

Der p 1 and Der p 2 induce less severe late asthmatic responses than native Dermatophagoides pteronyssinus extract after a similar early asthmatic response

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Summary

Background The models for exposure to house dust in research and clinical practice are selected with respect to their role in IgE-mediated immediate hypersensitivity. The use of isolated major allergens instead of complex allergen extracts is becoming increasingly popular as it offers some important advantages for quantitative measures in diagnosis and research.

Objective To compare house dust mite extract and isolated mite major allergens with respect to their ability to induce early and late asthmatic responses and bronchial hyperreactivity.

Methods Bronchial responses to house dust mite (HDM, *Dermatophagoides pteronyssinus*) extract and isolated major allergens from HDM (*Der p 1* and *Der p 2*) were compared in a double-blind, randomized, cross-over study in 20 patients with mild to moderate asthma who were allergic to HDM. Allergen was titrated to a standardized early asthmatic response. Bronchial hyper-responsiveness to histamine (PC₂₀histamine) was determined before and after allergen inhalation to assess allergen-induced bronchial hyper-responsiveness and IL-5 was measured in serum. In addition, the allergens were applied in intracutaneous skin tests and activation of basophil leucocytes and proliferation of peripheral blood mononuclear cells was tested *in vitro*.

Results After a similar early asthmatic response (mean Δ forced expiratory volume in 1 s (FEV₁)_{max} –29.4 (SD 7.2) vs. –33.1 (8.6) %; mean difference 3.6 (95% CI –0.9 to 8.2) %), the late asthmatic response (mean Δ FEV₁ –45.9 (21.9) vs. –32.7 (22.3) %; mean difference 13.2 (3.8–22.3) %), the degree of allergen-induced bronchial hyper-responsiveness (mean Δ PC₂₀histamine, 1.8 (1.0) vs. 1.2 (0.9) doubling dose; mean difference 0.6 (0.2–1.1) doubling dose) and serum IL-5 at 6 h were found to be significantly higher after bronchial challenge with HDM extract than after challenge with an isolated HDM major allergen. Likewise, there was an increased late skin reaction with HDM compared with isolated major allergen after a similar early skin reaction.

Conclusion Constituents of HDM extract, other than *Der p 1* or *Der p 2*, with no significant influence on the IgE-mediated early asthmatic response contribute significantly to the allergen-induced late asthmatic response and bronchial hyper-reactivity.

Keywords: house dust mites, major allergens, bronchial provocation, late asthmatic response

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Introduction

There is a close relationship between the exposure to house dust, containing allergens of the house dust mite (HDM, *Dermatophagoides pteronyssinus*), and the severity and prevalence of bronchial asthma [1–3]. For diagnostic and research purposes, HDM extracts are used as a model for exposure to house dust. The rationale to study asthma with HDM extracts is based on the presence of IgE-binding components and their role in immediate type hypersensitivity. However, HDM extract contains considerable amounts of non-IgE binding components with potential immunological activity. It is uncertain if other characteristics of HDM than those related to IgE-mediated mast cell activation contribute the chronic bronchial inflammation as found in asthma [4]. Non-IgE binding antigens may contribute to the activation of inflammatory cells during the late-phase reaction.

Major allergens in allergen extracts are solely defined according to their IgE binding properties. The use of major allergen, isolated by physicochemical methods, affinity chromatography or obtained by recombinant techniques, offers a number of advantages for diagnosis and research [5,6]. In general, biologic activity of major allergens is evaluated by their capability to induce immediate allergic reactions [7], but less is known about their properties to induce late allergic reactions.

In experimental bronchial allergen challenge of sensitized patients, an early asthmatic response (EAR) can occur which resolves spontaneously within 90 min after allergen inhalation [8]. The EAR is considered to be predominantly the result of IgE-mediated mast cell activation with subsequent bronchial smooth muscle cell stimulation [9], as indicated by complete reversibility of the EAR by β_2 -adrenoceptor agonists [10]. In some patients, the EAR can be succeeded by a late asthmatic response (LAR) which is found from 3 h up to 24 h after allergen inhalation [11]. In contrast to the EAR, the LAR is only partially reversible by β_2 -adrenoceptor agonists but can be inhibited by anti-inflammatory medication (i.e. corticosteroids) [10]. As a model, the LAR is considered to be more related to bronchial asthma than the EAR. First, descriptive studies of the LAR using bronchoalveolar fluid and bronchial biopsies showed cellular influx, comprising predominantly activated eosinophils and T lymphocytes, resembling the pathological characteristics as found in chronic asthma [12–15]. Second, the LAR is accompanied by the induction of bronchial hyper-responsiveness, which is a hallmark of asthma [16,17]. Third, LAR is experienced significantly more often by patients suffering from allergic asthma compared with allergic rhinitis patients, whereas no differences in EARs are found between these two groups of patients [18,19].

