

Comparison of airway immunopathology of eosinophilic bronchitis and asthma

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Background: Eosinophilic bronchitis is a condition characterised by a corticosteroid responsive cough, sputum eosinophilia, and normal tests of variable airflow obstruction and airway responsiveness. We performed a detailed comparative immunopathological study to test the hypothesis that the different airway function in patients with eosinophilic bronchitis and asthma reflects differences in the nature of the lower airway inflammatory response.

Methods: Exhaled nitric oxide was measured and induced sputum, bronchoscopy, bronchial wash (BW), bronchoalveolar lavage (BAL), and bronchial biopsy were performed in 16 subjects with eosinophilic bronchitis, 15 with asthma, and 14 normal controls.

Results: Both eosinophilic bronchitis and asthma were characterised by an induced sputum, BW and BAL eosinophilia, an increased number of epithelial and subepithelial eosinophils, and increased reticular basement membrane thickness. The median concentration of exhaled nitric oxide was higher in those with eosinophilic bronchitis (12 ppb) or asthma (8.5 ppb) than normal controls (2 ppb) (95% CI of the difference 5 to 16, $p < 0.0001$ and 2 to 11.3, $p = 0.004$, respectively). There were no group differences in epithelial integrity or the number of subepithelial T lymphocytes, mast cells or macrophages.

Conclusion: With the exception of our previously reported association of smooth muscle mast cell infiltration with asthma, the immunopathology of eosinophilic bronchitis and asthma are similar which suggests that eosinophilic airway inflammation, increased exhaled nitric oxide, and increased basement membrane thickening are regulated independently of airway hyperresponsiveness.

The development of sputum induction has provided a safe non-invasive method for assessing airway inflammation.^{1–4} One of the most interesting early observations made using this method was the identification of a group of patients with a sputum eosinophilia identical to that seen in asthma, but with none of the functional abnormalities associated with asthma.^{5,6} Patients with this condition, known as eosinophilic bronchitis, typically present in middle age with a corticosteroid responsive dry or minimally productive cough. Wheeze and dyspnoea are not prominent and tests of variable airflow obstruction and airway responsiveness are normal. We⁷ and others⁸ have shown that eosinophilic bronchitis is a common cause of cough in patients presenting to a respiratory specialist.

Previous studies have suggested that the different association between airway inflammation and dysfunction in asthma and eosinophilic bronchitis is not due to localisation of the inflammatory process in the upper airway in eosinophilic bronchitis,^{6,9} differences in the state of activation of the inflammatory process as assessed by induced sputum inflammatory mediator concentrations,¹⁰ or differences in Th2 type cytokine expression.^{11,12} We have recently reported that the functional differences between eosinophilic bronchitis and asthma may be due to infiltration of airway smooth muscle by mast cells in asthma.¹³ In this report no differences were found in the number of EG2+ eosinophils, T cells, or mast cells in the bronchial submucosa. However, no study has compared in detail the immunopathology from different compartments of the lower airway in these conditions. We have undertaken a comparative immunopathological study of induced sputum, bronchial wash, bronchoalveolar lavage (BAL) fluid, and bronchial mucosal biopsies from patients with eosinophilic bronchitis, symptomatic asthma, and normal controls.

METHODS

Subjects

Sixteen subjects with eosinophilic bronchitis, 15 with asthma, and 14 normal controls were recruited from Glenfield Hospital outpatients, staff, and by local advertising. The diagnostic criteria for asthma and eosinophilic bronchitis were as previously described.¹³ All subjects were non-smokers with a past smoking history of less than 10 pack years. None had taken inhaled or oral corticosteroids for at least 6 weeks before the study. Data on the lavage and biopsy cytokine and cell surface marker expression in this study population has been presented previously,¹² as has the number of submucosal T cells and mast cells and the reticular lamina and basement membrane thickness from eight controls, 13 subjects with eosinophilic bronchitis, and eight subjects with asthma.¹³ The Leicestershire ethics committee approved the study and all patients gave their written informed consent.

