, idiopathic thrombocytopenic purpura, and minimal lesion nephrotic syndrome.¹ Daily glucocorticoid administration, usually in the form of prednisone, has many adverse side effects, of which an infectious disease can be the most catastrophic. The predisposition to infection can be avoided by using alternate-day prednisone therapy;² however, this regimen does not always control the underlying disease.³ Thus, continued delineation of the mechanisms by which this predilection to infection occurs is of more than academic interest. Reports that neutrophils and monocytes show diminished skin window responses,⁴⁻⁶ adherence,^{7,8} chemotaxis,⁹ and bactericidal activity^{9,10} after in vitro and in vivo exposure to glucocorticosteroids indicate that defects in leukocyte function may

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exist at many different levels. Unfortunately, most of the *in vitro* studies have used concentrations of steroids higher than those generally obtained *in vivo*.⁷⁻¹⁰ We designed our study to measure leukocyte function *in vitro* at various time periods after *in vivo* administration of prednisone.

Leukocyte adherence to vascular endothelium and chemotaxis through interstitial spaces are prerequisite for the exudative response in natural inflammatory processes (e.g., wound healing, infection, etc.). Artificial situations like the Rebutck skin window assay do not measure these phenomena independently. Since we wished to investigate the effects of prednisone on human granulocyte function in these early but distinct inflammatory events, we measured adherence to nylon-wool columns and used the Zigmond-Hirsch chemotaxis assay.¹¹

MATERIALS AND METHODS

Materials were obtained as follows: Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.); Hypaque (Winthrop Laboratories, New York, N.Y.); *Escherichia coli* endotoxin 0127:B8 lipopolysaccharide B (Difco Laboratories, Detroit, Mich.); 3- μ cellulose nitrate filters (Sartorius, Göttingen, West Germany, distributed by Beckman Instruments, Science Essentials, Mountainside, N.J.); Hanks' tissue culture medium (pH 7.2) without calcium or magnesium (National Institutes of Health, Media Supply Section, Bethesda, Md.); Gey's tissue culture medium (pH 7.2) containing 2% bovine serum albumin and 2% penicillin and streptomycin (Microbiological Associates, Bethesda, Md.); scrubbed nylon fiber, 3 denier, 4 cm, type 200 (Fenwal Laboratory, Morton Grove, Ill.); 1-cc tuberculin syringes (Monoject Sherwood Medical Industries, Deland, Fla.).

Isolation of Human Eosinophils or Neutrophils

For all experiments healthy normal volunteers or patients with hypereosinophilia of unknown etiology¹²⁻¹³ who had greater than 70% eosinophilia were administered prednisone (60 mg) orally, and blood samples were drawn in heparinized syringes (20 units heparin/cc blood) at 0 hr (just prior to prednisone administration) and then at 4, 24, and 48 hr thereafter. All subjects were hospitalized at the National Institutes of Health, and all other medications were withheld from the hypereosinophilic subjects during these studies. The heparinized blood was diluted 1:2 in phosphate-buffered saline (PBS); 30-ml aliquots of this PBS-blood suspension were layered on 12 ml of 10.7% Hypaque-6.3% Ficoll cushions and spun at 900 *g* for 40 min at 20°C. The serum-mononuclear cell interface and Hypaque-Ficoll layers were aspirated and discarded. The pellets that contained neutrophils or eosinophils and erythrocytes were pooled, resuspended to the original blood volume in fresh homologous plasma diluted 1:4 in PBS, and then sedimented for 30-45 min in an equal volume of 3% dextran at 24°C. After the erythrocytes had sedimented, the upper granulocyte layer was aspirated and centrifuged at 200 *g* for 10 min at 4°C. The remaining erythrocytes were lysed with 0.2% sodium chloride at 4°C, agitated for 20 sec by vortex, and brought to 0.9% saline by addition of an equal volume of 1.6% saline. The mixture containing only eosinophils and/or neutrophils was then washed once with Hanks' medium without Ca⁺⁺ or Mg⁺⁺ and then resuspended in Gey's medium to a final cell concentration of 2.3×10^6 /ml for use in the chemotactic assays. For the studies in this report, neutrophils were obtained from the normal subjects, and eosinophils were obtained from patients with hypereosinophilia of unknown etiology.

