

The late asthmatic response is associated with baseline allergen-specific proliferative responsiveness of peripheral T lymphocytes *in vitro* and serum interleukin-5

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Summary

Background Increasing insights into the mechanism underlying the allergen-induced late asthmatic response (LAR) have been gained with implication of activated eosinophils and CD4⁺ T lymphocytes. However, the patient characteristics that indicate the individual capacity to develop a LAR are not well-defined.

Methods In 22 subjects with mild to moderate house dust mite-allergic asthma, we investigated the relationship between the LAR and two other models of late-phase allergic inflammation, i.e. the allergen-specific proliferative response of peripheral blood T lymphocytes *in vitro* and the late cutaneous response. Non-specific bronchial responsiveness (PC₂₀ histamine), lung function (FEV₁), peripheral blood eosinophil count, early phase allergic skin sensitivity, and levels of total and specific immunoglobulin E (IgE) were determined prior to bronchial allergen challenge. Serum levels of interleukin-5 (IL-5) were measured before and at several time points after allergen inhalation.

Results A significant correlation was found between the magnitude of the LAR and the allergen-specific proliferative response of peripheral T lymphocytes ($r = 0.44$, $P = 0.04$) but not the late cutaneous response. Stepwise-multiple linear regression of the magnitude of the LAR on the parameters analysed at baseline, resulted in a model combining PC₂₀ histamine, early phase allergic skin sensitivity, and the allergen-specific proliferative response of peripheral T lymphocytes ($R^2 = 0.84$, $P < 0.001$). No contribution of the late cutaneous response to the prediction of the LAR was found. Serum levels of IL-5 increased significantly at 6 h ($P = 0.01$) and 24 h ($P = 0.003$) after bronchial allergen challenge and correlated with the allergen-specific proliferative response of peripheral T lymphocytes *in vitro* ($\rho = 0.48$, $P = 0.02$).

Conclusions The findings in this study point to a role of TH2-lymphocyte responses in the development of the allergen-induced LAR. In allergic asthmatic patients, allergen-specific responsiveness of peripheral T-lymphocytes *in vitro* may serve as a model to determine the individual capacity to develop a LAR after allergen inhalation.

Keywords: late asthmatic response, T lymphocytes, house dust mite, interleukin-5

Clinical and Experimental Allergy, Vol. 29, pp. 217–227. Submitted 9 December 1997; revised 13 May 1998; accepted 20 July 1998.

Introduction

After bronchial allergen challenge of appropriately sensitized patients, an early and a late phase bronchial obstructive response can occur [1]. The early asthmatic response (EAR)

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is found 10 min after allergen inhalation and is considered to be predominantly the result of immunoglobulin (IgE)-mediated mast cell activation with subsequent mediator release and bronchial smooth muscle cell stimulation [2]. In some patients, the EAR can be succeeded by a late asthmatic response (LAR) that develops from approximately 4 h after allergen inhalation [1]. LAR is found to be related to late inflammatory changes in the bronchial wall with influx of activated eosinophils and T lymphocytes [3–5]. These histological characteristics of LAR resemble those found in spontaneous-occurring asthma [6,7]. Moreover, LAR is associated with the induction of bronchial hyperresponsiveness, which is a hallmark of asthma [8], and a LAR is found significantly more often in patients suffering from allergic asthma compared with patients with allergic rhinitis [9]. Hence, as a model, LAR has received increasing interest, especially with respect to the pathogenesis of allergic asthma [1].

There are strong indications for the role of CD4⁺T 'helper' lymphocytes (TH cells) in the development of bronchial eosinophilia that characterizes LAR [1,3,4]. A subset of TH cells (TH2 cells), next to their essential role for isotype switching of B cells for the production of IgE [10], can produce cytokines with specific pro-inflammatory effects on eosinophils [i.e. interleukin 5 (IL-5)] [11,12]. In cultures of peripheral blood mononuclear cells (PBMCs) *in vitro*, CD4⁺T lymphocytes have been found to be the predominant proliferating cells. Hence, PBMC culture assays have extensively been used as a model to determine the responsiveness of peripheral CD4⁺T lymphocytes to allergen [13–15]. Higher proliferative responses to allergen have been found in atopic asthmatic patients, compared with non-atopic controls or atopic patients without symptoms of asthma [13–15], which can be attributed to higher frequencies of peripheral allergen-specific T lymphocytes [16]. In allergic patients, the responding allergen-specific T cells in PBMC cultures appear to be TH2 'skewed' with predominant production of cytokines as IL-4 and IL-5 but not interferon- γ [17–19]. Recently, this cytokine producing response of peripheral allergen specific TH2 lymphocytes has been found to correlate with disease severity [15,19]. We therefore postulated that in well-defined allergic asthmatic patients, the allergen-specific responsiveness of peripheral TH lymphocytes may indicate the individual capacity to develop LAR, as was shown in relationship with EAR by others [20].

