

The kinetics of change in cytokine production by CD4⁺ T cells during conventional allergen immunotherapy

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Background: The effect of conventional allergen immunotherapy on allergen-specific T lymphocyte cytokine production is incompletely understood, particularly during the initial phase of treatment.

Objective: The purpose of this study was to prospectively follow the kinetics of change in CD4⁺ T cell cytokine secretion during the course of conventional immunotherapy.

Methods: Six allergic individuals were treated with extracts of *Dermatophagoides farinae*/*Dermatophagoides pteronyssinus* or with rye grass pollen (*Lolium perenne*) allergen, but not both, by using an internally controlled conventional immunotherapy protocol. CD4⁺ T cells from peripheral blood were examined in vitro at varying intervals after the initiation of immunotherapy by stimulation with *D farinae* or *L perenne* group I antigen. The quantity of IL-4 and IFN- γ produced and its relationship to clinical improvement was determined.

Results: The ratio of allergen-specific IL-4/IFN- γ production by CD4⁺ T cells from 4 of 6 individuals receiving immunotherapy greatly increased during the period when the dose of allergen was increasing. However, after high-dose maintenance therapy was achieved, this ratio decreased in subjects responding clinically to, but not in those failing, immunotherapy. In addition, late-phase skin reactions and allergen-specific IgE levels in responding, but not in nonresponding, subjects diminished over the course of immunotherapy.

Conclusion: Conventional immunotherapy may initially exacerbate allergic disease by increasing allergen-specific IL-4 and allergen-specific IgE production. Later clinical improvement is associated with a reduction in allergen-specific IL-4 production and in allergen-specific serum IgE. (J Allergy Clin Immunol 1999;103:468-75.)

Key words: Allergy, allergen desensitization, immunotherapy, desensitization, IL-4

Abbreviations used

APC: Antigen-presenting cell

PMA: Phorbol myristate acetate

The clinical efficacy of allergen-specific immunotherapy has been demonstrated in numerous controlled clinical trials.¹⁻⁴ This form of allergen desensitization, in which increasing doses of allergen are administered to allergic individuals, results in reduced symptoms on natural exposure to allergen. Clinical improvement occurs after the subjects receive the highest tolerated subcutaneous (maintenance) dose of allergen.⁵ However, the mechanisms by which immunotherapy accomplishes these effects are still incompletely understood.

We and others have previously shown that clinical improvement in allergic individuals receiving allergen immunotherapy is associated at the completion of therapy with decreased IL-4 production in allergen-specific T_H cells⁶ and by increased synthesis of IFN- γ and IL-2.^{7,8} T_H cells can be segregated into at least 2 effector phenotypes on the basis of their patterns of cytokine production and their function.^{7,9-13} T_{H1} cells produce IL-2 and IFN- γ and are involved primarily in cell-mediated immune responses, whereas T_{H2} cells produce IL-4, IL-5, IL-6, and IL-10 and are responsible primarily for humoral immune responses. Because IL-4 promotes isotype switch to IgE in B cells and IL-5 promotes eosinophil growth and differentiation, T_{H2} cells play a critical role in the pathogenesis of allergic disease by enhancing and prolonging IgE-mediated eosinophilic inflammation. Allergen immunotherapy appears to accomplish its immunomodulatory beneficial effects by converting T_{H2}-dominated immune responses into T_{H1}-dominated responses. However, the kinetics of the cytokine changes during the course of conventional allergen immunotherapy have not been studied.

To monitor cytokine changes induced by immunotherapy, we prospectively followed allergen-specific IL-4 and IFN- γ production by CD4⁺ T cells in subjects receiving immunotherapy with house dust mite or rye grass allergen extracts. Here we confirm that immunotherapy converts the T_{H2} pattern of cytokine synthesis in allergen-specific cells into a predominantly T_{H1} cytokine profile in responding subjects. In addition, we show that in the majority of subjects, IL-4 production in CD4⁺ T cells increased dur-

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TABLE I. Patient characteristics

