

T-cell cytokine pattern at three time points during specific immunotherapy for mite-sensitive asthma

M. MAJORI, A. CAMINATI, M. CORRADI, E. BRIANTI*, S. SCARPA* and A. PESCI

*Istituto di Clinica delle Malattie dell'Apparato Respiratorio dell'Università degli Studi and *Centro di Allergologia e Immunologia Clinica dell'Azienda Unità Sanitaria Locale, Parma, Italy*

Summary

Backgrounds Several lines of evidence indicate that specific immunotherapy may act by modifying the patterns of cytokines produced by helper T cells. However, different protocols have been used and different results obtained.

Objectives To quantify the effect of specific immunotherapy on the TH1/TH2 T-cell cytokine pattern at the single cell level.

Methods We examined the interferon- γ /interleukin-4 ratio in peripheral blood CD4⁺ and CD8⁺ T cells from 12 subjects with house dust mite-sensitive asthma using a flow cytometric method of intracellular cytokine detection. Cytokine production was determined following stimulation with phorbol myristate acetate/ionomycin, a polyclonal activator. Subjects were examined at three occasions: before specific immunotherapy, after the 3-months dose increase phase and after 1 year of treatment. During the treatment year patients kept a diary in which they recorded: (a) symptoms of asthma according to a 0–3 grading (0 = absent, 1 = mild, 2 = moderate, 3 = severe); (b) number of puffs (100 μ g) per day of salbutamol required to control symptoms; and (c) peak expiratory flow.

Results Specific immunotherapy improved clinical indices of disease activity including symptom scores and medication use during the treatment year, and had a marked effect in increasing the interferon- γ /interleukin-4 ratio in peripheral blood CD4⁺ T cells already after the dose increase phase (5.47 ± 1.5 vs $4.07 \pm 1.49\%$, $P = 0.03$) with and a further rise after 1 year's treatment (16.12 ± 2.8 vs 4.07 ± 1.49 and 16.12 ± 2.8 vs $5.47 \pm 1.5\%$, $P = 0.001$ and $P = 0.002$, respectively). There were no significant changes in the interferon- γ /interleukin-4 ratio in peripheral blood CD8⁺ T cells at the three times of the study.

Conclusions These data add to view that the efficacy of specific immunotherapy may be attributed to a modified cytokine secretion of CD4⁺ T cells.

Keywords: asthma, cytokines, flow cytometry, immunotherapy, peripheral blood, T cells

Clinical and Experimental Allergy, Vol. 30, pp. 341–347. Submitted 12 February 1999; revised 16 April 1999; accepted 14 May 1999.

Introduction

Despite nearly a century of successful treatment of allergic disease with specific immunotherapy (IT), the precise molecular mechanisms underlying this therapeutic modality remain elusive [1,2]. Whereas earlier works focused on circulating antibodies [3,4], newer studies reporting the modulation of interleukin (IL)-4 and/or interferon (IFN)- γ release by allergen-specific T cells have suggested a

correlation between successful IT and the induction of a phenotypic switch from a T helper 2 (TH2) to a TH1 cytokine phenotype [5–13]. However, these studies performed using allergen stimulation vary considerably in the details of this shift. Some report that IFN γ increases while IL-4 diminishes [5], other authors found a decrease in both cytokines IFN γ and IL-4 [6]; in some cases, IL-4 was reported to diminish, whilst IFN γ was not altered [7–10], whilst in others, IFN γ increased whilst IL-4 remained unchanged [11–13].

In particular, Lack *et al.* [13] observed an increase in

Correspondence: M. Majori, Istituto di Clinica delle Malattie Apparato Respiratorio, Via Rasori no. 10, 43100 Parma, Italy.

IFN γ production by peripheral blood CD4⁺ T cells after reaching maintenance of rush IT for house dust mite (HDM)-sensitive asthma using a flow cytometric method which permits analysis of cytokine production at the single cell level [14]. The aim of the present study was to determine whether conventional IT for HDM-sensitive asthma had the same effect on the TH1/TH2 T-cell cytokine pattern in peripheral blood of the treated subjects. For this purpose, using the FastImmune Cytokine System (Becton Dickinson, Mountain View, CA, USA) [15], we examined the IFN γ and IL-4 expression in peripheral blood CD4⁺ and CD8⁺ T cells from 12 subjects with HDM-sensitive asthma at three occasions: before IT, after the 3-months' dose increase phase and after 1 year's treatment.

