

Grass Pollen Immunotherapy Induces Mucosal and Peripheral IL-10 Responses and Blocking IgG Activity¹

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T regulatory cells and IL-10 have been implicated in the mechanism of immunotherapy in patients with systemic anaphylaxis following bee stings. We studied the role of IL-10 in the induction of clinical, cellular, and humoral tolerance during immunotherapy for local mucosal allergy in subjects with seasonal pollinosis. Local and systemic IL-10 responses and serum Ab concentrations were measured before/after a double-blind trial of grass pollen (*Phleum pratense*, Phl P) immunotherapy. We observed local increases in IL-10 mRNA-positive cells in the nasal mucosa after 2 years of immunotherapy, but only during the pollen season. IL-10 protein-positive cells were also increased and correlated with IL-10 mRNA⁺ cells. These changes were not observed in placebo-treated subjects or in healthy controls. Fifteen and 35% of IL-10 mRNA signals were colocalized to CD3⁺ T cells and CD68⁺ macrophages, respectively, whereas only 1–2% of total CD3⁺ cells and 4% of macrophages expressed IL-10. Following immunotherapy, peripheral T cells cultured in the presence of grass pollen extract also produced IL-10. Immunotherapy resulted in blunting of seasonal increases in serum allergen Phl p 5-specific IgE, 60- to 80-fold increases in Phl p 5-specific IgG, and 100-fold increases in Phl p 5-specific IgG4. Post-immunotherapy serum exhibited inhibitory activity, which coeluted with IgG4, and blocked IgE-facilitated binding of allergen-IgE complexes to B cells. Both the increases in IgG and the IgG “blocking” activity correlated with the patients’ overall assessment of improvement. Thus, grass pollen immunotherapy may induce allergen-specific, IL-10-dependent “protective” IgG4 responses. *The Journal of Immunology*, 2004, 172: 3252–3259.

Specific allergen immunotherapy (IT)³ is an effective prophylactic treatment for atopic IgE-mediated disease, in particular for severe seasonal allergic rhinitis (1–4). IT is also indicated in patients who develop anaphylaxis due to venom of stinging insects (5, 6). A variety of immunologic changes have been reported following IT. These include a modest reduction in serum allergen-specific IgE (7) and increases in allergen-specific IgG, particularly of the IgG4 isotype (8–10). Effector cells including eosinophils, mast cells, and basophils are reduced at allergic mucosal sites (11). There is also suppression of allergen-induced mediator secretion and reduced basophil reactivity to allergen (12). Given the importance of Th2-type (Th) cells in directing allergic responses (13), there has been particular interest in modification of T cell responses following IT. Some (14–17), but not all (18), studies have described decreased peripheral blood T cell responses to allergen and/or immune deviation following treatment. Following IT with grass pollen extracts, allergen provocation of the skin and nasal mucosa before/after IT has been associated with enhanced IFN- γ to allergens, suggesting that IT modifies the local

mucosal T lymphocyte response with preferential deviation of Th2 in favor of Th1 responses (18–21).

The inhibitory cytokine IL-10 has been shown to block costimulatory signaling via CD28 in T cells, an event which may lead to T cell unresponsiveness (anergy) (22). IL-10 also favors B cell switching to IgG4 in the presence of IL-4 (23). Venom IT has been linked to the induction of a population of peripheral blood T cells that produce IL-10 on restimulation in vitro with venom allergen (24). Neutralization of this endogenous IL-10 in vitro restored venom-induced T cell proliferation and cytokine production that had been inhibited following IT. These observations are reminiscent of those reported in murine models of respiratory allergy (25), delayed-type hypersensitivity (26), and graft rejection (27), where “regulatory” T cells, which produce IL-10 and TGF- β have been associated with suppression of immunopathology.

Allergic rhinitis and asthma, unlike venom hypersensitivity, are primarily mucosal diseases characterized by inhaled allergen-dependent chronic inflammation of the upper and lower respiratory tract. These conditions are often amenable to topically applied treatment such as corticosteroids, which have profound immunosuppressive activity on nasal mucosal responses to allergen (28, 29). In light of these studies, we hypothesized that during successful grass pollen IT the enhanced production of IL-10 locally within the nasal mucosa results in either down-regulation of allergen-specific Th2 T lymphocyte responses (11, 17) and/or immune deviation in favor of Th1 responses (18–20).

In a double-blind placebo-controlled trial of grass pollen IT (4), nasal mucosal biopsies were collected before and after 2 years of treatment. Biopsies were examined for IL-10 mRNA-expressing cells by in situ hybridization and IL-10 protein-positive cells by immunohistochemistry. We also examined the effects of IT on IL-10 responses by cultured PBMC in response to grass pollen allergen.

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³ Abbreviations used in this paper: IT, immunotherapy; Phl p, *Phleum pratense*.

Finally, to examine the effects of IT on possible IL-10-driven effector responses *in vivo*, sera from IT patients were tested for allergen-specific IgG and for the presence of Abs with "blocking" activity against the binding of allergen-IgE complexes to B cells. B cells are known to facilitate preferential Th2 (and hence IgE) responses to allergens, a putative mechanism being the IgE-facilitated CD23-dependent presentation of allergen (following binding and internal processing) to T cells (30).

