

Topical glucocorticoids inhibit activation by allergen in the upper respiratory tract

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We have studied the effect of a topically administered glucocorticoid, fluticasone propionate (FP), on infiltration and activation of eosinophils in the nasal mucosa after provocation with allergen. Forty-four patients with seasonal allergic rhinitis entered a double-blind, crossover study in which they underwent treatment with either FP (200 µg once daily) or identical placebo for 2 weeks. Patients then underwent nasal-allergen provocation followed by nasal lavage and biopsy at one of several time points between 0 and 8 hours. Patients subsequently received the alternate treatment for 2 weeks before repeat allergen provocation, nasal lavage, and biopsy, as before. Biopsy specimens of nasal mucosa obtained during the immediate allergic response demonstrated an influx of eosinophils (stained by monoclonal antibody EG1) of similar magnitude during both FP and placebo treatment. Significantly, fewer eosinophils in these biopsy specimens were activated (stained by monoclonal antibody EG2) after treatment with FP compared with that after placebo treatment (median values, 8.8 and 36.6 cells per square millimeter, respectively; $p < 0.02$). The concentration of eosinophil cationic protein in nasal lavage fluid was significantly elevated above baseline from 2 to 8 hours after allergen, and this increase was abolished by treatment with FP. These results suggest that topical glucocorticoids inhibit allergen-induced activation of eosinophils in allergic rhinitis. (J ALLERGY CLIN IMMUNOL 1992;89:951-7.)

Key words: Glucocorticoids, eosinophil, allergen, rhinitis, ECP, activation

The eosinophil is regarded as important in the pathogenesis of AR^{1,2} and asthma.³ In AR, numbers of eosinophils in nasal secretions are increased during natural exposure to allergen,¹ and a similar increase occurs during both the immediate and late-phase response^{2,4} to nasal provocation with allergen. Nasal secretions in AR contain eosinophil-derived MBP⁵ that is cytotoxic for nasal epithelial cells⁶ and leukotriene C₄⁷ that increases nasal airway resistance⁸ and that is the major leukotriene product of activated eosinophils.⁹

Glucocorticoids are the most effective treatment for AR.¹⁰ Their effects include a reduction in numbers of

Abbreviations used

AR:	Allergic rhinitis
FP:	Fluticasone propionate
MBP:	Major basic protein
MAB:	Monoclonal antibody
BU:	Biological unit
NAR:	Nasal airway resistance
ECP:	Eosinophil-cationic protein

eosinophils in nasal secretions,^{2,4} but it is not clear whether they affect the state of activation of eosinophils in vivo.

In the present study, we have assessed the effects of treatment for 2 weeks with a topically administered glucocorticoid, FP (Glaxo Laboratories, Ltd., Greenford, Middlesex, England), on the activation of eosinophils in biopsy specimens of the nasal mucosa obtained before and after nasal provocation with allergen. We used a double-blind, placebo-controlled crossover study design and immunocytochemical techniques incorporating MAbs EG1 and EG2 to as-

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sess total and activated eosinophil numbers, respectively.

MATERIAL AND METHODS

Patients' characteristics

We studied 44 patients with seasonal AR to grass pollen. All patients demonstrated an immediate reaction on skin prick testing with mixed grass pollens (Pharmacia, Ltd., Milton Keynes, U.K.). There were 18 men and 26 women, aged 19 to 47 years (mean, 25.5 years). All subjects were studied outside the grass-pollen season and did not have nasal symptoms at the time of study. We excluded subjects with perennial rhinitis, subjects currently taking regular treatment for nasal symptoms, and cigarette smokers. All patients gave informed consent, and approval was obtained from the Ethics Committee of St. Bartholomew's Hospital.

Allergen used for nasal provocation

Freeze-dried extracts of mixed grass pollen (Pharmacia, Ltd.) were made up in normal saline to result in final concentrations of 250, 2500, 25,000, and 125,000 BU/ml⁻¹.

