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Immunotherapy Suppresses both Th1 and Th2 Responses by Allergen Stimulation, but Suppression of the Th2 Response is a More Important Mechanism Related to the Clinical Efficacy of Immunotherapy for Perennial Allergic Rhinitis

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Increased attention has recently been directed at the possibility that the clinical efficacy of immunotherapy might be elaborated by alteration of T-cell reactivity. However, there is no general agreement among different investigators regarding the effect of immunotherapy on Th-cell reactivity. Peripheral blood mononuclear cells (PBMCs) from 15 nonatopic subjects and 76 patients with perennial allergic rhinitis (18 untreated patients and 58 patients on immunotherapy) were cultured in the absence and in the presence of a major *Dermatophagoides farinae* allergen, Der f 1, and the levels of IgE, interleukin-5 (IL-5), interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) in the culture supernatants were determined. The difference between the absence and presence of Der f 1 was calculated to consider the Der f 1-dependent synthesis of IgE, IL-5, IFN-γ and TNF-α. The levels of Der f 1-dependent synthesis of IgE, IL-5 and TNF-α were significantly higher in the untreated group than in the nonatopic group, whereas Der f 1-dependent synthesis of IFN-γ was significantly lower in the untreated group than in the nonatopic group. Immunotherapy decreased the enhanced Der f 1-dependent synthesis of IgE, IL-5 and TNF-α, and further decreased the suppressed Der f 1-dependent synthesis of IFN-γ as the therapy proceeded. The levels of Der f 1-dependent synthesis of IgE and IL-5 did not differ between nonatopic individuals and patients whose duration of immunotherapy was 10 or more years. The levels of Der f 1-dependent synthesis of IgE and IL-5, but not of IFN-γ and TNF-α, were correlated significantly with the levels of symptom scores. In addition, the levels of Der f 1-dependent synthesis of IgE and IL-5, but not of IFN-γ and TNF-α, differed significantly between good and poor responders. In conclusion, immunotherapy for perennial allergic rhinitis may possibly work via induction of tolerance or anergy of both Th1- and Th2 cells. However, our study is likely to support a view that the mechanisms responsible for the clinically beneficial effects of immunotherapy principally involve the tolerance of Th2- rather than Th1 cells. In addition, suppression of IgE synthesis is also likely to be linked to the clinical efficacy of immunotherapy for perennial allergic rhinitis.

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INTRODUCTION

There is increasing evidence that airway allergic diseases such as allergic rhinitis and asthma represent a specialized form of cell-mediated immunity, in which $CD4^+$ T helper (Th) cells and their products, various cytokines, play a central part in the orchestration and persistance of chronic eosinophil inflammation [1]. Murine Th cells have been categorized into at least two distinct subsets, based on their cytokine secretion profiles [2]. One type of Th cells (Th1 cells) secrete interleukin-2 (IL-2), interferon-gamma (INF-γ) and tumour necrosis factor-β (TNF-β), whereas the other type (Th2 cells) produce IL-4, IL-5, IL-6

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and IL-10, but not IL-2 or INF-γ. Other cytokines such as IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and TNF-α are produced by both types of Th cells. In humans, a clear functional dichotomy in $CD4^+$ T cells has not been definitely documented [3]. In a recent study, however, CD4-positive T cells that exhibit Th1 or Th2 subsets could be isolated from human peripheral blood or tissues in several diseases and conditions [4].

The phenotype of cytokine production by Th cells from peripheral blood and target tissues of atopic subjects has been extensively investigated. An accumulation of Th2-like cells at allergic inflammatory sites and increased levels of Th2 cell-derived cytokines in culture supernatants from peripheral blood lymphocytes have also been found [5, 6]. Cytokines derived from Th2-like cells could mediate allergic inflammation through activating eosinophils, promoting mast cell development, regulating immunoglobulin isotype switching to IgE and modulating adhesion molecule expression [7–10]. In the current concept, atopic allergy might be defined as a Th2 cell- or Th2 response-predominant inflammatory disease.

Dermatophagoides farinae (*D. farinae*) is the most important allergen causing perennial allergic rhinitis in Japan. Allergen-specific immunotherapy has been used widely in the treatment of perennial allergic rhinitis, and recent double-blind placebo-controlled trials have clearly shown the efficacy of immunotherapy for perennial allergic rhinitis [11–13]. Although several mechanisms for the clinical benefit of immunotherapy have been proposed, the exact working mechanisms behind the clinical efficacy still remain unclear. Th cells, especially Th2 cells and the cytokines secreted by them, are likely to play an important key role in the pathogenesis of perennial allergic rhinitis. Increased attention has recently been directed at the possibility that the clinical efficacy of immunotherapy might be elaborated by alteration of T-cell reactivity and cytokine profiles. Recent studies have shown that cytokine production could be modified by immunotherapy [14–16], but the effect of immunotherapy on cytokine production has been the subject of much debate and still remains unclear.

The aim of this study was, therefore, to evaluate the immunological mechanisms of immunotherapy for perennial allergic rhinitis, from a comparative study of cytokine production in response to the major *D. farinae* allergen, Der f 1 among nonatopic controls, untreated patients and patients on immunotherapy, with special reference to the clinical effect.

MATERIALS AND METHODS

Subjects. This study included 15 nonatopic healthy volunteers (7 females and 8 males; nonatopic group) and 76 patients (36 females and 40 males) with perennial allergic rhinitis due to *D. farinae*, who gave informed consent for participation. Each subject in the nonatopic group was chosen on the basis of the following criteria: (1) no history of allergic disease, (2) no physical findings indicative of atopic disease 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 molds.

