

Immunodeficiency and other clinical immunology

Increases in IL-12 messenger RNA+ cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy

Quatyba A. Hamid,^a Elsa Schotman,^a Mikila R. Jacobson,^b Samantha M. Walker,^b and Stephen R. Durham^b *Montreal, Quebec, Canada, and London, England*

IL-12, a novel cytokine produced by tissue macrophages and B lymphocytes, stimulates proliferation of T_{H1}-type T lymphocytes. We recently showed that in patients with summer hay fever, immunotherapy was effective and was associated with inhibition of allergen-induced late skin responses and increases in local interferon- γ messenger RNA-positive cells. In this study 10 patients were reassessed after 4 years of immunotherapy and compared with 10 untreated patients with hay fever. Intradermal grass pollen challenge was performed, the late response was measured, and biopsies were performed at 24 hours. In situ hybridization of biopsy sections was performed by using a riboprobe coding for IL-12 mRNA. When immunotherapy and control subjects were compared, there was a marked reduction in the size of the late skin response ($p = 0.0001$). Significant increases in allergen-induced IL-12 mRNA+ cells in cutaneous biopsy specimens occurred only in the immunotherapy-treated group (all 10 patients, $p = 0.002$). At allergen-challenged sites, IL-12+ cells correlated positively with interferon- γ + cells ($r = 0.64$, $p < 0.05$) and inversely with IL-4+ cells ($r = -0.67$, $p < 0.05$). The principal cell source (55% to 80%) of IL-12 message was the tissue macrophage (CD68+ cells). We suggest that IL-12 may promote T_{H1} responses and inhibit late-phase responses after successful immunotherapy. (J Allergy Clin Immunol 1997;99:254-60.)

Key words: Immunotherapy, cytokines, late responses IL-12

Immunotherapy for summer hay fever is effective, although efficacy must be balanced against side effects.^{1,2} We recently reported a double-blind, placebo-controlled trial in which grass pollen immunotherapy was highly effective in reducing clinical symptoms and requirements for topical and oral rescue medication.³ After 9 months of immunotherapy, a marked reduction in the size of the allergen-induced late cutaneous response was observed. Immunohistologic examination of cutaneous biopsy specimens obtained at 24 hours demonstrated a decrease in CD4+ T lymphocytes and eosinophils.⁴ Unexpected findings included significant increases in tissue macrophages (CD68+ cells) and

Abbreviations used

BU: Biological units
IFN- γ : Interferon- γ

highly significant increases in class II (HLA-DR) expression and IL-2 receptor (CD25)-bearing cells. Interferon- γ represents a major stimulus for HLA-DR, and IL-2 enhances IL-2 receptor expression. In situ hybridization studies subsequently revealed increases in messenger RNA+ cells for interferon- γ (IFN- γ) and IL-2 only in the immunotherapy-treated group. We recently confirmed allergen-induced increases in IFN- γ + cells during inhibition of late nasal responses after immunotherapy.⁵ These observations suggested that immunotherapy might act by inducing a local "T_{H1}-type" T-lymphocyte response to allergen with consequent inhibition of local T_{H2}-mediated events, such as tissue eosinophilia and IgE production by B cells.⁴⁻⁶

IL-12 is a novel cytokine, which may be involved in protective immunity against viruses.⁷ IL-12 is produced by macrophages and B lymphocytes and has the ability to induce proliferation of T_{H1}-type cells.⁸⁻¹⁰ In this study we

From ^athe Department of Medicine and Pathology, Meakins Christie Laboratory, McGill University, Montreal; and ^bUpper Respiratory Medicine, Imperial College London at the National Heart and Lung Institute, London.

Supported by the Medical Research Council, United Kingdom and Montreal Chest Institute, Network Centre of Excellence for Respiratory Disease, Montreal, Canada.

Received for publication Oct. 4, 1995; revised Jan 25, 1996; accepted for publication Mar. 7, 1996.

Reprint requests: Stephen R. Durham, Upper Respiratory Medicine, Imperial College London at the National Heart and Lung Institute, Dovehouse St., London SW3 6LY, England.

Copyright © 1997 by Mosby-Year Book, Inc.

