

Allergen-specific increase in interleukin (IL)-4 and IL-5 secretion from peripheral blood mononuclear cells during birch-pollen immunotherapy

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The mechanisms behind the effects of immunotherapy (IT) with birch-pollen extract are largely unknown. In this pilot study, we measured the cytokine secretion *in vitro* from peripheral blood mononuclear cells (PBMC) obtained from birch-pollen-allergic patients undergoing IT treatment ($n=4$) or placebo administration ($n=4$), collected before treatment, 1 and 4 weeks after start of treatment, and during and just after the pollen season (12–14 weeks after start of treatment). The PBMC were stimulated with birch-pollen extract *in vitro* for 7 days, followed by restimulation with the mitogens phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) for 24 h, to enhance the production of cytokines. The supernatants were analyzed with ELISA and radioimmunoassay for interferon-gamma (IFN- γ), interleukin (IL)-4, and IL-5. In the therapy group, we noted an increased secretion of IL-4 and IL-5 from PBMC collected at 4 weeks after the start of treatment (IL-4: 29 ± 21 pg/ml [day 0] to 374 ± 448 pg/ml [week 4], mean $\pm$ SD; IL-5: 95 ± 48 pg/ml to 1147 ± 697 pg/ml). No increase was seen in the placebo group. During the pollen season, we noted a trend toward increased IL-4 and IL-5 secretion in both groups. We conclude that the temporary increase in serum IgE observed in many IT studies may be a consequence of increased IL-4 production due to the allergen exposure.

Key words: birch-pollen allergens; immunotherapy; interleukin-4; interleukin-5; peripheral blood mononuclear cells; T helper cells.

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Allergy is a disease which seems to be increasing in Western countries. Much is unknown about the mechanisms of allergic development, but T helper (Th) cells are thought to have a central role. It is known that human Th cells can be divided into different subsets by their cytokine synthesis pattern (1). The cells in the Th1 subset secrete high amounts of interferon-gamma (IFN- γ) and tumor necrosis factor-beta (TNF- β), but no or small amounts of interleukin (IL)-4 and IL-5. On the other hand, T helper cells belonging to the Th2 subset secrete IL-4 and IL-5, but only small amounts of Th1-type cytokines. While Th1 cells are involved in the defense against intracellular bacteria and viruses, Th2 cells induce B cells to secrete IgE involved in allergic reactions and in the immunoreaction to parasitic helminths (1). Th0 cells are an intermediate subset of T cells, producing both Th1- and Th2-type cytokines (1). The Th2 cytokines IL-4 and

IL-5 are important factors in the development of atopic inflammation. IL-4 has been shown to be crucial for the regulation of allergy development and maintenance due to its important role in inducing B cells to produce IgE antibodies (2). IL-4 is also important for the Th2 development of naive T cells (3), and sufficient IL-4 can be produced by the naive T cells themselves, as has been shown in *in vitro* experiments (4). Damage of the lung epithelial cells is thought to contribute to the course of bronchial atopic asthma, and is mediated by IL-5-stimulated infiltration and differentiation of eosinophils into lung tissue (5). IFN- γ , produced by Th1 cells, has been shown to downregulate the development of Th2 cells *in vitro* (3), and is therefore important in the regulation of the development of atopy.

One effective treatment for many patients suffering from atopic diseases is allergen-specific

immunotherapy (IT). IT was introduced more than 80 years ago, but the mechanisms of its effect are still largely unknown.

This pilot study aimed to find a method to study early changes in cytokine secretion from allergen-specific T cells isolated from birch-pollen-allergic patients undergoing IT. We followed eight patients during their early treatment period (increment in dosing of allergen extract) and during the subsequent pollen season. In a double-blind, placebo-controlled study, four of the patients received birch-pollen IT, and the other four placebo injections. The production of IL-4, IL-5, and IFN- γ was measured after *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with birch-pollen allergen and the mitogens phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA).