Similar to the late bronchial reactions after allergen inhalation, late cutaneous responses can occur after allergen challenge of the skin. It has been suggested that the evolution of late allergic skin responses correlates better with the outcome of immunotherapeutic interventions than parameters of early allergic responses [20].

In this double-blind, randomized cross-over study, we established the dose of major allergens *Der p* 1 and *Der p* 2 necessary to obtain a standardized EAR (PD₂₀ allergen) and intraindividually compared the subsequent LAR and the level of allergen-induced bronchial hyper-responsiveness after inhalation of HDM extract vs. an isolated major IgE binding component of HDM (major allergen, *Der p* 1 or *Der p* 2) [6]. We performed standardized bronchial allergen challenge procedures with careful titration of the allergen dose to obtain an EAR within a narrow range. In this way the contribution of HDM characteristics besides those related to the early allergic reaction in the induction of late bronchial reactions could be determined.

Methods

Study subjects

Twenty subjects with mild to moderate asthma [21] (nine female; mean age 28 years (range 18–45 years)) were investigated by the same investigator (M.J.V.) in a double-blind, randomized, cross-over study. All subjects were sensitized to house dust mite (*D. pteronyssinus*) as determined by skin prick test and radioallergosorbent test (RAST) (HDM-specific IgE > 1 IU/mL). The subjects were investigated in a stable phase of their disease and had a forced expiratory volume in 1 s (FEV₁) > 70% of predicted on all study days. Patient characteristics are shown in Table 1. Anti-asthma medication consisted of inhalation therapy with short-acting β_2 -adrenoceptor agonists and/or corticosteroids and was ceased 12 h and 6 weeks prior to allergen challenge, respectively. Patients did not have a history of respiratory tract infection or obvious changes in allergen exposure for at least 6 weeks prior to each 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 given by all subjects.

Allergens

An acetone-precipitated HDM extract was derived from a *D. pteronyssinus* culture (ALK, Hørsholm, Denmark). The major allergens (MA) *Der p* 1 and *Der p* 2 were isolated from this HDM extract by hydrophobic interaction

Table 1 Patient characteristics

	Patient	Age, years	Sex, M/F	FEV ₁ pred. %	PC ₂₀ histamine, mg/mL	Total IgE, IU/mL	IgE anti-HDM, IU/mL	IgE anti-MA IU/mL	Other allergies
<i>Der p 1</i>	1	27	F	89	0.04	191	55.4	14.3	G,T
	2	45	F	72	0.07	1033	99.4	28.1	C,G
	3	25	M	103	0.3	291	57.5	19.9	C,G,T
	4	18	M	83	0.3	244	51.7	20.2	T
	5	24	F	70	0.5	347	64.0	37.3	C,G,T
	6	27	F	117	0.6	22	7.4	3.3	C
	7	19	F	105	1.0	2079	528.7	115.7	
	8	42	M	104	4.2	98	22.5	5.7	
	9	24	M	107	4.4	91	18.6	5.9	
	10	32	M	95	5.9	67	18.2	5.5	
Mean (SD)		28 (9)		94.5 (15.7)	0.6* (5.5)	205* (3.8)	44.6* (3.2)	14.6* (3.0)	
<i>Der p 2</i>	11	20	F	95	0.7	276	17.7	17.5	T
	12	23	M	93	1.0	409	84.6	66.7	
	13	35	F	113	1.3	16	4.8	4.1	
	14	28	M	106	2.0	43	19.0	16.4	
	15	24	F	91	2.6	178	26.6	18.8	
	16	28	M	118	2.8	87	4.2	4.2	G,T
	17	26	M	90	2.9	181	14.6	6.9	
	18	35	M	87	3.0	78	19.9	6.7	C,G
	19	23	F	94	5.5	45	4.7	4.6	G,T
	20	28	M	75	6.2	1186	47.7	44.9	G,T
Mean (SD)		27 (5)		96.2 (12.8)	2.3* (2.0)	125* (3.5)	15.8* (2.8)	11.9* (2.7)	

FEV₁ pred., baseline value of FEV₁ expressed as percentage of predicted value; HDM, house dust mite; MA, major allergen used for bronchial challenge; T, tree pollen; C, cat dander; G, grass pollen.

