Allergic asthma: Environmental factors challenging the immune system
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Increase in allergen-specific IgE and ex vivo-Th2 responses after a single bronchial challenge with house dust mite in allergic asthmatics

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ABSTRACT

Background: Airway responsiveness to allergen in patients with allergic asthma is studied by bronchial allergen challenge. Although the typical features of the early and late response on lung function and bronchial inflammation after allergen challenge are well known, little has been reported as yet on any changes in systemic allergic and immunologic parameters after 4-6 weeks.

Methods: In a clinical study, 27 subjects with allergic asthma and house dust mite (HDM) allergy underwent a bronchial allergen challenge with HDM. Blood samples were collected before and 5 weeks after allergen challenge. Serum levels of total IgE and allergen-specific IgE were measured and peripheral blood mononuclear cells were isolated and stimulated ex vivo with HDM to determine the allergen-specific T-cell cytokine response.

Results: Five weeks after bronchial allergen challenge with HDM the amount of circulating IgE against HDM and the major allergenic components Der p1 and Der p2 was significantly increased (10.8% and 8.8%, respectively). IgE antibodies against other environmental allergens decreased (grass pollen) or remained unchanged (cat dander). Allergen-induced Th2-cytokine production was also significantly increased (p < 0.001, p = 0.014 and p = 0.006 for IL-4, IL-5 and IL-13, respectively). The increase in Der p1- and Der p2-specific IgE in serum correlated with increased numbers of Th2-cytokine-producing cells (Rs = 0.56, p = 0.002 and Rs = 0.54, p = 0.004 for IL-4 and IL-13, respectively).

Conclusions: A single bronchial allergen challenge with HDM is accompanied by increased levels of allergen-specific IgE for HDM in serum and an enhanced Th2 response to HDM still detectable 5 weeks after challenge.

INTRODUCTION

Bronchial allergen challenge is an investigational tool to provide insight into the airway inflammatory processes and airway pathology caused by inhaled allergens. The challenge model is well standardized and can be used in clinical studies to evaluate the efficacy of (new) asthma therapies. The airway-related symptoms of the early and late asthmatic response (LAR) after bronchial allergen challenge are transient and return to baseline within weeks. Little is known yet about any systemic effects of the allergen challenge after 4-6 weeks.

Inhalation of allergen into the lower airways by sensitized subjects results in an immunoglobulin (Ig) E-mediated acute bronchoconstriction (early asthmatic response, EAR), which is maximal 15-30 min after exposure and resolves by about 2 h. At the same time many cytokines, such as interleukin (IL)-4, IL-5 and IL-13, are released both locally and systemically. Whereas IL-4 and IL-13 are important for IgE secretion, IL-5 plays a pivotal role in the recruitment, activation and survival of eosinophils in the airways. This allergen-induced eosinophilic airway inflammation is associated with the development of
a second episode of bronchoconstriction (LAR), which occurs 3-8 h after inhalation of allergen, and an increased airway hyperresponsiveness (AHR), which may last for up to several days or weeks.8

In several studies, concerning the safety and reproducibility of bronchial allergen challenge, no significant differences were found in EAR, LAR and eosinophilic inflammation between challenges that were repeated after a minimum of 2 weeks.4,9-11 From these results it was concluded that the experimental challenge model is safe and reproducible and that all of the allergen-induced effects of the challenge return to baseline within a few weeks. These results are in contrast with other studies that report a persistent increase in LAR and AHR after repeated bronchial challenges.12,13 Cockcroft et al.12 mentioned a so-called priming effect of allergen challenge that did not occur in all patients and suggested a possible association with the development of naturally occurring asthma. However, the mechanism underlying this priming response is still unclear.