Protocol and clinical measurements

Subjects attended on two occasions. At the first visit the severity of the symptoms cough, breathlessness and wheeze was measured on a 100 mm visual analogue scale from no symptoms to worst ever, as previously described.¹⁴ Exhaled nitric oxide, spirometric parameters, allergen skin prick tests, and methacholine airway responsiveness were measured, followed on recovery by a sputum induction test. End exhaled nitric oxide (eNO) was measured by a chemiluminescent technique (Logan, UK). Subjects exhaled at a flow rate of 250 ml/s with a sampling rate of 250 ml/min. Spirometric tests were performed using a dry bellows spirometer (Vitalograph, Buckingham, UK) with forced expiratory volume in 1 second (FEV₁) recorded as the best of successive readings within 100 ml. Allergen skin prick tests were performed to *Dermaphagoides pteronyssinus*, cat fur, grass pollen, and *Aspergillus fumigatus* solutions with normal saline and histamine controls

Table 1 Clinical characteristics of subjects

	Eosinophilic bronchitis (n=16)	Asthma (n=15)	Normal (n=14)
Mean (SE) age	48 (3)	46 (4)	37 (5)
Male	10	8	8
Atopy	9	10	4
Mean (SE) IgE (kU/l)	90 (17)	106 (29)	36 (10)
Mean (SE) blood eosinophils ($\times 10^9/l$)	0.44 (0.08)	0.33 (0.06)	0.2 (0.03)
Cough VAS (mm)†	39 (10–92)	19 (5–75)	0
Wheeze VAS (mm)†	0 (0–6)	10 (0–28)	0
SOB VAS (mm)†	0 (0–10)	5 (0–48)	0
PC ₂₀ FEV ₁ (mg/ml)†	94 (18–128)	0.8 (0.16–4.6)	128 (16–128)
Mean (SE) FEV ₁ (% pred)	100 (2.6)	99 (3.2)	100 (3.7)
Mean (SE) FEV ₁ /FVC (%)	80 (1.4)	72 (1.9)	79 (1.8)
Nitric oxide (ppb)†	12 (5–30)*	8.5 (2–32)*	2 (1–9)

FEV₁=forced expiratory volume in 1 second; FVC=forced vital capacity; PC₂₀FEV₁=concentration of methacholine provoking a fall in FEV₁ of 20% or more; SOB=shortness of breath.

* $p < 0.01$, Kruskal-Wallis test.

†Median (range).

(Bencard, UK). A positive response to an allergen on the skin prick tests was recorded by the presence of a weal of >2 mm more than the negative control. The methacholine challenge was performed using the tidal breathing method¹⁵ with doubling concentrations of methacholine from 0.03 to 128 mg/ml nebulised via a Wright nebuliser. After patients had recovered from the methacholine challenge, sputum was induced and processed as previously described.¹³

At the second visit 1 week later the subjects underwent bronchoscopy using an Olympus fibreoptic bronchoscope (Olympus Company, Tokyo, Japan) in accordance with recent BTS guidelines.¹⁶ A 20 ml bronchial wash of prewarmed normal saline into the bronchus intermedius was performed followed by 180 ml BAL fluid into the middle lobe in 60 ml aliquots. Bronchial mucosal biopsy specimens were taken from the right middle and lower lobe carinae. All subjects received nebulised 2.5 mg salbutamol 20 minutes before bronchoscopy and had appropriate sedation as required (midazolam 0–5 mg iv). Lignocaine (1–4%) was used for local anaesthesia and continuous oxygen was given via nasal cannulae throughout the procedure.

Mucosal biopsy specimens were immediately transferred into ice cooled acetone containing the protease inhibitors iodoacetamide (20 mM) and PMSF (2 mM) for fixation, stored at –20°C for 24 hours, and then processed into the water soluble resin glycol methacrylate (GMA) (Polysciences, Northampton, UK) for embedding.

Immunohistochemistry

Two μ m sections were cut, floated on 0.2% ammonia solution in water for 1 minute, and dried at room temperature for 1–4 hours. The following mouse IgG, monoclonal antibodies were used: CD3 (Dako Ltd, High Wycombe, UK), CD4 (Becton Dickinson, Oxford, UK), CD8 (Dako Ltd), AA1 to mast cell tryptase (Dako Ltd), MBP to eosinophil major basic protein (Bradsure Biologicals, Loughborough, UK), NE to neutrophil elastase (Dako Ltd), CD45 panleukocyte marker (Dako Ltd), CD14 to macrophages (Dako Ltd), and CD56 to natural killer cells (Dako Ltd). The technique of immunostaining applied to GMA embedded tissue has been described previously.¹⁷

Assessment and quantification of immunohistochemical staining

Subepithelial mucosa and epithelium were identified morphologically and the area calculated using a computer analysis system (Scion Image). Nucleated immunostained cells present in coded sections of the submucosa and epithelium were counted and the numbers of cells expressed per mm². Basement membrane width was measured as the mean of 50 measurements made at 20 μ m intervals as previously

described.¹⁸ In two subjects with asthma and one normal control a biopsy specimen was either not obtained or was insufficient to quantify, and one subject with asthma and two with eosinophilic bronchitis had a basement membrane length of <1 mm.

