Delayed Type of Asthmatic Response to Allergen Challenge and Cytokines in the Peripheral Blood

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Key Words

Allergic bronchial asthma • Delayed asthmatic response • Cytokines • Peripheral blood

Abstract

Background: Patients with allergic bronchial asthma can develop various types of asthmatic response to bronchial challenge with allergens, such as immediate (IAR), late (LAR) or delayed (DYAR) response, due to different immunological mechanisms. **Objective:** The DYAR, beginning within 26–32 h after the challenge, reaching its maximum between 32 and 48 h and resolving within 56 h ($p < 0.001$), differs from IAR and LAR regarding the clinical features, diagnostic and immunological parameters. **Methods:** The repeated DYAR ($p < 0.001$) in 28 patients was supplemented with recording of cytokine concentrations in the serum before and up to 72 h after the bronchial challenge by means of enzyme-linked immunoassay. **Results:** The DYAR was accompanied by a significant increase in the serum concentration ($p < 0.05$) of IL-2 at 24, 36 and 48 h; IL-10 at 12, 24, 36 and 48 h; IL-18 at 12 and 24 h; IFN- γ at 24, 36, 48 and 56 h; G-CSF at 1 and 12 h; TNF- α at 12, 36 and 48 h and TGF- β at 12 and 36 h, and a significant decrease in the concentration ($p < 0.05$) of IL-7 at 36 and 48 h and IL-12p70 at 12 h, as compared both with the prechallenge and with PBS control values. No significant changes in the serum cytokines were recorded during the PBS controls ($p > 0.2$). **Conclusions:** These results support the evidence for an active involvement of the Th₁ cells, neutrophils, monocytes, macrophages, epithelial cells and endothelial cells, upon cooperation of other cell types, in the immunological mechanism(s) underlying the DYAR.

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Introduction

Allergic bronchial asthma can be qualified as a multifactorial disorder, where various immunological mechanisms may play a causal role [1–3]. Besides the already established immediate hypersensitivity upon participation of IgE antibodies, mast cells/basophils, eosinophils and Th₂ lymphocytes, other hypersensitivity mechanisms can also be involved in this condition [1–11]. However, our knowledge of the role of the other mechanisms in this disorder is still relatively limited [1–3, 6, 11–14].

Patients with bronchial asthma can respond to bronchial allergen challenge with various types of asthmatic response, such as immediate (IAR), late (LAR) or dual late (DLAR) response. The IAR, LAR and DLAR have already been investigated extensively from various clinical, immunological and pharmacological points of view [2, 3, 5, 7, 8, 10, 11, 13–26]. In some of the bronchial asthma patients examined at our clinic, an unusual asthmatic response appearing 26–56 h after the bronchial challenge with various inhalant allergen was recorded [27–29]. This response, displaying clinical and immunological features different from those of the IAR and the LAR, was designated as a ‘delayed asthmatic response’ (DYAR) [27–29].

The purpose of this study was to search the possible changes in concentrations of particular cytokines in peripheral blood (PB) during the DYAR and hereby assess the possible involvement of individual cell types in this type of response.

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Fig. 1. Mean percentage changes in the FEV₁ values calculated from 28 DYARs and 28 PBS control challenges. ALL = Allergen challenge; I = initial (baseline) values. Bars = Means $\pm$ SEM.

Patients and Methods

Patients

Twenty-eight bronchial asthma patients having been referred to our Department of Allergology and Immunology (Institute of Medical Sciences 'De Klokkenberg', Breda, The Netherlands) between 1999 and 2000 for more extensive diagnostic and therapeutic analysis and developing an unusual asthmatic response appearing later than 24 h after the bronchial challenge with allergen (fig. 1), volunteered to participate in this study.

These patients, with an age range of 20–45 years and suffering from reversible bronchial constriction alternating with symptom-free periods, showed pulmonary function without any restrictive changes (Global Initiative for Asthma) [30]. They had no airway infections and did not use oral corticosteroids or immunotherapy. They were examined by routine diagnostic procedure, serving also as an inclusion-exclusion criterium, consisting of a number of diagnostic parameters and including 56 bronchial provocation tests (BPTs) with allergens and 28 PBS control challenges (tables 1–4). An interval of at least 5 days was always inserted between the end of one routinely performed BPT and/or PBS challenge and the start of the next. All BPTs were performed in a period without manifest bronchial complaints, outside the allergen-relevant season and during hospitalization of the patients under standard and controlled conditions in an allergen-free environment.

Inhalation glucocorticosteroids ($n = 15$) and long-acting β_2 -sympathomimetics ($n = 18$) were withdrawn 4 weeks, disodium cromoglycate ($n = 5$), nedocromil sodium ($n = 7$), leukotriene antagonists ($n = 1$) and xanthine derivatives ($n = 1$) 2 weeks, while other drugs were withdrawn 48 h prior to the BPTs. If the FEV₁ or both FEV₁ and FVC values decreased after allergen challenge with 50% or more, with respect to the predicted values, the pa-

tients ($n = 2$) were treated with a single dose of 200–400 μ g aerosolized Salbutamol. The supplementary parameters and the cytokine array were determined in all patients studied and in control subjects as a single test during the routine procedure (tables 1, 3, 4).

The BPTs with the inhalant allergens producing the DYAR (table 2) and the PBS control challenges were repeated 3 (range 2–6) weeks later (fig. 1) and supplemented with the recording of cell counts and cytokines in the PB before as well as 1, 12, 24, 36, 48, 56 and 72 h after the challenge (tables 5, 6). The local ethical committee approved this study and informed consent was obtained from all participants.

