

House dust mite immunotherapy results in a decrease in Der p 2-specific IFN- γ and IL-4 expression by circulating T lymphocytes

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Summary

Background Allergen-specific immunotherapy (IT) can be an important adjunctive therapy in the treatment of allergic disorders. Although it has now been used for over 80 yr, the precise mechanism of action remains unclear. Recently a number of studies have shown that cytokine production may be modified by IT, but different protocols have been used and different results obtained.

Objectives The aims of the present study were: (1) to document the allergen-specific expression of interleukin-4 (IL-4) and interferon- γ (IFN- γ) by peripheral blood cells in both untreated house dust mite (HDM) allergic patients (non-IT) and following at least 10 months of HDM-specific IT (post-IT); and (2) to determine whether alterations in these critical regulatory cytokines correlated with the clinical outcome of IT.

Methods IT was undertaken with nine fortnightly subcutaneous injections of increasing amounts of a *Dermatophagoides pteronyssinus* (Dpt) extract, reaching a final dose of 10 000 PNU. This was followed by 6- to 8-monthly maintenance injections of 5000 PNU. For cytokine measurement, mononuclear cells were separated from peripheral blood and stimulated with the major Dpt allergen, Der p 2, for 18 h, after which mRNA was isolated and IL-4 and IFN- γ cDNA were amplified by polymerase chain reaction (PCR). The presence of the particular cytokine was determined by visualization following electrophoresis on an agarose gel. The study was observational in nature being open and without a placebo group.

Results Fifteen Dpt-sensitive patients who had not received HDM IT (non-IT), and 16 who had, were studied. In the non-IT group, 80% expressed IL-4 and 75% expressed IFN- γ . In those post-IT, only 12.5% expressed IL-4 and 19% IFN- γ . The two patients still expressing IL-4 post-IT had had very little clinical response. Six patients were studied both pre- and post-IT. Prior to IT, three were positive for both cytokines, two positive for IL-4 alone and one for IFN- γ . Post-IT, all six were negative for IL-4 and five were negative for IFN- γ .

Conclusion Allergen-specific IT results in a reduction in expression of the critical cytokines IL-4 and IFN- γ in circulating lymphocytes. It is possible that this is a contributory mechanism in the beneficial effect of IT.

Keywords: house dust mite, interleukin-4, interferon- γ , cytokine expression, Der p 2, allergen-specific immunotherapy

Clinical and Experimental Allergy, Vol. 27, pp. 46–51. Received 7 November 1995; revised 22 December 1995; accepted 28 April 1996.

Introduction

Allergen-specific immunotherapy (IT) has been used for many years in the treatment of allergic diseases and results in alterations of a number of immunological parameters including a decrease in allergen skin test reactivity and specific IgE titre, an increase in specific IgG4 titre, the development of auto-anti-idiotypic antibodies and a reduction in allergen-specific lymphocyte proliferation and IL-2 release [1,2]. Efficacy has been confirmed in numerous studies and a recent meta-analysis has shown an overall significant benefit from house dust mite IT in asthma [3]. The mechanism for the improvement is not well understood and the alteration in serologic reactivity, in particular, does not correlate well with the clinical outcome [4]. More recently, attention has been directed to immunoregulatory mechanisms and changes in the cytokine profile of allergen-reactive T cells.

Secrist *et al.* reported a decrease in allergen-induced IL-4 production following IT, but no alteration in IL-2 or IFN- γ production by CD8-depleted PBMC [5]. In this study, though, the actual stimulus for cytokine production involved an 18 h culture with the mitogen PHA. In contrast, MacDonald *et al.*, studying cytokine release from PBMC in patients pre- and post-ragweed IT, showed a highly significant reduction in the production of IFN- γ , but no significant difference in IL-4 levels [6]. Varney *et al.*, using *in situ* hybridization with labelled riboprobes, investigated cytokine mRNA expression within inflammatory cells in allergen-induced late-phase cutaneous responses. An increase in hybridization for IL-2 and IFN- γ was only detected in those who had received grass pollen IT, and not in controls. There was no significant change in IL-4 expression [7]. A later study by the same group investigated cytokine mRNA expression in nasal mucosal cells during allergen induced late responses. Again, a significant increase in IFN- γ mRNA was detected; however, no significant alteration was detected in mRNA for IL-4 or IL-5 [8].

