

Dermatophagoides pteronyssinus II-induced interleukin-4 and interferon- γ expression by freshly isolated lymphocytes of atopic individuals

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Summary

Cytokines are known to play a major role in mediating many of the immunological and pathological features of allergic disease. Much of our understanding of cytokine production in response to allergens has come from studying allergen-specific T cell clones following long-term *in vitro* culture. This has largely been due to the lack of sufficiently sensitive assays to measure allergen-induced cytokine production by freshly isolated peripheral blood mononuclear cells (PBMCs). Here we have used the polymerase chain reaction to amplify reverse transcribed interleukin-4 (IL-4) and IFN γ mRNA expressed by allergen-stimulated PBMCs from a variety atopic individuals. Using *Der p* II, a major allergen of the house dust mite (HDM) *Dermatophagoides pteronyssinus*, we have demonstrated that cells from HDM-sensitive atopic patients ($n = 12$), can be induced to express either IL-4 alone (three patients), IL-4 and IFN γ (six patients), IFN γ alone (two patients) or neither cytokine (one patient). Cells from 13 non-atopic control individuals were also stimulated with *Der p* II and cytokine mRNA production was studied. None expressed IL-4, while seven of 13 transcribed IFN γ . Our results suggest that atopic individuals have allergen-reactive T cells at various stages of differentiation, with respect to the cytokines they produce. The use of this technique will aid in the further understanding of specific cellular hypersensitivity in allergic disease.

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Introduction

T cell derived cytokines have been implicated in mediating many of the immunological manifestations of allergic disease. Interleukin-4 (IL-4), for instance, induces both the production of IgE [1], as well as the growth and development of mast cells [2], both of which are central to the allergic response. IL-5 is associated with allergic disease by acting as a growth and activation factor for eosinophils [3], cells critically involved in atopic asthma. On the other hand, interferon- γ (IFN γ) antagonizes many of the effects of IL-4, including the induction of IgE [4].

Studies of aberrant cytokine production in allergic disease have been generally based on the use of non-specific mitogens, such as phytohaemagglutinin and *Con A* [5,6]. Certainly allergen-specific T cell responses are likely to have more relevance than mitogen-induced T cell reactivity and thus we have concentrated on the use of allergens to stimulate cytokine production. To date the lack of suitably sensitive cytokine assays, together with the crude allergen extracts used has hampered previous investigations into allergen-induced cytokine production. Recently, several groups have investigated cytokine production from allergen-specific T cell clones [7,8], demonstrating that clones obtained from atopic individuals were of the TH-2 phenotype, producing IL-4, IL-5, IL-6 and IL-10 upon activation, whereas clones from non-atopic individuals were of the TH-1

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phenotype, producing IL-2, IFN γ and tumour necrosis factor- β (TNF- β).

In this manuscript we report on studies undertaken primarily to determine whether these allergen-specific T cell clone responses occur *in vivo* in patients with allergic disease, or whether they are artificially created during *in vitro* cloning. We have investigated in both atopic and non-atopic individuals the expression of two cytokines from the two different T helper cell phenotypes, namely IL-4 from TH-2 and IFN γ from TH-1. These cytokines also have important roles in the regulation of IgE. The expression of IL-4 and IFN γ was studied in freshly isolated PBMC's which were co-cultured with a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, Der p II.

Materials and methods

Subjects and samples

Heparinized blood (10 ml) was collected from 12 consenting atopic individuals attending either the Allergy Clinic or the Dermatology Clinic of the Royal Melbourne Hospital. Patients were defined as being atopic if they were skin-prick or RAST positive to one or more common allergens, all having asthma and/or rhinitis and/or eczema. Asthma was diagnosed according to the criteria of the American Thoracic Society [9]. Rhinitis was diagnosed by the presence of typical symptoms of sneezing, rhinorrhoea, pruritis and nasal obstruction, all patients with rhinitis had perennial symptoms which occurred on at least 10 days per month. Atopic eczema was diagnosed according to the criteria of Hanifin and Rajka [10]. All patients recruited were symptomatic at the time of sampling.

The clinical severity of patients referred to the Allergy Clinic with asthma and/or rhinitis was recorded. Immediate-type hypersensitivity by skin-prick testing was performed with extracts purchased from Dome/Hollister Laboratories (Sydney, Australia). Allergens used included house dust mite, cat and dog hair and danders, mould spores and a number of grass, weed and tree pollens. Patients with atopic eczema were unable to be skin-prick tested. Severity of eczema was scored numerically from 0 to 5 for erythema, scale, vesiculation, lichenification, excoriation and degree of pruritis. Addition of the individual scores gave overall classifications of mild (0–10), moderate (11–20) and severe (21–30).

