

Grass pollen immunotherapy: Symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season

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Background: Tissue eosinophilia and infiltration by T_H2-type T cells are characteristic features of allergic rhinitis both after allergen challenge and during natural allergen exposure. Specific immunotherapy inhibits allergen-induced nasal eosinophilia.

Objectives: We sought to assess, in the context of a randomized trial, the relationships between symptomatic improvement after immunotherapy and eosinophil numbers and IL-5 expression in the nasal mucosa during the pollen season.

Methods: Nasal biopsy specimens were taken from 37 adults with severe summer hay fever at baseline (out of season) and at peak season after 2 years of treatment with a depot grass pollen extract or placebo. Biopsy specimens were processed for immunohistochemistry by using mAbs against eosinophils (EG2), T cells (CD3), and IL-2 receptor-positive cells (CD25), as well as for in situ hybridization by using a sulfur 35-labeled antisense riboprobe directed against IL-5.

Results: Immunotherapy significantly reduced symptoms (49%, $P = .01$) and medication requirements (80%, $P = .007$) compared with placebo. There was a 400% increase ($P = .004$) in eosinophils during the pollen season in placebo-treated patients, which was inhibited in the immunotherapy group (20% increase, $P = .04$ between groups). Seasonal increases were also observed for CD25⁺ cells ($P = .002$), CD3⁺ cells ($P = .02$), and IL-5 mRNA-expressing cells ($P = .03$) in the placebo group but not in the immunotherapy group. A significant correlation was observed between eosinophils and IL-5 expression ($r = 0.5$, $P < .05$). Both eosinophils ($r = 0.6$, $P < .02$) and IL-5 ($r = 0.6$, $P < .02$) correlated with symptoms after immunotherapy. **Conclusion:** Improvement in symptoms after grass pollen immunotherapy may result, at least in part, from inhibition of IL-5-dependent tissue eosinophilia during the pollen season. (*J Allergy Clin Immunol* 2001;107:971-6.)

Key words: Allergic rhinitis, eosinophils, IL-5, immunotherapy

Tissue eosinophilia is a characteristic feature of seasonal allergic rhinitis.¹ IL-5 is an eosinophil-specific cytokine that promotes the maturation, activation, and prolonged survival of eosinophils.² We previously reported increases in eosinophils and IL-5 in the nasal mucosa of patients with hay fever both after allergen provocation³ and during natural pollen exposure.⁴ A close correlation was observed between IL-5 mRNA and eosinophilia. Colocalization studies with dual immunohistochemistry-in situ hybridization confirmed that mucosa T lymphocytes are the principle source of IL-5 mRNA.^{4,5} These observations highlight the critical role of T_H2 T lymphocytes in human allergic responses, as previously determined in murine studies⁶ and studies of human peripheral blood T lymphocyte responses.⁷

We⁸ and others⁹ have shown that subcutaneous grass pollen immunotherapy is highly effective in treating hay fever, even in patients unresponsive to conventional pharmacotherapy. The recent demonstration that grass pollen immunotherapy may confer long-term benefit for at least 3 years after discontinuation¹⁰ highlights the prophylactic value of this therapy. The mechanism of immunotherapy is not completely understood. Earlier studies demonstrated increases in allergen-specific IgG antibodies in the serum.¹¹ We and others have reported decreases in effector cells, including mast cells¹² and eosinophils.¹³ More recent work suggests that immunotherapy may act by modifying allergen-specific T-cell responses, either through immune deviation in favor of T_H1 responses or through inhibition of T_H2 responses by, for example, induction of T-cell anergy.^{10,14}

We recently reported that grass pollen immunotherapy was effective in patients with seasonal allergic rhinitis complicated by seasonal asthma. We observed a marked reduction in symptoms and rescue medication requirements, an improvement in quality of life, and a decrease in bronchial hyperresponsiveness during the pollen season.¹⁵ In the same patients we have now tested the hypothesis that immunotherapy inhibits nasal mucosa eosinophilia during the pollen season and that this results from a decrease in IL-5 expression.