Material and methods

Patients and study design

This pilot study comprised eight birch-pollen-allergic patients. All of the patients suffered from seasonal allergic asthma, and the selection for the study was based on history of clinical symptoms during the birch-pollen season and positive results from both skin prick testing and specific IgE measurements (Table 1). The study was performed as a double-blind, placebo-controlled trial, and the patients were randomized into two blocks of fours. Thus, four of the patients received specific IT (therapy group) with birch-pollen extract Alutard SQ (ALK, Hørsholm, Denmark), and four patients received placebo (placebo group). All patients reported symptom scores during the study period.

The patients followed a semirush IT regimen with two injections once a week with increasing doses of birch extract or placebo, until the maintenance volume of 1 ml (representing 100 000 SQ units) was reached after 3 months. Blood was drawn from the patients before the start of treatment (day 0), 1

week after (week 1), and 4 weeks after (week 4). Blood was also collected during the subsequent pollen season (season) 12–14 weeks after the start of IT, and around 1 month after the end of the pollen season (15–17 weeks after the start of IT). Serum levels of specific IgE and IgG to birch pollen were determined for all samples with the Pharmacia CAP System (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). One patient in the placebo group (patient G) did not continue the IT after the pollen season; therefore, his last blood sample is missing.

Antigens

Crude extract of birch pollen (BPE) was prepared by extracting birch pollen (*Betula verrucosa*) (Allergon, Angelholm, Sweden) in Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} for 2 h, followed by filtration through filter paper and 0.2- μm sterile filter. The protein concentration was determined by the method of Bradford (6). The allergen extract was then stored at -20°C until use. Tetanus toxoid (TT) was obtained from SBL Vaccin AB, Stockholm, Sweden.

Preparation of PBMC

PBMC were separated from heparinized blood by Ficoll-Paque gradient centrifugation at 850 g for 30 min (Pharmacia Biotech, Uppsala, Sweden), washed twice in PBS, resuspended in 7.5% dimethyl sulfoxide (DMSO; Fluka Chemie, Buchs, Switzerland) in fetal calf serum (HyClone, Logan, UT, USA), and frozen. The cells were stored at -135°C until use.

Culture methods

We used a culture method similar to an earlier published method (7), but we used PHA and PMA instead of OKT3 antibodies to restimulate the cells. Briefly, PBMC were thawed and washed twice in

Table 1. Clinical data of patients studied

Patient	Age (years)/sex	Treatment	Skin prick test ^a	Birch-specific IgE ^b	Clinical symptoms
A	23/M	IT	9×8	11.2	Seasonal allergic asthma ^c
B	46/F	IT	10×7	19.6	Seasonal allergic asthma
C	37/F	IT	9×5	79.3	Seasonal allergic asthma
D	22/F	Placebo	12×7	20.3	Seasonal allergic asthma
E	32/F	IT	6×5	6.1	Seasonal allergic asthma
F	30/M	Placebo	9×6	61.5	Seasonal allergic asthma
G	26/M	Placebo	15×7	7.7	Seasonal allergic asthma
H	29/M	Placebo	5×8	12.8	Seasonal allergic asthma

^aDiameters of wheals (mm).

^bSpecific IgE in kU/l.

^cDefined as bronchial hyperreactivity (BHR) with $\text{PC}_{20} < 8$ mg/ml during pollen season.

PBS and resuspended at $1 \times 10^6/\text{ml}$ in conditioned medium (CM) composed of F-DMEM (National Veterinary Institute, Uppsala, Sweden), including 4 mM L-glutamine, supplemented with 10% heat-inactivated human AB+ sera and 50 $\mu\text{g}/\text{ml}$ gentamycin sulfate (Biological Industries, Israel). The cells were cultured in 24-well, flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) in a humidified chamber at 37°C in 7% CO_2 , in the presence of 0.2 $\mu\text{g}/\text{ml}$ BPE or 5.0 $\mu\text{g}/\text{ml}$ TT. These concentrations had previously been shown to stimulate optimal proliferation in PBMC cultures (data not shown). After antigen stimulation for 7 days, the cells were harvested, washed, and restimulated with 10 $\mu\text{g}/\text{ml}$ PHA (Pharmacia Biotech) and 10 ng/ml PMA (Sigma, St Louis, MO, USA) in CM, at a cell density of $5 \times 10^5/\text{ml}$. After restimulation for 24 h, the culture supernatants were harvested by centrifugation and frozen at -20°C .

