

Sputum inflammatory cells and allergen-induced airway responses in allergic asthmatic subjects

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Abstract

Background: Allergen inhalation causes early and late bronchoconstrictor responses, airway hyperresponsiveness and airway inflammation in allergic asthmatics. The role of airway inflammatory cells in causing allergen-induced bronchoconstriction and airway hyperresponsiveness is controversial. The objective of this study was to examine the relationships between allergen-induced increases in airway inflammatory cells, early and late bronchoconstrictor responses and methacholine airway hyperresponsiveness.

Methods: Allergen inhalation challenge was conducted in 50 allergic asthmatics. Changes in the forced expired volume in 1 s (FEV₁%) were followed for 7 h, induced sputum was obtained at 7 and 24 h, and the provocative concentration of methacholine causing a 20% fall in FEV₁ (MCh PC₂₀) was measured at 24 h.

Results: There was a significant negative correlation between the baseline methacholine PC₂₀ and baseline sputum eosinophils ($r = -0.512$, $P = 0.0001$). Allergen-induced changes in methacholine PC₂₀ were also significantly negatively correlated to allergen-induced change in sputum eosinophils at 24 h ($r = -0.434$, $P = 0.002$), but not to changes in any other inflammatory cells. There were no significant correlations between sputum eosinophils or other inflammatory cells and the allergen-induced early or late asthmatic responses.

Conclusion: Allergen-induced increases in airway eosinophils in asthmatic dual responders may contribute to allergen-induced changes in methacholine PC₂₀, but not the late asthmatic responses.

In sensitized asthmatics, inhaled allergen binds to the high affinity IgE receptors on mast cells, releasing preformed mediators including histamine and newly generated eicosanoids, particularly the cysteinyl leukotrienes, causing acute bronchoconstriction within 10–15 min (1). This is called the early asthmatic response (EAR). The EAR usually resolves within 1–2 h, but may be followed by a late asthmatic response (LAR), typically beginning between 2 and 4 h after allergen inhalation (2). The LAR is accompanied by an influx of inflammatory cells into the airways, which are predominantly eosinophils (3), but also includes an increase in airway neutrophils (4) and basophils (5). The allergen-induced changes in inflammatory cells can be most easily measured in induced sputum. Allergen-induced bronchoconstriction is largely, but not completely resolved, at 24 h, at which time there is usually an increase in airway responsiveness mea-

sured by a reduction in the provocative concentration of methacholine causing a 20% fall in FEV₁ (MCh PC₂₀) (6).

The importance of the allergen-induced increase in airway eosinophils, in causing allergen-induced LAR and airway hyperresponsiveness remains unclear. We have previously reported that pretreatment with both an anti-histamine and a leukotriene receptor antagonist abolishes both the EAR and LAR (7). As mast cells or basophils are the only cell types able to produce both histamine and cysteinyl leukotrienes, and there is an increase in airway basophils during the LAR (5), these results have suggested that basophils recruited into the airway, may be responsible for the LAR. In addition, a previous study has examined the ability of a humanized monoclonal antibody (hMab) directed against IL-5 (mepolizumab) to prevent allergen-induced responses in human subjects (8). These authors demonstrated that

mepolizumab completely inhibited allergen-induced sputum eosinophilia, but did not attenuate either the allergen-induced EAR or LAR. Unfortunately, these researchers could not demonstrate any allergen-induced airway hyperresponsiveness during placebo treatment, so could not make any comment on the role of eosinophils in mediating this more prolonged airway response after inhaled allergen. Thus, the importance of the allergen-induced increase in airway eosinophils in causing allergen-induced bronchoconstrictor responses and airway hyperresponsiveness is unclear.

The purpose of this study was to identify the relationship between allergen-induced changes in sputum cell counts, and allergen-induced bronchoconstrictor responses and the increase in methacholine airway responsiveness.