None of the patients recruited were on oral steroids or other immunosuppressive medication at the time of sampling, although some were on either intranasal, inhaled or topical steroids.

Blood was also collected from 13 healthy laboratory volunteers who had no history of asthma, rhinitis or eczema, and whose sera were radioallergen sorbent test (RAST) negative to house dust mite.

PBMC isolation and allergen induction

PBMC's were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. Cells were suspended in RPMI 1640 + 5% autologous serum supplemented with glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μ g/ml). Cells were stimulated with affinity-purified Der p II, a major allergen of *Dermatophagoides pteronyssinus* (from Dr W. Thomas, Princess Margaret Hospital, Perth, Australia). The concentration used (0.5 μ g/ml) was previously found to be optimal as determined by allergen-induced proliferation assays [11]. Cells were also cultured with PHA (10 μ g/ml) as a positive control for T cell activation. The cells were then incubated at 37°C with 5% CO₂ for 18 h after which total ribonucleic acid (RNA) was isolated by a modified method of Chomczynski and Sacchi [12].

Reverse transcription-PCR

RNA from 10⁵ cells was resuspended in diethyl pyrocarbonate (DEPC) treated water and incubated at 65°C for 5 min. After cooling the samples on ice, a mixture containing 500 μ M dNTPs, 0.5 μ g oligo dT₁₅ primers, 200 U M-MLV reverse transcriptase, 100 μ g/ml acetylated BSA and 40 U RNasin in reverse transcription (RT) buffer (50 mM Tris, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT) were added. The tubes were incubated at 37°C for 1 h after which they were boiled for 5 min. Sterile distilled water (70 μ l) was then added to each sample. Two microlitres of the reverse transcribed sample was used for amplification. The polymerase chain reaction (PCR) was performed in the presence of cytokine-specific 3' and 5' primers (25 pmol) (IL-4: 3' ATTTCTCTCATGATCGTC, 5' GTTCTTCCTGC-TAGCATGTG IFN γ : 3' CTCCTTTTTCGCTTCC-CTGT, 5' ATATCTTGGCTTTTCAGCTC), 200 μ M dNTPs and 1 unit of Taq polymerase in PCR buffer (10 mM Tris, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatine). The mixture was overlaid with a drop of light mineral oil. Both negative (no DNA) and positive controls (cloned cDNA) were included with each reaction. β 2-microglobulin (β 2MG) was also amplified as a control for the RNA isolation and transcription. PCR was performed in a DNA thermal cycler (Perkin-Elmer) for 33 cycles: 45 s 94°C, 45 s 60°C, 2 min 72°C

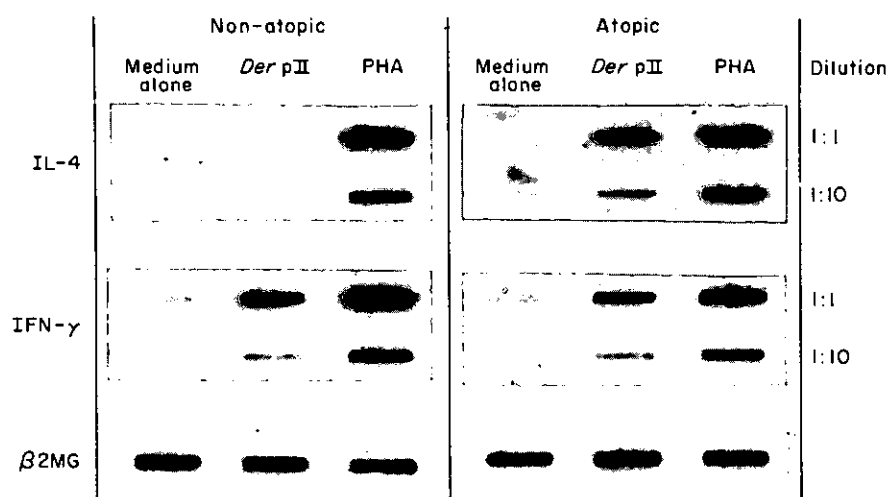


Fig 1. *Der p* II-induced cytokine expression in PBMCs from atopic and non-atopic individuals. PCR amplified products were diluted and blotted onto a nylon membrane. Digoxigenin-labelled probes to IL-4, IFN γ and β 2MG were sequentially hybridized and detected by chemiluminescence.

followed by a final 7 min at 72°C. Dilutions of each experiment were blotted onto a nylon membrane (Hybond-N+, Amersham, UK). Membranes were sequentially hybridized with digoxigenin-labelled oligonucleotide probes specific for each cytokine. Visualization was accomplished by a chemiluminescent method (AMPPD, Boehringer-Mannheim) performed according to the manufacturers instructions. Although not qualitative, differences in cytokine expression between cells which had been stimulated vs those which had not, were obvious visually (Fig. 1) and scored as a positive result.

