

Degranulation of eosinophils from pollen-atopic patients with asthma is increased during pollen season

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The secretion of granule proteins from eosinophils and neutrophils was studied in isolated cells, obtained from 11 pollen-atopic patients with asthma, twice during and twice outside pollen season. Granulocytes were stimulated with serum-opsonized Sephadex particles, and the released amount of eosinophil cationic protein (ECP), eosinophil protein X (EPX), and myeloperoxidase (MPO) were measured by means of specific radioimmunoassay (RIA). Eosinophils from the pollen-atopic patients obtained during pollen season released significantly more ($p < 0.02$) ECP and EPX than cells from the same patients obtained before pollen season. The released amount of ECP and EPX was correlated ($r = 0.54$; $p < 0.003$) to the total pollen count. The release of MPO from neutrophils was only raised ($p < 0.01$) at the end of the pollen season. Serum concentrations of ECP and EPX and blood eosinophil counts were significantly raised ($p < 0.002$, $p < 0.001$, and $p < 0.009$, respectively) before pollen season and increased further at the end of the pollen season. There were no changes in lung function during pollen season and consequently no discernible relationships to eosinophil and neutrophil degranulation. We conclude that eosinophils and, to some extent, neutrophils from birch pollen-atopic subjects have an increased propensity to secrete their granule proteins during a pollen season. We suggest that these cells have been primed as a consequence of allergen exposure. (J ALLERGY CLIN IMMUNOL 1992;89:131-9.)

Key words: Degranulation, ECP, eosinophil, eosinophil protein X, myeloperoxidase, neutrophil, patients with asthma, pollen-atopic patients, pollen season

During recent years, a role for the eosinophil granulocyte in asthma has been suggested by a number of studies. The eosinophil granulocytes contain several highly cytotoxic proteins, such as ECP, EPO, EPX (eosinophil-derived neurotoxin), and MBP.^{1, 2} ECP and MBP have been demonstrated to cause damage to airway epithelium resembling the histopathologic

Abbreviations used

ECP:	Eosinophil cationic protein
EPX:	Eosinophil protein X
MPO:	Myeloperoxidase
RIA:	Radioimmunoassay
EPO:	Eosinophil peroxidase
MBP:	Major basic protein
PEFR:	Peak expiratory flow rate
IL:	Interleukin

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findings in asthma.³ The eosinophil is a potent proinflammatory cell with the capacity to produce the potent bronchoconstrictory agents, leukotriene C₄ and platelet-activating factor.^{4, 5} Eosinophil accumulation in the lung is a common finding of patients with asthma, as demonstrated in bronchoalveolar lavage after allergen challenge in those patients who experience a late asthmatic reaction.⁶ Blood eosinophilia is a distinctive feature in patients with asthma, and several studies

have demonstrated a relationship between the extent of blood eosinophilia and the severity of asthma.^{7, 8} Also, the raised serum levels of eosinophil granule proteins reveal a relationship to the reactivity of the asthmatic lung.⁹ These raised serum levels may partly reflect the ongoing inflammation in the asthmatic lung. However, another explanation would be the increased propensity of the circulating eosinophils to degranulate. Thus, in a recent study, eosinophil granulocytes from patients with asthma demonstrated an increased propensity to release their granule proteins, which was suggested to be a consequence of priming of these cells.¹⁰

The question is whether this increased propensity is a constitutional abnormality of the asthmatic eosinophils or a consequence of allergen exposure. To test this, we have investigated the propensity of the eosinophils to release ECP and EPX *in vitro* in a group of pollen-atopic patients during a pollen season. The patients were examined once before, twice during, and once after the birch-pollen season. For comparison, the release of MPO from neutrophils was also measured to test the cellular specificity of this phenomenon.

MATERIAL AND METHODS

Patients

Eleven birch pollen-atopic patients with asthma, seven women and four men, with a mean age of 33 years (range, 18 to 50 years), were selected for the study. The patients were considered atopic as a result of positive skin tests to birch allergen. None of the patients were smokers. FEV₁ was $\geq 70\%$ of predicted, and all patients had a positive methacholine test. During pollen season, the patients were divided into three groups, based on the amount of treatment needed to control their asthmatic symptoms: (1) using inhaled β_2 -agonists and/or sodium cromoglycate, six patients; (2) using inhaled β_2 -agonists, inhaled steroids, and sodium cromoglycate, four patients; and (3) using a combination of inhaled β_2 -agonists, inhaled steroids, and oral β_2 -agonists or theophylline, one patient. One patient, out of the pollen season, used no medical treatment for asthmatic symptoms (group 0), seven patients belonged to group 1, and three patients belonged to group 2.

