

Allergen Induced mRNA Expression of Interleukin-5, but Not of Interleukin-4 and Interferon- γ , in Peripheral Blood Mononuclear Cells Obtained Before the Pollen Season Predicts the Clinical Efficacy of Immunotherapy for Seasonal Allergic Rhinitis

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The primary aim of this study was to investigate whether the allergen-induced synthesis of cytokines by peripheral blood mononuclear cells (PBMCs) obtained immediately before the pollen season could predict the clinical efficacy of immunotherapy during the following pollen season. PBMCs (1.0×10^6 cells/ml) were obtained from 17 nonatopic subjects and from 60 patients receiving immunotherapy for seasonal allergic rhinitis (caused by Japanese cedar pollens) immediately before the pollen season of 1998, and were cultured for 24 h in the presence of 10 mg/ml of Cry j 1, a major allergen of Japanese cedar pollens, at 37 °C in a fully humidified 5% CO₂ atmosphere. Total cellular RNA was extracted from the PBMCs, and the allergen-induced interleukin (IL)-4, IL-5 and interferon- γ (IFN- γ) mRNA expression was determined using a reverse transcription–polymerase chain reaction. According to the nasal symptoms during the pollen season of 1998, the 60 patients on immunotherapy were divided into 36 good responders (who had no nasal symptoms and no requirement for rescue medications) and 24 poor responders who needed rescue medications to control nasal symptoms. Neither IL-4 mRNA nor IL-5 mRNA was expressed in any of the 17 nonatopic individuals. By contrast, IL-4 mRNA was expressed in 26 good responders and in 22 poor responders, and IL-5 mRNA was expressed in eight good responders (22.2%) and in 23 poor responders (95.8%). IFN- γ mRNA was expressed in four nonatopic subjects, in nine good responders and in seven poor responders. The expression of IFN- γ did not differ significantly among the nonatopic subjects, the good responders and the poor responders. The mRNA expression of IL-5 ($P < 0.0001$), but not of IL-4 ($P = 0.0999$) and IFN- γ ($P = 0.7713$), differed significantly between the good and poor responders. Therefore, our study has highlighted that positive expression of IL-5 mRNA in PBMCs sampled immediately before the pollen season could be predictive of a poor clinical outcome of immunotherapy during the following pollen season and that the down-regulation of IL-5 mRNA expression in PBMCs could be an important mechanism of pollen immunotherapy related to the clinical efficacy.

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INTRODUCTION

Most recent studies have clearly demonstrated that immunotherapy for seasonal allergic rhinitis caused by pollens is clinically effective [1, 2], and that immunotherapy with pollen

extracts significantly improves symptoms and decreases the need for antiallergic medications such as antihistamine tablets. In Japan, Japanese cedar (*Cryptomeria japonica*) is the most important producer of pollen allergens responsible for seasonal allergic rhinitis. It pollinates heavily during early spring, from

February to April. For several decades, immunotherapy has been used in the treatment of seasonal allergic rhinitis caused by the pollen. Indeed, several studies have demonstrated clinical efficacy of immunotherapy for patients with this pollen induced seasonal allergic rhinitis. Some patients receiving the pollen immunotherapy have no seasonal aggravation during the pollen season and require no additional antiallergic medications, whereas others experience seasonal aggravation or need rescue medication during the pollen season [3, 4].

To identify, before the start of pollen season, those who need antiallergic medication to control seasonal aggravation and those who have no seasonal aggravation, would improve the quality of life of patients receiving immunotherapy and would also have a significant impact on the cost of health care. However, the objective criterion to predict the need for rescue medications during the pollen season has not currently been established.

