Allergen Immunotherapy Decreases Interleukin 4 Production in CD4+ T Cells from Allergic Individuals

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Summary

Allergen specific CD4+ T cell clones generated from allergic individuals have been shown to produce increased levels of the cytokine interleukin 4 (IL-4), compared to allergen specific clones generated from nonallergic individuals. This difference between CD4+ T cells from allergic and nonallergic individuals with regard to cytokine production in response to allergen is thought to be responsible for the development of allergic disease with increased IgE synthesis in atopic individuals. We examined the production of IL-4 in subjects with allergic rhinitis and in allergic individuals treated with allergen immunotherapy, a treatment which involves the subcutaneous administration of increasing doses of allergen and which is highly effective and beneficial for individuals with severe allergic rhinitis. We demonstrated that the quantity of IL-4 produced by allergen specific memory CD4+ T cells from allergic individuals could be considerably reduced by in vivo treatment with allergen (allergen immunotherapy). Immunotherapy reduced IL-4 production by allergen specific CD4+ T cells to levels observed with T cells from nonallergic subjects, or to levels induced with nonallergic antigens such as tetanus toxoid. In most cases the levels of IL-4 produced were inversely related to the length of time on immunotherapy. These observations indicate that immunotherapy accomplishes its clinical effects by reducing IL-4 synthesis in allergen specific CD4+ T cells. In addition, these observations indicate that the cytokine profiles of memory CD4+ T cells can indeed be altered by in vivo therapies. Thus, the cytokine profiles of memory CD4+ T cells are mutable, and are not fixed as had been suggested by studies of murine CD4+ memory T cells. Finally, treatment of allergic diseases with allergen immunotherapy may be a model for other diseases which may require therapies that alter inappropriate cytokine profiles of memory CD4+ T cells.

Although allergen immunotherapy is recognized as highly effective in the treatment of patients with severe allergic rhinitis (1–4), the specific immunologic mechanism(s) by which immunotherapy achieves its effects has not been fully elucidated. Several studies have demonstrated that immunotherapy, which involves the subcutaneous administration of increasing doses of allergen, induces a rise in IgG blocking antibodies, particularly of IgG4 (3, 5). Other studies have demonstrated that CD8+ suppressor cells are generated during immunotherapy which modulate allergen-specific IgE production (6). However, both of these observations remain controversial.

Recent advances in our understanding of the immunological basis of allergic disease suggest that the production by CD4+ T cells of increased quantities of cytokines such as IL-4, which is critical in the induction of IgE synthesis (7–9), and IL-5, which is an important eosinophil differentiation factor (10–12), determines whether allergic disease develops in a given individual (13). Thus, it has been demonstrated that allergen-specific CD4+ T cells isolated from patients with allergic diseases such as asthma and allergic rhinitis produce higher levels of IL-4 and IL-5 compared to allergen-specific CD4+ T cells obtained from nonallergic individuals (14–18). The purpose of our study therefore was to investigate whether allergen immunotherapy in allergic individuals causes a reduction in the amount of IL-4 produced by allergen specific CD4+ T cells.

Materials and Methods

Study Population. 10 patients on immunotherapy were studied, and all had histories of severe seasonal allergic rhinitis before the start of immunotherapy, with positive immediate skin test reactivity to dust mite antigen *Dermatophagoides farinae* (*Der. f.*)[1]

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[1] *Abbreviations used in this paper: Der. f., Dermatophagoides farinae* (dust mite); *Lol. p., Lolium perrene* (rye grass pollen); SI, stimulation index; TT, tetanus toxoid.

and/or to rye grass pollen allergen *Lolium perenne* (*Lol. p.*) before initiation of immunotherapy. These patients were treated with aqueous extracts of *Dermatophagoides farinae/Dermatophagoides pteronyssinus* and/or aqueous extracts of grass pollen administered subcutaneously, and were receiving maintenance (highest) doses of allergen (500–1,000 Au/dose) once every 4–6 wk. 10 allergic, untreated, matched control subjects had a similar history of severe seasonal allergic rhinitis, as well as positive skin test reactivity to the same allergens. All patients gave informed consent, and experimental protocols were approved by the Stanford University Administrative Panel on Human Subjects in Medical Research.

