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Differences in IL-4 release by PBMC are related with heterogeneity of atopy

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SUMMARY

Atopy is heterogeneous and the IgE immune response of patients allergic to a single allergen (monosensitized) differs from that of those allergic to multiple allergens (polysensitized). Since interleukin-4 (IL-4) and interferon-γ (IFN-γ) regulate human IgE synthesis *in vitro*, we determined whether cytokines may be involved in the heterogeneity of atopy by comparing the serum IgE and sCD23 titres to the cytokine profile of T lymphocytes from 44 atopic patients (13 mono- and 31 polysensitized) and seven non-atopic subjects. Monosensitized patients were allergic to grass or cypress pollens or house dust mites, and polysensitized ones to many pollen species ($n=5$) or many allergens ($n=26$). Total serum IgE was lower in the control group than in both atopic groups and in the monosensitized group than in the polysensitized one. IgE immunoblots to orchard grass pollen and house dust mites were less heterogeneous in the monosensitized group than in the polysensitized one. IL-4 production by *in vitro*-activated peripherial blood mononuclear cells (PBMC) was significantly higher in the polysensitized group than in the monosensitized, and marginal in the control group. In contrast, IFN-γ production was strongly reduced in both atopic groups, and IL-2 production comparable in the three groups. IgE and soluble CD23 (sCD23) release was higher in the atopic groups than in the control, and higher in the polysensitized group than in the monosensitized one. This study shows that PBMC of mono- and polysensitized subjects have a different IL-4 and sCD23 profile and suggests that human beings may be classified into high and low IgE responders on the basis of IL-4 production.

INTRODUCTION

The immune response to environmental allergens depends on genetic and environmental factors.[1] Exposed to a common environment, only certain individuals develop an IgE-mediated immune response differing from subject to subject. Some patients are sensitized to a single allergen whereas the majority of patients have IgE antibodies against many allergens. For a single allergen species, the IgE-mediated immune response is heterogeneous, as observed in patients sensitized to grass and birch pollens[2,3] or house dust mites.[4] Allergic patients to grass pollens differ immunologically and clinically from those allergic to many pollen species, monosensitized individuals having a lower total and specific IgE response, as assessed by the serum IgE titres and IgE immunoblots to orchard pollens, than polysensitized individuals.[3] In addition, specific immunotherapy was effective in grass pollen-monosensitized patients and less effective in those allergic to multiple pollens.[3]

IgE synthesis is regulated by several factors, among which cytokines play a central role. Interleukin-4 (IL-4) induces IgE secretion[5-8] by promoting isotype switch from IgM to IgE on resting B cells,[9,10] whereas interferon-γ (IFN-γ) exhibits suppressive effects on both IL-4-induced and spontaneous IgE synthesis.[6,7,11,12] Other factors synergize with IL-4 to enhance IgE synthesis, among which the soluble part of the low-affinity receptor for IgE (sCD23) plays a key role.[7,13,14] In mice, two subsets of $CD4^+$ T lymphocytes have been identified producing, besides many cytokines, either IL-2 and IFN-γ (Th1) or IL-4 and IL-5 (Th2).[15] Th1 and Th2 T-cell subsets are likely to exist in the human[16] and regulate IgE production since it was reported that tetanus toxoid- or *Dermatophagoides pteronyssimus* (*Der p*)-specific T-cell clones established from allergic and non-allergic individuals produced different cytokine profiles.[17-19]

These studies suggest that the T-cell cytokine patterns of mono- and polysensitized patients could differ according to their sensitization. In order to test this hypothesis, peripheral blood mononuclear cells (PBMC) of non-allergic and allergic

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Abbreviations: A23187, calcium ionophore A23187; CSN, culture supernatant; Der p, *Dermatophagoides pteronyssimus*; mAb, monoclonal antibody; sCD23, soluble form of the low affinity receptor for IgE; SPT, skin prick test.

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patients (mono- and polysensitized) were studied. The IgE-mediated immune response was assessed by total and specific IgE titres and IgE immunoblots. Cytokine production was determined in the culture supernatants (CSN) of polyclonal agent-activated PBMC.