We had previously attempted to study the allergen-specific production and release of cytokines from freshly isolated PBMC using highly sensitive biochemical assays. Due presumably to the low numbers of allergen-reactive cells present, these assays were only weakly positive at best, and frequently unreliable. Because of this, we developed a reverse transcriptase (RT) PCR for detection of allergen-specific IL-4 and IFN- γ mRNA expression in freshly isolated PBMC [9]. The major HDM allergen Der p 2 was used for specific cellular stimulation. Using this assay system in a group of nine patients sensitive to *Dermatophagoides pteronyssinus* (Dpt), all transcribed IL-4 when stimulated for 18 h with Der p 2, and 67% transcribed IFN- γ . In contrast, in 13 non-atopic control subjects, none transcribed IL-4 and seven transcribed IFN- γ [9]. As an alteration in T-cell reactivity

and/or cytokine release is likely to be one of the important mechanisms of action of IT, and as there is no clear consensus on the effect of IT on profiles of cytokine production, we studied the Der p 2-induced expression of IL-4 and IFN- γ mRNA in freshly isolated PBMC from Dpt-sensitive individuals, both in non-IT and post-IT patients.

Patients and methods

Patients

Adult patients attending the allergy clinics at either the Royal Melbourne Hospital or Western Hospital Footscray were asked to participate in this study. Patients selected were all symptomatic, with either rhinitis and/or asthma, and were all sensitive to Dpt by skin prick test. Fifteen patients were studied who had not received IT and six of these 15 were studied again after at least 10 months of Dpt IT. A further 10 patients were studied who had already received IT. There was no difference in severity or range of symptoms or skin test findings between those patients who went on to receive IT and those who did not. None of the patients were on oral steroids or anti-histamines at the time blood was drawn.

IT was undertaken by subcutaneous administration using Allpyral Dpt extracts (Bayer, Australia) and patients received nine fortnightly injections, all reaching the final dose of 1 ml of the 10 000 PNU/ml extract. Monthly maintenance injections of 0.5 ml of the 10 000 PNU/ml extract were then commenced and six to eight injections were given prior to the post-IT cytokine assays. All post-IT patients had received therapy for at least 10 months and none had received an injection within 3 weeks of blood sampling.

Symptoms of rhinitis and asthma, and medication usage, were assessed in patients pre- and post-IT, irrespective of whether cytokine assays were performed prior to IT. An overall score was obtained for a 2-month period prior to the IT and again prior to the lymphokine assays in the post-IT group. Patients were provided with a diary card and asked to record symptoms of sneezing, rhinorrhoea, nasal blockage, night cough, day wheeze and day breathlessness, on a scale from 0 to 3. Use of a nose spray or taking one or more antihistamines a day each had a score of 2, for that day. In asthmatics, the frequency of use of bronchodilator therapy was also recorded and a score of 0.5 was added for each separate use. The mean daily symptom-medication score for the 2-month period was compared in patients before and following IT. Response to IT was graded on a scale from 0 (no change), 1+ (minor improvement), 2+ (good response) and 3+ (excellent response) as follows: score post-IT > 75% of score pre-IT, response of 3+; score 50–75%, response of 2+; score 25–50%, response of 1+; score < 25%, response of 0.