Materials and Methods

Participants

All subjects had severe seasonal rhinitis and a positive skin test reaction (wheal >5 mm) to *Phleum pratense* (Phl p) (AquaGen ALK, Abelló Hørsholm, Denmark) as previously described (4). Thirty-seven of the 44 participants consented to nasal biopsy before/after 2 years of IT. IT- and placebo-treated patients were matched for gender (male, 10/20; female, 9/17), age (median, 32 years; range, 26–36 years; median, 34 years; range, 29–34 years), and skin prick test responses to grass pollen extract (median, 51 mm²; range, 33–62 mm²; median 53 mm², range 27–66 mm²), respectively. Control biopsies were obtained during the pollen season from 9 non-atopic healthy control subjects. The Ethics Committee of the Royal Brompton Hospital approved the study and all subjects gave written informed consent.

Study design

The details of the IT protocol and the clinical response to treatment have previously been reported (4). Briefly, active treatment involved a modified "cluster" regimen of injections with a standardized aluminum hydroxide-adsorbed extract of Phl p (Alutard SQ; ALK Abelló). The injections were given twice a week for 4 wk, between October 1996 and January 1997, followed by monthly depot maintenance injections until December 1998. Maintenance injections of 1 ml contained 100,000 standard quality units, which included 20 µg of the major grass pollen allergen Phl p 5. Placebo injections contained 0.01 mg/ml histamine acid phosphate (in PBS) in allergen diluent. Serum samples were taken before the start of treatment in February/March 1996 and at the height of the subsequent pollen season in May/June 1996. After 2 years of treatment, further serum samples were obtained during the peak pollen season (May/June 1998) and at the end of the study out of the season (October 1998). Nasal biopsies were taken before treatment/outside and after 2 years of treatment in the pollen season.

Nasal biopsy, *in situ* hybridization, and immunocytochemistry

Local anesthesia of the inferior nasal turbinate was achieved using 1 ml of 10% cocaine solution. A 2.5-mm biopsy was taken 10 min later using Gerritsma forceps. Biopsies were cut into two halves. One half was fixed in 4% paraformaldehyde, dehydrated in 15% sucrose, then snap frozen and stored for *in situ* hybridization studies. The other half was snap frozen and stored for immunohistology. Immunohistology was performed on 6-µm cryostat sections (fixed for 7 min in 60:40 acetone:methanol), using phenotype-specific markers CD3 (T cells) and CD68 (macrophages) (Dako Cytomation, Cambridge, U.K.) and the modified alkaline phosphatase-anti-alkaline phosphatase method as described previously (19). IL-10 protein was identified using mAb to human IL-10 (Santa Cruz Biotechnology, Santa Cruz, CA) and an avidin-biotin system (Vector Laboratories, Burlingame, CA). *In situ* hybridization was performed on 6-µm cryostat sections as described elsewhere (31). Riboprobes, both antisense (complementary to mRNA) and sense (identical sequence to mRNA), were prepared from cDNA encoding IL-10 cDNAs and inserted into different pGEM vectors and linearized with restriction enzymes before transcription. Transcription was performed in the presence of ³⁵S-uridine triphosphate and the appropriate T7 or SP6 RNA polymerase. Controls included sense probes (having an identical sequence to mRNA) and antisense probes following pretreatment of sections with RNase. Specific hybridization was recognized as clear dense deposits of silver grains in the photographic emulsion overlaying tissue sections (31).

Colocalization

Colocalization was performed on paraformaldehyde-fixed sections by sequential immunohistochemistry using phenotype-specific markers CD3 or CD68 and an alkaline phosphatase anti-alkaline phosphatase technique, developed using Fast Red (Sigma-Aldrich, St. Louis, MO). This was followed by *in situ* hybridization using a ³⁵S-labeled IL-10 antisense probe. Only double-positive cells were counted.

IL-10 production by peripheral T cells

To determine whether IL-10 was also detectable from peripheral circulatory cells, 20 ml of peripheral venous blood was collected from 15 patients who had received at least 12 mo of specific grass pollen IT, 14 patients with seasonal allergic rhinitis but who had not received IT, and 11 non-atopic normal control subjects. All samples were collected and processed during the U.K. grass pollen season. PBMC were isolated by density gradient centrifugation over Histopaque (Sigma-Aldrich), resuspended at 10⁶ cells/ml, and incubated at 37°C in the presence or absence of Phl p extract (20 µg/ml; kind gift from ALK Abelló). After 6 days of culture, supernatants were harvested and assayed for IL-10 by ELISA using paired Abs (BD PharMingen, Oxford, U.K.), sensitive above 5 pg/ml.

Serum allergen-specific Ab concentrations (IgE, IgG, and IgG4)

Serum allergen-specific IgE was measured by a radioallergosorbent test using Phl p 5 allergen coupled to Sepharose and detected by radiolabeled anti-IgE. Allergen-specific IgG was measured using purified ¹²⁵I-labeled Phl p 5 allergen. A two-step procedure was used: the serum was preincubated with the solid phase and nonbound Abs were removed before adding the labeled Ag. Serum (1.25 µl/test) was incubated with cyanogen bromide-activated Sepharose-coupled protein G (0.5 mg/test; Pharmacia, Uppsala, Sweden) and ~1 ng iodinated allergen was added. After overnight incubation, Sepharose-bound radioactivity was measured. High samples were retested at appropriate dilutions. The results were converted into units by comparison with dilutions of a reference serum. Allergen-specific IgG4 was measured in a similar way, but instead of Sepharose-coupled protein G we used Sepharose-coupled monoclonal anti-IgG4. The results were expressed as arbitrary units as compared with known standards.

