

Eosinophil markers in seasonal allergic rhinitis

Intranasal fluticasone propionate inhibits local and systemic increases during the pollen season

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Background The purpose was to study activation markers of the eosinophil granulocytes in seasonal allergic rhinitis, and the impact of topical steroid therapy thereupon.

Methods Sixty-three rhinitis patients with monoallergy to grass were examined before and at peak pollen season. Blood eosinophil count, eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) in serum and nasal lavage fluid were measured. During the season, patients were randomized to treatment with intranasal fluticasone propionate 0.1 mg o.d. ($n=26$), 0.2 mg o.d. ($n=25$), or placebo ($n=12$). Six healthy persons served as controls.

Results During the season, all parameters, except nasal lavage ECP, increased in the placebo group ($P<0.001$ – $P<0.05$). Significant differences were seen between the steroid groups and the placebo group for all parameters ($P<0.001$ – $P<0.05$). Higher eosinophil count ($P<0.05$), serum EPO ($P<0.02$), and nasal lavage EPO ($P<0.05$) were found in patients before season than in controls. The following winter, 44 patients returned for repeated measurement. Lower levels of nasal lavage EPO were observed for patients than levels at the beginning of the season ($P<0.0001$).

Conclusions Intranasal fluticasone propionate reduced inflammation of the nasal mucosa, demonstrated locally by nasal lavage ECP and EPO, and systemically by blood eosinophils, serum ECP, and serum EPO. EPO seemed more sensitive than ECP as indicator of allergic inflammation. EPO demonstrated some perennial eosinophil activity in hay fever patients, increasing locally during spring.

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A major feature of allergic inflammation is cellular infiltration by eosinophil granulocytes (1–3). The number of eosinophils may be of less importance in the pathogenesis of allergic inflammation than their state of activation, as measured by the capability of degranulating cytotoxic proteins (4, 5). Four major cytotoxic proteins are recognized in the granules of eosinophil granulocytes; i.e., major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil protein X/eosinophil-derived neurotoxin

(EPX/EDN). ECP and EPO are highly specific for the eosinophil granulocyte, making them useful as markers of eosinophil activation (6).

In a previous study of grass-pollen-induced rhinitis, we found a seasonal increase in serum and nasal lavage fluid ECP (7). Furthermore, data indicated eosinophil activation to be present from the very beginning of the pollen season before massive allergen exposure.

The purpose of the present study was to investigate further the cytotoxic granular proteins

of eosinophil granulocytes in allergic inflammation, using the nasal mucosa of hay fever patients as a model. The main points of interest were timing of events, correlation to symptoms, and influence of intranasal steroid treatment with fluticasone propionate (FP). Moreover, any difference between ECP and EPO was evaluated, according to previous data suggesting a difference of releasability between them (8).

Earlier data have indicated activation of eosinophils in the preseasonal period (7). Therefore, in the present study, eosinophils and their granule proteins were measured at a visit during the following winter, with a guaranteed absence of pollen from the environment.

Material and methods

Study population

Sixty-three adult patients (36 men, 27 women) aged 19–59 years participated in the study. They were all monoallergic to grass pollen, as documented by positive skin prick test or RAST, and had experienced symptoms of allergic rhinitis during at least the two most recent grass-pollen seasons. They had not received any corticosteroid treatment for 2 months. Six healthy adults (four men, two women) aged 31–45 years, without any history or signs of allergic disease, served as normal controls.

Study design

The study consisted of four visits. The first (visit 1) took place 2 weeks before the grass-pollen season of 1995, the second (visit 2) at the beginning of the season, and the third (visit 3) 3 weeks later. A fourth visit (visit 4) was made approximately 8 months after visit 3, at the end of a long, frosty period.

At visit 2, patients were randomized in a double-blind manner to receive 3 weeks of treatment with either FP 100 µg o.d. (FP100), FP 200 µg o.d. (FP200), or placebo administered as nasal aqueous spray. The patients could use antihistamine (acrivastine) and eye-drops (chromoglycate) as relief medication, apart from 12 h before each study visit.

Blood samples were drawn at each visit, while nasal lavages were performed at visits 1, 3, and 4. Between visits 1 and 3, the patients kept a diary, in which they scored daily for three different symptoms, i.e., sneezing, runny nose, and blocked nose, according to a linear scale ranging from 0 (no symptoms) to 3 (disabling symptoms). Eye symptoms and use of antihistamines were recorded daily.