Symptom Scoring. Allergy symptoms of the subjects were scored in terms of (*a*) episodes of sneezing and/or nasal pruritis, (*b*) rhinorrhea and/or nasal blockage, and their frequency of medication use, both (*c*) oral and (*d*) topical nasal spray medication. A score between 0 and 10 was given for each parameter and the total symptom score represented the sum of the individual scores. Patients on immunotherapy were evaluated with both a pre-therapy and current symptom score whereas untreated control allergic donors had only current symptoms evaluated.

Antigens. Allergenic extract of *Lol. p.* pollen (Berkeley Biologicals, Berkeley, CA), or purified *Lol. p.*, group 1 antigen (NIAID, Bethesda, MD) was used at a final concentration of 50 μg/ml. Partially purified house dust mite (*Der. f.*) antigen preparation was generously provided by S. Dreborg (Pharmacia; Stockholm, Sweden) at 10^5 BE/ml, and was used at a final concentration of 1:100 (vol/vol) in culture. The tetanus toxoid (TT) antigen preparation (Mass. Dept. of Public Health) was used in culture at 5 μg/ml.

Quantitation of Serum IgE. The amount of total IgE in the serum of subjects was determined by ELISA, as previously described (9).

Proliferation Assay. PBMC were isolated from heparinized blood by flotation over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO), as previously described (9). PBMC (2 × 10^5/well) from each donor were cultured in triplicate in 96-well plates in media alone or with appropriate concentrations of antigen. At day 5, cultures were pulsed with [^{3}H]thymidine and harvested 18 h later. Incorporation of [^{3}H]thymidine (cpm) was then determined by scintillation counting.

Depletion of CD8-Positive Cells. PBMC were suspended in RPMI-1630 at 12.5 × 10^6 cells/ml and depleted of CD8$^+$ cells by addi-

Table 1. *Patient Characteristics*

Donor #	Yrs on therapy	Age	Sex	Atopic status	Skin test reactivity			
					Immediate		Late-phase	
					Lol.p.	*Der.f.*	*Lol.p.*	*Der.f.*
Allergic untreated controls								
1	0	21	M	rhinitis	+	−	−	na
2	0	41	M	rhinitis, dermatitis	+	+	+	+
3	0	24	M	rhinitis	−	+	na	±
4	0	45	F	rhinitis, asthma	+	+	+	+
5	0	21	F	rhinitis	+	−	+	na
6	0	32	M	rhinitis	+	−	+	na
7	0	23	M	rhinitis	+	−	+	na
8	0	17	F	rhinitis, asthma	−	+	nd	nd
9	0	36	F	rhinitis	+	−	+	na
10	0	42	F	rhinitis	+	−	+	na
	mean age = 30.2 years							
Immunotherapy patients								
1	0.9	37	F	rhinitis	+	+	nd	nd
2	2	29	F	rhinitis	+	+	−	−
3	3	41	F	rhinitis	+	+	−	−
4	3	33	F	rhinitis, asthma	+	+	−	−
5	4	44	M	rhinitis	+	+	−	−
6	8	39	M	rhinitis, asthma	+	+	−	−
7	3	18	M	rhinitis, asthma	+	+	−	−
8	17	44	F	rhinitis, dermatitis	+	+	−	−
9	7	18	M	rhinitis, asthma	+	+	nd	nd
10	3	36	M	rhinitis, asthma	+	+	±	±
	mean age = 33.9 years							

na, not applicable
nd, not done

tion of anti-OKT8 (anti-CD8) mAb followed by the addition of rabbit complement (Pel-Freez; Brown Deer, WI) as previously described (9). Cells treated in this manner were <3% CD8 positive and >60% CD4 positive.