Table 1. *Der p* II-induced IL-4/IFN γ expression in non-atopic individuals

Non-atopic	Serum IgE	<i>Der p</i> II induced IL-4	<i>Der p</i> II induced IFN
1	< 15	Negative	Negative
2	63	Negative	Negative
3	< 15	Negative	Negative
4	48	Negative	Negative
5	< 15	Negative	Negative
6	38	Negative	Negative
7	75	Negative	Positive
8	121	Negative	Positive
9	32	Negative	Positive
10	< 15	Negative	Positive
11	24	Negative	Positive
12	19	Negative	Positive
13	58	Negative	Positive

Total and specific IgE

Total serum IgE was determined by an immunonephelometric method performed on an automated nephelometer according to the manufacturers instructions (Behring, Marburg, Germany). The standard used was calibrated against WHO reference material. HDM-specific IgE was determined by the RAST assay (Pharmacia, Uppsala, Sweden).

Results

Representative results of *Der p* II-induced IL-4 and IFN γ expression by PBMCs of atopic and non-atopic individuals are shown in Fig. 1. RNA isolation and reverse transcription was confirmed to be successful by the uniform presence of β 2MG in each experiment. All samples from each of the groups responded to PHA by transcribing both IL-4 and IFN γ . Table 1 shows the individual results of the 13 non-atopic PBMCs tested. The results are reported as negative or positive according to the absence or presence of a detectable band after RT-PCR and specific hybridization. As seen, none of the PBMCs from the 13 non-atopic individuals expressed IL-4 in response to *Der p* II. This was also found to be true at both 6 and 48 h after initiation of allergen stimulation (data not shown). IFN γ on the other hand was found to be expressed in seven of the 13 non-atopic individuals tested.

Table 2 shows results obtained from the 12 atopic individuals. Of these patients, eight had serum IgE levels above the reference range (110 IU/ml) and nine were found to be RAST positive to house dust mite (HDM) indicating the presence of specific IgE in their sera. The patients with negative RASTs to HDM all had positive responses by skin-prick test, suggesting a lower level of

Table 2. Individuals with atopic disease

Patient	Disease	Clinical severity	Skin sensitivities	RAST to HDM	Serum IgE	Der p II induced IL-4	Der p II induced IFN
1	Asthma	Mild	HDM	Negative	226	Negative	Negative
2	Rhinitis	Mild	HDM	Negative	112	Negative	Positive
3	Rhinitis	Mild	HDM	Negative	80	Negative	Positive
4	Eczema	Moderate	ND	Positive	2107	Positive	Positive
5	Eczema	Mild	ND	Positive	10,000	Positive	Positive
6	Eczema	Moderate	ND	Positive	11,000	Positive	Positive
7	Rhinitis/asthma	Moderate	HDM	Positive	207	Positive	Positive
8	Rhinitis/asthma	Moderate	Multiple	Positive	91	Positive	Positive
9	Rhinitis	Moderate	HDM	Positive	405	Positive	Positive
10	Rhinitis/asthma	Severe	Multiple	Positive	404	Positive	Negative
11	Rhinitis/asthma	Severe	Multiple	Positive	600	Positive	Negative
12	Eczema	Severe	ND	Positive	86	Positive	Negative

ND. Not done.

sensitization. In comparing IL-4 and IFN γ expression in response to Der p II by each of the PBMC's of the atopic individuals, six were found to express both cytokines, one expressed none, while three and two expressed either, IL-4, or IFN γ alone, respectively. A small but detectable expression of IL-4 was seen in cells from six of the 12 atopic subjects, cultured with medium alone (not shown), whereas no such finding was obvious in any of the cells from non-atopic group. Although not quantifiable, this was indeed a reproducible observation in each of the particular patients. Such a finding may be explained by either pre-activation occurring *in vivo*, or in constitutive IL-4 expression.

Of the three atopic individuals who were RAST negative to HDM, none expressed IL-4 when their PBMC's were co-cultured with Der p II. All those who were RAST positive expressed IL-4 in response to allergen. IFN γ , on the other hand, displayed a range of responses in relation to the individuals RAST result. In patients who were RAST negative, two of the three expressed IFN γ in response to Der p II while six of the nine who were RAST positive to HDM expressed IFN γ .