Delivery of solutions

One hundred microliters of allergen (or normal saline control) solutions was nebulized at an airflow rate of 7 L/min from a cuvette with a modified airbrush (Humbrol, Hull, England). Allergen was always delivered to the left nostril. During nebulization of allergen, patients were instructed to hold breath at the end of inspiration for approximately 3 seconds, and the opposite nostril was gently compressed by external finger pressure; these maneuvers were done with the aim of preventing the passage of solutions to the lower respiratory tract and opposite nostril.

Measurement of NAR

NAR was measured in the left nostril by active anterior rhinomanometry with a NR6 rhinomanometer (Mercury Electronics, Glasgow, Scotland). Subjects rested for 15 minutes before measurements were made, and a minimum of eight readings were taken at each time point. The mean of these eight readings was taken as the value of NAR for the left nostril. Two measurements separated by an interval of 15 minutes were made before nasal provocation, and the mean of these measurements was taken as the baseline value.

Nasal lavage and measurement of ECP concentration in lavage fluid

Nasal lavage was performed immediately before biopsy of the nasal mucosa. Five milliliters of normal saline at 37° C was instilled into the left nostril with the patient's neck extended and soft palate closed. After 10 seconds, the neck was flexed, and secretions were collected with a funnel held below the nose. The concentration of ECP in lavage fluid was subsequently measured with a double-antibody RIA (Pharmacia, Ltd.).

Biopsy, fixation, and staining of nasal mucosa

After the topical application of 4% lignocaine spray, cup forceps were used to take one biopsy specimen from the anterior end of the left inferior turbinate.

Biopsy specimens were immediately placed in Carnoy's fixative (absolute alcohol, 60 ml; chloroform, 30 ml; glacial acetic acid, 10 ml) for 3 hours. Specimens were then removed, placed in an automatic tissue processor (Shandon Southern Products, Ltd., Runcorn, England), and subsequently were embedded in paraffin wax before section at 3 µm thickness by use of a rotary microtome (Reichert-Jung Optische Werke AG, Vienna, Austria). Sections were dewaxed in xylene and rehydrated before staining.

Staining for mast cells with toluidine blue

A 0.5% solution of toluidine blue (BDH, Ltd., Pool, Dorset, England) was made up in 0.5 molar hydrochloric acid, and the tissue sections were incubated in this solution for 15 minutes. Sections were then washed, dehydrated, and mounted in DPX mountant (BDH, Ltd.) before examination for mast cells.

Staining for eosinophils with MAbs EG1 and EG2

To block endogenous peroxidase activity, sections were immersed in 0.5% hydrogen peroxide in methanol. Sections were then treated with 0.1% trypsin (Sigma Chemical Co., Poole, Dorset, England) for 3 minutes to expose epitopes of ECP. This procedure was followed by incubation with 1% normal horse serum for 15 minutes to block nonspecific binding of MAbs EG1 and EG2. Adjacent sections from each subject were incubated separately in mouse MAbs EG1 or EG2 solutions (Pharmacia, Ltd.) (diluted 1/20 and 1/40, respectively) for 16 hours at 4° C. Sections were then washed, incubated for 30 minutes with biotinylated rabbit antimouse antibody (DAKOPATTS A/S, Glostrup, Denmark), and conjugated by incubation with avidin-biotin-peroxidase complexes (DAKOPATTS A/S) for 30 minutes. Sections were then incubated for up to 5 minutes with freshly prepared diaminobenzidine-hydrogen peroxide substrate (Sigma Chemical Co.), washed, and counterstained with hematoxylin.

In negative control sections, the primary MAb was substituted by nonimmune sera. Positive control sections were provided by use of each staining procedure on sections of intestine affected by Crohn's disease.

Assessment of cellular infiltration in nasal biopsy specimens

All assessments of biopsy specimens were made by an observer (J. W.) who was unaware of the treatment period during which the biopsy specimen was taken. This observer counted the total number of specifically stained cells per tissue section. The Imagen computerized image analysis

system (Kompira, Ltd., Salsburgh, Scotland) was used to assess the area of tissue sections, and cellular infiltration was expressed as the number of cells per square millimeter of biopsy section.