Preparation of Antigen-presenting Cells. APC were prepared by incubating fresh PBMC (5–8 × 10⁶ cells/ml) in 60-mm plastic dishes in complete medium for 2 h at 37°C. In some experiments, PBMC which were previously frozen and stored in liquid N_2, were thawed and used as a source to isolate APC. Nonadherent cells were removed by gently washing the dishes three times with warm RPMI 1630 media containing 5% FCS. The remaining adherent cells were then harvested with cold PBS, washed three times, irradiated at 2,500 rad and used as APC.

Cell Cultures. CD8-depleted PBMC were cultured in 24-well plastic dishes at 10⁶/ml (Nunc, Naperville, IL) in 90% air, 10% CO_2 humidified environment at 37°C with the appropriate antigen: TT, dust mite antigen, or rye grass pollen antigen, as previously described (17). At day 7 of culture, cells were washed once in PBS and recultured with fresh APC, fresh antigen, and 10 U/ml hrIL-2 (Amgen, Thousand Oaks, CA). Between days 7 and 14, cultures were expanded as necessary. Previous studies (17, and Secrist, H., unpublished observations) have demonstrated that antigen/allergen specific CD4⁺ cells are preferentially expanded in such cultures. At day 14 of culture, cells were washed three times in PBS and resuspended in complete medium at 2 × 10⁶/ml with PHA-P (Difco Laboratories Inc., Detroit, MI; 1:200 [vol/vol]) and 1 ng/ml of PMA. After 18 h, supernatants were collected and kept at −80°C until assayed for cytokine content.

Quantitation of Cytokines. hIL-2 was measured by biological assay, using the murine indicator HT-2 cell line (generously provided by Dr. Sam Strober, Stanford), as previously described (9). hIL-4 and hIFN-γ were quantified by ELISA, as previously described (17).

Results

Clinical Efficacy of Immunotherapy Based on Skin Test Reactivity and Symptom Scoring. The characteristics of patients on allergen immunotherapy and untreated allergic control subjects are presented in Table 1. The mean age of immunotherapy patients and the untreated controls was 34 and 30, respectively. The duration of immunotherapy for patients ranged from 1 to 17 yr (mean, 5 yr). All donors exhibited positive immediate skin-test reactivity to appropriate allergens. None of the immunotherapy patients exhibited late-phase skin test reactivity (19), which is known to diminish with immunotherapy (20), whereas seven of the nine untreated allergic subjects tested exhibited positive late-phase skin test reactions (Table 1). Subjects in both treated and untreated groups had similar pre-therapy symptom scores, and those on immunotherapy treatment exhibited a marked decrease in allergy symptoms (Fig. 1). Donors were matched for serum IgE levels (Fig. 2), using data obtained currently for all subjects (post-therapy levels for immunotherapy patients), with untreated allergic controls having a mean IgE level of 271 IU/ml, and the treated immunotherapy patients having a mean level of 340 IU/ml. Subjects with serum IgE levels <50 IU/ml were excluded from study to ensure that selected individuals had severe allergic disease.

Proliferative Responses of PBMC to Allergen and Nonallergen. The results of proliferation studies of freshly isolated PBMC from immunotherapy patients and untreated allergic controls
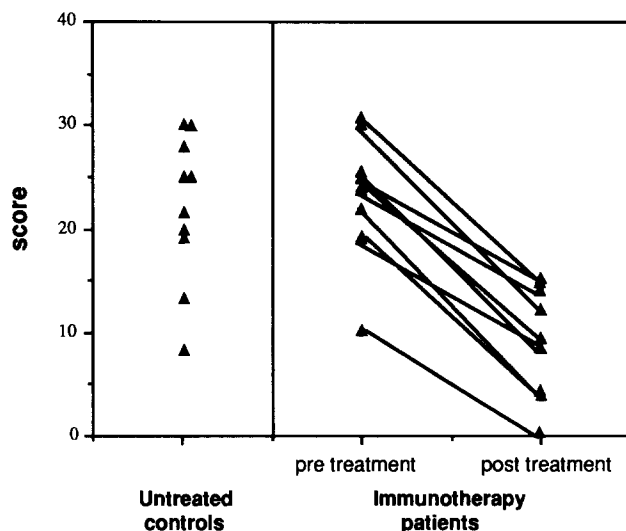