Control Subjects

Four groups of control subjects volunteered to participate in this study: 20 asthmatics demonstrating an isolated IAR to allergen challenge, 18 asthmatics developing an isolated LAR, 15 asthmatics showing DLAR and 48 healthy subjects (tables 1–4).

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 1,000–3,000 BU/ml for BPTs (table 2). The recommended concentrations by the manufacturer were 500 BU/ml for skin tests and 5,000 BU/ml for the BPTs.

Skin Tests

Scratch tests with allergenic extracts in concentrations of 500 BU/ml were performed and evaluated after 20 min. If they were negative, intracutaneous tests in a concentration of 100 BU/ml and then of 500 BU/ml were carried out and evaluated 20 min and then 6, 12, 24, 36, 48, 72 and 96 h after the intradermal injection. Histamine diphosphate was used as a positive and PBS as a nega-

Table 1. Characteristics of patients and control subjects

	Patients DYAR (n = 28)	Control subjects			
		IAR (n = 20)	Patients with asthma LAR (n = 18)	DLAR (n = 15)	Healthy subjects (n = 16)
Age, years	31 ± 4	30 ± 5	33 ± 3	28 ± 6	30 ± 6
Gender, M/F	12/16	7/13	10/8	6/9	8/8
Disease history, years	4.1 ± 2.0	4.9 ± 1.3	4.5 ± 1.7	5.3 ± 1.5	0
Asthmatic attacks per month	3 ± 1	4 ± 1	3 ± 1	4 ± 2	0
FEV ₁ , % predicted	95.3 ± 8.1	92.8 ± 5.3	91.4 ± 7.4	93.0 ± 5.2	99.7 ± 4.4
FVC, % predicted	98.5 ± 6.3	99.0 ± 4.7	101.2 ± 5.8	102.1 ± 6.4	104.2 ± 7.5
Blood leukocyte count (× 10 ⁹ /l) ^a	11.7 ± 0.4	7.7 ± 0.9	8.3 ± 0.5	8.5 ± 0.6	7.0 ± 0.3
Blood neutrophil count (× 10 ⁹ /l) ^b	6.3 ± 2.9	7.0 ± 3.3	5.5 ± 2.1	6.0 ± 2.5	5.8 ± 2.6
Blood eosinophil count (× 10 ⁶ /l) ^c	325 ± 32	541 ± 78*	523 ± 53*	534 ± 69*	216 ± 25
Bronchial histamine threshold ^d , mg/ml					
≤2.0	0	3	1	2	0
4.0	1	6	2	3	0
8.0	3	4	4	2	0
16.0	10	3	6	4	0
32.0	5	2	3	3	1
>32.0	9	2	2	1	15
BPT positive/total ^e	1/1–2	1/2	1/3	1/2–3	0
Ratio Th ₁ /Th ₂ in blood ^f , %	8.4 ± 2.7	7.1 ± 3.0	7.6 ± 3.1	7.2 ± 3.8	8.0 ± 2.4
IFN-γ, pg/ml – PBMC ^g	208 ± 79	171 ± 52	177 ± 63	175 ± 49	190 ± 55
IL-4, pg/ml – PBMC ^g	18.4 ± 6.9	24.6 ± 7.1	20.5 ± 4.8	22.3 ± 4.1	18.9 ± 5.2

Values = means ± SD. * p < 0.05 (statistical significance compared with healthy control subjects).

^a Normal value = 4.0–10 × 10⁹/l. ^b Normal value = 2.0–7.2 × 10⁹/l. ^c Normal value = <300 × 10⁶/l. ^d Normal value >32.0 mg/ml. ^e BPTs with allergens (per patient = positive/total). ^f Determined by means of flow cytometry (mean ± SEM). ^g After in vitro stimulation of PBMCs with PMA (mean ± SEM).

tive control. A skin wheal reaction (>7.0 mm in diameter) occurring after 20 min was qualified as a positive immediate skin response, skin infiltration appearing between 6–12 h as a late skin response and skin induration recorded later than 48 h was considered a delayed skin response [27–29].

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 a 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 μm. The BPTs, being a modification of the European standard technique [31], were performed according to the following schedule: (1) initial (baseline) values recorded at 0, 5 and 10 min; (2) PBS control values recorded at 0, 5 and 10 min after a 10-minute PBS inhalation; (3) inhalation of allergen aerosol for 2 × 5 min, with inserted spirometry measurement, followed by the recording of the FEV₁ and FVC values at 0, 5, 10, 20, 30, 45, 60, 90 and 120 min and every hour up to the 12th hour, every second hour during the 22nd and 38th, the 46th and 62nd and at the 72nd hour interval [32–34]. The PBS control challenge was performed by the same schedule as that of the BPTs with allergens.

Supplementary Parameters

Th₁/Th₂ ratio (%) values in PB were determined by means of flow cytometry. The intracellular IFN-γ and IL-4 were estimated in the blood mononuclear cells (PBMC) stimulated with Phorbol 12-myristate 13-acetate (PMA) by means of an enzyme-linked immunoassay (ELISA) kit, as described previously [27].

Determination of Cytokines Released from Unstimulated Circulating Cells into the Serum

Collected samples of 4 ml venous blood were kept for 1 h at room temperature and then centrifuged at 3,000 g at 4°C for 10 min. Aliquots of the serum were stored at –70°C until analysis. The cytokines were determined by means of commercial immunoassay (ELISA, EIA) kits, according to the manufacturers' recommendations. All measurements were performed in duplicate. The detection limits in pg/ml are reported in parentheses. 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), GM-CSF (<3.0), G-CSF (0.8), TNF-α (6.0) and TGF-β (6.0) were measured by means of R&D Systems kits (Minneapolis, Minn., USA), IL-4 (0.6), IL-8 (1.3), IL-12p70 (2.1), IL-18 (9.2) and IFN-γ (1.0) by Bender MedSystems kits (Wien, Austria) and IL-12p40 (3.9) by Becton Dickinson kits (San Jose, Calif., USA). The interassay as well as intraassay coefficients of variations for these kits were less than 10%.

