

Allergen-induced inflammation in the nose: a comparison of acute and repeated low-dose allergen exposure

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To investigate allergic rhinitis induced by two experimental provocation models, we compared local inflammation with markers of eosinophil activity in peripheral blood. Patients with strictly seasonal allergic rhinitis were selected and investigated outside the pollen season. An acute challenge with increasing doses of allergen every 15 min until symptoms occurred was performed in nine patients. Nasal lavage and blood samples were taken before and 4 and 24 h after challenge. After a 6-week washout period, 10 patients were submitted to 7 days of repeated low allergen exposures. One small dose ($\approx 1/100$ of the acute dose) was given each day. Blood and lavage samples were taken prior to and after the period. As control four patients were challenged with diluent only. The acute challenge resulted in sneezing and nasal discharge and blockage and was accompanied by a rise in histamine and eosinophil cationic protein (ECP) in lavage fluid after 4 h and continuing after 24 h, when there also was a rise in the number of eosinophils and ECP in peripheral blood. The repeated low-dose exposures caused very few symptoms but produced increased ECP in the lavage fluid and a trend toward increased histamine concentration. There were no changes in ECP, intracellular EG2 binding, or number of eosinophils in the blood. No changes were seen in the control group. Our findings show that changes in eosinophil mediator release in nasal lavage can be seen after very low, but repeated, allergen exposures despite no, or minimal, clinical symptoms.

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Inflammation of the airways is considered a cardinal sign in nasal and bronchial hyperresponsiveness. Many cells play a role including mast cells and eosinophils. In the immediate allergic response, mast cells release histamine and other mediators. Histamine and tryptase have been measured in nasal lavages and brush samples, and their concentrations have been used as markers of mast-cell activation (1–3). Allergen applied topically on the nasal mucosa in subjects with allergic rhinitis produces an immediate reaction involving itching, irritant, secretory, and exudative effects. Frequently, this initial response becomes a more sustained inflammatory process in the airway mucosa with influx and activation of cells, predominantly eosinophils (4–6). In allergic, parasitic, and some other diseases, eosinophil number is increased in both peripheral blood and affected tissue. Blood-and-tissue eosinophilia is often found in asthma and rhinitis, and

activated eosinophils, which appear to be hypodense, probably as a result of degranulation, have been demonstrated in increased numbers (7, 8). Release of toxic granule proteins from the eosinophil granulocytes probably plays a crucial role in the development of allergen-induced lesions of the nasal mucosal function, leading to changes in responsiveness to specific and nonspecific stimuli. Eosinophil cationic protein (ECP) and the number of eosinophils in peripheral blood correlate with the severity of asthma and allergic rhinitis (9–12), and there is an elevated level of ECP in plasma and nasal and bronchoalveolar lavage fluid after allergen provocation (6, 7, 13). Together with other eosinophil products, ECP has been suggested as a major factor in allergic inflammation.

A new method for evaluating eosinophil activity in peripheral blood has recently been developed whereby eosinophils in unseparated peripheral

blood can be analyzed by flow cytometry with a cell-membrane permeabilization procedure (14), the FOG method (15, 16). The aim of the present study was to assess the possibility of a correlation between allergic inflammation in the nasal mucosa and signs of activated eosinophils in peripheral blood measured as the expression of intracellularly bound EG2 antibodies and levels of ECP in serum. We also wanted to investigate whether this inflammation could be induced with two different types of allergen challenge. The first was a conventional allergen challenge with rapidly increasing allergen doses until symptoms occurred, and the second was a challenge model that more resembles natural allergen exposures with repeated low doses of allergen for a longer period.

Material and methods

Patients

Eleven nonsmokers (eight women and three men) (mean age 27.8 years, range 23–41 years) entered the trial. They all had a history of seasonal allergic rhinitis, positive skin prick test, and RAST greater than class 2 (Pharmacia CAP System RAST FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden) to birch pollen, and they had no clinical manifestations of their atopic disease during the study period. The control group comprised four of the patients. All the patients had been free from any medication for at least 3 weeks before entering the study and no drugs were allowed during the trial. The study was performed outside the pollen season and there was no history of respiratory infection for at least 3 weeks before the study period. The patients gave their informed consent and the study was approved by the ethics committee of Karolinska Hospital.

Study design

Part I. Acute challenge. A nasal lavage procedure was performed in nine patients prior to and 4 and 24 h after a nasal challenge, which was given with increasing doses of allergen until symptoms of itching and sneezing occurred. Before each nasal lavage, blood samples for differential counts, ECP, and intracellularly bound EG2 were drawn.