Because it was suggested that the clinical severity of an allergic disease is more related to the balance between the TH1 (IFN γ) and TH2 (IL-4), than to the absolute levels of TH2 cytokines [16], we focused our attention on the changes of the IFN γ /IL-4 ratio.

Methods

Subjects

The study population was composed of 12 subjects suffering from perennial asthma (6 men and 6 women, mean age 27.4 ± 2.9 years) living in the Parma area. The criteria for selecting patients for this study were: (1) diagnosis of asthma based on criteria listed by the American Thoracic Society [17]; (2) poor symptom control in previous years despite regular antiallergic treatment that included inhaled corticosteroids and sodium cromoglycate and antihistamines; (3) symptoms of perennial bronchial asthma alone, with no evidence of rhinitis or skin allergies. Patients with seasonal bronchial asthma were excluded from this study so as to avoid variations in parameters studied. Patients with rhinitis (which often coexists with asthma) were excluded, since variations in symptoms of rhinitis could have falsely altered the patient's perception of asthma symptom scores; (4) elevated serum immunoglobulin (Ig) E levels (normal value <100 IU/mL); (5) positive skin-prick tests (2+ or more) to *Dermatophagoides pteronyssinus* alone; and (6) positive RAST to *Dermatophagoides pteronyssinus*.

The use of inhaled corticosteroids was stopped 4 weeks before the start of the study. Theophylline, anticholinergic, oral corticosteroids, long-acting inhaled bronchodilators and oral bronchodilators were not used. Respiratory symptoms were controlled with inhaled short-acting bronchodilators (salbutamol) on a continuous basis or on demand.

Patients presenting with one or more of the following conditions – chronic asthma, asthma requiring one or more hospitalizations in the previous year, current smoking, previous treatment with IT, systemic illness, pregnancy –

were also excluded. The study protocol had the approval of the local ethics committee. Each subject gave informed and signed consent to participate in the study.

Study design

After selection, each patient was given instructions regarding appropriate avoidance measures towards HDM that included removal of carpets, frequent vacuuming, change of bedding to synthetic material such as foam and removal of soft toys. All measures suggested were strictly scrutinized and audited at each clinic visit, so that they would, as far as possible, result in uniform benefits. HDM measurements were included in the initial protocol for this study. However, social objections from the first few patients resulted in forced abandonment of such measurements from patient's homes.

Patients were asked to keep a diary in which they recorded: (a) symptoms of asthma according to a 0–3 grading (0 = absent, 1 = mild, 2 = moderate, 3 = severe); (b) number of puffs (100 μ g) per day of salbutamol required to control symptoms; and (c) PEF as the best of three pretreatment measurements taken twice daily using a mini-Wright portable peak flow meter (Markos, Monza, Italy). From October 1997 to October 1998 each subject was submitted to IT with an aluminium hydroxide-absorbed HDM-extract (Bayer SpA, Milan, Italy). The dose scheduled was administered subcutaneously and was similar in all subjects. The initial dose was 2.5 AU (activity units) of alpha fraction – 200 AU = SARAH (skin activity reference allergen/histamine 1%) – and it was first increased weekly up to a maintenance dose of 800 AU. The maintenance dose was reached after approximately 3 months, and then this dose was administered every 2 weeks for 1 month and thereafter every 4 weeks.

Peripheral venous blood sample was taken for lymphocyte analyses at three occasions: before IT (September 1997; T0), after the 3-month period of dose-increase with weekly injections (December/January 1998; T1) and after 1 year's treatment with the maintenance dose (monthly injections; October 1998; T2).

Patients underwent pulmonary function testing (FEV₁) (Spiroflow, Morgan, UK), skin-prick tests to *Dermatophagoides pteronyssinus* (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) and peripheral venous blood sample was taken for measurements of HDM-specific IgE levels (RAST score, Pharmacia Diagnostics, Uppsala, Sweden) before the beginning of IT (T0) and following 1 year's treatment (T2).

