

Intracellular production of IL-2, IL-4 and IFN- γ by peripheral blood CD3⁺ cells in intermittent allergic rhinitis

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Abstract. *Background:* Allergic inflammation is mainly driven by type 2 T helper cells. The aim was to assess the changes in production of type 1 and 2 cytokines by CD3⁺ T cells dependent on natural exposure to allergens in subjects with intermittent allergic rhinitis (IAR) and in non-atopic subjects. *Material:* A total of 13 patients with IAR and 13 healthy non-atopics were recruited into the study. 11 patients with IAR were examined during the grass pollen season and 11 patients outside the season, 9 of them were assessed on both occasions.

Methods: A flow cytometric assessment of intracellular expression of IL-2, IL-4 and IFN- γ by CD3⁺ cells was performed. For statistical analysis non-parametric tests were used.

Results: A tendency to decreased production of IL-4 outside the season was observed (6.94% [3.42–13.33] in season vs. 2.06% [0.7–3.6] out of season). The production of IL-4 was higher in the rhinitic group in the season than in the control group (1.93% [1.07–4.97], $p=0.0034$) and production of IL-2 was higher both in and outside the season (9.1% [3.94–15.09] and 10.0% [4.79–25.35] vs. 3.64% (2.64–5.03), $p=0.037$ and 0.045, respectively). IL-4/IL-2 and IL-4/IFN- γ ratios were higher in the IAR group in the season than outside the season.

Conclusion: A tendency towards a switch from a predominant type 2 response during natural allergen exposure to its suppression outside the season was found, together with a stable type 1 response.

Key words: Flow cytometry – Intermittent allergic rhinitis – Intracellular cytokines – Th1/Th2

Introduction

The Th2 hypothesis in allergy assumes that cytokines released by activated Th2 cells participate in induction and maintenance of allergic inflammation and dominate over

cytokines released by Th1 cells. The distinction between type 1 and type 2 T cells so far has been based mainly on analysis of secreted proteins or expressed mRNA by cultured T cells from peripheral blood or from sites of allergic inflammation after either non-specific or allergenic stimulation in vitro. However, results of such experiments largely depend on culture conditions and the phenotype in vitro is not necessarily the same as in vivo. Definition of functional T-cell subsets characterized by Th1 or Th2 cytokine patterns may also rely on flow cytometric measurement of intracellular cytokine secretion expression, in which unseparated whole blood is used. This method mostly approximates the conditions in vivo [1–3].

Allergic rhinitis is characterized by an inhalant allergen-dependent chronic inflammation, involving eosinophils, mast cells, and basophils, immunologically driven by T cells [4, 5]. The participation of Th1 and Th2-cytokines in the pathophysiology of intermittent allergic rhinitis has been examined employing various methods, but not by flow cytometric analysis of intracellular cytokine expression on a single-cell level [6–8]. So we decided to use flow cytometric method to assess whether the intracellular expression of the type 1 cytokines, IL-2 and IFN- γ , and the type 2 cytokine, IL-4, by CD3⁺ T cells changes as a result of natural exposure to allergens in intermittent allergic rhinitis and whether it differs from the production in non-atopics.

Material and methods

Subjects

Patients with intermittent allergic rhinitis (IAR, $n=13$, 6 male, median age 27 years [25–31]) were recruited from the District Outpatient Clinic of Allergology. Healthy, non-atopic subjects ($n=13$, 3 male, median age –29 years [24–32]) formed a control group (CON). Diagnosis of intermittent allergic rhinitis with sensitization to grass and rye pollens was established based on clinical history, skin prick test results and serum levels of allergen specific IgE against the panel of common inhalant and food allergens. Eleven patients of the IAR group were evaluated in a symptomatic period, during natural exposure to aeroallergens (from May to June) and 11 patients were evaluated in an asymptomatic

period, in autumn or winter. 9 of them were evaluated twice, both in and out of the pollen season. Non-atopic state of the control subjects was confirmed by SPT results and estimation of as-IgE. None of the participants were taking antihistamines or corticosteroids drugs before blood sampling.

The study was approved by the local Ethics Committee at the Silesian School of Medicine, Katowice and written informed consent was obtained from all subjects.

