Delayed Asthmatic Response to Bronchial Challenge with Allergen-Mediators, Eicosanoids, Eosinophil and Neutrophil Constituents in the Blood and Urine

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Key Words

Delayed asthmatic response • Bronchial provocation tests with allergen • Eicosanoids • Eosinophil constituents • Neutrophil constituents

Abstract

Background: In patients with allergic bronchial asthma, different immunologic mechanisms may participate and lead to different types of asthmatic response to allergen challenge, such as immediate/early (IAR/EAR), late (LAR) or delayed asthmatic response (DYAR). **Objectives:** In 55 of 397 asthmatics, DYAR has been recorded ($p < 0.001$) and confirmed by repeated bronchial challenge with the same allergen ($p < 0.001$). DYAR began between 26 and 32 h, reached a maximum between 32 and 48 h and resolved within 56 h after the challenge. DYAR was associated with various clinical symptoms and diagnostic parameters having diverged from those recorded during the IARs/EARs and LARs. **Methods:** In 25 of 55 patients, repeated DYAR has been supplemented with the recording of leukotriene B₄ (LTB₄), LTC₄, LTE₄, prostaglandin D₂ (PGD₂), PGE₂, PGF_{2α}, thromboxane B₂, lipoxin A₄, eosinophil cationic protein, eosinophil-derived neurotoxin/eosinophil protein X, eosinophilic peroxidase, myeloperoxidase, histamine and tryptase in peripheral blood, and of LTC₄, thromboxane B₂, eosinophil-derived neurotoxin and 9α,11β-

PGF₂ in urine, before and up to 72 h after the bronchial allergen challenge, by means of enzyme-linked immunoassay (ELISA/EIA) or ImmunoCAP. **Results:** DYAR was accompanied by a significant increase in the plasma concentrations of LTB₄ ($p < 0.05$) and myeloperoxidase ($p < 0.05$) at 24, 36 and 48 h after the challenge, whereas the plasma/serum or urine concentrations of the other factors did not demonstrate any significant changes ($p > 0.05$). **Conclusions:** These results would indicate an active and prominent involvement of neutrophils, in addition to the previously demonstrated role of the Th1 lymphocytes, in the clinical DYAR.

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Introduction

Bronchial asthma should be qualified as a multifactorial disorder due to the complex immunologic mechanisms [1–3]. Two basic components, an allergic and a non-specific hyperreactivity component, may participate simultaneously in this disorder, however, to various degrees and in variable ratios [1, 4, 5]. The allergic component can be due to different types of the hypersensitivity mechanism, such as immediate, late or delayed type [1–10].

Each type of the hypersensitivity mechanism, with its own characteristics and including specific parts, such as

Fig. 1. DYAR to allergen challenge; PBS control challenge. The mean percentage changes in the FEV₁ values calculated from 25 DYAR and 25 PBS control challenges. ○ = Initial DYAR; ● = repeated DYAR; * = initial PBS; × = repeated PBS; I = initial (baseline) values; ALL = allergen challenge; bars = means ± SEM.

particular cell types, individual immunoglobulin classes, specific mediators, chemotactic factors, cytokines, chemokines, CD molecules, adhesion molecules, enzymes, activated cellular and tissue receptors, may lead to a specific clinical manifestation of the lower asthmatic airways [1–43].

Patients with allergic bronchial asthma being challenged with an allergen may develop different types of asthmatic response, such as immediate (IAR/EAR) [1–4, 10, 14, 16, 20, 23–25, 32, 42], late (LAR) [1–3, 5, 8, 10, 15, 16, 19–25] and recently reported delayed asthmatic response (DYAR) [6, 7], probably due to different hypersensitivity mechanisms [1–43].

The purpose of this study, being a continuation of our previous work concerning DYAR [6, 7], was to investigate some immunologic features of clinical DYAR, especially possible changes in the blood concentrations of some typical products of activated mast cells, basophils, eosinophils and neutrophils, such as eicosanoids and intracellular constituents, and thus, to contribute to the clarification of the mechanism(s) underlying this clinical phenomenon.

Methods

Patients

Twenty-five of the 55 bronchial asthma patients visiting our Department of Allergology and Immunology, Institute of Medical Sciences 'De Klokenberg', Breda, The Netherlands, between

1999 and 2000 and developing DYAR (fig. 1) during the routine bronchial provocation tests (BPTs) with inhalant allergens [7], volunteered to participate in this study.

These patients, 20–45 years of age, showed reversible bronchial constriction alternating with symptom-free periods without any restrictive changes of their lung function. They did not suffer from respiratory infections. They had not been treated with oral corticosteroids or immunotherapy. They were examined using a routine diagnostic procedure, serving also as inclusion-exclusion criteria (tables 1, 2) [7], consisting of (1) a general part: disease history, physical examination, basic laboratory tests, X-ray of the chest and sinuses, lung function, blood gas determination, bacteriological examination of the sputum; (2) an allergologic part: skin tests, bronchial histamine threshold [44], blood leukocyte differential count, determination of the serum immunoglobulins; (3) control challenge with phosphate-buffered saline (PBS; n = 25) followed by bronchial challenges with allergens (BPTs; n = 58). The allergens for BPTs were chosen with respect to disease history and skin tests (table 3). The initial BPTs have always been performed with allergenic extracts diluted 1:10 and if they were negative, then BPTs with undiluted extracts (our standard) followed (table 3).

In these 25 patients demonstrating DYAR during the routine procedure (amplitude $44 \pm 8\%$, mean $\pm$ SD) (fig. 1), the BPTs with the same allergens (table 3) and PBS controls were repeated 2–6 weeks later. The repeated DYARs and PBS challenges (fig. 1) were supplemented with the recording of various in vivo and in vitro response-related diagnostic parameters and particular factors in the blood serum/plasma before and up to 72 h after the allergen challenge (table 4). The local ethical committee approved this study, and an informed consent was obtained from all study participants.

