

Specific induction of interleukin-4-producing cells in response to *in vitro* allergen stimulation in atopic individuals

S. GABRIELSSON, S. PAULIE, S. RAK*, E. LAGGING†, M. VAN HAGE-HAMSTEN†, B. HÄRFÄST† and M. TROYE-BLOMBERG

Department of Immunology, Stockholm University, *Allergy Unit, Sahlgrenska Hospital and †Clinical Immunology, Karolinska Hospital, Stockholm, Sweden

Summary

Background and Objective CD4⁺ T cells can be divided into two major subsets, T helper (TH)₁ and TH₂ cells. Interleukin-4 (IL-4) is produced by TH₂ cells and induces switching of immunoglobulin (Ig) M/IgG to IgE. Interferon-γ (IFNγ) produced by TH₁ cells counteracts the IgE-promoting effects of IL-4. In this study we wanted to investigate whether the number of IL-4-producing cells could be a direct measurement of allergen exposure *in vitro*, and whether this was correlated to the elevated serum IgE-levels seen in atopic persons.

Methods We compared the number of IL-4- and IFNγ-producing cells using an enzyme-linked immunospot assay (ELISPOT) in response to allergens from birch and cat in peripheral mononuclear cells from atopic and healthy individuals.

Results In the two sensitized groups there was an increase in the number of IL-4-producing cells in response to the specific allergen which was not seen in the healthy group (1/20 000 cells and 1/200 000 cells, respectively, $P < 0.001$ for birch). In criss-cross experiments where birch-sensitized individuals were stimulated with cat allergen, no IL-4-producing cells were seen, indicating a high degree of specificity. In individual subjects, the elevated numbers of IL-4-producing cells were significantly correlated with their allergen-specific serum IgE levels. When allergen was combined with a suboptimal dose of PHA, there was a synergistic increase in the number of allergen-induced IL-4-producing cells (1/4 000 cells) in the atopic donors, which was not seen with the number of IFNγ-producing cells.

Conclusions Allergen-specific IL-4 producing cells in a peripheral blood mononuclear cell (PBMC) culture can be detected by ELISPOT and the response can synergistically be enhanced by suboptimal concentrations of PHA.

Keywords: allergen-specific, atopy, ELISPOT, IFNγ, IL-4

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Introduction

The acute cause of most symptoms in Type 1 allergies is the IgE-induced release of histamine, prostaglandins and other mediators by mast cells. In allergic individuals, there is an elevated level of IgE in serum [1]. The cytokine IL-4 has been shown to initiate switching to IgE in resting [2,3] and LPS-activated B-cells [4,5] and is also needed for maintaining IgE-production [6]. IL-13 is another cytokine that, although less potently, promotes IgE-production [7].

IL-4 is produced by a certain subset of CD4⁺ T-helper (TH) cells. Mosmann and Coffman first proposed the division of murine TH cells into TH₁ and TH₂ cells based on the cytokines that they secrete upon antigen stimulation [8]. TH₁ cells produce IFNγ, TNFα, TNFβ and IL-2, while TH₂ cells produce IL-4, IL-5 and IL-6. There is also an intermediate or non-differentiated TH cell called TH₀, producing IL-2 and IFNγ, as well as IL-4 [9]. Although not as clear-cut, recent data suggest that human CD4⁺ T cells also are heterogenous with regard to cytokine production [10]. TH₁ cell clones provide help for cell-mediated responses and play an important role in delayed type hypersensitivity, while TH₂ cells are most potent in delivering stimuli to B

Correspondence: S. Gabrielsson, Department of Immunology, Stockholm University, S-106 91 Stockholm, Sweden.

cells, leading to antibody production [11]. The cytokines of TH₁ and TH₂ cells reciprocally counteract each other, e.g. IL-4 inhibits IFN γ production and IL-4-induced IgE-synthesis is blocked by addition of IFN γ [12]. In murine experimental infections there is now good evidence that the balance between the two response patterns determines the type and efficiency of the immune response.