Antibodies and reagents

CD3 FITC, mouse Ig₂G1 PE, CD69 PE were purchased from Becton Dickinson, anti-IL-4 PE, anti-IFN- γ PE, anti-IL-2 PE from Coulter Immunotech, phorbol 12-myristate acetate (PMA) and ionomycin from Sigma, RMPI from BioWhittaker, IntraPrep Reagents 1 and 2 from DAKO.

Intracellular cytokine expression

Whole heparinized blood was diluted with RPMI and calf serum and then after addition of 25 μ l of PMA (25 ng/ml), 20 μ l of ionomycin (1 μ g/ml) and 10 μ l of monensin (2.5 μ M) incubated in water bath for 4 h at 37°C in tightly capped tubes. Stimulated whole blood was distributed in 100 μ l aliquots into tubes containing 10 μ l antihuman CD3-FITC, vortexed and incubated for 20 min at room temperature in the dark. To fix the cells 100 μ l of IntraPrep Reagent 1 was added to the samples, followed by vortexing, incubation for 15 min at room temperature in the dark, and washing with 2 ml of Cell-wash solution. Simultaneous permeabilization of the white cells and lysis of red cells were then undertaken by addition of 100 μ l IntraPrep Reagent 2 and incubation. Intracellular staining procedure included addition of 20 μ l of anti-IL-2, anti-IL-4 and anti-IFN- γ monoclonal antibodies or isotype control mouse antibodies conjugated with PE.

Flow cytometric assessment with FACSscan flow cytometer (Becton Dickinson) was performed the same day. The percentage of CD3⁺ cells producing IL-2, IL-4 and IFN- γ was determined. Quadrants were set based upon the isotype controls. The acquisition was performed both on samples stimulated with PMA and ionomycin, and on non-stimulated samples, in order to estimate the residual intracellular expression of the cytokines. Activation of the cells was confirmed by estimation of the CD69 expression, which was over 95% in all samples. Acquisition and analysis were performed with CellQuest software package (Becton Dickinson).

Statistical analysis

Results were expressed as median and interquartile ranges and non-parametric tests (Mann-Whitney rank sum test and Wilcoxon signed rank test) were used. All analyses were performed with a software package (The Quick STATISTICA PL.) and p values less than 0.05 were considered significant.

Results

Percentages of CD3⁺ cells producing IL-2, IL-4 and IFN- γ in the IAR group and in the control group are shown in Table 1. Production of all examined cytokines by CD3⁺ cells was comparable in and outside the pollen season. A tendency to less production of IL-4 by CD3⁺ cells outside the season as compared to during the season was observed, though non-significant. The expression of IL-2 by CD3⁺ cells was higher in the IAR group than in the CON group both in and outside the pollen season ($p = 0.037$ and 0.045 , respectively).

Table 1. Percentages of CD3⁺ cells producing IL-2, IL-4 and IFN- γ in intermittent allergic rhinitis patients in and out of the pollen season and in the control group (IAR – the intermittent allergic rhinitis group, CON – the control group).

	IAR group		CON group
	In season	Out of season	
CD3 ⁺ IL-2 ⁺	9.1 (3.94–15.09)*	10.0 (4.79–25.35) [†]	3.64 (2.64–5.03)
CD3 ⁺ IL-4 ⁺	6.94 (3.42–13.33) [‡]	2.06 (0.7–3.6)	1.93 (1.07–4.97)
CD3 ⁺ IFN- γ	7.76 (2.83–11.58)	4.37 (3.52–16.69)	5.78 (4.53–10.51)

* IAR – in the pollen season vs. CON $p = 0.037$.

[†] IAR – outside the pollen season vs. CON $p = 0.045$.

[‡] IAR – in the pollen season vs. CON $p = 0.0034$.

