
Delayed-type asthmatic response to bronchial challenge with allergen, I: clinical features

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Background: Patients with allergic asthma being challenged with allergen may develop different types of asthmatic response, such as immediate asthmatic response (IAR), late asthmatic response (LAR), or dual late asthmatic response (DLAR), because of different immunologic mechanisms.

Objectives: To investigate the clinical features of delayed asthmatic response (DYAR), its reproducibility, and its association with other in vivo and in vitro diagnostic parameters and to contribute to the understanding of the possible mechanism(s) underlying this unusual clinical phenomenon.

Methods: In 51 asthma patients developing 51 DYARs, the bronchial challenges with the same allergens were repeated and supplemented with additional diagnostic parameters. Control groups consisted of asthma patients developing IAR (n = 56), LAR (n = 43), and DLAR (n = 31) and healthy individuals (n = 48).

Results: The DYAR began at 26 to 32 hours, reached a maximum at 32 to 48 hours, and resolved within 56 hours after the challenge. DYAR was statistically highly significant ($P < .001$) compared with phosphate-buffered saline controls. The differences between the initial and repeated DYAR were not significant ($P = .14$). The DYAR was associated with dyspnea, wheezing, tiredness, increased peripheral blood leukocyte count, lymphocytosis, neutrophilia but not eosinophilia, significant changes in the T_H1/T_H2 ratio in peripheral blood in favor of T_H1 cells, and significant increase in the intracellular concentration of interferon γ but not interleukin 4 or 5.

Conclusions: In addition to the previously established IAR, LAR, and DLAR, existence of another, the so-called DYAR to allergen challenge, has been demonstrated. In this type, the T_H1 cells, together with neutrophils, may well play the predominant causal role.

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INTRODUCTION

The allergic component involved in patients with allergic bronchial asthma has classically been attributed to the immediate hypersensitivity mechanism(s) on involvement of IgE antibody, mast cells and basophils, eosinophils, and T_H2 cells.¹ Patients with bronchial allergy may develop different types of bronchial response to allergen challenge, such as an immediate asthmatic response (IAR), late asthmatic response (LAR), or dual late asthmatic response (DLAR), as already described by several authors and analyzed in our previous studies.^{2–6}

Some patients with allergic asthma examined at our department using bronchial provocation tests (BPTs) developed an unusual asthmatic response appearing later than 24 hours after the allergen challenge. This response type, different from the IAR and LAR, has been designated by us as the delayed asthmatic response (DYAR). The existence of DYAR had already been reported in our preliminary articles.⁶ The purposes of this study were to investigate the clinical features of DYAR, its reproducibility, and its association with

other in vivo and in vitro diagnostic parameters and to contribute to the understanding of the possible mechanism(s) underlying this unusual clinical phenomenon.

METHODS

Patients

Fifty-five of 397 patients with allergic asthma who had been referred to the Department of Allergology & Immunology, Institute of Medical Sciences “De Klokkenberg,” Breda, the Netherlands, between 1999 and 2000 for more extensive diagnostic and therapeutic analysis had developed DYAR during the BPTs performed as a part of the routine diagnostic procedure. Fifty-one of these 55 patients who did not need restricted rescue treatment volunteered to participate in this study. These patients, who were 20 to 45 years of age, showed reversible bronchial constriction alternating with symptom-free periods without any restrictive changes of their pulmonary function (Table 1). They did not have any airway infections and did not use oral corticosteroids or immunotherapy. They were examined using a routine diagnostic procedure (acting also as an inclusion-exclusion check) consisting of (1) general examination (disease history, physical examination, basic laboratory tests, x-ray examination of the chest and sinuses, lung function, blood gases determination, and bacteriologic examination of the sputum), (2) allergologic examination (skin tests, bronchial histamine threshold,⁷ blood leukocyte differential count, and determination of the

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Table 1. Characteristics of the Patients and Controls^a

Characteristics	DYAR patients (n = 51)	Asthmatic patients			Healthy controls (n = 48)
		IAR (n = 56)	LAR (n = 43)	DLAR (n = 31)	
Age, y	34 (5)	29 (7)	31 (9)	30 (4)	32 (6)
Sex, M/F, No.	22/29	25/31	19/24	14/17	23/25
Disease history, y	4.2 (1.6)	5.3 (0.9)	5.0 (1.2)	6.2 (2.0)	0
Asthmatic attacks per month	3 ± 1	5 ± 1	3 ± 2	2 ± 1	0
FEV ₁ , % predicted	94.2 (7.8)	91.5 (5.6)	94.7 (4.1)	96.1 (3.5)	98.4 (6.2)
FVC, % predicted	99.0 (5.3)	102.3 (3.9)	97.9 (8.1)	101.2 (7.2)	105.6 (4.7)
Blood leukocyte count, ×10 ⁹ /L ^b	10.2 (0.7)	7.3 (0.3)	8.4 (0.5)	7.8 (0.4)	7.7 (1.1)
Blood eosinophil count, ×10 ⁶ /L ^c	311 (27)	514 (63) ^e	472 (85) ^e	499 (71) ^e	201 (32)
Blood neutrophil count, ×10 ⁹ /L ^f	5.7 (0.8)	6.1 (1.0)	5.5 (0.7)	6.3 (0.5)	6.0 (1.2)
Blood thrombocyte count, ×10 ⁹ /L ^g	338 (49)	294 (31)	317 (54)	323 (22)	342 (58)
Bronchial histamine threshold ^h					
≤2.0 mg/mL	1	3	4	2	0
4.0 mg/mL	2	4	2	3	0
8.0 mg/mL	5	13	5	6	0
16.0 mg/mL	14	15	9	1	1
32.0 mg/mL	13	11	12	8	1
>32.0 mg/mL	16	10	11	2	46
BPT, total No./No. positive	2–3/1	2–3/1–2	2–3/1	2/1–2	0
T _H 1/T _H 2, %	8.1 (3.0)	7.2 (2.7)	7.8 (3.9)	8.0 (3.6)	8.6 (3.5)
IFN-γ, pg/mL	169 (91)	175 (103)	194 (110)	182 (85)	203 (98)
IL-2, pg/mL	3.1 (0.1)	<3.0	<3.0	<3.0	<3.0
IL-4, pg/mL	20.6 (4.4)	23.2 (5.3)	21.8 (6.5)	19.2 (5.5)	24.7 (4.1)
IL-5, pg/mL	3.8 (1.0)	7.1 (1.2)	8.5 (1.0) ^d	8.7 (1.3) ^d	4.2 (2.3)