References

The reference group consisted of 14 (seven women and seven men) healthy, nonallergic, laboratory employees and their relatives. This group had no allergic symptoms and had serum concentrations of total IgE within the reference range. Nine of the reference group were tested repeatedly during the study, that is two to four times. There was no statistical difference in this group before, during, and after pollen season.

Design of the study

Tests were performed before (test period I), twice during pollen season 1989 (test period II and III), and before pollen season 1990, from Jan. 22 to Feb. 26 (test period IV). All lung-function tests and blood sampling were performed between 9 and 11 AM. The tests included methacholine provocation, FEV₁, FEV₁ in percent of predicted, PEFr, and blood sampling for measurement of blood eosinophil number, eosinophil and neutrophil degranulation, and serum levels of ECP, EPX, and MPO. Total pollen was counted as described below.

Methacholine provocation and FEV₁

Nonspecific bronchial reactivity was assessed by a methacholine challenge test. A stock solution of 2% methacholine was used, and the test was started with tidal-volume inhalation of 3 ml of physiologic saline delivered from a DeVilbiss (DeVilbiss Co., Somerset, Pa.) 346 nebulizer (driven by air at a flow rate of 6 L/min) for 3 minutes. This was followed by inhalations of methacholine solutions in doubling concentrations at 3-minute intervals until FEV₁ had decreased $<80\%$ of the lowest postsaline solution value. The starting concentration of methacholine was either 0.125 or 1.25 mg, depending on the patients' estimated airway hyperreactivity. FEV₁ was measured on Vitalograph (Vitalograph Ltd., Buckingham, England) dry-wedge type of spirometer at 30 seconds and 90 seconds after each inhalation. The nebulizer containing the solution was weighed before and after each inhalation, and the cumulative dose of methacholine was calculated. The provocation dose of methacholine causing a 20% decrease in FEV₁ was estimated from a log dose-response curve by linear interpolation of the last 2 points when FEV₁ had dropped 20%.

PEFRs

PEFRs were measured with a Wright peak-flow meter. The highest value of three attempts was recorded.

Measurement of eosinophil and neutrophil degranulation

The release assays were performed according to the method of Winqvist et al.¹¹ with some minor modifications, as previously described in detail.¹⁰ Granulocytes were isolated from heparinized blood by dextran sedimentation.¹² The granulocyte mixture obtained by this procedure had a purity of $80\% \pm 6\%$ (SD) (references and pollen-atopic patients). The relative amount of eosinophils was, on average, 3% (range, 1% to 6%) in the granulocyte populations obtained from references and 6% (range, 1% to 22%) from pollen-atopic patients. The final concentration of granulocytes in the assay was 1.0×10^9 /L. Incubation was performed with washed serum-treated Sephadex G-15 (Pharmacia Biosystems AB, Uppsala, Sweden) particles (167 gm/L) for 0, 5, and 20 minutes at 37° C. All incubations were made in duplicate. For the measurement of the total cellular content of granule proteins, 300 μ l of granulocytes,