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METHODS

Patients

Forty-four patients were recruited from the allergy clinic of the Royal Brompton Hospital, London, United Kingdom, or by means of advertisement in a local newspaper. All subjects had a history of severe summer hay fever that was not controlled by antiallergic drugs and a positive skin test reaction (wheal >5 mm) to *Phleum pratense* (Timothy grass pollen). Patients were excluded if they had multiple allergies or had received immunotherapy within the preceding 5 years. The study was performed with the approval of the local ethics committee and with the patients' written informed consent.

Study design

This was a randomized, double-blind, placebo-controlled, parallel group study, as previously described.¹⁵ After one summer of symptom monitoring (1996) and before randomization to immunotherapy or placebo injections, baseline nasal biopsies were performed in October and November of 1996 at a time when patients were asymptomatic. After 2 years of treatment, a second biopsy specimen was taken at the peak of the grass pollen season from the 37 subjects remaining in the study. Throughout the summer (May-August), subjects kept symptom diaries as in previously reported studies,^{1,4,8,10} recording their eye (itch, redness, swelling, and streaming), nose (sneeze, blockage, and running), throat (itch and dryness), and chest (breathlessness, cough, wheeze, and chest tightness) symptoms daily, each on a scale of 0 to 3. Area-under-the-curve scores were calculated for each subject for the 11 weeks corresponding to the pollen season.

Additional biopsy specimens were taken from 9 nonatopic control subjects at the same time as the second (peak seasonal) set of study biopsy specimens to control for the effects of seasonal pollen exposure in nonsensitive patients.

Nasal biopsy

Local anaesthesia was achieved by using a small pledget of cotton wool that was soaked in 10% cocaine solution and placed adjacent to the planned biopsy site 2 cm distal to the anterior end of the inferior turbinate. After 10 minutes, a 2.5-mm biopsy specimen was obtained by using cup and ring (Gerritsma) forceps.¹⁶ A cotton wool plug soaked in 1:1000 adrenaline was then placed adjacent to the biopsy site for 10 minutes, and any residual bleeding was controlled by means of cautery with a silver nitrate stick. Biopsy specimens were immediately cut in half and processed separately for immunohistochemistry and in situ hybridization.

Immunohistochemistry

Half of each biopsy specimen was immediately mounted in OCT (BDH Merck, Dagenham, United Kingdom) compound, snap-frozen by immersion in isopentane precooled in liquid nitrogen, and then stored at -80°C. Immunohistochemistry was performed on 6-μm cryostat sections fixed in acetone/methanol (60:40) by using the alkaline phosphatase antialkaline phosphatase method, as previously described.¹⁷ Briefly, sections were incubated for 30 minutes (overnight for CD25) at room temperature with the primary antibody followed by 30-minute incubations each with rabbit anti-mouse antibody and the alkaline phosphatase antialkaline phosphatase complex (both from DAKO Ltd, Cambridge, United Kingdom). The reaction was developed with Fast Red stain, giving positive cells a red appearance. Monoclonal antibodies used were EG2 (Pharmacia and Upjohn diagnostics, Uppsala, Sweden) for activated eosinophils, CD25 (Becton Dickinson, San Jose, Calif) for activated T lymphocytes, and CD3 (DAKO) for total T lymphocytes.

In situ hybridization

The remaining half of each biopsy specimen was immediately placed in 4% paraformaldehyde for 2 hours and then in 15% sucrose in PBS for 1 hour and again overnight before being mounted in OCT and snap-frozen as described above. Slides were stored at -80°C until used.

Riboprobes, both antisense and sense, were prepared from cDNA for IL-5. cDNA was inserted into different pGEM vectors and linearized with appropriate enzymes before transcription. Transcription was performed in the presence of sulfur 35 (³⁵S)-labeled uridine triphosphate and the appropriate T7 or SP6 RNA polymerases. In situ hybridization was performed on 6-μm cryostat sections on polylysine slides (BDH Merck). Sections were permeabilized with Triton X-100 in PBS followed by proteinase K digestion. Sections were treated with iodoacetamide and N-ethylmaleimide and then acetic anhydride-triethanolamine to inhibit nonspecific binding of ³⁵S. As a negative control, sections were either hybridized with the sense probe or treated with ribonuclease A solution before the prehybridization step with antisense probes.