Biochemistry

The number of eosinophil granulocytes in peripheral blood (EOS) was determined by counting in a Fuchs-Rosenthal chamber. Briefly, peripheral blood was diluted (1:18) in a hypotonic solution containing acetone as stabilize and eosin for staining of eosinophils. Between 20 min and 2 h later, the number of eosinophils was counted in a Fuchs-Rosenthal chamber with a volume of 3.2 µl. All counts were performed in duplicate.

For measurements of serum ECP (sECP), serum EPO (sEPO), and serum human neutrophil lipocalin (sHNL), serum was obtained after allowing venous blood to clot for 60 min at 20°C, followed by centrifugation (10 min, 4°C, 1600 g). The resulting serum samples were kept at –80°C until analysis.

Nasal lavage was performed as described previously (7). After centrifugation (10 min, 4°C, 1600 g), the supernatant was kept at –80°C until analyzed for ECP, EPO, and HNL.

ECP was measured by Pharmacia CAP System® ECP FEIA (Pharmacia & Upjohn Diagnostics AB), according to the instructions of the manufacturer. The inter- and intra-assay coefficients of variation were less than 8% and the detection limit was 0.5 µg/l.

EPO and HNL were assessed by prototype immunofluorometric assays utilizing the Pharmacia CAP System®. Briefly, EPO purified by a previously described method (9), with some modifications, was used as standard, ranging from 0.5 to 200 g/l. HNL, purified as earlier described (10), was used as standard, ranging from 20 to 600 µg/l.

Monoclonal antibodies (mAbs) were produced by standard hybridoma technique by fusion of spleen cells from, respectively, EPO and HNL immunized Balb/c mice with Sp2/0 mouse myeloma cells, followed by cloning. Supernatants were screened by ELISA for activity against purified EPO and HNL. Selected hybridomas were expanded and antibodies purified from these were of the IgG1 subtype.

The assay procedure was identical to that of Pharmacia CAP System ECP FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala Sweden). One mAb was covalently bound to the ImmunoCAP® (Pharmacia & Upjohn Diagnostics AB), and the other mAb was labeled with the enzyme β-galactosidase. Briefly, specific antibodies coupled to the solid phase react with EPO or HNL in standard or sample, and bound protein is detected by means of the enzyme-labeled mAb followed by the addition of the development solution. The fluorescent product is eluted with Stop solution and measured in the FluoroCount96. The fluorescence detected is

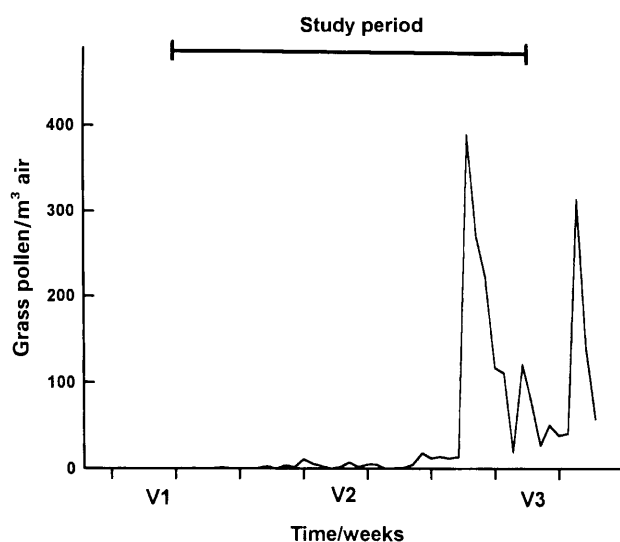


Fig. 1. Daily grass-pollen counts in Aarhus (summer 1995). V: visit.

then directly proportional to the amount of EPO or HNL in the standard and sample. Cross-reactivities with ECP and myeloperoxidase (MPO) in the EPO assay were <0.3 and $<0.01\%$, respectively. The detection limit of the EPO assay was 0.5 g/l , and the intra- and interassay coefficients of variation were less than 10% . Five serum samples were diluted serially two, four, and eight times in phosphate buffer, pH 7.5, containing 0.2% CTAB, 10 mM Na-EDTA, 20% glycerol, and 5% FCS to test for parallelism, with the standard curve as an indication of specificity. The mean ratio between obtained and expected levels was 1.02 (range 88 – 112%).