Statistical Analysis

The initial and repeated DYAR and PBS controls were statistically evaluated by means of fitting polynomials to the mean curves over time; 8 time points within 120 min and 25 time points up to 72 h after the allergen or PBS challenge. The hypotheses were tested by the generalized multivariate analysis of the variance model (MANOVA) [32]. The postchallenge cytokine values, recorded at each of the time points during the repeated DYAR and PBS control in individual patients, were compared with their prechallenge values and statistically analyzed by a Wilcoxon matched-pair signed-rank test. The mean post-challenge cytokine values measured at each of the time points during the repeated DYAR were compared with corresponding PBS values and statistically evaluated by a Mann-Whitney U test. A p value < 0.05 was considered to be statistically significant.

Results

The clinical course of the DYAR recorded in 28 patients, beginning within 26–32 h, reaching a maximum within 32–48 h and resolving within 56 h after the allergen challenge (fig. 1), differed substantially from that of IAR and LAR [25–28, 44]. The DYARs were statistically highly significant both in the comparison of the postchallenge with the prechallenge FEV₁ values ($p < 0.01$) and when compared with the PBS control values ($p < 0.001$). No significant differences were found in the appearance of DYAR (in the patients studied) or the other asthmatic response types (in the control patients) with respect to the individual allergens ($p > 0.1$) or between the seasonal and perennial allergens ($p > 0.1$) (table 2). The DYAR patients demonstrated an allergen-relevant positive immediate skin response in 46%, a late skin response in 11% and a delayed skin response in 43%, a decreased bronchial histamine threshold in 71% and nonincreased serum concentrations of particular immunoglobulins in 100% (tables 1, 3).

The DYAR was accompanied by increased blood leukocyte, neutrophil and lymphocyte counts, significant changes in the Th₁/Th₂ cell ratio in PB in favor of Th₁ cells ($p < 0.01$) and a significant increase in the intracellular concentration of IFN- γ ($p < 0.05$), but not of IL-4 ($p > 0.05$) (table 5).

The repeated DYAR was also highly significantly positive when comparing the postchallenge and prechallenge FEV₁ values ($p < 0.001$) and compared to the PBS controls ($p < 0.001$) (fig. 1). No statistically significant differences were found between the initial and the repeated DYAR ($p > 0.05$).

The prechallenge serum concentrations of the IL-2 and IFN- γ were slightly elevated ($p \leq 0.05$) in DYAR pa-

Table 2. Allergens causing particular types of asthmatic response

Allergen	Concentration BU/ml	DYAR (n = 28)	IAR (n = 20)	LAR (n = 18)	DLAR (n = 15)
<i>Dermatophagoides pteronyssinus</i>	1,000	6	8	5	5
<i>Dermatophagoides farinae</i>	1,000	1	0	1	1
Animal danders					
Dog	3,000	1	2	2	1
Cat	1,000	2	2	1	2
Horse	2,000	1	0	1	0
Hamster	2,000	0	1	0	0
<i>Aspergillus fumigatus</i>	1,000	1	0	0	1
Pollen					
Grass mix I	1,000	6	3	4	2
Grass mix II	1,000	3	2	2	0
Tree mix	3,000	2	1	0	1
Weed mix	1,000	1	0	0	1
Birch	1,000	2	1	1	1
Poplar	2,000	1	0	0	0
Ragweed giant	1,000	1	0	1	0

BU/ml = Biologic units per milliliter (Allergopharma, Reinbek, Germany).

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*.

tients compared with the healthy control subjects. Those of other cytokines did not differ significantly ($p > 0.05$) (table 4), but they differed from the prechallenge profiles recorded in the control patients developing the IAR, LAR or DLAR (table 4).

The DYAR was accompanied by statistically significant changes ($p < 0.05$) in the serum concentrations of the following cytokines: (1) an increased serum concentration of IL-2 at 24, 36 and 48 h; IL-10 at 12, 24, 36 and 48 h; IL-18 at 12 and 24 h; IFN- γ at 24, 36, 48 and 56 h; G-CSF at 1 and 12 h; TNF- α at 12, 36 and 48 h and TGF- β at 12 and 36 h and (2) a decreased serum concentration of IL-7 at 36 and 48 h, and IL-12p70 at 12 h when compared both with their prechallenge values and with the PBS control values (fig. 2, table 6). No significant changes in the serum concentrations of cytokines were measured during the PBS control challenge ($p > 0.2$).

Table 3. Survey of the single diagnostic parameters

	Patients DYAR (n = 28)	Control subjects			
		IAR (n = 20)	Patients with asthma LAR (n = 18) DLAR (n = 15)		Healthy subjects (n = 16)
Positive skin response (i.c.)					
Immediate	13	18	5	6	0
Late	3	2	13	9	0
Delayed	12	0	0	0	0
Increased total IgE in serum ^a	0	2	0	1	1
Increased specific IgE in serum ^b	0	9	3	8	0
Increased total IgG in serum ^c	0	0	11	6	0
Increased IgG sub-classes in serum					
IgG ₁ > 5.0 g/l	0	0	1	1	0
IgG ₂ > 2.6 g/l	0	0	0	0	0
IgG ₃ > 0.4 g/l	0	0	3	2	1
IgG ₄ > 0.5 g/l	0	0	5	1	0
Increased total IgM in serum ^d	0	0	0	0	0
Increased total IgA in serum ^e	0	0	0	0	0
Thorax X-ray ^f	1	0	1	0	0

i.c. = Intracutaneous tests.