Study design

All visits were made outside the grass-pollen season.

Assessment of nasal reactivity to grass pollen. Normal saline was instilled into the left nostril, and measurements were made of NAR in this nostril at 5-minute intervals for 15 minutes. Subsequently, provocation of the same nostril was performed with increasing concentrations of grass pollen (250, 2500, 25,000, and 125,000 BU/ml⁻¹) until NAR in the left nostril during the 15 minutes after provocation was three times greater than the maximum value after normal saline. Patients were excluded from entry into the study when this provocation with grass pollen produced a rise in NAR that was less than three times greater than the maximal value produced by normal saline. A positive response was observed in 44 patients who were studied as described below. Two subjects failed to complete the study (see RESULTS section).

Patients were randomly assigned to receive, under double-blind conditions, either FP aqueous nasal spray (50 µg per spray) or identical placebo, two sprays into each nostril daily for 2 weeks. The last dose was taken on the day preceding the next visit.

Allergen provocation and nasal biopsy (after 2 weeks of treatment). On this visit, which was made after 2 weeks of treatment, seven patients underwent nasal biopsy, but not provocation with grass pollen, to provide a baseline.

The remaining 35 patients first underwent provocation of the left nostril with grass pollen in the concentration found to produce a threefold rise in NAR. NAR was subsequently measured in the left nostril at 5-minute intervals for the first 30 minutes, at 10-minute intervals for the second 30 minutes, and subsequently, every hour, until the time of the individual's nasal biopsy. Biopsy specimens were obtained from the left nostril (immediately after nasal lavage) at one of the following times after allergen: 30 minutes ($n = 10$), 1 hour ($n = 9$), 2 hours ($n = 9$), or 8 hours ($n = 7$) (Fig. 1). Record was made of the number of sneezes during the 10 minutes after allergen provocation.

Patients then underwent a washout period of 4 weeks before starting the alternate treatment that was taken for 2 weeks.

Allergen provocation and nasal biopsy (after 2 weeks of alternate treatment). This visit was made after 2 weeks of the alternate treatment. Each patient underwent the same procedure, including nasal lavage and biopsy, at the same time point, as on the visit after 2 weeks of treatment (see above).

Analysis of results

The Wilcoxon's signed-rank test was used to compare cell counts (and NAR values) in the FP-treatment period

with cell counts obtained from the same patients, at the same time point, after allergen, in the placebo-treatment period.

The Mann-Whitney U test was used to compare cell counts (and NAR values) obtained at intervals after allergen provocation, with baseline values from the patients in the same treatment period who had not undergone provocation.

Results obtained from biopsy specimens taken at 30 minutes and 1 hour after allergen provocation were pooled to provide early phase cell counts. Cell counts obtained from biopsy specimens at 2 hours and 8 hours were pooled to provide late-phase cell counts.

RESULTS

Two patients were withdrawn, both during the placebo-treatment period. In one case, withdrawal was necessitated by the development of undue upper respiratory tract symptoms and headache, and in the other case, because nasal biopsy was poorly tolerated. We analyzed these data of the remaining 42 patients, who completed the study satisfactorily.

Values of NAR were significantly ($p < 0.02$) higher than baseline at each time point at which measurement was made, up to and including 2 hours after allergen provocation, in both the active-treatment and placebo-treatment periods. There was no significant difference between placebo- and FP-treatment periods in the values of NAR at any time point before or after allergen. The comparison between placebo- and FP-treatment periods, with respect to the maximal NAR value observed in each patient during the early and late phases, is summarized in Table I.

The median number of sneezes during the 10 minutes after allergen provocation did not differ significantly between the placebo-treatment period (median value, 3; range, 0 to 21) and the FP-treatment period (median value, 3; range, 0 to 13) ($p > 0.10$).

ECP concentration in nasal lavage fluid was significantly ($p < 0.02$) elevated above baseline values during placebo treatment in the late, but not early, phase reaction. During treatment with FP, this late-phase rise in ECP concentration was abolished (Fig. 2).