*Geometric mean and SD.

chromatography on Phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). The *Der p 2* preparation was further purified by gel filtration with forced pressure liquid chromatography. All allergen preparations were freeze-dried, diluted in sterile PBS and stored in small aliquots at -70°C before use. The concentration of HDM major allergens (*Der p 1*, *Der p 2*) was determined by using radioimmunoassays as described before [6]. The HDM extract preparation contained 65.2 μg *Der p 1*/mg and 17.0 μg *Der p 2*/mg [6]. SDS-PAGE of the purified *Der p 1* and *Der p 2* showed single protein bands without evidence of contamination with other proteins. With the sensitive immunoassays cross-contamination of the *Der p 1* preparation with *Der p 2*, and the *Der p 2* preparation with *Der p 1* was found to be 0.1% and 0.008%, respectively. The content of endotoxin was measured by using a Limulus amoebocyte lysate assay (Endosafe, Charleston, SC, USA) [22]. On the day before each allergen challenge, series of two-fold dilutions were made of the allergen preparations

in PBS, 0.03% HSA, 0.5% phenol (ALK Benelux, Groningen, The Netherlands). The same batch of allergens was used for all tests *in vivo* and *in vitro*.

Bronchial allergen challenge

Bronchial allergen challenge was performed as described by Sterk *et al.* [23] with modifications as described by us using a reservoir aerosol delivery system [24]. 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. In each patient, two bronchial allergen challenges (HDM vs. *Der p 1* or *Der p 2*) were performed with a median interval of 6.9 weeks (IQR

4.9–10.1). The major allergen to which a significant level of specific IgE was detected was selected for MA provocation (*Der p* 1, $n = 10$; *Der p* 2, $n = 10$). In 12 patients, the first allergen challenge was performed with HDM extract, in eight patients with MA. The starting dose for allergen challenge was determined by the relation between skin prick test threshold and PC₂₀histamine [23]. Doubling doses of allergen were inhaled with a minimal interval of 10 min until a fall in FEV₁ $\geq 20\%$ (best of two measurements) relative to baseline FEV₁ (mean of three measurements within 5%) occurred 10 or 20 min after inhalation as measured with a dry rolling-seal spirometer (SensorMedics BV, Bilthoven, The Netherlands). If the FEV₁ dropped between 15% and 20%, the last dose of allergen was administered again. After the last dose of allergen, FEV₁ was measured every 10 min until 60 min ($t = 60$ min) and at 90 min ($t = 90$ min). Hereafter, FEV₁ was measured hourly until 13 h ($t = 13$ h) with a portable spirometer (Micromedical diarycard; SensorMedics BV). Spirometers were mutually calibrated. The day before each allergen challenge, a control bronchial challenge was performed with three doses of diluent (PBS, 0.03% HSA, 0.5% phenol; ALK Benelux) and diurnal variation in FEV₁ was determined. The results of the temporal bronchial response to allergen were 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) [23] 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}). For comparison with the ratio of threshold dosages in skin test and basophil histamine release test the total dose of inhaled allergen was expressed as the dose of major allergens (*Der p* 1 and *Der p* 2) and the cumulative dose causing a 20% fall in FEV₁ (PD₂₀allergen) was calculated by interpolation of the logarithmic converted dose–response curve [23]. The total dose of inhaled endotoxin was found to be low in both types of allergen challenge (geometric mean 2.3 vs. 0.67 pg for HDM extract and MA challenges, respectively).

Bronchial histamine challenge

Bronchial histamine challenge was performed 48 h before and 24 h after allergen challenge according to the method described by Sterk *et al.* [23]. Non-specific bronchial hyper-responsiveness was expressed as the concentration of histamine (range 0.015–32 mg/mL) causing a 20% fall in FEV₁ (PC₂₀histamine), calculated by log-linear interpolation. Allergen-induced bronchial hyper-responsiveness was expressed as the 2-log of the ratio

PC₂₀histamine-*before* to PC₂₀histamine-*after* allergen challenge, i.e. the difference in number of doubling doses of histamine (Δ PC₂₀histamine).