^a Total IgE in the serum (PRIST)-normal value = <500 IU/ml. ^b Positive specific IgE in the serum (RAST) = >0.70 U/ml (= more than class 1). ^c Total IgG in the serum (single radial immunodiffusion = Mancini technique and ELISA); normal value = <15.0 g/l. ^d IgM normal value in the serum = <3.8 g/l (<1.5). ^e IgA normal value in the serum = <4.0 g/l (<3.2). ^f Thorax X-ray = Bronchovascular markings.

Table 4. Prechallenge values of cytokine concentrations in the serum, pg/ml

	Patients DYAR (n = 28)	Control subjects			
		IAR (n = 20)	Patients with asthma LAR (n = 18) DLAR (n = 15)		Healthy subjects (n = 16)
IL-1 β	1.5 $\pm$ 0.3	1.8 $\pm$ 0.6	2.0 $\pm$ 0.9	1.6 $\pm$ 0.3	1.6 $\pm$ 0.4
IL-2	4.1 $\pm$ 1.5*	<3.0	<3.0	3.1 $\pm$ 0.1	3.2 $\pm$ 0.2
IL-3	<31.2	<31.2	<31.2	<31.2	<31.2
IL-4	2.5 $\pm$ 0.8	5.0 $\pm$ 2.5**	3.1 $\pm$ 1.1	5.3 $\pm$ 1.4**	2.9 $\pm$ 0.5
IL-5	3.8 $\pm$ 0.7	6.9 $\pm$ 1.3**	6.0 $\pm$ 0.9**	4.4 $\pm$ 1.5	3.3 $\pm$ 0.2
IL-6	<4.0	5.3 $\pm$ 1.2	5.0 $\pm$ 0.9	6.7 $\pm$ 1.3**	<4.0
IL-7	1.2 $\pm$ 0.6	2.4 $\pm$ 0.3**	1.8 $\pm$ 0.7	2.5 $\pm$ 0.7**	0.8 $\pm$ 0.3
IL-8	5.8 $\pm$ 1.7	4.9 $\pm$ 1.3	7.2 $\pm$ 2.3**	6.1 $\pm$ 1.9	4.4 $\pm$ 0.5
IL-10	3.9 $\pm$ 0.4	<3.0	4.8 $\pm$ 0.7	5.0 $\pm$ 0.6	3.6 $\pm$ 1.1
IL-12p40	7.0 $\pm$ 2.0	6.6 $\pm$ 1.7	7.1 $\pm$ 4.9	7.6 $\pm$ 2.3	6.8 $\pm$ 1.6
IL-12p70	8.8 $\pm$ 1.3	9.1 $\pm$ 1.9	10.1 $\pm$ 0.8	9.0 $\pm$ 2.1	9.3 $\pm$ 2.4
IL-13	<3.0	4.5 $\pm$ 0.4*	3.5 $\pm$ 0.3	3.9 $\pm$ 0.7	<3.0
IL-18	10.9 $\pm$ 0.6	11.5 $\pm$ 0.8	10.2 $\pm$ 3.1	12.0 $\pm$ 2.4	10.5 $\pm$ 2.7
IFN- γ	1.3 $\pm$ 0.2*	<1.0	<1.0	<1.0	<1.0
GM-CSF	<3.0*	3.5 $\pm$ 0.2	4.8 $\pm$ 0.6*	4.1 $\pm$ 0.9	3.7 $\pm$ 0.5
G-CSF	<0.8	<0.8	<0.8	<0.8	<0.8
TNF- α	7.1 $\pm$ 0.4	<6.0	7.1 $\pm$ 0.9	<6.0	6.8 $\pm$ 0.6
TGF- β	9.5 $\pm$ 4.2	8.5 $\pm$ 2.2	13.3 $\pm$ 5.1*	11.7 $\pm$ 3.3	9.0 $\pm$ 4.1

Values = means $\pm$ SEM. * p $\leq$ 0.05; ** p < 0.05 (statistical significance compared with values of healthy subjects i.e. controls).

Table 5. Differential counts in PB, Th₁/Th₂ ratio, IFN- γ and IL-4 during the DYAR and PBS

