Factors in clinical expression of allergic airways disease

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CHAPTER 5

Allergen-induced bronchial inflammation in house dust mite allergic patients with or without asthma

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Summary

Background: It is presently unknown which factors determine the occurrence and persistence of asthma in house dust mite allergic individuals. The level of allergen specific IgE antibodies does not seem to be decisive for asthmatic symptoms. Moreover, levels of exposure to mite allergens do not seem to differ significantly between asthmatic and non-asthmatics individuals.

Aim of this study: It was hypothesised that the presence or absence of asthmatic symptoms in house dust mite allergic patients is associated with quantitative or qualitative differences in the cellular bronchial inflammatory response during the late phase of the allergic reaction. This hypothesis was tested in the bronchial allergen challenge model.

Material and methods: Whole lung challenges with house dust mite extract were performed in 52 house dust mite allergic subjects of whom 26 had asthma and 26 had perennial rhinitis without asthmatic symptoms. Primary outcomes were parameters for bronchial inflammation in serial samples of induced sputum (cell differentials, eosinophil cationic protein [ECP], Interleukin-8 [IL-8], myeloperoxidase [MPO]). In addition, lung function, non-specific bronchial hyperresponsiveness and serial blood samples (eosinophils and IL-5) were analysed.

Results: At baseline sputum eosinophils and ECP were similar in both groups but neutrophils and IL-8 were higher in asthmatics. The early bronchoconstriction after allergen challenge was similar in asthma and non-asthmatic rhinitis (median decrease in FEV₁: asthma -31.7% vs. non-asthmatics -29.1%, p>0.1). The late phase bronchoconstriction was significantly greater in asthma (median decrease in FEV₁: asthma -27.6% vs. non-asthmatics -18.9%, p=0.02). Induction of bronchial hyperresponsiveness was similar in both groups. Bronchial allergen challenge elicited significant increases in sputum eosinophils and ECP, which were indistinguishable for both groups (p>0.1 and p=0.07, respectively). In contrast, higher numbers of neutrophils persisted in asthma 24 hrs after challenge and were accompanied by significant increases in IL-8 and MPO, which were absent in non-asthmatics (difference between groups p=0.007 and p=0.05, respectively).
Conclusion: Allergen challenge induced very similar increases in eosinophils and ECP in induced sputum in allergic asthmatics and in allergic non-asthmatic patients. The difference in bronchial inflammation between asthma and non-asthmatic rhinitis appeared to be more closely related with indices for neutrophilic inflammation.
Introduction

Subjects allergic to common inhalant allergens are prone to develop symptoms of the upper airways as well as asthmatic symptoms. It remains to be established which factors determine the symptomatic involvement of the lower airways in atopic individuals. Previous studies showed that this was not explained by differences in the level of allergen-specific IgE. Immediate allergic skin reactions and early bronchial reactions appeared to be similar in rhinitics and asthmatics\(^1\)\(^-\)\(^3\). In addition, there are no indications for persistent differences in exposure to environmental allergens\(^4\). There is agreement in literature on the presence of some degree of eosinophilic bronchial inflammation in non-asthmatic atopy\(^5\), but data on quantitative differences with bronchial inflammation in asthma are inconclusive\(^6\)\(^-\)\(^8\).

Patients with allergic rhino-conjunctivitis show variable levels of non-specific bronchial hyperresponsiveness but this is more pronounced in allergic asthmatics\(^9\).

In spite of similar early asthmatic reactions (EAR), late asthmatic reactions (LAR) appear to be increased in asthmatics after inhalation provocation\(^10\). We hypothesised that quantitative or qualitative differences in the late cellular bronchial inflammatory response after allergen exposure might be associated with the development or persistence of asthmatic symptoms. To test the hypothesis we studied baseline bronchial inflammation and allergen induced changes in bronchial inflammation after a standardised whole lung challenge as a model for the chronic bronchial inflammatory processes in allergic individuals.