We hypothesized that a single bronchial allergen challenge, apart from the known early and late responses on lung function and inflammatory parameters, which occur within 24 h, might also enhance longer-lasting systemic B- and T-cell responses to allergen. To test this hypothesis, we assessed the allergen-specific responses in blood samples from house dust mite (HDM)-allergic asthmatics that were collected before and 5 weeks after a single bronchial allergen challenge. Total IgE and allergen-specific IgE levels were measured in serum. Peripheral blood mononuclear cells (PBMC) were isolated and stimulated ex vivo with HDM to compare the allergen-specific T-cell responses.

METHODS

Study population and design

For a clinical intervention study, assessing the effect of synbiotics on allergic asthma, adult patients with intermittent to mild persistent asthma and HDM allergy, as determined by positive skin prick test and ImmunoCAP-system (IgE to Dermatophagoides pteronyssinus > 0.5 kU/L), were included. Patients were not allowed to use immunosuppressive medication, long-acting β2-agonists, oral antihistamines and (inhaled) corticosteroids for 4 weeks prior to and during the study. Usage of short-acting β2-agonists was allowed until 12 hours before each visit. A detailed description of the study population is published elsewhere.14

The study had a double-blind, placebo-controlled, parallel design and started with a baseline visit: on day one blood and sputum samples were collected and baseline bronchial hyperresponsiveness was determined with PC20methacholine according to standardized procedures3; on day two patients were challenged with HDM, blood was collected at 1, 6 and 24 h (day 3) after HDM challenge, sputum was induced at 6 and 24 h
(day 3) and PC20 methacholine at 24 h (day 3). Subsequently, subjects were randomly allocated to receive a food supplement with or without synbiotics for 4 weeks. After intervention, patients were subjected to the same schedule as for baseline visit. Whenever cosensitization to pollen was observed, the study was performed outside the relevant pollen season.

Subjects were recruited via advertisement on internal billboards in the Academic Medical Center (AMC) in Amsterdam and gave written informed consent. The study was approved by the Medical Ethical Committee of the AMC.

**Bronchial allergen challenge**

All subjects underwent a bronchial allergen challenge, inhaling doubling doses of a standardized *D. pteronyssinus* extract (50,000 BU/ml, containing 98 µg/ml Der p1 and 7 µg/ml Der p2, ALK Abelló, Nieuwegein, the Netherlands) with 10-min intervals via a reservoir aerosol delivery system until a decline in forced expiratory volume in 1 s (FEV1) of 20% from baseline (median of three measurements within 5%) was reached

Changes in FEV1 were measured every 10 min for the first hour, then hourly for at least 6 h.

**Analysis of blood samples**

Blood was collected on day 1 of the challenge schedule: 24 h before and 5 weeks after baseline allergen challenge. After blood clotting for 30-60 min, samples were centrifuged and serum was stored in aliquots at −80°C until analysis. Serum levels of total IgE and allergen-specific IgE antibodies against HDM, Der p1, Der p2, grass pollen and cat dander were determined using the ImmunoCAP-system (Phadia, Uppsala, Sweden).

Peripheral blood mononuclear cells (PBMC) were isolated within 2 h from heparinized blood by standard density gradient techniques and stored in aliquots in liquid nitrogen until analysis.

**Cell stimulation assays**

We performed ELISPOT assays to determine the number of cells producing Th1 and Th2 cytokines after allergen-specific stimulation and quantified the allergen-induced cytokine production by PBMC in a Luminex assay as described in detail earlier

with minor modifications. Briefly, we first optimized the stimulation assays to ensure the cytokine response was allergen specific and not influenced by, for example, endotoxin contamination of the HDM extract (1 EU/µg). Stimulation of PBMC with 0.2 µg HDM/ml induced a typical allergen-specific cytokine response. Then, cryopreserved PBMC were thawed, washed and incubated in culture medium with phytohemagglutinin (PHA, 0.1 µg/ml) as a positive control, HDM (0.2 µg/ml) or medium alone. We used cytokine-
specific caption and detection antibodies for IL-4, IL-13, IL-10, IL-12 and IFNγ from Mabtech AB (Nacka Strand, Sweden), according to the manufacturer’s protocol, and incubated the cells at 37°C and 5% CO₂ for 72 h in 96-well PVDF-based membrane plates (Millipore, Billerica, MA, USA). After visualization of the spots with streptavidin-ALP and BCIP/NBT substrate (Sigma-Aldrich, St.Louis, MO, USA), the numbers of cytokine-producing cells were determined by ELISpot analysis software from A.EL.VIS GmbH (Hannover, Germany) and were expressed as positive cells per 10⁵ stimulated cells.