All the patients were selected from our outpatients who satisfied all of the following conditions before treatment: (1) well-documented history of severe typical perennial allergic rhinitis according to Okuda's criteria [17] but no history of asthma, (2) positive skin test to *D. farinae* according to Okuda's criteria [17], (3) positive nasal provocation test to *D. farinae* according to Okuda's criteria [17] and (4) eosinophilia in nasal smears according to Okuda's criteria [17]. Fifty-eight (28 females and 30 males) of the 76 patients had undergone variable periods (mean, 6.9 ± 4.8 years; range, 1–17 years) of immunotherapy using the Hollister–Stier standardized *D. farinae* extracts (Miles Inc., Spokane, WA, USA; immunotherapy group). Every patient of the immunotherapy group received maintenance injections at the time of participation of the present study. The patients in the immunotherapy group were further divided into four groups according to the duration of immunotherapy: those who had been treated with immunotherapy for 2 or fewer years (short-IT group; 7 females and 7 males), those who had been treated with immunotherapy for 3–5 years (medium-IT group; 6 females and 7 males), those who had been treated with immunotherapy for 6–9 years (long-IT group; 6 females and 7 males), and those who had been treated with immunotherapy for 10 or more years (longest-IT group; 9 females and 9 males). The remaining 18 patients (9 females and 9 males) had not been treated previously with immunotherapy, and were not receiving steroid treatment (untreated group). Age did not differ significantly among the groups (nonatopic group versus untreated group: $P = 0.7010$, nonatopic group versus short-IT group: $P = 0.1147$, nonatopic group versus medium-IT group: $P = 0.1732$, nonatopic group versus long-IT group: $P = 0.4320$, nonatopic group versus longest-IT group: $P = 0.1238$).

Mode of immunotherapy. The patients were given subcutaneous injections of Hollister–Stier standardized *D. farinae* extracts, with prior informed consent. Taking into consideration the end-point concentration determined by the threshold skin test and serum concentrations of specific IgE, the individual initiative dose was determined. Doses were increased by 50–100% at weekly intervals until the highest tolerated doses or 3000 allergen units (AU) were reached. The maximum tolerated dose was attained in 4–8 months with the once weekly program. The maximum tolerated dose was 3000 AU in 54 of the 58 patients, and 300 AU in the remaining 4 patients. Weekly injection of this dose was then 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 in the absence of an episode of symptomatic relapse. After each injection, the patient was asked to remain under our supervision for a minimum of 30 min and to report any symptoms they may have experienced. No concomitant medication affecting nasal symptoms was admitted throughout the period, with prior informed consent. All the patients intentionally continued the treatment, to avoid possible relapse even though they could alleviate nasal symptoms completely.

Study design. This study design followed the principals outlined in the Declaration of the Helsinki [18]. Peripheral blood was collected from each subject between September and October 1997, and serum concentrations of IgE and *D. farinae*-specific IgE were determined by the use of a lumiward immunoassay system [19]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-density gradient centrifugation. PBMCs were washed twice in PBS and resuspended in RPMI 1640 (Flow Laboratories, Meckenheim, Germany), containing 10% FCS (JRH Biosciences, Lenexa, CA, USA), 100 μg/ml streptomycin (Sigma, St Louis, MO, USA), 100 IU/ml penicillin and

2 mM L-glutamine. Two sets of PBMC cultures at a final concentration of 1.0×10^6 cells/ml in 12-well plates (Iwaki glass, Tokyo, Japan) were prepared from each subject.

Spontaneous synthesis of IgE and cytokines. One of PBMCs at a final concentration of 1.0×10^6 cells/ml from each subject was cultured in the absence of the allergen for 96 h at 37°C in a fully humidified, 5% CO_2 atmosphere. The culture supernatants were then harvested by centrifugation and stored at −80°C until measurement of IgE, IL-5, IFN-γ and TNF-α.

Synthesis of IgE and cytokines in the presence of Der f 1. The other PBMCs at a final concentration of 1.0×10^6 cells/ml from each subject were cultured for 96 h in the presence of 30 μg (10 μg/ml) of Der f 1 (Asahi Co., Ltd, Tokyo, Japan), a major allergen of *D. farinae*. The culture supernatants were also harvested by centrifugation and stored at −80°C until measurement of IgE, IL-5, IFN-γ and TNF-α.

Der f 1-dependent synthesis of IgE and cytokines. Der f 1-dependent synthesis of IgE and cytokines was calculated using the following formulae:

(Der f 1-dependent synthesis)
= (synthesis in the presence of Der f 1)
− (spontaneous synthesis)

Determination of IgE, IL-5, IFN-γ and TNF-α in culture supernatants. IgE was determined by the use of a lumiward immunoassay system [19]. IL-5, IFN-γ and TNF-α were measured by ELISA using commercially available kits (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan); the details and justification of the methods employed in this study have been described elsewhere [20–22].

In brief, microtitration plates were coated with anti-IL-5 monoclonal antibody (MoAb, JES1-39D10 at 2 μg/ml), anti-IFN-γ MoAb (ANOC 1611 at 10 μg/ml) or anti-TNF-α MoAb (ANOC 706 at 2 μg/ml) in 100 μl/well of PBS, pH 7.4. Following overnight incubation at 4°C, the wells were blocked with a 1% skim milk in PBS solution for at least 1 h at room temperature and washed three times with PBS containing 0.05% Tween-20 (Tween-20–PBS, washing was always performed with this buffer). One hundred μl of samples or standard in 0.1% BSA–PBS were added to the wells and the plate was incubated at 4°C for 24 h. The plates were then washed three times, and 100 μl of rabbit IL-5 antibodies (MoAb JES1-5A10: diluted to 1/500 with 0.1% BSA–PBS–Tween-20), rabbit anti-IFN-γ antibodies (OCT 1601: diluted to 1/2000 with 0.1% BSA–PBS–Tween-20) or biotin-labeled anti-TNF-α antibodies (MoAb ANOC 705: diluted to 1/5000 with 0.1% BSA–PBS–Tween-20) were added to each well and the plates were incubated for 2 h at 23°C. Subsequently, the plates were washed, supplemented with 100 μl of HRP-labeled Avidin (diluted to 1/10000 with 0.1% BSA–PBS–Tween-20) for IL-5 assay and with 100 μl of HRP-labeled goat anti-rabbit IgG (diluted to 1/10000 with 0.1% BSA–PBS–Tween-20), and incubated at 23°C for 2 h. Finally, 100 μl of enzyme substrate (1 mg/ml *O*-phenylene diamine and 0.03% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0) was added to each well and the plates were incubated at room temperature for 10 min. The reaction was stopped by adding 100 μl of H_2SO_4 to each well and the absorbance at 492 nm was determined using a Titertek Multiscan. The lowest sensitivity of the method employed is 10 pg/ml for IL-5 and 5 pg/ml for IFN-γ and TNF-α, and it does not measure the presence of other cytokines.