Patients (n = 28)	Before the challenge	After the challenge, h								
		1	6	12	24	36	48	56	72	
Leukocytes ^a										
DYAR	8.5 ± 0.6	10.4 ± 0.7	10.8 ± 0.5	11.3 ± 1.0*	11.8 ± 0.6*	13.9 ± 0.4**	12.8 ± 0.5**	11.2 ± 0.8*	11.1 ± 0.4	
PBS	7.8 ± 0.4	6.9 ± 0.5	7.5 ± 1.1	9.3 ± 0.8	7.2 ± 0.5	8.3 ± 1.1	9.0 ± 1.1	7.4 ± 0.9	8.2 ± 0.7	
Eosinophils ^b										
DYAR	280 ± 31	301 ± 25	310 ± 26	325 ± 40	279 ± 30	275 ± 20	265 ± 33	225 ± 21	294 ± 37	
PBS	271 ± 31	290 ± 35	284 ± 23	300 ± 32	291 ± 40	269 ± 27	283 ± 24	279 ± 24	288 ± 26	
Neutrophils ^c										
DYAR	6.9 ± 0.7	6.6 ± 0.5	7.1 ± 0.8	9.6 ± 0.9**	8.9 ± 0.5**	8.8 ± 1.0**	7.7 ± 0.4*	7.5 ± 0.7*	6.4 ± 0.5	
PBS	5.6 ± 1.0	6.3 ± 0.7	6.8 ± 0.6	6.5 ± 0.9	6.8 ± 0.5	7.0 ± 0.9	6.9 ± 0.8	6.6 ± 0.3	6.5 ± 0.4	
Thrombocytes ^d										
DYAR	324 ± 45	340 ± 29	331 ± 33	352 ± 41	355 ± 44	488 ± 50*	400 ± 37	317 ± 23	312 ± 21	
PBS	265 ± 20	324 ± 25	301 ± 29	288 ± 27	363 ± 30	322 ± 25	323 ± 28	271 ± 26	282 ± 21	
<i>Differential count^e</i>										
Basophils										
DYAR	0.05	0.08	0.10	0.08	0.10	0.06	0.09	0.07	0.15	
PBS	0.06	0.10	0.05	0.07	0.06	0.09	0.04	0.09	0.05	
Eosinophils										
DYAR	0.35	0.5	0.45	0.6	0.38	0.55	0.25	0.4	0.25	
PBS	0.22	0.35	0.5	0.25	0.20	0.38	0.15	0.25	0.15	
Neutrophils										
DYAR	6.8	6.3	6.7	7.5	8.8**	9.5**	8.9**	8.7**	7.1	
PBS	6.1	6.0	6.5	5.7	6.1	6.7	5.9	7.0	5.6	
Monocytes										
DYAR	0.3	0.4	1.0	1.8**	1.9**	0.9	1.1	0.6	0.4	
PBS	0.25	0.2	0.7	0.6	0.5	0.37	0.3	0.25	0.6	
Lymphocytes										
DYAR	2.5	3.3	2.8	3.5	4.5**	4.9**	3.2	2.1	2.4	
PBS	2.2	2.0	3.5	3.8	2.6	3.0	2.7	2.4	2.8	
Th ₁ /Th ₂ ratio ^f , %										
DYAR	8.1 ± 1.8	8.5 ± 2.3	9.7 ± 4.0	13.8 ± 4.4**	15.5 ± 4.5**	17.3 ± 4.0**	16.0 ± 4.4**	15.9 ± 4.5**	9.4 ± 3.0	
PBS	7.2 ± 3.0	8.4 ± 3.1	9.5 ± 4.1	7.7 ± 4.4	8.9 ± 4.7	9.2 ± 4.5	10.0 ± 3.2	9.1 ± 4.3	7.9 ± 4.6	
IFN-γ, pg/ml ^g										
DYAR	212 ± 109	195 ± 90	231 ± 117	220 ± 114	398 ± 122**	445 ± 127**	420 ± 111**	380 ± 123**	225 ± 100	
PBS	165 ± 110	200 ± 125	219 ± 110	195 ± 123	219 ± 113	185 ± 115	188 ± 122	170 ± 104	191 ± 120	
IL-4, pg/ml ^g										
DYAR	23.3 ± 2.1	22.9 ± 3.0	28.0 ± 4.0	33.1 ± 4.2	29.0 ± 4.6	27.3 ± 4.7	26.5 ± 3.4	30.1 ± 4.5	28.3 ± 4.6	
PBS	24.8 ± 4.0	25.7 ± 3.4	28.0 ± 3.5	29.8 ± 3.2	30.1 ± 3.1	28.6 ± 3.0	25.9 ± 4.7	28.8 ± 3.3	25.4 ± 4.5	

Values = means $\pm$ SEM: * $p \leq 0.05$, ** $p < 0.05$ (statistical significance compared with values of healthy subjects i.e. controls).

^a Normal value 4.0–10.0 $\times 10^9$ /l. ^b Normal value $<300 \times 10^6$ /l. ^c Normal value 2.0–7.2 $\times 10^9$ /l. ^d Normal value 150–400 $\times 10^9$ /l.

^e Normal values ($\times 10^9$ /l): B = <0.2 ; E = <0.50 ; N = 2.0–7.2; M = 0.2–1.0; L = 1.0–4.0 $\times 10^9$ /l. ^f Determined by means of flow cytometry.

^g After in vitro stimulation of PBMCs with PMA.

Control patients with IAR demonstrated significant elevated serum concentration ($p < 0.05$) of IL-4, IL-5 and IL-7, patients with LAR significant elevation ($p < 0.05$) of IL-5 and IL-8, and DLAR patients significantly elevated concentrations ($p < 0.05$) of IL-4, IL-6 and IL-7 compared with the healthy control subjects (table 4).

Discussion

The allergen-induced DYAR differs substantially from the IAR and LAR in clinical and immunological features [2, 3, 5, 7, 13–29]. The DYAR is associated with factors and changes different from those recorded during the IAR

Fig. 2. Repeated DYAR ($n = 28$) ($\square$) and the mean percentage changes in the concentrations of particular cytokines in the serum. ALL = Allergen challenge; I = initial (baseline) value.

and LAR [27–29]. Cytokines, compounds produced by various cell types and tissues, represent an important part of the immunological processes [4–6, 8–10, 12, 17–27, 33–43]. They are involved in these processes at various steps and fulfil not only the signal-transmitting role, but also a regulatory (stimulation vs. inhibition), effector and

target role [4, 8–10, 12, 23, 33, 34, 41, 43]. They act closely with other factors participating in the immunological mechanisms, such as mediators, chemokines, cell surface molecules (CD), adhesion molecules and cellular constituents released by various cell types [1, 4–6, 8–12, 18–23, 27, 29, 33, 34, 36, 39, 41, 43].

