

Effect of bronchial allergen challenge on *in vitro* cytokine release by peripheral blood mononuclear cells of atopic patients

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Background During the pollen season, peripheral blood mononuclear cells (PBMC) from allergic patients produce increased levels of Th2 cytokines after stimulation with allergen *in vitro*. We have studied the effect of a single bronchial provocation test (BPT) of allergic patients to determine whether allergen challenge *in vivo* modulates cytokine production by PBMC, after subsequent stimulation with the same allergen *in vitro*.

Methods Twelve atopic asthmatic patients were challenged with the relevant allergen, and their PBMC, isolated before (T0) or 6 (T6) or 24 h (T24) after BPT, respectively, were cultured for 120 h in the presence or absence of the same allergen, after which cytokine production was measured by ELISA.

Results Allergen-specific activation of the PBMC at T0 resulted in interleukin (IL)-5 and IL-13 production, but not in detectable levels of interferon-gamma and IL-4. BPT did not induce the secretion of the latter cytokines. However, IL-5 and IL-13 production was significantly decreased at T24, as compared to T0. No statistically significant differences were found between the production of IL-10 before and after BPT.

Conclusions In contrast to the effects of natural challenge with allergen, a decrease in the production of some Th2 cytokines by peripheral blood T cells was observed 24 h after BPT, suggesting a concomitant decrease in the frequency of allergen-specific T cells in the circulation.

Key words: allergy; bronchial allergen challenge; cytokines; Th2 cells.

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Human CD4⁺ T helper cells (Th) have been classified into at least two subsets, Th1 and Th2, according to differences between their cytokine production profiles, as well as their functional properties (reviewed in References 1, 2). Human Th1 clones produce mainly interferon-gamma (IFN- γ) upon activation and no interleukin (IL)-4 and IL-5, whereas Th2 clones secrete IL-4 and IL-5, but low amounts of IFN- γ . Although IL-10 and IL-13 are produced by either subpopulation of human CD4⁺ Th clones (3, 4), according to their functional properties, both cytokines are considered to be Th2 cytokines, and their production *in vivo* may indeed be restricted. The heterogeneity of cytokine production profiles observed among human and mouse Th cell lines has cast some doubt on the categorization of discrete Th1 and Th2 subsets, in view of the independent regulation of cytokine gene expression in individual cells.

Therefore, it has been argued that the restricted production of cytokines by either subpopulation represents only the two extremes of a large spectrum of intermediate cytokine production profiles (5). Cytokines produced by Th2 cells are strongly implicated in atopy and allergic inflammation. IL-5 is likely to play a central role in promoting selective tissue accumulation and activation of eosinophils in allergic diseases (6), whereas IL-4 and IL-13, which have largely overlapping functions, are crucial for the differentiation of B cells into IgE-producing plasmocytes.

A large number of studies have aimed at the characterization of T-cell cytokine profiles in allergic patients. Because of the poor sensitivity of enzyme-linked immunosorbent assay (ELISA) methods for cytokine detection, combined with the very low levels of cytokine production after allergen-specific stimulation, many investigators

have used polyclonal agents alone (7–13) or as a boosting step, in association with allergenic stimulation (14–16). Nevertheless, a few studies have reported that peripheral blood mononuclear cells (PBMC) of atopic patients produce detectable levels of IL-4 and IL-5 after primary culture with allergen (17, 18). The frequency of allergen-specific T cells in peripheral blood is low as compared to their frequency in various target organs in allergic diseases, such as atopic skin and inflamed lung epithelium (19).

However, it has been shown that allergen exposure during the pollen season increased the production of Th2 cytokines by PBMC *in vitro* (9, 10, 12, 20), suggesting the presence of enhanced levels of allergen-specific T cells in blood, probably due to enhanced recirculation of these cells. In contrast, it has been suggested that allergen-specific provocation of asthmatic patients induced the recruitment of T cells into tissue during the late-phase reaction (21–24), although it is not known whether this procedure resulted in the presence of increased numbers of allergen-specific T cells in the circulation as well. To test the latter hypothesis, we challenged asthmatic patients with a single dose of allergen and analyzed the cytokine response of their PBMC after allergen-specific stimulation *in vitro*.

Material and methods

Patients

Twelve patients (11 men, one woman), ranging in age from 18 to 28 years (mean age: 24 years), were recruited, on the basis of clinical history of allergy to various allergens. All patients presented:

- 1) positive skin prick test (SPT), performed as previously described (25), to at least one of the standardized allergen extracts
- 2) a positive bronchial provocation test (BPT) to the most reactive extract and the presence of specific IgE (Phadebas CAP System, Pharmacia Upjohn, Uppsala, Sweden).

