

Original Article

Effect of modified immunotherapy with an allergen–pullulan conjugate in patients with Japanese cedar pollinosis

Ruby Pawankar,¹ Ryuta Takizawa,¹ Minoru Goto,¹ Yuichi Goto,¹ Minoru Okuda,² Shigeo Yamagishi,¹ Kimihiro Ohkubo,¹ Manabu Nonaka,¹ Hirokuni Ohtsuka³ and Toshiaki Yagi¹

¹Department of Otolaryngology, Nippon Medical School, ²Japan Asthma and Allergy Clinic, Shinbashi, Tokyo and ³Ohtsuka Clinic, Kanagawa, Japan

ABSTRACT

Immunologic mechanisms of allergen immunotherapy are still incompletely understood. Immunotherapy of a higher maximum tolerance dose with reduced risks of systemic reactions, such as anaphylaxis, needs to be developed. Seasonal allergic rhinitis (SAR) is a type I allergic disease characterized by typical seasonal symptoms of rhinitis and an increase in mast cells and T helper (Th) 2-type cells, as well as tissue eosinophilia. The present study was designed to investigate the effect of a modified immunotherapy (IT) with an allergen–pullulan conjugate (CS-560) in patients with SAR to Japanese cedar pollen (Japanese cedar pollinosis) using objective parameters, such as analyzing the alteration in the proportion of effector cells like mast cells, eosinophils and T cells, and the Th2 and Th1 cytokine profile. We also analyzed the efficacy of modified IT on the severity of symptoms, using subjective parameters, such as the symptom–medication score. Immunotherapy for 15 months duration with the modified drug CS-560 in patients with Japanese cedar pollinosis induced an immune deviation from a Th2- to a Th1-type cytokine profile and inhibited the in-season

increase in the proportion of intraepithelial mast cells and eosinophils. These changes were associated with a reduction in the symptom–medication score. These data suggest that this modified IT with an allergen–pullulan conjugate (CS-560) is an effective mode of treatment of patients with Japanese cedar pollinosis.

Key words: eosinophil, immunotherapy, Japanese cedar pollinosis, mast cell, seasonal allergic rhinitis, Th1 cytokine, Th2 cytokine.

INTRODUCTION

Cross-linking of allergen-specific IgE bound to the high-affinity IgE receptor expressed on the surface of mast cells with multivalent antigen results in the release of inflammatory mediators, such as histamine, leukotrienes and prostaglandins etc.,^{1–4} and in the clinical expression of allergic disease. Seasonal allergic rhinitis (SAR) to Japanese cedar pollen is a prototype of a type I allergic disease that affects approximately 10–20% of the general population in Japan. It is now evident that allergic diseases are characterized by an increase in CD4⁺ T cells that secrete interleukin (IL)-4, IL-5, IL-13 (T helper (Th) 2-type cells), which not only promotes the synthesis of IgE but also contributes to the recruitment of effector cells, such as eosinophils and basophils, into the site of allergic inflammation. Several studies have demonstrated the predominance of IL-4-, IL-5- and IL-13-secreting Th2-like activated T lymphocytes and mast cells in the nasal

Correspondence: Dr R. Pawankar, Department of Otolaryngology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. Email: susan@nms.ac.jp

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mucosa of patients with atopic rhinitis and bronchial mucosa or bronchoalveolar lavage of patients with atopic asthma.⁵⁻⁹ Upregulation of Th2-type cytokines and a seasonal increase in intraepithelial mast cells in patients with SAR is also well documented.¹⁰⁻¹² Japanese cedar pollinosis (JCP) is treated mainly with antiallergic drugs started 1-2 weeks before the season and continued until the end of the season. Pharmacotherapy can efficiently suppress the symptoms of pollinosis in patients with JCP, but its effect is only symptomatic and does not alter the course of the disease.

Ever since 1911, when Noon and Freeman attempted to modify the symptoms of patients with allergic rhinitis and asthma, allergen immunotherapy (IT) has been widely used in the treatment of allergic disorders.¹³⁻¹⁸ Allergen injection IT involves the stepwise subcutaneous injection of increasing doses of specific allergens to which a patient is sensitive. Although the precise mechanisms involved in IT are not well known, IT attempts to alter the degree of the patient's sensitivity to the natural allergen (immunologic tolerance). This may result through the induction of blocking antibodies, particularly IgG4 antibodies.^{15,19} Others have demonstrated that CD8⁺ suppressor T cells are generated during IT, which can modulate the levels of allergen-specific IgE production.²⁰ In addition, a reduction in intraepithelial metachromatic cells after IT has also been reported.²¹ More recently, it is believed that IT results in an alteration of cytokine profile from a predominant Th2 response in favor of a Th1 response (interferon (IFN)- γ) resulting in a decrease in tissue eosinophilia and specific IgE.

Despite its beneficial effects, IT has the problem of rare but dangerous adverse systemic reactions, such as anaphylaxis. Novel approaches currently being explored include the use of T cell-reactive peptides, which may circumvent the risk of anaphylaxis, and the use of adjuvants, such as IL-12 or mycobacterial vaccines, to potentiate the effects of allergen in inducing immune deviation. Previously, we and others had demonstrated an increase in mast cells, T cells, eosinophils and Th2-type cells (IL-4/IL-13) in the epithelial compartment of the nasal mucosa of patients with SAR (in season). In the present study, we assessed the efficacy of a modified IT with an allergen-pullulan conjugate (CS-560) in patients with JCP by analyzing the proportion of mast cells, T cells, eosinophils and Th2/Th1-type (IL-4/IFN- γ) cells in the epithelial compartment of the nasal mucosa, serum antibodies and the clinical expression of disease.

METHODS

Allergen extract

The allergen extract (CS-560) used in the present study was made by the conjugation of pullulan (α -1,4'- α -1,6'-glucan), a neutral polysaccharide, with Cry j 1 and 2 (purified allergenic substances from Japanese cedar pollen), by the collaboration of Hayashibara Biochemical Institute (Tokyo, Japan) and Sankyo Pharmaceutical Co. (Tokyo, Japan) and is characterized by weak allergenicity, but strong antigenicity, having an immunologic profile of allergen and stable antigen titre. This extract is supplied as a solution of 200 ng CS-560 (Cry j1 : Cry j2 = 4.1) and 0.3 mg human serum albumin/mL solution.