Allergen-IgE binding to B cells and effects of sera from IT patients

B cells within PBMC of an atopic donor were EBV transformed and enriched for CD23⁺ cells using anti-CD23 mAb (BD PharMingen) in conjunction with anti-IgG-coated magnetic beads (CELLlection Pan Mouse IgG kit; Dynal, Oslo, Norway). B cells were CD19⁺, CD20⁺, CD23⁺, HLA-DR⁺, CD14⁺, CD25⁺, and CD3⁺ (BD PharMingen). Serum (40% dilution in PBS) from an atopic grass pollen allergic patient containing high concentrations of allergen-specific IgE was preincubated alone or along with Phl p allergen extract (3 µg/ml) at 37°C for 1 h before adding EBV-transformed B cells at 2 × 10⁵ cells/sample and incubated for an additional 1 h at 4°C. Cells were then washed and surface binding of allergen-IgE complexes was detected using a FITC-labeled anti-IgE polyclonal Ab (Dako Cytomation). For each sample a minimum of 5000 cells was analyzed using a FACScan flow cytometer (BD Biosciences, U.K.) (32).

The effects of sera from IT or placebo patients on allergen-IgE binding to B cells were tested in this system. Briefly, the initial preincubation of IgE-containing serum (40% dilution) with Phl p allergen extract was conducted with the additional presence of coded sera (also 40% dilution) from patients undergoing IT or placebo treatment. Samples were subsequently processed and analyzed as described above.

To determine whether the inhibitory activity of postimmunotherapy serum resided within the IgG4 fraction, 5 ml of serum with confirmed inhibitory activity was run over an anti-IgG4 Sepharose column (62 mg monoclonal anti-human IgG4 (MH164-4; CLB, Amsterdam, The Netherlands) coupled to 3 g Sepharose). After washing with excess PBS, IgG4 was eluted with a buffer containing 0.1 M glycine-HCl (pH 2.5) and 0.1 M NaCl. The purified IgG4 fractions were compared with the serum fractions without IgG4 and the original serum sample in the allergen-IgE B cell-binding assay.

Statistical analysis

Within group comparisons were performed using the Wilcoxon matched pairs signed-rank test. Between-group comparisons were performed using the Mann-Whitney *U* test. For measurements of IL-10 in the nasal mucosa, the predetermined primary analysis was used to compare between groups and the within-group changes before/after treatment, thereby eliminating baseline variability between subjects. Correlation coefficients were obtained using Spearman's rank method. All analyses were performed using a commercial statistics software package (Minitab, State College, PA). All tests were two tailed and values of *p* < 0.05 were considered to be statistically significant.

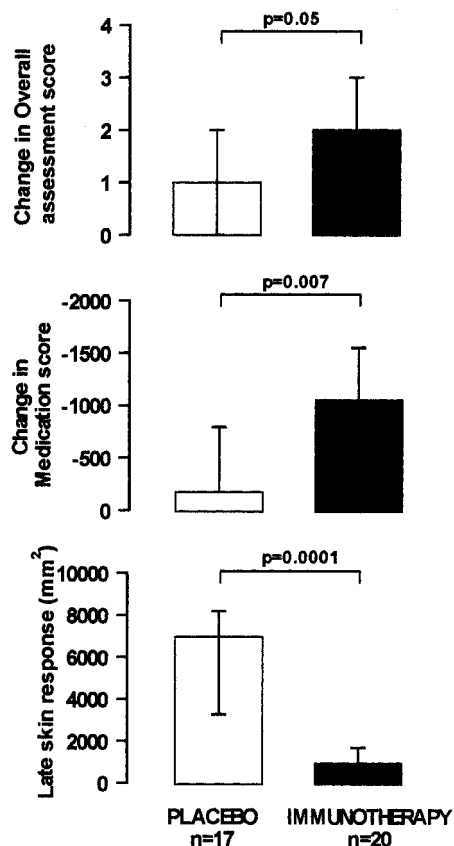


FIGURE 1. Grass pollen injection IT for severe seasonal allergic rhinitis. Results are expressed as change in overall assessment score (*upper panel*), change in medication score (*middle panel*), and late skin response after 2 years of IT or placebo treatment. Bars represent median $\pm$ interquartile range.

Results

Grass pollen IT is clinically effective

Two years of treatment with a depot grass pollen extract was highly effective in reducing seasonal symptoms, rescue medication, and bronchial hyperresponsiveness during the pollen season (4). Clinical results have been reported previously. Results for the 37 of the 44 patients who consented to undergo repeated nasal biopsies are presented in Fig. 1. There was an approximate improvement in overall subjective symptom scores by 50% ($p =$

0.05), use of rescue medication by 80% ($p = 0.007$), and a 70% decrease in the late-phase skin responses ($p = 0.0001$) after 2 years of treatment. IT was well tolerated and no immediate systemic reactions were observed during either the up-dosing or maintenance phases of IT.