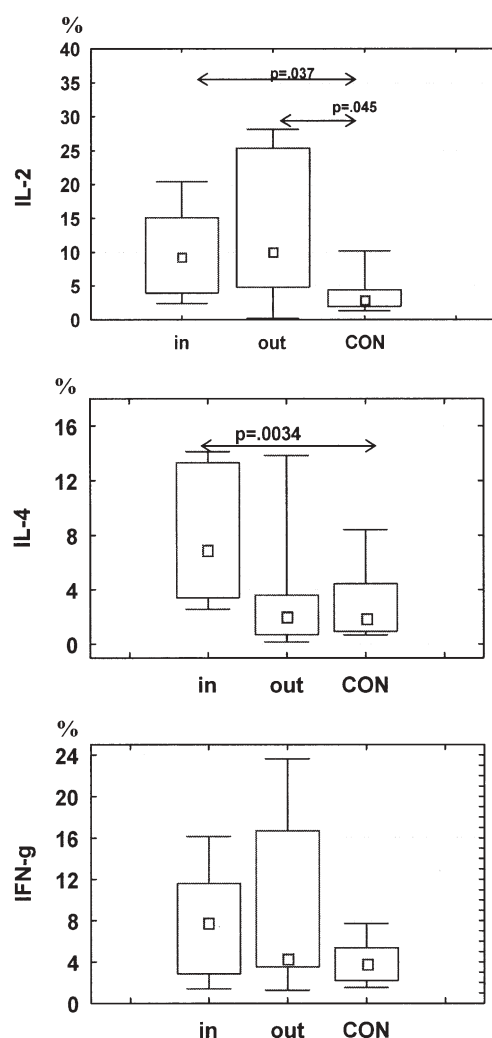


Fig. 1. The intracellular expression of IL-2, IL-4 and IFN- γ by CD3⁺ cells in the intermittent allergic rhinitis group in ("in") and outside ("out") the pollen season and in the control group ("CON"). The Mann-Whitney U test was used for comparison between groups and the Wilcoxon test was used for comparison within the SAR group. Squares represent median values, boxes – interquartile ranges, and whiskers – total range.

Percentage of IL-4 producing CD3⁺ cells was higher in the IAR in the season than in the CON group ($p = 0.0034$).

Ratios between type 2 and type 1 cytokines expressed in CD3⁺ cells were calculated both for values obtained in the pollen season and outside the season, and in the control group as well (table 2). In the IAR group, IL-4⁺CD3⁺/IL-2⁺CD3⁺ and IL-4⁺CD3⁺/IFN- γ ⁺CD3⁺ ratios were higher in the pollen season than outside the pollen season ($p = 0.008$ and $p = 0.08$, respectively). The ratio IL-4⁺CD3⁺/IFN- γ ⁺CD3⁺ in the IAR

group in the pollen season was higher than in the control group ($p = 0.002$).

Discussion

In the present study we estimated the cytokine production at a single cell level in intermittent allergic rhinitis using the flow cytometric method. We compared the cytokine profile in this disease to the healthy, non-atopic state, and assessed the changes in and outside the grass pollen season.

We observed a higher production of the type 2 cytokine, IL-4, by CD3⁺ cells in the IAR group as compared to the CON group, however only in the grass pollen season. There was also a tendency to decreased production of IL-4 by CD3⁺ cells outside the grass pollen season as compared with values observed in the season. Wosinska [9] analysed cytokine production by CD4⁺ cells from patients with rhinoconjunctivitis and sensitization to birch pollen and found the significantly higher percentage of IL-4⁺ lymphocytes (CD3⁺CD8⁻) than outside the birch pollen season. The higher serum level of IL-4 in patients with intermittent allergic rhinitis than in

Table 2. Ratios between intracellular expression of IL-4/IL-2 and IL-4/IFN- γ by CD3⁺ cells in the examined groups (IAR – the intermittent allergic rhinitis group, CON – the control group).

	IAR – in	IAR – out	CON
IL-4/IL-2	0.8 (0.69–0.88)	0.44 (0.07–0.58) [†]	0.54 (0.33–1.16)
IL4/IFN- γ	0.97 (0.8–1.35)	0.15 (0.12–0.69) [‡]	0.35 (0.24–0.68)*

* IAR in the pollen season vs. CON $p = 0.002$.

[†] IAR in the pollen season vs. IAR outside the pollen season $p = 0.008$.

[‡] IAR in the pollen season vs. IAR outside the pollen season $p = 0.08$.