Figure 1. Symptom scores of untreated allergic control subjects and of immunotherapy treated patients. Derivation of scores is as described in the Materials and Methods. The mean score ± SE in the untreated control group was 22.1 ± 2.33, while the mean score of the immunotherapy group was 22.9 ± 1.9 (before therapy) and 9.1 ± 1.6 (post therapy).

in response to *Der. f.*, and *Lol. p.* antigens are presented in Fig. 3 *A*. Although the mean stimulation index to allergen of immunotherapy patients was somewhat lower than that of the untreated controls, as has been previously demonstrated (21), allergen-specific T cells were present in both groups of donors. PBMC from subjects who were not allergic to one of the two allergens, as assessed by immediate skin test reactivity, did not proliferate to that allergen (stimulation index ≤1) (Fig. 3 *A*).

Proliferative responses of PBMC from subjects to TT were also assessed (Fig. 3 *B*). The mean stimulation indices (31 ± 9.7 for untreated control allergic subjects vs. 24.3 ± 4.6
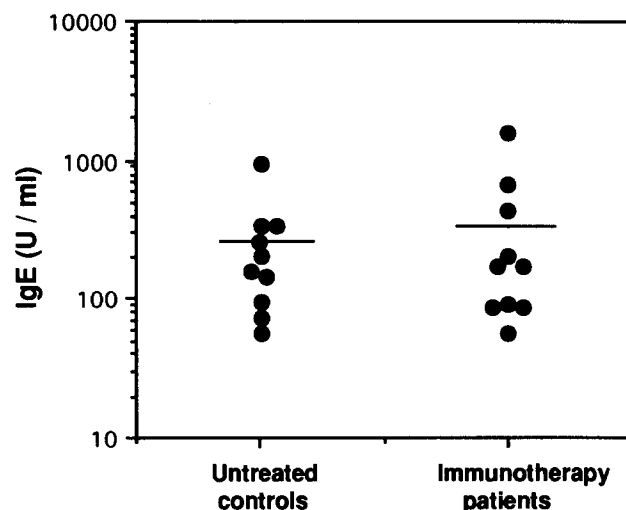