Counts of mast cells in the early (30 minutes and 1 hour) or late-phase (2 and 8 hours) biopsy specimens were not significantly different from counts in the baseline biopsy specimens (Table II). There was no significant difference between placebo- and FP-treatment periods in the counts of mast cells for the early or late-phase biopsy specimens (Table II).

Counts of eosinophils stained with MA6 EG1 were significantly greater in the early and late phase compared with that of baseline biopsy specimens in both

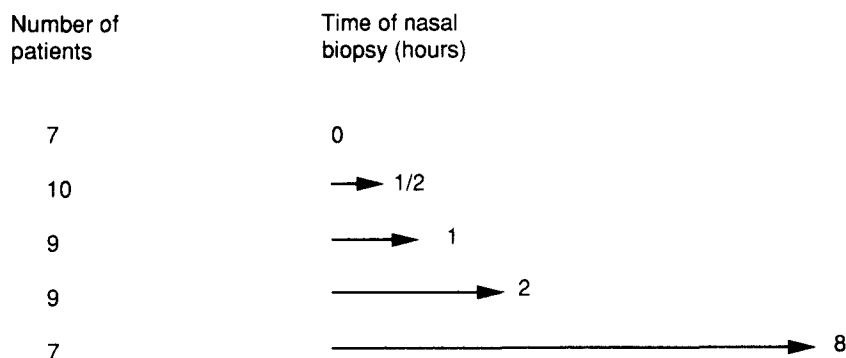


FIG. 1. Time between allergen provocation and biopsy of the nasal mucous membrane in 35 patients. Seven patients with biopsies at time 0 did not undergo provocation.

TABLE I. Maximal NAR value (Pa/ml) observed in each patient during the early (5 minutes to 1 hour inclusive after allergen) and late (readings made thereafter up to 8 hours) phases

	Baseline NAR	Early phase NAR (max)	Late-phase NAR (max)
Placebo	0.437 (0.161-3.66)	1.641 (0.328-15.87)	0.799 (0.254-6.17)
FP	0.433 (0.212-1.343)	1.46 (0.283-17.07)	0.868 (0.324-2.21)

Max, Maximum.

There was no significant difference between placebo- and FP-treatment periods in the values of NAR at any time point before or after allergen.

the FP- and placebo-treatment periods (Table II). There was no significant difference between placebo- and FP-treatment periods in the numbers of MAb EG1-stained cells for early or late-phase biopsy specimens (Table II and Fig. 3).

Counts of eosinophils stained with MAb EG2 were significantly greater in early and late-phase biopsy specimens compared with that of baseline (Table II). However, counts were substantially lower in early phase biopsy specimens obtained during treatment with FP compared with counts during placebo treatment ($p < 0.02$) (Table II and Fig. 3).

There was no significant difference between the placebo- and FP-treatment periods, in baseline counts of mast cells, or MAbs EG1- or EG2-stained eosinophils (Table II).

DISCUSSION

This study demonstrates that pretreatment for 2 weeks with the topical corticosteroid FP inhibits activation of eosinophils infiltrating the nasal mucosa during the early phase response to allergen. In accordance with this observation, the concentration of ECP in nasal lavage fluid after provocation with allergen was reduced during treatment with FP.

We used an immunocytochemical method using MAb EG2 to stain activated eosinophils. This antibody binds to ECP only during secretion¹¹; it does not stain normal eosinophils but does demonstrate eosinophils after phagocytosis of zymosan C3b particles or after incubation with eosinophil cytotoxicity-enhancing factor.¹¹ MAb EG1 recognizes both the storage and secreted forms of ECP and was used to assess the total number of (activated and nonactivated) eosinophils. We found that treatment with topical glucocorticoids reduced the number of activated (MAb EG2-stained) eosinophils in the nasal mucosa during the early phase allergic reaction. This effect indicates inhibition of activation, rather than migration, of eosinophils, since their total (MAb EG1-stained) number was unaltered by treatment.