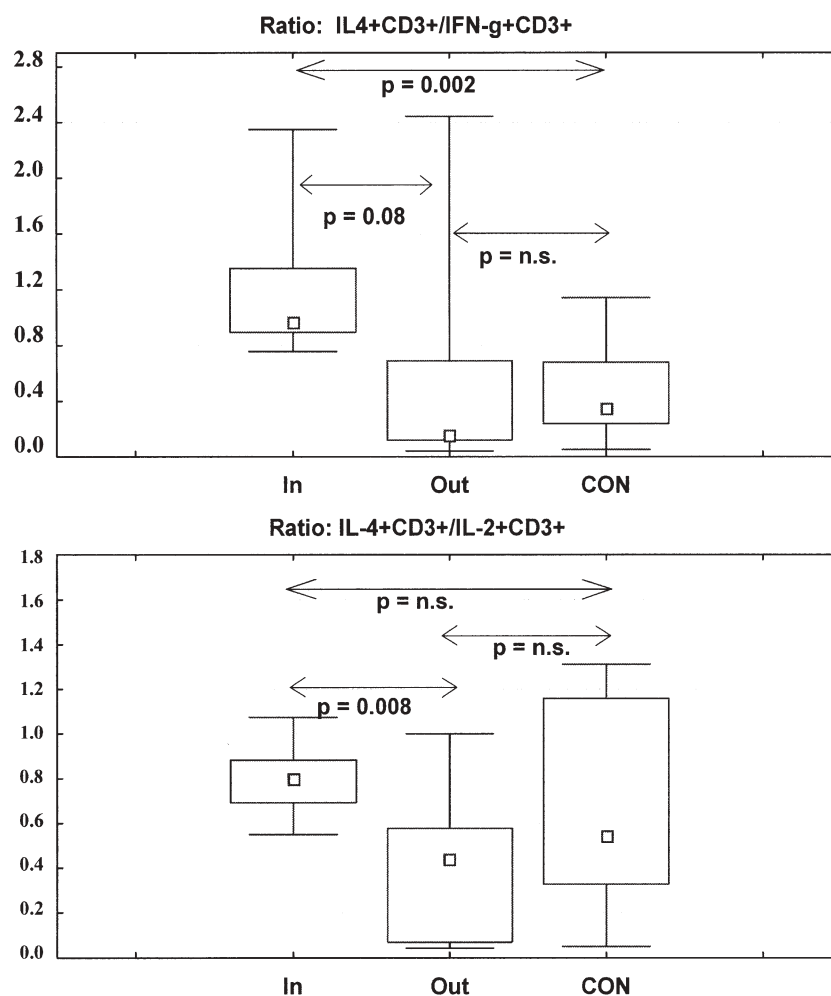


Fig. 2. Ratios of IL-4/IL-2 and IL-4/IFN- γ in ("In") and outside ("Out") the pollen season in the IAR group and in the control group (CON). The Mann-Whitney U test was used for comparison between groups and the Wilcoxon test was used for comparison within the SAR group. Squares represent median values, boxes – interquartile ranges, and whiskers – total range.

non-atopic volunteers, even outside the pollen season has been documented previously [10, 11]. Furthermore, increased synthesis of IL-4 in nasal mucosa in intermittent allergic rhinitis was also confirmed both in a morphological study and by RT-PCR analysis [12, 13].

In our study we observed an increased production of the type 1 cytokine, IL-2, by CD3⁺ cells in patients suffering from intermittent allergic rhinitis as compared to non-atopic subjects. The production remained elevated regardless of the exposure to the natural allergens. IL-2 is secreted mainly by Th1 cells, but also by cytotoxic/suppressor T cells. IL-2 stimulates the proliferation of T cells and their differentiation towards cytotoxic T cells. IL-2 together with IFN- γ enhances activity of natural suppressor cells. In our study the intracellular expression of IFN- γ by CD3⁺ did not differ between rhinitic and healthy subjects, and did not change within the IAR group in and outside the grass pollen season. Similar results were obtained by Lagier et al. [10] who found that the pollen season did not affect IFN- γ production by peripheral mononuclear cells stimulated with polyclonal agents and measured by ELISA. The lack of differences between intracellular expression of IFN- γ in allergic patients and control subjects is consistent with a previous report of comparable levels of IFN- γ in supernatants of cultured PBMC of untreated patients with intermittent allergic rhinitis and of non-atopic subjects [14].