In this prospective experimental study we investigated the late allergic inflammatory reaction in the bronchi after challenge with house dust mite extract (HDM, *D.pteronyssinus*) in 52 HDM allergic individuals of whom 26 had asthma and 26 had perennial rhinitis without any evidence for current or past asthmatic symptoms. Primary outcomes were bronchial influx of eosinophils, neutrophils and changes in levels of eosinophil cationic protein (ECP), myeloperoxidase (MPO) and interleukin-8 (IL-8) as detected in induced sputum 24 hours after a standardised early asthmatic reaction. Secondary outcomes were induction of bronchial hyperresponsiveness, and changes in eosinophil counts and IL-5 levels in peripheral blood. The study was powered to detect a 25% difference in sputum eosinophils between groups.
Materials and methods

Study subjects
Fifty-two subjects sensitised to HDM (D. Pteronyssinus) as determined by skin prick test (SPT) and Radio Allergosorbent Test (RAST) were studied by the same investigator (CL). Asthma was diagnosed according to the American Thoracic Society (ATS) criteria and included a documented history of recurrent episodes of wheezing, chest tightness and dyspnoea and a normal lung function between asthmatic attacks\textsuperscript{11}. Asthma severity before institution of treatment ranged from Grade I (episodic) to Grade III (moderate persistent), according to the staging as proposed in the Global Initiative for Asthma\textsuperscript{12}. Rhino-conjunctivitis was not an exclusion criterion for the asthma group. Non-asthmatic patients had perennial rhinitis characterised by recurrent sneezing, watery rhinorrhea, pruritus in the nose, eyes and palate and nasal obstruction\textsuperscript{13}. Patients with past or present asthmatic symptoms, a diagnosis of asthma and/or any previous use of bronchodilators were excluded from the non-asthmatic perennial rhinitis group. The participating patients were recruited via the outpatient departments of Pulmonology and Otorhinolaryngology of the Academic Medical Center.

The following inclusion criteria applied to all participants: a) FEV\textsubscript{1} ≥ 70% of predicted value, b) specific IgE to HDM > 0.5 kU/l, c) able to stop short acting β\textsubscript{2}-adrenoceptor agonists for at least 8 hours, oral anti-histamines for 2 weeks and inhaled corticosteroids 6 weeks prior to the start of the study, d) no significant change in environmental allergen exposure for one year (not moved home, no allergen avoidance procedures), e) in the case of co-sensitisation against pollen, patients participated in the study outside the relevant pollen season.

Exclusion criteria were: a) history of immunotherapy, b) respiratory tract infection within 6 weeks prior to the study, c) other medication or comorbidity, d) smoking.

The study was approved by the local Medical Ethics Committee and all subjects gave written informed consent.
Figure 1 Schematic representation of the study design.
Study design
A summary of the study design is shown in figure 1. Prior to the inclusion all subjects were screened by RAST and skin prick test (SPT) with a standard panel of allergens and by spirometry. Corticosteroids were withheld for 6 weeks. On the first day of the study period, duplicate skin prick tests (SPT) with two-fold dilutions of the house dust mite extract (HDM) were performed and blood samples were obtained. On the second day baseline sputum induction was performed followed by PC_{20} histamine, venepuncture and control bronchial challenge with diluent. On the third day, bronchial allergen challenge was performed with a starting dose of allergen based upon the skin test threshold and PC_{20} histamine according to the method described by Cockcroft et al.\textsuperscript{14}.

Patients were observed for at least 8 hours and remained in the hospital if a late asthmatic reaction with more than 20% decrease in FEV\textsubscript{1} persisted after 12 hours. On the next day and one week later sputum induction, PC_{20} histamine and venepuncture were repeated.

Allergen extract
A single batch of a standardised house dust mite extract (1 mg dry weight/ml, equivalent to 10^6 SQ units/ml and 50,000 BU/ml; ALK, Diephuis, Houten, The Netherlands) was used for all assays. This extract contained 85.1 μg Der p1/mg protein and 9.7 μg Der p2/mg protein. It was kept at -20°C. Allergen dilutions were made freshly from stock in PBS with 0.03% human serum albumin (HSA) and 0.5% phenol (ALK, Diephuis, Houten, The Netherlands) immediately before use.