Discussion

Studies into allergen-specific T cell clones isolated from atopic individuals have shown that these cells produce cytokines of the TH-2 phenotype, that is, IL-4, IL-5, IL-6 and IL-10 in response to specific allergen [7,8]. This cytokine profile correlates well with the pathological manifestations of allergic disease, including raised serum

levels of IgE, eosinophilia and reduced production of mitogen-induced IFN γ . Our results, using freshly isolated PBMC's from atopic individuals, support to a large extent the findings of these cloning studies.

We have found that cells of atopic patients whose sera contained HDM-specific IgE responded to Der p II by expressing IL-4. Of these, 66% also expressed IFN γ . The presence of allergen-reactive T cells in atopic individuals underscores the importance of these cells in the pathogenesis of allergic disease. Thus, when presented with allergen, these cells can react by producing IL-4 to induce neighbouring B cells to switch to IgE production. Continued exposure to allergen stimulation could lead to the preferential development of IgE antibodies having high binding affinities with the allergen.

The observation that PBMC's of some atopic individuals express both IL-4 and IFN γ in response to Der p II, could indicate the presence of a single T cell responding to the allergen by expressing both cytokines. This cytokine profile would resemble that of the TH-0 phenotype which produces IL-2, IL-4 and IFN γ upon activation. It has been postulated that this phenotype is an intermediate in the differentiation of T helper cells toward either a TH-1 or TH-2 phenotype [13]. Alternatively, the expression of IL-4 and IFN γ may suggest that more than one allergen-specific cell population exists in these individuals, with some responding in a TH-2-like manner expressing IL-4, and others responding as a TH-1. We are currently addressing this question using *in situ* hybridization techniques on allergen stimulated PBMC populations.

The fact that cells from seven of 13 asymptomatic, non-atopic individuals responded to *Der p* II with the expression of IFN γ indicates that prior immune sensitization has occurred. In these individuals the immune response has been directed or deviated toward the TH-1 phenotype with an inhibition of the generation of cells capable of mediating allergic hypersensitivity. This observation was also evident in allergen-specific T cell cloning studies where all HDM-specific clones from non-atopics were of the TH-1 type [7]. Cells from six of the 13 non-atopic individuals expressed neither IL-4, nor IFN γ in response to *Der p* II. This may indicate a very low prior level of exposure without sensitization [14], or alternatively, T cell anergy may have been induced.

Of the PBMC's from atopic individuals whose sera was RAST positive to HDM three responded by expressing IL-4 alone, typical of the TH-2 phenotype. It could be hypothesized that over time, these cells have fully differentiated into TH-2-like cells without any TH-1 cells remaining in the circulation. How and why T cells differentiate through differing T helper phenotypes remains unknown. Bendelac and Schwartz [15] suggests that murine thymocytes leaving the thymus have the ability to produce IL-4 in response to antigen. For this cell type to continue to fully differentiate into a TH-2-like T cell, they must have repeated or continued antigen exposure with the presence of IL-4. It is well recognized that a critical level of exposure must exist before sensitization can occur. Furthermore, it has been demonstrated that higher levels of HDM allergen exists in mattress dust from asthmatic than from non-asthmatic controls [16]. The second requirement for the development toward a TH-2 phenotype, that is IL-4, may come from other allergen-induced TH-O cells, producing both IL-4 and IFN γ , or by activated mast cells which have recently been shown to produce IL-4 when activated by cross-linking receptor bound IgE [17].

In relation to this preceding point, in patients with asthma and/or rhinitis, it is of particular interest to note that in patients considered to have the most severe clinical disease, their PBMC's expressed exclusively IL-4 without IFN γ in response to *Der p* II. Another group of patients had only mild symptoms, negative RAST's for HDM with positive skin tests for HDM alone, suggesting only a low level of allergic sensitivity. In this group, cells expressing IL-4 to *Der p* II could not be detected. Cells from patients with moderate clinical severity expressed both IL-4 and IFN γ .

It may be that dose or length of time of the allergen exposure determines whether the T cells in atopic individuals respond in a TH-O or TH-2 manner. It is tempting to postulate that cytokine therapy, in the form of IFN γ , may be of use in those individuals with

allergen-reactive TH-O cells predominating. IFN γ , found to be reduced in mitogen-activated PBMC's from atopic individuals [18], is known to preferentially direct T cell clonal development toward the TH-1 phenotype, while inhibiting both IL-4 production and TH-2 cell growth [19]. Perhaps this form of therapy would be of greatest benefit during desensitization procedures when antigen activation of T cells is occurring. IFN γ trials are currently underway for the treatment of atopic eczema [20] and inhaled IFN γ has been shown to turn off pulmonary hypersensitivity responses in murine models of asthma [21]. Perhaps knowledge of whether the atopic individual has predominantly allergen-reactive TH-O, or TH-2-like T cells in the circulation may be of benefit in selecting and monitoring patients for this form of therapy.

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