Total and specific IgE

Levels of total IgE in serum samples obtained by venepuncture 24 h before allergen challenge were measured as described before [6]. Levels of specific IgE were determined in these serum samples by using the RAST [6]. In the RAST, 20 µg HDM extract and 1.0 µg MA per test were bound to solid phase. A correction was made for possible differences in the amount of allergen bound [6]. In short, sera were tested in a dilution resulting in approximately half maximal binding of radioactivity (¹²⁵I-sheep anti-human IgE) and with two additional dilutions of the solid phase (i.e. 1:3, 1:9). RAST results were expressed as the mean of duplicate measurements in international units per millilitre by using an in-house 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) [25].

Intracutaneous test

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

Basophil histamine release assay

Isolation and incubation of human leucocytes with series of 10-fold dilutions of HDM extract, *Der p* 1, and *Der p* 2 and sheep polyclonal anti-human IgE (100 ng/mL; CLB SH25P01, Amsterdam, The Netherlands) was performed as described before [6]. Histamine release in the supernatant of the samples was measured with an automated fluorometric method. After subtraction of spontaneous release, histamine release was calculated as the percentage of total cellular histamine content. The allergen threshold

concentration for a positive response was calculated as the concentration eliciting 10% histamine release as found by log-linear interpolation [6] and expressed as the major allergen concentration.

Serum IL-5 measurements

Venepuncture was performed 24 h before, and 6 h, and 7 days after inhalation of allergen. Levels of serum IL-5 were measured with a specific solid-phase sandwich ELISA as described elsewhere (detection limit 0.25 pg/mL) [26,27].

Peripheral blood mononuclear cell proliferation assay

This test was performed as described previously [28]. Heparinized blood was obtained by venepuncture before the allergen challenge procedures. Peripheral blood mononuclear cells (PBMC) 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). PBMC 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 $1-2 \times 10^5$ cells/well was assayed in a 96-well flat-bottomed 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 and isolated major allergens (concentrations in well 50–0.2 µg/mL HDM and 5–0.02 µg/mL MA), *Candida albicans* (10 µg/mL) and tetanus toxoid (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 ct/min of triplicate cultures to the mean ct/min found in triplicate cultures in the absence of antigen.

Statistical analysis

Data analysis was performed with SPSS 6.1 software (Chigaco, IL, USA). Paired data analysis was performed with Student's *t*-test. Allergen and histamine thresholds were analysed after log-transformation to obtain normal distribution and differences were expressed as the mean ratio of HDM extract to MA-related values. Paired analysis of serum IL-5 data and PBMC proliferation assays were performed with the Wilcoxon signed ranks

test. Relationships between (log-)normal distributed parameters were calculated using linear regression. Two-tailed *P*-values < 0.05 were considered significant.

Results

Patient characteristics are shown in Table 1. Baseline lung function (mean FEV₁ 94.7 (SD 14.7) vs. 96.0 (14.8) % predicted) and baseline non-specific bronchial hyper-responsiveness (geometric mean PC₂₀histamine, 1.18 vs. 1.21 mg/mL; mean ratio 1.0 (95% CI 0.7–1.3)) did not differ between HDM extract and MA challenges. In addition, no differences in these parameters were found between first and second bronchial allergen challenges (results not shown). Specific IgE directed to *Der p* 1 and *Der p* 2 was detected in all patients and accounted together for 51–100% of IgE directed to HDM extract. Specific IgE levels measured before the first and second allergen challenge did not differ significantly.

In some allergen challenges (HDM, *n* = 7; MA, *n* = 1, Fisher's exact test *P* = 0.04), inhalation of a short-acting β₂-adrenoceptor agonist (Salbutamol) was required during the LAR. Six times (HDM, *n* = 5, MA, *n* = 1) salbutamol was taken after *t* = 10 h, in two patients escape medication was required earlier (HDM, *n* = 2 at *t* = 9 and *t* = 8 h). In these patients the last FEV₁ measured before intake was used for evaluation of later time points.