0091-6749/97 \$5.00 + 0 1/173616

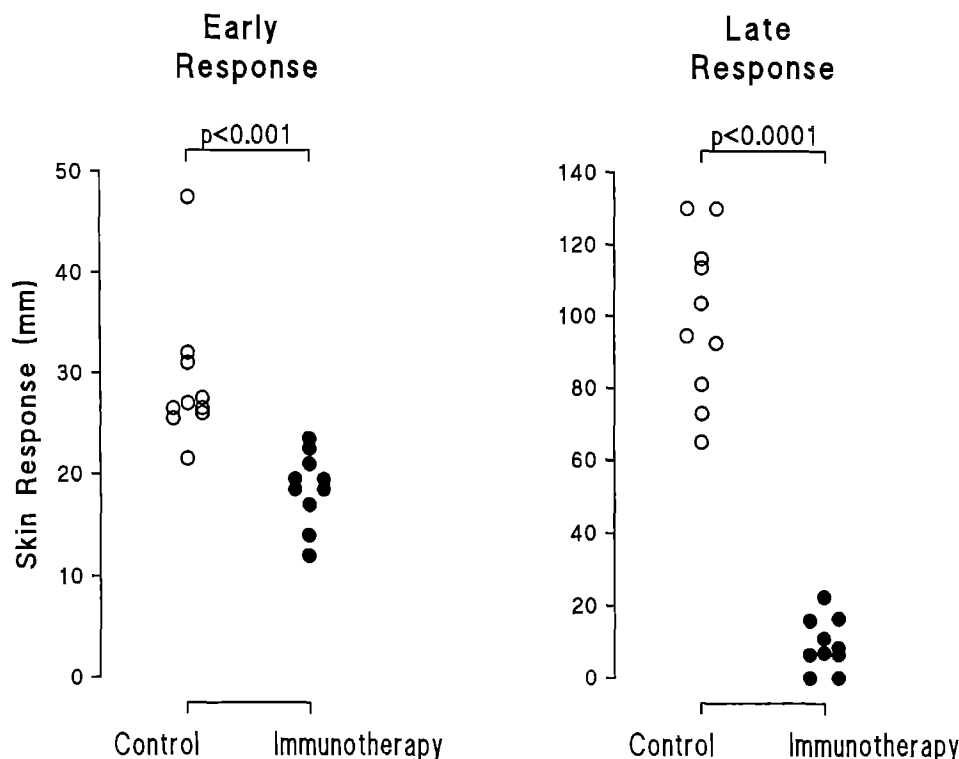


FIG. 1. Size of early (15-minute) and late (24-hour) skin responses after intradermal allergen (30 BU *Phleum pratense*) in control (open circles) and immunotherapy-treated (filled circles) patients. Between-group comparisons were done with unpaired *t* test.

hypothesize that T_{H1} responses after immunotherapy may occur as a consequence of increased production of IL-12. We have therefore performed intradermal allergen challenge (and a control challenge) in patients after 4 years of grass pollen immunotherapy.¹¹ In situ hybridization studies were performed by using an antisense riboprobe coding for IL-12 mRNA on cutaneous biopsy specimens obtained at 24 hours. Results were compared with a control group of untreated grass pollen-sensitive subjects.

METHODS

Patients

Patients who had previously received 4 years of grass pollen immunotherapy for treatment of severe summer hay fever were studied.^{3,11} As a control group, 10 grass pollen-sensitive subjects with hay fever of comparable severity (when compared with immunotherapy-treated patients before they had received treatment) were recruited from the Allergy Clinic at Royal Brompton Hospital, London (Table I). Subjects were excluded from the study if they had a history of asthma, eczema, or other significant medical or immunologic disease. Immunotherapy-treated patients had received 4 years of treatment with Alutard SQ *Phleum pratense* (ALK, Horsholm, Denmark), an aluminum hydroxide-precipitated depot grass pollen extract. After routine twice weekly dose increases, to a maintenance dose of 100,000 SQ units in 1 ml, patients continued to receive monthly maintenance injections for 4 years.