Part II. Repeated low-dose challenge. After a wash-out period of 6 weeks, 10 subjects, of whom eight had participated in part I, were submitted to a series of single allergen challenges repeated on seven successive weekdays. The allergen doses used were approximately 1/100 of the cumulative dose, for each individual, used in part I (range 13–260 SQ) in accordance with Ihre & Zetterström (17) and Ihre et al. (18). The two patients that did not

participate in part I (nos. 9 and 10) were given 260 SQ. The same allergen dose was given each day. The trial started and ended with a nasal lavage procedure, and blood samples for differential counts, ECP, and intracellularly bound EG2 were drawn.

Part III. Control challenge. Control challenges with diluent (Aquagen, ALK, Copenhagen, Denmark) in accordance with parts I and II were done in four of the patients. The controls 1–4 correspond to patients 8, 9, 5, and 7.

Nasal lavage technique

A nasal lavage technique described in detail by Naclerio et al. (3) was employed. With their heads bent back, the patients kept 5 ml of isotonic room-temperature saline solution in each nasal cavity for 10 s. The lavage fluid was then returned into a plastic cup and immediately chilled and centrifuged at 4°C at 300 g for 8 min. The sol phase was separated from the cellular phase, and the supernatant was aliquoted for subsequent analysis.

Nasal challenge procedure

Directly after the initial lavage, the subjects were challenged with birch pollen extract (Aquagen, ALK, Copenhagen, Denmark) dissolved in an albumin diluent (Aquagen, ALK, Copenhagen, Denmark). In part I, the dose was increased 10-fold every 15 min until the subject had a positive (immediate) reaction measured on clinical symptoms such as nasal blockage, itching, secretion, and sneezing. The doses used were 26, 260, 2600, or 26 000 SQ delivered with a mechanical pump spray delivering 0.13 ml of the solution (two puffs were sprayed into the right nostril).

Symptom registration

Before each lavage during parts I and II, and every day during part II, the participants recorded their symptoms, using a detailed symptom score sheet. The number of sneezes was counted. The symptoms of blockage and secretion were assessed using a 4-point scale ranging from 0 to 3 where 0 represented no symptoms and 3 represented almost intolerable symptoms.

Eosinophil cationic protein in nasal lavage fluid

ECP was measured in duplicate in unconcentrated nasal lavage fluid with a commercially available double-bound radioimmunoassay (Pharmacia ECP RIA, Pharmacia Diagnostics AB, Uppsala,

Sweden). ECP in the nasal fluid competes with ^{125}I -labeled ECP for binding sites of specific antibodies. Bound and free ECP is separated by adding a second antibody immunosorbent. After centrifugation and decanting, the radioactivity measured in the pellet is inversely proportional to the quantity of ECP in the nasal fluid. ECP levels are expressed in $\mu\text{g/l}$. Levels of ECP under $2 \mu\text{g/l}$ are undetectable.

Histamine

Histamine was analyzed in unconcentrated nasal lavage fluid with a commercially available radioimmunoassay (histamine, radioimmunoassay kit, IMMUNOTECH International SA, Marseilles, France). In the assay, modified histamine competes with iodinated histamine tracer for the binding to an antihistamine antibody coated on a test tube. Standard solution or sample was mixed with acylation buffer and incubated for 30 min. Acylated standard or sample and ^{125}I -radiolabeled histamine were then added to antihistamine-antibody-coated tubes. The total radioactivity was determined in uncoated tubes. After incubation for 18 h at $2-6^\circ\text{C}$, the coated tubes were aspirated, and the bound radioactivity was counted. The histamine levels are expressed in ng/ml . Levels of histamine under 0.05 ng/ml are undetectable.

Eosinophil counts

The percentage and number of eosinophils were enumerated by routine differential counting using a Coulter STKS (five-part differential count) at the Department of Clinical Chemistry, Karolinska Hospital.

Serum eosinophil cationic protein

ECP levels were measured by a commercially available fluoroimmunoassay (Pharmacia ECP System CAP FEIA, Pharmacia Diagnostics AB, Uppsala, Sweden). The sera were collected and handled according to the instructions by the manufacturer and then stored at -20°C until analyzed.

