

Immunotherapy abrogates the generation of eosinophil and neutrophil chemotactic activity during pollen season

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In a group of 40 birch pollen-allergic patients with a history of rhinoconjunctivitis and wheezing during the pollen season, 20 were immunotherapy (IT) treated preseasonally with birch-pollen extract (Pharmacia, Uppsala, Sweden). Blood samples for determination of the levels of heat-labile eosinophil chemotactic activity (HL-ECA), heat-labile neutrophil chemotactic activity (HL-NCA), and heat-stable neutrophil chemotactic activity were collected before the season, at the beginning of the study, at the start of the season, at the peak, at the end, and after the birch-pollen season. The symptoms from rhinoconjunctivitis and airways, peak expiratory flow, and use of medication were recorded throughout the season. Significant increases of HL-ECA and HL-NCA were observed in untreated compared with IT-treated patients at the start of the season ($p < 0.0001$ for both activities) and at the peak of the birch-pollen season ($p < 0.0005$ and $p < 0.01$, respectively). At the end of the season, HL-ECA levels were not significantly different between the patient groups, whereas HL-NCA levels were still higher in untreated patients ($p < 0.005$). We conclude that IT completely abrogates the generation of HL-ECA and HL-NCA during a pollen season. (J ALLERGY CLIN IMMUNOL 1990;86:706-13.)

It is generally agreed that asthma is an inflammatory disease. Both cells and mediators contribute to epithelial cell damage in the airways and participate in the development of bronchial hyperreactivity. Eosinophils and neutrophils are found in biopsy specimens of skin during late allergic reaction,¹ although eosinophils dominate in lung tissue from patients with asthma in postmortem material² and in bronchoalveolar lavage fluid from patients with asthma.³

The number of eosinophils also increases in bronchoalveolar lavage during LAR after allergen challenge.^{4,5} It is probably by the action of NCF and ECF that both cell types are recruited to the site of inflam-

Abbreviations used

IT:	Immunotherapy
HL-ECA:	Heat-labile eosinophil chemotactic activity
HL-NCA:	Heat-labile neutrophil chemotactic activity
HS-NCA:	Heat-stable neutrophil chemotactic activity
SPT:	Skin prick test
NPT:	Nasal provocation test
PC ₂₀ :	Provocative concentration causing a 20% fall in FEV ₁
LAR:	Late asthmatic reaction
BU:	Biological units
CV:	Coefficient of variation
PEF:	Peak expiratory flow
NCF:	Neutrophil chemotactic factor
ECF:	Eosinophil chemotactic factor
HMW:	High molecular weight

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mation. ECFs have been identified from guinea pig and human lung fragment perfusates on an IgE-dependent reaction; this chemotactic activity could be probably attributable, in part, to two tetrapeptides and the cellular origin to mast cells.⁶ In vitro ECA was

also generated from alveolar macrophages⁷ and T-lymphocytes.⁸

In humans, ECA was found in patients with urticaria,⁹ in antigen-induced reaction in the skin,¹⁰ and, recently, during early and late phase of antigen-induced asthmatic response.¹¹

We have previously demonstrated that HL-NCA occurs after allergen challenge of patients with asthma and that this activity is closely related to the ensuing LAR.¹² We also demonstrated the generation of such activity during the pollen season and, in addition, the generation of HL-ECA.¹³ The results from the latter study also suggested that these activities are all due to one and the same molecule. A HMW HS-NCF was found after antigen challenge and described by Atkins et al.¹⁴ It increased simultaneously with histamine, and the activity correlated with the degree of bronchospasm.

Disodium cromoglycate prevented HMW HS-NCF increments after antigen challenge¹⁵; thus, a mast cell origin was assumed. The factor was later found in exercise-induced asthma¹⁶ during LAR,¹⁷ food-induced bronchospasm,¹⁸ and aspirin-induced asthma.¹⁹

Treatment with allergenic extracts has been used for many years to cure or at least diminish the symptoms of allergy. However, the mechanism by which IT relieves atopic symptoms in most individuals is largely unknown. In an attempt to gain further insight into the mechanism of IT, we have followed the generation of ECA and NCA in 40 atopic individuals. Half the patients were allocated to treatment with IT preseasonally, and the other half served as control subjects. Control subjects rather than a placebo-treated group was used because the main objective of the study was not to investigate the efficacy of the treatment but rather its effect on some biochemical factors.