Subjects

Ten patients with JCP (four males and six females; all Japanese) ranging in age from 19 to 45 years were selected after careful screening and definite diagnosis of SAR to Japanese cedar pollen. All patients were selected on the basis of a well-documented history of seasonal attacks of sneezing, rhinorrhea and nasal obstruction in the season of JCP (from the first week of February to the end of April), no history of asthma, clinical examination by anterior rhinoscopy and allergy tests, including *in vitro* tests for specific IgE (capsulated hydrophobic carrier polymer-radioallergosorbent (CAP-RAST) test), skin-prick tests and nasal provocation test. Any associated nasal diseases, such as sinusitis, were excluded by clinical and X-ray examination. These 10 patients were given IT with an allergen-pullulan conjugate after their informed consent had been obtained. No concomitant treatment with pharmacotherapeutic agents was allowed in patients in the IT group except in-season when the symptoms were intolerable, in which case these patients were given oral antihistamines (clemastine fumarate; Sandoz, Basel, Switzerland) as rescue medication. In order to exclude the influence of this rescue medication on the results of IT, we included a pharmacotherapy group comprising 10 patients with JCP (five males and five females; all Japanese) ranging in age from 19 to 41 years and selected by the same criteria as for the IT group. Patients in the control (pharmacotherapy) group were prescribed only oral antihistamines (clemastine fumarate or pemirolast; Tokyo Tanabe, Tokyo, Japan). None of the patients in the control (pharmacotherapy) group had previously been treated with IT or was receiving steroids. All patients with JCP were symptomatic in-season and had a CAP-RAST

score greater than grade 3. Two of 10 patients with JCP in the IT group and one of the patients in the control (pharmacotherapy) group had associated nasal allergy to house dust mite (HDM).

Study design

Immunotherapy was commenced in January 1996 for a period of 2 years. The period of this study was from December 1995 to June 1997 (until 18 months post-IT), covering two seasons of JCP (1996 and 1997). Specimens of the nasal epithelial layer (nasal scrapings) were collected by gently scraping the anterior surface of the inferior turbinates (bilaterally) with a small surgical curette (cup size 2 x 3 mm). Nasal scrapings were collected over two seasons, twice off-season (before starting IT and 13 months after starting IT) and in-season (3 and 15 months after starting IT) and finally off-season at 18 months after IT. Nasal scrapings were also collected from the control group at the same time points. Analysis of the proportion of mast cells, lymphocytes, eosinophils and IL-4- (Th2-type cytokine) and IFN- γ -expressing (Th1-type cytokine) cells in nasal scrapings (epithelial compartment) was performed by immunohistochemistry. Specimens were coded and blinded. Venous blood was collected twice from each patient, before and during the season, for the analysis of serum-specific IgE and IgG4. The clinical expression of disease was analyzed using subjective parameters, such as the symptom score and the symptom-medication score, as described later, and analyzed from the patient's record of an allergy diary. In the present study, we included a pharmacotherapy group as a control, because we had no placebo controls due to difficulty in getting informed consent.

Mode of IT

Patients were given subcutaneous injections of CS-560 with prior informed consent. Taking into consideration the end-point concentration determined by the intracutaneous titration technique using a series of 10-fold dilutions of CS-560 at concentrations of 0.002–200 ng/mL, the individual initial dose was determined. Doses were increased weekly from 0.1, 0.3 and 0.5 mL at the threshold concentration to the highest tolerated concentration (usually 0.5 mL of 200 ng/mL). The maximum tolerated dose was attained in approximately 3 months with the once a week program. Weekly injection of this dose was continued for the first few months after attainment of the

maintenance dose. Patients then received injections of this dose on a bi-weekly basis for the next 4 months and then monthly injections. After each injection, the patient was kept under supervision for a minimum of 30 min.

Monoclonal antibodies

The anti-IL-4 monoclonal antibody (mAb; DNAX, Palo Alto, CA, USA), antitryptase (Chemicon, Temecula, CA, USA), anti-CD3/anti-CD4 (Dako Patts, Palo Alto, CA, USA); anti-IFN- γ (Genzyme, Cambridge, MA, USA) and anti-EG2 (Pharmacia, Uppsala, Sweden), and mouse IgG₁/rat IgG₁ negative controls (Dako Patts) were obtained commercially.

Estimation of the levels of serum-specific IgE and IgG4 antibodies

The levels of serum IgE (CAP-RAST; Pharmacia) and IgG4 antibodies were estimated by sandwich ELISA.

Collection of specimens and experimental schedule

Specimens were collected from the IT group as shown in Fig. 1, off-season before starting IT in December 1995, at the peak of the JCP season 3 months after starting IT (first week of March 1996), off-season at 13 months after starting IT (January 1997) and at the peak of the second JCP season 15 months after IT (first week of March 1997), and then off-season 18 months after starting IT. Specimens were also collected from the control group off-season and in-season in 1996 and 1997. Nasal scrapings were obtained by gently scraping the anterior surface of the nasal inferior turbinates (bilaterally) using a small surgical curette (cup size 2 x 3 mm). The scrapings were fixed in periodate polylysine paraformaldehyde (PLP) for 15 min, washed in phosphate-buffered saline (PBS; pH 7.4) with 15% sucrose, cytospinned onto silane-coated slides and stored air-tight at -80°C until further use.

Immunohistochemistry for the analysis of mast cells, T cells and eosinophils

Analysis of the proportion of mast cells (tryptase-positive cells), T lymphocytes (CD3⁺ cells) and eosinophils (EG2-positive cells) was performed by staining with the relevant mAbs using the alkaline phosphatase antialkaline phosphatase (APAAP) method (Dako APAAP kit). Briefly, for the