TABLE I. Clinical data of patients studied

	Control group (n = 10)	Immunotherapy-treated group (n = 10)
Age (yr $\pm$ 56)	32.5 (3.5)	44.9 (3.2)
Sex (M/F)	6/4	4/6
Skin prick test to grass pollen	9.2 (0.6)	9.4 (0.8)
No. of patients with hay fever severity score*		
A	4	5
B	6	5

*Severity score A = skin prick test >16 mm; positive conjunctival provocation test at <300 BU/ml; history of symptoms for >10 weeks associated with asthma and previous oral or injected corticosteroids, or both. Severity score B = patients with moderate to severe hay fever who did not fulfill all of above criteria.

Study design

The study was approved by the Royal Brompton Hospital Ethics Committee and performed with the patients' written informed consent. Intradermal challenges were performed on the extensor aspect of the forearm at the same time of day with 30 biological units (BU) (0.02 ml) of intradermal grass pollen extract and the allergen diluent (0.02 ml) as a control. The grass pollen extract used (*Phleum pratense*, ALK) was a biologically standardized and partially purified freeze-dried extract, which

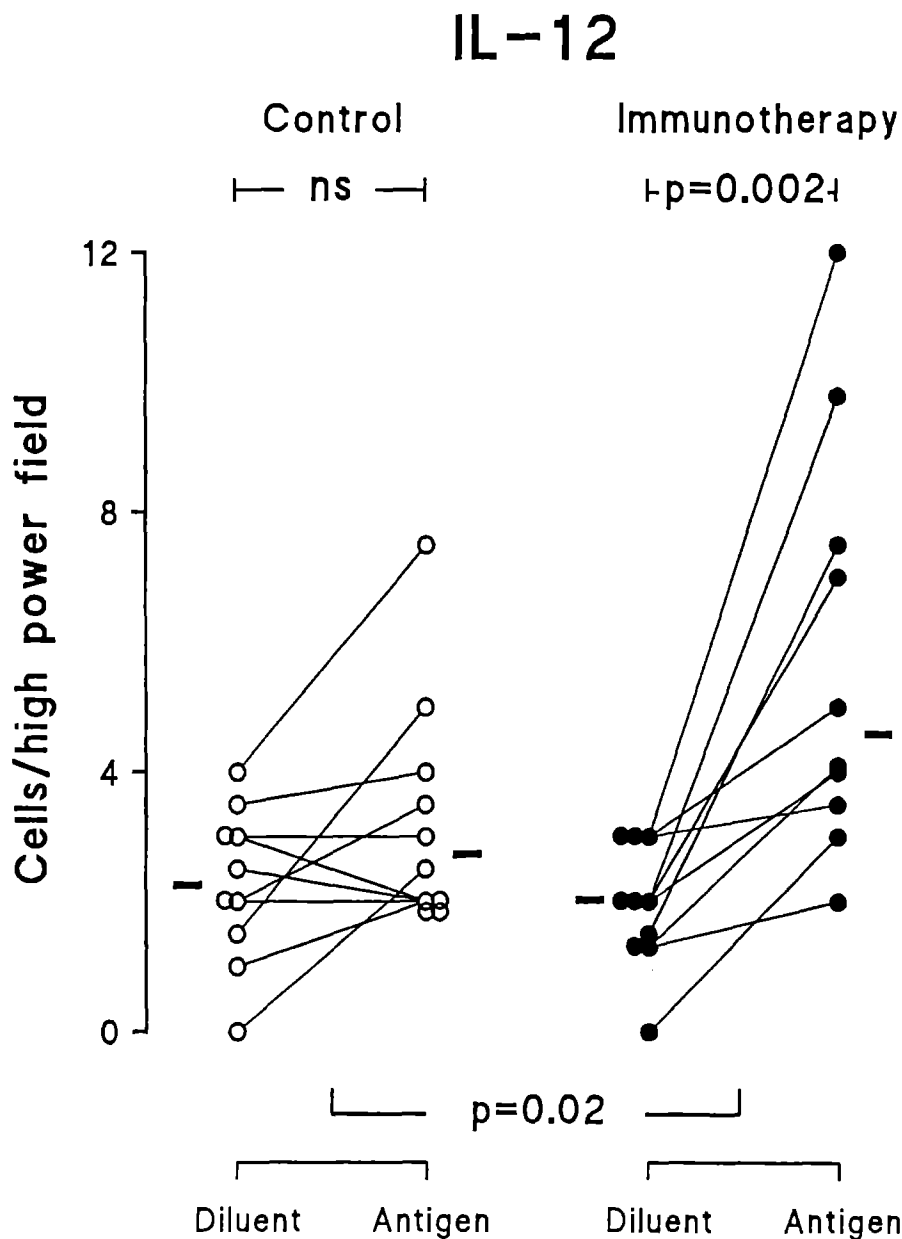


FIG. 2. In situ hybridization studies of IL-12 mRNA+ cells in cutaneous biopsy specimens 24 hours after control (allergen diluent) and allergen (30 BU grass pollen extract) in untreated grass pollen-sensitive patients (*open circles*) and immunotherapy-treated patients (*filled circles*). Within- and between-group comparisons were performed by using paired and unpaired *t* tests.

