

Original article

Expression of eotaxin in induced sputum of atopic and nonatopic asthmatics

Background: The chemokine eotaxin has been implicated in airway eosinophilia in atopic asthma. We have compared airway eosinophils and eotaxin expression in induced sputum from well-matched atopic and nonatopic asthmatics.

Methods: Eosinophil numbers, eosinophil cationic protein (ECP), and the expression of eotaxin were examined in induced sputum from atopic asthmatics (AA=11), nonatopic asthmatics (NAA=11), and atopic (AC=12) and normal (NC=10) controls. Slides were prepared for differential cell counts by Romanowsky stain, and ECP levels were measured by RIA. Eotaxin expression was detected by *in situ* hybridization, with ³⁵S-labelled riboprobes and immunocytochemistry.

Results: The numbers of eosinophils and ECP concentration were increased in the sputum of AA and NAA compared with AC and NC ($P<0.05$). The numbers of eotaxin mRNA⁺ and immunoreactive cells were increased in NAA, but not AA, when compared with controls ($P<0.05$). Eotaxin immunoreactive cells in NAA were significantly higher than in AA ($P<0.05$).

Eotaxin was expressed predominantly by macrophages, eosinophils, and epithelial cells. In NAA, but not AA, the numbers of eotaxin mRNA⁺ cells were correlated with histamine PC₂₀ ($r=-0.81$, $P<0.01$) and eosinophil numbers in sputum ($r=0.7$, $P<0.05$).

Conclusions: Eotaxin production by macrophages, eosinophils, and epithelial cells may play a more pronounced role in airway eosinophilia in nonatopic than in atopic asthma.

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Asthma is a multifactorial syndrome with different causes, both allergic (extrinsic) and nonallergic (intrinsic). Eosinophilic inflammation of the airway is a common feature of both types of the disease (1, 2). This recruitment of eosinophils to the site of inflammation is mediated by a broad network of CC chemokines which acts in collaboration with other cytokines and contributes to tissue damage by the release of toxic granule proteins and reactive oxygen species (3, 4). Among those, eotaxin is an unusual selective chemokine (5), and several clinical studies have implicated it in the pathogenesis of allergic inflammation. An increased expression of eotaxin mRNA and immunoreactive protein has been observed within the airways of atopic asthmatic subjects (6, 7) and in nasal biopsy specimens from individuals with allergic rhinitis (8). In addition, an increase in the local expression of eotaxin within epithelial and subepithelial layers of the nasal mucosa has been found in allergic and nonallergic sinusitis (8, 9). The role of eotaxin in nonallergic asthma has been studied recently, and eotaxin was found to be elevated in the bronchial mucosa of asthmatics,

regardless of atopic status. That study found eotaxin to be expressed predominantly by endothelial and epithelial cells. However, asthmatic patients were not stringently matched, since the nonatopic asthmatics were significantly older than their atopic counterparts (10).

In the present study, we examined the expression of eotaxin in the airway of well-matched allergic and nonallergic asthmatics in induced sputum. In these samples, eotaxin expression was more prominent in nonatopic than atopic asthma.

Material and methods

Study subjects

Induced sputum was obtained from 11 atopic asthmatics (AA=11), 10 nonatopic asthmatics (NAA=10), 10 nonasthmatic atopic controls (AC=10), and 12 nonatopic nonasthmatic controls (NC=12). The patients studied were recruited from the Royal Brompton Hospital, London, UK, while five nonatopic