Table 1. Characteristics of DYAR patients and control subjects

	DYAR patients (n = 25)	Control subjects		healthy subjects (n = 38)
		asthmatics		
		IAR (n = 36)	LAR (n = 31)	
Age, years	32 ± 4	29 ± 7	25 ± 9	30 ± 6
Gender, male/female	12/13	15/21	17/14	22/16
Disease history, years	4.9 ± 1.5	5.3 ± 0.9	5.0 ± 1.2	0
Asthmatic attacks per month	3 ± 1	5 ± 1	3 ± 2	0
FEV ₁ , % predicted	95.1 ± 7.3	91.5 ± 5.6	94.7 ± 4.1	98.4 ± 6.2
FVC, % predicted	98.7 ± 5.1	102.3 ± 3.9	97.9 ± 8.1	105.6 ± 4.7
BPT per patient, total/positive	2–3/1	2–3/1–2	2–3/1	0
Blood erythrocyte count, × 10 ¹² /l	5.3 ± 0.3	5.8 ± 0.2	5.5 ± 0.5	5.9 ± 0.4
Blood leukocyte count, × 10 ⁹ /l	11.4 ± 0.6	7.0 ± 0.3	8.4 ± 0.5	6.7 ± 1.1
Blood eosinophil count, × 10 ⁶ /l	324 ± 29	514 ± 63 ^b	472 ± 85 ^b	201 ± 32
Blood thrombocyte count, × 10 ⁹ /l	313 ± 38	285 ± 35	340 ± 25	290 ± 30
Blood differential count, × 10 ⁹ /l				
Basophils	0.1	0.1	0.15	0.12
Eosinophils	0.25	0.7 ^a	0.6 ^a	0.3
Neutrophils	6.1	4.1	4.8	4.5
Monocytes	0.3	0.2	0.15	0.18
Lymphocytes	1.8	1.0	1.2	1.1
Erythrocyte sedimentation rate	10	9	15	10
Blood Th1/Th2, %	8.5 ± 2.9	7.2 ± 2.7	7.8 ± 3.9	8.6 ± 3.5
Interferon-γ, pg/ml – PBMC	173 ± 94	175 ± 103	194 ± 110	203 ± 98
Interleukin-4, pg/ml – PBMC	20.1 ± 4.2	23.2 ± 5.3	21.8 ± 6.5	24.7 ± 4.1
Plasma				
LTB ₄ , pg/ml	39.9 ± 5.1	58.7 ± 6.3 ^a	61.4 ± 5.9 ^a	33.6 ± 4.2
LTC ₄ , pg/ml	8.1 ± 0.5	14.9 ± 0.4 ^a	13.5 ± 0.9 ^a	8.7 ± 0.3
LTE ₄ , pg/ml	56.5 ± 3.5	52.2 ± 4.1	68.0 ± 2.3 ^a	49.4 ± 3.9
PGD ₂ , pg/ml	400 ± 32	368 ± 39	455 ± 20	348 ± 47
PGE ₂ , pg/ml	810 ± 38	1,125 ± 45	1,570 ± 80 ^b	1,085 ± 65
PGF _{2α} , pg/ml	385 ± 11	577 ± 39 ^b	605 ± 56 ^a	411 ± 25
TXB ₂ , pg/ml	325 ± 33	318 ± 41	482 ± 29 ^b	295 ± 52
LXA ₄ , ng/ml	28.0 ± 3.5	29.3 ± 4.4	41.6 ± 4.5	30.3 ± 1.9
MPO, ng/ml	54.5 ± 16.0	42.8 ± 11.3	50.0 ± 23.6	47.9 ± 13.5
Serum				
ECP, μg/l	4.9 ± 0.6	9.5 ± 1.1 ^b	11.7 ± 0.9 ^b	5.4 ± 1.0
EDN/EPX, ng/ml	8.1 ± 2.1	8.8 ± 1.9	11.9 ± 1.4 ^a	7.0 ± 0.8
EPO, μg/l	15.5 ± 2.0	23.5 ± 1.8 ^a	19.2 ± 2.1	16.7 ± 1.6
Tryptase, μg/l	3.8 ± 0.7	6.9 ± 1.4 ^a	5.3 ± 1.0	4.1 ± 1.2
Histamine, ng/ml	1.2 ± 0.5 (4.1%)	3.6 ± 0.7 ^b (7.5%)	2.5 ± 0.5 ^a (6.9%)	1.1 ± 0.3 (4.3%)
Urine ¹				
LTE ₄ , pg/mg C	52.3 ± 7.1	123.5 ± 11.0 ^a	89.2 ± 9.4	56.7 ± 5.0
9α,11β-PGF ₂ , pg/mg C	31.0 ± 4.8	42.8 ± 9.3	37.5 ± 8.0	33.2 ± 6.3
TXB ₂ , pg/mg C	25.6 ± 3.5	27.1 ± 5.5	43.9 ± 6.5 ^a	21.6 ± 3.5
EDN/EPX, ng/mmol C	11.0 ± 5.2	35.4 ± 7.0 ^a	20.3 ± 5.7	16.5 ± 4.2

Values are means ± SD. PBMC = Peripheral blood mononuclear cells. Statistically significant difference (either of normal values or of values measured in healthy control subjects): ^a $p \leq 0.05$; ^b $p < 0.05$. Normal values: blood erythrocyte count 4.0–6.0 × 10¹²/l; blood leukocyte count 4.0–10.0 × 10⁹/l; blood eosinophil count <300 × 10⁶/l; blood thrombocyte count 150–400 × 10⁹/l; blood differential count: 0.0–0.15 × 10⁹/l (basophils), 0.0–

0.50 × 10⁹/l (eosinophils), 2.0–7.2 × 10⁹/l (neutrophils), 0.15–0.9 × 10⁹/l (monocytes), 1.0–4.0 × 10⁹/l (lymphocytes); erythrocyte sedimentation rate <20 mm/1 h; histamine ≤1.5 ng/ml, percentage of 'completes' (total potential content of histamine in the blood, mostly in basophils).

¹ Concentrations of particular factors in urine are related to urine creatinine (C).