Another potential model of the late-phase allergic response in the airways is the late cutaneous response [21,22]. Several histological similarities between the late cutaneous response and LAR have been described [23,24]. However, a relationship between the capacity to develop a late allergic response in the skin and airways is uncertain.

In asthmatic patients sensitized to house dust mite

(*Dermatophagoides pteronyssinus*), we investigated the allergen-induced LAR in relationship with the allergen-specific proliferative response of peripheral T lymphocytes *in vitro* and the late cutaneous response. The role of these and other baseline patient characteristics (i.e. non-specific bronchial hyperresponsiveness, peripheral blood eosinophil count, allergic sensitivity, baseline lung function) in the prediction of LAR was determined by using multiple linear regression. As both LAR and the late cutaneous response will depend on the extent of the immediate allergic reaction, early phase allergic responses in the skin and airways were standardized to be within narrow range by titration of the allergen dose. Serum levels of IL-5 were measured at baseline and at several time points after bronchial allergen challenge because IL-5 appears to be a main pro-inflammatory cytokine involved in LAR which is predominantly produced by CD4⁺T cells [11,25,26].

Materials and methods

Study subjects

Twenty-two subjects with mild to moderate asthma [27] (10 female; mean age: 27.1 years, range 18–45 years) were investigated by the same investigator (MV). All patients were sensitized to house dust mite (*D. pteronyssinus*) as determined by skin prick test and radioallergosorbent test (RAST). Patient characteristics are in Table 1. All subjects were investigated in a stable phase of their disease and had a forced expiratory volume in 1 s (FEV₁) $\geq$ 70% of predicted on all study days. Medication by oral antihistamines, inhalation therapy with short-acting β_2 -adrenoceptor agonists and corticosteroids was ceased 1 week, 12 h and 6 weeks prior to allergen challenge, respectively. None of the patients had received house dust mite-specific immunotherapy. Patients did not have a history of respiratory tract infection or obvious changes in allergen exposure for at least 6 weeks prior to allergen challenge. Concomitant inhalant allergies were determined by RAST and patients sensitized to grass or tree pollen were investigated outside the relevant pollen season. The study was approved by the AMC Medical Ethical Committee and written informed consent was obtained from all patients.

House dust mite extract

An acetone precipitated house dust mite (HDM) extract was derived from a *D. pteronyssinus* culture (ALK, Hørsholm, Denmark) and the same batch was applied in all assays *in vitro* and *in vivo* described in this study. The HDM extract was freeze dried, diluted in sterile phosphate-buffered saline (PBS) and stored in -70°C before use. The content of

Table 1. Patient characteristics

Patient	Age (years)	Sex (M/F)	FEV ₁ (% pred)	PC ₂₀ histamine (mg/mL)	Total IgE (IU/L)	IgE anti-HDM (IU/mL)	Other allergies	Baseline eosinophil count ($\times 10^6/L$)
1	27	F	89	0.04	191	55	G, T	340
2	45	F	72	0.07	1033	99	C, G	220
3	24	F	70	0.2	432	59	C, G, T	510
4	18	M	83	0.3	244	52	T	470
5	25	M	106	0.6	257	54	C, G, T	230
6	20	F	95	0.7	276	18	T	130
7	27	F	115	0.9	26	6.0	C	200
8	19	F	105	1.0	2079	529		340
9	35	F	114	1.1	14	4.6		300
10	23	M	95	1.3	432	101		490
11	28	M	106	2.0	43	19		220
12	42	M	98	2.2	89	19		300
13	24	F	91	2.6	178	17		380
14	28	M	118	2.8	87	4.2	G, T	150
15	26	M	90	2.9	181	15		210
16	35	M	87	2.9	78	20	G, T	370
17	24	M	106	4.2	106	20		330
18	23	F	94	5.5	45	4.7	G, T	150
19	22	M	102	6.6	237	1.0	G, T	580
20	28	M	71	8.0	893	36	G, T	200
21	31	M	114	>32	378	49	C, G	50
22	22	F	104	>32	382	48	C, G, T	350

HDM, house dust mite; C, cat; G, grass pollen; T, tree pollen.

HDM major allergens (i.e. Der p 1 and Der p 2) was determined by radioimmunoassay as described elsewhere [28] and found to be 65.2 µg Der p 1/mg and 17.0 µg Der p 2/mg. The concentration of endotoxin was measured by using a Limulus amoebocyte lysate assay (Endosafe, Charleston, SC, USA) and found to be <1 ng/mg. For use in allergen challenge procedures, the HDM extract was diluted to a 1:10 stock solution in PBS, 0.03% HSA, 0.5% phenol (ALK Benelux, Groningen, The Netherlands) and stored for a maximum of 3 months at 4 °C. On the day before each allergen challenge, series of twofold dilutions of the allergen preparations were made from this stock solution using the same dilution buffer.