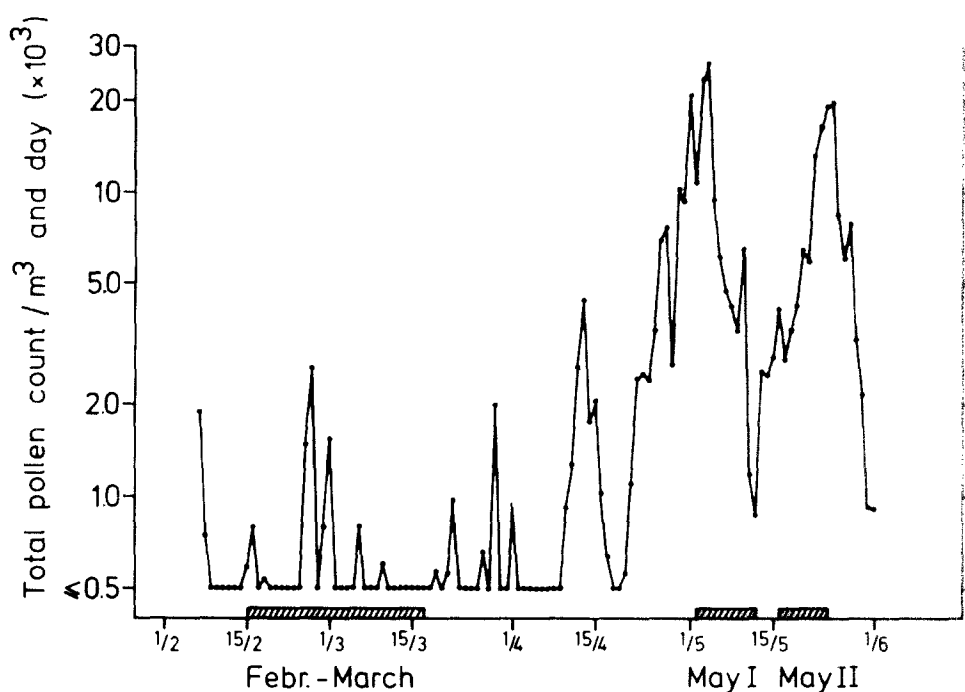


FIG. 1. Total pollen count in 1989 and test periods of the study. *Febr.-March*, period I; *May I*, period II; *May II*, period III.

$3 \times 10^9/L$, were mixed with 1.5 ml of 0.5% *N*-cetyl-*N,N,N*-trimethylammonium bromide in 0.15 mol/L of NaCl and incubated for 1 hour at room temperature, followed by centrifugation at 600 *g* for 10 minutes at 4° C. A volume of 1.5 ml of the supernatant was removed and stored for measurement of granule proteins.

RIA of ECP, EPX, and MPO

The serum concentration, cellular contents, and released amount of ECP, EPX, and MPO were assayed by means of specific RIAs.^{10, 13, 14} The coefficients of variation of the RIAs for ECP, EPX, and MPO were 7%, 4%, and 5%, respectively. There was no cross-reactivity of the MPO assay with EPO.¹⁵

Calculation of released amount of ECP, EPX, and MPO

The amount of released ECP, EPX, and MPO was expressed as percent of the total cellular content, which was calculated from a standard curve of serial dilutions of the respective cell extract. The amount of protein in control incubations was subtracted from the amounts obtained in incubations with particles. Results were calculated with regression analysis.

Blood eosinophil count

The number of eosinophil granulocytes in blood was counted according to the method of Forsham et al.¹⁶

Total pollen count

The total number of pollen was counted by Palynological Laboratory, Swedish Museum of Natural History, Stockholm, Sweden, during 1989, between Feb. 7 and Aug. 31. In 1990, the counting of total number of pollen was started Feb. 7, with Burkard (Burkard Manufacturing Co., Ltd., Rickmansworth, Hertfordshire, England) 7-day recording volumetric spore trap.¹⁷ A sucking rate of 10 L/min was used to collect the airborne pollen and spores. The trap was placed on the roof of a house in the central part of Stockholm.¹⁸ The height of the orifice above the ground level was 20 m. The exposed tape was cut into daily segments and embedded in glycerine jelly. Twelve transverse strips for every second hour were counted.¹⁹ The analyzed pollen grains are expressed as the total number of pollen per day and per cubic meter (Fig. 1).

Statistical evaluation

Wilcoxon's signed-rank test, Mann-Whitney U test, linear correlation analysis, and Spearman's rank correlations were used. All statistical calculations were performed on a personal computer by means of the statistical package, Statgraphics (STSC, Inc., Rockville, Md.).