CD4⁺ T-helper (Th) cells have been categorized into at least two distinct subsets of Th1 and Th2, based on their profiles of cytokine secretion [5]. Cytokines such as interleukin (IL)-4 and IL-5 derived from Th2 cells are involved in immunoglobulin E (IgE) synthesis and allergic inflammation, whereas Th1-type cytokines, such as interferon- γ (IFN- γ), inhibit IgE synthesis and the development of Th2 clones. A current hypothesis is that imbalances between the cytokines produced by Th1 and Th2 cells appear to be responsible for the development of allergic conditions. Recent studies have suggested that IL-5, in particular, is implicated in the allergic inflammatory events and airway hyper-responsiveness, and might be a determinant cytokine linked to clinical manifestation of allergic rhinitis and asthma [6–8]. In our previous study, peripheral blood mononuclear cells (PBMCs) were obtained during the pollen season, and PBMCs from symptomatic individuals synthesized higher levels of IL-5 under allergen stimulation than those from asymptomatic individuals sensitized to the pollen [8]. Therefore, synthesis of the pollen allergen-induced IL-5 during the pollen season is probably indicative of seasonal aggravation in seasonal allergic rhinitis. In our previous study, however, PBMCs were obtained during the pollen season. It remains unclear whether the pollen allergen-induced synthesis of IL-5 by PBMCs obtained immediately before the pollen season is linked to the nasal symptoms that occur during the pollen season. The present study was designed to investigate whether the pollen allergen-induced synthesis of cytokines by PBMCs obtained immediately before the pollen season could predict the possible seasonal aggravation of nasal symptoms and subsequent need for rescue medications during the following pollen season. In addition, our study was also aimed at elucidating the underlying working mechanism of clinically successful immunotherapy for patients with seasonal allergic rhinitis caused by the pollen.

MATERIALS AND METHODS

Subjects. The study design followed the principles outlined in the Declaration of the Helsinki [9]. This study included 17 nonatopic healthy volunteers (six females and 11 males, age range: 18–45 years, nonatopic

group) and 97 adult patients with seasonal allergic rhinitis caused by Japanese cedar pollens (rhinitis group: 49 females and 48 males, age range: 16–48 years) who gave informed consent for participation. Seventeen subjects in the nonatopic group were chosen on the basis of the following criteria:

- (1) no history of allergic diseases;
- (2) no physical findings indicative of allergic diseases; and
- (3) negative serum IgE antibodies specific to the major allergens in Japan, such as house dust mites, Japanese cedar pollen, ragweed, Japanese cypress pollen and moulds.

The 97 patients in the rhinitis group were subdivided further into the following two groups: an untreated group and an immunotherapy group. The untreated group included 37 patients who fulfilled the following criteria: a well-documented history of typical seasonal allergic rhinitis caused by Japanese cedar pollens; a positive skin test result to Japanese cedar pollens, according to Okuda's criteria [10]; and a positive nasal provocation test to pollens, according to Okuda's criteria [10]. The immunotherapy group included 60 patients receiving immunotherapy who fulfilled the following criteria: a well-documented history of typical seasonal allergic rhinitis caused by Japanese cedar pollens before immunotherapy; a positive skin test result to Japanese cedar pollens before immunotherapy; a positive nasal provocation test to pollens before immunotherapy; and immunotherapy with the pollen extracts for at least 9 months. The levels of specific and total IgE in serum before the commencement of immunotherapy were 28.3 ± 26.6 U/ml (range: 0.8–100.0 U/ml) and 254.7 ± 226.7 IU/ml (range: 25.6–1178.0 IU/ml), respectively.

Study design. Pollination of Japanese cedars in 1998 occurred from the second week of February to the second week of April. Peripheral blood was collected from each subject prior to the cedar pollen season (during January in 1998). Serum concentrations of the pollen-specific IgE were determined by using the immunoCAP. PBMCs were isolated from heparinized blood samples by Ficoll density-gradient centrifugation. PBMCs were washed twice in phosphate-buffered saline (PBS) and resuspended in RPMI-1640 (Flow Laboratories, Meckenheim, Germany), containing 10% inactivated fetal calf serum (FCS; JRH Biosciences, Lenexa, KA., USA), 100 mg/ml of streptomycin (Sigma, St Louis, MO, USA), 100 IU/ml of penicillin and 2 mM L-glutamine. PBMCs from each subject were cultured and stimulated with 10 mg/ml of Cry j 1, a major allergen of Japanese cedar pollens, for 24 h at a final concentration of 1.0×10^6 cells/ml in 12-well plates (Iwaki glass, Tokyo, Japan) at 37 °C in a fully humidified 5% CO₂ atmosphere. Cry j 1 protein was isolated from Japanese cedar pollens by affinity chromatography using a monoclonal antibody (MoAb) specific for Cry j 1. The justification of this method for the preparation of Cry j 1 and the quality and potency of Cry j 1 used in this study have been described previously [11, 12].