Abbreviations: BPT, bronchial provocation tests with allergens; DLAR, dual late asthmatic response; DYAR, delayed asthmatic response; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IAR, immediate asthmatic response; IFN-γ, interferon γ; IL, interleukin; LAR, late asthmatic response.

^a Data are presented as mean (SD) unless otherwise indicated.

^b Reference range, 4.0 to 10.0 × 10⁹/L.

^c Reference range, less than 300 × 10⁶/L.

^d *P* ≤ .05.

^e *P* < .05.

^f Reference range, 2.0 to 7.2 × 10⁹/L.

^g Reference range, 150 to 400 × 10⁹/L.

^h Reference range, greater than 32.0 mg/mL.

serum immunoglobulins), and (3) control challenge with phosphate-buffered saline (PBS) (n = 51) followed by bronchial challenges with allergens (BPT; n = 126). The allergens for BPTs were chosen on the basis of disease history and skin tests (Table 2). The initial BPTs have always been performed with allergenic extracts diluted 1:10, and if the results were negative, then BPTs with undiluted extracts (our standard) followed (Table 2).

In 51 patients demonstrating a DYAR during the routine procedure (Figure 1A), the BPTs with the same allergens and the PBS controls were repeated 2 to 6 weeks later and supplemented with various in vivo and in vitro diagnostic parameters before and repeatedly after the challenge (Tables 1, 3, and 4). A 5-day interval was always inserted between the end of the preceding test and the begin of the following test to prevent the carryover effects and to allow patient recovery. All BPTs were performed under standard conditions in a period without manifest bronchial symptoms outside the allergen-relevant season and during hospitalization.

Inhaled glucocorticosteroids and long-acting β₂-sympathomimetics were withdrawn at 4 weeks; cromolyn, nedocromil sodium, leukotriene modifiers, and xanthine derivatives at 2 weeks; and short-acting β₂-sympathomimetics and histamine₁-receptor antagonists at 48 hours before each of the BPTs. The forced expiratory volume in 1 second (FEV₁) decrease of 50% or more, with respect to the predicted values, was treated with 200 to 400 μg of salbutamol. Four of the 55 patients requiring treatment with intravenous glucocorticosteroids were excluded from the study. The local ethical committee approved this study, and informed oral consent was obtained from all study participants.

Control Groups

Fifty-six patients demonstrating an IAR to allergen challenge, 43 asthmatic patients developing an LAR, 31 asthma patients demonstrating a DLAR (IAR plus LAR), and 48 healthy individuals, who were known to be not have any form of

Table 2. Survey of the Allergens Causing Particular Types of Asthmatic Response^a

Allergen	Concentration, BU/mL	DYAR (n = 51)	NAR (n = 75)	IAR (n = 56)	LAR (n = 48)	DLAR (n = 31)
<i>Dermatophagoides pteronyssinus</i>	1,000	14	21	19	18	12
<i>Dermatophagoides farinae</i>	1,000	1	3	0	1	1
Animal danders						
Dog	3,000	2	5	5	3	1
Cat	1,000	3	7	6	4	2
Horse	2,000	1	1	0	1	0
Guinea pig	2,000	1	2	1	1	0
Hamster	2,000	0	1	1	0	1
Feathers						
Pigeon	3,000	1	3	0	2	0
Parakeet	3,000	1	3	1	0	0
Canary	3,000	0	1	0	1	0
Parrot	3,000	0	0	1	0	1
<i>Aspergillus fumigatus</i>	1,000	1	2	0	1	1
<i>Aspergillus niger</i>	1,000	1	1	0	0	1
Pollen						
Grass mix 1	1,000	11	13	10	8	5
Grass mix 2	1,000	5	4	6	2	1
Tree mix	3,000	2	3	3	0	1
Weed mix	1,000	1	2	0	1	1
Birch	1,000	3	0	2	1	2
Poplar	2,000	1	2	0	1	0
Ragweed short	1,000	1	0	1	2	0
Ragweed giant	1,000	1	1	0	1	1

Abbreviations: DLAR, dual late asthmatic response; DYAR, delayed asthmatic response; IAR, immediate asthmatic response; LAR, late asthmatic response; NAR, negative asthmatic response.

^a Grass pollen mix 1 contained *Dactylis glomerata*, *Lolium perenne*, *Phleum pratensis*, and *Poa pratensis*. Grass pollen mix 2 contained *Festuca pratensis*, *Holcus lanatus*, *Agrostis alba*, and *Anthoxanthum odoratum*. Tree pollen mix contained *Betula pendula*, *Corylus avellana*, *Juniperus communis*, and *Salix alba*. Weed pollen mix contained *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, and *Taraxacum officinale*.

allergy, volunteered to participate in this study as controls (Tables 1 and 5 and Figure 1B–D).

Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) diluted in PBS were used in concentrations of 100 to 500 BU/mL for skin tests and 1,000 to 3,000 BU/mL for BPTs (Table 2). The manufacturer recommended concentrations of 500 BU/mL for the skin tests and up to 5,000 BU/mL for the bronchoprovocations.