RESULTS

Eosinophil and neutrophil degranulation

Eosinophil degranulation was measured by means of the release of ECP and EPX. Eosinophils from the

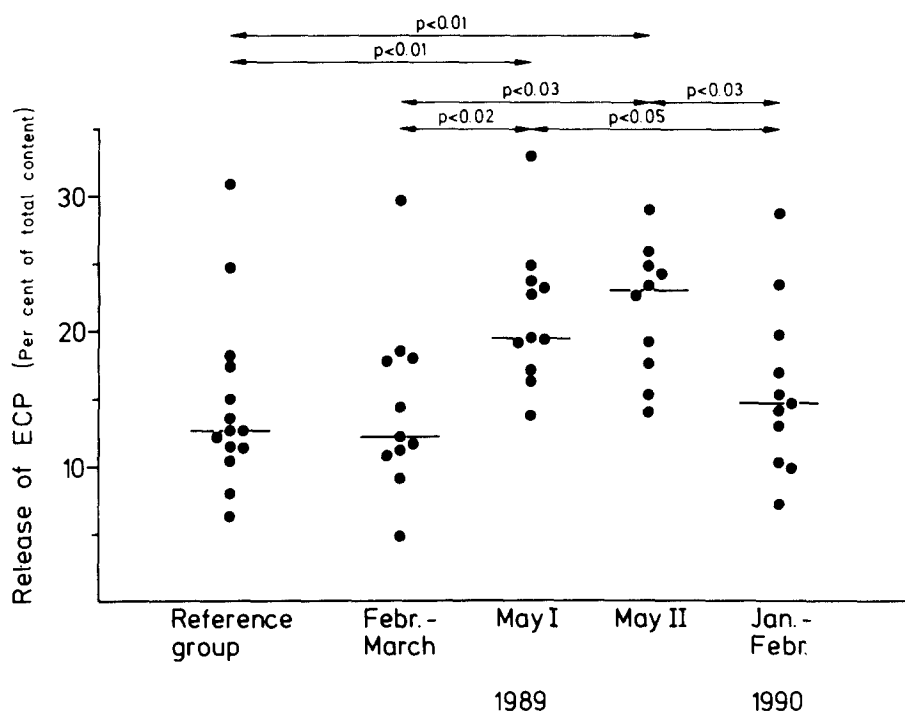


FIG. 2. Release of ECP from eosinophils of pollen-atopic patients ($N = 11$) and reference group ($N = 14$) after 20 minutes of incubation with washed serum-opsonized particles. Respective median values and significant difference between the two groups as determined by means of Mann-Whitney U test are indicated. Significant differences within the pollen-atopic group as determined by means of Wilcoxon's signed-rank test are also indicated.

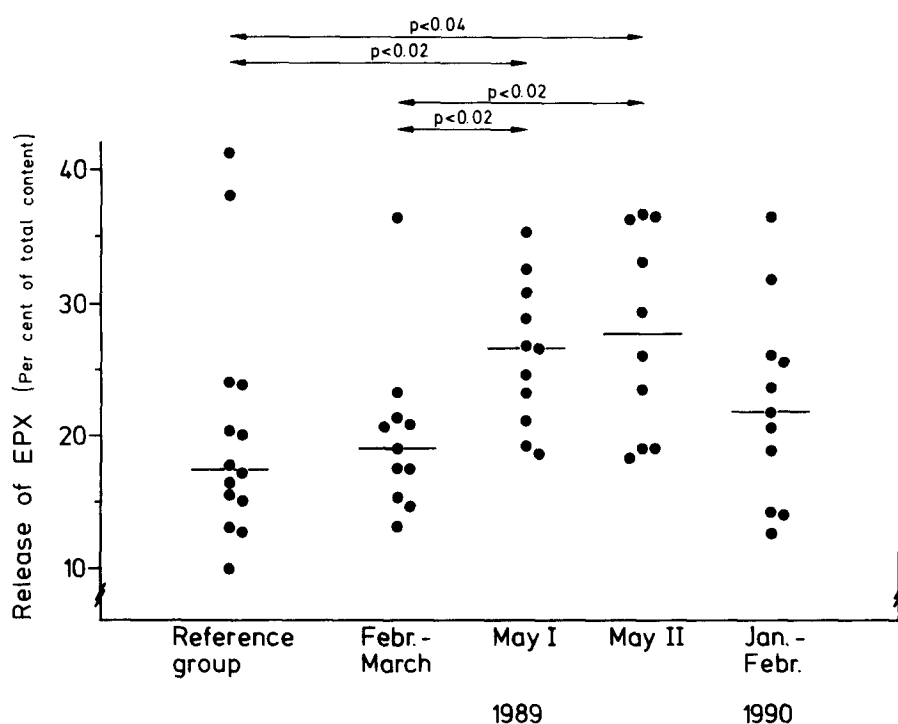


FIG. 3. Release of EPX from eosinophils of pollen-atopic patients ($N = 11$) and reference group ($N = 14$) after 20 minutes of incubation with washed serum-opsonized particles. Respective median values and significant difference between the two groups as determined by means of Mann-Whitney U test are indicated. Significant differences within the pollen-atopic group as determined by means of Wilcoxon's signed-rank test are also indicated.