PATIENTS AND METHODS

Forty birch pollen-allergic patients participated in the study. There were 26 female and 14 male patients with an age range from 18 to 49 years, mean, 31 years.

Diagnosis of birch allergy was based on a positive history of rhinoconjunctivitis and wheezing during the birch-pollen season. SPT, NPT, and histamine challenge were performed. For inclusion in the study, positive SPT and NPT, baseline lung function with FEV₁ >70% of predicted, and histamine sensitivity <16 mg/ml of histamine concentration outside the season were required. Patients who had received IT during the last 12 months or steroid treatment during the last 6 months and pregnant or lactating women were excluded from the study. The presence of immunologic or cardiovascular diseases was considered contraindicating.

SPT was performed according to the Nordic guidelines²⁰

with a standardized panel of antigens with birch, alder, hazel, grass, animal dander, mite, and mold represented. SPT was regarded positive if the diameter of the wheal was at least >3 mm at 10,000 BU/ml of antigen concentration. For reproducibility, antigen sensitivity in the skin was tested on both forearms simultaneously.

NPT was performed with a series of dilutions from 10 BU up to 10,000 BU of birch extract. The symptoms obtained were graded from 0 to 3 for each symptom with 0 representing no symptoms; 1, slight; 2, moderate; and 3, severe. Symptoms counted were sneezing (number of sneezes), rhinorrhea, and congestion at each concentration up to 10,000 BU/ml of birch-pollen concentration.²¹

Among the patients, 15 were also sensitive to grass. (Grass season does not interfere with birch pollen, which is earlier in the spring.) Seventeen patients were also sensitive to animal dander, the reason for requiring no animals at home, at the nearest neighborhood, or at close relatives. No patients with mite or mold allergy were admitted to the study.

The patients had mild asthmatic symptoms, well controlled with β_2 -agonist spray occasionally. Histamine-provocation challenge was performed according to standardized method,²² and the patients demonstrated a wide range of histamine sensitivity, as expressed in PC₂₀ histamine values.

After admission testing, 20 patients were allocated to IT with birch-pollen extract (Pharmalgen, Pharmacia, Uppsala, Sweden) in the winter preceding the pollen season. The other 20 patients represented the control group. There were 11/20 women in the IT-treated group and 15/20 in the untreated group, respectively. The patients were of similar age (32.2 ± 7.4 SD) in the IT-treated group and (30.3 ± 8.1) in the control group, respectively. The degree of specific birch sensitivity measured with SPT and NPT (details in RESULTS) was similar in both groups. When bronchial hyperresponsiveness was considered, the groups were matched so that the ranges of PC₂₀ histamine were similar for both groups as well. For ethical reason, no placebo was administered to the control subjects. All treated patients started IT and reached maintenance dose before the start of the season.

During the season, clinical data were collected with diary cards. All patients recorded daily score of symptoms from eyes, nose, and bronchi (graded from 0 to 3). PEF was measured morning and evening. Medication was also noted (number of tablets and puffs of sprays). Decongestants and local steroids were allowed for the treatment of eyes and nose symptoms and β_2 -agonist spray or tablets for asthmatic symptoms.

Design

SPT and NPT were performed before the season and after 10 to 12 months of treatment after the season. Blood was collected before the season, at test day 1 (beginning of the birch-pollen season), at test day 2 (the peak of the pollen count), at test day 3 (the end of the birch-pollen season), and after the season. Symptom scores and medication were