In this study we wanted to investigate whether the elevated levels of allergen-specific IgE in serum seen in allergic patients were reflected by an increased number of IL-4-producing cells after activation with allergen. To permit a minimum of *in vitro* manipulation we used the highly sensitive enzyme-linked immunospot (ELISPOT) assay which allows analysis at the single-cell level of *in vitro* activated bulk cultured mononuclear cells.

Materials and methods

Reagents

The monoclonal antibodies used in the ELISPOT were: MoAb 1-DIK and 7B6-1 to human IFN γ and MoAb 82-4 (IL-4-1) and 12-1 (IL-4-2) to human IL-4 (all MoAb were obtained from MABTECH, Stockholm, Sweden). The specific allergens used were Aquagen-SQ birch and cat, 1×10^6 SQ-U/mL (ALK, Denmark). The mitogen was PHA (Murex Diagnostics Ltd, Dartford, UK).

Subjects

The sensitized groups comprised asthma patients positive in radio-allergosorbent test (RAST, > 4 kU/L or $>$ class 2) for birch ($n = 17$, aged 27–60, mean 40.1) and/or cat ($n = 12$, aged 22–34, mean 29.1). All birch patients were studied > 3 months after the pollen season. The controls were 17 age-matched volunteers negative in RAST for birch and cat.

Preparation and stimulation of PMNC

Mononuclear cells from heparinized peripheral blood were isolated by Ficoll-Paque (Pharmacia, Stockholm, Sweden) gradient centrifugation. The cells were then diluted to 2 million cells/mL in tissue-culture media (TCM) consisting of RPMI-HEPES with Gentamicin ($25 \mu\text{g/mL}$), 2 mM L-glutamine and 10% heat-inactivated fetal calf serum. The cells were processed within 24 h of bleeding, or frozen and used later. For freezing, PBMC were suspended (10^7 to 10^8 cells/mL) in TCM containing 10% Dimethyl sulphoxide (DMSO) and frozen gradually 1°C/min to -70°C in a freezing container (Nalgene Cryo 1°C , Nalge Company, USA). Thereafter, tubes were stored in liquid nitrogen. Before use, the cells were thawed quickly in a 37°C waterbath and diluted and washed in TCM. Half or 1 mL cell suspension