Methods

Subjects

The subjects consisted of 28 female and 22 male, nonsmoking, mild allergic asthmatics, with no other lung disease, ranging in age from 18 to 56 years (Table 1). These were 50 sequential subjects undergoing a screening allergen challenges prior to entry into trials of novel therapies for allergic asthma. The baseline FEV₁ was >70% of predicted normal in all subjects and the provocative concentration of methacholine causing a 20% fall in FEV₁ (MCh PC₂₀) was <16 mg/ml. Subjects had no lower respiratory tract infection, worsening of asthma for 6 weeks before study. The subjects were all studied outside allergen season and were requested to avoid any exposure to sensitizing allergens such as cats and dogs. Subjects used inhaled β_2 -agonist infrequently and were steroid naïve. They refrained from β_2 -agonist and caffeinated beverages before visits. All subjects had a positive skin prick test to known common allergens and the specific allergen administered. All had previously demonstrated >20% fall in FEV₁ 10 min after the inhalation of doubling concentrations of a specific allergen and had >15% fall in FEV₁ at 7 h.

Table 1 Subject characteristics

Subjects	50
Age (year)	25.98 $\pm$ 1.39
Gender, male/female	22/28
FEV ₁ % predicted	91.39 $\pm$ 1.72
Methacholine PC ₂₀	1.79 (GSEM 1.15)
Allergen	Cat (15), House dust mite DP/DF (17/4), Alternaria (2), Grass (4), Ragweed (8)
Sputum eosinophil median	0.9% (range 0.0–35.3%)
Sputum neutrophil median	36.0% (range 4.8–96.3%)

DF, *D. Farinae*; DP, *D. Pteronyssinus*; PC₂₀, provocative concentration of methacholine producing a 20% fall in FEV₁.

Summary statistics are shown as arithmetic mean and standard error of mean, except for PC₂₀ which is shown as geometric mean and geometric standard error of mean.

Study design

The subjects attended the laboratory on three sequential study days. On the first study visit, baseline lung function was measured and methacholine inhalation challenge performed and subsequently, sputum was induced. On the second study visit, the following morning, baseline lung function was measured and allergen-inhalation challenge was performed. The FEV₁ was measured before allergen challenge and for 7 h after allergen. At 7 h, the bronchoconstriction was reversed with an inhaled β_2 -agonist and sputum was induced. On the third study visit, 24 h after allergen lung function was measured, methacholine inhalation challenge was performed, and sputum was induced.

Laboratory procedures

Methacholine inhalation

Methacholine inhalation challenge was performed as described by Cockcroft (9), using tidal breathing, from a Wright nebulizer (Roxon Meditech, Montreal, PQ, Canada). The test was terminated when a fall in FEV₁ of at least 20% of the baseline value occurred, and the methacholine provocative concentration causing a 20% fall in FEV₁ (Mch PC₂₀) was calculated.

Allergen inhalation challenge

Allergen inhalation challenge was conducted as described by O'Byrne et al. (2). The starting concentration of allergen extract was determined using the magnitude of the skin test response in combination with the Mch PC₂₀ (10). The allergen was diluted in 0.9% saline and the starting dose was calculated at two doubling doses below that predicted to induce a 20% drop in FEV₁. Incremental doubling concentrations of allergen were repeated until a 20% decrease in FEV₁ was reached 10 min postallergen inhalation. The FEV₁ was measured at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 420 min after allergen challenge. The EAR was measured as the largest decrease in FEV₁ within the first 120 min. The LAR was measured as the decrease in FEV₁ at 7 h after allergen inhalation.

Sputum induction and analysis

Sputum was induced and processed in a standardized manner as previously described (11). Subjects inhaled 200 μ g albuterol and then inhaled 3%, then 4%, and then 5% saline for 7 min consecutively. The induction was continued until the all three saline inductions were complete. The FEV₁ was measured after each saline concentration and the induction terminated if the FEV₁ declined by 20%. Cell plugs were selected and weighed and diluted with four times their weight of 0.1% dithiothreitol (Sputolysin; Calbiochem Corp., San Diego, CA, USA) and then with four times their weight of Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies Inc., Grand Island, NY, USA). The cell suspension was filtered through 52- μ m nylon gauze (BNSH Thompson, Scarborough, ON, Canada) to remove debris. The total cell count was determined in a hemocytometer chamber

(Neubauer Hausser Scientific, Blue Bell, PA, USA) and expressed as the number of cells per milligram. The cells were suspended in DPBS at $0.75\text{--}1.0 \times 10^6/\text{ml}$. Cytospin preparations were made on glass slides and differential cell counts were obtained. Two slides were stained with Diff-Quik (American Scientific Products, San Diego, CA, USA) and 400 cells were counted per slide. The results were expressed both as % total cells and as cells/g sputum. The mean value of the two slides was used. Mast cells, basophils and T-cells are uncommon in induced sputum, require special stains, and were not quantified for this analysis.