All patients had respiratory symptoms during allergen exposure. None of the patients had received immunotherapy of any form during the 5 years preceding the study. None of the patients had been treated by systemic or inhaled corticosteroid, sodium cromoglycate, nedocromil sodium, theophylline, or ketotifene for the previous 3 months in view of the possible immunomodulatory effects of these drugs. None of the patients were symptomatic at the time of BPT. The study was approved by the ethics committee of the University of Montpellier (France), and written consent was obtained from each patient. All patients were polysensitized to

various allergens, whereas four patients reacted mainly to the house-dust-mite allergens Der p and Der f, three patients to the cat allergen *Fel d*, and five patients to the grass-pollen allergen *Lol p*.

Reagents

Purified recombinant IL-4, IL-5, and IFN- γ were purchased from R&D Systems (Abingdon, UK). The two anti-IFN- γ monoclonal antibodies (mAb) A35 and B27, kind gifts from Dr F. Rousset (Schering-Plough, Dardilly, France), were prepared for use in ELISA, as previously described (26). Purified mouse anti-human IL-4 (clone 8D4-8, used at 1 μ g/ml), biotinylated rat anti-human IL-4 (clone MP4-25D2, 0.25 μ g/ml), purified rat anti-mouse/human IL-5 (clone TRFK5), and biotinylated rat anti-human IL-5 mAbs were purchased from PharMingen (San Diego, CA, USA). Streptavidin-alkaline phosphatase conjugate was obtained from Southern Biotechnology Associates (Birmingham, AL, USA) and used at a dilution of 1:2500. Commercial ELISAs for the detection of human IL-10 and IL-13 were from Diaclone (Besançon, France). Lyophilized and standardized grass-pollen, house-dust-mite, and cat-allergen extracts were from ALK (Hørsholm, Denmark), and a mix of five grasses from the Laboratoire des Staller-gènes (Fresnes, France).

BPT were performed by using the appropriate extract in a lyophilized form that was freshly reconstituted before each test at concentrations varying from 1 to 10⁵ SQU/ml. Pulmonary function tests were carried out with Pneumoscreen equipment (E. Jaeger Laboratories, Würzburg, Germany). Administration of all drugs that might interfere with performance of BPT, especially β_2 -agonists, was interrupted for at least 12 h. The baseline forced expiratory volume at 1 s (FEV₁) was always over 70% of predicted values. Diluent or allergen extracts were delivered with an automatic, inhalation-synchronized dosimeter jet nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland) (27). The reproducibility of the generation of the aerosols was controlled by a constant aerosolization pressure of 2 bar. The dosimeter recorded the number of inhalations taken by each patient. The bronchial response to aerosol challenge was assessed by measuring serially FEV₁. Inhalations were continued until the presence of an immediate reaction, measured when the drop of FEV₁ surpassed 20%.

Stimulation of PBMC

Peripheral blood was obtained by venapuncture immediately before (T0), and 6 (T6) and 24 h (T24)

after the immediate reaction. PBMC were isolated by centrifugation on Ficoll Paque (Pharmacia) and cultured in the presence or absence of 1 µg/ml of relevant allergen or (for some patients) with phorbol-12-myristate-13-acetate (PMA, 10 ng/ml) and the calcium ionophore A23187 (500 ng/ml; both from Calbiochem, San Diego, CA, USA) at a concentration of 2×10^6 cells/ml in 24-well, flat-bottom, tissue-culture plates (Nunc, Roskilde, Denmark). All cultures were carried out in Yssel's medium (28) (Irvine Scientific, Santa Ana, CA, USA), supplemented with 1% human AB⁺ serum. Supernatants were collected after 24 h and 120 h of culture and frozen at -20°C until analysis for cytokine content.

Analysis of cytokine production

IL-4, IL-5, and IFN-γ production was measured by ELISA, as described previously (29). IL-10 and IL-13 production was measured by commercial ELISA as specified by the manufacturer. The limits of sensitivity of the assays were as follows: 10 pg/ml for IL-4, IL-5, and IL-13; 5 pg/ml for IL-10; and 20 pg/ml for IFN-γ.

Statistical analysis

Statistical analysis was performed by nonparametric tests. The Wilcoxon signed rank test was used to compare the cytokine production before and after BPT.

Results

PBMC from allergic patients, taken before BPT (T0), did not produce detectable levels of the cytokines IL-4, IFN-γ, IL-5, IL-10, and IL-13. In addition, BPT did not induce *in vitro* production of any of these cytokines, measured at T6 and T24, respectively, by PBMC cultured in medium only (Fig. 1).