Fig. 2 (continued)

The role of cytokines in allergic bronchial asthma has been studied from various points of view [1, 4–6, 8–10, 12, 17–27, 33–44], mostly dealing with their single determination in bronchial asthma patients at various stages [23, 33, 34, 36, 40, 41, 43].

In some of the studies, the bronchial allergen challenge has been utilized as the central event to which the appearance and the changes of cytokines in various media [blood, sputum, bronchoalveolar lavage (BAL) fluid, tissue biopsies] were related [5, 7, 18–20, 22, 26, 38, 39]. However, in most cases cytokines have been investigated in the BAL fluid collected after the segmental challenge with allergen [5, 7, 20, 24–26, 38, 39].

There are not many studies dealing with the measurement of the cytokine changes in the PB during individual types of asthmatic response to allergen challenge [5, 17, 18, 24, 38]. In most of them, the cytokine concentrations were measured after ‘in vitro’ stimulation of the cells isolated from PB, induced sputum or BAL fluid [5, 17, 22–24, 26, 33–37, 40, 41] and those investigating the cytokine appearance in the PB after the BPTs with inhaled allergens are rare [17, 18]. Nevertheless, the data concerning the appearance of cytokines in patients with bronchial asthma presented in the literature show relatively high variations [1, 5, 7, 11, 18, 20, 25, 26, 35–37, 43]. We recorded interesting data in control patients, namely significantly elevated se-

rum concentrations of IL-4, IL-5 and IL-7 in IAR patients, increased concentrations of IL-5 and IL-8 in LAR patients and elevated concentrations of IL-4, IL-6 and IL-7 in DLAR patients compared to the healthy subjects (table 4).

These findings are partly consistent with other investigators’ data of increased levels of IL-4 and IL-6 in the serum or in BAL fluid of patients developing an IAR [18, 22, 25, 26] or of increased levels of IL-3, IL-4 and IL-5 in the blood, induced sputum or BAL fluid of patients demonstrating a LAR [5, 7, 19, 20, 22, 24, 25, 38] to bronchial or segmental challenge with allergens. In contrast to other reports [5, 17–20, 22, 24–26, 36, 38], we measured no increased blood levels of other cytokines in our control patients.

The differences between our results and those of other studies may be explained by a number of factors, e.g. (1) variations in the asthmatic patient populations, (2) existence of various phenotypes of bronchial asthma, (3) natural variations of cytokine concentrations both in the same medium and among the media themselves, (4) differences in the bronchial challenge techniques, (5) differences in the material collection techniques and (6) variations of the methods and kits used for the cytokine assays [18, 19, 25, 36].

The bronchial challenge techniques have a number of advantages and disadvantages. The advantage of the seg-

Table 6. Serum concentrations of particular cytokines recorded during DYAR and PBS control tests