Bronchial allergen challenge, early asthmatic reaction

All patients showed a EAR (Δ FEV₁ < –20%) with both allergen challenges. The magnitude of the EAR was found to be similar for HDM and MA challenges (mean Δ FEV_{1,max} –29.4 (SD 7.2) vs. –33.1 (8.6) % (mean AUC_{EAR} 20.9 (SD 5.9) vs. 24.5 (8.4) Δ FEV₁*h). The difference in Δ FEV_{1,max} and AUC_{EAR} was not statistically significant (mean difference 3.6 (95% CI –0.9 to 8.2) % and 3.6 (–0.5 to 7.7), respectively).

On a weight basis, the cumulative dose of allergen to obtain the EAR was 10 times less for isolated MAs compared with HDM extract (geometric mean of dose 0.26 µg vs. 2.6 µg, mean ratio 9.9 (95% CI 7.0–14.0)). The allergen doses expressed as the cumulative amount of major allergen *Der p* 1 and *Der p* 2 were very similar (0.18 vs. 0.21 µg, mean ratio 1.2 (95% CI 0.7–1.8)). The PD₂₀ of isolated major allergen was two-fold higher (mean ratio 2.0 (95% CI 1.6–2.5)) than the PD₂₀ of the same major allergen in the whole HDM extract (Fig. 1).

Bronchial allergen challenge, late asthmatic reaction

In contrast to the EAR, a significantly greater LAR was found after inhalation of HDM extract compared with MA

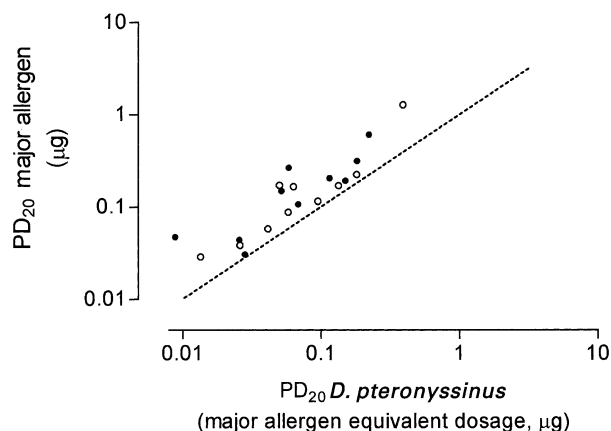


Fig. 1. Dose of *Dermatophagoides pteronyssinus* extract (HDM) and isolated major allergen (MA) to obtain a 20% fall in forced expiratory volume in 1 s (FEV_1) (PD_{20} allergen), expressed as dose of the relevant purified major allergen and the dose of the same major allergen in whole *D. pteronyssinus* extract. Broken line indicates line of identity. Mean ratio of MA to HDM is 2.0 (95% CI 1.6–2.5). ●, *Der p* 1; ○, *Der p* 2.

challenges (mean $\Delta FEV_{1,max}$ –45.9 (SD 21.9) vs. –32.7 (22.3) %, mean difference –13.2 (95% CI –3.4 to –23.0) %) (mean AUC_{LAR} 231.1 (SD 134.4) vs. 141.8 (117.1), mean difference 89.3 (95% CI 37.6–141.1), ΔFEV_1^*h). Significant differences in LAR between HDM extract and MA challenges were found when groups of *Der p* 1- and *Der p* 2-challenged patients were analysed separately, indicating an effect independent of the major allergen used. In the *Der p* 1-challenged group the AUC_{LAR} with HDM was 319.3 (SD 104.3) ΔFEV_1^*h and with *Der p* 1 205.5 (SD 127.7) ΔFEV_1^*h with a mean difference 113.8 (95% CI 5.8–211.7) ΔFEV_1^*h . In the *Der p* 2-challenged group the AUC_{LAR} with HDM was 142.8 (SD 88.7) ΔFEV_1^*h and with *Der p* 2 78.0 (SD 40.1) ΔFEV_1^*h with a mean difference of 64.9 (95% CI 1.6–128.1) ΔFEV_1^*h . Possible contribution of variation in the dimension of the EAR (ΔAUC_{EAR}) to the difference found in the LAR (ΔAUC_{LAR}) is unlikely, because no significant regression of the ΔAUC_{LAR} on the ΔAUC_{EAR} was found ($r^2 = 0.08$, $\beta = 0.28$, $P = 0.23$). In Fig. 2, the results are depicted of the allergen challenges with HDM extract and isolated MA in the 17 patients with a definite LAR in at least one allergen challenge ($\Delta FEV_{1,max} < -15\%$). In these patients, the mean difference in $\Delta FEV_{1,max}$ and AUC_{LAR} of the LAR was –15.4 (95% CI –4.2 to –26.7) % and 105.0 (47.0–163.0) ΔFEV_1^*h , respectively. A significant inverse relationship was found between the cumulated dose of allergen and the differences in the LAR (regression of ΔAUC_{LAR} on log-dose of HDM extract, $r^2 = 0.28$, $\beta = 0.52$, $P = 0.02$), indicating that differences in LARs were most pronounced in patients receiving the lowest dose of allergen.