Antibodies and reagents

Anti-IFN γ -conjugated to phycoerythrin (PE), anti-IL-4 PE, anti-CD3 (T cells) peridin chlorophyll protein (PerCP),

anti-CD4 (T helper-inducer) FITC (fluorescein isothiocyanate) and PE, anti-CD8 (T suppressor-cytotoxic) FITC and PE, anti-CD25 (p55 IL-2 receptor) PE, anti-HLA-DR (Class II MHC) PE, anti-CD23 (B cells expressing the low affinity Fc IgE receptor) PE, isotype-matched, directly conjugated (FITC, PE and PerCP) control antibodies, FACS Lysing Solution and FACStm Permeabilizing Solution were obtained from Becton Dickinson.

Phorbol myristate acetate (PMA), ionomycin, brefeldin A, paraformaldehyde, L-glutamine, RPMI 1640 and AB serum were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Processing of cells and staining procedure

For surface marker analysis of peripheral blood lymphocytes, 100 μ L of heparinized whole blood was incubated with 10 μ L of one of the FITC-labelled antibodies along with 10 μ L of one of the PE-labelled antibodies at room temperature for 20 min. Erythrocytes were lysed by adding 2 mL lysing solution for 10 min. Cells were washed twice with phosphate-buffered saline (PBS) containing 2% foetal calf serum and 0.1% sodium azide.

For intracellular cytokine detection, 500 μ L of heparinized whole blood was added to RPMI 1640 with 2 mmol/L L-glutamine. Cells were stimulated with PMA (25 ng/mL) and ionomycin (1 μ g/mL) in the presence of brefeldin-A (10 μ g/mL) and kept in tubes (Falcon, Becton Dickinson) for 4 h at 37 °C in a humidified atmosphere at 5% CO₂ in air. As previously described [18], brefeldin-A was used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells.

Ten microlitres of anti-CD3 PerCP and 10 μ L of anti-CD8 FITC were added to 100 μ L of stimulated whole blood and incubated for 15 min at room temperature. Erythrocytes were lysed by adding 2 mL lysing solution for 10 min. Cells were washed with PBS containing 0.5% albumin bovine serum and 0.1% sodium azide and subsequently resuspended in 500 μ L FACStm permeabilizing solution. After 10 min, the cells were washed with PBS and incubated with saturating concentrations of anti-IFN γ or anti-IL-4 PE for 30 min at room temperature. After a final wash in PBS, cells were resuspended in 500 μ L paraformaldehyde 1% and kept at 4 °C for no more than 24 h in the dark until analysed. Negative controls consisted of unstimulated cells.

Flow cytometric analysis

A FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm), and PerCP (> 650 nm) was used.

For surface marker analysis of peripheral blood lymphocytes, 10 000 cells were computed in list mode and analysed

using appropriate software (Lysis, Becton Dickinson). Simultaneous T-cell subset analysis (CD4⁺ and CD8⁺ T cells) and activation study (expression of activation markers CD25 and HLA-DR) were performed on peripheral blood lymphocytes.

For intracellular cytokine detection, 10 000 cells were computed and analysed with the same software. An electronic gate was set on the lymphocytes on the forward and side-scatter plot, and CD3⁺(PerCP⁺) cells falling within the gated area were then identified and selected in a second gate. As previously described [18], cytokine production was analysed by detection of FITC and PE staining, and the number of cells staining for each cytokine was expressed as a percentage of CD4⁺ and CD8⁺ T cells. CD4⁺ T cells were identified as CD3⁺ CD8⁺ cells because of the downregulation of CD4 after phorbol ester stimulation [19]. Specific binding of mAb was controlled by subtraction of isotype-matched mouse immunoglobulins.

In order to quantify the cytokine profile the IFN γ /IL-4 ratios were calculated.

Statistical analysis

Results were expressed as means $\pm$ SEM. Wilcoxon's signed rank test was used to compare changes in immunological and clinical parameters at the different times of the study. A *P*-value less than 0.05 was considered significant. To further analyse whether associations exist between the changes that occur in various parameters during IT, data were recorded separately for each patient and logarithmically transformed, and then multiple Spearman correlation analyses were done.