pollen-atopic patients during pollen season (test periods II and III) released, after 20 minutes of incubation, significantly higher amounts of both ECP ($p < 0.02$ and $p < 0.03$, respectively) and EPX ($p < 0.02$ and $p < 0.02$, respectively) than eosinophils from the same patients taken before pollen season. There was no difference between the released amounts of ECP and EPX from pollen-atopic patients before and after pollen season compared with the released amount of the same proteins of eosinophils from the reference group (Figs. 2 and 3).

Neutrophil degranulation was measured by means of MPO release. Neutrophils from the pollen-atopic patients obtained during pollen season (test period III) released, after 20 minutes of incubation, significantly higher ($p < 0.01$) amounts of MPO compared with neutrophils from the same patients taken before or after pollen season. There was no difference between the released amount of MPO from pollen-atopic patients before and after pollen season compared with the released amount of MPO of neutrophils from the reference group (Fig. 4).

The cellular contents of ECP and EPX

The amount of ECP and EPX per eosinophil was estimated. There was no difference between the content of either ECP or EPX in eosinophils from the pollen-atopic patients during test periods I to IV compared with content of eosinophils from the reference group (Table I). As presented in Table I, the cellular contents of both ECP and EPX were higher in period III compared with period IV in the pollen-atopic subjects.

Serum concentrations of ECP and EPX

In the pollen-atopic group, the mean serum concentration of ECP was 50.5 $\mu\text{g/L}$ (range, 8.8 to 118 $\mu\text{g/L}$) and of EPX, 37.7 $\mu\text{g/L}$ (range, 15.7 to 66.5 $\mu\text{g/L}$) before pollen season. Both values were significantly higher ($p < 0.002$ and $p < 0.001$, respectively) than the mean concentration of ECP and EPX in the reference group (Table II). As presented in Table II, the serum levels of ECP increased further during late pollen season and decreased again after pollen season. There was a correlation between serum levels of EPX ($r = 0.42$; $p < 0.02$) and degranulation of EPX after 20 minutes incubation in vitro (Table III).

Blood eosinophil counts

In the pollen-atopic group, the mean blood eosinophil count was $283 \times 10^6/\text{L}$ (range, 110 to $730 \times 10^6/\text{L}$) before season, which was significantly higher than the mean blood count in the reference group ($p < 0.009$). The eosinophil counts stayed unaltered during and after the pollen season (Table IV).

TABLE I. Cellular contents of ECP and EPX

	Mean ($\pm$ SEM) $\mu\text{g}/10^6$ eosinophils	Range
ECP		
Ref	19.2 ± 2.8 (N = 14)	7.0-41.2
P I	14.8 ± 2.1 (N = 10)	6.8-27.5
P II	14.5 ± 1.9 (N = 11)	7.8-25.6
P III	$21.8 \pm 3.6^*$ (N = 10)	5.4-44.8
P IV	11.6 ± 1.4 (N = 11)	4.4-19.9
EPX		
Ref	12.2 ± 1.6 (N = 14)	4.4-21.5
P I	9.8 ± 1.6 (N = 11)	4.8-23.1
P II	11.0 ± 1.7 (N = 11)	5.4-19.2
P III	$16.0 \pm 2.8^*$ (N = 10)	5.4-34.5
P IV	8.3 ± 1.2 (N = 11)	3.4-14.7

Ref, Reference group; P, period.

The cellular contents for the pollen-atopic patients during periods I to IV and the reference group are presented. The statistical comparison of the values of the reference group and the pollen-atopic group during periods I to IV, respectively, as calculated means of Mann-Whitney U test, revealed no significant differences.

