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Increases in eotaxin-positive cells in induced sputum from atopic asthmatic subjects after inhalational allergen challenge

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Key words: allergen challenge; asthma; chemokine; eosinophil; sputum.

Background: Eosinophils are believed to be critical proinflammatory cells in airway mucosal damage in asthma. Eotaxin is a C-C chemokine with selective activity for eosinophils and basophils. Previous studies have shown increased expression of eotaxin in the airways of asthmatics at baseline. We aimed to investigate eotaxin expression during the late-phase reaction to allergen inhalation in atopic asthmatics.

Methods: Sputum induction was performed before and 24 h after inhalational allergen challenge in atopic asthmatics, and eotaxin protein was detected immunocytochemically.

Results: Thirteen patients with a mean decrease in forced expiratory volume in 1 s of 28% (± 1.5) during the early asthmatic reaction, and 39% (± 4.7) during the late asthmatic reaction produced sufficient sputum for study. The percentage of eosinophils in sputum was increased 24 h after allergen challenge ($P < 0.004$), and eosinophil percentages in sputum after challenge correlated with the magnitude of the late-phase reaction ($r = 0.56$, $P = 0.05$). The percentage of eotaxin-positive cells increased from 12.6% (range 2–43.8) to 24.3% (8.1–47.1, $P < 0.005$). Allergen-induced increases in eotaxin-positive cells correlated with increases in eosinophils ($r = 0.63$, $P < 0.01$).

Conclusions: These findings suggest that eotaxin may contribute to allergen-induced recruitment of eosinophils to the airway in asthmatic subjects.

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Allergen inhalation by sensitized atopic asthmatic subjects results in a rapid onset bronchoconstriction which resolves within 1–2 h. This is followed by a delayed response occurring 3–8 h later, termed the late asthmatic reaction (LAR). Study of the inflammatory processes occurring during

the LAR have shown increased eosinophil numbers in peripheral blood (1), bronchoalveolar lavage (BAL) (2, 3), and bronchial biopsies (4), which were related to the size of the bronchoconstrictor response during the LAR.

Sputum induction is a validated, reproducible, and noninvasive technique for investigating airway inflammation in asthma (5). Analysis of induced sputum after aerosolized allergen challenge in atopic asthmatics showed an increase in eosinophils, eosinophil granule proteins (6), and cytokines that may contribute to eosinophil accumulation and activation, such as interleukin-5 (IL-5) and tumour necrosis factor alpha (TNF- α) (7).

The C-C chemokine eotaxin is a potent and selective eosinophil chemoattractant, which signals via the CCR3 receptor (8, 9). Elevated levels of eotaxin protein and mRNA have been demonstrated in the airway of asthmatic patients at baseline (10–12). There is no information on the effect of allergen inhalation on eotaxin expression in human volunteers.

Material and methods

Twenty-two patients were screened and selected on the basis of a demonstrated LAR to inhalational allergen challenge. Sufficient sputum to perform differential cell counts and immunocytochemistry was produced on each occasion by 13 of these patients. The inclusion criteria included a history of mild asthma precipitated by an inhaled allergen, a positive skin prick test to at least one common aeroallergen, and a forced expiratory volume in 1 s (FEV₁) greater than 70% predicted. All patients were nonsmokers, and none had had an upper respiratory tract infection in the 6 weeks preceding the trial, or had used inhaled or oral corticosteroids, or other treatment, apart from inhaled short-acting β_2 -agonists, for at least 12 weeks before entering the trial. No patient had undergone allergen immunotherapy, and all were studied out of their allergen season. The study was approved by the East London and City Health Authority Ethics Committee.

At the first screening visit, a full history; an examination, skin prick tests with house-dust mite, grass, cat, dog, and diluent control (Soluprick, ALK, Hørsholm, Denmark); and lung-function tests were performed. One week later, the patient attended for baseline sputum induction by a protocol based on that described by Pin et al. (6). The following day, the patient attended for an inhalational allergen challenge performed by a standardized protocol (13). FEV₁ was measured at 5 and 10 min