Patients (n = 28)	Before the challenge	After the challenge, h						
		1	12	24	36	48	56	72
IL-1 β , pg/ml								
DYAR	2.1 $\pm$ 0.6	1.8 $\pm$ 0.3	2.3 $\pm$ 1.0	2.5 $\pm$ 0.7*	2.4 $\pm$ 0.4	2.3 $\pm$ 0.5	2.0 $\pm$ 0.5	2.4 $\pm$ 0.5
PBS	1.9 $\pm$ 0.4	1.0 $\pm$ 0.2	2.2 $\pm$ 0.4	2.0 $\pm$ 0.2	2.0 $\pm$ 0.6	2.2 $\pm$ 0.4	2.0 $\pm$ 0.3	2.1 $\pm$ 0.1
IL-2, pg/ml								
DYAR	3.5 $\pm$ 0.5	4.5 $\pm$ 1.2	4.9 $\pm$ 1.0*	6.8 $\pm$ 0.7**	7.2 $\pm$ 1.3**	6.2 $\pm$ 0.6**	5.3 $\pm$ 1.1*	4.1 $\pm$ 0.8
PBS	3.6 $\pm$ 0.3	4.0 $\pm$ 0.6	4.2 $\pm$ 0.3	4.0 $\pm$ 0.5	4.3 $\pm$ 0.4	4.1 $\pm$ 0.8	3.7 $\pm$ 0.6	4.0 $\pm$ 0.5
IL-3, pg/ml								
DYAR	31.4 $\pm$ 0.1	31.7 $\pm$ 0.5	32.8 $\pm$ 1.1	33.1 $\pm$ 1.5	32.5 $\pm$ 0.7	31.2 $\pm$ 1.0	32.1 $\pm$ 0.8	32.0 $\pm$ 0.5
PBS	31.5 $\pm$ 0.4	<31.2	31.7 $\pm$ 0.3	32.9 $\pm$ 0.6	30.0 $\pm$ 1.0	<31.2	<31.2	31.4 $\pm$ 0.2
IL-4, pg/ml								
DYAR	3.3 $\pm$ 0.5	3.1 $\pm$ 0.1	3.0 $\pm$ 0.3	3.2 $\pm$ 0.2	3.0 $\pm$ 0.6	3.1 $\pm$ 0.2	3.0 $\pm$ 0.4	3.2 $\pm$ 0.7
PBS	3.2 $\pm$ 0.3	3.4 $\pm$ 0.1	3.2 $\pm$ 0.6	3.1 $\pm$ 0.5	3.3 $\pm$ 0.3	3.0 $\pm$ 0.1	3.3 $\pm$ 0.2	3.4 $\pm$ 0.9
IL-5, pg/ml								
DYAR	4.2 $\pm$ 0.8	3.9 $\pm$ 0.4	4.6 $\pm$ 1.0	4.3 $\pm$ 0.8	4.5 $\pm$ 1.0	4.7 $\pm$ 1.3	4.4 $\pm$ 1.0	4.0 $\pm$ 0.6
PBS	3.5 $\pm$ 0.7	3.7 $\pm$ 0.6	3.3 $\pm$ 0.2	4.1 $\pm$ 1.5	3.8 $\pm$ 0.3	4.1 $\pm$ 1.7	4.0 $\pm$ 0.5	4.1 $\pm$ 0.9
IL-6, pg/ml								
DYAR	4.3 $\pm$ 0.2	<4.0	4.9 $\pm$ 0.7*	4.3 $\pm$ 0.3	<4.0	<4.0	4.4 $\pm$ 0.4	<4.0
PBS	<4.0	<4.0	4.2 $\pm$ 0.1	<4.0	4.3 $\pm$ 0.1	4.0 $\pm$ 0.0	4.2 $\pm$ 0.2	<4.0
IL-7, pg/ml								
DYAR	2.0 $\pm$ 0.3	1.8 $\pm$ 0.2	2.1 $\pm$ 0.2	1.9 $\pm$ 0.5	<0.1**	<0.1**	1.8 $\pm$ 0.3*	2.0 $\pm$ 0.2
PBS	2.0 $\pm$ 0.4	1.9 $\pm$ 0.2	2.0 $\pm$ 0.5	2.0 $\pm$ 0.3	1.9 $\pm$ 0.2	2.1 $\pm$ 0.1	2.2 $\pm$ 0.4	2.0 $\pm$ 0.5
IL-8, pg/ml								
DYAR	7.4 $\pm$ 1.9	7.1 $\pm$ 1.3	12.5 $\pm$ 0.5*	11.9 $\pm$ 0.4*	6.9 $\pm$ 1.1	7.5 $\pm$ 0.8	7.6 $\pm$ 1.4	7.3 $\pm$ 0.7
PBS	7.2 $\pm$ 1.6	7.5 $\pm$ 2.7	6.9 $\pm$ 2.2	7.0 $\pm$ 1.8	7.1 $\pm$ 2.0	6.7 $\pm$ 1.0	6.2 $\pm$ 0.9	7.4 $\pm$ 0.5
IL-10, pg/ml								
DYAR	3.2 $\pm$ 0.1	3.4 $\pm$ 0.3	4.8 $\pm$ 1.2**	8.1 $\pm$ 1.0**	9.5 $\pm$ 1.5**	7.4 $\pm$ 1.7**	3.8 $\pm$ 0.7	3.1 $\pm$ 0.1
PBS	3.5 $\pm$ 0.4	<3.0	<3.0	<3.0	3.3 $\pm$ 0.2	<3.0	3.0 $\pm$ 0.0	<3.0
IL-12p40, pg/ml								
DYAR	6.9 $\pm$ 3.1	8.0 $\pm$ 5.2	6.1 $\pm$ 1.5	7.3 $\pm$ 2.7	7.0 $\pm$ 0.5	7.2 $\pm$ 0.9	7.1 $\pm$ 1.4	6.8 $\pm$ 1.2
PBS	7.0 $\pm$ 2.1	7.4 $\pm$ 1.3	7.0 $\pm$ 0.6	7.2 $\pm$ 2.3	7.3 $\pm$ 1.1	7.5 $\pm$ 1.6	6.5 $\pm$ 1.1	6.9 $\pm$ 0.5
IL-12p70, pg/ml								
DYAR	10.1 $\pm$ 3.2	9.8 $\pm$ 2.7	6.0 $\pm$ 3.1**	7.5 $\pm$ 4.2*	8.9 $\pm$ 2.6	10.7 $\pm$ 2.3	9.5 $\pm$ 4.0	10.4 $\pm$ 0.7
PBS	10.3 $\pm$ 2.7	11.1 $\pm$ 1.7	11.9 $\pm$ 2.2	10.8 $\pm$ 4.3	9.3 $\pm$ 2.8	9.0 $\pm$ 2.0	10.3 $\pm$ 1.7	8.1 $\pm$ 1.0
IL-13, pg/ml								
DYAR	<3.0	<3.0	3.5 $\pm$ 0.5	<3.0	3.1 $\pm$ 0.1	3.2 $\pm$ 0.1	<3.0	3.3 $\pm$ 0.2
PBS	<3.0	4.0 $\pm$ 0.5	3.5 $\pm$ 0.4	3.3 $\pm$ 0.3	<3.0	<3.0	3.7 $\pm$ 0.5	3.5 $\pm$ 0.4
IL-18, pg/ml								
DYAR	10.8 $\pm$ 1.0	12.5 $\pm$ 2.3	15.9 $\pm$ 2.1**	16.5 $\pm$ 2.0**	13.7 $\pm$ 0.8	12.9 $\pm$ 1.6	13.0 $\pm$ 0.4	9.5 $\pm$ 1.0
PBS	11.1 $\pm$ 3.9	12.0 $\pm$ 1.5	12.1 $\pm$ 1.9	11.0 $\pm$ 2.2	11.1 $\pm$ 1.6	10.5 $\pm$ 1.3	10.8 $\pm$ 1.2	10.2 $\pm$ 1.4
IFN- γ , pg/ml								
DYAR	2.3 $\pm$ 0.5	2.9 $\pm$ 1.1	3.8 $\pm$ 0.5*	9.4 $\pm$ 2.7**	8.9 $\pm$ 4.1**	8.5 $\pm$ 3.8**	6.3 $\pm$ 1.4**	2.1 $\pm$ 1.0
PBS	2.0 $\pm$ 0.1	1.7 $\pm$ 0.5	2.5 $\pm$ 1.0	1.8 $\pm$ 0.7	2.0 $\pm$ 0.7	2.1 $\pm$ 1.1	2.2 $\pm$ 1.2	1.9 $\pm$ 0.4
GM-CSF, pg/ml								
DYAR	3.3 $\pm$ 0.3	3.1 $\pm$ 0.3	5.5 $\pm$ 2.6*	5.1 $\pm$ 2.3*	4.9 $\pm$ 1.0*	3.1 $\pm$ 0.5	3.4 $\pm$ 0.9	3.7 $\pm$ 0.4
PBS	4.0 $\pm$ 1.5	3.6 $\pm$ 1.0	3.9 $\pm$ 0.5	4.0 $\pm$ 1.1	3.2 $\pm$ 0.6	3.9 $\pm$ 1.0	3.5 $\pm$ 0.7	4.1 $\pm$ 0.9
G-CSF, pg/ml								
DYAR	<0.8	3.7 $\pm$ 1.3**	4.3 $\pm$ 0.8**	1.5 $\pm$ 0.6*	<0.8	<0.8	<0.8	1.0 $\pm$ 0.4
PBS	<0.8	1.1 $\pm$ 0.3	1.2 $\pm$ 0.3	1.0 $\pm$ 0.2	0.9 $\pm$ 0.1	<0.8	<0.8	<0.8
TNF- α , pg/ml								
DYAR	7.4 $\pm$ 1.1	8.1 $\pm$ 2.7	9.7 $\pm$ 1.3**	8.0 $\pm$ 2.2	9.8 $\pm$ 3.0**	9.9 $\pm$ 2.6**	8.4 $\pm$ 1.5*	7.1 $\pm$ 1.2
PBS	7.0 $\pm$ 2.5	7.3 $\pm$ 1.0	6.6 $\pm$ 0.9	6.9 $\pm$ 1.1	7.6 $\pm$ 2.4	7.3 $\pm$ 2.0	7.0 $\pm$ 0.8	6.5 $\pm$ 1.3
TGF- β , pg/ml								
DYAR	10.3 $\pm$ 3.1	13.6 $\pm$ 2.3	18.5 $\pm$ 5.7**	14.1 $\pm$ 3.8	15.0 $\pm$ 2.5**	12.0 $\pm$ 1.4	10.8 $\pm$ 1.1	10.0 $\pm$ 2.3
PBS	9.9 $\pm$ 1.2	10.9 $\pm$ 1.8	9.3 $\pm$ 2.2	10.4 $\pm$ 3.0	10.9 $\pm$ 1.7	11.3 $\pm$ 1.5	12.4 $\pm$ 2.7	11.8 $\pm$ 0.5