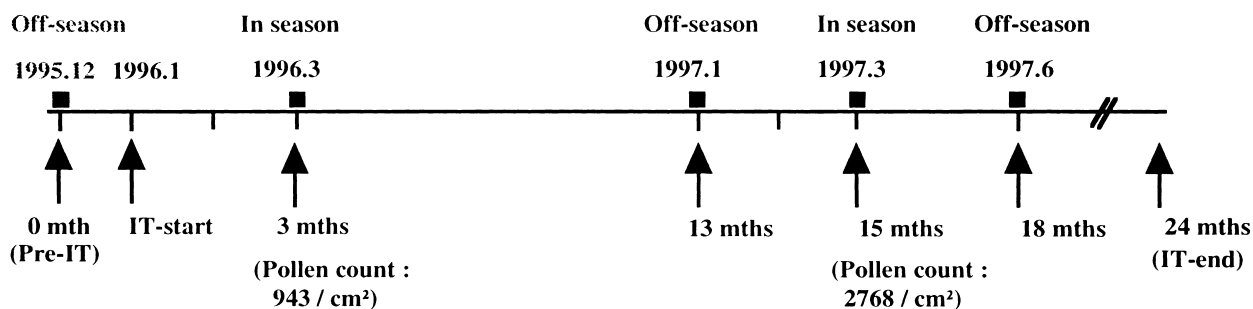


Fig. 1 Experimental schedule for the collection of specimens. Specimens of nasal scrapings were collected at the indicated time points, off-season before starting immunotherapy (IT), at the peak of the Japanese cedar pollinosis (JCP) season 3 months after starting IT, off-season at 13 months after starting IT, at the peak of the JCP season 15 months after IT and, finally, off-season 18 months after IT.

staining of mast cells, eosinophils and T lymphocytes, specimens were air-dried, rehydrated in Tris-buffered saline (TBS; pH 7.6), pretreated with 10% normal rabbit serum for 15 min and then incubated in saturating concentrations of the relevant primary mAbs for 1 h. Subsequently, specimens were incubated in rabbit anti-mouse IgG for 30 min and then with the APAAP reagent for 30 min. Between each incubation, the specimens were rinsed in TBS. The reaction was developed by incubating specimens in the substrate Naphthol AS-MX Fast-Red TR (Dako Patts, Palo Alto, CA, USA). Finally, specimens were rinsed in distilled water, counterstained with Mayer's hematoxylin and mounted in Dako gel. Negative controls were obtained by substituting the primary mAb with an isotype-matched unrelated mAb (Mouse myeloma IgG1; Dako).

Immunohistochemistry for the analysis of IL-4- and IFN- γ -positive cells

Analysis for the proportion of IL-4⁺ and CD4⁺ cells was performed by double staining with the relevant mAbs using the avidin-biotin and indirect immunofluorescence method. Briefly, specimens were air-dried, permeabilized by incubation in PBS supplemented with 25 mM HEPES (hemisodium salt) and 0.1% saponin (PBS-saponin), pretreated with 10% normal rabbit serum for 15 min and then incubated overnight at 4°C with the relevant primary mAbs (anti-IL-4/IFN- γ mAb), stained by the biotin-streptavidin horseradish peroxidase method (ABC kit; Vector Laboratories, Palo Alto, CA, USA) as described previously.^{22,23} Specimens were then treated with 10% normal goat serum, the anti-CD4 mAb and fluorescence isothiocyanate (FITC)-conjugated antimouse IgG. Between each incubation, specimens were rinsed twice in PBS-saponin.

Finally, specimens were rinsed in distilled water (DW) and mounted in Dako gel. Negative controls were obtained using isotype-matched unrelated mAbs. Results were examined with a Nikon fluorescent microscope (Tokyo, Japan).

Cell counting

The proportion of mast cells, T cells and eosinophils in nasal scrapings was assessed by counting the numbers of tryptase-positive, CD3⁺ and EG2-positive cells and total infiltrating cells in an area of 0.202 mm² ($\times 200$ h.p.f.) using an objective micrometer and then calculating the percentage of each cell type per hundred infiltrating cells. To determine the proportion of CD4⁺ IL-4-positive and CD4⁺ IFN- γ -positive cells, we counted the number of double-positive cells and CD4⁺ cells in an area of 0.202 mm² ($\times 200$ h.p.f.) using an objective micrometer and calculated the percentage of CD4⁺ IL-4-positive or CD4⁺ IFN- γ -positive cells per hundred CD4⁺ cells. An average of six fields per specimen was taken as the final value.

Assessment of clinical expression of disease

Nasal symptoms were scored on the basis of the number of sneezes, the number of nose blowings and the degree of nasal obstruction, as described previously.^{24,25} Briefly, patients were asked to maintain an allergy diary to note their daily symptoms and the grading of severity was assessed on a scale from 1 to 4 as described previously:²⁴ 1+, mild; 2+, moderate; 3+, severe; and 4+, very severe. The medication score was calculated on the basis of the kinds and combination of drugs the patient was taking (score 1, second generation antihistamines or anticholinergics/vasodilators; score 2, topical steroids;

Table 1 Background of the subjects

Group	Sex		Age (years)	slgE (IU/mL)	Skin test	
	No. males	No. females			JCP	HD
IT (<i>n</i> = 10)	4	6	28.6 ± 9.8 (19–45)	30.1 ± 16.2	1.9 ± 0.7 (1–3)	1
Controls (<i>n</i> = 10)	5	5	30.6 ± 8.6 (19–41)	33.3 ± 12.8	2.1 ± 0.7 (1–3)	1

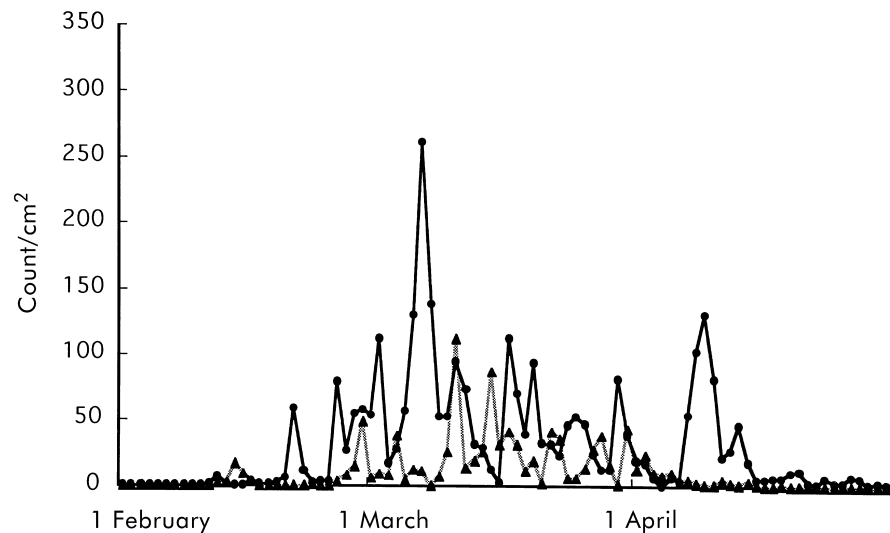
Where appropriate, data are the mean ± SD with the range given in parentheses.

