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Cytokines Released from Allergen-Stimulated Blood Cells during the Delayed Asthmatic Response to Allergen Challenge

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Abstract

Background: Bronchial asthma patients may develop various asthmatic response types to bronchial challenge with allergen, such as immediate (IAR), late (LAR), dual (DAR) or delayed (DYAR), due to different immunologic mechanisms. The DYAR, beginning between 26 - 32 hrs and lasting up to 56 hrs after the allergen challenge, differs from the IAR, LAR and DAR in clinical, diagnostic and immunologic aspects. The aim of this study was to investigate the concentrations of the particular intracellular cytokines released by blood cells stimulated with relevant allergens "*in vitro*", before and during the DYAR. Methods: In 23 patients, the repeated DYAR ($p < 0.001$) was supplemented with cytokine determination in the supernatants of the blood cells stimulated with relevant allergens before and up to 72 hours after the bronchial challenge, by means of enzyme-linked immunoassay. Results: The significantly elevated pre-challenge concentrations ($p < 0.05$) of IL-2, IL-17, IFN-γ and G-CSF released by allergen-stimulated blood cells "*in vitro*" were recorded in the DYAR patients as compared with healthy controls. The significantly increased post-challenge concentrations ($p < 0.05$) of IL-1β, IL-2, IL-8, IL-12p70, IL-18, IFN-γ and TNF-α, whereas decreased concentrations of IL-4, IL-6 and IL-17, were released by blood cells stimulated with relevant allergens "*in vitro*", as compared both with their pre-challenge concentrations and with the corresponding PBS control values. Conclusions: The profiles of cytokines released by allergen-stimulated peripheral blood cells during the DYAR would suggest an activation of Th_1 cells, neutrophils, monocytes and probably also bronchial macrophages, epithelial and endothelial cells and their involvement in the immunologic mechanism(s) underlying the clinical DYAR.

Keywords

Delayed Asthmatic Response; Cytokines; Allergen-Stimulated Peripheral Blood Cells

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1. Introduction

In allergic bronchial asthma various immunologic mechanisms can be involved [1]-[8]. The causal role of immediate hypersensitivity upon participation of IgE antibody, mast cells/basophils, eosinophils and Th2-lymphocytes, in this condition, has already been established [1]-[8]. Although, some evidence for participation of the other immunologic mechanisms has already been gathered, our knowledge of the involvement of the non-IgE mechanisms in the bronchial asthma remains still incomplete [1] [5]-[11]. Patients with bronchial asthma being challenged with allergens may develop different asthmatic response types, such as immediate (IAR), late (LAR) or dual (DAR) response, having been already studied from various points of view [3] [5] [7]-[22]. Recently, we have described an asthmatic response appearing between 26 - 56 hours after the bronchial challenge with various inhalant allergens, designated as a "delayed asthmatic response" (DYAR) [23] [24]. This response type exhibited different clinical, immunologic and pharmacologic features from the IAR, LAR and DAR [12] [13] [23] [24]. The purpose of this study, was to investigate: 1) the cytokine profiles released by peripheral blood cells of patients developing the DYAR after the "*in vitro*" stimulation with relevant allergens; 2) the possible changes in the particular cytokines released bythe "*in vitro*" stimulated blood cells with allergen during the DYAR; 3) the activation degree of particular blood cell types during the DYAR.

2. Material and Methods

2.1. Patients

Twenty-three asthmatics examined at our Department of Allergology & Immunology, Inst. Med. Sci. "De Klokkenberg", Breda, The Netherlands during a period 1998-1999 and demonstrating DYAR after the bronchial allergen challenge (**Figure 1**) volunteered to participate in this study. These patients, 20 - 51 years of age, suffered from reversible bronchial constriction alternating with symptom-free periods, but without any restrictive changes of their pulmonary function (**Table 1**). They did not use oral corticosteroids or immunotherapy and had no airway infections. They were examined by routine diagnostic procedure, serving also as inclusion-exclusion criteria, including various diagnostic parameters (**Table 1**), among others also 47 bronchial provocation tests with inhalant allergens (BPT) (**Table 2**) and 23 phosphate buffered saline (PBS) control challenges. All BPTs were performed in a period without manifest bronchial complaints, outside the allergen-relevant season and during hospitalization. Inhalation corticosteroids (n = 9) and long-acting β2-sympathomimetics (n = 5) were

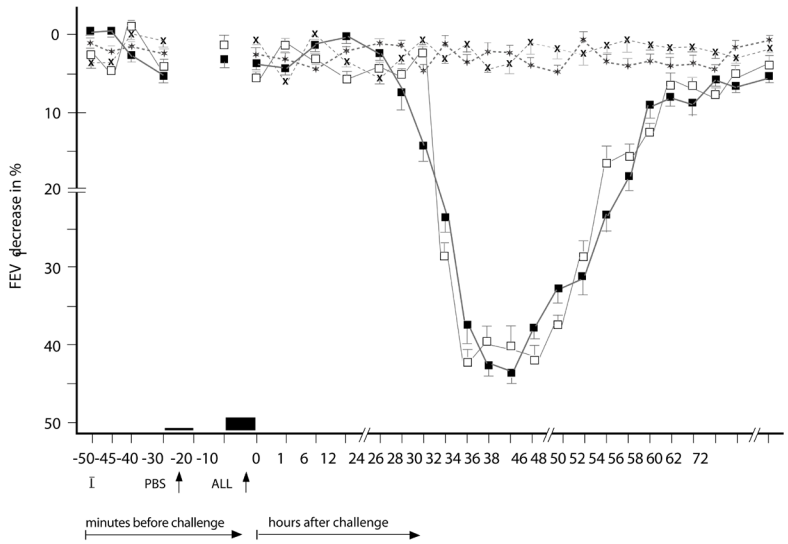


Figure 1. Delayed asthmatic response to allergen challenge (DYAR) and phosphate buffered saline (PBS) control challenge. The mean percentage changes in the FEV_1 values calculated from 23 DYARs and 23 PBS control challenges; (■) = the initial DYAR; (□) = the repeated DYAR; (*) = the initial PBS; (x) = the repeated PBS; I = initial (baseline) values; ALL = allergen challenge; PBS = phosphate buffered saline; Bars = means ± SEM.

Table 1. Characteristics of the patients and control subjects.