Table 2. Survey of the allergens causing particular types of asthmatic response

Allergen	Concentration BU/ml	DYAR (n = 25)	IAR (n = 36)	LAR (n = 31)
<i>Dermatophagoides pteronyssinus</i>	1,000	7	9	8
<i>Dermatophagoides farinae</i>	1,000	1	0	1
Animal danders				
Dog	3,000	1	5	3
Cat	1,000	1	3	2
Horse	2,000	0	0	1
Guinea pig	2,000	1	1	1
Feather				
Pigeon	3,000	1	0	2
Parakeet	3,000	1	1	0
Canary	3,000	0	0	1
<i>Aspergillus fumigatus</i>	1,000	1	0	1
Pollen				
Grass mix I	1,000	5	5	4
Grass mix II	1,000	2	6	2
Tree mix	3,000	1	3	0
Weed mix	1,000	1	0	1
Birch	1,000	1	2	1
Ragweed short	1,000	0	1	2
Ragweed giant	1,000	1	0	1

Danders = Hairs and squamae; grass pollen mix I (equal parts) = *Dactylis glomerata*, *Lolium perenne*, *Phleum pratensis*, *Poa pratensis*; grass pollen mix II (equal parts) = *Festuca pratensis*, *Holcus lanatus*, *Agrostis alba*, *Anthoxanthum odoratum*; tree pollen mix (equal parts) = *Betula pendula*, *Corylus avellana*, *Juniperus communis*, *Salix alba*; weed pollen mix (equal parts) = *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, *Taraxacum officinale*.

Control Subjects

Three groups of control subjects volunteered to participate in this study: 36 asthmatics demonstrating an isolated IAR/EAR (amplitude $32 \pm 11\%$), 31 asthmatics developing an isolated LAR (amplitude $35 \pm 9\%$) and 38 healthy subjects (tables 1, 2).

Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) diluted in PBS were used in concentrations of 100–500 biologic units (BU)/ml for skin tests and 1,000–3,000 BU/ml for BPTs (table 3). The recommended concentrations by the manufacturer were 500 BU/ml for skin tests and up to 5,000 BU/ml for bronchoprovocation.

Skin Tests

Scratch tests with allergenic extracts in a concentration of 500 BU/ml were carried out and evaluated after 20 min. If negative, then intradermal tests were performed in concentrations of 100 BU/ml, and then 500 BU/ml, and evaluated 20 min, 6, 12, 24, 36,

48, 72 and 96 h after the intracutaneous injection. A skin wheal reaction (>7.0 mm in diameter) appearing 20 min after the injection was considered to be a positive immediate skin response, skin infiltration occurring between 6 and 12 h to be a late skin response, and skin induration recorded later than 48 h was designated a delayed skin response [4–7].

Bronchial Provocation Tests (BPT)

The BPTs were performed by means of spirometry (Spirograph D-75, Lode, Groningen, The Netherlands) recording the forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV₁) values. The FEV₁ values and FEV₁/FVC ratio were considered to be indicative parameters for bronchial obstruction. The aerosolized allergen extracts and PBS were administered to the patients by means of the Wiesbadener Doppel Inhalator at an airflow of 10 l/min. The nebulizer output was 0.12–0.14 ml/min and the aerosol particles had a median mass diameter of 2.8–3.6 μ m.

The BPTs [4–7, 44] were performed according to the following schedule: (1) baseline values recorded at 0, 5 and 10 min; (2) PBS control values recorded at 0, 5 and 10 min after a 10-min PBS inhalation; (3) inhalation of the allergen aerosol for 2×5 min, with inserted FEV₁ and FVC measurement, followed by recording of the spirometric values at 0, 5, 10, 20, 30, 45, 60, 90 and 120 min and every hour up to the 12th hour and every second hour during the 22nd and 38th and the 46th and 56th hour interval [6]. A 5-day interval was always inserted between the end of the preceding test and the beginning of the following test to prevent carry-over effects and to allow patient recovery.

All BPTs were performed in a period without manifest bronchial complaints, outside the allergen-relevant season and during hospitalization under standard conditions. Inhaled glucocorticosteroids (n = 15) and long-acting β_2 -sympathomimetics (n = 2) were withdrawn 4 weeks, cromolyn (n = 1), nedocromil sodium (n = 5), anti-leukotrienes (n = 1) and xanthine derivatives (n = 1) 2 weeks, while short-acting β_2 -sympathomimetics and histamine H₁ receptor antagonists 48 h before each BPT. The FEV₁ decrease of $\geq 50\%$, with respect to the predicted values, was treated with inhalation of a single dose of 200–400 μ g salbutamol.

Other Diagnostic Parameters

In vivo and in vitro diagnostic parameters were recorded either singly (tables 1, 2) or repeatedly during the repeated DYARs and PBS controls (table 4). Th1/Th2 ratio (%) values in peripheral blood were determined by means of flow cytometry, and the intracellular cytokines in the peripheral blood mononuclear cells, interferon- γ and interleukin-4 were estimated by means of immunoassay ELISA kits, as described in detail previously [7]. Histamine serum concentrations, so-called 'blanks', were measured by Siraganian's fluorometric method [7, 45].

Supplementary Immunologic Parameters

Blood

The samples of venous blood were obtained before and then at 1, 12, 24, 36, 48, 56 and 72 h after the allergen challenge. Venous blood samples (5 ml) were collected into separator tubes (S-Monivette, Sarstedt, Germany), kept at room temperature for 1 h and then centrifuged at $3,000 \times g$ for 10 min at 4°C. The serum supernatants were removed, stored at 2–8°C and processed with-

Table 3. Survey of the single diagnostic parameters

	DYAR patients (n = 25)	Control subjects		
		asthmatics		healthy subjects (n = 38)
		IAR (n = 36)	LAR (n = 31)	
Positive skin response ¹				
Immediate	10	27	11	1
Late	1	9	19	0
Delayed	14	0	0	0
Increased total IgE in the serum	0	3	1	0
Positive specific IgE in the serum	0	12	3	0
Increased total IgG in the serum	0	1	13	0
Increased IgG subclasses in the serum				
IgG ₁	0	0	2	0
IgG ₂	0	1	0	0
IgG ₃	0	0	5	1
IgG ₄	0	0	12	0
Increased total IgM in the serum	0	0	1	0
Increased total IgA in the serum	0	0	0	0

Normal values: total IgE (PRIST) <500 IU/ml; positive allergen-specific IgE (ImmunoCAP) >0.70 U/ml (>class 1); total IgG (single radial immunodiffusion and ELISA) <15.0 g/l; IgG₁ < 5.0 g/l; IgG₂ <2.6 g/l; IgG₃ <0.4 g/l; IgG₄ <0.5 g/l; IgM <3.8 g/l (<1.5); IgA <4.0 g/l (<3.2).