Expression of the EG2-epitope on intracellular ECP in eosinophils

The method used for fixation and cell membrane permeabilization of peripheral blood leukocytes (the FOG method) has been described in detail elsewhere (15, 16). Briefly, permeabilization is obtained by treating paraformaldehyde-fixed leukocytes with n -octyl- β -D-glucopyranoside (OG). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody EG2 (Pharmacia Diagnostics AB, Uppsala,

Sweden) was added to the permeabilized leukocytes and analyzed for the expression of antibody bound to intracellular ECP, using flow cytometry. The expression of the EG2 epitope on ECP was measured in mean fluorescence intensity (MFI) units.

Statistical analysis

Statistical evaluation of potential differences between before and after challenge was performed with the nonparametric Wilcoxon signed ranks test for paired observations, as the data did not come from a normal distribution. Results are presented as medians with interquartiles.

Results

All subjects in part I completed the study. One of the patients from part I was not able to continue into part II.

I. Acute allergen challenge

Symptoms. All patients had immediate symptoms of sneezing and/or secretion and itching after the acute challenge.

Nasal lavages. ECP in lavage fluid was significantly increased from 3.3 (i.q.r. $2.7-5.1$) to 7.6 (i.q.r. $6.2-12.0$) $\mu\text{g/l}$ 24 h after challenge ($P < 0.05$). Histamine levels increased significantly from 0.2 (i.q.r. $0.05-0.6$) before to 0.6 (i.q.r. $0.12-1.0$) ng/ml ($P < 0.03$) 4 h and 0.4 (i.q.r. $0.3-0.6$) ng/ml ($P < 0.05$) 24 h after the acute allergen challenge. (Figs. 1 and 2).

Eosinophil counts and s-ECP in peripheral blood. There was a statistically significant change in the number of eosinophils ($P < 0.03$) from baseline 0.2 (i.q.r. $0.1-0.3$) $\times 10^9/\text{l}$ to 0.4 (i.q.r. $0.2-0.4$) $\times 10^9/\text{l}$ 24 h after challenge and in ECP ($P < 0.05$) 24 h after the allergen exposure. Baseline s-ECP values were 7.4 (i.q.r. $5.6-9.8$) $\mu\text{g/l}$ compared with 11.5 (i.q.r. $9.4-13.5$) $\mu\text{g/l}$ 24 h after challenge (Figs. 1 and 3).

Intracellular expression of the EG2 epitope on ECP. There was no statistically significant change in the intracellular expression of the EG2 epitope on ECP 4 or 24 h after allergen challenge.

II. Low-dose allergen exposure

Symptoms. No or minimal clinical symptoms were observed.

Nasal lavages. Concerning ECP in lavage fluid, the level was significantly higher (6.8 [i.q.r. $2.8-16.0$]

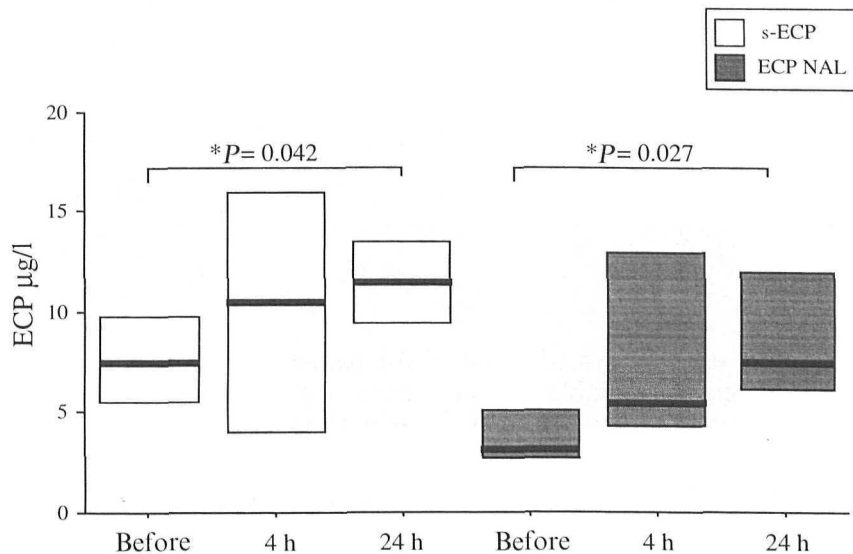


Fig. 1. s-ECP and ECP in nasal lavage fluid (NAL) before and after acute allergen challenge (part I). Boxes cover values within interquartile ranges. Horizontal bold line is at median.