Quantitation

Positively stained cells were counted at 200× magnification by using an Olympus BH2 microscope (Olympus Optical Company Ltd, Tokyo, Japan) with an eyepiece graticule. Sections were counted to one grid depth beneath the epithelium, and results were expressed as the number of positive cells per square millimeter.

Statistical analysis

Statistical analysis was performed by using a commercial software package (Minitab release 7; Minitab Inc, State College, Pa). Comparisons between measurements obtained before and after treatment in the same subject were analyzed by using the Wilcoxon matched-pairs signed-rank test. Comparisons between immunotherapy and placebo-treated patient groups were performed by using the Mann-Whitney *U* test. Correlations were performed by using the Spearman rank correlation method. A *P* value of less than .05 was considered to be statistically significant.

RESULTS

Clinical response to immunotherapy

Thirty-seven (20 receiving immunotherapy and 17 receiving placebo) of the 44 subjects completed the clinical trial. Of the 7 (2 receiving immunotherapy and 5 receiving placebo) subjects who withdrew, 4 did so because of moving out of the area, 2 because of increased work commitments, and 1 because of a series of colds. The immunotherapy and placebo groups, respectively, were well matched at baseline for age (mean, 32 years; range, 22-64 years vs mean, 32 years; range, 23-59 years), sex (12 women and 10 men vs 9 women and 13 men), immediate skin prick test response size (median, 50 mm²; interquartile range, 33-62 mm² vs median, 43 mm²; interquartile range, 27-86 mm²), and visual analogue score (out of 10) during the baseline (1996) pollen season (median, 4.4; interquartile range, 3.2-5.4 vs median, 4.5; interquartile range, 3.5-5.6).

Detailed results have been reported previously.¹⁵ Briefly, in the immunotherapy-treated patients, there were reductions of 49% in symptoms and 80% in medication requirements compared with 15% (*P* = .01 between groups) and 18% (*P* = .007), respectively, in the

TABLE I. Immunohistochemistry and in situ hybridization of nasal mucosa biopsy specimens

	Immunotherapy		Placebo		Control subjects	Point estimate (95% CI)	P value*
	Before treatment	Peak season	Before treatment	Peak season			
IMH							
EG2	12 (3.2-21)	14.2 (9.4-27)†	7.5 (2.8-28.8)	39.2 (16.4-76)‡	3.4 (1.3-26.25)	17 (1.1-39.1)	.04
CD3	477 (263-615)	346 (189-537)	317 (153-444)	553 (293-616)†	443 (309-483)	106 (−68-255)	.2
CD25	2.5 (0-20)	7.4 (0.7-33.5)	0.9 (0-3.3)	11.4 (2-19)‡	0.3 (0-1.3)	−0.5 (−15-8)	.8
ISH							
IL-5	0 (0-0)	0.7 (0-4.57)	0 (0-1.45)	2.2 (0-17)†	0 (0-3.3)	1.25 (0-7.09)	.16

Data are given as cells per square millimeter (median [interquartile range]).

IMH, Immunohistochemistry; ISH, in situ hybridization.

*Between-group comparisons (peak season) were performed by using the Mann-Whitney *U* test.

[†]*P* < .05 and [‡]*P* < .01. Within-group comparisons were performed by using the Wilcoxon matched-pairs signed-rank test.

placebo group. Clinical improvements were accompanied by a marked reduction in the late-phase response to allergen in the skin.

Eosinophils, T cells, and IL-5

In the placebo-treated patients, when compared with baseline, there was a highly significant, greater than 4-fold increase in the number of EG2⁺ eosinophils in the nasal mucosa during the pollen season (*P* = .004, Fig 1 and Table I) compared with a 20% increase in the immunotherapy-treated patients (*P* = .03). The peak seasonal values were significantly different between groups (*P* = .04). There were virtually no eosinophils seen in the corresponding biopsy specimens taken from nonatopic control subjects.