asthmatics came from Sotiria Hospital, Athens, Greece. All participants gave informed consent before entering the study. Asthmatic subjects had a clear history of asthma requiring intermittent inhaled β_2 -agonists, and documented reversible airway obstruction (20% improvement in FEV₁ either spontaneously, or after administration of inhaled β_2 -agonist) and a histamine PC₂₀ provocation test of ≤ 6 mg/ml in the previous 2 weeks. Atopy was defined as a positive skin prick test (wheal at 15 min of > 3 mm in diameter in the presence of positive histamine and negative diluent controls) to more than one extract of the common local aeroallergens. Nonatopic subjects had a negative skin test to a wide range of local common aeroallergens, serum IgE concentration within the normal range (0–150 IU), and negative RAST (Pharmacia Diagnostica, Uppsala, Sweden) to 25 common aeroallergens (intrinsic asthmatics from Greece were also tested with olive pollen). For all subjects, total serum IgE and RAST measurements were performed in the same laboratory. All subjects were nonsmokers and had not taken oral (2 months) or inhaled (2 weeks) glucocorticoids before the study.

None of the volunteers had taken oral corticosteroids for at least 2 months before inclusion in the study. All but three asthmatics were not currently using inhaled corticosteroids (and had not done so for at least 3 months). Two nonatopic asthmatics discontinued inhaled corticosteroids 2 weeks before sputum induction, and one atopic asthmatic had stopped inhaled corticosteroids for 4 weeks before sputum induction. None of the atopic patients were receiving nasal corticosteroids.

Exclusion criteria included age under 18 or over 65 years, FEV₁ of $< 60\%$ of the predicted value on the study day, evidence of acute or chronic infection, pregnancy, or any chronic medical illness other than asthma. The study was approved by the ethics committees of the Royal Brompton Hospital, London, UK, and Sotiria Hospital, Athens, Greece.

Sputum induction and processing

Sputum was induced by a protocol based on that described by Pin et al. (11). Briefly, volunteers were pretreated with 200 μ g salbutamol and then inhaled a mist of 3%, 4%, and then 5% hypertonic saline generated by a low output ultrasonic nebulizer (Sonix 2000, Medix Ltd, Harlow, UK; output 0.9 ml/min; median mass diameter 5.6 μ m) for 7 min each.

Sputum was processed within 2 h by the method described by Popov et al. (12), with some modifications. Plugs of sputum were selected and dispersed with three 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 on a bench-rocker, an equal volume of

cooled D-PBS was added, and the sample was filtered through a 48- μ m nylon gauze. The filtered sample was centrifuged at 2000 revolutions per minute for 10 min, and then cells were resuspended at 1×10^6 cells/ml in D-PBS. Cell viability and total cell count were estimated by trypan blue staining (Sigma). Sputum samples were considered acceptable if cell viability was more than 50%, and the contamination with squamous cells was less than 10%. The median viability of cells was over 65%, while the contamination with squamous cells was under 5% in all cases. No significant differences were detected between the four groups.

Differential cell counts were performed on cytocentrifuge preparations stained with Romanowsky (13). In all cases, 400 nucleated nonsquamous cells were counted, and the results are expressed as percentage of total nonsquamous cells. The concentration of ECP was measured by RIA (Pharmacia).

In situ hybridization (ISH)

The eotaxin riboprobes (sense and antisense) were generated from 308-bp of human eotaxin (14) (3' UTR region 893–1201 bp, a kind gift from Dr J Rottman, LeukoSite, Inc., Cambridge, MA, USA). The DNA template was inserted into PCR Bluescript. Permeabilization, prehybridization, and hybridization protocols were as described previously (7, 15, 16). Incubation in *N*-ethyl maleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the ³⁵S-UTP-labelled probes. Furthermore, the experiments were performed under very high stringency conditions (hybridization at 50°C, and post-hybridization washing at 60°C, 0.1 SSC) to minimize nonspecific hybridization. Negative controls employed hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, UK) before hybridization with the antisense probe. For autoradiography, slides were dipped into K-5 emulsion (Ilford, Basildon, UK) and exposed at 4°C for 2 weeks in absolute darkness in a desiccated environment. The slides were developed (D-19 developing solution; Eastman Kodak Co., Rochester, NY, USA), rinsed, and counterstained with Harris haematoxylin. Dense deposits of silver grains on autoradiographs were present over cells expressing eotaxin mRNA. Slides were counted in duplicate, blind to the patients' clinical status, with an eyepiece graticule. The results were expressed as the number of positive cells per 400 cells. The coefficient of variability of the duplicate counts obtained from all slides was less than 5%.