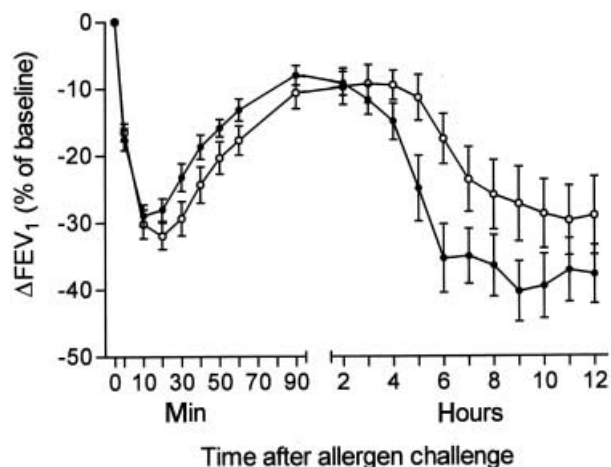


Fig. 2. Bronchial allergen challenges: house dust mite extract (●) vs. isolated major allergen (○). Results expressed as the relative differences to baseline forced expiratory volume in 1 s (FEV_1) as found in the 17 patients with a definite late asthmatic response in at least one of the challenge procedures (mean and SEM; *Der p* 1, $n = 10$; *Der p* 2, $n = 7$).

In addition to the LAR, the level of allergen-induced bronchial hyper-responsiveness was higher after HDM extract challenges compared with MA challenges ($\Delta PC_{20} \text{histamine}$: mean 1.8 (SD 1.0) vs. 1.2 (0.9) doubling dose; mean difference 0.6 (95% CI 0.2–1.1) doubling dose).

Skin test and basophil histamine release test

The differences between the LAR as found after bronchial allergen challenge with HDM extract vs. isolated MA were confirmed in the intracutaneous test. After a similar early cutaneous response (mean weal 15.5 (SD 2.9) vs. 15.3 (2.2) mm, mean difference 0.2 (95% CI –1.2–1.6) mm) (mean flare 48.3 (SD 6.8) vs. 46.4 (5.7) mm, mean difference 1.9 (95% CI –1.9–5.8) mm), significantly higher late cutaneous responses were found with HDM extract compared with MA (mean 42.6 (SD 11.3) vs. 35.5 (12.8) mm, mean difference 7.1 [95% CI 1.2–13.0) mm).

No differences between HDM extract and MA were found with regard to the allergen thresholds, expressed as MA content, for immediate allergic reactions in the bronchial allergen challenge test (geometric mean PD_{20} allergen, 0.16 vs. 0.13 μg ; mean ratio 1.2 (95% CI 0.8–1.9)), the intracutaneous test (geometric mean skin test threshold 0.009 vs. 0.011 ng/mL; mean ratio 0.9 (95% CI 0.4–1.7)) and the basophil histamine release assay (geometric mean histamine release threshold 0.44 vs. 0.23 ng/mL; mean ratio 1.8 (95% CI 0.5–6.8); $n = 17$). In the latter assay, basophils of three patients were found not to respond to any of the stimuli including the control anti-IgE reagent,

Table 2 Serum IL-5 levels after allergen challenge

Challenge	<i>Dermatophagoides pteronyssinus</i>	Major allergen (<i>Der p</i> 1 or <i>Der p</i> 2)	
Time after challenge	Serum IL-5 (pg/mL) Median (IQR)	Serum IL-5 (pg/mL) Median (IQR)	Wilcoxon rank test for paired differences <i>P</i> -value
-24 h	0.9 (< 0.25–5.0)	0.8 (< 0.25–3.1)	0.26
6 h	6.4 (3.3–12.2)	3.9 (1.1–6.8)	0.019
7 days	1.1 (< 0.25–1.85)	1.0 (< 0.25–2.7)	1.0

IQR, Interquartile range.

which is in agreement with the percentage of non-responders reported in the literature [6].