A significant (75%) increase was observed in the number of total (CD3⁺) T lymphocytes in the placebo group during the pollen season (*P* = .02). This was not observed in the immunotherapy group (*P* = .3). There was an 11-fold increase in the number of activated (CD25⁺) presumed T cells in placebo-treated patients (*P* = .002) compared with a less than 3-fold increase in immunotherapy-treated patients (*P* = .3). These between-group differences were not, however, significant (Fig 2 and Table I). In placebo-treated patients there was a significant increase in the number of cells expressing IL-5 mRNA during the pollen season (*P* = .03) that was not seen in the immunotherapy group (*P* = .2), although this difference failed to achieve statistical significance (*P* = .16, Fig 2 and Table I).

Relationships among seasonal symptom scores, eosinophils, and IL-5

In patients who had received grass pollen immunotherapy, a highly significant correlation was observed between their numbers of peak-seasonal mucosa eosinophils and their symptoms recorded in the 2 days preceding their peak-seasonal biopsy (*r* = 0.74, *P* < .01; Fig 3). When total seasonal symptoms expressed as area under the curve were considered, similar correlations were found among seasonal symptom scores, mucosa eosinophils, and IL-5 mRNA⁺ cells (Table II).

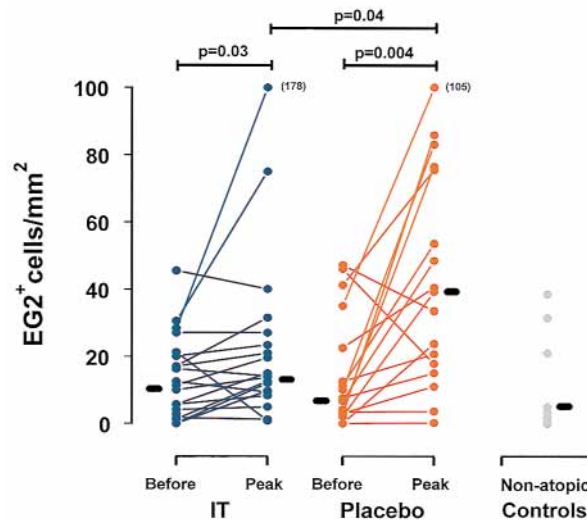


FIG 1. Effect of 2 years of immunotherapy on numbers of EG2⁺ eosinophils in the nasal submucosa. Data shown are for baseline (*Before*) and peak-seasonal (*Peak*) nasal biopsy specimens in immunotherapy- and placebo-treated patients and nonatopic control subjects (peak only). Horizontal lines represent median values. Within-group comparisons between baseline and peak-seasonal counts were performed by using the Wilcoxon matched-pairs signed-rank test, and comparisons between immunotherapy- and placebo-treated patients were performed by using the Mann-Whitney *U* test.

DISCUSSION

We have confirmed our previous observation⁴ that natural grass pollen exposure in seasonal allergic rhinitis is associated with nasal mucosa eosinophilia and increased expression of mRNA encoding for the proeosinophilic cytokine IL-5. In addition, we have shown that specific allergen immunotherapy, like topical corticosteroid therapy,⁴ suppresses this seasonal tissue eosinophilia and IL-5 mRNA expression. Additionally, we showed that these mucosa changes correlated with each other and with the positive clinical response to immunotherapy. We have