Cross-reactivity in the HNL assay with MPO, elastase, lactoferrin, and cathepsin G was $<0.8\%$, and with EPO and lysozyme $<0.4\%$. The detection limit of the HNL assay was $20 \mu\text{g/l}$. The intra- and interassay coefficients of variation were less than 8% .

Pollen count

The grass-pollen levels were measured daily throughout the study period. Collection was done

in the study area with a Burkard trap and expressed as number/ m^3 air (Fig. 1).

Statistics

The statistical analyses were carried out with SAS software (version 6.11). For data measured on a ratio scale, analysis of variance was used. Data were log transformed, if the normal distribution assumption was not met. If log transformation of the data did not achieve normal distribution, non-parametric statistics were used (Wilcoxon rank sum test).

For data measured on an ordinal scale, the Wilcoxon rank sum test was used.

Ethics

The study was approved by the local scientific ethics committee (County of Aarhus, Denmark). All patients gave informed consent in writing before entering the study.

Results

Study population

Sixty-three patients were randomized. Twenty-six patients constituted the FP100 group, 25 patients the FP200 group, and 12 patients received placebo. The baseline characteristics of patients and controls are listed in Table 1. The patients with allergic rhinitis had significantly higher levels of total serum IgE ($P<0.01$) and allergen-specific serum IgE ($P<0.001$) than the healthy controls.

Blood eosinophils

Blood eosinophils (Fig. 2) increased during the study period in placebo-treated patients, whereas declining levels were observed in steroid-treated patients. When we compared the changes, a significant difference was seen between placebo and each of the steroid groups ($P<0.05$).

EOS in the nonallergic control group was significantly lower at visit 1 than in the combined patient

Table 1. Baseline characteristics of study population (*mean $\pm$ SEM at study entry)

	Placebo	FP100	FP200	P	Controls	P
n	12	26	25		6	
Age (years) mean (range)	32.4 (21–53)	37.5 (23–56)	34.2 (19–59)	NS	36.8 (31–44)	NS
Sex (M/F)	6/6	19/7	11/14	NS	4/2	NS
Serum IgE (kU/l)*	81.5 ± 16.4	91.6 ± 14.3	95.7 ± 17.1	NS	24.0 ± 5.1	<0.01
Grass IgE (PRU/l)*	8.51 ± 1.64	9.03 ± 1.50	8.42 ± 1.39	NS	0.03 ± 0.01	<0.001

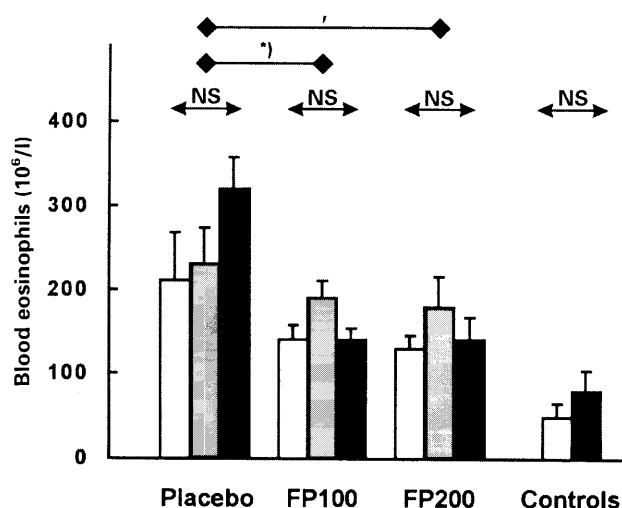


Fig. 2. Number of eosinophils in peripheral blood. Comparison of intra- (◀→) and intertreatment (◆◆) changes. □ visit 1; ▒ visit 2; ■ visit 3 (mean ± SEM; NS: not significant; * P <0.05).

group (P <0.05). During the season, this difference was accentuated between nonallergic controls and the placebo group (P =0.001), while no differences were observed between controls and either group treated with topical steroid.

ECP/EPO in serum

Significant increases were demonstrated for sECP (P <0.01) and sEPO (P <0.001) in the placebo group during the season. Only minor, nonsignificant changes were observed in the two groups receiving active treatment (Fig. 3). Intertreatment comparison of the changes revealed a significant difference between placebo and each of the FP-treated groups (P <0.001).