Subject	Age (y)	Sex	Atopy*	Skin test reagent	Skin testing				Positive MAST-specific IgE
				Treatment antigen control	Before treatment		Maintenance treatment		Before treatment
					Immediate reaction (mm)	Late reaction (mm)	Immediate reaction (mm)	Late reaction (mm)	
1	42	M	AR	Rye grass Histamine	5 × 5 5 × 5	4 × 4 Negative	4 × 6 5 × 5	Negative Negative	Rye grass, <i>D farinae</i> , Bermuda grass, Johnson grass, Dog, Oak
2	36	F	AR	Rye grass Histamine	14 × 32 5 × 5	6 × 6 Negative	Negative 5 × 5	Negative Negative	Rye grass, <i>D farinae</i> , Olive, Oak, Bermuda grass, Russian Thistle
3	27	M	AR	Rye grass Histamine	5 × 10 4 × 4	8 × 11 Negative	5 × 5 4 × 4	Negative Negative	Rye grass, <i>D farinae</i> , Bermuda grass, Oak
4	30	M	AR, AS	<i>D farinae</i> Histamine	5 × 6 5 × 5	3 × 5 Negative	4 × 10 5 × 5	Negative Negative	Rye grass, <i>D farinae</i> , Johnson grass
5	24	M	AR, AS	Rye grass Histamine	7 × 10 6 × 6	6 × 6 Negative	7 × 7 5 × 5	Negative Negative	Rye grass, <i>D farinae</i> , Cat, <i>Alternaria</i> spp
6	46	F	AR, AS	<i>D farinae</i> Histamine	5 × 5 5 × 5	5 × 5 Negative	4 × 8 3 × 3	11 × 11 Negative	Rye grass, <i>D farinae</i> , Johnson grass, Cat, Dog

*Atopic history: AR, allergic rhinitis; AS, asthma.

ing the period when the dose of allergen was increasing and then fell when the highest dose was achieved during the maintenance phase of immunotherapy. In a subject who failed immunotherapy because of repeated adverse reactions associated with increasing allergen doses, the level of allergen-specific IL-4 production remained elevated after more than 1.5 years of therapy.

METHODS

Study population

Six subjects were selected for immunotherapy on the basis of a history of seasonal allergic rhinitis and/or asthma, with positive immediate skin test reactivity to house dust mite antigen (*Dermatophagoides farinae*) and to rye grass pollen allergen (*Lolium perenne*). Immunotherapy was performed according to a standard conventional immunotherapy protocol. The subjects were given subcutaneous injections of aqueous extracts of *D farinae*/*D pteronyssinus* or aqueous extract of grass pollen (ALK Laboratories, Inc, Wallingford, Conn). Doses were increased once or twice weekly until the highest tolerated dose (0.5 mL of 500 to 1000 AU/mL) was reached. The maximum tolerated dose was attained within 6 to 10 months, and weekly injections were continued for the first few weeks after attainment of the maintenance dose. Subjects subsequently received injections of this dose every 2 to 3 weeks for the next few months and then once every 4 weeks. All subjects gave informed consent, and experimental protocols were approved by the Stanford University Administrative Panel on Human Subjects in Medical Research. The characteristics of the subjects are presented in Table I.

Examination of cytokine production

Heparinized peripheral venous blood was collected before treatment and every 2 to 4 months until attainment of the maintenance dose. PBMCs were isolated by centrifugation through Ficoll-Hypaque (Sigma, St Louis, Mo). To obtain a purified CD4⁺ T-cell population, PBMCs were suspended in RPMI-1640 culture media with 1% human albumin at 100 × 10⁶ cells/mL and depleted of

CD8⁺ cells by addition of anti-OKT8 (anti-CD8) mAb followed by the addition of rabbit complement (Pel-Freez, Brown Deer, Wis) as previously described.⁶ Cells treated in this manner were less than 3% CD8 positive and greater than 60% CD4 positive.

Preparation of antigen-presenting cells

Antigen-presenting cells (APCs) were prepared by incubating fresh PBMCs (5 to 8 × 10⁶ cells/mL) in 60-mm plastic dishes in complete media for 1.5 hours at 37°C. Nonadherent cells were removed by gently washing the dishes 3 times with warm RPMI-1640 media containing 5% FCS. The remaining adherent cells were then harvested with cold PBS, washed 3 times, irradiated (2000 rads), and used as APCs.

In vitro antigens

Purified *L perenne* group I antigen (National Institute of Allergy and Infectious Diseases, Rockville, Md) was used at a final concentration ranging from 1 µg/mL to 10 µg/mL. Partially purified house dust mite (*D farinae*) antigen preparation was generously provided by Dr S. Dreborg (Pharmacia, Stockholm, Sweden) at 10⁵ biologic equivalent/mL and was used at a final concentration ranging from 1:100 to 1:1000 (vol/vol). Tetanus toxoid (Connaught Laboratories, Swiftwater, Pa) was used in the range of 0.5 µg/mL to 5 µg/mL in culture.