* $p < 0.02$, statistical difference of the values at period III compared with IV was calculated by means of Wilcoxon's signed-rank test.

Relationship between the released amount of ECP, EPX, and MPO and total pollen count

In the pollen-atopic group, the released amounts of ECP, EPX, and MPO, respectively, were correlated ($p < 0.003$, $p < 0.003$, and $p < 0.02$, respectively) to the total pollen count (Table V).

Relationship between the medical treatment of the patients and the degranulation of eosinophils and neutrophils

An attempt was made to determine whether there was any relationship between the degranulation of the respective protein in vitro and the extent of medication. For this purpose, the individual values obtained during periods I to III were correlated. As presented in Table VI, a relationship was suggested between degranulation in vitro of ECP and EPX and the extent of medical treatment needed to control the patients'

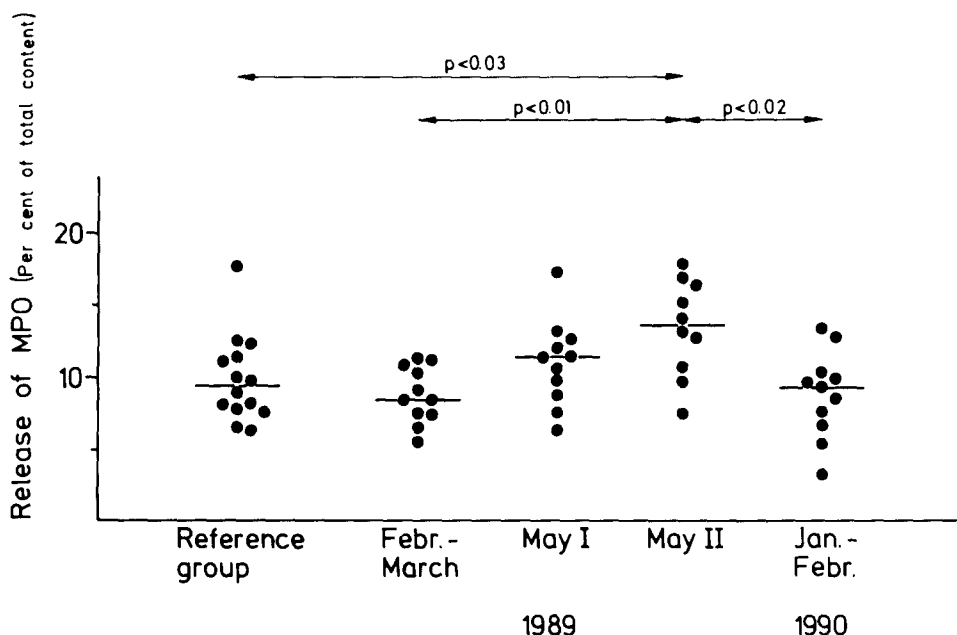


FIG. 4. Release of MPO from neutrophils of pollen-atopic patients ($N = 11$) and reference group ($N = 14$) after 20 minutes of incubation with washed serum-opsonized particles. Respective median values and significant difference between the two groups as determined by means of Mann-Whitney U test are indicated. Significant differences within the pollen-atopic group as determined by means of Wilcoxon's signed-rank test are also indicated.

asthmatic symptoms. No such relationship was, however, found between medical treatment and neutrophil degranulation (Table VI).

Relationship between the clinical conditions of the patients and the degranulation of eosinophils and neutrophils

The released amount of ECP, EPX, and MPO and the serum concentrations of ECP and EPX were evaluated in relation to the patients' lung function, measured as PEFR (not presented), FEV₁ in percent predicted, and methacholine provocation. There were no changes in lung function during pollen season (Table VII), and no discernible relationships to eosinophil or neutrophil degranulation.

DISCUSSION

The present investigation has demonstrated that eosinophils and, to some extent, neutrophils from the peripheral blood of pollen-atopic patients with asthma have an increased propensity to secrete their granule proteins during a pollen season. This finding supports the previous demonstration in a group of subjects with chronic, perennial, asthma.¹⁰ The results also indicate that the propensity to degranulate is related to actual allergen exposure rather than being a constitutional abnormality of the cells. Indeed, we found a signifi-

cant correlation between the pollen load and the propensity to degranulate. The use of specific marker assay for eosinophil and neutrophil degranulation allowed us to measure the activity of these cells in mixed cell population. This approach is probably more relevant than the use of a highly purified population in which the risk of enrichment of certain cell subpopulation is obvious.