MATERIALS AND METHODS

Reagents and cell lines

Purified recombinant IL-4 ($1{\cdot}0 \times 10^7$ U/mg) was a gift of Dr J. E. De Vries (DNAX Research Institute, Palo Alto, CA), IFN-γ ($1{\cdot}0 \times 10^6$ IU/mg) and IL-2 from Professor G. Garotta (Hoffmann LaRoche and Co., Basel, Switzerland), the anti-IL-4 monoclonal antibody (mAb) 11B4 and the polyclonal goat anti-IL-4 from Dr J. Banchereau (Schering-Plough, Dardilly, France), and the anti-IFN-γ mAb A35 and B27 and the anti-IgE mAb I-27 also from Dr J. E. De Vries. The immunoglobulin (Ig) fractions were purified from the ascites fluids and biotinylated as previously reported.[13] The CTLL-2 cells were from Dr J. Banchereau. Standardized allergen extracts were prepared by the Laboratoires des Stallergènes (Fresnes, France) and have been described previously.[20]

Patients

Forty-four allergic patients (21 men and 23 women, aged 8–63 years) volunteered to enter the study after informed consent. It was approved by the Ethical Committee of the Montpellier University. All were selected on (1) a suggestive history of allergic diseases (asthma and/or rhinitis), (2) positive skin prick tests (SPT) to at least one of the aeroallergens of the Montpellier area and (3) the presence of orchard grass pollen- and house dust mite-specific IgE (Phadebas CAP System®; Pharmacia Diagnostics, Uppsala, Sweden). None of the patients had received specific immunotherapy or had been treated by corticosteroids for the previous 3 months. Seven non-allergic subjects (four men and three women, aged 24–34 years) were included as controls.

Patients were placed in the monosensitized group when SPT and specific IgE were positive for one allergen only (13 patients reacted to grass or cypress pollens or to house dust mites of the species *D. pteronyssinus* and *farinae*) and in the polysensitized group when SPT and specific IgE were positive for many allergens (31 patients: five to multiple pollen species only and 26 to many allergens including pollens and mites).

Preparation of PBMC

Fifty millilitres of peripheral venous blood was collected and PBMC isolated as previously described.[13] PBMC were resuspended in Iscove's modified Dulbecco's culture medium (Gibco, Paisley, U.K.), supplemented with 5% autologous serum (for IL-2, IL-4 and IFN-γ production) or 5% fetal calf serum (for IgE and sCD23 production), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

Cytokine and IgE production

PBMC were plated at $2{\cdot}0 \times 10^6$ cells/ml in duplicate, in 48-well cluster plates (Costar, Cambridge, MA), in 1·0 ml/well, at 37° and 5% CO_2 humidified atmosphere. Cells were stimulated with 10 μg/ml phytohaemagglutinin (PHA; Flow Laboratories, Irvine, U.K.) plus 10 ng/ml phorbol myristate acetate (PMA; Sigma Chemicals, St Louis, MO) or 10 ng/ml PMA plus 100 ng/ml calcium ionophore A23187 (A23187; Sigma).[12,21] After a 48-hr incubation, the culture supernatants (CSN) were harvested, centrifuged at +4° for 5 min and stored at −20° for quantifying IFN-γ and IL-2 release. The cells were incubated for an additional 10-day period with 20 IU/ml IL-2 and 1 μg/ml PHA and fed every 3 days by replacing 0·5 ml of the CSN with IL-2- and PHA-containing fresh medium. At day 10, the CSN were collected for IgE and sCD23 determination and the PBMC restimulated with the same activating agents as initially. CSN were harvested after 48-hr incubation for IL-4 quantification.

ELISA for IL-4 and IFN-γ

ELISA were performed according to methods previously described in detail[13] and modified as follows. For IL-4 quantification, microplates were coated with a purified Ig fraction of a polyclonal goat anti-IL-4 antiserum. Dilutions of the CSN and calibration curves were added to the plates for 18 hr at +4°. The rat anti-IL-4 mAb 11B4 was added to the wells for 4 hr at room temperature. The sensitivity of the assay was 40 pg/ml. IFN-γ was measured by using two anti-IFN-γ mAb as catcher (mAb A35) and tracer antibodies (mAb B27), respectively. The biotinylated mAb B27 was added for 4 hr at room temperature. The sensitivity of the assay was 20 pg/ml.