IT, immunotherapy; slgE, soluble IgE; JCP, Japanese cedar pollinosis; HD, house dust.

Table 2 Classification of the symptom–medication score

Type of medication		Nasal symptoms				
1	No medication	0	1	2	3	4
2	Oral antihistamines/mast cell stabilizers	1	2	3	4	5
3	Topical steroids	2	3	4	5	6
4	Oral antihistamines/mast cell stabilizers + topical steroids	3	4	5	6	7
5	4 + others (oral steroids)	4	5	6	7	8

Fig. 2 Pollen counts for the Japanese cedar pollinosis (JCP) season in 1996 (▲) and 1997 (●). Total pollen counts in-season, in the Tokyo area from where the patients came was 943 cm² for 1996 and 2768 cm² for 1997.



and score 4, oral steroids), as described previously.²⁶ Finally, the symptom–medication score was calculated from the symptom and medication score as shown in Table 1 and as described previously.²⁶

Statistical analysis

Results are expressed either as the mean ± SD or as the median and interquartile range. For intergroup comparison, the Mann–Whitney *U*-test (STAT View, Berkeley, CA, USA) was used. For comparison of values within a group, Wilcoxon's signed-rank test (STAT View) was used. The correlation coefficient was estimated by Spearman's rank correlation analysis. Statistically significant differences were accepted when *P* < 0.05.

RESULTS

Background of the subjects

There were no significant differences in sex or age between the 10 patients in the IT group and the 10 patients in the control group (Table 2). Neither was there any statistically significant difference in the levels of serum-specific IgE or skin tests between the two groups.

Total pollen counts in 1996 and 1997

The total pollen count in-season in the Tokyo area from where the patients came was 943 cm² for 1996 and 2768/cm² for 1997 (Fig. 2).

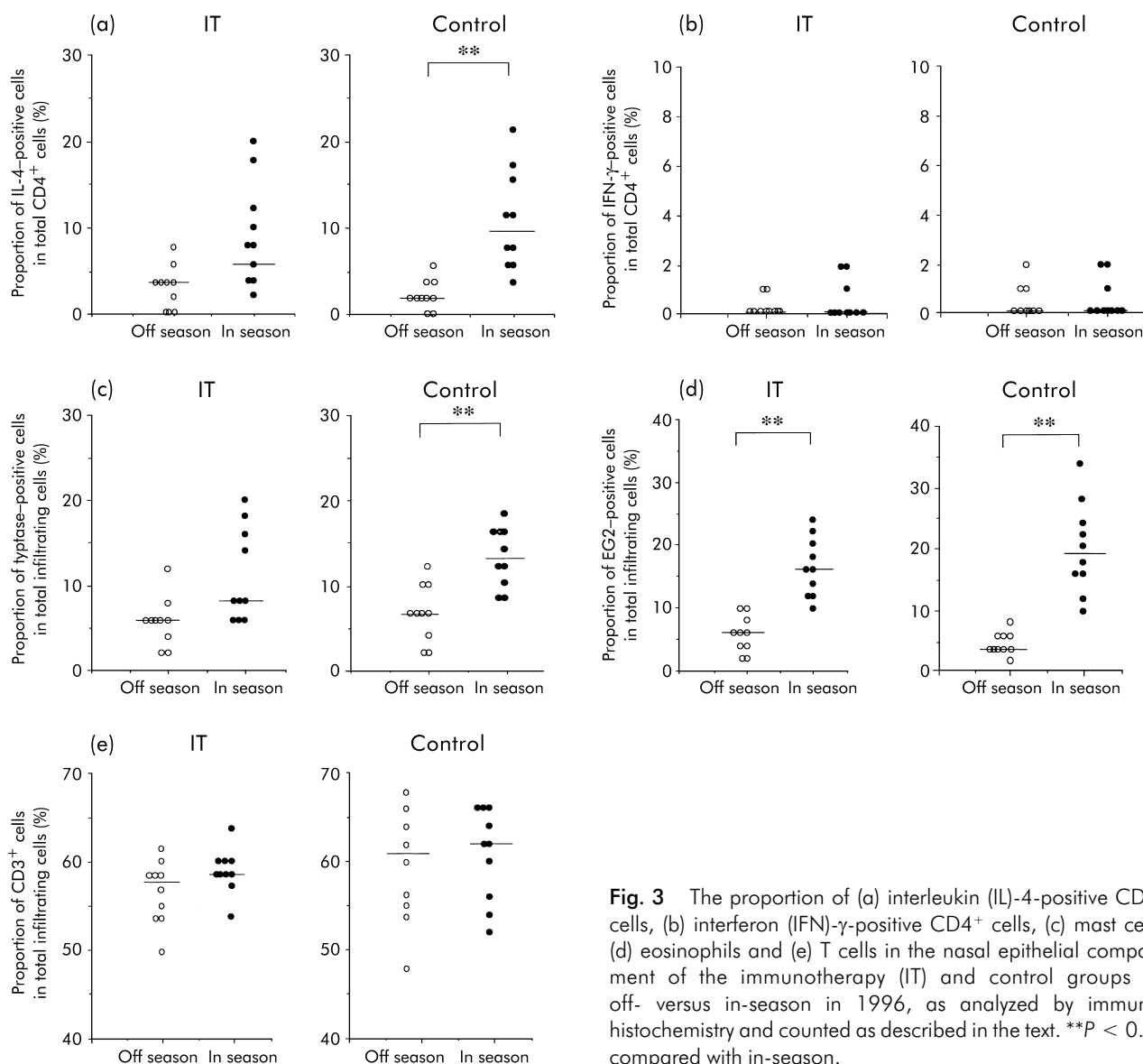


Fig. 3 The proportion of (a) interleukin (IL)-4-positive CD4⁺ cells, (b) interferon (IFN)-γ-positive CD4⁺ cells, (c) mast cells, (d) eosinophils and (e) T cells in the nasal epithelial compartment of the immunotherapy (IT) and control groups for off- versus in-season in 1996, as analyzed by immunohistochemistry and counted as described in the text. ***P* < 0.01 compared with in-season.