Bronchial allergen challenge

Bronchial allergen challenge was performed as described by Sterk *et al.* [29] by using an aerosol delivery system with a large-volume aerosol reservoir [30]. An allergen aerosol was generated from a 0.5 mL sample with a highly efficient nebulizer (Mallinckrodt Diagnostica, Petten, The Netherlands) into a collapsible reservoir of approximately 30 L, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA). Subsequently, the entire volume of the reservoir was inhaled by tidal breathing in approximately 3 min through a three-way valve system with the nose clipped. The starting dose for allergen challenge was determined by the relation between skin-prick test threshold and PC₂₀ histamine [29]. Doubling doses of allergen were inhaled with 10 min intervals until a fall in FEV₁ ≥ 20% (best of two measurements) relative to baseline FEV₁ (mean of three measurements within 5%) occurred 10 min or 20 min after inhalation, as measured with a dry rolling-seal spirometer (SensorMedics BV, Bithoven, The Netherlands). If the FEV₁ dropped between 15–20%, the last dose of allergen was administered again, in an effort to keep the EAR within narrow range. After the final dose of allergen, FEV₁ was measured every 10 min up to 60 min ($t = 60$ min) and at 90 min ($t = 90$ min). Subsequently, FEV₁ was measured hourly until 13 h after allergen inhalation ($t = 13$ h) with a portable spirometer (Micromedical Diary-card, SensorMedics BV, Bithoven, The Netherlands). The day before allergen challenge, a control bronchial challenge was performed with three volumes of diluent (PBS, 0.03% HSA, 0.5% phenol; ALK Benelux, Groningen, The Netherlands) and diurnal variation in FEV₁ was determined. The temporal bronchial response to allergen was expressed as the percentage change from baseline FEV₁ as measured with the corresponding spirometer and corrected for diurnal variation in FEV₁ as found after control challenge. The area under the curve (AUC) [29] was calculated from $t = 1$ min to $t = 60$ min for the EAR (AUC_{EAR}) and from $t = 6$ h to $t = 12$ h for the LAR (AUC_{LAR}).

Bronchial histamine challenge

Bronchial histamine challenge was performed 48 h before allergen challenge according to the 2-min tidal breathing method as described by Sterk *et al.* [29]. A DeVilbiss 646 nebulizer attached to a three-way valve box was used, calibrated to an output of 0.13 mg/min (flow 5.8 L/s). Non-specific bronchial responsiveness was expressed as the concentration histamine diphosphate in PBS (range, 0.015–32 mg/mL) causing a 20% fall in FEV₁ (PC₂₀ histamine) as calculated by log-linear interpolation. In two patients (patients 21 and 22), PC₂₀ histamine before allergen challenge exceeded the upper threshold value and 64 mg/mL was used for analysis.

PBMC proliferation assay

The test was performed as previously described [31]. Heparinized blood was obtained by venepuncture 24 h before allergen challenge. PBMCs were isolated by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway) and washed three times in Hanks' Balanced Salt Solution (HBSS) with 2.5% fetal calf serum plus supplier (Hyclone, Logan, UT, USA). PBMCs were resuspended in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK), supplemented with 5% pooled, complement-inactivated normal human serum and gentamycin (80 µg/mL; Duchefa, Haarlem, The Netherlands). Proliferation of $1\text{--}2 \times 10^5$ cells/well was assayed in a 96-well flat-bottom culture plate after 6 days of culture in the presence of stimulants at 37 °C in a humidified atmosphere containing 5% CO₂. Stimulants used were doubled concentrations of HDM extract (concentration in well, 0.2–50 µg/mL), *C. albicans* (concentration in well, 10 µg/mL) and tetanus toxoid (concentration in well, 1.5 Lf/mL). The cultures were terminated by a 16 h [³H]TdR-pulse (Amersham International, Amersham, UK). Subsequently, the incorporation of [³H]TdR was determined by liquid scintillation spectroscopy. Proliferation at each stimulus concentration was expressed as the stimulation index (SI) representing the ratio of mean counts/min of triplicate cultures to the mean counts/min as found in triplicate cultures in the absence of antigen.