Activation of eosinophils leads to the generation of inflammatory mediators. These include preformed highly cationic proteins, such as MBP and ECP from the eosinophil granules,¹² and nonpreformed products, such as leukotriene C₄⁹ and platelet-activating factor.¹³ Our observation that FP inhibits eosinophil activation in vivo is compatible with the inhibition by glucocorticoids in vitro of the release from eosinophils of granule peroxidase,^{14, 15} ECP,¹⁶ and superoxide an-

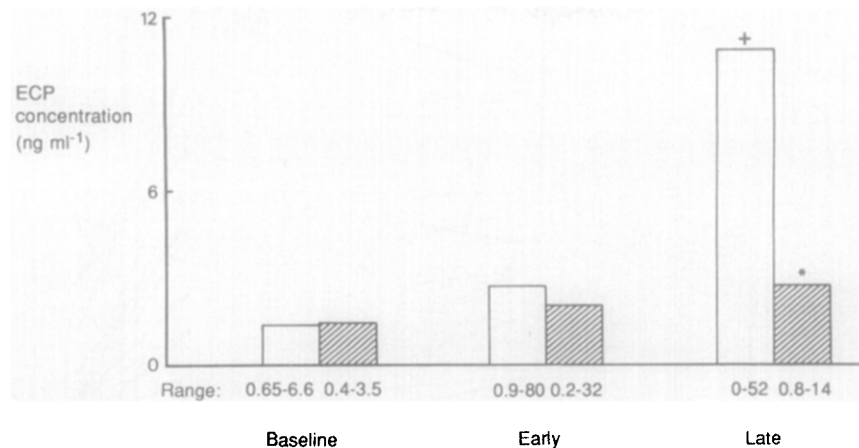


FIG. 2. ECP concentration in nasal lavage fluid during treatment with placebo (□) or FP (▨). Baseline samples were obtained from six patients in whom no allergen provocation was made. Early phase samples were obtained immediately before nasal biopsy was performed in 18 patients at 30 minutes or 1 hour after allergen. Late-phase samples were obtained immediately before nasal biopsy was performed in 15 patients at 2 or 8 hours after allergen. +, $p < 0.02$, comparison with baseline values; * $p < 0.01$, comparison between FP and placebo.

TABLE II. Median values (range) for cell counts (cells per square milliliter) in biopsy specimens of the nasal mucous membrane

Cell type	Baseline	Early phase	Late phase
Mast cells			
Placebo	34.7 (10.1-125.8)	33.3 (15.3-140)	32.7 (16.1-79.5)
FP	42.8 (6.4-83.6)	34.1 (9.57-93.1)	31.3 (9.98-117.4)
MAb EG1-stained eosinophils			
Placebo	5.2 (2.1-49.5)	45.75* (2.4-106.4)	21.4† (8.1-46.3)
FP	6.7 (1.8-41.8)	31.45* (3.6-102.1)	19.2* (7.3-45.9)
MAb EG2-stained eosinophils			
Placebo	2.1 (0-3.5)	36.6* (1.6-96.5)	11.2* (1.2-65.4)
FP	0 (0-24.2)	8.8*‡ (0.9-57.5)	9.1† (0-24.9)

Early and late-phase biopsy specimens were obtained at 30 minutes to 1 hour and 2 to 8 hours, respectively, after allergen provocation. A significant difference compared with that of baseline values for the same treatment phase is indicated:

* $p < 0.02$.

† $p < 0.05$.

A significant difference between FP and placebo treatment for the same time period is indicated:

‡ $p < 0.02$.

ion.¹⁵ Our findings suggest that the effect of corticosteroid treatment in reducing levels of ECP,^{17, 18} as well as MBP⁵ and eosinophil-derived neurotoxin⁵ in nasal secretions in AR, is likely to be due not only to reduced numbers of eosinophils in the nasal

secretions^{2, 4, 5} but also to inhibition of their activation. This suggestion is compatible with the observation that, in asthma, inhaled corticosteroid therapy reduced the concentration of ECP but not numbers of eosinophils in bronchoalveolar lavage fluid.¹⁹

