

# Priming of eosinophil adhesion in patients with birch pollen allergy during pollen season: Effect of immunotherapy

Lena Håkansson, PhD,<sup>a, b</sup> Christina Heinrich, MD,<sup>c</sup> Sabina Rak, MD, PhD,<sup>b, d</sup> and Per Venge, MD, PhD<sup>a, b</sup> Uppsala, Västerås, and Gothenburg, Sweden

The adhesion of eosinophil granulocytes to E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) was investigated before and during birch pollen season in 24 patients allergic to birch pollen who had rhinoconjunctivitis and, in half of the cases, asthma during season. Half of the patients were undergoing specific immunotherapy for birch pollen allergy. Increased adhesion to VCAM-1 and ICAM-1 ( $p < 0.05$ ) during season as compared with before season was demonstrated by eosinophils of patients in the control group and by eosinophils of the patients without asthma treated with immunotherapy, but not by eosinophils from the immunotherapy-treated patients with asthma. Eosinophils from the control group of patients demonstrated increased cell surface expression of CD18 and CD49d ( $p < 0.05$  and  $p < 0.01$ , respectively) during season as compared with before season, and eosinophils from the immunotherapy-treated patients showed increased cell surface expression of CD49d ( $p < 0.01$ ) during season. Simultaneous measurement of neutrophil adhesion revealed increased adhesion to E-selectin and ICAM-1 ( $p < 0.01$ ) during season compared with before season in the immunotherapy-treated group of patients. Neutrophils from the control subjects without asthma showed increased adhesion to E-selectin ( $p < 0.05$ ) during season. In conclusion, eosinophils from patients allergic to birch pollen demonstrated priming of the adhesion to VCAM-1 and ICAM-1 during birch pollen season. Immunotherapy treatment prevented the priming of eosinophil adhesion during pollen season in the patients allergic to birch pollen who had asthma, but not in those without asthma. In contrast, neutrophils from the immunotherapy-treated patients, both with and without asthma, demonstrated priming of the adhesion to E-selectin and ICAM-1 during season. The latter results indicate that immunotherapy, in case of the patients allergic to birch pollen with asthma induced a shift from the production of primarily eosinophil priming agents to primarily neutrophil priming agents, which may be caused by a shift from Th2 to Th1 lymphocytes. (*J Allergy Clin Immunol* 1997;99:551-62.)

**Key words:** Eosinophil, neutrophil, adhesion, pollen season, immunotherapy, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, very late activation antigen, macrophage antigen-1

Accumulation of eosinophil granulocytes is a common finding in lung tissue of patients with asthma, especially during an active state of the disease.<sup>1, 2</sup> The accumulated eosinophils are considered to play an important role in the inflammatory process of the asthmatic disease. Several studies have demonstrated a relationship between the accumulation of activated eosinophils in the lungs of patients with asthma and the severity and symptoms of asthma.<sup>3-7</sup> The mechanisms of the specific eosinophil accumulation in lung tissue of patients with asthma are not completely clarified. There are, however, some conceivable mechanisms such as production of eosinophil chemotactic factors that attract the eosinophils, priming of eosinophil migratory responses, priming of eosinophil adhesion, and induction of adhesion molecules on the endothelial cells in the blood vessels of the lung.

Previous investigations of chemotactic activities in bronchoalveolar lavage (BAL) fluid obtained from patients with asthma who were allergic to pollen indicated that eosinophil chemoattractants are produced in the lungs of patients with asthma.<sup>8</sup> The chemotactic activity was, however, not entirely specific for eosinophils but also attracted neutrophils. Studies on the migration of eosinophil granulocytes have demonstrated increased chemotactic and chemokinetic responses of eosinophils from patients with asthma, which indicates a priming of eosinophil migratory responses.<sup>9, 10</sup> The specific priming of eosinophil migratory responses and the production of granulocyte chemoattractants thus probably support eosinophil accumulation in lung tissue of patients with asthma, but are not likely to be the only mechanisms.

Adhesion of granulocytes to endothelial cells and the subsequent transendothelial migration of granulocytes are mediated by the induction and activation of adhesion molecules on endothelial cells and granulocytes.<sup>11-13</sup> Eosinophils and neutrophils are able to adhere to the endothelial cell adhesion molecules P-selectin, E-selectin, and intercellular adhesion molecule-1 (ICAM-1), whereas only eosinophils bind to vascular cell adhesion molecule-1 (VCAM-1) because they express the counter receptor very late activation antigen-4 (VLA-4) (CD49d/CD29).<sup>14, 15</sup> Adhesion to ICAM-1 is mediated through

From the Department of Clinical Chemistry<sup>a</sup> and the Asthma Research Center,<sup>b</sup> University Hospital, Uppsala; Department of Otolaryngology,<sup>c</sup> Central Hospital, Västerås; and Department of Lung Medicine,<sup>d</sup> Sahlgrenska Hospital, Gothenburg, Sweden.

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Reprint requests: Lena Håkansson, PhD, Laboratory for Inflammation Research, Department of Clinical Chemistry, University Hospital, S-751 85 Uppsala, Sweden.

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*Abbreviations used*

|         |                                    |
|---------|------------------------------------|
| BAL:    | Bronchoalveolar lavage             |
| ECP:    | Eosinophil cationic protein        |
| ICAM-1: | Intercellular adhesion molecule-1  |
| MPO:    | Myeloperoxidase                    |
| PBS:    | Phosphate-buffered saline solution |
| PEF:    | Peak expiratory flow rate          |
| VCAM-1: | Vascular cell adhesion molecule-1  |
| VLA-4:  | Very late activation antigen-4     |

the adhesion molecules leukocyte function-associated antigen-1 (CD11a/CD18) and macrophage antigen-1 (CD11b/CD18) on granulocytes, adhesion to P-selectin by P-selectin glycoprotein ligand-1, and adhesion to E-selectin by sialyl-Lewis<sup>x</sup>.<sup>16, 17</sup> Studies on biopsy samples of bronchial tissue from patients with allergic asthma have demonstrated expression of VCAM-1 and E-selectin and increased expression of ICAM-1 on endothelial cells in capillaries and venules compared with findings in biopsy samples from healthy persons, which only show a low expression of ICAM-1.<sup>18-20</sup> Soluble VCAM-1 has also been found in BAL fluid from patients with allergic asthma after allergen challenge, indicating expression and activation of VCAM-1 during the allergic inflammatory reaction.<sup>21</sup> A previous investigation of adhesion of eosinophils from patients with asthma demonstrated increased adhesion to ICAM-1 and VCAM-1, as compared with that of eosinophils from healthy persons.<sup>22</sup> The priming of eosinophil adhesion was, however, not entirely specific because neutrophil adhesion to ICAM-1 was also increased in the patients with asthma compared with the references. The combination of increased expression of VCAM-1 and ICAM-1 on endothelial cells in bronchial capillaries and venules and the priming of eosinophil adhesion to VCAM-1 and ICAM-1, accordingly, is one of the probable mechanisms behind eosinophil accumulation in lung tissue of patients with asthma.