Figure 2. Serum IgE levels of untreated allergic control subjects and of immunotherapy treated patients. Mean IgE levels of the two groups were 271 ± 85 and 340 ± 140 (*P* >0.1, Mann-Whitney test), respectively.
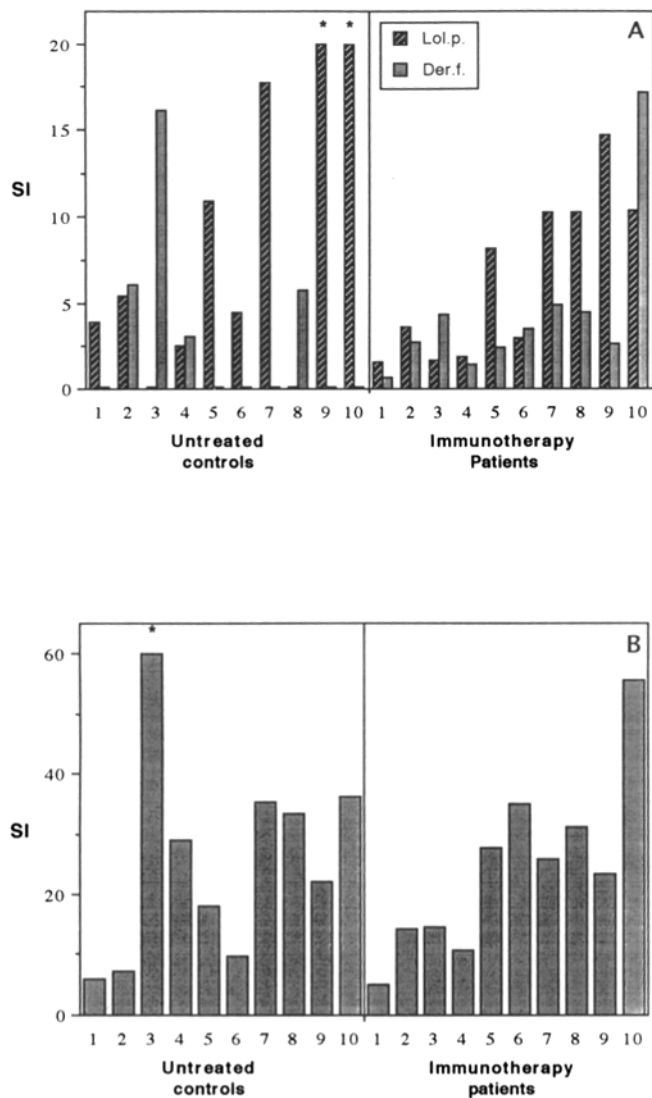
in vitro requires two stimulations with antigen/allergen (17), the CD8-depleted PBMC were stimulated twice with allergen, after which the cytokine profile of the cells was determined. As depicted in Fig. 4 A, CD8-depleted PBMC from untreated allergic controls produced significantly more IL-4 in response to allergen (7,919 ± 2,480 pg/ml) than that of patients on immunotherapy (477 ± 169) (P <0.002). Interestingly, with one exception, immunotherapy patients exhibiting IL-4 levels >300 pg/ml (range: 528-2,090 pg/ml) were on immunotherapy for the least amount of time (1-3 yr) whereas patients demonstrating IL-4 levels below 300 pg/ml were on
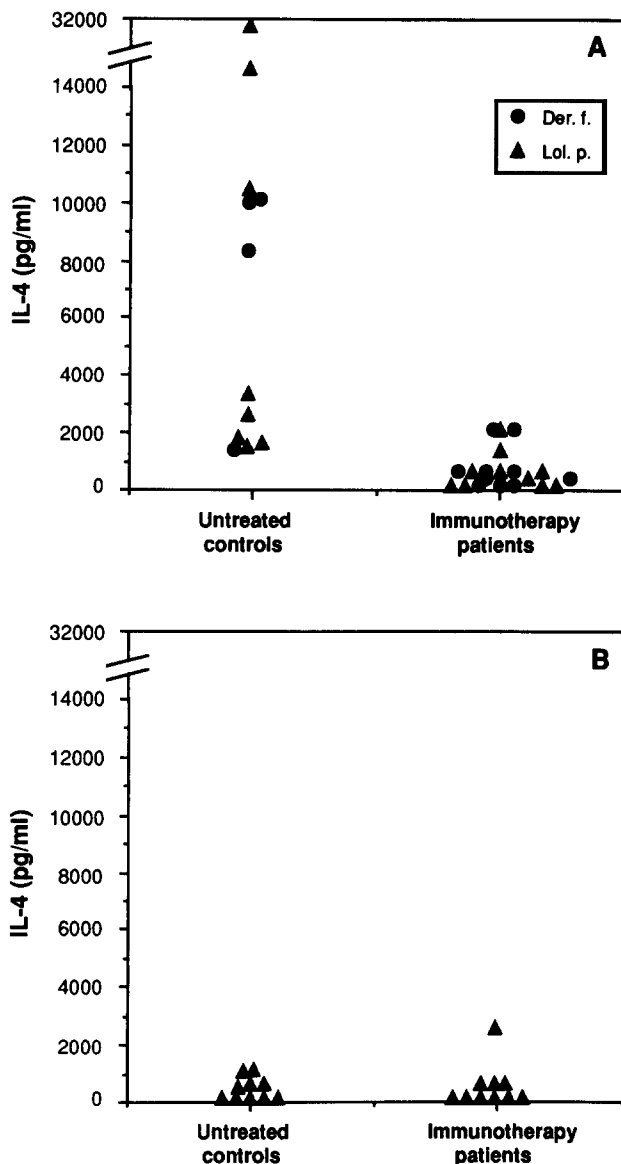
Figure 3. Proliferative responses of PBMC to allergen (*A*) and to the nonallergen TT (*B*). Values are presented as the stimulation index (*SI*), calculated by dividing the cpm of antigen-stimulated cultures by the cpm of unstimulated cultures. Mean SI ± SE for the untreated group was 12.1 ± 3.7 and for the immunotherapy group 5.5 ± 1.0 in response to allergen (*P* <0.04, Mann-Whitney test) while the mean SI for the untreated group in response to TT was 31 ± 9.7 and for the immunotherapy group was 24.3 ± 4.6 (*P* >0.3, Mann-Whitney test). (*) Actual values for untreated control donors #9 and #10 in response to allergen are 47 and 22.6, respectively. Actual value for untreated control donor #3 in response to TT is 112.2.

