

Cytokine production by peripheral blood mononuclear cells following birch-pollen immunotherapy

Robert Movérare^{a,b,*}, Lena Elfman^b, Eythor Björnsson^a, Gunnemar Stålenheim^a

^a Department of Medical Sciences, Respiratory Medicine and Allergology, Uppsala University, Uppsala, Sweden

^b Pharmacia and Upjohn Diagnostics AB, SE-751 82 Uppsala, Sweden

Received 23 March 2000; accepted 10 May 2000

Abstract

We studied the Th2/Th1 balance by short-term stimulation of peripheral blood mononuclear cells (PBMC) isolated during the pollen season from seven allergic patients treated with conventional birch-pollen immunotherapy (IT) for 18 months, eight matched allergic control patients and 10 non-atopic individuals. The PBMC were cultured for 7 days with birch-pollen extract (BPE) or tetanus toxoid (TT), and then restimulated with PHA and PMA to induce high IL-5, IL-10 and IFN- γ production. The serum levels of birch-pollen-specific IgG and IgG4 were significantly elevated after IT treatment. The proliferative response to BPE was significantly enhanced in the allergic control group, but not in the IT-treated group, compared to the non-atopic group ($P < 0.05$). Birch-pollen-specific IL-5 production was significantly enhanced in both the IT-treated group and the allergic control group ($P < 0.01$ – 0.05). Furthermore, both the IT-treated group and the allergic control group had a cytokine profile to BPE significantly more Th2 polarized (high IL-5/IFN- γ ratio) than to TT ($P < 0.05$ and $P < 0.01$, respectively). No differences in IL-10 production between the three study groups were observed. The Th2/Th1 balance in vitro correlated with the serum concentrations of birch-pollen-specific IgE ($r = 0.60$, $P < 0.05$), and in the IT-treated group, also with the IgG and IgG4 levels ($r = 0.79$, $P < 0.05$ and $r = 0.86$, $P < 0.05$, respectively). We conclude that conventional birch-pollen IT does not lead to changes in the cytokine profile of the circulating pool of allergen-specific T cells during birch-pollen season. However, induction of peripheral T-cell tolerance and increased production of specific IgG and IgG4 might be part of the mechanisms of IT. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Allergy; Birch–pollen allergen; IFN- γ ; IL-5; IL-10; Immunotherapy; PBMC; Th2/Th1

1. Introduction

Birch-pollen allergy is one of the most common seasonal allergies in Northern Europe [1]. Allergen-specific immunotherapy (IT) has been shown to be highly effective in treating birch-pollen allergic rhinoconjunctivitis and asthma [2]. It has been proposed that successful IT is associated with a change from allergen-specific T cells with a Th2-like phenotype producing high amounts of IL-4 and IL-5, to T cells with a Th1-like profile producing high amounts of IFN- γ and no or only low levels of IL-4 and IL-5 [3]. Recently, Akdis et al. [4] reported that the immunoregula-

tory cytokine IL-10 seems to play an important role in the development of T-cell anergy during IT.

Different studies have shown that allergen-specific stimulation in vitro of peripheral blood mononuclear cells (PBMC) isolated from birch-pollen allergic patients reveals a skewed cytokine profile towards a Th2-like phenotype [5–7]. Only few studies, however, have been performed concerning the Th2/Th1 balance during birch-pollen-specific IT [8–10]. In these studies, the early immunological changes during the first months of IT was concerned, and to our knowledge, no group has studied the Th2/Th1 balance for more than 1 year of birch-pollen-specific IT.

In this investigation, performed during the birch-pollen season, we studied the proliferative response and the in vitro production of IL-5, IL-10, and IFN- γ by PBMC isolated from birch-pollen allergic patients

* Corresponding author. Tel.: +46-18-163753; fax: +46-18-166465.

E-mail address: robert.moverare@eu.pnu.com (R. Movérare).

treated with allergen-specific IT for 18 months. We also studied the serum levels of birch-pollen-specific IgE, IgG and IgG4. Two control groups were included, namely a group of allergic patients without IT treatment and a group of non-atopic individuals. We found no significant differences regarding cytokine synthesis between the allergic patients, with or without IT treatment. However, induction of peripheral T-cell tolerance was indicated after IT, together with increased serum levels of IgG and IgG4.