The aim of the present investigation was to further investigate eosinophil adhesion in patients with allergic asthma. Adhesion to E-selectin, VCAM-1, and ICAM-1 of eosinophils from patients allergic to birch pollen was studied before and during birch pollen season to investigate whether the immunologic and inflammatory processes induced by pollen exposure affect the adherence function of eosinophils. In addition, eosinophil adhesion was compared with neutrophil adhesion, and the amount of the adhesion molecules CD11b, CD18, and CD49d expressed on the cell surface of eosinophils and neutrophils was measured by flow cytometry. Furthermore, the influence of immunotherapy on eosinophil adhesion during birch pollen season was compared with that of the standard treatment, topical nasal steroids, which was used as a control. For ethical reasons it was impossible to include a control group of this kind of patients without any topical steroid treatment.

**METHODS****Patients**

Twenty-four patients allergic to birch pollen participated in the study. There were 11 men and 13 women with a mean age of 31 years (range 21 to 42 years). They all had a history of rhinoconjunctivitis during birch pollen season, and 11 of 24 also had a history of asthma during birch pollen season. The diagnosis of allergy was based on positive results on a skin prick test of >3 mm with birch pollen extract (*Betula verrucosa*, ALK, Hørsholm, Denmark) and RAST. Some patients were sensitized to grasses and animal dander but none was exposed to pets. Sensitivity to mites and molds was not allowed. Patients with asthma showed bronchial hyperresponsiveness of a PC<sub>20</sub> of methacholine <16 mg/ml outside the season. The disease in these patients necessitated the use of inhaled  $\beta_2$ -agonists occasionally. The patients were not allowed to use inhaled steroids during the study. None of the patients was treated with immunotherapy earlier in life.

The study was approved by the local ethics committee, and the patients gave their informed consent.

**Healthy subjects**

Nine healthy persons aged 22 to 43 years (mean 31 years) with no symptoms of asthma or allergy, no bronchial hyperresponsiveness, and negative skin prick test results, recruited via the European Community Respiratory Health Survey,<sup>22, 23</sup> served as a reference group.

**Treatment design**

The study was a double-blind controlled group comparison, in which active treatment was subcutaneous immunotherapy with birch pollen extract Alutard (ALK, Hørsholm, Denmark) and the control treatment was topical nasal steroid administration (budesonide), 400  $\mu$ g once daily covering 7 weeks pre-seasonally and the whole birch pollen season. The immunotherapy treatment was initiated after preseasonal testing with skin prick test, RAST, methacholine challenge, and blood sampling. All patients reached a maintenance dose of 1 ml of 100,000 standardized quality units/ml before the start of the birch pollen season. The patients received between one and four maintenance doses, and the last dose before the season was given to the patients at approximately the same time; a further dose was given after 6 weeks (at the end of the season).

Methacholine challenge and blood sampling were done again at the peak of the birch pollen season.

Throughout the season patients recorded symptoms of rhinoconjunctivitis, morning and evening peak expiratory flow rate (PEF) measurements with use of a Wright peak flow meter (in triplicate), and use of medication. Antihistamines and eye drops of levocabastin and antazolin were allowed for treatment of rhinoconjunctivitis and inhaled  $\beta_2$ -agonists for treatment of asthma symptoms.

**Methacholine challenge**

Methacholine challenge was done as described elsewhere.<sup>24</sup> Briefly, methacholine chloride was diluted in saline solution and delivered in nebulized form by a Pari Inhalerboy nebulizer (output 0.75 ml/min) (Paul Ritzau Pari Werken, Starnberg, Germany) via mouthpiece with tidal breathing. Lung function measurements (Vitalograph, England) were taken at the start of the test, then saline inhalation was conducted for 2 minutes followed by methacholine solution delivery starting from 0.03 mg/ml and increasing at 5-minute intervals. FEV<sub>1</sub> values were

measured 2 and 4 minutes after each dilution until a fall of 20% was obtained.

## Procedures

**Isolation of granulocytes.** Granulocytes were isolated according to a modification of the method described by Hansel et al.<sup>25</sup> and Håkansson et al.<sup>26</sup> The isolated granulocytes were adjusted to a concentration of  $1 \times 10^9$ /L in the assay buffer containing NaCl (140 mmol/L), KCl (5 mmol/L),  $MgCl_2$  (1 mmol/L),  $CaCl_2$  (1 mmol/L), glucose (5.6 mmol/L), human serum albumin (30 mg/L), and HEPES (10 mmol/L), and with a pH of 7.4. The purity of the granulocytes was  $\geq 99\%$ .

**Adherence assay.** Adherence of eosinophil and neutrophil granulocytes to E-selectin, VCAM-1, and ICAM-1 was measured according to a recently described method<sup>26</sup> with the use of cell lines of hamster fibroblasts that selectively express E-selectin, VCAM-1, and ICAM-1. The nontransfected and transfected cell lines were maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Paisley, Scotland) supplemented with 10% fetal calf serum, penicillin 100 U/ml, streptomycin 100  $\mu$ g/ml, and glucose 4.5 gm/L. Methotrexate (Lederle, Puerto Rico) was added to the medium of transfected cell lines to obtain a final concentration of 1  $\mu$ mol/L. The fibroblasts were cultured at 37°C in a 5%  $CO_2$  atmosphere and routinely passaged every third or fourth day. From the continuously cultured fibroblast cell lines 96-well microtiter plate cultures were prepared for adhesion measurements. The fibroblasts were seeded at  $5 \times 10^3$  per well and cultured overnight before performance of the adhesion assay.

The fibroblast monolayers in 96-well microtiter plates were washed three times with assay buffer, and  $1 \times 10^5$  granulocytes in assay buffer with 0.5 mmol/L of  $MnCl_2$  was added to the wells and incubated 1 hour at 37°C. After incubation nonadherent granulocytes were removed by washes with buffer. The adherent cells were treated with a detergent solution containing N,N,N-cetyl-triammonium bromide (5 gm/L) and NaCl (8.8 gm/L) for 1 hour at room temperature. The supernatants were collected after centrifugation at 600g for 10 minutes. The amounts of eosinophil cationic protein (ECP) and myeloperoxidase (MPO), respectively, were assayed by radioimmunoassay (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden) and the percentage of adherent neutrophils and eosinophils of the total amount that had been added to the wells was calculated from a standard curve, consisting of serial dilutions of an extract of the initial amount of added granulocytes prepared by N,N,N-cetyl-triammonium bromide treatment as previously described.