		Control subjects			
	Patients	Patients with asthma			Healthy subjects
	DYAR n = 23	IAR n = 24	LAR n = 16	DAR n = 17	n = 25
Age (years)	27 ± 8	30 ± 4	29 ± 6	28 ± 4	30 ± 7
Gender (M/F)	11/12	10/14	7/9	9/8	12/13
Disease history (years)	4.8 ± 2.2	4.5 ± 2.7	5.1 ± 2.1	4.8 ± 2.9	0
Asthmatic attacks per month	3 ± 1	4 ± 1	3 ± 2	3 ± 2	0
FEV 1 (% predicted)	95.2 ± 6.1	97.5 ± 6.3	97.2 ± 3.5	99.2 ± 3.1	104.3 ± 2.5
FVC (% predicted)	98.7 ± 4.1	98.3 ± 3.3	99.3 ± 2.0	98.2 ± 4.4	103.4 ± 5.3
Blood leukocyte count ($\times 10^9$/L)$^{\circ}$	10.1 ± 0.9^{+}	6.3 ± 1.1	8.0 ± 1.7	8.2 ± 0.7	7.1 ± 0.5
Blood eosinophil count ($\times 10^6$/L)$^{\circ\circ}$	373 ± 45	569 ± 71*	544 ± 35*	551 ± 53*	265 ± 24
Blood thrombocyte count ($\times 10^9$/L)$^{\circ\circ\circ}$	324 ± 58	311 ± 24	346 ± 35	279 ± 39	306 ± 28
Bronchial histamine threshold$^{\square}$					
≤2.0 mg/mL	2	3	2	3	0
4.0 mg/mL	1	4	1	2	0
8.0 mg/mL	2	6	5	3	0
16.0 mg/mL	7	3	3	4	0
32.0 mg/mL	5	5	3	3	1
>32.0 mg/mL	6	3	2	2	24
Positive skin response (i.c.)					
Immediate	8	21	5	10	0
Late	2	3	11	7	0
Delayed	13	0	0	0	0
Increased total IgE in serum$^{\blacksquare}$	0	6	3	7	0
Positive specific IgE in serum$^{\blacksquare\blacksquare}$	0	15	10	11	0
Increased total IgG in serum$^{\blacksquare\blacksquare\blacksquare}$	0	0	9	8	0
Increased IgG sub-classes in serum$^{\Delta}$					
IgG_1	0	0	2	1	0
IgG_2	0	0	0	1	0
IgG_3	0	0	3	2	0
IgG_4	0	0	5	2	0
Increased total IgM in serum$^{\Delta\Delta}$	0	0	0	0	0
Increased total IgA in serum$^{\Delta\Delta\Delta}$	0	0	0	0	0
Ratio Th_1 /Th_2 (%) in blood$^{\blacktriangle}$	8.5 ± 2.1^{+}	6.4 ± 2.2	6.6 ± 2.0	6.8 ± 2.4	7.0 ± 2.3
IFN-γ (pg/mL)-PBMC$^{\blacktriangle\blacktriangle}$	377 ± 45^{+}	219 ± 30	236 ± 42	198 ± 51	232 ± 46
IL-4 (pg/mL)-PBMC$^{\blacktriangle\blacktriangle}$	17.1 ± 6.3	23.5 ± 4.0^{+}	21.6 ± 4.0	24.4 ± 2.5^{+}	16.8 ± 3.0

DYAR = delayed asthmatic response; IAR = immediate asthmatic response; LAR= late asthmatic response; DAR = dual late asthmatic response; Values = mean ± SD; Statistical significance as compared with healthy control subjects =$^{+}$ $p \leq 0.05$, * $p < 0.05$; normal value = 4.0 - 10 × 10^9/L; $^{\circ\circ}$normal value = <300 × 10^6/L; $^{\circ\circ\circ}$normal value= 150 - 400 × 10^9/L; $^{\square}$= normal value< 32.0 mg/mL(according to the European and Dutch criteria) [54] [55]; (i.c.) = intracutaneous tests; $^{\blacksquare}$Total IgE in the serum (PRIST)-normal value ≤ 500 IU/mL; $^{\blacksquare\blacksquare}$Positive specific IgE in the serum (RAST) ≥ 0.70 U/mL (= more than class 1); $^{\blacksquare\blacksquare\blacksquare}$Total IgG in the serum (Single radial immuno-diffusion =Mancini technique and ELISA)-normal value ≤ 15.0 g/L; $^{\Delta}$Normal values: IgG_1 < 5.0 g/L; IgG_2 < 2.6 g/L; IgG_3 < 0.4 g/L; IgG_4 < 0.5 g/L; $^{\Delta\Delta}$ Normal values: IgM ≤ 3.8 g/L; $^{\Delta\Delta\Delta}$Normal values: IgA ≤ 4.0 g/L; $^{\blacktriangle}$= measured by flow-cytometry after stimulation with PMA(phorbol 12-myristate 13-acetate) [24]; $^{\blacktriangle\blacktriangle}$= measured by ELISA in supernatants of PMA-stimulated peripheral blood mononuclear cells [24]; PBMC = peripheral blood mononuclear cells.

withdrawn 4 weeks, cromolyn (n = 3) and nedocromil sodium (n = 5) 2 weeks and other treatments 48 hours prior the BPTs. Post-challenge FEV1 decrease by 50% or more with respect to the pre-challenge values (n = 3) was treated with a single dose of 200 - 400 mcg Salbutamol aerosol. In all study participants a single determination of cytokines released from peripheral blood cells after an "*in vitro*" stimulation with corresponding allergens was performed (**Table 2**). In the DYAR patients the BPTs and PBS controls were repeated 2 - 6 weeks later (**Figure 1**) and supplemented with recording of cytokines released by isolated peripheral blood cells after the "*in vitro*" stimulation with relevant allergens before and at 1, 12, 24, 36, 48, 56 and 72 hours after the challenge. The local ethical committee approved this study and an informed consent was obtained from all participants.

2.2. Control Subjects

The 24 patients demonstrating an IAR, 16 developing LAR, 17 showing DAR and 25 healthy subjects volunteered to participate as control subjects (**Tables 1-3**).

2.3. Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) diluted in PBS were used in concentrations of 100 - 500 BU/mL for skin tests and 1000 - 3000 BU/mL for BPTs (**Table 3**). The concentrations recommended by the manufacturer were 500 BU/mL for skin tests and 5000 BU/mL for the BPTs [24].

2.4. Skin Tests

Skin prick tests (SPT) with allergenic extracts in concentrations of 500 BU/mL were evaluated after 20 minutes. If they were negative, intracutaneous tests in concentration of 100 BU/mL and then 500 BU/mL were performed and evaluated 20 minutes, 6, 12, 24, 36, 48, 72 and 96 hours after the intradermal injection. A skin wheal (>7.0 mm in diameter) occurring after 20 minutes was qualified as immediate skin response, skin infiltration appearing between 6 - 12 hours as a late skin response and skin induration observed later than 48 hours was considered a delayed skin response [24].