Cell cultures

CD8-depleted PBMCs were cultured in 24-well plastic dishes at 10⁶ cells/mL (Nunc, Naperville, Ill) in 90% air, 10% CO₂ humidified environment at 37°C with the appropriate antigen (*D farinae* antigen or *L perenne* pollen antigen and tetanus toxoid antigen). To maximize IL-4 production, the cells were washed once on day 6 of culture in PBS and recultured with fresh antigen, 5 U/mL hrIL-2 (Amgen, Thousand Oaks, Calif), and fresh APCs isolated from blood drawn from the original donor on day 6. Between days 6 and 12, cultures were expanded and split to prevent overcrowding as previously described.^{14,15} At day 12 of culture, cells were washed 3 times in PBS and resuspended in complete media at 10⁶ cells/mL with PHA-P (Difco Laboratories Inc, Detroit, Mich) 1:200 (vol/vol)

and 1 ng/mL phorbol myristate acetate (PMA). After 18 hours, supernatants were collected and kept at -80°C until assayed for cytokine content. The validity of this culture system, in which T cells are stimulated several times for optimal induction of IL-4 synthesis and in which IL-4 production reflects the allergic status of the donor, has been demonstrated.^{6,15} This system reflects the fact that IL-4 production in resting T cells requires repeated stimulation with antigen and several cycles of cell division.^{16,17}

Quantitation of cytokines

hIL-4 and hIFN- γ levels in the supernatants were measured by using a sandwich ELISA technique. The antibodies used for the IL-4 assay were hIL-42 and hIL-44 (monoclonal coating antibody; M. Nahm, Washington University, St Louis, Mo), rabbit anti-hIL-4 (second antibody; Genzyme, Cambridge, Mass), and goat anti-rabbit IgG (H+L) (third antibody; Jackson Labs, West Grove, Pa). The antibodies used for the IFN- γ assay were 8700 (monoclonal coating antibody, ATCC), rabbit anti-hIFN- γ (second antibody, Genzyme), and goat anti-rabbit IgG (H+L) (third antibody, Jackson Labs). The lower limit of sensitivity for each of the ELISAs was 78 pg/mL (IL-4) and 25 pg/mL (IFN- γ).

Quantitation of serum IgE

The amount of total IgE in the serum of subjects was determined by ELISA. The ELISA was performed in 96-well microtiter plates (Dynatech Laboratories Inc, Chantilly, Va) coated with 2 anti-human IgE mAbs (1 $\mu\text{g/mL}$; obtained from Dr Ruben Siraganian, National Institute of Dental Health, Bethesda, Md). After washing with PBS with 0.1% Tween 20, the plates were blocked with a 0.1% BSA solution for 1 hour and washed, and 0.1 mL of appropriately diluted serum was added to the wells. After 18 hours, the plates were again washed, and biotinylated goat anti-human IgE (Tago Inc, Burlingame, Calif) was added for 120 minutes. The plates were washed, and horseradish peroxidase-labeled streptavidin (Southern Biotech Associates, Birmingham, Ala) was added for 60 minutes. Bound enzyme was quantitated by using the substrate *o*-phenylenediamine (Sigma Chemical Co) for 10 minutes, and the absorbance at 493 nm was read on a SpectraMax (Molecular Devices Corp, Sunnyvale, Calif) automatic ELISA reader. Standard curves for IgE were performed by using purified IgE (Pharmacia AB). The lower limit of this assay is 0.06 IU/mL.

Quantitation of serum allergen-specific IgE

Serum allergen-specific IgE to different allergens was determined by using the MAST CLA allergy system (MAST Immunosystems, Mountain View, Calif), which is a nonisotopic modification of the RAST method. The MAST CLA allergen-specific IgE assay was performed by filling a MASTpette test chamber with patient serum. IgE in the serum bound to the allergen-coated cellulose threads during incubation. The test chamber was then washed with buffer to remove unbound serum components. Enzyme-labeled anti-IgE was then added to the chamber and coupled with the serum IgE bound to the threads. After a second wash, the test chamber was filled with a photoreagent mixture that reacted with the labeled antibody to produce chemiluminescence. The luminometer measures light emission in luminescence units. The amount of light emitted by each thread was directly proportional to the amount of allergen-specific IgE in the subject serum. The allergens included *D farinae*, Kentucky bluegrass, Johnson grass, Bermuda grass, Acacia, Ash, Birch, Elm, Oak, Olive, Western Ragweed, Cockroach, *Aspergillus* spp, *Cladosporium* spp, and others. For rye grass-specific IgE, subject sera were analyzed by using Pharmacia CAP IgE FE1A (Pharmacia & Upjohn, Kalamazoo, Mich).