Grass pollen IT is associated with seasonal increases in nasal mucosal IL-10 mRNA expression during natural allergen exposure

Nasal mucosal biopsies were obtained from IT- and placebo-treated patients at baseline and during the peak pollen season after 2 years of treatment. The combination of 2 years of IT and natural seasonal exposure to grass pollen was associated with increases in the number of IL-10 mRNA-expressing cells ($p = 0.002$) and these increases were significant when compared with the corresponding changes for the placebo group ($p < 0.03$). In contrast, in the placebo group, the numbers of IL-10 mRNA-expressing cells in the nasal mucosa did not change ($p = 0.9$; Fig. 2 and Table I). These increases in IL-10 mRNA⁺ cells in season were accompanied by a trend for an increase in IL-10 protein-positive cells in 8 of 11 IT-treated patients for whom biopsy material was available (median before, 0; after, 2.65/mm², $p = 0.1$) compared with placebo-treated patients (median before, 1.6; after 1.0/mm², $p = 0.7$; Fig. 3). Following IT, there was also a strong correlation between the number of IL-10 mRNA⁺ cells and IL-10 protein⁺ cells ($r = 0.82$, $p = 0.003$).

To determine whether seasonal increases in nasal mucosal IL-10 could occur in response to allergen exposure per se, independent of the atopic phenotype, we obtained biopsies during the peak pollen season from normal nonatopic controls (Fig. 2). Only low numbers of IL-10 mRNA⁺ cells were observed in normal volunteers, which implies that nasal mucosal expression of IL-10 is not a feature of the "normal" immune response to grass pollen.

We also sought to determine whether or not IT-induced local increases in IL-10 mRNA⁺ cells following systemic administration of the vaccine could be detected in the absence of natural allergen exposure, outside the pollen season. In contrast to the findings during natural pollen exposure, no significant increases in IL-10 mRNA⁺ cells ($p = 0.9$) above baseline were observed in biopsies taken during the winter months at the end of the study (Fig. 2). The in/out of season differences for IL-10 following 2 years of IT were also significant ($p = 0.03$).

Sequential immunohistochemistry and in situ hybridization of nasal biopsies were performed to determine what proportion of IL-10 mRNA⁺ cells were expressed by CD3⁺ T lymphocytes and

FIGURE 2. IL-10 mRNA⁺ cells in the nasal mucosa of IT-treated patients (●), placebo-treated (PL) patients (○), and normal non-atopic control subjects (△). Results are expressed as the number of cells per square millimeter. Pretreatment biopsies were taken outside the pollen season and posttreatment biopsies were taken during the peak pollen season after 2 years of treatment. Biopsies were also taken from IT patients after treatment, outside the pollen season.

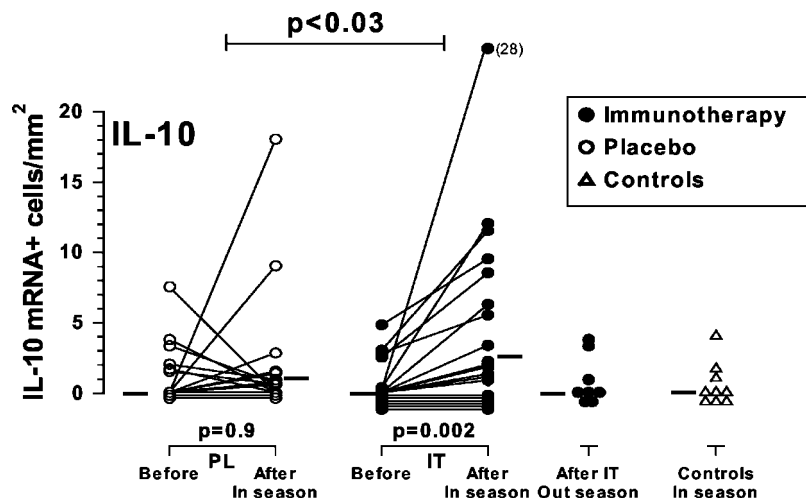


Table I. Local nasal cytokines, serum Ab concentrations and serum blocking activity for allergen-IgE binding to B cells

	IT		Placebo		Point Estimate (95% Confidence Interval)	<i>p</i> ^a
	Before (Winter 1995)	After (Summer 1998)	Before (Winter 1995)	After (Summer 1998)		
IL-10 mRNA/mm ²	0 (0, 0.3)	2.0** (0, 7.9)	0 (0, 1.9)	0.7 (0, 2.1)	-2.2 (-5.0, -0.1)	0.03
Serum IgE Phl p 5	15.8 (10.7, 25.9)	19.5 (11.8, 27.6)	15.4 (11.3, 27.6)	24.1 (20.4, 31.0)	-2.2 (-9.6, 6.4)	0.71
Serum IgG Phl p 5	1.2 (0.4, 3.5)	81.4*** (75.8, 86.5)	1.7 (0.8, 2.9)	2.3 (1.1, 3.7)	79.6 (76.5, 83.0)	0.0001
Serum IgG4 Phl p 5	0.07 (0, 0.4)	67.2*** (57.5, 69.2)	0.31 (0.06, 0.94)	0.42 (0.14, 1.15)	66.1 (59.0, 67.8)	0.001
Allergen-IgE binding to B cells (% Total)	44.8 (40.3, 47.9)	7.5*** (3.4, 19.0)	44.3 (37.6, 51.7)	45.8 (41.2, 52.5)	-35.1 (-39.8, -30.3)	0.001

^a Values refer to between-group differences for within-group (before/after IT) comparisons.