Assessment of symptom scores. Nasal symptoms at PBMC sampling were evaluated by our criteria using daily symptom diary cards. The details of the method employed in this study have been described elsewhere [23]. In brief, all the patients were asked to complete daily symptom diary cards in the 14 days before the assessment of nasal symptom scores. The daily symptom card includes the number of sneezing attacks, the number of nose blowings, and the degree of nasal obstruction. A careful survey of the diary card graded the three nasal symptoms (sneeze, rhinorrhea and obstruction) on a scale of 0–3, depending on severity. The average daily scores of sum total of the three nasal symptom scores were used as symptom scores (maximum: 9). The levels (mean ± SD) were 7.6 ± 1.2 (range: 5–9) in the untreated group, 4.1 ± 2.1 (range: 0–7) in the short-IT group, 1.8 ± 2.0 (range: 0–5) in the medium-IT group, and 0.3 ± 0.8 (range: 0–2) in the long-IT group, respectively. The symptom score in every patient in the longest-IT group was 0. Based upon the symptom scores, the 58 patients in the immunotherapy group were subdivided into a good response group (36 patients) whose symptom score was 0 and a poor response group (22 patients) whose symptom score was 1 or more.

Statistical analysis. If the level of IgE, IL-5, IFN-γ or TNF-α in particular patients was below the sensitivity of the respective assay, the lowest detectable value (IgE: 0.3 IU/ml; IL-5: 10 pg/ml; IFN-γ: 5 pg/ml; TNF-α: 5 pg/ml) obtained for that assay was arbitrarily used for statistical analysis.

For comparisons between different groups, the Mann–Whitney *U*-test was used. For comparisons of paired values, Wilcoxon signed-rank test was used. Differences were considered significant when $P < 0.05$. Spearman's correlation coefficient (r_S) was calculated to determine the degree of correlation between two different parameters, and significant correlation was accepted when $P < 0.05$ on Spearman's rank correlation analysis.

RESULTS

IgE in serum

The serum levels of IgE at the sampling of PBMC in the untreated group ($P = 0.0034$), the short-IT group ($P < 0.0001$) and the medium-IT group ($P = 0.0098$) were significantly higher than those in the nonatopic group. However, the serum levels of IgE at the sampling of PBMC in the long-IT group ($P = 0.2052$) as well as the longest-IT group ($P = 0.0576$) were not significantly different from those in the nonatopic group. The serum levels of IgE at the sampling of PBMC in the untreated group were not significantly different from those in the short-IT group ($P = 0.5433$), the medium-IT group ($P = 0.7639$), long-IT group ($P = 0.0599$) or the longest-IT group ($P = 0.0665$). Also, none of the IgE serum levels before immunotherapy in the short-IT group ($P = 0.4704$), the medium-IT group ($P = 0.1386$), the long-IT group ($P = 0.7793$) or the longest-IT group ($P = 0.4107$) were significantly different from those in the untreated group at the sampling of PBMC (Table 1).

Specific IgE in serum

Specific IgE was not detected in the serum of any individual in the nonatopic group at the sampling of PBMC. However, the concentration of specific IgE to *D. farinae* in each patient was above the lower limit of detection. The specific IgE levels at the sampling of PBMC did not differ significantly among the untreated group, the short-IT group and the medium-IT group (untreated group versus short-IT group: $P = 0.5942$; untreated

Table 1. IgE levels in serum (IU/ml)

	At the time of PBMC sampling				Before immunotherapy			
	Mean	SD	SEM	Range	Mean	SD	SEM	Range
Nonatopic group	92.9	96.3	24.9	6.6–345.7				
Untreated group	578.3	701.6	165.4	5.3–2300.0				
Immunotherapy group	424.2	574.4	75.4	13.0–2505.6	686.7	504.5	66.2	58.0–2140.0
Short-IT group	732.3	726.8	194.3	83.9–2505.6	766.7	669.2	178.8	60.8–2140.0
Medium-IT group	663.2	759.1	210.5	13.0–2500.0	798.7	551.1	152.9	78.0–1880.0
Long-IT group	182.5	207.0	57.4	14.0–660.0	578.1	377.6	104.7	58.0–1217.0
Longest-IT group	186.5	196.9	46.4	15.0–730.0	622.2	409.4	96.5	253.0–1896.0
Good response group	271.7	430.0	71.7	15.0–2500.0	664.9	427.2	72.9	58.0–1896.0
Poor response group	673.8	694.4	148.0	13.0–2505.6	722.5	608.3	129.7	60.8–2140.0

group versus medium-IT group: $P = 0.3566$). The specific IgE levels at the PBMC sampling in the long-IT group ($P = 0.0411$), as well as the longest-IT group ($P = 0.0028$), were significantly lower than those at the PBMC sampling in the untreated group. However, none of the serum levels of specific IgE before immunotherapy in the short-IT group ($P = 0.4704$), the medium-IT group ($P = 0.5750$), the long-IT group ($P = 0.9198$) or the longest-IT group ($P = 0.4107$) were significantly different from those in the untreated group at the PBMC sampling (Table 2).

Spontaneous synthesis in culture supernatant

The levels of spontaneous synthesis of IgE and cytokines are summarized in Table 3. The levels of spontaneous synthesis of IgE in the untreated, short-IT, medium-IT and poor response groups, but not in the long-IT, longest-IT and good response groups, were significantly higher than those in the nonatopic group. The levels of spontaneous synthesis of IL-5 did not differ significantly between the groups. The levels of spontaneous synthesis of IFN-γ in the medium-IT, longest-IT and good response groups, but not in the untreated, short-IT, long-IT and poor response groups, were significantly lower than those in the nonatopic group. The levels of spontaneous synthesis of TNF-α in the longest group alone were significantly lower than those in the nonatopic group.