**Flow cytometry.** Granulocytes, at a concentration of  $5 \times 10^9$ /L, isolated according to the procedure previously described herein, were incubated with monoclonal antibodies against CD11b, CD18, and CD49d (Immunotech, Marseilles, France) for 30 minutes at 4°C. After two washes with phosphate-buffered saline solution (PBS) the granulocytes were further incubated with phycoerythrin-conjugated goat anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) for 30 minutes at 4°C. After two further washes with PBS the granulocytes were further incubated with fluorescein-isothiocyanate-conjugated anti-CD9 (Dakopatts). After two final washes the cells were resuspended to a concentration of  $1 \times 10^9$ /L in PBS with human serum albumin (1 gm/L) and analyzed in an EPICS Profile II flow cytometer (Coulter Company Inc., Hialeah, Fla.). On the basis of the pattern of forward and side scatter a granulocyte gate was set. Eosinophils were identified as CD9-positive granulocytes<sup>27,28</sup> and neutrophils as CD9-negative granulocytes. The mean specific fluorescence of CD11b, CD18,

and CD49d was calculated by subtracting the mean background fluorescence of eosinophils/neutrophils labeled with an irrelevant primary antibody and the secondary phycoerythrin-labeled antibodies from the value obtained with anti-CD11b, anti-CD18, and anti-CD49d, respectively.

**Measurement of concentrations of ECP and MPO.** Serum samples obtained for the measurement of ECP and MPO were handled according to the instructions of the manufacturer. The serum concentrations of ECP and MPO were assayed by means of double antibody radioimmunoassays (Kabi Pharmacia Diagnostics AB). The intraassay and interassay coefficients of variation are  $<11\%$  in the case of ECP and  $<13\%$  in the case of MPO. The detection limit is  $<2 \mu$ g/L in the case of ECP and  $<8 \mu$ g/L in the case of MPO.

## Pollen count

The number of pollen was counted by the Palynological Laboratory, Swedish Museum of Natural History, Stockholm, between March 1 and June 30, 1993. Pollen recording was done with the use of a Burkhard 7-day recording volumetric spore trap.<sup>29,30</sup> The trap was placed on the roof of the hospital of Eskilstuna. The exposed tape was cut into daily segments and embedded in glycine jelly. Twelve transverse strips for every second hour were counted. The analyzed pollen grains were expressed as the mean number of pollen per cubic meter per 2-hour interval over the day (Fig. 1).

## Statistics

Results are expressed as median and lower and upper quartiles, or median and range, respectively. Statistical evaluations were made by Mann-Whitney U test and Wilcoxon's matched pair test, and Spearman's rank order correlation test was used to analyze relationships between different variables, all by the use of CSS Statistica software (StatSoft Inc., Tulsa, Okla.).

## RESULTS

### Lung function and blood cell counts

Lung function estimated as  $FEV_1$  and PEF values and the bronchial reactivity in the patients measured as  $PC_{20}$  of methacholine before and during season are demonstrated in Table I. The patients in the control group demonstrated a slight but significant decrease in  $FEV_1$  and PEF values and a pronounced decrease in the  $PC_{20}$  of methacholine during season compared with before season. The immunotherapy-treated patients did not demonstrate any significant changes of  $FEV_1$ , PEF, or  $PC_{20}$  of methacholine during season.

Half of the patients had, at the start of the study, a diagnosis of asthma and these patients were equally distributed between the two treatment groups. Data on the lung function and bronchial reactivity of the patients in the different groups are shown in Table II. The patients without asthma in both treatment groups showed a significant decrease in  $PC_{20}$  of methacholine during as compared with before season. Five of seven patients without asthma in the control group had the development of bronchial hyperreactivity during season as did four of six patients without asthma in the immunotherapy-treated group. In the control group the patients with asthma demonstrated a lower  $PC_{20}$  of methacholine than the patients without asthma during season,

## Pollen season of 1993

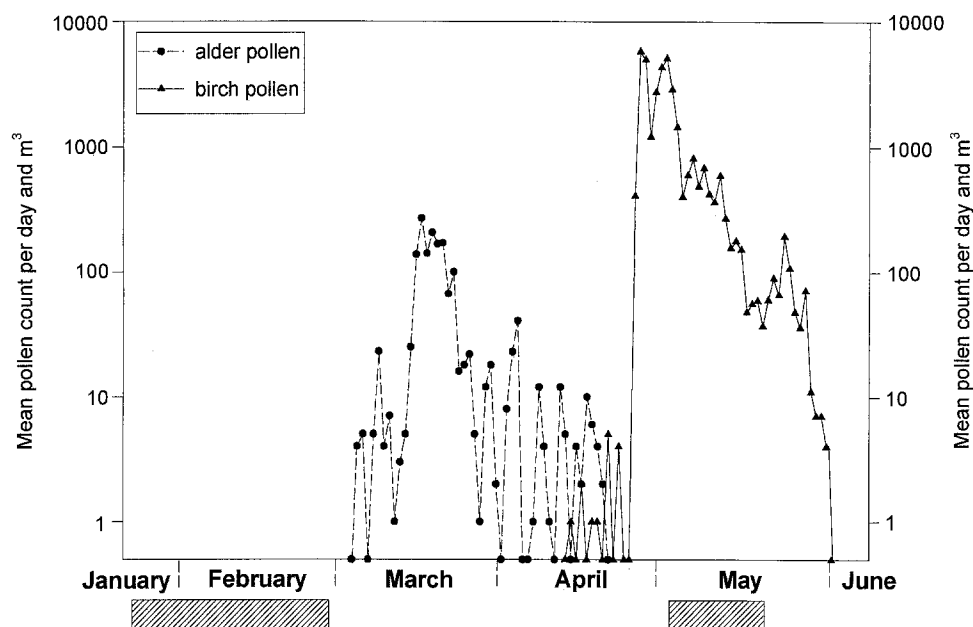


FIG. 1. Mean count of alder and birch pollen in 1993 during period of study. Test period before season (during January and February) and test period during season (first half of May) are indicated.

TABLE I. Lung function in patients before and during birch pollen season and in healthy subjects

| Group                     | FEV <sub>1</sub> (%) | PEF (%)      | PC <sub>20</sub> methacholine (mg/ml) |
|---------------------------|----------------------|--------------|---------------------------------------|
| Control subjects (n = 12) |                      |              |                                       |
| Preseason                 | 105 (78-122)         | 102 (80-132) | >16 (0.05->16)                        |
| Season                    | 94 (69-107)*         | 99 (66-120)* | 0.74 (0.12->16)*                      |
| IT patients (n = 12)      |                      |              |                                       |
| Preseason                 | 97 (80-122)          | 108 (94-132) | 10.7 (0.09->16)                       |
| Season                    | 95 (76-119)          | 102 (90-138) | 1.6 (0.24->16)                        |
| Health subjects (n = 11)  | 82 (74-122)          | 104 (70-115) | >16                                   |

The presented values for FEV<sub>1</sub> and PEF were obtained at the respective days of blood sampling. The results represent medians and ranges of the different groups.

IT, Immunotherapy-treated.

\*Significant difference between results during and before season ( $p < 0.01$ ).

whereas there was no significant difference between the immunotherapy-treated patients with and without asthma during season.

The number of blood eosinophils increased in both groups during season compared with before season, whereas the number of neutrophils and the serum concentrations of ECP and MPO were not significantly changed (Table III). The patients in the control group both without asthma and with asthma and the asthma-free patients in the immunotherapy-treated group showed an increase of the blood eosinophil count during season, in contrast to the immunotherapy-treated patients with asthma who did not show any significant change in the blood eosinophil count (Table IV).