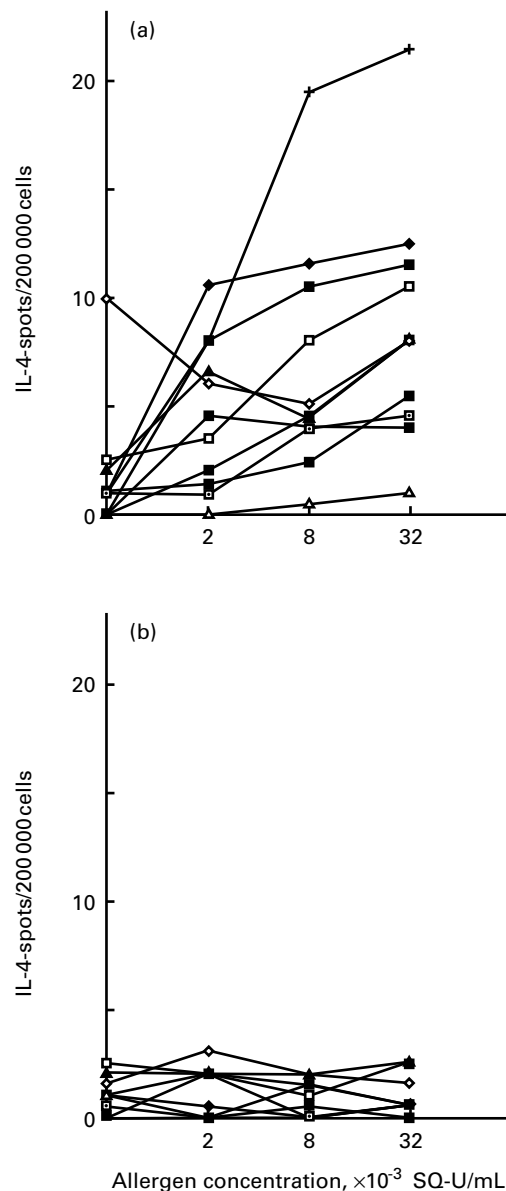


Fig. 1. The number of IL-4-producing cells in (a) 11 birch sensitive and (b) nine healthy individuals in response to birch allergen. Peripheral mononuclear cells were cultured for 48 h in medium containing different concentrations of birch allergen. Results represent the mean values of duplicate determinations.

was transferred to 5 mL-culture tubes (Falcon, Becton-Dickinson, New Jersey, USA) and allergens and/or PHA were added at different concentrations. The tubes were incubated at 37°C in a humid atmosphere containing 5% CO_2 for 4 h. After the incubation, the cells were resuspended, and $100 \mu\text{L}$ of the cell suspension were added to each coated ELISPOT-well (200 000 cells/well) in duplicates and transferred to ELISPOT-plates. In the case of IFN γ , cells were first diluted ten times (20 000 cells/well).

Table 1. The number of IL-4-producing cells/200 000 cells from *in vitro* activated PBMC measured by ELISPOT*

Stimuli	Birch sensitive (17) Mean $\pm$ SD	Birch negative (9) Mean $\pm$ SD	<i>t</i> -test, unpaired <i>P</i>	<i>t</i> -test, paired <i>P</i>
w/o	1.3 $\pm$ 2.3	1.1 $\pm$ 0.8	NS†	
Ag	7.9 $\pm$ 5.6	0.9 $\pm$ 1.0	0.001	0.0035
PHA	101.2 $\pm$ 58.4	46.5 $\pm$ 30.2	0.020	
PHA + Ag	149.5 $\pm$ 98.9	39.3 $\pm$ 26.9	0.034	0.0013

* PBMC were cultured for 48 h in medium or medium containing 0.25 μ g/mL PHA and/or 32 000 SQ-U birch allergen. Results from each individual represent the mean values of duplicate determinations.

† not significant.

ELISPOT

The reverse ELISPOT assay is based upon the ELISA-plaque assay for detection of antibodies [13] and was performed mainly as described elsewhere [14,16]. Briefly, the anti-human-IFN γ monoclonal antibody 1-DIK or anti-human IL-4 monoclonal antibody 82-4 were diluted to a concentration of 15 μ g/mL in sterile, filtered (0.45 μ m) phosphate buffered saline (PBS), and 100 μ L per well were added onto nitrocellulose plates (Millititer, Millipore Corp., Bedford, Massachusetts, USA). Plates were incubated overnight at 4°C and thereafter unbound antibodies

were washed away by six successive washings with filtered PBS; 100 μ L of cell suspension were added to each well and the plates were incubated 40–42 h at 37°C whereafter the cells were washed away and 100 μ L of the biotinylated IFN γ MoAb 7B6-1 or IL-4 MoAb 12-1 (1 μ g/mL) were added and incubated for 2 h at room temperature (RT). Thereafter, the plates were washed and incubated for 90 min at RT with 100 μ L of streptavidin-alkaline-phosphatase (MABTECH, Stockholm, Sweden) diluted 1/1000. Unbound conjugate was removed by another series of washings and finally 100 μ L of BCIP/NBT substrate solution (Bio-Rad, Richmond, USA) were added and incubated at RT until dark spots emerged (1/2 h for IFN γ and 1 h for IL-4). The colour development was stopped by three washings with 200 μ L of tap-water/well. After drying, the spots were counted in a dissection microscope ($\times 40$).

Statistical analysis

The statistical analyses performed were paired student's *t*-test between w/o and allergen stimulated cultures and unpaired student's *t*-test between the sensitive and healthy group. Linear regression analysis on logarithmic values was also performed.