for immunotherapy patients) were not significantly different between the two groups of subjects, although the proliferative responses to TT were generally greater than that to allergens.

Patients on Immunotherapy Exhibit Decreased Production of IL-4 in Response to Allergen. To directly examine cytokine production by CD4⁺ T cells in untreated control subjects and immunotherapy patients, PBMC were depleted of CD8⁺ T cells and cultured with allergen (*Der. f., Lol. p.*) or TT. Since we have previously shown that the peak production of IL-4

Figure 4. IL-4 production in response to allergen (*A*) and to the nonallergen TT (*B*). CD8-depleted cells were stimulated with allergen or antigen on day 0, and restimulated on day 7 with the same allergen/antigen. On day 14, the cultures were harvested, and the cytokine profiles of cells determined. Mean IL-4 production was 7,919 ± 2,480 pg/ml in the untreated control group and 477 ± 169 pg/ml in the immunotherapy group (*P* <0.002, Mann-Whitney test).

immunotherapy for more than three years. In other words, IL-4 levels were inversely related to the length of time on immunotherapy.

The PBMC from the untreated control donors produced significant quantities of IL-4 in response only to allergens to which they were allergic. Thus, little or no IL-4 was produced in response to allergens when allergen-specific IgE was absent in the sera of the donor (negative immediate skin test), or in response to a control nonallergenic antigen, TT (Fig. 4 *B*, and reference [17]). The low but detectable levels of IL-4 from CD8-depleted PBMC in response to TT were comparable in both donor groups (Fig. 4 *B*), and were not statistically different from the IL-4 levels produced by CD8-depleted PBMC from immunotherapy patients in response to allergens. This indicated that immunotherapy could reduce allergen-specific IL-4 synthesis to levels observed with nonallergenic antigens.
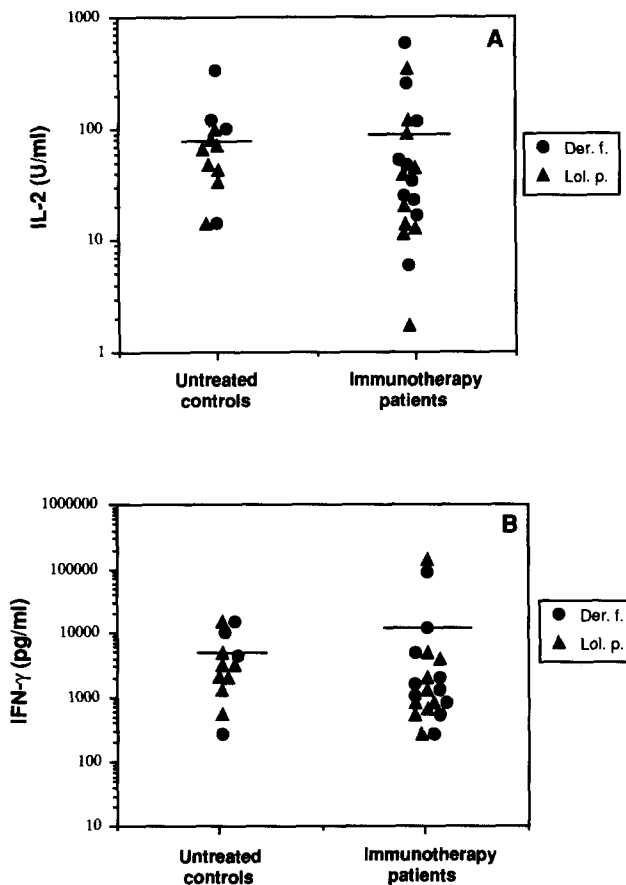