IL-2 titration

IL-2 activity was quantified by measuring the proliferation-inducing activity of the CSN on the IL-2-dependent murine T-cell line CTLL-2.[12] Briefly, the CSN and IL-2 were introduced in parallel in flat-bottomed 96-well culture microplates (Nunc, Roskilde, Denmark). CTLL-2 cells were added at 6×10^6/ml. After 36-hr incubation, 37 kBq/well [^{3}H]thymidine (Amersham France SA, Les Ulis, France) was added for 4 hr.

ELISA for sCD23

sCD23 was quantified by using a sandwich ELISA kit (The Binding Site, Birmingham, U.K.).

IgE titration

Serum IgE concentrations and IgE released in the CSN were determined by ELISA as previously described.[11,13] The coating antibody was a rabbit anti-IgE (Dakopatts, Glostrup, Denmark) and the calibration curve was made of a standard IgE serum (Phadexact® Serum; Pharmacia Diagnostics AB). The anti-IgE mAb I-27 was used as the tracer antibody. The sensitivity of the assay was 75 pg/ml.

IgE immunoblots

IgE immunoblots to the orchard grass pollen or *Der p* extracts were performed according to a method previously described in detail.[22] The immunoblots were classified depending on the number of protein bands revealed from 1 to 4, according to a previous study.[3] Sera from non-atopic and non-pollen-sensitive individuals were used as controls.

Statistical analysis

Statistical analysis was performed by means of non-parametric tests. The Mann–Whitney *U*-test was used to compare each individual group. The Spearman rank test was used for correlations.

RESULTS

Serum total IgE and IgE immunoblots

The levels of serum IgE were greater in polysensitized patients (mean ± SD, 607 ± 696 ng/ml) than in the control subjects (56 ± 39 ng/ml), whereas monosensitized patients (135 ± 171 ng/ml) presented intermediate IgE levels (Fig. 1). These differences were significant between mono- and polysensitized patients but not significant between normal and monosensitized subjects (Fig. 1).

The patterns of IgE immunoblots to orchard grass pollen and *Der p* are presented in Fig. 2a and the distribution of these among patients in Fig. 2b. Most of the monosensitized patients presented a low number of proteins revealed (pattern of 1–2), with the exception of one patient who displayed a pattern of 4. In contrast, most polysensitized patients presented a high number of proteins revealed (pattern of 3–4).

Serum sCD23

The levels of serum sCD23 were increased in most allergic patients (mean ± SD: polysensitized, 2·09 ± 1·51 ng/ml; monosensitized, 1·83 ± 1·36 ng/ml) compared with non-allergic subjects (1·12 ± 0·71 ng/ml), and more particularly in polysensitized ones (not shown), but results did not reach significance.

IL-4 and IFN-γ production by PBMC from allergic and non-allergic donors

PBMC from all non-allergic donors and most allergic patients did not spontaneoulsy produce IL-4, with the exception of a few allergic patients (eight out of a total of 44) producing low or marginal amounts of IL-4 (Table 1). IL-4 production was not enhanced when the culture period was increased to 72 and 96 hr (not shown). The activation of the PBMC *in vitro* in the presence of combinations of PHA + PMA or PMA + A23187 resulted, in most experiments, in the production of relatively low amounts of IL-4 (Table 1). In order to optimize the IL-4 production by PBMC, the cells were cultured for an additional 10–12 day period in the presence of recombinant IL-2 plus PHA and a second stimulation was performed for 48 hr with the same activators (Table 1), such a stimulation inducing a significant IL-4 production. The IL-4 production obtained in the presence of PHA + PMA was comparable to that obtained in the presence of PMA + A23187. On the other hand, a strong IFN-γ production was obtained with a single activation procedure with either PHA + PMA or PMA + A23187 (Table 1). When the cells were cultured in the presence of IL-2 plus PHA for 10 days and restimulated, it did not result in an increased production of IFN-γ (Table 1).

Subsequently, according to these results, the phenotype of T-cell subsets in mono- and polysensitized patients was assessed by determining the production of IFN-γ after a first 48-hr stimulation of freshly prepared PBMC and that of IL-4 after a second 48-hr stimulation of prolonged cultures in PHA and IL-2.