2. Materials and methods

2.1. Study design

This study included 15 patients with a history of birch-pollen allergy with rhinitis, conjunctivitis, and occasional broncho-obstructive symptoms. They were all IgE-positive for birch-pollen allergens measured by skin prick testing and/or Pharmacia CAP System. Besides sensitized to birch pollen, most patients were also sensitized to grass and/or weed pollen (80%). Other sensitizations were to animal dander (60%), and in a few cases to house dust mite or fungi (20%). Seven patients, four men and three women, aged 26–51 years (median age 32 years) received conventional IT treatment (IT-treated group). Eight patients, four men and four women, aged 26–42 years (median age 33.5 years), had never received IT and served as controls (allergic control group). The sensitization patterns of the IT-treated group and the allergic control group were similar. Ten non-atopic persons, one man and nine women, aged 24–48 (median age 38.5 years) were also included as healthy controls (non-atopic group). They lacked symptoms of allergy and were negative in Phadiatop-test and for specific IgE to birch pollen.

The IT was performed by weekly subcutaneous injections with increasing doses of Alutard SQ Birch (ALK Abello, Hørsholm, Denmark) until reaching the maintenance dose of 100 000 SQ units, which was then administered every 6 weeks for 3 years. The treatment was well tolerated. The patients were allowed to use anti-histamines, inhaled and nasal corticosteroids, sodium chromoglycate and β_2 -agonists when needed. None of the patients were using oral or parenteral corticosteroids. Before each injection the patients were questioned about side effects of the previous injection and about their general health. Peak Expiratory Flow (PEF) measurements were performed before and after each injection. Blood samples were collected from all subjects during the peak of the second birch-pollen season after start of IT (1998, a relatively strong season), when the cumulative birch-pollen counts exceeded 2000 grains/m³. The IT-treated group had at that point of time received IT for 18 months. The fresh PBMC

were cultured as described below and serum samples were frozen at -20°C until use.

2.2. Clinical evaluation

The patients were retrospectively asked about their symptoms and medication during the birch-pollen seasons before and after start of IT. The questions were standardized and handled by the research nurse. For much worse symptoms, the improvement score was set to one (1), worse symptoms (2), no change (3), less symptoms (4), much less symptoms (5), and no symptoms (6). A similar system for scoring changed consumption of rescue medication was used. For much more medication the improvement score was set to one (1), more medication (2), no change (3), less medication (4), much less medication (5), and no use of rescue medication (6).

2.3. Antibody measurements

Specific serum IgE, IgG and IgG4 to birch pollen were measured with Pharmacia CAP System (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Specific IgE to aeroallergens as a test for atopy was performed with Phadiatop (Pharmacia and Upjohn Diagnostics).

2.4. Skin prick testing

For skin prick testing, extracts of birch pollen, timothy pollen, mugwort pollen, cat dander, dog dander, horse dander, *Dermatophagoides pteronyssinus*, *Alternaria* and *Cladosporium* were used (Soluprick SQ, ALK Abello). Positive (histamine 10 mg/ml) and negative (buffer) controls were included. A reaction was considered positive if the mean diameter of the wheal was at least 3 mm.

2.5. Cell cultures

The cultures for cytokine production and proliferation measurements were performed as described elsewhere [7]. Briefly, PBMC were stimulated at $1 \times 10^6/\text{ml}$ with birch-pollen extract (BPE) or tetanus toxoid (TT) for 7 days, harvested and washed, and restimulated at $5 \times 10^5/\text{ml}$ for 24 h with the mitogens PHA and PMA. This restimulation step means an improved control of culture conditions during the cytokine production. It predominantly stimulates cytokine synthesis by the antigen-activated T-cell blasts that are enriched during the antigen-specific prestimulation step (unpublished observation). The supernatants were centrifuged and frozen until cytokine analysis. The proliferative response was measured in separate cultures ($2 \times 10^6/\text{ml}$) stimulated with the antigens for 6 days. The last 4 h of

culture, 1 μCi ^3H -labeled thymidine was added, and the rate of incorporated nucleotide was determined. The stimulation index (SI) was calculated as counts per min (cpm) in antigen-stimulated cultures divided with cpm in non-stimulated cultures.

2.6. Cytokine assays

Concentrations of IL-5, IL-10 and IFN- γ in the cell-culture supernatants were measured by using Quantikine ELISA (R&D Systems, Minneapolis, MN, USA).

