

Effects of prednisone on the cellular responses and release of cytokines and mediators after segmental allergen challenge of asthmatic subjects

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Background: Systemic glucocorticoids are a major therapy for the management of allergic inflammation and asthma; however, information about their effects *in vivo* are limited.

Objective: This study was performed to examine the effects of prednisone on inflammatory mediators, cytokines, and cellular responses in the model of segmental allergen challenge (SAC) of allergic asthmatic subjects.

Methods: The effects of a 3-day pretreatment with oral prednisone (30 mg twice daily) on the physiologic and inflammatory responses to SAC were studied in 10 allergic asthmatic subjects in a double-blind, placebo-controlled, crossover protocol.

Results: Prednisone improved baseline FEV₁ by 10% and modestly inhibited the SAC-induced fall in FEV₁ at 30 minutes and at 6 to 8 hours. Five minutes after challenge, levels of histamine, PGD₂, 9 α ,11 β -PGF₂, and thromboxane B₂ increased in bronchoalveolar lavage fluid (median increase, 5- to 14-fold); prednisone did not inhibit these responses. Prednisone inhibited (median decrease, 66%-97%) the total influx of inflammatory cells, specifically eosinophils, basophils, and some subsets of T lymphocytes (CD4, CD45RA, and CD45RO cells) assessed 19 hours after SAC, but it did not inhibit the influx of neutrophils. Increases in soluble E-selectin, kinins, and albumin were also inhibited by the glucocorticoid (median decrease, 36%-74%). Prednisone treatment inhibited the appearance of mRNA, protein, or both for T_H2 cytokines (IL-4 and IL-5), as well as for IL-2 and transforming growth factor α , but did not inhibit increases of immunoreactive GM-CSF in bronchoalveolar lavage fluid.

Conclusion: These studies indicate that prednisone suppresses multiple components of allergic airway inflammation, including cell recruitment, adhesion molecule expression or release, airway permeability, and production of cytokines potentially

involved in airway immunity or remodeling. (*J Allergy Clin Immunol* 2001;108:29-38.)

Key words: Glucocorticoids, inflammation, asthma, cytokines

Glucocorticoids have been available for the treatment of asthma for almost 50 years and are still the most effective medication for this condition. Knowledge of the mechanism of glucocorticoid action in asthma has increased tremendously.¹ Early studies demonstrated that glucocorticoids caused an improvement in FEV₁ within hours of administration, and these drugs are now routinely used for the treatment of severe exacerbations of asthma. Prolonged treatment with oral or inhaled glucocorticoids has been shown to improve airway hyperreactivity and reduce inflammatory cells in the airways, including mast cells, eosinophils, and lymphocytes.²⁻⁴ Because asthma is now recognized to be a disease of inflammation, this reduction in inflammatory cells is believed to be a key glucocorticoid effect. Other effects of glucocorticoids on the pathology of asthma, which may or may not result from a reduction of inflammatory cells, include reduced deposition of matrix proteins (eg, tenascin) in the region of the basement membrane, reduced vascular leak and appearance of macromolecules in the airways, and reduced inflammatory responses of resident cells (eg, endothelial cells, epithelial cells, and others).⁵⁻⁷

The mechanism or mechanisms by which glucocorticoids inhibit the recruitment and accumulation of inflammatory cells in the airways is still a matter of debate. One hypothesis is that glucocorticoids disrupt this process by suppressing the activation of cells that produce the cytokines that mediate the process, including those that activate endothelium (eg, IL-1, TNF, IL-4, and IL-13), those that prime eosinophils and maintain their survival (eg, IL-5, IL-3, and GM-CSF), and those that induce the migration of lymphocytes, eosinophils, and basophils into the airway (eg, the chemokines RANTES; monocyte chemoattractant protein 3; monocyte chemoattractant protein 4; eotaxin 1; and perhaps eotaxin 2).⁸ Although there are some studies that provide support for this hypothesis, more information is needed. Robinson et al⁹ showed that prednisone treatment reduced the appearance

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Abbreviations used

BAL:	Bronchoalveolar lavage
FVC:	Forced vital capacity
PNU:	Protein nitrogen units
RANTES:	Regulated upon activation, normal T cell expressed and secreted
SAC:	Segmental allergen challenge
TGF- α :	Transforming growth factor α

of cells expressing mRNA for IL-4 and IL-5, as determined with *in situ* hybridization. The bulk of these cells were probably T cells, as determined with double staining, with some eosinophils and mast cells also positive for these mRNAs. Wang et al^{10,11} have shown a reduction of GM-CSF and RANTES in the airways, as detected by means of immunohistochemistry, in glucocorticoid-treated subjects. A study by Sim et al,¹² using a challenge model in the upper airways, has demonstrated a reduction of several inflammatory cytokines after glucocorticoid treatment (including IL-1 β , GM-CSF, macrophage inflammatory protein 1 α , IL-8, and RANTES). A more recent study by Naseer et al¹³ has shown that glucocorticoid treatment of asthmatic subjects results in a decrease in IL-13-expressing cells and an increase in IL-12-expressing cells, as detected with immunohistochemistry.¹³ This finding is of great interest because IL-12 has been shown to be a potent and effective inhibitor of allergic airways responses, probably in part as a result of its ability to induce large quantities of IFN- γ .^{14,15}

The purpose of the present study was to use a segmental lung antigen challenge model to further delineate the effects of oral glucocorticoids on inflammatory cell populations and the appearance of inflammatory mediators, especially cytokines, in the lungs.¹⁶ A randomized, double-blind, placebo-controlled, crossover trial of the effects of treatment with prednisone, 30 mg twice daily, for 3 days on antigen-induced inflammation was performed to assess the effects of glucocorticoids. Inflammation was induced experimentally by means of segmental allergen challenge (SAC), and the response was assessed by means of bronchoalveolar lavage (BAL). This study demonstrates that glucocorticoids have profound effects on the influx of inflammatory cells and plasma proteins into the airways and that some, but not all, cytokines and chemical mediators of inflammation are dramatically diminished by glucocorticoids.