Chemotaxis

A modification of the method described by Zigmond and Hirsch¹¹ for neutrophil chemotaxis was used to measure both neutrophil and eosinophil chemotaxis. For eosinophil chemotaxis only leukocyte preparations containing more than 80% eosinophils were used because of the difficulty of doing differential counts within the filters. Cellulose nitrate filters (3- μ pores, 140 μ thick) were placed in modified Boyden chambers; the cells and samples were introduced, and the chambers were incubated for 35 min at 37°C in 100% humidity and 5% CO₂. After incubation, the filters were rinsed in normal saline, fixed in methanol, and stained with Mayer's hematoxylin and eosin. The filters were dehydrated with increasing concentrations of ethanol, cleared with xylene, and mounted on glass slides with immersion

oil. All samples were assayed in duplicate. Cells were counted at 10- μ intervals within the filters by turning the microscope micrometer to the desired level, and in most experiments the cell numbers at each 10- μ interval beyond 40 μ into the filter were summed. Three high-power fields per filter were counted in this way. The cell counts from duplicate filters were pooled, and the mean and standard error were determined.¹⁴ The means of the sums from these six high-power fields were compared using Student's *t* test. Initial studies established that this technique provided data similar to those seen with more conventional assays of chemotaxis.¹⁵

Endotoxin-Activated Serum

In order to generate chemotactically active components of human serum, *E. coli* endotoxin was added to a 1:10 dilution of fresh frozen serum in veronal buffer (0.145-*M* NaCl, 0.001-*M* sodium barbital, 3×10^6 -*M* barbituric acid, 5×10^{-4} -*M* Mg⁺⁺, 7×10^{-4} -*M* Ca⁺⁺), 30 μ g to each milliliter for a 5% endotoxin-activated serum final preparation. A control solution consisted of a mixture of 30 μ g of endotoxin added to each milliliter of veronal buffer. Both solutions were incubated at 37°C for 1 hr, then at 56°C for 30 min. The endotoxin-activated serum and the control buffer were cooled in ice, and then an equal volume of Gey's tissue culture medium was added to each solution before assaying for chemotactic activity.

Adherence Assay

Adherence was measured by determining the number of leukocytes that passed through a standardized nylon-wool column, as modified from the assay of MacGregor et al.⁷ Each column was prepared by packing 40 μ g of nylon wool into a 0.4-cc volume of a tuberculin syringe. Then 5 cc of whole blood were drawn in a heparinized (20 units/cc) syringe. A 1-cc aliquot was set aside for total white blood cell (WBC) counts and differential counts, and the remaining 40 cc were divided into four equal aliquots, each poured over a separate nylon-wool column. After the columns were allowed to stand 15 min, samples of the effluents and original aliquot were run through a Coulter counter for determination of total leukocytes per milliliter. Additional samples were used for determination of the differential cell counts. The numbers of adherent neutrophils and eosinophils were determined for each of the four aliquots, expressed as mean and standard error of the mean (SEM). Means at 4, 24, and 48 hr after prednisone administration were compared to the means prior to drug administration by the paired-sample *t*-test.

RESULTS

Eosinophil Chemotaxis

The data in Table 1 show that the day-to-day variation in the chemotaxis assay system used was within reasonable limits for both neutrophils and eosinophils. Figure 1 is a representative experiment of eosinophil chemotaxis in response to endotoxin-activated serum before (0 hr) and 24 and 48 hr after prednisone administration. Eosinophil chemotaxis at 4 hr could not be assayed because marked blood eosinopenia occurs at that time. The number of eosinophils per high-power

Table 1. Variation of Chemotaxis From Day to Day

Cell Type*	Days			
	1	2	3	4
Eosinophils	129 \pm 25†	106 \pm 4	111 \pm 15	105 \pm 6
Neutrophils				
A	131 \pm 12	153 \pm 18	—	—
B	178 \pm 18	175 \pm 26	239 \pm 24	—
C	155 \pm 19	193 \pm 18	145 \pm 10	—

*The horizontal rows indicate cells studied from a given donor on the days specified.

†Data are means \pm SEM of six determinations of cells per high-power field that had migrated beyond 40 μ into a 3.0- μ cellulose nitrate filter in response to *E. coli* endotoxin-activated sera.