Table 2. Allergens caused particular types of asthmatic response.

Allergen	Concentration BU/mL	DYAR n = 23	IAR n = 14	LAR n = 16	DAR n = 17	Healthy subjects n = 25
Dermatophagoides pteronyss	1000	6	9	3	5	0
Dermatophagoides farinae	1000	1	1	2	0	0
Animal danders						0
Dog	3000	1	2	1	1	0
Cat	1000	1	1	1	2	0
Horse	2000	0	0	1	0	0
Hamster	2000	1	0	1	0	0
Aspergillus fumigatus	1000	1	0	1	1	0
Pollen						0
Grass mix I	1000	5	4	3	3	0
Grass mix II	1000	2	3	1	0	0
Tree mix	3000	1	1	0	1	0
Weed mix	1000	1	1	0	1	0
Birch	1000	2	1	1	1	0
Poplar	2000	1	0	0	1	0
Ragweed giant	1000	0	1	1	1	0

BU/mL = biologic units per 1 mL Grass pollen mix I = *Dactylis glomerata*, *Lolium perenne*, *Phleum pratensis*, *Poa pratensis*; Grass pollen mix II = *Festuca pratensis*, *Holcus lanatus*, *Agrostis alba*, *Anthoxanthum odoratum*; Tree pollen mix = *Betula pendula*, *Corylus avellana*, *Juniperus communis*, *Salix alba*; Weed pollen mix = *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, *Taraxacum officinale.*

Table 3. Single determination of cytokine concentrations in the supernatants of peripheral blood cells stimulated "*in vitro*" by relevant allergens (pg/mL).

		Control subjects			
	Patients	Patients with asthma			Healthy subjects
	DYAR n = 23	IAR n = 24	LAR n = 16	DAR n = 17	n = 25
IL-1β	4.0 ± 1.1	5.5 ± 0.8	4.7 ± 0.3	6.0 ± 0.9	3.7 ± 0.6
IL-2	6.9 ± 0.4*	<3.0	3.1 ± 0.1	<3.0	<3.0
IL-3	<31.2	37.4 ± 0.9*	36.2 ± 2.3*	39.1 ± 0.7*	<31.2
IL-4	3.7 ± 1.0	24.6 ± 2.4*	9.1 ± 1.5^{+}	12.3 ± 1.0*	3.2 ± 0.5
IL-5	4.8 ± 0.7	11.0 ± 3.0*	13.5 ± 2.8*	12.6 ± 2.2*	<3.0
IL-6	5.0 ± 0.8	11.6 ± 1.8*	16.3 ± 2.0*	18.8 ± 3.3*	<4.0
IL-7	4.8 ± 0.5	5.2 ± 0.3	7.9 ± 1.1^{+}	5.5 ± 0.4	4.0 ± 0.5
IL-8	10.2 ± 2.1	9.5 ± 2.5	14.1 ± 1.9*	10.4 ± 3.2	8.9 ± 2.4
IL-10	7.7 ± 2.2	15.8 ± 1.4*	11.3 ± 2.0	12.9 ± 1.6	8.0 ± 1.3
IL-12p40	7.8 ± 0.6	19.3 ± 3.5*	16.7 ± 2.9*	15.8 ± 1.4^{+}	8.5 ± 2.2
IL-12p70	15.3 ± 1.6	17.8 ± 3.1^{+}	15.0 ± 1.0	14.8 ± 2.4	12.7 ± 3.1
IL-13	<3.0	5.0 ± 0.4	8.8 ± 2.0^{+}	6.7 ± 1.2	<3.0
IL-16	59.6 ± 2.4	55.4 ± 3.8	59.2 ± 2.9	54.0 ± 2.5	56.8 ± 3.1
IL-17	12.3 ± 3.0*	7.4 ± 2.0	6.8 ± 1.7	8.2 ± 1.3	6.6 ± 1.4
IL-18	20.1 ± 1.3	19.6 ± 3.8	20.5 ± 4.1	20.7 ± 3.0	18.1 ± 1.9
IFN-γ	397 ± 101*	176 ± 29	210 ± 35	235 ± 42	211 ± 25
GM-CSF	12.1 ± 0.7^{+}	6.7 ± 1.1	7.0 ± 0.4	7.3 ± 0.7	8.2 ± 0.5
G-CSF	8.1 ± 0.6*	3.9 ± 0.6	4.2 ± 0.2	4.1 ± 0.5	3.8 ± 0.9
TNF-α	12.4 ± 1.1	10.1 ± 2.7	11.3 ± 3.0	10.6 ± 2.5	9.2 ± 1.6
TGF-β	16.1 ± 1.3	21.3 ± 4.1	24.8 ± 3.7^{+}	23.0 ± 3.5^{+}	15.0 ± 1.7

DYAR = delayed asthmatic response; IAR = immediate/early asthmatic response; LAR = late asthmatic response; DAR = dual late asthmatic response; Values = mean ± SEM; Statistical significance as compared with healthy subject values: $^{+}$= $p \leq 0.05$, *= $p < 0.05$; $^{\Delta}$= blood cells of the healthy subjects were stimulated with: D. pteronyssinus, Grass mix I, Grass, mix II dog and cat allergens.

2.5. Bronchial Provocation Tests (BPT)

The BPTs were performed by means of spirometry (Spirograph D-75 Lode, Groningen, The Netherlands) recording the FVC and FEV1 values. The aerosolized allergen extracts and PBS were inhaled using the Wiebadener Doppel-Inhalator at an airflow of 10 L/min. The nebulizer output was 0.12 - 0.14 mL/min and the aerosol particles were of a median mass diameter of 2.8 - 3.6 µ. The BPTs, being a modification of the European standard [27], were performed by the following schedule: 1) initial (baseline) values recorded at 0, 5 and 10 minutes; 2) PBS control values recorded at 0, 5 and 10 minutes after a 10-minute PBS inhalation; 3) inhalation of allergen aerosol for 2 × 5 minutes, with inserted spirometric value measurement, followed by the recording of the FEV1 and FVC values at 0, 5, 10, 20, 30, 45, 60, 90 and 120 minutes and the every hour up to 12th hour and every second hour during the 22nd and 38th, the 46th and 62nd and 72nd hour interval [23]-[26]. The PBS control challenge was performed according the same schedule as that applied to the BPTs with allergens. A 5-day interval has always been inserted between the consecutive tests [23]-[26].