¹ Intradermal skin test with allergen causing DYAR.

in 1 h. Other venous blood portions were collected in vacutainers containing EDTA, kept for 1 h at room temperature and then centrifuged at $2,500 \times g$ for 10 min at 4°C. The plasma supernatants were removed and processed within 1 h. The resting aliquots were stored at -70°C.

The serum/plasma levels of appropriate factors were measured by using commercially available kits, following manufacturers' recommendations. Tryptase, eosinophil cationic protein (ECP), eosinophilic peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) were estimated in the serum, whereas all other factors were determined in the plasma. All measurements were performed in duplicate by a double-blind schedule. The intra-assay as well as the inter-assay coefficients of variation for all the assay kits employed were <10%. (1) Leukotrienes B₄ (LTB₄), LTC₄, LTE₄ – EIA kits (Cayman Chemical Company, Ann Arbor, Mich., USA). Detection limits (DLs) for LTB₄, LTC₄ and LTE₄ were 4.8, 2.0 and 3.7 pg/ml, respectively. (2) Prostaglandin D₂ (PGD₂), PGE₂, PGF_{2α} – EIA kits (Cayman Chemical). DLs for PGD₂, PGE₂ and PGF_{2α} were 200, 15 and 12.8 pg/ml, respectively. (3) Thromboxane B₂ (TXB₂) – EIA kit (Cayman Chemical). DL for TXB₂ was 11 pg/ml. (4) Lipoxin A₄ (LXA₄) – ELISA kit (Neogen Corporation, Lansing, Mich., USA). DL for LXA₄ was 20 pg/ml. (5) ECP – ImmunoCAP (Pharmacia Diagnostics, Uppsala, Sweden). DL for ECP was 2 µg/l. (6) EDN – ELISA kit (MBL International Corp., Woburn, Mass., USA). DL for EDN/EPX was 0.62 ng/ml. (7) EPO – immunofluorometric assay ImmunoCAP (Pharmacia Diagnostics). DL for EPO was 0.5 µg/l. (8) Tryptase – ImmunoCAP (Pharmacia Diagnostics). DL for tryptase was 1.0 µg/l. (9) Myeloperoxidase (MPO) – ELISA kit (Oxis International Inc., Portland, Oreg., USA). DL for MPO was 25 ng/ml.

Urine

The samples of urine were obtained before and then at 1, 12, 24, 36, 48, 56 and 72 h after the bronchial allergen challenge.

Urine samples were centrifuged at $1,350 \times g$ for 10 min at 4°C, aliquoted (20–30 ml each), transferred to plastic tubes, stored at 4°C and analyzed within 1 h. The concentrations of particular factors were measured by using commercially available kits, following the manufacturers' recommendations and related to urine creatinine determined by the alkaline picrate method (Grainer Diagnostics AG, Langenthal, Switzerland). All measurements were performed in duplicate by a double-blind schedule. The intra-assay as well as the inter-assay coefficients of variations for all the assay kits employed were <10%. (1) LTE₄ – EIA kit (Cayman Chemical). DL for LTE₄ was 25 pg/ml. (2) 9α,11β-PGF₂ – EIA kit (Cayman Chemical). DL for 9α,11β-PGF₂ was 5.5 pg/ml. (3) TXB₂ – EIA kit (Cayman Chemical). DL for TXB₂ was 11 pg/ml. (4) EPX/EDN – ELISA kit (MBL International). DL for EPX/EDN was 0.62 ng/ml.

Statistical Analysis

The BPTs were statistically evaluated by means of fitting polynomials to the mean curves over time: 8 time points within 120 min and 20 time points up to 56 h after the allergen challenge. The hypotheses were tested by generalized multivariate analysis of variance (MANOVA) [46]. The post-challenge values of particular factors recorded at each of the time points during DYAR as well as the PBS control test in individual patients were compared with their pre-challenge values and evaluated by the Wilcoxon matched-pair signed rank test. The mean post-challenge values measured at each of the time points during DYAR were