Values = means $\pm$ SEM. * $p \leq 0.05$; ** $p < 0.05$ (statistical significance as compared with the prechallenge i.e. baseline values).

mental challenge is the provocation of the limited lung area with a small allergen amount and direct access to the immunological reaction site. Its disadvantage is an exclusion of the large part of the respiratory system (from the mouth to the secondary bronchi) which is regularly the site of the initial stages of the immunological process leading to the asthmatic response. This method is relatively laborious, requires an anesthetic agent and the less natural BAL technique can burden the patient. The advantage of the inhalation technique is its simulation of the natural allergen exposure upon including of all parts of the airways, the relatively low strain of the patient and the low risk of postintervention complications besides bronchoconstriction. Its disadvantage is the need of a rather large allergen dose to be effective [31].

It is also important how frequently cytokines in bronchial asthma patients were measured. A single estimation of cytokine concentrations in the PB, sputum or BAL fluid in asthmatics [23, 33–37, 40, 41] which is not related directly to a specific and controlled event, such as provocation test with allergen, seems us to be of a limited scientific value. Being related only to a certain time interval, it does not generate any information concerning the dynamic aspects of the cytokine changes through the whole immunological process and clinical response. This can only be achieved by repeated measurements of these factors over time.

Unfortunately, there is also a dearth of comprehensive information concerning the cytokine and chemokine profiles in the PB, sputum and BAL fluid in healthy subjects which could be exploited as a reference standard [44].

The DYAR patients did not differ significantly from the healthy subjects with regard to prechallenge serum

concentrations of cytokines. The cytokine profile associated with the DYAR differ distinctly from the cytokine profiles found by other investigators in patients developing either the IAR or the LAR [5, 7, 17–19, 22, 24–26]. The different cytokine profiles would indicate involvement of different immunological mechanisms in the DYAR and those in the IAR and the LAR.

Our results of significant changes in the serum concentrations of IL-2, IL-10, IL-12p70, IL-18, IFN- γ , G-CSF, TNF- α , TGF- β , in the blood counts of neutrophils, monocytes and lymphocytes, and in the Th₁/Th₂ ratio during the DYAR, together with our previous findings [28, 29], would suggest the prominent role of Th₁ lymphocytes, neutrophils, monocytes, endothelial and epithelial cells and probably also macrophages and NK in the mechanisms underlying the DYAR. This assumption would also be supported by the unchanged blood concentrations of IL-4, IL-5, IL-8 and IL-13 during the DYAR.

Unfortunately, our results cannot be confirmed by other findings, as we found no comparable data in the literature. Taken together, an involvement of the cell-mediated hypersensitivity mechanisms or at least some of their parts in the clinical DYAR may be suspected. However, further concurrent research will be needed to fully clarify the clinical phenomenon DYAR and the underlying mechanisms.

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