The enhanced degranulation may reflect the fact that the cells have been primed by some unknown factors. In other studies, we demonstrated that the responsiveness of the eosinophils to chemotactic and chemokinetic stimuli was enhanced in patients with asthma,²⁰ which indicates that the enhanced responsiveness is not restricted to a single function of the eosinophil but a more general phenomenon.

One putative principle involved in the priming of eosinophils is IL-5. This cytokine has been demonstrated to enhance degranulation *in vitro* by a mechanism related to granule processing of the proteins (Carlson M. In preparation). IL-5 enhances oxidative metabolism as well as the chemotactic responsiveness of mature eosinophils and promotes differentiation of immature eosinophils.^{21,22} Apart from the fact that IL-5 is a B cell growth factor, the activities directed against eosinophils are apparently selective. This knowledge therefore is in contrast to some of the ob-

TABLE II. Serum levels of ECP, EPX, and MPO (expressed as micrograms per liter; mean [$\pm$ SEM])

	ECP	EPX	MPO
Ref	13.0 $\pm$ 1.7 (N = 14)	16.1 $\pm$ 1.3 (N = 14)	181 $\pm$ 32.0 (N = 14)
P I	50.5 $\pm$ 10.3* (N = 11)	37.7 $\pm$ 5.4† (N = 11)	271 $\pm$ 52.3 (N = 11)
P II	63.2 $\pm$ 20.3† (N = 11)	41.0 $\pm$ 5.8† (N = 11)	302 $\pm$ 65.2 (N = 11)
P III	96.6 $\pm$ 38.3† (N = 10)	52.9 $\pm$ 12.9† (N = 10)	332 $\pm$ 97.4 (N = 10)
P IV	38.3 $\pm$ 9.8* (N = 11)	31.6 $\pm$ 4.3* (N = 11)	186 $\pm$ 37.1 (N = 11)

Ref, Reference group; P, period.

The statistical differences between the reference and pollen-atopic group during periods I to IV were calculated by means of Mann-Whitney U test:

* $p < 0.01$.

† $p < 0.001$.

‡ $p < 0.05$.

§Statistical difference as calculated by Wilcoxon's signed-rank test.

TABLE III. Correlation between released amounts of ECP, EPX, and MPO after 20 minutes of incubation during periods I to III and the serum levels of the ECP, EPX, and MPO

Released granule protein	Serum granule protein	N	r	p
ECP	S-ECP	32	0.22	NS
EPX	S-EPX	32	0.42	0.02
MPO	S-MPO	32	0.18	NS

S, Serum; NS, not significant.

The coefficient of correlation and the significance of the correlations were calculated by Spearman's rank-correlation test.

TABLE IV. Blood eosinophil count (expressed as mean [$\pm$ SEM] $\times 10^6/L$)

Ref	P I	P II	P III	P IV
134 $\pm$ 19 (N = 14)	283 $\pm$ 57* (N = 10)	341 $\pm$ 45† (N = 11)	368 $\pm$ 61* (N = 10)	351 $\pm$ 62* (N = 11)

Ref, Reference group; P, period.

Blood eosinophil counts for the pollen-atopic patients before and during pollen season and the reference group are presented. The statistical differences between the reference group and the pollen-atopic patients during periods I to IV were calculated by means of Mann-Whitney U test.

* $p < 0.01$.

† $p < 0.001$.

servations made in our present study and suggest that other mechanisms of priming of the eosinophils are involved as well. Thus, we observed a priming of the neutrophils in addition to that of the eosinophils. Furthermore, there was a dissociation between the extent of priming of the eosinophils and blood eosinophil number, since the patients, already before season, had a significant eosinophilia, despite a normal propensity

to degranulate. Other priming factors involved could therefore be cytokines, such as granulocyte-macrophage-colony-stimulating factor and IL-3, since both these molecules will promote growth of eosinophils and prime both neutrophils and eosinophils with respect to receptor enhancement and cytotoxicity.²³⁻²⁷

One of the proposed consequences of priming of eosinophils is the change in density of the cells. In

TABLE V. Correlation between the released amounts of granule proteins ECP, EPX, and MPO after 20 minutes of incubation and log-total pollen count during periods I to III

Released granule protein	N	r	p
ECP	32	0.54	<0.003
EPX	32	0.54	<0.003
MPO	32	0.43	<0.02

The coefficient of correlation and the significances of the correlations were calculated by Spearman's rank-correlation test.