METHODS**Subjects**

Ten allergic asthmatic subjects, 6 men and 4 women (4 African American and 6 white subjects) ranging in age from 22 to 44 years, were studied in a protocol approved by the Johns Hopkins Bayview Medical Center Institutional Review Board. All subjects had mild disease defined clinically by a history of episodic dyspnea and wheezing provoked by exercise, allergen exposure, or both and controlled with intermittent use of inhaled bronchodilators. Subjects were reactive to methacholine at a concentration of less than 2.5 mg/mL, had a positive immediate-type skin test response to the allergen used for local airway challenge, and were not preselected for a late-phase response.

Allergen sensitivity, determined by endpoint skin test titration to achieve a wheal of 5 to 10 mm and erythema of 21 to 30 mm, ranged from 10⁻⁵ to 10 protein nitrogen units (PNU)/mL (mean log PNU per milliliter, -2.1 ± 0.7). In the ragweed preparation 1 PNU is equivalent to 0.005 μ g of ragweed antigen E (Amb a 1).

Bronchoscopy with BAL and SAC

Bronchoscopy was performed in accordance with National Institute of Health guidelines and previous studies.¹⁶ Briefly, subjects were premedicated with intravenous atropine (0.6 mg) and fentanyl (0.1 mg) after achievement of local anesthesia with lidocaine (4%) inhaled through a nebulizer. By using instilled 2% lidocaine, an Olympus BF 10 bronchoscope was wedged into the anterior segment of the right upper lobe, and BAL was performed with five 20-mL aliquots of normal saline followed by immediate aspiration of each aliquot (baseline). To assess the immediate response to challenge with normal saline, the bronchoscope was wedged into a segment of the lingula, 5 mL of normal saline was instilled, and, after 5 minutes, a small lavage sample of 20 to 40 mL was obtained. To assess the immediate response to allergen, the bronchoscope was wedged into a segment of the right middle lobe, 5 mL of ragweed (Greer, Lenoir, NC) or Timothy grass (Miles Inc, Elkhart, Ind) allergen at a concentration of 20 or 100 PNU/mL was instilled, and, after 5 minutes, a small lavage sample of 20 to 40 mL was obtained. Each subject was challenged with the same dose of antigen during both placebo and prednisone periods. A total of 9 subjects were challenged with ragweed (4 with 100 PNU/mL and 5 with 20 PNU/mL), and 1 subject was challenged with Timothy grass (20 PNU/mL). Allergen dose was reduced after challenge of the initial 4 subjects because of concerns about provoking asthma symptoms and an unacceptable decline in lung function in 2 subjects. After 18 to 21 hours, a second bronchoscopy was performed to assess the late inflammatory responses to challenge, and the segments previously challenged with normal saline and allergen underwent lavage with 5 or 6 $\times$ 20-mL aliquots of normal saline.

Glucocorticoid study protocol

In this double-blind, placebo-controlled, crossover study subjects received 3 days of study drug, prednisone 30 mg twice daily, or placebo at 8 AM and 8 PM, as randomized by an investigator who did not participate in the study. All measurements were made without prior knowledge of drug administration. Before starting the drug, blood was obtained for a complete blood count, differential, and total eosinophil count. After 5 doses, bronchoscopy with SAC was performed between 1 and 4 PM, and the last dose of drug was taken at 8 PM. The final dose of study drug was taken 5.3 ± 0.2 hours after antigen challenge during the placebo period and 5.6 ± 0.2 hours after challenge during the prednisone period. Repeat bronchoscopy was performed the next morning between 9 AM and 12 PM, 18 to 21 hours after SAC. The time between bronchoscopies for challenge and BAL averaged 19.4 ± 0.3 hours for placebo and 19.3 ± 0.2 hours for prednisone. Blood for complete blood counts, differentials, and total eosinophil counts was obtained just before each bronchoscopy. Blood T-cell analysis by means of flow cytometry was performed on cells obtained at the time of the second bronchoscopy. After a 5- to 7-week washout period (5.7 ± 0.2 weeks), subjects were crossed over to the other drug, and the entire protocol was repeated.

Two subjects reported fevers the evening after the second bronchoscopy and were treated with amoxicillin. Two subjects had a significant decline in lung function (FEV₁ fell greater than 50% of predicted shortly after the first bronchoscopy with allergen challenge), which responded promptly to inhaled bronchodilator administration. One of these individuals was treated with prednisone (40 mg daily) and amoxicillin for 3 days after completion of each part of the study without further complications.

Serial pulmonary function tests

Spirometry was performed by using a Stead-Wells, water-sealed, volume-displacement spirometer (Warren E. Collins, Braintree, Mass), recording the best of 3 attempts at each time. Measurements were taken before and after each bronchoscopy, every hour for 8 hours, and then again at 18 hours after SAC.

Cell analysis in blood and BAL fluids

EDTA-treated blood was sent to the Johns Hopkins Bayview Medical Center clinical laboratory for complete blood count, a differential cell count with Wright stain, and a total eosinophil count (Unipette method). PBMCs separated by means of Ficoll Hypaque density gradient centrifugation were analyzed for T-cell numbers and subpopulations by means of flow cytometry, as described below.

BAL cell differential and mast cell-basophil enumeration

The volume of fluid recovered from all lavages was recorded, and the fluid was filtered through 2 layers of gauze. Analysis of cellular components was only performed on large, baseline, or late-phase lavages of 100 to 120 mL. Small BAL samples of 20 to 40 mL obtained 5 minutes after saline and allergen challenge were examined only for histamine and prostanoids. Cell enumeration on BAL fluids was performed as previously described.¹⁶ Differential cell counts were performed after Diff-Quik staining (American Scientific Products, McGaw Park, Ill) of cytocentrifuge preparations (Cytospin; Shandon Southern Instruments, Inc, Sewickley, Pa). Separate cytocentrifuge slides were stained with alcian blue for mast cell-basophil enumeration. In this method 10,000 to 15,000 cells are scanned, and the number of alcian blue-positive cells is identified.

Lymphocyte enumeration by means of flow cytometry

Monoclonal antibodies that recognize the following surface markers were used to evaluate the lymphocyte subpopulations in the blood and BAL fluid: CD2, CD3, CD4 (T-helper population), CD8 (T-suppressor population), CD45 (panleukocyte marker), CD45RO (memory cells), CD45RA (naïve cells), and IgG₁ and IgG_{2a} controls (Becton Dickinson, San Jose, Calif).