Immunocytochemistry

Immunocytochemistry was performed by the alkaline phosphatase antialkaline phosphatase (APAAP) method, as previously described (7). Rabbit antimouse immunoglobulin (Ig), APAAP, and control IgG1 were

purchased from Dako (High Wycombe, UK). Anti-human eotaxin mAb (2G6) was a kind gift from Dr Walter Newman (Leukosite, Inc., Cambridge, MA, USA). Omission or substitution 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 (7), were used as a positive control.

Statistical analysis

Data were analysed with the aid of a commercially available statistical package (Minitab for Windows, Minitab Release 9.2, Minitab, Inc., State College, PA, USA). The Mann-Whitney U test was then used for intergroup comparison (with Bonferroni's correction). Correlation coefficients were obtained by Spearman's rank-order method. A *P* value of less than 0.05 was accepted as statistically significant.

Results

Study population

Adequate samples for examination were obtained from 43 of 65 subjects studied. In 22 volunteers, the small volume of sputum, the low cell viability, and/or contamination with squamous cells precluded analysis. Five of the atopic asthmatic patients and three atopic nonasthmatics had seasonal symptoms; all were studied out of season.

There were no significant clinical differences between the subjects who produced adequate sputum samples and those who did not, nor between the intrinsic asthmatics from the two centres. The atopic and nonatopic subjects with asthma were well matched in terms of age and asthma severity, reflected by per cent predicted FEV₁ and histamine PC₂₀. Sputum induction was well tolerated by all subjects, none of whom developed any significant untoward effects. The mean

fall in FEV₁ (% predicted) after sputum induction in NC was 4% (range 2–10), 7% (range 5–15) in AC, 15% (range 12–23) in AA, and 18% (range 17–28) in NAA, respectively. In two asthmatic patients, FEV₁ dropped more than 20%, but returned to normal after inhalation of salbutamol at the end of the procedure (maximum after 20 min).

Asthmatics, regardless of atopic status, had elevated blood eosinophils compared with nonasthmatic controls (*P* < 0.005). The atopic subjects, whether they had asthma or not, had clearly elevated median total serum IgE concentrations as compared with the nonatopic control subjects (*P* < 0.05). Details of the study population are shown in Table 1.

Differential cell count in induced sputum

The median numbers of cells retrieved in the sputum of NAA (30.3×10^5 , range $3\text{--}80 \times 10^5$) and AC (27.4×10^5 , range $2\text{--}70 \times 10^5$) were significantly increased compared with AA (9.4×10^5 , range $1.5\text{--}82 \times 10^5$) and NC (9.5×10^5 , range $2.4\text{--}69 \times 10^5$) (*P* < 0.05). No other significant differences in viability or squamous cell contamination were detected between the four groups.

The median percentage of sputum eosinophils in both the NAA (7%, range 0–44) and the AA groups (4%, range 0–12.5) was significantly increased compared with the AC (2.5%, range 0–10) and NC (0.5%, range 0–2.5) (*P* < 0.005). No differences were detected between the atopic and nonatopic asthmatics.

Similarly, the absolute number of eosinophils was significantly increased in NAA (0.62×10^5 , range 0.0036– 15.4×10^5) and AA (0.47×10^5 , range 0.0007– 5.9×10^5), compared to AC (0.41×10^5 , range 0.0002– 1.86×10^5) and NC (0.035×10^5 , range 0.0003– 1.38×10^5).

A significant positive correlation was found between sputum and peripheral blood eosinophils in the NAA group (*P* < 0.01, *r* = 0.88).

A significant increase in the percentage of epithelial cells was detected in asthmatics (AA + NAA), when compared to nonasthmatic controls (*P* < 0.005).