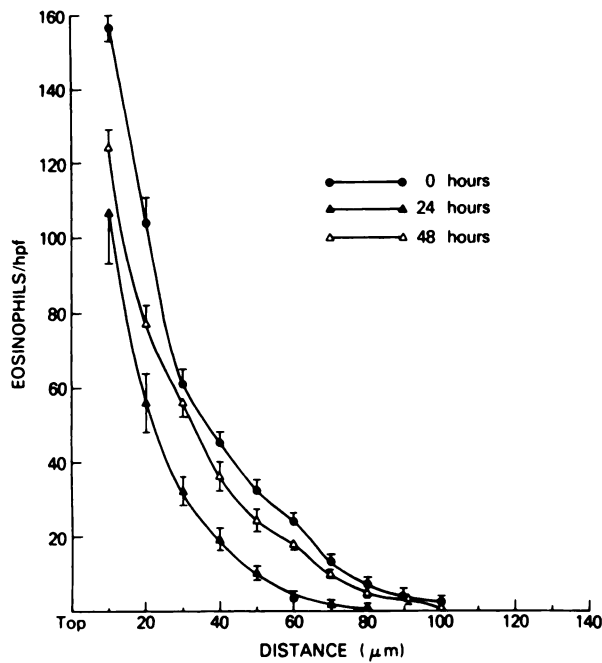


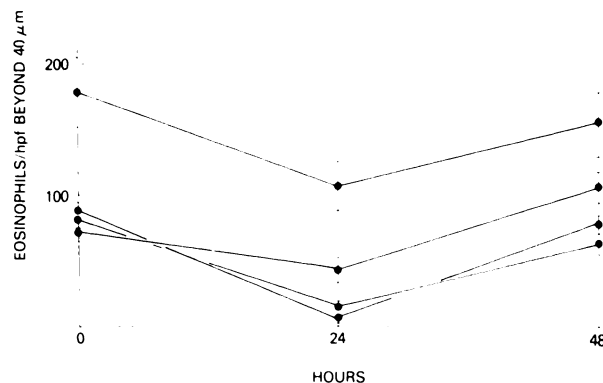
Fig. 1. In vitro eosinophil chemotaxis to *E. coli* endotoxin-activated serum before (0 hr) and 24 and 48 hr after administration of prednisone. Curves represent numbers of cells at varying depths in a 3.0- μ cellulose nitrate filter after 35 min incubation. Mean \pm SEM, six measurements, one experiment.

field that migrated to each level in the filter were markedly decreased 24 hr after prednisone exposure as compared with the number before its administration. By 48 hr the eosinophil migration had almost returned to normal. In Fig. 2 the results from four similar experiments using different patients with hypereosinophilia are compiled. In every case the migration of eosinophils to endotoxin-activated serum was depressed 24 hr after prednisone, but it returned to baseline activity by 48 hr. The depressed locomotion 24 hr after prednisone administration was significant ($p < 0.01$, paired-sample *t*-test).

Eosinophil Adherence

Adherence to nylon-wool columns of eosinophils from the same 4 patients with hypereosinophilia was measured before and after prednisone administration (Fig. 3). At 4 hr the mean eosinophil adherence had decreased significantly ($p < 0.02$),

Fig. 2. Chemotactic responsiveness of human eosinophils obtained from 4 patients with hypereosinophilia before and 24 or 48 hr after prednisone administration (60-mg oral dose). *E. coli* endotoxin-activated serum was the stimulant. Each point represents the mean \pm SEM of six determinations.



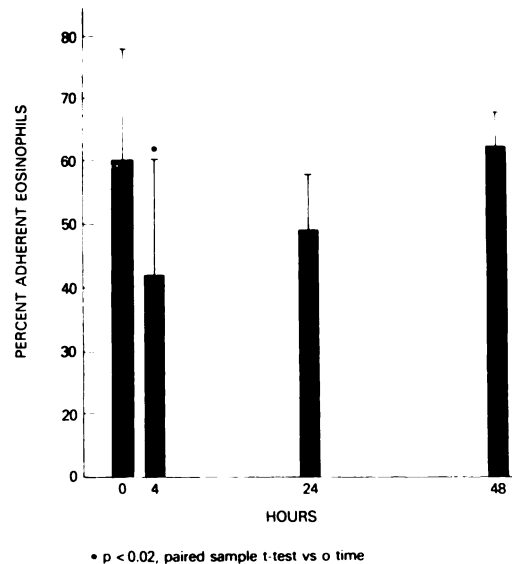


Fig. 3. Adherence of eosinophils present in heparinized peripheral blood to nylon-wool columns before and after administration of prednisone (60 mg/ml). Mean \pm SEM of data obtained using peripheral blood from the same 4 patients with hypereosinophilia as in Fig. 2.

but by 24 hr partial recovery had occurred, and by 48 hr adherence had returned to normal.

Neutrophil Chemotaxis

Although no clear pattern of neutrophil chemotaxis after prednisone administration was observed after testing 7 subjects, one interesting pattern is shown in Fig. 4. This individual's neutrophils showed increased migration to endotoxin-activated serum at both 4 and 48 hr after prednisone administration, and at 24 hr a slight depression from the baseline level is noted. The enhancement at 4 hr and 48 hr was so marked that the cells showed prominent en masse migration into the filter despite the short (35 min) incubation time. This result was confirmed in repeated testing.