Serum IL-5 measurements and blood eosinophil counts

Venepuncture was performed at five time points; 24 h before, and 0.5 h, 6 h, 24 h and 7 days after inhalation of the last dose of allergen. Total and differential leucocyte counts were performed in ethylenediaminetetraacetic acid–blood by using an automated fluorometric method (H₃-RTX, Bayer-Technicon, Tarrytown, NY, USA). Filtered serum samples were obtained after 1 h of clotting on glass at room temperature, centrifugation for 10 min at 1000 g, to be stored at –20 °C. Measurement of IL-5

levels in the serum samples was performed by using a specific solid-phase sandwich enzyme-linked immunosorbent assay as described elsewhere (detection level, 1 pg/mL) [32].

Total and specific IgE

Total IgE levels in the serum samples obtained 24 h before allergen challenge were measured as described before [28]. Specific IgE levels were determined by using the RAST [28]. In the RAST, 20 µg HDM extract was bound to solid phase (CNBr-activated Sepharose 4B; Pharmacia, Uppsala, Sweden). RAST results were expressed as the mean of duplicate measurements IU/mL using a standard with mouse/human chimeric antibodies directed to Der p 2 and house dust mite solid phase, calibrated against the World Health Organization standard (detection level 0.1 IU/mL) [33].

Intracutaneous test

Intracutaneous tests (ICTs) with series of tenfold dilutions of the allergen preparation used for bronchial challenge were performed on the day before allergen challenge as described before [28]. The longest (A) and orthogonal (B) weal and flare diameters were determined at 15 min and mean diameters were calculated as $(A + B)/2$. In 20 patients, late cutaneous responses were investigated on the allergen concentration of a factor 100 above the lowest allergen concentration eliciting a skin reaction with surrounding erythema. After 6 h, the late cutaneous response was determined as the area of erythematous skin induration [22] and expressed as the mean diameter, calculated as described above. The early phase ICT allergen-threshold concentration (ICT threshold) eliciting a flare diameter of 10 mm was calculated by log-linear interpolation [28].

Statistical analysis

Statistical analysis was performed by using SPSS 6.1 software (Chigaco, IL, USA). Relations between normally distributed data (after log-transformation where applicable) were determined by using parametric correlation (Pearson's r) and stepwise multiple linear regression (P -limits: in <0.05 , out >0.1). Differences between IL-5 levels were determined using Friedman's non-parametric repeated measures test and correlations with IL-5 data by using rank-correlation (Spearman's ρ). All P -values corresponded to two-tailed tests.

Results

All patients tolerated the bronchial allergen inhalation challenge without complication. As a result of careful allergen titration during bronchial allergen challenge, variability in the magnitude of EAR was small [mean

$FEV_{1\max_{EAR}}, -28.7$ (SD 7.5)%; mean $AUC_{EAR}, 20.1$ (SD 6.0) $\Delta FEV_1 \times h$] (Fig. 1, Table 2). By contrast, large variability in the magnitude of the late asthmatic response (LAR) was found [mean $\Delta FEV_{1\max_{LAR}}, -41.8$ (SD 26.2)%; mean $AUC_{LAR}, 185.6$ (SD 140.2) $\Delta FEV_1 \times h$] (Fig. 1, Table 2), with 17 (77%) patients having a definite LAR (i.e. $\Delta FEV_{1\max_{LAR}} \geq 15\%$). The mean peripheral eosinophil count increased after bronchial allergen challenge with a significant peak at 24 h relative to baseline [mean $\Delta eos, 66.2$ (95% confidence interval, 36.0–96.4)%] (Fig. 2). Levels of IgE directed to the HDM extract used for bronchial challenge, were found to be within a range of three orders of a magnitude for the group of patients analysed [median IgE anti-HDM, 19.9 (range 1.0–528.7) IU/mL] (Table 1). After a standardized and similar early cutaneous response [mean diameter (weal), 15.5 (SD 2.9) mm, $n = 20$], late cutaneous responses were found in all patients tested [mean diameter, 42.6 (SD 11.3) mm, $n = 20$] (Table 2). In the PBMC proliferation assay, the highest proliferation rate was found with one of the three top concentrations of HDM extract used in each patient tested (results not shown). For analytical purposes, we applied the mean SI as found on these three concentrations (SI mean) (Table 2). Similar results were found when the highest SI, the SI found on any of the three top concentrations, or the calculated threshold concentration of HDM extract for obtaining a SI of three was used in bivariate correlation and multiple linear regression (results not shown). The Spearman rank correlation between SI mean and threshold concentration for SI = 3 was 0.92.