For quantification of allergen-induced cytokine production, we stimulated PBMC in round-bottom 96-well plates for 24 h with the same culture medium and stimuli as used for the ELISpot assay. The amount of several cytokines (IL-4, IL-5, IL-10, IL-12, IL-13 and IFNγ) was determined in the supernatant in a Luminex assay with antibodies from Biosource (Camarillo, CA, USA). Responses from PHA-stimulated cells were treated as positive control and medium-only culture as negative control. All T-cell responses from stimulated cultures were adjusted for background by subtracting the responses from the medium-only culture. All paired samples were processed in parallel, and analyses were performed batchwise to limit inter-assay variation. In addition, in every batch we took along control PBMC, which yielded similar results each time.

**Statistical analysis**

Allergen-induced changes in IgE within patients were analysed using the Student’s t-test. The role of possible confounding factors (including the intervention with synbiotics) that might be related to the changes in IgE or HDM-induced cytokine responses was studied in general linear models (ANOVA and repeated-measures ANOVA). When data were not normally distributed, either log transformation was performed (PC₂₀ methacholine and total allergen dose) to obtain normal distribution or the non-parametric Mann-Whitney U-test was used (changes in cytokine-producing cells and cytokine production). Correlations were determined with the Spearman’s rank test. Two-sided p-values of < 0.05 were considered statistically significant. We used SPSS version 15.0.1 (SPSS Inc., Chicago, IL, USA) for statistical analyses.

**RESULTS**

**Subject characteristics**

We obtained blood from twenty-seven allergic asthmatic patients who underwent a bronchial allergen challenge with HDM [geometric mean cumulative dose (range): 321 BU (20-1718)] before and 5 weeks after challenge. Subject characteristics of these patients are described in table 1. The challenges were performed year-round and, when co-sensitization to pollen was observed, outside the relevant pollen season. None of the
Table 1. Subject characteristics

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<td>HDM-specific IgE (kU/L) ‡</td>
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Values are expressed as *median (range), †mean (SD), ‡geometric mean (GSD).

FEV1, forced expiratory volume in 1 s; PC_{20} methacholine, provocative concentration of methacholine causing a decline in FEV1 of 20% from baseline; Δmax, maximum decline during the first hour after allergen challenge (early asthmatic response) and from 2-6 h after allergen challenge (late asthmatic response); IgE, Immunoglobulin E; HDM, house dust mite; EAR, early asthmatic response; LAR, late asthmatic response.

patients posed changes that could affect their environmental allergen exposure, 6 months prior to the start and during the study.

Allergen challenge-induced changes in serum levels of IgE

Five weeks after bronchial allergen challenge with HDM, the amount of circulating IgE antibodies against HDM and against the major HDM allergen components Der p1 and Der p2 was significantly increased (+10.8% and +8.8%, respectively, p < 0.05, fig. 1A). In contrast, levels of specific IgE against other environmental allergens to which the subjects were sensitized remained unchanged (cat dander) or decreased (grass pollen, −6.5%, p < 0.05). The difference between changes in IgE to HDM and IgE to control allergens was statistically significant. Serum levels of total IgE were also increased but did not reach statistical significance (+8.1%, p = 0.055). The increase in HDM-specific IgE was associated with PC_{20} methacholine (Rs = 0.734, p < 0.001, fig. 1B), and was not influenced by the intervention with synbiotics.

