

Airway eosinophilia in chronic cough, asthma  
and chronic obstructive pulmonary disease: an  
immunopathological feature of disease and a  
marker of response to corticosteroids

Thesis submitted for the degree of  
Doctor of Philosophy  
At the University of Leicester  
by

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# **Airway eosinophilia in chronic cough, asthma and chronic obstructive pulmonary disease: an immunopathological feature of disease and a marker of response to corticosteroids**

Shortened title: *Airway inflammation in eosinophilic bronchitis, asthma and chronic obstructive pulmonary disease*

Contents	i
Abstract	v
Acknowledgements	vi
Publications arising from this thesis	vii
List of tables and figures	ix
<b>1. Introduction</b>	
<b>1.1 Asthma</b>	
1.1.1 Immunopathology of Asthma	1
1.1.2 Eosinophils in Asthma	
1.1.2.1 An historic perspective	2
1.1.2.2 The Biology of Eosinophils	3
1.1.2.3 Eosinophilic Airway Inflammation	4
1.1.2.4 Eosinophils and the Relationship to Disease Severity	8
1.1.2.5 Anti-Inflammatory Drugs in Asthma	12
1.1.2.6 Novel Anti-Eosinophil Therapies	14
1.1.3 Mast Cells in Asthma	14
1.1.4 T-lymphocytes in Asthma	17

1.2	<b>Eosinophilic Bronchitis without Asthma</b>	
1.2.1	Sputum as a measure of airway inflammation	21
1.2.2	A common and treatable cause of chronic cough?	22
1.2.3	What does eosinophilic bronchitis tell us about asthma?	23
1.2.4	Immunopathology of eosinophilic bronchitis	24
1.2.5	Aetiology and natural history of eosinophilic bronchitis	25
1.3	<b>Chronic Obstructive Pulmonary Disease</b>	
1.3.1	Airway inflammation in COPD	28
1.3.2	The effect of treatment on airway inflammation	30
1.4	<b>Hypothesis and Aims</b>	32
2.	<b>Methods</b>	
2.1	<b>Clinical</b>	
2.1.1	Peripheral blood tests, skin tests	34
2.1.2	Spirometry	34
2.1.3	Airway responsiveness	34
2.1.4	Sputum induction	35
2.1.5	Bronchoscopy	38
2.1.6	Nasal Lavage	38
2.1.7	Capsaicin cough challenge	38
2.1.8	Chronic respiratory disease health status questionnaire	39
2.1.9	Incremental shuttle walk test	39
2.1.10	Symptom visual analogue scores	39
2.2	<b>Laboratory</b>	
2.2.1	<b>Cells</b>	
2.2.1.1	Peripheral blood mononuclear cell separation	40

2.2.1.2	Sputum, bronchial wash and bronchoalveolar lavage processing	40
2.2.1.3	Flow cytometry T-cell activation markers and chemokine receptors	43
2.2.1.4	Flow cytometry T-cell intracellular cytokines	44
2.2.1.5	Cytoblocks	45
2.2.2	<b>Supernatants from sputum</b>	
2.2.2.1	Elastase spectrofluorimetric assay	45
2.2.2.2	IL-8, cysteinyl leukotrienes enzyme immunoassay	46
2.2.2.3	ECP fluoroimmunoassay	46
2.2.2.4	Prostanoids GC-MS-NICI	47
2.2.2.5	Histamine radioenzymic assay	47
2.2.3	<b>Generation of probes for in-situ hybridisation</b>	
2.2.3.1	mRNA extraction	47
2.2.3.2	Reverse transcriptase polymerase chain reaction (PCR)	48
2.2.3.3	Generating primers	49
2.2.3.4	PCR	50
2.2.3.5	Asymmetric PCR	53
2.2.4	<b>Biopsies</b>	
2.2.4.1	Fixation, processing and embedding in glycomethacrylate and paraffin	54
2.2.4.2	Immunohistochemistry	54
2.2.4.3	In-situ hybridisation	57

### 3. **Studies**

3.1	<b>Clinical and Immunopathological Characterisation of Eosinophilic Bronchitis</b>	
3.1.1	Eosinophilic Bronchitis Is an Important Cause of Chronic Cough	60
3.1.2	Induced Sputum Inflammatory Mediator Concentrations in	72

	<b>Eosinophilic Bronchitis and Asthma</b>	
3.1.3	Comparison of the immunopathology of eosinophilic bronchitis and asthma	83
3.1.4	Th2 cytokine expression in bronchoalveolar lavage T-lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis	98
3.1.5	Mast cell infiltration of the airway smooth muscle defines the asthmatic phenotype	118
3.2	<b>Airway Eosinophilia: A Marker Of Response To Corticosteroids</b>	
3.2.1	Airway inflammation, airway responsiveness and cough before and after inhaled budesonide in patients with eosinophilic bronchitis	133
3.2.2	Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial	147
4.	<b>Conclusion</b>	
4.1	Summary of findings	162
4.2	Techniques developed and problems encountered	163
4.3	Criticisms	163
4.4	Future studies	165
<b>Appendix I</b>	Subject characteristics of subjects with eosinophilic bronchitis	167
<b>AppendixII</b>	Repeatability bronchial biopsy cell counts and reticular lamina thickness	168
<b>Appendix III</b>	Repeatability of induced sputum, visual analogue scores and chronic respiratory disease questionnaire.	169
<b>References</b>		170

# **Airway eosinophilia in chronic cough, asthma and chronic obstructive pulmonary disease: an immunopathological feature of disease and a marker of response to corticosteroids**

Christopher Brightling

## **Abstract**

Eosinophilic bronchitis is a condition characterised by chronic cough and a sputum eosinophilia without the variable airflow obstruction or airway hyperresponsiveness characteristic of asthma. This thesis describes the incidence of eosinophilic bronchitis as a cause of chronic cough and determines whether the presence of a sputum eosinophilia is associated with a favourable response to corticosteroids in eosinophilic bronchitis and chronic obstructive pulmonary disease (COPD). This is the first study to investigate in detail the immunopathology of patients with eosinophilic bronchitis compared to asthma. Understanding differences in the immunopathology will inform our understanding of the development of the disordered airway physiology observed in asthma. We demonstrated that eosinophilic bronchitis is a common cause of chronic cough and that a sputum eosinophilia predicts a good response to corticosteroids in this condition, asthma and COPD. We found sputum, bronchial wash and bronchoalveolar lavage (BAL) eosinophilia and bronchial submucosal evidence of eosinophilic airway inflammation, increased Th2 cytokine expression and basement membrane thickening and increased constitutive intracellular expression of IL-4 from BAL derived T-cells in subjects with eosinophilic bronchitis to the same degree as those with asthma. Thus, the immunopathology of eosinophilic bronchitis is very similar to asthma questioning the importance of these classical pathological characteristics of asthma in the development of abnormal airway physiology. In addition, we quantified the inflammatory cell infiltration of the airway smooth muscle and found a striking increase in the number of mast cells within the airway smooth muscle in asthma compared to eosinophilic bronchitis and normal subjects. Our findings suggest that in asthma the microlocalisation of mast cells within the airway smooth muscle is a key factor in the development of variable airflow obstruction and airway hyperresponsiveness. Thus, specific targeting of the mast cell-smooth muscle interaction may provide a novel approach to the effective treatment for asthma.

## **Acknowledgements**

I would like to thank the patients and volunteers who participated in the studies.

I would like to thank my co-supervisors Ian Pavord and Andy Wardlaw. They both have offered support and guidance, but yet have encouraged me to be autonomous allowing me to develop as an independent researcher. Peter Bradding's technical assistance in the laboratory in setting up many of the techniques together with his help as an additional mentor has been invaluable. Fiona Symon has been pivotal in developing flow cytometry in the laboratory and has taught me to control for everything. Several of the clinical studies would have not been possible without technical support from Richard Ward, Will Monteiro, Debbie Parker, Simon Barlow in the laboratory with sputum processing, supernatant analysis and immunohistochemistry. Patient recruitment and careful clinical characterisation is essential and I would like to thank Ruth Green, Surinder Birring, Bev Hargadon, Sue McKenna for their help. Mike Morgan, Professor Stockley and Sally Singh offered thoughtful advice in the design of the protocol for the COPD study. The Respiratory Physiology and Pharmacy Departments also supported this study.

I would like to thank my collaborators Ryzard Dworski and James Sheller for their help with the assessment of sputum mediators, Stephen Holgate who collaborated on the mast cells in airway smooth muscle study, Jim Campbell for his expertise in flow cytometry and T-cell trafficking and Howard Pringle for his help with the in-situ hybridisation.

This research was supported by grants from The National Asthma Campaign and Trent Regional R&D. Astra-Zeneca provided technical support in the assessment of ECP and supplied Pulmicort Turbohalers to study the effects of inhaled corticosteroids in eosinophilic bronchitis. The Woodhouse Eaves Ladies Group kindly raised funds towards the purchase of an ultramicrotome.

A special thanks to Michelle and our children Ellen, Joe and Isobel.

## **Publications arising from this thesis**

### **1 Papers**

CE Brightling, P Bradding, FA Symon, ST Holgate, AJ Wardlaw, ID Pavord  
Mast cell infiltration of airway smooth muscle characterises the asthmatic phenotype (New Eng J Med in press)

CE Brightling, FA Symon, S Barlow, P Bradding, AJ Wardlaw, ID Pavord  
Comparison of the immunopathology of eosinophilic bronchitis and asthma (Am J Respir Crit Care under submission)

CE Brightling, FA Symon, SS Birring, P Bradding, ID Pavord, AJ Wardlaw  
Th2 cytokine expression in bronchial mucosa and bronchoalveolar lavage T-lymphocytes is a feature of asthma and eosinophilic bronchitis without asthma (J Allergy Clin Immunol under submission)

CE Brightling, W Monteiro, RH Green, D Parker, MD Morgan, AJ Wardlaw, ID Pavord  
Induced sputum and other outcome measures in chronic obstructive pulmonary disease; safety and repeatability. *Respir Med.* 2001; 95: 999-1002.

Campbell JJ, Brightling CE, Symon FA, Qin S, Murphy KE, Hodge M, Andrew DP, Wu L, Butcher EC, Wardlaw AJ. Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. *J Immunol.* 2001 15;166(4):2842-2848

Brightling CE, Monterio W, Ward R, Parker D, Morgan MDLM, Wardlaw AJ, Pavord ID  
Sputum eosinophilia and the response to prednisolone in chronic obstructive pulmonary disease *Lancet* 2000. 28; 356 (9240): 1480-5

Brightling CE, Ward R Woltmann G, Bradding P, Sheller JR, Dworski R, Pavord ID  
Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma *Am J Respir Crit Care Med* 2000; 162: 878-882

Brightling CE, Ward R, Wardlaw AJ, Pavord ID Airway inflammation, airway responsiveness and cough before and after inhaled budesonide in patients with eosinophilic bronchitis *Eur Respir J* 2000 Apr;15(4):682-6

Brightling CE, Ward R, Goh KL, Wardlaw AJ, Pavord ID Eosinophilic bronchitis is an important cause of cough *Am J Resp Crit Care* 1999 160: 406-410

Pavord ID, Brightling CE, Woltmann G, Wardlaw AJ Non-eosinophilic, corticosteroid unresponsive asthma *Lancet* 1999 353: 2213-2214

## **2 Editorials and reviews**

Eosinophils in asthma and other allergic diseases Wardlaw AJ, Brightling CE, Green RH, Woltmann G, Pavord ID *Br Med Bull* 2000; 56: 843-864

Eosinophilic bronchitis: a common cause of prolonged cough Brightling CE, Pavord ID *Annals Med* 2000; 32 (7): 446-51

Eosinophilic bronchitis what is it and why is it important? Brightling CE, Pavord ID *Clin Exp Allergy* 2000 30 (1): 4-7

## **3 Case report**

Development of irreversible airflow obstruction in a patient with eosinophilic bronchitis Brightling CE, Woltmann G, Ward R, Wardlaw AJ, Pavord ID *Eur Respir J* 1999; 14 (5); 1228-1230

## **4 Abstracts**

20 abstracts published or in press in *Thorax*, *Eur Respir J*, *Am J Resp Crit Care Med* and *Journal Allergy and Clinical Immunology*

## List of tables

- 2.1 Antibody source and concentration used for flow cytometry immunohistochemistry
- 2.1 Antibody source and concentration used for immunohistochemistry
- 3.1 Clinical features, investigations and treatment of chronic persistent cough
- 3.2 Causes of isolated chronic cough
- 3.3 Characteristics of patients with eosinophilic bronchitis
- 3.4 Subject details, sputum characteristics and mean (SEM) sputum supernatant mediator concentrations
- 3.5 Subjects' clinical characteristics
- 3.6 Median (range) differential cell counts in sputum, bronchial wash and BAL
- 3.7 Median (range) submucosal cell counts/mm<sup>2</sup>
- 3.8 Subjects' clinical characteristics
- 3.9 Median (range) of the proportion (%) of CD4+ (CD3+CD8-) and CD8+ (CD3+CD8+) peripheral blood T-lymphocytes expressing cytokine staining
- 3.10 Median (range) of the proportion (%) of CD4+ (CD3+CD4+) and CD8+ (CD3+CD4-) T-cells that express chemokine receptors and activation markers in bronchoalveolar lavage in subjects with eosinophilic bronchitis, asthma and normal individuals
- 3.11 Subjects' clinical characteristics
- 3.12 Median (range) cells/mm<sup>2</sup> submucosa and cells/mm epithelium
- 3.13 Characteristics of subjects at diagnosis
- 3.14 Patient characteristics before and after inhaled budesonide
- 3.15 Mean (SEM) outcome measures before and after treatment
- 3.16 Baseline characteristics after stratification into tertiles by baseline eosinophil counts
- 3.17 Mean (95% CI) change in secondary outcomes after prednisolone treatment compared with placebo

## List of Figures

- 1.1 Change in lung function and sputum eosinophilia over two-years with the development of fixed airflow obstruction in a patient with eosinophilic bronchitis.
- 2.1 Sputum induction protocol
- 2.2 Sputum processing protocol
- 2.3 Forward and reverse primers for IFN- $\gamma$  and IL-4
- 2.4 PCR bands for IFN- $\gamma$  from cDNA derived from stimulated PBMC and IL-4 band from IL-4 bluescript (gift)
- 2.5 Bands for IFN- $\gamma$  and IL-4 probes from asymmetric PCR
- 2.6 IFN- $\gamma$  mRNA expression by (a) stimulated PBMC and (b) tonsil tissue, sense and anti-sense
- 3.1 Diagnostic algorithm for patients with chronic persistent cough
- 3.2 Geometric mean (log SEM) concentrations of ECP, cysteinyl-leukotriene, histamine and mean (SEM) PGD<sub>2</sub> concentrations in each group of subjects
- 3.3 An example of a sputum and BAL eosinophilia
- 3.4 Examples of positive submucosal staining for eosinophils and neutrophils and basement and reticular lamina thickening
- 3.5 Submucosal cell counts/mm<sup>2</sup> eosinophils (MBP) and neutrophils (NE) and reticular lamina and basement membrane thickness in subjects with asthma, eosinophilic bronchitis and normal controls
- 3.6 Examples of submucosal staining for IL-4 (3H4 and 4D9), IL-5 and IFN- $\gamma$
- 3.7 Submucosal cell counts/mm<sup>2</sup> expressing IL-4 (3H4 and 4D9), IL-5 and IFN- $\gamma$  in subjects with asthma, eosinophilic bronchitis and normal controls
- 3.8 Example dot-plots from an individual normal control (A) a subject with eosinophilic bronchitis (B) and asthma (C) for IFN- $\gamma$  intracellular cytokine staining and isotopic control in resting and stimulated PBMC T-lymphocytes
- 3.9 Example dot-plots from an individual normal control (A) a subject with eosinophilic bronchitis (B) and asthma (C) for IL-4 intracellular cytokine staining and isotopic control in resting and stimulated BAL T-lymphocytes

- 3.10 Individual data for the proportion (%) of CD4 (CD3+CD8-) expressing intracellular IL-4 and IFN- $\gamma$  in resting and stimulated BAL T-lymphocytes
- 3.11 Submucosal cell counts/mm<sup>2</sup> for mast cells (AAI) and eosinophils (EG2) and basement membrane thickness in subjects with asthma, eosinophilic bronchitis and normal controls
- 3.12 Mast cells in bronchial smooth muscle in asthma
- 3.13 Mast cells in bronchial smooth muscle/mm<sup>2</sup> in subjects with asthma, eosinophilic bronchitis and normal controls
- 3.14 Sputum and nasal lavage eosinophil count before and after inhaled budesonide
- 3.15 Capsaicin concentration causing two coughs and five coughs before and after inhaled budesonide
- 3.16 Correlation between fold decrease in sputum eosinophil count after treatment with inhaled budesonide and cough sensitivity assessed as the doubling fold increase in capsaicin concentration required to cause five coughs
- 3.17 Trial profile
- 3.18 Mean (SEM) absolute increase in primary outcomes for each tertile after prednisolone compared with placebo

## 1.1 Asthma

### 1.1.1 Immunopathology of Asthma

Asthma is a condition characterised by variable airflow obstruction and airway hyperresponsiveness in association with airway inflammation that usually has an eosinophilic component. Pathologically, together with the accumulation of eosinophils there is an increase in CD4<sup>+</sup> lymphocytes, dendritic cells and macrophages with activation of mast cells and epithelial cells (Kay 1996) (Djukanovic et al. 1990a). Inflammatory mediators secreted by these and other cells contribute to changes in airway function and structure. The structural changes include thickening of the sub-epithelial collagen layer and submucosal matrix deposition (Roche et al. 1989), hyperplasia and hypertrophy of goblet cells (Fahy 2001) with mucus hypersecretion and smooth muscle hypertrophy and hyperplasia (Knox et al. 2000). These structural changes form the basis of the 'airway remodelling', which is a further important characteristic of asthma. In addition epithelial desquamation, or at least fragility, has been considered a hallmark of the disease and recently, it has been suggested that epithelial fragility is a feature of atopic but not non-atopic asthma (Amin et al. 2000). However, detailed studies of the pathology of asthma deaths have called this dogma into question (Carroll et al. 1993).

Thus, although there is increasing recognition that the airway inflammation in asthma is a complex interaction between a number of inflammatory cells it is attractive to propose that one particular type of inflammatory cell might 'orchestrate' the inflammatory process. There is a growing consensus that the early differentiation of T-cells into those producing Th2 type cytokines can lead onto the development of eosinophilic inflammation and the structural changes typical of the asthma (Robinson 2000). However, the relative importance of different inflammatory cells in the development of airway dysfunction in asthma is unclear.

Asthma can be clinically subdivided into atopic and non-atopic asthma (Rackeman 1947). Those with non-atopic asthma have negative skin tests, normal IgE and no specific IgE antibodies to common allergens. They account for about 10-20% of patients with asthma, usually present in later life often with

a more severe clinical course. Although non-atopic and atopic asthma are different clinical entities they have a similar immunopathology. In a series of bronchial biopsy studies using in situ hybridisation and immunohistochemistry, alone or in combination, both variants of disease were characterised by infiltration of eosinophils and Th2-type cells secreting IL-4 and/or IL-5, the presence of CC chemokines and FcεRI cells (Bentley et al. 1992), (Humbert et al. 1996a), (Ying et al. 1997), (Humbert et al. 1996b), (Humbert et al. 1997). Due to the pathological similarity between non-atopic and atopic asthma the further descriptions of asthma in this introduction will address both clinical subtypes as a single disease.

I shall summarise the evidence for the role of the eosinophil, mast cell and T-cell in asthma. Although the relative importance of all of these cells in the development of disordered airway physiology is addressed in this thesis, more emphasis is placed upon the eosinophil in terms of its role in cough and its value as a marker of response to corticosteroid treatment thus I have given more weight to the eosinophil in this introduction.

### **1.1.2 Eosinophils in Asthma**

#### **1.1.2.1 An Historic perspective**

Eosinophils are a characteristic feature of the pathology of asthma. The eosinophil was discovered by Jones in 1846 but its propensity to stain with aniline dyes was first described by Paul Ehrlich in 1879 (Ehrlich 1879). Recognized and named for this quality, eosinophils possess an abundance of highly basic proteins within their granules which confer their affinity for acidic dyes. An association between a sputum eosinophilia and asthma has been recognised since the last half of the 19<sup>th</sup> century following the description of Charcot-Leyden crystals and Curschmann spirals (Charcot & Robin 1853) (Leyden 1871)(Curshmann 1882). Ideas about their role in asthma have fluctuated with a view in the 1970's that they were an ameliorating influence. Since the 1980's there has been a consensus that they are important pro-inflammatory cells. This view evolved from evidence that eosinophils were potentially cytotoxic. In the 1970's it was shown that eosinophils could

effectively kill the larval stage of helminthic parasites (Butterworth et al. 1975). This was followed by evidence demonstrating a toxic effect on mammalian cells, particularly bronchial epithelium, mediated by the eosinophil specific basic proteins (Frigas, Motojima, & Gleich 1991). Eosinophils were shown to produce large amounts of the sulphidopeptide leukotrienes (LTC<sub>4</sub>/D<sub>4</sub> and E<sub>4</sub>) and platelet activating factor (PAF), which were thought to be involved in causing bronchospasm in asthma. Furthermore it had long been known that one of the most noticeable effects of glucocorticoids in asthma was their ability to reduce the blood and airway eosinophilia. Further impetus for the hypothesis that eosinophils were inextricably involved in the asthma process was provided by the development of the hypothesis in the 1990's that allergic disease is due to inappropriate activation of allergen specific Th2 lymphocytes (Corrigan & Kay 1992). Th2 cells through elaboration of IL-4 and IL-5 are inextricably linked with the development of blood and tissue eosinophilia with IL-5 being the major eosinophil specific growth factor (Sanderson 1992) and IL-4 causing selective upregulation of eosinophil specific endothelial adhesion and chemoattractant pathways (Wardlaw 1999). The view of asthma as a disease caused by an airway eosinophilia leading to airway hyperresponsiveness has now become firmly embedded in the literature.

#### **1.1.2.2 The Biology of Eosinophils**

Eosinophils are end stage cells derived from the bone marrow under the influence of GM-CSF, IL-3 and the late differentiation factor IL-5, which in humans is only active on eosinophils and basophils (Denburg 1999). In terms of their ontogeny they are closely related to the basophil rather than the neutrophil or monocyte. The selective recruitment of eosinophils into the airway is mediated by a multi-step process directed by Th2 cytokine producing T cells (Wardlaw 1999). The first step is increased production and release of eosinophils from the bone marrow under the influence of the IL-5 and specific chemoattractants such as eotaxin. Secondly the target organ vasculature has increased adhesiveness for eosinophils through the specific effects of locally generated IL-4 and IL-13. These cytokines induce expression of VCAM-1 which binds eosinophils through

VLA-4, a receptor not expressed by neutrophils, and P-selectin to which eosinophils bind with greater avidity than neutrophils (Woltmann et al. 2000) (Edwards et al. 2000) (Symon et al. 1996). CC chemokines such as eotaxin, which bind CCR3, a receptor almost exclusively expressed by eosinophils and basophils, attract eosinophils into tissue where they survive for prolonged periods as a result of locally generated IL-5 and GM-CSF.

The relatively eosinophil specific basic proteins, which are stored in the distinctive secondary granules, are major basic protein (MBP) eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN). All of these proteins are toxic to the larval stages of many helminthic parasites as well as bronchial epithelial cells. Inhaled MBP can also induce airway hyperresponsiveness in primates (Gundel, Letts, & Gleich 1991), and inhibit the actions of down regulatory M2 muscarinic receptors (Costello et al. 1997). Eosinophils, with mast cells and basophils, are the most prominent source of sulphidopeptide leukotrienes produced mainly by enzymes present in lipid bodies (Bozza et al. 1997). They also produce significant amounts of PAF although this is not specific to the eosinophil. Many of the diverse range of cytokines produced by eosinophils are released only in small amounts, which may nonetheless have important autocrine effects (Lacy & Moqbel 2000). For example the small amounts of GM-CSF produced by the eosinophil is sufficient to prolong its own survival. The production of cytokines such as TGF $\alpha$  and TGF $\beta$  by eosinophils has broadened the range of eosinophil functions emphasising their potential importance in, for example, wound healing.

The physiological triggers, which lead to eosinophil mediator release in airway diseases remain uncertain. Engagement of Fc $\gamma$  and Fc $\alpha$  receptors are the most reliable physiological triggers for degranulation of eosinophil basic proteins, especially when presented on the surface of a large particle, or in the context of co-engagement of the adhesion receptor Mac-1 (Kaneko et al. 1995). Priming with eosinophil active growth factors such as IL-5 greatly enhances degranulation via Fc receptors and also causes degranulation directly (Kita et al. 1992). PMA is an effective, albeit non-physiological, stimulus for superoxide production but not for degranulation or cytokine release. Calcium ionophore is a good stimulus for lipid mediator production. Chemoattractants binding via G

protein linked serpentine receptors stimulate superoxide production, but are not a good stimulus for other mediators. Eosinophils undergo piecemeal degranulation in most *in vivo* settings (Dvorak & Weller 2000). This involves the eosinophil releasing its granule products through pores in the plasma membrane without leading to cell death. With more vigorous stimuli, for example after allergen challenge, cytolysis is often prominent (Erjefalt et al. 1999).

### 1.1.2.3 Eosinophilic Airway Inflammation in Asthma

#### **Post-mortem studies**

Eosinophils and their granule products have been identified to be increased in the airways in post mortem specimens (Filley et al. 1982) (Azzawi et al. 1992), although a small proportion of patients dying of asthma, particularly those with sudden death, do not have an airway eosinophilia, (Sur et al. 1993). The inflammatory response in asthma deaths has been noted to affect both the large and small airways (Carroll, Cooke, & James 1997). Post-mortem studies inevitably suffer from small numbers, lack of appropriate controls, a paucity of clinical details and the difficulty in controlling for the effects of treatment.

#### **Bronchoscopy studies**

In the early 1980's it was appreciated that bronchoscopy in asthma could be carried out safely as long as appropriate precautions were taken (Djukanovic et al. 1991). It then became apparent that even in very mild asthma there was evidence of airway inflammation and that the most obvious feature of the inflammatory response was the increased number of eosinophils in BAL fluid without an increase in neutrophils (Wardlaw et al. 1988), reviewed in (Djukanovic et al 1990a). These studies demonstrated a considerable variability in the degree of BAL eosinophilia ranging from 1% to up to 30% (the normal being less than 1%). There was evidence that the eosinophils were actively secreting mediators with increased amounts of the eosinophil granule proteins and leukotrienes in BAL fluid (Wardlaw et al. 1989) (Broide et al. 1991) and sputum (Pavord et al. 1999a), although data regarding mediators in BAL fluid should always be treated with caution because of the lack of a good denominator

to control for recovery of airway lining fluid. BAL eosinophils express the putative activation receptor CD69, have reduced expression of L-selectin, and increased expression of Mac-1 and ICAM-1 when compared to peripheral blood eosinophils. These changes in phenotype can be mimicked *in vitro* by treatment with IL-5 (for > 24hours) (Hartnell et al. 1993), and suggest an activated phenotype.

Studies of BAL were followed by biopsy studies which allowed a more detailed immunopathological analysis of the inflammatory response in the bronchial tree (Djukanovic et al. 1990b) (Azzawi et al. 1990) (Poston et al. 1992). One advantage of biopsy studies is that they allow accurate quantification of bronchial lymphocytes and mast cells which are anchored within the epithelium and do not migrate into the lumen so do not appear in BAL fluid (or sputum) in representative numbers. These studies confirmed the consistent increase in the number of eosinophils in the airway submucosa without an increase, in most cases, in neutrophils. Eosinophils, which are very infrequent in the normal airway, are enriched by up to 100 fold in the airways of asthmatic subjects compared to neutrophils. All these studies involve a cross-sectional analysis and tells us little to nothing about the kinetics of migration of these cells into the airways. Endobronchial biopsy studies also demonstrated increased numbers of CD4 T cells in asthmatic airways. Although most bronchoscopy studies have been undertaken in young adults with mild atopic asthma, increased numbers of eosinophils have also been reported in intrinsic asthma (Bentley et al. 1992), and occupational asthma due to Western Red Cedar and toluene diisocyanate (Frew et al. 1995) (Saetta et al. 1992), as well as aspirin sensitive asthma where more eosinophils were seen than in non-aspirin sensitive asthmatics (Nasser et al. 1996). In severe corticosteroid dependent asthma two patterns were observed. Out of 34 severe oral glucocorticoid dependent asthmatics, in fourteen patients eosinophils were absent in endobronchial biopsies whereas in 20 subjects eosinophils were increased. Neutrophils were increased in both groups. The eosinophilic group had been intubated more often (Wenzel et al. 1997). Few bronchoscopy studies have been undertaken in children in asthma although increases in both eosinophils and neutrophils have been reported (Kim et al. 2000) (Marguet et al. 1999). Eosinophils in bronchial biopsies often have a partially degranulated appearance (Djukanovic et al. 1992a)

and after nasal allergen challenge were shown to have undergone degranulation through a combination of cytolysis and piecemeal degranulation (Erjefalt et al 1999). Tissue eosinophils express a range of cytokines as shown by both in situ hybridization and immunohistochemistry (Broide, Paine, & Firestein 1992). The anti-ECP antibody EG2 has been widely used as a marker of eosinophil activation although the validity of this has recently been called into question (Nakajima et al. 1999). An airway eosinophilia is a consistent feature of the late response to allergen challenge in the bronchial and nasal mucosa as well as the skin (Aalbers et al. 1993) (Varney et al. 1992) (Tsicopoulos et al. 1994). This is often more marked than the often modest number seen in chronic disease. However the extent to which allergen challenge is a model for clinical disease remains controversial.