Table 4. Survey of DYAR-related diagnostic parameters

Patients (n = 25)	Before the challenge	After the challenge						
		1 h	12 h	24 h	36 h	48 h	56 h	72 h
Bronchial complaints ¹								
Dyspnea	÷	÷	÷	++ ^b	++++ ^c	+++ ^b	++ ^b	÷
Wheezing	0	0	0	+	++ ^b	+++ ^c	++ ^b	0
Cough	0	0	0	0	+	÷	0	0
Expectoration	0	0	0	0	0	0	0	0
General complaints ¹								
Pressure on the chest	0	0	0	+	+	+	0	0
Chills	0	0	0	÷	÷	0	0	0
Tiredness	0	0	0	0	0	++ ^b	++ ^b	++ ^b
Headache	0	0	0	0	0	+ ^b	0	0
Increased total IgE in the serum	0	–	0	–	0	–	0	–
Positive specific IgE in the serum	0	–	0	–	0	–	0	–
Increased total IgG in the serum	0	–	0	–	0	–	0	–
Blood leukocyte count, × 10 ⁹ /l	8.1 ± 0.7	9.6 ± 0.4	10.9 ± 0.5	11.7 ± 0.6 ^b	13.1 ± 0.5 ^b	12.6 ± 0.4 ^b	11.5 ± 0.4 ^b	10.1 ± 0.2
Blood eosinophil count, × 10 ⁶ /l	268 ± 31	300 ± 27	332 ± 18	274 ± 25	296 ± 31	290 ± 28	288 ± 29	279 ± 28
Blood thrombocyte count, × 10 ⁹ /l	254 ± 41	312 ± 21	287 ± 39	300 ± 45	280 ± 37	265 ± 33	304 ± 30	260 ± 37
Blood neutrophil count, × 10 ⁹ /l	5.8 ± 0.7	6.3 ± 0.5	6.0 ± 0.8	8.4 ± 0.6 ^b	8.1 ± 0.8 ^b	7.7 ± 1.0 ^a	6.5 ± 0.7	7.0 ± 0.4
Blood differential count, × 10 ⁹ /l								
Basophils	0.12	0.07	0.15	0.11	0.05	0.15	0.05	0.05
Eosinophils	0.4	0.2	0.3	0.5	0.2	0.3	0.25	0.2
Neutrophils	4.9	5.7	7.6 ^a	8.9 ^b	8.3 ^b	8.5 ^b	7.4 ^a	5.5
Monocytes	0.8	1.0	0.9	1.6 ^b	0.8	0.5	1.3 ^a	0.9
Lymphocytes	2.4	1.9	3.8 ^b	4.1 ^c	3.9 ^b	4.2 ^c	3.0	2.8
Th1/Th2 ratio (%)	7.7 ± 3.0	9.4 ± 4.0	9.9 ± 4.1	14.6 ± 5.0 ^a	19.7 ± 4.5 ^c	16.9 ± 5.1 ^b	15.3 ± 4.8 ^b	10.9 ± 5.1 ^a
Interferon-γ, pg/ml	165 ± 109	205 ± 131	288 ± 131 ^a	370 ± 115 ^b	444 ± 169 ^b	483 ± 151 ^c	345 ± 104 ^b	207 ± 113
Interleukin-4, pg/ml	24.1 ± 2.0	30.2 ± 5.1	30.3 ± 3.7	22.6 ± 5.0	23.4 ± 4.9	28.3 ± 4.2	20.9 ± 5.4	21.2 ± 4.5

Data are means ± SD. 0 = Normal (negative, no changes); – = not determined; ÷ = doubtful value. Statistical significance: ^a $p \leq 0.05$; ^b $p < 0.05$; ^c $p < 0.01$. Normal values: total IgE <500 IU/ml; specific IgE <0.70 U/ml; total IgG <15.0 g/l; blood leukocyte count 4.0–10 × 10⁹/l; blood eosinophil count <300 × 10⁶/l; blood thrombocyte count 150–400 × 10⁹/l; blood neutrophil count 2.0–7.2 × 10⁹/l; blood differential count <0.2 × 10⁹/l (basophils),

<0.5 × 10⁹/l (eosinophils), 2.0–7.0 × 10⁹/l (neutrophils), 0.2–1.0 × 10⁹/l (monocytes), 1.0–3.0 × 10⁹/l (lymphocytes).

¹ Bronchial and general complaints (author's modified scoring system): 0 = absent, ÷ = very slight/incidental, + = slight, ++ = moderate/intermittent, +++ = pronounced/regularly, ++++ = very pronounced/distinct/frequent.

compared with PBS control values recorded at the corresponding time points and analyzed by the Wilcoxon matched-pair signed rank test. A p value < 0.05 was considered to be statistically significant.

Results

DYAR recorded in 25 asthma patients began within 26–32 h, reached a maximum within 32–48 h and resolved within 56 h (fig. 1). DYAR was statistically highly significant as compared with the PBS control challenge

($p < 0.001$). Repeated DYAR (fig. 1) was also statistically highly significant both in comparing the post-challenge with the pre-challenge FEV₁ values ($p < 0.01$) and as compared with the PBS control challenge ($p < 0.001$).

No significant differences were found between the initial and the repeated DYARs ($p > 0.1$).

The initial as well as the repeated DYAR were associated with dyspnea, wheezing, increased blood leukocyte, neutrophil and lymphocyte counts, non-increased eosinophil count, significant changes in the Th1/Th2 cell ratio in peripheral blood in favor of Th1 cells ($p < 0.01$) and a significant increase in the intracellular concentration of

Table 5. Plasma/serum concentrations of particular factors (mean $\pm$ SEM) recorded during DYAR and PBS control tests (n = 25)