Bronchial allergen challenge
Bronchial allergen challenge was performed by using a reservoir aerosol delivery system as described previously\textsuperscript{15,16}. Shortly, a collapsible reservoir of approximately 30 litre, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA) and filled with dry air, was connected to a highly efficient jet nebulizer (Mallinckrodt Diagnostica, Petten, The Netherlands) producing an allergen aerosol from 0.5 ml allergen solution. The entire volume of the reservoir was inhaled by tidal breathing through a 3-way valve system with the nose clipped. The day before to the bronchial allergen challenge, a control challenge was performed with 3 doses of diluent (PBS, 0.03% HSA, 0.5% phenol) instead of allergen. The bronchial response to allergen
expressed as the percentage change from baseline FEV\(_1\) was corrected for the diurnal variation in FEV\(_1\) as determined during the control challenge day.

**Bronchial histamine challenge**

Bronchial histamine challenge was performed 24 hours before, 24 hours after and 1 week after bronchial allergen challenge according to the two-minute tidal breathing method described by Sterk et al.\(^\text{17}\). Non-specific bronchial hyperresponsiveness was expressed as the concentration of histamine diphosphate in PBS (range: 0.015 to 32 mg/ml) causing a 20% fall in FEV\(_1\) (PC\(_{20}\) histamine) as calculated by log-linear interpolation.

**Sputum induction**

Sputum induction was performed as described earlier\(^\text{18}\). In short, hypertonic saline was nebulised by an Aerodyne Omega Ultrasonic nebulizer (Kendall, Neustadt/Donau, Germany). The mass median aerodynamic diameter of the aerosol was 4.5 \(\mu\)m. No sputum induction was performed when the FEV\(_1\) was less than 80% of the baseline value or there were complaints of dyspnoea. Sputum induction started with 3% saline inhalation for 15 minutes, thereafter the saline concentrations were raised to 4% and 5% at 15 minutes intervals. Subjects were encouraged to expectorate through several attempts of forced expiration following every inhalation interval. Sputum was collected in plastic tubes and kept on ice until processing\(^\text{19}\).

**Sputum processing**

Sputum was processed according to the method described by Nocker et al.\(^\text{18}\). An equal volume of dithiotreitol (10mM DTT in 135 mM Tris buffer, pH 8.0) was added to the sputum (DTT; Sigma Chemical Company, St. Louis, Missouri, USA). The sample was incubated in a shaking bath of 37°C for 15 minutes with periodically gently manual mixing to ensure complete homogenisation. Cell numbers were counted in a Bürker counting chamber. Slides were stained with Jenner-Giemsa staining and Romanovsky (Diff-Quick). Sputum samples that contained less than 20% non-squamous cells or less than 50% viable cells were excluded from analysis. For cell differentials a total number of 500 non-squamous cells were examined. If less than 2% eosinophils was found another 500 non-squamous cells were counted in order to decrease the
confidence limits of low eosinophil percentages. All differential cell counts were performed by one investigator blinded for patient identity and sample time point.

**Protein assays**
Levels of ECP were determined in the sputum supernatants with an ELISA described previously\(^\text{29}\). The detection limit was 15 pg/ml. Assay reagents (Rabbit-anti-human ECP antiserum and anti-ECP antibody-biotin) were kindly donated by dr. A. Zuurbier (CLB Sanquin, Amsterdam, The Netherlands). A standard dilution curve of ECP was derived from Pharmacia & Upjohn (Uppsala, Sweden). Measurements of Der p1 and Der f1 in mattress dust were performed as described before\(^\text{1}\). MPO\(^\text{21}\), IL-5\(^\text{22}\), IL-8\(^\text{23}\) and Alpha-2-macroglobulin (A2M)\(^\text{18,24}\) were measured by ELISA as described earlier. Albumin was measured by an immunoturbidimetric assay with a Cobas Bio analyser (Roche, Diagnostics, Nutley, NJ, USA). As a standard “N protein” standard serum for nephelometry was used (Behring, Marburg, Germany).