Results

All the patients with HDM-sensitive asthma completed the treatment period and no local or systemic side-effects were reported.

The comparison of symptom scores and salbutamol consumption during the treatment year showed a statistically significant decrease for both symptom scores (from 4.41 ± 0.45 to 3.82 ± 0.39 score, *P* = 0.003) and salbutamol consumption (from 4.7 ± 0.38 to 4.25 ± 0.39 puffs/day, *P* = 0.01). Owing to the poor symptom control in previous years patients had stopped the use of inhaled corticosteroids 4 weeks before the start of the study. Patients were found to have kept inaccurate records of PEF measurements during the treatment year and therefore in view of incomplete data, this has not been incorporated in the evaluation of the results.

There were no significant differences in the FEV₁ (from 86.58 ± 1.77 to $88.25 \pm 1.18\%$ of predicted value, NS) and titres of specific IgE (from 3.41 ± 0.19 to 3.16 ± 0.16 score, NS) at T2 when compared with T0. Skin-prick tests to

Dermatophagoides pteronyssinus were significantly reduced after the treatment year in all patients (from 2.83 ± 0.11 to 1.08 ± 0.31 score, $P = 0.002$).

Since if blood is taken shortly after giving the injection with an aluminium hydroxide-absorbed extract, there is a non-specific stimulation of lymphocyte proliferation and cytokine release, peripheral venous blood sample was taken for lymphocyte analyses immediately before the first injection of IT, at least 48 h after maintenance was achieved and at least 48 h after the completion of the treatment year.

Flow cytometric analysis of peripheral blood lymphocytes showed no significant differences in either the percentage or absolute numbers of $CD3^+$, $CD19^+$ and $CD23^+$ cells at T1 when compared with T0, and at T2 when compared with T0 and T1. There were also no differences in the percentage of $CD3^+$ cells expressing CD4 or CD8, or the expression of CD25 and HLA-DR in either $CD4^+$ or $CD8^+$ T cells.

No cytokine levels were detectable without stimulation, and appropriate isotype-matched control antibodies showed no positive staining. An increased production of $IFN\gamma$ by peripheral blood $CD4^+$ T cells was observed at T1 when compared with T0 (36.93 ± 6.55 vs $26.9 \pm 3.93\%$, $P = 0.003$), while there was no significant difference in the production levels of this cytokine at T2 when compared with T0 (34.38 ± 5.5 vs $26.9 \pm 3.93\%$, NS) and T1 (34.38 ± 5.5 vs $36.93 \pm 6.55\%$, NS) (Fig. 1). A decreased secretion of IL-4 by peripheral blood $CD4^+$ T cells was observed at T2 when compared with T0 (6.12 ± 3.58 vs $19.59 \pm 6.56\%$, $P = 0.04$) and T1 (6.12 ± 3.58 vs $12.22 \pm 2.96\%$, $P = 0.04$) (Fig. 2).

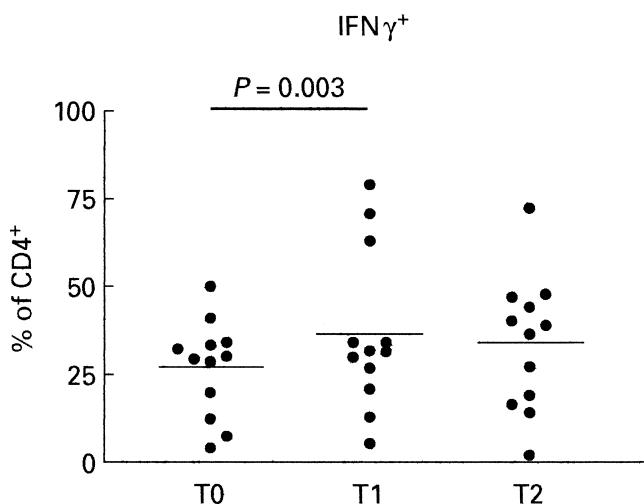


Fig. 1. Percentages of peripheral blood $CD4^+$ T cells with positive intracellular staining for $IFN\gamma$ from patients with HDM-sensitive asthma before IT (T0), after the 3 months' dose increase phase (T1) and after 1 year's treatment (T2). Horizontal bars represent group mean values