Table 2 is a compilation of the seven experiments done on normal volunteers, with the results reported in neutrophils migrating beyond 40μ into the filter in one high-power field. When the means \pm SEM of migration before and after prednisone administration were compared, statistically significant enhancement was

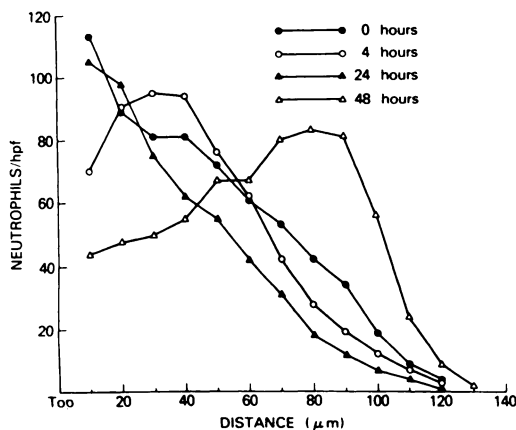


Fig. 4. Neutrophil chemotactic responsiveness to *E. coli* endotoxin-activated serum of cells obtained from a normal subject before and following oral prednisone (60 mg). Data are mean numbers of neutrophils (six determinations) migrating to the indicated depths of a $3.0\text{-}\mu$ cellulose nitrate filter.

Table 2. Neutrophil Chemotaxis After In Vivo Prednisone*

Subject	Hours After Prednisone			
	0	4	24	48
1	297 ± 26	517 ± 39†	199 ± 8	282 ± 6
2	75 ± 8	199 ± 15‡	79 ± 8	261 ± 12‡
3	131 ± 12	151 ± 8	119 ± 15	218 ± 18†
4	314 ± 14	251 ± 32	248 ± 10†	440 ± 13‡
5	145 ± 10	110 ± 20	145 ± 13	147 ± 15
6	153 ± 18	543 ± 23‡	322 ± 33‡	227 ± 16§
7	499 ± 23	387 ± 30§	454 ± 20	596 ± 49

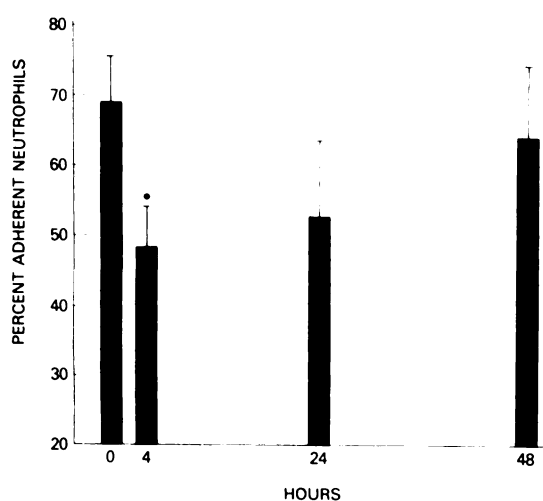
*Neutrophil chemotactic responses to *E. coli* endotoxin-activated sera before (0 hr) and at indicated times after 60 mg of prednisone. Data are quantitated as numbers of cells migrating beyond 40 μ into cellulose nitrate filters (see Methods); mean \pm SEM, six measurements.

† $p < 0.02$; ‡ $p < 0.01$; § $p < 0.001$; significance level of difference of chemotactic response after prednisone compared with the 0-hr control, Student's *t* test.

observed at 48 hr as compared with that before prednisone administration (0 hr) ($p < 0.05$ by the paired *t*-test). At 4 hr after prednisone administration 3 of 7 subjects had significant enhancement of neutrophil chemotaxis, whereas 4 of 7 subjects had no significant change in neutrophil migration. When chemotaxis was observed at 24 hr after prednisone administration, 6 of 7 subjects had either no change or slight diminution in neutrophil migration, and 1 subject had enhanced neutrophil migration.

Neutrophil Adherence

Neutrophil adherence was run in parallel with the chemotaxis assays previously reported, and the data are summarized in Fig. 5. The percentage adherent neutrophils is the mean \pm SEM adherence of blood leukocytes from the 7 normal volunteers. As demonstrated with eosinophil adherence, the neutrophil adherence 4 hr after prednisone administration was significantly depressed ($p < 0.01$), and partial recovery was seen at 24 hr and total recovery at 48 hr.



• $p < 0.01$, paired sample *t*-test vs 0 time

Fig. 5. Adherence of peripheral blood neutrophils to nylon-wool columns before and following oral prednisone (60 mg). Mean \pm SEM from seven subjects.