RESULTS

Clinical success of specific immunotherapy

The characteristics of subjects treated with allergen immunotherapy are presented in Table I. The mean age of these subjects was 34.1 years (range, 24 to 48 years). For the study, the duration of treatment with allergen immunotherapy ranged from 10 to 53 months. All subjects had a history of allergic rhinitis and/or asthma and exhibited positive immediate skin test reactivity to rye grass (*L perenne*) and house dust mite (*D farinae*) antigens. In addition, all 6 subjects exhibited markedly positive late-phase skin test reactivity to treatment antigens, but not to histamine. Immunotherapy was performed with 2 allergens: house dust mite antigen and rye grass pollen antigen. Each subject received immunotherapy to 1, but not the other, of the 2 study antigens to which they were sensitive. Therefore 4 subjects received rye grass antigen but not house dust mite extract, and 2 subjects received house dust mite extract but not rye grass antigen. All but 1 of the 6 subjects markedly improved after receiving immunotherapy in terms of clinical symptoms. In addition, all of the responder subjects lost late-phase skin test reactivity to the study antigen with which they were treated (Table I). The one nonresponder (subject 6) who did not clinically improve had no change in skin test reactivity to the study antigen. Significant large local reactions to the injections developed in this patient, and the patient had 1 episode of mild anaphylaxis.

Changes in allergen-specific IL-4/IFN- γ ratio during immunotherapy

To investigate the effects of allergen immunotherapy on the cytokines produced by T_H2 cells, $CD4^+$ T cells were isolated from peripheral blood of subjects before and during immunotherapy. The cells were cultured for 12 days with relevant antigens and APCs in a system that we have shown to optimize IL-4 production and in which the quantity of IL-4 produced reflects the allergic status of the donor.^{6,15} After 12 days, culture supernatants were generated and collected and assayed for IL-4 and IFN- γ by ELISA. To normalize cytokine production over the several year time period of the study, we calculated the ratio of IL-4 to IFN- γ found in the culture supernatants.

Table II shows that just before the start of immunotherapy, which was designated phase I, the ratio of IL-4 to IFN- γ produced by $CD4^+$ T cells from the 6 study subjects in response to the treatment allergen ranged from 0.62 to 94.0. The value of the ratio was not always greater than 1 because in our experimental system the final T-cell stimulus was PHA and PMA, which induced IFN- γ not only in allergen-specific T cells but in all T cells. Because IL-4 produced in this system came primarily from allergen-specific T cells, differences in IL-4 production in these cultures reflected the allergic status of the T-cell donor (and the viability of cells in culture). On the other hand, IFN- γ induced by this method varies from person to person, as well as with the viability of the cells in culture, accounting for the large differences in the IL-4 to IFN- γ ratio between

TABLE II. Changes in allergen-specific IL-4/IFN- γ ratio during immunotherapy

Subject no	IL-4/IFN- γ ratio			IT efficacy
	Phase I	Phase II	Phase III	
1	5.08	237	0.37	Response
2	0.62	19.2	0.41	Response
3	1.92	4.89	0.68	Response
4	94.0	6.53	19.5	Response
5	9.16	0.07	0.37	Response
6	1.01	241	9.0	Failure

CD4⁺ T cells (10^6 cells/mL) were stimulated with Lol p 1 (1 to 10 μ g/mL) or *D farinae* antigen (1:100 to 1:1000 vol/vol) for 12 days. IL-4 and IFN- γ were measured in culture supernatants collected 18 hours after stimulation with PHA-P and PMA as described in the Methods section. During phases II and III, 2 to 3 determinations were performed. For phase II, the highest ratio is presented (generally corresponded to when the highest doses were administered). For phase III, the average of 2 to 3 determinations is given. Phase I results for subjects 1 to 5 were significantly different from phase III results (subjects 1 to 5) ($P = .02$, Wilcoxon matched-pair signed-rank test). Phase I was defined as the period immediately before initiation of immunotherapy (IT), phase II included the dose-escalation period, and phase III was defined as the maintenance phase of IT.

individuals. During phase III, the maintenance phase of immunotherapy, the 5 subjects who clinically responded to immunotherapy had significant reductions in the IL-4/IFN- γ ratio in response to the treatment allergen compared with that of the initial phase I ratio ($P = .02$, Wilcoxon matched-pair signed-rank test). These results are consistent with previous studies demonstrating that successful allergen immunotherapy is associated with a decrease in allergen-specific IL-4 production and an increase in allergen-specific IFN- γ production.^{6,7} During phase II of the study, however, when antigen dose escalation was occurring, the IL-4/IFN- γ ratios increased in 4 of the 6 subjects in response to the treatment allergen but declined in the other 2 subjects. One of the subjects whose IL-4/IFN- γ ratio rose had severe allergic reactions on immunotherapy during the escalation phase (subject 6) and did not clinically improve on immunotherapy. In addition, the IL-4/IFN- γ ratio in this subject remained elevated in phase III, as well as compared with that in phase I.