The antigen-driven proliferation of the PBMC was tested separately by culturing the cells at $1 \times 10^6/\text{ml}$ in CM in the presence or absence of the antigens at optimal concentrations. The cultures were incubated in 96-well, flat-bottom tissue culture plates (Costar) in four replicates for 6 days. For the final 4 h of culture, 1.0 μCi of ^3H -labeled thymidine (Amersham, Buckinghamshire, UK) was added to each well. The cultures were then harvested on glass fiber disks, and the incorporated radioactivity was measured in a beta-counter (Beckman, Fullerton, CA, USA).

Flow cytometry

After 7 days of stimulation with BPE or TT, the cells were harvested by centrifugation and resuspended in PBS at $1\text{--}2 \times 10^6/\text{ml}$. Aliquots (50 μl) of the cell suspensions were stained with 10–20 μl fluorescent-conjugated monoclonal antibodies, as recommended by the manufacturer. The antibodies were anti-CD3-PE, anti-CD4-FITC, anti-CD8-FITC, and appropriate control antibodies from Becton-Dickinson (San Jose, CA, USA). After incubation in the dark for 15 min at room temperature (RT), 2 ml ice-cold PBS was added, and the suspensions were centrifuged. The supernatants were discarded, and the pellets were resuspended in 0.5 ml ice-cold PBS and immediately analyzed in a Coulter EPICS XL flow cytometer (Coulter, Miami, FL, USA).

Measurements of cytokines

IL-4 and IFN- γ concentrations in the supernatants were determined by the commercial ELISAs Quantikine human IL-4 immunoassay and Quantikine human IFN- γ immunoassay, respectively (R&D Systems, Minneapolis, MN, USA). IL-5 was measured

by an in-house-developed microtiter sandwich radioimmunoassay, as described elsewhere (8).

Statistical analysis

The number of patients was too low to allow reliable statistical analyses of changes within the patient groups (Wilcoxon signed ranks test). The unpaired nonparametric Mann-Whitney U-test (two-tailed *P* value) was used to identify significant differences between the patient groups. Only *P* values <0.05 were considered significant.

Results

Clinical efficacy

The birch-pollen season was of medium intensity. The patients suffered a mild, nonsignificant increase in symptoms during the season. No significant differences were observed between the two study groups (IT-treated and placebo) when symptom scores of rhinoconjunctivitis or lower airways were calculated.

Allergen-specific PBMC activation and proliferation in vitro during IT

All patients' PBMC responded by proliferation to BPE or TT stimulation as measured by ^3H -labeled thymidine incorporation (data not shown). No significant changes in proliferation were seen during IT or during the birch-pollen season compared to before the start of treatment. We also performed flow cytometry analysis on some of the cultures. Activation and proliferation led to increased numbers of large lymphocytes, and, by gating CD3+CD4+ cells (T helper cells) or CD3+CD8+ cells (cytotoxic T cells), we were able to analyze blast formation in the two cell populations. Fig. 1 is a representative graph of the fraction of blast cells in the CD4+ as well as in the CD8+ T-cell populations in a culture stimulated with BPE for 7 days. We always found more activation within the CD4+ T-cell population. The fraction of blast cells in this population varied between 6 and 43% (mean 27%, $n=7$), compared to 3 and 22% (mean 11%, $n=7$) in the CD8+ T-cell population. This indicates a predominant activation and expansion of antigen-specific T helper cells in the PBMC cultures in this study.