### **Induced sputum**

Bronchoscopy is invasive, potentially hazardous and expensive. Small numbers of individuals with generally mild disease are usually studied and repeat measurements are difficult. Measurement of the eosinophil count in induced sputum has overcome some of these problems (see 1.2.1 for more details on the development of sputum induction). Eosinophil counts in sputum correlate well with eosinophil counts in bronchial biopsies, washings and BAL with the eosinophilia being often more marked in sputum (Maestrelli et al. 1995) (Fahy et al. 1995a). The normal value for non-smokers has been reported as 0.4% with a 90<sup>th</sup> percentile of up to 1.1% with atopics higher than non-atopics (Belda et al. 2000). We have found similar values. The range of eosinophil counts in asthma is wide from 0% through to 50% or more. We have found that most, though not all, asthmatics have a raised eosinophil count. Taking a cut off of 1% as indicating a raised sputum eosinophil count when compared with normal airways a sputum eosinophilia as a test for asthma (defined by a Pc20 of <8.0mg/ml or a significant improvement in FEV<sub>1</sub> after  $\beta$ 2 agonists) gives a sensitivity of over 80% and a specificity of 95%. This falls to 70% and 80% when compared with subjects with respiratory symptoms and a preliminary diagnosis of asthma, who subsequently turn out to have other conditions. In both cases this is considerably better than peak flow variability and much more sensitive than improvements in

FEV<sub>1</sub>. Interestingly in our experience while the majority of patients with acute severe asthma have a very high eosinophil count, a small proportion had no eosinophils. Raised numbers of neutrophils in acute severe asthma have been reported by others (Fahy et al. 1995b) (Norzila et al. 2000) although in the Fahy study about a third had been taking oral glucocorticoids which may have influenced the findings. Up to 64% of neutrophils are found in the normal airway, which makes interpretation of a raised neutrophil count in asthma more difficult. A sputum eosinophilia is a feature of occupational asthma and may be useful in diagnosis (Lemiere et al. 1999). As discussed below the use of induced sputum has allowed a much more detailed assessment of the relationship between asthma and airway inflammation in terms of severity, asthma phenotype, bronchial hyperresponsiveness and response to treatment. For example bronchoscopy studies have suggested that whereas asthma is characterised by an airway eosinophilia, COPD is associated with a neutrophilia (Jeffery 1999), although eosinophils were noted to be present during exacerbations (Saetta et al. 1994). However it is clear from sputum studies in which larger numbers of more severe patients can be studied that up to a third of patients with stable irreversible smoking associated COPD have a significant airway eosinophilia (Pizzichini et al. 1998) (Balzano et al. 1999). In addition an airway eosinophilia is commonly associated with chronic cough, which occurs in the absence of variable airflow obstruction and airway hyperresponsiveness, a condition labelled Eosinophilic Bronchitis (Gibson et al. 1989b) (eosinophilic bronchitis is described in detail in 1.2). Increased numbers of eosinophils compared to controls are seen in the airways of atopics without asthma although the increase is less marked than in asthmatics (Djukanovic et al 1992a) (Belda et al 2000) (Foresi et al. 1997).

#### **1.1.2.4 Eosinophils and the relationship to disease severity**

##### **Symptoms and lung function**

If eosinophils were important in causing the pathophysiology of asthma and related diseases then a correlation between the degree of tissue eosinophilia and disease severity might be expected. Early studies of peripheral blood eosinophil counts had suggested a correlation with lung function both in clinical disease (Horn et al. 1975) and after allergen challenge (Durham & Kay 1985). However

blood eosinophils are an insensitive and imprecise marker of tissue inflammatory responses (Pizzichini et al. 1997a). In asthma deaths the eosinophilic inflammation, particularly in the proximal bronchial tree, is more intense than in mild to moderate asthma (Synek et al. 1996). In the early bronchoscopy studies the presence of eosinophils in BAL fluid was associated with symptomatic asthma and was not seen in asthmatics in remission (Wardlaw et al 1988). There was also a broad correlation between clinical severity and degree of airway eosinophilia (Bousquet et al. 1990). In 17 patients with mild asthma Walker *et al* reported a correlation between the degree of BAL eosinophilia, lung function and airway hyperresponsiveness. This study also found a correlation between the numbers of eosinophils and activated T cells, as did a bronchial biopsy study by Bradley *et al* emphasising the relationship between T cells and eosinophils in allergic disease (Bradley et al. 1991). Lim *et al* found that there was a weak correlation between mucosal eosinophils and lung function in 16 asthmatics, half of which were taking inhaled glucocorticoids (Lim et al. 2000). Most bronchoscopy studies have investigated mild asthma so it is difficult to get a spectrum of asthma severity in terms of symptoms and lung function. Induced sputum can be obtained safely even in moderate to severe asthmatics and has allowed a more detailed analysis of the relationship between eosinophilic airways inflammation and asthma (Rosi & Scano 2000).

In an early sputum study Pin et al found an inverse correlation between FEV<sub>1</sub> and sputum eosinophil counts (Pin et al. 1992a). In contrast another study of 20 asthmatics after an eight-week course of high dose inhaled steroids no correlation between sputum eosinophils and clinical markers of severity was observed although there was a weak correlation with airway hyperresponsiveness (Gibson, Saltos, & Borgas 2000). In 20 atopic asthmatic children on inhaled steroids with moderate to severe chronic asthma a weak correlation was observed between sputum eosinophil numbers and asthma severity in terms of lung function and an asthma severity index (Grootendorst et al. 1999). In a study, which compared the degree of airways inflammation in mild and moderate asthma the number of eosinophils, as well as other markers of inflammation, were more marked in the more severe disease group (Vignola et al. 1998). In a study of induced sputum in 74 asthmatics ranging from mild to severe persistent disease, asthma severity as assessed by lung function, symptoms scores and airway hyperresponsiveness,

correlated with the degree of airway eosinophilia. A weak correlation was also seen between sputum neutrophilia and symptom scores (Louis et al. 2000). In a study of 43 mild to severe asthmatics the sputum eosinophil count was greater in the severe compared to the mild and moderate asthmatics as defined by the clinical Aas score, but only weak correlation's with FEV<sub>1</sub> and airway hyperresponsiveness were observed (Ronchi et al. 1997). In an interesting study by Jatakanon et al withdrawing inhaled steroids in a subset of subjects with asthma induced an exacerbation. Baseline sputum eosinophilia was a predictor of subsequent exacerbations and the degree of sputum eosinophilia correlated with falls in PEF and FEV<sub>1</sub> (Jatakanon, Lim, & Barnes 2000). Probably more important than numbers of eosinophils is the amount of mediators they are generating. In a study of 36 patients with asthma ranging in severity from mild to severe there was a higher concentration of ECP in the severe asthmatics compared to the mild to moderate patients. Taking all the subjects ECP levels correlated with symptom scores and inversely with PEF (Fujimoto et al. 1997). Similarly Virchow et al found that in 14 patients with asthma not taking corticosteroids sputum ECP was a better marker of severity in terms of lung function than sputum eosinophil counts (Virchow, Jr., Holscher, & Virchow, Sr. 1992).

Studying the relationship between various markers of asthma severity and airway inflammation is difficult. Symptoms are not objective and peak flow measurements are unreliable. Cross-sectional studies involving a single measurement of FEV<sub>1</sub> and eosinophil count, in a disease like asthma which is defined in terms of its variable severity are crude, particularly when the sampling errors involved in measuring the degree of airway eosinophilia are taken into account. There is a paucity of longitudinal studies correlating asthma severity with airway inflammation on an individual basis. Most of the reported studies are relatively underpowered and treatment with anti-inflammatory drugs adds another important variable.

### **Eosinophils and airway hyperresponsiveness**

Airway hyperresponsiveness refers to the increased sensitivity of asthmatics to irritant inhaled stimuli. This is non-specific in the sense that it not antigen dependent and can be caused by a number of diverse agents ranging from smoke

and dust through to cold air, exercise and perfumes. Agents such as methacholine and histamine that act directly on smooth muscle to cause bronchoconstriction or agents such as cold air that are thought to act indirectly possibly through a neural reflex can therefore cause it. Airway responsiveness is a hallmark of asthma and measurement of airway responsiveness, for example by constructing a histamine or methacholine dose response curve, is the most sensitive and specific test for asthma. Airway hyperresponsiveness is regarded as a necessary ingredient for the integrity of asthma models either in humans or animals. However the extent to which the increased bronchial reactivity seen after allergen challenge and in animal models is related to the airway hyperresponsiveness seen in clinical disease is debatable. Despite intensive research over the last twenty years the cause of airway hyperresponsiveness is still unknown. In particular the extent to which airway hyperresponsiveness is caused by, or interacts with, airway inflammation and especially eosinophilic inflammation remains contentious. Airway hyperresponsiveness and eosinophilic inflammation generally occur together. In the early bronchoscopy studies although there was a clear association between airway hyperresponsiveness and an airway eosinophilia there was little evidence of a correlation between the severity of the airway hyperresponsiveness and the number of BAL eosinophils (Wardlaw et al 1988). Similarly in a study by Foresi et al of 15 asthmatics and 30 patients with seasonal allergic rhinitis a good correlation ( $p < 0.005$ ) was seen between sputum eosinophils and airway hyperresponsiveness, but this was skewed by the inclusion of patients with seasonal rhinitis without asthma (Foresi et al 1997). In a much larger study of 71 asthmatics no relationship was seen between sputum eosinophilia and airway hyperresponsiveness although the eosinophil count did inversely correlate with lung function (Crimi et al. 1998). Some studies have seen a correlation. For example Jatakanon et al found a weak inverse correlation ( $r = -0.4$ ) between the sputum eosinophil count and  $PC_{20}$  in 35 stable asthmatics taking only  $\beta_2$  agonists. A stronger inverse correlation was seen with nitric oxide (NO) concentrations in exhaled air (Jatakanon et al. 1998). In our own experience of over 200 stable asthmatics attending our routine outpatient clinics (46% atopic and 44 % taking inhaled steroids) there was no relationship between log sputum eosinophil count and log  $PC_{20}$  in the non-atopic group. However,

there was a significant although weak inverse correlation in the atopic group ( $r=-0.365$ ,  $P<0.002$ ) in both patients taking inhaled steroids and those on  $\beta_2$  agonists alone. Dissociation between airway hyperresponsiveness and airway eosinophilia has been observed in animal studies. For example Henderson *et al* found that an antibody against the  $\alpha_4$  integrin when given intra-peritoneally was able to inhibit eosinophil migration into the airways after ovalbumin challenge, but had no effect on airway hyperresponsiveness. Whereas both airway hyperresponsiveness and the airway eosinophilia were inhibited by nasal delivery of the antibody (Henderson, Jr. et al. 1997).

Current evidence supports the idea that airway hyperresponsiveness and eosinophilic airway inflammation are independently regulated but closely interrelated, a view supported by a factor analysis undertaken by Rosi et al in 99 mild asthmatics (Rosi et al. 1999). This would predict that in a cross-section of patients, for a given degree of inflammation, marked differences in airway hyperresponsiveness could result. This is consistent with the observation that eosinophilic inflammation can occur without airway hyperresponsiveness as in Eosinophilic Bronchitis and marked airway hyperresponsiveness can occur in the context of minimal airway eosinophilia. It would also suggest however, that within an individual, changes in airway hyperresponsiveness might mirror changes in eosinophilic airway inflammation to the extent that airway inflammation could be used longitudinally to guide asthma management.

#### 1.1.2.5 Eosinophils and Anti-inflammatory Drugs in Asthma

If eosinophils are to be plausibly implicated in causing asthma, drugs, which are effective in their treatment, should also reduce eosinophil numbers and mediator release. With the exception of  $\beta_2$  agonists and anti-cholinergic bronchodilators this appears to be the case as is most clearly seen with corticosteroids, the most effective anti-inflammatory treatment for asthma. Corticosteroids cause a marked eosinopenia when given orally and both oral and topical corticosteroids reduce the tissue eosinophilia in a dose dependent manner. In contrast they have little effect on neutrophils, actually increasing the peripheral blood neutrophil count, and are less effective in most lung diseases characterised by neutrophilic inflammation. Corticosteroids increase the rate of

eosinophil apoptosis, although this effect is modest in degree and only seen at relatively high concentrations of steroids with an IC<sub>50</sub> of about 10<sup>-6</sup> molar whereas they prolong neutrophil lifespan (Meagher et al. 1996). The molecular basis for this difference is unknown. It is likely that the principal action of steroids in asthma is to inhibit cytokine and chemokine production by both leucocytes and resident airway cells, such as epithelial cells, fibroblasts and bronchial smooth muscle (Bentley et al. 1996). The evidence that corticosteroids reduce eosinophil counts in asthma is consistent. For example 6 days of treatment with oral glucocorticoids reduced the sputum eosinophil count (and ECP level) in 24 asthmatics from 14% to 1% whereas no change was observed in the placebo group. Moreover the increase in peak flow associated with steroid treatment correlated with the fall in the eosinophil count (Claman et al. 1994). Oral prednisolone caused a fall in the sputum eosinophil count and ECP level in patients with severe exacerbation's of their disease. The improvement in sputum eosinophils and ECP levels correlated with improvement in lung function (Pizzichini et al. 1997b). In a bronchial biopsy study of ten asthmatics, treatment with 2000µg of beclomethasone dipropionate for 6 weeks resulted in an improvement in lung function, symptoms, PC<sub>20</sub> and a reduction in markers of inflammation including the eosinophil count (Djukanovic et al. 1992b). Inhaled budesonide resulted in a fall in eosinophil counts which correlated with the improvement in PC<sub>20</sub> in 14 asthmatics (Lim et al. 1999). Oral prednisolone given for two weeks caused a significant fall in eosinophil counts which was not seen in the placebo limb of the study (Bentley et al 1996). In all these studies comparable effects were also seen on mast cell and T cell counts emphasising the broad spectrum of anti-inflammatory actions of corticosteroids, although neutrophil numbers were unaffected. However the eosinophil count does appear to be a useful marker of steroid responsiveness in asthma, COPD and rhinitis (Pavord et al 1999b) (Little et al. 2000) (Pizzichini et al 1998) (Fujimoto et al. 1999) (Kita et al. 2000). In all these studies the airway eosinophilia was not abolished and in some patients only a modest fall was seen. It was striking that in our clinic population many patients still had a significant sputum eosinophilia despite being on inhaled corticosteroids, although whether this was due to poor compliance, insufficient dose or corticosteroid resistance is not clear. As well as

corticosteroids leukotriene antagonists modestly reduce the eosinophil count in asthma. Interestingly they also promote eosinophil apoptosis (Pizzichini et al. 1999a) (Lee et al. 2000). Cyclosporin and a thromboxane A2 antagonist have been shown to reduce eosinophil counts in asthmatic airways although whether this is related to their benefit in asthma is not clear as they have a wide range of other actions (Hoshino et al. 1999) (Khan et al. 2000).

#### **1.1.2.6 Novel Anti-Eosinophil Therapies**

The evidence supporting an important role for the eosinophil in causing allergic disease has led to a number of pharmaceutical companies developing specific anti-eosinophil therapies. The most advanced of these is anti-IL-5 antibody therapy. In the allergen challenge study the antibodies were well tolerated and effective in reducing the peripheral blood and to a lesser extent sputum eosinophilia, but no effect was seen on either the early or late response or on the severity of airway hyperresponsiveness in these very mild subjects (Leckie et al. 2000). Caveats to this study are the extent to which allergen challenge is a good model for asthma and the reliance on sputum so that we cannot be sure that the eosinophil counts in the bronchial submucosa was also inhibited. Whether eosinophils cause asthma or features of the disease requires detailed long-term studies with specific anti-eosinophilic therapies. Although, we await these studies with interest the preliminary observations suggest that eosinophilic airway inflammation may not be important in the development of the disordered airway physiology in asthma. This leads us to question whether there is an alternative or perhaps hitherto undescribed immunopathology which is critical for the development of airway hyperresponsiveness and variable airflow obstruction in asthma.

#### **1.1.3 Mast cells in asthma**

Since mast cells were first described in 1877 by Ehrlich (Ehrlich 1878), scientists have struggled with identifying a role for these cells. In the past suggestions have included storage cells, detoxification and ion exchange, but following the discovery of IgE in the 1960s (Porter 1991) the mast cell's function

has thought to be centred on their pathologic role in allergic disease. Although, this dogma is still strong there is emerging a potentially wider role of the mast cell as both an effector and immunoregulatory cell.

Mast cells originate from progenitor cells in the bone marrow, circulate as undifferentiated mononuclear cells in the peripheral circulation and subsequently, following migration into tissue mature under the influence of locally derived growth factors and cytokines including stem cell factor (Bradding & Holgate 1996). Mast cells are widely distributed throughout the body in both connective tissue and at mucosal surfaces. Pulmonary mast cells are located in the airway lumen, bronchial epithelium, submucosa and lung parenchyma. The predominant mast cell subtype in the lung contains tryptase (MC<sub>T</sub>), although tryptase and chymase mast cells are also present (MC<sub>TC</sub>) (Irani et al. 1986). Mast cell activation occurs when IgE bound to the high affinity IgE Fc receptor (FcεRI) on mast cells is cross-linked by allergen triggering mast cell degranulation (Wedemeyer & Galli 2000). This results in the release of preformed granule-derived mediators, the synthesis and release of newly formed lipid products and the transcription of numerous cytokines. In addition to this conventional IgE-dependent mast cell activation recent studies indicate that common allergens, many of which are serine proteases, can induce mast cell histamine and cytokine secretion directly through an IgE-independent mechanism (Dudler T et al. 1995; Machado DC et al. 1996).

In asthma mast cells are present in an “activated” secretory state within the asthmatic bronchial mucosa with on-going mediator release, cytokine synthesis, and widespread mast cell degranulation evident by electron microscopy (Beasley et al. 1989; Broide et al 1991; Ying et al. 1997). These activated mast cells have the capacity to contribute to the development and maintenance of the airway inflammation and associated disordered airway physiology, which characterises asthma.

A number of studies, reviewed in (Wedemeyer & Galli 2000), have described the role of the mast cell in atopic asthma and described the contribution of mast cell products in the early and late asthmatic reactions following allergen challenge. It is established that the early asthmatic reaction is most likely a result

of the release of preformed mediators from mast cells including histamine, the autocoid mediators PGD<sub>2</sub> and LTC<sub>4</sub> which can induce bronchoconstriction, mucus secretion and mucosal oedema. This is supported by three lines of evidence. Firstly, the kinetics of IgE-dependent mediator release *in vivo* parallels that of purified mast cells *in vitro* (Liu et al. 1990). Secondly, the presence of mast cell activation during the early asthmatic reaction is confirmed by the rapid increase in concentrations of the preformed mast cell-specific protease, tryptase, recovered by bronchoalveolar (BAL) within minutes following local bronchial allergen challenge (Sedgwick et al. 1991; Wenzel, Fowler, & Schwartz 1988). Thirdly, both sodium cromoglycate and  $\beta$ -agonists such as salbutamol, known inhibitors of mast cell degranulation *in vitro* (Church & Hiroi 1987), completely abolish the early reaction and the associated increase in plasma histamine levels (Howarth et al. 1985; Pepys et al. 1968). In contrast to the early asthmatic reaction there is little direct evidence to suggest that mast cells play a direct role in the late asthmatic reaction, but the mast cell secretes many cytokines and proteases which have the potential to orchestrate the late asthmatic reaction and the development of chronic airway inflammation. The proinflammatory cytokines synthesised and secreted by mast cells includes IL-4, IL-5, and IL-13, which regulate both IgE synthesis, Th2 lymphocyte differentiation and the development of eosinophilic inflammation (Bradding P. 1996; Bradding & Holgate 1996). In addition, the mast cell produces several cytokines and neutral proteases including TGF $\beta$ , basic FGF, tryptase and chymase, which may contribute to airway wall remodelling by activating myofibroblasts (Bradding & Holgate 1996). Recently a number of reports have identified a key role for prostaglandin (PG)D<sub>2</sub> as an important chemokine for eosinophils acting on the DP<sub>2</sub> receptor and for T helper type 2 (Th2) cells and eosinophils via the chemoattractant receptor-homologous molecule for Th2 cells (CRTH2) receptor (Hirai et al. 2001), (Fujitani et al. 2002), (Monneret et al. 2001).

The importance of the mast cell in asthma has been questioned by the relatively poor clinical benefit of “mast cell stabilising” drugs such as sodium cromoglycate, nedocromil sodium and the  $\beta_2$ -adrenoceptor agonists. However, both cromoglycate and nedocromil sodium have weak effects on mediator release from lung mast cells and exhibit tachyphylaxis (Okayama et al. 1992) and

chronic administration of  $\beta_2$ -adrenoceptor agonists, in contrast to attenuating mast cell secretion, can lead to enhanced release of mediators (Cho, Hartleroad, & Oh 2001; Swystun et al. 2000). The importance of the mast cell in asthma has been reconfirmed by the development of novel specific monoclonal anti-IgE antibody therapy, which has been shown to suppress the allergen-induced late-phase reaction, to enable patients to decrease their corticosteroid usage (Milgrom et al. 1999) and to reduce exacerbations (Busse et al. 2001).

There is increasing evidence that in asthma the mast cell as well as contributing to airway inflammation plays a role in the development of disordered airway physiology. Strong correlations have been observed between the severity of airway hyperresponsiveness and mast cell numbers, histamine concentrations, and spontaneous histamine release in BAL (Casale TB, Wood D, & Richerson HB 1987; Casolaro V, Galeone D, & Giacummo A 1989; Wardlaw et al 1988). In addition Ammit et al reported that *ex-vivo* bronchial rings that demonstrated contractile responses to allergen, contained more mast cells within the smooth muscle than those that were unresponsive (Ammit et al. 1997) suggesting that microlocalisation of mast cells within airway smooth muscle may be related to the development of variable airflow obstruction and airway hyperresponsiveness. Whether mast cell infiltration into the airway smooth muscle is important in asthma needs to be clarified.

Thus, in addition to its widely accepted role in the early asthmatic reaction evidence is emerging which suggests that the mast cell may be important in the development and maintenance of chronic airway inflammation and disordered airway physiology in asthma. Specific therapies targeted at mediators released by mast cells will be informative on their relative contribution to the pathophysiology of asthma but perhaps a greater understanding of the microlocalisation of mast cells within the airway may provide novel insights into the immunopathology of asthma.

#### 1.2.4 T-lymphocytes in asthma

The lung is an important route of sensitization for airborne antigens. Consistent with this function, there is an extensive network of hilar and mediastinal lymph

nodes that are anatomically and functionally connected to the lung. In addition, the normal adult human lung contains large numbers of lymphocytes. These are comprised largely of memory  $\alpha^{\beta}$ TCR T cells, which are found principally in two compartments, the bronchial lamina propria, where they form the major leukocyte subtype, and the peripheral alveolar and interstitial regions of the lung.

T cells recirculate from the blood to tissue and back to the blood. Naive T cells exclusively traffic through secondary lymphoid tissue such as lymph nodes and bronchus-associated lymphoid tissue via specialized postcapillary venules called high endothelial venules (HEV). In contrast, memory T cells, in addition to migrating into lymph nodes, can also transmigrate through the nonspecialized postcapillary venules of the systemic circulation. Whereas naive T cells do not appear to show any regional preference in their migratory habits, memory T cells preferentially return, or "home," to regions or microenvironments of the body similar to those where antigen was initially encountered (Butcher et al. 1999). Lymphocyte homing is controlled by adhesion molecules and chemoattractant signals expressed by HEVs and postcapillary venular endothelium in an organ-specific manner. Interactions between homing T cells and the endothelium follow the now well-established multistep paradigm of leukocyte adhesion to endothelium, which creates the considerable combinatorial diversity necessary to direct the different patterns of leukocyte emigration that characterize various inflammatory responses (Butcher 1991). This involves an initial capture or tethering step mediated by selectins and their ligands as well as the  $\alpha_4$  integrins  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ , an activation step mediated by chemoattractants, which for T cells appear to be largely chemokines, a firm arrest step mediated principally by  $\beta_2$  integrins, and a transmigration step, which for T cells again is likely to be controlled principally by chemokines. Chemokines bind to one or more chemokine receptors (Murphy 1994) expressed by the lymphocytes. The exact molecular signals that control T cell trafficking to the lung either during normal homeostasis or in disease are unclear.

The concept of asthma as a disease associated with activation of T-lymphocytes, principally T-helper type 2 (Th-2) cells, was proposed in the early 1990's with the observation that human as well as mouse memory T cell clones could be committed towards cells releasing IL-4 and IL-5 (Th2) or IFN $\gamma$  and IL-2

(Th1) (Romagnani S 1997). IL-4 which promotes IgE synthesis and eosinophil recruitment (Vercelli D et al. 1988) (Wardlaw 1999) and IL-5 the major eosinophil cytokine (Wardlaw et al. 2000), are of obvious relevance to asthma. A number of observations support this hypothesis. In patients with asthma, infiltration of the bronchial submucosa by activated lymphocytes was increased (Azzawi et al 1990). By using in situ hybridization, up to 50% of BAL T cells from asthmatics, but very few from normal subjects, expressed mRNA for IL-4 with no difference between asthmatics and non-asthmatics in mRNA expression for IFN $\gamma$  (Robinson et al. 1992). Recently, high concentrations of mRNA for GATA-3, a transcription factor confined to Th2 cells, were observed in bronchial biopsies from subjects with asthma. The importance of the T-cell in asthma is further supported by the efficacy of a monoclonal anti-CD4 (Kon et al. 1998) and cyclosporin A (Khan et al 2000) in the treatment of chronic corticosteroid-dependent asthma.

The molecular signals directing selective lymphocyte homing in asthma have in part been investigated in two reports. We have reported the characterization of the chemokine receptors expressed by lung derived and BAL T cells (Campbell et al. 2001). There were no differences in chemokine expression or T-cell activation markers between the healthy individuals or those with asthma. In particular, we saw no bias in subjects with asthma toward the putative Th2-linked chemokine receptors CCR3 and CCR4. The only receptors expressed to a high level on BAL T cells, and which therefore might be candidates for an adhesion-triggering receptor, were CCR5 and CXCR3. In contrast in another report following allergen challenge CCR4 was expressed on virtually all T cells in bronchial biopsies of subjects with asthma (Panina-Bordignon et al. 2001) supporting the view that Th-2 specific chemokine receptors play an important role in asthma.

Although the concept of asthma as a Th2 disease is now widely supported there is surprisingly little data showing that T cells in asthma are producing increased amounts of Th2 cytokines. Indeed Krug *et al* (Krug et al. 1996), using intracellular flow cytometry found an increase in IFN $\gamma$  but not IL-5 or IL-4 expression by BAL T cells from asthmatics after stimulation with PMA or ionomycin. They observed that only a mean of about 2% of cells expressed IL-4

even after stimulation, raising the possibility that the increase in mRNA expression seen by in situ hybridization was not being translated into protein. Similarly, in a study using flow cytometry to investigate cytokine expression in peripheral blood an increase in IL-4 expression was related to atopy, but IFN $\gamma$  production, mainly in the CD8 population of T cells, was more closely allied to the asthma phenotype (Magnan et al. 2000). Although Till *et al* (Till SJ et al. 1998) observed increased production of IL-5 by BAL T cells from asthmatics this was after allergen challenge and IL-4 production was not measured. In addition there was almost as much IFN $\gamma$  produced as IL-5 providing limited evidence for a Th2 bias. Increased amounts of IL-4 and IL-5 expression have been found by immunohistochemistry in asthma, but this is mainly present in mast cells and eosinophils (Bradding et al. 1994) (Moller et al. 1996) (Nonaka M et al. 1995).

The relative importance of Th2 and Th1 cytokines in driving the inflammatory process in asthma is beginning to be established by the emergence of specific monoclonal antibody therapies to individual cytokines. Anti-interleukin (IL)-5 and recombinant IL-12 both markedly decrease the peripheral blood and sputum eosinophilia after allergen challenge (Leckie M et al 2000) (Bryan SA et al. 2000), suggesting that both IL-5 and the Th1 versus Th2 balance are important in the development of eosinophilic airway inflammation. However neither therapy influenced airway hyperresponsiveness or airflow obstruction questioning the importance of these cytokines in the development of the asthma phenotype.

Therefore, the current evidence does support the hypothesis that airway inflammation in asthma can be driven by Th2 type cytokines. However, the relationship between airway inflammation and disordered airway physiology in asthma is unclear. Thus, to describe asthma as a Th2 disease is oversimplistic. The lack of a relationship between airway inflammation and disordered airway physiology is exemplified by the condition eosinophilic bronchitis, which is the focus of the next section in this introduction.

## **1.2 Eosinophilic bronchitis without asthma**

### **1.2.1 Sputum as a measure of airway inflammation**

Clinicians have been interested in the macroscopic and microscopic appearance of sputum in asthma since the end of the 19<sup>th</sup> century when Curshman spirals and Charcot-Leyden crystals were first described as being associated with a sputum eosinophilia (Pavord et al 1997). In the 1950s Morrow-Brown demonstrated that asthmatic patients with a sputum eosinophilia, identified on a sputum smear, had a good response to corticosteroids (Morrow Brown 1958). In the 1970s in addition to the identification of inflammatory cells several sputum mediators began to be assessed including histamine and slow reacting substance of anaphylaxis (Turnbull LS et al. 1977). Since then, the array of proteins, proteases, inflammatory mediators and cytokines that have been measured in sputum have rapidly increased.

Early analysis of sputum was from spontaneous samples. This had three major shortcomings: difficulty in obtaining samples from some subjects, high squamous cell contamination and poor repeatability due to inadequate cell dispersal. The introduction of sputum induction where sputum production is facilitated by the use of ultrasonically nebulised hypertonic saline has enabled sputum to be obtained successfully in about 80% of attempts (Pavord et al 1997). Manoeuvres such as washing out the mouth and blowing the nose after each nebulisation and selection of sputum plugs has reduced squamous cell contamination. The use of a mucolytic dithiothreitol (DTT), which produces mucolysis by opening disulphide bonds which cross-link glycoprotein fibres has improved cell dispersion during the sputum processing (Wooten & Dulfano 1978).

The development of this safe, non-invasive method to assess airway inflammation has increased our understanding of the relationship between airway inflammation and disease (Hunter et al. 1999; Pavord et al 1997; Pizzichini et al. 1996a; Pizzichini et al 1997b). One of the most interesting observations was the identification of a group of patients with a sputum eosinophilia identical to that seen in asthma, but none of the functional abnormalities associated with asthma (Gibson et al 1989b; Gibson et al. 1995). Patients with this condition, known as

eosinophilic bronchitis, typically present in middle age with a dry or minimally productive cough. Wheeze and dyspnoea are not prominent and tests of variable airflow obstruction and airway responsiveness are normal. Patients are usually non-smokers and atopy is no more common than in the general population. Like asthma occupational exposure can occasionally cause eosinophilic bronchitis (Lemiere, Efthimiadis, & Hargreave 1997). Despite being recognised as a cause of chronic cough for ten years eosinophilic bronchitis remains largely unreported. This is because the diagnosis depends on measurement of airway inflammation, which is not routinely undertaken in most clinics. We suspect that the condition is often inappropriately labelled as asthma or given the unsatisfactory label of corticosteroid responsive cough after a positive response to a trial of steroid treatment. In contrast to asthma little is known about the incidence, cause, immunopathology, natural history or response to treatment of eosinophilic bronchitis.