Total cellular RNA was extracted from the PBMCs using the ISOGEN (Nippon Gene, Tokyo, Japan) method, and total RNA was dissolved in 20 ml of RNase-free water. One milligram of RNA, 1 ml of 50 pmol/ml random primer (hexadeoxyribonucleotide mixture; Takara Biochemicals, Tokyo, Japan) and H₂O were mixed in a total volume of 11 ml. Annealing reactions were performed at 65 °C for 10 min, and then chilled on ice for 5 min. Four millilitres of 5 $\times$ first-strand buffer (Gibco BRL, Rockville, MD, USA), 2 ml of 10 mM dNTPs, 2 ml of 0.1 M dithiothreitol (DTT; Gibco BRL), 0.5 ml of 200 U/ml M-MLV reverse transcriptase (Gibco BRL) and 0.5 ml of RNase inhibitor were added to the tube. Reverse transcription (RT) was performed at 37 °C for 60 min, and incubated at 99 °C for 10 min. Then, 30 ml of H₂O were added to the RT products.

Five millilitres of each of the RT products was amplified by using the polymerase chain reaction (PCR). In this study, oligonucleotide primers for the three cytokines and an initial marker were designed to amplify the cDNA fragments. For IL-4, the sense primer was 5'-CGGCAACTTTGACCACGGACACAAGTGCGATA-3' (117–148), and the antisense primer was 5'-ACGTACTCTGGTTGGCTTCCTTCACAGGACAG-3' (429–460). For IL-5, the sense primer was 5'-GCTTCTGCATTTGAGTTT GCTAGCT-3' (9–33), and the antisense primer was 5'-TGGCCGTCATGTA TTTCTTTATTAAG-3' (276–302). For IFN- γ , the sense primer was 5'-ATGA AATATACAAGTTA TATCTTGGCT TT-3' (127–155), and the antisense primer was 5'-GATGCTCTTCGACCTCGAAACAG CAT-3' (595–620). For β -actin, the sense primer was 5'-AAGAGAGGCATCCTCACCC T-3' (222–241), and the antisense primer was 5'-TACATGGCTGGGGTGTGAA-3' (420–439). cDNA was amplified in a 20-ml reaction mixture containing 0.125 ml of 5 U/ml of Ex *Taq* DNA polymerase (Takara Biochemicals, Tokyo, Japan), 2.5 ml of 10 $\times$ Ex *Taq* Buffer (Takara Biochemicals), 2 ml of 2.5 mM dNTPs, 1.25 ml of 10 mM each primer, and H₂O. For IL-4, the PCR profile consisted of 40 cycles of 0.5 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C; for IL-5 and β -actin, 33 cycles of 0.5 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C, for IFN- γ , 33 cycles of 0.5 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C, respectively. Ten millilitres of each of the PCR products were run on a 1.5% agarose gel and visualized with ethidium bromide staining. IL-4, IL-5, IFN- γ and β -actin cDNA fragments were detected at 344 bp, 294 bp, 494 bp and 218 bp, respectively. The results were divided into negative or positive expression of cytokine mRNA (Fig. 1).

In the information recorded of nasal symptoms during the pollen season of 1998, all of the untreated group had symptoms typical of seasonal allergic rhinitis and needed antiallergic medications to control nasal symptoms. Twenty-four patients in the immunotherapy group experienced nasal symptoms and/or needed rescue medications to control nasal symptoms (poor responders). The remaining 36 patients in the immunotherapy group had no nasal symptoms during the pollen season and no requirement for any rescue medications (good responders).

Fisher's exact probability test and the Mann–Whitney *U*-test were used for statistical analysis, and a significant difference was accepted in either analysis when $P < 0.05$. To exclude possible bias or prejudice, the information recorded of nasal symptoms during the pollen season of 1998 and detection of cytokine mRNA expression were performed by different doctors. In addition, no information about the background of each sample was given to the doctors who were involved in the detection of cytokine mRNA expression.