### Eosinophil adhesion and cell surface expression

Eosinophils from both treatment groups showed significantly enhanced adhesion to VCAM-1 during as compared with before season and as compared with those in healthy subjects (Fig. 2). The mean level of eosinophil adhesion to VCAM-1 during season was, however, significantly higher in the control group than in the immunotherapy group. The increase of eosinophil adhesion to VCAM-1 during season was 21.4% (quartile range: 11.1% to 26.1%) in the control group and 9.8% (quartile range: 2.9% to 14.5%) in the immunotherapy group ( $p < 0.01$ ). Eosinophil adhesion to ICAM-1 was significantly increased in the control group during as compared with before season and as compared with that in the reference group.

**TABLE II.** Lung function of patients with and without asthma in the two treatment groups before and during birch pollen season

| Group                                           | FEV <sub>1</sub> (%) | PEF (%)      | PC <sub>20</sub> methacholine (mg/ml) |
|-------------------------------------------------|----------------------|--------------|---------------------------------------|
| Control patients without asthma ( <i>n</i> = 7) |                      |              |                                       |
| Preseason                                       | 103 (94-122)         | 101 (80-113) | >16                                   |
| Season                                          | 94 (74-107)*         | 100 (66-112) | 2.6 (0.5->16)*                        |
| Control patients with asthma ( <i>n</i> = 5)    |                      |              |                                       |
| Preseason                                       | 107 (78-111)         | 103 (80-132) | 1.1 (0.05-2.6)†                       |
| Season                                          | 94 (69-100)          | 97 (67-120)  | 0.39 (0.12-2.3)‡                      |
| IT patients without asthma ( <i>n</i> = 6)      |                      |              |                                       |
| Preseason                                       | 101 (95-122)         | 112 (95-132) | >16 (13.5->16)                        |
| Season                                          | 104 (83-117)         | 103 (90-138) | 4.6 (0.76->16)*                       |
| IT patients with asthma ( <i>n</i> = 6)         |                      |              |                                       |
| Preseason                                       | 91 (80-114)          | 105 (94-124) | 1.32 (0.09->16)†                      |
| Season                                          | 87 (76-119)          | 100 (92-120) | 1.6 (0.24->16)                        |

Median values and ranges of the values from the different groups are shown. *IT*, Immunotherapy treated.

\*Significant difference within the same patient group between values during and before season (*p* < 0.05).

†Significant difference between patients with and without asthma within same treatment group at same time point (*p* < 0.01).

‡Significant difference between patients with and without asthma within the same treatment group at same time point (*p* < 0.05).

**TABLE III.** Neutrophil and eosinophil blood counts and serum concentrations of ECP and MPO

| Group                             | Neutrophils (×10 <sup>9</sup> /L) | Eosinophils (×10 <sup>6</sup> /L) | Serum MPO (μg/L) | Serum ECP (μg/L) |
|-----------------------------------|-----------------------------------|-----------------------------------|------------------|------------------|
| Control ( <i>n</i> = 12)          |                                   |                                   |                  |                  |
| Preseason                         | 2.9 (1.2-5.5)                     | 145 (90-210)                      | 388 (243-575)    | 7.4 (3.3-13.5)   |
| Season                            | 2.5 (1.8-4.9)                     | 425 (130-1090)*                   | 329 (240-421)    | 17.7 (3.9-45.1)  |
| IT patients ( <i>n</i> = 12)      |                                   |                                   |                  |                  |
| Preseason                         | 3.2 (2.2-4.8)                     | 135 (100-560)                     | 451 (279-1011)   | 9.5 (4.4-24.3)   |
| Season                            | 2.8 (1.9-4.3)                     | 280 (90-770)*                     | 363 (247-622)    | 10.3 (4.1-36.5)  |
| Healthy subjects ( <i>n</i> = 11) | 3.3 (2.5-3.8)                     | 110 (40-190)                      | 373 (279-829)    | 9.9 (5.7-30.7)   |

The presented results are medians and ranges of the different groups. *IT*, Immunotherapy-treated.

\*Significant changes during season compared with before season (*p* < 0.01).

**TABLE IV.** Eosinophil blood count and serum concentration of ECP in patients with and without asthma in the two treatment groups before and during season

| Group                                           | Eosinophils (×10 <sup>6</sup> /L) | Serum ECP (μg/L) |
|-------------------------------------------------|-----------------------------------|------------------|
| Control patients without asthma ( <i>n</i> = 7) |                                   |                  |
| Preseason                                       | 170 (130-180)                     | 7.4 (3.6-13.5)   |
| Season                                          | 380 (210-1090)*                   | 9.9 (3.9-41.8)   |
| Control patients with asthma ( <i>n</i> = 5)    |                                   |                  |
| Preseason                                       | 110 (90-210)                      | 6.6 (4.1-10.3)   |
| Season                                          | 560 (150-850)*                    | 28.2 (5.3-31.4)  |
| IT patients without asthma ( <i>n</i> = 6)      |                                   |                  |
| Preseason                                       | 145 (100-330)                     | 11.1 (4.4-24.3)  |
| Season                                          | 315 (140-470)*                    | 11.1 (4.7-36.5)  |
| IT patients with asthma ( <i>n</i> = 6)         |                                   |                  |
| Preseason                                       | 120 (100-560)                     | 8.8 (5-16.4)     |
| Season                                          | 235 (90-770)                      | 10.5 (4.1-26.6)  |

The values represent median values and ranges of the different patient groups. *IT*, Immunotherapy-treated.

\*Significant difference before and during season within the same patient group (*p* < 0.05).

In the immunotherapy-treated patient group eosinophil adhesion to ICAM-1 during season was not significantly changed compared with before season, but was significantly higher than that of eosinophils from the reference group (Fig. 2). Eosinophils from the immunotherapy-treated patients also demon-

strated enhanced adhesion to E-selectin during season compared with before season, but not compared with eosinophils from the reference group.

Eosinophils from the control group showed increased expression of CD18 and CD49d during as compared with before season and as compared with reference