and compared to the post-diluent baseline. Increasing concentrations of allergen were inhaled under identical conditions until a decrease in FEV₁ greater than or equal to 20% was recorded. FEV₁ was then monitored at 5-min intervals for 45 min and 15-min intervals for 2 h, and then at 30-min intervals for 12 h after the early asthmatic reaction was established. FEV₁ recorded 0–2 h after allergen challenge were recorded as the EAR, while FEV₁ recorded at 2–12 h represent the LAR. Twenty-four hours after allergen challenge, sputum induction was repeated and processed as described by Popov et al. (14). Plugs of sputum were selected and dispersed with four volumes of cooled 0.1% dithiotreitol (DTT) (Sigma Pharmaceuticals, Dorset, UK) freshly diluted in Dulbecco's phosphate-buffered saline (D-PBS) (Sigma). After 15 min, an equal volume of cooled D-PBS was added, and the sample was filtered through a 48- μ m nylon gauze and centrifuged at 2000 revolutions per minute for 10 min. Cells were then resuspended at 1×10^6 cells/ml in D-PBS. Cell viability and total cell count were estimated by trypan blue staining (Sigma). Differential cell counts were performed on cytocentrifuge preparations stained with Romanowsky's stain (15) by two observers blind to clinical details. In all cases, 400 nucleated nonsquamous cells were counted, and results were expressed as percentage of total nonsquamous cells.

Immunocytochemistry was performed by the alkaline phosphatase antialkaline phosphatase (APAAP) method, as previously described. Rabbit antimouse immunoglobulin (Ig), APAAP, and control IgG1 were purchased from Dako (High Wycombe, UK). Antihuman eotaxin mAb (2G6) was a kind gift from Dr Walter Newman (Leukosite, Inc., Cambridge, MA, USA). Omission or replacement of the primary antibody with an irrelevant antibody of the same species was used as a negative control. Isolated human umbilical vein endothelial cells (HUVEC), which are known to express eotaxin (11), were used as a positive control.

Data are expressed as median (with minimum to maximum range), or as mean ($\pm$ standard error of the mean), as appropriate. Statistical analyses were performed with the aid of a commercially available statistical package (Minitab for Windows, Minitab Release 9.2, Minitab, Inc., State College, PA, USA). Statistical analyses of comparisons before and after the allergen inhalation were performed with Wilcoxon's signed-rank tests. Spearman's rank correlation coefficient test was used to examine the association between inflammatory parameters and airway function. A *P* value of less than 0.05 was accepted as statistically significant.

Results

Sputum induction was well tolerated by all but one of the 22 patients. However, one patient developed a fall in FEV₁ of 53% during the induction attempt before allergen challenge, and was unable to produce a sample. This bronchoconstriction was rapidly reversed by 2.5 mg nebulized salbutamol. The median (and interquartile range) decrease in FEV₁ after sputum induction was 1.44% (4.27–1.28) before and 0.0% (–3.56 to 3.45) after allergen challenge. Thirteen patients with a mean decrease in FEV₁ of 28% (± 1.5) during the EAR, and 39% (± 4.7) during the LAR produced sufficient sputum on each occasion for comparison between before and after allergen challenge. There were no significant clinical differences between the 13 patients who produced adequate sputum volumes and the nine who did not either at baseline or during the allergen challenge (Table 1). The median total cell number recovered in sputum before allergen challenge was 0.8×10^6 cells (range 0.17–2.4), vs 1.3×10^6 cells (range 0.17–9.2) 24 h after challenge, and no significant changes were observed in total cell numbers after the allergen inhalation.

There was a significant increase in the median percentage of eosinophils in induced sputum seen 24 h after allergen challenge from 1.2% (range 0–15.9) before, to 17.1% (3.8–34.4) after challenge ($P < 0.004$) (Fig. 1a). The percentage of macrophages decreased significantly from 57.2% (4.3–80) before to 32.7% (5.4–75.2) after allergen inhalation ($P < 0.05$).

There was no significant change in the median percentages of neutrophils (31.3% [14.4–95.3] vs 41.6% [15.9–81.3], epithelial cells (0% [0–13.2] vs 0.7% [0–34.1], or lymphocytes (1.1% [0–2.7] vs 1% [0–4.5]) before and after the allergen inhalation. There was a significant correlation between the increase in eosinophil percentage in sputum after allergen challenge and the maximal decrease in FEV₁ during the LAR ($r = 0.55$, $P = 0.05$).