Table 1. Clinical characteristics of study subjects

Subjects	Age (years)	Sex (M:F)	FEV ₁ % (predicted)	Histamine PC ₂₀ (mg/ml)	IgE (IU/ml)	Peripheral blood eosinophils ($\times 10^9/l$)
AA (<i>n</i> = 11)	28 (20–54)	4:7	100 (74–120)	1.16 (0.096–4)	353** (43–831)	0.3 ⁺ (0.2–0.9)
NAA (<i>n</i> = 10)	32 (23–56)	5:5	91.5 [†] (76–110)	2 (1.9–5)	17.5 (1–83)	0.3 ⁺ (0.1–1.2)
AC (<i>n</i> = 10)	26 (20–35)	7:3	105 (80–124)	> 16	103* (15–166)	0.15 (0.1–0.7)
NC (<i>n</i> = 12)	31 (22–51)	7:5	108 (94–140)	> 16	20 (6–150)	0.1 (0.1–0.3)

* *P* < 0.01, AC vs NC/NAA.

** *P* < 0.005, AA vs AC/NC/NAA.

[†] *P* < 0.005, AA/NAA vs NC.

[‡] *P* < 0.05, NAA vs NC.

Values are expressed as medians (ranges in parentheses).

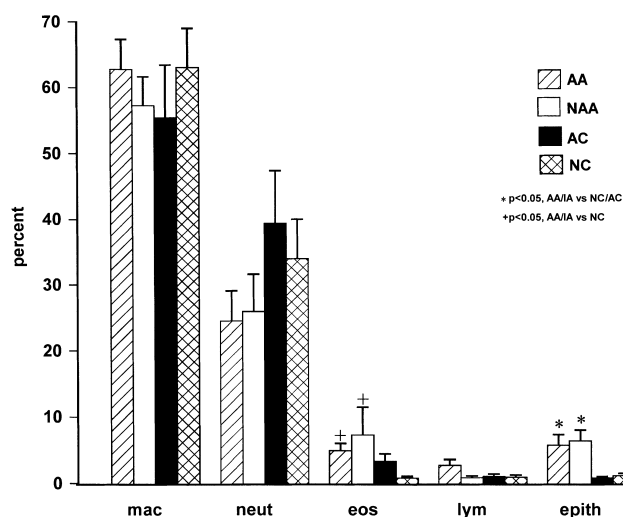


Figure 1. Cellular profile of sputum from atopic asthmatics (AA), nonatopic asthmatics (NAA), and atopic (AC) and normal controls (NC). Cell counts are expressed as percentage of total nonsquamous cells, and median values are shown.

No significant differences were found in the percentages of macrophages, neutrophils, and lymphocytes between the four groups (Fig. 1).

Five of the nonatopic asthmatics were from Greece, and the remainder of the study volunteers were from the UK. The Greek subjects were similar to the UK asthmatics in terms of clinical features (median FEV₁ 90% predicted [range 83–110] for the Greek asthmatics,

and median 97% [76–106] for the UK asthmatics). Sputum eosinophil counts tended to be lower in the Greek subjects (median absolute eosinophil count 1×10^4 [0.6–154 $\times 10^4$]) than in the UK asthmatics (median 12.5×10^4 eosinophils [7.2–56 $\times 10^4$]), although these differences were not statistically significant.

ECP concentration in sputum

The concentration of eosinophil cationic protein (ECP) in cell-free supernatant was significantly elevated in the asthmatic group, regardless of atopic status, compared to normal controls ($P < 0.05$). Increased ECP was also seen in two atopic subjects without asthma. No significant differences were detected between the asthmatic or the control groups (Fig. 2).

In the asthmatic group (AA + NAA), a significant inverse correlation was found between histamine PC₂₀ and either the percentage of sputum eosinophils or ECP concentration in sputum ($P < 0.05$, $r = -0.5$). Moreover, in the asthmatic group (AA + NAA), sputum ECP concentration was correlated with the percentage of eosinophils in peripheral blood ($P < 0.05$, $r = 0.67$).