**Peripheral blood parameters**
Venepuncture was performed 24 hours before, 24 hours after and 7 days after inhalation of allergen. Total and differential leukocyte counts were performed in EDTA-blood by using an automated fluorometric method (H\(_3\)-RTX, Bayer-Technicon, Tarrytown, NY). Filtered serum samples were obtained after 1 hour of clotting on glass at room temperature and subsequent centrifugation for 10 min at 1000 g for detection of IL-5.
Statistical analysis

SPSS, version 7.5.3 Statistics U.K. (Chicago, IL, USA) was used for statistical analyses. When applicable, data were log-transformed for parametric analyses. Data were summarised by (geometric) mean and standard error (SE) or by median and interquartile range (IQR) in the absence of a (log) normal distribution. Within group comparisons were analysed with paired t-test and results verified with Wilcoxon test. Between groups comparisons were analysed with t-test and verified with the Mann-Whitney test. Analysis was limited to the non-parametric tests when the assumption that data arrived from a (log) normal distribution was rejected by Kolmogorov-Smirnov and Shapiro-Wilk tests (serum IL-5, blood eosinophils, EAR and LAR). Comparison of allergen induced changes in outcome parameters between groups was done with analysis of covariance with post-challenge values as dependent variable, patient group as a fixed factor and baseline values as covariate. Correlations were determined with the Spearman rank test. All p-values were two-tailed and p-levels of less than 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Table 1 Summary of patient characteristics.</th>
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<tbody>
<tr>
<td>Non-asthmatic rhinitis</td>
</tr>
<tr>
<td>Age (mean, range)</td>
</tr>
<tr>
<td>Gender (m/f)</td>
</tr>
<tr>
<td>FEV₁ (% predicted)¹</td>
</tr>
<tr>
<td>PC₂₀ histamine (mg/ml)²</td>
</tr>
<tr>
<td>Total IgE (KU/l)²</td>
</tr>
<tr>
<td>HDM specific IgE (KU/l)²</td>
</tr>
</tbody>
</table>

¹, mean and standard error; ², geometric mean and standard error.
* p<0.05; ** p<0.01.
Results

A summary of demographic and clinical parameters of both patient groups is shown in table 1. There were no significant differences in gender, age and total IgE between asthmatics and non-asthmatics. The majority of asthmatics (19/26) also appeared to have symptoms of perennial rhinitis. Asthmatics had slightly higher levels of specific IgE to house dust mite. Since specific IgE may contribute directly to the late allergic inflammation by antibody-facilitated antigen presentation and subsequent activation of allergen-specific T lymphocytes and indirectly by its role in the immediate allergic reaction parametric analyses of primary outcomes were supplemented by inclusion of specific IgE as a covariate. As expected, the level of non-specific bronchial hyperresponsiveness was significantly higher in asthmatics. Baseline Forced Expiratory Volume in one second (FEV1) expressed as percentage of predicted value was significantly lower in the asthma group. Differences in lung function and non-specific bronchial hyperresponsiveness persisted after adjustment for the level of specific IgE.

Inflammatory parameters at baseline

In seventeen asthmatics and sixteen non-asthmatic rhinitics induced sputum was recovered successfully at baseline. The differences in eosinophil related parameters (percentage and total number of eosinophils and the level of ECP) in induced sputum did not reach significance (table 2). In contrast, the total number of neutrophils and the level of IL-8 were significantly higher in asthmatics. Similar results were obtained when considering only the subgroup of non-asthmatics with an EAR.

In peripheral blood no significant differences in baseline IL-5 and eosinophils were found between both groups (median asthma IL-5-level 0.5 pg/ml [IQR 2.8]; non-asthmatics 0.5 pg/ml [IQR 3.3], p>0.2; median peripheral blood eosinophil count: asthma 260x10⁶/l [235], non-asthmatics 195x10⁶/l [215], p>0.2).
Table 2  Analysis of changes in induced sputum from baseline to 24 hour post-challenge.