Lymphocytes from PBMCs and BAL fluid were selected on the basis of CD45, CD3, and appropriate scatter characteristics. T-lymphocyte subsets were identified by means of dual immunofluorescent cell staining with mAbs directly conjugated to either FITC or phycoerythrin. Aliquots containing approximately 1×10^6 cells were incubated in PBS with 4 mg/mL human IgG to minimize nonspecific binding to FcγR. After antibody labeling, the cells were washed and fixed with 1% paraformaldehyde before analysis. An EPICS profile flow cytometer was used to analyze fluorescent signals after excitation at 488 nm, and at least 1000 lymphocyte events were analyzed for each marker.

The percentage of T lymphocytes was determined as the ratio of CD3⁺ or CD2⁺ cells per ungated CD45⁺ cell (total leukocytes).

The total number of T lymphocytes was calculated as the product of the percentage of T cells determined by means of flow cytometry times the total number of cells on the basis of hemacytometer counts (see above). Total numbers of T-lymphocyte subsets were similarly calculated.

Measurement of prostanoids, histamine, kinins, and albumin

These measurements were performed as previously described.¹⁶ After centrifugation (10 minutes at 400g), 1- to 4-mL aliquots of cell-free BAL fluid were spiked with 1.0 to 4.0 ng of 3,3,4,4-tetradeuterated analogs of PGE₂, PGD₂, PGF_{2α}, thromboxane B₂, and 6-keto-

PGF_{1α} as internal standards for quantitation of prostanoids by using previously published methods involving solid-phase extraction, derivatization, and analysis with combined capillary gas chromatography-negative ion chemical ionization mass spectrometry.¹⁶ Histamine was measured by using a radioenzymatic assay with a lower limit of detection of 0.1 ng/mL. Kinins were measured by using an RIA with a limit of detection of 40 pg/mL. Albumin was quantified by using an RIA with an assay sensitivity of 1 ng/mL.

Cytokine measurements

Concentration of BAL was performed by using Centrprep 3 ultrafiltration units (1000-d molecular weight cutoff; Amicon, Lexington, Mass). BAL fluids were concentrated 6- to 34-fold (16.5 ± 0.9 -fold [mean $\pm$ SD], $n = 53$) from starting volumes of 9 to 47 mL (25.6 ± 1.3 mL, $n = 53$). In one patient BAL returns were so poor that no concentrated fluids were available; one baseline sample was lost in processing. Hence only paired samples of concentrated BAL fluids from 9 subjects were available for analysis. Note that all values derived from concentrated samples were corrected and expressed per milliliter of unconcentrated BAL fluid.

Cytokines were measured in BAL fluids by using either in-house or commercial ELISA or RIA methods. Transforming growth factor α (TGF- α), GM-CSF, and IL-5 were measured in concentrated BAL fluids ($n = 9$ subjects) by using assays with mean limits of detection of 7, 4, and 2 pg/mL, respectively. IL-4 was measured in samples of unconcentrated BAL fluid ($n = 10$ subjects) by using an ultrasensitive IL-4 ELISA with a mean limit of detection of less than 1 pg/mL. IL-2 was measured in samples of concentrated BAL fluids ($n = 7$ subjects) by using an ELISA with a mean limit of detection of less than 1 pg/mL. IFN- γ was measured in samples of concentrated BAL fluid ($n = 6$ subjects) by using an ELISA with a mean limit of detection of less than 0.6 pg/mL BAL. Assay kits used were as indicated in parentheses: TGF- α (RIA; Biomedical Technologies Inc, Stoughton, Mass); GM-CSF (R&D Inc, Minneapolis, Minn); IFN- γ (Biosource, Camarillo, Calif); IL-5 (RIA; Dr David Proud, reagents kindly provided by Dr Richard Cook, SmithKline Beecham); and IL-4 (Biosource). Each fluid was assayed at 1 to 3 dilutions in duplicate, and the result for each sample was calculated by averaging the results obtained from all dilutions, followed by correction for concentration. When levels of cytokines were not detected, half the lower limit of detection was used for statistical analysis.

Competitive RT-PCR analysis of cytokine gene expression for IL-2, IL-4, IL-5, and IFN- γ

In 6 subjects total RNA was extracted from BAL cells by using the RNeasy method (Tel-Test, Friendswood, Tex). Cytokine-specific mRNA expression was analyzed by performing RT-PCR in which a known amount (1 pg) of cytokine-specific internal standard cRNA (competitor) was added to 1 μ g of total RNA extracted from BAL cells (target). Specific reagents, RT-PCR conditions, and detailed methods for analysis of cytokine gene expression by using competitive RT-PCR with cytokine-specific cRNA internal standards for IL-4, IL-5, and IFN- γ have been described previously.¹⁷ Molecular sizes of cytokine-specific PCR products and internal standards, respectively, were as follows: IL-2, 458 and 630 bp; IL-4, 456 and 696 bp; IL-5, 293 and 386 bp; and IFN- γ , 494 and 627 bp. In some cases during IL-4 mRNA analysis, a second target band of smaller size was observed. Only the major band was used for analysis of target/competitor ratios. Samples were initially screened for integrity of mRNA by using RT-PCR amplification of β -actin, which was easily detected in all samples.

E-selectin assay

Concentrated BAL fluids ($n = 9$ subjects) were analyzed for E-selectin by using a sandwich ELISA. The sandwich ELISA was per-

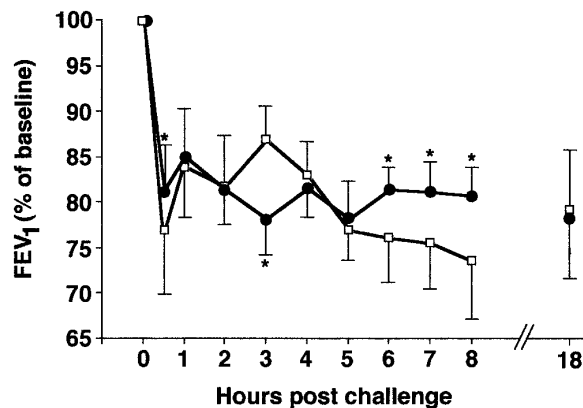


FIG 1. Pulmonary function after SAC of subjects treated with placebo (open squares) or prednisone (filled circles). Data are means $\pm$ SEM at indicated times from 9 subjects. * $P < .05$, prednisone versus placebo.

formed by using 2 specific anti-E-selectin antibodies (3B7 and 7A9) that define nonoverlapping epitopes of the molecule, as previously described.¹⁸ Results are expressed as picograms per milliliter of unconcentrated BAL fluid.