Figure 5. IL-2 production (*A*) and IFN-γ production (*B*) in response to allergen. Cultures were performed as described for Fig. 4. Mean IL-2 production was 81 ± 23 U/ml in the untreated control group and 88 ± 30 U/ml in the immunotherapy group (*P* >0.3, Mann-Whitney test). Mean IFN-γ production was 5,069 ± 1,577 pg/ml in the untreated control group and 12,320 ± 7,050 pg/ml in the immunotherapy group (*P* >0.1, Mann-Whitney test).

IFN-γ and IL-2 Production Is Not Significantly Altered in Immunotherapy Patients. Since in some murine models the level of IL-4 production was inversely related to the level of IFN-γ production (22, 23), we next asked whether the decrease in allergen-specific IL-4 production demonstrated in immunotherapy patients was accompanied by a corresponding increase in allergen-specific IL-2 and IFN-γ production. As demonstrated in Fig. 5, production of IL-2 and IFN-γ by CD8-depleted cells from both untreated control and immunotherapy patients was variable, but no statistical differences (*P* >>0.1) were demonstrated in IL-2 or IFN-γ production between the two groups. The amount of IFN-γ produced by allergen stimulated cultures was similar to that observed in response to TT. Thus, while immunotherapy treatment specifically downregulates IL-4 production in response to allergenic stimulation, there was no discernable effect on the production of IL-2 and IFN-γ.

Discussion

We demonstrated that treatment of individuals suffering from allergic rhinitis with allergen immunotherapy was associated with a significant reduction in allergen induced IL-4 synthesis in vitro. The reduced IL-4 synthesis in vitro from PBMC of immunotherapy patients was allergen-specific and a consistent finding associated with improved clinical symptoms. The subjects in our patient and untreated control groups were closely matched for symptom severity before immunotherapy as well as for total IgE in serum. Patients on immunotherapy with low total IgE in serum were not included in our study, even though such levels have been reported to decrease over time with immunotherapy (24, 25). We conclude therefore from our retrospective study, that immunotherapy accomplishes its clinical effects by reducing IL-4 synthesis in allergen specific CD4$^+$ T cells to levels that approximate those from nonallergic subjects, or to levels observed in response to nonallergens such as TT. Consistent with this idea is our observation that IL-4 production by T cells from individuals on immunotherapy varies inversely with the length of time on immunotherapy (Fig. 4, and Secrist, H., and C.J. Chelen, manuscript in preparation). However, a prospective placebo controlled study examining cytokine production in CD4$^+$ T cells before, during, and after immunotherapy is necessary to confirm our results.