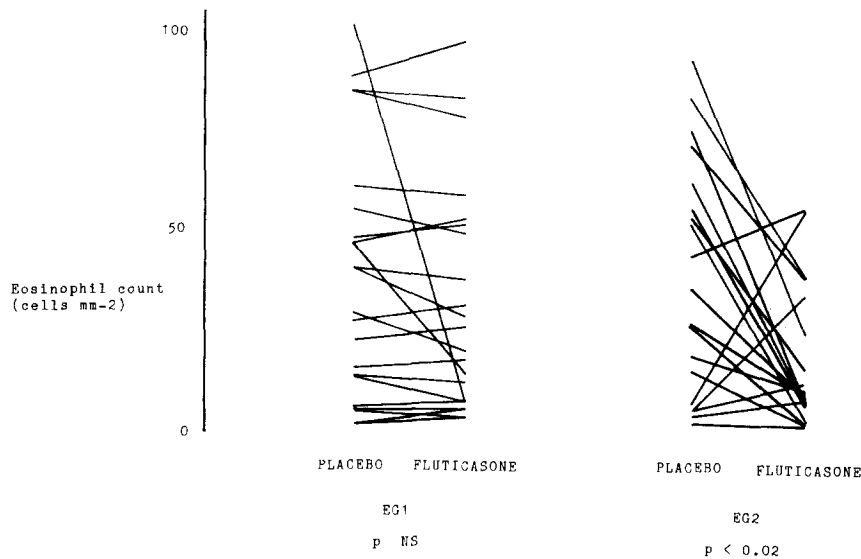


FIG. 3. Numbers of eosinophils stained with MAbs EG1 and EG2 in biopsy specimens of nasal mucous membrane obtained during the early phase allergic reaction. During treatment with FP, numbers of eosinophils stained with MAb EG2 were significantly smaller than during treatment with placebo.

The infiltration of eosinophils into the nasal mucosa itself during the early phase allergic reaction has been demonstrated previously.²⁰ Although staining with MAb EG2 demonstrated that many of these eosinophils were activated, a substantial rise in nasal lavage ECP was not observed until the late-phase reaction. This difference probably reflects the time necessary for the secretion of ECP after eosinophil activation. We also found that numbers of eosinophils in the mucosa remained elevated above baseline values after the early phase reaction, during the period 2 to 8 hours after provocation. This finding is in keeping with the previous observation that there is an influx of eosinophils into nasal secretions during the late-phase reaction,^{2, 4} an influx that is inhibited by pretreatment with topical glucocorticoids.⁴

Previous studies, with substantially smaller numbers of patients, have demonstrated variable effects on the early phase increase in NAR as a result of pretreatment with topical glucocorticoids.²¹⁻²³ We found that, although pretreatment with topical FP inhibited allergen-induced activation of eosinophils, it had no effect on the early phase increase in NAR nor on the number of sneezes recorded during this phase. This finding is not unexpected, since the early phase response to nasal challenge is believed to reflect activation of mast cells,²⁴ and glucocorticoids have been found to have no inhibitory effect *in vitro* on their degranulation.²⁵ Furthermore, pretreatment *in vivo* with topical glucocorticoids does not have a significant

effect on the influx of inflammatory cells, such as basophils, into the nasal secretions during the early phase allergic response,⁴ and although release of inflammatory mediators, such as histamine, has been found to be inhibited, it was not abolished.²⁴

The absence of inhibition by topical glucocorticoids of the early phase increase in NAR in our patients, who were studied out of season, is not incompatible with their clinical efficacy during sustained natural exposure to allergen. In the present study, there was no effect on baseline numbers of mast cells or eosinophils in accordance with previous observations made out of season.^{4, 26} However, during sustained natural exposure to allergen, each of these cell types becomes more numerous,²⁷ and treatment with topical glucocorticoids in these circumstances results in a reduction in numbers of mast cells and eosinophils in the nasal mucosa.²⁸⁻³⁰ This effect of topical corticosteroids observed during sustained allergen exposure might be important for their action in alleviating symptoms of rhinitis and reducing NAR.^{30, 31}

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