Comparison of IL-4-positive CD4⁺ cells (Th2), IFN-γ-positive CD4⁺ cells (Th1), mast cells, eosinophils and T lymphocytes in nasal epithelial compartment of IT and control groups in 1996 (off-season vs in-season)

The proportion of IL-4-positive CD4⁺ and tryptase-positive cells in nasal scrapings (nasal epithelial compartment) were modestly but not significantly increased in-season (3 months after starting IT) compared with levels observed during the off-season (before starting IT; Fig. 3a,c). In contrast, the proportion of IFN-γ-positive

CD4⁺ and CD3⁺ cells was not increased in-season compared with the off-season (Fig. 3b,e). The proportion of EG2-positive cells (eosinophils) in the nasal epithelial compartment was increased in-season (3 months after starting IT) compared with off-season (Fig. 3d).

In the control group, the proportion of IL-4-positive CD4⁺ cells, tryptase-positive cells (mast cells) and EG2-positive cells (eosinophils) was increased in-season compared with the off-season (Fig. 3). No significant differences were observed in the percentage of IFN-γ-positive CD4⁺ cells or CD3⁺ cells (T cells).

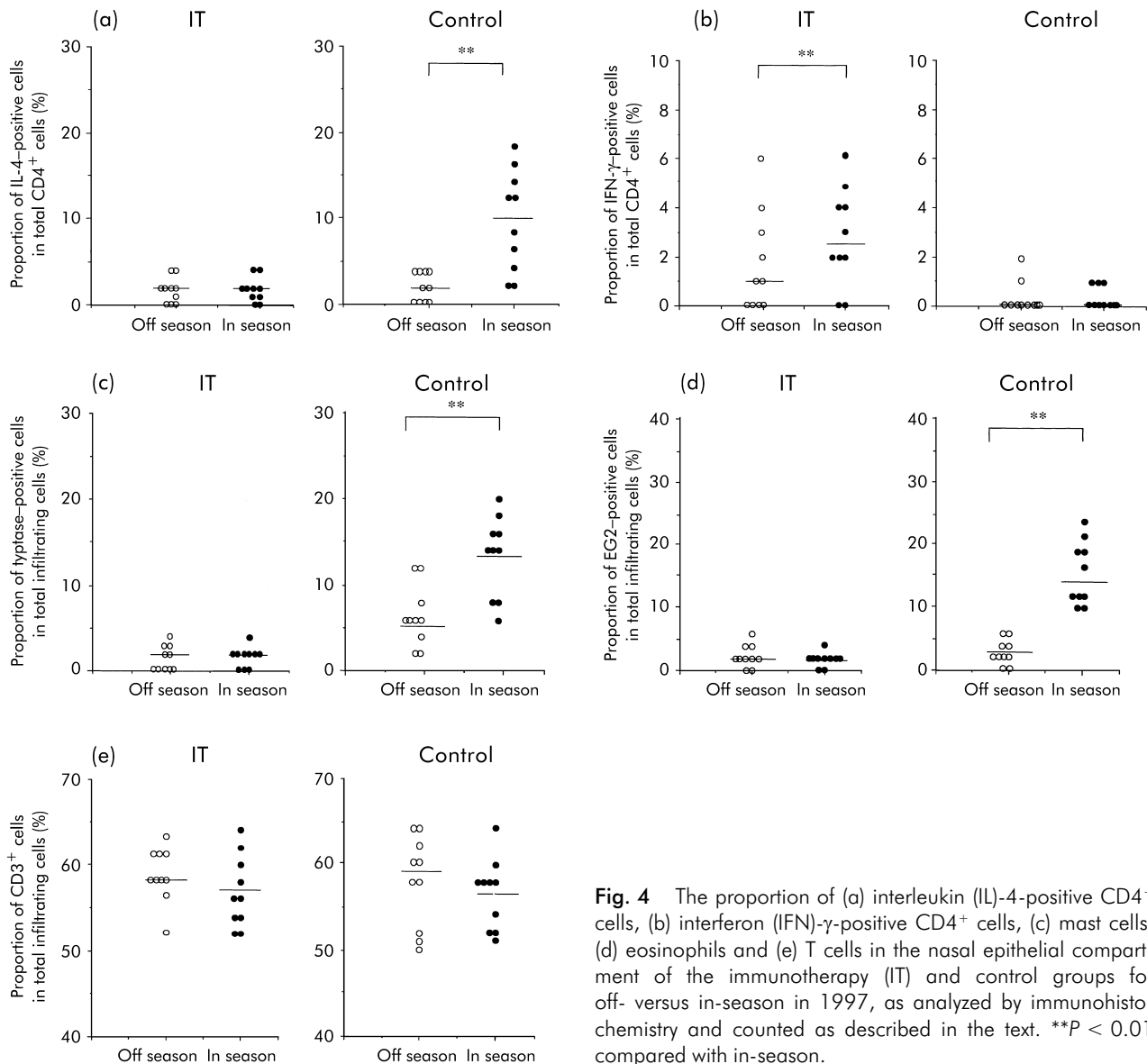


Fig. 4 The proportion of (a) interleukin (IL)-4-positive CD4⁺ cells, (b) interferon (IFN)- γ -positive CD4⁺ cells, (c) mast cells, (d) eosinophils and (e) T cells in the nasal epithelial compartment of the immunotherapy (IT) and control groups for off- versus in-season in 1997, as analyzed by immunohistochemistry and counted as described in the text. $**P < 0.01$ compared with in-season.

Comparison of IL-4-positive CD4⁺ cells (Th2), IFN- γ -positive CD4⁺ cells (Th1), mast cells, eosinophils and T lymphocytes in the nasal scrapings of the IT and control groups in 1997 (off-season vs in-season)

In-season in 1997 (15 months after starting IT), the proportion of IL-4-positive CD4⁺, tryptase-positive, EG2-positive and CD3⁺ cells was not increased in-season compared with the off-season (Fig. 4a,c,d). In contrast, the proportion of IFN- γ -positive CD4⁺ cells was increased

in-season (15 months after starting IT) compared with the off-season.

In the control group, the percentage of IL-4-positive CD4⁺, tryptase-positive and EG2-positive cells increased significantly in-season compared with the off-season (Fig. 4). No significant changes were observed in the proportion of IFN- γ -positive CD4⁺ cells or CD3⁺ cells in-season compared with the off-season.