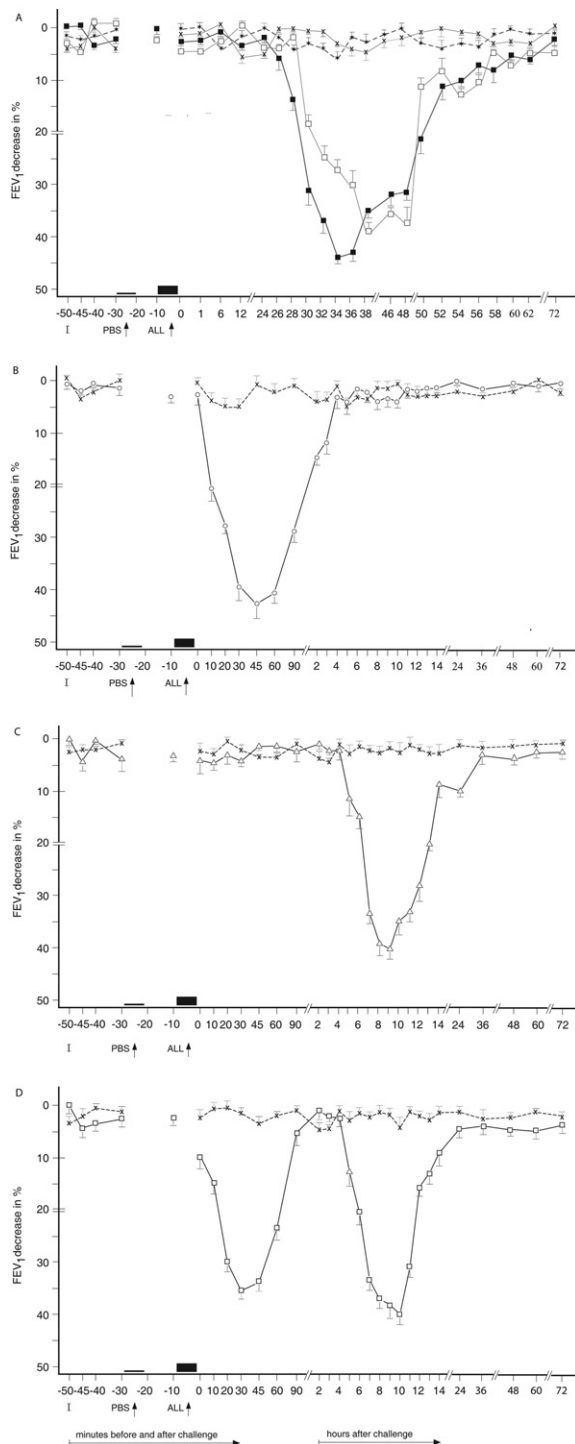
Skin Tests

Scratch tests with allergens in concentrations of 500 BU/mL were performed and evaluated after 20 minutes. If the results were negative, then intracutaneous tests were performed in concentrations of 100 BU/mL and 500 BU/mL and evaluated at 20 minutes and 6, 12, 24, 36, 48, 72, and 96 hours after the injection. A skin wheal (>7.0 mm in diameter) appearing 20 minutes after the injection was considered to be a positive immediate skin response, the skin infiltration observed between 6 and 12 hours to be a late skin response, and the skin induration recorded later than 48 hours was designated a delayed skin response.^{4,5}

Bronchial Provocation Tests

The BPTs were performed by means of spirometry (Spirograph D-75; Lode, Groningen, the Netherlands) recording the forced vital capacity (FVC) and FEV₁ values.⁷ The aerosols of allergenic extracts and PBS were administered by means of the Wiesbadener Doppel inhalator (Wiesbadener Inhalatoren-Vertrieb, Wiesbaden, Germany) at an airflow of 10 L/min. The nebulizer output was 0.12 to 0.14 mL/min. The aerosol particles had a median mass diameter of 2.8 to 3.6 μ m. The following schedule was applied in performing the BPTs: (1) baseline values recorded at 0, 5, and 10 minutes; (2) PBS control values recorded at 0, 5, and 10 minutes after a 10-minute inhalation; (3) inhalation of the allergen aerosol for 2 $\times$ 5 minutes, with inserted spirometric value measurement, followed by recording of the spirometric parameters at 0, 5, 10, 20, 30, 45, 60, 90, and 120 minutes and then every hour up to the 12th hour and every second hour during the 22nd to 38th hours, the 46th to 56th hours, and if necessary up to the 72nd-hour interval.^{4–6} The BPTs were evaluated according to the following criteria: (1) the decrease in FEV₁ of less than 10% with respect to the prechallenge values as negative, from 10% to 20% as doubtful, and of 20% or more

as positive; (2) the decrease in FEV₁ values recorded at least at 3 consecutive intervals was considered to be a positive asthmatic response; and (3) the asthmatic response appearing later than 24 hours after the allergen challenge was considered a positive DYAR.⁴⁻⁶



Other Diagnostic Parameters

In vivo and in vitro diagnostic parameters were recorded either singly (Tables 1 and 5) or repeatedly during the DYAR (Tables 3 and 4).

Supplementary Diagnostic Parameters

Histamine in the serum. Histamine serum concentrations, so-called blanks, were measured using the Siraganian fluorometric method.⁸ The total histamine content was obtained by lysing the cells in whole blood with 2.0% perchloric acid, the so-called completes, and also measured by fluorometric assay. All measurements were duplicated. The spontaneously released histamine in blanks was expressed in nanograms per milliliter and as a percentage of the total histamine content in completes.