Allergen induction and cytokine detection by (RT)PCR

PBMC were isolated by centrifugation on Ficoll-Hypaque, suspended in medium containing 5% autologous human serum and cultured with affinity-purified Der p 2 at 0.5 µg/ml for 18 h. Detailed methods for cell culture, RT PCR and cytokine detection have previously been described [9]. Briefly, total RNA was isolated after culture with either Der p 2, PHA as a positive control or medium alone, and PCR was used to amplify the reverse transcribed cytokines. Both negative (no DNA) and positive (cloned cDNA) controls were included with each reaction. β 2-microglobulin was also amplified as a control for the RNA isolation and reverse transcription. Following 35 cycles of PCR, 15 µl of the amplified product was electrophoresed on a 2% agarose gel. The gel was then stained with ethidium bromide and visualized under UV light and results were unequivocally positive or negative.

Results

Fifteen allergic symptomatic individuals who had not received IT (non-IT) and 16 who had (post-IT) were studied, six patients being studied both pre- and post-IT. Results for all patients are presented in Table 1. In the non-IT group, 12/15 (80%) expressed IL-4 and 9/12 (75%) expressed IFN- γ , with six expressing both cytokines, three expressing IL-4 alone and three IFN- γ alone. In those post-IT, only 2/16 (12.5%) expressed IL-4 and 3/16 (19%) IFN- γ . Cells from all patients expressed both cytokines following PHA stimulation. Of the six patients studied both pre- and post-IT, three were positive for both cytokines, two positive for IL-4 and one for IFN- γ , prior to IT. Post-IT, all six were negative for IL-4, one patient continued to express IFN- γ . Figure 1 shows results of cytokine analysis for IL-4 and IFN- γ from patient 1, both pre- and post-IT.

In Table 2 the symptom-medication scores, pre- and post-IT, are presented for those patients who received treatment. Cytokine expression was determined in the first six patients prior to IT, and in all patients post-IT. Concerning patients 16 and 21, who still expressed IL-4 post-IT, the former had made no clinical response and the latter made only a minor improvement. Both patients 16 and 21 had received the full IT course and seven and eight maintenance injections. Of the remaining 14 post-IT patients who did not express IL-4, nine (64%) had made a good or excellent response. Table 3 presents a summary of the correlation between clinical response and cytokine expression post-IT.

Discussion

The principal findings from this study are that circulating mononuclear cells from the majority of symptomatic HDM

sensitive patients, who have not received immunotherapy, express both IL-4 and IFN- γ mRNA following allergen-specific stimulation *in vitro*. After IT, only 12.5% and 19% of patients expressed IL-4 and IFN- γ messages respectively. Moreover, of the 56% of patients who made a good or excellent response to IT, none expressed IL-4. However, among the 44% who made only a minor or no improvement, 29% expressed IL-4. It is important to stress that this was a non-randomized, open study, without a placebo control group and, hence, it remains possible that factors other than the IT may have influenced the outcome, although this is highly unlikely. A double-blind placebo controlled trial would be required for more definitive proof.

The fact that both IL-4 and IFN- γ expression were substantially reduced post-IT suggests that the immunotherapy was not acting predominantly by switching the allergic immune reactivity from a Th2-type response to a Th1-type (i.e. immune deviation). Rather, these results suggest that the IT was acting, at least in part, by reducing the overall allergen-specific production of inflammatory cytokines, including both IL-4 and IFN- γ . This may be due to either the induction of peripheral T cell tolerance/anergy or possibly deletion of allergen-reactive lymphocytes. The possible importance of this down-regulation of cytokine expression is highlighted by the fact that cytokine expression tended to persist in those patients who had a less beneficial clinical response to IT. A number of studies