Although no significant differences were detected in the proportion of IL-4-positive CD4⁺, tryptase-positive and EG2-positive cells between the IT and control

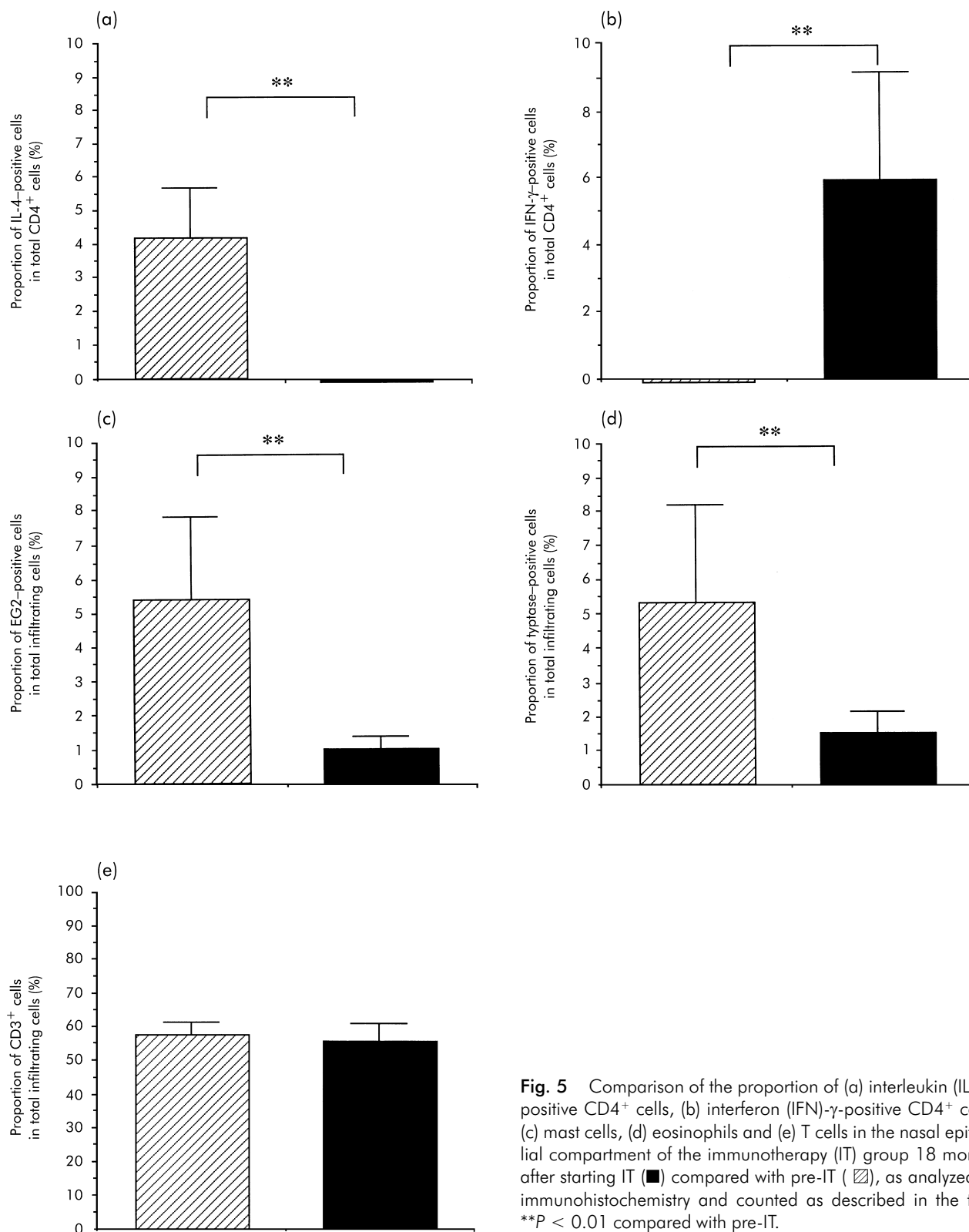


Fig. 5 Comparison of the proportion of (a) interleukin (IL)-4-positive CD4⁺ cells, (b) interferon (IFN)-γ-positive CD4⁺ cells, (c) mast cells, (d) eosinophils and (e) T cells in the nasal epithelial compartment of the immunotherapy (IT) group 18 months after starting IT (■) compared with pre-IT (▨), as analyzed by immunohistochemistry and counted as described in the text. ***P* < 0.01 compared with pre-IT.

groups at baseline, the selective increase of these cell types in the control group suggests that IT inhibited

the seasonal increase in these cell types in the IT group.

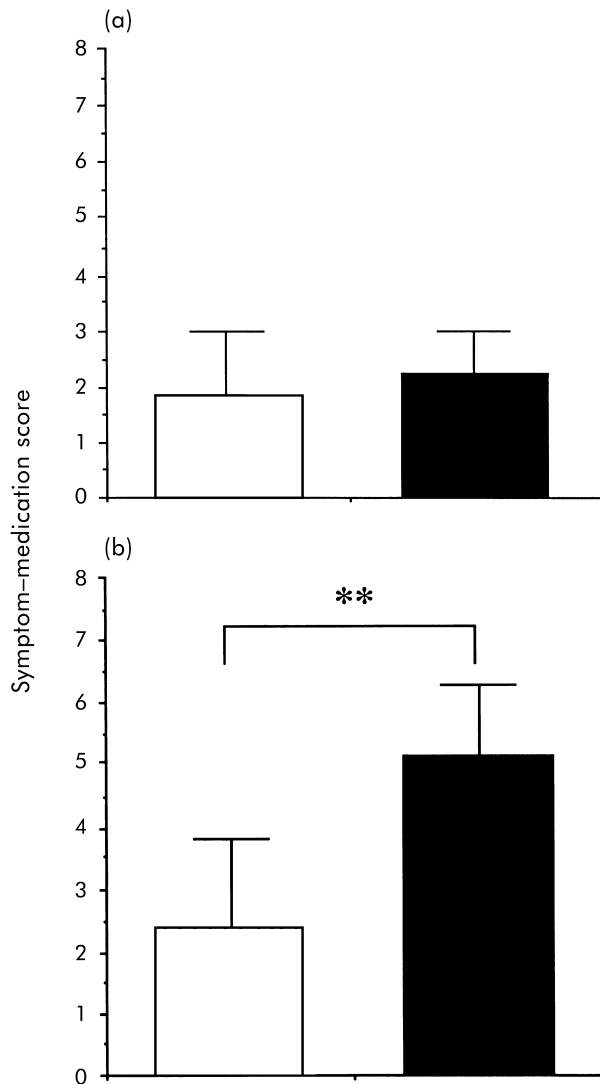


Fig. 6 Symptom-medication (S-M) score in the immunotherapy (IT; □) and control (■) groups. The S-M score was calculated as described previously.²³ (a) The S-M score in the IT group at the first season in 1996 (3 months after starting IT) was not statistically different from that of controls. (b) However, the S-M score in the IT group in the second season, in 1997 (15 months after starting IT), was significantly lower compared with that of the control group. ** $P < 0.01$ compared with the control group.