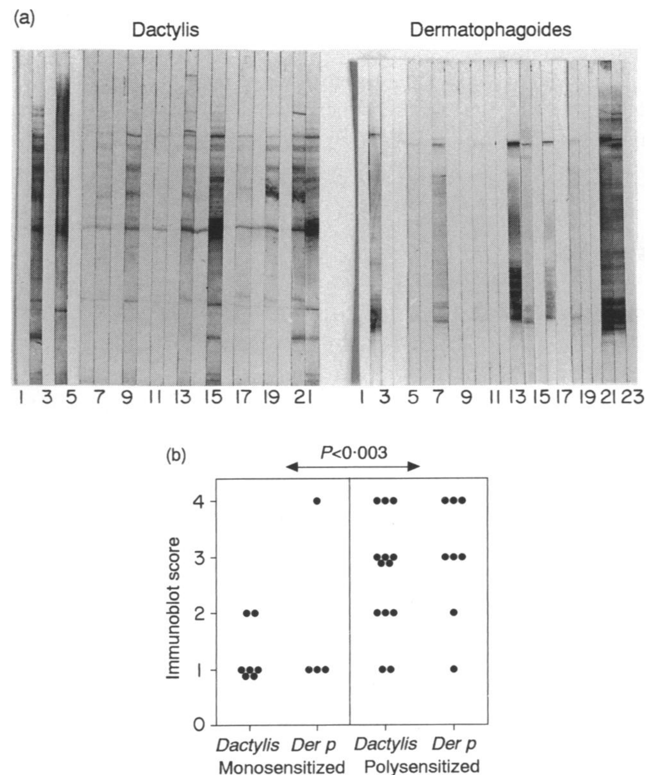

Figure 2. (a) IgE immunoblots against orchard grass pollen (*Dactylis glomerata*) and *Der p*. Dactylis: blot 1, control; blot 2, India ink protein staining; blot 3, serum control; blots 6, 10, 11, 14, 18, score 1; blots 7, 9, 17, 19, score 2; blot 13, score 3; blots 4, 15, 21, 22, score 4. *Dermatophagoides*: blot 1, control; blot 2, India ink protein staining; blot 3, serum control; blots 5, 10, score 1; blot 18, score 2; blots 7, 13, 14, 15, score 3; blots 21, 22, score 4. (b) Immunoblot scores of mono- and poly-sensitized patients. Statistical analysis for the whole group by Mann–Whitney *U*-test.

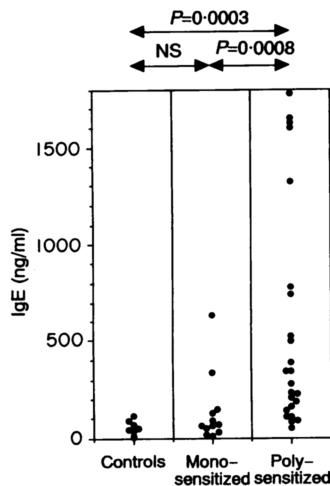

Figure 1. Comparison of the total IgE concentrations in sera of allergic and non-allergic subjects. Serum IgE was titrated by ELISA as indicated in the Materials and Methods. Statistical analysis is according to the Mann–Whitney *U*-test. NS, not significant.

Table 1. Effects of culture conditions on the induction of IL-4 and IFN-γ production by PBMC of mono- and polysensitized patients

Exp.	Group (patient no.)	Culture conditions PHA+PMA* (48 hr)	PMA+A23187† (48 hr)	IL-2+PHA‡ (10 days)	IL-4 (ng/ml)	IFN-γ (ng/ml)
1	Control (38)	−	−	−	≤0·04	0·43
		+	−	−	0·25	129
		−	+	−	0·10	222
		+	−	+	0·53	155
		−	+	+	0·28	173
2	Mono (2)	−	−	−	≤0·04	≤ 0·04
		+	−	−	0·19	6·00
		−	+	−	≤0·04	22·05
		+	−	+	4·00	15·00
		−	+	+	4·05	10·23
3	Mono (4)	−	−	−	≤0·04	≤0·04
		+	−	−	≤0·04	9·13
		−	+	−	0·21	11·86
		+	−	+	1·27	15·40
		−	+	+	1·93	14·65
4	Poly (12)	−	−	−	0·12	≤0·04
		+	−	−	0·82	12·67
		−	+	−	0·21	22·17
		+	−	+	7·14	6·0
		−	+	+	9·29	3·93
5	Poly (24)	−	−	−	≤0·04	≤0·04
		+	−	−	0·92	2·10
		−	+	−	0·62	3·46
		+	−	+	5·24	0·31
		−	+	+	4·21	0·15

*PBMC were activated *in vitro* with PHA plus PMA for 48 hr; then, culture supernatants were collected and the synthesis of IL-4 and IFN-γ determined by ELISA.