T_H1/T_H2 ratio in peripheral blood. Heparinized venous blood (500 μ L) was added to RPMI 1640 medium (Sigma-Aldrich, St Louis, Missouri) with 2-mmol/L l-glutamine (Gibco, Paisley, England), 100 IU/mL of penicillin, and 100 μ g/mL of streptomycin. Cells were stimulated with 25 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1- μ mol/L ionomycin (Sigma-Aldrich) in the presence of 1 μ g/mL of Brefeldin A (Sigma-Aldrich) and cultured in tubes (Becton, Dickinson & Co [BD], Franklin Lakes, New Jersey) for 4 hours at 37°C with 5% carbon dioxide. Activated lymphocytes were confirmed with 96% expression of activation marker CD69 using phycoerythrin (PE)-conjugated CD69 specific monoclonal antibody (MoAb; BD). Activated cultures were aliquoted and stained with 20 μ L of peridinin chlorophyll protein-conjugated CD4-specific MoAb (BD) for 15 minutes at room temperature. Erythrocytes were lysed by adding 2 mL of fluorescence-activated cell-sorting (FACS) lysing solution (BD) for 10 minutes. After centrifugation, the cells were washed in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide and subsequently resuspended in 500 μ L of FACS permeabilizing solution (BD) for 10 minutes at room temperature in the dark. The cells were washed twice with PBS and incubated with saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated interferon γ (IFN- γ) specific MoAb (BD) or PE-conjugated interleukin 4 (IL-4) specific MoAb (BD) for

Figure 1. Asthmatic responses. A, Delayed asthmatic response (DYAR) to allergen challenge (ALL) and phosphate-buffered saline (PBS) control challenge. The mean percentage changes in the forced expiratory volume in 1 second (FEV₁) values calculated from 51 DYAR and 51 PBS control challenges. $\square$, Initial DYAR; $\blacksquare$, repeated DYAR; *, initial PBS; x, repeated PBS; and I, initial (baseline) values. Bars indicate means $\pm$ SEM. B, Isolated immediate asthmatic response (IAR) to allergen challenge and PBS control challenge. The mean percentage changes in the FEV₁ values calculated from 56 IAR ($\circ$) and 56 PBS control challenges (x). C, Isolated late asthmatic response (LAR) to allergen challenge and PBS control challenge. The mean percentage changes in the FEV₁ values calculated from 43 LAR (Δ) and 43 PBS control challenges (x). D, Dual late asthmatic response (DLAR) to allergen challenge and PBS control challenge. The mean percentage changes in the FEV₁ values calculated from 31 DLAR ($\square$) and 31 PBS control challenges (x).

Table 3. T_H1/T_H2 Ratio in Peripheral Blood and Intracellular Concentrations of IFN- γ , IL-2, IL-4, and IL-5^a

Parameter	Before the challenge	After the challenge, h						
		1	12	24	36	48	56	72
T_H1/T_H2 ratio, %								
DYAR	8.4 $\pm$ 3.2	10.1 $\pm$ 4.5	9.1 $\pm$ 4.3	12.5 $\pm$ 5.2	18.9 $\pm$ 4.8 ^b	17.5 $\pm$ 5.4 ^c	13.0 $\pm$ 4.1 ^c	11.6 $\pm$ 5.7
PBS	7.5 $\pm$ 3.5	9.0 $\pm$ 5.2	10.4 $\pm$ 4.8	8.8 $\pm$ 4.0	9.7 $\pm$ 3.9	10.1 $\pm$ 4.6	9.0 $\pm$ 5.5	8.2 $\pm$ 4.5
IFN- γ , pg/mL								
DYAR	184 $\pm$ 116	205 $\pm$ 143	197 $\pm$ 122	211 $\pm$ 102	418 $\pm$ 155 ^c	457 $\pm$ 143 ^b	339 $\pm$ 119 ^c	232 $\pm$ 127 ^d
PBS	162 $\pm$ 121	181 $\pm$ 111	178 $\pm$ 145	198 $\pm$ 133	185 $\pm$ 106	189 $\pm$ 127	165 $\pm$ 93	177 $\pm$ 124
IL-2, pg/mL								
DYAR	<3.0	<3.0	4.2 $\pm$ 0.7	5.5 $\pm$ 1.0 ^d	6.1 $\pm$ 0.9 ^d	5.8 $\pm$ 0.5 ^d	5.3 $\pm$ 0.8 ^d	4.0 $\pm$ 0.6
PBS	3.1 $\pm$ 0.1	<3.0	<3.0	3.4 $\pm$ 0.3	3.2 $\pm$ 0.1	<3.0	3.3 $\pm$ 0.2	<3.0
IL-4, pg/mL								
DYAR	22.3 $\pm$ 2.1	31.2 $\pm$ 5.5	28.6 $\pm$ 4.4	20.5 $\pm$ 6.1	25.0 $\pm$ 5.7	30.3 $\pm$ 4.9 ^d	26.5 $\pm$ 6.7	25.4 $\pm$ 5.0
PBS	27.5 $\pm$ 6.4	20.4 $\pm$ 3.2	25.6 $\pm$ 5.3	24.8 $\pm$ 5.6	29.1 $\pm$ 4.8	23.5 $\pm$ 5.3	28.2 $\pm$ 3.0	24.9 $\pm$ 4.5
IL-5, pg/mL								
DYAR	4.2 $\pm$ 0.8	3.8 $\pm$ 0.4	4.7 $\pm$ 1.0	5.3 $\pm$ 0.8	4.5 $\pm$ 0.9	5.0 $\pm$ 1.3	5.4 $\pm$ 1.0	4.8 $\pm$ 0.6
PBS	3.5 $\pm$ 0.7	3.0 $\pm$ 0.0	3.3 $\pm$ 0.2	4.1 $\pm$ 1.5	3.8 $\pm$ 0.3	4.4 $\pm$ 0.7	4.6 $\pm$ 0.5	4.2 $\pm$ 0.4

Abbreviations: DYAR, delayed asthmatic response; IFN- γ , interferon γ ; IL, interleukin; PBS, phosphate-buffered saline.