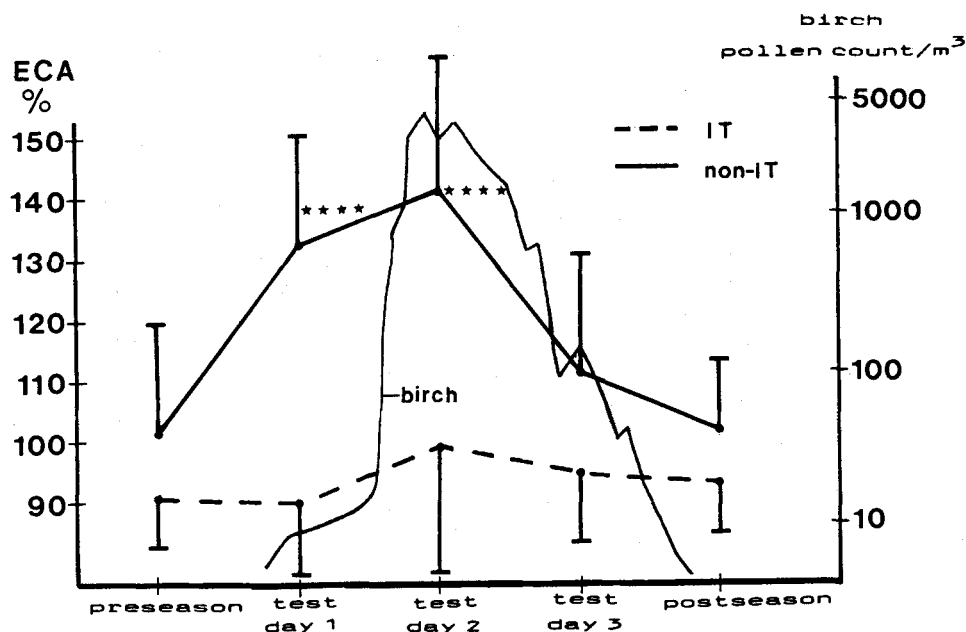


FIG. 1. Levels of HL-HCA in percent of normal pooled sera (means and SD) before, during, and after the birch-pollen season; comparison between untreated and IT-treated groups at test day 1, **** $p < 0.001$; at test day 2, **** $p < 0.001$.

TABLE I. HS-NCA in serum of untreated and IT-treated atopic patients before, during, and after birch-pollen season

Sample day	Chemotactic activity ($\mu\text{m/hr}$) (mean $\pm$ SD)	
	Untreated patients	IT-treated patients
Preseason	14 $\pm$ 12*	6.8 $\pm$ 5.4‡
During season		
Period 1	13 $\pm$ 14†	6.2 $\pm$ 11
Period 2	13 $\pm$ 14†	9.3 $\pm$ 14
Period 3	19 $\pm$ 17 †	7.7 $\pm$ 11‡
Postseason	-6.2 $\pm$ 4.8*	4.2 $\pm$ 6.3§
References	3.6 $\pm$ 1.9	

Evaluation was by means of Student's *t* test.

* $p < 0.001$, differences between normal and atopic individuals.

† $p < 0.01$, differences between normal and atopic individuals.

‡ $p < 0.02$, differences between untreated and IT-treated patients.

§ $p < 0.001$, differences between untreated and IT-treated patients.

counted for each 2-week period in the season, corresponding to the related test day and were numbered 1 to 3. Serum was collected and stored at -70°C until it was assayed.

The assay of ECA and NCA in serum was performed as described in detail by Håkanson et al.²³ All assays were run without knowledge of the patients' protocol.

Chemotactic assay

The chemotactic activity of serum was assayed with a modification of the Boyden chamber method.^{12, 23} Granulocytes were isolated from heparinized blood from apparently healthy blood donors by means of dextran sedimentation.²⁴ The granulocyte suspension obtained contained $85\% \pm 5\%$ (SD) granulocytes of which $2\% \pm 2\%$ (SD) were eosinophil granulocytes. The granulocytes were diluted to a final concentration of 1.5×10^6 granulocytes per liter in Gey's solution²⁵ with or without albumin (2 gm/L, AB Kabi, Stockholm, Sweden) added. The migration assays were performed with micropore filters of either 3 or 5 μm pore size (Millipore Corp., Bedford, Mass.). Incubation was performed for 1 hour at 37°C . The procedure used to stain both neutrophils and eosinophils was a modification of the method earlier described by Kay.²⁶ Migration of eosinophils and neutrophils was assayed by means of the leading-front technique.²⁵

HL-NCA and HL-ECA in serum were assayed in fresh-frozen (-70°C) serum diluted 1:20. The granulocytes were diluted in Gey's solution. Filters with 5 μm pore size were used. The NCA and ECA were calculated in relation to the response to normal serum (100%). The chemotactic response of neutrophils to normal serum was 90 ± 19 (SD) $\mu\text{m/hr}$, and of eosinophils, 66 ± 16 (SD) $\mu\text{m/hr}$. Migration toward Gey's solution was used as a negative control, 24 ± 4 (SD) $\mu\text{m/hr}$ (neutrophils), and 20 ± 4 (SD) $\mu\text{m/hr}$ (eosinophils), respectively.