Mode of immunotherapy. The mode of immunotherapy employed in the study has been described previously [13]. The patients were given subcutaneous injections of Japanese cedar pollen extracts (Torii Co, Ltd, Tokyo, Japan), which is only commercially available in Japan at present. The aqueous extracts were prepared from native pollens. Immunotherapy was started exclusively outside the pollen season (between May and July). Individual initial doses were determined by threshold skin tests and the titres of specific IgE in serum. Doses were increased by 50–100% at weekly intervals until the highest tolerated dose or 0.1 ml of 1 : 100 wt/vol was reached. The maximum tolerated dose was 0.1 ml of 1 : 100 wt/vol in 57 out of the 60 patients, and 0.1 ml of 1 : 1000 wt/vol in the remaining three patients. The maximum tolerated dose was attained within 4–8 months, and weekly injection was continued for the first few months after attainment of the maintenance dose. The patients then received injections of this dose on a biweekly basis for the next few months, and then monthly injections. Finally, the interval between injections was gradually increased up to 3 months. After each injection, the patient was asked to remain under our supervision for a minimum of 30 min and to report any symptoms that they may have experienced.

RESULTS

Specific IgE in serum

Specific IgE was not detected in any patient of the nonatopic group. The levels of specific IgE in serum did not differ

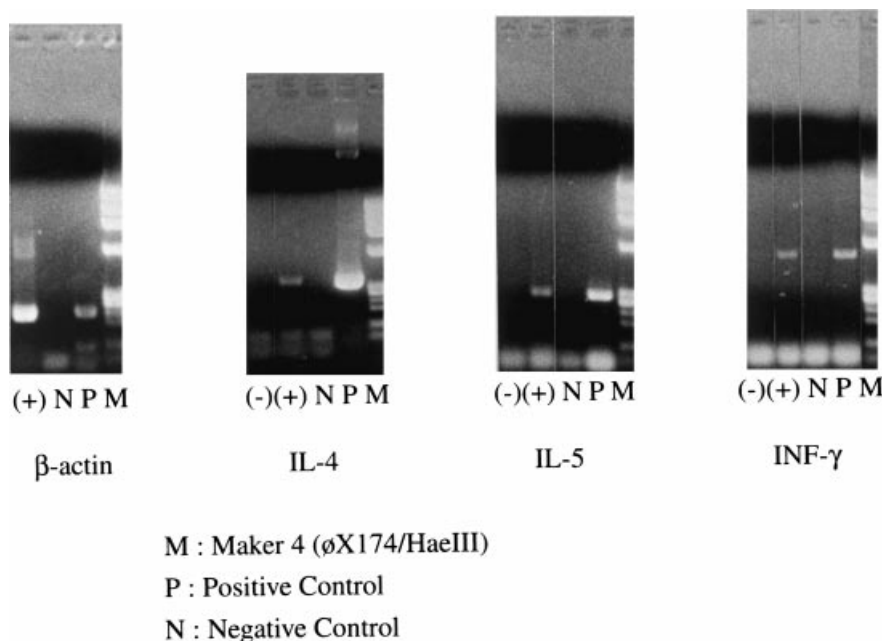


Fig. 1. mRNA analysis using reverse transcription–polymerase chain reaction (RT–PCR). The test results obtained following RT–PCR were divided into negative or positive expression of cytokine mRNA. (–), a subject with negative results; (+), a subject with positive results, M, marker 4; N, negative control; P, positive control.

significantly among the untreated group, the good responders and the poor responders (untreated group: 14.9 ± 15.8 U/ml; good responders: 17.8 ± 19.2 U/ml; poor responders: 15.6 ± 10.5 U/ml; untreated group versus good responders: $P = 0.3773$; untreated group versus poor responders: $P = 0.2433$; good responders versus poor responders: $P = 0.7801$, Fig. 2).

β -actin mRNA expression

Cry j 1-induced β -actin mRNA expression was detected in each participant.

IL-4 mRNA expression

Cry j 1-induced IL-4 mRNA expression was detected in no subjects in the nonatopic group. IL-4 mRNA was expressed in 32 of the 37 patients in the untreated group, in 26 of the 36 good responders and in 22 of the 24 poor responders (Figs 3 and 4).

IL-4 mRNA expression differed significantly between the nonatopic group and each of the rhinitis groups (untreated group, $P < 0.0001$; good responders, $P < 0.0001$; poor responders, $P < 0.0001$). However, IL-4 mRNA expression did not differ significantly among the untreated group, the good responders and the poor responders (untreated group versus good responders, $P = 0.1567$; untreated group versus poor responders $P = 0.6936$; good responders versus poor responders, $P = 0.0999$; Table 1).