The IL-4/IFN- γ ratio presented in Table II generally reflects the changes in the absolute amount of IL-4 produced in culture, which is shown in Table III. However, the IL-4 data (Table III) are somewhat less reliable than the ratio of IL-4/IFN- γ provided in Table II because the absolute amount of IL-4 and IFN- γ produced in this biologic system varied due to fluctuation in the viability of the cultured cells. Because the IFN- γ that was produced by antigen-nonspecific cells in the culture reflected the viability of these cells, the ratio of IL-4 to IFN- γ normalized the data for comparison over the 1- to 4-year study period. Therefore we believe that the allergen-specific IL-4/IFN- γ ratio (Table II), rather than allergen-specific IL-4 (Table III), reflects the allergic status of our patients and the cytokines produced by allergen-specific CD4⁺ T cells and is useful for tracking the status of cytokine production over the course of the study.

TABLE III. Changes in allergen-specific IL-4 during allergen immunotherapy

Subject no	IL-4 (pg/mL)			IT efficacy
	Phase I	Phase II	Phase III	
1	4560	54,672	5838	Response
2	1381	12,888	1051	Response
3	4998	5391	4598	Response
4	9400	6444	6082	Response
5	15,360	128	1046	Response
6	9800	46,286	18,643	Failure

CD4⁺ T cells (10^6 cells/mL) were stimulated with Lol p 1 (1 to 10 μ g/mL) or *D farinae* antigen (1:100 to 1:1000 vol/vol) for 12 days. IL-4 was measured in culture supernatants collected 18 hours after stimulation with PHA-P and PMA as described in the Methods section. During phases II and III, 2 to 3 determinations were performed. For phase II, the highest ratio is presented (generally corresponded to when the highest doses were administered). For phase III, the average of 2 to 3 determinations is given.

TABLE IV. Changes in the IL-4/IFN- γ ratio during allergen immunotherapy in response to control allergens

Subject no	IL-4/IFN- γ ratio			IT efficacy
	Phase I	Phase II	Phase III	
1	0.67	1314	0.12	Response
2	0.36	0.88	0.20	Response
3	0.43	1.04	0.13	Response
4	1.67	0.07	0.15	Response
5	1.06	0.04	1.52	Response
6	1.17	204	4.58	Failure

Subjects were allergic to the control allergen (either *L perenne* or *D pteronyssinus*) as determined by skin testing, but they were not treated with control allergen. CD4⁺ T cells were stimulated with Lol p 1 (1 to 10 μ g/mL) or *D farinae* antigen (1:100 to 1:1000 vol/vol). IL-4 and IFN- γ were measured as described in the Methods section. Phase I results for subjects 1 to 5 were not significantly different from Phase III results for subjects 1 to 5 (Wilcoxon matched-pair signed-rank test). Phase I results for subjects 1 to 5 compared with phase II results were not significantly different by the same test.

Changes in allergen-specific IL-4/IFN- γ ratio in response to nontreatment allergen

To examine the specificity of changes in cytokine production during allergen immunotherapy, we examined cytokine production in response to an allergen to which the subjects were allergic but with which they were not treated. Because the subjects were not equally allergic to both study allergens, they were generally treated with the allergen to which they had greater symptoms. Therefore the phase I IL-4/IFN- γ ratios for the control allergen were often lower than those for the treatment allergen. Nevertheless, Table IV demonstrates that, in contrast to the results in Table II, the phase III ratio of IL-4/IFN- γ for the nontreatment control allergen was not dramatically reduced from that observed during phase I of immunotherapy ($P > .2$, Wilcoxon matched-pair signed-rank test). Similarly, the phase III ratio of IL-4 to IFN- γ in response to tetanus toxoid was not reduced from that observed during phase I (Table V). This indicated that the allergen-specific IL-4/IFN- γ ratio was reduced at the completion of allergen immunotherapy only when specific allergen was provided during immunotherapy.

TABLE V. Changes in the IL-4/IFN- γ ratio during allergen immunotherapy in response to control antigen tetanus toxoid

Subject no	IL-4/IFN- γ ratio			IT efficacy
	Phase I	Phase II	Phase III	
1	0.41	20.0	0.02	Response
2	0.43	0.54	0.06	Response
3	0.29	1.39	0.33	Response
4	0.37	0.11	0.23	Response
5	0.91	0.01	2.55	Response
6	0.16	171	12.5	Failure

CD4⁺ T cells were stimulated with tetanus toxoid (0.5 to 5 μ g/mL). IL-4 and IFN- γ were measured as described in the Methods section. Phase I results for subjects 1 to 5 were not significantly different from phase II or phase III results for subjects 1 to 5 (Wilcoxon matched-pair signed-rank test).