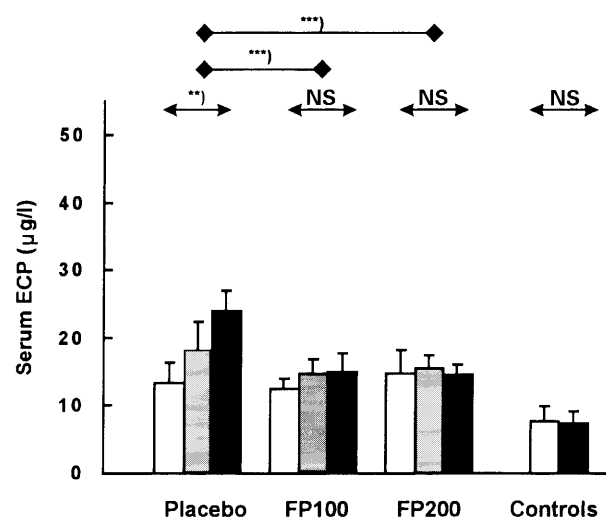


Fig. 3. Serum ECP and serum EPO. Comparison of intra- (◀→) and intertreatment (◆◆) changes. □ visit 1; ▒ visit 2; ■ visit 3 (mean ± SEM; NS: not significant; ** P <0.01; *** P <0.001).

At visit 1, sEPO was markedly elevated in the combined patient group compared to the controls (P <0.02). No significant difference was observed regarding sECP. At visit 3, sECP was higher in all three treatment groups than in controls; however, levels of significance were reached only for placebo (P <0.01) and FP200 (P <0.05). Regarding sEPO, all patient groups had significantly higher levels during the season than the controls (P <0.01).

ECP/EPO in nasal lavage

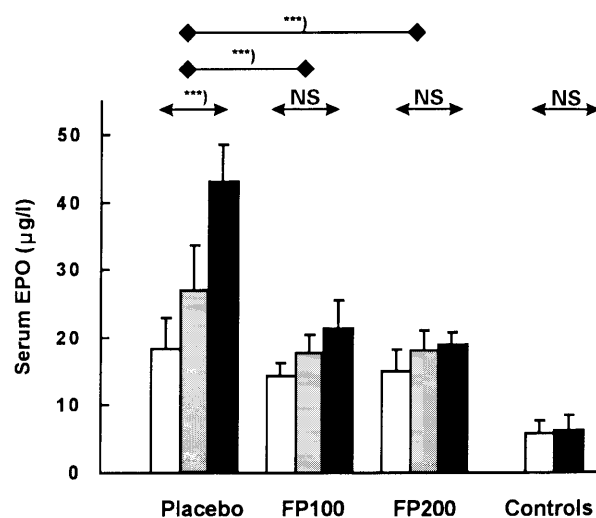
nECP (Fig. 4) increased slightly during the season in the placebo group (NS), while a marked elevation was seen in nEPO (P <0.05). These increases were significant, compared to the minute changes in the steroid-treated groups (P <0.05 and P <0.01, respectively). The levels of nECP in patients were not statistically different from those of controls, either before or during the season. nEPO was significantly higher before the season in the combined patient group (P <0.05) and in the placebo group during the season (P <0.02) than in the controls.

HNL in serum and nasal lavage

In both serum and nasal lavage fluid, the neutrophil marker HNL showed no significant differences, either within or between groups (data not shown). Moreover, no differences were seen from controls.

Symptoms

The mean score for the combined nasal symptoms in the placebo group increased 2.13 units from baseline (P <0.01), while nonsignificant changes