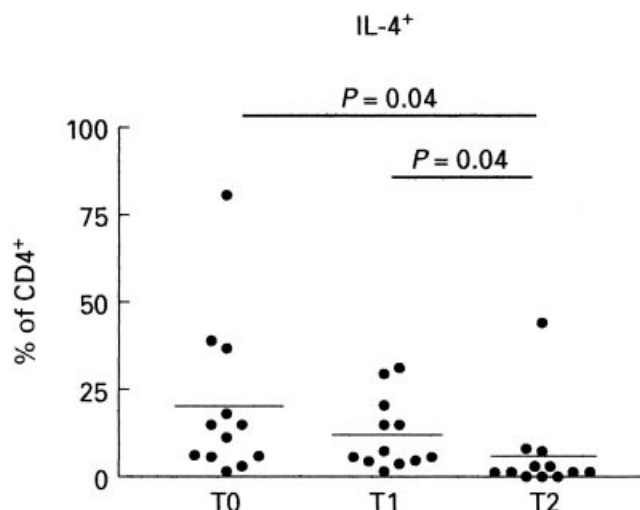


Fig. 2. Percentages of peripheral blood $CD4^+$ T cells with positive intracellular staining for IL-4 from patients with HDM-sensitive asthma before IT (T0), after the 3 months' dose increase phase (T1) and after 1 year's treatment (T2). Horizontal bars represent group mean values

There was an increased $IFN\gamma/IL-4$ ratio among peripheral blood $CD4^+$ T cells from the subjects with HDM-sensitive asthma at T1 when compared with T0 (5.47 ± 1.5 vs 4.07 ± 1.49 ratio, $P = 0.03$) and at T2 when compared with T0 (16.12 ± 2.8 vs 4.07 ± 1.49 ratio, $P = 0.001$) and T1 (16.12 ± 2.8 vs 5.47 ± 1.5 ratio, $P = 0.002$) (Fig. 3).

In contrast, there was no significant difference between the different times of the study in the $IFN\gamma/IL-4$ ratio or single cytokine production among peripheral blood $CD8^+$ T cells.

No relationship was found between the interval between the allergen injection and the blood drawing and the height of the $IFN\gamma$ produced (data not shown).

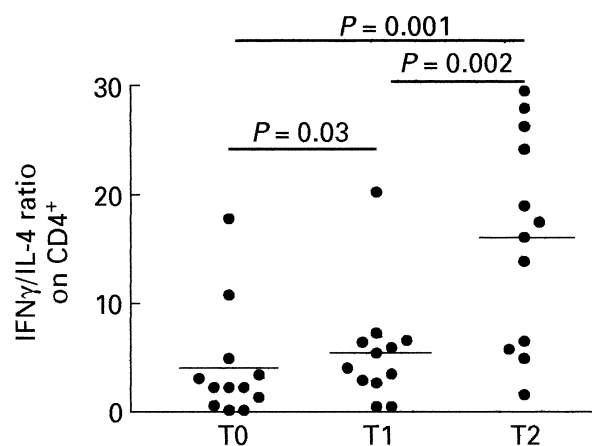


Fig. 3. Ratio $IFN\gamma/IL-4$ on peripheral blood $CD4^+$ T cells from patients with HDM-sensitive asthma before IT (T0), after the 3 months' dose increase phase (T1) and after 1 year's treatment (T2). Horizontal bars represent group mean values

No correlation was found between changes in clinical and immunological parameters.

Discussion

This study shows a continuous shift toward a TH1-type cytokine profile of peripheral blood CD4⁺ T cells in patients with HDM-sensitive asthma during the course of IT. A significant rise in the IFN γ /IL-4 ratio among peripheral blood CD4⁺ T cells was already present after the dose increase phase of the treatment and we observed a further rise in this parameter after 1 year's therapy. This immune deviation was due to an early increase in IFN γ production after the 3 months' dose increase phase, and to a selective decrease of IL-4 during the second phase of the treatment year. Cytokine production was determined following stimulation with phorbol myristate acetate/ionomycin, a polyclonal activator.