†PBMC were activated with PMA plus calcium ionophore A23187 for 48 hr and treated as above.

‡Cells were activated with PHA plus PMA or PMA plus A23187, washed and grown for 10 days in IL-2 and PHA. At day 10, the cells were restimulated with the same combinations of activators as initially.

Comparison of cytokine production by PBMC from allergic and non-allergic individuals

With respect to IL-4 production induced in the presence of PMA + A23187 (Fig. 3a), significant differences were observed among the three groups of subjects, polysensitized patients releasing the greatest amounts and control subjects the smallest. The production of IL-4 by PBMC from polysensitized patients was significantly higher (mean ± SD, 5·77 ± 2·05 ng/ml) than that of monosensitized donors (1·85 ± 1·41 ng/ml), which itself was significantly higher than that of control non-allergic individuals (0·27 ± 0·22 ng/ml). In contrast, IFN-γ production by PBMC in the presence of PMA + A23187 (Fig. 3b) was highly and significantly reduced in both groups of allergics (polysensitized, 12·44 ± 18·81 ng/ml; monosensitized, 16·46 ± 23·26 ng/ml) compared to that of healthy donors (225 ± 67 ng/ml). For both IL-4 and IFN-γ production, similar results were obtained when PBMC were stimulated in parallel with PHA + PMA (not shown).

For the three groups of donors, comparable amounts of IL-2 were synthesized following a single 48-hr step activation whatever the degree of allergenic sensitivity (mean ± SD, polysensitized, 25·4 ± 20·61; monosensitized, 17 ± 16·74 ng/ml; controls, 33·75 ± 10·27 ng/ml).

Comparison of IgE and sCD23 production by PBMC from allergic and non-allergic individuals

There was a difference in the production of IgE and sCD23 by PBMC of the three groups of subjects (Fig. 4). IgE synthesis was significantly higher in both groups of allergic patients (mean ± SD, polysensitized, 46·72 ± 23·38 ng/ml; monosensitized, 17·52 ± 14·7 ng/ml) than in the control group (5·46 ± 4·18 ng/ml) and in the polysensitized group than in the monosensitized one (Fig. 4a). sCD23 release was significantly higher in the two groups of allergic individuals (polysensitized, 17·49 ± 11·46 ng/ml; monosensitized, 12·73 ± 12·43 ng/ml) than in the controls (3·91 ± 3·0 ng/ml) but differences did not reach significance between poly- and monosensitized patients (Fig. 4b).

Correlations between the different biological markers

Table 2 shows the correlations between the markers studied by Spearman rank test. Culture conditions were similar for IL-4,

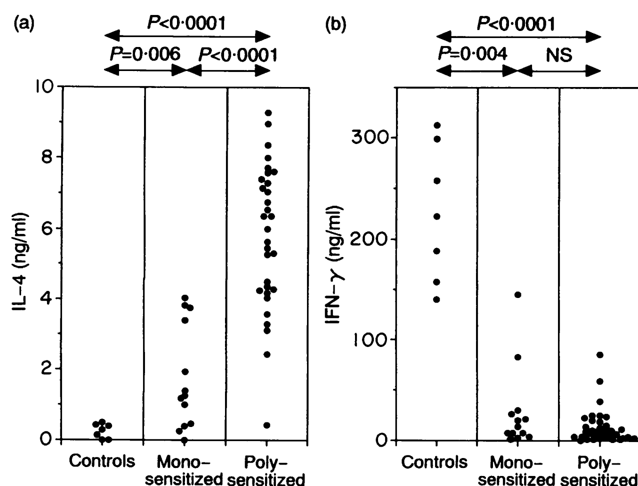

Figure 3. Comparison of the effect of PMA plus calcium ionophore on the IL-4 and IFN-γ synthesis by PBMC of mono- and polysensitized patients. PBMC were isolated and activated in the presence of PMA plus calcium ionophore A23187 and the release of IL-4 and IFN-γ was quantified as described in the Materials and Methods. IFN-γ was measured after a 48-hr single step activation procedure whereas IL-4 was quantified in the CSN following a two-step procedure assay, as described in the Materials and Methods. (a) IL-4 production; (b) IFN-γ production. Statistical analysis is according to the Mann–Whitney U-test. NS, not significant.