was reconstituted immediately before intradermal use. The sizes of the immediate (15-minute) and late-phase (24-hour) responses were recorded as the means of the longest and the perpendicular diameters. After local anesthesia was induced with 1% lignocaine, 24-hour skin biopsy specimens were obtained from the center of allergen and diluent (control) sites by using a 3 mm disposable punch.⁴

In situ hybridization

Biopsy specimens were immediately placed in 4% paraformaldehyde in 0.1 molar phosphate-buffered saline for 1 hour, followed by 15% sucrose-phosphate-buffered saline for 1 hour, and then subsequently in 15% sucrose-phosphate buffered saline overnight. Biopsy specimens were then snap-frozen and

stored at -80°C . Cryostat sections ($10\text{ }\mu\text{m}$) were cut and placed on poly-L-lysine-coated microscope slides. An antisense riboprobe for IL-12 mRNA was generated from IL-12 complementary DNA (p35 isoform).¹² In situ hybridization was performed as previously described by using sulfur 35-labeled probes.^{4,13} Allergen-challenged biopsy specimens from immunotherapy-treated patients were also hybridized with antisense probes against IL-4 and IFN- γ (Glaxo Biogen, Geneva). Slides were counted in blinded fashion by using an Olympus BH2 microscope (magnification $\times 200$) with an eyepiece graticule. The upper edge of the grid was placed at the epidermal junction. Messenger RNA+ cells were expressed as cells per field, excluding areas containing sweat ducts and hair shafts where only intervening dermal regions were counted. Within-

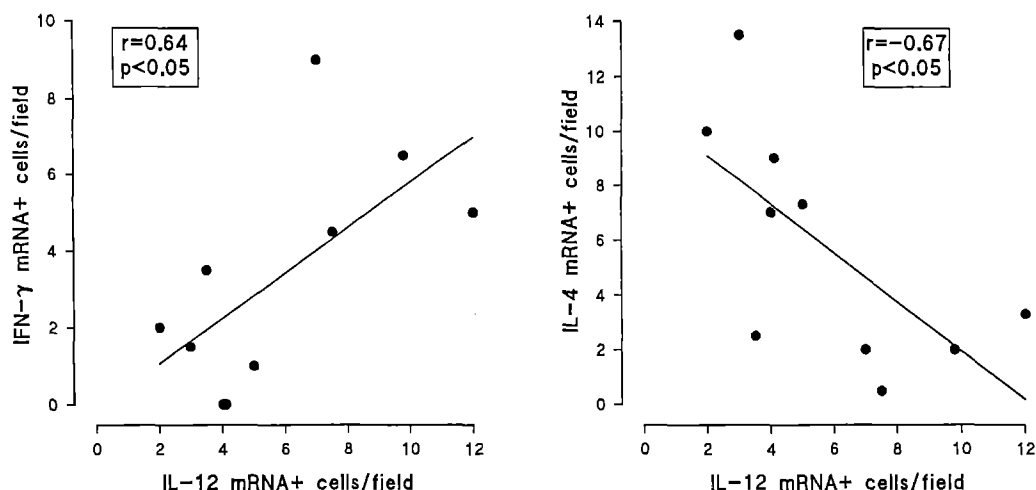


FIG. 3. Correlations between IL-12 mRNA+ cells and IFN- γ + and IL-4+ cells at allergen-challenged sites in patients who had received immunotherapy. Pearson correlation coefficient was used. Levels of statistical significance are shown.