### **1.2.2 Eosinophilic bronchitis: a common and treatable cause of chronic cough?**

Chronic cough is one of the commonest causes of presentation to general practice. At any one time 20% of the UK population have a troublesome cough and sufferers consume 75 million doses of over the counter anti-tussive medication annually (Fuller & Jackson 1990). Most cases are acute and self-limiting although a significant minority are referred for a specialist opinion with an isolated persistent chronic cough. This is traditionally defined as a cough lasting for more than 3 weeks with no overt clinical and radiological evidence of lung disease. In many cases a treatable cause can be identified using a simple 'anatomical diagnostic' protocol originally proposed by Irwin *et al* 20 years ago (Irwin, Corrao, & Pratter 1981). Rhinitis with post-nasal drip, cough variant asthma and gastro-oesophageal reflux are the most important causes of isolated chronic cough and several large series have shown that one or more of these conditions may be responsible for as many as 90% of cases where a cause is identified (Irwin, Corrao, & Pratter 1981; Irwin, Curley, & French 1990; McGarvey *et al.* 1998).

None of these series included an assessment of airway inflammation, so eosinophilic bronchitis might have been missed, particularly as a negative

methacholine inhalation test has been said to obviate the need for a corticosteroid trial (McGarvey et al 1998). A small study by Carney *et al* using sputum induction in the assessment of cough have identified eosinophilic bronchitis as the primary cause of chronic cough in three patients out of a series of 30 (Carney et al. 1997).

One uncontrolled study has shown that patients with eosinophilic bronchitis have an improvement in their symptoms and a significant fall in their sputum eosinophil count following inhaled corticosteroids (Gibson et al 1995).

### **1.2.3 What does eosinophilic bronchitis tell us about asthma?**

Most asthmatics have a combination of eosinophilic airway inflammation, airway hyperresponsiveness and variable airflow obstruction to the extent that many definitions of asthma now include these three features (this is described in more detail in 1.1.1 and 1.1.2). How these features are related remains controversial. Recent studies have shown generally a weak correlation in patients between various measures of airway dysfunction and airway inflammation particularly when large and heterogeneous populations are studied (Brusasco, Crimi, & Pellegrino 1998). This contrasts to the situation within patients where treatment or allergen associated changes in eosinophilic airway inflammation are associated with a parallel and proportionate change in airway responsiveness (Pin et al. 1992b).

The complex relationship between eosinophilic airway inflammation and airway responsiveness may be because features are independently regulated, or because there are functionally important differences in site, nature or intensity of eosinophilic airway inflammation between patients. Patients with eosinophilic bronchitis represent an extreme example by having inflammation with no hyperresponsiveness and study of this group may particularly inform our understanding of the relationship between airway inflammation and airway responsiveness.

#### 1.2.4 Immunopathology of eosinophilic bronchitis

The particular reasons why patients with eosinophilic bronchitis, who often have a dramatic sputum eosinophilia, do not have airway hyperresponsiveness or variable airflow obstruction are unclear. One possibility is that eosinophilic airway inflammation does increase airway responsiveness in eosinophilic bronchitis, but within the normal range. A case report of a patient with eosinophilic bronchitis, who developed worsening symptoms and airway hyperresponsiveness during an exacerbation of eosinophilic airway inflammation, supports this view (Wong et al. 1996).

Upper airway symptoms are common in patients with eosinophilic bronchitis (Gibson et al 1995) and it has been speculated that inflammation maybe confined to the upper airway. However, Gibson *et al* (Gibson et al. 1998) have shown a similar degree of bronchoalveolar lavage eosinophilia and GM-CSF and IL-5 gene expression in patients with asthma and eosinophilic bronchitis. This observation suggests that the site of inflammation in eosinophilic bronchitis is mainly in the lower airway.

Another possibility is that there is a difference in the 'activation' of the inflammation in eosinophilic bronchitis and asthma. This has been assessed by measurement of cytokines (Berlyne et al. 1999) in induced sputum supernatant and nitric oxide in exhaled air (Berlyne et al. 2000), but no important differences were identified. In a small study sputum IL-5 concentration was not different between patients with asthma and eosinophilic bronchitis (Berlyne et al 1999). This suggests that the difference in airway function in eosinophilic bronchitis and asthma is not due to differences in cytokine production, but whether there are differences in the degree of activation of other important effector mediators is unknown.

Elevated exhaled nitric oxide concentration due to increased inducible nitric oxide synthetase (iNOS) expression and activity in the bronchial epithelium is a feature of untreated asthma (Kharitonov et al. 1994). Nitric oxide is raised in eosinophilic bronchitis to the same degree as seen in asthma (Berlyne et al 2000), suggesting that activation of the bronchial epithelium occurs to a similar degree in eosinophilic bronchitis and asthma.

It is important to further compare the nature and degree of the airway inflammation in eosinophilic bronchitis. A detailed comparative study using bronchoscopic techniques might reveal more subtle, but important differences.

#### 1.2.5 Aetiology and Natural history of eosinophilic bronchitis

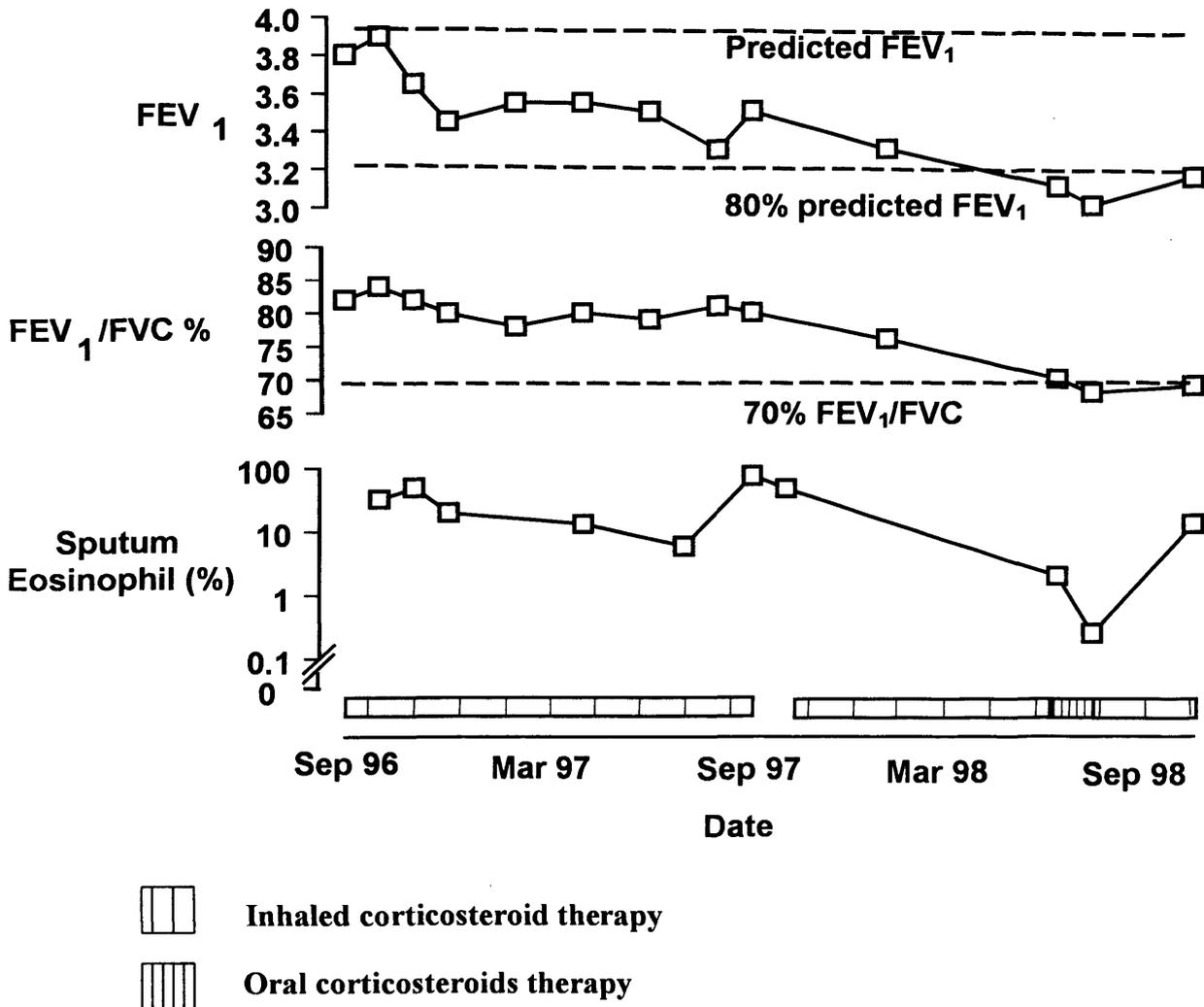
The aetiology of eosinophilic bronchitis is unknown. Like asthma subjects with eosinophilic bronchitis can be atopic or non-atopic, although those with non-atopic disease have only been investigated using a limited number of known allergens. Thus, it would seem likely that eosinophilic bronchitis in many instances has an allergic basis, but other possibilities include a hitherto unrecognised allergen, infections (perhaps viral) and autoimmune processes.

The natural history of eosinophilic bronchitis is unclear. In one cross-sectional study it has been observed that 30-40% of patients with COPD without a history of asthma and with no bronchodilator reversibility have sputum evidence of an airway eosinophilia (Pizzichini et al 1998). Over 12 months Turnbull *et al* (Turnbull et al 1977) analysed sputum samples every 6-weeks and showed that all of 23 subjects with chronic bronchitis had a sputum eosinophilia on at least one occasion (the airway inflammation in COPD and its response to treatment is expanded upon in 1.3). One possible explanation for the presence of eosinophilic airway inflammation in COPD without apparent pre-existing asthma could be that a number of patients with eosinophilic bronchitis develop COPD. If this is true it has important implications in the early diagnosis and successful treatment of eosinophilic bronchitis.

In support of this hypothesis we have recently described (Brightling et al. 1999a) a patient with eosinophilic bronchitis who over a two-year period developed fixed airflow obstruction. The patient, a 48 year old who had never smoked, presented with an isolated chronic cough. He had normal spirometric values, peak flow variability and airway responsiveness at the time of diagnosis. His induced sputum eosinophil count was 33% and a diagnosis of eosinophilic bronchitis was made. The cough improved with inhaled corticosteroids, but the sputum eosinophilia persisted. Over two-years he developed airflow obstruction, which did not improve following nebulised bronchodilators and a two-week course of prednisolone 30mg once daily sufficient to return the sputum

eosinophilia to normal (0.5%). Figure 1.1 illustrates the change in spirometric values over a twenty-five month period. The progressive irreversible airflow obstruction may have been due to remodelling of the airway secondary to the persistent eosinophilic airway inflammation in spite of corticosteroid therapy. There is an urgent need to define the natural history of eosinophilic bronchitis, as this condition may be a hitherto unknown and potentially treatable cause of COPD.

**Figure 1.1** Change in lung function and sputum eosinophilia over two-years with the development of fixed airflow obstruction in a patient with eosinophilic bronchitis.



### **1.3 Airway inflammation in chronic obstructive pulmonary disease (COPD).**

#### **1.3.1 Airway inflammation in COPD**

There are several difficulties facing researchers interested in measuring lower airway inflammation in COPD. Firstly the functionally important inflammatory response in the small airways and surrounding lung parenchyma is in the lung periphery and is therefore inaccessible. Secondly the various techniques used to assess airway inflammation differ markedly in their properties and in the profile of inflammatory cells they measure suggesting they are accessing different lung compartments (Keatings et al. 1997a).

Despite these problems, a number of inflammatory cells have been shown consistently to be present in increased numbers in the airways in COPD and to relate to the severity of airflow obstruction suggesting a causal role. The evidence is perhaps strongest for the CD8 positive T lymphocyte and the neutrophil. In a pathological study of the peripheral airways of smokers with and without COPD undergoing lung resection, Saetta *et al* (Saetta et al. 1999) found an increased proportion of CD8 positive T-lymphocytes in the smokers with COPD and a significant negative correlation between the cell count and the FEV<sub>1</sub> % predicted. O'Shaughnessy *et al* (O'Shaughnessy et al. 1997) made similar observations in central airway biopsies. The mechanism of CD8 positive lymphocyte recruitment and its functional significance remains to be determined.

Increased neutrophil numbers are particularly obvious in patients with established airflow obstruction. Bronchial biopsy and induced sputum studies have consistently shown a correlation between the severity of airflow obstruction and neutrophil counts and in some studies the correlation has been close (Di Stefano et al. 1998) (Keatings et al. 1996) (Stanescu et al. 1996). Interestingly an airway neutrophilia is also a feature of severe asthma with a degree of fixed airflow obstruction (Wenzel et al 1997). A pathogenic role for the neutrophil in COPD is supported by the findings of Stanescu *et al* (Stanescu et al 1996) who showed that the induced sputum neutrophil differential count was higher in patients who had a more rapid decline in FEV<sub>1</sub> over a 15 year follow up period. Furthermore the protease-antiprotease hypothesis (Stockley 1995) offers a

biologically plausible mechanism for the tissue destruction seen in COPD in association with neutrophilic airway inflammation.

Less attention has been paid to the presence of eosinophilic airway inflammation in stable COPD although there is bronchial biopsy and sputum evidence of prominent eosinophilic airway inflammation in patients studied during exacerbations of COPD (Saetta et al 1994). A sputum eosinophilia has been observed in 20-40% of patients with COPD (Saetta et al 1994) (Confalonieri et al. 1998) (Pizzichini et al 1998) (Turnbull et al 1977). One bronchial biopsy study has reported an increased number of eosinophils in patients with chronic bronchitis and COPD but lower bronchoalveolar lavage (BAL) concentrations of eosinophilic cationic protein (ECP) than in asthmatics suggesting that eosinophils are present but are less activated in COPD (Lacoste et al. 1993). Turnbull *et al* (Turnbull et al 1977) found that other mediators histamine and slow reacting substance of anaphylaxis were found in concentrations in the sputum in subjects with chronic bronchitis similar to asthma and that these significantly correlated with peak flow variation over a year (Turnbull et al 1978).

The origin of eosinophilic airway inflammation in COPD is unclear although it is widely assumed that it indicates an asthmatic component to the fixed airways obstruction (Barnes 1998). This is unlikely to be the case as most studies on patients with COPD rigorously exclude subjects with variable airflow obstruction and those with clinical features suggesting asthma. It is more likely that smoking and other mechanisms that recruit neutrophils into the airway mucosa in COPD may in turn cause a minor degree of eosinophil influx. However, it is difficult to explain the very high levels of sputum eosinophilia observed in some of our subjects. An alternative and intriguing possibility is that eosinophilic COPD starts as eosinophilic bronchitis. This is a common cause of chronic cough in middle age characterised by a sputum eosinophilia but no symptoms and functional evidence of variable airflow obstruction or airway hyperresponsiveness (Gibson et al 1989b). Although characterised by normal spirometric values at the time of diagnosis this has been associated with an accelerated decline in FEV<sub>1</sub> and the development of COPD (Brightling et al 1999a). This hypothesis that eosinophilic COPD originates in middle age as eosinophilic bronchitis differs importantly from the 'Dutch hypothesis' which

suggests that atopy and airway hyperresponsiveness are risk factors for the development of COPD (Orie, Sluiter, & De Vries K 1961).

### 1.3.2 The effect of treatment on airway inflammation

Intervention studies examining the effects of treatment on airway inflammation in COPD have generally used induced sputum to assess airway inflammation and inhaled or oral corticosteroids as the putative anti-inflammatory agent. Most studies have particularly focused on cell and molecular markers of neutrophilic airway inflammation. One placebo controlled study has investigated the effect of inhaled beclomethasone on bronchoscopic markers of airway inflammation with largely negative findings, although there was some evidence of a treatment related decrease in an airway inflammation score and in the BAL concentration of various molecular markers of microvascular leak (Thompson et al 1992).

Of the three studies examining the effect of inhaled corticosteroids on cellular markers of airway inflammation in induced sputum in COPD two found no evidence of an effect of short-term inhaled treatment on the sputum neutrophil count or on sputum supernatant concentrations of markers of neutrophilic inflammation (Keatings et al. 1997b) (Culpitt et al 1999). However one unblinded small study showed that treatment with beclomethasone dipropionate was associated with significant 21.3% fall in the differential neutrophil count (Confalonieri et al 1998). This study examined the effect of two months treatment so the differences between studies may be related to the duration of treatment. Larger, placebo controlled double blind studies examining the effects of longer-term treatment on airway inflammation are required to fully address this issue.

One small single blinded study has shown that following treatment with a short-course of prednisolone there was no evidence of a treatment-associated change in the sputum neutrophil count or in the sputum supernatant concentration of myeloperoxidase or elastase (Pizzichini et al 1998). They observed that oral corticosteroid treatment is associated with a significant fall in the sputum eosinophil count and in the sputum supernatant concentration ECP. Furthermore the improvement in FEV<sub>1</sub> and quality of life scores with treatment was significantly greater in those with a significant sputum eosinophilia (>3%)

(Pizzichini et al 1998). This beneficial effects of oral corticosteroid treatment needs to be confirmed in a double blind placebo controlled trial and the relationship between the degree of eosinophilic airway inflammation and magnitude of response needs to be assessed.

In conclusion, although there are difficulties sampling the functionally important site of airway inflammation in COPD, the available techniques have identified some consistent abnormalities that relate to the degree of functional impairment and the rate of progression of this impairment. The evidence is strongest for neutrophilic airway inflammation assessed using induced sputum and this is therefore the most promising target for potential anti-inflammatory agents. Studies examining the effects of corticosteroids on airway inflammation in COPD have shown no consistent effect on the sputum neutrophil count. In contrast oral corticosteroids appear to effectively suppress sputum markers of eosinophilic airway inflammation. Whether the beneficial effects of treatment are related to the degree of eosinophilic airway inflammation needs to be further clarified.

## 1.4 Hypothesis

I hypothesise that eosinophilic bronchitis is a common cause of chronic cough and that both the cough and airway eosinophilia will respond to corticosteroids. Similarly, I postulate that the presence of a sputum eosinophilia will predict a good response to corticosteroids in COPD.

I hypothesise that there is a fundamental difference between the immunopathology of asthma and eosinophilic bronchitis and that this difference is responsible for the different clinical phenotype observed in asthma of variable airflow obstruction and airway hyperresponsiveness, which are absent in eosinophilic bronchitis. I believe the difference may be related to the degree of activation or anatomical site of the inflammatory response or possibly due to differences in the orchestration of inflammatory cell recruitment.

## Aims

### 1) Clinical and immunopathological characterisation of eosinophilic bronchitis.

#### *Airway eosinophilia: an immunopathological feature of disease.*

- To describe the incidence of eosinophilic bronchitis as a cause of chronic cough amongst patients referred to a specialist clinic.
- To assess the contribution of upper airway inflammation to the sputum eosinophilia observed in eosinophilic bronchitis.
- To assess the degree of activation of the inflammatory response in eosinophilic bronchitis in terms of the presence of important effector mediators in airway secretions.
- To compare the proportion of inflammatory cells in bronchoalveolar lavage in eosinophilic bronchitis and asthma.
- To compare the cell counts in the epithelium, submucosa and smooth muscle and the basement membrane thickness in eosinophilic bronchitis and asthma.
- To evaluate the balance between Th1/Th2 cytokines in BAL T-cells and bronchial biopsies.
- To characterise the T-cell phenotype in eosinophilic bronchitis with respect to CD4/CD8 ratio and chemokine receptor expression.

### 2) *Airway eosinophilia a marker of response to corticosteroids.*

- To assess the response to corticosteroids in:
  - Eosinophilic bronchitis
  - COPD

## 2. **Methods**

### 2.1 **Clinical**

#### 2.1.1 **Peripheral blood tests and skin tests**

Venous blood samples were taken to measure peripheral blood eosinophil count, total IgE. Atopy was assessed either by radioallergosorbent tests to *Dermatophagoides pteronyssinus*, cat fur and grass pollen or allergen skin prick tests to *Dermatophagoides pteronyssinus*, cat fur, grass pollen and *Aspergillus fumigatus* solutions with normal saline and histamine controls (Bencard, UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control.

#### 2.1.2 **Spirometry**

Spirometry was performed with a Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Bronchodilator reversibility was assessed 15 minutes after administration of 2.5mg salbutamol nebulised via a Flaem Nuova Type II nebuliser (Deva Medical, Runcorn, Cheshire) with a median particle size of 2 µm and the patient breathing tidally or 200µg salbutamol inhaled via a volumatic. FEV<sub>1</sub> was recorded as the better of two successive readings within 100 mL.

#### 2.1.3 **Airway responsiveness**

Using the standard tidal breathing method the concentration of methacholine causing a 20% fall in FEV<sub>1</sub> was recorded as the PC<sub>20</sub>FEV<sub>1</sub> (Juniper, Cockcroft, & Hargreave 1994). In brief, following the measurement of the baseline FEV<sub>1</sub> subjects inhaled saline followed by doubling concentrations of methacholine (or

histamine) 0.03-16 mg/ml via a Wright's nebulizer (flow 0.13ml/min driven by dry compressed air) (gift from Fisons, Leicestershire, UK). Additional concentrations of methacholine were used in some studies of 32, 64, 128 mg/ml. The subject was instructed to breathe quietly (tidal breathing) for 2 min with a noseclip. The FEV<sub>1</sub> is measured 30 and 90 s after the nebulization is completed. If the FEV<sub>1</sub> falls less than 20% the procedure was repeated with the next highest concentration. If the FEV<sub>1</sub> fell more than 20% from baseline (or the highest concentration has been given), no further methacholine was given. Methacholine (histamine) PC<sub>20</sub>FEV<sub>1</sub> concentration was calculated by linear interpolation of log dose response curve.

#### 2.1.4 Sputum induction

##### **Instructions for Patients**

Prior to commencing the induction the procedure was fully explained to the patient including:

- Instruction on spitting out saliva generated during inhalation of saline into a "discard" vessel.
- Instruction about blowing their nose and rinsing their mouth and swallowing the water prior to trying to expectorate sputum. (It is important that the subject moves quickly through this procedure to prevent loss of sputum due to swallowing).
- Instruction on how to expectorate effectively. It is necessary to explain and demonstrate the technique for coughing up sputum and moving sputum from the back of the throat, forward to the specimen container.
- A reminder not to swallow the sputum as it comes up the bronchial tree.
- Guidance on posture: sitting straight upright during nebulisation, and leaning forward during expectoration.

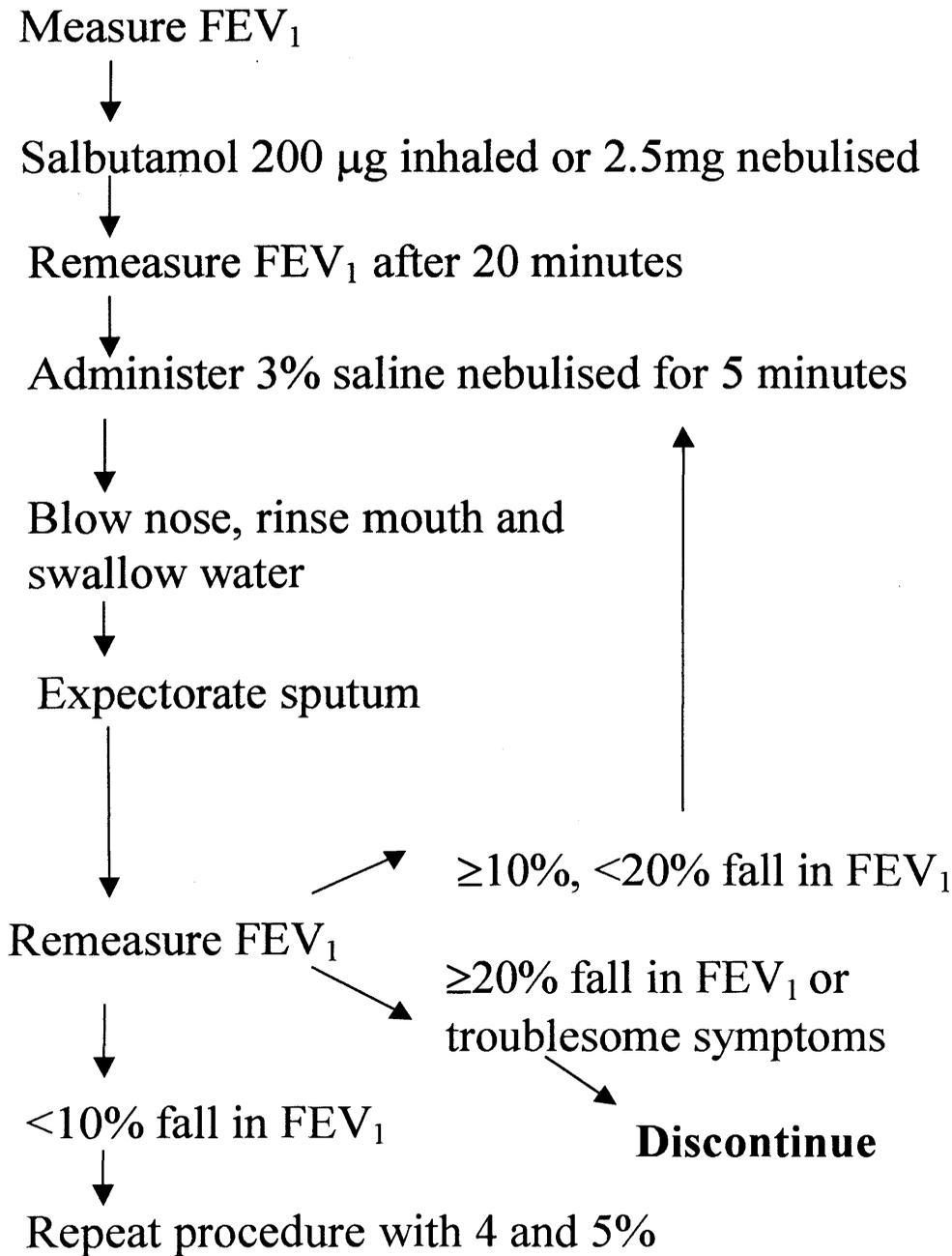
## **Protocol**

Subjects were pretreated with either inhaled salbutamol 200 µg or nebulised salbutamol 2.5mg 10-30 minutes before sputum induction to minimise bronchoconstriction. Sputum was induced using 3, 4 and 5% saline inhaled in sequence for five minutes via an ultrasonic nebuliser (Medix, Harlow, UK; output 0.9 ml/min; mass median diameter 5.5 µm). Tidal breathing was employed, taking a slightly deeper breath every minute. After each inhalation patients blew their noses and rinsed their mouths to minimise nasal contamination and expectorated sputum into a sterile pot. FEV<sub>1</sub> was measured after each inhalation. If the FEV<sub>1</sub> fell by more than 10% but less than 20%, the same concentration of saline is administered. If the FEV<sub>1</sub> fell by more than 20% of the best post-bronchodilator value, or if significant symptoms occurred, the nebulisation was stopped and the patients were treated with repeat short-acting β-agonist. (Figure 2.1)

## **Safety Procedures During the Induction**

Inhaled hypertonic saline is a bronchoconstrictor stimulus so sputum induction using ultrasonically nebulised hypertonic saline should be carried out with care. The usual laboratory resuscitation apparatus plus nebulised salbutamol was readily available. A doctor either performed the procedure or was nearby during each procedure.

**Figure 2.1 Sputum induction protocol**



### 2.1.5 **Bronchoscopy**

Bronchoscopy was performed as a day case procedure in line with the British Thoracic Society guidelines (British Thoracic Society 2001). After obtaining written informed consent subjects were premedicated with nebulised 2.5mg salbutamol. Topical lignocaine gel is applied inside the nose. Sedation was offered to the subjects and was given in boluses of 2.5mg midazolam to a maximum of 5mg. Supplemental oxygen was supplied and pulse oximetry monitored throughout the procedure. 4ml of 4% lignocaine was administered via the bronchoscope to the vocal cords and 3 aliquots of 2ml of 2% was applied to the trachea, right and left main bronchi. A 20ml bronchial wash was performed in the right bronchus intermedius and an 180ml bronchoalveolar lavage (BAL) in 60ml aliquots was performed into the middle lobe. The fluid is recovered by gentle aspiration. Four-six bronchial biopsies were taken from the segmental and subsegmental carina from the right lung using a 20-cupped biopsy forceps.

### 2.1.6 **Nasal lavage**

Nasal lavage was obtained by instilling 5 mL of normal saline into each nostril, with the subjects head extended and their soft palate closed (Bucca et al. 1995a). After 10 s the patient flexed their neck and expelled the lavage into a sterile container.

### 2.1.7 **Capsaicin cough challenge**

Cough sensitivity was assessed using the capsaicin cough challenge (Choudry & Fuller 1992; O'Connell et al. 1994). Subjects inhaled at just below their functional residual capacity doubling concentrations of capsaicin (0.5-500  $\mu$ M) in a sequential order at 1-min intervals via a nebulizer attached to a breath-activated dosimeter delivering 8 mL. The number of coughs in response to each concentration was counted and recorded. The challenge was stopped when the concentration elicited 5 coughs or the highest dose of capsaicin was reached. The

concentration of capsaicin required to cause two coughs ( $C_2$ ) and five coughs ( $C_5$ ) were calculated by linear interpolation of log dose response curve. Cough paroxysms of more than five coughs were not possible to quantify so a censored value of 10 coughs was assigned.

#### **2.1.8 Chronic respiratory disease health status questionnaire**

Health status was assessed using the chronic respiratory disease (CRQ) questionnaire (Guyatt et al. 1987) consisting of 20 questions measuring four domains: dyspnoea, fatigue, emotions and mastery. The dyspnoea domain was examined by the subject specifying five frequent activities in which they experienced breathlessness and scoring the intensity of this symptom on a seven-point Likert scale. The same activities were used at each subsequent visit. The other domains were similarly assessed using a seven-point Likert scale.

#### **2.1.9 Incremental shuttle walk test**

The incremental shuttle walk test assesses a subject's functional capacity (Singh et al. 1992). All subjects had a practice walk, as the test was reproducible after a single practice walk. In brief, subjects walked around the 10m course at a speed indicated by a beep from a cassette player. After each minute the speed increased. The end point of the test is when the subject was unable to maintain the required speed. The total distance of the completed shuttles was recorded.