*, *p* = 0.06; **, *p* = 0.002; ***, *p* = 0.0001 (within-group comparison, before/after IT).

CD68⁺ macrophages (Figs. 3 and 4). Results from biopsies from three individuals demonstrated that the mean (SD) of 15% (12) of IL-10-expressing cells was CD3⁺ T cells and overall 1% (1) T cells expressed mRNA for IL-10. Similarly, 35% (11) of IL-10 mRNA⁺ cells were CD68⁺ macrophages, whereas 4% (2) macrophages expressed mRNA for IL-10 (Fig. 4).

Peripheral IL-10 responses are also increased by IT

PBMC were purified from peripheral blood obtained during the grass pollen season. Six-day cultures in the presence/absence of allergen were assayed for IL-10 production. No differences among patients and controls were observed in levels of IL-10 produced spontaneously in culture. In contrast, stimulation with grass pollen allergen resulted in significantly higher IL-10 production in the IT group compared with both atopic rhinitis subjects who had not received IT (*p* < 0.01) and nonatopic controls (*p* < 0.001; Fig. 5). IL-10 production by PBMC out of season was not different from that observed in season (data not shown).

IT inhibits seasonal increases in allergen-specific IgE and increases allergen-specific IgG and IgG4 concentrations

When compared with baseline, 2 years of IT was associated with blunting of seasonal increases in the Phl p 5-specific IgE, whereas

this was not observed in the placebo group (Fig. 6). IT resulted in ~60- to 80-fold increases in Phlp 5-specific IgG (*p* < 0.001) and >100-fold increases in Phl p5-specific IgG4 (*p* < 0.001) that were evident both in and out of the pollen season (Fig. 6 and Table I). When Ab concentrations were related to the clinical response to IT, a significant correlation was observed between the level of allergen-specific IgG after 2 years of treatment and the subjects' seasonal symptom scores (*r* = -0.63, *p* = 0.005) and their overall assessment scores (*r* = 0.58, *p* = 0.01), whereas this was not observed for allergen-specific IgE concentrations.

Post-immunotherapy serum inhibits IgE-facilitated allergen binding to B cells

To demonstrate activity of blocking Abs, a system was used based on allergen-dependent IgE binding to CD23⁺ B cells (32). The mechanism behind this phenomenon involves formation of allergen-IgE complexes that show enhanced CD23 binding resulting from multiple adjacent Fcε regions. Sera from all IT patients but not from one single placebo-treated patient markedly inhibited the formation of allergen-IgE complexes as evidenced by suppression of allergen-IgE binding to B cells (*p* < 0.0001; Fig. 7 and Table I). This IgG blocking activity was related to the patients' overall assessment of efficacy of IT. A trend was observed for those with

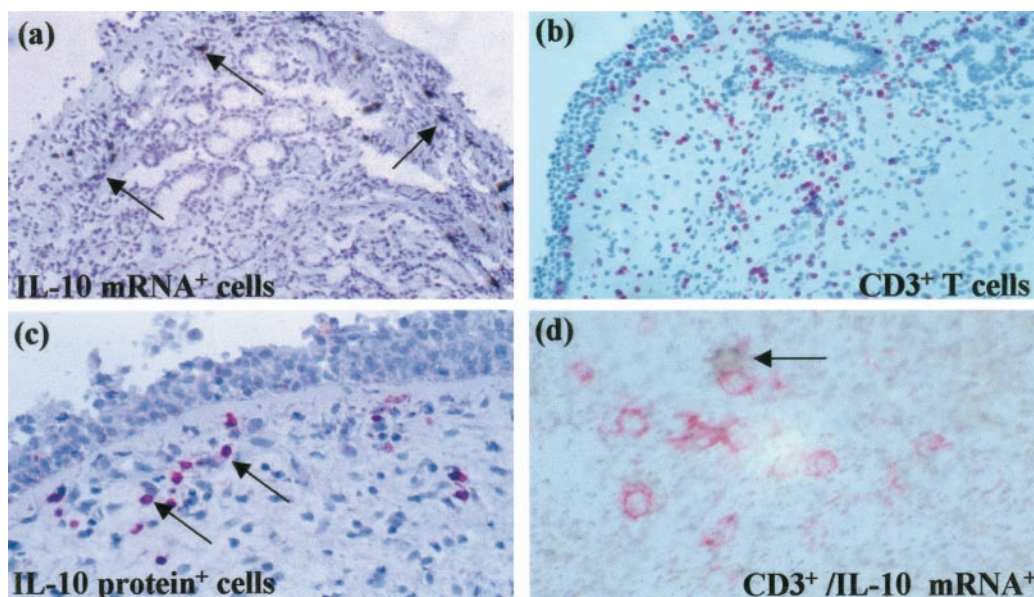


FIGURE 3. *a*, IL-10 mRNA⁺ cells detected by in situ hybridization (³⁵S-labeled riboprobe; magnification, ×40). *b*, CD3⁺ T lymphocytes detected by immunohistochemistry (alkaline phosphatase anti-alkaline phosphatase technique; magnification, ×40). *c*, IL-10 protein-positive cells detected by immunohistochemistry (avidin-biotin technique; magnification, ×40). *d*, Colocalization of IL-10 mRNA to CD3⁺ T cells by sequential immunohistochemistry followed by in situ hybridization (magnification, ×100). Arrows show individual positive cells.