Immunocytochemistry detected expression of eotaxin at baseline (median 12.6%, range 2–34), and the percentage of cells expressing eotaxin significantly increased after the allergen inhalation (24.3%, range 8–47) ($P = 0.002$) (Fig. 1b). A significant positive correlation was evident between the increase in sputum eosinophil percentages and the increase in eotaxin expression after allergen challenge ($r = 0.63$, $P < 0.01$) (Fig. 1c). Additionally, increased eotaxin expression correlated significantly with the maximum decrease in FEV₁ during the LAR ($r = 0.4$, $P < 0.01$).

Comparison of the structure of cells counterstained with haematoxylin after immunostaining to that of cells stained with Romanowsky (Fig. 2) showed that most sputum cells positive for eotaxin appeared to be macrophages (65%, range 52–84; 67%, range 50–78% of the total eotaxin-immunoreactive cells before and after allergen challenge, respectively). Eosinophils accounted for 5% (range 0–12) before challenge, increasing to 15% (range 0–23) after allergen inhalation ($P = 0.04$). Epithelial cells accounted for 6.5% (range 3–16) and 5% (range 0–51), and lymphocytes

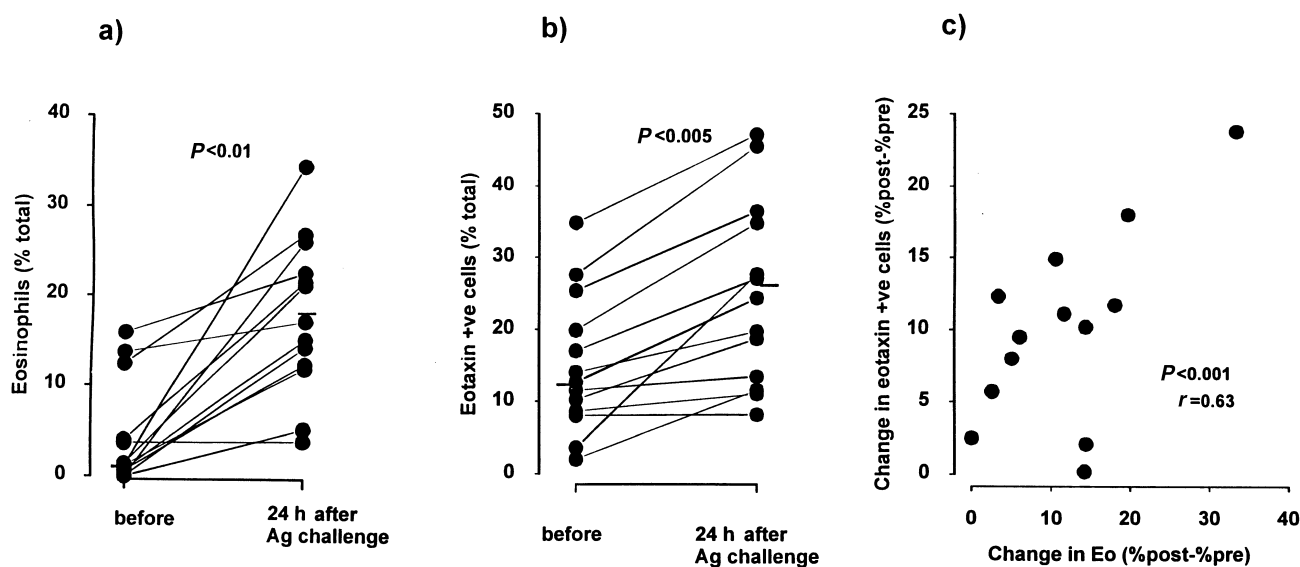


Figure 1. a) Percentage of eosinophils in induced sputum before and 24 h after allergen challenge. b) Percentage of cells immunoreactive to eotaxin before and 24 h after allergen challenge. Individual and median values shown. c) Relationship between allergen-induced changes in induced sputum, percentage of eosinophils, and eotaxin expression. Results expressed as (percentage of eosinophils in induced sputum after allergen) – (percentage of eosinophils in induced sputum before allergen) vs (percentage of eotaxin-positive induced sputum cells after allergen challenge) – (percentage of eotaxin-positive induced sputum cells before allergen challenge). r and P values correspond to Spearman's rank correlation coefficient.