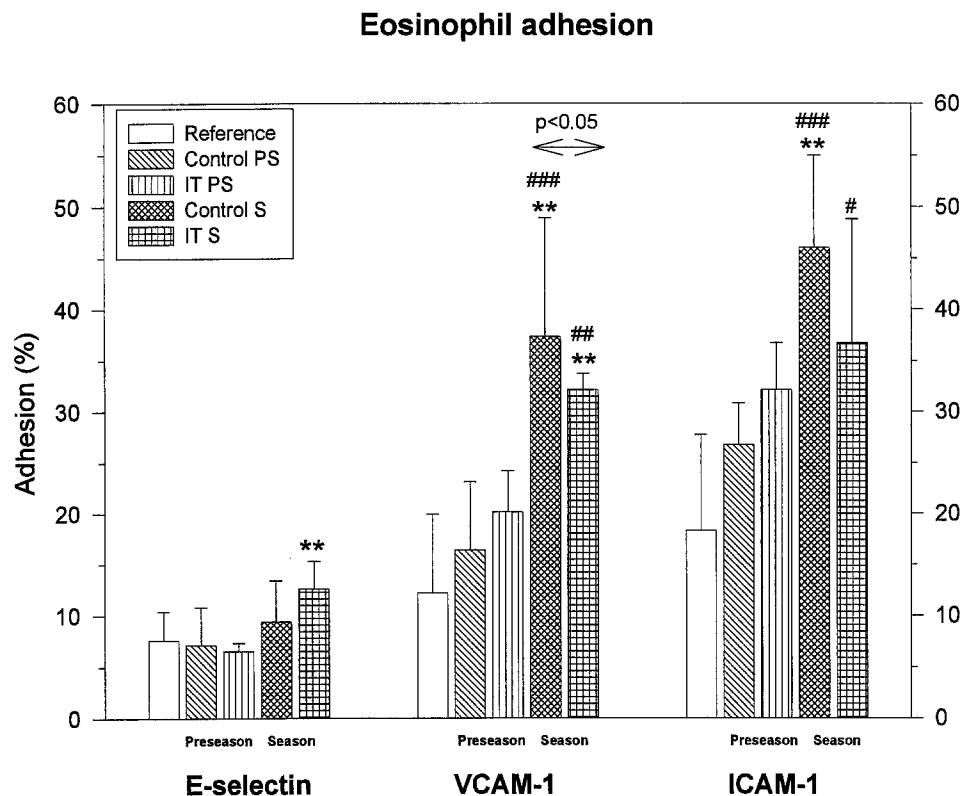


**FIG. 2.** Adhesion to E-selectin, VCAM-1, and ICAM-1 of eosinophil granulocytes from reference group and from control and immunotherapy-treated (IT) patients before season (PS) and during season (S). Significant changes of eosinophil adhesion during season compared with before season are indicated by asterisks (\*\* $p < 0.01$ ) and significant differences between patients and reference subjects are indicated by pound symbols (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Furthermore, significant difference between two patient groups in eosinophil adhesion to VCAM-1 during season is indicated. Results shown are median values and upper quartile ranges. Zero level represents basic adhesion to nontransfected baby hamster kidney fibroblasts.

eosinophils (Fig. 3). In the immunotherapy-treated patient group the eosinophils demonstrated increased expression of CD49d during season compared with before season and compared with those of the reference group. There were no significant relationships between eosinophil adhesion and eosinophil cell surface expression (results not shown).

#### Neutrophil adhesion and cell surface expression

Neutrophils from both treatment groups demonstrated enhanced adhesion to E-selectin and ICAM-1 during as compared with before season (Fig. 4). The increase of neutrophil adhesion to E-selectin during season was, however, significantly higher ( $p < 0.05$ ) in the immunotherapy group (22.3%; quartile range: 14.3% to 27.6%) than in the control group (12.8%; quartile range: 7.0% to 18.7%). As compared with neutrophils from the reference group, neutrophil adhesion to E-selectin was higher in both treatment groups during season and the neutrophil adhesion to ICAM-1 was higher in the immunotherapy group during season (Fig. 4).

The neutrophil cell surface expression of CD11b and CD18 was unchanged during as compared with before

season in both groups and was not different from that of reference neutrophils (Fig. 5).

#### Eosinophil and neutrophil adhesion during season in relation to asthma

Eosinophils from the control group showed increased adhesion to VCAM-1 and ICAM-1 during as compared with before season and as compared with references ( $p < 0.02$ ), irrespective of whether the patients had a diagnosis of asthma (Fig. 6). In the immunotherapy group eosinophils from patients without asthma demonstrated enhanced adhesion to VCAM-1 and ICAM-1 during season, whereas eosinophils from the patients with asthma did not show any significant changes of adhesion to VCAM-1 or ICAM-1. Eosinophils from the immunotherapy-treated patients without asthma also demonstrated increased adhesion to VCAM-1 and ICAM-1 during season as compared with eosinophils from reference subjects ( $p < 0.02$ ).

Neutrophils from the immunotherapy-treated patients showed increased adhesion to E-selectin and ICAM-1 during season compared with before season irrespective of whether the patients had asthma (Fig. 7). Compared with references, neutrophils from immunotherapy-

### Eosinophil cell surface expression

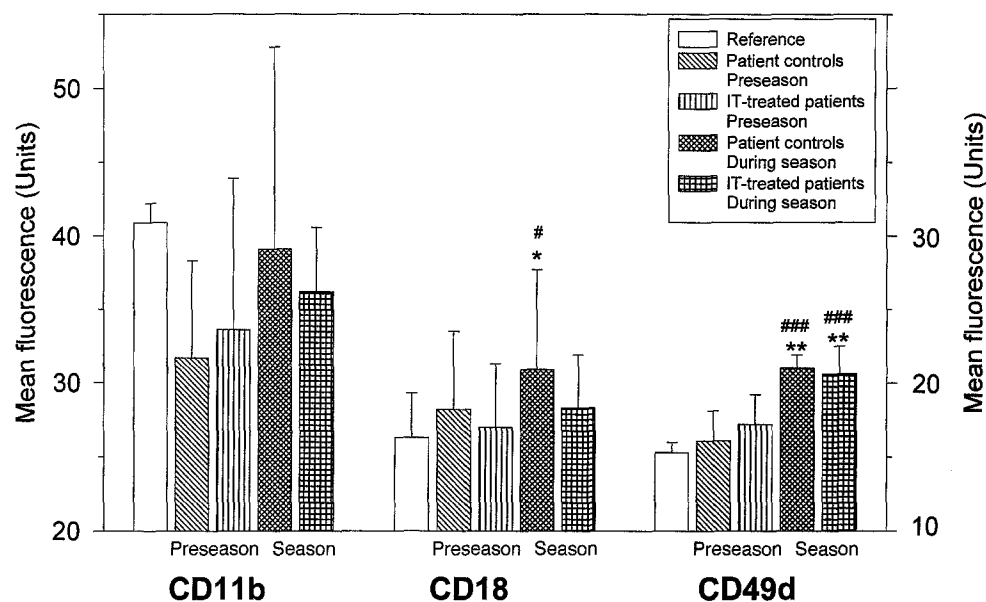


**FIG. 3.** Specific cell surface expression of CD11b, CD18, and CD49d on eosinophils from reference group and from control and immunotherapy-treated (IT) patients before season (PS) and during season (S). Scale on left y-axis denotes expression of CD11b and CD18 and scale on right y-axis expression of CD49d. Significant changes of eosinophil cell surface expression during season compared with before season are indicated by asterisks (\* $p < 0.05$ , \*\* $p < 0.01$ ) and significant differences between patients and reference subjects are indicated by pound symbols (# $p < 0.05$ , ### $p < 0.001$ ). Results shown are median values and upper quartile ranges. Zero level represents background fluorescence of eosinophils labeled with an irrelevant monoclonal antibody of the same isotype.

treated patients with asthma showed increased adhesion to E-selectin and ICAM-1 during season ( $p < 0.05$ ), whereas neutrophils from the immunotherapy-treated patients without asthma showed increased adhesion to ICAM-1 during season ( $p < 0.02$ ). In the control group neutrophils from patients without asthma demonstrated increased adhesion to E-selectin but not to ICAM-1 during season compared with before season, whereas neutrophils from the patients with asthma did not show any changes of adhesion to E-selectin or ICAM-1 during season. As compared with neutrophils from reference subjects, neutrophils from control patients with and without asthma did not show any increased adhesion to E-selectin or ICAM-1 during season.