Prediction of the LAR

For analysis of relationships with LAR, the area under the curve (AUC_{LAR}) was used as a more appropriate parameter for the extent of LAR as compared with the maximal fall in FEV_1 ($\Delta FEV_{1\max_{LAR}}$). However, associations as found in bivariate correlation and multiple linear regression were even more pronounced when the latter parameter was used (results not shown). In some allergen challenges ($n = 7$), inhalation of a short-acting β_2 -adrenoceptor agonist (salbutamol) was required during the LAR, but never before $t = 10$ h and the last FEV_1 measured before intake was used for evaluation of later time points. Analysis of LAR defined as area under the curve from 6–10 h after the challenge, excluding extrapolated data from patients who received rescue medication, gave identical results. In addition, exclusion of the two patients with extrapolated PC_{20} histamine values (>32 mg/mL) from the group of patients analysed (i.e. patients 21 and 22) resulted in similar findings as presented in the following section.

A strong association was found between the AUC_{LAR} and the degree of non-specific bronchial hyperresponsiveness

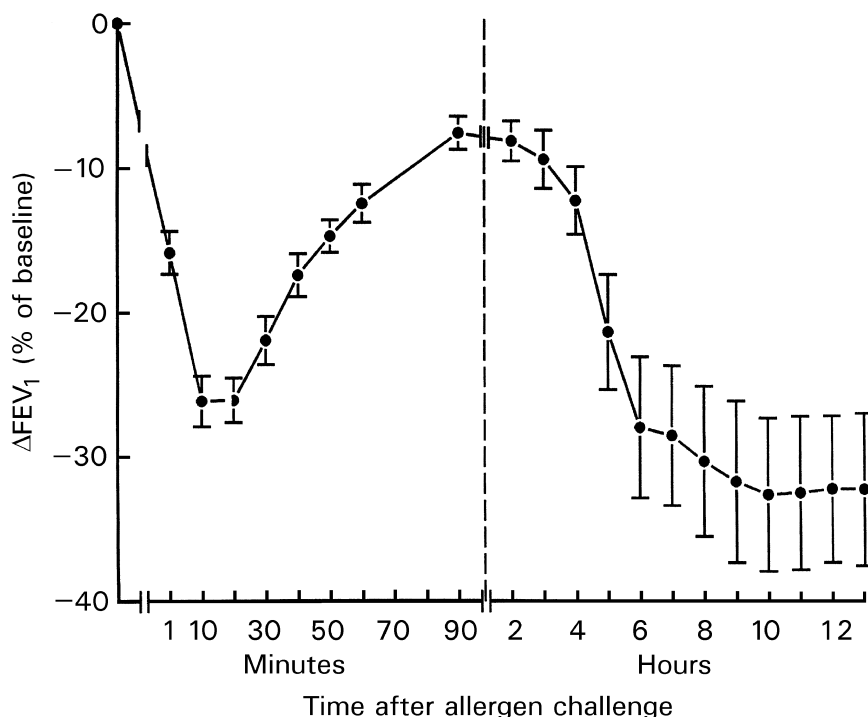


Fig. 1. Results of bronchial allergen challenge with *D. pteronyssinus* showing the functional airway response expressed as the relative difference to baseline lung function (mean Δ FEV₁ and SEM, $n = 22$).

prior to bronchial allergen challenge (log PC₂₀ histamine, $r = -0.78$, $P < 0.001$) as well as the allergen threshold dose for the EAR (log PD₂₀ allergen, $r = -0.68$, $P = 0.001$). The latter as the result of the recognized strong relationship between PD₂₀ allergen and PC₂₀ histamine ($r = 0.71$, $P < 0.001$). A significant correlation was found between the magnitude of LAR and the index of allergen-specific lymphocyte proliferation as found in the PBMC assay (log SI mean, $r = 0.44$, $P = 0.04$). Furthermore, significant bivariate correlations were found between the AUC_{LAR} and the magnitude of the EAR (AUC_{EAR}, $r = 0.47$, $P = 0.03$) and indices of IgE-mediated hypersensitivity to HDM (log IgE anti-HDM, $r = 0.46$, $P = 0.03$, log ICT threshold, $r = -0.47$, $P = 0.03$). By contrast, no correlations (i.e. $r < 0.15$) were found between the AUC_{LAR} and the magnitude of the late cutaneous response, the baseline blood eosinophil count, the level of total IgE (log total IgE), and baseline lungfunction (FEV₁% predicted).

Stepwise-multiple regression with all baseline parameters resulted in a model for the prediction of the magnitude of LAR combining PC₂₀ histamine, SI mean, and ICT-threshold (Table 3). This model was confirmed by using forward and backward regression. Allergen threshold dose (log PD₂₀ allergen), early reaction (AUC_{EAR}), specific and total IgE (log IgE anti-HDM, log total IgE), baseline lung function (FEV₁% pred.), baseline blood eosinophil count and magnitude of the late cutaneous response had no additional contribution to the prediction of LAR. In addition, no contribution of the SI on the control stimulants (i.e.

C. albicans, tetanus toxoid), to the model was found, indicating an allergen-specific response as detected in the PBMC proliferation assay (results not shown).