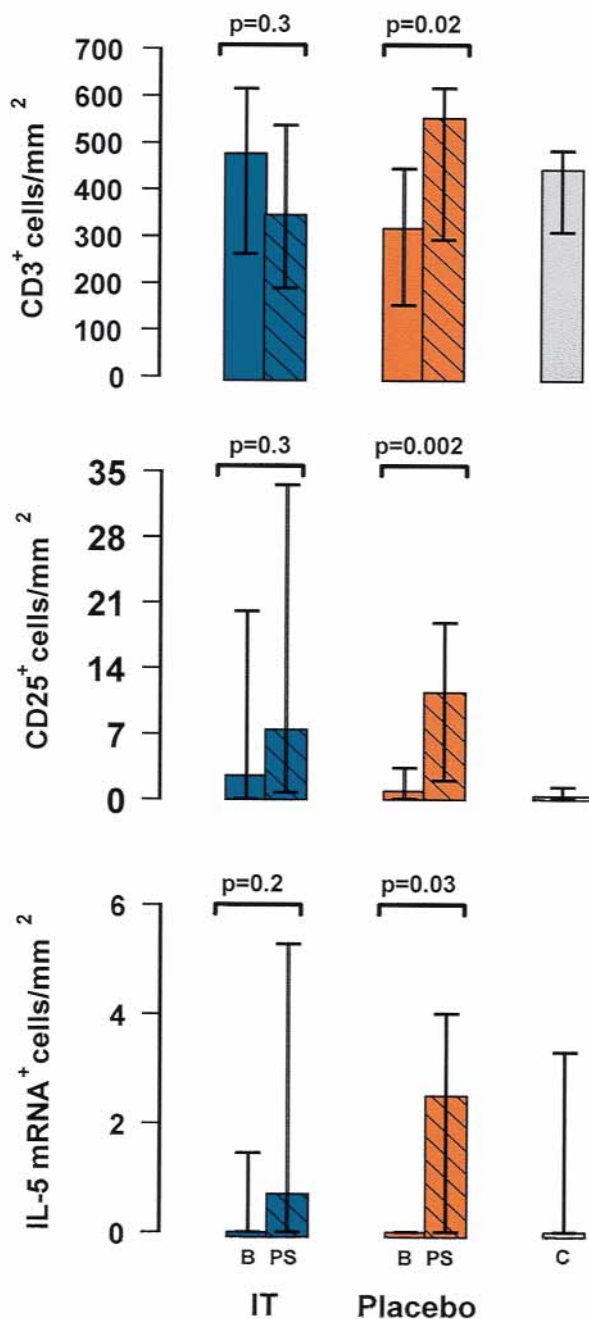


FIG 2. Effect of 2 years of immunotherapy on numbers of CD3⁺ T lymphocytes, CD25⁺ T lymphocytes, and IL-5 mRNA-expressing cells (medians and interquartile ranges) in the nasal submucosa. Data shown are for baseline (B) and peak seasonal (PS, hatched bars) nasal biopsy specimens in immunotherapy- and placebo-treated patients and nonatopic control subjects (C; peak only). Within-group comparisons between baseline and peak-seasonal counts were performed by using the Wilcoxon matched-pairs signed-rank test.

also shown increased numbers of total (CD3⁺) T lymphocytes, IL-2 receptor-positive (CD25⁺) cells (presumed T cells) in the nasal mucosa during the pollen season, a phenomenon not observed in patients who had received immunotherapy.

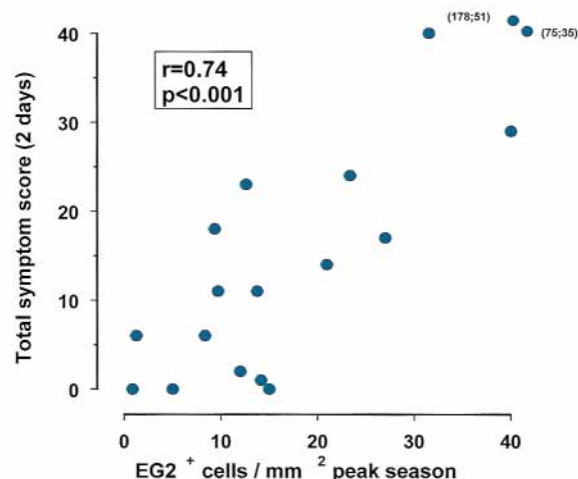


FIG 3. Correlation between symptom scores at the time of nasal biopsy and numbers of EG2⁺ eosinophils in the nasal mucosa at the peak of the pollen season after 2 years of immunotherapy treatment. Correlation was performed by using the Spearman rank correlation method.

TABLE II. Relationships among clinical response to immunotherapy, nasal eosinophils, and IL-5

Correlation	r Value*	P value*
Eosinophils vs seasonal symptoms	0.6	<.02
IL-5 vs seasonal symptoms	0.6	<.02
Eosinophils vs IL-5	0.5	<.05

*Spearman rank correlation.