The intraday variation of the method was 7.8% (CV), and the interday variation was 12.8% (CV).

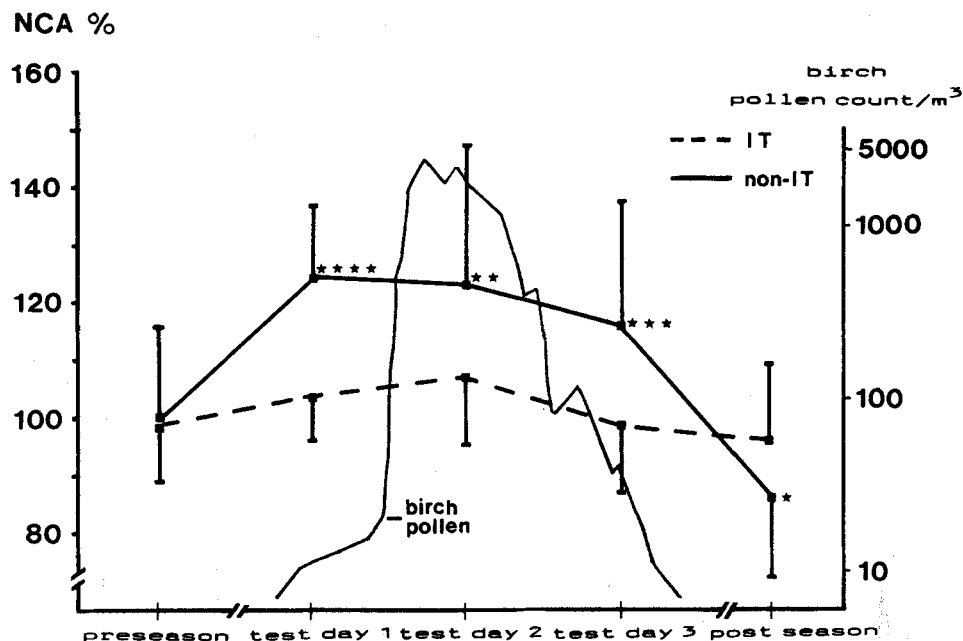


FIG. 2. Levels of HL-NCA in percent of normal pooled sera (means and SD) before, during, and after the birch-pollen season; comparison between the untreated and IT-treated groups at test day 1, **** $p < 0.001$; at test day 2, ** $p < 0.01$; at test day 3, *** $p < 0.005$; and postseasonally, * $p < 0.05$.

HS-NCA in serum was assayed in serum pretreated at 56° C for 30 minutes and diluted 1:40. The granulocytes were diluted in Gey's solution supplemented with albumin (2 g/L). The filter pore size was 3 μ m. The chemotactic activity was expressed as the migration (micrometers per hour) exceeding that toward Gey's solution only.

The intraday variation of the method was 6.7% (CV), and the interday variation, 11.0% (CV).

Gel filtration was performed on a column (2.6 by 90 cm) of Sephacryl S-200 superfine (Pharmacia LKB Biotechnology, Uppsala, Sweden). Two milliliters of freshly frozen serum was applied to the column and eluted with Gey's solution with ϵ -aminocaproic acid at an elution rate of 10 ml/hr. The volume of the fractions was 2.5 ml. The NCA and ECA were measured in fractions diluted 1:5 and 1:10, before and after heat treatment of the fraction for 30 minutes at 56° C. The granulocytes were diluted in Gey's solution with 2 g of albumin per liter. The chemotactic activity (units) was expressed as the maximum migration obtained with one of the dilutions (1:5 or 1:10) of the particular fraction minus the migration toward Gey's solution (random migration).

RESULTS

HL-ECA rose significantly during the pollen season in the untreated group. In the IT-treated patients, however, the activity stayed completely unaltered during the observation period (Fig. 1).

HL-NCA also demonstrated significant rise during the pollen season in the untreated group, whereas the activity in the IT-treated group stayed unaltered (Fig. 2).

HS-NCA was unaltered in both the untreated and the IT-treated groups. However, a significant difference between the two groups was observed outside the season (Table I).