Serum interleukin-5

At both baseline and $t = 0.5$ h, serum levels of IL-5 were above detection level (>1 pg/mL) in only two patients (patients 2 and 3) (Fig. 3). In contrast, at $t = 6$ h and $t = 24$ h, serum IL-5 levels were detectable in eight and 11 patients, respectively, and found to be significantly increased relative to baseline ($t = 6$ h, $P = 0.01$; $t = 24$ h, $P = 0.003$). After 7 days, the serum IL-5 levels were below detection level in all but one patient. Significant rank-correlations were found between IL-5 levels at $t = 24$ h and both the SI mean ($r = 0.48$, $P = 0.02$) and the AUC_{LAR} ($r = 0.44$, $P = 0.04$). In contrast, no rank-correlations were found between IL-5 levels at $t = 24$ h and both absolute eosinophil count or the increase in peripheral eosinophils relative to baseline observed at this time point ($r = 0.19$, $P = 0.40$; $r = 0.26$, $P = 0.24$, respectively).

Discussion

In this study, we investigated the allergen-induced LAR in allergic asthmatic patients in relationship with two other models of late phase allergic inflammation, i.e. the allergen-specific proliferative response of T lymphocytes *in vitro* and the late cutaneous response. The contribution of these and

Table 2. Results of bronchial allergen challenge, skin test and PBMC-proliferation assay

Patient	Bronchial allergen challenge				Intracutaneous test			PBMC-assay
	PD ₂₀ allergen (µg)	AUC _{EAR} (Δ FEV ₁ × h)	max Δ FEV ₁ LAR (% fall)	AUC _{LAR} (Δ FEV ₁ × h)	ICT threshold (pg/mL)	Early (100 × threshold) weal (mm)	Late (100 × threshold) (mm)	
1	0.1	13.8	57.9	351.4	131	16	45	11.1
2	0.4	24.1	63.3	478.6	14	21	46	17.1
3	1.0	24.8	36.3	222.0	965	16	43	0.8
4	0.4	33.4	69.4	388.4	13	19	36	1.7
5	0.8	27.9	47.4	196.0	9	16	59	6.0
6	1.5	24.7	37.5	158.9	131	16	32	4.6
7	1.8	26.2	55.2	318.6	126	17	74	11.7
8	2.3	12.3	74.8	417.9	1	10	33	21.0
9	7.9	21.3	54.6	279.7	1051	11	36	29.8
10	0.8	26.2	28.0	170.8	117	14	42	11.4
11	2.4	26.7	57.8	248.3	131	13	33	26.3
12	0.9	21.7	46.4	150.7	121	13	50	8.8
13	2.9	13.3	25.1	71.0	105	ND	ND	5.2
14	3.4	18.9	46.0	173.6	734	15	39	6.4
15	5.6	18.0	17.1	68.5	142	15	63	1.8
16	3.7	14.5	15.4	66.8	103	16	42	1.4
17	3.4	19.4	39.1	192.5	150	ND	ND	7.5
18	22.9	14.3	10.5	31.0	121	13	37	0.9
19	162.4	11.8	3.7	- 5.3	9479	16	33	4.8
20	10.7	18.0	9.9	28.3	800	16	35	3.1
21	56.7	15.9	9.5	25.1	98	16	41	11.2
22	1.6	14.8	11.2	49.9	11	21	32	6.1

PD₂₀ allergen, accumulated dose of allergen resulting in fall of 20% in FEV₁ during EAR; AUC, area under the curve; EAR/LAR, early and late asthmatic response; ICT threshold, intracutaneous test allergen threshold; SI mean, mean stimulation index.

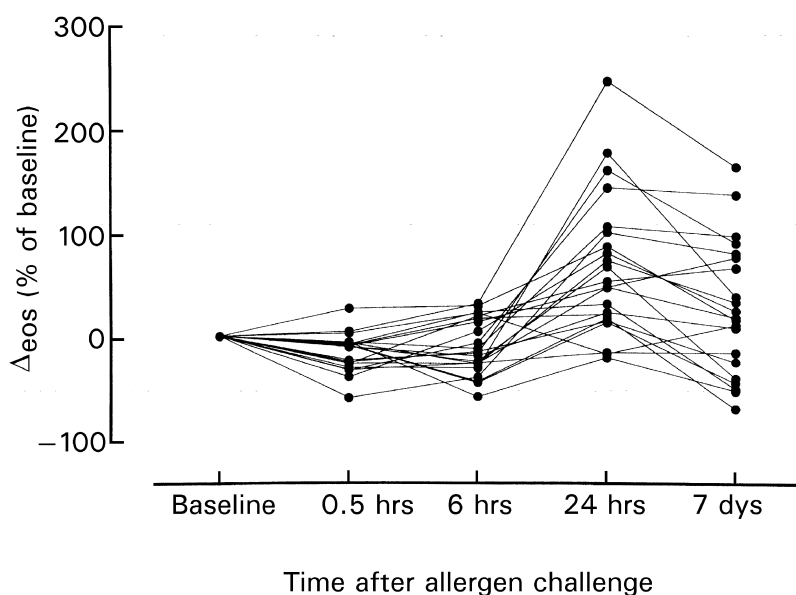


Fig. 2. Peripheral eosinophil counts relative to baseline at several time points after inhalation of the last dose of allergen ($n = 22$).