Patients (n = 25)	Before the challenge	After the challenge						
		1 h	12 h	24 h	36 h	48 h	56 h	72 h
LTB ₄ , pg/ml								
DYAR	31.2 ± 3.7	27.5 ± 4.1	39.1 ± 4.4	78.9 ± 5.5 ^b	89.8 ± 5.2 ^b	72.3 ± 4.6 ^b	58.3 ± 3.9 ^a	35.2 ± 3.0
PBS	33.5 ± 2.9	32.0 ± 3.4	35.5 ± 3.2	29.7 ± 2.6	30.1 ± 3.0	31.5 ± 2.8	32.7 ± 3.5	30.8 ± 4.2
LTC ₄ , pg/ml								
DYAR	9.2 ± 2.7	10.1 ± 1.3	10.9 ± 2.0	9.8 ± 1.6	11.5 ± 3.3	10.4 ± 2.9	10.7 ± 1.5	10.3 ± 1.8
PBS	9.7 ± 2.6	12.3 ± 2.2	10.5 ± 3.0	10.7 ± 2.5	10.0 ± 1.9	9.2 ± 1.6	8.8 ± 1.7	9.9 ± 2.0
LTE ₄ , pg/ml								
DYAR	44.8 ± 4.6	42.0 ± 3.3	47.5 ± 4.3	38.9 ± 4.0	40.2 ± 3.4	45.5 ± 5.2	39.7 ± 3.8	40.3 ± 2.9
PBS	41.1 ± 5.5	36.5 ± 4.1	39.0 ± 3.9	51.3 ± 4.4	46.5 ± 5.1	40.0 ± 4.7	43.2 ± 4.0	38.7 ± 4.2
PGD ₂ , pg/ml								
DYAR	363 ± 32	410 ± 52	445 ± 47	398 ± 40	361 ± 26	425 ± 38 ^a	403 ± 44	387 ± 35
PBS	339 ± 31	394 ± 27	325 ± 41	371 ± 33	415 ± 39	369 ± 30	355 ± 32	343 ± 26
PGE ₂ , pg/ml								
DYAR	962 ± 53	1,176 ± 89	1,236 ± 93 ^a	989 ± 55	1,064 ± 72	1,109 ± 65	910 ± 54	1,010 ± 48
PBS	1,031 ± 67	1,079 ± 61	872 ± 45	1,100 ± 83	967 ± 58	1,032 ± 43	1,056 ± 69	994 ± 56
PGF _{2α} , pg/ml								
DYAR	362 ± 28	389 ± 35	400 ± 41	357 ± 35	312 ± 29	333 ± 25	377 ± 42	326 ± 31
PBS	343 ± 25	311 ± 19	354 ± 23	360 ± 43	325 ± 32	315 ± 29	335 ± 22	338 ± 20
TXB ₂ , pg/ml								
DYAR	414 ± 31	457 ± 45	431 ± 33	402 ± 28	429 ± 35	457 ± 37	444 ± 25	421 ± 32
PBS	450 ± 29	429 ± 34	448 ± 49	463 ± 54	446 ± 28	425 ± 30	410 ± 21	433 ± 24
LXA ₄ , pg/ml								
DYAR	29.3 ± 3.7	21.6 ± 4.0	25.7 ± 3.1	30.6 ± 2.0	23.8 ± 3.9	19.8 ± 3.3	24.2 ± 3.6	27.1 ± 2.7
PBS	27.1 ± 4.2	25.5 ± 3.5	22.4 ± 2.6	28.8 ± 3.8	30.0 ± 2.5	26.9 ± 3.0	21.4 ± 2.9	25.6 ± 2.3
ECP, µg/l								
DYAR	5.5 ± 1.2	7.1 ± 2.2	6.3 ± 1.9	8.9 ± 3.0	7.5 ± 2.6	6.2 ± 2.1	6.7 ± 2.0	5.9 ± 2.3
PBS	6.1 ± 1.3	5.9 ± 1.7	5.6 ± 1.5	6.4 ± 2.1	6.0 ± 3.0	5.7 ± 2.3	5.8 ± 1.4	5.4 ± 1.6
EDN, ng/ml								
DYAR	5.9 ± 0.8	4.6 ± 1.5	6.7 ± 2.2	7.3 ± 2.1	7.0 ± 1.9	6.6 ± 2.0	6.1 ± 2.7	5.5 ± 0.9
PBS	5.0 ± 1.0	5.1 ± 1.3	5.4 ± 1.8	6.0 ± 2.5	6.2 ± 2.9	5.3 ± 1.5	4.8 ± 1.1	5.1 ± 1.4
EPO, µg/l								
DYAR	17.2 ± 2.5	15.5 ± 3.2	18.1 ± 2.9	21.1 ± 1.8	19.4 ± 2.3	20.2 ± 1.9	17.8 ± 2.1	18.5 ± 2.4
PBS	19.0 ± 2.9	21.2 ± 2.7	21.8 ± 1.6	18.0 ± 2.2	16.9 ± 1.5	17.4 ± 2.6	18.1 ± 1.7	17.9 ± 2.0
MPO, ng/ml								
DYAR	65.8 ± 12.6	58.0 ± 10.3	61.5 ± 11.1	115.4 ± 13.9 ^a	165.5 ± 27.0 ^b	161.0 ± 19.0 ^b	98.9 ± 17.1	71.3 ± 14.8
PBS	70.1 ± 13.4	73.5 ± 9.6	76.8 ± 8.1	61.7 ± 10.0	68.3 ± 5.6	75.2 ± 11.0	67.2 ± 9.8	69.0 ± 9.5
Tryptase, µg/l								
DYAR	4.3 ± 1.4	5.1 ± 1.0	4.5 ± 0.8	5.0 ± 1.7	4.4 ± 0.9	5.3 ± 1.0	4.9 ± 1.3	4.7 ± 1.1
PBS	4.5 ± 1.7	4.0 ± 0.5	5.2 ± 1.1	5.5 ± 1.0	5.1 ± 1.4	4.8 ± 0.6	4.4 ± 0.7	4.3 ± 0.9
Histamine, ng/ml ¹								
DYAR	0.6 ± 0.3	1.4 ± 0.5	1.1 ± 0.4	1.2 ± 0.3	0.9 ± 0.6	0.5 ± 0.2	1.3 ± 0.5	0.8 ± 0.3
	5.5%	6.2%	6.0%	5.7%	5.1%	4.6%	4.9%	5.0%
PBS	1.0 ± 0.5	0.9 ± 0.5	1.6 ± 0.7	0.8 ± 0.4	1.3 ± 0.5	1.1 ± 0.3	1.4 ± 0.6	1.2 ± 0.5
	5.7%	4.3%	6.1%	4.5%	4.8%	5.2%	5.1%	4.7%

^a p $\leq$ 0.05; ^b p < 0.05.¹ Normal value in serum <1.5 ng/ml; percentage of 'completes' (total potential content of histamine in the blood, mostly in basophils).

Table 6. Concentrations of particular factors recorded in urine (mean $\pm$ SEM) during the DYAR and PBS control tests (n = 25)