TABLE VI. Serum total IgE concentration and the efficacy of allergen immunotherapy

Subject no	Serum total IgE (U/mL)			IT efficacy
	Phase I	Phase II	Phase III	
1	766 $\pm$ 6	822 $\pm$ 7	728 $\pm$ 4	Response
2	837 $\pm$ 1	1306 $\pm$ 3	914 $\pm$ 3	Response
3	207 $\pm$ 1	165 $\pm$ 1	133 $\pm$ 1	Response
4	1039 $\pm$ 6	616 $\pm$ 5	1125 $\pm$ 8	Response
5	446 $\pm$ 3	643 $\pm$ 1	1297 $\pm$ 3	Response
6	231.3 $\pm$ 2	94 $\pm$ 3	42 $\pm$ 2	Failure

Patient serum total IgE was analyzed by ELISA. The results are presented as the means $\pm$ SD.

During the phase II dose-escalation period, we observed that the IL-4/IFN- γ ratio for the control allergen and tetanus toxoid increased in 4 of the 6 subjects (3 of 5 responder subjects). This suggests that bystander effects were prominent in these subjects, such that IL-4 produced in response to treatment allergen enhanced IL-4 production in response to nontreatment control allergen and to tetanus toxoid. However, it is possible that the increased IL-4/IFN- γ ratios were in vitro effects due to increased spontaneous IL-4 production in culture, rather than a true in vivo phenomenon. On the other hand, our observations are similar to those described in murine systems, in which true bystander effects occur, such that ongoing T_{H2}-dominated immune responses to 1 antigen enhance T_{H2} cytokine production in response to other antigens that do not ordinarily induce T_{H2} cytokines.¹⁸

Modulation of serum total IgE and allergen-specific IgE

Total and allergen-specific IgE were measured in serum obtained from the subjects before immunotherapy (phase I), during the course of immunotherapy (phase II, during escalation of allergen dose), and while receiving maintenance doses (phase III). There was no correlation with efficacy of allergen immunotherapy and change in the level of serum IgE (Table VI). At the conclusion of the study, serum total IgE concentration was increased in 3 of 5 subjects in the responder group but decreased in 2

subjects. However, the nonresponder subject demonstrated a decrease in total IgE concentration with immunotherapy. The inconsistency with changes in total serum IgE is possibly due to the fact that the study subjects were allergic to multiple allergens and treated for some but not all of these allergens. Alternatively, it is possible that total IgE is not a sensitive parameter that changes with effective immunotherapy, or it is possible that too few subjects were studied.

In contrast to the results with total serum IgE, the results with serum allergen-specific IgE reflected the clinical course in the 2 patients in which serum was available. Thus Fig 1 shows that serum rye grass-specific IgE (treatment allergen) fell significantly during the course of allergen immunotherapy in subject number 1, whereas *D farinae*-specific (nontreatment antigen) IgE did not. This response correlated precisely with the loss of late-phase skin reactivity to the treatment, but not the nontreatment, allergen. In the nonresponder person (subject number 6), serum *D farinae*-specific IgE (treatment allergen) rose during the course of immunotherapy (Fig 2), whereas grass-specific IgE (nontreatment control antigen) did not change after more than a year of maintenance therapy. Unfortunately, sera from other subjects were not available. Nevertheless, the unique internally controlled study design, in which subjects were treated with 1, but not the other, of the 2 study antigens strengthens the results of these 2 subjects. The results thus suggest that allergen-specific IL-4/IFN- γ ratios and allergen-specific IgE decrease over the course of immunotherapy in responder, but not in nonresponder, subjects.

DISCUSSION

A principal finding of this study is that clinical improvement from allergen immunotherapy is associated with a significant reduction in allergen-specific IL-4 production in CD4⁺ T cells and with a reduction in the ratio of allergen-specific IL-4 to IFN- γ produced in culture. Equally important are our findings that during the dose-escalation phase of allergen immunotherapy, the IL-4/IFN- γ ratio increased in 4 of 6 subjects and that untoward allergic reactions resulting in failure of immunotherapy were associated with persistently elevated allergen-specific IL-4/IFN- γ ratios and persistently elevated allergen-specific IgE. In contrast, clinical improvement during the course of immunotherapy was associated with a reversal of, and a decrease in, the elevated allergen-specific IL-4/IFN- γ ratios and with a reduction in allergen-specific IgE. Although our observations involved only a small number of subjects and should be confirmed with studies involving larger numbers of individuals, our unique study design maximized the validity of the observations through the use of internal controls: each subject received immunotherapy for only 1 of the 2 study allergens, although the subjects were all allergic to both allergens.