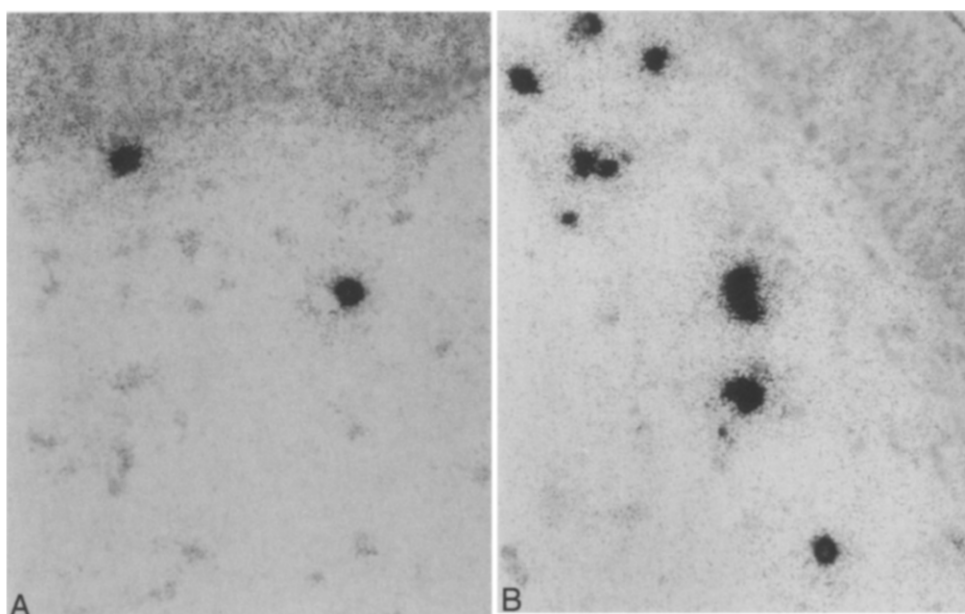


FIG. 4. Autoradiographs of skin biopsy sections obtained from control (diluent-challenged) site (A) and allergen-challenged site (B) obtained from an immunotherapy-treated patient. Sections were hybridized with an ^{35}S -labeled antisense riboprobe for IL-12. IL-12-positive signals are evident as discrete cells overlaid by black silver grains.

observer coefficient of variation for repeat measurements was less than 5%. Negative controls included use of an IL-12 sense probe and pretreatment of sections with RNase before use of the antisense probe.

For co-localization of IL-12 message, double immunocytochemistry/in situ hybridization was performed as described previously.¹⁴ Immunocytochemistry was performed (alkaline phosphatase-anti-alkaline phosphatase technique) by using a monoclonal antibody directed against CD68 (Becton Dickinson, Cowley, Oxford, U.K.), followed by in situ hybridization with an ^{35}S -labeled antisense IL-12 riboprobe.

Analysis of results within and between group comparisons

were made by using Student's paired and unpaired t tests. Correlations were obtained by using Pearson's test.

RESULTS

Control and immunotherapy-treated patients (before treatment) were well-matched for gender, summer hay fever severity, and grass pollen sensitivity as determined by skin prick tests. There was a small significant difference in age between the groups ($p < 0.05$) (Table I). After intradermal testing with grass pollen, 30 BU, the size of the early (15-minute) and late (24-hour) skin

TABLE II. Co-localization of IL-12 mRNA+ cells to macrophages (CD68+)

Patient No.	Total IL-12 mRNA+ cells/hpf	IL-12 mRNA+ CD68+ cells	IL-12 mRNA+ cells that are CD68+ (%)
1	16	9	55
3	22	14.5	65
12	13	10	79
15	15	12	80

Data are from biopsy specimens of allergen-challenged sites in four patients after immunotherapy.

hpf, High-power field.

responses were highly significantly reduced in immunotherapy-treated patients compared with control patients ($p < 0.0001$) (Fig. 1).