Statistical analysis

Subject characteristics were described using descriptive statistics. Exhaled nitric oxide concentration, differential cell counts, and epithelial integrity were expressed as median (range) values. Basement membrane width was described as mean (SE). Comparisons between the three groups were undertaken using the Kruskal-Wallis test and the Mann-Whitney U test was used to compare between groups with non-parametric data when a difference was identified and by ANOVA and unpaired *t* tests for parametric data. A *p* value of <0.05 was considered statistically significant.

RESULTS

Characteristics of the study subjects are shown in table 1. The median exhaled nitric oxide concentration was higher in patients with eosinophilic bronchitis (12 ppb, 95% CI of difference 5 to 16, $p < 0.001$) and asthma (8.5 ppb, 95% CI 2 to 11.3, $p = 0.004$) than in normal controls (2 ppb). There were no differences in the nitric oxide concentration between patients with eosinophilic bronchitis and those with asthma (table 1).

Differential inflammatory cell counts in sputum, bronchial wash (BW), and BAL fluid are shown in table 2. Induced sputum, BW, and BAL fluid eosinophil counts were significantly higher in subjects with eosinophilic bronchitis (sputum: 95% CI of difference 4 to 13.3%, $p < 0.0001$; BW: 95% CI 1.1 to 3.5%, $p < 0.0001$; BAL: 95% CI 0.25 to 2.2%, $p = 0.006$) and asthma (sputum: 95% CI 0.2 to 5%, $p = 0.01$; BW: 95% CI 0 to 4.1%, $p = 0.01$; BAL: 95% CI 0 to 1.7%, $p = 0.02$) than in normal subjects. There were no differences in the eosinophil counts between subjects with asthma and those with eosinophilic bronchitis, and no differences were seen in other differential cell counts between the groups (table 2).

The median MBP+ cells/mm² subepithelium were significantly higher than controls in both subjects with eosinophilic bronchitis (95% CI of difference 12 to 40, $p = 0.0004$) and those with asthma (95% CI 4 to 35, $p = 0.01$). There were no differences in the subepithelial eosinophil counts between eosinophilic bronchitis and asthma. The median NE+ cells/mm² subepithelium in the submucosa was higher in subjects with eosinophilic bronchitis than in those with asthma (95% CI of difference 0.2 to 29, $p = 0.046$) and normal controls (95% CI 1 to 28, $p = 0.02$). No differences were observed in the other submucosal cell counts (table 3); counts in atopic and non-atopic subjects within groups were similar.

Table 2 Median (range) differential cell counts (%) in sputum, bronchial wash, and BAL fluid

	Eosinophilic bronchitis	Asthma	Normal
Induced sputum			
Eosinophil	9.75 (3.3–68)**	3.4 (0–33.5)**	0.35 (0–2.75)
Neutrophil	48 (9–83)	25 (0–77)	46 (8–84)
Macrophage	27 (0–83)*	64 (1–91)	50 (12–90)
Lymphocytes	0.5 (0–2)	0.4 (0–2)	1 (0–4)
Epithelial cell	0.8 (0–11)	3 (1–5)	2 (1–15)
Squamous contamination	5 (0–20)	9 (0–24)	2 (0–7)
Viability	71 (34–94)	62 (18–86)	58 (37–84)
Total cell count	2.7 (0.8)	2.5 (0.6)	1.8 (0.3)
Bronchial wash			
Eosinophil	2.4 (0.5–25)**	1.4 (0–40.5)**	0 (0–1)
Neutrophil	47 (7.5–73.5)	24 (2.6–85.6)	43 (4.3–65.7)
Macrophage	26 (5–60)	30 (3–64)	43 (7–90)
Lymphocytes	0.7 (0–4)	0 (0–2)	0.6 (0–3)
Epithelial cell	17 (0–65)	32 (1–81)	20 (6–34)
Viability	54 (28–79)	49 (20–82)	46 (12–74)
Recovery (%)	35 (20–40)	30 (0–50)	31 (20–50)
Total cell count $\times 10^6$	0.5 (0.2–1.6)	0.5 (0–3)	0.5 (0.06–2.5)
BAL fluid			
Eosinophil	1.6 (0–13)*	1.5 (0–4)*	0.5 (0–2)
Neutrophil	5.4 (0.2–36)	5.6 (0–46)	3.1 (0–24)
Macrophage	74 (35–94)	80 (38–98)	84 (43–94)
Lymphocytes	7.8 (2–28)	7.8 (0.2–18)	5.7 (1–15)
Epithelial cell	4.4 (1–22)	5 (1–33)	3 (0–26)
Viability	82 (65–92)	86 (64–94)	78 (49–86)
Recovery (%)	27 (17–40)	27 (19–31)	28 (8–47)
Total cell count $\times 10^6$	6.5 (3–9)	6.2 (2–15)	6.7 (0.8–19)