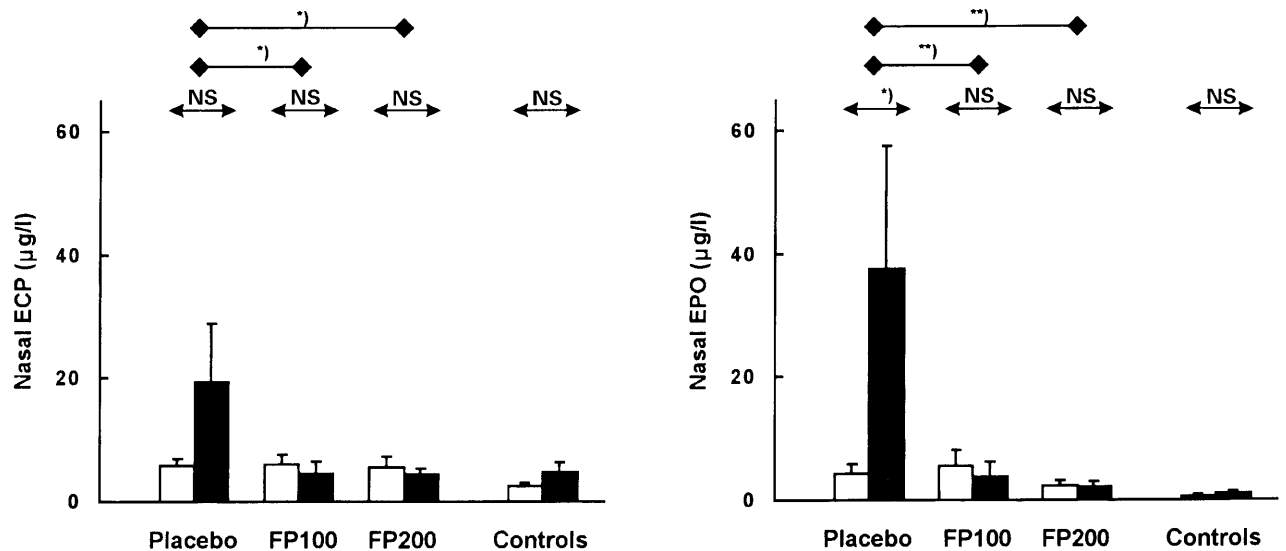


Fig. 4. ECP and EPO content in nasal lavage fluid. Comparison of intra- (↔) and intertreatment (◊↔◊) changes. □ visit 1; ■ visit 3 (mean ± SEM; NS: not significant; * $P<0.05$; ** $P<0.01$).

were observed in the two active treatment groups; 0.06 and 0.22 units, respectively. Similar observations were made for each of the three nasal symptoms (data not shown). The between-group comparison revealed significant differences in favor of the two FP-treated groups, with no difference between the two groups for each of the nasal symptoms, sneezing ($P<0.01$), runny nose ($P<0.05$), and nasal blockage ($P<0.05$), as well as the combined symptom score ($P<0.02$).

Antihistamine intake was increased only in the placebo group during the season (0.42 tablets/day [$P<0.05$]), whereas this was unchanged in the two FP-treated groups (0.02 and -0.04 tablets/day, respectively). The intake of antihistamine during the season in the FP100 and FP200 groups was signifi-

cantly lower than in the placebo group ($P<0.05$ and $P<0.01$, respectively). Eye symptoms and the use of eye-drops (data not shown) evolved equally in all three treatment groups during the season.

Eosinophil proteins out of the season

Forty-four of the patients returned for a winter visit (visit 4) to measure blood eosinophils and concentration of their granule proteins in serum and nasal lavage fluid out of the season. This enabled comparison with visit 1 values, immediately before season and treatment, and pooling of the results of all patients irrespective of treatment group. None of the patients showed signs of nasal disease or had nasal complaints at visit 4.