Chromatographic characterization of the NCA and ECA in serum obtained during pollen season from one patient in the untreated group and from one patient in the IT-treated group is illustrated in Figs. 3 and 4. In serum from the untreated patients, major NCA was eluted in the void volume and at molecular weights corresponding to 50,000 and 100 to 200,000. The activity at 100 to 200,000 apparent molecular weight was HL. In serum from IT-treated patient, the NCAs were markedly reduced. The ECA in serum from untreated and IT-treated patient demonstrated approximately the same pattern as the NCA. ECA was eluted in the void volume and at apparent molecular weights of 50,000 and 100 to 200,000, and the activities were reduced in serum from IT-treated patients.

SPT demonstrated comparable wheal size with birch extract, 70 mm² (range, 33 to 122 mm³) in control subjects and 68 mm² (range, 19 to 144 mm²)

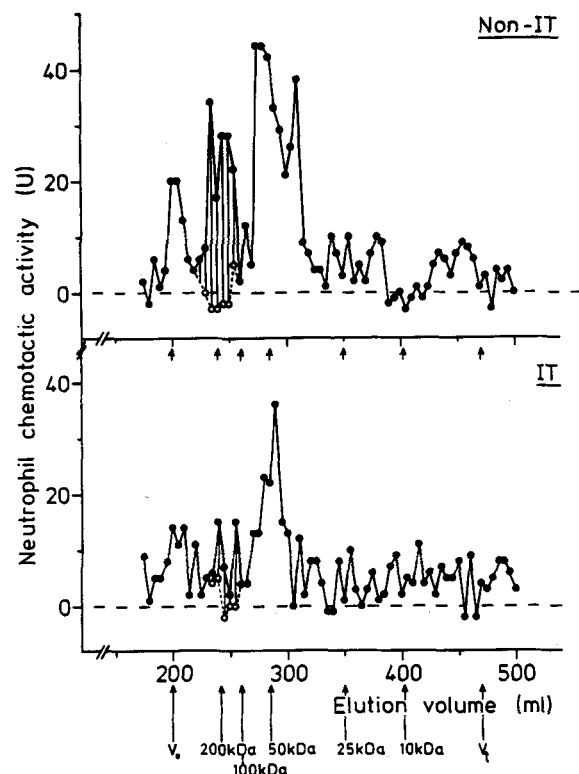


FIG. 3. NCA in serum separated on Sephacryl S-200. Activity in serum obtained during pollen season from one untreated birch pollen-allergic patient (*non-IT*) and one IT-treated (*IT*) patient is illustrated. The *dashed line* represents the random migration of neutrophils. Chemotactic activity of >10 units (*U*) indicates an activity significantly different from random migration. *Open circles* represent the activity after heat treatment (56° C for 30 minutes) of the respective fraction. Calculated elution volumes of molecules with certain molecular weights are also listed; V_o , void volume; V_t , total volume. Chromatograms are representative for several other chromatograms not presented.

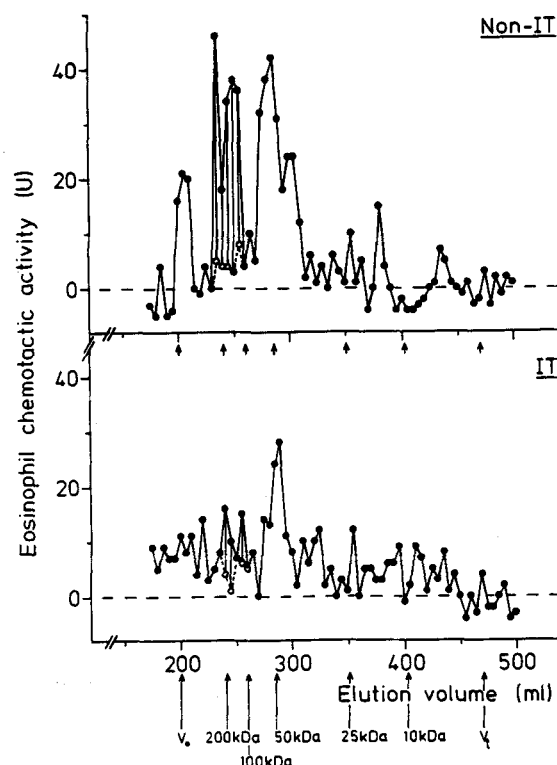


FIG. 4. ECA in serum separated on Sephacryl S-200. Activity in serum obtained during pollen season from one untreated birch pollen-allergic patient (*non-IT*) and one IT-treated (*IT*) patient is illustrated. The *dashed line* represents the random migration of eosinophils. Chemotactic activity of >10 units (*U*) indicate an activity significantly different from random migration. *Open circles* represent the activity after heat treatment (56° C for 30 minutes) of the respective fraction. Calculated elution volumes of molecules with certain molecular weights are also listed; V_o , void volume; V_t , total volume. Chromatograms are representative for several other chromatograms not presented.