Cytokine production after allergen stimulation in vitro

Birch-pollen-allergen-specific stimulation *in vitro* and restimulation with PHA and PMA resulted in detectable levels of IL-4 and IL-5 in the cell

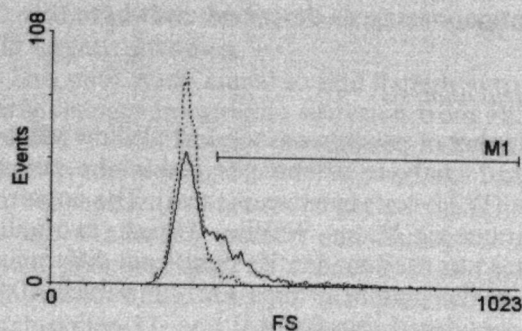


Fig. 1. Forward scatter graph showing activated blast cells (M1) identified as CD3+CD4+ T cells (solid line) or CD3+CD8+ T cells (dotted line). Graph is representative of typical BPE-stimulated culture.

supernatants. Low levels of IL-4 and IL-5 were found in all BPE-prestimulated cultures of PBMC collected from the patients before the start of treatment. During the first month of treatment, the production of IL-4 and IL-5 increased in the therapy group (IL-4: 29 ± 21 pg/ml [day 0] to 374 ± 448 pg/ml [week 4], mean $\pm$ SD; IL-5: 95 ± 48 pg/ml to 1147 ± 697 pg/ml) (Fig. 2A and B). These increases were not seen in the placebo group.

During the birch-pollen season, there was a trend toward increased IL-4 and IL-5 secretion in both the therapy group (IL-4: 29 ± 21 pg/ml [day 0] to 136 ± 167 pg/ml [season], mean $\pm$ SD; IL-5: 95 ± 48 pg/ml to 794 ± 540 pg/ml) and the placebo group (IL-4: 22 ± 12 pg/ml to 238 ± 420 pg/ml, mean $\pm$ SD; IL-5: 70 ± 90 pg/ml to 876 ± 1512 pg/ml) (Fig. 2A and B). This trend of increased IL-4 and IL-5 secretion during the pollen season was maintained also 1 month after the peak of the pollination season.

High levels of secreted IFN- γ were found in all cultures. When PBMC isolated before start of treatment (day 0) were cultured with BPE followed by mitogenic restimulation, we found 749–6510 pg/ml IFN- γ in the cell-culture supernatants. On the other hand, we could not find any major changes in IFN- γ secretion during the study period in either of the two patient groups (Fig. 2C).

TT was used as a control antigen to study the specificity of the allergen-driven activation and expansion of T cells in our cultures. The increase in IL-4 and IL-5 secretion after BPE stimulation and restimulation with PHA and PMA, observed in the therapy group, was absent in the TT-prestimulated PBMC cultures. This indicates that the increase in Th2-type cytokine production was birch-pollen specific (data not shown).

We also analyzed the cell-culture supernatants after stimulation with the allergen/antigen without restimulation with PHA and PMA. We found a

cytokine pattern in these supernatants very similar to that of the restimulated cultures, but with much lower levels of the cytokines produced (data not shown).

Changes in cytokine ratios during IT

We also calculated the IFN- γ /IL-4 and IFN- γ /IL-5 ratios for the cultures; the results are shown in Tables 2 and 3, respectively. There were large decreases in the IFN- γ /IL-4 and IFN- γ /IL-5 ratios in the BPE- and PHA/PMA-stimulated cell cultures after 1 month of IT (therapy group), indicating a shift toward a more Th2-like phenotype of the T cells initially during IT treatment. There were also decreases in the IFN- γ /IL-4 and IFN- γ /IL-5 ratios during and after the pollen season in both patient groups after BPE stimulation. We then wanted to see whether BPE and TT induced different cytokine profiles; consequently, we compared their cytokine ratios at two different time points: before the start of IT (day 0) and during the pollen season. When comparing BPE and TT stimulation, we did not observe any differences in the PHA/PMA-induced cytokine ratios which could reflect their potency to induce a Th0 phenotypic T-cell response (see Discussion).