2.6. Determination of Cytokines

Samples of 6 mL heparinized peripheral blood were centrifuged at 1800 × g for 5 minutes at 4˚C and plasma aliquots were stored at −70˚C. The unseparated blood cells were washed in RPMI 1640 (Sigma-Aldrich, St.

Louis, USA), centrifuged at 2000 × g for 10 minutes at 4˚C and re-suspended in RPMI 1640 with penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (2 mmol/L) at a concentration of 5×10^6 leukocytes/mL. The cell viability was confirmed by trypan blue dye exclusion. The cell suspension was divided into 2 equal portions. The first portion stimulated with the allergen, identical to that causing the DYAR, in a concentration of 50 BU/mL and the second non-stimulated control portion were cultured for 24 hours at 37˚C under 5% CO_2 in a humidified incubator. The supernatants were collected by centrifugation at 2000 × g for 15 minutes and processed within 1 hour. The cytokines in the supernatants were determined by immunoassay (ELISA) kits, following the manufacturers' recommendations. All measurements were performed in duplicate. The detection limits in *pg/mL* are reported in parenthesis. The cytokines Il-1β (1.0), IL-2 (<3.0), IL-3 (31.2), IL-5 (3.0), IL-6 (4.0), IL-7 (0.1), IL-10 (<3.0),IL-13 (<3.0), IL-16 (31.2), GM-CSF (<3.0), G-CSF (0.8), TNF-α (6.0), TGF-β (6.0) were measured using the R & D System (Minneapolis/MN, USA) kits, IL-4 (0.6), IL-8 (1.3), IL-12p70 (2.1), IL-18 (9.2) and IFN-γ (1.0) by Bender MedSystems (Wien, Austria), IL-17 (<5.0) by BioSource International Inc (Camarillo, USA) and IL-12p40 (3.9) by Becton Dickinson (San Jose, USA) kits. The inter-assay as well as intra-assay coefficients of variations for these kits were less than 10%. The blood cells of the healthy controls were stimulated separately with D. pteronyssinus, Grass mix I + II, dog and cat allergens in concentrations of 50 BU/mL and the total mean cytokine values were then calculated.

2.7. Statistical Analysis

The initial and repeated DYAR and PBS controls were statistically analyzed by means of fitting polynomials to the mean curves over time; eight times points within 120 minutes and twenty-five times points up to 72 hours after the challenge. The hypotheses were tested by the generalized multivariate analysis of the variance model (MANOVA) [28].

The post-challenge cytokine values during the repeated DYAR and PBS controls in individual patients were compared with their pre-challenge values and evaluated by Wilcoxon matched-pair signed rank test. The mean post-challenge values of particular cytokines during the repeated DYARs were compared with the corresponding mean PBS values and evaluated by Mann-Whitney *U* test. A p value < 0.05 was considered to be statistically significant.

3. Results

The initial DYARs as well as the repeated DYARs, (**Figure 1**) were significantly positive both in the comparison of the post-challenge with the pre-challenge FEV1 values ($p < 0.001$, $p < 0.001$ respectively) and as compared with the PBS control values ($p < 0.001$, $p < 0.001$, respectively). No significant differences were found between the initial and the repeated DYAR ($p > 0.2$) nor between the DYAR appearance and the individual allergens ($p > 0.1$) (**Table 2**).

The DYAR was associated with decreased bronchial threshold in 73%, immediate skin response in 35% and delayed skin response in 57% (**Table 1**). The DYAR was accompanied by increased blood leukocyte, neutrophil, monocyte and lymphocyte counts, changed Th1/Th2 cell ratio in peripheral blood in favour of Th1 cells ($p < 0.01$) and increased intracellular concentration of IFN-γ ($p < 0.01$) but not of IL-4 ($p > 0.05$) (**Table 4**). The DYAR patients, as compared with healthy subjects, demonstrated increased concentrations of IL-2, IL-17, IFN-γ and G-CSF ($p < 0.05$) released by the allergen-stimulated blood cells "*in vitro*". The cytokine profiles of the DYAR patients differed also from those measured in the control patients demonstrating IAR, LAR and DAR (**Table 3**). The DYAR was associated with following post-challenge cytokine concentrations released by peripheral blood cells stimulated "*in vitro*" with relevant allergens, as compared both with their pre-challenge and with the corresponding PBS control values: (I). A slight increase ($p \leq 0.05$) in IL-13, G-CSF and a significant increase ($p < 0.05$) in IL-1β, IL-2, IL-8, IL-12p70, IL-18, IFN-γ, TNF-α; (II). A slight decrease ($p \leq 0.05$) in IL-5, GM-CSF, and a significant decrease ($p < 0.05$) in IL-4, IL-6, IL-17 (**Table 5**, **Figures 2(a)** and **(b)**). Most of these changes occurred 24 - 56 hours after the allergen challenge, which is the time of the maximal DYAR performance. No significant changes in the cytokine concentrations ($p > 0.1$) were found in supernatants during the PBS controls.

Control Subjects

The IAR, LAR or DAR control patients, as compared with healthy control subjects, demonstrated significant

Table 4. Differential count in peripheral blood and some cytokines in the serum during the DYAR and PBS.