IL-5 mRNA expression

Cry j 1-induced IL-5 mRNA expression was detected in no subjects in the nonatopic group. IL-5 mRNA was expressed in 34 of the 37 patients in the untreated group, in eight of the 36 good responders and in 23 of the 24 poor responders (Fig 3 and 4).

IL-5 mRNA expression differed significantly between the nonatopic group and each of the rhinitis groups (untreated group, $P < 0.0001$; good responders, $P = 0.0438$; poor responders, $P < 0.0001$). IL-5 mRNA expression did not differ significantly between the untreated group and the poor responders ($P > 0.9999$). IL-5 mRNA expression was less frequently observed in the good responders than in the untreated group ($P < 0.0001$) or in the poor responders ($P < 0.0001$, Table 1).

IFN- γ mRNA expression

Cry j 1-induced IFN- γ mRNA was expressed in four of the 17 subjects in the nonatopic group, in 10 of the 37 patients in the untreated group, in nine of the 36 good responders and in seven of the 24 poor responders, respectively (Figs 3 and 4). IFN- γ mRNA expression did not differ significantly among the nonatopic group, the untreated group, the good responders and the poor responders (Table 1).

DISCUSSION

The primary aim of this study was to investigate a possible correlation between the seasonal aggravation of nasal symptoms and the allergen-induced mRNA expression of three cytokines (IL-4, IL-5 and IFN- γ) in PBMCs obtained immediately before the pollen season from patients on immunotherapy for seasonal allergic rhinitis. Nonatopic volunteers and untreated patients with seasonal allergic rhinitis were recruited as controls.

Our study has demonstrated that there is a strong probability (96.6%) that the patients on immunotherapy whose PBMCs did not express IL-5 mRNA under allergen stimulation prior to the pollen season neither experience nasal symptoms nor need any rescue medications during the following pollen season.

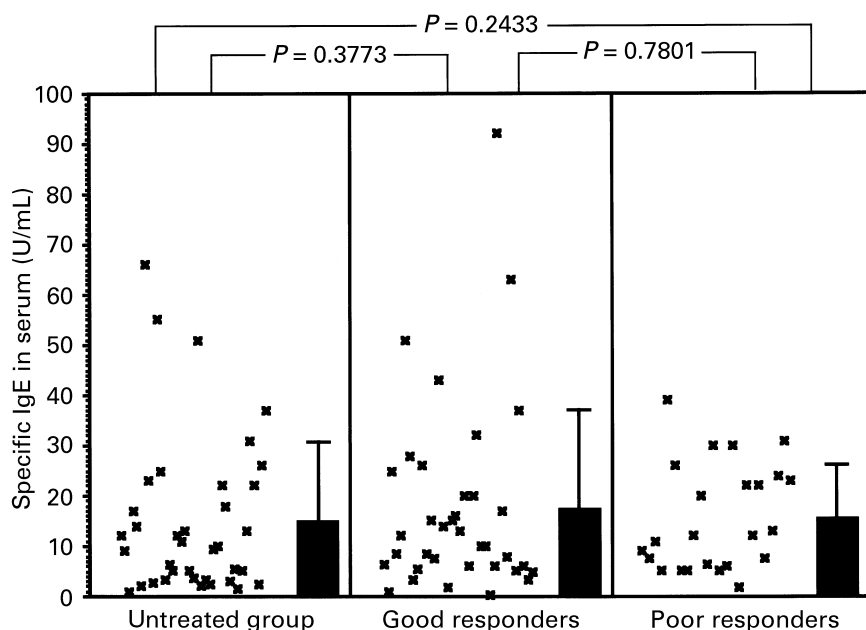


Fig. 2. Specific immunoglobulin E (IgE) level. The levels of specific IgE in serum before the pollen season were 14.9 ± 15.8 U/ml in the untreated group, 17.8 ± 19.2 U/ml in the good responders and 15.6 ± 10.5 U/ml in the poor responders, respectively. The levels of specific IgE did not differ significantly among the nonatopic group, the good responders and the poor responders.