^a Data are presented as mean $\pm$ SD. The concentration values of IL-2 under the detection limits were assigned for statistical purposes to be 1.5 pg/mL.

^b $P < .01$.

^c $P < .05$.

^d $P \leq .05$.

30 minutes at room temperature. After final wash in PBS, cells were resuspended in 500 μ L of paraformaldehyde (1%) and stored at 4°C for maximally 24 hours until subjected to the flow cytometry analysis. The FITC-conjugated mouse IgG₂ and PE-conjugated mouse IgG₁ were used as controls. Negative controls consisted of unstimulated cells.

The data were generated from the 3-color FACS-Calibur flow cytometer (BD), equipped with a 15-mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm), and peridinin chlorophyll protein (>650 nm), and the percentage of IFN- γ - and IL-4-producing cells were analyzed using CellQuest software (BD). The typical forward and side scatter gate for lymphocytes together with a CD4 gate (logical gate) were set to exclude contaminating monocytes from the analysis. Subsequently, within this gated area, a second gate was further selected. The analyses were performed on the cells in the second gate. All determinations were accompanied by parallel incubations with FITC- and PE-conjugated irrelevant antibodies matched for isotypes of the anticytokine antibodies. The cutoff level for definition of positive cells was set at the less than 1% level. In this way, less than 1% of irrelevant antibody-stained cells were positive.

Cytokines from activated peripheral blood mononuclear cell cultures. The peripheral blood mononuclear cells were separated by Hypaque-Ficoll density-gradient centrifugation (Pharmacia, Stockholm, Sweden) and cultured at a concentration of 5×10^6 cells/mL in the presence of 50 ng/mL of phorbol 12-myristate 13-acetate (Sigma) and 1 μ g/mL of ionomycin for 24 hours. After culture, the cells were centrifuged and viability was determined using trypan blue dye exclusion. Supernatants were stored at -80°C. The concen-

trations of cytokines were measured in the centrifuged supernatants by means of enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, Minnesota) in accordance with the manufacturer's instructions. The minimal detectable limits were as follows: IFN- γ , 8.0 pg/mL; IL-2, less than 3.0 pg/mL; IL-4, less than 10.0 pg/mL; and IL-5, less than 3.0 pg/mL. The intra-assay coefficients of variations of these assays were less than 9%, and the inter-assay coefficients of variations were less than 10%.

Statistical Analysis

The BPT results were statistically analyzed by means of fitting polynomials to the mean curves over time: 8 time points within 120 minutes and 20 time points up to 56 hours after the allergen challenge. The hypotheses were tested by means of the generalized multivariate analysis of variance.⁹ The changes of diagnostic parameters accompanying the DYAR and PBS controls were analyzed by Mann-Whitney *U* test. The ELISA and flow cytometric data obtained at various postchallenge time points were compared with the prechallenge values and evaluated by Wilcoxon signed-rank pair test. $P < .05$ was considered statistically significant for all statistical methods.

RESULTS

Fifty-five patients developed 55 DYARs to bronchial challenge with allergens in concentrations listed in Table 2. The preceding BPTs with diluted allergenic extracts (1:10), the 75 BPTs with other allergens, and the 55 PBS controls all had negative results ($P > .01$, $P > .01$, and $P > .001$, respectively).

Table 4. Survey of the DYAR-Related Diagnostic Parameters^a

Parameter	Before the challenge	After the challenge, h						
		1	12	24	36	48	56	72
Bronchial symptoms								
Dyspnea	÷	÷	÷	++ ^c	++++ ^d	+++ ^d	++ ^d	÷
Wheezing	0	0	0	+	++ ^c	+++ ^d	++ ^c	0
Cough	0	0	0	0	+	±	0	0
Expectoration	0	0	0	0	0	0	0	0
General symptoms								
Pressure on the chest	0	0	0	+	+	+	0	0
Chills	0	0	0	+	±	0	0	0
Tiredness	0	0	0	0	0	+	++ ^c	++ ^c
Headache	0	0	0	0	0	+ ^c	0	0
Increased body temperature ^e	0	0	0	0	+	+	+	0
Blood pressure								
Increase, systolic/diastolic	−/−	−/−	−/−	−/−	+/−	+/+	−/−	−/−
Decrease, systolic/diastolic	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Pulse rate								
Tachycardia	0	0	0	0	+	+	0	0
Bradycardia	0	0	0	0	0	0	0	0
Increased total IgE in the serum ^f	1	...	1	...	1	...	1	...
Positive specific IgE in the serum ^g	0	...	0	...	0	...	0	...
Increased total IgG in the serum ^h	0	...	0	...	0	...	0	...
Blood leukocyte count, ×10 ⁹ /L ⁱ	10.8 ± 0.9	10.4 ± 0.5	10.5 ± 0.3	11.0 ± 0.8 ^b	12.6 ± 0.7 ^c	12.8 ± 0.5 ^c	11.9 ± 0.6 ^b	11.1 ± 0.3
Blood eosinophil count, ×10 ⁶ /L ^j	295 ± 33	305 ± 17	283 ± 25	301 ± 29	307 ± 22	291 ± 30	297 ± 24	302 ± 19
Blood neutrophil count, ×10 ⁹ /L ^k	6.2 ± 0.9	5.9 ± 1.1	6.5 ± 1.4	7.3 ± 0.7	7.8 ± 1.0 ^b	7.9 ± 0.6 ^b	7.5 ± 1.2	7.0 ± 0.8
Blood thrombocyte count, ×10 ⁹ /L ^l	315 ± 40	332 ± 29	303 ± 36	435 ± 48 ^b	458 ± 43 ^b	275 ± 41	300 ± 25	320 ± 33