The mean ratios of HDM to MA threshold dosages did not differ significantly between the three tests.

Serum IL-5

Serum IL-5 levels were similar but low before both allergen challenges. With both allergen sources there was a significant increase at 6 h. During the LAR at $t = 6$ h, serum IL-5 was significantly higher after HDM challenge than after MA challenge (Table 2).

PBMC proliferation assay

In contrast to the early allergic reactions, where on a weight basis HDM was 10 times less potent than purified MA (and equipotent on the basis of MA content), SIs in the PBMC proliferation assays *in vitro* were significantly higher with HDM compared with MA, based on MA content but also on a weight basis (Table 3). Significant proliferation responses ($SI > 2$) were found with *Der p* 1 (5 µg/mL) in 7/20, with *Der p* 2 (5 µg/mL) in 3/20, with HDM (5 µg/mL) in 10/20 and with HDM (50 µg/mL) in 16/20 patients. The median and ranges of the SIs in the total group of 20 patients were 1.2 (0.5–5.1) for *Der p* 1 (5 µg/mL), 1.0 (0.6–6.3) for *Der p* 2 (5 µg/mL), 1.6 (0.7–14.3) for HDM (5 µg/mL) and 9.4 (0.6–36.2) for HDM (50 µg/mL).

Discussion

In allergic asthmatic patients, the LAR and the level of allergen-induced bronchial hyper-responsiveness were found to be increased after experimental bronchial challenge with HDM extract compared with an isolated major IgE-binding allergen of HDM (major allergen, MA).

The baseline level of non-specific bronchial hyper-responsiveness and the level of the EAR were similar in both types of allergen challenge. The HDM extract did not induce bronchus obstruction in asthmatic control patients who were not allergic to HDM, thereby excluding a significant non-specific trigger activity. There was a similar increase in serum tryptase at $t = 0.5$ h between both challenges (not shown). Moreover, the ratio of threshold dosages of HDM extract and MA to induce early asthmatic responses in the bronchial challenge test was very similar to the ratios of threshold dosages for induction of IgE-mediated immediate allergic responses in the skin test and in the basophil histamine release assay. Taken together, these data suggest that both allergen sources elicited a similar degree of early mast cell release after bronchial challenge.

Hence, the increased late bronchial reactions as found after inhalation of HDM extract seem to be determined by HDM characteristics unrelated to *Der p* 1 or *Der p* 2 and with no significant influence on the IgE-mediated early asthmatic response.

In spite of the higher level of allergen-specific IgE relevant for the HDM challenge (HDM sp. IgE, median 21.2 (IQR 41.6) IU/mL vs. MA sp. IgE, 15.4 (20.5) IU/mL) the PD₂₀ allergen of HDM was not lower than the PD₂₀ MA, neither on a weight basis nor on the basis of major allergen content. This is in accordance with our earlier studies in which we found that at levels > 3 IU/mL the contribution of the concentration of specific IgE to the prediction of the early asthmatic response rapidly levels off [7]. The higher amount of relevant spIgE in the case of the HDM challenge may contribute to an increased LAR. However, other mechanisms than early mast cell activation are likely to be involved. Antibody-facilitated antigen presentation and subsequent activation of allergen-specific T lymphocytes might represent such a mechanism [29].

In accordance with the *in vitro* basophil histamine

Table 3 Allergen induced proliferation of peripheral blood mononuclear cells *in vitro*

Stimulus	<i>Dermatophagoides pteronyssinus</i>	Major allergen (for bronchial challenge)	Wilcoxon rank test for paired differences <i>P</i> -value
	SI Median (IQR)	SI Median (IQR)	
Concentration			
5 µg/mL (weight)	1.9 (1.2–5.7)	1.1 (0.9–2.0)	0.009
5 µg/mL (major allergen*)	10.0 (3.4–20.2)	1.1 (0.9–2.0)	< 0.0001

IQR, Interquartile range; SI, stimulation index.