Comparison between spontaneous synthesis vs synthesis in the presence of Der f 1 in culture supernatant

The levels of synthesis of IgE and cytokines in the presence of Der f 1 are summarized in Table 4. Statistical comparison between the spontaneous synthesis and the synthesis in the presence of Der f 1 in each group was performed to evaluate whether the synthesis in the presence of Der f 1 was simply a spontaneous synthesis or a real Der f 1-dependent synthesis. The levels of IgE synthesis in the presence of Der f 1 were not significantly different from those of spontaneous synthesis of IgE in the nonatopic, long-IT and longest-IT groups. The levels of IL-5 synthesis in the presence of Der f 1 were not significantly different from those of spontaneous synthesis of IL-5 in the nonatopic, medium-IT, long-IT, longest-IT and good response groups. The levels of IFN-γ and TNF-α in the presence of Der f 1

Table 2. *D. farinae*-specific IgE levels in serum (IU/ml)

	At the time of PBMC sampling				Before immunotherapy			
	Mean	SD	SEM	Range	Mean	SD	SEM	Range
Untreated group	39.3	36.7	8.6	0.07–100.0				
Immunotherapy group	22.1	31.0	4.1	0.06–100.0	46.1	32.2	4.2	2.3–100.0
Short-IT group	43.8	36.0	9.6	0.1–100.0	49.6	30.0	8.0	6.1–100.0
Medium-IT group	29.0	39.7	11.0	0.06–100.0	45.5	34.6	9.6	2.3–98.0
Long-IT group	14.2	20.5	5.7	0.07–61.0	48.1	38.2	10.6	4.4–100.0
Longest-IT group	6.0	8.6	2.0	0.1–34.0	42.3	29.6	7.0	8.6–100.0
Good response group	11.8	18.0	3.0	0.06–70.4	45.9	32.5	5.4	5.2–100.0
Poor response group	39.0	39.8	8.5	0.07–100.0	46.3	32.4	6.9	2.3–100.0

Table 3. Spontaneous synthesis (mean ± SD) of IgE and cytokines

	IgE (IU/ml)	IL-5 (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)
Nonatopic group	0.30 ± 0.00	10.00 ± 0.00	17.59 ± 21.27	6.96 ± 3.08
Untreated group	0.42 ± 0.13*	10.44 ± 1.89	10.31 ± 9.46	9.02 ± 10.93
Short-IT group	0.45 ± 0.22*	10.00 ± 0.00	9.23 ± 8.81	7.32 ± 4.58
Medium-IT group	0.43 ± 0.21*	10.54 ± 1.45	5.87 ± 3.13†	5.51 ± 1.74
Long-IT group	0.33 ± 0.09	10.92 ± 2.25	10.73 ± 14.05	6.38 ± 3.53
Longest-IT group	0.32 ± 0.05	10.56 ± 2.36	5.00 ± 0.00†	5.29 ± 1.23*
Good response group	0.38 ± 0.19	10.67 ± 2.14	7.07 ± 8.69†	5.64 ± 2.31
Poor response group	0.37 ± 0.11*	10.23 ± 1.07	8.21 ± 7.44	6.78 ± 3.90

* Significantly higher than the level in nonatopic group.
† Significantly lower than the level in nonatopic group.

Table 4. Synthesis (mean ± SD) of IgE and cytokines in the presence of Der f 1

	IgE (IU/ml)	IL-5 (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)
Nonatopic group	0.33 ± 0.10	15.49 ± 18.64	450.23 ± 362.12	35.15 ± 18.10
Untreated group	1.02 ± 0.55	70.58 ± 53.49	126.68 ± 187.31	253.83 ± 288.97
Immunotherapy group	0.81 ± 1.03	20.04 ± 22.28	70.48 ± 128.11	99.09 ± 141.86
Short-IT group	1.65 ± 1.52	42.09 ± 35.16	158.86 ± 217.08	168.10 ± 219.14
Medium-IT group	0.92 ± 1.02	11.94 ± 4.31	52.23 ± 94.43	113.06 ± 118.67
Long-IT group	0.43 ± 0.23	16.95 ± 14.60	43.78 ± 47.88	87.38 ± 97.92
Longest-IT group	0.36 ± 0.13	10.96 ± 3.17	34.13 ± 49.45	63.08 ± 91.13
Good response group	0.66 ± 0.85	13.31 ± 9.43	43.97 ± 57.79	91.15 ± 103.82
Poor response group	1.06 ± 1.26	20.81 ± 31.60	105.66 ± 186.62	121.33 ± 189.05

Table 5. Comparison of spontaneous synthesis and synthesis in the presence of Der f 1

	IgE synthesis	IL-5 synthesis	IFN-γ synthesis	TNF-α synthesis
Nonatopic group	NO	NO	YES	YES
Untreated group	YES	YES	YES	YES
Short-IT group	YES	YES	YES	YES
Medium-IT group	YES	NO	YES	YES
Long-IT group	NO	NO	YES	YES
Longest-IT group	NO	NO	YES	YES
Good response group	YES	NO	YES	YES
Poor response group	YES	YES	YES	YES

YES = synthesis in the presence of Der f 1 is significantly higher than spontaneous synthesis.
NO = synthesis in the presence of Der f 1 is not significantly different from spontaneous synthesis.

were significantly higher than the levels of spontaneous IFN-γ and TNF-α synthesis in any group (Table 5).

Der f 1-dependent synthesis of IgE in supernatant

The levels of Der f 1-dependent IgE synthesis in the untreated group, the short-IT group and the medium-IT group were significantly higher than those in the nonatopic group. However, the levels of Der f 1-dependent IgE synthesis in the long-IT group and the longest-IT group were not significantly different from those in the nonatopic group. The geometric mean of Der f 1-dependent IgE synthesis in the short-IT group (1.20±