Patients n = 23	Before the challenge	After the challenge (hrs)							
		1	6	12	24	36	48	56	72
Leukocytes[□]									
DYAR	8.9 ± 0.5	10.1 ± 0.8	10.5 ± 0.4	11.9 ± 0.9[+]	12.7 ± 0.5[*]	13.5 ± 0.6[*]	12.9 ± 0.7[*]	11.6 ± 0.3[+]	10.0 ± 0.5
PBS	8.3 ± 0.4	7.9 ± 0.7	7.8 ± 1.0	8.2 ± 0.9	7.4 ± 0.5	8.4 ± 1.0	8.0 ± 0.6	7.9 ± 0.5	8.1 ± 0.4
Eosinophils[□□]									
DYAR	293 ± 27	332 ± 35	318 ± 24	343 ± 30	281 ± 29	272 ± 27	301 ± 30	247 ± 25	288 ± 34
PBS	285 ± 21	309 ± 45	293 ± 26	268 ± 22	311 ± 47	256 ± 22	295 ± 29	287 ± 34	276 ± 23
Neutrophils[□□□]									
DYAR	6.4 ± 0.3	6.7 ± 0.9	6.6 ± 0.5	10.1 ± 0.8[*]	10.5 ± 0.7[*]	9.4 ± 1.1[*]	8.7 ± 0.4[*]	7.1 ± 0.3	6.2 ± 0.6
PBS	5.9 ± 1.1	6.0 ± 0.8	6.9 ± 0.4	6.1 ± 0.5	6.5 ± 0.9	7.1 ± 0.4	6.8 ± 0.7	6.2 ± 0.5	6.4 ± 0.3
Thrombocytes[○]									
DYAR	343 ± 41	320 ± 25	329 ± 23	347 ± 40	368 ± 34	415 ± 53	386 ± 31	319 ± 27	333 ± 29
PBS	298 ± 17	336 ± 37	323 ± 25	299 ± 16	354 ± 27	318 ± 21	349 ± 38	301 ± 20	285 ± 26
Differential count[○○]									
Eosinophils									
DYAR	0.41	0.52	0.40	0.58	0.39	0.53	0.36	0.38	0.29
PBS	0.34	0.37	0.39	0.34	0.26	0.43	0.25	0.26	0.19
Neutrophils									
DYAR	6.4	6.8	6.5	8.5[*]	9.4[*]	9.8[*]	9.1[*]	8.9[*]	7.0
PBS	6.0	6.3	6.7	5.5	6.6	6.9	5.8	7.1	5.9
Basophils									
DYAR	0.06	0.09	0.10	0.05	0.10	0.05	0.09	0.06	0.15
PBS	0.07	0.03	0.05	0.10	0.08	0.09	0.04	0.09	0.05
Monocytes									
DYAR	0.55	0.6	1. 0	1.9[*]	1.8[*]	1.7[*]	1.1[+]	0.5	0.6
PBS	0.35	0.2	0.7	0.6	0.5	0.35	0.45	0.25	0.5
Lymphocytes									
DYAR	2.8	3.1	3.8[+]	3.5	4.7[*]	4.8[*]	3.9[+]	2.5	2.4
PBS	2.4	2.2	3.4	3.7	2.9	3.0	2.6	2.3	2.5
Th_1/Th_2 ratio (%)[Δ]									
DYAR	8.0 ± 1.5	8.6 ± 2.1	9.9 ± 3.0[+]	14.7 ± 4.0[*]	16.2 ± 3.5[*]	17.1 ± 3.0[*]	16.4 ± 3.4[*]	15.6 ± 4.5[*]	9.1 ± 2.2
PBS	7.3 ± 3.1	8.2 ± 3.0	9.4 ± 2.1	7.5 ± 3.4	8.8 ± 3.7	9.0 ± 4.1	9.3 ± 3.1	8.9 ± 4.2	7.6 ± 2.6
IFN-γ (pg/mL)									
DYAR	2.4 ± 0.5	2.7 ± 1.0	4.9 ± 0.4[+]	9.2 ± 1.7[*]	9.0 ± 3.1[*]	8.8 ± 3.2[*]	7.1 ± 1.3[*]	3.1 ± 1.0	2.9 ± 0.3
PBS	2.1 ± 0.2	1.9 ± 0.8	2.2 ± 0.6	1.9 ± 0.8	2.4 ± 0.5	2.2 ± 1.0	2.5 ± 1.0	2.4 ± 0.5	2.3 ± 0.2
IL-4 (pg/mL)									
DYAR	3.0 ± 0.1	3.2 ± 0.1	3.5 ± 0.4	3.7 ± 0.6	3.2 ± 0.4	3.4 ± 0.3	3.5 ± 0.5	3.3 ± 0.5	3.5 ± 0.4
PBS	3.1 ± 0.2	3.0 ± 0.2	3.3 ± 0.1	3.4 ± 0.5	3.5 ± 0.2	3.0 ± 0.2	3.4 ± 0.7	3.5 ± 0.6	3.2 ± 0.1

DYAR = Delayed asthmatic response; PBS=Phosphate buffered saline (control); Values = mean ± SEM; Statistical significance as compared with healthy subject values: [+]= $p \leq 0.05$, [*]= $p < 0.05$, Automated counts: [□]= normal value 4.0 - 10.0 × 10^9/L; [□□]= normal value <300 × 10^6/L; [□□□]= normal value 2.0 - 7.2 × 10^9/L; [○]= normal value 150 - 400 × 10^9/L; Manual counts: [○○]=normal values (×10^9/L): E < 0.50; N = 2.0 - 7.2; B < 0.2; M = 0.2 - 1.0; L = 1.0 - 4.0; [Δ]= measured by flow-cytometry after stimulation with PMA (Phorbol 12-myristate 12-acetate).

Table 5. Cytokine released by peripheral blood cells (PMN and PBMC) stimulated "*in vitro*" with relevant allergens (means ± SEM) during the DYAR and PBS controls.