Cutaneous biopsy specimens were processed for in situ hybridization by using an IL-12 antisense riboprobe (Fig. 2). There was no significant difference in the number of IL-12 mRNA+ cells after control (diluent) challenge for either group of patients. A significant increase in the number of IL-12 mRNA+ cells was observed only in the immunotherapy-treated group ($p < 0.006$). This difference (allergen minus diluent sites) was significant ($p = 0.02$) when compared with the corresponding values for the untreated patients. The number of IL-12 mRNA+ cells at allergen-challenged sites for the immunotherapy-treated group was related to the corresponding number of cells mRNA+ for IFN- γ and IL-4 (Fig. 3). A significant positive correlation was observed between IL-12 mRNA+ cells and IFN- γ + cells ($r = 0.64$, $p < 0.05$) and a significant inverse relationship between IL-12+ and IL-4+ cells ($r = -0.67$, $p < 0.05$). A significant negative correlation was also found between the number of IL-4 and IFN- γ mRNA+ cells ($r = -0.72$, $p < 0.02$, data not shown).

Examples of hybridization studies of IL-12 mRNA+ cells in cutaneous biopsy specimens are shown in Fig. 4. IL-12 mRNA+ cells were distributed around blood vessels and scattered throughout the dermis. Individual mRNA+ cells were evident as discrete, dark-staining cells with a low background signal under the conditions used when compared with experimental controls.

Sequential immunostaining/in situ hybridization of four allergen-challenged biopsy specimens from the immunotherapy-treated group showed that 55% to 80% of positive IL-12 mRNA hybridization signals were co-localized to macrophages (CD68+ cells) (Table II).

DISCUSSION

In agreement with previous studies^{3, 15, 16} we have shown that prolonged immunotherapy in patients with hay fever resulted in almost complete inhibition of allergen-induced late cutaneous responses. In situ hybridization demonstrated highly significant increases in IL-12 mRNA+ cells at allergen-challenged sites, which correlated with the number of IFN- γ + cells. An inverse correlation was noted between IL-12+ cells and IL-4+

cells. Taken together, these observations suggest that successful grass pollen immunotherapy may modify the T-lymphocyte response to subsequent allergen exposure with an additional T_{H1} response (with local increases in IFN- γ), which may be driven by IL-12.

Immunotherapy-treated and control patients were well-matched for gender, disease severity, and allergen sensitivity, although by chance there was a small increase in the mean age of the immunotherapy-treated group. However, increases in IL-12 mRNA were observed in all 10 immunotherapy-treated patients irrespective of their age, such that age was unlikely to account for the marked differences observed in local IL-12 mRNA expression. Co-localization studies, in which previously validated methods were used, confirmed the macrophage as the principal source of IL-12. The remainder may be either B lymphocytes or possibly other cells not examined in this study. Increases in IL-12 were only observed in immunotherapy-treated patients and not in the untreated group, which suggests that increases were unlikely to be explained by any contaminants such as excipients or endotoxin, which could have been present in the allergen extract. The latter is unlikely because the allergen extract used was manufactured under sterile conditions, stored freeze-dried in sterile vials, and re-constituted immediately before use.

Previous studies of the mechanism of immunotherapy have focused on changes in peripheral blood. A blunting of seasonal increases in IgE¹⁷ and marked increases in allergen-specific IgG concentrations¹⁸ have been observed. A recent study demonstrated a reduction in mast cell numbers in nasal epithelial scrapings after immunotherapy in house dust mite-sensitive patients.¹⁹ Immunotherapy has also been shown to decrease mast cell mediators²⁰ and reduce eosinophil infiltration²¹ after local nasal allergen provocation. Our previous placebo-controlled studies of late responses in the skin⁴ and nose⁵ after grass pollen immunotherapy and studies of peripheral blood mononuclear cells after mite²² and venom^{23, 24} immunotherapy support our original hypothesis⁶ that immunotherapy may act by inducing T_{H1} responses. It is of interest that normal nonatopic individuals also mount immune responses to grass pollen extract. In vitro studies confirm that T lymphocytes from normal subjects proliferate^{22, 25, 26} in response to grass pollen and produce increased IFN- γ ²⁶ and decreased IL-4^{22, 26} when compared with grass pollen-sensitive subjects with atopic rhinitis. In this sense, immunotherapy may simply be amplifying the "normal" T_{H1} response to grass pollen present in nonatopic subjects, which, in patients with atopic rhinitis, competes with the "abnormal" T_{H2} response. It would be of interest to observe whether nonatopic individuals also produce IL-12 in response to grass pollen, although this was not examined in this study.