In vitro stimulation of PBMC, isolated before BPT, with 10 µg/ml of relevant allergen to which the patients were allergic, resulted in the production of IL-5 and IL-13. In contrast, production levels of IFN-γ and IL-4 were below the detection limit of the assay. The failure to produce the latter cytokines was not due to suboptimal stimulation conditions, since culture of PBMC in the presence of higher concentrations of allergen (up to 50 µg/ml) did not induce their production either (results not shown). Moreover, PBMC were cultured for as long as 120 h (Fig. 2), but stimulation with allergen for shorter periods of time also did not result in detectable levels of IFN-γ or IL-4. IL-10 production levels, which were barely detectable in PBMC

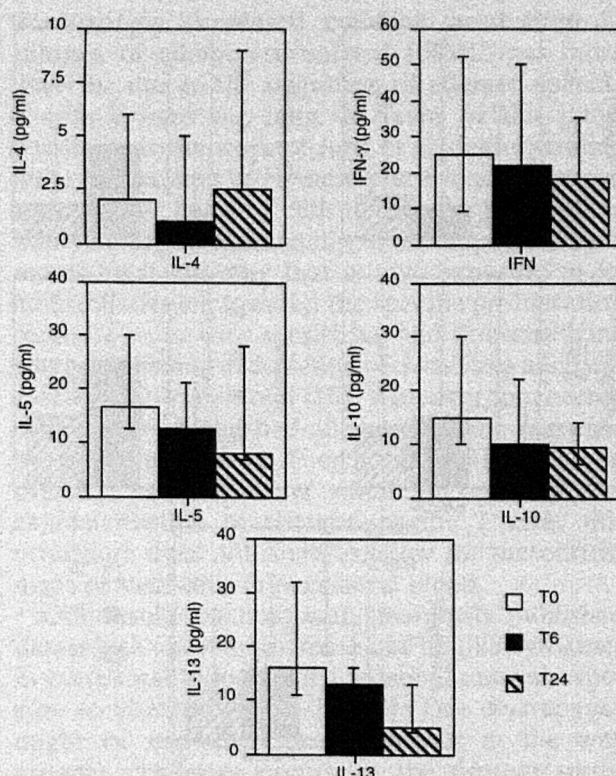


Fig. 1. Spontaneous cytokine production by PBMC before and after bronchial provocation. PBMC, isolated from 12 atopic patients immediately before (T0) and 6 (T6) and 24 h (T24) after BPT, were cultured for 120 h in medium alone, and IL-4, IFN-γ, IL-5, IL-10, and IL-13 production in culture supernatants was measured by ELISA. Results are expressed as median value of $n=12$, and bars represent 25–75 percentiles.

cultured in medium only, increased slightly, but not significantly, after stimulation of the PBMC with allergen.

Furthermore, as shown in Fig. 2, BPT had no effect on the lack of production of IL-4 and IFN-γ by allergen-stimulated PBMC, isolated at T6 and T24 after provocation. However, BPT had a down-regulatory effect on the median release of IL-5 (Fig. 3A) and IL-13 (Fig. 3B) by PBMC isolated after BPT. Whereas, at T6, the variation of IL-5 and IL-13 production was highly heterogeneous from donor to donor as compared to T0 (data not shown), production of IL-5 decreased in 8/12 patients and that of IL-13 in 9/12 patients, measured in PBMC that were isolated at T24, and this effect of the BPT was significant ($P < 0.05$ for both cytokines). IL-10 levels also decreased in 7/12 patients, but the overall diminution between T0 and T24 was not significant.

BPT had no effect on the intrinsic capacity of the PBMC to produce cytokines, since 24-h stimulation with PMA and calcium ionophore induced the following production of IL-4 (mean values $\pm$ SD in pg/ml at T0, T6, and T24, respectively: 367 ± 349 ,