Results

Estimation of the number of IL-4-producing cells in birch atopic persons and in controls

The ELISPOT assay was used to determine the number of IL-4 producing cells induced in response to specific allergen. For this purpose mononuclear cells from birch allergic patients or healthy non-allergic individuals were stimulated with three different doses of birch allergen. Sera from all allergic patients were classified as being RAST class 3 or more. As can be seen in Fig. 1a, there was a response to

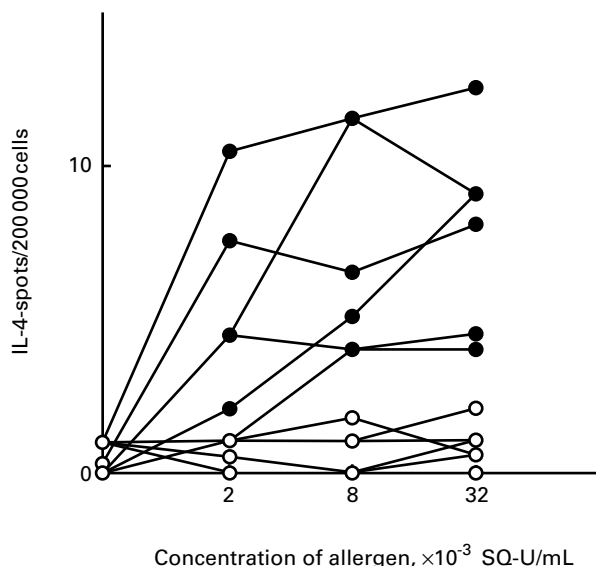


Fig. 2. Test of crossreactivity to cat allergen in six birch-sensitive individuals. ●, birch (specific allergen), ○, cat (unrelated allergen). Peripheral mononuclear cells were cultured for 48 h in medium containing different concentrations of birch or cat allergen. Results represent the mean values of duplicate determinations.

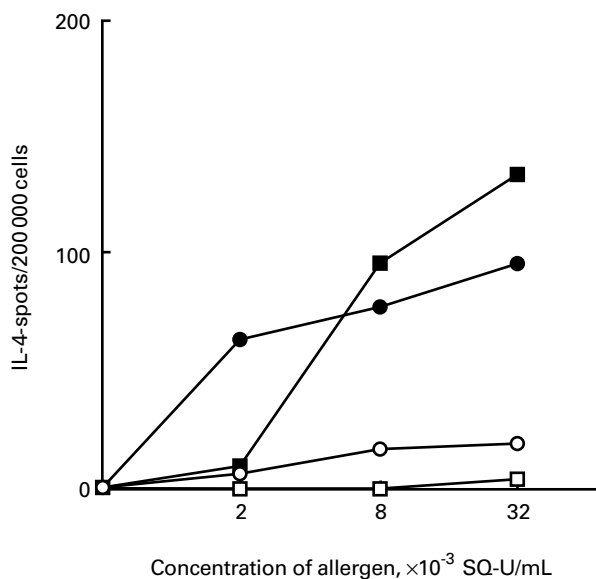


Fig. 3. IL-4-producing cells in two representative birch sensitive individuals out of 11. A suboptimal dose of PHA in combination with specific allergen induces a synergistic effect on the number of IL-4-producing cells. The number of spots produced without allergen stimuli has been subtracted. Peripheral mononuclear cells were cultured for 48 h in medium containing PHA (0.25 µg/mL) and different concentrations of birch allergen. The number of PHA induced spots was for donor 1 and 2, 65 and 170 spots/200 000 cells respectively. Results represent the mean values of duplicate determinations. ■, Patient 1 w. PHA □, Patient 1 w/o PHA, ●, Patient 2 w. 2PHA, ○, Patient 1 w/o PHA.

birch allergen in all except one birch allergic patient, with the highest dose (32 000 SQ-Units/mL) inducing the highest number of IL-4-producing cells. Therefore, the last six individuals tested were only stimulated with the highest allergen dose. The response was significantly higher than to medium only ($P = 0.0035$, paired t -test). This response was specific

and not seen in the non-allergic group, where no or very few IL-4-producing cells were seen (Fig. 1b). The difference of spots in response to allergen between the two groups was statistically highly significant ($P < 0.001$, Table 1).