Statistical methods

By using the skewness and kurtosis test for normality, about 60% of the data were not normally distributed. Therefore comparisons of data from measures of cells, mediators, cytokines, and adhesion molecules were performed by using a nonparametric Wilcoxon signed-rank test. Results are presented as medians with full or interquartile ranges or as means $\pm$ SEM as specified. A P value of .05 or less was considered significant. Correlations were assessed by using a Spearman rank correlation coefficient. Differences in pulmonary function tests and serial spirometry after SAC were analyzed by means of ANOVA, followed by post-hoc paired t tests with super ANOVA (Abacus Concepts, Inc, Berkeley, Calif).

RESULTS

Lung function

Spirometry improved during prednisone treatment, with FEV₁ increasing (placebo, 3.02 ± 0.24 L and $79.2\% \pm 4.3\%$ predicted; prednisone, 3.33 ± 0.20 L and $88.1\% \pm 3.5\%$ predicted; $P = .007$), forced vital capacity (FVC) remaining unchanged (placebo, 4.13 ± 0.27 L and $89.4\% \pm 3.6\%$ predicted; prednisone, 4.17 ± 0.24 L and $92.0\% \pm 4.7\%$ predicted), and FEV₁/FVC percentage increasing (placebo, $72.8\% \pm 2.5\%$; prednisone, $79.9\% \pm 1.5\%$; $P = .013$).

Changes in lung function after SAC are displayed in Fig 1. These data are expressed as the percentage of baseline just before SAC during each treatment to correct for improved lung function during prednisone treatment. During placebo treatment, mean FEV₁ fell 23% immediately after SAC, improved to a maximum within 14% of baseline at 3 hours, and fell steadily from hours 4 to 8 to a nadir of 74% of baseline at 8 hours. This pattern was reminiscent of a dual response, with the late phase observed after whole-lung allergen challenge. Prednisone treatment had only a modest effect on the early response, slightly dampening both the acute fall and recovery of

FEV₁ in the first 3 hours. The clearest effect of prednisone on lung function was a significant improvement in FEV₁ at hours 6 to 8, which is consistent with inhibition of the late-phase response to allergen challenge. FVC revealed significant improvements during prednisone treatment at hours 1, 2, 6, 7, and 8 (not shown). In 8 normal, nonallergic subjects challenged with ragweed, mean FEV₁ and FVC fell 6% and 10%, respectively, immediately after SAC (data not shown). In previous studies of BAL alone in normal, allergic rhinitic, and allergic asthmatic subjects, mean FEV₁ fell less than 6%.¹⁹ These data suggest that most of the change in lung function after SAC of allergic asthmatic subjects is due to specific allergen-induced changes in sensitized individuals.

Mediators

A number of mediators of inflammation were monitored in BAL fluids after airway challenge. Mean fluid recoveries in BAL performed 5 minutes after challenge ranged from 17% to 25%, with mean recoveries of BAL fluid performed at 19 hours ranging from 35% to 38%. As shown in Tables I and II, histamine, PGD₂, 9 α ,11 β -PGF₂, and thromboxane B₂ levels were increased within 5 minutes of SAC, and prednisone pretreatment had no significant effect on the appearance of these mediators. The increase in PGE₂ with prednisone treatment is of doubtful significance because of the low levels detected. Similarly, levels of PGD₂, PGE₂, PGF_{2 α} , 9 α ,11 β -PGF₂, and thromboxane B₂ were increased during the late response to SAC but were largely unaffected by prednisone. The significant decrease in 9 α ,11 β -PGF₂ after prednisone is of questionable significance because of the low levels measured. Levels of kinins and albumin were also increased by SAC (Table II). Kinins are peptide mediators that can induce vascular permeability, the appearance of which is also linked to plasma leakage. Albumin can serve as a marker of plasma exudation in the lung. SAC caused increases in median levels of albumin and kinin of 6- and 10-fold, respectively, and prednisone treatment inhibited these increases by 60% ($P = .01$) and 74% ($P = .03$), respectively. Taken together, the results suggest that the protective effect of steroids on plasma exudation during SAC is not caused by inhibition of histamine or prostanoid mediators but is more likely related to the protective effects on target cells for these mediators, inhibition of inflammatory cell influx, or both.

Blood cells

As a measure of adherence to the medication protocol, peripheral blood leukocytes were quantitated. Total blood leukocyte counts were increased approximately 80% by glucocorticoid treatment (6.9 ± 0.6 vs $12.2 \pm 1.2 \times 10^3/\text{mm}^3$) as a result of steroid-induced neutrophilia of nearly 3-fold. Lymphocyte numbers were suppressed by 56%, and eosinophil numbers were suppressed by greater than 99% (total eosinophils, 332 ± 49 vs $2 \pm 1/\text{mm}^3$ after placebo and prednisone, respectively). At the time of the post-SAC BAL, the total leukocytes had increased (29%) from the level before challenge in the placebo period,

TABLE I. Effects of prednisone on BAL mediators 5 minutes after allergen challenge (n = 9 subjects)

	Placebo		Prednisone	
	Saline	Allergen	Saline	Allergen
Histamine (ng/mL)	2.1 (0.8-7.9)	9.4* (0.4-58.8)	2.3 (0.6-5.0)	7.5* (2.9-28.4)
Eicosanoids (pg/mL)				
PGD ₂	6.3 (0.8-49.2)	51.9* (0.6-1474.5)	2.1 (0-38.5)	17.6* (0-1338.4)
PGE ₂	0 (0-8.2)	0.2 (0-2.8)	0 (0-2.3)	0.1* (0-21.2)
PGF _{2α}	0.7 (0-4.6)	1.4 (0-4.2)	1.2 (0-5.4)	2.1 (0-7.3)
9α,11β-PGF ₂	0.2 (0-5.6)	2.7* (0.2-43.9)	0.3 (0-4.8)	0.9* (0-117.7)
6-keto-PGF _{1α}	0 (0-0.1)	0 (0-2.8)	0 (0-0)	0 (0-0.8)
Thromboxane B ₂	0 (0-14.6)	3.4* (0-33.7)	1.0 (0-5.9)	2.2 (0-30.3)

*P < .05, allergen versus saline.