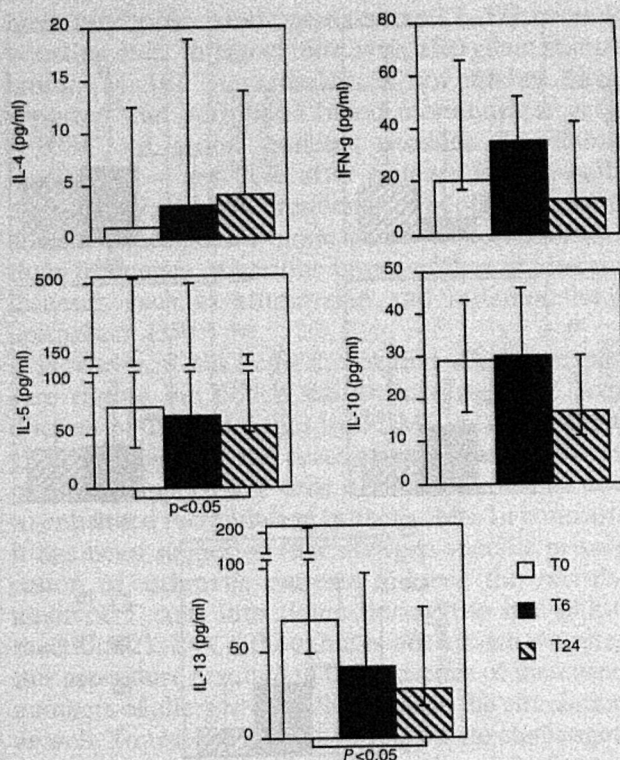


Fig. 2. Net cytokine production by allergen-stimulated PBMC before and after bronchial provocation. PBMC, isolated from 12 atopic patients immediately before (T0) and 6 (T6) and 24 h (T24) after BPT, were stimulated *in vitro* with optimal concentrations of specific allergen, and production of IL-4, IFN- γ , IL-5, IL-10, and IL-13 in supernatants was measured by ELISA after 120 h of culture. Results are expressed as median value of $n=12$, and bars represent 25–75 percentiles.

738 $\pm$ 488, and 350 $\pm$ 369); of IL-5 (789 $\pm$ 749, 1122 $\pm$ 762, and 784 $\pm$ 562); of IL-10 (41 $\pm$ 50, 98 $\pm$ 90, and 35 $\pm$ 41); of IL-13 (165 $\pm$ 176, 359 $\pm$ 261, and 186 $\pm$ 169);

and of IFN- γ (553 $\pm$ 483, 716 $\pm$ 341, and 112 $\pm$ 79), at levels which were comparable between PBMC isolated at T0, T6, and T24, respectively.

Discussion

Allergen-specific CD4⁺ T cells play an important role in the onset and maintenance of allergic diseases, as a result of their production of the Th2 cytokines IL-4, IL-5, and IL-13. Although an accumulation of allergen-specific T cells might be expected in the target organs of allergic diseases, such as skin and bronchial airways, their frequency in peripheral blood is low (19), and the production of Th2 cytokines, especially that of IL-4, is rarely detected in peripheral blood after allergen-specific stimulation of peripheral blood T cells of atopic patients. However, it has been reported that allergen-specific stimulation *in vitro* of CD4⁺ T cells isolated from atopic subjects during the pollen season resulted in enhanced levels of IL-4 (10, 12) and IL-5 production (20). From these observations, it can be speculated that after natural exposure the numbers of allergen-specific T cells in peripheral blood might be increased, resulting in an increased production of cytokines.

However, the results of the present study show that an allergen-induced BPT in asthmatic patients does not permit the detection of allergen-induced cytokine production by peripheral blood T cells *in vitro*, indicating that bronchial provocation does not seem to increase the number of allergen-specific T cells in peripheral blood. Moreover, in contrast to observations after natural exposure to allergen during the pollen season, BPT resulted in a decrease, rather than an

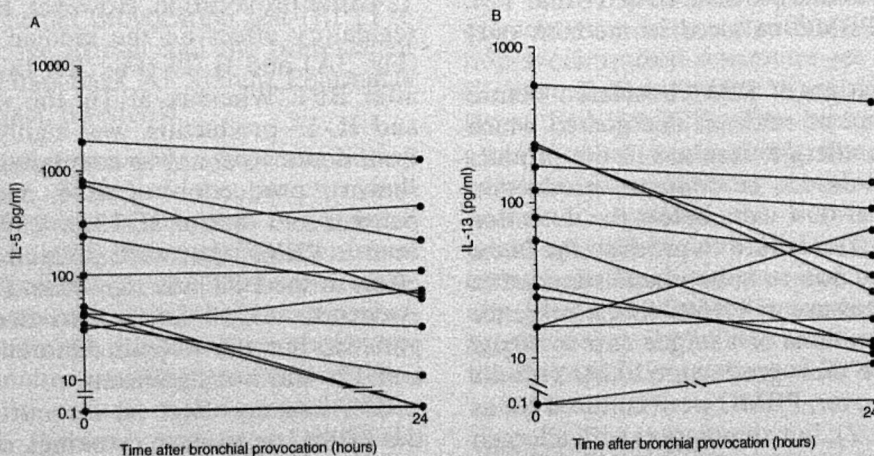


Fig. 3. Production of IL-5 and IL-13 by allergen-stimulated PBMC before and after bronchial provocation. PBMC, isolated from 12 atopic patients immediately before (T0) and 24 h after (T24) BPT, were stimulated *in vitro* with optimal concentrations of specific allergen, and production of IL-5 (A) and IL-13 (B) was measured in culture supernatants after 120 h of culture.

increase, in the frequency of allergen-specific T cells in the circulation. This effect was observed for IL-5 and IL-13, the only cytokines detectable in the culture supernatants of *in vitro* allergen-stimulated PBMC. PBMC, isolated 6 h after BPT, reduced their production of both cytokines, as compared to the production of these cells before BPT, an effect which was even more pronounced at 24 h after provocation (T24).