Statistical analysis

Results were expressed as mean $\pm$ SEM, except for methacholine PC₂₀, which was expressed as geometric mean $\pm$ SEM. Mch PC₂₀ was log₂ transformed to fit a normal distribution before analysis. The allergen-induced changes in sputum eosinophils from baseline to 7 or 24 h after allergen were initially analyzed as quartiles. The allergen-induced changes from baseline in FEV_{1%} predicted, Mch PC₂₀, sputum total cell numbers and sputum differential numbers were also analyzed using a repeated measures analysis of variance (within group: baseline vs 7 vs 24 h; Statistica 7.0; Statsoft, Tulsa, OK, USA). Correlations were analyzed by simple regression (STATISTICA 7.0; STATSOFT). A $P < 0.05$ was considered to be statistically significant.

Results

Allergen inhalation challenge resulted in both an EAR and an LAR in all subjects; the mean maximal fall in FEV₁ during the EAR was 37.26% and during the LAR was 24.35%. In addition, allergen inhalation resulted in a significant decrease in Mch PC₂₀ values, from 1.79 mg/ml before, to 0.67 mg/ml at 24 h after allergen (Table 2), as well as a significant increase in sputum eosinophils (expressed as percentage of total cells and as absolute cell numbers), an increase in sputum neutrophils (expressed as percentage of total cells) at both 7 and 24 h after allergen, and a significant decrease in sputum macrophages (expressed as percentage of total cells

and as absolute cell numbers) at 7 h after allergen (Table 2). Sputum eosinophils increased in 48/50 subjects at 7 h and remained elevated in 47/50 at 24 h; sputum neutrophils increased in 33/50 subjects and remained elevated in 33/50 at 24 h; and airway macrophages decreased in 41/50 at 7 h and remained decreased in 41/50 at 24 h.

There were no significant correlations with sputum cell counts at baseline or at 7 h and the FEV₁ at baseline, the allergen-induced fall in FEV₁ during the EAR, or the LAR. There was a trend toward the highest quartile of the allergen-induced change in sputum eosinophils at 24 h being associated with the largest decline in FEV₁ at 24 h (the decline in FEV₁ in the lowest quartile being $-4.8 \pm 2.4\%$, and in the highest quartile $-14.2 \pm 3.2\%$). There was no significant correlation between the allergen-induced change in sputum eosinophils and the FEV_{1%} at 24 h ($P = 0.192$).

There was a significant negative correlation between the log baseline Mch PC₂₀ and baseline sputum eosinophils, either expressed as % total cells ($r = -0.476$, $P = 0.0005$) or absolute number ($r = -0.512$, $P = 0.0001$). There were no significant correlations between baseline log Mch PC₂₀ and other sputum cells.

The magnitude of change in Mch PC₂₀ from baseline to 24 h was significantly greater in the third and fourth quartiles for the changes in sputum eosinophils, being -0.84 ± 0.21 in the first quartile and -1.80 ± 0.23 in the third and -1.96 ± 0.23 in the fourth quartile ($P < 0.05$ for both) (Fig. 1A). In addition, there was a significant negative correlation between the allergen-induced change in Mch PC₂₀ from baseline to 24 h and the change in sputum eosinophils between baseline and 24 h ($r = -0.434$, $P = 0.002$) (Fig. 1B). There were no significant correlations between the allergen-induced change in Mch PC₂₀ and any other sputum cell type.