### DISCUSSION

The present investigation of eosinophil adhesion in patients allergic to birch pollen was done during the pollen season of 1993, which was a season with extremely high birch pollen counts preceded by high alder pollen counts. The peak mean birch pollen count was about 6000 per cubic meter, whereas the peak count of a "normal" high season in the same area is about 2000 per cubic meter.<sup>8, 30</sup> This strong allergen challenge resulted in a significant increase of the bronchial reactivity to methacholine in the nonasthmatic groups of both the immunotherapy-treated patients and the control pa-

tients. The majority (5/7) of the patients in the control group who had no diagnosis of asthma before season had bronchial hyperreactivity during season. The blood eosinophil counts increased in both treatment groups during season, but a separate analysis of findings in patients with and without asthma demonstrated a lack of significant increase in the immunotherapy-treated patients with asthma in contrast to results in all the other groups. The serum concentration of ECP did not show any significant change in any of the patient groups. In the case of the immunotherapy-treated patients this is in agreement with findings of previous investigations that demonstrated an unaltered serum level of ECP in immunotherapy-treated patients during season in contrast to the raised serum ECP level in untreated patients.<sup>31</sup> The lack of an increased level of ECP in serum from the present control group might be explained by the fact that the samples were obtained during the first half of the birch pollen season, whereas the increase of the serum ECP level in the previous investigations was shown to occur during the second half of the birch pollen season.<sup>30, 31</sup> Another contributing cause might be that the patients in the present control group were treated with topical nasal steroids, because at least local inhalation treatment with steroids has been shown to decrease the serum level of ECP.<sup>32, 33</sup>

The massive allergen challenge during the birch pol-

## Neutrophil adhesion

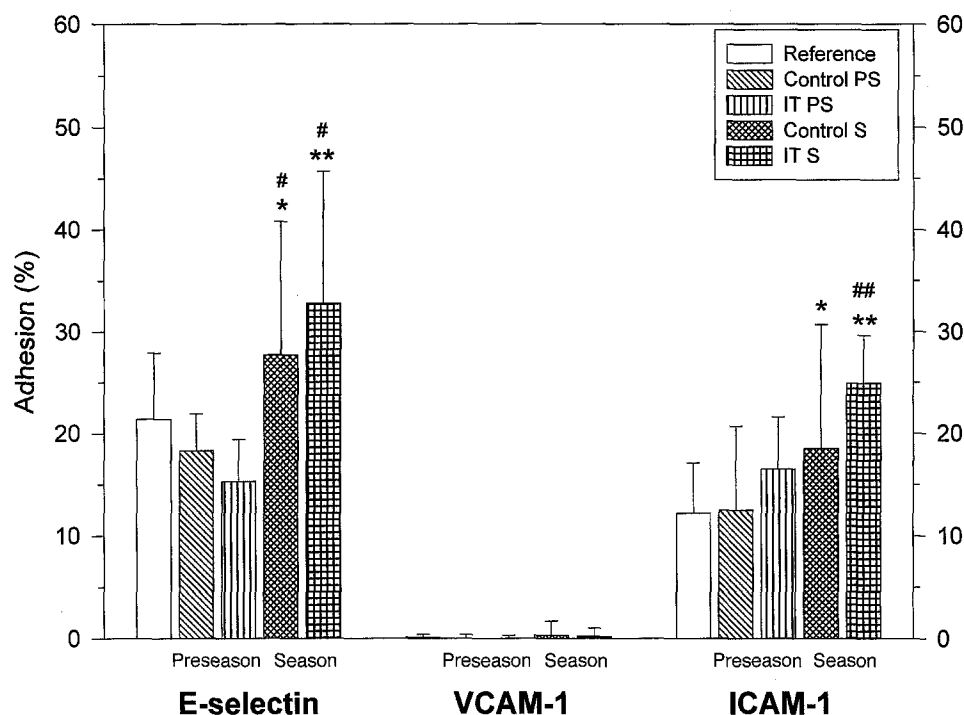


**FIG. 4.** Adhesion to E-selectin, VCAM-1, and ICAM-1 of neutrophil granulocytes from reference group and from control and immunotherapy-treated (IT) patients before season (PS) and during season (S). Significant changes of neutrophil adhesion during season compared with before season are indicated by asterisks (\* $p < 0.05$ , \*\* $p < 0.01$ ) and significant differences between patients and reference subjects are indicated by pound symbols (# $p < 0.05$ , ## $p < 0.01$ ). Results shown are median values and upper quartile ranges. Zero level represents basic adhesion to nontransfected baby hamster kidney fibroblasts.

len season induced a priming of adhesion to VCAM-1 of eosinophils from both the control group and the immunotherapy-treated patients, although the increase of adhesion to VCAM-1 during season was significantly higher in the control group than in the immunotherapy-treated group of patients. The increase of eosinophil adhesion to VCAM-1 was probably partly a result of the increase of the adhesion molecule VLA-4 on the cell surface of the eosinophils. There was, however, no direct relationship between the adhesion to VCAM-1 and the cell surface expression of the  $\alpha$  chain of VLA-4 (CD49d), which indicates that the functional change of the VLA-4 receptor leading to an enhanced adhesiveness is the most important part of the increased eosinophil adhesion to VCAM-1. A closer examination of the changes of eosinophil adhesion to VCAM-1 during season demonstrated not only that immunotherapy dampened the priming of eosinophil adhesion, but also that the immunotherapy treatment inhibited the increase of eosinophil adhesion to VCAM-1 in the patients with asthma. Eosinophil adhesion to ICAM-1 showed the same pattern. Allergen exposure induced a priming of eosinophil adhesion to ICAM-1 in both patients with and those without asthma in the control group and in the immunotherapy-treated patients with-

out asthma. In contrast, immunotherapy treatment of the patients with asthma prevented the priming of eosinophil adhesion to ICAM-1 during season. The parallel, but noncorrelated, increase of eosinophil adhesion to ICAM-1 and the eosinophil cell surface expression of CD18 in the control group indicate that also in this case the enhanced adhesion was caused by a combination of an increased number and functional capacity of receptors.