The immunologic mechanisms by which immunotherapy induces clinical improvement has been studied for

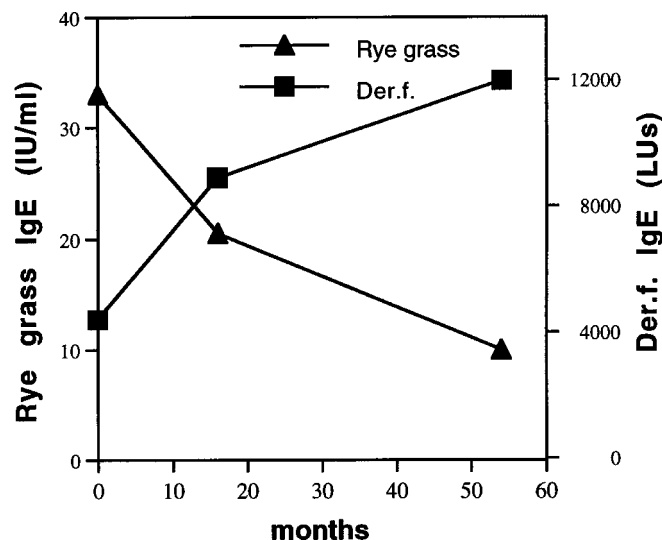


FIG 1. Serum allergen-specific IgE during conventional immunotherapy. Allergen-specific IgE to treatment allergen (rye grass allergen) decreased during the course of therapy in a responder patient (subject number 1). Results represent international units per milliliter by using the Pharmacia CAP IgE FEIA assay. Allergen-specific IgE against a nontreatment allergen *D farinae* (Der. f.) in the same patient rose during immunotherapy. The results represent luminescence units by using the MAST CLA allergy system.

many years.^{19,20} We and others have shown that allergen-specific IL-4 production in T cells from allergic subjects who have completed immunotherapy and are clinically improved is greatly downregulated.^{6,7,12,21-23} These previous studies, however, did not examine the kinetics of change in allergen-induced IL-4 production, particularly during the dose-escalation period of conventional allergen immunotherapy. In addition, allergen-specific IL-4 production has not been studied in patients who fail to improve with allergen immunotherapy. Jutel et al⁷ found that during rush immunotherapy with bee venom, in which maintenance therapy is reached within 4 to 6 hours of initiation of therapy, bee venom-specific IL-4 production fell rapidly (ie, within days).⁷ In contrast, in the majority of our subjects (4 of 6 total subjects and 3 of 5 responder subjects) allergen-specific IL-4 production and IL-4 to IFN- γ ratios initially rose.

In retrospect this rise in the IL-4/IFN- γ ratio during the escalation phase was not surprising because it is known that during this phase of immunotherapy patients frequently develop increased allergic symptoms and have IgE levels that rise initially before falling.^{24,25} Clinical improvement during immunotherapy and reduction in IL-4 production in CD4⁺ T cell cytokine profiles requires administration of high antigen doses of allergen, which clearly can, at least during the dose-escalation phase, increase allergic symptoms and, as we have shown, increase allergen-specific IL-4/IFN- γ ratios. This increase in IL-4/IFN- γ ratios was not observed in patients undergoing rush immunotherapy with bee venom,⁷ perhaps because the immunologic mechanisms that occur during rush immunotherapy differ from those in conventional immunotherapy and may

include apoptosis or anergy, as well as later reversal of cytokine production.^{13,26-28}

Another important observation in this study is that allergen-specific IL-4/IFN- γ ratios remained persistently elevated in one subject (subject 6) who failed allergen immunotherapy because of recurrent systemic and local reactions to injected allergen. This is the first report describing cytokine profiles in a patient who failed immunotherapy. In addition, subject number 6 had increased allergen-specific IgE to the treatment allergen but not to allergens that she did not receive but to which she was allergic (Fig 2). These IgE levels correlated with the increased allergen-specific IL-4/IFN- γ ratios in phase II and phase III (Table II). Although we studied cytokine production in only 1 subject who failed immunotherapy (only 1 subject continued to receive allergen injections in the face of significant local and systemic reactions to the injections), we believe our observations showing increased IL-4/IFN- γ ratios are clinically relevant. Using a unique study design in which each subject had internal controls (each subject received immunotherapy for only 1 of the 2 study allergens), we showed that in the nonresponder subject, IgE and IL-4/IFN- γ levels remained elevated for the treatment, but not for the nontreatment, allergen.