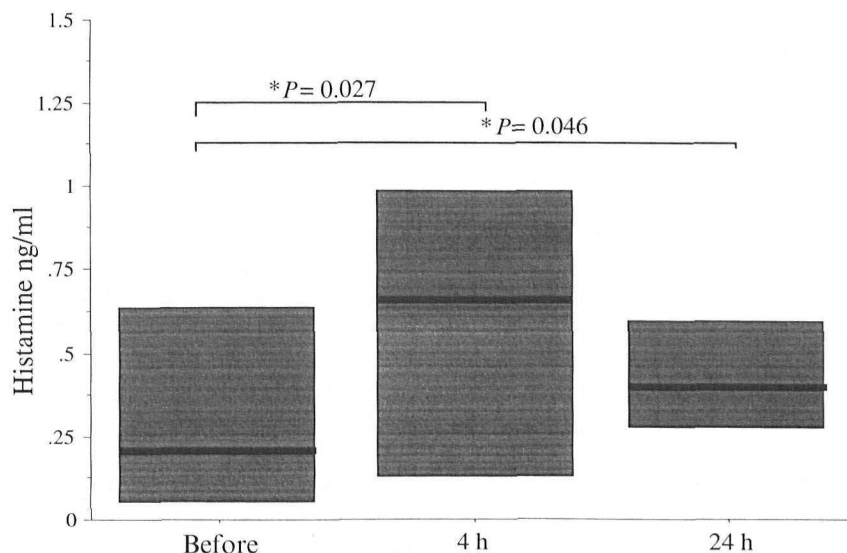


Fig. 2. Histamine in nasal lavage fluid before and after acute allergen challenge (part I). Boxes cover values within interquartile ranges. Horizontal bold line is at median.

$\mu\text{g/l}$) ($P < 0.02$) after the 7 days of low-dose allergen exposure compared to baseline values (3.5 [i.q.r. 1.5 – 4.8] $\mu\text{g/l}$) (Fig. 4). After 7 days of low doses of allergen exposure, there were no changes in the levels of histamine 6.2 (i.q.r. 2.7 – 45.0) ng/ml before and 12.5 (i.q.r. 2.5 – 40.0) ng/ml after the challenge period.

Eosinophil counts and ECP in peripheral blood. No statistically significant changes were observed.

Intracellular expression of the EG2 epitope on ECP. No significant changes were detected in expression of the intracellularly bound EG2 epitope on ECP after 1 week of low-dose allergen exposure. Table 1 shows the individual data.

III. Control challenge

In the control group, no symptoms or significant changes were found in the amount of histamine and ECP recovered in the nasal lavage fluid after the challenge periods. No changes were observed in eosinophil counts and ECP in peripheral blood. Tables 2 and 3 show the individual data.

Discussion

Tissue and blood eosinophilia have been regarded as a hallmark of allergy, but the degree of activation of the eosinophils *in vivo* in man and their role in allergic inflammation is still largely speculative.

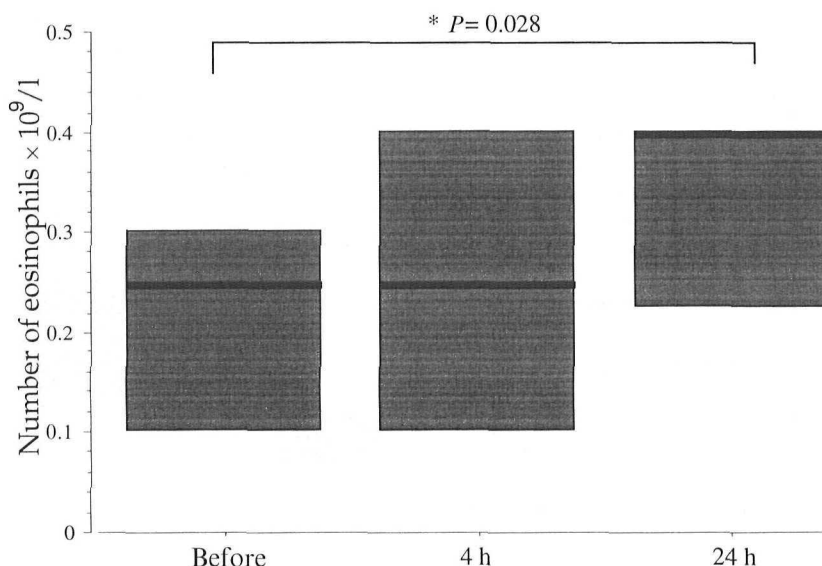


Fig. 3. Number of eosinophils in peripheral blood before and after acute allergen challenge (part I). Boxes cover values within interquartile ranges. Horizontal bold line is at median.