Table 4. Continued

Parameter	Before the challenge	After the challenge, h						
		1	12	24	36	48	56	72
Blood differential count, ×10 ⁹ /L ^m								
Basophils	10.1	0.15	0.05	0.1	0.03	0.05	0.1	0.1
Eosinophils	0.2	0.3	0.25	0.4	0.4	0.2	0.3	0.3
Neutrophils	5.8	6.3	7.2	7.7 ^b	8.2 ^c	8.5 ^c	7.6 ^b	6.2
Monocytes	0.3	1.1	0.7	0.5	1.0	0.4	0.6	0.8
Lymphocytes	1.1	1.2	3.3	3.1	3.8 ^c	4.1 ^d	3.7 ^c	2.6
Erythrocyte sedimentation rate ⁿ	11	14	10	15	10	13	12	14
Thorax x-ray findings ^o	3	...	...	...	40	...	...	9
Histamine in the serum, ng/mL ^p								
DYAR	0.9 ± 0.4 (6.1%)	1.3 ± 0.3 (5.3%)	1.4 ± 0.6 (6.5%)	0.8 ± 0.3 (4.9%)	1.2 ± 0.5 (5.7%)	0.5 ± 0.1 (5.2%)	1.1 ± 0.4 (5.0%)	0.7 ± 0.3 (6.0%)
PBS	1.0 ± 0.0 (5.7%)	0.9 ± 0.5 (4.3%)	1.6 ± 0.7 (6.1%)	0.8 ± 0.4 (4.5%)	1.3 ± 0.5 (4.8%)	1.1 ± 0.3 (5.2%)	1.4 ± 0.6 (5.1%)	1.2 ± 0.5 (4.7%)

Abbreviations: DYAR, delayed asthmatic response; PBS, phosphate-buffered saline.

^a Data are presented as mean ± SD unless otherwise indicated. 0 indicates normal (negative, no change); . . ., not determined; ÷, borderline value; +, positive; and −, negative.

^b $P \leq 0.05$.

^c $P < 0.05$.

^d $P < 0.01$.

^e More than 37°C (98.6°F) axillary.

^f Reference range, less than 500 IU/mL.

^g Reference range, less than 0.70 U/mL.

^h Reference range, less than 15.0 g/L.

ⁱ Reference range, 4.0 to 10.0 ×10⁹/L.

^j Reference range, less than 300 ×10⁶/L.

^k Reference range, 2.0 to 7.2 ×10⁹/L.

^l Reference range, 150 to 400 ×10⁹/L.

^m Reference ranges: basophils, less than 0.2 ×10⁹/L; eosinophils, less than 0.5 ×10⁹/L; neutrophils, 2.0 to 7.0 ×10⁹/L; monocytes, 0.2 to 1.0 ×10⁹/L; and lymphocytes, 1.0 to 3.0 ×10⁹/L.

ⁿ Reference range, less than 20 mm/1 h.

^o Number of patients demonstrating pronounced bronchovascular markings on the radiographs.

^p Reference range, less than 1.5 ng/mL; percentage of completes (total potential content of histamine in blood, which means in basophils).

Table 5. Survey of the Single Diagnostic Parameters

Parameter	Controls					
	DYAR patients		Asthmatic patients			Healthy controls
	DYAR	NAR	IAR	LAR	DLAR	
Positive skin response ^a						
Immediate	34	62	53	19	27	1
Late	2	10	3	24	4	0
Delayed	15	3	0	0	0	0
Increased total IgE in the serum ^b	1	1	3	1	3	0
Positive specific IgE in the serum ^c	0	2	17	3	11	0
Increased total IgG in the serum ^d	0	0	1	18	7	0
Increased IgG subclasses in the serum ^e						
IgG ₁	0	0	0	2	1	0
IgG ₂	0	0	1	0	0	0
IgG ₃	0	0	0	5	3	1
IgG ₄	0	0	0	13	2	0
Increased total IgM in the serum ^f	0	0	0	1	0	0
Increased total IgA in the serum ^g	0	0	0	0	0	0
Thorax x-ray examination ^h	3	3	1	5	0	0

Abbreviations: DLAR, dual late asthmatic response; DYAR, delayed asthmatic response; IAR, isolated immediate asthmatic response; LAR, late asthmatic response; NAR, negative asthmatic response.

^a Intracutaneous (intradermal) skin test.

^b Total IgE in the serum (PRIST); reference range, less than 500 IU/mL.

^c Positive allergen specific IgE result in the serum (ImmunoCAP); reference range, greater than 0.70 U/mL (more than class 1).

^d Total IgG in the serum (single radial immunodiffusion and enzyme-linked immunosorbent assay); reference range, less than 15.0 g/L.

^e Reference ranges: IgG₁, less than 5.0 g/L; IgG₂, less than 2.6 g/L; IgG₃, less than 0.4 g/L; and IgG₄, less than 0.5 g/L.

^f IgM reference range, less than 3.8 g/L (<1.5).

^g IgA reference range, less than 4.0 g/L (<3.2).

^h Number of patients with bronchovascular markings on their radiographs before bronchial provocation tests (during the inclusion-exclusion stages).

The DYAR began within 6 to 32 hours, reached the maximum within 32 to 48 hours, and resolved mostly within 56 hours after the allergen challenge (Figure 1A). The clinical course of DYAR differed from the clinical course of IAR, LAR, and DLAR (Figure 1B-D). All DYARs recorded during the routine BPTs were significantly positive, comparing the postchallenge FEV₁ values with the prechallenge FEV₁ values ($P < .001$), as well as comparing with the PBS control values ($P < .001$).