<table>
<thead>
<tr>
<th></th>
<th>Non-asthmatic rhinitis</th>
<th>Asthma</th>
<th>Rhinitis vs. Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>24 hrs</td>
<td>24 hrs</td>
</tr>
<tr>
<td></td>
<td>Mean* (SE)</td>
<td>Mean* (SE) 24 hrs vs. baseline</td>
<td>Mean* (SE) 24 hrs vs. baseline</td>
</tr>
<tr>
<td>Eosinophils (% )</td>
<td>8.8 (3.0)</td>
<td>33.7 (5.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Eosinophils (10^4/g)</td>
<td>1.3 (1.8)</td>
<td>9.4 (1.9)</td>
<td>0.006</td>
</tr>
<tr>
<td>ECP (ng/ml)</td>
<td>48.1 (1.3)</td>
<td>204.2 (1.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>32.6 (4.3)</td>
<td>20.5 (2.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Neutrophils (10^5/g)</td>
<td>1.3 (1.4)</td>
<td>0.63 (1.8)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>248.8 (1.4)</td>
<td>277.1 (1.3)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>MPO (ng/ml)</td>
<td>211.6 (1.4)</td>
<td>166.8 (1.4)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>A2M $ (μg/ml)</td>
<td>2.28 (1.5)</td>
<td>3.07 (1.4)</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Albumin (μg/ml)</td>
<td>25.4 (1.2)</td>
<td>23.7 (1.2)</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

*, geometric mean and SE for ECP, IL-8, MPO, A2M, Albumin and cell counts.
$§$, alfa-2-macroglobulin
+ , paired t test
**+, t test
+++, analysis of covariance: 24 hours post-challenge values adjusted for baseline values signifying differences in allergen induced changes.
Correlations between baseline bronchial inflammation, lung function parameters and symptoms

Overall, baseline histamine thresholds showed a significant association with sputum ECP (R spearman: \(-0.428, p=0.013\)), and sputum eosinophils (%eosinophils: \(R_s=-0.42, p=0.015\) and number of eosinophils: \(R_s=-0.55, p=0.001\)), but not with other sputum parameters. By contrast, presence and severity of asthmatic symptoms graded by the staging proposed in the Global Initiative for Asthma (0-III) was associated with sputum IL-8 (\(R_s=0.35, p=0.046\)) and total neutrophils (\(R_s=0.43, p=0.013\)) but not with other sputum parameters.

Bronchial allergen challenge

All patients tolerated the allergen inhalation without complications. Short-acting beta-2-agonists for relieve of symptoms were never used before 12 hours after challenge. Twenty-four hours after challenge none of the patients had airway symptoms and residual bronchoconstriction was less than 15% decrease from baseline FEV\(_1\) in all. However, some asthmatic patients had to restart maintenance treatment for asthma in the first week after challenge because of exacerbation of symptoms.

All asthma patients and twenty-one subjects of the non-asthmatic perennial rhinitis group experienced an early asthmatic reaction (EAR) defined by at least 20% fall in FEV\(_1\). In 5 non-asthmatic patients early decrease in FEV\(_1\) did not reach 20% with the highest dose of HDM delivered (2400 BU, drop in FEV\(_1\) from baseline: range \(-6.5\%\) to \(-19.5\%\)). The maximal drop in FEV\(_1\) during the EAR was slightly but not significantly less in non-asthmatic patients as compared with asthmatics (median early decrease in FEV\(_1\): asthma \(-31.7\%[IQR 11.5]\) versus non-asthmatics \(-29.1\%[IQR 12.0], p=0.11\) (fig.2). Nineteen asthmatics and eleven non-asthmatics developed a late asthmatic reaction (LAR) defined by at least 20% fall in FEV\(_1\) between 4 and 12 hours after challenge. Two non-asthmatic patients without an EAR did have a LAR. The maximal decrease in FEV\(_1\) during the phase of the LAR was significantly greater in asthma than in non-asthmatics (median late decrease in FEV\(_1\): asthma \(-27.6\%[IQR 34.1]\) versus non-asthmatics \(-18.9\% [IQR 21.0], p=0.01\).