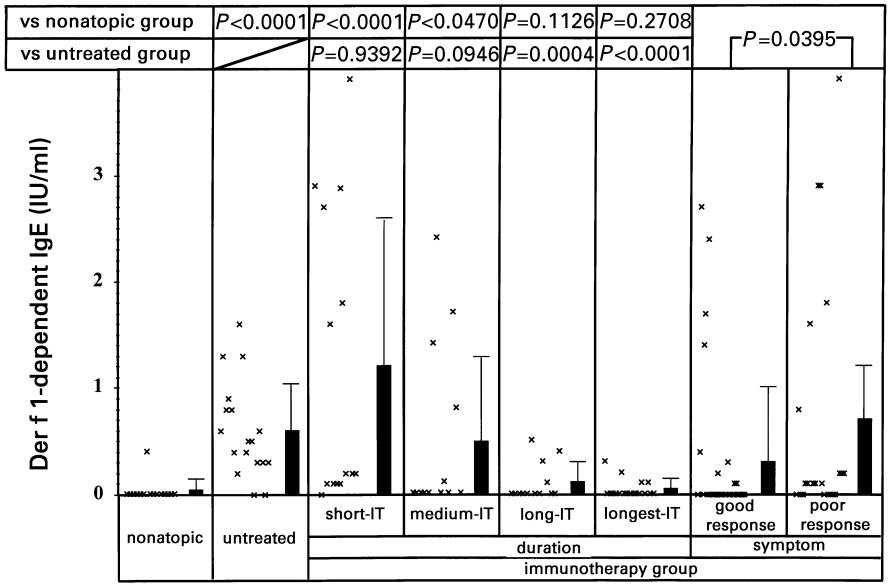

Fig. 1. Der f 1-dependent IgE synthesis in the culture supernatants. The level in the untreated group was significantly higher than that in the nonatopic group. The level was rather higher in the short-IT group than that in the untreated group. However, the level became lower in the medium-IT, long-IT and longest-IT groups as the treatment proceeded. The level in the good response group was significantly lower than in the poor response group.

1.39 IU/ml) was approximately two-fold higher compared with that in the untreated group (0.60 ± 0.45 IU/ml), but the levels of Der f 1-dependent IgE synthesis did not differ significantly between the untreated group, the short-IT group and the medium-IT group. By contrast, the levels of Der f 1-dependent IgE synthesis in the long-IT group and the longest-IT group were significantly lower than those in the untreated group. The levels of Der f 1-dependent IgE synthesis in the good response group were significantly lower than those in the poor response group (Fig. 1). The levels of Der f 1-dependent IgE synthesis in the 58 patients receiving immunotherapy were significantly correlated with the symptom scores ($r_S = 0.439$, $P = 0.0264$), and were inversely correlated with the duration (years) of immunotherapy ($r_S = -0.380$, $P = 0.0001$).

Der f 1-dependent synthesis of IL-5 in supernatant

The levels of Der f 1-dependent IL-5 synthesis in the untreated group and the short-IT group were significantly higher than those in the nonatopic group. However, the levels of Der f 1-dependent IL-5 synthesis in the medium-IT, the long-IT and the longest-IT groups were not significantly different from those in the nonatopic group. The levels of Der f 1-dependent IL-5 synthesis in any of the four immunotherapy groups were significantly lower than those in the untreated group. The levels of Der f 1-dependent IL-5 synthesis in the good response group were significantly lower than those in the poor response group (Fig. 2). The levels of Der f 1-dependent IL-5 synthesis in the 58 patients receiving immunotherapy were significantly correlated with the symptom

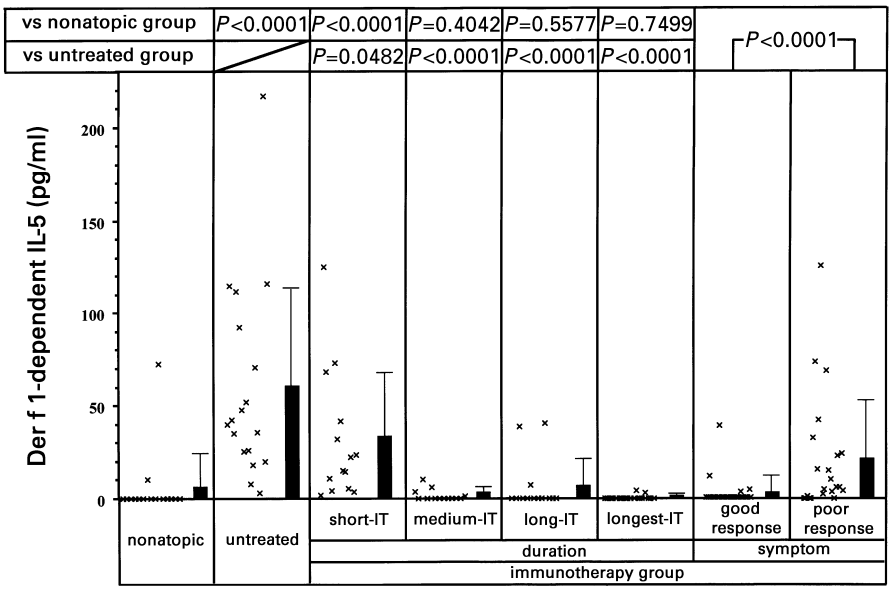

Fig. 2. Der f 1-dependent IL-5 synthesis in the culture supernatants. The level in the untreated group was significantly higher than that in the nonatopic group. The levels became lower in the immunotherapy group as the therapy proceeded. The level in the medium-IT, long-IT and longest-IT group was not different from that in the nonatopic group. The level in the good response group was significantly lower than that in the poor response group.

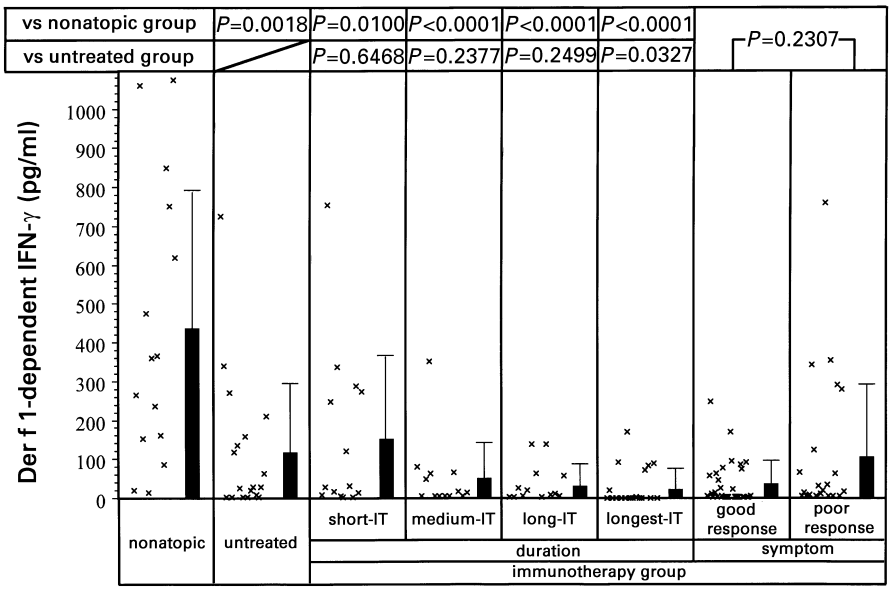

Fig. 3. IFN-γ synthesis in the culture supernatants. The level in the untreated group was significantly lower than that in the nonatopic group. The level became much lower in the immunotherapy group as the therapy proceeded. However, the levels did not differ between the good and poor response groups.

scores ($r_S = 0.708$, $P < 0.0001$), and were inversely correlated with the duration (years) of immunotherapy ($r_S = -0.478$, $P < 0.0001$).