Eotaxin expression in sputum of atopic, nonatopic asthmatics, and atopic and normal controls

The numbers of cells in sputum encoding mRNA for eotaxin were significantly elevated in nonatopic asthmatics, but not atopic asthmatics compared with subjects without asthma ($P < 0.05$). Immunocytochemistry revealed significantly increased numbers of immunoreactive cells to eotaxin in nonatopic asthmatics compared with atopic asthmatics, and atopic and normal controls ($P < 0.05$) (Fig. 3).

The numbers of eotaxin-positive cells (mRNA and protein) were significantly correlated with the percentage of sputum eosinophils in the NAA, but not the AA group. In the same group of patients, a significant inverse correlation was found between the percentage of eotaxin mRNA⁺ cells and histamine PC₂₀ ($P < 0.01$, $r = -0.81$). No other significant correlations between eotaxin expression and clinical or sputum characteristics were detected between the groups.

Comparison of the structure of cells counterstained with haematoxylin after immunostaining to that of cells stained with Romanowsky showed that the majority of eotaxin immunoreactive cells in sputum in all groups appeared to be macrophages. The median percentage of macrophages (per total immunoreactive cells) was 53% (range 35–55) in NAA, 63% (range 30–85) in AA, 68% (range 45–90) in AC, and 58% (range 25–80) in NC.

On the other hand, eosinophils accounted for 12% (range 0–30) in NAA, 12% (range 0–34) in AA, 17% (range 0–37) in AC, and 25% (range 0–40) in NC. Epithelial cells accounted for 35% (range 17–60) in NAA, 25% (range 0–45) in AA, 15% (range 5–20) in

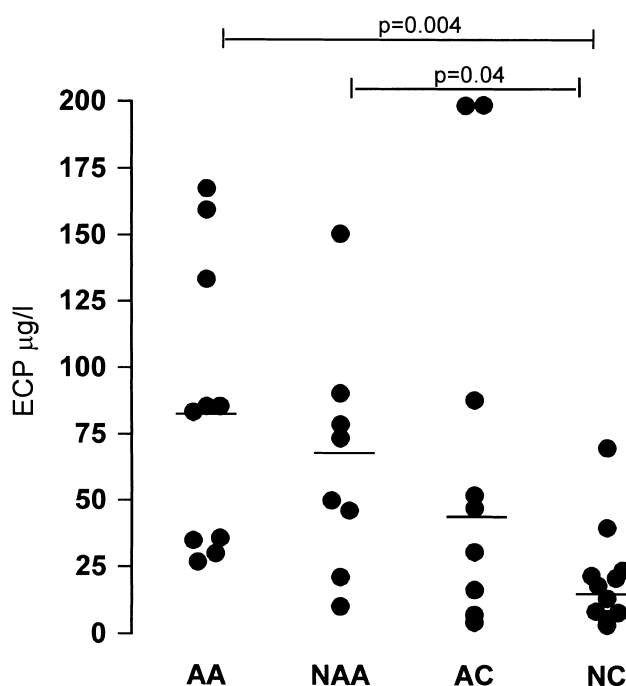


Figure 2. ECP concentration ($\mu\text{g/l}$) in induced sputum from atopic asthmatics (AA), nonatopic asthmatics (NAA), atopic nonasthmatics (AC), and normal controls (NC). Comparison between groups was by Mann–Whitney U test, with Bonferroni's correction.