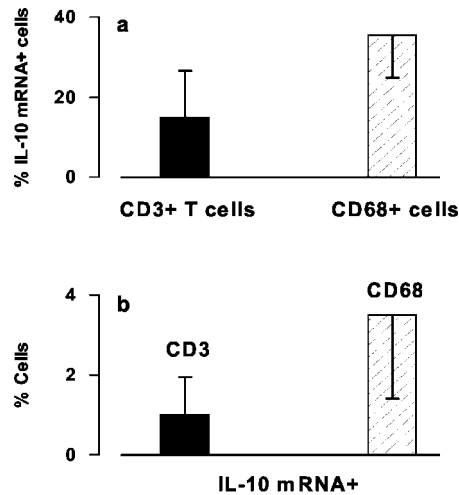


FIGURE 4. The percentages of IL-10 mRNA-expressing cells that are CD3⁺ T lymphocytes (■) or CD68⁺ macrophages (▨, top panel). The percentage of CD3⁺ T lymphocytes (■) and CD68⁺ macrophages (▨) that express IL-10 mRNA (bottom panel). Bars represent mean $\pm$ SD.

the lowest overall assessment scores (0 or 1, absent or only slight improvement) to show a lower inhibition of allergen-dependent IgE binding to B cells in response to treatment when compared with those patients scoring 2 or 3 (moderate or marked improvement; $p = 0.058$).

Since IT is associated with increases in allergen-specific IgG4, we purified the IgG4 fraction from post-immunotherapy sera using affinity chromatography. Fig. 8 demonstrates that this IgG4 fraction could inhibit allergen IgE binding to B cells to a similar extent as whole post-immunotherapy serum, whereas the IgG4-depleted fraction had markedly reduced inhibitory activity.

Discussion

We report the effects of a course of conventional grass pollen IT, of proven clinical efficacy, on the expression of IL-10 within the nasal mucosa. Increases in numbers of IL-10 mRNA-expressing cells were observed during the pollen season after IT and correlated with the numbers of IL-10 protein-expressing cells. PBMC isolated from grass pollen IT patients but not allergic or nonallergic controls also produced IL-10 when stimulated in vitro with grass pollen allergen. These findings suggest that IT induces a systemic population of cells able to produce IL-10 when stimu-

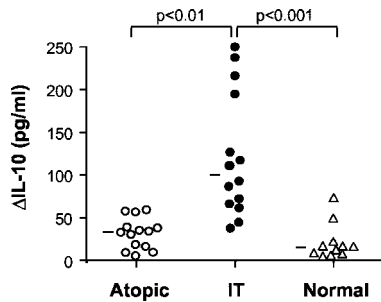


FIGURE 5. IL-10 production by PBMC isolated from patients treated with grass pollen IT (●), untreated atopic rhinitic control (○), and normal non-atopic control (△) subjects. PBMC were stimulated with 20 μ g/ml Phl p extract for 6 days and IL-10 was measured in culture supernatants by ELISA. Values represent concentrations in allergen-stimulated cultures minus concentrations in unstimulated cultures (background). The unstimulated PBMC cultures produced an average of 9.9 ± 3.1 pg/ml. Values of p were determined by Kruskal-Wallis with a Dunn post test.

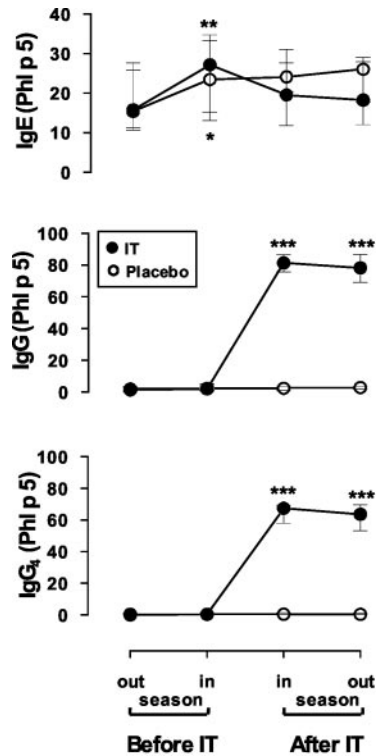


FIGURE 6. Serum Phl p 5-specific IgE, IgG, and IgG4 concentrations for IT (●)- and placebo-treated (○) patients before (Before IT) and after 2 years of treatment (After IT) before (out) and during (in) the pollen season. Results are expressed as median $\pm$ interquartile ranges. *, Values of p for IgE measurements represent statistical differences within groups (** = 0.005, * = 0.04) as determined by the Wilcoxon test and for IgG and IgG4 measurements differences between groups as determined by the Mann-Whitney U test (*** = 0.001).