IL-12 appears to be the principal cytokine responsible for proliferation of T_{H1} -type cells in response to antigen challenge.⁷⁻⁹ IL-12 has also been shown to inhibit proliferation of T_{H2} (IL-4-producing) T lymphocytes and inhibit IL-4-dependent B-cell switching from IgG to

IgE production.¹⁰ Production of IL-4 by cloned cells, however, is at best only minimally affected by the presence or absence of IL-12.²⁷ Our findings of increases in IL-12 mRNA+ cells, which correlate directly with IFN- γ + and inversely with IL-4+ cells, provide further support for the view that T_{H1} responses after immunotherapy may be IL-12-driven.

There are practical implications to our findings. Human allergic responses would appear to result from a local mucosal imbalance of T_{H2}/T_{H1} responses.²⁸ Local increases in IFN- γ may also be consistent with the induction of T-cell anergy.²⁹ However, our finding of an increase in IL-12 would support the induction of T_{H1} responses rather than T-cell anergy as responsible for inhibition of the late response after immunotherapy. Recent animal studies also provide evidence that strategies involving topical IFN- γ or IL-12 might represent a viable therapeutic approach in patients with allergy, although clearly detailed safety studies would first be required. For example, IFN- γ administered by inhalation in mice was effective in inhibiting IgE production and allergen-induced increases in airway responsiveness.³⁰ When vaccination of rodents with *Schistosoma* eggs was combined with IL-12, there was a marked reduction in hepatic granuloma formation and fibrosis after subsequent natural infection with *Schistosoma* worms.³¹ This raises the possibility that IL-12 in combination with allergen might have the potential to inhibit T_{H2}-mediated allergic responses while reducing the dose of allergen (and attendant risks of side effects) required for effective immunotherapy.

We thank Dr. H. Lowenstein, ALK Denmark, for the supply of allergen extracts for diagnosis and immunotherapy. We also thank Qui Meng for her help in preparation of the IL-12 probe. IL-12 cDNA was a generous gift from Genetics Institute (Cambridge, Mass.).