#### **2.1.10 Symptom visual analogue scores**

Symptom scores were recorded using a 100mm visual analogue scale fixed at both ends by no symptom to the worst symptom ever in a number of studies and the symptoms recorded included dyspnoea, cough, sputum production and wheeze.

## 2.2 Laboratory

### 2.2.1 Cells

#### 2.2.1.1 Peripheral blood mononuclear cell (PBMC) separation

Twenty milliliters of blood were taken from the subjects undergoing bronchoscopy and the mononuclear cell fraction was obtained by centrifugation 1300rpm for 30 mins at room temperature on Ficoll (Histopaque 1077). The mononuclear cells were then washed twice with phosphate buffered saline (PBS) and 0.5% bovine serum albumin (BSA) before immunostaining.

#### 2.2.1.2 Sputum, bronchial wash and bronchoalveolar lavage processing

##### Protocol sputum processing

Sputum free from salivary contamination was selected and weighed. To the selected sputum was added 4X volume/weight of 0.1% dithiothrietol (DTT) (Sigma, Poole Dorset). The sputum was dispersed by gentle aspiration into a Pasteur pipette, vortex for 15 s and 15 mins rocking on a bench spiromix. After the addition of an equal volume of Dulbecco's phosphate buffered saline (D-PBS) (Sigma, Poole, Dorset) the sputum suspension was filtered through 48 $\mu$ m nylon gauze and centrifuged 2000rpm (790g) for 10 mins. The sputum supernants was removed and stored at -80°C for future mediator assay. The cell pellet was resuspended in a small volume of PBS. An aliquot was removed and a total cell count, squamous cell contamination and viability were assessed using a Neubauer haemocytometer by the trypan blue exclusion method. The cell suspension was adjusted with PBS to 0.5-0.75  $\times 10^6$  cells/ml and cytopspins were prepared from 75 $\mu$ l aliquots at 450rpm (18.1g) for 6 mins using a Shandon III cytocentrifuge) (Shandon, UK). The cytopspins were stained in neat Romanowski stain for 5 mins and fixed in dilute stain for 25 mins. A differential cell count was obtained by counting >400 non-squamous cells on a Romanowski stained cytopspin (Figure 2.2).

**Romanowski stain preparation:**

Dissolve 1.5g Azure-B-thiocyanate in DMSO at 37°C and 0.5g Eosin in 300ml methanol at room temperature. Slowly add the Azure blue solution to the Eosin and store away from light.

Dilute Romanowski stain:

62 ml 10mM HEPES buffer pH 7.2

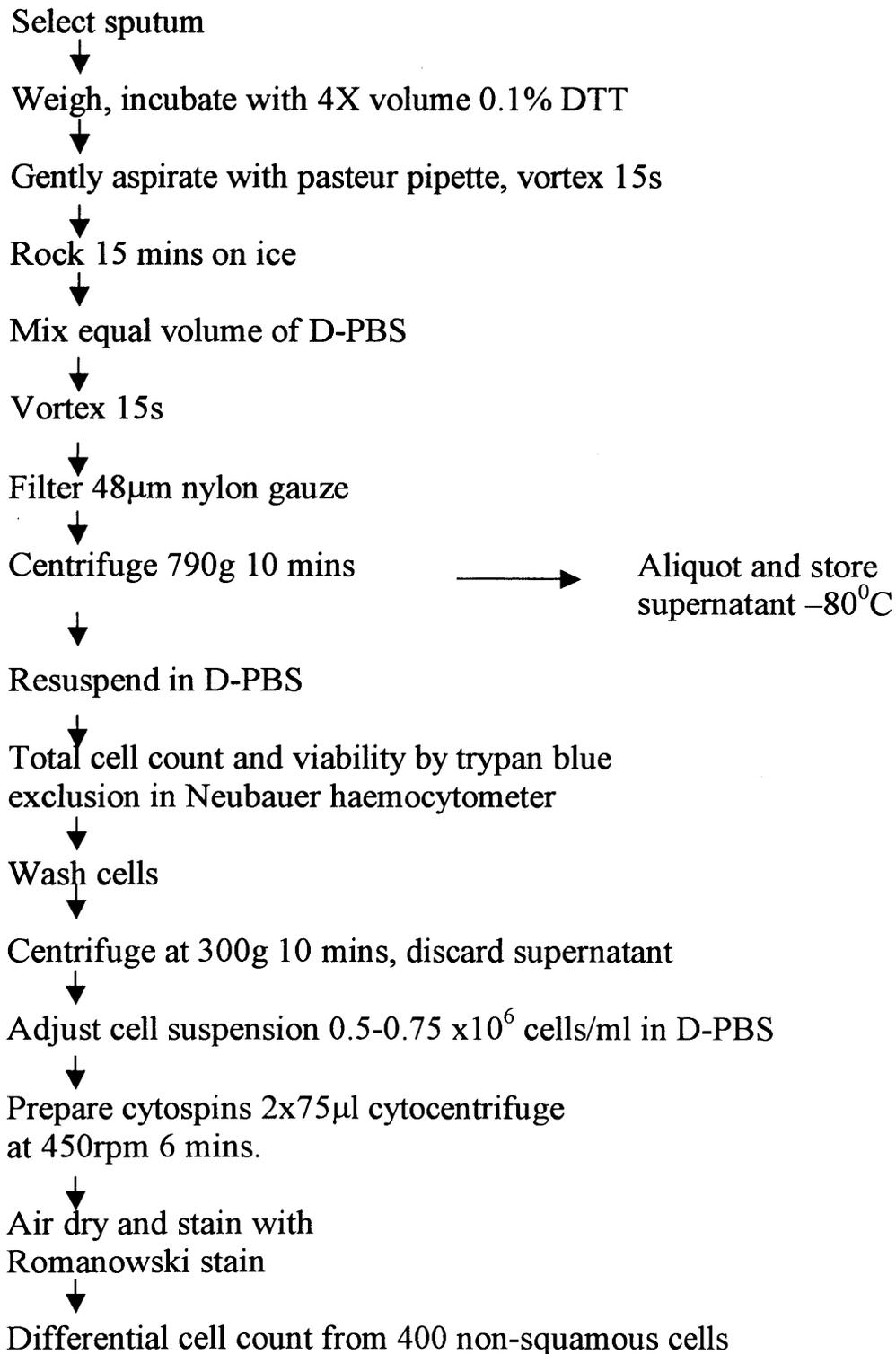
3.5 ml DMSO

4.6 ml Romanowski stain

**Protocol bronchial wash and BAL sample processing**

It was not necessary to disperse bronchial wash and BAL cells with DTT so they were filtered and centrifuged with the supernatant stored and the cell pellet resuspended to produce stained cytopins as per the sputum protocol. The remaining BAL cells were assessed by flow cytometry.

## Figure 2.2 Sputum processing protocol



### **2.2.1.3 Flow cytometry T-cell activation markers and chemokine receptors**

#### **Protocol**

Unlabelled first antibodies at appropriate concentrations (Table 2.1) were added to 100µl cells ( $1-5 \times 10^6$  cells/ml) per tube in PBS, 0.5% bovine serum albumin (BSA) and incubated on ice for 15-30min. The cells were washed with 1ml PBS, 0.5% BSA per tube and centrifuged at 1200rpm for 8min at 4°C. The supernatant was discarded and cells resuspended in 100µl of 5% FITC-labelled Rabbit anti-Mouse Ig antibody in buffer. Following incubation for 15-30min on ice the cells were washed, centrifuged and resuspended with 10% mouse serum in PBS, 0.5% BSA per tube and incubated for a further 15min on ice. Directly labelled anti-CD3-RPE (5µl) and anti-CD4-PerCP (10µl) was added to each tube for 30min on ice. Cells underwent a final wash and were resuspended in 300µl PBS, 0.5% BSA. Control tubes included an unlabelled isotypic control and cells labelled with a single colour FITC, RPE or Per-CP for every experiment.

**Table 2.1** Antibody source and concentration used for immunohistochemistry

Mab	Concentration	Source
MoIgG	1:10	(Dako)
CD18	1:10	(Dako)
CD8	1:20	(Pharmingen)
CCR3 (7D11) (1:10dil)	1:10	(Millenium)
CCR5	1:20	(Pharmingen)
CD103	1:10	(Dako)
CD25	1:10	(Dako)
CD49a	1:20	(Serotec)
HLA-DR	1:10	(Dako)
CXCR3	1:50	(R+D)
CCR6	1:50	(R+D)
MoIgG-RPE	1:50	(Dako)
CD3-RPE	1:20	(Dako)
CD4-PerCP	1:10	(Becton Dickenson)
Rabbit anti-Mouse Ig-FITC	1:10	(DAKO)

#### 2.2.1.4 Flow cytometry T-cell intracellular cytokines

##### Protocol

Blood and BAL samples prepared as above (methods 2.2.1.1 and 2.2.1.2) were resuspended at  $1 \times 10^6$  cells/ml in culture medium RPMI, 10% FCS, Hepes, Glutamax. The cell suspension was divided and half were stimulated with the remaining cells unstimulated or resting. Cells were stimulated with PMA (5ng/ml), calcium ionophore (250ng/ml) and Brefeldin A (10 $\mu$ g/ml) was used to prevent release of intracellular cytokines (final concentrations) and all cells were incubated in a tissue culture plate for 4hr at 37°C in a CO<sub>2</sub> incubator.

Following incubation harvested cells were washed with PBS, 0.5% BSA and centrifuged at 1200rpm for 10min at 4°C. The cell pellet was resuspended in 4% paraformaldehyde; PBS and the cells were fixed for 15 min on ice. After washing the cells a further 2x and centrifuging at 1400rpm the cells were stored overnight at 4°C in PBS, BSA.

The cells were resuspended in fresh staining buffer and incubated with directly labelled CD3-FITC/RPE and CD4-PerCP or isotopic controls for 15min on ice. After a further wash the cells were fixed and permeabilised in 4% paraformaldehyde, 0.1% saponin for 15 min on ice. The cells underwent two further washes with PBS, 0.5% BSA, 0.1% saponin. Following incubation of the cells with PBS, BSA, 10% mouse serum for 15 minutes to block non-specific binding direct-labelled IL-4-RPE and IFN- $\gamma$ -FITC or isotopic controls were added for 45min at 4°C. The cells were washed 2x with PBS, 0.5% BSA, 0.1% saponin and resuspended in a final volume of 300 $\mu$ l of PBS, 0.5%BSA ready for analysis by flow cytometry. Three-colour flow cytometry was performed using a FACScan and CellQuest software version 3.1 (BD Biosciences). Control experiments showed that DMSO, fixation or overnight storage did not alter expression of intracellular cytokines.

### **2.2.1.5 Cytoblocks**

#### **Protocol**

Cells were fixed for 15 minutes in 4% paraformaldehyde and processed into cytoblocks as per manufacturers instructions (Shandon, UK).

### **2.2.2 Supernatants from sputum**

#### **2.2.2.1 Elastase spectrofluorimetric assay**

Sputum elastase was measured by a spectrofluorimetric assay, in which methoxy-succinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-amino-methylcoumarin (Sigma, Poole, UK) was used as the substrate (Janoff, Raju, & Dearing 1983).

We added 50 µL of the substrate solution (0.1 mg/mL) with appropriate negative controls to 50 µL of elastase standards (Elastin Products, Owensville, MI, USA), samples, and quality-control samples. The plate was read every 5 min with a fluorimeter (Victor Wallac, Perkin Life Sciences, Cambridge, UK) that used an excitation wavelength of 340 nm and emission of 455 nm over 2 h at 37°C. We calculated the standard curve and unknown values from the maximum slope of fluorescence compared with time curves. The limit of detection for elastase was 1.5 µg/g of sputum, respectively, and the between-assay and within-assay variabilities were between 5% and 10%. Spiking experiments confirmed complete recovery of mediators added to the cell-free supernatant.

#### **2.2.2.2 Interleukin (IL)-8, cysteinyl leukotrienes enzyme immunoassay**

We measured interleukin (IL)-8 with a commercial ELISA (OptEIA Set, Becton Dickinson, Cowley, Oxford, UK). The standard curve was unaffected by the addition of DTT. The limit of detection for IL-8 was 30 pg/g of sputum and the between-assay and within-assay variabilities were between 5% and 10%. LTC<sub>4</sub>/ D<sub>4</sub>/E<sub>4</sub> was measured by enzyme immunoassay employing a cysteinyl-leukotriene polyclonal antiserum. (Cayman Chemical, Ann Arbor, MI) (Pavord et al 1999a; Sladek et al. 1994). The intra-assay coefficient of variability of the cysteinyl-leukotriene assay was 5 to 10% and the interassay coefficient of variability was 10 to 15% across the range of concentrations measured. Our collaborators Drs Sheller and Dworski performed the measurement of cysteinyl-leukotrienes reported in this thesis.

#### **2.2.2.3 Eosinophilic cationic protein (ECP) fluoroimmunoassay**

The concentration of eosinophilic cationic protein (ECP) was measured using a commercial fluoroimmunoassay (Unicap; Pharmacia, Milton Keynes, UK), which produces results very similar to the previously described radioimmunoassay (Pizzichini et al 1996a). The limit of detection for ECP was 18ng/g of sputum and the between-assay and within-assay variabilities were between 5% and 10%.

Spiking experiments confirmed complete recovery of ECP added to the cell-free supernatant. Our collaborators at Astra Charnwood, UK performed the measurement of ECP.

#### 2.2.2.4 Prostanoids GC-MS-NICI

The concentrations of the prostanoids PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, and TXB<sub>2</sub> in the sputum supernatant were determined by modified stable isotope dilution assays that used gas chromatography-negative ion chemical ionization-mass spectroscopy (GC-NICI-MS) (Pavord et al 1999a) (Sladek et al 1994). These measurements were performed by our collaborators Drs Sheller and Dworski at Vanderbilt University, Nashville, USA.

#### 2.2.2.5 Histamine radioenzymic assay

Histamine was measured using a sensitive radioenzymic assay based on the conversion of histamine to [<sup>3</sup>H] methyl histamine by the enzyme, histamine-*N*-methyl transferase, in the presence of [<sup>3</sup>H] S-adenosyl methionine as the methyl donor (Liu et al 1990). The intra-assay coefficient of variability of the histamine assay was 5 to 10%.

### 2.2.3 Generation of probes for in-situ hybridisation

#### 2.2.3.1 mRNA extraction

##### Buffers and solutions

Lysis binding buffer:

100mM Tris HCl pH 8.0  
500mM LiCl  
10mM EDTA pH 8.0  
1% LiDS (SDS)  
5mM dithiothreitol (DTT)

Wash buffer with LiDS:

10mM Tris HCl pH8.0  
0.15 M LiCl  
1mMEDTA  
0.1% LiDS

Washing buffer without LiDS:  
 10mM Tris.HCl pH8.0  
 0.15M LiCl  
 1mM EDTA

**Protocol**

Cell pellets from stimulated PBMC were resuspended in cell lysis buffer at a concentration of  $0.5 \times 10^6$  cells/ml. Cell lysate (100 $\mu$ l) was incubated with proteinase K/DEPC to a final concentration of 50 $\mu$ g/ml at 37°C for 1 hour. DNA was sheared by passing through a 21G, followed by 25G needle in a 1ml syringe, 3 to 5 times. To each sheared lysate 30 $\mu$ l of conditioned Dynabeads oligo(dT)<sub>25</sub> (Dyna, UK) were added and allowed to bind for 5 minutes at room temperature. Dynabeads were pelleted using a Dynal Magnetic particle concentrator (MPC) and washed 2x with wash buffer with LiDS and 2x with wash buffer without LiDS. The beads were resuspended in 30 $\mu$ l DEPC water and stored at 4°C until the RT reaction was performed.

**2.2.3.2 Reverse transcriptase polymerase chain reaction (PCR)**

**Protocol**

Reverse transcriptase (RT) reactions were carried out on mRNA extracted onto dynabeads with and without the reverse transcriptase enzyme to produce positive and negative RT products. The reactions were set-up as shown below and incubated for 1 hour at 42°C, using a hybaid PCR machine and stored at 4°C.

	+RT reaction	-RT reaction
mRNA/ beads preparation	10 $\mu$ l	10 $\mu$ l
5x AMV buffer	5 $\mu$ l	5 $\mu$ l
10mM dNTPs/ DEPC	2.5 $\mu$ l	2.5 $\mu$ l
RNasin (25U) (Promega,UK)	0.62 $\mu$ l	0.62 $\mu$ l
DEPC water	6.38 $\mu$ l	6.88 $\mu$ l
AMV-RT (5U) (Promega, UK)		0.5 $\mu$ l -

### 2.2.3.3 Generating primers

The gene sequence for IL-4 and IFN- $\gamma$  was derived from BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and primers generated to be ~20 bases in length with GC % ~50% and probe length to be 200-500 bases in length using software available free on the internet ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)). The forward and reverse primer sequences are shown for IL-4 and IFN- $\gamma$  (Figure 2.3). These sequences were checked again using BLAST. The biotinylated and unbiotinylated primers were produced by GibcoBRL Custom Primers (Life Technologies, Paisley, Scotland).

#### **Figure 2.3** Forward and reverse primers for IL-4 and IFN- $\gamma$

##### **IFN- $\gamma$ forward primer**

Sequence (5' to 3') GAG TGT GGA GAC CAT CAA GGA

%GC 52%

##### **IFN- $\gamma$ reverse primer**

Sequence (5' to 3') GGA TTA AGT GAG ACA GTC ACA GG

%GC 50%

##### **IL-4 forward primer**

Sequence (5' to 3') TCT CAC CTC CCA ACT GCT TC

%GC 55%

##### **IL-4 reverse primer**

Sequence (5' to 3') TGA GGA TGC TTC TGC ATT TG

%GC 50

#### 2.2.3.4 Polymerase chain reaction (PCR)

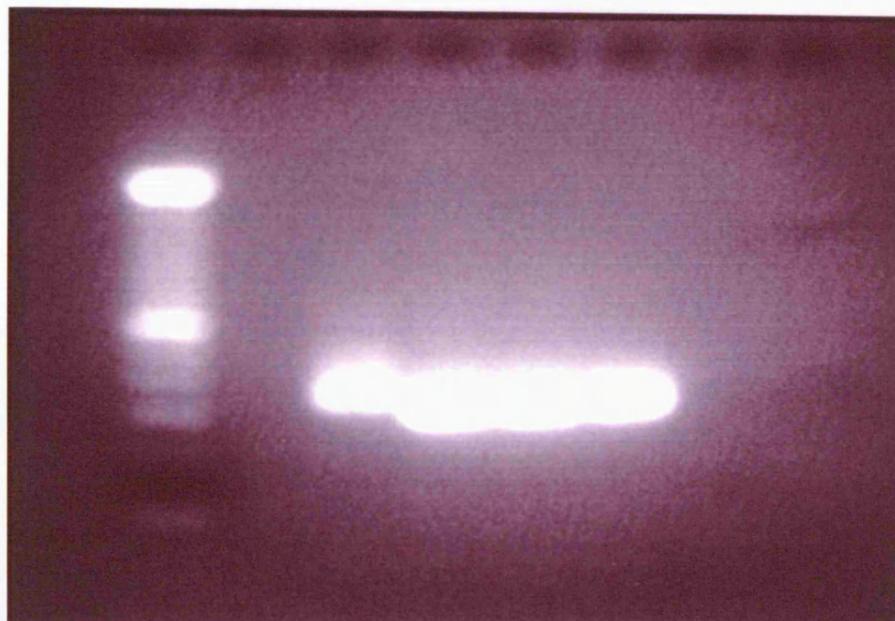
##### Protocol

To cDNA from stimulated PBMC were added appropriate forward and reverse primers in AJ buffer with positive DNA control, negative DNA control and negative no template control. Following initial denaturation at 98°C for 3 mins. Taq polymerase (2µl) diluted 1 in 10 in 1x AJ buffer was added at the annealing temperature. The reaction was amplified over 30 cycles with the denaturation temperature at 94°C for 30s, annealing of primers at 60°C for 30s and extension of sequences at 72°C for 30s. PCR products and controls were assessed on agarose gels. PCR products are as shown for IFN- $\gamma$  (Figure 2.4a) and IL-4 bluescript at various concentrations (gift from Colin Hewitt) (Figure 2.4b). An adequate band for IL-4 was not identified from stimulated PBMC. For the asymmetric PCR (Methods 2.2.3.5) the antisense probe was prepared using the forward biotinylated PCR product and the sense probe with the reverse biotinylated PCR product. These PCR products were checked on an agarose gel and produced a strong band (Figure 2.5).

**Figure 2.4** PCR bands for IFN- $\gamma$  from cDNA derived from stimulated PBMC and IL-4 from IL-4 bluescript (gift)



GAPDH	cDNA	Negative	Negative
Genomic	PBMC	DNA	no
DNA	IFN- $\gamma$	only	template
		control	control



GAPDH	10ng	1ng	0.1ng	Negative	Negative
cDNA	IL-4	bluescript		DNA	no
PBMC	IL-4	primers		only	template
				control	control

**Figure 2.5** Bands for IFN- $\gamma$  and IL-4 biotinylated and non-biotinylated PCR products



1 2 3 4 5 6 7

- 1 GAPDH cDNA stimulated PBMC (positive control)
- 2 IFN- $\gamma$  forward biotinylated primer (template for antisense probe)
- 3 Negative DNA only control
- 4 IFN- $\gamma$  reverse biotinylated primer (template for sense probe)
- 5 IL-4 forward biotinylated primer (template for antisense probe)
- 6 Negative no template control
- 7 IL-4 reverse biotinylated primer (template for sense probe)

### 2.2.3.5 Asymmetric PCR

#### Buffers

2x B & W buffer:

1M Tris.HCl pH 7.6    100 $\mu$ l

0.5 M EDTA            20 $\mu$ l

#### Protocol

The template DNA for asymmetric PCR was provided by PCR reactions (Methods 2.2.3.4). The PCR product had been checked on an agarose gel and produced a strong band (Figure 2.4) (Figure 2.5).

The antisense probe was prepared using the reverse primer and forward biotinylated PCR product and the sense probe with the forward primer and reverse biotinylated PCR product. To the appropriate PCR product (1 $\mu$ l) and non-biotinylated primer (100pM) was added 10xAJ buffer (5 $\mu$ l), 10x dNTP mix (0.5 $\mu$ l) and 1mM DNP-11-dUTP. Initial denaturation was carried out at 98°C for 3 mins and Taq polymerase (2ml) in AJ buffer was added whilst the reaction was held at the annealing temperature at 60°C. The PCR reaction was performed for 20 cycles with denaturation for 30s at 94°C, annealing of primers for 30s at 60°C and extension of sequences for 30s at 72°C.

Removing biotinylated double-stranded DNA with Dynal streptavidin beads and washed in 2x B & W buffer purified the single stranded DNP-labelled probe.

## 2.2.4 Biopsies

### 2.2.4.1 Fixation, processing and embedding in glycomethacrylate and paraffin

#### Protocol glycomethacrylate blocks

Mucosal biopsies were immediately transferred into ice-cooled acetone containing the protease inhibitors (20mM) (PMSF) (2mM) for fixation, stored at -20°C for 24h. The fixative was replaced with acetone followed by methyl benzoate at room temperature for 15 min each. The biopsies were infiltrated with 5% methyl benzoate in glycol methacrylate (GMA solution A, Polysciences, Northampton, UK) at 4°C, 3x2 hours and then embedded in a solution of GMA A 10mls: GMA solution B 250µls: Benzoyl peroxide 45mg (which acts the catalyst for polymerisation). The blocks were polymerised at 4°C overnight and kept in dry airtight boxes at -20°C.

#### Protocol paraffin blocks

One-two mucosal biopsies were fixed for 4 hours at room temperature in 4% paraformaldehyde then transferred into sterile d-PBS overnight at 4°C. The biopsies were processed through a series of alcohol washes at 36°C 70% 30 mins, 90% 2x 30 mins, absolute alcohol 2x 30 mins, clearing solvent 2x 45mins and wax 2x 40 mins at 60°C. Following processing the biopsies were embedded in separate paraffin blocks.

### 2.2.4.2 Immunohistochemistry

#### Buffers and solutions

Tris Buffered Saline pH 7.6

Sodium chloride	80g
Tris	6.05g
1M hydrochloric acid	38mls
Distilled water	10L

Mix buffer salts and acid in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6

#### Blocking medium

Dulbecco's modified Eagles medium	80mls
Fetal calf serum	20mls
Bovine serum albumin	1g

#### Tris HCl Buffer pH 7.6

0.2M Tris	12mls
0.1M hydrochloric acid	19mls
Distilled water	19mls

Mix all reagents together, adjust pH to 7.6

#### Protocol

Two-micrometer sections were cut, floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993). Briefly, slides were pretreated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide to inhibit endogenous peroxidase. After 2X 5min washes in TBS pH 7.6, blocking medium consisting of Dulbecco's MEM, 10% FCS and 1% BSA was applied for 30 min. Sections were then incubated with the primary antibody for 16-20h overnight at room temperature at appropriate concentrations (Table 2.2). Bound antibodies were labelled with biotinylated rabbit anti-mouse Fab fragments (Dako Ltd., Ely, Cambridgeshire, UK) during a 2h incubation, and demonstrated using the streptavidin-biotin-peroxidase detection system (Dako Ltd). Aminoethylcarbazole (AEC) was applied as the chromogen, which gives a red reaction product. Sections were counterstained with Mayers haematoxylin. Appropriate control sections were similarly treated either with the primary mAb omitted or in the presence of an unrelated antibody of the same isotype (IgG1 Dako, Ltd).

**Table 2.2** Antibody source and concentration used for immunohistochemistry

Antibody	Clone	Epitope Stained	Source	Dilution
CD3	UCHT1	T lymphocytes	DAKO	1:1000
CD4	Leu 3a/3b	T helper cells	BD	1:10
CD8	DK25	T suppressor cells	DAKO	1:100
MBP				
EG2		ECP	PHARMACIA	1:200
Tryptase	AA1	Mast cell tryptase	DAKO	1:1000
NE	NP57	Neutrophils	DAKO	1:1000
CD14	TUK4	Macrophages	DAKO	1:8
CD45	2B11+P D7/26	Leucocyte common antigen	DAKO	1:150
CD56			DAKO	
IL-4	4D9	IL-4 (intracellular)	AMS	1:30
IL-4	3H4	IL-4 (activated)	AMS	1:50
IL-5	MAB7	IL-5	Glaxo (gift)	1:100
IFN $\gamma$		IFN $\gamma$	R+D	1:25
IgG1		Isotope control Ig	SIGMA	1:60
Biot-rab antiMo		Second stage antibody	DAKO	1:300
StABC-HRP		Third stage antibody	DAKO	1+1:200

### 2.2.4.3 In-situ hybridisation

#### Special note

All glassware and reagents used prior to hybridisation stage was treated with diethyl pyrocarbonate DEPC to destroy RNase activity.

#### Buffers and solutions

Prehybridisation solution:

dH<sub>2</sub>O

5M NaCl/ DEPC

10x PE (mod)/DEPC

50% dextrose sulphate

ssDNA/ DEPC

Formamide 50%

#### Protocol

Slides cut from paraffin blocks were pre-treated by immersing sequentially for 5 minutes in xylene, xylene, 99% alcohol, 99% alcohol, 95% alcohol and DEPC water and then covered with 100µl proteinase K solution in 50mM Tris HCl pH 7.6/ DEPC at the appropriate concentration and incubated at 37°C. Tonsil tissue used for controls was incubated for 1 hour with 5µg/ml proteinase K and cell cytoblocks and bronchial biopsies were incubated for 30 minutes with 2 µg/ml proteinase K solution.

The sections were washed in DEPC water 2x 5 minutes and incubated at 37°C for 1 hour with 100µl prehybridisation solution. Sense and anti-sense probes at varying concentrations (25-500ng/ml, optimised at 100ng/ml for IFN-γ) and prehybridisation control were applied to the sections overnight at 37°C overnight.

After a wash with 20x SSC preheated to 37°C for 2x 10 minutes and 10 mins with TBS, 3% BSA, 0.1% triton (TBSBT) the slides were incubated with F(ab') rabbit anti-DNP alkaline phosphatase conjugate diluted 1 in 50 in TBSBT for 30

minutes at room temperature. Alkaline phosphatase activity was demonstrated using NCIP/BCT detection system (Dako) at room temperature for 4-6 hours.

IFN- $\gamma$  mRNA was demonstrated on stimulated PBMC cytoblocks and tonsil as a positive control (Figure 2.6). A suitable positive control for IL-4 mRNA expression was not identified in spite of attempts using stimulated PBMC, tonsil, lung resection tissue and stimulated human mast cell line. MRNA expression for both IFN- $\gamma$  and IL-4 was not clearly demonstrated on bronchial biopsies with high background staining. For these reasons in-situ hybridisation was not successfully performed.

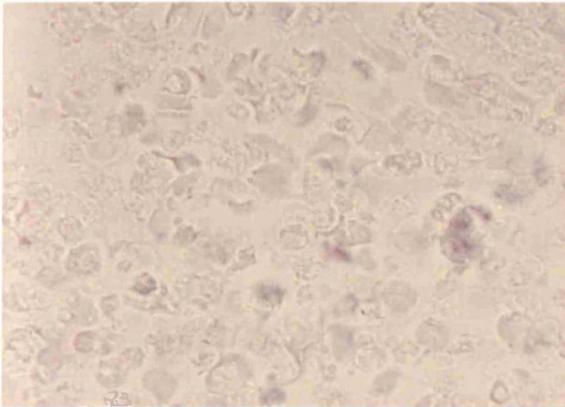
There a number of possible explanations why this technique was unsuccessful. It would seem unlikely to include problems with the primers as bands of an appropriate size and good intensity were produced following the asymmetric PCR. The uptake of the DNP label by the IFN- $\gamma$  probe was adequate to result in good positive controls with stimulated PBMC and tonsil tissue. Thus, the labelling of the IFN- $\gamma$  probe was working appropriately, but this may have not been the case with the IL-4 probe. Good staining on the bronchial biopsy tissue was not achieved with either of the probes. Thus, the shortcoming may have been related to probe concentration, its sensitivity or to the fixation and processing of the biopsy tissue.