TABLE II. Effects of prednisone on BAL mediators 19 hours after allergen challenge (n = 9 subjects)

	Placebo		Prednisone	
	Saline	Allergen	Saline	Allergen
Histamine (ng/mL)	1.1 (0.3-4.5)	1.6 (0.7-5.0)	0.8 (0.4-1.9)	1.6* (0.6-4.5)
Eicosanoids (pg/mL)				
PGD ₂	2.6 (0-28.4)	8.2* (0.2-190.8)	1.4 (0-10.7)	4.9* (0-144.5)
PGE ₂	0.1 (0-1.9)	1.5* (0-35.6)	0.1 (0-0.8)	0.3 (0-2.7)
PGF _{2α}	0.5 (0-1.3)	1.4* (0-22.1)	0.5 (0-1.7)	0.7 (0-2.7)
9α,11β-PGF ₂	0.2 (0-1.0)	0.6* (0-10.5)	0.2 (0-0.5)	0.2† (0-4.6)
6-keto-PGF _{1α}	0 (0-0.1)	0 (0-14.3)	0 (0-0)	0 (0-1.7)
Thromboxane B ₂	0.9 (0-6.6)	3.8* (0-43.1)	0.7 (0-6.3)	2.4 (0-59.9)
Kinins (pg/mL)	285 (49-608)	2734* (423-31,250)	408 (49-770)	710† (405-4055)
Albumin (μg/mL)	53 (16-120)	291* (55-1625)	51 (18-111)	117*† (18-405)

*P < .05, allergen versus saline.

†P < .05, prednisone versus placebo.

which was caused largely by a 55% increase in neutrophils. No SAC-induced neutrophilia was observed during the prednisone period.

Analysis of blood T-cell subsets by means of flow cytometry demonstrated a fall in the percentage of CD3⁺ T cells from 51.0% ± 5.2% with placebo to 42.5% ± 4.6% with prednisone (*P* = .018), which was caused largely by a fall in CD4⁺ T helper cells from 31.2% ± 3.1% to 24.1% ± 2.9% (*P* = .011). CD8⁺ suppressor-cytotoxic T cells were unchanged (placebo, 12.7% ± 1.6% vs prednisone, 9.7% ± 1.8%). CD45RA levels were slightly suppressed (52.4% ± 4.4% vs 42.7% ± 3.7%, *P* = .066), and CD45RO numbers were slightly enhanced (34.1% ± 3.8% vs 46.5% ± 4.0%, *P* = .05) in subjects treated with prednisone.

BAL cells

The pattern of all cell types observed in BAL fluids at baseline and 19 hours after SAC is displayed in Fig 2. For reference, total cell recovery in baseline BAL during placebo was 13.6 ± 3.1 × 10⁶ cells (median, 9.3 × 10⁶; range, 4.4-35.6 × 10⁶), consisting of 84.8% ± 3.1% macrophages, 10.7% ± 2.2% lymphocytes, 1.9% ± 0.3% neutrophils, 2.1% ± 0.8% eosinophils, 0.6% ± 0.02% bronchial cells, 0.068% ± 0.015% mast cells, 0.006% ± 0.003% indeterminate cells, and 0.005% ± 0.003% basophils (Fig 2, *top left*). Prednisone treatment had no significant effects on cell numbers in baseline BAL fluid.

In saline-challenged sites the number of neutrophils was increased compared with baseline values during both treatment periods, which is consistent with a nonspecific effect of saline challenge or the bronchoscopic procedure itself (Fig 2, *top right*). Results of basophil and mast cell enumeration in baseline and normal saline controls are not shown because they were negligible (see above).

Allergen challenge induced marked changes in cellular infiltrate (Fig 2, *lower panels*), with increases in medians of total cells (4-fold), neutrophils (8-fold), eosinophils (47-fold), lymphocytes (3-fold), and basophils (65-fold) compared with that of normal saline control challenge. Prednisone pretreatment reduced the median number of total cells infiltrating the airways after antigen challenge by 38% (*P* = .04), mainly because of the 95% reduction of eosinophils (*P* = .005). The median basophil number was reduced by 97% (*P* = .005). Analysis of T-cell subsets revealed no changes from baseline in saline-challenged sites; however, SAC caused a 4-fold increase in the median number of T cells, which was accounted for largely by a 5-fold increase in the median number of CD4⁺ cells, predominantly of the CD45RO⁺ memory type (Fig 3). Prednisone treatment suppressed the increase in lymphocytes in BAL fluids; this effect was significant for CD4⁺, CD45RO⁺, and CD45RA⁺ subsets of lymphocytes (*P* = .02-.04) and approached significance for total T cells (CD3⁺) and CD8⁺ cells (*P* = .066).