Der f 1-dependent synthesis of IFN-γ in supernatant

The levels of Der f 1-dependent IFN-γ synthesis in any of the patient groups were significantly lower than those in the nonatopic group. The levels of Der f 1-dependent IFN-γ synthesis in the short-IT, the medium-IT and the long-IT groups were not significantly different from those in the untreated group, but the levels of Der f 1-dependent IFN-γ synthesis in the longest-IT group were significantly lower than those in the untreated group. The levels of allergen-dependent IFN-γ did not differ significantly between the good response and the poor response groups (Fig. 3). The levels of Der f 1-dependent IFN-γ synthesis in the 58 patients receiving immunotherapy were not significantly correlated with the symptom scores ($r_S = 0.328$, $P = 0.1015$), but were inversely correlated with the duration (years) of immunotherapy ($r_S = -0.279$, $P = 0.0179$).

Der f 1-dependent synthesis of TNF-α in supernatant

The levels of Der f 1-dependent TNF-α synthesis in the untreated group and the short-IT group were significantly higher than those in the nonatopic group. However, the levels of Der f 1-dependent TNF-α synthesis in the medium-IT, the long-IT and the longest-IT groups were not significantly different from those in the

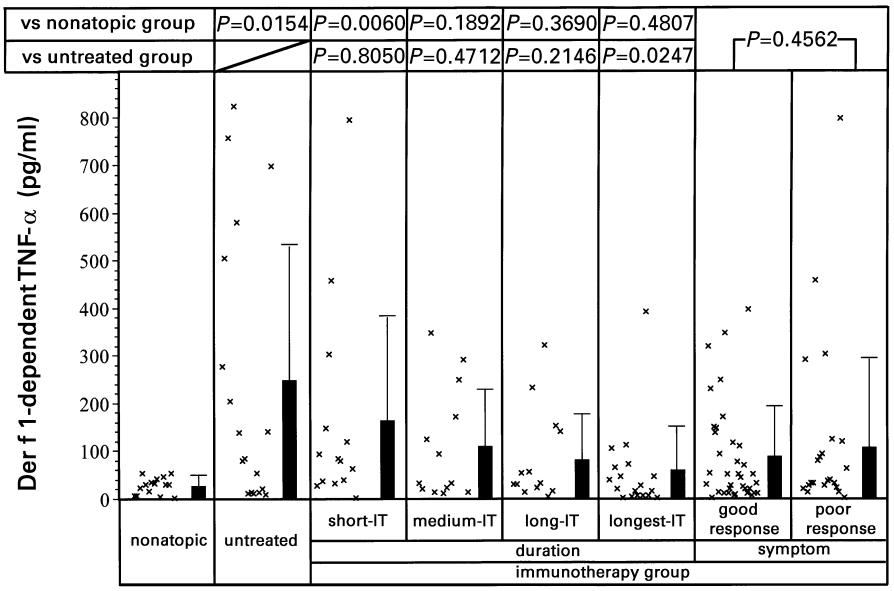

Fig. 4. TNF-α synthesis in the culture supernatants. The level in the untreated group was significantly higher than that in the nonatopic group. Immunotherapy decreased the level as the therapy proceeded. However, the levels did not differ between the good and poor response groups.

nonatopic group. The levels of Der f 1-dependent TNF-α synthesis in the short-IT, the medium-IT and the long-IT groups were not significantly different from those in the untreated group, but the levels of Der f 1-dependent TNF-α synthesis in the longest-IT group were significantly lower than those in the untreated group. The levels of Der f 1-dependent TNF-α synthesis did not differ significantly between the good response and the poor response groups (Fig. 4). The levels of Der f 1-dependent TNF-α synthesis in the 58 patients receiving immunotherapy were not significantly correlated with the symptom scores ($r_S = 0.230$, $P = 0.3400$), but were inversely correlated with the duration (years) of immunotherapy ($r_S = -0.316$, $P = 0.0160$).

DISCUSSION

The main aim of the study was to investigate whether immunotherapy could affect spontaneous, as well as allergen-induced, synthesis of IgE and cytokines by PBMCs in patients with perennial allergic rhinitis and whether the effect was related to the clinical efficacy. Spontaneous IgE synthesis was significantly enhanced in the untreated group, but spontaneous synthesis of IL-5, IFN-γ and TNF-α did not differ between the untreated and the nonatopic groups. Our findings suggest that untreated patients with perennial allergic rhinitis may differ fundamentally from nonatopic individuals in IgE synthesis system even in the absence of allergens. Since the levels of spontaneous IgE synthesis in the short-IT and medium-IT groups, but not in the long-IT and longest-IT groups, were significantly higher than those in the nonatopic group, immunotherapy was likely to decrease spontaneous IgE synthesis. The levels of spontaneous IgE synthesis in the good response group were not different from those in the nonatopic group, but those in the poor response group were significantly higher than those in the nonatopic group. Therefore, the suppression of spontaneous IgE synthesis might be, at least partly, involved in the working mechanisms of immunotherapy related to the clinical efficacy.