The PC\(_{20}\)histamine showed a significant decrease in both groups 24 hours after challenge, but this was not different between the two groups (change in doubling doses of histamine: asthma \(-1.6 [SE 0.3]\) versus non-asthmatics \(-2.2 [SE 0.4], p>0.2\). One week after challenge a slight
but statistically significant decrease in PC_{20} histamine remained detectable in both groups without a difference between groups (change in doubling doses: asthma -0.8 [SE 0.2] vs. non-asthmatics –0.7[SE 0.2], p>0.2).

Figure 2 Mean (SE) changes in lung function after bronchial allergen challenge in asthma and non-asthmatic rhinitis. There is a significant difference during the phase of the Late Asthmatic Reaction (LAR) between both groups after a similar Early Asthmatic Response (EAR).
Allergen-induced changes in bronchial inflammation

Twenty-four hours after allergen challenge induced sputum was successfully recovered from 16 asthmatics and 17 non-asthmatics. Paired analysis of baseline and post challenge values demonstrated significant increases in percentage eosinophils, number of eosinophils and ECP levels in both groups (table 2). However, neither the percentage and number of eosinophils at 24 hours after challenge nor the change from baseline of eosinophils (table 2) differed significantly between groups. The level of ECP was significantly higher in the asthma group at 24 hrs but the difference did not reach statistical significance after correction for baseline values of sputum ECP.

Interestingly, the differences in numbers of neutrophils and IL-8 level present at baseline between both groups were even more pronounced 24 hours after challenge. Both IL-8 and MPO increased significantly in the asthma group but not in non-asthmatic rhinitis (fig.3). Analysis of the 24 hr post challenge time point with correction for baseline levels confirmed that there was a significant difference in the changes in the levels of IL-8 and MPO between asthma and non-asthmatic rhinitics (table 2). Adjustment for the difference in the levels of specific IgE to HDM did not influence these results. Plasma protein leakage into the airway lumen as indicated by alpha-2-macroglobulin and albumin in sputum, did not differ between asthma and non-asthmatics at any of the time points. One week after challenge induced sputum was successfully recovered from 17 asthmatics and 15 non-asthmatic rhinitics. At this time point values had returned to baseline levels and no significant differences with baseline remained detectable in either group. Between groups comparisons did not reveal significant differences.
**Figure 3** Allergen induced changes in IL-8 and MPO in asthma and non-asthmatic rhinitis. There is a significant increase in IL-8 and MPO in asthma only. Between groups comparison shows a significant difference in changes in IL-8 and MPO (p = 0.007 and p = 0.05).

**Correlations between allergen-induced bronchial inflammation and lung function parameters**

Both the degree of late phase bronchoconstriction and the increase in non-specific bronchial hyperresponsiveness at 24 hour showed an association with the increase in sputum eosinophils (Rs = 0.50, p = 0.007 and Rs = 0.41, p = 0.03, respectively) but not with the change in other inflammatory markers in sputum. Multiple linear regression analysis was applied to predict the degree of late phase bronchoconstriction by baseline non-specific bronchial hyperresponsiveness in combination with changes in markers of inflammation in induced sputum. Besides baseline PC_{20} histamine, the increase in sputum eosinophil counts at 24 hours appeared to be the only other independent variable to contribute significantly. (model R=0.75; standardized coefficients: PC_{20} histamine β = 0.48 [p = 0.003] and increase in number of eosinophils β = -0.48 [p = 0.003]).
**Allergen-induced changes in peripheral blood**

Both serum IL-5 (median values before challenge and at 24 hours after challenge: asthma: 0.5 and 17.6 pg/ml, p<0.0001; non-asthmatics: 0.5 and 18.5 ng/ml, p<0.0001) and blood eosinophils (median values before challenge and at 24 hours: asthma: 240 and 405 10^6/l, p<0.0001; non-asthmatics: 195 and 345 10^6/l, p<0.0001) showed a significant increase 24 hours after challenge but without a statistical significant difference between groups. Increase in serum IL-5 was significantly associated with the increase in sputum eosinophils at 24 hours after challenge (Rs = 0.46, p = 0.015 for eosinophil numbers and Rs = 0.57, p = 0.002 for % eosinophils). No statistically significant associations between changes in serum IL-5 and changes in peripheral blood eosinophils were found.