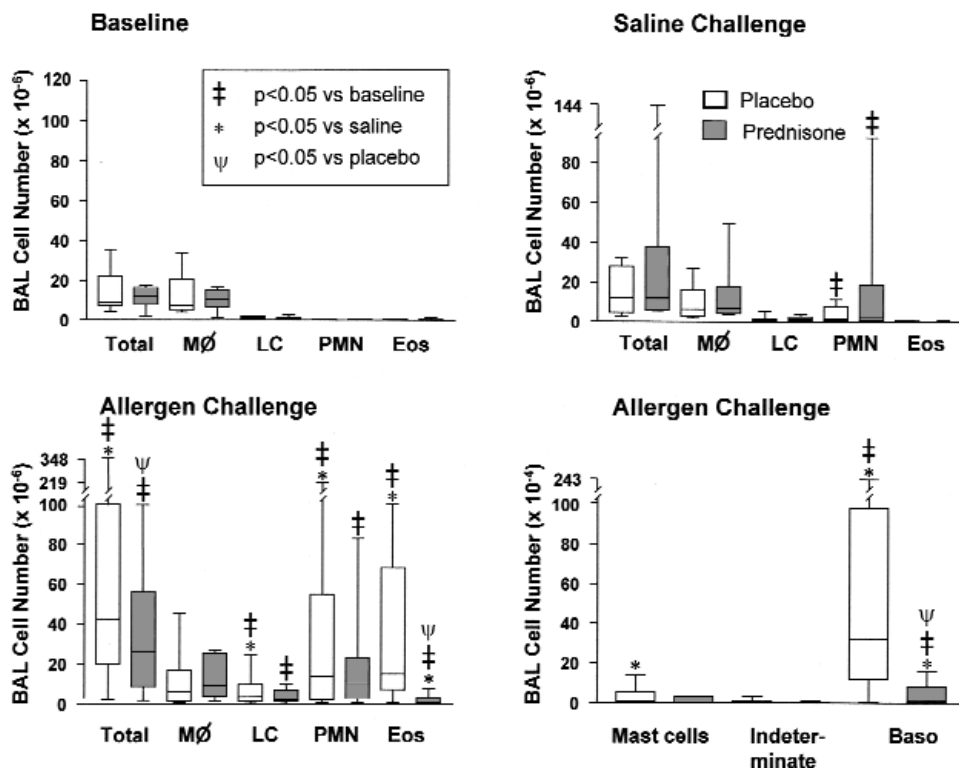


FIG 2. Cells recovered in BAL fluids from placebo- and prednisone-treated individuals before challenge (baseline) and 19 hours after challenge with saline or allergen. Data shown are median and interquartile ranges of values from 10 subjects. *Mφ*, Macrophages; *LC*, lymphocytes; *PMN*, neutrophils; *Eos*, eosinophils; *Baso*, basophils.

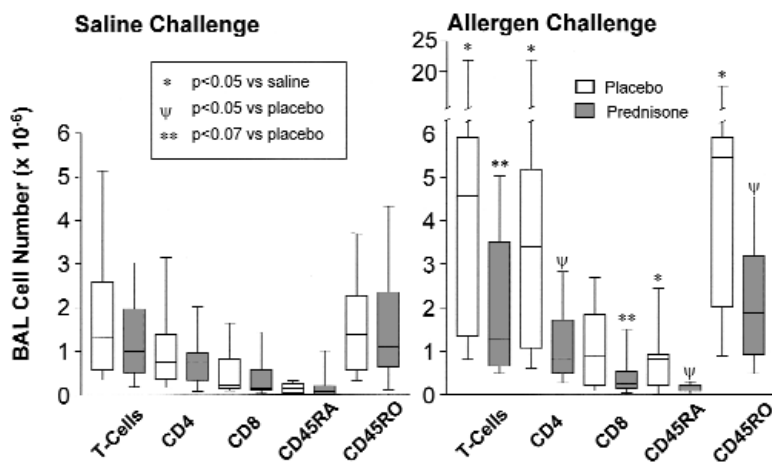


FIG 3. Flow cytometric analysis of lymphocytes in BAL fluid after saline (left) or allergen (right) challenge of placebo- and prednisone-treated individuals. Data shown are median and interquartile ranges of values from 9 subjects.

Cytokines and soluble E-selectin

Molecules with putative roles in cellular recruitment (E-selectin), fibrosis (TGF- α), eosinophil survival (GM-CSF), and immunoregulation (the T_H1 products IL-2 and IFN- γ and the T_H2 products IL-4 and IL-5) were assessed by measurements of protein in BAL fluids and mRNA

expression in BAL cells (Fig 4). SAC resulted in increased levels of IL-2, IL-4, and IL-5 protein in BAL fluids, reaching statistical significance for IL-2 and IL-5 (Fig 4, *second row*). IFN- γ levels were undetectable in BAL fluid. The released cytokine levels were characterized by large variations among individuals, and comparison of mean

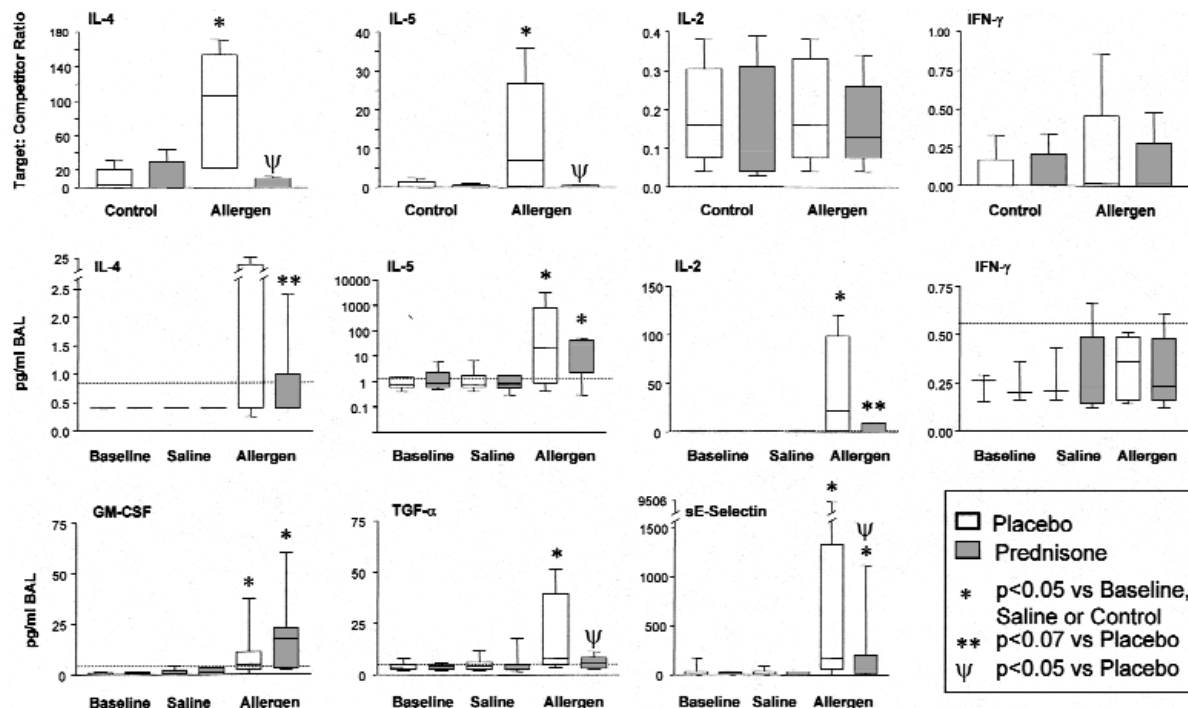


FIG 4. Analysis of mRNA (*top row*) and protein levels (*middle and bottom rows*) of cytokines and soluble E-selectin in BAL fluids at baseline and after challenge with saline or allergen. Data shown are median and interquartile ranges of a total of 6 to 10 values for proteins and 6 values for mRNAs. Dotted lines indicate the lower limit of detection for protein levels. For determinations of mRNA, *control* refers to either baseline or saline data.