Synthesis of IgE and cytokines by PBMCs were investigated in the presence, as well as in the absence, of 10.0 μg/ml of Der f 1, and the difference was calculated to evaluate Der f 1-dependent synthesis. The level of Der f 1-dependent IgE synthesis was significantly higher in the untreated group than in the nonatopic group, which suggested that IgE synthesis in response to allergen stimulation was enhanced in patients with perennial allergic rhinitis. The level of Der f 1-dependent IgE synthesis was inversely correlated with the duration of immunotherapy. Indeed, the levels of Der f 1-dependent IgE synthesis in the long-IT and longest-IT groups were not significantly different from those in the nonatopic group. The levels of Der f 1-dependent IgE synthesis were correlated with the symptom scores, and those in the good response group were significantly lower than those in the poor response group. Taking these results together, an enhanced Der f 1-dependent synthesis of IgE can be suppressed by immunotherapy as the therapy proceeds, and the suppression is probably involved in the working mechanisms of immunotherapy behind the clinical efficacy.

Different lines of evidence for the importance of Th2-type cytokines in the pathogenesis of allergic inflammation have emerged [1]. Of the cytokines secreted by activated Th2-like cells, IL-5 is particularly implicated in the pathogenesis of allergic inflammatory diseases of the airway, because it selectively promotes the differentiation, priming, activation and survival of eosinophils *in vitro* [24]. Expression of IL-5 mRNA has been detected in bronchial biopsies of asthmatics, and the expression of IL-5 mRNA has tended to increase in severe disease [25]. IL-5 is a key cytokine linked to symptomatic episodes of allergic rhinitis and asthma in sensitized individuals [26, 27]. In addition, treatment with anti-IL-5mAb could inhibit the development of hyperresponsiveness [28]. In cultures of mouse B cells, IL-5 also promoted IgE production in the presence of IL-4 [29]. Not only the Th2, but also the Th1, response is considered to differ between atopic and nonatopic individuals. IFN-γ secreted by Th1-like cells is generally considered to play a reciprocal role against the activities of Th2-type cytokines, because IFN-γ inhibits IgE synthesis by B lymphocytes and the development of Th2 clones [30]. Indeed, stimulated peripheral blood T lymphocytes from atopic individuals are reported to secrete significantly less IFN-γ than those from normal controls [31]. However, some authors have demonstrated increased spontaneous and allergen-stimulated IFN-γ production in atopic individuals [32, 33], and IFN-γ may even up-regulate IL-4 dependent IgE production [33]. TNF-α is produced by not only Th1 cells but also Th2 cells and monocytes, which are involved in switching B lymphocytes from IgG to IgE production [34], and is also implicated in allergic inflammation through the increase of the expression of intercellular adhesion molecule-1 [35]. Thus, we studied the Der f 1-induced synthesis of three kinds of cytokines, IL-5 produced by Th2 cells, IFN-γ produced by Th1 cells and TNF-α produced by Th1 cells, Th2 cells and monocytes.

In untreated patients, Der f 1-dependent synthesis of IL-5 and TNF-α was more enhanced and allergen-dependent synthesis of IFN-γ more suppressed than in nonatopic individuals. Therefore, our results suggest that in untreated patients with perennial allergic rhinitis due to *D. farinae*, the Th2 response is predominantly enhanced and Th1 response is rather suppressed in response to the allergen stimulation. It is also of clinical interest whether the Der f 1-dependent abnormal cytokine synthesis in patients with perennial allergic rhinitis could be modulated by immunotherapy. An alteration in T-cell reactivity and cytokine production is likely to be one of the key working mechanisms of immunotherapy, but there is no clear consensus on the effect of immunotherapy on cytokine synthesis or Th responses. We therefore investigated Der f 1-dependent synthesis of IL-5, IFN-γ and TNF-α by PBMCs in patients on immunotherapy. Allergic rhinitis is an extremely complex disease, in which a variety of immunological factors as well as other pathogenesis are involved. Immunotherapy modulates and affects many different immunological phenomena to produce clinical efficacy, and clinical improvement is likely to be a consequence of different mechanisms working over time [14, 23, 36–38]. Therefore,

production of different cytokines might be modulated by immunotherapy at different phases of the immunotherapy course. The possible modulation of profiles of cytokine production by immunotherapy was evaluated in four subgroups of patients whose duration of immunotherapy was different.

Our comparative analysis clearly demonstrated that immunotherapy suppressed Der f 1-dependent synthesis of IL-5 and that the suppression became more apparent as the therapy proceeded. In particular, Der f 1-dependent synthesis of IL-5 in the patients whose duration of immunotherapy was 3 or more years was not different from that in the nonatopic group. Der f 1-dependent synthesis of IL-5 was significantly correlated with the symptom scores, and Der f 1-dependent synthesis of IL-5 was significantly lower in the good response group than in the poor response group. Our findings therefore suggest that the enhanced synthesis of IL-5 or increased reactivity of Th2 cells in response to the allergen stimulation can be down-regulated by immunotherapy and that the suppression of the Th2 response is critically implicated in the mechanisms of immunotherapy linked to the clinical efficacy.

Since the levels of IgE and IL-5 synthesis in the presence of Der f 1 were not different from the levels of spontaneous IgE and IL-5 synthesis in the nonatopic group, the synthesis of IgE and IL-5 in the presence of Der f 1 might not be allergen-dependent but simply a spontaneous release. It is thus probable that in nonatopic individuals neither IgE nor Th2 responses occurred even under the allergen stimulation. Similarly, IgE response under Der f 1 stimulation was not apparent in patients whose duration of immunotherapy was 6 or more years, and Th2 response under Der f 1 stimulation was not positive in patients whose duration of immunotherapy was 3 or more years. Therefore, it is likely that immunotherapy can induce the disappearance of the Th2 response and then the disappearance of the IgE response under the allergen stimulation.

The level of Der f 1-dependent synthesis of IFN-γ was inversely correlated with the duration of immunotherapy, and the level of Der f 1-dependent synthesis of IFN-γ in the longest-IT group was significantly lower than those in the untreated group, which suggested that the suppressed synthesis of IFN-γ or the suppressed reactivity of Th1 cells in patients with perennial allergic rhinitis could be further suppressed as immunotherapy proceeded. However, the level of Der f 1-dependent synthesis of IFN-γ was not significantly correlated with the symptom scores, and additionally the level of Der f 1-dependent synthesis of IFN-γ did not differ significantly between the good and poor response groups. These findings are likely to suggest that suppression of Th1 cells by immunotherapy might be simply an epiphenomenon unrelated to the working mechanism of clinically successful immunotherapy. Similarly, the increased Der f 1- dependent synthesis of TNF-α in patients with perennial allergic rhinitis was gradually suppressed as immunotherapy proceeded. However, Der f 1-dependent synthesis of TNF-α was not correlated with the symptom scores, and did not differ significantly between the good and poor response groups. Our findings therefore suggest that the suppression of TNF-α synthesis is not likely to be a major working mechanism of immunotherapy related to the clinical efficacy.