Birch-pollen-specific IgE and IgG serum levels

The concentrations of birch-pollen-specific IgE in the study group varied between 6.1 and 79 kU/l at the beginning of the study (Table 1). No changes in specific serum IgE levels were seen before the pollen season and during treatment, but, during the birch-pollen season, there was a small increase in specific IgE in most of the patients (data not shown). The increases varied between 32% and 216%. No clear differences could be seen between the therapy group and the placebo group in specific IgE.

During the birch-pollen season, there was a trend toward an increase in birch-pollen-specific IgG, which varied between 64% and 842%, in the sera from the therapy group (data not shown). This increase was completely absent in the placebo group. The difference between the groups in specific serum IgG during the pollen season was almost statistically significant ($P=0.057$).

Discussion

Although allergen-specific IT for patients suffering from atopic diseases was introduced over 80 years ago, the mechanisms behind its beneficial effects are still a matter of speculation. The increase in blocking IgG antibodies or decrease in specific IgE to inhalant allergens does not always seem to

Increased IL-4 and IL-5 secretion during IT

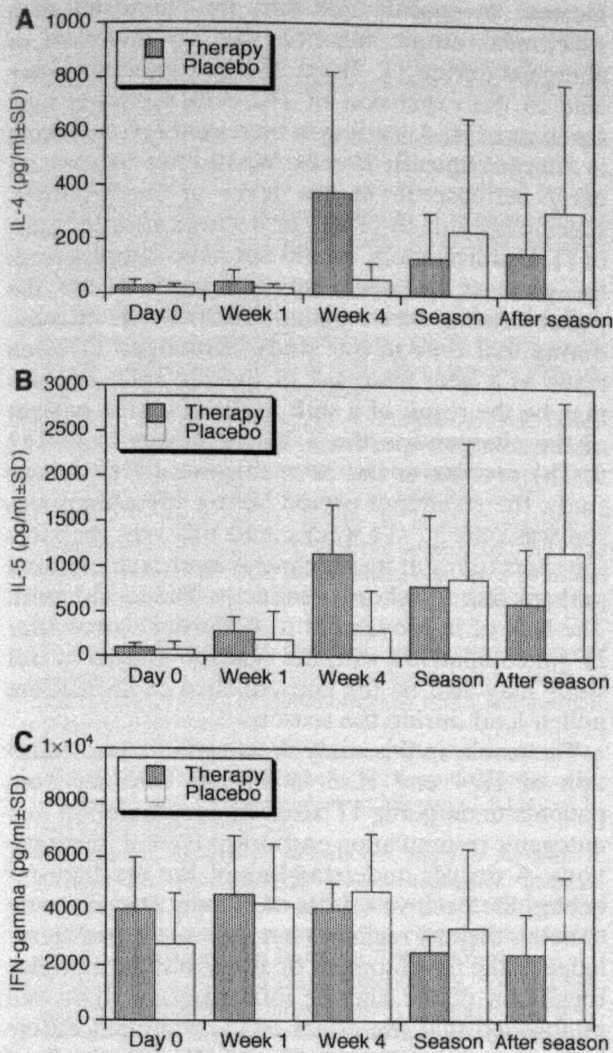


Fig. 2. IL-4 (A), IL-5 (B), and IFN- γ (C) concentrations in BPE-stimulated cultures, restimulated with PHA and PMA. Data are presented as mean concentration $\pm$ standard deviation (SD) for therapy group (hatched bars) and placebo group (open bars).

correlate with clinical improvement (9–13). Induction of T-cell tolerance has been suggested as a possible mechanism (14, 15), and new data have shown that IT inhibits allergen-induced late-phase responses in association with a decreased recruitment of CD4⁺ T cells and activated eosinophils (16). Although some research has been done on cytokine production during IT, the early changes in cytokine secretion during IT treatment of inhalant allergy are largely unknown.