Discussion

This study has demonstrated that allergen-induced changes in methacholine airway responsiveness at 24 h after allergen challenge, in asthmatic subjects who developed an allergen-induced EAR and LAR, were significantly correlated with

Table 2 Allergen-induced responses

	Baseline	7 h	24 h
FEV _{1%} predicted (%)	91.39 $\pm$ 1.72	68.95 $\pm$ 1.36*	82.77 $\pm$ 1.90*††
Methacholine PC ₂₀ (mg/ml)	1.79 (0.22–12.46)	–	0.67* (0.06–5.27)
Total cell ($\times 10^6/\text{g}$)	3.70 $\pm$ 0.46	4.58 $\pm$ 0.50	4.41 $\pm$ 0.45
Eosinophil (%)	3.70 $\pm$ 0.96	15.61 $\pm$ 2.20**	15.06 $\pm$ 2.01**
Neutrophil (%)	41.30 $\pm$ 3.91	54.26 $\pm$ 3.62**	47.05 $\pm$ 3.47†
Macrophages (%)	54.35 $\pm$ 3.84	29.68 $\pm$ 3.32**	37.66 $\pm$ 3.43**†
Eosinophil ($\times 10^6/\text{g}$)	0.11 $\pm$ 0.03	0.71 $\pm$ 0.11**	0.73 $\pm$ 0.13**
Neutrophil ($\times 10^6/\text{g}$)	2.00 $\pm$ 0.42	2.74 $\pm$ 0.42	2.28 $\pm$ 0.34
Macrophages ($\times 10^6/\text{g}$)	1.57 $\pm$ 0.16	1.11 $\pm$ 0.14*	1.40 $\pm$ 0.15

* $P < 0.05$ vs baseline, ** $P < 0.01$ vs baseline, † $P < 0.05$ vs 7 h, †† $P < 0.01$ vs 7 h.

All values expressed as mean $\pm$ SEM, except methacholine PC₂₀ (mg), expressed as geometric mean (minimum – maximum).

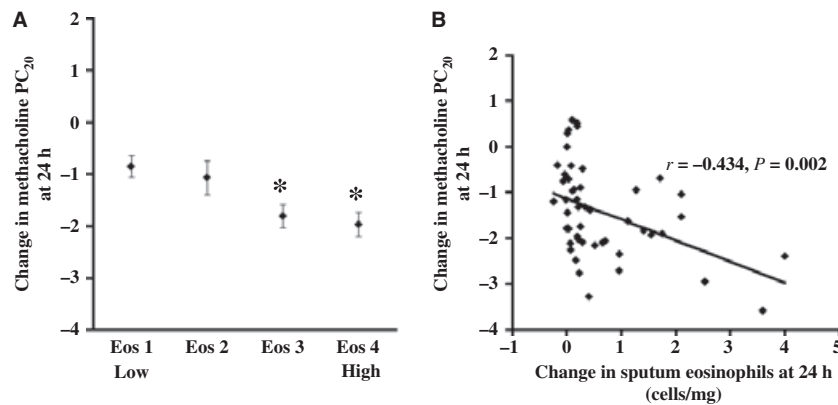


Figure 1 (A) Changes in sputum eosinophils 24 h after inhaled allergen expressed as quartiles (minimum–maximum) related to the allergen-induced change in methacholine PC₂₀ at 24 h. The magnitude of change in methacholine PC₂₀ from baseline to 24 h was significantly greater in the third and fourth quartiles for the changes in

sputum eosinophils (* $P < 0.05$). (B) The relationship between the changes in sputum eosinophils 24 h after inhaled allergen and the allergen-induced changes in methacholine PC₂₀ at 24 h. There is a significant correlation between the variables ($r = -0.434$, $P = 0.002$).

the allergen-induced increases in sputum eosinophils, but not sputum neutrophils or macrophages. In addition, the baseline Mch PC₂₀ was significantly correlated with the baseline sputum eosinophils. There were no significant correlations between sputum inflammatory cells and any other allergen-induced changes in lung function, including the EAR and LAR.