lated by grass pollen allergen and that these cells circulate or are recruited to the nasal mucosa where they are activated during the pollen season. As a putative regulatory cytokine, IL-10 has a number of properties that could be relevant to induction of hyporesponsiveness to allergen following IT, including inhibition of T

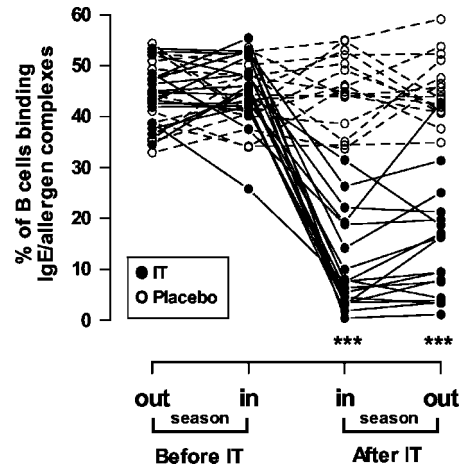


FIGURE 7. Inhibition of allergen-IgE binding to B cells by sera from IT-treated (●) but not placebo-treated (○) patients before (Before IT) and after 2 years of treatment (After IT) before (out) and during (in) the pollen season. * Values of p represent statistical differences within groups as determined by the Wilcoxon test and between groups as determined by the Mann-Whitney U test (*** = 0.0001).

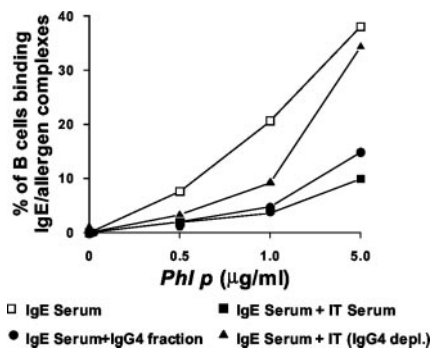


FIGURE 8. Serum obtained following IT was separated by affinity chromatography. The IgG4 fraction, the eluted fraction from which IgG4 had been removed, and whole serum were compared for their ability to inhibit the binding of allergen-IgE complexes to B cells, as detected by flow cytometry.

cell responses by blocking CD28 signaling (22) as well as modulation of B cell Ig class switching in favor of IgG4 production (23). Consistent with IL-10-dependent immune regulation *in vivo*, strong allergen-specific IgG4 responses were seen in the sera of all IT patients. Moreover, in a functional assay, IgG4-containing sera from IT- but not placebo-treated patient was shown to block formation of allergen-IgE complexes required for binding and processing of allergen via cell surface CD23.

Previous studies have shown that IT for bee venom (33), house dust mite (34), and pollen allergy (35) is accompanied by increases in IL-10 production by peripheral blood CD4⁺CD25⁺ T cells, presumed regulatory T cells, following *in vitro* stimulation with the relevant allergen. More recently, cutaneous challenge of bee venom patients with bee venom allergen was associated with increased numbers of IL-10-expressing cells accompanied by inhibition of the late skin response (*i.e.*, 24 h after provocation) (36). The present study represents the first description of local nasal IL-10 expression in response to conventional IT with pollens. Moreover, it is the first to report that IT results in increased expression of IL-10 within the respiratory mucosa only during the pollen season, which implies that mucosal contact with the relevant allergen is required for either recruitment and/or activation of these cells. This is also the first study to link local mucosal and systemic IL-10 production and, in the same individuals, to link increased production of the regulatory cytokine IL-10 with increases in serum allergen-specific IgG and IgG4 concentrations. These changes were also associated with an increase in blocking activity in serum, which inhibited allergen-IgE binding to B cells in an IgG-dependent fashion that correlated with the clinical response to IT.

Colocalization of the cell sources of IL-10 within the nasal mucosa revealed major contributions from CD68⁺ macrophage (35%) and CD3⁺ T cells (15%). A significant proportion of IL-10 mRNA⁺ cells remain unaccounted for. Likely candidates include dendritic cells and/or B cells. Unfortunately, we were unable to colocalize IL-10 mRNA to these cell types because their detection requires tissue fixation using acetone:methanol, whereas IL-10 mRNA detection requires paraformaldehyde-fixed tissue.

The immunologic mechanisms by which cutaneous allergen IT gives rise to cells that are able to produce IL-10 on subsequent restimulation is entirely unknown. However, similar phenomena are well documented in murine models of immune tolerance (37, 38) with immunosuppressive properties being linked to induction of CD4⁺CD25⁺ regulatory T cells producing IL-10 and TGF- β (26, 39). There is some evidence that dendritic cells may be critical

for induction of these regulatory T cells: in murine asthma models, T cell tolerance and protection against airway pathology can be achieved by intranasal exposure to aeroallergen and is associated with the development of IL-10 producing pulmonary dendritic cells (25, 40). These dendritic cells not only stimulated development of IL-10-producing T cells *in vitro* (a phenotype consistent with regulatory T cells) but also could adoptively transfer protection against allergen to other animals. Thus, IL-10 production by T cells is associated with tolerogenic responses in animal models of immunopathology, and induction of dendritic cell subsets may be a primary event in this process. Based on these observations, we speculate that following injection IT, dendritic cells might take up allergen and undergo maturation into tolerogenic phenotypes that drive allergen-specific T cells circulating through lymph nodes to differentiate into IL-10-producing cells.