other baseline patient characteristics (i.e. non-specific bronchial hyperresponsiveness, peripheral blood eosinophil count, allergic sensitivity, baseline lung function) to the prediction of LAR was assessed using multiple linear regression. In addition, serum levels of IL-5 were determined at several time points after bronchial allergen challenge.

In line with previous reports [21,34], a strong relationship was found between the extent of the allergen-induced LAR (i.e. area under the curve, AUC_{LAR}) and the degree of non-specific bronchial responsiveness (PC_{20} histamine) prior to allergen challenge. Interestingly, a significant association was found between the AUC_{LAR} and an index of the proliferative responsiveness of peripheral T lymphocytes to allergen *in vitro*. By using stepwise multiple linear

regression, a model was found comprising PC_{20} histamine, the allergen-specific proliferative response of peripheral T lymphocytes and an index of IgE-mediated hypersensitivity (i.e. skin test allergen threshold) that explained 84% of variation in the AUC_{LAR} . By contrast, no contribution of the extent of the late cutaneous response to the prediction of the LAR was found. The regression model for prediction of the LAR as proposed by Crimi *et al.* [34], combining non-specific airway hyperresponsiveness, serum specific IgE, and baseline airway calibre, was confirmed in the present study (resulting model: $R^2 = 0.78$, $P < 0.001$, $n = 22$). However, using Akaike Information Criteria [35], this model was found to be inferior to our model that was found by stepwise regression.

Our finding of an association between LAR and the allergen-specific proliferative response of peripheral blood lymphocytes *in vitro* is in accordance with the study of Wasserman *et al.* [36] who found a similar association in ovalbumin-sensitized rats. It suggests a role of allergen-induced T lymphocyte responses in the genesis of the LAR, which is in accordance with mechanisms underlying the LAR that are postulated by several investigators (recently reviewed by Bentley *et al.* [1]). Activation of $CD4^+$ T cells has been described in the airways of allergic asthmatic individuals that developed LAR after allergen inhalation [4]. Moreover, after the exposure to allergen egress of peripheral $CD4^+$ lymphocytes into the airways has been described [37–39]. There are strong indications that in allergic individuals the allergen-specific $CD4^+$ T cells belong to the TH2 subtype [7]. These TH2-cells may specifically contribute to the local recruitment and activation of eosinophils through their production of the

Table 3. Stepwise multiple linear regression of the LAR

Dependent variable: AUC_{LAR}			
Independent variables	B (SE)	β	P
Constant	32.8 (40.0)		
Log PC_{20} histamine	-126.3 (17.1)	-0.71	<0.001
Log SI mean	100.9 (29.7)	0.33	0.003
Log ICT threshold	-51.7 (16.5)	-0.31	0.006

Model: $R^2 = 0.84$; R^2 adjusted = 0.81; $P < 0.001$, $n = 22$.

AUC, area under the curve; LAR, late asthmatic response; PD_{20} histamine, concentration of histamine resulting in fall of 20% in FEV_1 ; ICT threshold, intracutaneous test allergen threshold; SI mean, mean stimulation index with house dust mite extract.