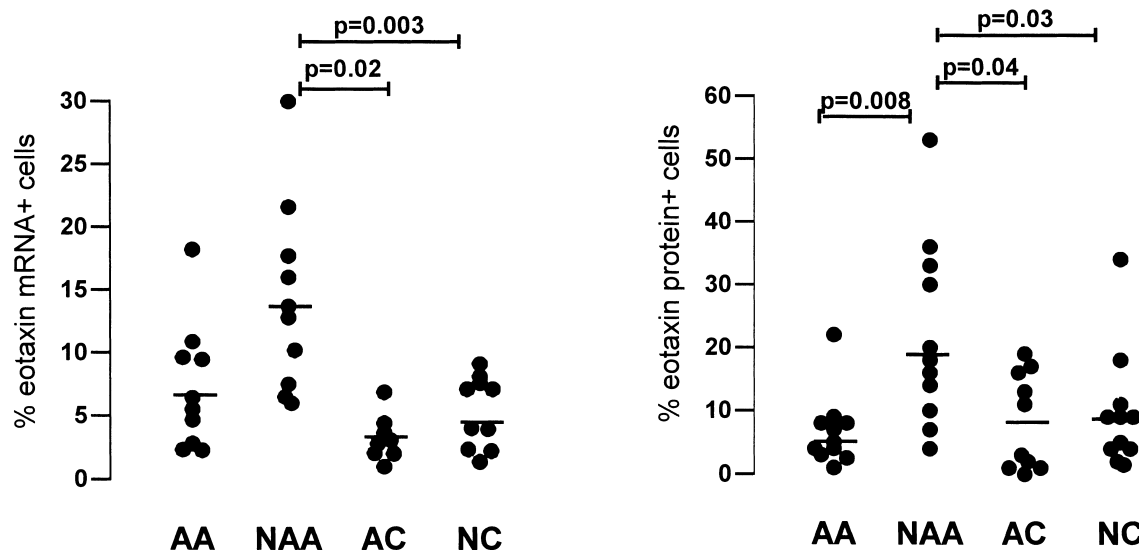


Figure 3. Percentage of cells expressing mRNA and protein product for eotaxin in sputum from atopic asthmatics (AA), nonatopic asthmatics (NAA), atopic controls (AC), and normal controls (NC). Comparison between groups was by Mann-Whitney U test, with Bonferroni's correction.

AC, and 17% (range 0–40) in NC. No differences in the expression of eotaxin by the various cell types were detected between the groups (Fig. 4).

Discussion

Although there are clinical differences between atopic and nonatopic asthmatics, eosinophil accumulation into the airway seems to be an invariable feature of asthma of diverse pathogenesis (1, 2). In the present study, we examined one of the mechanisms of

eosinophil recruitment to the airways of atopic and nonatopic asthmatics by studying eotaxin expression in sputum. The data presented here show elevated numbers of sputum eosinophils and eotaxin expression in nonatopic asthmatics, and a correlation between them. These findings suggest a more prominent role for eotaxin in promoting airway eosinophilia in nonatopic than atopic asthmatics.

Both asthmatic groups shed a significantly higher number of epithelial cells than normal subjects, confirming previous findings of considerable bronchial epithelial cell disruption in asthma (17–19). Similarly, ECP, the cytotoxic eosinophil granule protein, was elevated in the sputum of both atopic and nonatopic asthmatics compared to controls, and was similar to those reported from atopic asthmatics in other studies (20, 21), although the mechanism(s) by which ECP is released into the sputum is unknown. The observed inverse correlation between sputum ECP and bronchial hyperactivity in asthmatics (AA+NAA) suggests similarities in activation and degranulation of eosinophils in both types of the disease. It is of note that two atopic patients without asthma had elevated ECP in sputum. We did not measure serum ECP, but since these two subjects were sensitized to house-dust mite, the measured sputum ECP might reflect systemic levels. Atopy appears to influence ECP levels; elevated serum ECP has been detected in atopic patients and correlated with serum IgE (22).