Our data suggest that IL-10 at least may have effects at two levels following IT: first, repeated injection of grass pollen allergen may result in IL-10 production by T cells within the lymph nodes which subsequently traffic through the target organ via peripheral blood. Within these lymph nodes, IL-10 may reduce IL-4-induced IgE synthesis, while increasing allergen-specific γ 4 transcription and IgG4 production by B cells with an elevated IgG4:IgE ratio. IL-10 has proanergic effects on allergen-specific T cells as a result of its capacity to block CD28 tyrosine phosphorylation (22). Second, direct expression of IL-10 also occurs locally within the nasal mucosa on exposure to inhaled aeroallergen. Since Abs such as IgE may be produced within the nasal and bronchial mucosae (41–44) and mucosal T cell responses to allergen are thought to be CD28 dependent (45), local IL-10 production also has the potential to direct local IgG4 synthesis. Furthermore, IL-10 may modulate the capacity of APCs such as dendritic cells to activate T cells to produce cytokines both directly (46) and indirectly through inducing allergen-specific IgG4 Abs that modulate IgE-dependent Ag processing and presentation.

Following IT, deviation of allergen-specific Th2 responses in favor of Th1 cytokine responses have been described in allergen-exposed tissues (19, 20) and in some studies, but not others, in peripheral blood (18). An important and unanswered question concerns the relationship between a Th2 to Th1 shift and induction of IL-10 responses. Although IL-10 could arise through separate and superimposed mechanisms, we believe that it is more likely that these changes occur interdependently. For example, IL-10 blockade of CD28 costimulation may render both Th2 and Th1 cells anergic and such cells have been reported to preferentially express Th1 cytokines when “rescued” *in vitro* by IL-2 or IL-15 (47).

Alternatively, Th2-Th1 immune deviation may occur through alternative non-IL-10-dependent mechanisms. For example, we previously showed that T cells with characteristic Th2 phenotype harvested from bronchoalveolar lavage fluid during human allergen-induced late pulmonary responses remain susceptible to inhibitory cytokines such as IL-12 (48). We further showed that suppression of late cutaneous responses following IT was accompanied by local enhanced IL-12 production, which correlated directly with Th1 (IFN- γ) expression and inversely with Th2 (IL-4) expression in the tissue (49). In the presence of IL-12 after IT and following mucosal contact with grass pollen, such cells may be reactivated in favor of Th1-dominant responses. Support for this concept comes from our previous observations (18, 20) that immune deviation to Th1 responses (with enhanced IFN- γ) only occurs during mucosal contact with allergen (18, 20) and not outside the pollen season (18). An alternative explanation for the local increases in IL-10⁺ cells following IT may be production of IL-10

by "bystander" cells such as macrophages, rather than from T regulatory cells. Macrophage secretion of IL-10 might occur in response to enhanced IFN- γ production possibly following immune deviation in favor of local Th1 responses (18).

Other immunologic changes described in association with IT are reductions in mucosal recruitment of inflammatory cells (11), moderate reductions in IgE, and increases in allergen-specific IgG Abs, particularly of the IgG4 isotype (5–10). In one study, the ratio of serum IgG:IgE after pollen IT correlated inversely with clinical symptoms (8). However, in the majority of studies, increases in IgG failed to predict the clinical response to treatment, raising the possibility that IgG may reflect high allergen exposure rather than play a causal role in successful IT (5–10). In our study, we confirmed blunting of seasonal increases in IgE and substantial and highly significant increases in immunoreactive IgG, particularly IgG4. The inhibitory activity of post-immunotherapy serum on allergen-IgE binding to B cells, a functional measure of IgG activity, was largely confined to the IgG4 subclass and was associated with clinical improvement. We did not observe a correlation between IL-10 mRNA expression and the reduction in symptoms following IT (data not shown). However, a single measurement of IL-10 during the peak pollen season is unlikely to be predictive. Repeated measurements of the time course of changes in local and systemic IL-10 production, immunoreactive IgG and IgG blocking activity during the up-dosing and early maintenance phases of IT, are needed to further assess their possible predictive role in the clinical response to IT.

In summary, we show that conventional high-dose grass pollen injection IT induces a peripheral population of cells that produce IL-10 when activated by specific allergen, and that this is accompanied by modification of subsequent responses to allergen exposure in the nasal mucosa in favor of local IL-10 expression. These responses are paralleled by inhibition of seasonal increases in IgE and increases in serum allergen-specific IgG and in particular IgG4. IT also induces a serum-blocking activity, presumed to be allergen-specific IgG that blocks formation of allergen-IgE complexes and binding to CD23⁺ B cells. A likely consequence of this is inhibition of IgE-facilitated allergen presentation to T cells. Future studies should address the mechanisms, possibly at the level of dendritic cell phenotype or state of activation/maturation, that underlie the induction of IL-10 production. Such studies should allow us to refine antiallergic therapies based around IT.

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