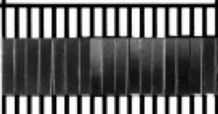
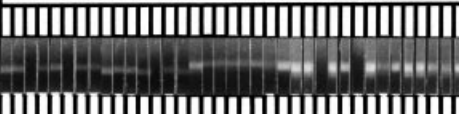
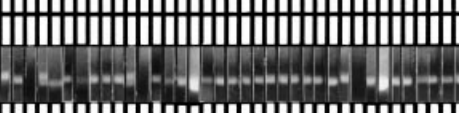
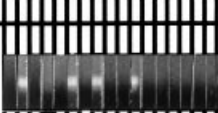
	nonatopic group (n=17)	untreated group (n=37)
IL-4		
IL-5		
IFN-γ		

Fig. 3. Cry j 1-induced mRNA expression in the nonatopic and untreated groups. Interleukin (IL)-4 mRNA expression was detected in no subjects in the nonatopic group, whereas IL-4 mRNA expression was positive in 32 of the 37 patients in the untreated group. None of the nonatopic group expressed IL-5 mRNA, whereas 34 of the 37 patients in the untreated group expressed IL-5 mRNA. Interferon- γ (IFN- γ) mRNA expression was detected in four subjects of the nonatopic group and in 10 of the 37 patients in the untreated group, respectively.

Therefore, the negative expression of allergen-induced IL-5 mRNA immediately before the pollen season could be an objective predictor of a good clinical outcome of immunotherapy during the coming pollen season. On the contrary, the allergen-induced mRNA expression of IL-5 in PBMCs prior to the pollen season probably indicates the likelihood of seasonal aggravation of nasal symptoms during the following pollen season and the requirement for preventive medications.

The working mechanism of immunotherapy has recently been discussed from the point of view of T-cell responses or cytokine production, and a variety of immunological, as well as non-immunological, mechanisms are probably involved [14]. Most studies have investigated the effect of immunotherapy on the synthesis of IL-4 and IFN- γ . Secrist *et al.* demonstrated that the production of IFN- γ by allergen-specific CD4⁺ T cells was not affected in grass pollen- and mite-allergic subjects receiving immunotherapy, whereas IL-4 production was reduced by immunotherapy and the levels of IL-4 were found to be inversely related to the length of time on immunotherapy [15]. A similar effect of immunotherapy on allergen-induced T-cell

reactivity was reported by Söderlund *et al.* [16]. They analysed allergen-induced IL-4 and IFN- γ mRNA expression in PBMCs of birch-allergic patients. The patients receiving immunotherapy expressed less IL-4 mRNA than the patients receiving placebo injections during the pollen season, whereas no significant differences in the IFN- γ mRNA expression could be observed between them [16]. In our previous serological study, both pollen- and mite immunotherapy reduced serum levels of IL-4 with treatment over several years [3, 17]. On the other hand, an important role of IFN- γ in the mechanism of immunotherapy has also been demonstrated. Varney *et al.* and Durham *et al.* reported a significant increase in IFN- γ expression, without a significant change in IL-4 expression, in experiments using *in situ* hybridization to inflammatory cells in the skin or nasal mucosa in patients who received grass pollen immunotherapy [18, 19]. Conversely, MacDonald *et al.* showed a significant reduction in the production of IFN- γ , but no significant changes in IL-4, in patients receiving ragweed immunotherapy [20]. O'Brien *et al.* stimulated PBMCs (with Der p 2) from patients sensitive to house dust mite, and demonstrated that immunotherapy resulted

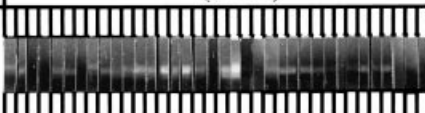
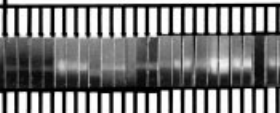
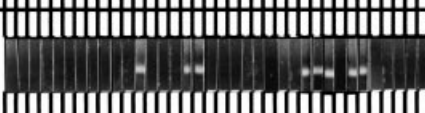
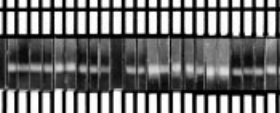
	immunotherapy group (n=60)	
	good responders (n=36)	poor responders (n=24)
IL-4		
IL-5		
IFN-γ		

Fig. 4. Cry j 1-induced mRNA expression in the good and poor responders. Interleukin (IL)-4 mRNA expression was detected in 26 of the 36 good responders and in 22 of the 24 poor responders. Eight of the 36 good responders and 23 of the 24 poor responders expressed IL-5 mRNA. Interferon- γ (IFN- γ) mRNA expression was detected in nine of the 36 good responders and in seven of the 24 poor responders, respectively.