The profile of cytokines produced by antigen-specific CD4$^+$ T cells has been proposed to determine the type or form of the immune response that occurs in vivo. For example, protective immunity against intracellular pathogens (e.g., *Mycobacteria*) requires the development of Th cells with cytokine profiles (i.e., IL-2 and IFN-γ synthesis [Th1 profile] [26–29]) that are different from the profiles required for protective immunity against extracellular bacteria (e.g., *Streptococcus pneumonia*, requiring IL-4 and IL-5 synthesis [Th2 profile] [9, 26, 30–32]). It has been proposed that the development of inappropriate cytokine profiles gives rise to abnormal (nonprotective) immunity resulting in the dissemination of infection, or in the development of allergic disease. The idea that disease may be caused by the development

of inappropriate cytokine profiles and inappropriate forms of immunity, suggests that therapies for such diseases might involve modification of the cytokine profile of primed or memory T cells. In a number of model systems in mice, treatment of the mice before immunization or infection with "immune response modifying agents", such as cytokines or anticytokine monoclonal antibodies, have been shown to indeed alter the profile of cytokines that develop in such unprimed T cells (22, 29, 33). Although such agents (e.g., anti-IL-4 mAb or anti-IL-4 receptor mAb) have been proposed for the treatment of allergic diseases (8, 34), it appears that the cytokine profiles of memory CD4$^+$ T cells (as well as sIgE$^+$ B cells) are resistent to modification with such agents (35, 36). Nonetheless, our study of allergen-specific CD4$^+$ T cells from allergic individuals demonstrates that allergen specific IL-4 synthesis can be inhibited with immunotherapy, and that immunotherapy, in contrast to treatment with anti-IL-4 mAb, can indeed alter the cytokine profiles of memory T cells.

Although all of our patients on immunotherapy continued to have positive immediate (IgE mediated) skin tests to allergens, it is likely that the production of allergen-specific IgE diminished as the amount of allergen-specific IL-4 synthesis decreased with immunotherapy. We believe this to be the case because none of the immunotherapy patients had persistence of allergen-specific late phase cutaneous responses (19, 20), and because the production of smaller quantities of IL-4 was associated with lower levels of allergen specific IgE in the serum of the T cell donor (data not shown).

Further studies are in progress to determine the precise mechanisms by which immunotherapy modifies cytokine synthesis in CD4$^+$ T cells. Since natural exposure to allergen occurs generally by inhalation whereas treatment with immunotherapy involves subcutaneous administration of allergen,

it has been postulated that it is the route of antigen exposure rather than the physical-chemical structure of the allergen that significantly affects the cytokine profiles that develop in antigen/allergen specific T cells (37). In addition, since the amount of allergen administered during immunotherapy is much greater than the amount inhaled, the antigen dose may also affect the cytokine profile, possibly by altering the type of APC or monokines involved (38–41). As shown by our proliferation studies, allergen specific CD4$^+$ T cells remained in the peripheral blood of immunotherapy patients, and therefore such cells appeared not to be deleted or tolerized during immunotherapy. Although our study suggests that immunotherapy actively modifies the cytokines produced by allergen-specific CD4$^+$ T cells, it is not clear whether immunotherapy alters the profile of a currently existing CD4$^+$ (or CD8$^+$) T cell population or expands an entirely new population of CD4$^+$ T cells.

Although there have been considerable recent improvements in the symptomatic treatment of patients with allergic rhinitis with the availability of nonsedating H1 histamine antagonists and nasal corticosteroids, treatment with immunotherapy, which appears to alter the underlying immunological basis of the disease in an allergen-specific way, may be in the long run a more beneficial modality. Studies such as ours, which are directed at understanding the underlying mechanisms of immunotherapy, are likely to lead to modifications in the technique, resulting in methods that are even safer and more effective in achieving clinical and immunological effects. Furthermore, immunotherapy with modified forms of antigen may be applicable to other diseases in which inappropriate cytokine profiles in memory CD4$^+$ T cells require alteration.

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