Specificity was further shown by stimulating cells from six birch allergic patients with birch allergen and for these patients, an unrelated cat allergen. As can be seen in Fig. 2, a response was only seen to the specific allergen ($P = 0.0017$, paired t -test for the highest allergen dose).

Suboptimal polyclonal activation synergistically increases the frequency of allergen-specific IL-4-producing cells in atopic patients

In this study and in agreement with previously reported [17], polyclonal activation by PHA induced more IL-4-producing cells in the atopic group than in the group of healthy individuals. In individual donors the dose of PHA required to induce optimal number of IL-4-producing cells varied between 1 and 10 µg/mL (not shown). Also when stimulating with lower concentrations (0.25 µg/mL), the atopics responded with more IL-4-producing cells (Table 1).

Although there was an increase in the sensitized patients in IL-4-producing cells after stimulation with the allergen, the total number of IL-4-producing cells was generally low. However, when combining suboptimal doses of PHA (0.25 µg/mL) with the allergen, there was a synergistic induction of IL-4-producing cells in the birch sensitive individuals (Fig. 3, Table 1). In birch sensitive, the difference between PHA + allergen and PHA alone was 48 IL-4-spots/200 000 cells. The difference between allergen and medium only (without the PHA-synergy) was 7 IL-4-spots/200 000 cells. No increase was seen in healthy individuals.

Cat atopics

The response to the cat allergen in cat allergic patients was

Table 2. The number of IL-4-producing cells/200 000 cells from *in vitro* activated PBMC measured by ELISPOT*

Stimuli	Cat atopics (12) Mean ± SD	Cat negative (8) Mean ± SD	<i>t</i> -test, unpaired <i>P</i>	<i>t</i> -test, paired <i>P</i>
w/o	0.7 ± 0.7	0.5 ± 0.6	NS†	
Ag	10.0 ± 12.1	2.0 ± 1.8	NS	0.016
PHA	98.8 ± 100.9	47.9 ± 28.6	NS	
PHA + Ag	133.0 ± 147.1	56.4 ± 30.1	NS	0.017

* PBMC were cultured for 48 h in medium or medium containing 0.25 µg/mL PHA and/or 32 000 SQ-U cat allergen. Results from each individual represent the mean values of duplicate determinations.

† not significant.