In accordance with a number of previously published studies, we were unable to detect IL-4 production by PBMC (7, 11–13, 15, 16). However, the failure to detect IL-4 production by allergen-stimulated PBMC, isolated at different time points after BPT, indicates that bronchial challenge with allergen does not enhance the frequency of IL-4-producing, allergen-specific T cells in the circulation. The observation that production of IL-5 and IL-13, but not IL-4, by allergen-stimulated peripheral blood T cells can be detected was probably due to IL-4 production levels in culture supernatants that were below the detection limit of the assay rather than to the inability of these cells to make IL-4 at all. Indeed, stimulation of PBMC with phorbol ester and calcium ionophore induced high levels of IL-4 production. In addition, the lack of detectable IL-4 production was not due to consumption of this cytokine by proliferating T cells, since similar results were obtained when culture supernatants were analyzed at different time points after stimulation with allergen (results not shown). Therefore, the results agree with the general notion that IL-4 is likely to act locally at relatively high concentrations, but that levels secreted in culture supernatants may be too diluted to be detected by ELISA. No differences were observed in production levels of IL-10 before and after BPT. However, although IL-10 is produced at high levels by antigen-specific Th1 and Th2 clones (3), IL-10 levels in cultures of PBMC are mostly the result of production by monocytes (30). Finally, little IFN- γ was induced after allergen-specific stimulation, confirming the notion that atopy is a Th2-cell-associated disease (2, 22).

The strongly decreased production of IL-5 and IL-13 by PBMC isolated 24 h after BPT might be due to the induction of a state of nonresponsiveness in allergen-specific T cells. Since stimulation of PBMC with PMA and calcium ionophore, which acts independently of the signal transduction pathway, involving T-cell receptor/CD3 complex triggering, resulted in the production of high levels of cytokines, including IL-4, IL-5, IL-13, and IFN- γ , it cannot be excluded that allergen-specific T cells were still present in the circulation, but were no longer able to respond to stimulation with allergen. Although the latter possibility cannot be excluded,

the strongly decreased cytokine production in cultures of allergen-stimulated PBMC was more likely be due to the migration of allergen-specific T cells toward the lung. In favor of this latter possibility is the work of Jung et al. demonstrating that s.c. injections of Hymenoptera venom allergen attract CD4⁺ helper T cells to the allergen application site (31). Additionally, it is supported by a recent study showing that *in vivo* exposure to an inhalant allergen lowered the specific proliferation response to *in vitro* sensitizing and nonsensitizing allergens, leading to depletion of circulating allergen-specific Th2 from blood (16). Whatever the mechanism of the diminished allergen-specific response by peripheral blood T cells after BPT, from a practical point of view, when one aims at the characterization of allergen-specific T cells, this procedure does not seem to allow for the enrichment of such cells in peripheral blood.

Our results contrast with previously published data which show an increase in Th2-like cytokines in peripheral blood during seasonal natural exposure to allergen (9, 10, 12, 20). This discrepancy might be explained by differences in the way allergen challenge occurs. In the present study, patients were challenged with a single, defined dose of allergen, aimed to induce an allergic crisis. However, exposure to allergen during the pollen season is long-lasting, whereas amounts of allergen are likely to differ considerably. Therefore, continuous natural challenge could lead to a constant recirculation of T cells and might mask cell migration from the blood toward target organs, as observed after a single challenge with allergen.

It is possible that bronchial provocation induces the proliferation of allergen-specific T cells in lymphoid tissues that would then go to the blood before their migration into the local sites of allergic inflammation, and there could be a critical period within 24 h after bronchial provocation during which this process might take place. In addition, the time for migration to take place may vary from one patient to another, since, at 6 h after bronchial provocation, cytokine secretion was found to increase in certain patients, whereas it decreased in others, as compared to the production of cytokines before allergen challenge (data not shown). Therefore, a more precise follow-up of IL-5 and IL-13 release as a function of time in the hours after bronchial provocation may yield information on the kinetics of the migration of allergen-specific T cells between blood and tissues.

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