IgE and sCD23 production but not for IFN-γ. There was a highly significant positive correlation between the production of IL-4 by PBMC and total serum IgE, PBMC IgE and PBMC sCD23. The production of sCD23 by PBMC was significantly correlated with serum IgE and PBMC IgE. There was a

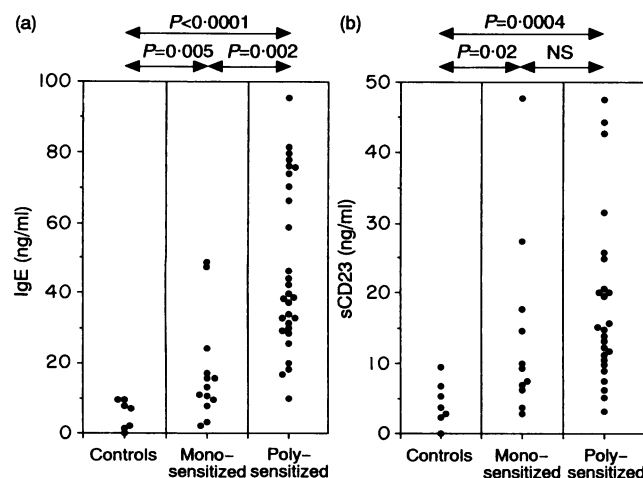

Figure 4. Comparison of the effect of PMA plus calcium ionophore on the IgE and sCD23 synthesis by PBMC of mono- and polysensitized patients. PBMC were isolated and activated in the presence of PMA plus calcium ionophore A23187 for 48 hr, washed and grown in IL-2 plus PHA for 10 days as indicated in the Materials and Methods. At day 10, culture supernatants were harvested and IgE and sCD23 were quantified by ELISA (see the Materials and Methods). (a) IgE production; (b) sCD23 production. Statistical analysis is according to the Mann–Whitney U-test. NS, not significant.

Table 2. Correlations between the different biological markers

Markers*		Rho	P value
IL-4	Serum total IgE	0·46	⩽0·003
	sCD23	0·50	⩽0·0006
	IgE	0·57	⩽0·0001
IFN-γ	Serum total IgE	−0·328	⩽0·04
	IgE	−0·47	⩽0·001
sCD23	Serum total IgE	0·34	⩽0·035
	IgE	0·495	⩽0·0007
IgE	Serum total IgE	0·75	⩽0·0001

*IL-4, IFN-γ, sCD23 and IgE refer to the production of these markers in the CSN at the time-points indicated in the Materials and Methods. Serum total IgE refers to the IgE quantified in the sera.

significant negative correlation between the production of IFN-γ and serum IgE or PBMC IgE. Finally, there was a significant correlation between the IgE immunoblot results and the production of IL-4 by PBMC ($P \leqslant 0{\cdot}02$, Kendall's τ test).

DISCUSSION

Data presented here show that the heterogeneity of atopy between mono- and polysensitized subjects is associated with a different quantitative and qualitative IgE immune response and may be related to a difference in IL-4 production. In addition, a good correlation between the sensitization of the patients and sCD23 release by PBMC was observed. Thus, PBMC of mono- and polysensitized patients have a different IL-4 and sCD23 profile, suggesting that humans may be classified into high and low IgE responders on the basis of IL-4 production and that IL-4 may be involved in the regulation of IgE-dependent responses.

To determine the basis of the heterogeneity of atopy clearly, it was important to characterize the subjects entering in this study as precisely as possible on the basis of clinical observations. As previously shown,[23] serum IgE levels and IgE immunoprints were found to be in agreement with the classification of the patients as mono- or polysensitized, indicating that they differ by the amounts of total serum IgE and that allergen-specific IgE differ qualitatively.