DISCUSSION

After glucocorticosteroid treatment of patients with allergic rhinitis or asthma, blood eosinophil counts fall, and tissue localization of eosinophils diminishes.¹⁶ One possible explanation for this resolution of blood and tissue eosinophilia is inhibition of the release of mediators that attract eosinophils, such as eosinophil chemotactic factor of anaphylaxis (ECF-A)¹⁷ and histamine.¹⁴ Glucocorticosteroids, however, do not inhibit the release of mediators from basophils and mast cells,¹⁸ the major reservoir of the eosinophil chemoattractant agents.^{19,20}

Another possible mechanism for the reduction in tissue and blood eosinophils seen after glucocorticoid therapy is inhibition of their response to chemoattractants, either through a direct inhibition of adherence and/or locomotion or a general depression of cell metabolism. Therefore the adherence and chemotaxis of eosinophils from several patients with the hypereosinophilic syndrome¹² were assayed *in vitro* before and 4, 24, and 48 hr after prednisone administration. As shown in Figs. 3 and 4, prednisone causes a profound effect on both adherence and chemotaxis of eosinophils. Whether this is a direct effect or is secondary to a circulating mediator released by prednisone is not known. Furthermore, additional studies are necessary to determine whether the *in vivo* effects of prednisone represent drug effects on the cell or enrichment within the circulation of a subpopulation of nonmigrating cells. In this regard, it is of interest that prednisone *in vitro* inhibits eosinophil adherence and chemotaxis, although relatively high concentrations are required.²¹

The hematologic kinetics of neutrophils after glucocorticoid administration are quite distinct from the response seen with eosinophils in that a brisk neutrophilia occurs within 4 hr after prednisone administration.²² Despite this rise in blood neutrophils there is a profound decrease in tissue neutrophil accumulation to infectious and inflammatory insults.^{5,22,23} Both of these steroid effects, however, might be explained by diminished neutrophil adherence to endothelial cells. Over 20 yr ago several *in vivo* studies demonstrated that corticosteroids reduce granulocyte adherence to vascular endothelium.^{23,24} Recently, granulocytes from patients and volunteers who had received prednisone have been shown *in vitro* to have reduced adherence to nylon-wool columns for 2–4 hr after drug administration.⁷ The results shown in Fig. 5 confirm that *in vivo* prednisone reduces *in vitro* neutrophil adherence for 24 hr. Reduced tissue localization of neutrophils also might be the result of sluggish neutrophil movement. Whether or not steroids affect neutrophil migration has been a matter of controversy for a decade, but most recent studies show little or no effect when using *in vitro* chemotactic chambers and normal pharmacologic concentrations of glucocorticoids.^{25,26} However, other studies from our laboratory have shown that steroids block chemotactic-factor-induced changes in neutrophil surface charge,²⁷ which may be important in increased cell adhesiveness caused by chemotactic factors.^{28,29} Our findings in Fig. 4 confirm that prednisone has little inhibitory effect on *in vitro* neutrophil chemotaxis.

Although some cell-to-substrate adherence is probably necessary for cell migration, these findings suggest that a significant decrease in adherence can occur without interfering with chemotaxis. Why this dichotomy should exist in neutrophil activity but not in eosinophil function is not known; however, reduced tissue localization of both cell populations is most likely secondary to their reduced adherence to endothelial cells. The clinical effect of the decreased eosinophil localization is not known since their general function is poorly understood;³⁰

however, the absence of a neutrophil exudate in patients treated with glucocorticoids probably contributes significantly to their inability to cope with infection.

It should be emphasized that the eosinophils used in the present study were taken from patients with the hypereosinophilic syndrome, which may in effect be a reflection of abnormal eosinophils. However, since it is extremely difficult because of technical constraints to obtain eosinophils in sufficient numbers from normal individuals, the present source of eosinophils was used of necessity. Still, useful information regarding the differential effects of glucocorticoids on eosinophils and neutrophils was obtained by this approach.

The different patterns of *in vitro* chemotaxis of eosinophils from patients with hypereosinophilia and that of neutrophils from normal subjects may relate to the eosinopenia and neutrophilia seen after glucocorticoid administration. For example, patient eosinophils, but not normal neutrophils, may lose their ability to leave the marrow after steroid therapy, and this would result in eosinopenia. This mechanism of eosinopenia, in fact, has been suggested in a horse model, since an increase in bone marrow eosinophils was noted after cortisol infections.³¹ Alternatively, glucocorticoids may have different effects on the margination of subpopulations of neutrophils³² and eosinophils.³³ Delineation of the mechanisms of the observed glucocorticoid effects on neutrophil and eosinophil functions is currently under study.

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