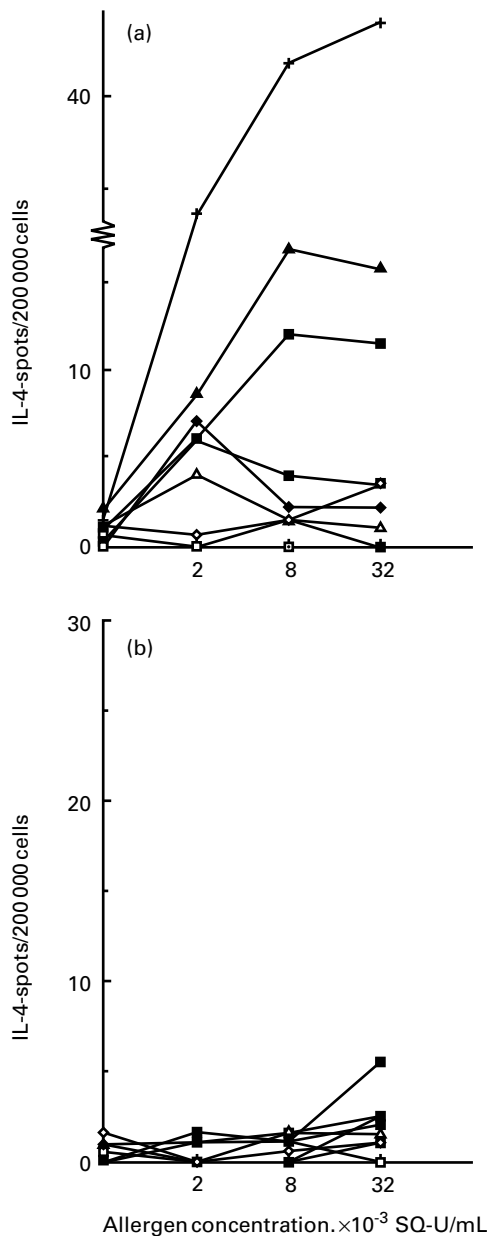


Fig. 4. The number of IL-4-producing cells in (a) eight cat sensitive and (b) eight healthy individuals in response to cat allergen. Peripheral mononuclear cells were cultured for 48 h in medium containing different concentrations of cat allergen. Results represent the mean value of duplicate determinations.

also statistically significant ($P = 0.0035$ for allergen only and $P = 0.0013$ for the combination with PHA. Table 2, Fig. 4). However, the differences between the cat allergics and healthy individuals were not significant probably because of the large variation in the absolute numbers of IL-4-producing cells. Interestingly, one patient reporting allergic symptoms to cat but low in specific IgE and thereby excluded from the

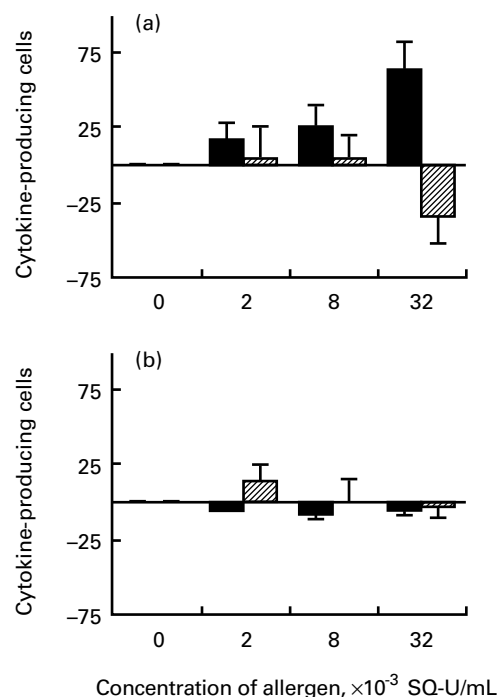


Fig. 5. The number of (■) IL-4-producing cells/200 000 cells and (▨) IFN- γ -producing cells/20 000 cells from (a) 11 birch sensitive and (b) nine healthy individuals in response to allergen. The number of spots produced without allergen stimuli has been subtracted. Mononuclear cells were cultured for 48 h in medium containing PHA (0.25 μ g/mL) and different concentrations of birch allergen. The number of PHA-induced spots varied between 40 and 270 spots/200 000 cells and 130 and 950 spots/20 000 cells for IL-4 and IFN- γ , respectively. Results represent the mean values of 11 individuals + SE.

study, responded to stimulation with allergen + PHA and one cat sensitive individual not responding to only allergen responded to the combination. Two non-allergic individuals responded weakly to allergen + PHA (not shown), and one to the allergen only (Fig. 4).

Comparison of IL-4- and IFN- γ -production after stimulation with allergens

To determine how the numbers of IL-4 producing cells in response to PHA combined with increased concentrations of allergen were related to the number of IFN- γ -producing cells, ELISPOT analyses were also performed for this cytokine. While the number of IL-4-producing cells increased, the number of IFN- γ -producing cells decreased after stimulation with the highest dose of allergen (Fig. 5a). Similar analyses made with non-allergic individuals did not show any effect of the allergen (Fig. 5b). There was no IFN- γ -production spontaneously or after only allergen stimulation (not shown) in either groups.