Patients n = 23	Before the challenge	After the challenge (hrs)						
		1	12	24	36	48	56	72
IL-1*β* (pg/mL)								
DYAR	4.1 ± 1.1	5.0 ± 0.7	6.8 ± 1.5*	7.0 ± 0.6*	6.9 ± 0.5+	5.0 ± 0.2	5.1 ± 1.0	4.6 ± 0.7
PBS	2.9 ± 0.8	4.1 ± 0.8	4.2 ± 0.3	3.9 ± 0.5	3.4 ± 1.0	3.0 ± 0.5	3.5 ± 0.4	3.3 ± 0.2
IL-2 (pg/mL)								
DYAR	6.9 ± 0.4	5.2 ± 0.1	9.6 ± 1.0*	11.3 ± 0.4*	11.8 ± 1.7*	10.4 ± 0.6*	8.9 ± 0.2*	<3.0
PBS	4.8 ± 0.8	5.0 ± 0.9	7.1 ± 0.4	6.0 ± 0.3	4.8 ± 0.6	5.3 ± 0.2	4.9 ± 0.4	4.5 ± 0.4
IL-3 (pg/mL)								
DYAR	<31.2	<31.2	<31.2	32.1 ± 0.4	<31.2	<31.2	<31.2	<31.2
PBS	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2
IL-4 (pg/mL)								
DYAR	3.7 ± 1.0	5.0 ± 0.7	4.1 ± 0.9	2.0 ± 0.3	<0.6*	<0.6*	<0.6*	3.3 ± 0.2
PBS	2.9 ± 0.3	2.7 ± 1.0	3.6 ± 0.8	3.4 ± 0.5	3.7 ± 0.6	2.2 ± 0.3	2.6 ± 0.8	2.5 ± 1.0
IL-5 (pg/mL)								
DYAR	6.9 ± 1.0	6.7 ± 0.8	6.5 ± 1.3	5.0 ± 0.4+	5.1 ± 0.7+	6.3 ± 1.1	7.0 ± 1.0	6.4 ± 0.9
PBS	6.5 ± 0.3	7.5 ± 1.4	7.0 ± 0.7	7.0 ± 1.1	7.2 ± 0.6	7.1 ± 0.4	6.3 ± 0.5	6.6 ± 1.0
IL-6 (pg/mL)								
DYAR	5.0 ± 0.8	5.8 ± 0.6	8.1 ± 1.1*	4.6 ± 0.4	<4.0*	<4.0*	4.5 ± 0.4	5.0 ± 0.5
PBS	5.1 ± 0.5	5.1 ± 1.0	5.2 ± 0.6	5.9 ± 1.3	5.0 ± 1.0	5.4 ± 0.3	4.7 ± 0.5	4.5 ± 0.4
IL-7 (pg/mL)								
DYAR	4.8 ± 0.5	5.7 ± 1.0	6.2 ± 0.8	6.5 ± 0.4	5.9 ± 1.0	6.6 ± 0.7	6.3 ± 0.4	5.8 ± 0.3
PBS	4.4 ± 0.4	4.9 ± 0.7	5.5 ± 0.9	4.8 ± 0.9	6.0 ± 1.0	6.3 ± 0.8	4.6 ± 0.5	5.3 ± 0.6
IL-8 (pg/mL)								
DYAR	11.2 ± 2.1	13.6 ± 1.8	15.5 ± 1.0+	16.9 ± 1.6*	17.0 ± 1.4*	15.8 ± 1.0*	14.9 ± 1.1+	12.8 ± 1.1
PBS	10.5 ± 0.4	11.5 ± 0.6	12.4 ± 0.5	12.6 ± 1.0	13.0 ± 1.0	12.5 ± 0.7	11.5 ± 2.0	10.2 ± 1.0
IL-10 (pg/mL)								
DYAR	10.9 ± 2.2	10.3 ± 0.5	11.5 ± 0.8	11.2 ± 0.3	11.4 ± 1.3	11.7 ± 0.5	11.0 ± 1.0	10.3 ± 0.4
PBS	9.5 ± 0.6	10.0 ± 0.3	9.8 ± 0.6	10.9 ± 1.1	11.0 ± 0.7	10.8 ± 0.6	9.7 ± 0.3	10.0 ± 0.7
IL-12p40 (pg/mL)								
DYAR	11.7 ± 0.6	12.3 ± 1.1	12.6 ± 2.0	12.6 ± 0.9	11.5 ± 1.2	11.9 ± 0.7	11.6 ± 1.0	12.2 ± 0. 6
PBS	11.0 ± 1.4	12.0 ± 2.0	12.2 ± 1.0	11.7 ± 0.7	12.1 ± 2.0	11.5 ± 0.5	11.9 ± 0.8	11.4 ± 1.1
IL-12p70 (pg/mL)								
DYAR	15.3 ± 1.6	15.7 ± 1.1	19.4 ± 2.1*	23.5 ± 2.5*	24.4 ± 2.6*	20.1 ± 3.0*	22.3 ± 1.8*	16.0 ± 1.0
PBS	15.6 ± 1.4	15.0 ± 1.0	16.3 ± 1.2	16.0 ± 0.6	17.6 ± 1.4	17.1 ± 0.8	16.5 ± 1.0	17.0 ± 1.4

Continued

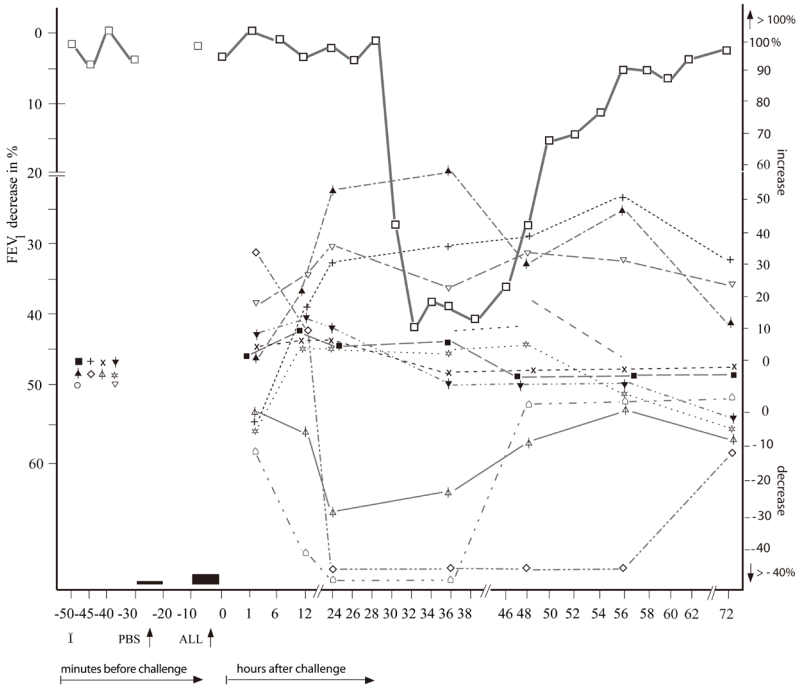
IL-13 (pg/mL)								
DYAR	5.7 ± 0.5	6.1 ± 0.8	7.2 ± 0.6^{+}	7.1 ± 0.4^{+}	6.3 ± 1.0	6.2 ± 1.0	5.5 ± 0.4	5.8 ± 0.6
PBS	5.0 ± 0.7	5.5 ± 1.0	7.3 ± 0.8	7.3 ± 1.0	6.6 ± 1.3	6.1 ± 1.2	5.9 ± 0.7	5.6 ± 0.4
IL-16 (pg/mL)								
DYAR	59.6 ± 2.4	66.3 ± 3.7	68.0 ± 3.2	65.0 ± 2.9	58.6 ± 4.5	61.4 ± 3.1	58.5 ± 2.7	57.0 ± 2.5
PBS	60.7 ± 4.1	56.5 ± 3.0	57.7 ± 2.8	60.2 ± 3.3	59.9 ± 4.3	55.4 ± 3.8	61.2 ± 3.1	58.2 ± 2.9
IL-17 (pg/mL)								
DYAR	12.3 ± 3.0	11.6 ± 2.8	7.4 ± 3.9^{+}	<5.0*	<5.0*	12.6 ± 1.5	12.9 ± 2.2	12.4 ± 2.1
PBS	12.0 ± 2.3	12.5 ± 3.1	11.4 ± 2.5	10.9 ± 2.2	11.7 ± 2.8	10.0 ± 2.6	11.5 ± 2.9	11.2 ± 1.5
IL-18 (pg/mL)								
DYAR	20.1 ± 1.3	19.6 ± 1.0	23.4 ± 0.8	26.7 ± 1.5^{+}	27.5 ± 1.2*	28.0 ± 2.6*	30.1 ± 4.0*	26.3 ± 2.7
PBS	19.9 ± 0.6	20.0 ± 2.1	22.1 ± 2.0	21.8 ± 1.2	22.4 ± 0.9	21.9 ± 0.6	21.0 ± 0.4	20.2 ± 1.1
IFN-γ (pg/mL)								
DYAR	385 ± 101	403 ± 111	538 ± 107^{+}	591 ± 122*	593 ± 120*	513 ± 94*	489 ± 106*	414 ± 108^{+}
PBS	381 ± 97	377 ± 98	398 ± 90	345 ± 116	355 ± 121	358 ± 121	383 ± 95	374 ± 101
GM-CSF (pg/mL)								
DYAR	12.1 ± 0.7	13.6 ± 0.5	10.3 ± 1.0	9.8 ± 1.2^{+}	9.9 ± 2.0 $^{+}$	12.0 ± 0.9	12.5 ± 1.2	12.6 ± 0.7
PBS	11.4 ± 0.5	11.9 ± 0.8	12.5 ± 1.1	12.9 ± 1.0	11.8 ± 1.3	11.6 ± 0.5	11.8 ± 1.0	11.2 ± 0.9
G-CSF (pg/mL)								
DYAR	7.1 ± 0.6	8.4 ± 1.0	8.9 ± 0.7^{+}	9.3 ± 2.2^{+}	9.0 ± 1.6^{+}	8.6 ± 0.7	7.5 ± 1.2	7.8 ± 1.1
PBS	7.8 ± 0.5	7.0 ± 0.4	7.9 ± 0.5	8.6 ± 1.1	8.0 ± 0.3	8.2 ± 1.4	8.5 ± 0.9	7.7 ± 0.9
TNF-α (pg/mL)								
DYAR	12.4 ± 1.1	14.2 ± 0.9	17.8 ± 1.0*	18.5 ± 0.6*	18.8 ± 0.4*	19.0 ± 2.0*	17.7 ± 1.1*	16.1 ± 1.0^{+}
PBS	13.0 ± 0.6	13.9 ± 1.1	14.4 ± 1.0	15.0 ± 1.3	14.7 ± 1.3	14.0 ± 2.0	15.0 ± 1.0	14.2 ± 0.7
TGF-β (pg/mL)								
DYAR	17.9 ± 1.3	18.5 ± 2.2	19.7 ± 2.0	18.3 ± 1.0	19.1 ± 1.4	17.8 ± 1.5	18.4 ± 1.0	17.3 ± 0.5
PBS	18.3 ± 1.0	18.2 ± 0.6	19.5 ± 0.4	19.0 ± 1.0	20.2 ± 1.3	19.5 ± 0.7	18.9 ± 0.6	19.1 ± 0.4