**House dust mite allergen in mattress dust**

The mean levels of Der p1 and Der f1 as measured in mattress dust, which was sampled in a standardised way in all patients, did not differ between asthmatics and non-asthmatic rhinitics (geometric mean [SE] Der p1, asthma 0.4 [1.7] vs non-asthmatic rhinitis 0.7 [1.7] µg/gram; Der f1, asthma 2.9 [1.6] vs. non-asthmatic rhinitis 5.2 [1.6] µg/gram).
Discussion

In this study on allergen induced bronchial inflammation in HDM allergic patients we found that the difference between asthma and non-asthmatic rhinitis, in addition to the well-known functional differences in non-specific bronchial hyperresponsiveness and FEV₁, appeared to be the higher neutrophil counts and levels of IL-8 in induced sputum in asthma. After bronchial allergen challenge with a standardised EAR the difference in neutrophil numbers found at baseline persisted and was now accompanied by a significant increase in the level of IL-8 and MPO in the asthma group only. Similar numbers of eosinophils and the eosinophil product ECP were found at baseline and the significant increases in percentage and numbers of eosinophils after challenge were statistically indistinguishable for asthmatic and non-asthmatic patients.

The majority of HDM allergic asthmatics also had symptom of perennial rhinitis which is in keeping with literature. Actual natural exposure to HDM, as estimated from the concentration of Der p 1 and Der f 1 in mattress dust obtained from all patients, was found to be similar in the asthma and the non-asthmatic rhinitis group.

The validity of the classification as being asthmatic or non-asthmatic was affirmed by the significant differences in baseline non-specific bronchial hyperresponsiveness and baseline FEV₁, parameters that were not available for inclusion into either group. In accordance with literature non-specific bronchial hyperresponsiveness was increased in the group of non-asthmatic patients with allergic rhinitis although less so than in asthma. The level of specific IgE to HDM was slightly higher in asthmatics, but the difference in spIgE may have been underestimated in this study because low spIgE (<0.5 KU/l) was applied as an exclusion criterion. Correction for the difference in specific IgE by introducing it as a covariate in the statistical analysis did not change the results of the primary outcomes.

We, like others, reported that the level of non-specific bronchial hyperresponsiveness and the level of specific IgE are the two main determinants of the allergen threshold for reaching an EAR. Due to the higher degree of non-specific bronchial responsiveness as well as the higher level of HDM specific IgE in asthma the dose of allergen administered in asthma was lower than in non-asthmatic rhinitis. A possible difference in allergen induced influx of eosinophils into the airways in favour of asthma after similar allergen exposure may therefore have been obscured by
this phenomenon. For the same reason, the difference in IL-8, MPO, ECP and the number of neutrophils after challenge, with higher levels in asthma in spite of lower allergen exposure, is likely to be underestimated in this study.

In both groups induced sputum was successfully recovered in similar numbers of patients. Moreover, comparison of patients who did or did not produce valid sputum did not reveal significant differences in severity of complaints, lung function values or other indicators for disease severity. Therefore, bias of the study results based on a difference in the capability to produce induced sputum samples between the groups is unlikely. We, like others, found a significant association between non-specific bronchial hyperresponsiveness at baseline and baseline eosinophils and ECP in induced sputum. Interestingly, in this study presence of asthma symptoms appeared to be associated with neutrophil counts and the level of IL-8 in sputum and not with eosinophils or ECP. The complete overlap in the extent of eosinophilic bronchial inflammation in asthma and non-asthmatic perennial rhinitis at baseline as well as after allergen challenge suggests that other mechanisms in addition to eosinophilic bronchial inflammation may contribute to the development or persistence of asthmatic symptoms in allergic individuals.