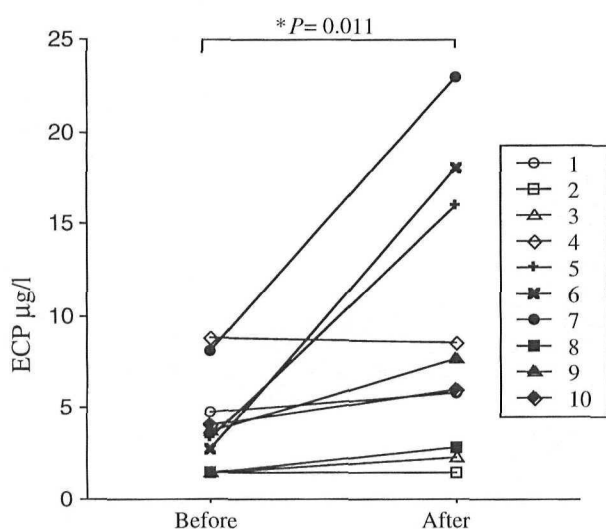


Fig. 4. ECP measured in lavage fluid before and after 7 days of low-dose allergen exposure (part II).

Others have previously shown (6) an early influx of probably activated eosinophil granulocytes, and the rise in ECP in nasal fluid found in our study after allergen challenge fits with such an influx. We also found a significant increase of histamine in the lavage fluid which lasted for 24 h after the acute challenge. Since histamine is rapidly destroyed and probably cleared by nasal discharge, the long-lasting histamine response cannot be due solely to the initial cell response from the mast cells. It has been reported that basophils are recruited to the mucosa after allergen exposure in both skin, and nose and bronchi (19–21). These cells could be a source of this histamine.

The allergen challenge in the first part of the

study triggered a significant influx of eosinophils into peripheral blood as well as an increase in s-ECP (but still within normal range). This could reflect either the fact that the number of eosinophils is increased or a general increase in eosinophil activity. However, no direct evidence of eosinophil activity was seen in peripheral blood measured by the EG2 epitope on ECP. It is interesting that an allergen challenge given to the limited area of nasal mucosa produced detectable changes in the systemic circulation.

In the second part of the present trial, we did not perform a conventional nasal provocation test in which relatively high allergen doses are administered. Instead, we gave repeated low “subclinical” doses of allergen that probably more resemble natural allergen exposure during a normal pollen season. In spite of the lack of, or minimal, clinical symptoms after this challenge period, we demonstrated an increase in ECP in lavage fluid, which is probably a result of eosinophil influx and release. However, no changes were found in number of eosinophils or ECP level in peripheral blood. The results contrast with findings in atopic subjects with ongoing symptoms from bronchi and skin (22, 23), where serum ECP levels were elevated and correlated to the severity of disease. This difference could be explained by the intensity of inflammation and size of the nasal mucosa. In parallel, no change in eosinophil activity in peripheral blood could be detected with the FOG method and EG2 antibodies after repeated low doses of allergen exposure, in contrast to that previously found by Ihre et al. (24). In their study, five of six asthmatic patients exhibited activated eosinophils in the circulation, as

Table 1. Individual data: low-dose allergen challenge. s-ECP and number of eosinophils in peripheral blood. ECP and histamine in nasal lavage fluid. Measurements before and after 7 days of low-dose allergen challenges

Patient	Allergen	s-ECP		EOS		EG2		ECP NAL		HIST	
	Birch	Before	After	Before	After	Before	After	Before	After	Before	After
	SQ	$\mu\text{g/l}$	$\mu\text{g/l}$	$10^9/\text{l}$	$10^9/\text{l}$	MFI	MFI	$\mu\text{g/l}$	$\mu\text{g/l}$	ng/ml	ng/ml
1	26	4.7	2.6		0.1	35.1	30.7	4.8	5.8	0.32	0.25
2	260	3.2	5.2	0.1	0.2	31.8	22.9	1.5	1.5	3.4	1.5
3	13	11	7.8	0.5	0.3	24.3	28.4	1.5	2.3	4.5	4.0
4	26	17	21	0.2	0.2	36.8	25.1	8.8	8.6	0.27	1.3
5	26	7.2	8.3	0.2	0.3	39.1	29	3.4	16	0.05	0.2
6	260	25	18	0.8	0.5	27.1	28.3	2.7	18	0.35	1.2
7	26	11	23	0.4	0.5	31.6	27.3	8.1	23	0.9	0.5
8	260	13	14	0.2	0.2	25.8	24.9	1.5	2.8	27.0	40.0
9	260	6.1	10	0.3	0.4	25.9	26	3.7	7.7	12.3	11.0
10	260	3.7	5.1	0.2	0.3	23.3	27	4.1	6	0.13	0.05

EOS: eosinophils; HIST: histamine; NAL: nasal lavage fluid; MFI: mean fluorescence intensity units.