DYAR = Delayed asthmatic response; PBS= Phosphate buffered saline (control); Values of cytokines = means ± SEM; Statistical significance of the cytokine concentrations as compared with their pre-challenge (baseline) values: $^{+}= p \leq 0.05$; $^{*}= p < 0.05$.

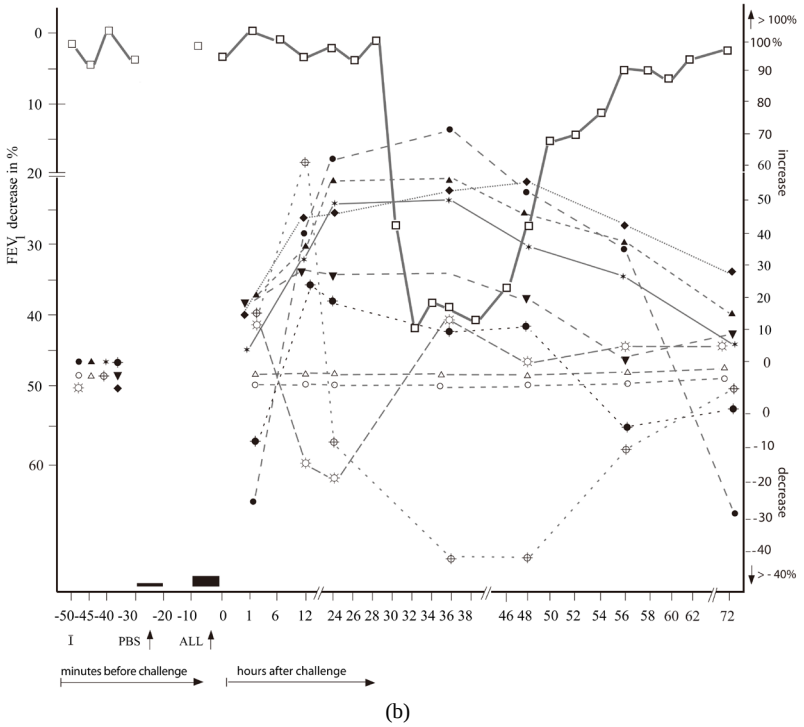
changes in the (pre-challenge) concentrations of various cytokines in the supernatants of the blood cells stimulated "*in vitro*" with relevant allergens, differing also from concentrations measured in the DYAR patients (**Table 3**).

4. Discussion

The DYAR differs from IAR, LAR and DAR in clinical course and accompanying features, such as cellular changes and different profiles of eicosanoids, adhesion molecules and cytokines in peripheral blood [1] [3]-[8] [12]-[15] [20] [23]-[26]. These differences suggest involvement of different immunologic mechanisms in the particular asthmatic response types. Cytokines expressing manifold biologic effects, including those on cellular signal transmission, stimulation, inhibition, chemotaxis and cell migration, transmembrane metabolism and



(a)



(b)

Figure 2. The repeated delayed asthmatic response to allergen challenge (DYAR) (□—□) and the mean percentage changes in the concentrations of particular cytokines released from isolated blood cells stimulated with allergen "*in vitro*". (a) Δ = IL-3; ◇ = IL-4; ▲ =IL-5; ✲ = IL-10; **x** = IL-12p40; ▲ = IL-12p70; ▼ = IL-16; ⌂ = IL-17; + = IL-18; ■ = TGF-*β*; (b) ○ = IL-1*β*; ● = IL-2; ⊕ = IL-6; ▽ = IL-7; ▲ = IL-8; ● =IL-13; ✲ = IFN-*γ*; ◆ =TNF-*α*; ☼ = GM-CSF; ▼= G-CSF; I = Initial (baseline) value; ALL = Allergen challenge; PBS= Phosphate buffered saline.

maturation of various cell types, represent important parts of the immunologic mechanisms [1] [4] [6] [9] [14] [16] [22] [29] [30]. Their role in the immunologic mechanisms underlying the allergic bronchial asthma has already been investigated from various points of view [4] [6] [8]-[11] [14]-[20] [24] [25] [29]-[52].