Our data nicely fit with those of Lack *et al.* [13] who demonstrated an increased frequency of CD4⁺ T cells producing IFN γ after reaching maintenance of rush IT for HDM-sensitive asthma using a flow cytometric method of intracellular cytokine detection. Our study confirms these data and extends the phenomenon to conventional IT. In agreement with the studies by Secrist *et al.* [7], Ebner *et al.* [8] and Van Bever *et al.* [9], we observed a selective decrease of IL-4 production after several months of IT.

We confirm these data using the flow cytometric method of intracellular cytokine detection which permits the simultaneous detection of cytokine product and T-cell surface markers in unseparated samples at the single cell level and thus provides an insight into the frequency of T cells capable of producing different cytokines. In this regard, most of the studies on the underlying mechanisms of IT have used the technique of *in situ* hybridization to detect the expression of mRNA for the IFN γ and IL-4 [6,11,12]. However, cytokine gene transcription into mRNA does not necessarily parallel translation into protein or its subsequent secretion [20]. On the other hand, our own technique does not study secretion but only accumulation of cytokines in the cell. In this regard, previous study [21] showed that accumulation in the cell and secretion for IFN γ may not be tightly linked. On this account, *in situ* hybridization, intracellular cytokine detection and techniques assessing cytokine secretion should be combined. The studies which examined the variations in cytokine secretion following IT [7–10] evaluated the products of antigen-specific T cells. Antigen stimulation requires peripheral blood mononuclear cell fractionation with resuspension in synthetic tissue culture environment and it has demonstrated that the cytokine profile of cloned T cells may be skewed by the culture conditions needed to expand the clones [22]. Our method permits the reduction of sample manipulation and

biohazard risk and more accurately represents *in vivo* conditions.

Because it is possible that there are distinct time courses for changes in individual cytokines after IT we examined the IFN γ and IL-4 expression in peripheral blood CD4⁺ and CD8⁺ T cells from subjects with HDM-sensitive asthma at three occasions: before IT, after the 3 month dose increase phase and after 1 year of treatment. In this regard, most of the previous studies on the underlying mechanisms of IT examined only a single time point after IT, and the kinetics of IFN γ and IL-4 modulation with IT remain unknown. Only Ebner *et al.* [8] evaluated immunological changes during IT of grass pollen allergy before IT, after the 3 month dose increase phase and after 1 year of treatment. Lack *et al.* [13] studied their patients' T cells after reaching maintenance, Pène *et al.* [10] examined the effects of IT after 6 weeks of therapy; whereas O'Brien *et al.* [6], Secrist *et al.* [7], Van Bever *et al.* [9], Varney *et al.* [11] and Durham *et al.* [12] investigated patient T cells after several months or years of IT. Varney *et al.*, Durham *et al.* and Lack *et al.* found increases in IFN γ production, Secrist *et al.*, Ebner *et al.*, Van Bever *et al.* and Pène *et al.* demonstrated a decrease in IL-4, and O'Brien *et al.* found a decrease in both cytokines IFN γ and IL-4. Akdis *et al.* [23] also found – following 2 months of rush desensitization with bee venom – a decrease in both cytokines IFN γ and IL-4. Whereas MacDonald *et al.* [24], studying cytokine release from peripheral blood mononuclear cells 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. It is possible that the conflicting results of these studies [6–13] are due to the different time-points used. Another possible explanation for these differing findings may be the different cell source used (i.e. from the tissue or from peripheral blood). 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 stimulus used for cytokine production, i.e. mitogen or antigen, may also be responsible for some of the variations in results.

The mechanism by which IT modifies cytokine production in CD4⁺ T cells is not defined. It has been postulated that the route of antigen exposure, rather than the specific properties of the allergen, may determine the nature of cytokine responses that develop in T cells [25].

Normal exposure to aeroallergens occurs by inhalation, whereas during IT allergen is injected subcutaneously. The amount of allergen administered during IT is much greater than the amount inhaled, and the differences in local concentrations of antigen may alter the nature of the interaction between antigen-presenting cells and T cells [26]. Different concentrations of antigen presented by antigen-presenting cells may alter the relative production of IFN γ and IL-4 by T cells [27].