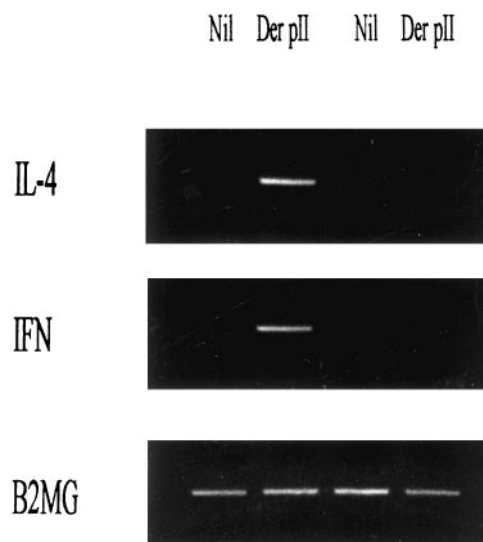


Fig. 1. Ethidium bromide stained gel of PCR products from patient 1, pre- and post-immunotherapy (pre: lanes 1 and 2; post: lanes 3 and 4). PBMC were cultured with medium alone, Der p 2 or PHA for 18 h and amplification was performed using primers specific for IL-4, IFN- γ or β 2-microglobulin. Results for PHA samples are not shown but all were positive for both IL-4 and IFN- γ .

Table 1. Der p 2-specific IL-4 and IFN- γ expression by PBMC in untreated Dpt-allergic patients and in those post-immunotherapy

Patient no.	Age (yr)	Clinical features*	Disease duration (yr)	Cutaneous sensitivities†	IL-4‡	IFN- γ
<i>Untreated allergic patients</i>						
1§	18	R/C	12	Dpt 3+, cat 2+	P	P
2	25	R	7	Dpt 2+	P	P
3	22	A, R/C	8	Dpt 3+, cat 2+, pollen 3+	P	P
4	31	A, R, E	14	Dpt 2+, pollen 2+, mould 2+, cat 1+	P	N
5	27	A, R	10	Dpt 3+, mould 1+, pollen 1+	P	N
6	42	R/C	21	Dpt 2+, cat 1+, dog 1+, pollen 2+	N	P
7	20	A, R	14	Dpt 2+, pollen 3+, cat 1+	P	—
8	33	R	7	Dpt 1+	P	P
9	37	A, R/C	16	Dpt 4+	P	P
10	19	R/C	5	Dpt 3+, mould 2+, pollen 2+	P	N
11	23	A, R/C, U	14	Dpt 4+, mould 3+, pollen 2+	N	P
12	29	A/R	6	Dpt 2+, pollen 2+	P	—
13	22	A, R	3	Dpt 3+, pollen 2+, mould 2+	P	—
14	25	R, E	12	Dpt 3+, mould 2+, cat 2+	P	P
15	36	A	19	Dpt 1+, GP 2+, mould 1+	N	P
<i>Post-immunotherapy patients</i>						
1	18	R/C	12	Dpt 3+, cat 2+	N	N
2	25	R	7	Dpt 2+	N	N
3	22	A, R/C	8	Dpt 3+, cat 2+, pollen 3+	N	P
4	31	A, R, E	14	Dpt 2+, pollen 2+, mould 2+, cat 1+	N	N
5	27	A, R	10	Dpt 3+, mould 1+, pollen 1+	N	N
6	42	R/C	21	Dpt 2+, cat 1+, dog 1+, pollen 2+	N	N
16	21	A, R, E	14	Dpt 3+, pollen 2+, mould 1+	P	N
17	16	R	7	Dpt 4+, pollen 3+, mould 2+	N	N
18	24	A, R/C	12	Dpt 2+, cat 2+, dog 2+	N	N
19	28	A, R	16	Dpt 3+, cat 2+	N	N
20	19	R/C	6	Dpt 1+	N	N
21	29	A, R	13	Dpt 3+, pollen 3+, mould 1+	P	P
22	27	A, R	8	Dpt 2+, cat 2+, dog 1+, pollen 2+	N	N
23	20	R	6	Dpt 4+, mould 3+, pollen 1+	N	N
24	28	R	9	Dpt 3+, cat 2+, pollen 2+	N	N
25	22	A, R	14	Dpt 2+, pollen 3+	N	P

* R = rhinitis; C = conjunctivitis; A = asthma; E = eczema; U = urticaria.