In order to address these possible problems future work could include further experiments to optimise the probe concentration. However, this was attempted and was unsuccessful. An alternative solution would be to collaborate with a group experienced in identifying IFN- $\gamma$  and IL-4 gene expression in bronchial biopsy tissue, whereby the sensitivity of our technique may be improved by altering the probe and or detection system perhaps using full length riboprobes and or radioisotopic detection systems. If the problem is found to be due to biopsy fixation and processing this could only be overcome by performing an additional study requiring recruitment and characterisation of further subjects.

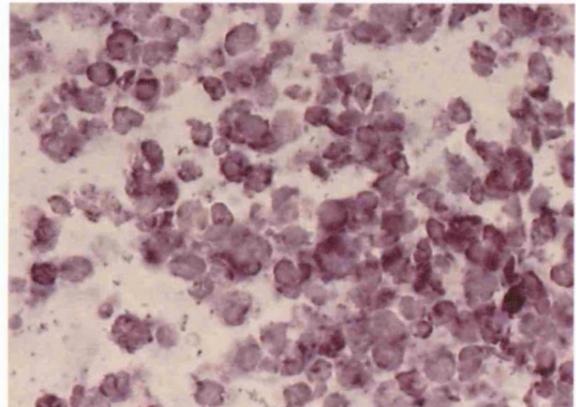
**Figure 2.6** IFN- $\gamma$  mRNA expression by (a) stimulated PBMC and (b) tonsil tissue sense and antisense x400. Most of the stimulated PBMC demonstrate positive staining for IFN- $\gamma$  mRNA expression and expression seems to be in clusters for the tonsil tissue.

**A**

**Sense**

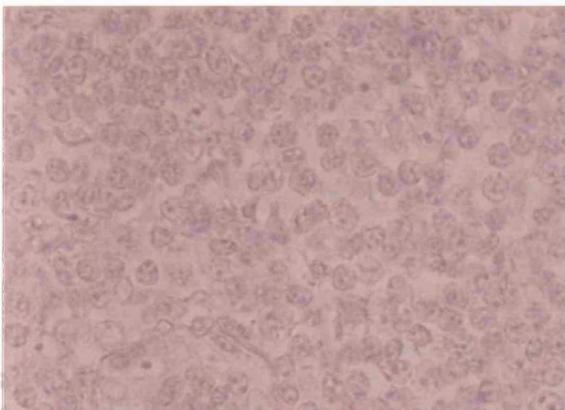


**Antisense**

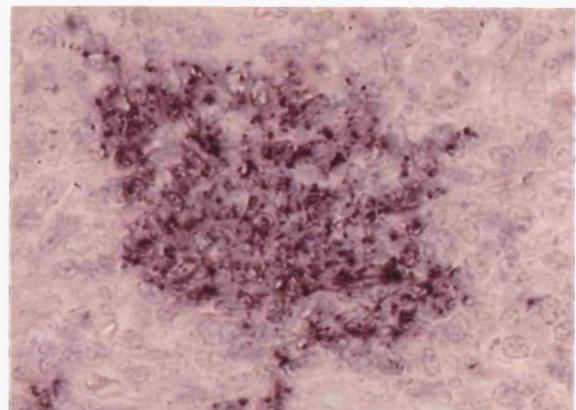


**B**

**Sense**



**Antisense**



### **3. Studies**

#### **3.1 Clinical and immunopathological characterisation of eosinophilic bronchitis**

##### **3.1.1 Eosinophilic Bronchitis Is an Important Cause of Chronic Cough**

###### **Abstract**

Eosinophilic bronchitis presents with chronic cough and sputum eosinophilia, but without the abnormalities of airway function seen in asthma. It is important to know how commonly eosinophilic bronchitis causes cough, since in contrast to cough in patients without sputum eosinophilia, the cough responds to inhaled corticosteroids. We investigated patients referred over a 2-yr period with chronic cough, using a well-established protocol with the addition of induced sputum in selected cases. Eosinophilic bronchitis was diagnosed if patients had no symptoms suggesting variable airflow obstruction, and had normal spirometric values, normal peak expiratory flow variability, no airway hyperresponsiveness (provocative concentration of methacholine producing a 20% decrease in FEV<sub>1</sub> ([PC<sub>20</sub>] > 8 mg/ml), and sputum eosinophilia (> 3%). Ninety-one patients with chronic cough were identified among 856 referrals. The primary diagnosis was eosinophilic bronchitis in 12 patients, rhinitis in 20, asthma in 16, post-viral-infection status in 12, and gastroesophageal reflux in seven. In a further 18 patients a diagnosis was established. The cause of chronic cough remained unexplained in six patients. In all 12 patients with eosinophilic bronchitis, the cough improved after treatment with inhaled budesonide 400 µg twice daily, and in eight of these patients who had a follow-up sputum analysis, the eosinophil count decreased significantly, from 16.8% to 1.6%. We conclude that eosinophilic bronchitis is a common cause of chronic cough, and that sputum induction is important in the investigation of cough.

## **Introduction**

Gibson and colleagues (Gibson et al 1989b) (Gibson et al 1995) have described a group of patients with corticosteroid-responsive chronic cough who had sputum evidence of an eosinophilic bronchitis but normal spirometry, no evidence of airway hyperresponsiveness (AHR), and normal peak expiratory flow (PEF) variability. The features of this condition were distinct from those of asthma, and Gibson and colleagues suggested that it should be known as eosinophilic bronchitis. It is important to know how commonly eosinophilic bronchitis causes chronic cough, since in contrast to cough in patients without sputum eosinophilia (Pizzichini et al. 1999b), the cough responds well to inhaled corticosteroids (Gibson et al 1995). Current algorithms for investigating chronic cough do not include assessment of airway inflammation, and would not allow recognition of these patients.

We have modified a well-validated protocol (Irwin, Corrao, & Pratter 1981) (Irwin, Curley, & French 1990) for investigating chronic cough by adding a differential inflammatory cell count on induced sputum if a diagnosis has not been reached after a history, clinical examination, chest radiography, spirometry, serial measurement of PEF, and a methacholine inhalation test. We used this modified protocol to prospectively look for evidence of eosinophilic bronchitis in new patients with chronic cough of more than 3 wk duration who were seen by a single consultant (Dr Pavord) between January 1996 to December 1997.

## **Methods**

### **Subjects**

Patients with an isolated chronic cough lasting more than 3 wk were identified from new patient referrals made between January 1996 and December 1997 to a single respiratory physician by primary care physicians in both rural and urban areas. Patients were between 28 and 76 yr of age, and other than having a chronic cough, had no clinical or radiologic evidence of significant lung disease at the time of referral. Subjects gave full informed consent to participate in the study. The protocol was approved by the Leicestershire Health Authority ethics

committee. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

## Measurements

Spirometry was done with a rolling-seal spirometer (Vitalograph, Buckingham, UK), with recording of the greater of successive readings within a 200 ml range. If the FEV<sub>1</sub>/FVC ratio was < 70%, spirometry was repeated 15 min after inhalation of 200 µg salbutamol. Allergen skin sensitivity was measured by skin prick testing with *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and *Aspergillus fumigatus* solutions, with controls consisting of normal saline and histamine (Bencard, Brentford, UK). PEF was measured twice daily as the best of three expirations, using a mini-Wright peak flow meter (Clement Clarke Ltd., London, UK). Airway responsiveness was measured according to the tidal breathing method (Juniper, Cockcroft, & Hargreave 1994) (Methods 2.1.3). Sputum was induced and processed as suggested by Pizzichini and colleagues (Pizzichini et al 1996a) (Methods 2.1.4, 2.2.1.2).

## Protocol

The cause of chronic cough in each subject was investigated according to the anatomic-diagnostic protocol suggested by Irwin and colleagues (Irwin, Corrao, & Pratter 1981) (Irwin, Curley, & French 1990), with modifications similar to those suggested by O'Connell and coworkers (O'Connell et al 1994) (Table 3.1, Figure 3.1). All patients had an initial clinical assessment consisting of a history, physical examination, chest radiograph, allergen skin prick tests, twice-daily measurement of peak expiratory flow for at least 2 wk, and, if appropriate, spirometry with reversibility studies following inhalation of 200 µg of salbutamol (Figure 3.1) (Methods 2.1.2). A trial of treatment was begun only if patients had one or more symptoms suggesting an underlying diagnosis, together with at least one positive finding on examination or investigation. The clinical features and abnormalities found in the study, and the investigations and therapies used, are outlined on Table 3.1. Patients were followed up after 6 to 8 wk and asked if their cough had improved. The primary diagnosis was accepted if pretreatment criteria were fulfilled and the cough improved with specific therapy. In situations in which more than one disease process was thought to be contributing to the cough,

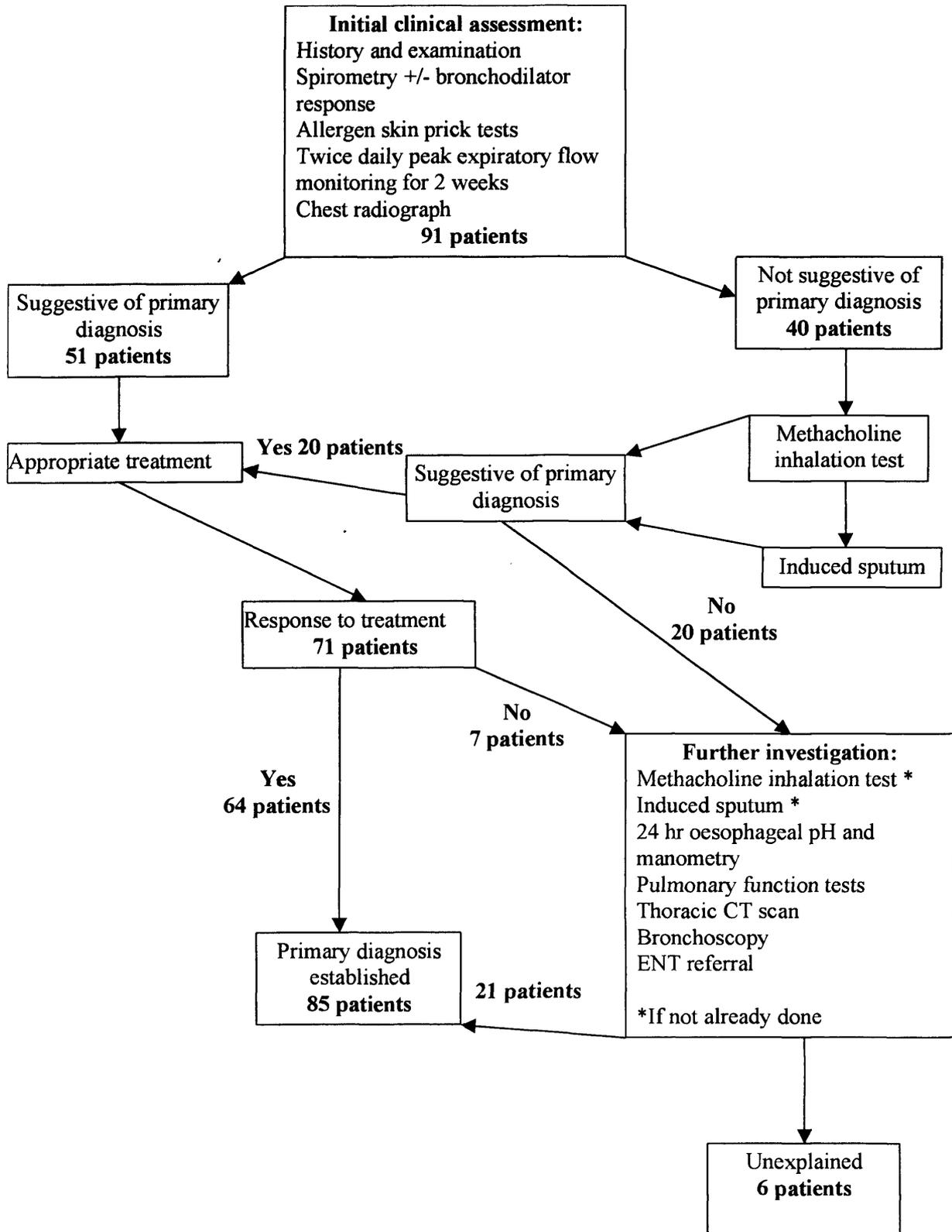
therapy aimed at all potential causes was begun and the most important cause in the opinion of the responsible clinician was designated the primary diagnosis. If no initial cause for the cough could be identified, or if the initial treatment did not alleviate the cough, the patient had a methacholine inhalation test followed by sputum induction. Other investigations were dictated by the individual clinical picture (Table 3.1, Figure 3.1). Eosinophilic bronchitis was diagnosed if a patient had a cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal peak PEF variability (percent difference of the maximum within-day amplitude of < 20% from the mean over a 2-wk period), a provocative concentration of methacholine causing a 20% decrease in FEV<sub>1</sub> (PC<sub>20</sub>) > 8 mg/ml, and sputum eosinophilia of > 3% nonsquamous cells. We chose 3% in order to be consistent with Carney and colleagues (Carney et al 1997) definition of eosinophilic bronchitis, and because this is > 3SD outside the normal range in our laboratory (our normal range is 0 to 1%).

**Table 3.1** Clinical features, investigations and treatment of chronic persistent cough

Diagnosis	History	Examination	Investigations	Treatment
Rhinitis	Rhinorrhea, nasal obstruction, sinus pain, sneezing, nasal itch, postnasal drip	Nasal secretions, nasal or pharyngeal mucosal inflammation	Sinus X-ray/CT showing mucosal thickening and/or fluid level	Topical budesonide/BDP 100 µg twice daily. In selected cases: topical ipratropium bromide 40 µg twice daily, topical xylometazoline HCL 0.1%, oral antibiotics, oral antihistamine
Eosinophilic bronchitis	No wheezing, dyspnea	No signs of airflow obstruction	FEV <sub>1</sub> > 80% pred. FEV <sub>1</sub> /FVC > 75%. Maximum within-day PEF variability over 2 wk < 20%. PC <sub>20</sub> > 8 mg/ml. Sputum eosinophil count > 3%	Inhaled budesonide/BDP 400 µg twice daily with prednisolone 30 mg daily for 14 d in selected cases
ACE inhibitor-induced cough	Cough onset temporarily related to starting ACE inhibitor			Drug withdrawal. Substitution of alternative if appropriate.
Gastroesophageal reflux	Heartburn, flatulence, waterbrash		Barium swallow, endoscopy, and 24 h esophageal manometry and pH in selected cases	Weight reduction, elevation of head of bed, avoidance of eating within 2 h of bedtime, acid suppression. Prokinetic agent in selected cases
Asthma	Episodic wheezing, dyspnea, and/or chest tightness	Polyphonic expiratory wheeze	One or more of the following: > 15% increase in FEV <sub>1</sub> after inhaled salbutamol 200 µg, maximum within-day PEF variability over 2 wk > 20%, PC <sub>20</sub> < 8 mg/ml	Inhaled budesonide/BDP 400 µg twice daily with prednisolone 30 mg daily for 14 d in selected cases. Inhaled β <sub>2</sub> -agonist as required
Postviral	Onset following viral upper respiratory tract infection			Observation
Chronic bronchitis	Productive morning cough > 3 mo/yr for more than 1 yr. Smoking history	Coarse crackles		Cessation of smoking

*Definition of abbreviations:* ACE = angiotensin converting enzyme; BDP = beclomethasone dipropionate; CT = computed tomography; PC<sub>20</sub> = provocative dose of methacholine causing a 20% decrease in FEV<sub>1</sub>.

**Figure 3.1** Diagnostic algorithm for patients with chronic persistent cough



## **Analysis**

Methacholine PC<sub>20</sub> was calculated by linear interpolation of the log dose-response curve. The sputum eosinophil count was not normally distributed, and was log transformed and described as a geometric mean. Changes in sputum eosinophil count were expressed as fold differences with a 95% confidence interval (CI).

## **Results**

Ninety-one patients with chronic cough were identified from 856 new referrals seen between January 1996 and December 1997 (10.6%). A diagnosis leading to successful treatment was reached in 93% of cases. Fifty-one (56%) patients had findings at presentation suggestive of a primary diagnosis, which was successfully treated in 44 (48%) patients. Four of the remaining seven patients, and the 40 (44%) patients who did not have suggestive clinical features, had a methacholine challenge and sputum induction. This led to a diagnosis in a further 20 patients. The remaining 27 patients had further investigations, resulting in a diagnosis in 21 cases, and thus leaving six (7%) patients with unexplained cough (Figure 3.1). Ten patients (11%) were thought to have more than one explanation for their cough. In seven patients gastroesophageal reflux was one of the codiagnoses. Sputum suitable for processing was obtained from 40 of the 44 patients who had sputum induction. Of the four patients in whom sputum induction was unsuccessful, two had AHR and were diagnosed as having asthma. The other two patients had no explanation for their cough. All of the patients with unexplained cough had empirical treatment directed toward asthma, eosinophilic bronchitis, rhinitis, and gastroesophageal reflux, without clinical improvement in any case.

The causes of cough are shown in Table 3.2. Eosinophilic bronchitis was the primary diagnosis for cough in 12 (13.2%) patients. The characteristics of these patients are shown in Table 3.3. After treatment with budesonide 400 µg given via a turbobaler, cough improved in all 12 patients. In eight patients repeat sputum induction was performed from 4 to 8 wk after treatment was begun, and showed a significant decrease in the sputum eosinophil count, from a geometric mean of 16.8% to 1.6% (10.7-fold difference; 95% CI: 4.1 to 27.7;  $p < 0.01$ ).

**Table 3.2 Causes of isolated chronic cough (n=91)**

<b>Primary Cause of Cough</b>	<b>No. of Patients (%)</b>
Rhinitis	20 (24%)
Asthma	16 (17.6%)
Postviral	12 (13.2%)
Eosinophilic bronchitis	12 (13.2%)
Gastroesophageal reflux	7 (7.7%)
Unexplained	6 (6.6%)
COPD	6 (6.6%)
Bronchiectasis	5 (5.5%)
ACE inhibitor-induced cough	4 (4.4%)
Lung cancer	2 (2.2%)
Cryptogenic fibrosing alveolitis	1 (1.1%)

Definition of abbreviations: ACE = angiotensin converting enzyme; COPD = chronic obstructive pulmonary disease.

**Table 3.3** Characteristics of patients with eosinophilic bronchitis

No. of subjects	12
Age*	52 (28-76)
Male	2
FEV <sub>1</sub> , % predicted*	107 (95-133)
FEV <sub>1</sub> /FVC, %*	80 (72-94)
Methacholine PC <sub>20</sub> < 16, mg/ml	0
Atopy	5
Sputum eosinophils, % †	10.5 (4.7-38)
Peripheral blood eosinophil count (cells × 10 <sup>9</sup> /l) †	0.18 (0.09-1.1)
Symptoms of rhinitis	4
Cough duration, months*	26.3 (2-120)
Current smokers	0
Pack-yr > 5	1

\*Mean and range.

†Geometric mean, range in brackets.

## **Discussion**

We successfully identified a cause or causes for chronic cough in most patients, confirming the diagnostic value of the anatomic-diagnostic approach suggested by Irwin and colleagues (Irwin, Corrao, & Pratter 1981). Our treatment success rate of 93% was very similar to those reported by Irwin and colleagues (Irwin, Curley, & French 1990) (Irwin, Corrao, & Pratter 1981) and others (Poe et al. 1989) in a similar patient population, and was slightly higher than that reported by O'Connell and coworkers (O'Connell et al 1994) and McGarvey and associates (McGarvey et al 1998) in patients referred to a tertiary referral center. We have confirmed that rhinitis and gastroesophageal reflux are common causes of chronic cough in this clinical setting. Our modified protocol allowed us to recognize eosinophilic bronchitis in 13% of patients. This estimate of the incidence of eosinophilic bronchitis is similar to that reported by Carney and associates (Carney et al 1997), who used diagnostic criteria identical to ours and found three cases in 30 patients with chronic cough.

Our data and those of Carney and associates suggest that assessment of airway inflammation is an important addition to the algorithm for investigating chronic cough. We chose to assess airway inflammation with induced sputum, since this method is noninvasive and has been shown to be successful in the majority of patients with asthma (Hunter et al 1999). Sputum differential cell counts have been shown to be valid and repeatable in patients with asthma (Pizzichini et al 1996a). We have shown that sputum induction is also successful in most patients with chronic cough, and that sputum eosinophilia is the only significant finding in 13% of cases of such cough. Although we chose to analyze induced sputum, spontaneous sputum could be used if patients have a productive cough. Differential cell counts are similar with the two methods, but the cell viability is greater and squamous cell contamination less with induced sputum, resulting in better quality cytospin preparations (Pizzichini et al. 1996b).

The patients with eosinophilic bronchitis in our study presented with a cough without wheezing, dyspnea, or objective evidence of variable airflow obstruction,

and thus did not meet conventional criteria for the diagnosis of asthma (Hunter et al 1999). They had a subjective improvement in their cough and a significant decrease in their sputum eosinophil count after treatment with inhaled corticosteroids, which resembled the findings of Gibson and coworkers (Pizzichini et al 1999b). We did not formally assess the time course of the response to corticosteroids, although anecdotally, improvement began 2 to 3 wk after inhaled corticosteroids were begun.

Most previous studies have not identified patients with eosinophilic bronchitis as a distinct subgroup among patients with chronic cough. Although one cannot discount the possibility that eosinophilic bronchitis is a new condition, we feel that the failure to recognize it in previous studies is more likely to reflect differences in referral pattern, or less stringent criteria for the diagnosis of asthma. Many patients, particularly tertiary referrals, are likely to have received a trial of corticosteroids before referral, and those patients who responded to corticosteroids may have been diagnosed as having asthma before further tests were done or irrespective of the results of objective tests of variable airflow obstruction or airway responsiveness. We diagnosed asthma in patients with consistent symptoms and objective evidence of variable airflow obstruction and/or AHR. Our criteria for an objective demonstration of variable airflow obstruction and AHR are widely accepted (Juniper, Cockcroft, & Hargreave 1994) (Jamison & McKinley 1993) (Taylor 1997), and particularly in the case of methacholine inhalation testing are sensitive markers of currently symptomatic asthma. In common with previous reports, our diagnosis of asthma was subjectively supported by successful response to treatment (McGarvey et al 1998) (Irwin et al. 1997). It has been proposed that a negative histamine or methacholine challenge rules out asthma and therefore obviates the need for a trial of inhaled steroids (McGarvey et al 1998). We agree that normal airway responsiveness makes a diagnosis of asthma very unlikely, but disagree that this precludes the need for a trial of inhaled corticosteroids, since this approach would deny effective treatment to a significant number of patients.

Typically, the patients with eosinophilic bronchitis in our study presented in middle age with a dry cough or a cough productive of small amounts of viscid sputum in the morning. Few were smokers, perhaps reflecting a selection bias,

since primary-care physicians are likely to attribute a chronic cough in smokers to chronic bronchitis, and would therefore not refer these patients for a specialist's opinion. Four of our patients with eosinophilic bronchitis had symptoms of rhinitis, although the absence of other clinical or radiographic features and lack of improvement in cough following treatment of rhinitis by their primary-care physicians lead us to perform further investigations and to conclude that eosinophilic bronchitis was the primary cause of cough in these patients. We have not studied the natural history of this condition, but the long duration of symptoms before referral suggests that it is a chronic problem.

The recognition of patients with sputum eosinophilia without variable airflow obstruction (eosinophilic bronchitis) has important implications for understanding the role of airway inflammation in asthma, and suggests that in some cases, the conventional view of a direct relationship between eosinophilic airway inflammation and AHR (Barnes 1989) is overly simplistic. Possible explanations for the relative absence of a functional effect of eosinophilic airway inflammation in our patients include differences in the site or state of activation of the inflammatory response. An alternative possibility is that AHR is increased by the airway inflammation in eosinophilic bronchitis, but stays within the normal range because baseline airway responsiveness is far to the right of the normal range. We have recently observed such a phenomenon in a patient with eosinophilic bronchitis studied during an exacerbation of eosinophilic airway inflammation (Wong et al 1996). Further research is required into the cause of and relationship between airway inflammation, AHR, and cough in patients with eosinophilic bronchitis.

We conclude that eosinophilic bronchitis is a common cause of chronic cough in patients referred for a specialist's opinion. Recognition of patients with a cough caused by eosinophilic bronchitis is important, since effective treatment is possible. We suggest that assessment of airway inflammation be added as an important component of the investigation of cough.

### 3.1.2 Induced Sputum Inflammatory Mediator Concentrations in Eosinophilic Bronchitis and Asthma

#### Abstract

Eosinophilic bronchitis is a common cause of chronic cough, which like asthma is characterized by sputum eosinophilia, but in contrast to asthma there is no variable airflow obstruction or airway hyperresponsiveness. Our hypothesis was that the differences in airway pathophysiology maybe due to less active airway inflammation in eosinophilic bronchitis, with reduced release of important effector mediators. We measured the concentration of various proinflammatory mediators in induced sputum cell-free supernatant in eight patients with eosinophilic bronchitis, 17 patients with asthma matched for sputum eosinophil count, and 10 normal subjects. Cysteinyl-leukotrienes (cys-LT) were measured by enzyme immunoassay, eosinophilic cationic protein (ECP) by fluoroimmunoassay, prostanoids (PGE<sub>2</sub>, PGD<sub>2</sub>, TXB<sub>2</sub>, and PGF<sub>2α</sub>) by gas chromatography-negative ion chemical ionization-mass spectroscopy, and histamine by radioenzymic assay. The geometric mean sputum eosinophil count was similar in asthma (13.4%) and eosinophilic bronchitis (12.5%). Sputum cys-LT and ECP were a mean (95% CI) 1.6-fold (1.1, 2.5) and 6.4-fold (1.4, 28) higher in eosinophilic bronchitis and 1.9-fold (1.3, 2.9) and 7.7-fold (1.2, 46) higher in asthma compared with that in control subjects (geometric mean, 5.9 and 95 ng/ml, respectively). In eosinophilic bronchitis the mean concentration of sputum PGD<sub>2</sub> (0.79 ng/ml) and histamine (168 ng/ml) were significantly higher than in asthma (mean absolute difference in PGD<sub>2</sub> concentration, 0.47 ng/ml [95% CI, 0.19 to 0.74] and mean-fold difference in histamine concentration, 6.7 [95% CI 1.7 to 26]) and normal subjects (0.64 ng/ml [0.36 to 0.90] and 11-fold [3.3 to 36]), respectively. In conclusion, eosinophilic bronchitis is associated with active airway inflammation with increased release of vasoactive and bronchoconstrictor mediators.

## **Introduction**

Eosinophilic bronchitis presents with chronic cough and is characterized by sputum eosinophilia similar to that seen in asthma, but unlike asthma the patients have no symptoms or objective evidence of variable airflow obstruction or airway hyperresponsiveness (Gibson et al 1989b) (Gibson et al 1995). We (Brightling et al. 1999b) and others (Carney et al 1997) have shown that eosinophilic bronchitis is a common cause of cough in patients presenting to a respiratory specialist. The aetiology of eosinophilic bronchitis and the reason for the absence of lower airway hyperresponsiveness in this disease is unknown. One possible explanation is that the eosinophilic airway inflammation is less active than asthma, with less release of important effector mediators.

We and others have shown that various proinflammatory mediators, including cysteinyl-leukotrienes (cys-LT), prostanoids (PG) (Pavord et al 1999a), and eosinophilic cationic protein (ECP) (Pizzichini et al 1996a) can be measured in the induced sputum cell-free supernatant repeatedly. We have used this technique to test our hypothesis by measuring eicosanoids, ECP, and histamine in the cell free supernatant from induced sputum in patients with eosinophilic bronchitis or asthma and in normal subjects.

## **Methods**

### **Subjects**

Patients with eosinophilic bronchitis or asthma, and healthy volunteers were recruited from respiratory outpatient clinics and from staff at The Glenfield Hospital. The subjects with eosinophilic bronchitis ( $n = 8$ ) had an isolated cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal peak expiratory flow (PEF) variability (maximum within-day amplitude, % mean < 20% over 2 wk), a methacholine  $PC_{20} > 16$  mg/ml, a normal chest radiograph, and a sputum eosinophilia (> 3% nonsquamous cell). None had received oral or inhaled corticosteroids for at least 1 mo before entry into the study. Subjects with asthma ( $n = 17$ ) gave a suggestive history and had objective evidence of variable airflow obstruction, as indicated by one or more of the following: (1) methacholine airway hyperresponsiveness ( $PC_{20} < 8$  mg/ml);

(2) > 15% improvement in FEV<sub>1</sub> 10 min after 200 µg albuterol; or (3) PEF (> 20% maximum within-day amplitude from twice daily PEF measurements over 14 d). The subjects with asthma were clinically stable and treated, as required, with either β-agonists only (n = 9) or with inhaled corticosteroids and, as required, β-agonists (n = 8). All the subjects with asthma had a sputum eosinophil count > 3%. The healthy subjects (n = 10) gave no history of respiratory diseases, had negative allergen skin prick tests, normal spirometry, and normal airway responsiveness. The sputum eicosanoid levels in the normal and asthmatic subjects have been previously reported (Pavord et al 1999a). All subjects gave written informed consent to participate in the study. The protocol was approved by the Leicestershire Health Authority ethics committee. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

### **Protocol and Clinical Measurements**

Subjects attended on two occasions. At the first visit they had spirometry and allergen skin prick tests. At the second visit the subjects had a methacholine inhalation test followed on recovery by sputum induction. Spirometry was performed using a dry bellows spirometer (Vitalograph, Buckingham, UK) with the FEV<sub>1</sub> recorded as the best of successive readings within 100 ml. Allergen skin prick tests were performed to *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and *Aspergillus fumigatus* solutions with normal saline and histamine controls (Bencard, Brentford, UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal > 2 mm more than the negative control (Methods 2.1.1). The methacholine challenge was performed using the tidal breathing method (Juniper, Cockcroft, & Hargreave 1994) with doubling concentrations of methacholine (0.03 to 16 mg/ml) nebulized via a Wright nebulizer (Methods 2.1.3). Sputum was induced and processed as previously described (Pizzichini et al 1996a) (Pavord et al 1997) (Methods 2.1.4, 2.2.1.2).

## **Mediator Measurements**

There was insufficient sputum supernatant to measure histamine in seven asthmatics and one normal subject, ECP in eight asthmatics and two normal subjects, and prostanoids in two normal subjects.

The concentration of the eicosanoids PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , TXB<sub>2</sub> and LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> and histamine and eosinophilic cationic protein (ECP) were measured in the sputum supernatant (Methods 2.2.2.2, 2.2.2.3, 2.2.2.4, 2.2.2.5).