A more comprehensive explanation for the mechanisms operative in IT provides the combination of a cytokine shift with the induction of a specific T-cell tolerance. In this regard, recent data show a crucial role for IL-10 in tolerance induction during IT [28]. In the present study, however, we have not investigated the expression of this cytokine.

Although in our previous study [29] we showed a down-regulation of T-cell activation in peripheral blood from asthmatic subjects after 1 year's IT for grass pollen-sensitive asthma, in the present study we were unable to show any significant difference in the expression of CD25 and HLA-DR in either peripheral blood CD4⁺ or CD8⁺ T cells during the study. This discrepancy may be due to the fact that in the previous study we examined subjects with grass pollen-sensitive asthma out of the pollen season, whereas at present we examined asthmatic patients undergoing continuous exposure to the sensitizing antigen. Bonno *et al.* [30] demonstrated an upregulation of CD25 expression on peripheral blood CD8⁺ T cells from subjects with HDM-sensitive asthma after IT. This discrepancy with our study may be explained by the different population of asthmatic patients evaluated (children) or by different methodology used (analysis of allergen-specific CD8⁺ T cells after a 6 day culture with *Dermatophagoides farinae*).

We found that HDM-specific IgE in the serum were not affected by 1 year's IT, while skin-prick tests to *Dermatophagoides pteronyssinus* were significantly reduced after the treatment year. It could be argued that this is also a measure of specific IgE. On the other hand, the meaning of these parameters and their relationship with the clinical outcome is otherwise not clear and established [31]. Paradoxically, the rise in allergen-specific IgE in serum, early in the course of IT, occurs at a time when cutaneous reactivity is diminished [32]. The absence of a significant reduction of specific serum IgE antibodies as a consequence of the decreased production of IL-4 after 1 year's IT in our patients may be explained by the existence of other non-IL-4-regulated mechanisms involved in maintaining the formation of HDM-specific IgE.

The reduction in symptoms and need for drugs during the year's treatment of our study confirmed the clinical efficacy of IT in HDM-sensitive asthma [33]. Since a variety of cells and mediators can contribute to the inflammatory processes in asthma, the lack of correlation between the changes in flow cytometric and clinical and physiologic data was not unexpected.

In conclusion, this study shows a continuous shift toward a TH1-like immune response of peripheral blood CD4⁺ T cells in patients with HDM-sensitive asthma during the course of IT. These data add to view that the efficacy of IT may be attributed to a modified cytokine secretion of CD4⁺ T cells.

Further research into the precise mechanisms of specific

IT is clearly required. In particular, it will be interesting to see whether the immunological changes reported in our study persist following treatment discontinuation, thereby offering the possibility of long-term modification of the course of the disease.

Acknowledgements

We would like to thank Iris Spanevello for her expert technical assistance.

This study was partially supported by a grant from Ministero dell'Università, Ricerca Scientifica e Tecnologica, Rome, Italy.