Patients (n = 25)	Before the challenge	After the challenge						
		1 h	12 h	24 h	36 h	48 h	56 h	72 h
LTE ₄ , pg/mg C								
DYAR	51.3 ± 9.1	57.3 ± 4.5	60.4 ± 3.3	45.7 ± 6.0	55.0 ± 3.9	63.2 ± 7.7	54.6 ± 5.3	52.2 ± 3.8
PBS	54.5 ± 6.0	44.9 ± 5.7	49.0 ± 4.2	53.8 ± 7.5	49.2 ± 6.1	54.8 ± 5.4	50.2 ± 4.1	45.5 ± 4.6
9α,11β-PGF ₂ , pg/mg C								
DYAR	29.4 ± 5.2	25.6 ± 2.8	33.5 ± 4.4	27.9 ± 3.1	26.6 ± 5.0	32.5 ± 4.8	28.0 ± 2.7	24.3 ± 4.0
PBS	30.9 ± 7.5	31.8 ± 4.6	28.7 ± 5.3	21.0 ± 5.2	27.7 ± 6.5	25.4 ± 7.0	26.2 ± 4.4	21.7 ± 5.5
TXB ₂ , pg/mg C								
DYAR	23.2 ± 8.0	31.7 ± 8.5	32.9 ± 6.5	27.2 ± 4.9	25.5 ± 3.8	20.6 ± 3.0	25.5 ± 3.9	23.0 ± 3.1
PBS	20.8 ± 4.1	22.2 ± 1.8	26.4 ± 5.5	24.0 ± 3.8	20.7 ± 4.3	25.9 ± 5.1	28.7 ± 4.8	20.6 ± 2.2
EPX/EDN, ng/mmol C								
DYAR	15.6 ± 6.4	13.4 ± 1.5	11.9 ± 2.0	16.3 ± 4.2	14.0 ± 2.7	12.8 ± 3.1	12.5 ± 2.9	14.4 ± 3.0
PBS	12.8 ± 3.5	12.3 ± 2.3	11.6 ± 1.4	13.6 ± 2.8	12.0 ± 2.1	13.7 ± 2.5	11.8 ± 3.0	12.9 ± 2.4

Concentrations of particular factors in urine are related to the urine creatinine concentration (C).

interferon- γ ($p < 0.05$) but not interleukin-4 ($p > 0.05$) cytokines (table 4).

Repeated DYAR has been associated with a significantly increased plasma concentration of LTB₄ at 24 h ($p < 0.05$), 36 h ($p < 0.05$), 48 h ($p < 0.05$) and doubtfully at 56 h ($p \leq 0.05$) after the allergen challenge as compared both with the pre-challenge values and relevant PBS control values (table 5). Almost parallel, also the plasma concentration of MPO increased doubtfully at 24 h ($p \leq 0.05$), but significantly at 36 h ($p < 0.05$) and 48 h ($p < 0.05$) after the allergen challenge as compared both with the pre-challenge values and with the relevant PBS control values (table 5).

No significant changes in the concentrations of LTC₄, LTE₄, PGD₂, PGE₂, PGF_{2 α} , TXB₂, LXA₄, ECP, EDN/EPX, EPO, tryptase and histamine were recorded in peripheral blood during DYAR as compared with the pre-challenge values ($p > 0.05$ to $p > 0.1$) as well as with the relevant PBS control values ($p > 0.05$; table 5). No significant differences were found between the duplicated measurements of these factors in the blood ($p > 0.1$). No significant concentration changes of these factors were measured in the peripheral blood during the PBS controls ($p > 0.05$; table 5).

The concentrations of LTE₄, 9 α ,11 β -PGF₂, TXB₂ and EDN/EPX recorded in the urine did not demonstrate any significant changes during DYAR or PBS control challenge (table 6). No significant differences were found between the values of these factors recorded in the duplicates of urine ($p > 0.05$).

No statistically significant differences in the pre-challenge concentrations of these factors were recorded in the peripheral blood of the control patients developing IAR and LAR, or in healthy subjects as compared with those measured in the DYAR patients ($p > 0.05$, > 0.1 and > 0.01 , respectively) (table 1).

Discussion

DYAR differs from IAR/EAR and LAR in its clinical course and features, association with various in vivo and in vitro diagnostic parameters, immunologic features and hypersensitivity types underlying these asthmatic responses [4–8, 10, 15, 16, 20–25, 42].

Each of the hypersensitivity mechanisms is characterized by typical involvement of particular cell types, releasing a complex of mediators, chemotactic factors, eicosanoids, cytokines, chemokines, immunoglobulins enzymes and expressing typical adhesion molecules and receptors (CD molecules). The individual cell types, activated by various intercellular signal mechanisms, follow specific traffic patterns on various levels in the blood and bronchial tissue (epithelium, mucosal and submucosal layers, smooth muscles and secretory glands), leading to a variety of inflammatory processes, bronchoconstriction, increased sputum production and airway remodeling [2–43, 47–58].

Additionally, a number of other factors, such as particular clotting system, metabolic system, hormones and

genetic factors, can directly or indirectly contribute to the individual hypersensitivity mechanisms underlying the particular types of asthmatic response [1–3].

Despite our extensive knowledge, the detailed extent of all immunologic and metabolic mechanisms involved in the particular types of asthmatic response to allergen challenge is not yet fully clarified [1–5, 7, 9–13, 17, 19, 26, 27, 48, 59, 60].

Most of the clinical and research data have been gathered from asthma patients without further differentiation of the particular types of asthmatic response by means of BPTs [18, 19, 26, 29, 31, 33–39, 41, 43, 49–51, 58, 61–67]. Such an approach resulted in the collection of data which cannot be properly related to specific and well-defined types of hypersensitivity and the underlying immunologic processes. The BPT with allergen is the only method demonstrating the existence and participation of a certain type of asthmatic response in the individual patient to which the other *in vivo* and *in vitro* diagnostic parameters and immunologic aspects can be related [4–8, 10, 14–16, 19–25, 30, 32, 52–57].

In most of the *in vivo* studies, either the single estimation of these factors has been performed in the (induced) sputum [19, 26, 47, 51, 61–67], bronchoalveolar lavage (BAL) fluid [18, 50], blood [14, 35, 40–41, 51, 59, 65, 67], lung biopsy [26, 43], urine [35] or exhaled breath condensate [58], or their concentrations were measured repeatedly in BAL fluid [8, 14, 15, 20, 21, 42, 53, 54], induced sputum [22, 28, 31, 55], blood [7, 15, 16, 30, 39, 52], exhaled breath condensate [56], urine [23, 24, 30, 32, 57] or lung biopsy [15] after a segmental/endobronchial provocation with allergen. However, the changes of these factors in sputum, BAL fluid, urine or exhaled breath condensate were usually measured within a relatively short period of time, usually 1–2 h [8, 53, 54], and exceptionally up to 12 h [16, 20, 23] or 24 h [15, 21, 22, 52] after the allergen provocation.