We calculated the ratios of IL-4/IFN- γ and IL-4/IL-2-positive CD3⁺ cells. We showed that the investigated "T2/T1" ratios were higher in the grass pollen season and decreased outside the season. A previous study has shown an increased ratio of IL-4/IFN- γ in stimulated CD4⁺ T cells from subjects sensitive to birch pollen at the peak of the birch pollen season compared with the alder season, the beginning of the birch season and outside the tree pollen season [9]. The changes in intracellular production of IL-4 and the stable intracellular production of IFN- γ and IL-2 by CD3⁺, irrespective of the allergen exposure, indicated the predominance of type 2 reactivity during the natural exposure to the aeroallergens and the decrease of this reactivity outside the pollen season. Interestingly, type 1 reactivity was not influenced by natural exposure to grass pollens. The explanation of this observation may be the possible participation of T regulatory cells in the balance among different sub-populations of T cells. Th1/Th2 dichotomy seems not to be a true "dichotomy", but T regulatory cells (Tr1 and Th3), releasing IL-10 and/or TGF- β , respectively, may play an important role. Tr1 and IL-10 have been implicated in the mechanism of immunotherapy in bee venom and grass pollen allergy [15, 16]. No changes in the intracellular expression of type 1 cytokines by CD3⁺ cells irrespective of the allergen exposure, indicates a stable role of type 1 T cells in the pathogenesis of the intermittent allergic rhinitis.

One of the limitations of our study is the assessment of cytokine production only by CD3⁺ cells, without distinction between CD4⁺ and CD8⁺ cells. The Th2 hypothesis in allergy indicates the existence of a functional dichotomy within the CD4⁺ T cell population. However, it has been documented that also CD8⁺ T cells [17] and even NK cells [18] may be divided into two subsets according to the dominating profile of their secreted cytokines.

In some studies, cytokine production by different T cell subpopulations was assessed in other allergic disorders by

means of similar methods. Some analyses were limited to CD3⁺ cells, but in others the separate analysis of CD4⁺ and CD8⁺ cells was performed. Results of some studies provide a rationale for estimation of only CD3⁺ T cells. For example, Cho et al. [19] found comparable frequencies of CD8⁺ T cells producing type 1 and type 2 cytokines and CD4⁺ T cells producing the same cytokines in atopic asthmatic patients. Nakazawa et al. [20] observed consistently higher intracellular production of IL-4 and lower production of IFN- γ both by CD4⁺ and CD8⁺ cells in atopic dermatitis patients as compared to healthy controls. However, they did not compare cytokine production by the two populations of T cells. Moreover, others observed that changes in percentages of CD4⁺ and CD8⁺ type 2-cytokine-producing T cells induced by treatment, e.g. by immunotherapy in asthmatic children, occurred in parallel [21].

On the contrary, some studies indicate functional differences between CD4⁺ and CD8⁺ T cells. A higher production of IL-4 and IL-5 by CD4⁺ T cells as compared to CD8⁺ T cells was observed in asthmatic patients [22]. However, in that study cytokine production was assessed in culture supernatants of purified cells isolated from peripheral blood by means of ELISA, not by flow cytometry. As a result of specific immunotherapy for mite-sensitive asthma the IFN- γ /IL-4 ratio in peripheral blood CD4⁺ T cells increased, but not in CD8⁺ T cells [23].

Antunez et al. found an increased percentage of CD4⁺ T cells producing IL-13, IL-4 and TNF- α in patients with acute atopic dermatitis, but not in patients in the chronic phase of the disease. In contrast, the cytokine production by CD8⁺ cells both in an acute and in a chronic phase of the disease was comparable to that of healthy subjects [24].

Consequently, further studies on intracellular production of cytokines by T cells in allergic disorders should comprise both CD4⁺ and CD8⁺ T cell populations, because the results of previous studies are equivocal. Moreover, the relative role of CD4⁺ and CD8⁺ cells producing type 1 and type 2 cytokines in intermittent allergic rhinitis needs further elucidation.

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