**Der p* 1 and/or *Der p* 2.

release and the skin test, on a weight basis 10 times more HDM than MA was required for a similar EAR, but the allergen doses were very similar when expressed as the amount of MA. Apparently, the remaining IgE-binding allergens in the HDM extract (non-*Der p* 1 and 2 allergens) did not contribute significantly to the EAR. This is compatible with the finding that *Der p* 1 and *Der p* 2 quantitatively represent the majority of allergens in the HDM extract (18% of total HDM protein) and that IgE to *Der p* 1 and 2 dominates the IgE response to HDM (> 50% of sIgE to HDM).

It is possible that the remaining allergens or the combination of *Der p* 1 and *Der p* 2 in the whole HDM extract do play a role during the late asthmatic events by antibody-dependent or direct activation of inflammatory cells (e.g. T lymphocytes). Again, an effect on early mast cell activation seems unlikely, as this would have also influenced the EAR.

There was an inverse relationship between the dose of inhaled HDM allergen and the differences in LAR, indicating that non-specific factors will probably not explain the differences in late bronchial reactions as found in this study. Although a role of endotoxins cannot be excluded completely, their contribution is unlikely because very low cumulative doses of endotoxin were inhaled in both types of allergen challenge, with total doses of at least a factor 1000 below the dose that is described to induce bronchial responses in asthmatic patients [30].

Based on the above, we consider a specific late inflammatory mechanism the most likely explanation for the differences in the LAR described in this study. There are strong indications for a pivotal role of T helper (Th) lymphocytes in the bronchial inflammation that is found in the airways of asthmatic patients [31–33]. The Th2 subset of T helper lymphocytes, apart from being essential for the isotype switch of B cells for the production of IgE [34], may contribute directly to the late allergic response by the production of cytokines with

specific proinflammatory effects on eosinophils (i.e. IL-5) [13,15,34–36].

Our finding that the early increase in serum IL-5 was significantly higher after HDM challenge than after MA challenge is compatible with an increased activation of IL-5-producing T lymphocytes. Nevertheless, other cell types (mast cells, eosinophils) may contribute to this IL-5 production.

In spite of the, on a weight basis, 10-fold lower potency of whole HDM to induce immediate IgE-dependent allergic reactions, the potency to stimulate *in vitro* proliferation of PBMC was even higher than purified MA. The significantly higher proliferation rate *in vitro* of allergen-specific T lymphocytes induced by HDM compared with isolated MAs fits in with a higher capability of HDM extract to activate antigen-specific T lymphocytes.

We postulate that the differences in late bronchial reactions described in this study will be related to the increased multiplicity of T cell epitopes present in HDM extract compared with an isolated major allergen preparation. After inhalation of HDM extract, a more heterogeneous population of Th lymphocytes in the bronchial mucosa may become activated with subsequent amplification of the bronchial inflammatory response [37,38]. Further detailed studies will have to be performed to identify the characteristics of house dust mite that determine the induction of late bronchial responses after allergen inhalation.

The current opinion that allergic reactions are predominantly determined by specific IgE antibody levels and the level of exposure to IgE-binding allergens probably holds true for immediate allergic reactions, but is less certain for late-phase allergic reactions. The present study demonstrates that characteristics of HDM not related to IgE-mediated early allergic responses contribute significantly to the late asthmatic response and allergen-induced bronchial hyper-responsiveness after allergen inhalation. This finding points to a role of additional mechanisms

besides IgE-mediated mast cell activation in the induction of late allergic bronchial inflammation. It is generally accepted that late allergic bronchial events bear a closer relationship to chronic asthma than early asthmatic reactions. Therefore, our findings question the validity of anaphylactic models in asthma diagnosis and research. Differences in reactivity to components or characteristics of house dust mite that specifically contribute to late bronchial reactions might explain some of the differences in symptomatology as found in patients with similar levels of specific serum IgE and similar early allergic responses [18,19].

In conclusion, characteristics of house dust mite extracts without detectable influence on IgE-mediated early allergic reactions were found to contribute significantly to the late asthmatic response and induction of bronchial hyperreactivity after allergen inhalation. This finding has implications for the models of the exposure to house dust that are used in asthma research and diagnosis and for the evaluation of immunotherapeutic interventions. For these purposes, allergen sources should represent not only their role in immediate IgE-mediated allergic reactions, but also their capacity to induce late-phase allergic reactions. Application of isolated major allergens may underestimate the role of some of the mechanisms which are relevant for the development of allergic asthma.

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