In patients with allergic rhinitis, the nasal mucosa contains increased numbers of inflammatory cells, particularly eosinophils, both after allergen challenge¹⁷ and during natural seasonal pollen exposure.¹ Activated eosinophils release a multitude of cytotoxic granule proteins, including eosinophil cationic protein and major basic protein, which disrupt the basement membrane, leading to epithelial desquamation and ciliostasis.¹⁸ In addition, eosinophils are a primary source of proinflammatory mediators, such as leukotrienes, integrins, selectins, and the T_H2 cytokines IL-4¹⁹ and IL-5.²⁰ Eosinophils are therefore a pivotal component of the inflammatory response to allergen.

IL-5 is the predominant proeosinophilic T_H2 cytokine and is secreted mainly by T_H2-polarized T lymphocytes,⁵ although eosinophils, mast cells, and natural killer T cells²¹ are alternative sources. IL-5 exerts its effect through the IL-5 receptor, which is present on eosinophils at both early²² and late²³ stages of their development. The effects of IL-5 on eosinophils include promotion of their release from the bone marrow,²⁴ activation,^{25,26} adhesion,²⁷ degranulation,²⁸ and survival.²⁹

IL-5 acts as a specific chemoattractant for peripheral blood eosinophils in vitro.³⁰ Application of recombinant human IL-5 to the allergic nasal mucosa results in significantly increased numbers of eosinophils and eosinophil cationic protein in nasal lavage fluid,³¹ and the transfer of an

ovalbumin-specific IL-5–producing T-cell clone to the lungs of unsensitized mice results in pulmonary eosinophilic inflammation after inhalational ovalbumin challenge.³² In contrast, IL-5–deficient sensitized mice undergoing aeroallergen challenge do not have tissue eosinophilia or bronchial hyperreactivity.³³ Thus eosinophilic inflammation appears to be IL-5 dependent.

IL-5 and eosinophils are both present in increased amounts in nasal wash fluid taken from patients with seasonal allergic rhinitis during the pollen season,³⁴ and cells positive for IL-5 mRNA are present in the bronchial mucosa of asthmatic subjects³⁵ and in the nasal mucosa of subjects with allergic rhinitis during the pollen season.⁴ Also present in the nasal mucosa are CD34⁺/IL-5R α ⁺ progenitor cells,³⁶ which undergo terminal differentiation to eosinophils within the mucosa under the influence of IL-5.³⁷ The increase in eosinophil numbers that we have observed during the pollen season may therefore be due either to the infiltration of mature eosinophils from the circulation under the influence of adhesion factors (eg, intercellular adhesion molecule 1), IL-5,³⁸ and other chemotactic factors (eg, eotaxin)²³ or to the local differentiation of eosinophil progenitor cells under the control of locally secreted IL-5. The seasonal increase in both total and activated T cells that we have observed in the placebo-treated patients is consistent with the hypothesis that allergen-specific T_H2-type T cells resident within the nasal mucosa, recruited from the circulation, or both are activated in the presence of allergen. Such activated T cells will secrete T_H2-type cytokines, including IL-5, resulting in tissue eosinophilia by either or both of the above mechanisms.

Grass pollen immunotherapy is highly effective in reducing hay fever symptoms and medication requirements during the pollen season.³⁹ This clinical improvement is associated with a reduction in both immediate and late cutaneous sensitivity to allergen, a reduction in allergen-induced nasal eosinophils and T lymphocytes, and an increase in the number of cells positive for the T_H1 cytokine IFN- γ .⁴⁰ In this study we have shown that the reduction in seasonal nasal mucosa eosinophilia after immunotherapy is closely associated with inhibition of the mucosa expression of mRNA for IL-5, a pivotal T_H2 cytokine.

In conclusion, we have demonstrated, for the first time, that after immunotherapy for seasonal allergic rhinitis, there is a close correlation between symptoms, tissue eosinophilia, and IL-5 mRNA expression during natural pollen exposure. These data suggest that changes in the local cytokine milieu induced by immunotherapy lead directly to reduced eosinophil infiltration and symptomatic improvement. These data also strengthen the argument that IL-5 is crucial in the pathogenesis of allergic rhinitis and suggest that strategies against IL-5 (eg, using an mAb against IL-5)⁴¹ may represent an alternative, more targeted approach to treatment.

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