in IT-treated subjects, respectively, before the season. After 10 to 12 months of IT, a significant decrease in wheal size was observed ($p < 0.001$) in the IT-treated group, 30 mm² (range, 17 to 50 mm²), but not in the control subjects, 59 mm² (range, 22 to 109 mm²). The mean birch pollen-extract concentration required to evoke a positive NPT increased from 2100 BU/ml (range, 320 to 10,000 BU) initially to 10,000 BU/ml (range, 3200 to 100,000 BU) ($p < 0.001$) after 10 to 12 months of IT treatment but remained unchanged, 1900 BU/ml (range, 100 to 10,000 BU) before the season and 1900 (range, 320 to 10,000 BU) after the season in the control subjects.

The median PC₂₀ values were 1.69 mg/ml (range, 0.048 to 15.5 mg/ml) in IT-treated and 1.25 (range,

0.021 to 10.27 mg/ml) in untreated patients pre-season. The largest increase was observed in the third period of the season, 0.075 (range, 0.0004 to 0.38 mg/ml) and 0.01 (0.0004 to 1.8 mg/ml), in the respective groups. The difference between the groups was $p = 0.07$. No correlations between PC₂₀ values and chemotactic activities were found in any group.

The symptoms from nose and eyes increased significantly during period 1 compared with the values recorded during the week before the start of the season ($p < 0.05$). An additional increase was noted toward the peak of the pollen count where symptoms of rhinoconjunctivitis also peaked. The decrease of eye and nose symptoms was markedly delayed during the third period, despite decreasing pollen count in the un-

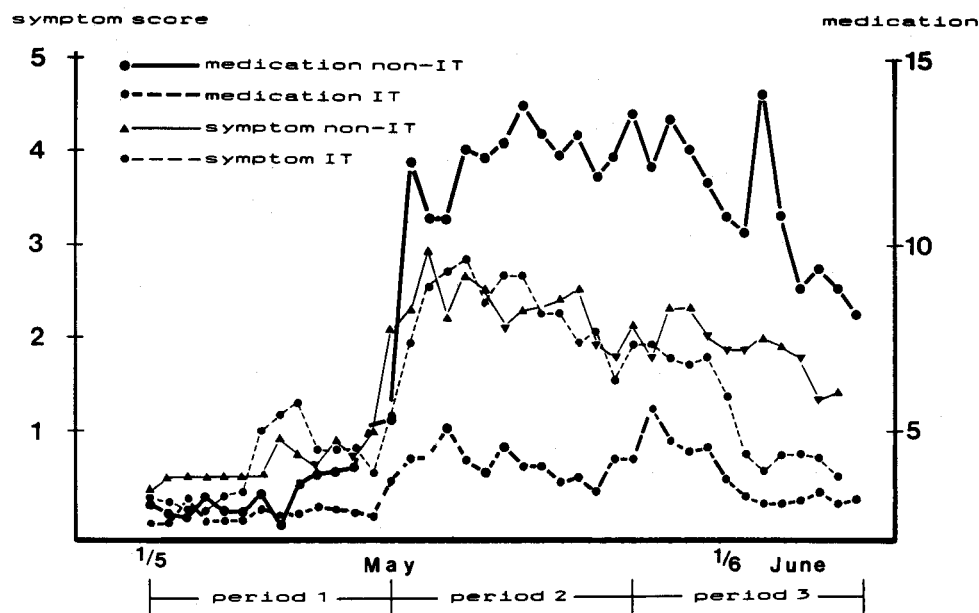


FIG. 5. Score of symptoms from eyes and nose, and medication during birch-pollen season for both patient groups. Means of the score and medication used for each group were counted biweekly (periods 1 to 3). The differences in symptom score between the groups were significant at period 3, $**p < 0.01$, and in medication during all three periods, $**p < 0.01$.

treated patients. The difference between the groups reached a significant level ($p < 0.01$), to the advantage of the IT-treated group (Fig. 5).