cytokine levels after SAC revealed the highest levels of IL-5 (549 ± 337 pg/mL BAL), followed by IL-2 (38 ± 17 pg/mL BAL) and IL-4 (4.5 ± 2.6 pg/mL BAL). Levels of these cytokines were not significantly correlated among individuals. Levels of GM-CSF, TGF- α , and E-selectin were all increased by SAC (Fig 4, *lowest row*).

With prednisone treatment, there were reductions of mean cytokine levels of 84% to 95% of the released cytokines IL-2, IL-4, and IL-5; however, median levels for IL-4 and IL-5 were similar before and after prednisone treatment. Of these 3 cytokines, only IL-5 was statistically significantly increased by allergen after prednisone treatment ($P = .008$). The difference between prednisone and placebo for these cytokines did not reach statistical significance ($.06 < P < .21$), probably because of the small number of subjects and large interindividual variations. Median GM-CSF levels were actually increased by 3-fold with prednisone, but the difference was not statistically significant. TGF- α was completely suppressed by prednisone treatment ($P = .008$), and median levels of soluble E-selectin were reduced by 73% ($P = .04$). As with the cytokine protein measurements, SAC resulted in clear increases in mRNA for IL-4 and IL-5 in BAL cells, with no changes in mRNA for IL-2 and IFN- γ (Fig 4, *upper row*). Prednisone treatment significantly inhibited the SAC-induced expression of mRNA for IL-4 and IL-5 but did not reduce the low or undetectable levels of mRNA for IL-2 and IFN- γ (Fig 4, *upper row*).

Overall, the protein and mRNA data were in agreement, showing the same directional changes, although levels were not correlated. IL-2 was an exception where cytokine protein was detected, but mRNA expression was not. Although this could suggest that the source of IL-2 protein may be cell types other than those found in the BAL fluid, recent studies at 4 hours after SAC have revealed increases in IL-2 mRNA, suggesting that IL-2 mRNA may appear only transiently (data not shown).

DISCUSSION

The purpose of this study was to further characterize the effects of glucocorticoids on various parameters of inflammation after experimental allergen challenge in the lungs of allergic asthmatic individuals. Relatively few studies are available describing the effects of glucocorticoids *in vivo* in human subjects in whom lavage has been performed with or without antigen challenge in the airways.^{6,9,20-23}

Glucocorticoids have been shown to be effective inhibitors of the late-phase reaction but relatively ineffective against the early reaction unless applied topically for an extended period of time.^{24,25} In the present study segmental antigen challenge caused a fall in whole-lung FEV₁ and FVC that was modestly inhibited by prednisone. The mechanism of the change in pulmonary function induced by local allergen challenge is not clear

but could involve local release of mediators, including perhaps cytokines, which results in increased systemic inflammatory stimuli for noninvolved areas of the lung. Other possible explanations include neural reflex mechanisms stimulated by local irritation causing generalized bronchoconstriction or release of circulating bronchoconstrictive substances affecting airway function diffusely. Whatever the mechanism by which local challenge induces whole-lung physiologic responses, it appears to be relatively glucocorticoid insensitive.

The initial response after antigen challenge is characterized by mast cell degranulation and the appearance of mast cell mediators, including histamine and PGD₂. Although this response was somewhat dampened by glucocorticoid treatment, the inhibitory effects of prednisone were not statistically significant (Fig 1 and Tables I and II). The failure of glucocorticoids to inhibit mast cell mediator release in vivo in the present studies is similar to results obtained in nasal and cutaneous challenge model systems and agrees with a failure of glucocorticoids to inhibit in vitro degranulation and generation of newly synthesized mediators by human mast cells derived from lung and other tissue sources.^{26,27} The effects of glucocorticoids on mast cells are complicated and have been reviewed.²⁸ Although studies by Pipkorn et al,²⁹⁻³¹ Jeffery et al,³ Djukanovic et al,⁴ and Laitinen et al³² have demonstrated that topical glucocorticoid treatment can diminish mast cell numbers in the human lung, the modest decrease in BAL histamine and PGD₂ in the present study suggests that minimal effects on mast cells resulted from 3 days of treatment with prednisone.

Metabolites of arachidonic acid are known to be capable of modifying lung function. Glucocorticoids have been shown to inhibit the release of arachidonic acid and its metabolites from a variety of tissues.³³ We monitored the appearance of a host of metabolites of arachidonic acid. As previously reported, antigen challenge increased the levels of several prostanoids in the airways.^{16,23} In a previous study with human lung fragments and purified human lung mast cells, we found that in vitro treatment with dexamethasone did not inhibit the anti-IgE-induced release of the mast cell-derived prostanoids PGD₂ and thromboxane B₂ but did inhibit the release of secondary prostanoids arising from other cells in the tissue, including PGE₂, PGF_{2α}, and the prostacyclin metabolite 6-keto-PGF_{1α}.²⁶ The same pattern of effect was observed in the present in vivo study. The antigen-induced release of PGE₂, PGF_{2α}, and 9α,11β-PGF₂ was suppressed in the prednisone-treated subjects, whereas the release of (presumably mast cell derived) PGD₂ was not inhibited by prednisone.