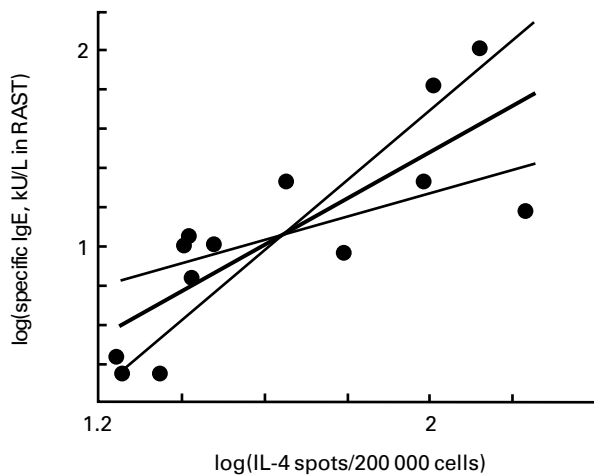


Fig. 6. Linear regression analysis between the logarithm of specific serum IgE (measured by RAST) and the logarithm of the number of IL-4-producing cells in response to specific allergen ($\rho = 0.80$, $P = 0.001$) in 13 birch- or cat-allergic individuals. Peripheral mononuclear cells were cultured for 48 h in medium containing PHA only or PHA together with birch or cat allergen. The number of spots produced without allergen stimuli has been subtracted. PHA alone gave 40–270 IL-4-spots/200 000 cells. Results represent the mean value of duplicate determinations.

Specific IL-4 response vs specific IgE

To investigate if the IL-4-response was correlated to allergen-specific serum IgE-levels, the numbers of allergen-specific IL-4-producing cells were compared with the individual allergen-specific IgE levels (Fig. 6). Linear regression analysis revealed a statistically significant relationship between the two measures ($\rho = 0.80$, $P = 0.001$). This was in contrast to what was seen between IL-4-response to PHA and total IgE in serum (not shown).

Discussion

Resting T cells are poor producers of IL-4, but when stimulated *in vitro* with mitogens or anti-CD3 antibodies in the presence of IL-2 and IL-4, they can develop into IL-4-producing cells. Still, very low frequencies of IL-4 producing cells are seen after stimulation compared to several other cytokines, e.g. IL-2 [18]. Here, we have used the ELISPOT-method, since this method has been shown to be more sensitive than conventional ELISAs [19] and the frequency of cytokine producing cells can be measured. In ELISPOT the cytokine is measured close to the cell where it is produced and not in the supernatant, where it will be diluted or can bind to cell-bound or soluble receptors. As few as one cytokine producing cell in 200 000 cells can be detected by this assay. In ELISPOT the true frequency of cytokine-producing cells might be

underestimated if more IL-4 is produced later than 44 h or if the MoAbs or cytokine molecules dissociate during the time of the incubation. An alternative sensitive method to detect cytokine producing cells is RT-PCR. However, since the IL-4 production is transient and RNA is degraded faster than protein, the time-points are more critical [20].

Type 1 allergic responses are TH₂-dependent [10,21]. Thus, cells from atopic patients produce more IL-4 after polyclonal stimulation than healthy people [17,22]. The IL-4 production is also higher in polysensitized patients compared with monosensitized patients [17]. In agreement with this, in the present study many more IL-4-producing cells in response to PHA were found in the atopic group compared to the healthy donor group.