Table 2. Individual data: control group. Acute allergen challenge

Patient	s-ECP			EOS			EG2			ECP NAL			HIST		
	Before	4 h	24 h	Before	4 h	24 h	Before	4 h	24 h	Before	4 h	24 h	Before	4 h	24 h
	$\mu\text{g/l}$	$\mu\text{g/l}$	$\mu\text{g/l}$	$10^9/\text{l}$	$10^9/\text{l}$	$10^9/\text{l}$	MFI	MFI	MFI	$\mu\text{g/l}$	$\mu\text{g/l}$	$\mu\text{g/l}$	ng/ml	ng/ml	ng/ml
1	16.0	12.0	11.0	0.2	0.1	0.2	24.7	27.6	22.4	3.3	1.5	3.5	29.00	15.00	13.0.0
2	3.9	14.0	10.0	0.1	0.1	0.1	15.8	16.9	15.9	1.5	1.5	1.5	0.07	0.21	0.05
3	10.0	5.8	8.9	0.2	0.2	0.2	26.0	23.4	24.6	2.7	1.5	5.0	0.17	0.07	0.43
4	12.0	7.5	7.2	0.1	0.1	0.1	13.7	13.8	17.5	5.1	3.5	27.0	1.20	1.50	2.20

Abbreviations as in Table 1.

Table 3. Individual data, control group. Low-dose allergen exposure

Patient	s-ECP		EOS		EG2		ECP NAL		HIST	
	Before	After	Before	After	Before	After	Before	After	Before	After
	$\mu\text{g/l}$	$\mu\text{g/l}$	$10^9/\text{l}$	$10^9/\text{l}$	MFI	MFI	$\mu\text{g/l}$	$\mu\text{g/l}$	ng/ml	ng/ml
1	5.5	8.7	0.2	0.2	28.4	24.6	3.4	2.6	21.00	9.40
2	11.0	5.8	0.1	0.1	16.3	13.6	2.6	1.5	0.23	0.17
3	9.5	13.0	0.2	0.2	22.6	25.7	2.2	5.2	0.08	0.27
4	4.1	8.0	0.4	0.3	29.3	25.1	9.0	8.7	3.20	0.48

Abbreviations as in Table 1.

detected with the FOG method after 7 days of inhalation of repeated low-allergen doses. This could be because Ihre et al. used a bronchial allergen challenge model instead of a nasal model. If slightly higher doses had been used in our repeated challenges, more symptoms and signs might have been noted. Thus, neither in the acute exposure nor after repeated low-dose exposure could systemic activation of eosinophils (EG2) be seen. The fact that EG2 did not increase might be explained by the relatively small challenge organ. However, since ECP rose in serum after the acute allergen exposure, this is less likely.

It is natural that changes could be seen more easily at the actual site of the inflammation. In this

study, we tried to count eosinophils in lavage fluid, but few and very variable levels were seen even though the cytopspin method was used and staining was performed by a trained technician. Thus, ECP in lavage fluid seems to be a more useful measure of local inflammation.

In conclusion, our results demonstrate a significant increase of ECP measured in nasal fluid with two different models of allergen challenge. Even without significant clinical symptoms, as during and after the period of low-dose exposure, there was an increase of nasal ECP as a sign of ongoing eosinophil infiltration in the tissue. In the two experimental challenge models, local inflammation was inducible with both methods; thus, challenge with repeated

low doses of allergen may be valuable as a clinical experimental model that more resembles natural pollen exposure for investigating the development of local inflammation. This agrees with Ihre & Zetterström (17), who showed an increase in bronchial hyperresponsiveness in 11/13 patients after a week of inhalation with repeated low allergen doses. However, we were not able to show changes in activity markers in peripheral blood when using the low-dose exposure model. The explanation of this is probably that too low doses of allergen were used.

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