## **Analysis**

Sputum differential eosinophil counts and eicosanoid concentrations (corrected for the sputum dilution and expressed as nanograms per milliliter sputum) were log-transformed and described as geometric mean (log SEM). Other differential cell counts, total cell counts, viability, and squamous cell contamination were described as median and interquartile range. Sputum supernatant PGD<sub>2</sub> concentration was normally distributed and was described as mean (SEM). Sputum eosinophil count and supernatant mediator concentrations were compared between groups by one-way analysis of variance (ANOVA), and differences were expressed as fold or absolute change with 95% CI. Other sputum indices were compared by the Kruskal-Wallis test. Mediator concentrations were compared between subjects with asthma treated with inhaled corticosteroids and those treated with  $\beta$ -agonist alone by Student's unpaired *t* test. Significance was accepted at the level of 95% and the Student-Newman-Keuls procedure was used to adjust for multiple comparisons. The number of subjects with eosinophilic bronchitis selected was determined by the number available and willing to participate in the study. No power calculation was performed.

## **Results**

The groups were well matched for age and sex (Table 3.4). Atopy was more common in the subjects with asthma than in those with eosinophilic bronchitis. There was no difference in the cysteinyl-leukotriene, ECP, PGD<sub>2</sub>, or histamine concentrations between the asthma subjects treated with inhaled corticosteroids and those treated with  $\beta$ -agonist alone (Figure 3.2). The sputum cell counts and cell-free sputum supernatant mediator concentrations were as shown (Table 3.4

and Figure 3.2). The subjects with asthma and eosinophilic bronchitis were well matched for sputum eosinophilia. Sputum cysteinyl-leukotriene and ECP were a mean (95% CI) 1.6-fold (1.1 to 2.5) ( $p < 0.05$ ) and 6.4-fold (1.4 to 28) ( $p < 0.01$ ) higher in eosinophilic bronchitis and 1.9-fold (1.3 to 2.9) and 7.7-fold (1.2 to 46) ( $p < 0.01$ ) higher in asthma than in control subjects. Sputum  $\text{PGD}_2$  and histamine concentrations were significantly higher in eosinophilic bronchitis than in asthma (mean absolute difference in  $\text{PGD}_2$  concentration, 0.47 ng/ml [95% CI, 0.19 to 0.74],  $p < 0.01$  and mean-fold difference in histamine concentration 6.7 [95% CI, 1.7 to 26],  $p < 0.01$ ), and normal subjects (0.64 ng/ml [0.36 to 0.90],  $p < 0.01$  and 11-fold [3.3 to 36],  $p < 0.01$ ), respectively. The concentration of the other prostanoids were greater in the subjects with eosinophilic bronchitis and asthma than in normal subjects, but these differences did not reach statistical significance (Table 3.4).

**Table 3.4** Subject details, sputum characteristics and mean (SEM) sputum supernatant mediator concentrations

	Eosinophilic Bronchitis	Asthma	Normal
Number	8	17	10
Male	3	9	4
Age, mean (range)	53 (28-70)	42 (15-62)	41 (19-57)
Atopy	3	11	0
On inhaled steroids	0	8	0
% FEV <sub>1</sub> <sup>†</sup>	113 (93, 133)	82 (74, 90) <sup>‡</sup>	105 (92, 117)
% FEV <sub>1</sub> /FVC <sup>†</sup>	81 (76, 85)	70 (65, 75) <sup>‡</sup>	85 (79, 90)
PC <sub>20</sub> (mg/ml) <sup>§</sup>	> 16	1.22 (0.24)	> 16
TCC × 10 <sup>6</sup> /ml <sup>#</sup>	2.8 (2.1)	3.4 (3.7)	2.3 (2.0)
Squamous cell % <sup>#</sup>	4.2 (3.3)	3.2 (3.9)	5.1 (4.1)
Viability, % <sup>‡</sup>	70 (31)	65 (26)	68 (30)
Sputum eosinophils, % <sup>§</sup>	12.5 (0.14) <sup>¶</sup>	13.4 (0.11) <sup>¶</sup>	0.2 (0.11)
Sputum macrophages, % <sup>#</sup>	22.5 (18.3) <sup>‡</sup>	35 (34.4) <sup>‡</sup>	59 (44.2)
Sputum neutrophils, % <sup>#</sup>	64 (39)	41.9 (45.3)	36.7 (47.6)
Sputum lymphocytes (%) <sup>#</sup>	0.15 (0.85)	0.6 (1.7)	0.8 (1.2)
Sputum epithelial cells (%) <sup>#</sup>	2 (2.8)	1.7 (1.1)	2.9 (6.1)
ECP, ng/ml <sup>§</sup>	604 (2.2) <sup>¶</sup>	735 (2.8) <sup>¶</sup>	95 (1.4)
LTC <sub>4</sub> /D <sub>4</sub> /E <sub>4</sub> , ng/ml <sup>§</sup>	9.27 (0.08) <sup>‡</sup>	11.1 (0.08) <sup>‡</sup>	5.86 (0.04)
Histamine, ng/ml <sup>§</sup>	168 (0.19) <sup>¶</sup>	25.1 (0.2)	15.5 (0.16)
PGD <sub>2</sub> , ng/ml <sup>§</sup>	0.79 (0.11) <sup>‡</sup>	0.32 (0.06)	0.15 (0.05)
PGE <sub>2</sub> , ng/ml <sup>§</sup>	1.95 (0.07)	1.36 (0.06)	1.22 (0.10)
PGF <sub>2α</sub> , ng/ml <sup>§</sup>	0.60 (0.11)	0.53 (0.06)	0.40 (0.07)
TXB <sub>2</sub> , ng/ml <sup>§</sup>	1.58 (0.07)	0.94 (0.10)	0.70 (0.10)

*Definition of abbreviations:* %FEV<sub>1</sub> = percent predicted FEV<sub>1</sub>; %FEV<sub>1</sub>/FVC = percent predicted FEV<sub>1</sub>/FVC; ECP = eosinophilic cationic protein.

<sup>†</sup>Mean with range shown in parentheses.

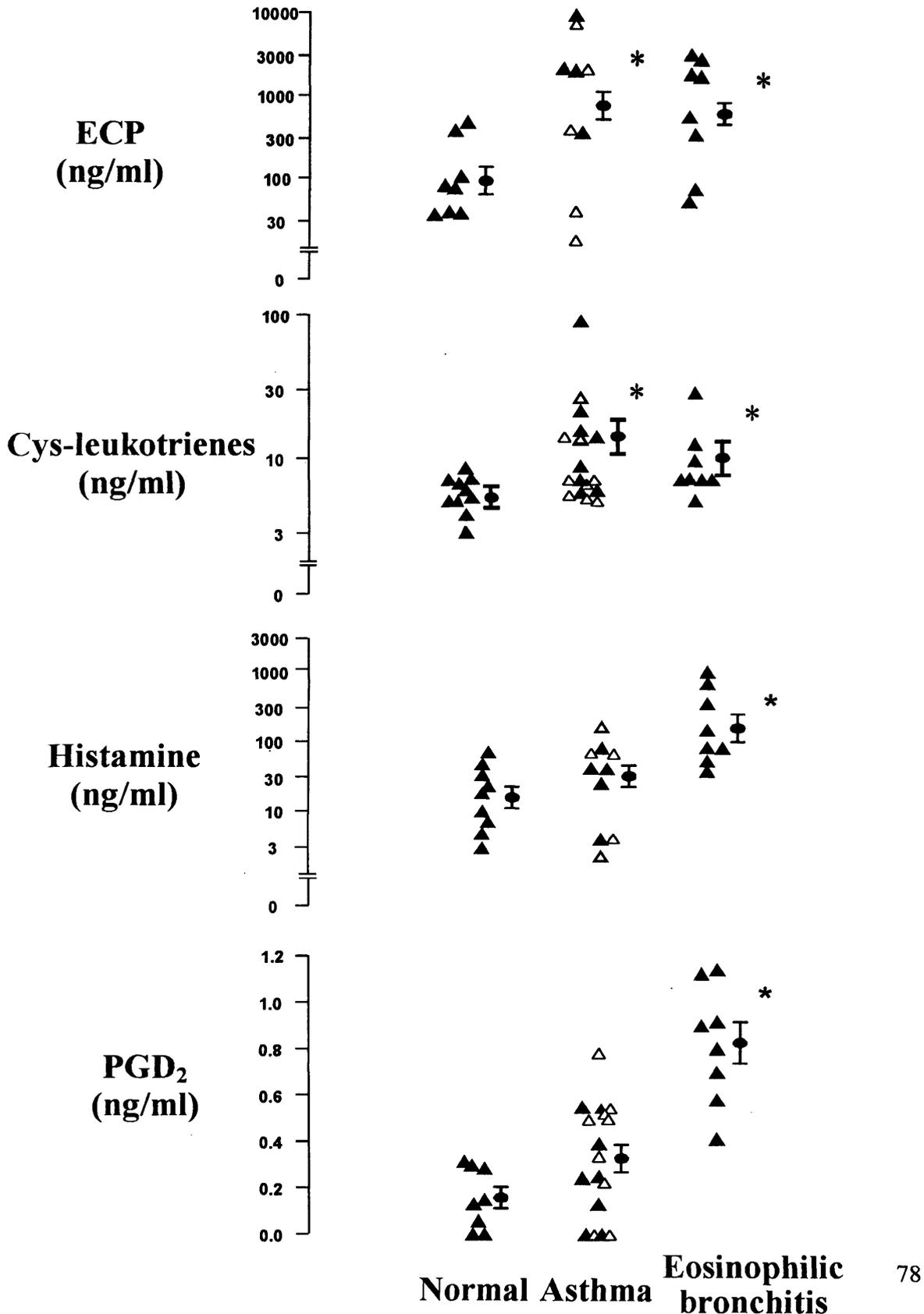
<sup>‡</sup>p < 0.05, <sup>¶</sup> p < 0.01 versus normal subjects by ANOVA.

<sup>§</sup> Geometric mean with log SEM shown in parentheses.

<sup>#</sup>Median with interquartile range shown in parentheses.

**Figure 3.2** Geometric mean (log SEM) concentrations of ECP, cysteinyl-leukotriene, histamine and mean (SEM) PGD<sub>2</sub> concentration in each group of subjects

(*closed triangles*) subjects with asthma treated with corticosteroids (*open triangles*) treated with  $\beta$ -agonist alone. \* $p < 0.05$



## Discussion

We have demonstrated for the first time that induced sputum cysteinyl-leukotrienes and eosinophilic cationic protein concentrations are increased in subjects with eosinophilic bronchitis when compared with normal subjects. The increase was comparable to that seen in subjects with asthma with a similar degree of eosinophilic airway inflammation. Sputum histamine and PGD<sub>2</sub> concentrations were raised only in eosinophilic bronchitis. Thus there is evidence of active eosinophilic airway inflammation in eosinophilic bronchitis with ongoing release of vasoactive, bronchoconstrictor and airway damaging mediators. This strongly suggests that the difference in airway function in eosinophilic bronchitis and asthma are not due to differences in mediator production.

We have measured a wide spectrum of mediators with different functions representing the major effector and airway-damaging mediators in asthma. Cysteinyl-leukotrienes, produced by eosinophils and mast cells, are potent airway smooth-muscle contractile agonists and increase mucus production and vascular permeability and may directly increase eosinophilic airway inflammation (Laitinen et al. 1993b). PGD<sub>2</sub> and histamine produced by mast cells have similar effects on airway smooth muscle, although they are less potent. ECP is directly toxic to epithelial cells *in vitro* (Ayars et al. 1989). PGE<sub>2</sub> may increase cough sensitivity by a direct effect on the cough receptors (Choudry, Fuller, & Pride 1989). The lack of an increase in PGE<sub>2</sub> concentration in eosinophilic bronchitis suggests this is not the mechanism of increased cough sensitivity previously observed in this condition (Brightling et al. 2000a).

We chose to estimate airway mediator production using induced sputum supernatant since this is noninvasive, simple (Pavord et al 1999a), safe (Hunter et al 1999), and responsive (Pizzichini et al 1997b), and previous studies have shown that sputum ECP and eicosanoid concentrations are significantly higher than in bronchoalveolar lavage (Sladek et al 1994) (Liu et al 1990) and can be measured repeatably (Pavord et al 1999a) (Pizzichini et al 1996a). We have considered potential problems that may lead to an exaggeration of the between-category differences observed in this study. Cell viability was not different between groups, indicating that the differences in mediator levels measured were

unlikely to be due to cytotoxic release. Furthermore, leukotrienes and prostanoids are not stored preformed, unlike histamine and ECP, and so their presence in sputum provides evidence of ongoing synthesis and release. We have previously shown that the concentrations of eicosanoids in sputum treated with agents blocking *ex vivo* production and breakdown are not different from untreated sputum (Pavord et al 1999a), so significant *ex vivo* production of eicosanoids is unlikely to have affected our findings. It is possible that the mediator concentrations in the sputum from subjects with asthma or eosinophilic bronchitis may have been increased by the effect of hypertonic saline on mast cells and other mediator-producing cells. We consider this unlikely as subjects were pretreated with salbutamol before sputum induction, which would be expected to attenuate acute mediator release (Church & Hiroi 1987).

This study illustrates that, like asthma, there is active airway inflammation in eosinophilic bronchitis with release of bronchoconstrictor mediators. Why then might an apparently similar and equally active airway inflammation in eosinophilic bronchitis be associated with different abnormalities of airway function? It is possible that the site of the airway inflammation is different in the two diseases. We (Brightling et al 1999b) and others (Gibson et al 1995) have noted that upper airway symptoms are common in patients with eosinophilic bronchitis and have speculated that inflammation is confined to the upper airway. However, eosinophilic bronchitis is not typically associated with a nasal wash eosinophilia or upper airway hyperresponsiveness (Brightling et al 2000a). Furthermore, Gibson and colleagues (Gibson et al 1998) have shown a similar degree of bronchoalveolar lavage (BAL) eosinophilia and GM-CSF and IL-5 gene expression in patients with asthma and eosinophilic bronchitis. These observations suggest that the site of inflammation in eosinophilic bronchitis is mainly in the lower airway.

One possible reason for the difference in airway responsiveness is that in eosinophilic bronchitis the epithelium is intact. With asthma the degree of airway responsiveness is correlated with loss of epithelial structure (Jeffery et al. 1989) and the appearance of epithelial cells in bronchoalveolar lavage fluid (Beasley et al 1989). In asthma the partial loss of an epithelial barrier may allow greater amounts of bronchoconstricting mediators to reach the smooth muscle, or there

may be a reduction in bronchoprotective substances such as PGE<sub>2</sub> (Pavord & Tattersfield 1995). We found no differences in epithelial cell count or PGE<sub>2</sub> concentration between groups, which would not support this view. Whether sputum epithelial counts reflect epithelial integrity is unclear and further bronchial biopsy studies are required to fully address this question.

An alternative explanation for the difference in airway responsiveness is that airway responsiveness may be increased by the airway inflammation in eosinophilic bronchitis but stays within the normal range because baseline airway responsiveness is far to the right of the normal range (Wong et al 1996). Alternatively airway hyperresponsiveness and variable airflow obstruction may develop later in the course of the disease in eosinophilic bronchitis, perhaps as a result of airway remodeling. However, we have observed a patient with severe eosinophilic bronchitis who developed an accelerated decline in FEV<sub>1</sub>, presumably secondary to airway remodeling, but did not develop airway hyperresponsiveness or other features of asthma (Brightling et al 1999a).

Interestingly, although we found the cysteinyl-leukotriene and ECP sputum concentrations to be increased in asthma and eosinophilic bronchitis, the sputum histamine and PGD<sub>2</sub> concentrations were only significantly elevated in subjects with eosinophilic bronchitis. This difference was not due to some of the asthmatic subjects being treated with inhaled corticosteroids as there was no difference between the asthmatic subjects treated with inhaled corticosteroids and those treated with  $\beta$ -agonist alone. The elevation of histamine in combination with PGD<sub>2</sub> is highly suggestive of mast cell activation since basophils, which also produce histamine, do not produce PGD<sub>2</sub> (Schulman et al. 1983). Thus, mast cell activation appears to be a feature of eosinophilic bronchitis. In support of this finding, bronchial brushings have revealed a greater number of mast cells with eosinophilic bronchitis than with asthma (Gibson et al 1998). It has been demonstrated that in bronchial rings recovered from thoracic surgery, those demonstrating contractile responses to allergen contain more mast cells within the smooth muscle than those that are unresponsive (Ammit et al 1997). It is not known whether mast cells infiltrate the bronchial smooth muscle in asthma or eosinophilic bronchitis. One possibility is that mast cells are more localized to the epithelium, so that mediators reach airway smooth muscle in lower

concentrations than in asthma, but are present in high concentrations in epithelial lining fluid and the sputum.

In conclusion, there is active eosinophilic airway inflammation with release of vasoactive, bronchoconstrictor mediators in patients with eosinophilic bronchitis. Further detailed and systematic comparison of the immunopathologic features of the eosinophilic airway inflammation in asthma and eosinophilic bronchitis will be important in identifying particular features of the inflammatory process that are functionally important.

### 3.1.3 Comparison of the immunopathology of eosinophilic bronchitis and asthma

#### Abstract

Eosinophilic bronchitis is a condition characterised by a corticosteroid responsive cough, a sputum eosinophilia and normal tests of variable airflow obstruction and airway responsiveness. We have tested the hypothesis that the different association between airway inflammation and airway dysfunction in eosinophilic bronchitis and asthma reflects differences in the nature of the lower airway inflammatory response by performing a detailed comparative immunopathological study.

We performed bronchoscopy with bronchial wash (BW), bronchoalveolar lavage (BAL) and bronchial biopsy in 16 subjects with eosinophilic bronchitis, 15 with asthma and 14 normal controls.

Both disease groups were characterised by an induced sputum, BW and BAL eosinophilia, increased number of submucosal major basic protein (MBP)<sup>+</sup> cells and increased basement membrane thickness. There were no significant differences between disease categories in these measures. The median submucosal MBP<sup>+</sup> cells/mm<sup>2</sup> were 8, 20 (95% CI of difference from normal 4, 35; p=0.01) and 32 (95% CI 12, 40; p=0.0004) and the mean basement membrane thickness (µm) 7.2, 9 (95% CI of difference from normal 0.2, 3.4; p=0.03) and 10.7 (95% CI 1, 6; p=0.01) in normal, asthma and eosinophilic bronchitis respectively.

Eosinophilic bronchitis and asthma are associated with similar degrees of eosinophilic airway inflammation and basement membrane thickening implying that these pathological changes are not important in the genesis of variable airflow obstruction and airway hyperresponsiveness in asthma.

## **Introduction**

We (Brightling et al 1999b) and others (Carney et al 1997) have shown that eosinophilic bronchitis is a common cause of cough in patients presenting to a respiratory specialist. The sputum features are similar to asthma although in eosinophilic bronchitis there is no variable airflow obstruction or airway hyperresponsiveness. Previous studies have suggested that the different association between airway inflammation and dysfunction in asthma and eosinophilic bronchitis are not due to localisation of the inflammatory process in the upper airway in eosinophilic bronchitis (Brightling et al 2000a) or differences in the state of activation of the inflammatory process as assessed by induced sputum inflammatory mediator concentrations (Brightling et al. 2000b). However no study has compared in detail the lower airway immunopathology of these conditions. We have undertaken a comparative immunopathological study of induced sputum, bronchoalveolar lavage (BAL) and bronchial mucosal biopsies from patients with eosinophilic bronchitis, symptomatic asthma and normal controls.

## **Methods**

### **Subjects**

Sixteen subjects with eosinophilic bronchitis, 15 with asthma, 14 normal controls were recruited from Glenfield Hospital outpatients, staff and local advertising. Subjects with asthma gave a suggestive history and had objective evidence of variable airflow obstruction as indicated by one or more of the following: 1) methacholine airway hyperresponsiveness ( $PC_{20} FEV_1 < 8 \text{ mg/ml}$ ); 2)  $>15\%$  improvement in  $FEV_1$  10 minutes after  $200 \mu\text{g}$  inhaled salbutamol or 3)  $>20\%$  maximum within day amplitude from twice daily peak expiratory flow (PEF) measurements over 14 days). The subjects with eosinophilic bronchitis had an isolated cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal PEF variability (maximum within day amplitude % mean  $<20\%$  over two weeks), a methacholine  $PC_{20} >16 \text{ mg/ml}$ , a normal chest radiograph and a sputum eosinophilia ( $>3\%$  non-squamous cell). Normal subjects were asymptomatic and had no evidence of variable airflow obstruction or airway hyperresponsiveness. All were non-smokers with a past

smoking history of less than 10 pack years. None of the subjects had taken inhaled or oral corticosteroids for at least six weeks prior to the study. The study was approved by the Leicestershire Ethics Committee and all patients gave their written informed consent. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

### **Protocol and clinical measurements**

Subjects attended on two occasions. At the first visit we measured exhaled nitric oxide, spirometry, allergen skin prick tests and methacholine airway responsiveness. This was followed on recovery by a sputum induction. End exhaled nitric oxide (eNO) was measured by a chemiluminescent technique (Logan Research, Kent, UK). Subjects exhaled at a flow rate of 250ml/s with a sampling rate of 250ml/min. Spirometry was performed using a dry bellows spirometer (Vitalograph, Buckingham, UK) with the FEV<sub>1</sub> recorded as the best of successive readings within 100ml. Allergen skin prick tests were performed to *Dermatophagoides Pteronyssinus*, cat fur, grass pollen and *Aspergillus Fumigatus* solutions with normal saline and histamine controls (Bencard, Newark, Notts., UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control (Methods 2.1.1). The methacholine challenge was performed using the Tidal breathing method (Juniper, Cockcroft, & Hargreave 1994) with doubling concentrations of methacholine (0.03 to 128mg/ml) nebulised via a Wright nebuliser (Methods 2.1.3). Sputum was induced and processed as previously described (Pavord et al 1997) (Methods 2.1.4, 2.2.1.2)

At the second visit the subjects underwent bronchoscopy using an Olympus fibre-optic bronchoscope (Olympus Company, Tokyo, Japan) in line with the most recent BTS guidelines (British Thoracic Society 2001). A 20ml bronchial wash of pre-warmed normal saline into the bronchus intermedius was performed followed by a 180ml BAL into the middle lobe in 60ml aliquots. Bronchial mucosal biopsies were taken from the right middle and lower lobe carinae. All subjects received nebulised 2.5mg salbutamol 20 minutes prior to bronchoscopy and had appropriate sedation as required of midazolam 0-5mg i.v. Lignocaine

(1-4%) was used for local anaesthesia and continuous oxygen given via nasal cannulae throughout the procedure (Methods 2.1.5).

Mucosal biopsies were immediately transferred into ice-cooled acetone containing the protease inhibitors iodoacetamide (20mM) and PMSF (2mM) for fixation, stored at -20°C for 24h, and then processed into the water soluble resin, glycolmethacrylate (GMA) (Polysciences, Northampton, UK) for embedding (Methods 2.2.4.1).

### **Immunohistochemistry**

Two micrometer sections were cut, floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The following mouse IgG<sub>1</sub> monoclonal antibodies were used: CD3 (Dako Ltd, High Wycombe, UK), AA1 to mast cell tryptase (Dako Ltd), MBP to eosinophil major basic protein (Research Diagnostics, New Jersey, USA), NE to neutrophil elastase (Dako Ltd), CD45 panleukocyte marker (Dako Ltd), CD14 to macrophages (Dako Ltd), CD56 to natural killer cells (Dako Ltd). The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993) (Methods 2.2.4.2).

### **Assessment and quantification of immunohistochemical staining**

Sub-epithelial mucosa was identified morphologically and the area calculated using a computer analysis system (Scion Image, Maryland, USA). Nucleated immunostained cells present in coded sections were enumerated in the submucosa and numbers of cells expressed as the number/mm<sup>2</sup> of submucosa. Basement membrane and lamina reticularis thickness was measured as the mean of 50 measurements made at 20µm intervals (Sullivan P et al. 1998). Epithelial integrity was assessed as the length of intact epithelium as a percentage of the total length of basement membrane. In 2 subjects with asthma and 1 normal control biopsies were either not obtained or were insufficient to quantify and 1 subject with asthma and 2 with eosinophilic bronchitis had a basement membrane length of <1mm.

## Statistical Analysis

Subject characteristics were described using descriptive statistics. Exhaled nitric oxide concentration, differential cell counts and epithelial integrity were expressed as medians (ranges). Basement membrane and lamina reticularis thickness was described as mean (SEM). Comparisons across 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 and by ANOVA and unpaired t-tests for parametric data. A value of  $p < 0.05$  was taken as being statistically significant. The number of subjects recruited was based on power calculations from previous findings on the repeatability of cell counts from bronchial biopsies, which found that approximately 15 subjects per group would be an optimal number (Richmond et al. 1996).

## Results

The subject characteristics are as shown (Table 3.5). The median exhaled nitric oxide concentration (ppb) was higher in eosinophilic bronchitis 12 and asthma 8.5 than normal controls 2 (95% CI of the difference 5, 16,  $p < 0.0001$  and 2, 11.3;  $p = 0.004$  respectively) (Table 3.5).

Sputum, bronchial wash (BW) and BAL differential inflammatory cell counts were as shown on table 3.6. Induced sputum, bronchial wash and BAL eosinophil counts were significantly higher in subjects with eosinophilic bronchitis (sputum 95% CI of the difference 4, 13.3%,  $p < 0.0001$ ; BW 1.1, 3.5%;  $p < 0.0001$ , BAL 0.25, 2.2%;  $p = 0.006$ ) and asthma (sputum 0.2, 5%,  $p = 0.01$ ; BW 0, 4.1%,  $p = 0.01$ ; BAL 0, 1.7%,  $p = 0.02$ ) compared to normal subjects. An example cytospin of a sputum and BAL eosinophilia is shown Figure 3.3. There were no differences in the other differential cell counts between groups (Table 3.6).

**Table 3.5** Subjects' clinical characteristics

	Eosinophilic Bronchitis	Asthma	Normal
Number	16	15	14
Age#	48 (3)	46 (4)	37 (5)
Male	10	8	8
Atopy	9	10	4
IgE (KU/l)#	90 (17)	106 (29)	36 (10)
Blood Eosinophil count (x10 <sup>9</sup> /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 <sub>20</sub> FEV <sub>1</sub> (mg/ml)	94 (18-128)	0.8 (0.16-4.6)	128 (16-128)
FEV <sub>1</sub> % predicted#	100 (2.6)	99 (3.2)	100 (3.7)
FEV <sub>1</sub> /FVC %#	80 (1.4)	72 (1.9)	79 (1.8)
Nitric oxide (ppb)^	12 (5-30)*	8.5 (2-32)*	2 (1-9)

# mean (SEM), ^median (range), \*p<0.01 Kruskal-Wallis test

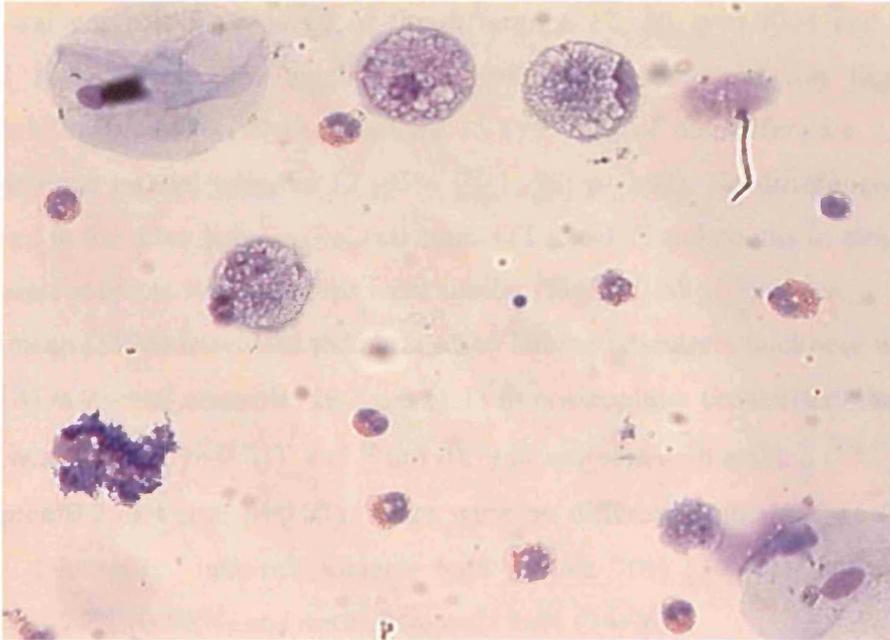
**Table 3.6** Median (range) (%) differential cell counts in sputum, bronchial wash and BAL

	Eosinophilic Bronchitis	Asthma	Normal
<b>Induced sputum</b>			
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)
<b>Bronchial wash</b>			
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 x10 <sup>6</sup>	0.5 (0.2-1.6)	0.5 (0-3)	0.5 (0.06-2.5)
<b>BAL</b>			
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 x10 <sup>6</sup>	6.5 (3-9)	6.2 (2-15)	6.7 (0.8-19)

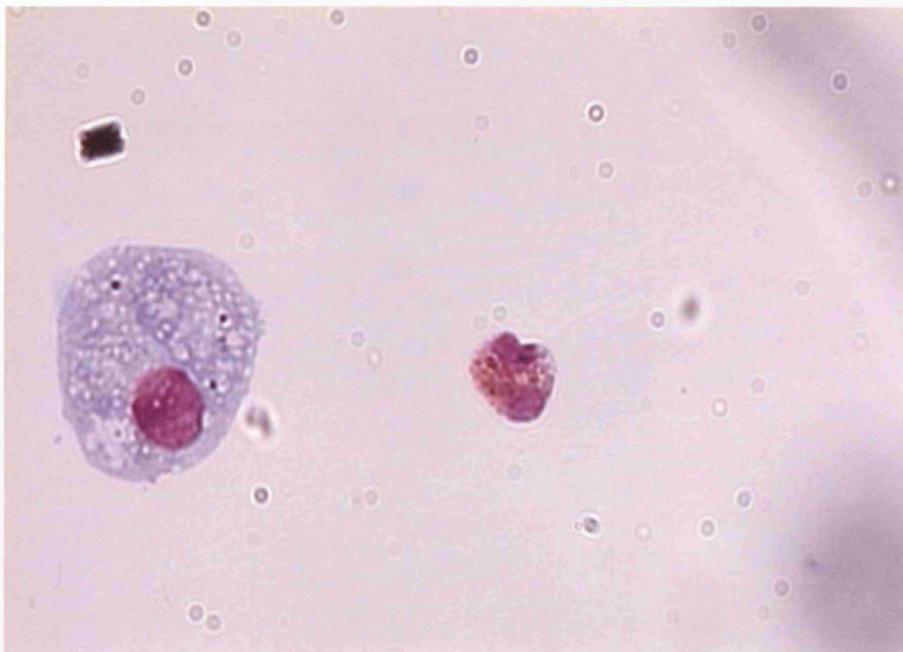
\*p<0.05, \*\*p<0.01 Kruskal-Wallis test

**Figure 3.3** An example of (a) sputum eosinophils (x200) and (b) BAL eosinophil (x400)  
Eosinophilic granules highlighted by red staining.