* $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test.**Table 3** Median (range) subepithelial and intraepithelial cell counts per mm^2

	Eosinophilic bronchitis	Asthma	Normal
Subepithelium			
MBP	32 (12–430)**	20 (4–114)**	8 (0–24)
NE	20 (4–70)*	15 (2–26)	12 (0–84)
CD3	47 (24–122)	46 (15–155)	52 (30–255)
CD4	31 (9–131)	27 (8–95)	27 (8–51)
CD8	14 (6–58)	13 (0–73)	21 (8–51)
AA1	30 (13–78)	22 (5–82)	16 (11–67)
CD45	52 (20–192)	56 (13–129)	71 (7–239)
CD14	10 (0–40)	9.6 (2–31)	3.2 (0–36)
CD56	3.4 (0–9.3)	2.2 (0–14)	5.3 (0–16)
Epithelium			
MBP	11.6 (0–288)**	16.7 (0–33.3)**	0 (0–5.2)
AA1	19.6 (0–125)	16.7 (0–57)	8.3 (3.9–40)
CD3	25 (0–257.1)	33.3 (0–150)	19.2 (0–140)

* $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test.

The intraepithelial median eosinophil count/ mm^2 epithelium was significantly different in subjects with asthma (16.7) and with eosinophilic bronchitis (11.6) from those in normal controls (0; 95% CI of difference 0 to 25, $p = 0.015$; 95% CI 1 to 50, $p = 0.007$, respectively), but there were no differences between the two disease groups. There were no between group differences in the median number of epithelial T cells ($p = 0.77$) or mast cells ($p = 0.33$, table 3).

The mean (SE) basement membrane width was 7.2 (0.4) μm in normal controls, 10.7 (1.1) μm in subjects with eosinophilic bronchitis (95% CI of difference 1 to 6, $p = 0.01$), and 9 (0.7) μm in subjects with asthma (95% CI of difference 0.2 to 3.4, $p = 0.03$). There was no difference in the basement membrane and reticular lamina thickness between the two disease groups. There were no differences in epithelial integrity between subjects with asthma (median 70% (range 2–96)), eosinophilic bronchitis (79% (0–98)), and normal controls (86% (34–96)).

DISCUSSION

Sputum, bronchial wash, and BAL fluid eosinophilia, epithelial and submucosal evidence of eosinophilic airway inflammation, increased eNO levels, and increased basement membrane thickening were found in subjects with mild asthma. These findings are entirely consistent with previous studies in this patient group.^{13 19 20} Importantly, very similar abnormalities were found in subjects with eosinophilic bronchitis, a condition characterised by the absence of variable airflow obstruction and airway hyperresponsiveness.^{5 6} The implication of our findings is that none of these features is important in the genesis of the disordered airway function observed in asthma.

Airway inflammation was assessed using a variety of complementary techniques that are likely to sample different parts of the bronchial tree.²¹ There were no significant differences in eosinophil counts in any samples, suggesting that differences in the localisation of the eosinophilic airway inflammation is

unlikely to explain the different functional associations seen in asthma and eosinophilic bronchitis. The trend towards an increase in sputum eosinophil count in eosinophilic bronchitis compared with asthma may represent a selection bias since a count of >3% was part of the entry criteria in eosinophilic bronchitis but not asthma. Fujimura *et al* have described a group of patients with atopic cough who have an eosinophilic tracheobronchitis without BAL fluid eosinophilia.²² Our study confirms a previous report¹¹ that BAL fluid eosinophilia is a feature of eosinophilic bronchitis and provides further evidence that atopic cough and eosinophilic bronchitis are distinct conditions.

Our findings add to a growing body of evidence questioning a direct causal association between eosinophilic airway inflammation and airway responsiveness in asthma. Recent large observational studies have found at best a weak correlation between the induced sputum eosinophil count and methacholine airway responsiveness in subjects with atopic asthma.²³ Furthermore, early studies with anti-interleukin (IL)-5 antibodies have shown an effective reduction in the peripheral blood and sputum eosinophilia seen following allergen challenge, but no effect on either the early or late response or on the severity of airway hyperresponsiveness.²⁴ These observations suggest either that there is an important component of airway hyperresponsiveness in asthma that is independent of eosinophilic airway inflammation, or that there are other functionally important aspects of the inflammatory response that, although closely linked to eosinophilic airway inflammation, can be dissociated from it. One aspect of the inflammatory response that might be particularly important is the localisation of mast cells since they are present within the airway smooth muscle in asthma but not in eosinophilic bronchitis.¹³