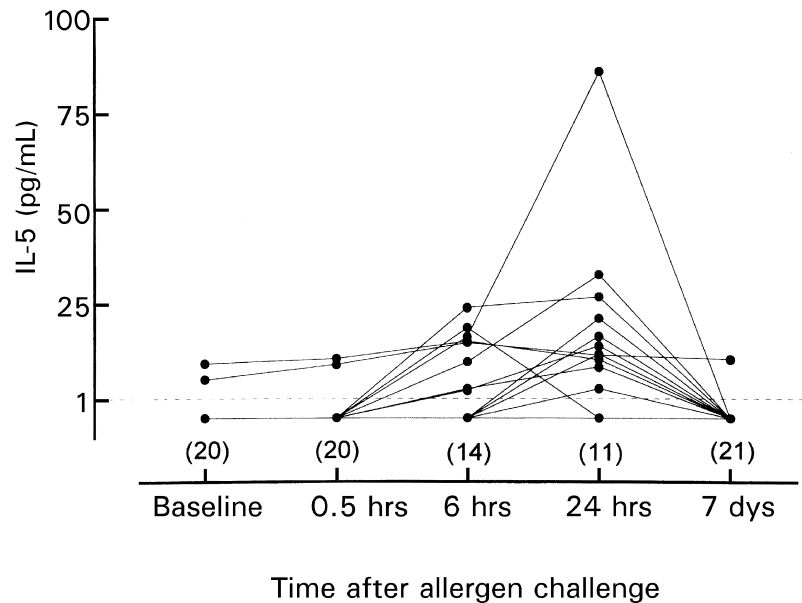


Fig. 3. Serum levels of IL-5 at several time points relative to allergen inhalation. The number of measurements below detection level are indicated at each time point ($n = 22$).

eosinophil active cytokines IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor [11,12]. There is accumulating evidence to show that IL-5 especially is important in the development of the eosinophilic inflammatory response that characterizes LAR [25,26]. In the present study, we found an increase in serum IL-5 at 6 and 24 h after allergen inhalation. This is in line with a recent report of an increase in IL-5 in induced sputum obtained 24 h after bronchial challenge [40]. The rise in serum IL-5 correlated significantly with the allergen-specific proliferative response of peripheral T lymphocytes *in vitro*, suggesting a role of activated TH2-type lymphocytes as the cellular source of this IL-5 *in vivo*. A possible relationship between activated CD4⁺T lymphocytes and serum IL-5 was described previously in patients with exacerbations of asthma [41]. However, it should be noted that several other structural components present in the airways of allergic asthmatic individuals can produce IL-5 upon stimulation with allergen, including mast cells, fibroblasts, endothelial cells, epithelial cells, and eosinophil itself [42]. In the present study, circulating eosinophils were considered unlikely candidates as the cellular source of the serum IL-5 in view of the absent relationship between levels of IL-5 and the number of peripheral eosinophils.

In this study, we investigated the proliferative response of PBMCs after stimulation with allergen as a model of allergen-specific responsiveness of T lymphocytes. However, in addition to the increased frequency and/or responsiveness of allergen-specific T cells [16], the responsiveness of antigen-presenting cells (e.g. dendritic cells) or other cells potentially contributing to T-cell activation (e.g. basophils [43]) also should be considered. Furthermore, it is

suggested that qualitative variations in allergen-specific T-cell reactivity (i.e. cytokine profiles) as opposed to differences in the quantitative response (i.e. proliferation rate) will ultimately reflect allergic responder phenotype [19]. This was underscored by the finding of proliferative responses of peripheral T lymphocytes after stimulation with allergen in up to 100% of subjects, irrespective of atopic status using serum-free culture medium [44]. However, in allergic individuals, the allergen-specific response of T lymphocytes can be considered qualitatively similar (i.e. 'TH2 skewed') with the predominant production of cytokines as IL-4 and IL-5 but not IFN- γ [15,17,19,45]. The proliferative response as determined in the present study may therefore express the individual responsiveness of allergen-specific T cells quantitatively.

In a study by Boulet *et al.* [21], a relationship was found between the capacity to develop a late cutaneous response and the ability to develop LAR after allergen inhalation. In the present study, we found no association between the extent of the late allergic response in the skin and airways as induced by significant and standardized early allergic responses. The bronchial response after allergen challenge is determined functionally by the decrease in airway calibre, whereas in skin tests the response is assessed directly by the area of macroscopic tissue alteration. Furthermore, in the present study, rather low between-patient variation in the magnitude of the late cutaneous response was found, which in combination with the considerable variation in the measurement of the magnitude of the late cutaneous response [46] may have obscured a relationship between the allergen-induced late inflammatory response in the skin and airways.

In summary, in allergic asthmatic patients, a relationship was found between baseline allergen-specific proliferative responsiveness of peripheral T lymphocytes *in vitro* and the late bronchial obstructive response after allergen inhalation. In addition, an increase in serum IL-5 was found after allergen challenge, which correlated significantly with the peripheral T-lymphocyte proliferative responsiveness *in vitro*. These findings point to a role of allergen-specific TH2-lymphocyte responses in the allergen-induced LAR. We conclude that, in allergic asthmatic patients, the allergen-specific responsiveness of peripheral T lymphocytes *in vitro* may serve as a model to determine individual capacity to develop LAR after allergen inhalation.

Acknowledgements

This study was supported by a research grant from the Netherlands Asthma Foundation (grant 93.47). The *D. pteronyssinus* preparation used in this study was a generous gift of ALK, Hørsholm, Denmark. We thank Dr T. Out and Dr M.L. Kapsenberg for their comments on the manuscript.

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