Table 1. Subject characteristics and allergen-induced airway responses

Subject no.	Age (years)	Sex	% Predicted FEV ₁	Blood eosinophils ($\times 10^9/l$)	Inhaled allergen	EAR % decrease FEV ₁	LAR % decrease FEV ₁
Patients producing adequate sputum cells for differential counts and immunocytochemistry							
1	28	F	88	0.21	Der p	23	62.5
2	48	F	75	0.23	Der p	32.5	52.5
3	24	F	104	0.04	Grass	26	30
4	28	M	91	0.55	Cat	35	63.4
5	27	M	114	0.13	Der p	32	48
6	22	F	102	0.2	Der p	25	16
7	40	F	92	0.2	Der p	30	44
8	30	F	70	0.09	Der p	30	49
9	30	F	98	0.16	Der p	21	15
10	20	M	102	0.09	Cat	21	26.4
11	27	M	89	0.12	Der p	20.2	51.2
12	28	F	115	0.09	Grass	33.7	27.5
13	38	M	94	0.65	Dog	33.3	20.5
Mean ($\pm$ SEM)	30 ($\pm$ 2.15)		94.9 ($\pm$ 3.7)	0.2 ($\pm$ 0.05)		27.9 ($\pm$ 1.5)	38.9 ($\pm$ 4.74)
patients unable to produce adequate number of sputum cells							
1	32	M	71.3	0.1	Der p	21.4	28.6
2	27	F	98.6	0.2	Cat	66	52.5
3	22	F	91	0.18	Cat	22	19.4
4	28	M	101.7	0.02	Der p	78	29
5	24	M	88	0.46	Der p	32.5	50
6	28	F	104	0.05	Der p	22.3	44.8
7	22	F	109	0.15	Der P	28.4	23.2
8	30	F	82	0.16	Cat	26.7	37.8
9	24	F	98.4	0.16	Cat	25.5	20
Mean ($\pm$ SEM)	26.3 ($\pm$ 1.18)		93.8 ($\pm$ 3.96)	0.16 ($\pm$ 0.05)		35.9 ($\pm$ 7)	33.9 ($\pm$ 4.26)

accounted for 2.5 % (range 2–6) and 1.5 % (range 0–1.7) before and after challenge, respectively. The percentage of total macrophages expressing eotaxin increased from 12.5 % (range 2–28) to 22.5 % (range 7–38), as did the percentage of eosinophils, increasing from 4.5 % (range 0–17) to 11 % (range 5–28), and epithelial cells, increasing from 26 % (range 0–35) to 39 % (range 0–90). Neutrophils were not stained positive to eotaxin.

Discussion

To our knowledge, this is the first time that an increase in airway eotaxin expression in response to inhaled allergen in asthmatic patients has been demonstrated. We have also shown that this increase in eotaxin expression correlates

both with the influx of eosinophils and with the magnitude of the decrease in FEV₁ seen during the LAR.

There were no significant differences in the clinical characteristics or allergen-induced spirometric changes between those 13 patients who produced sufficient sputum for our study and those nine who did not. This yield from sputum induction is similar to that reported in previous studies (5–7) and does not appear to have resulted in any selection bias. Our study has shown very similar sputum differential cell counts at baseline, and in response to allergen challenge to those shown by other groups (5, 6). The low percentage of eosinophils detected at baseline in these subjects probably reflects their mild asthma and the lack of current symptoms. We selected 24 h after allergen inhalation as a time point for study of eotaxin, as increased eosinophil numbers in sputum (6), BAL (2), and bronchial biopsies (4) have been previously demonstrated at 24 h, and