Table 1. Comparison of cytokine mRNA expression among four groups of subjects

	Nonatopic group	Untreated group	Good responders	Poor responders
Nonatopic group		IL-4: $P < 0.0001$ IL-5: $P < 0.0001$ IFN- γ : $P > 0.9999$	IL-4: $P < 0.0001$ IL-5: $P = 0.0438$ IFN- γ : $P > 0.9999$	IL-4: $P < 0.0001$ IL-5: $P < 0.0001$ IFN- γ : $P = 0.7364$
Untreated group	IL-4: $P < 0.0001$ IL-5: $P < 0.0001$ IFN- γ : $P > 0.9999$		IL-4: $P = 0.1567$ IL-5: $P < 0.0001$ IFN- γ : $P > 0.9999$	IL-4: $P = 0.6936$ IL-5: $P > 0.9999$ IFN- γ : $P > 0.9999$
Good responders	IL-4: $P < 0.0001$ IL-5: $P = 0.0438$ IFN- γ : $P > 0.9999$	IL-4: $P = 0.1567$ IL-5: $P < 0.0001$ IFN- γ : $P > 0.9999$		IL-4: $P = 0.0999$ IL-5: $P < 0.0001$ IFN- γ : $P = 0.7713$
Poor responders	IL-4: $P < 0.0001$ IL-5: $P < 0.0001$ IFN- γ : $P = 0.7364$	IL-4: $P = 0.6936$ IL-5: $P > 0.9999$ IFN- γ : $P > 0.9999$	IL-4: $P = 0.0999$ IL-5: $P < 0.0001$ IFN- γ : $P = 0.7713$	

in a reduction in expression of both IL-4 and IFN- γ in circulating lymphocytes [21]. Thus, there is no general agreement among different investigators regarding the effect of immunotherapy on IL-4 and IFN- γ . The exact reason for these different results is unclear, but some factors, such as different types of allergies (seasonal or perennial), different length of immunotherapy, different target organs (peripheral blood or inflamed tissue) and different experimental protocols (*in vitro* or *in vivo*) among different studies will probably underlie the contradictory results.

Recent studies have suggested that IL-5 is deeply implicated in the allergic inflammatory events and airway hyper-responsiveness, and might be a determinant cytokine linked to clinical manifestation of allergic rhinitis and asthma [6–8]. However, there is limited published information on the effect of immunotherapy on IL-5 synthesis. Söderlund *et al.* investigated the effect of immunotherapy on the IL-5 mRNA expression in PBMCs of birch-allergic patients [16]. They failed to find any significant effect of immunotherapy on the IL-5 mRNA expression. We have, more recently, studied allergen-induced synthesis of IL-5 from PBMCs of patients with perennial and seasonal allergic rhinitis [22, 23]. The levels of Der f 1-induced synthesis of IL-5 were significantly correlated with the nasal symptom scores and were also inversely correlated with the duration of immunotherapy [22]. In addition, the levels of Cry j 1-induced synthesis of IL-5 by PBMCs obtained during the pollen season were significantly related to the clinical efficacy of immunotherapy for seasonal allergic rhinitis caused by Japanese cedar pollens [23]. In our present study, the allergen-induced mRNA expression of IL-5, but not of IL-4 and IFN- γ , differed significantly between the good and poor responders to immunotherapy. Our study thus implies that the suppression of allergen-induced IL-5 synthesis is an important mechanism of immunotherapy behind the clinical efficacy for seasonal allergic rhinitis.

In conclusion, our study has highlighted that the allergen-induced negative expression of IL-5 mRNA in PBMCs

sampled prior to the pollen season could be predictive of a good clinical outcome of immunotherapy during the following pollen season, and that the down-regulation of IL-5 mRNA expression in PBMCs could be an important mechanism of pollen immunotherapy related to the clinical efficacy.

Furthermore, we investigated the allergen-induced synthesis of IL-4, IL-5 and IFN- γ at the mRNA level of PBMCs obtained immediately prior to the pollen season. The results obtained by RT-PCR were qualitative, not quantitative. A quantitative measurement of these other cytokines, such as IL-12, using an enzyme-linked immunosorbent assay would be necessary to confirm the important role of IL-5 in seasonal allergic rhinitis.

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