Allergen-specific IL-4 can be induced in atopic individuals. However, in these instances even smaller amounts of IL-4 are produced than after polyclonal stimulation. Several authors have reported that in order to be able to detect antigen-specific IL-4-production, repeated antigen stimulation [23–25] or *in vitro* pulsing with polyclonal stimuli [26] are needed. Other reports on allergen-specific IL-4-production have been performed using specific T-cell clones [27,28]. Here, we demonstrate that by using the ELISPOT assay it is possible to detect IL-4-producing cells directly after a short incubation with specific allergen. Thus, when PBMC obtained from birch allergic patients were *in vitro* activated with the allergen, the numbers of IL-4-producing cells significantly increased over non-stimulated cultures. As analyses were performed out of season for birch pollen allergics these *in vitro* tests seem to reflect a secondary response. In similar cultures obtained from healthy individuals, birch allergen did not induce any IL-4-secretion. Moreover, in the atopic individuals the number of IL-4 spots was significantly correlated to their specific serum IgE levels.

In patients with atopic dermatitis, an inflammatory skin disease associated with high IgE-levels in serum, a decreased IFN γ -production as compared with that in normal individuals in response to non-specific stimulation has been reported, suggesting a switch to TH₂ in the atopic patients [29,30]. This was also the case in our study, where an increased number of IL-4-producing cells and a reduced number of IFN γ -producing cells were found. The fact that there was no reduction of IFN γ -producing cells in the healthy individuals after allergen stimulation suggests that the TH₁-cells are suppressed by the IL-4 induced in the atopic individuals, leading to a reduced number of IFN γ -producing cells.

When PBMC were obtained from cat allergic patients not all of them responded to cat allergen. Since the number of IL-4-producing cells in some cat sensitized individuals was as high as for the birch sensitized group, the lack of response

is not likely due to fewer T-cell epitopes in the cat allergen preparation. Rather the reason for the low response in some individuals is probably due to that cat allergen remains airborne for a long while, and thus the patients have been exposed without actually being in contact with the animal. This might lead to migration of the specific T cells into the target organ, and the cells remaining in the periphery would be difficult to stimulate further. The migration of activated cells from the periphery has been demonstrated in malaria [31]. The reason why two non-allergic individuals responded to the cat allergen is unknown. The response is very low and only to the highest dose of allergen, and could be due to a subclinical allergy or the crude nature of the allergen.

When PHA and allergen were used together, a synergistic increase in the number of IL-4-producing cells was seen in the atopic group. The reason for the synergy is most likely that PHA stimulates T cells, hereby leading them to aggregate and to gain cell-cell contact, facilitating interaction between cell-surface molecules. Several cytokines will also be produced, e.g. IL-2, which is known to be a costimulatory factor necessary for IL-4-production, since anti-IL-2 completely inhibits IL-4-production [32]. The frequency of allergen stimulated IL-4-producing cells with the PHA probably reflects better the *in vivo* situation in the lung, where the cells more easily reach contact with other cells than in a culture tube *in vitro*. Furthermore, additional cytokines produced *in vitro* by the PHA could *in vivo* be produced by other cells not present in the *in vitro* PBMC culture. The synergistic increase was not seen in the number of IFN γ -producing cells. Thus, this suggests an increased activation of TH₂ cells, or alternatively of other cells producing a TH₂-like cytokine pattern in atopic individuals. However, our data does not exclude the possibility of existing allergen specific TH₁ or TH₀ type of cells. Cells producing TH₂-cytokines have been shown to include CD8⁺ T cells [33], $\gamma\delta$ T cells [34], CD4-CD8- $\alpha\beta$ T cells [35] as well as non-B non-T cells [36]. However, recent data suggest that the IgE initiating factor most likely is CD4⁺ derived IL-4 [37]. The synergistic effect observed with PHA is not specific for TH₂ cells since in psoriasis, a TH₁-associated disease, the synergistic effect of PHA is seen as an increased number of IFN γ -producing cells [38].

In conclusion, the results demonstrate an increased number of allergen-induced IL-4-producing cells in the atopic individuals which was not seen in the healthy individuals. The number of IL-4-producing cells was significantly correlated with presence of allergen-specific serum IgE-levels. At present we do not know whether this IL-4 is produced by T or non-T cells. Despite this, the ELISPOT assay was shown to be a useful tool for detection of allergen-specific IL-4-producing cells in a PBMC-culture.

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