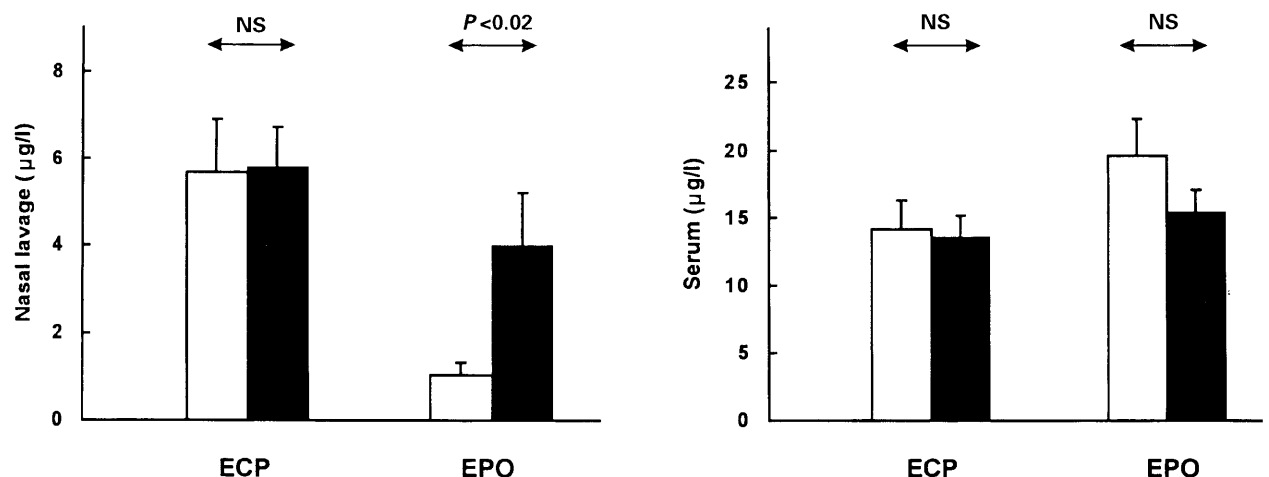


Fig. 5. ECP and EPO content of nasal lavage fluid and serum during winter (visit 4) and 2 weeks before season (visit 1). □ visit 4 ($n=44$); ■ visit 1 ($n=60$ for nasal lavage fluid and $n=63$ for serum) (mean ± SEM; NS: not significant).

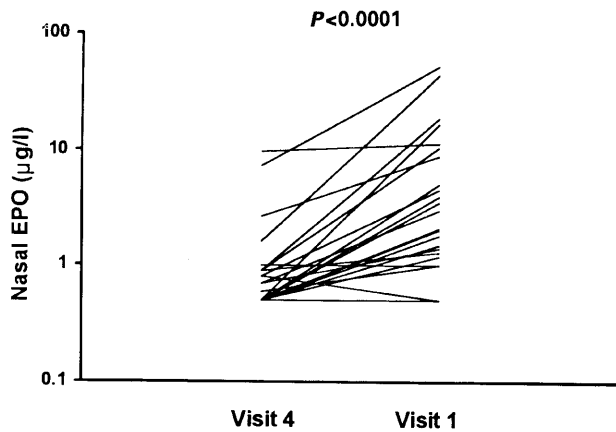


Fig. 6. Comparison of change between nasal lavage fluid EPO in patients examined at visit 4 and visit 1 ($n=41$).

EOS was almost equal at visit 4 and at visit 1; i.e., $209 \pm 38 \times 10^6/l$ and $149 \pm 25 \times 10^6/l$ (NS). SECP, sEPO, and nECP were not significantly different between visits 4 and 1 (Fig. 5). However, nEPO was significantly lower at visit 4 than visit 1 ($P < 0.02$). This finding was accentuated by paired analysis of nEPO (Fig. 6) in patients seen at both visits ($P < 0.0001$).

Discussion

Eosinophil proteins in allergic rhinitis

Recruitment of eosinophil leukocytes from the bone marrow and their activation in the airway mucosa are a hallmark of allergic inflammation when sensitized patients are exposed to inhaled allergens.

In the present study, we observed an increase during the grass-pollen season in blood eosinophil count, as well as serum and nasal lavage concentration of ECP and EPO. These findings are in accordance with earlier reports of a seasonal increase in EOS and systemic and lavage levels of eosinophil granule proteins (11–13). Our data emphasize the importance of considering not only the number of eosinophils, but also their degree of activation, as illustrated by the relatively higher increase of activation markers when compared to the eosinophil count.

Using carefully selected monoallergic patients, we found higher EOS in patients than controls before the pollen season. More interesting, we also found an increased level of sEPO at the same time point. Thus, signs of systemic eosinophil recruitment and activation were present in patients without known allergen exposure. We can merely speculate about the explanation.

Previous data have indicated that an eosinophil inflammation may be self-perpetuating by autocrine

secretion-activation of inflammatory cells; e.g., eosinophil recruitment by release of interleukin-5 from eosinophils (14).

Exposure to food allergens represents an alternative explanation. Regular exposure to small amounts of cross-reacting food allergens may induce inflammation without causing obvious clinical symptoms.

Furthermore, pollen-allergic persons could, genetically or otherwise constitutionally, be predisposed to a higher degree of eosinophil "readiness".

While the cause of the increase in eosinophil markers in pollen-allergic subjects outside the season remains speculative, previous findings make it highly unlikely that this is an incidental finding (7, 15).

It was also noteworthy that an increase in the systemic levels of eosinophils as well as their granular proteins was seen in the period just before the pollen season. Considering the eosinophil markers at the inflammatory site, i.e., the nasal mucosa, no signs of inflammation was observed in winter, but nEPO was increased significantly at visit 1. A possible explanation would be a reaction with earlier occurring allergens, but this finding could also suggest a cyclic variation throughout the year with increasing eosinophil activity toward the season.