2.7. Statistical analysis

The Mann–Whitney *U*-test (two-tailed *P* value) was used to identify significant differences between the groups, and the Wilcoxon signed ranks test to identify differences between BPE and TT stimulation within the groups. Cytokine ratios were log-transformed prior to statistical analysis. Spearman rank correlation test was used to evaluate correlation between immunological parameters. Only *P* values less than 0.05 were considered significant.

Table 1
Serum levels (mean $\pm$ SD) of birch-pollen-specific antibodies

	IgE (kU/l)	IgG (mg/l)	IgG4 (mg/l)
IT-treated group	24.4 $\pm$ 22.3*	19.4 $\pm$ 11.7**,***	9.2 $\pm$ 7.0****
Allergic control group	27.8 $\pm$ 32.8*	5.2 $\pm$ 1.4	0.4 $\pm$ 0.2
Non-atopic group	<0.35 $\pm$ 0	3.5 $\pm$ 2.3	0.2 $\pm$ 0.3

* *P* < 0.0001 compared to the non-atopic group.

** *P* < 0.01 compared to the allergic control group.

*** *P* < 0.001 compared to the non-atopic group.

**** *P* < 0.001 compared to the allergic control group and to the non-atopic group.

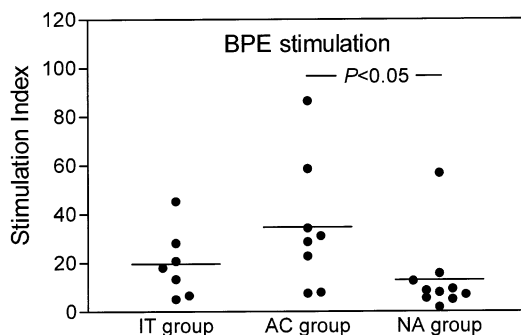


Fig. 1. Stimulation index (SI) of BPE-stimulated PBMC isolated during pollen season from IT-treated patients (IT group), allergic control patients (AC group), and healthy non-atopic individuals (NA group). The incorporation of ^3H -labeled thymidine was determined after 6 days of culture. The individual and mean SI:es are shown.

The study was approved by the local Ethics Committee of the Medical Faculty of Uppsala University (Sweden) and the patients gave their informed consent.

3. Results

3.1. Clinical efficacy

The IT was successful. All patients reported clinical improvements during the birch-pollen seasons following start of IT. Regarding allergic symptoms, the improvement scores for the second and third year were 4.2 ± 0.5 (mean $\pm$ SD) and 5.0 ± 0.8 , respectively. The corresponding improvement scores for medication were 4.6 ± 1.1 and 5.2 ± 0.7 .

3.2. Serum antibodies

There was no difference in serum concentrations of birch-specific IgE between the IT-treated group and the allergic control group during the birch-pollen season (Table 1). However, in the IT-treated group, the IgG levels were significantly higher than in the allergic and the non-atopic control groups. The mean IgG4 concentration in the IT-treated group was much higher compared to the allergic control group ($26 \times$) and the non-atopic group ($38 \times$).

3.3. Antigen-specific proliferation

The birch-pollen-specific proliferative response, measured during the pollen season, was significantly higher in the allergic control group compared to the non-atopic group (Fig. 1). The mean proliferative response to BPE in the IT-treated group was in-between the responses of the two control groups, but not significantly different from either of them. The TT-specific proliferative response was similar in all three groups (data not shown).

3.4. Cytokine production

The BPE-specific IL-5 production was significantly increased in both the IT-treated group and the allergic control group compared to the non-atopic group during the birch-pollen season (Fig. 2A). In the allergic control group, prestimulation with BPE induced significantly higher IL-5 production than with TT. There were no significant differences in IFN- γ production between the three groups or the two antigens studied (Fig. 2B). However, a clear trend towards decreased IFN- γ production was found in the allergic control group after prestimulation with BPE compared to TT (*P* = 0.055). When calculating the IL-5/IFN- γ ratios, both the IT-treated group and the allergic control