The correlation between baseline sputum eosinophils and MCh PC₂₀ described in this study is not a novel observation. We previously identified a relationship in mild stable asthmatic subjects between bronchoalveolar lavage (BAL) eosinophils and MCh airway hyperresponsiveness (12). Other studies described this relationship with sputum eosinophils (13, 14), while another study described this relationship with eosinophil counts in bronchial biopsies (15). However, not all studies have agreed that such a relationship exists (16, 17). The correlation between the allergen-induced change in sputum eosinophils and change in MCh PC₂₀ has not been previously described. Oddera et al. (17) did identify a correlation between BAL eosinophils and allergen-induced changes in MCh PC₂₀; however, Shaver et al. (18) did not demonstrate a correlation with BAL. In addition, previous studies have not examined the allergen-induced inflammatory changes and the associated physiological changes at several time periods during the allergen response, so could not comment on the associations between the allergen-induced inflammatory responses the EAR and LAR. The correlation between the change in sputum eosinophils and the change in methacholine PC₂₀ suggests that 19% of the variance in the change in PC₂₀ can be explained by the sputum eosinophilia. This supports the hypothesis that methacholine airway hyperresponsiveness consists of both a component of airway inflammation and airway structural changes (19). The median baseline levels of sputum eosinophils were normal for the group as a whole, and there was no relationship between the baseline eosinophil numbers and any of the allergen-induced responses.

Despite the long history demonstrating an increase in airway eosinophils associated with inhaled allergen in asthmatic subjects, their role in mediating the allergen-induced airway responses remains unclear. Only one study has used an eosinophil specific approach, with an anti-IL-5 hMab (mepolizumab), to test the importance of eosinophils in these responses (8). Mepolizumab did successfully attenuate allergen-induced airway eosinophilia, but did not influence the magnitude of the LAR. This result is consistent with our observations that there is no correlation between the magnitude of allergen-induced airway eosinophilia and the LAR. This supports the speculation, derived from the pharmacological interventions with ant-histamines and anti-leukotrienes (7), that basophil activation may be more important in causing this response, as reported for nasal allergen-induced responses (20). Unfortunately, the study utilizing mepolizumab could not demonstrate an allergen-induced change in MCh PC₂₀ in the placebo treatment arm, so could not make any comment on the ability of mepolizumab to attenuate this response (8). The results of the current study suggest that an increase in sputum eosinophils accounts for part of the variance in the allergen-induced change in MCh PC₂₀, and that an eosinophil specific treatment would attenuate this response.

Increases in sputum neutrophils were also seen in 66% of the subjects studied, which although less frequent than the increases in sputum eosinophils (96% of subjects), was greater than we had expected. Increases in both cells types were seen in the majority of subjects. There was no relationship between the magnitude of the neutrophilic response and any of the allergen-induced changes in airway responses. Moreover, a significant increase in sputum neutrophils was only demonstrated at 7 h after allergen, when expressed as percentage of cells and not as absolute cell number. This can be explained by the decrease in percentage of sputum macrophages that was identified at 7 h after allergen. Other studies have reported that BAL and/or bronchial biopsy neutrophils

increase after local endobronchial allergen instillation (21, 22), within 4 h of the challenge (4). The increases are, in part, mediated by the neutrophil chemoattractant leukotriene (LT)_{B4} (23). Other investigators have suggested that the neutrophil influx is a non-specific consequence of the bronchoscopy procedure, rather than a specific effect of the allergen inhalation (24).

The number of airway macrophages fell after allergen challenge in most subjects (82%), both expressed as percentage of total cells (where a decrease might be expected as the percentage of eosinophils and neutrophils increased), and as cell numbers per mg sputum, at 7 h after allergen inhalation. Macrophages are known to express both the high affinity (FcεR1) (25) and low affinity (CD23) IgE receptors (26), and have been implicated in inducing allergen-induced airway inflammation (27–29). However, this is the first study to identify a significant reduction in airway macrophages after allergen inhalation. Previous studies using BAL did not find such a reduction (18, 28). There was no relationship between the absolute number of macrophage and the severity of the LAR.

In conclusion, allergen inhalation by allergic asthmatic subjects, who develop allergen-induced dual asthmatic responses,

causes an increase in sputum eosinophils, sputum neutrophils (when expressed as percentage of total cells) and a fall in sputum macrophages. Only the changes in sputum eosinophils correlated with allergen-induced changes in MCh PC₂₀ supporting a possible role for airway eosinophils in causing this allergen-induced response.

Author contributions

HI, RW, TS, GO, KW all contributed to the collection of the data. HI, GG, KK and POB contributed to the data analysis and its interpretation. All authors have contributed to the writing of the paper and have approved its submission.

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Conflict of interest

No author has any conflict of interest to identify in relation to this study.

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