Priming of eosinophil adhesion to VCAM-1 and ICAM-1 was demonstrated in a previous investigation of patients with asthma.<sup>22</sup> In that case, the enhanced eosinophil adhesion was related to the disturbance of the lung function measured as PEF variability in a group of patients with essentially mild asthma. In that previous investigation no increase in the eosinophil cell surface expression of CD18 or CD49d was detectable, which indicated that the increased adhesion in that case was caused by increased functional capacity of the receptors. In the present study the mean level of eosinophil adhesion to ICAM-1 and VCAM-1 during season in the control group was, however, approximately 10% higher than that of the patients with asthma in the previous investigation. A comparison of the two studies suggests that a lower dose of priming substances induces an

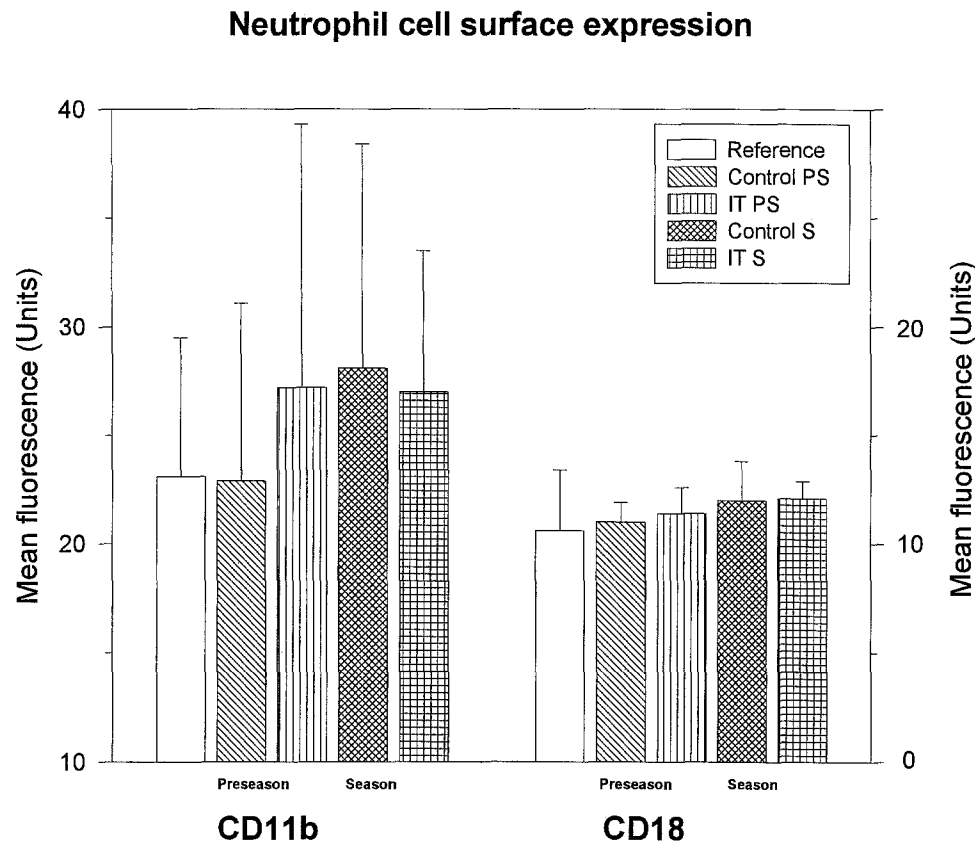


**FIG. 5.** Specific cell surface expression of CD11b and CD18 on neutrophils from reference group and from control and immunotherapy-treated (*IT*) patients before season (*PS*) and during season (*S*). Scale on left y-axis denotes expression of CD11b and scale on right y-axis expression of CD18. No significant changes of neutrophil cell surface expression were observed during season compared with before season. Results shown are median values and upper quartile ranges. Zero level represents background fluorescence of neutrophils labeled with irrelevant monoclonal antibody of same isotype.

enhanced adhesiveness of the receptors, whereas higher doses in addition induce an increased expression of the receptors.

Priming of eosinophils during pollen season has previously been demonstrated in a study of eosinophil degranulation that showed an increased release of ECP and eosinophil protein X during pollen season compared with before and after season.<sup>30</sup> The priming substance or substances can only be hypothesized, but on the basis of previous investigations IL-5 is a strong candidate. IL-5 is known to be a relatively specific primer of eosinophil function in vitro and is known to be produced in vivo during the allergic inflammatory reaction.<sup>34-37</sup> Furthermore, IL-5 has been shown to prime eosinophil adhesion to endothelial cells in vitro.<sup>38</sup> RANTES is a newly discovered eosinophil primer that has been shown to stimulate eosinophil adhesion,<sup>39</sup> but its relation to allergic inflammation has not been extensively investigated. The source of IL-5 is probably the subpopulation of T cells designated Th2 cells.<sup>40-44</sup> The Th2 and Th1 cells were originally described in mice.<sup>45</sup> These T cell clones differ in their patterns of cytokine secretion. Th2 cells produce IL-4, IL-5, IL-6, and IL-10,

whereas Th1 cells produce IL-2 and interferon- $\gamma$ , and both cell types produce IL-3 and granulocyte/macrophage colony-stimulating factor.<sup>46</sup> T cells with the same kind of cytokine production patterns have also been characterized in human beings. Evidence suggests that Th2 cells participate in allergic reactions, for instance, IL-4 induces B cells to switch to IgE production.<sup>47</sup>

Neutrophil adhesion before and during season revealed a different pattern compared with that of the eosinophils. When evaluated in general neutrophils from both the control group and the immunotherapy-treated patients demonstrated priming of adhesion to E-selectin and ICAM-1 during season. A closer examination of the results, however, demonstrated a priming of the adhesion to E-selectin and ICAM-1 of neutrophils from the immunotherapy-treated patients, both those with and without asthma, during season. In contrast, only neutrophils from the control subjects without asthma showed increased adhesion during season and, in this case, only to E-selectin. Neutrophil expression of the  $\alpha$  and  $\beta$  chains of macrophage antigen-1 was not influenced by the pollen season.