Allergen-induced cytokine production by PBMC

Compared with the HDM-induced cytokine response of ex vivo-stimulated PBMC from blood taken before challenge, we found a significant increase in the amount of Th2 cytokines, produced by PBMC that were isolated 5 weeks after challenge (p < 0.001, p = 0.014 and p = 0.006 for IL-4, IL-5 and IL-13, respectively, fig. 2). Moreover, we found a
Figure 1. (A) Five weeks after allergen challenge, the amount of specific IgE against house dust mite (HDM) and the HDM-allergenic components Der p1 and Der p2 in serum was significantly increased (+10.8% and +8.8%, respectively). Serum levels of specific IgE against other environmental allergens did not change (cat dander, □) or decreased (grass pollen, -6.5%, ○). Data are expressed as percentage change from baseline. *p < 0.05. (B) The increase in HDM-specific IgE significantly correlated with the PC_{20} methacholine, determined one day before allergen challenge.

Figure 2. Five weeks after allergen challenge, the allergen-induced Th2-cytokine production was significantly increased by peripheral blood mononuclear cells (PBMC) that were stimulated ex vivo with house dust mite for 24 h. This also applied to the IL-10 production. No significant changes were found in Th1-cytokine production. The positive controls by allergen-independent phytohemagglutinin stimulation (open bars) are provided for comparison. Data are corrected for medium control and expressed as picogram per ml supernatant.
significant increase in the production of IL-10 (p = 0.002). There were no changes in the allergen-induced production of Th1 cytokines, such as IL-12 and IFNγ.

Consistent with the enhanced allergen-induced Th2-cytokine production, we found a trendwise increase in the number of cells producing IL-4 or IL-13 (p = 0.079 and p = 0.061, respectively) after stimulation of PBMC with HDM (fig. 3A). This trend was not found for cells producing IL-10, IL-12 or IFNγ. Allergen-independent stimulation with PHA showed that there was no difference in the total number of responsive PBMC at both time points for these cytokines (data not shown). The increase in the number of Th2-cytokine-producing cells correlated with the increase in Der p1- and Der p2-specific IgE in serum (Rs = 0.56, p = 0.002 and Rs = 0.54, p = 0.004 for IL-4 and IL-13 respectively, fig. 3B). The increase in HDM-specific IgE also correlated with the increase in IL-4 production (Rs = 0.455, p = 0.017), but not with the increased production of other cytokines. When corrected for intervention, which we found to reduce Th2-cytokine production, the increase in production of all Th2-cytokines was significantly correlated to the increase in HDM-specific IgE.

**DISCUSSION**

To test our hypothesis that a single bronchial allergen challenge might enhance systemic B- and T-cell responses to allergen at a time point when the local allergen-induced effects on lung function and bronchial inflammation are normally resolved, we analyzed the allergen-specific responses in blood samples, taken before and 5 weeks after a bronchial allergen challenge during a clinical intervention study. Here, we show that a single bronchial allergen challenge with HDM in sensitized patients slightly, but significantly increased the HDM-specific IgE levels in serum. This increase was paralleled by an enhanced Th2 response of PBMC to HDM.

Because patients did not move house, change the interior of their houses or change pets 6 months prior to and during the study, and participated year-round, a significant change in environmental allergen exposure is an unlikely cause of the changes in HDM-specific IgE. Moreover, Der p1 exposure during the challenge [geometric mean cumulative dose (range): 629 ng (39-3367)] was relatively high compared with the expected natural exposure. The nightly exposure to Der p1 is approximately 6 ng as suggested by Gore et al.\(^\text{16}\). As an intra-individual control we measured specific IgE levels against allergens that were not used for challenge. The lack of an increase in the IgE levels against these control allergens strongly argues for an HDM-specific effect. Therefore, we conclude that the increase in HDM-specific IgE was most likely the result of the bronchial allergen challenge.