In this pilot study, we examined the mechanisms of IT by studying the kinetics of cytokine production in PBMC cultures from birch-pollen-allergic patients. We used a culture method like that of Jutel et al. (7). To be able to compare the cytokine production in different cultures, we first stimulated

Table 2. IFN- γ /IL-4 ratio (mean $\pm$ SD) for PBMC stimulated with antigen (BPE or TT) for 7 days and restimulated with PHA and PMA for 24 h

Culture	Day 0	Week 1	Week 4	Season	After season
IT; BPE-stimulated	170 $\pm$ 97	164 $\pm$ 113	23 $\pm$ 17	43 $\pm$ 38	44 $\pm$ 35
PL; BPE-stimulated	122 $\pm$ 36	208 $\pm$ 103	124 $\pm$ 71	90 $\pm$ 60	48 $\pm$ 53
IT; TT-stimulated	135 $\pm$ 107	191 $\pm$ 205	120 $\pm$ 127	87 $\pm$ 57	110 $\pm$ 89
PL; TT-stimulated	164 $\pm$ 166	145 $\pm$ 115	36 $\pm$ 40	53 $\pm$ 43	76 $\pm$ 104

IT=therapy group; PL=placebo group.

Table 3. IFN- γ /IL-5 ratio (mean $\pm$ SD) for PBMC stimulated with antigen (BPE or TT) for 7 days and restimulated with PHA and PMA for 24 h

Culture	Day 0	Week 1	Week 4	Season	After season
IT; BPE-stimulated	46 $\pm$ 23	19 $\pm$ 11	5.3 $\pm$ 4.6	18 $\pm$ 32	12 $\pm$ 19
PL; BPE-stimulated	73 $\pm$ 67	96 $\pm$ 89	46 $\pm$ 34	21 $\pm$ 14	11 $\pm$ 14
IT; TT-stimulated	65 $\pm$ 14	95 $\pm$ 29	48 $\pm$ 10	47 $\pm$ 25	47 $\pm$ 25
PL; TT-stimulated	124 $\pm$ 165	66 $\pm$ 64	46 $\pm$ 48	18 $\pm$ 16	22 $\pm$ 30

IT=therapy group; PL=placebo group.

the PBMC in an antigen-specific way, and then recultured them at 5×10^5 /ml in fresh media. PHA and PMA were added to promote cytokine production. This restimulation seemed to stimulate high cytokine production from already antigen-stimulated cells, and the cytokine pattern was very similar between restimulated cultures and cultures stimulated only with antigen (data not shown).

The results from this pilot study indicate that injections of allergen extracts after a semirush IT regimen (double injections, once a week) may initially lead to an increased production of IL-4 and IL-5, cytokines known to be involved in allergic reactions. This increased production of Th2-type cytokines was observed after stimulation *in vitro* with birch-pollen allergens followed by mitogenic restimulation in PBMC cultures from patients undergoing IT. However, this change in Th2-type cytokine secretion was not linked to a change in IFN- γ secretion. The increased production of IL-4 and IL-5 resulted in a shift in IFN- γ /IL-4 and IFN- γ /IL-5 ratios toward a more Th2-like response by birch pollen-specific T cells. The main source of the cytokines measured in our system was probably of the CD4⁺ phenotype, as indicated by a high rate of blast-cell formation within this T-cell population. Other sources may be CD8⁺ T cells (although they seem to be less activated), natural killer cells, or monocytes.