Previous investigations of the influence of immuno-

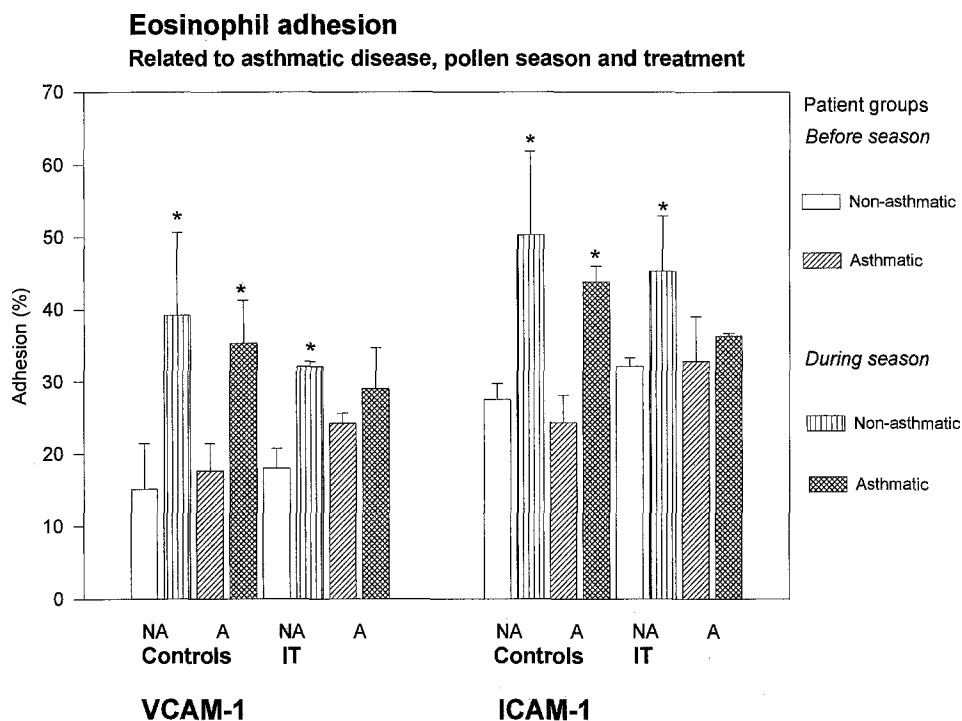


**FIG. 6.** Adhesion to VCAM-1 and ICAM-1 of eosinophils from patients with asthma (A) and those without asthma (NA) in the two treatment groups before and during season. Significant changes of eosinophil adhesion during season compared with before season are indicated by asterisk (\* $p < 0.05$ ). Results shown are median values and upper quartile ranges. IT, Immunotherapy-treated group.

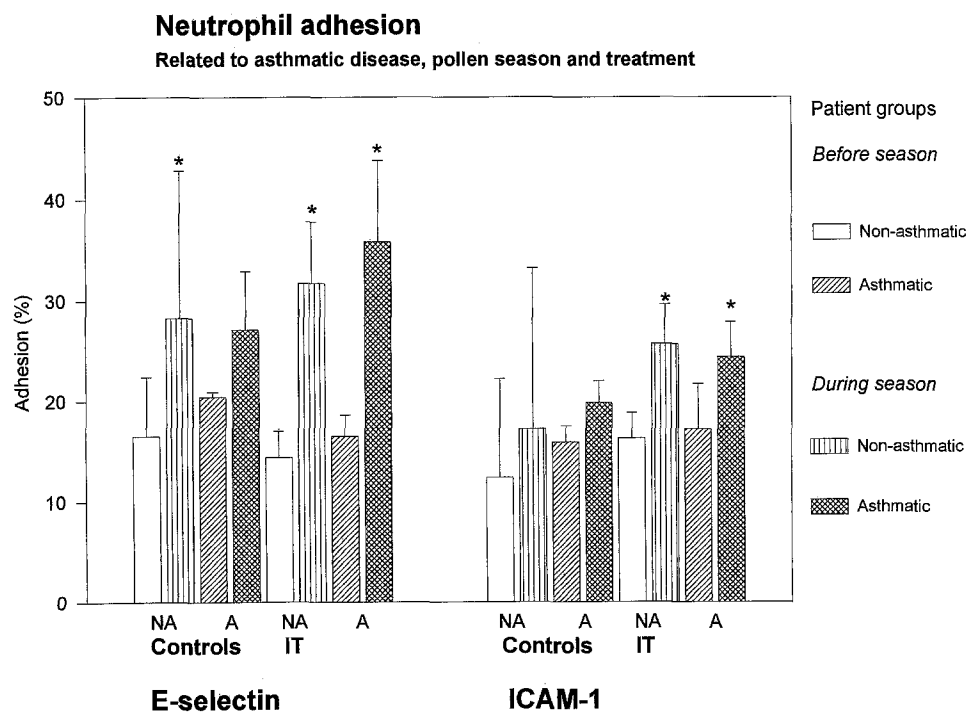


**FIG. 7.** Adhesion to E-selectin and ICAM-1 of neutrophils from patients with asthma (A) and those without asthma (NA) in the two treatment groups before and during season. Significant changes of neutrophil adhesion during season compared with before season are indicated by asterisk (\* $p < 0.05$ ). Results shown are median values and upper quartile ranges. IT, Immunotherapy-treated group.

therapy on eosinophil function have demonstrated an inhibition of the increase of the serum concentration of ECP, which in nontreated patients is induced by the pollen season.<sup>31</sup> Furthermore, the increase of eosinophil chemotactic activity in serum and BAL fluid from allergic patients with asthma during season has been shown to be abrogated by immunotherapy.<sup>8,48</sup> The present investigation, however, is the first to show that immunotherapy interferes with the priming of eosinophil function that occurs in patients with allergies during season. The mechanism by which immunotherapy inhibits eosinophil priming is unknown, but on the basis of the hypothesis that the priming is caused by IL-5 and other cytokines produced by Th2 cells, the effect of immunotherapy might be either to inhibit the activation of Th2 cells or to favor the activation of Th1 cells. Results from in vitro experiments demonstrate a mutual inhibition of the activities of Th1 and Th2 cells; for instance, interferon- $\gamma$  produced by Th1 cells inhibits IL-4 production by Th2 cells.<sup>49,50</sup> That priming of neutrophil adhesion during season was found mainly in the immunotherapy-treated patient group might suggest that immunotherapy induces a switch from Th2 cell to Th1 cell activation. Such a shift would diminish IL-5 production and eosinophil priming and the neutrophil priming effects of IL-3 and granulocyte/macrophage colony-stimulating factor would be more markedly expressed. A few previous investigations have demonstrated signs of a shift from Th2 cells to Th1 cells in patients with allergies treated with immunotherapy.<sup>51-55</sup> That a shift to priming of neutrophil adhesion by immunotherapy does not induce neutrophil accumulation in the lungs of the patients<sup>48</sup> is probably because of the absence of additional mechanisms favoring neutrophil accumulation. Previous investigations have demonstrated that immunotherapy abrogates the production of chemoattractants,<sup>48</sup> and the neutrophil migratory responses have been shown not to be primed in patients with asthma.<sup>9</sup>

The dampening effect of immunotherapy on eosinophil blood count and eosinophil adhesion was most pronounced in the patients with asthma. The same difference in the effect of immunotherapy on patients with and without asthma was observed throughout the whole study, for instance regarding eosinophil chemotactic activity, B cell number, and cell surface markers (data to be published). The reason for these results can only be speculated upon, but they might reflect differing pathophysiologic mechanisms behind rhinitis and asthma. Whether these results have any clinical implications cannot be proved from the present investigation. Probably the groups were too small to permit a comparison of clinical symptoms. The only clinical parameter that indicated a difference was the bronchial reactivity to methacholine, which was not further decreased in the patients with asthma treated with immunotherapy.

In conclusion, the present investigation has demonstrated that natural allergen challenge during birch pollen season induced priming of eosinophil adhesion in patients allergic to birch pollen. The priming resulted in

an increased adhesion to VCAM-1 and ICAM-1, which in combination with induction of VCAM-1 and ICAM-1 expression on endothelial cells in bronchial venules and capillaries might be an essential mechanism behind the accumulation of eosinophils in the lungs of patients with allergic asthma. Immunotherapy dampened the priming of eosinophil adhesion, probably by interfering with the production of priming factors.

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