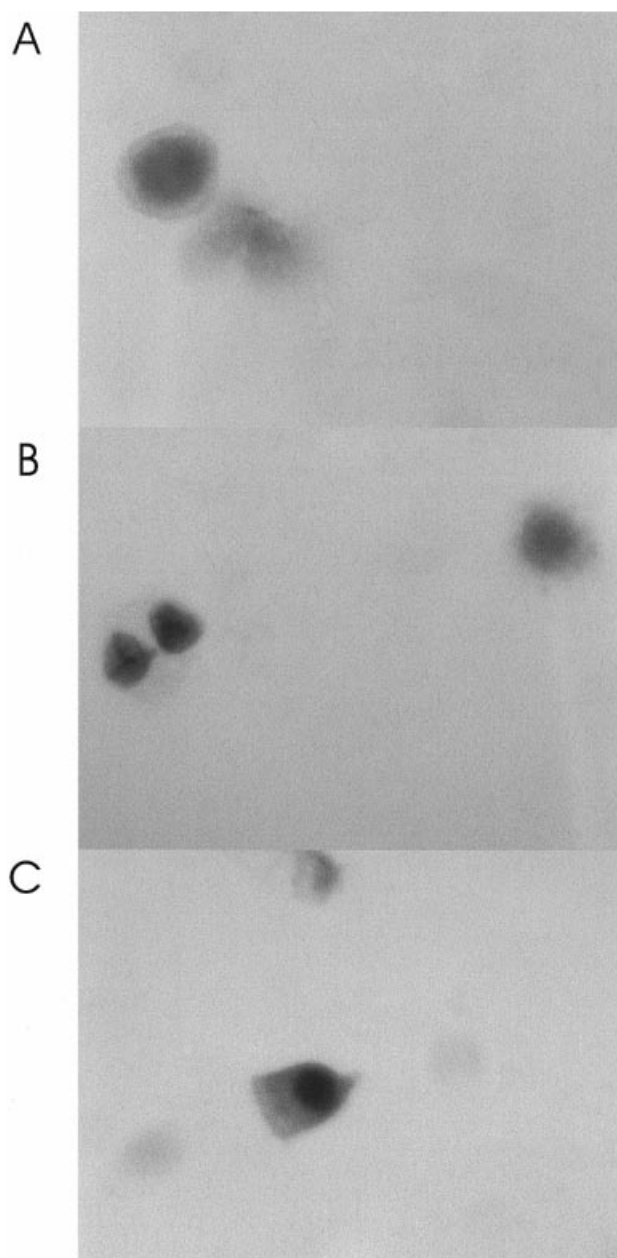


Figure 2. Photomicrograph of eotaxin-immunoreactive cells on sputum cytopspins ($\times 1000$). A: macrophage, B: eosinophil, C: epithelial cell.

eosinophil numbers in BAL and biopsies at 24 h have previously been shown to be proportional to the magnitude of the LAR (2, 4). We have confirmed these findings in sputum and have extended the work by showing the increase in the eosinophil/basophil-selective C-C chemokine, eotaxin.

We were not able to measure eotaxin in the liquid phase of induced sputum by ELISA, because dithiotreitol interferes

with detection of eotaxin by this method by disrupting disulphide bonds within the molecule, and this also prevented demonstration of chemotactic activity. Similarly, we did not have sufficient cells to examine RNA expression, but demonstration of an increased percentage of cells by immunocytochemistry is at least compatible with an increase in eotaxin expression in the airway after allergen challenge. The primary purpose of this study was to detect allergen-induced changes in eotaxin expression, rather than to identify eotaxin-producing cells. However, on morphologic grounds, it appeared that macrophages, eosinophils, epithelial cells, and lymphocytes all participate in eotaxin production in the airway, a finding which is in agreement with prior studies (12, 16, 17).

The mechanisms involved in upregulating eotaxin in different cell types *in vivo* have yet to be determined. As in our study, pulmonary levels of eotaxin in sensitized animals are known to increase after allergen exposure (18). Increased eotaxin expression may be mediated via IgE-dependent release of mast-cell mediators during the EAR (e.g., histamine, leukotrienes, or cytokines), or via T-cell cytokines. However, as both mast-cell and T-cell numbers are limited in sputum, this would be difficult to study by this technique. Another possibility is that eotaxin upregulation is controlled by factors involved in the innate immune response. For instance, it has been shown that both $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ stimulate eotaxin production in pulmonary epithelial cell lines *in vitro* (19). Keatings et al. (7) reported elevated concentrations of $\text{TNF-}\alpha$ and correlation with eosinophil numbers in the sputum of asthmatics 24 h after allergen challenge. Studies in animal models suggest co-operativity between eotaxin and IL-5 in eosinophil recruitment to the airway (20–22). Thus, eotaxin may be released in many inflammatory situations in which $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ are released, but IL-5 expression may also be required to augment and sustain eosinophilia.

In summary, allergen inhalation by mildly asthmatic subjects resulted in a significant increase in the percentage of cells in induced sputum expressing eotaxin, and this increase correlated with the increase in sputum eosinophilia and the magnitude of the LAR. These findings support the hypothesis that eotaxin contributes to allergen-induced airway eosinophil inflammation in asthma.

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