References

- Noon L, Cantab BO. Prophylactic inoculation against hay fever. *Lancet* 1911; 1:1572–3.
- Frankland AW, Augustin R. Prophylaxis of summer hay fever and asthma. *Lancet* 1954; 1:1055–7.
- Golden DB, Meyers DA, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom specific immunoglobulin G antibody level during immunotherapy. *J Allergy Clin Immunol* 1982; 69:489–93.
- Yan-der-Zee JS, Aalberse RC. The role of IgG in immediate-type hypersensitivity. *Eur Respir J Suppl* 1991; 13:91S–6S.
- 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.
- O'Brien RM, Byron KA, Varigos GA, Thomas WR. House dust mite immunotherapy results in a decrease in Der, p 2-specific IFN- γ and IL-4 expression by circulating T lymphocytes. *Clin Exp Allergy* 1997; 27:46–51.
- Secrist H, Chelen 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.
- Ebner C, Siemann U, Bohle B et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl, p 1, a major grass pollen allergen. *Clin Exp Allergy* 1997; 27:1007–15.
- Van Bever HP, Vereecke IF, Bridts CH, De Clerck LS, Stevens WJ. Comparison between the in vitro cytokine production of mononuclear cells of young asthmatics with and without immunotherapy (IT). *Clin Exp Allergy* 1998; 28:943–9.
- Pène J, Desroches A, Paradis L et al. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. *J Allergy Clin Immunol* 1998; 102:571–8.
- Varney VA, Hamid QA, Gaga M 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, Ying S, Varney VA 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.
- 13 Lack G, Nelson HS, Amran D et al. Rush immunotherapy results in allergen-specific alterations in lymphocyte function and interferon- γ production in CD4+ T cells. *J Allergy Clin Immunol* 1997; 99:530–8.
 - 14 Jung T, Schauer U, HeusSeries C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. *J Immunol Methods* 1993; 159:197–207.
 - 15 Majori M, Corradi M, Caminati A, Cacciani G, Bertacco S, Pesci A. Predominant Th1 cytokine pattern in peripheral blood from subjects with chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 1999; 103:458–62.
 - 16 Imada M, Estelle F, Simons R, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon- γ production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunology* 1995; 85:373–80.
 - 17 American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987; 136:225–44.
 - 18 Picker LJ, Singh MK, Zdraveski Z et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 1995; 86:1408–19.
 - 19 Petersen CM, Ilso Christensen EI, Andresen BS, Moller BK. Internalization, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. *Exp Cell Res* 1992; 201:160–73.
 - 20 Schall TJ, O'Hehir RE, Goeddel DV, Lamb JR. Uncoupling of cytokine mRNA expression and protein secretion during the induction phase of T cell anergy. *J Immunol* 1992; 148:381–7.
 - 21 Tang M, Kemp A. Production and secretion of interferon-gamma (IFN- γ) in children with atopic dermatitis. *Clin Exp Immunol* 1994; 95:66–72.
 - 22 Sad S, Mosmann TR. Single IL-2-secreting precursor CD4 T-cell can develop into either Th1 or Th2 cytokine secreting phenotype. *J Immunol* 1994; 153:3514–22.
 - 23 Akdis CA, Akdis M, Blesken T et al. Epitope-specific T cell tolerance to phospholipase A₂ in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996; 98:1676–83.
 - 24 MacDonald SM, Naclerio RM, Hamilton RG, Langdon JM, White JM, Lichtenstein LM. Cytokine measurements in patients receiving standard immunotherapy. *J Allergy Clin Immunol* 1994; 93:230.
 - 25 Saloga J, Renz H, Lack G et al. Development and transfer of immediate cutaneous hypersensitivity in mice exposed to aerosolized antigen. *J Clin Invest* 1993; 91:133–40.
 - 26 Parish CR, Liew FY. Immune response to chemically modified flagellin 3. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *J Exp Med* 1972; 135:298–311.
 - 27 DeKruyff RH, Fang Y, Umetsu DT. IL-4 synthesis by in vivo primed keyhole limpet hemocyanin-specific CD4+ T cells. I. Influence of antigen concentration and antigen-presenting cell type. *J Immunol* 1992; 149:3468–76.
 - 28 Akdis CA, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.
 - 29 Majori M, Bertacco S, Piccoli ML, Meley R, Pileggi V, Pesci A. Specific immunotherapy downregulates peripheral blood CD4 and CD8 T-lymphocyte activation in grass pollen-sensitive asthma. *Eur Respir J* 1998; 11:1263–7.
 - 30 Bonno M, Fujisawa T, Iguchi K et al. Mite-specific induction of interleukin-2 receptor on T lymphocytes from children with mite-sensitive asthma: modified immune response with immunotherapy. *J Allergy Clin Immunol* 1996; 97:680–8.
 - 31 Malling HJ, Weeke B. Immunotherapy position paper. *Allergy* 1993; (Suppl. 48): 9–35.
 - 32 Tipton WR, Nelson HS. Experience with daily immunotherapy in 59 adult allergic patients. *J Allergy Clin Immunol* 1982; 69:194–9.
 - 33 Abramson MJ, Puy RM, Weiner JM. Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am J Respir Crit Care Med* 1995; 151:969–74.