It is not clear why this individual did not respond to immunotherapy and why IL-4/IFN- γ ratios and IgE levels did not fall in this individual. It is possible that in some individuals, deficient production of IFN- γ may be responsible.²⁹ Such reduced IFN- γ production may be secondary to chronic corticosteroid use, which decreases production of IL-12 and results in reduced IFN- γ production³⁰ and which increases the relative frequency of IL-4-producing NK1.1⁺ T cells.³¹ Alternatively, failure

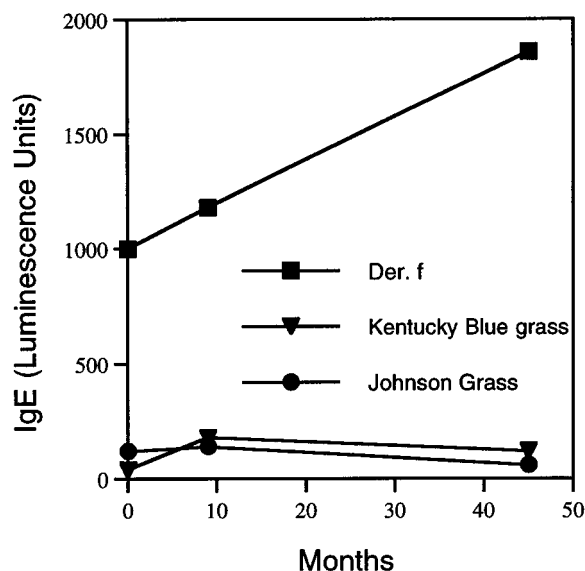


FIG 2. Serum allergen-specific IgE to a treatment allergen, *D farinae* (*Der. f.*), but not to a nontreatment allergen, *L perenne* (*Lol. p.*), increased during conventional immunotherapy in the nonresponder subject (subject number 6). Results represent luminescence units by using the MAST CLA allergy system.

of allergen immunotherapy may be secondary to suppression of IL-12 production by high levels of histamine³² or secondary to fixation of T-cell cytokine profiles in long-standing chronic allergic disease, or it may be due to a failure of immunotherapy to promote the expansion of regulatory T cells producing transforming growth factor (TGF)- β .^{33,34} In any case, conventional immunotherapy fails in 5% to 10% of patients, and improved, more efficacious methods of immunotherapy are required for allergic disease. Such novel methods might include allergen IL-12 fusion proteins³⁵ or naked DNA containing cDNA for allergen.³⁶⁻³⁸ The need for more efficacious therapies is particularly important because allergen immunotherapy is the only therapy available for allergic disease that actually changes the underlying immunology and changes the course of the disease. Thus the beneficial effects of immunotherapy persist for years after discontinuing therapy, whereas symptom-reducing medications that block the effects of mediators or cytokines (eg, corticosteroids, antihistamines, or antileukotrienes) require chronic administration.

The results of our study also suggest that elevated IL-4 production induced by allergen immunotherapy has bystander effects. Thus the administered allergens, particularly during the dose-escalation phase, enhanced IL-4 production in responding T cells but may also increase IL-4 production in T cells that respond to nontreatment allergens/antigens. For example, elevated IL-4 production induced during dose-escalation phase II for rye grass in subject 1 resulted in enhanced IL-4 production in T cells responding to dust mite allergen and to tetanus toxoid, antigens that were not used for injection therapy. To our knowledge, this is the first report to suggest that

bystander effects may occur during immunotherapy. The implications of these findings are that allergic reactions to one antigen may lead to allergic, T_H2 -dominated responses to other antigens, as has been suggested by studies in murine systems.¹⁸ The IL-4-enhancing bystander effect appeared to be present only during the dose-escalation phase and was present only in some subjects.

In conclusion, we found that during the course of successful immunotherapy, the ratio of IL-4 to IFN- γ produced in response to allergen increased in most subjects when the allergen dose was escalating but fell after the maintenance phase of therapy was achieved. We observed bystander effects during the escalation phase in which IL-4 produced by T cells responding to the treatment allergen may have enhanced IL-4 production in T cells responding to nontreatment allergens/antigens. Furthermore, unsuccessful immunotherapy was associated with a persistently elevated IL-4/IFN- γ ratios and persistently elevated allergen-specific IgE. To confirm these findings, larger randomized controlled trials must be performed.

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