In most of these studies, a single measurement of cytokines in bronchoalveolar lavage fluid (BAL), (induced) sputum or in the peripheral blood was performed [9]-[11] [17] [19] [32]-[34] [38] [40] [42] [43] [45]-[49] [51] [52]. Studies dealing with the cytokine determination after the allergen challenge, especially during the IAR, LAR or DAR, are not numerous [5] [6] [14] [15] [18] [20] [30] [31] [39] [41] [50]. The concentration changes of particular cytokines in asthma patients reported in the literature display relatively high variation. This result diversity is probably caused by factors related either to the investigated patient populations, such as different asthma phenotypes, extent of the diagnostic procedures and selection criteria or to the differences in the methods, such as medium studied, technique and timing of the material collection, bronchial provocation technique (inhalational, intra-bronchial, segmental challenge), and the processing of collected material [2] [5]-[8] [14]-[20] [22] [24] [29] [30] [32] [38] [40] [41] [45] [52]. Usually, the concentrations of cytokines released by the circulating or airway-related cells into the peripheral blood by "*in vivo*" inhaled allergens are lower than the cytokine amounts released by "*in vitro*" stimulation of the isolated cells with various agents/factors [8] [15] [20]. The "*in vitro*" stimulation of the isolated blood or the airway-related cells, can be performed with non-specific agents, e.g. PHA , PMA, fMLP, LPS [9] [10] [17] [18] [32] [48], bacterial enterotoxin, other cytokines [6] [34]-[37] [39] [44] [46], or with allergens [6] [9] [33] [34] [38] [40] [42] [43] [45]-[49] [51] [52].

Of the "*in vitro*" techniques, the stimulation of isolated cells with allergens produces relatively limited cytokine amounts, stimulation with cytokines can release larger cytokine amounts, and the stimulation with non-specific agents generates the largest cytokines amounts [11] [24] [45] [46] [48] [49]. The differences in the released cytokine amounts can be explained by the mode of action of the stimulating agents. The stimulation with the non-specific, but very powerful, stimulating agents results in a direct release of the almost complete intracellular cytokine potential, including even those portions which would not be released upon natural allergen inhalation. In contrast, stimulation with specific allergens results in a selective release of a part of the total intracellular cytokine capacity only. Moreover, the stimulation of the isolated cells with allergen lacks a number of factors and mechanisms involved in the "*in vivo*" process, such as antigen presentation by the antigen presenting cells, synergic and antagonistic effects of other cells and factors in the bronchial tissue as well as in the peripheral blood, and concomitant effects of other cytokines and chemokines [1] [4] [6] [8] [14] [18] [21] [34].

The measurement of the cytokines released by the allergen-stimulated blood cells "*in vitro*" is important for the evaluation of the ratio of particular cytokines released by allergen stimulation with respect to their total intracellular capacity released by their stimulation with non-specific agents, and should be interpreted as a model for studying of these processes. However, the cytokine determination in the natural media, such as blood or sputum, related to the allergen inhaled during the bronchial challenge would be more representative for the genuine processes involved in the clinical bronchial asthma. This fact may be supported by our , not yet published, results of higher cytokine concentrations released after the "*in vitro*" stimulation with PMA than cytokine amounts recorded in plasma after the bronchial allergen challenge as well as by similar findings of other investigators [40] [42] [45] [49]. Unfortunately, the discrepancy between the cytokine amounts released by the "*in vivo*" inhaled allergen and those released after the "*in vitro*" stimulation of the isolated BAL or peripheral blood cells with non-specific agents during particular asthmatic response types, has not yet been sufficiently investigated. Moreover, the cytokine profiles in the peripheral blood, BAL fluid or sputum as well as after stimulation of isolated blood cells with various agents, including allergens, in healthy subjects, which would served as reference data, have as yet been insufficiently investigated [9] [29] [32] [47] [49].

In majority of the papers, studying the cytokines in bronchial asthma patients, the single cytokine determination was performed [9] [10] [17] [33] [34] [38] [40] [42] [43] [45]-[49] [51] [52]. However, our data (**Table 5**) show that the significant changes of the cytokine concentrations were measured till 24 - 56 hours after the allergen challenge. This fact would emphasize the importance of cytokine measuring before and repeatedly after the bronchial allergen challenge, being the only method capable of following the dynamic course of the cytokine changes related to the allergen exposure [3] [5] [14] [15] [18] [20] [23]-[26] [30] [31] [39] [41] [50]. Results of this study, consistent with our previous findings [23]-[26] [53], suggest that immunologic mechanisms underlying the DYAR differ from those involved both in IAR and in LAR. The pre-challenge serum cytokine levels recorded in the DYAR patients [53] as well as those released from the allergen-stimulated blood cells "*in vitro*" (**Table 3**) differed from those measured in the IAR, LAR or DAR patients. The "*in vitro*" allergen-stimulated

blood cells released increased amount of IL-1β, IL-2, IL-8, IL-12p70, IL-13, IL-18, IFN-γ and TNF-α, while decreased amounts of IL-4, IL-6 and IL-17 (**Table 5**). These cytokine profiles differed from those recorded in the serum during the DYAR [53]. These differences may probably lie in various, above mentioned, factors and mechanisms participating in the cytokine release "*in vivo*", but lacking in the "*in vitro*" processes. An interesting aspect was a prevalence of changes of pro-inflammatory cytokines.

The post-challenge cytokine profiles released by the allergen-stimulated blood cells during the DYAR differed from other investigator findings reporting increased release of IL-1β, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-16, IFN-γ, or TFN-α by blood mononuclear cells of asthmatics due to the stimulation with inhalant allergens. However, in most of these studies, only a limited cytokine number has been measured and even without any relation to a particular asthmatic response type [11] [33] [34] [38] [45]-[49]. The cytokine profiles reported in this study, together with our previous results [23]-[26] [53], especially with the significant changes in the Th1/Th2 ratio in peripheral blood in favour of Th1 cells, increased intracellular concentrations of IFN-γ and IL-2, but not those of IL-4 or IL-5, increased blood leukocyte, neutrophil and monocyte, but not eosinophil, counts, increased plasma levels of of LTB4 and MPO and serum concentration changes of various soluble adhesion molecules would suggest involvement of the cell-mediated hypersensitivity upon participation of Th1 lymphocytes, neutrophils, monocytes, probably also macrophages, NK cells, epithelial and endothelial cells, in the DYAR. Nevertheless, more concurrent investigations should be performed to clarify the immunologic mechanisms underlying the DYAR.

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