A



B



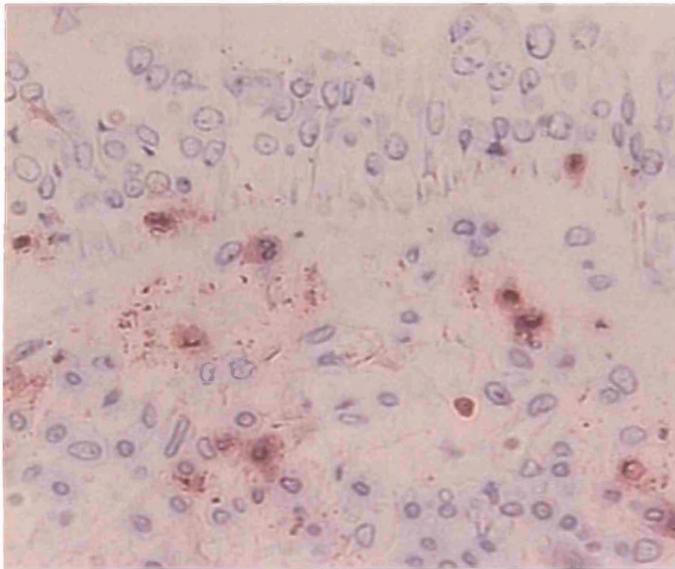
Examples of positive staining for eosinophils and neutrophils and reticular basement membrane thickening are shown Figure 3.4. The individual values for submucosal eosinophils (MBP+), neutrophils (NE+) and basement membrane width are as illustrated (Figure 3.5). The median MBP+ cells/mm<sup>2</sup> of submucosa were significantly higher in both eosinophilic bronchitis 32 and asthma 20 than in normal controls 8 (95% CI of the difference 12, 40; p=0.0004 and 4, 35; p=0.01 respectively). The median NE+ cells/mm<sup>2</sup> submucosa was higher in eosinophilic bronchitis 20 than asthma 15 (95% CI of the difference 0.2, 29; p=0.046) and normal controls 12 (95% CI 1, 28; p=0.02). No differences were observed in the other submucosal cell counts (Table 3.7) and counts in atopic and non-atopic subjects within groups were similar (Figure 3.5).

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

Repeatability of cell counts and reticular lamina thickening are shown in Appendix II.

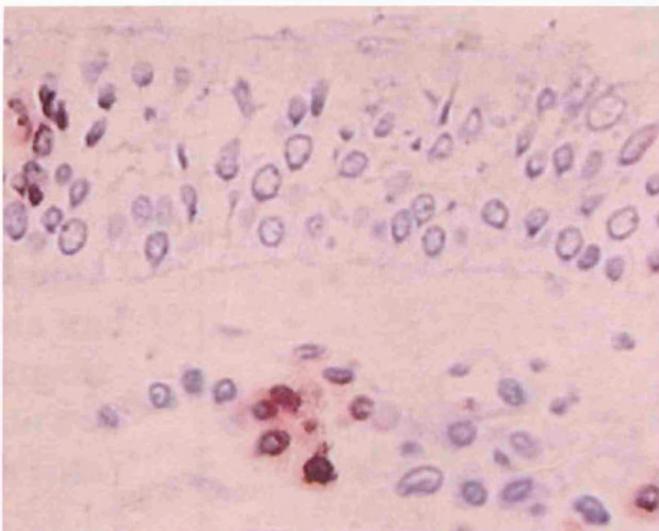
**Figure 3.4** Examples of submucosal staining for (a) eosinophils x100 and (b) neutrophils x100 from two subjects with eosinophilic bronchitis and (c) an eosinophil in a vessel which is excluded for quantification of cells in the submucosa. Note reticular basement membrane thickening in (a) and (b).

A

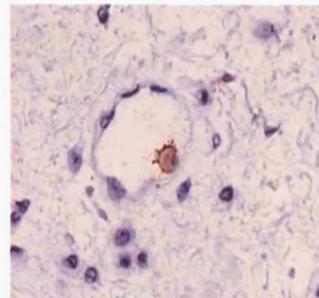


↑ Epithelium  
↓ Reticular lamina  
↓ basement membrane  
↑ Lamina propria submucosa

B



C





**Table 3.7** Median (range) other submucosal cell counts/mm<sup>2</sup>

	Eosinophilic Bronchitis	Asthma	Normal
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)
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)

\*p<0.05, \*\*p<0.01 Kruskal-Wallis test

## **Discussion**

This is the first study to investigate in detail the airway mucosal immunopathology of patients with eosinophilic bronchitis compared to asthmatic subjects and normals. We found a sputum, bronchial wash and BAL eosinophilia and bronchial submucosal evidence of eosinophilic airway inflammation and basement membrane thickening in subjects with eosinophilic bronchitis to the same degree as those with asthma. Thus, the immunopathology of eosinophilic bronchitis is very similar to asthma implying that these pathological changes are not important in the genesis of the disordered airway physiology observed in asthma.

Our findings add to a growing body of evidence questioning a direct causal association between eosinophilic airway inflammation and airway responsiveness in asthma. In a recent review it has been shown that 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 (Wardlaw et al 2000). Furthermore early studies with anti interleukin (IL)-5 antibodies have shown 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 (Leckie et al 2000). These observations suggest that either there is an important component of airway hyperresponsiveness in asthma that is independent of airway inflammation or that there are other functionally important aspects of the inflammatory response that, although closely linked to eosinophilic airway inflammation, can be disassociated from it.

Previous immunopathological studies of asthma have reported thickening of the sub-epithelial collagen layer, increased numbers of epithelial cells in the bronchial wash and a reduction in epithelial integrity in bronchial biopsies (Wardlaw et al 2000). Bronchial epithelial cells are activated in asthma as reflected by increased inducible nitric oxide synthetase (INOS) expression and elevated nitric oxide concentration in exhaled air (Kharitonov et al 1994). We observed thickening of the basement membrane and lamina reticularis in eosinophilic bronchitis and asthma and we confirmed that both conditions were associated with increased concentrations of exhaled nitric oxide (Berlyne et al 2000). There were no

differences in the number of epithelial cells in the bronchial wash or BAL between normal subjects and either disease groups and we found no differences in epithelial integrity suggesting that this is either not a consistent feature of asthma or that previous studies have identified an artefact, perhaps related to the biopsy technique (Ordonez et al. 2000). These findings demonstrate that structural abnormalities of the bronchial wall are a feature of both asthma and eosinophilic bronchitis, which suggests that together with eosinophilic airway inflammation they are not important in the development of disordered airway physiology in asthma.

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 demonstration 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 corticosteroids (Brightling et al 2000a) would be consistent with a causal association. There is some evidence that, like asthma, eosinophilic bronchitis is associated with an increased rate of decline in FEV<sub>1</sub> and the development of fixed airflow obstruction (Brightling et al 1999a) and it is possible that this important complication of chronic asthma is also related to eosinophilic airway inflammation.

We have previously reported that patients with eosinophilic bronchitis have increased concentrations of histamine and prostaglandin D<sub>2</sub> in their sputum when compared to subjects with mild asthma (Brightling et al 2000b) suggesting that mast cell activation is a particular feature of eosinophilic bronchitis. In support of this finding bronchial brushings have revealed a greater number of mast cells in eosinophilic bronchitis compared to asthma (Gibson et al 1998) and in this current study there was a non-significant trend towards more mast cells in the submucosa of patients with eosinophilic bronchitis. One possible explanation for these observations is that mast cells preferentially migrate to different sites with mast cells localised more superficially in the bronchial wall in eosinophilic bronchitis than asthma.

Neutrophil numbers were increased in the bronchial subepithelium in eosinophilic bronchitis compared to other groups. Bronchial submucosal neutrophilic inflammation is a feature of severe asthma (Wenzel et al. 1999) 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 neutrophilia (Brightling et al 2000b). Further work is required to investigate whether the difference in neutrophilic airway inflammation is functionally important.

In conclusion the main features of the immunopathology of eosinophilic bronchitis and asthma are very similar with both conditions characterised by eosinophilic airway inflammation and basement membrane thickening. This strongly suggests that eosinophilic airway inflammation, together with structural changes in the airway wall are regulated independently of airway hyperresponsiveness.

### **3.1.4 Th2 cytokine expression in bronchoalveolar lavage T-lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis**

#### **Abstract**

Asthma is characterised by variable airflow obstruction and airway hyperresponsiveness in association with airway inflammation under the influence of Th2 cytokines. Eosinophilic bronchitis has a similar immunopathology to asthma but without disordered airway physiology. Whether eosinophilic bronchitis is associated with increased expression of Th2 cytokines is unknown.

Th-2 cytokine expression from blood and bronchoalveolar lavage (BAL) T-cells and bronchial mucosal biopsies were assessed from subjects with eosinophilic bronchitis, asthma and normal controls.

The proportion of resting (stimulated) CD4 BAL T-cells expressing intracellular IL-4 was significantly higher in the subjects with eosinophilic bronchitis 7.2 (11.4)% and asthma 5.3 (5.5)% than normal controls 2.8 (3.9)% ( $p=0.03$ ). The number of cells/mm<sup>2</sup> of bronchial submucosa were significantly higher in the disease groups than normal controls for IL-4+ ( $p<0.001$ ) and IL-5+ ( $p=0.003$ ). Expression of intracellular IFN- $\gamma$  was significantly higher in stimulated blood CD8 T-cells from subjects with eosinophilic bronchitis 24% and asthma 17% than normal controls 5% ( $p=0.003$ ). There were no between group differences in expression of IFN- $\gamma$  in the BAL T-cells or in the bronchial submucosa.

These findings support the concept of asthma as a disease associated with activation of Th2 lymphocytes in the airway and provides evidence that these cytokines play a key role in the development of airway inflammation in eosinophilic bronchitis. This suggests that the release of Th2 cytokines is not sufficient for the elaboration of disordered airway physiology in asthma.

## **Introduction**

Asthma is a condition characterised by variable airflow obstruction and airway hyperresponsiveness in association with airway inflammation. Pathologically there is accumulation of eosinophils and CD4 lymphocytes, activation of the epithelium and smooth muscle, mucus hypersecretion, thickening of the sub-epithelial collagen layer, mast cell degranulation and smooth muscle hypertrophy and hyperplasia (Wardlaw et al 2000) (Kay 1996).

There is increasing evidence that the development and maintenance of the airway inflammation in asthma is due to T-lymphocyte activation with the production of Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 (Robinson 2000). These Th2 cytokines cause B cell switching to IgE, increased expression of VCAM-1 and P-selectin leading to selective eosinophil recruitment (Woltmann et al 2000) and mucus hypersecretion (Chung & Barnes 1999). However, the extent to which Th2 cytokine expression, eosinophilic airway inflammation and airway hyperresponsiveness are related is unclear.

A dissociation between airway inflammation and airway hyperresponsiveness is observed in patients with eosinophilic bronchitis a condition characterised by corticosteroid responsive cough and the presence of a sputum eosinophilia without airway hyperresponsiveness (Gibson et al 1989b) (Brightling et al 1999b) (Brightling et al 2000a). Studies suggest that the nature and state of activation of lower airway inflammation in asthma and eosinophilic bronchitis appear similar (Gibson et al 1998) (Brightling et al 2000b) (Berlyne et al 2000). Whether the airway inflammation in eosinophilic bronchitis is associated with activation of lymphocytes expressing Th2 cytokines or with expression of Th2 cytokines by other cells in the airway submucosa is unknown.

To address this question we have undertaken a comparative immunopathological study of the activation and cytokine expression of bronchoalveolar lavage (BAL) T-lymphocytes and Th-2 cytokine expression from bronchial mucosal biopsies from patients with eosinophilic bronchitis, symptomatic asthma and normal controls.

## Methods

### Subjects

Sixteen subjects with eosinophilic bronchitis, 12 with asthma, 14 normal controls were recruited from Glenfield Hospital outpatients, staff and local advertising. (The 16 subjects with eosinophilic bronchitis, 12 with asthma and 12 normal controls participated in the immunopathology study 3.1.3). The subjects' clinical characteristics are as shown (Table 3.8). Subjects with asthma gave a consistent history and had objective evidence of asthma as indicated by one or more of the following: 1) methacholine airway hyperresponsiveness ( $PC_{20} FEV_1 < 8 \text{ mg/ml}$ ); 2)  $>15\%$  improvement in  $FEV_1$  10 minutes after  $200 \mu\text{g}$  inhaled salbutamol or 3)  $>20\%$  maximum within day amplitude from twice daily peak expiratory flow (PEF) measurements over 14 days. The subjects with eosinophilic bronchitis had an isolated cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal PEF variability (maximum within day amplitude % mean  $<20\%$  over two weeks), a methacholine  $PC_{20} >16 \text{ mg/ml}$ , a normal chest radiograph and a sputum eosinophilia ( $>3\%$  non-squamous cell). Normal subjects were asymptomatic and had no evidence of variable airflow obstruction or airway hyperresponsiveness. All were current non-smokers with a past smoking history of less than 10 pack years. None of the subjects had taken inhaled or oral corticosteroids for at least six weeks prior to the study. The study was approved by the Leicestershire Ethics Committee and all patients gave their written informed consent. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

**Table 3.8** Subjects' clinical characteristics

	Eosinophilic Bronchitis	Asthma	Normal
Number	16	12	14
Age <sup>#</sup>	48 (3)	47 (5)	42 (5)
Male	10	6	8
Atopy	10	8	4
PC <sub>20</sub> FEV <sub>1</sub> <sup>^</sup>	94 (18-128)	1.2 (0.16-4.6)	64 (16-128)
FEV <sub>1</sub> % predicted <sup>#</sup>	100 (2.6)	100 (3.1)	100 (3.6)
FEV <sub>1</sub> /FVC % <sup>#</sup>	80 (1.4)	72 (2.3)	80 (1.9)

<sup>^</sup> median (range)

<sup>#</sup> mean (SEM)

### Protocol and clinical measurements

Subjects attended on two occasions. At the first visit we measured spirometry, allergen skin prick tests and methacholine airway responsiveness. This was followed on recovery by a sputum induction. Spirometry was performed using a dry bellows spirometer (Vitalograph, Buckingham, UK) with the FEV<sub>1</sub> recorded as the best of successive readings within 100ml (Methods 2.1.2). Allergen skin prick tests were performed to *Dermatophagoides pteronyssinus*, cat fur, grass pollen and *Aspergillus fumigatus* solutions with normal saline and histamine controls (Bencard, Newark, Notts., UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control (Methods 2.1.1). The methacholine challenge was performed using the Tidal breathing method with doubling concentrations of methacholine (0.03 to 128mg/ml) nebulised via a Wright nebuliser (Juniper, Cockcroft, & Hargreave 1994) (Methods 2.1.3). Sputum was induced and processed as previously described (Pavord et al 1997) (Methods 2.1.4, 2.2.1.2).

At the second visit the subjects underwent bronchoscopy using an Olympus fibre-optic bronchoscope (Olympus Company, Tokyo, Japan) in line with the

most recent BTS guidelines (British Thoracic Society 2001) (Methods 2.1.5). Bronchial mucosal biopsies were taken from the right middle and lower lobe carinae. Peripheral blood mononuclear cells (PBMC) from a 20ml venous blood sample and cells recovered from a 180ml bronchoalveolar lavage (BAL) of pre-warmed normal saline into the middle lobe were analysed from a subgroup of 10 subjects with eosinophilic bronchitis, 9 with asthma and 10 normal controls.

Mucosal biopsies were immediately transferred into ice-cooled acetone containing the protease inhibitors iodoacetamide (20mM) and PMSF (2mM) for fixation, stored at -20°C for 24h, and then processed into the water soluble resin, glycolmethacrylate (GMA) (Polysciences, Northampton, UK) for embedding (Methods 2.2.4.1).

### **Immunohistochemistry**

Two-micrometer sections were cut, floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The following mouse IgG<sub>1</sub> monoclonal antibodies were used: IL-4 (3H4) (AMS Biotechnology, Abingdon, UK), IL-4 (4D9) (AMS Biotechnology, Abingdon, UK), IL-5 (gift from GlaxoSmithKline, Harlow, UK), IFN- $\gamma$  (R&D Systems, Abingdon, UK). The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993) (Methods 2.2.4.2).

### **Assessment and quantification of immunohistochemical staining**

Sub-epithelial mucosa was identified morphologically and the area calculated using a computer analysis system (Scion Image, Maryland, USA). Nucleated immunostained cells present in coded sections were enumerated in the submucosa and numbers of cells expressed as the number/mm<sup>2</sup> of submucosa by an experienced-blinded observer.

### **Flow cytometry of surface receptor expression and intracellular cytokines**

The peripheral blood mononuclear cell (PBMC) fraction was obtained by centrifugation on Ficoll. After washing with FACS buffer (PBS, 0.5%BSA) PBMC and BAL cells were divided for analysis of surface receptor expression and intracellular cytokine staining.

For surface receptor expression cells were resuspended in FACS buffer at a concentration of between 0.5 and  $1 \times 10^6$ /ml depending on the number of cells available. Non-specific antibody binding was blocked using mouse IgG (Sigma, Poole, Dorset, UK). BAL cells and PBMC were stained with directly conjugated monoclonal antibodies against CD3-RPE (Dako, Ltd), CD4-PerCP (BD, Cowley, Oxford, UK) and to CD8 (Dako, Ltd.), activation markers CD25 (Dako, Ltd.), HLA-DR (Dako, Ltd.) and CD49a (Serotec, Oxford, UK) and chemokine receptors CCR3 (gift from Millenium, USA), CCR5 (BD), CCR6 and C-X-CR3 (R&D Systems) indirectly labelled with FITC with appropriate isotypic controls. Lymphocytes were gated for CD3 expression and then further subdivided by CD4 or CD8 expression and were analysed by three-colour flow cytometry on a FACScan (BD).

The remaining cells were either stimulated with phorbol 12-myristate 13-acetate (PMA) (5ng/ml) (Sigma, Poole, Dorset, UK), calcium ionophore (250ng/ml) (Sigma) and Brefeldin A (10 $\mu$ g/ml) (Sigma) or incubated in culture medium alone (resting) for 4hr at 37°C. The cells were fixed in 4% paraformaldehyde (Sigma) and stored overnight at 4°C in PBS, 0.5% BSA. The following day the cells were washed and incubated with CD3-FITC/RPE (Dako, Ltd.) and CD8-PerCP (BD), as CD4 becomes internalise after fixation, permeabilised in 4% paraformaldehyde, 0.1% saponin (Sigma) for 15 min on ice and labelled with IL-4-RPE (BD) and IFN- $\gamma$ -FITC (BD) or isotypic controls and analysed by three-colour flow-cytometry as for the surface receptor expression (Methods 2.2.1.4).

### **Statistical Analysis**

Subject characteristics were described using descriptive statistics. The number of submucosal cells expressing cytokines and the proportion of PBMC and BAL lymphocytes expressing chemokine receptors, activation markers and intracellular cytokines were expressed as median (range). Comparisons across the three groups were undertaken using the Kruskal-Wallis test and between groups using the Mann-Whitney test. A value of  $p < 0.05$  was taken as being statistically significant. The number of subjects recruited was based on power calculations from previous

findings on the repeatability of cell counts from bronchial biopsies, which found that approximately 15 subjects per group would be an optimal number (Richmond et al. 1996).

## Results

Examples of positive submucosal staining for IL-4, IL-5 and IFN- $\gamma$  are as shown (Figure 3.6). The individual values for the number of cells expressing IL-4 (3H4, 4D9), IL-5 and IFN- $\gamma$  in the submucosa are as shown (Figure 3.7).

The median cells/mm<sup>2</sup> submucosa were significantly higher in the subjects with eosinophilic bronchitis and asthma than normal controls for IL-4 (3H4)+ (6.5, 7.6, 0.5 respectively;  $p < 0.001$ ), IL-4 (4D9)+ (8.2, 9.6, 1.5 respectively;  $p < 0.001$ ) and IL-5+ (6.3, 6.4, 1.1 respectively;  $p = 0.003$ ). There were no differences seen in IL-4, 5 expression between those subjects with asthma or eosinophilic bronchitis. There were no between group differences in the median IFN- $\gamma$ + cells/mm<sup>2</sup> submucosa in eosinophilic bronchitis (3.9), asthma (1.9), normal control (2.4) ( $p = 0.8$ ). Counts in atopic and non-atopic subjects within groups were similar.

PBMC expression of intracellular IFN- $\gamma$  was increased in those subjects with eosinophilic bronchitis and asthma compared to normals in stimulated CD3+CD8+ ( $p = 0.003$ ) and CD3+CD8- cells ( $p = 0.024$ ) (Table 3.9). There were no differences in IFN- $\gamma$  expression between the disease groups in resting cells and no between group differences in IL-4 expression in resting or stimulated PBMC cells (Table 3.9). Example dot-plots illustrating expression in resting and stimulated cells of IFN- $\gamma$  in PBMC and IL-4 in BAL cells for each subject group are shown figure 3.8 and 3.9.

Expression of intracellular IL-4 by CD3+CD8- BAL cells was increased in eosinophilic bronchitis and asthma compared to normal controls in both the resting cells (7.2, 5.3, 2.8 respectively;  $p = 0.03$ ) and stimulated cells (11.4, 5.5, 3.9 respectively;  $p = 0.03$ ) (Figure 3.10). The median (range) of the proportion (%) of CD3+CD8+ BAL cells expressing intracellular IL-4 was significantly higher in eosinophilic bronchitis and asthma than normal controls in resting cells 6.1 (1.2-13), 5.3 (1.5-12.8), and 2.3 (0-5) ( $p = 0.017$ ) and stimulated cells 8 (3-16),

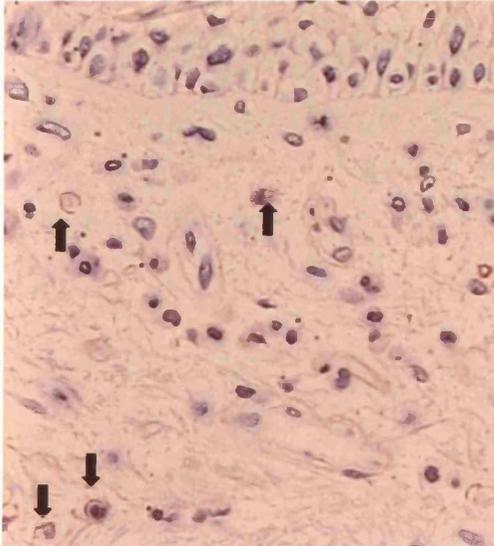
7.8 (3.6-17), and 3.8 (0-18) ( $p=0.05$ ) respectively. Intracellular IFN- $\gamma$  expression in CD3+ BAL cells markedly increased after stimulation compared with resting cells, but there were no between group differences in the proportion of BAL cells expressing intracellular IFN- $\gamma$ . Counts in atopic and non-atopic subjects within groups were similar (figure 3.10).

The median (range) of the proportion of CD3+ BAL cells expressing CD4 was similar in those subjects with asthma 57.5 (45-77), eosinophilic bronchitis 55(20-76) and normal controls 55.5 (36-78) ( $p=0.9$ ). No differences were seen in the proportion of CD3+CD4+ or CD3+CD4- cells expressing activation or chemokine receptors between the groups in PBMC (data not shown) and BAL cells (Table 3.10).

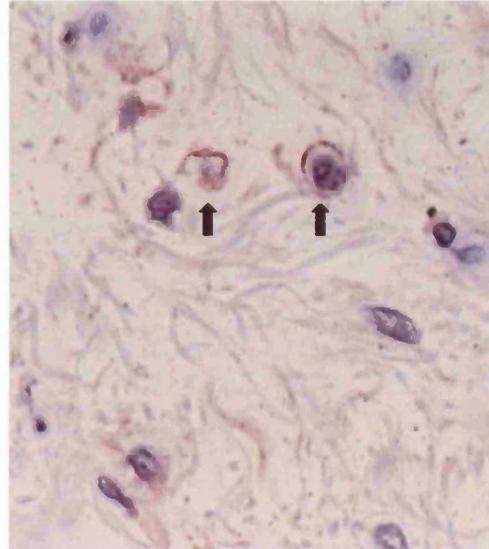
Repeatability of cell counts are shown in Appendix II.

**Figure 3.6a** Examples of submucosal staining for IL-4 (a) 3H4 and (b) 4D9; i) x200, ii) x400 from a subject with asthma and eosinophilic bronchitis. (Arrows highlight positive staining)

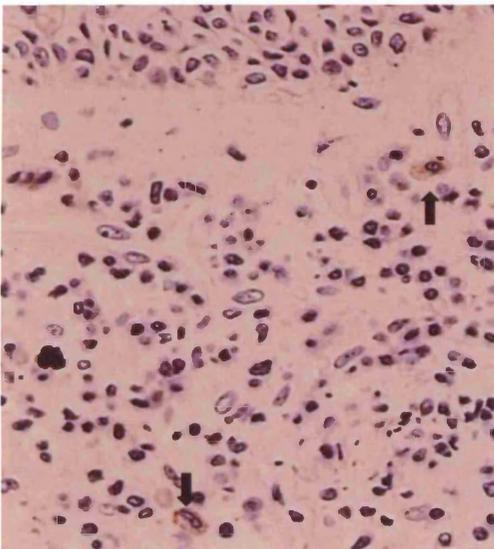
A i



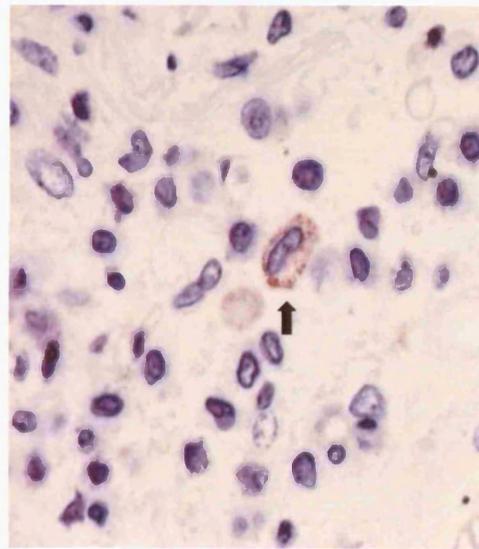
ii



B i

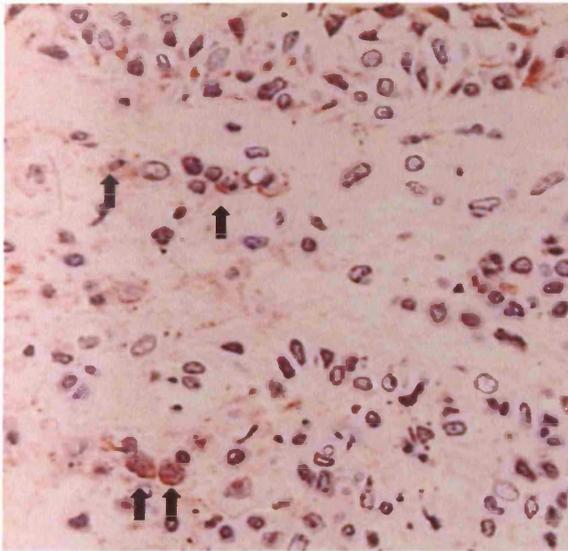


ii

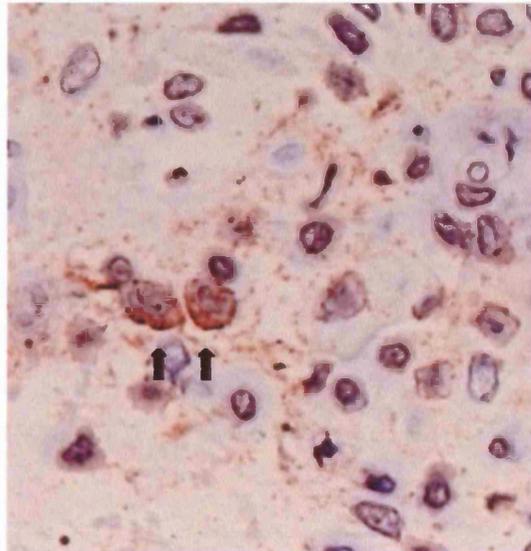


**Figure 3.6b** Examples of submucosal staining for (a) IL-5 (b) IFN- $\gamma$ ; i) x200, ii) x400 from a subject with asthma and eosinophilic bronchitis . (Arrows highlight positive staining)