TABLE VI. Correlation between the released amounts of ECP, EPX, and MPO after 20 minutes of incubation and the extent of medical treatment the patients needed to control their asthmatic symptoms during periods I to III

Released granule protein	N	r	p
ECP	32	0.44	<0.02
EPX	32	0.41	<0.03
MPO	32	0.32	NS

NS, Not significant.

The coefficient of correlation and the significance of the correlations were calculated by Spearman's rank-correlation test.

subjects with asthma, an increased number of hypodense cells have been demonstrated.²⁸ The granule protein content of these hypodense eosinophils is reduced, as compared to normodense cells. Our findings of a virtually unaltered or even slightly increased content of ECP and EPX in the eosinophils during season would therefore imply that the eosinophils of the patients in the present study had not been changed in this respect. Unaltered content in unseparated eosinophils of patients with asthma has, however, been demonstrated before and is therefore in keeping with the present results.²⁹

The serum levels of ECP and EPX were raised already before season and corresponded to the blood eosinophilia. During season, however, the serum levels increased further in spite of unaltered blood eosinophil counts. The increase was most obvious during late pollen season, which is compatible with earlier findings on ECP. The dissociation between blood eosinophil counts and serum ECP levels supports the hypothesis that ECP may be used to reflect

TABLE VII. Evaluation of lung function in pollen-atopic patients before and during pollen season, measured as relFEV₁ and PD₂₀ value

PD ₂₀	Median (mg)	Range
P I	1.10 (N = 11)	0.27-3.50
P II	1.10 (N = 11)	0.05-8.50
P III	0.37 (N = 9)	0.15-2.90
P IV	0.32* (N = 10)	0.09-3.50

relFEV ₁	Mean (± SEM) (%)	Range
P I	81.1 ± 4.1 (N = 11)	56-96
P II	82.2 ± 4.5 (N = 11)	54-102
P III	82.2 ± 3.6 (N = 11)	64-103
P IV	82.4 ± 4.5 (N = 10)	58-105

PD₂₀, Provocative dose of methacholine causing a 20% decrease in FEV₁; P, period; relFEV₁, FEV₁ percent predicted.

The statistical differences between the different periods were calculated by Wilcoxon's signed-rank test.

*Statistical difference compared with P I, *p* < 0.05.

both the activity and size of the eosinophil population.³⁰ Thus, even a correlation was found between eosinophil activity, as measured as the released amount of EPX and serum levels of EPX. The fact that this relationship was absent between the two ECP parameters could be explained by a different kinetics and elimination of ECP caused by the binding to proteins in the circulation, such as α₂-macroglobulin.³¹

There was no discernible relationship between the increased degranulation of eosinophils and neutrophils and the clinical activity of the disease of the pollen-atopic patients. The reason might be explained by the fact that all patients had a well-controlled disease with respect to their pulmonary functions. The asthma medication may also have modified and reduced the release of the granule proteins from both the eosinophils and neutrophils, since both β₂-agonists and glucocorticosteroids are known to do so.^{9, 32} The effect of the drugs could also have led to an underestimation of the extent of degranulation of the eosinophils and neutrophils in the patients. However, we found a relationship between the extent of medical treatment the patients needed to control their asthmatic symptoms and the

in vitro degranulation of ECP and EPX, which suggests that those patients with the most severe asthma are those with the most activated eosinophils. Whether these findings also relate to other atopic patients without asthma is at present unknown and warrants further investigation.

We conclude that the eosinophils, and, to a somewhat lesser extent, the neutrophils, obtained from a group of birch pollen-atopic patients with asthma, have an increased propensity to secrete their granule proteins during pollen season. This propensity was related to the allergen load and to the severity of their asthma. The mechanism of priming of the cells is at present unknown but may involve the production of cytokines, such as IL-5, granulocyte-macrophage-colony-stimulating factor, and other cytokines.

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