We also wanted to know whether there was a difference in cytokine profiles between the two stimuli used. BPE has been reported to stimulate expansion of T-cell clones *in vitro* of both Th2 and Th0 phenotypes from birch-pollen-allergics (17, 18),

while the Th0 phenotype predominates in non-allergics (19). TT preferably stimulates T cells of the Th0 phenotype (1). We did not observe any differences in cytokine ratios in the PBMC cultures stimulated with either BPE or TT. We analyzed the ratios at two different time points, before the start of IT (day 0) and during the pollen season, and they both gave the same results. This lack of discrepancy may be the consequence of the capability of both the antigens to induce a strong Th0 phenotypic T-cell response shadowing the Th2 response to BPE. In bulk cultures, it could also be difficult to observe minor differences in the T-cell capacity to produce cytokines in response to antigenic stimulation, at least, as in this case, when the cytokine production is enhanced by mitogenic restimulation.

When stimulation with TT was performed, no major change in IL-4, IL-5, or IFN- γ secretion was noted during the study period, indicating that IT with BPE specifically influenced the BPE-specific T cells, which consequently altered their cytokine production. This BPE-specific increased production of IL-4 and IL-5 due to IT is surprising, since a widely accepted theory of the mechanism in successful IT in allergic patients is that IT leads to a shift in the T-cell cytokine pattern from a Th2 to a Th1 profile, and thus to decreased production of IL-4 and IL-5, together with increased IFN- γ production (16). Various reports support this theory (20–23). However, the kinetics of this process is not clear. Our findings of increased IL-4 and IL-5 production *in vitro* during the first month of birch-pollen IT indicate an increased number of BPE-specific Th2 cells in peripheral blood, or a shift of BPE-specific T cells toward a more Th2-like phenotype. This is in contrast to earlier studies which reported an early fall in IL-4 and IL-5 production during bee-venom IT (20, 21).

The explanation of the discrepancies between these investigations and ours may be the type of allergy studied. Bee-venom allergy differs from inhalant allergy by the route of allergen entry. In venom allergy, the allergen enters the body systemically by the insect sting. In inhalant allergy, pollen reaches the mucosa of the eyes, nose, and lungs, leading to a local allergic inflammatory reaction. It may also be that the regimen used for allergen injections during IT could affect the early changes in cytokine pattern. Rush and ultrarush IT may lead to faster changes in cytokine secretion and decreased IL-4 and IL-5 production, while conventional IT leads to slower changes in cytokine pattern, as indicated in the study by McHugh et al. (21). The initial increase in Th2 cytokines during the first month of IT seen in our study fits well with earlier studies showing an initial increase in specific serum IgE during IT (13, 24, 25). This temporary

increase in specific IgE may be the result of a subclinical atopic reaction due to injection of allergens during IT. These allergen injections may lead to the expansion of Th2-cells secreting high amounts of IL-4, leading to increased IgE switching in allergen-specific B cells. We did not observe an early increase in serum level of birch-pollen-specific IgE due to IT at 1 or 4 weeks after the start of IT. Unfortunately, we did not have samples from the patients collected between week 4 and the pollen season, so we cannot rule out an increase during that time in our study. Prolonged IT often leads to a later decrease in specific IgE, and this may be the result of a shift in the cytokine pattern of the allergen-specific T helper clones from Th2 to Th1 profiles, as has been suggested (16). In our study, the treatment period before the pollen season was only 12–14 weeks, and this was probably too short to result in any clinical improvements and perhaps also too short to induce a Th2 to Th1 shift. The lack of improvement in symptom scores after IT (in comparison with the placebo group) in this study may also be the consequence of insufficient pollen load during the season.

The results in this study showing increased secretion of IL-4 and IL-5 in PBMC cultures from patients undergoing IT after BPE stimulation and mitogenic restimulation encourage further investigations. A deeper understanding of the mechanisms behind the positive effects of IT may lead not only to better therapy regimens but also to a better knowledge of the development of atopy and the complex regulation of the allergic inflammation. It is well established that the effect of IT treatment differs between patients and studies (9, 10, 26). Study of early changes in cytokine profiles during IT may help clinicians to monitor the effect of the therapy, and to distinguish between patients responding positively to IT and those with few or no positive effects.

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