Relatively few studies deal with the determination of these factors in BAL fluid [8, 21, 42], induced sputum [22, 28, 31, 55], blood [16, 25, 52, 68], urine [23, 24, 32, 57] or exhaled breath condensate [56] after a BPT performed by inhalation of allergen. In some of these studies, the recorded factor changes are related to the particular types of the asthmatic response, such as an IAR [4, 16, 23–25, 28, 42, 53, 57] or LAR [5, 15, 16, 21–25, 31, 55, 56].

In most of the *in vitro* studies, the changes of these factors were measured after a non-specific stimulation [14, 18, 33, 53, 54] or sporadically after an allergen-specific stimulation [39] of the particular cell types, e.g., eosinophils, basophils, neutrophils and alveolar macro-

phages, isolated from the BAL fluid, induced sputum or peripheral blood [14, 34, 36, 39]. The patients included in these studies mostly demonstrated the classical type of bronchial asthma attributed to the immediate hypersensitivity upon involvement of immunoglobulin E (IgE) antibody, mast cells/basophils, eosinophils and Th2 lymphocytes [1, 2].

However, the growing importance of LAR [1–3, 5, 8, 10, 15, 16, 20–25, 35, 42, 52], together with findings of absence of IgE antibody and/or eosinophilia, in some asthmatic patients, would indicate additional involvement of the so-called 'non-immediate' (non-IgE, IgE-independent) mechanisms in bronchial asthma [59, 60]. The later reported existence of the various phenotypes of bronchial asthma, such as the eosinophilic, non-eosinophilic, neutrophilic and paucigranulocytic type [9, 11–13, 19, 26–28, 50, 62–67], creates a necessity for more and focused research of the role of non-immediate hypersensitivity mechanisms in the clinical bronchial asthma.

The target of this clinical study was to investigate the possible participation of the mast cells, basophils, eosinophils and neutrophils, in addition to the already documented involvement of Th1 cells, in the mechanism(s) underlying DYAR [7, 68].

The role of LTs, PGs and other products of activated target cells such as eosinophils, neutrophils, mast cells, basophils, lymphocytes, alveolar macrophages and bronchial epithelial cells in bronchial asthma has already been investigated from various points of view [8, 14–26, 28–43, 47, 49–59].

Our findings of an increased count of neutrophils [7, 68] and increased secretion of their products, such as LTB₄ and MPO (table 5), in the peripheral blood during DYAR is partly consistent with the other investigators' reports of the existence of an asthma phenotype associated with increased neutrophil, but not eosinophil, count in peripheral blood [9, 11, 19, 30, 33, 65, 67], sputum or BAL fluid [8, 12, 19–21, 26–28, 50, 62–67]. Therefore, DYAR would represent a new phenotype of bronchial asthma, where the neutrophils and Th1 cells may play a pivotal role, besides the already reported other asthma phenotypes [1–3, 9, 11–17, 19, 26, 31, 38, 39, 47, 50, 59, 61–67].

In most patients with bronchial asthma, especially in those in whom the immediate hypersensitivity may play a predominant role, an increased count of circulating eosinophils and sometimes also basophils, increased concentrations of allergen-specific IgE, histamine, LTC₄, ECP, EPO, EDN and sometimes tryptase and PGs in blood have been reported [14–16, 20–22, 29, 31, 41, 51–55, 61].

These results are consistent with findings of increased concentrations of various factors in the BAL fluid and induced sputum of patients suffering from bronchial asthma due probably to the immediate hypersensitivity, such as histamine [8, 20, 42, 47, 53], tryptase [28], LTC₄ [8, 47, 53], LTB₄ [21, 36], LTE₄ [42], LTD₄ [47], PGD₂ [8, 42, 53, 54], PGF_{2α} [20], ECP [14, 18, 28, 31, 47, 51], TXB₂ [8, 20, 42, 53], or LXA₄ [40]. Also, increased concentrations of LTE₄ [30] and PGF_{2α} [30] were found in the urine of asthmatics. The comparable changes, mostly an increase in concentration of these factors, were observed in the BAL fluid or induced sputum after the BPT [16, 21, 22, 28, 31, 55] or the segmental provocation with allergen [15, 42, 53, 54] in asthmatics who have developed either IAR [31, 51, 55] or LAR [8, 15, 20–22, 28, 31, 53–55]. The increased concentrations of MPO and LTB₄ were found in blood [29, 35, 41], sputum and BAL [19, 37, 50] or in exhaled breath condensate [37, 49] of asthmatics.

However, significant changes in the concentrations of MPO or LTB₄ in the blood of the patients developing either IAR or LAR to allergen challenge were not found by us in the available literature.

In contrast, significantly increased concentrations of LTB₄ and MPO have been measured in the peripheral blood during DYAR (table 5). No significant changes in blood concentrations of LTC₄, LTE₄, PGD₂, PGE₂, PGF_{2α}, TXB₂, LXA₄, ECP, EDN, EPO or tryptase or in urine concentrations of LTE₄, TXB₂, 9α,11β-PGF₂ or EDN/EPX were recorded during DYAR (tables 5, 6).

However, some of the aspects concerning the possible mechanism(s) involved in DYAR may probably be detected and clarified by serial determination of the above-studied factors in repeated (induced) sputum, BAL and/or bronchial biopsies. Unfortunately, no reliable data concerning the appearance and changes of eicosanoids, eosinophil and neutrophil constituents in the (induced) sputum, BAL or lung biopsy during DYAR were available due to technical as well as ethical reasons. This fact may be regarded as a certain deficit of this study and a limitation of the above-presented results.

These findings would support evidence for an involvement of neutrophils, and possibly also monocytes and macrophages, in the immunologic mechanism(s) underlying DYAR. Together with evidence of an active role of Th1 lymphocytes in DYAR [7], these results suggest the possible involvement of delayed (cell-mediated) hypersensitivity mechanism(s) in DYAR. Nevertheless, further concurrent clinical and immunologic studies will be needed to clarify the immunologic mechanisms underlying this clinical phenomenon.

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