A difference was seen between the changes in eosinophil markers in the present study. EPO was earlier and more sensitive as a marker of eosinophil inflammation than ECP. A previous study has proposed selective ECP and EPO release from eosinophils, based upon the type of receptor stimulus; i.e., whether it is IgE or non-IgE mediated (8). According to this, EPO release might be mediated primarily by IgE, an effect which could explain the obvious difference between the two mediators in our allergic patients. Data demonstrating sECP levels to be higher in non-allergic asthma and rhinitis than in allergic disease could indirectly support this theory (16). However, other studies have, so far, failed to support the theory of selective release. Moreover, it seems reasonable that other mechanisms, i.e., cytokine priming of eosinophils, plays a major part in the release of granular proteins from eosinophils.

Tests for eosinophil activation

There is a need for a biochemical test for eosinophil inflammation. Studies (17–19) have clearly shown that EOS has too low a sensitivity and discriminative power to be useful. Our results indicate that the measurement of eosinophil markers, and especially EPO in allergic subjects, may be useful for

monitoring the effect of steroid treatment. The nose is an easily accessible organ, and it could be preferential to measure values as close as possible to the site of inflammation; i.e., in nasal lavage fluid. However, the scatter of values is considerable with lavage methods used at present, and further standardization is needed.

Steroid effect in allergic rhinitis

Intranasal steroids have a profound effect on symptoms of allergic rhinitis and nasal polyposis, which are both characterized by nasal eosinophilia (20). However, steroids have little or no clinical effect in infectious rhinosinusitis, which is characterized by neutrophilia (21).

In the present study, FP completely inhibited seasonal increase of eosinophil markers, but did not influence the neutrophil marker HNL, either in nasal lavage fluid or in serum. These findings emphasize the close association between eosinophil inflammation and steroid responsiveness. Possibly, measurement of nasal eosinophil markers can be used to predict responsiveness to steroids in non-allergic rhinitis. Moreover, the present results suggest the benefit of initiating treatment before the season, to abolish the building-up of the nasal inflammatory reaction.

Intranasal FP abolished the increase not only in nasal but also in circulating eosinophil markers. This new and most interesting observation is supported by recently published results (7).

Steroids can be absorbed from the nose (22), however, a recent publication has shown that FP is absorbed to a low degree (23). We find it unlikely that significantly reduced levels of systemic eosinophil markers were caused by a systemic absorptive effect of intranasal FP. We prefer to assume that the cause is an indirect anti-inflammatory mechanism, induced by a local steroid effect in the nose. When allergens are deposited in the nose, local allergen-IgE interaction probably recruits eosinophils from the bone marrow by release of cytokines and chemokines from T cells and other inflammatory cells in the nose (24, 25). We believe that it is a local steroid-induced inhibition of cytokine/chemokine generation which indirectly causes the effect on circulatory eosinophil markers. This view is supported by our observation of mucosal inflammation being the first step of the allergic reaction.

This hypothesis of a systemic consequence of a local anti-inflammatory activity may have important clinical implications. It indicates that intranasal anti-inflammatory treatment may have a beneficial effect on asthma by reducing circulating eosinophil number and activation. In fact, a number of clinical trials have shown intranasal steroid treatment to

have a clinically beneficial effect on bronchial hyper-responsiveness as well as asthma symptoms (26–29).

We were not able to demonstrate any difference between the two dosages of FP in the present study, either regarding impact on symptoms or relating to influence upon eosinophils and their markers. This finding illustrates the dramatic efficacy of even small steroid doses in the treatment of allergic inflammation and its consequences.

In Conclusion, the present results support the role of eosinophil granulocytes as one of the major effector cell types in allergic inflammation. Moreover, they indicate a possible role for eosinophil markers in diagnosis and monitoring of allergic rhinitis. The need to consider not only the number of eosinophils, but also their degree of activation, as illustrated by the granular proteins ECP and EPO, is emphasized. The data also demonstrate that EPO is a more sensitive measure of allergic inflammation than ECP.

Our results point to the presence of an increased degree of activation of systemic eosinophils in pollen-allergic rhinitis patients out of season, and that the mucosal reaction is first in line of eosinophil activation during allergic inflammation, with a feed-back for systemic eosinophils, when a certain stimulus level is reached.

Intranasal FP was able to decrease eosinophil activation not only in nasal mucosa, but also systemically, as illustrated by serum levels. Finally, present data demonstrate the efficacy of even small doses of intranasal steroid therapy in the treatment of seasonal allergic rhinitis.

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