The repeated BPTs ($n = 51$) resulted in the development of similar and statistically significant DYARs comparing the postchallenge with the prechallenge FEV₁ values ($P < .001$) and with the PBS controls ($P < .001$) (Figure 1A). No statistically significant differences were observed between the initial (routine) and the repeated DYARs ($P < .05$). The DYAR demonstrated therefore an excellent reproducibility (Figure 1A). No significant differences were found in the appearance of the DYAR with respect to the individual allergens ($P > .10$) or between the pollen and perennial allergens (Table 2).

Of the patients developing DYARs, 69% demonstrated a decreased bronchial histamine threshold, 67% demonstrated a positive immediate skin response, 4% developed a late skin response, and 29% developed a delayed skin response and

nonincreased serum concentrations of particular immunoglobulin classes (Tables 1 and 5).

The DYAR was accompanied by subjective concerns in almost 100% of patients; slightly increased body temperature in 86%; increased bronchovascular markings on the thorax x-ray film in 78%; increased blood leukocyte count, relative neutrophilia, and lymphocytosis in 98%; nonincreased eosinophil count in 98%; and nonincreased histamine serum concentration in 100% (Table 4).

The T_H1/T_H2 ratio in peripheral blood of the DYAR patients before the allergen challenge did not differ significantly from the control patients demonstrating IAR ($P > .05$), LAR ($P > .05$), DLAR ($P > .05$), or healthy individuals ($P > .05$) (Table 1). However, the T_H1/T_H2 ratio in peripheral blood changed significantly 36, 48, and 56 hours after the allergen challenge in 98% of cases ($P < .01$, $P < .01$, and $P < .05$, respectively) in favor of T_H1 cells (Table 3). In the cultures stimulated with peripheral blood mononuclear cells, significantly increased concentrations of IFN- γ were measured 36, 48, and 56 hours after the allergen challenge in 96% of cases ($P < .05$, $P < .01$, and $P < .05$, respectively). The intervals of the IFN- γ increase correspond with the clinical maximum of the DYAR (Table 3). No significant changes in the concentrations of IL-4 or IL-5 were measured in 96% of the DYAR cases ($P > .05$) (Table 3).

Control Patients

The IARs, LARs, and DLARs recorded in the control patients (Figure 1B-D) were associated with other *in vivo* and *in vitro* parameters in different degrees from those accompanying the DYAR (Tables 1 and 5). These differences concern mainly the skin tests, IgG and specific IgE in the serum, and the blood leukocyte and eosinophil counts. No significant changes in other diagnostic parameters were recorded in the healthy individuals (Tables 1 and 5).

DISCUSSION

BPTs with allergen are recognized as an important research tool and useful diagnostic procedure for confirmation of the role of allergic component in patients with asthma.^{2-5,7} During the BPTs, the bronchial tree is challenged directly with an allergen in a certain dose, and the bronchial response of a certain type can be recorded quantitatively in its dynamic course.²⁻⁷ The BPT is therefore a more reliable technique for the detection of a bronchial reaction and its clinical consequences than data from single diagnostic tests (eg, skin tests or radioallergosorbent tests). However, BPTs require certain special conditions and have some limitations and contraindications.³ We used spirometry for BPTs because of its simplicity, reliability, and excellent reproducibility. The recording of FEV₁ and FVC values is a well-established method for assessment of the bronchial obstruction.⁷ Our standard BPT schedule includes the recording of the spirometric values up to 56 hours after the challenge.⁴⁻⁶ According to our standard conditions, all BPTs were performed during hospitalization to exclude an unexpected or additional allergen exposure and with respect to the patient's safety (eg, potential complications).

The DYAR differs from the IAR, LAR, and DLAR in clinical course, features, association with various diagnostic parameters, and pharmacologic modulation.⁴⁻⁶ The DYAR began 26 to 32 hours after the allergen challenge (the time in which the LAR had almost been resolved), reached the maximum within 32 to 48 hours, and resolved within 56 hours (Figure 1). The DYAR was accompanied by a distinct dyspnea and wheezing, whereas the other bronchial symptoms appeared to a lesser degree (Table 5). The appearance of general concerns and increased bronchovascular markings on the thorax x-ray film during most DYAR cases (Table 4) may be indicative of involvement of larger inflammatory mechanism(s) in this response type.

Patients developing the DYAR and the DYAR itself were compared with control patients demonstrating other types of asthmatic response and healthy controls. However, because the mechanism underlying the DYAR is with high probability different from atopy, an important control group consisting of nonatopic asthmatic patients, who would receive the same BPT protocol as the healthy controls, has not been included for technical reasons. This fact would represent a certain limitation of this study.

The DYAR patients demonstrated positive immediate skin response to the relevant allergen in 67% and delayed skin response in 29% (Table 5). This statistically nonsignificant

correlation ($P > .10$) would suggest DYAR of a more local character.

The increased blood leukocyte count, including elevated lymphocyte and neutrophil counts, would suggest an active involvement of these cell types and larger inflammatory mechanism(s) in the DYAR. Our results are supported by other authors' findings reporting increased lymphocyte and/or neutrophil counts in blood,¹⁰ bronchoalveolar lavage (BAL) fluid and induced sputum,¹¹⁻¹⁶ or lung tissue biopsy¹⁷ of asthma patients and in blood and/or BAL fluid several hours after the allergen challenge.¹⁸⁻²¹ Similar results were reported from the animal models.²²⁻²⁴ Some authors suggested even a separate entity: "neutrophilic type of asthma."^{14,25-27}

The serum histamine concentration did not increase during the DYAR. This finding is consistent with other investigators' observation of nonincreased histamine levels in the blood or BAL fluid of some asthmatic patients,^{17,20,28} and it would not support an involvement of circulating basophils in the mechanism(s) underlying the DYAR. In contrast, we found changes in the histamine serum concentration during the IAR and the LAR.²⁹