† Skin prick test (mean of maximal diameter and its orthogonal) 1+, 3–5 mm > histamine (1 mg/ml) control; 2+, 6–9 mm; 3+, 10–14 mm; 4+, >15 mm.

‡ P, expression of mRNA for specified cytokine; N, no cytokine expression.

§ Patients 1 to 6 were studied both prior to IT and post-IT.

have now shown an uncoupling of cytokine mRNA expression and protein secretion during the induction phase of T cell anergy, although in these situations the mRNA continues to be expressed without concomitant protein secretion [10,11]. Thus in the patients reported here, the possibility exists that even some of those with detectable message may not be secreting the cytokine. The assay is qualitative and we have not undertaken studies on the actual frequency of allergen-specific cytokine expressing cells.

Our results, showing a reduction of allergen-specific IL-4 and IFN- γ expression post-IT, concur in part with those of MacDonald *et al.*, who reported a reduction in IFN- γ release by PBMC post-IT, but no significant change in IL-4 [6]. Different from the above, but still partly in agreement with our results, were the data of Secrist *et al.*, who showed a reduction in IL-4 post-IT, but no significant alteration in IFN- γ production [5]. Conversely, Varney *et al.* [7] and Durham *et al.* [8] reported no change in IL-4 but a

Table 2. Symptom-medication score and expression of the cytokines IL-4 and IFN- γ in patients pre- and post-immunotherapy.

Patient	Symptom-medication score		IL-4 expression		IFN- γ expression	
	Pre	Post	Pre	Post	Pre	Post
1	9	4	P	N	P	N
2	11	4	P	N	P	N
3	23	18	P	N	P	P
4	17	7	P	N	N	N
5	13	6	P	N	N	N
6	8	8	N	N	P	N
16	19	20		P		N
17	10	3		N		N
18	14	15		N		N
19	16	5		N		N
20	13	13		N		N
21	15	13		P		P
22	16	8		N		N
23	10	7		N		N
24	6	1		N		N
25	16	5		N		P

For patients 16 to 25, IL-4 and IFN- γ expression were not measured prior to IT.

Table 3. Correlation between clinical response to immunotherapy and cytokine production post-therapy

Clinical response*	No.†	IL-4 expression‡	IFN- γ expression‡
2+/3+	9	0	1
0/1+	7	2	2

* Responses are defined in the patients and methods section.

† Number of patients with the specified clinical response.

‡ Number of patients still expressing IL-4 or IFN- γ post-IT.

significant increase in IFN- γ expression, in experiments using *in situ* hybridization to inflammatory cells in the skin or nasal mucosa. The reason for these differing findings is unclear and it may well be that the pattern of T cell reactivity does differ between the peripheral blood and the site of allergic inflammation in the tissues. Nevertheless the different experimental protocols employed may also be responsible for some of the variations in results.

Concerning the results of Secrist *et al.*, the allergen-specific T cells may, in fact, have a decreased IFN- γ production but this was, unlike IL-4, masked by lymphokines produced by cells of other specificities during the mitogen stimulation [5]. Varney *et al.* use another protocol which only measures message hybridization in activated T

cells attracted to a skin-test site. However, it is not known if these cells are allergen-specific, are bystanders, or have specificities for other non-allergen components of the extract [7]. Although this project did not utilize target-organ cells, it is complementary to those that did and has the advantage of allowing allergen-specific studies on unselected fresh T cells, before the recruitment to inflammatory lesions. This report is, in fact, the first study to directly measure circulating T cells specific for a major HDM allergen and show unequivocally a reduction in both IL-4 and IFN- γ expression post-IT.

Acknowledgements

Doctors R. M. O'Brien and W. R. Thomas were supported in part by grants from the National Health and Medical Research Council, Australia. The authors thank Ms Lisa Paiman for excellent technical assistance and Mrs Linda Scott for assistance in the preparation of this manuscript.

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