There was a positive association between the increase in HDM-specific IgE and the baseline PC\(_{20}\) methacholine. The PC\(_{20}\) methacholine has been shown to be pivotal for the
Figure 3. (A) Five weeks after allergen challenge, we found an increased number of peripheral blood mononuclear cells (PBMC) that produced Th2 cytokines after stimulation for 72 h with house dust mite (HDM). (B) The increase in HDM-specific IgE (Der p1 + Der p2) in serum 5 weeks after allergen challenge with HDM significantly correlated with the increase in number of Th2-cytokine-producing PBMC after stimulation with HDM in an ELISpot assay. No changes were found for IL-10- and IFN-γ-producing cells. Data are corrected for medium control and expressed as number of cytokine-producing cells (spots) per 100,000 ex vivo-stimulated PBMC.

dose of HDM needed to reach a 20% decline in FEV1 during the challenge\textsuperscript{17} (the correlation between $PC_{20}$ methacholine and the total allergen dose delivered was $Rs = 0.609, p = 0.001$). Therefore, we assume that the total dose of allergen, which was highest in patients with relatively little AHR, is the main factor to determine the increase in specific IgE.

In an animal study, repeated intratracheal inoculation of *Dermatophagoides farinae* (Der f) in mice significantly increased both the total and Der f-specific IgE antibody concentrations in BALF and serum\textsuperscript{18}. After repeated challenges, the authors also observed chronic airway inflammation, characterized by increased numbers of lymphocytes and eosinophils and increased levels of IL-5 and IFN-γ in BALF.

Although we did find an increase in allergen-specific IgE and in the allergen-induced production of Th2 cytokines, this enhanced systemic sensitivity to HDM neither caused a detectable increase in baseline airway responsiveness at 5 weeks after challenge nor was
it accompanied by an enhanced allergen-induced response in airway inflammation and airway responsiveness 24 h after a second allergen challenge in the intervention study (table 2 in reference 14).

This is consistent with the aforementioned studies that investigated the safety and reproducibility of allergen challenges in asthmatics.\textsuperscript{4,9,10} Apparently, the persistent small changes in systemic allergic parameters, caused by the single allergen challenge, do not lead to a detectable aggravation of the increase in airway responsiveness and bronchial inflammation within the 24 h after a second allergen challenge. However, accumulation of such long-term effects by chronic or repeated experimental allergen exposure might resemble the effects of natural repeated exposure to allergen that results during, for example, the grass pollen season in the induction of bronchial hyperresponsiveness and inflammatory responses involving T cells, mast cells and eosinophils.\textsuperscript{19-21} In a 2-year follow-up study of starting laboratory animal workers, inhalation of a comparable cumulative dose of allergen resulted in the development of allergen specific IgE antibodies that was associated with the development of an allergen-specific IL-4 response.\textsuperscript{22} These changes in allergic and immunologic parameters also did not result in detectable changes in bronchial hyperresponsiveness, but may help to understand the mechanism of increasing airway responsiveness after repeated or prolonged allergen exposure.

To our knowledge, this is the first study to show effects of a single bronchial allergen challenge on systemic allergic and immunologic parameters 5 weeks after allergen challenge at a time point when bronchial responsiveness and inflammation already have returned to baseline levels. The increased levels of HDM-specific IgE and the enhanced sensitivity of PBMC to HDM at 5 weeks after a high-dose inhalation of HDM emphasize the role of B and T cells in the development and maintenance of allergy. It remains to be established how long the systemic sensitivity to allergen will be enhanced and whether multiple experimental challenges would lead to aggravation or induction of allergic diseases (resembling natural exposure). Moreover, it has to be confirmed whether these results only apply for HDM or also to other allergens. The enhanced systemic sensitivity to allergen after a single challenge should be taken into consideration when repeated allergen challenges are performed and when these challenges are used in clinical studies. The effect appears to be dependent on the total dose of allergen delivered during allergen challenge. Therefore, patients with relatively little airway hyperreactivity are at greatest risk to experience an increase in allergen-specific IgE as they will receive the highest dose of allergen in standard provocation protocols.
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REFERENCES