We found increased expression of eotaxin in the sputum of nonatopic, but not atopic, asthmatics. This high expression of eotaxin in luminal airway samples from these patients would explain the relative increase in numbers of eosinophils in nonatopic asthma, as has been shown for both bronchial biopsy and sputum

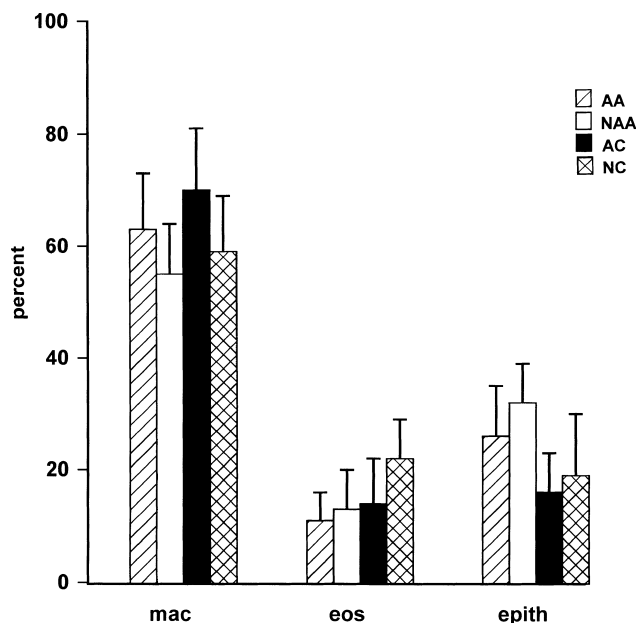


Figure 4. Phenotype of cells immunoreactive to eotaxin. Cell counts are expressed as percentage of cells immunoreactive to eotaxin; median values are shown.

specimens (10, 15). In previous studies, the expression of eosinophilic chemokines, such as RANTES, MCP-3, and MCP-4, in the bronchial mucosa of nonatopic asthmatics was not significantly different from that seen in atopic asthmatics (10, 15). Although examination of induced sputum has emerged as an important investigative tool in airway diseases because it is noninvasive (23), safe (24), and provides reproducible measurements (25), the sample obtained is substantially different from that in airway biopsies and bronchoalveolar lavage. One potential disadvantage of sputum induction is the low number of cells retrieved, as not all patients can produce adequate quantities (success rate: ~75%) (26, 27). In our study, adequate sputum samples were obtained from 43 out of 65 subjects studied (66%). In sputum samples, eotaxin was produced predominantly by macrophages with minor eosinophilic and epithelial component. On the other hand, in bronchial biopsy specimens the major cell types encoding mRNA and protein are endothelial and epithelial cells (6, 16). The different phenotype of cells expressing eotaxin between variable specimens may explain the apparent discrepancy between biopsy and sputum studies (10). We would argue that data from biopsies, BAL, and sputum should be considered together for an overall picture of airway inflammation in asthma. While sputum has the potential advantage of ease of repeated sampling, it may not fully reflect cellular changes lower in the airways.

The current data showing macrophage eotaxin production in nonatopic asthma reinforce the concept of macrophage differences between nonatopic and atopic asthma (1, 28). In support of this interpretation, Folkard et al., showed that the CC chemokine RANTES may be differentially produced by BAL macrophages in atopic and nonatopic asthma (29). The stimulus leading to eotaxin production in asthma remains to be established, but may include macrophage-derived cytokines such as TNF- α and IL-1 β (9).

Generally, nonatopic asthmatics present later in life than atopic asthmatics, and this has been reflected in the age of subjects included in previous studies. However, our subjects were well matched for age and disease severity. Although five of the nonatopic subjects came from a different ethnic group (Greece rather than the UK), we do not believe this affected our results, since the Greek nonatopic asthmatics had slightly lower sputum eosinophil counts than their UK counterparts, and this would have tended to reduce rather than accentuate the differences between atopic and nonatopic asthmatics.

In conclusion, the present study shows an increased expression of eotaxin in the airway luminal cells of nonatopic asthmatics, and suggests that this chemokine may play a more prominent role in promoting airway eosinophilia in this variant of the disease.

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