The principal findings of our study are that in untreated patients with perennial allergic rhinitis, the Th2 response and IgE synthesis were predominantly enhanced and the Th1 response was rather suppressed in response to the allergen stimulation and that immunotherapy suppressed the increase reactivity of Th2 cells and IgE synthesis and further suppressed the decreased reactivity of Th1 cells. The present evidence that both Th1 and Th2 responses were substantially suppressed by immunotherapy suggests that immunotherapy does not predominantly act through a shift in allergic immune reactivity from a Th2 response towards a Th1 response, but that immunotherapy induces tolerance or anergy to both types of Th cells.

The effect of immunotherapy on the Th1 and Th2 responses has recently been studied by several investigators. Secrist *et al*. [15] demonstrated that production of IFN-γ by allergen-specific $CD4^+$ T cells was not affected in grass pollen and mite allergic subjects on immunotherapy whereas IL-4 production was reduced by immunotherapy and that the levels of IL-4 produced are inversely related to the length of time on immunotherapy. We demonstrated previously that serum levels of IL-4 in patients with perennial allergic rhinitis were decreased by immunotherapy [23, 39]. In our previous serological study for patients with seasonal allergic rhinitis, pollen immunotherapy reduced a seasonal activation of Th2 response and additionally switched the seasonal preferential activation of Th2 cells to reciprocal activation of Th1 cells with treatment over several years [14]. However, an important role of IFN-γ in the mechanism of immunotherapy has also been demonstrated. Varney *et al*. [16] and Durham *et al*. [40] reported a significant increase in IFN-γ expression, without a significant changes 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. Conversely, MacDonald *et al*. [41], studying cytokine release from PBMCs in patients on ragweed immunotherapy, showed a highly significant reduction in the production of IFN-γ, but no significant changes in IL-4. Most recently, O'Brien *et al*. [42] stimulated PBMCs from patients sensitive to house dust mites with Der p 2, and measured cytokine production using a polymerase chain reaction. They demonstrated that immunotherapy results in a reduction in the expression of both IL-4 and IFN-γ in circulating lymphocytes. Our results are completely in agreement with their findings, in suggesting that immunotherapy suppresses both Th1 and Th2 responses during allergen stimulation.

Thus, there is no general agreement between different investigators regarding the effect of immunotherapy on Th cells or cytokine synthesis. 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 inflammed tissue) and different experimental protocols (*in vitro* or *in vivo*) between studies are likely to underlie the contradictory results. Although immunotherapy is a clinically effective treatment for atopic

patients, it is not effective for all of them. Nevertheless only a few studies have referred to a possible correlation between clinical efficacy and modification of Th responses by immunotherapy. In our previous study, the effect on Th2 response, but not on Th1 response, was related exclusively to the clinical efficacy, although pollen immunotherapy affected both Th1 and Th2 responses during the pollen season [14]. Similarly, in our present study the suppression of the Th2 response, but not of the Th1 response, is linked exclusively to the clinical efficacy or the symptom score, although mite immunotherapy affected both Th1 and Th2 responses during the allergen stimulation. Taking these results together, the suppression of the Th2 response is a key mechanism of action underlying the clinical efficacy of immunotherapy, and modulation of Th1 response is likely to be an epiphenonenon unrelated to the clinical efficacy. O'Brien *et al.* [42] also discussed a correlation between cytokine production and clinical efficacy of immunotherapy. Although their study was not quantitative, mRNA expression of both Th1 and Th2 types of cytokines in circulating lymphocytes tended to persist in patients who had a less beneficial clinical response to immunotherapy.

In conclusion, our study has demonstrated that immunotherapy for perennial allergic rhinitis may possibly be working through induction of tolerance or anergy of both Th1 and Th2 cells. However, our study is likely to support a view that the mechanisms responsible for the clinically beneficial effects of immunotherapy principally involve the tolerance of Th2 rather than Th1 cells (Fig. 5). In addition, suppression of IgE synthesis is also likely to be linked to the clinical efficacy of immunotherapy for perennial allergic rhinitis.

Our study design was not a double-blind placebo-controlled trial, because, for ethical reasons, it is not possible to perform such a time-consuming clinical trial in Japan. Some factors other than immunotherapy might possibly affect the result, although this was extremely unlikely. A double-blind placebo-controlled study would be necessary to definitely establish the correlation between the effect on T-cell reactivity and the clinical effect of immunotherapy. In addition, it would be necessary for the evaluation of whether the immunotherapy-induced suppression of Th cells was allergen-specific or a general immune suppression to investigate Th-cell responses to irrelevant antigens or nonaspecific stimulation such as tetanus toxoid and phytohemagglutinin (PHA).

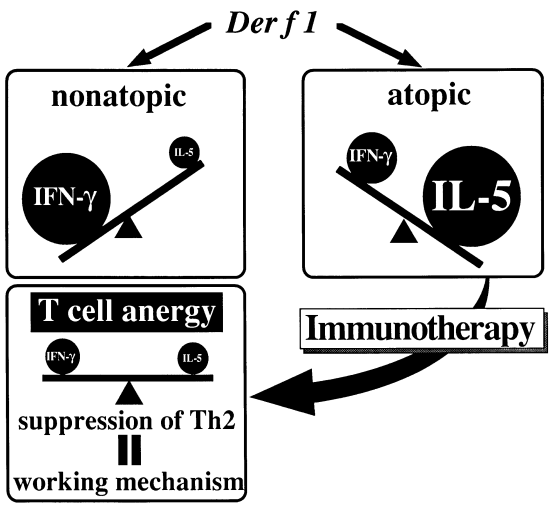

Fig. 5. Schematic presentation of the mechanism of immunotherapy. Immunotherapy can suppress both Th1 and Th2 responses during allergen stimulation, but the suppression of Th2 response is exclusively related to the clinical efficacy of immunotherapy.

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