One of the prominent effects of glucocorticoids in vivo is their ability to inhibit the migration of inflammatory cells to a reaction site. Part of this effect is probably related to changes in circulating cell numbers. Glucocorticoids have long been known to cause changes in circulating leukocyte counts, including an increase in neutrophils of 2- to 3-fold associated with an 80% to 100% decrease in the absolute numbers of circulating eosinophils and basophils and a modest decrease in the

numbers of lymphocytes and monocytes. In the lymphocyte compartment effects of glucocorticoids are confined to the recirculating, rather than the nonrecirculating, cells (ie, those that do not leave the bloodstream for the lymphatic system).³⁴ One interesting observation in the present study was an increase in CD45RO⁺ cells and a decrease in CD45RA⁺ cells in the peripheral blood associated with steroid treatment. Because CD45RO is considered to be a marker of memory T cells, this result suggests that glucocorticoids may modify trafficking of naive and memory T cells independently in human subjects. The T cells that migrated into the lung after antigen challenge were found to predominantly be CD4⁺ cells and were of the CD45RO (memory) phenotype. Prednisone treatment diminished lymphocyte influx to the airways after antigen challenge; this effect was largely caused by inhibition of CD4⁺ and CD45RO⁺ subtypes in the airways, although the influx of CD45RA⁺ cells was also inhibited. Robinson et al³⁵ have shown that the primary IL-4- and IL-5-producing cells in the airways are T cells. Thus the marked suppression of the influx of CD4⁺ cells into the airways by prednisone is likely to have had an important effect on the appearance of other cell types and cytokines in the airways.

The most impressive effect of prednisone on cellular infiltration was the near-complete inhibition of eosinophil and basophil migration after antigen challenge in the airways. In placebo-treated individuals eosinophils comprised on average 41% of the total leukocytes in the BAL fluid after antigen challenge. The inhibition of eosinophil recruitment to the lungs probably reflects in part the tremendous diminution of eosinophil numbers in the circulation. Studies in the nose indicate that topical glucocorticoids can inhibit the recruitment of eosinophils and other cells, however, suggesting that steroids can also dampen the local tissue-based mechanisms by which these leukocytes are recruited.^{30,36} Because prednisone also inhibited the influx of CD45RO⁺ lymphocytes in the late period in the present study, despite the fact that circulating CD45RO lymphocyte numbers were slightly increased, the possibility that glucocorticoids were exerting local inhibitory effects in the lung appears likely.

On the basis of a number of in vivo and in vitro studies, it has been suggested that glucocorticoids inhibit cellular recruitment, in large part because of their ability to inhibit the generation of chemoattractants, such as leukotrienes, platelet-activating factor, and chemokines, as well as their ability to inhibit the release of endothelial activators, including IL-1, TNF, IL-4, and IL-13.⁸ Inhibition of IL-1 and TNF release would be expected to result in diminished endothelial activation and in turn dampen recruitment of most or all leukocyte types. Previous studies have suggested that endothelial activation may be important in cellular recruitment in allergic reactions.¹⁸ Soluble E-selectin is a soluble form of an endothelial adhesion molecule that has been shown to be important in recruitment of a variety of leukocyte subtypes on the endothelium after inflammatory stimulation.³⁷ Monitoring of soluble E-selectin in BAL fluid revealed a clear

suppression of the level of this protein in BAL fluid from prednisone-treated individuals. This result suggests, but does not prove, that endothelial activation was inhibited by prednisone. This may occur in part by means of inhibition of the generation of endothelial-activating cytokines. The primary endothelial-activating cytokines in the lung are unknown. Borish et al³⁸ have shown that glucocorticoid treatment can diminish IL-1-forming cells in BAL fluid, and in vitro studies with human lung tissue have demonstrated inhibition of IL-1 formation by glucocorticoids.³⁹

Another prominent effect of glucocorticoids is a decrease in vascular permeability. This is believed to result from the direct effects of glucocorticoids on vascular cells, as well as indirectly by means of a decrease in the appearance of inflammatory cells and mediators that cause vascular leakage. In vivo studies have shown that treatment with glucocorticoids causes a marked decrease in markers of plasma exudation in asthmatic patients and decreases experimentally induced leakage of blood vessels in animals and human subjects.⁴⁰ The present finding, that prednisone decreases the appearance of albumin and bradykinin in BAL fluid, is consistent with the expectation that glucocorticoids inhibit vascular responses associated with allergen challenge in this model system.

Inhibition of cytokine generation by glucocorticoids is widely believed to be of central importance to their anti-inflammatory effects. In the present study 6 cytokines were monitored in BAL fluid: IL-2, IL-4, IL-5, IFN- γ , GM-CSF, and TGF- α . Prednisone treatment inhibited the appearance of TGF- α in BAL fluids, and IL-2, IL-4, and IL-5 showed clear trends toward inhibition, with an average decrease of 84% to 95%. Prednisone significantly reduced mRNA levels in BAL cells for both IL-4 and IL-5, reinforcing the conclusion that glucocorticoids suppress expression of both of these cytokines. As discussed above, one likely source of IL-4 and IL-5 mRNA and protein is CD4⁺ T lymphocytes. Eosinophils are likely a source of TGF- α in these studies (Liu et al, manuscript in preparation). The reduction in IL-2, IL-4, IL-5, and TGF- α may occur as a result of direct effects on the steroid-sensitive cells producing these cytokines or may occur after a decrease in the number of these cells. Interestingly, prednisone treatment failed to inhibit formation of GM-CSF, suggesting that it arises from a glucocorticoid-nonresponsive cell type. In the case of IFN- γ , no detectable level of protein was observed, and antigen challenge did not increase levels of this cytokine or its respective mRNA. IL-2 protein, but not mRNA, was detected, perhaps suggesting a transient production had occurred.

Results in this study suggest that glucocorticoids can disrupt the pattern of migration of inflammatory cells into the airways after exposure to antigen. In particular, glucocorticoid treatment suppressed the appearance of eosinophils, basophils, and CD4⁺ helper and memory T lymphocytes into the airways. This effect is associated with a decrease in numerous mediators and markers of the inflammatory response, including albumin and

bradykinin (markers of vascular leak), some prostanoids (perhaps secondary to effects on cellular recruitment), and several cytokines (IL-2, IL-4, IL-5, and TGF- α). Inhibition of the appearance of soluble E-selectin, a marker of endothelial activation, suggests that prednisone inhibited endothelial activation. We conclude that glucocorticoids have impressive anti-inflammatory effects in the lung in this experimental antigen challenge system and that their ability to suppress numerous interrelated but distinct aspects of the inflammatory response contributes to their anti-inflammatory efficacy.

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