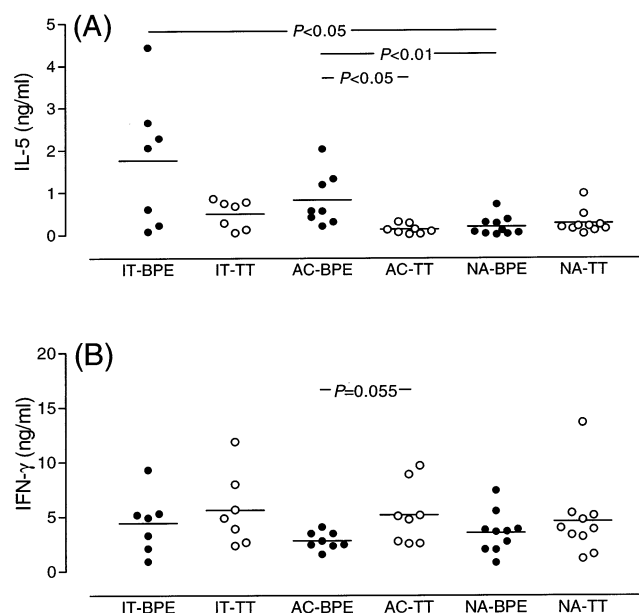


Fig. 2. Cytokine production by PBMC isolated during pollen season from IT-treated patients (IT), allergic control patients (AC) and healthy non-atopic individuals (NA). The cells were stimulated with birch-pollen extract (BPE) or tetanus toxoid (TT) for 7 days and restimulated for 24 h with PHA and PMA. The secreted cytokines were determined by ELISA. The individual and mean levels of IL-5 (A), and IFN- γ (B) are shown.

Table 2

Logarithmic IL-5/IFN- γ ratios (mean $\pm$ SD) in PBMC cultures stimulated with either BPE or TT for 7 days and restimulated with PHA/PMA for 24 h

	BPE	TT	P value
IT-treated group	$-0.59 \pm 0.60^*$	-1.15 ± 0.44	0.0156
Allergic control group	$-0.63 \pm 0.32^{**}$	-1.71 ± 0.35	0.0078
Non-atopic group	-1.54 ± 0.55	-1.30 ± 0.47	0.2754

* $P < 0.01$ compared to the non-atopic group.

** $P < 0.001$ compared to the non-atopic group.

group showed a more Th2-like cytokine profile to birch-pollen allergens in contrast to the bacterial antigen TT (Table 2). This Th2-like polarization of the T-cell response was, however, not found in the non-atopic group.

The IL-5/IFN- γ ratios in the BPE-prestimulated PBMC cultures from the allergic patients correlated with the serum concentrations of birch-pollen-specific IgE ($r = 0.60$, $P < 0.05$). In the IT-treated group, the IL-5/IFN- γ ratios not only correlated with specific IgE ($r = 0.86$, $P < 0.05$), but also with the birch-pollen-specific IgG and IgG4 levels ($r = 0.79$, $P < 0.05$ and $r = 0.86$, $P < 0.05$, respectively).

In this study, IL-10 was measured in the supernatants of the PBMC cultures prestimulated with BPE. The results showed no significant differences in IL-10 pro-

duction between the three study groups (Fig. 3). No correlation between the IL-10 production and the proliferative response was seen either ($r = 0.05$, $P = 0.81$).

4. Discussion

In the present investigation, we studied different immunological parameters during birch-pollen season after 18 months of successful IT of birch-pollen-allergic patients. Investigation of the proliferative response of PBMC showed that the response to birch-pollen allergens was significantly higher in the allergic control group compared to the non-atopic group. In the IT-treated group the proliferative response was hampered, suggesting that T-cell anergy or depletion of allergen-specific T-cell clones could be a significant mechanism of birch-pollen-specific IT. T-cell tolerance has also been suggested as a mechanism of IT by other groups [11–14].

It is well known that both IgE and IgG4 production is induced by the Th2-type cytokine IL-4 [15]. The large increases in specific IgG4 levels together with unaffected or only slightly decreased specific IgE levels observed in this and other IT studies [16,17] raises questions about the hypothesis of a shift from a Th2- to Th1-like phenotype of allergen-specific T cells during successful IT. However, diminished IL-5 production is probably more important than reduced IL-4 production, since IL-5 stimulates the infiltration and activation of eosinophils involved in the late-phase allergic reaction [18]. We therefore decided to measure IL-5 production by birch-pollen-specific T cells in vitro.