The increased late asthmatic reactions after a similar early asthmatic reaction in asthmatics as compared to non-asthmatic rhinitis confirms earlier reports\(^2,3,10,27\). The finding that non-specific bronchial hyperresponsiveness is the main determinant of the LAR independent of its contribution to the EAR is also in accordance with earlier reports\(^28\). In addition to baseline non-specific bronchial hyperresponsiveness, the increase in eosinophil numbers in induced sputum but, not of other markers of inflammation, was associated with the degree of the late phase bronchoconstriction and the increase in non-specific bronchial hyperresponsiveness. Late asthmatic reactions were found both in asthmatics and in non-asthmatics although the frequency was higher in asthma. Regarding the increased non-specific bronchial hyperresponsiveness at baseline in asthma it is not surprising to find increased late phase bronchoconstriction in this group in spite of induction of a similar degree of eosinophilic bronchial inflammation. As contrasted with changes in eosinophil numbers, neutrophil associated parameters did not show a statistically significant association with the LAR. In accordance with other studies, we found that the fold increase in percentage and numbers of eosinophils and the level of ECP was the main allergen induced change in bronchial inflammation in asthma as well as in non-asthmatic rhinitis,
but there was no significant difference between patients with and without asthma. These results suggest that late phase bronchoconstriction and to a lesser extent induction of non-specific bronchial hyperresponsiveness induced by the allergen challenge procedure is similarly associated with an increase in indices of eosinophilic inflammation in both groups, whereas the presence of clinical asthma seems to be more closely associated with increased indices for neutrophilic inflammation. This phenomenon may demonstrate one of the limitations of artificial allergen challenge as a model for chronic asthma.

The difference in baseline neutrophil counts and IL-8 levels persisted after allergen challenge and was accompanied by a significant increase in the main neutrophil chemotactic interleukin IL-8 and the neutrophil product MPO in asthmatics only. There may be various explanations for the significant allergen-induced increases in IL-8 and MPO in asthmatics in the absence of an increase in number of neutrophils as detected in the cell differentials. Increases in soluble markers may indicate a higher degree of neutrophil activation, but activation and subsequent lysis of neutrophils may render cells unrecognisable in cell differentials and may lead to an underestimation of cell numbers. Alternatively, increases in soluble markers in the absence of increases in number of cells in sputum may point to selective accumulation of neutrophils in the bronchial mucosa.

In contrast to baseline and 24 hours post challenge, the differences between asthma and non-asthmatic rhinitis did not reach statistical significance one week after challenge. Possibly, restarting of pulmonary medication in a number of asthma patients because of exacerbation of symptoms during the first week after allergen challenge may have contributed to this phenomenon.

Taken together these results suggest that neutrophilic bronchial inflammation may play an additional pathophysiological role in bronchial inflammation in asthma. The results are compatible with the hypothesis that the combination of allergen induced eosinophilic inflammation and an increased neutrophilic bronchial inflammation predisposes to the development or persistence of asthmatic symptoms in allergic individuals. The implication of neutrophils in the pathophysiology of severe asthma as reported in literature would be in keeping with this vision. The results are in line with our previous studies with segmental allergen challenge and sampling of bronchoalveolar lavage fluid at 4 hours after challenge, showing an
enhanced influx of neutrophils into the airway lumen in asthmatics as compared to allergic but non-asthmatic controls\textsuperscript{31}. The maximal increase in airway eosinophils is reported to occur approximately 24 hours after allergen challenge. In animal models influx of neutrophils after allergen challenge precedes influx of eosinophils\textsuperscript{32}. Therefore, studying bronchial inflammation 24 hour after allergen challenge may have underestimated the contribution of neutrophils to the allergen-induced bronchial inflammation in asthma. Additional studies are necessary to investigate the interaction between the eosinophilic and the neutrophilic component of the bronchial inflammation.

In conclusion, the results of this study in house dust mite allergic patients suggest that neutrophils contribute more to bronchial inflammation in allergic asthma than in non-asthmatic perennial rhinitis and allergen challenge leads to increased levels of IL-8 and MPO in asthma only. Presence of asthmatic symptoms does not seem to be determined by the number of eosinophils present in the airways. Further studies into a contribution of neutrophils and their products to the bronchial inflammation in asthma and the development or persistence of asthmatic symptoms are warranted.

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