REFERENCES

- BSACI Working Party. Position paper on allergen immunotherapy. *Clin Exp Allergy* 1993;23(suppl 3):1-44.
- Malling HJ, Weeke B. Immunotherapy. Position paper of the European Academy of Allergology and Immunology. *Allergy* 1993; 48(suppl 14):1-35.
- Varney V, Gaga M, Frew AJ, Aber VA, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hayfever uncontrolled by antiallergic drugs. *Br Med J* 1991;302: 265-9.
- Varney VA, Hamid QA, Gaga M, Sun Ying, Jacobson M, Frew AJ, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993;92:644-51.
- Durham SR, Sun Ying, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4⁺ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon- γ . *J Allergy Clin Immunol* 1996;97:1356-65.
- Durham SR, Varney VA, Gaga M, Jacobson MR, Frew AJ, Kay AB. Immunotherapy and allergic inflammation. *Clin Exp Allergy* 1991; 21(suppl 1):206-10.
- Clerici M, Lucey DR, Berzofsky JA, Pinto LA, Wynn TA, Blatt SP, et al. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science* 1993;262:1721-4.
- Hsieh C-S, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4⁺ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993;260:547-9.
- Manetti R, Parronchi P, Giudizi MG, Piccinini M-P, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin-12 {IL-12}) induces T helper type 1 (Th-1)-specific immune responses and inhibits the development of IL-4 producing Th cells. *J Exp Med* 1993;177:1199-204.
- Kiniwa M, Gately M, Gubler U, Fargeas C, Delespessis GJ. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J Clin Invest* 1992;90: 262-6.
- Walker SM, Varney VA, Jacobson MR, Durham SR. Grass pollen immunotherapy: efficacy and safety during a four year follow-up study. *Allergy* 1995;50:405-13.
- Bette M, Jin SC, Germann T, Schazer MK, Weike E, Rude E, et al. Differential expression of mRNA encoding interleukin-12 p35 and p40 subunits in situ. *J Immunol* 1994;10:2435-40.
- Chan-Palay V, Yasargil G, Hamid Q, Polak JM, Palay SL. Simultaneous demonstration of neuropeptide Y gene expression and peptide storage in single neurons of the human brain. *Proc Natl Acad Sci* 1988;88:3213-5.
- Hamid Q, Wharton J, Terenghi G, Hassall CJS, Aimi J, Taylor KM, et al. Localization of atrial natriuretic peptide mRNA and immunoreactivity in the rat heart and human atrial appendage. *Proc Natl Acad Sci* 1987;84:6760-4.
- Fling JA, Ruff ME, Parker WA, Whisman BA, Martin ME, Moss RB, et al. Suppression of the late cutaneous response by immunotherapy. *J Allergy Clin Immunol* 1989;83:101-9.
- Nish WA, Charlesworth EN, Davis TL, Whisman BA, Valtier S, Charlesworth MG, et al. The effect of immunotherapy on the cutaneous late phase response to antigen. *J Allergy Clin Immunol* 1994;93:484-93.
- Lichtenstein LM, Ishizaka K, Norman PS, Kagey-Sobotka A, Hill BM. IgE antibody measurements in ragweed hayfever. Relationship to clinical severity and the results of immunotherapy. *J Clin Invest* 1973;52:472-82.
- Djurup R. The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen specific immunotherapy. *Allergy* 1985;40:469-86.
- Otsuka H, Mezawa HA, Ohnishi M, Okubo K, Seki H, Okuda M. Changes in nasal metachromatic cells during allergen immunotherapy. *Clin Exp Allergy* 1991;21:115-9.
- Creticos PS, Adkinson NF Jr, Kagey-Sobotka A, Proud D, Meier HL, Naclerio RM, et al. Nasal challenge with ragweed in hayfever patients: effects of immunotherapy. *J Clin Invest* 1985;76:2247-53.
- Furin MJ, Norman PS, Creticos PS, Proud D, Kagey-Sobotka A, Lichtenstein LM, et al. Immunotherapy decreases antigen-induced eosinophil migration into the nasal cavity. *J Allergy Clin Immunol* 1991;88:27-32.
- Secrist H, Cheten CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin-4 production in CD4⁺ T cells from allergic individuals. *J Exp Med* 1993;178:2123-30.
- McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine response from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin Exp Allergy* 1995;25:828-38.
- Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen stimulated T cell cultures. *J Immunol* 1995;154:4187-94.
- Sallusto F, Quintieri F, Pugliese O, Reale G, Pini C, Di Felice G. T cells and antibody response to *Parietaria judaica* allergenic fractions in atopic and non-atopic individuals. *Allergy* 1993;48:37-44.
- Imada M, Simons FER, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon- γ production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunotherapy* 1995;85:373-80.
- Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P, Piccinini M, et al. Interleukin-12 induces stable priming for interferon- γ (IFN- γ) production during differential of human Thelper (Th) cells and transient IFN- γ production in established Th2 cell clones. *J Exp Med* 1994;179:1273-83.

28. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995;13:251-76.
29. O'Hehir RE, Yssel H, Verma S, et al. Clonal analysis of differential lymphokine production in peptide and superantigen induced T-cell allergy. *Int Immunol* 1991;3:819-26.
30. Lack G, Renz H, Saloga J, Bradley KL, Loader J, Leung DYM, et al. Nebulized but not parenteral IFN- γ decreases IgE production and normalises airways function in a murine model of allergen sensitisation. *J Immunol* 1994;152:2546-54.
31. Wynn TA, Cheever AQ, Jankovic D, Poindexter RW, Casper P, Lewis FA, et al. An IL-12 based vaccination method for preventing fibrosis induced by schistosome infection. *Nature* 1995;376:594-6.