In contrast to the non-atopic group, the response to birch-pollen allergens in both the allergic control group and the IT-treated group was skewed towards a more Th2-like cytokine profile in comparison with the bacterial antigen TT. Consequently, no normalization of the

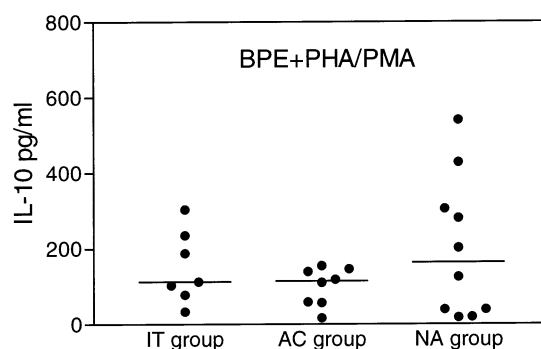


Fig. 3. IL-10 production (individual and mean levels) by PBMC isolated during pollen season from IT-treated patients (IT), allergic control patients (AC) and healthy non-atopic individuals (NA). The cells were stimulated with birch-pollen extract (BPE) for 7 days and restimulated for 24 h with PHA and PMA. The secreted IL-10 was determined by ELISA.

Th2/Th1 balance in the response to birch-pollen allergens after IT was observed. The Th2/Th1 balance (IL-5/IFN- γ ratio) in the response to birch-pollen allergens in vitro correlated with the serum concentrations of birch-pollen-specific IgE in the allergic patients. In the IT-treated group, the Th2/Th1 balance also correlated with birch-pollen-specific IgG and IgG4, indicating the role of Th2-type responses in humoral immunity.

The role of IL-10 in the immunological mechanism behind IT has been in focus since Akdis et al. [4] reported that IL-10 increases during the first weeks of bee-venom rush IT. In their system, IL-10 inhibited T-cell proliferation as well as the synthesis of both Th1 and Th2-type cytokines in vitro. We found no altered IL-10 production after conventional birch-pollen IT, and it was not correlated with the proliferative response. The discrepancy between the studies might be explained by the fact that different forms of allergic diseases were studied, i.e. bee-venom allergy versus birch-pollen allergy. It could also depend on the different time-phases of IT that were studied.

Rush or ultra-rush insect-venom IT seems to have a strong impact on the cytokine profile. The cytokine balance shifts early during treatment from a Th2 to a Th1-like profile [4,12,19]. During the early phase of conventional IT for treatment of allergy in the airways, we and others have shown an increased Th2-like phenotype of PBMC in vitro [8,20]. Later during IT, a shift towards a more Th1- or Th0-like cytokine response has been reported [21,22]. However, Till et al. [23] found no decrease in the IL-5 production by peripheral T cells from grass-pollen-allergic patients treated with conventional IT for as long as 6–7 years. Similar to the latter study, we could not observe a weakened Th2-like cytokine profile of BPE-stimulated PBMC from allergic patients treated with birch-pollen-specific IT for 18 months, arguing against a shift in the cytokine profile of peripheral allergen-specific T cells as the major mechanism of birch-pollen IT.

In vitro studies have shown that the allergen exposure during the pollen season influences the cytokine production by PBMC from allergic patients [24,25]. In the present investigation, the PBMC were collected during the peak of the relatively strong birch-pollen season. It is possible that newly developed naive T cells are involved in the response to the birch-pollen allergens during pollen season. The major effect of IT might take place in the pool of memory cells, and to be able to study these, the PBMC should be collected outside the pollen season. Furthermore, increased numbers of IFN- γ producing cells have been observed in the skin of grass-pollen IT-treated patients after intradermal allergen challenge [26]. It is quite plausible that the major immunological effect of IT is to be found at the site of allergic inflammation rather than in the pool of circulating T cells [10]. Therefore, it would be of great

interest to study the cytokine profile of T cells in the airways, e.g. in bronchoalveolar lavage, after successful IT.

In summary, our study shows increased serum levels of birch-pollen-specific IgG and IgG4, and an impaired proliferative response by PBMC after 18 months of successful birch-pollen-specific IT, indicating that T-cell tolerance is one of the immunological mechanisms. The IL-5 and IFN- γ production in the IT-treated group did not significantly differ from the non-treated allergic patients, neither did the IL-10 production. Shifts in the cytokine profile of memory T cells might have been masked by the relatively strong pollen season. However, immunological changes after birch-pollen-specific IT are probably confined mostly to the airways, i.e. the major site of inflammation in birch-pollen allergy.

Acknowledgements

We thank Ulrike Spets-Nyström for excellent technical assistance.

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