If eosinophilic airway inflammation is not important in the development of airway hyperresponsiveness, then how does it contribute to the pathophysiology of asthma? Both eosinophilic bronchitis and asthma are associated with cough, and it is possible that eosinophilic airway inflammation is directly responsible for this aspect of the asthmatic process. Our previous finding of a significant correlation between the improvement in cough reflex sensitivity and fall in induced sputum eosinophil count following treatment of subjects with eosinophilic bronchitis with inhaled corticosteroids⁹ would be consistent with a causal association. We have reported an increased rate of decline in FEV₁ and the development of fixed airflow obstruction in a patient with eosinophilic bronchitis,²⁵ and it is possible that this important complication of chronic asthma is also related to eosinophilic airway inflammation. Finally, eosinophilic airway inflammation could be causally associated with the occurrence of asthma exacerbations since corticosteroid withdrawal studies show that the sputum eosinophil count is an independent predictor of the development of an exacerbation,²⁶ and that an increase in the sputum eosinophil count occurs well before the exacerbation.²⁷

Previous immunopathological studies of asthma have reported thickening of the subepithelial collagen layer, increased numbers of epithelial cells in the bronchial wash,²⁸ and a reduction in epithelial integrity in bronchial biopsy specimens.¹⁹ Bronchial epithelial cells are also activated, as reflected by increased inducible nitric oxide synthetase (iNOS) expression²⁹ and an increased concentration of nitric oxide in exhaled air.²⁰ We found no differences in the number of epithelial cells in the bronchial wash or BAL fluid, and no difference in epithelial integrity or basement membrane width between subjects with eosinophilic bronchitis and those with asthma. Similarly, and as noted before,³⁰ both conditions were associated with increased concentrations of exhaled nitric oxide. Our findings, together with the recent identification of a subgroup of patients with severe asthma who have a normal basement membrane and lamina reticularis width and no bronchoscopic evidence of eosinophilic airway

inflammation,¹³ suggest that these epithelial abnormalities relate more closely to the presence of eosinophilic airway inflammation than the clinical expression of the disease. We found no differences between the normal control group and either disease group in epithelial integrity, which suggests that this is not a consistent feature of asthma or that previous studies have identified an artefact, perhaps related to the biopsy technique.³¹

Neutrophil numbers were increased in the bronchial subepithelium in those with eosinophilic bronchitis compared with the other groups. Bronchial submucosal neutrophilic inflammation is a feature of severe asthma³² and the differences we observed may be a reflection of our selection of mild asthmatics. The difference in subepithelial neutrophil numbers was small and could have arisen by chance, although the finding is consistent with our previous observations of a trend towards an increased sputum neutrophil count¹⁰ and a raised sputum concentration of the neutrophil chemokine IL-8 in eosinophilic bronchitis.³³ Further work is required to investigate whether the difference in neutrophilic airway inflammation is functionally important.

In conclusion, with the exception of our previously reported association of mast cell infiltration into the airway smooth muscle with asthma, the immunopathology of eosinophilic bronchitis and asthma is very similar with both conditions being characterised by eosinophilic airway inflammation, increased exhaled nitric oxide, and increased basement membrane thickening. This strongly suggests that these features of airway inflammation, together with structural changes in the airway wall, are regulated independently of airway hyperresponsiveness.

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LUNG ALERT

Anti-IgE therapy protects against peanut allergy

▲ Leung DY, Sampson HA, Yunginger JW, *et al*. Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med* 2003;**348**:986–93

Peanut induced anaphylaxis (believed to be an IgE mediated condition) may result in death. Mortality in this increasingly common illness is usually associated with accidental ingestion of the equivalent of one to two peanuts. This multicentred, double blind, randomised, controlled study investigated 84 patients with a known immediate hypersensitivity response to peanuts. Patients were allocated to receive placebo or a series of weekly doses of TNX-901 (150 mg, 300 mg, or 450 mg) over the 4 week study period. TNX-901 is a monoclonal antibody directed against IgE. Increasing doses of this antibody provided statistically significant protection ($p < 0.01$) against oral peanut challenge. At the highest dose (450 mg) protection was provided against the equivalent of nine peanuts (enough to guard against most cases of unintentional exposure) 4 weeks after the last administration. The antibody was well tolerated.

Until now the mainstay of treatment of peanut induced anaphylaxis has been self-administered adrenaline, but patients may forget to carry this. TNX-901 may prove to be a beneficial alternative therapeutic agent for this condition. However, the trial was performed over 4 weeks only and further long term studies are required.

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