As previously shown,[12,21] PBMC did not release IL-4 nor IFN-γ spontaneously, but had to be stimulated with combinations of T-cell polyclonal activating agents (PHA plus PMA or PMA plus A23187). Even under these conditions, a two-step activation procedure was required for obtaining consistent IL-4 production, as already reported.[21] An alternative method to analyse T-cell cytokine patterns is to generate allergen-specific T-cell clones. Using this methodology, immune responses preferentially associated with Th1 or Th2 T lymphocytes were differentiated.[17-19] However, it was important to determine the phenotype of crude T-cell populations and not only allergen-specific T-cell clones for the following reasons: (1) we analysed allergic and non-allergic patients; (2) many of our patients were

sensitized to allergens which are not available in a purified form; (3) only a few clones might have been selected, which could not completely reflect the original global T-cell population.[21,24]

IL-4 production was significantly enhanced in both allergic groups by comparison with non-allergic individuals and an inverse correlation was found for IFN-γ release. These results fit with those comparing PBMC from non-allergic donors and patients with high IgE levels[12] or PBMC from patients with atopic dermatitis[25–27] but disagree with those of Takahashi *et al.* which show that IL-4 and IFN-γ production were not different between patients with atopic dermatitis and control individuals and that no significant correlation was found between cytokine production and clinical symptoms.[28] However, the data of Tang *et al.* indicate a correlation between the degree of imbalance of IL-4 and IFN-γ synthesis and the severity of the disease.[26] Our data are also in accordance with the studies demonstrating that allergen-specific T-cell clones generated from allergic donors produce IL-4 but little or no IFN-γ whereas T-cell clones from non-allergic individuals or clones specific for non-allergenic antigens such as tetanus toxoid only produce IFN-γ.[17–19] These results suggest that Th1- or Th2-like cells are operational *in vivo* and the data presented herein suggest that the increasing sensitization of patients to allergens correlates with increasing potentials of T cells to differentiate into a Th2-like phenotype.

In the mouse, IL-2 is produced by the Th1 subset.[15] Surprisingly, no differences in IL-2 production between the three groups of donors were observed. However, these results are in line with those of Rousset *et al.*[12] showing that only IL-4 and IFN-γ are, respectively, enhanced and reduced in allergic subjects and those of Wierenga *et al.*[17] reporting that *Der p*-specific T-cell clones generated in non-atopic and atopic individuals could only be distinguished on the basis of IL-4 and IFN-γ production but not IL-2. On the other hand, Robinson *et al.*[29] reported that the mRNA for IL-2 was more expressed in T cells from asthmatic subjects than from the controls. Since this study was performed on bronchial material, it may indicate that the microenvironment of T cells in the bronchi could modulate their phenotype and functions.

A high correlation was observed between the production of IgE and IL-4 release by PBMC. Since IgE and sCD23 production has been shown to be up-regulated by IL-4[13,30] and since seric sCD23 is elevated in atopic disorders[31] it seems likely that these phenomena play an important role in the initiation and development of allergy.

Differences observed between mono- and polysensitized subjects based on the release of IL-4, but not IFN-γ, and qualitative and quantitative IgE production suggest that humans can be classified into 'high' and 'low' IgE responders and that these characteristics could be under the control of IL-4. In animal models, the IgE immune response differs considering the strains of mice: BALB/c mice are high IgE responders whereas SJL are low responders and this characteristic is controlled by a single autosomal gene not linked to the MHC complex.[32] Strains of mice differ greatly in their capacity to produce IL-4 and low and the high IgE responder trait may be determined depending on the level of IL-4 mRNA,[33] indicating that IL-4 production is involved, at least in part, in the IgE response in mice. These results suggest that there are at least two genetic mechanisms controlling the atopic status: the specific immune response genes associated with the histocompatibility complex[34] and a second control, not associated with it, involved in the regulation of IgE serum levels ('high' and 'low' responders).[35] Altogether, these data suggest that, in monosensitized patients (the 'low' IgE responders) the allergic sensitization depends closely on HLA-DR or DP molecules, whereas in polysensitized patients (the 'high' IgE responders) the same sensitization may develop regardless of HLA-DR or DP molecule expression, suggesting a degenerate binding of epitopes to MHC in this latter group.

The present study shows evidence that using polyclonal activators, mono- and polysensitized individuals are heterogeneous in terms of qualitative and quantitative IgE immune responses and that these differences are, at least in part, due to a difference in the IL-4 production. It provides useful tools to study allergen-specific stimulation and detection of other cytokines regulating the IgE synthesis.

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