Comparison of the proportion of IL-4-positive CD4⁺ cells (Th2), IFN- γ -positive CD4⁺ cells (Th1), mast cells, eosinophils and T cells in the nasal scrapings of the IT group 18 months after IT (off-season) compared with pre-IT

The percentage of IL-4-positive CD4⁺, tryptase-positive and EG2-positive cells was significantly less in the IT

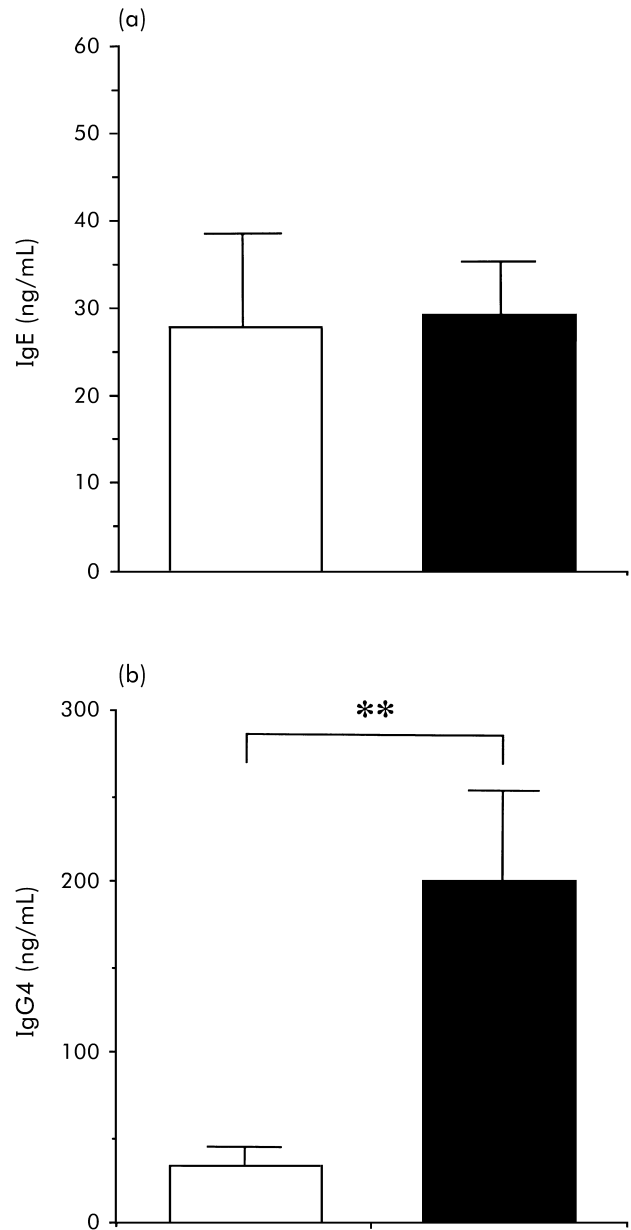


Fig. 7 Levels of serum (a) IgE and (b) IgG4 antibodies in the immunotherapy (IT) group at 18 months after IT (■) compared with pre-IT (□). The levels of serum IgE and IgG4 were analyzed by ELISA as described in the text. (a) Levels of serum IgE were not altered in the IT group 18 months after treatment compared with levels pre-IT. (b) In contrast, levels of serum IgG4 antibodies in the IT group were significantly increased 18 months after treatment compared with pre-IT. ** $P < 0.01$ compared with pre-IT.

group 18 months after IT compared with that before starting IT (pre-IT; $P < 0.01$; Fig. 5). In contrast, the proportion of IFN- γ -positive CD4⁺ cells in the IT group

increased significantly compared with pre-IT ($P < 0.01$). No significant difference was detected in the proportion of CD3⁺ cells 18 months after IT compared with pre-IT.

Clinical expression of disease in the IT and control groups

The symptom–mediation score (S-M score) in the IT group in-season in 1996, 3 months after starting IT, was not significantly different compared with that of the control group (Fig. 6a). However, the S-M score in the IT group in-season in 1997, 15 months after starting IT, was significantly lower compared with that of the control group (Fig. 6b).

Levels of serum IgE and IgG4 antibodies

The levels of serum-specific IgE in the IT and control groups were not different at baseline (Table 1). Even at 18 months after IT, no statistically significant difference was noted in the levels of serum-specific IgE compared with pre-IT (Fig. 7a). In contrast, the levels of IgG4 antibodies were significantly increased in the IT group at 18 months after starting treatment compared with levels pre-IT (Fig. 7b).

Correlation between the S-M score and immunologic parameters in the IT group

In order to assess any probable parameters that could reflect the clinical efficacy of IT, we examined the correlation between the S-M score and various immunologic parameters, such as the proportion of mast cells, eosinophils, T cells, IL-4- or IFN- γ -positive cells, serum IgE and IgG4. We only found a good correlation between the proportion of IL-4-positive CD4⁺ cells and the S-M score ($r = 0.76$, $P < 0.007$ in-season in 1996; $r = 0.6$, $P < 0.04$ in-season in 1997).

DISCUSSION

Specific allergen injection IT is highly effective in selected patients with IgE-mediated disease, including respiratory allergy and venom anaphylaxis.¹⁵ Research in this area provides insight into the immunologic basis of allergic disease. The present study was designed to evaluate whether modified IT with an allergen–pullulan conjugate (CS-560) could alter the local mucosal cytokine profile and cellular population and whether those changes

could reflect the clinical efficacy of treatment. Although in the present study we had no placebo controls due to difficulty in obtaining informed consent, we have included a pharmacotherapy group as the control group.