The use of medication for eyes and nose was significantly higher in untreated patients in all periods (1 to 3) of the season ($p < 0.01$) (Fig. 5).

The symptoms from airways were similar in treated and untreated patients at the start of the season and increased in both groups during the season. The level of airway symptoms was higher in the untreated group at period 3, but the difference between the groups was not significant at any period.

However, differences in PEF values and medication used (salbutamol, number of puffs) for airways were observed. The IT-treated group had significantly higher PEF values ($p < 0.01$) and used significantly less medication ($p < 0.05$).²⁷

DISCUSSION

The present study strongly suggests the clinical relevance of HL-NCA and HL-ECA. The increase of their levels in serum during the pollen season corresponded with the amount of antigen in the air and the rise of allergic symptoms, and the decrease corresponded with the falling amounts of birch pollen. The peak levels of the chemotactic activity preceded by 2 weeks the peak levels of the eosinophil degranulation product, eosinophil cationic protein, in the blood, which in turn was associated with the highest hista-

mine sensitivity values in the airways of allergic subjects with asthma during the season.²⁷

The IT-treated patients demonstrated a complete lack of increase of HL-ECA and HL-NCA during the pollen season. Chromatographic characterization indicated that the lack of HL-ECA and HL-NCA was due to a decrease of HL chemotactic activity of 100 to 200 kd molecular weight, compared with serum from an untreated patient. These results are in accordance with previous results²⁸ that suggested that HL-ECA and HL-NCA found in serum from allergic patients with asthma were due to one and the same molecule. Furthermore, it was suggested that the ECF and NCF of 100 to 200 kd comprised the HL-ECA and HL-NCA in serum from birch pollen-allergic patients with asthma during pollen season, as well as in serum from patients with asthma during allergen challenge.^{13, 28} The HS-NCA in serum of the untreated patients did not increase during pollen season but was increased already before season, compared with the IT-treated patients and references. Serum from the IT-treated patients did not demonstrate any increase of HS-NCA compared with that of the references. Chromatographic characterization demonstrated HS chemotactic activities in the void volume (>650 kd) and of ≈ 50 kd molecular weight, which were both decreased in serum from the IT-treated patient. The chemotactic activity that eluted in the void volume is probably identical to HMW HS-NCA previously

described by Atkins et al.,¹⁴ Lee et al.,¹⁶ and Nagy et al.,¹⁷ and has been demonstrated to be unique to serum from patients with asthma,²⁸ whereas the chemotactic activity of ≈ 50 kd molecular weight was also present in serum from references.

Thus, our results demonstrate that IT decreases the levels of both HL and HS chemotactic activities in serum, presumably by inhibition of the production of chemotactic factors in the lung. The HMW HS-NCA is assumed to originate from mast cells,¹⁵ and HL-NCA has, in one investigation, demonstrated a relationship to monocyte/macrophage activity.¹² The hypothesis based on the present results would thus be that IT by some mechanism interferes with the production of chemotactic factors from mast cells and macrophages/monocytes.

An increase of the symptoms during the pollen season was also noted in the IT-treated group; however, the increase was smaller than in the group of untreated patients. This finding may be explained by an unusually intensive pollen season, which, after preseasonal treatment, might override the benefit of IT. However, the treated group used significantly less medication ($p < 0.01$) during the whole season. The appearance of symptoms in treated patients, in spite of lack of increase in chemotactic activity during the season, invites some speculation as to the origin of symptoms. There are data suggesting that many different cells have the ability to release mediators after IgE-mediated stimulation,²⁹⁻³¹ thus contributing to clinical symptoms. Accordingly, our results suggest that the production of chemotactic factors is a process that is markedly sensitive to IT, whereas the mechanisms behind the production of other mediators of atopic symptoms are less sensitive or even unaffected by IT.

In conclusion, the present investigation demonstrated the increase of HL-ECA and HL-NCA in serum from birch pollen-allergic patients during pollen season, which was parallel to the amount of pollen in the air and the rise of the allergic symptoms. Furthermore, a hitherto unknown effect of IT is described, that is, the inhibition of the production of HL-ECA, HL-NCA, and NS-NCA. A prevention of the production of chemotactic factors would theoretically result in a decrease of the attraction of eosinophils and neutrophils to the lungs and thereby diminish the inflammatory process behind the asthmatic disease.

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