The nonincreased blood eosinophil count without significant changes during the DYAR would not support the active role of eosinophils in the mechanism(s) underlying the DYAR. It would suggest that these mechanisms may be different from those involved in the IAR and LAR, where eosinophils play an important role.^{1,4,5} This finding is in agreement with other investigators' observation of "non-eosinophilic asthma type."^{14,17,25,26,30,31} Similar results were reported from the experimental asthma model in animals.^{22,24,32-34}

The absence of changes in the serum concentration of immunoglobulins would exclude their involvement in the mechanism(s) underlying the DYAR. These findings would be consistent with other authors' reports of the non-IgE-mediated asthma in humans^{35,36} and animals.^{34,37} Nevertheless, the possible role of local antibodies in the bronchial mucosa without any substantial progression into blood circulation cannot be neglected. On the other hand, the DYAR was accompanied by significant changes in the T_H1/T_H2 ratio in peripheral blood in favor of T_H1 cells and significantly increased production of IFN- γ at 36 to 56 hours after the challenge, whereas the concentrations of IL-4 and IL-5 remained unchanged. These findings would indicate the possible involvement of T_H1 cells in the mechanisms underlying the DYAR. However, a participation of other lymphocyte types (eg, NK cells) in the increased blood lymphocyte counts cannot be excluded.^{38,39}

The transiently increased blood leukocyte count due to the increased neutrophil and lymphocyte counts, appearing simultaneously with the T_H1/T_H2 ratio and IFN- γ changes, would indicate an involvement of hypersensitivity mechanisms different from those having been classically presumed in the bronchial asthma on participation of the IgE antibody, mast cells, eosinophils, and T_H2 cells. Our results and hypothesis of the pivotal role of the T_H1 cells in the DYAR may

be supported by other investigators' findings in patients with bronchial asthma^{10,11,13-18,25-27,40-47} and in the animal models.^{22,23,32,33}

Kay et al⁴⁸ described a "tertiary peak in the airway resistance" at 48 hours after bronchial challenge in guinea pigs, accompanied by increased T lymphocytes in BAL fluid. Hutson et al⁴⁹ recorded 2-step bronchoconstriction at 17 and 72 hours after the bronchial allergen challenge in guinea pigs, accompanied by increased neutrophil and eosinophil counts in BAL fluid. Nishida et al⁵⁰ recorded delayed-type pulmonary reactions characterized by peribronchial infiltrates containing I-A⁽⁺⁾ dendritic cells and CD4⁺ T_H cells, followed by macrophage accumulation, 24 to 48 hours after the intratracheal antigen challenge in sensitized mice. Cui et al²² reported ability of the T_H1 cells to induce a severe lung inflammation dominated by neutrophils and airway hyperresponsiveness independent of IL-4 and IL-13 in the BAL fluid of sensitized mice 24 hours to 3 days after the antigen challenge. Pan et al²⁴ observed increased recruitment of neutrophils in the airways of sensitized mice 24 hours after antigen challenge induced by activated allergen-specific T_H1 cells through the upregulation of RANTES. Huang et al³⁰ demonstrated that allergen-specific T_H1 cells in rats down-regulated the efferent T_H2 cytokine-dependent bronchial hyperresponsiveness and BAL eosinophilia 18 to 24 hours after the antigen challenge via IFN- γ mechanisms. Nabe et al⁵¹ reported delayed-type asthmatic response induced by intratracheal challenge with toluene-2,4,-diisocyanate in guinea pig, peaking after 22 hours and resolving after 72 hours, associated with an infiltration of the airways by mononuclear cells, eosinophils, and neutrophils.

In humans, however, the clinical DYAR to occupational allergens was first suggested by Pepys and Hutchcroft.² The first evidence for the DYAR to bronchial challenge with inhalant allergens was probably reported by us.⁶ The association of DYAR with various bronchial and general concerns increased blood leukocyte, neutrophil, and lymphocyte counts, and changes within the CD⁺ phenotype cells could be indicative of involvement of more complex inflammatory mechanism than those underlying the IAR and LAR.^{4,5} The significant changes in the peripheral blood T_H1/T_H2 ratio in favor of T_H1 cells, together with significantly increased intracellular IFN- γ (Table 3), give evidence for allergen-dependent activation of the T_H1 cells, leading then to their active effector role in the immunologic mechanisms underlying the DYAR.

These findings may be consistent with other authors' results and suggestions.^{11,23-25,33,36,37,46-48,51,57} The effector role of T_H1 cells and their cytokines in the DYAR is probably different from their presumed regulatory role toward the T_H2 cells in classic atopic asthma due to immediate hypersensitivity (eg, IAR).^{11,18,19,26,30,32,33,41-43,46} Moreover, the possible involvement of chemokines, released by T_H1 and T_H2 cells, executing potent chemotactic activities toward various cell types (eg, neutrophils)^{34,52} should also be considered in the immunologic mechanisms underlying the DYAR.

The involvement of immunologic mechanisms in the DYAR, different from those underlying the IAR and the LAR, may also be supported by our preliminary results concerning the pharmacologic modulation of DYAR,⁶ which were different from those observed in the IAR and LAR cases.^{53,54} The DYAR was prevented to a highly significant degree by glucocorticosteroids, whereas disodium cromoglycate did not affect this response type. In contrast, the IAR was inhibited highly significantly by disodium cromoglycate but not by glucocorticosteroids. Finally, both the glucocorticosteroids and the disodium cromoglycate prevented significantly the LAR.

Although the immunologic mechanisms leading to the development of clinical DYAR are not yet fully identified, the present comprehensive results may suggest an involvement of the cell-mediated hypersensitivity mechanism in this response type. However, further studies will be necessary to clarify the DYAR and the underlying mechanisms.

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