One way in which IT may act is by modifying the T lymphocyte response to subsequent natural allergen exposure. Studies in peripheral blood and within the target organ have demonstrated a shift in the balance of T cell subsets away from the Th2-type (producing, particularly, IL-4 and IL-5) in favor of a Th1-type T lymphocyte response (with the preferential production of IFN- γ).^{27–29} Interleukin-4 is a key cytokine that is known to play a crucial role in the development and perpetuation of the allergic reaction through the synthesis of IgE, development of Th2-type cells, upregulation of adhesion molecules, such as vascular cell adhesion molecule-1 and upregulation of Fc ϵ R1 expression in mast cells.²² Our results exhibited an inhibition of the seasonal increase in the proportion of IL-4-positive cells. In contrast, IT induced an increase in IFN- γ -positive cells. Yet, no difference was observed in the proportion of lymphocytes before and after treatment. Using sugi basic protein (SBP)-specific T cell lines and clones from BALBc mice, it was recently shown that repeated cycles of stimulation of the cell lines with the SBP–pullulan conjugate resulted in a polarization of the T cell response towards a Th1-like pattern of cytokine production, essentially augmentation of IFN- γ production and inhibition of IL-4, IL-5 and IL-10 production.²⁴ Furthermore, IL-4 production by the Th2 clones was almost completely abrogated in response to SBP–pullulan. This was not due to anergy of these cells because significant proliferation was observed in these T cell lines in response to SBP.²⁷ Thus, the allergen–pullulan conjugate can downregulate IL-4 expression (Th2-type cells) and upregulate the IFN- γ (Th1) cells. These observations can be compared with our present data that IT with the allergen–pullulan conjugate did not affect the proportion of lymphocytes, but only inhibited the seasonal increase in IL-4-positive CD4⁺ cells (Th2-type cells), thereby inducing an immune deviation from a Th2 to a Th1 profile.

It has been shown that modification in the T cell receptor (TCR) contact residue of an antigen can cause qualitative differences in the effector phase of the immune response.²⁸ In view of this, one may speculate that when pullulan binds to an amino acid residue of SBP within or near the TCR contact region, the conjugate may transmit qualitatively different signals leading to quantitative and qualitative variations in cytokine production.

It is said that IT reduces allergen-induced immediate-phase symptoms and concentrations of inflammatory mediators, including histamine and prostaglandin D₂, in the nasal fluid of ragweed-sensitive patients.²¹ Similarly, nasal epithelial mast cell numbers have been reported to be decreased after IT.²⁹ A characteristic feature of IT is its ability to inhibit late-phase responses.^{30,31} In the nose, IT is accompanied by a decrease in eosinophil numbers in lavage during late responses.³² In the present study, we found that this modified IT inhibited the seasonal increase in mast cells and eosinophils, 15 months after starting IT (compared with controls). Because the mast cell is the central cell in the immediate-phase allergic reaction and the eosinophil is a key cell in the late-phase allergic reaction, these results suggest that IT with CS-560 can probably suppress the early as well as late-phase responses. Moreover, because mast cells are an important source of Th2-type cytokines (IL-4, IL-5, IL-6, IL-13), the reduction in mast cell numbers may contribute to reduced IL-5 production and, thereby, a reduction in tissue eosinophilia. Still further, the IT-induced inhibition in the seasonal increase of IL-4-positive cells and the IT-induced upregulation of IFN- γ -positive cells can also contribute to downregulating mast cell mediator release, because IL-4 upregulates Fc ϵ RI expression and mediator release from mast cells/basophils,^{22,33} whereas IFN- γ reciprocally downregulates it.³⁴ Most importantly, the alteration in immunologic parameters was associated with a reduction in the symptoms of the patients.

At baseline, in both the control and IT groups, IL-4-positive cells, eosinophils and mast cells were detectable in the nasal epithelial compartment. This could be explained by the more recent understanding of the concept of minimal persistent inflammation (MPI),³⁵ which is the existence of minimal levels of inflammation even in the absence of symptoms. Although some of the patients had associated nasal allergy to HDM, there was no major difference between those who had associated nasal allergy to HDM and those who did not. The present data, demonstrating a significant decrease in the proportion of IL-4-positive cells, eosinophils and mast cells in the nasal epithelial compartment at 18 months after IT compared with baseline values pre-IT, suggest that IT may also be effective in downregulating MPI.

It was previously postulated that IT could decrease the levels of IgE, but this reduction may require several years. Characteristic changes in serum immunoglobulins have been reported, with an initial increase in IgE followed by a blunting of seasonal increases in IgE in pollen-sensitive

patients and a gradual decline in allergen-specific IgE levels over several years.³⁶ This is accompanied by an increase in allergen-specific IgG (blocking antibodies), although neither appear to correlate closely with the clinical response to IT.³⁶ In the present study, we found no marked reduction in the level of serum-specific IgE, even at 18 months after starting treatment. However, the levels of IgG4-blocking antibodies was significantly increased from 15 to 18 months after starting IT. Yet, serial monitoring of the levels of IgE and IgG4 for several years is essential to precisely evaluate their clinical significance.

Although in the present study the total number of patients was too few to comment on the safety of IT, in a separate larger clinical trial using this modified IT it was found that the incidence of anaphylaxis with CS-560 was less than 0.3% (unpubl. data). This may be because the pullulan of the allergen–pullulan conjugate binds to one of the IgE molecules, thus preventing the cross-linking of two adjacent IgE molecules by the allergen. Thus, this modified IT can induce immunologic tolerance to the natural allergen, without the risk of causing systemic reaction. Moreover, in addition to the safety and efficacy of this modified IT on various immunologic parameters, in separate studies it has also been observed that this modified IT improves the quality of life of patients (unpubl. data).

Thus, taken together, these results suggest that this modified IT with the allergen–pullulan conjugate CS-560 is effective, as assessed by both objective and subjective parameters. Moreover, the clinical benefit of this IT can be attributed to an inhibition of the seasonal increase in the proportion of effector cells, such as mast cells and eosinophils, and an immune deviation from a Th2 to a Th1 cytokine profile and perhaps also a rise in blocking antibodies.

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