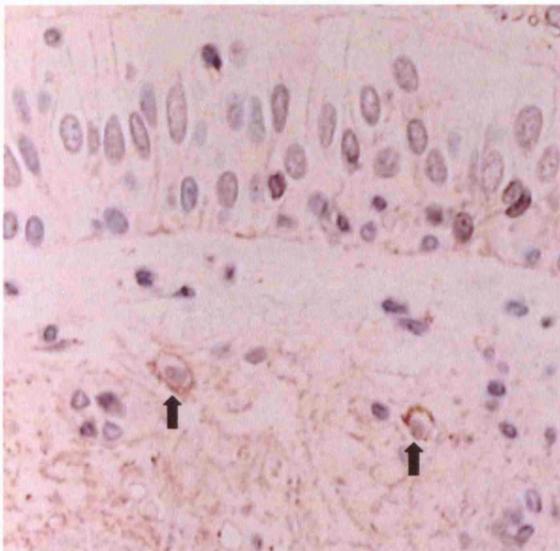
A i



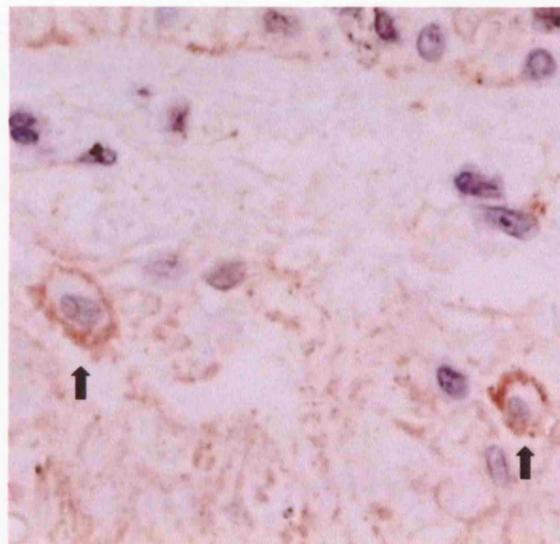
ii



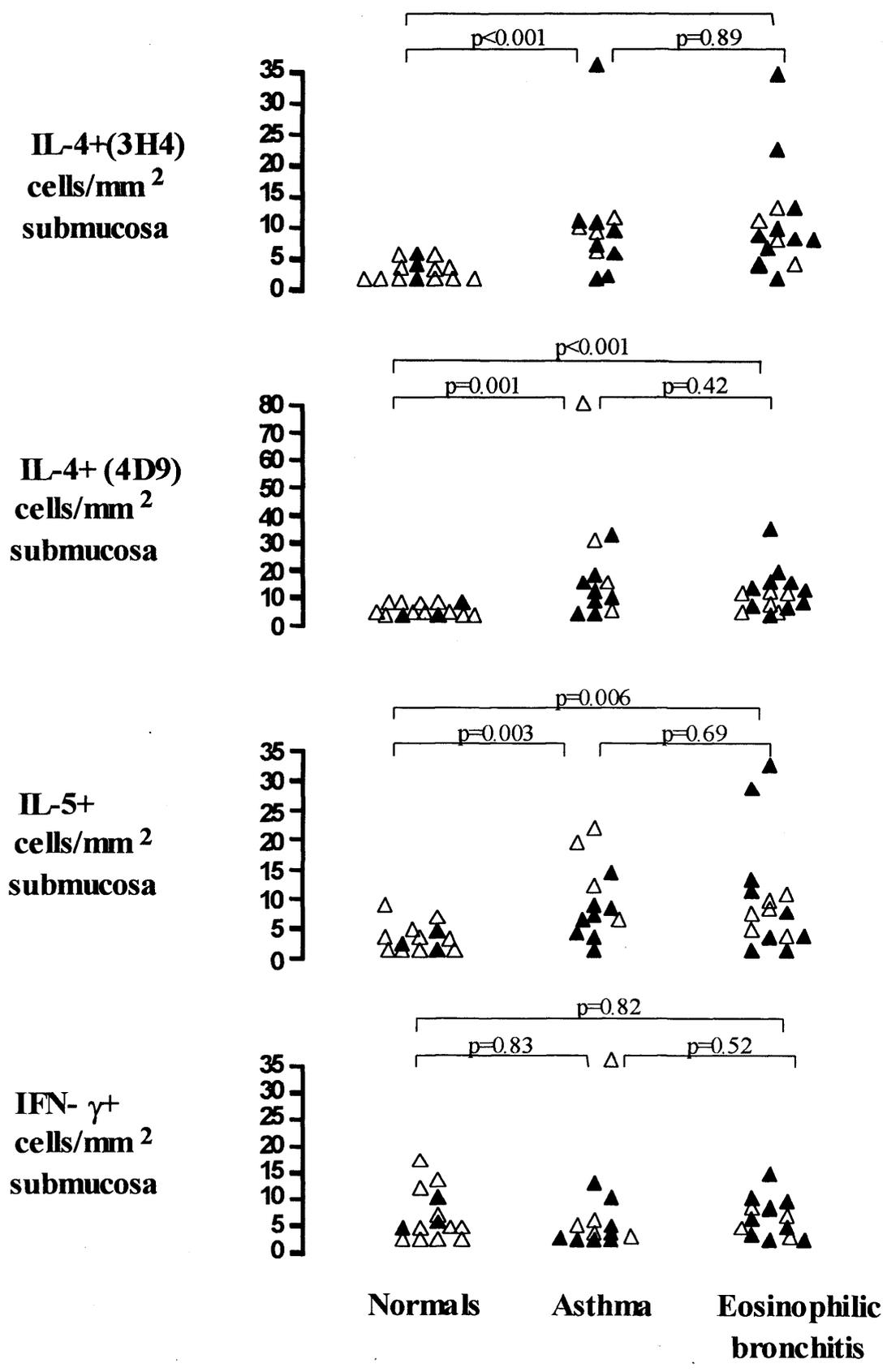
B i



ii



**Figure 3.7** Submucosal cell counts/mm<sup>2</sup> expressing IL-4 (3H4 and 4D9), IL-5 and IFN- $\gamma$  in subjects with asthma, eosinophilic bronchitis (EB) and normal controls  
 Closed triangles atopic, open triangles non-atopic.  
 P values derived by Mann-Whitney test  $p < 0.001$

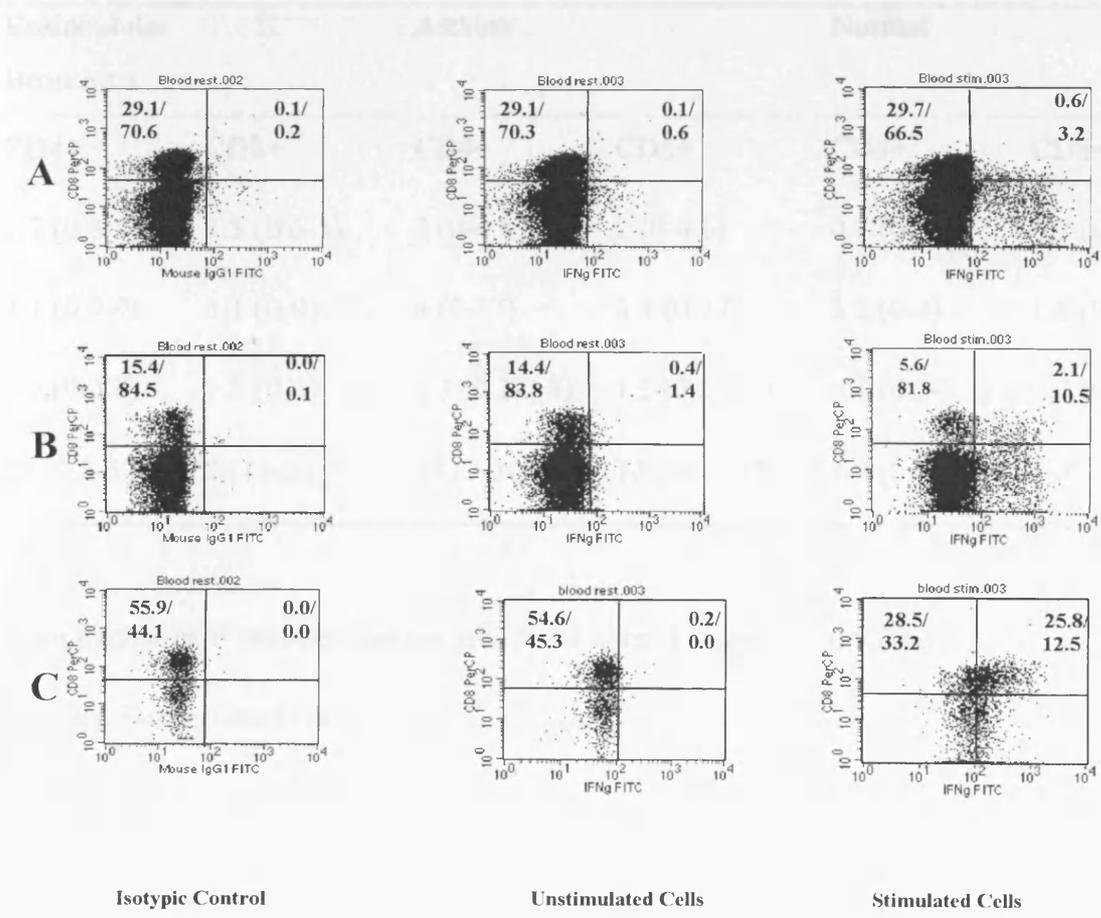


The median cells/mm<sup>2</sup> submucosa were significantly higher in the subjects with eosinophilic bronchitis and asthma than normal controls for IL-4 (3H4)+ (6.5, 7.6, 0.5 respectively; p<0.001), IL-4 (4D9)+ (8.2, 9.6, 1.5 respectively; p<0.001) and IL-5+ (6.3, 6.4, 1.1 respectively; p=0.003). There were no differences seen in IL-4, 5 expression between those subjects with asthma or eosinophilic bronchitis. There were no between group differences in the median IFN- $\gamma$ + cells/mm<sup>2</sup> submucosa in eosinophilic bronchitis (3.9), asthma (1.9), normal control (2.4) (p=0.8). Counts in atopic and non-atopic subjects within groups were similar.

PBMC expression of intracellular IFN- $\gamma$  was increased in those subjects with eosinophilic bronchitis and asthma compared to normals in stimulated CD3+CD8+ (p=0.003) and CD3+CD8- cells (p=0.024) (Table 3.9). There were no differences in IFN- $\gamma$  expression between the disease groups in resting cells and no between group differences in IL-4 expression in resting or stimulated PBMC cells (Table 3.9). Example dot-plots illustrating expression in resting and stimulated cells of IFN- $\gamma$  in PBMC and IL-4 in BAL cells for each subject group are shown figure 3.8 and 3.9.

Expression of intracellular IL-4 by CD3+CD8- BAL cells was increased in eosinophilic bronchitis and asthma compared to normal controls in both the resting cells (7.2, 5.3, 2.8 respectively; p=0.03) and stimulated cells (11.4, 5.5, 3.9 respectively; p=0.03) (Figure 3.10). The median (range) of the proportion (%) of CD3+CD8+ BAL cells expressing intracellular IL-4 was significantly higher in eosinophilic bronchitis and asthma than normal controls in resting cells 6.1 (1.2-13), 5.3 (1.5-12.8), and 2.3 (0-5) (p=0.017) and stimulated cells 8 (3-16), 7.8 (3.6-17), and 3.8 (0-18) (p=0.05) respectively. Intracellular IFN- $\gamma$  expression in CD3+ BAL cells markedly increased after stimulation compared with resting cells, but there were no between group differences in the proportion of BAL cells expressing intracellular IFN- $\gamma$ . Counts in atopic and non-atopic subjects within groups were similar (figure 3.10).

**Figure 3.8** Example dot-plots from an individual normal control (A) a subject with asthma (B) and eosinophilic bronchitis (C) for IFN  $\gamma$  intracellular cytokine staining and isotypic control in resting and stimulated PBMC T-lymphocytes.



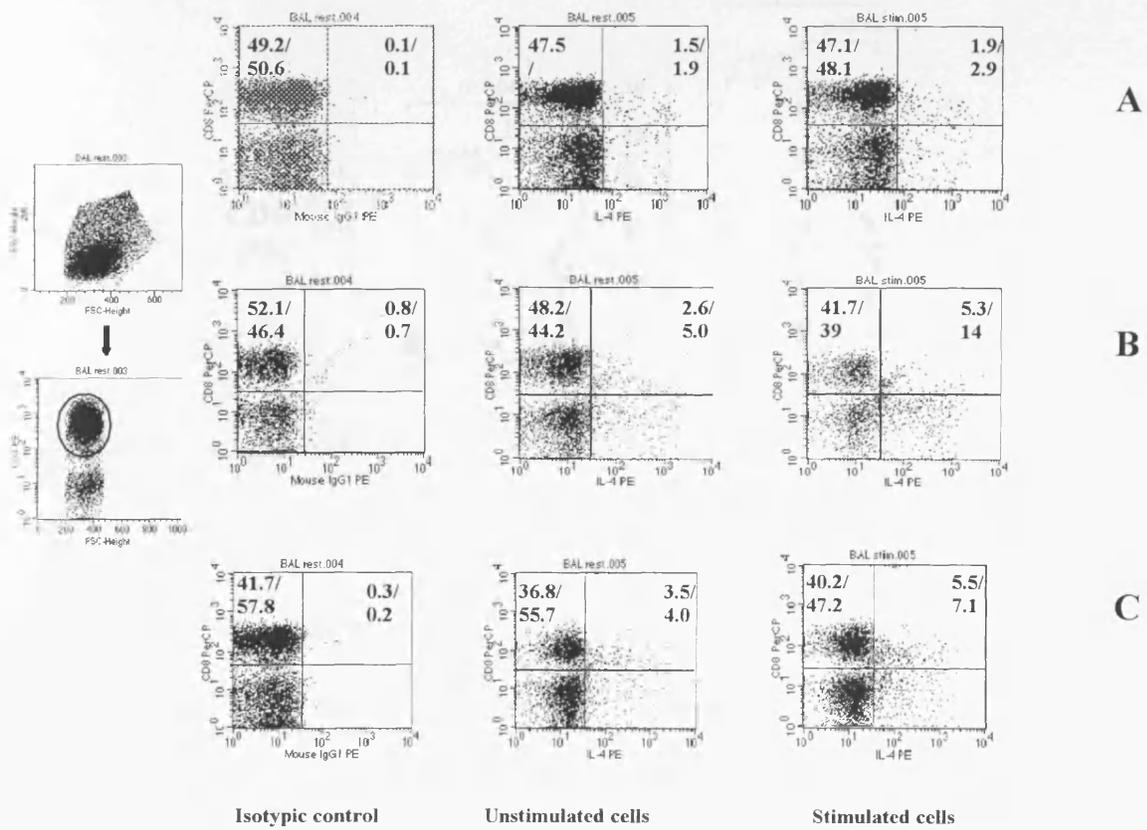
**Table 3.9** Median (range) of the proportion (%) of CD4+ (CD3+CD8-) and CD8+ (CD3+CD8+) peripheral blood T-lymphocytes cells expressing intracellular cytokine staining

	Eosinophilic Bronchitis		Asthma		Normal	
	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
IL-4 R	1.7 (0.8-5)	1.3 (0.6-5)	2 (0-4.6)	2 (0-4.6)	0.7 (0-3.6)	0.7 (0-3.6)
IL-4 S	3.1 (0.9-9)	3.1 (0-9)	4 (0-17)	3.4 (0-17)	2.2 (0-4)	1.8 (0-3.8)
IFN- $\gamma$ R	0.9 (0-5.8)	1.5 (0-6)	1.3 (0.2-3.8)	1.3 (0.2-3.8)	0.7 (0.3-2.5)	0.7 (0.1-6)
IFN- $\gamma$ S	14 (2.8-52)*	24 (5-52)*	15 (7-30)*	17 (10-57)*	3.9 (0.1-12)	5 (0.1-12)

\*p<0.05 for comparison between disease group and normal control Mann-Whitney test

R-resting cells, S-stimulated cells.

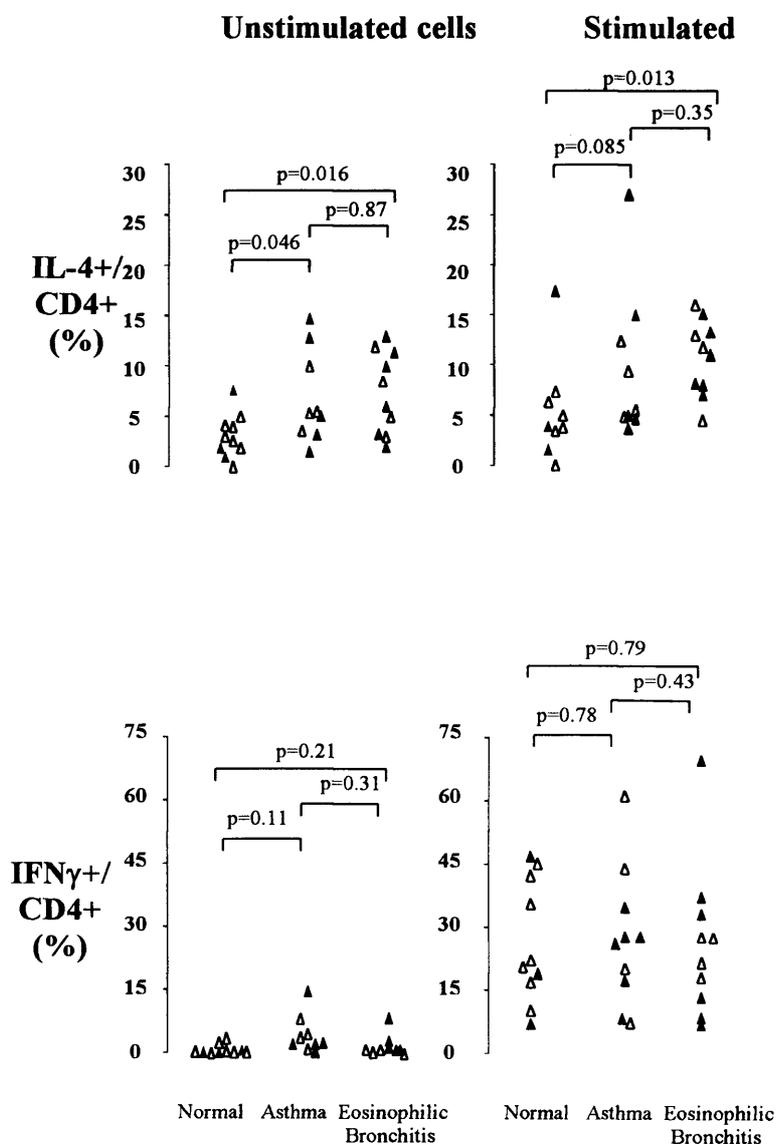
**Figure 3.9** Example dot-plots from an individual normal control (A) a subject with asthma (B) and eosinophilic bronchitis (C) for IL-4 intracellular cytokine staining and isotypic control in resting and stimulated BAL T-lymphocytes.



**Figure 3.10** Individual data for the proportion (%) of CD4 (CD3+CD8-) expressing intracellular IL-4 and IFN- $\gamma$  in resting and stimulated BAL T-lymphocytes.

Closed triangles atopic, open triangles non-atopic.

P values given for comparison between groups by Mann-Whitney test



**Table 3.10** Median (range) of the proportion (%) of CD4+ (CD3+CD4+) and CD8+ (CD3+CD4-) T-cells that express chemokine receptors and activation markers in bronchoalveolar lavage in subjects with eosinophilic bronchitis, asthma and normal individuals.

	EB		Asthma		Normal	
	CD4	CD8	CD4	CD8	CD4	CD8
CD25	36 (22-60)	20 (16-36)	38 (19-49)	21 (12-58)	50 (29-67)	25 (12-53)
CD49a	54 (30-78)	75 (60-86)	59 (42-95)	72 (60-90)	52 (12)	76 (23)
HLA-DR	87 (74-95)	81 (63-90)	79 (50-95)	71 (43-88)	72 (26)	66 (20)
CCR3	7 (3-43)	6 (5-48)	16 (3-54)	13 (3-43)	10 (3-23)	6 (3-20)
CCR5	94 (80-98)	93 (88-98)	94 (86-99)	94 (81-97)	92 (76-93)	94 (88-96)
CCR6	66 (50-78)	30 (25-46)	58 (38-94)	49 (14-89)	64 (30-86)	34 (16-46)
C-X-CR3	93 (78-98)	96 (92-98)	91 (53-98)	87 (17-99)	87 (43-94)	95 (80-98)

## Discussion

This study has made three important and novel observations. Firstly, we have demonstrated that there is increased constitutive expression of IL-4 protein, the classical Th2 cytokine, in T cells from the airways of asthmatics, supporting the concept of asthma as a disease associated with activation of Th2 lymphocytes. Secondly, we have shown that eosinophilic bronchitis is a disease characterised by increased expression of Th2 cytokines demonstrating a dissociation between T cell activation and the abnormalities in airway physiology that characterise asthma. Thirdly, we have shown that both asthma and eosinophilic bronchitis are characterised by priming of peripheral blood T cells for cytokine release after stimulation with PMA and ionomycin, suggesting there is a systemic T cell abnormality in these diseases.

The concept of asthma as a disease associated with activation of Th2 cells was proposed in the early 1990's (Romagnani S 1997). This hypothesis was supported by the observation using in situ hybridization, that up to 50% of BAL T cells from asthmatics, but very few from normal subjects, expressed mRNA for IL-4 with no difference between asthmatics and non-asthmatics in mRNA expression for IFN $\gamma$  (Robinson et al 1992). Although the concept of asthma as a Th2 disease is now widely supported there is surprisingly little data showing that T cells in asthma are producing increased amount of Th2 cytokines. Indeed Krug *et al* (Krug et al 1996), using intracellular flow cytometry found an increase in IFN $\gamma$  but not IL-5 or IL-4 expression by BAL T cells from asthmatics after stimulation with PMA or ionomycin. They observed that only a mean of about 2% of cells expressed IL-4 even after stimulation, raising the possibility that the increase in mRNA expression seen by in situ hybridization was not being translated into protein. Increased amounts of IL-4 and IL-5 expression have been found by immunohistochemistry in asthma, but this is mainly present in mast cells and eosinophils (Bradding et al 1994) (Moller et al 1996) (Nonaka M et al 1995). The current evidence for asthma being a Th2 disease is therefore limited.

Our study provides direct support for this hypothesis by demonstrating constitutive expression of IL-4 by a small percentage of both CD4 and CD8 cells in the airway lumen. This represented at most about 10% of the total CD3 population. Virtually no cells were constitutively expressing IFN $\gamma$  in any of the

groups although IFN $\gamma$  producing cells were easily the most frequent type of T cell after polyclonal stimulation in all three groups with no significant difference between them. Although at odds with the levels of mRNA expression seen in earlier studies it seems to us plausible that Th1 and Tc1 cells, presumably directed against respiratory viruses and bacteria, will make up the majority population of T cells in the adult lung and that allergen specific cells making Th2 cytokines will be in a minority. The crucial difference appears to be that in asthma the Th2 cells are actively secreting mediators whereas the Th1 cells are quiescent. It is of interest that we found increased numbers of IL-4 secreting T-cells in non-atopic as well as atopic subjects supporting the idea of intrinsic asthma as a Th2 associated condition (Humbert et al. 1996). Similar to previous studies we found increased expression of IL-4 and IL-5 but not IFN $\gamma$  in the airway mucosa, but we also found that the majority of these cells were mast cells and eosinophils (data not shown).

Eosinophilic bronchitis like asthma is characterised by eosinophilic airway inflammation, but unlike asthma there is no AHR or bronchoconstriction. Although it is a condition of interest in its own right, one of the reasons for studying the immunopathology of eosinophilic bronchitis was the hope that it would shed light on the specific pathological features that define the asthma phenotype. Perhaps the most interesting and important aspect of this study is our demonstration of dissociation between the activation of Th2 lymphocytes and the asthma phenotype. The observation that Th2 cells are also present in eosinophilic bronchitis strongly suggests that this disordered immunology, while plausibly causing an airway eosinophilia, cough and mucus hypersecretion, is not directly responsible for AHR and bronchoconstriction. In this and other recent reports it has been shown that the immunopathology of asthma and eosinophilic bronchitis are almost identical (Brightling et al 2000b) (Gibson et al 1998) (3.1.2, 3.1.3).

Another intriguing finding of our study was the increased expression of IFN $\gamma$  in stimulated peripheral blood T cells in both asthma and eosinophilic bronchitis. This has been previously noted by Magnan *et al* (Magnan et al 2000) where it was seen principally in the CD8 population. In contrast in our study it occurred in both CD4 and CD8 cells. In fact there was also the suggestion of an increase

in the IL-4 secreting T cells but our study was under-powered to investigate this definitively. In the study by Magnan *et al* (Magnan et al 2000) the increase in IL-4 producing cells was seen in the atopic subjects irrespective of whether they had asthma. At first sight there appears to be a discrepancy between constitutive activation of Th2 cells in the airway in asthma and eosinophilic bronchitis and increased numbers of T cells expressing IFN $\gamma$  in the blood. However in the blood the difference between subjects with airway inflammation and normal controls was seen only after stimulation, whereas in the airway we saw evidence of T cells constitutively producing IL-4. This suggests that increased IFN $\gamma$  production is due to a priming effect on peripheral blood T cells and is perhaps consistent with a systemic inflammatory process, which is present in subjects with mild disease and no obvious systemic features.

Against the concept of there being primed T cells in the blood of asthmatics we found no difference in the expression of activation markers on T cells between the three groups either in the blood or airway. One possible explanation is that these markers are not very sensitive. We also found no difference in the expression of chemokine receptors between the three groups. Indeed as we reported previously (Campbell et al 2000) high levels of expression of the putative Th1 chemokine receptors CCR5 and CXCR3 and low levels of expression of the putative Th2 chemokine receptor CCR3 were seen in the asthmatic as well as the eosinophilic bronchitis patients. However as the Th2 cells only make up about 10% of the BAL T cell population it is still possible that there are differences in chemokine receptor expression between the two T cell phenotypes which our techniques are not sufficiently discriminating to detect.

In summary therefore we have shown that eosinophilic bronchitis like asthma is a disease characterised by activation in the airways of Th2 lymphocytes. However it seems that while Th2 mediated cytokines are closely linked to eosinophilic inflammation they are not directly associated with the hallmarks of the asthmatic phenotype, airway hyperresponsiveness and variable airflow obstruction.

### **3.1.5 Mast cell infiltration of airway smooth muscle characterises the asthmatic phenotype**

#### **Abstract**

In asthma the extent to which disordered airway physiology and airway inflammation are related remains unclear. A lack of association is exemplified in eosinophilic bronchitis, a condition characterised by chronic cough and a sputum eosinophilia without variable airflow obstruction or airway hyperresponsiveness. In both diseases there is on-going mast cell activation, but interestingly patients with eosinophilic bronchitis have the highest sputum supernatant concentrations of histamine and prostaglandin D<sub>2</sub>. We therefore hypothesised that the microlocalisation of mast cells within the airway smooth muscle is an important factor determining the asthmatic phenotype.

We have undertaken a comparative immunohistochemical study in bronchial biopsies from 17 subjects with asthma, 13 with eosinophilic bronchitis and 11 normal controls recruited from two centres.

Both disease groups had a similar degree of submucosal and epithelial eosinophilia and reticular lamina thickening. In contrast, there was a striking increase in the number of tryptase<sup>+</sup> mast cells in the airway smooth muscle bundles of patients with asthma (median 5.1, range 0-33.3 mast cells/mm<sup>2</sup> of smooth muscle) compared to both eosinophilic bronchitis (median 0, range 0-4.8 mast cells /mm<sup>2</sup>) and normal controls (median 0, range 0-6.4 mast cells/mm<sup>2</sup>) ( $p < 0.0001$ ). There was a significant inverse correlation between the number of airway smooth muscle mast cells and the PC<sub>20</sub> methacholine in the subjects with asthma ( $r_s = -0.5$ ,  $p = 0.03$ ). In contrast T cells and eosinophils were not usually seen in the airway smooth muscle with no differences between groups.

These observations support the hypothesis that airway smooth muscle infiltration by mast cells is important in the pathogenesis of asthma .

#### **Introduction**

Asthma is a condition characterised by variable airflow obstruction and airway hyperresponsiveness in association with airway inflammation that usually has an eosinophilic component. Pathologically, in addition to accumulation of eosinophils

and CD4+ lymphocytes, there is activation of the epithelium and smooth muscle, mucus hypersecretion, thickening of the sub-epithelial collagen layer and submucosal matrix deposition, mast cell degranulation and smooth muscle hypertrophy and hyperplasia (Wardlaw et al 2000).

The extent to which airway inflammation and airway hyperresponsiveness are related remains controversial (Brusasco, Crimi, & Pellegrino 1998). A dissociation between airway inflammation and airway hyperresponsiveness can be clearly observed in patients with eosinophilic bronchitis (Gibson et al 1989b) (Brightling & Pavord 2000); a condition characterised by corticosteroid responsive cough and the presence of a sputum eosinophilia occurring in the absence of variable airflow obstruction or airway hyperresponsiveness (Brightling et al 1999b). The limited studies so far undertaken suggest that the nature and state of activation of lower airway inflammation in asthma and eosinophilic bronchitis are similar (Gibson et al 1998) (Brightling et al 2000b), although patients with eosinophilic bronchitis are reported to have higher concentrations of histamine and prostaglandin D<sub>2</sub> in their sputum when compared to asthma (Brightling et al 2000b). This finding has led us to hypothesise that there may be differences in the localisation of mast cells within the airway wall between the two conditions, specifically that in asthma there is an increase in the number of mast cells in the airway smooth muscle which is functionally linked to the development of airway hyperresponsiveness.

To test our hypothesis we have undertaken a comparative immunohistochemical study in bronchial mucosal biopsies obtained from symptomatic asthmatic subjects, patients with eosinophilic bronchitis and normal controls.

## **Methods**

### **Subjects**

Subjects were recruited from two centres. From Leicester 15 subjects with asthma, 16 with eosinophilic bronchitis and 14 normal controls and from Southampton 15 subjects with asthma and 8 normal controls were recruited. In total assessable airway smooth muscle was present in biopsies from 8 subjects with asthma, 13 with eosinophilic bronchitis and 8 normal controls from Leicester and from 9 subjects with asthma and 3 normal controls from Southampton. (The subjects from Leicester were included in the studies 3.13, 3.14, 3.15). The subject characteristics are as shown (table 3.11).

**Table 3.11** Subjects' clinical characteristics

	Eosinophilic bronchitis	Asthma	Normal
Number	13	17	11
Age <sup>^</sup>	45 (3)	40 (4)	33 (5)
Male	8	12	9
Atopy	7	14	2
PC <sub>20</sub> FEV <sub>1</sub> <sup>#</sup>	86 (18-128)	0.5 (0.03-4.6)	48 (16-128)
FEV <sub>1</sub> % predicted <sup>^</sup>	101 (3)	100 (4)	102 (5)

<sup>^</sup>Mean (SEM), <sup>#</sup>median (range)

Subjects with asthma gave a suggestive history and had objective evidence of variable airflow obstruction as indicated by one or more of the following: 1) methacholine airway hyperresponsiveness (PC<sub>20</sub> FEV<sub>1</sub><8mg/ml); 2) >15% improvement in FEV<sub>1</sub> 10 minutes after 200µg inhaled salbutamol or 3) PEF (>20% maximum within day amplitude from twice daily PEF measurements over 14 days). The subjects with eosinophilic bronchitis had a persistent isolated cough, no symptoms suggesting variable airflow obstruction whilst under regular review for at least two months, normal peak expiratory flow (PEF) variability (maximum within day amplitude % mean <20% over two weeks), a normal chest radiograph and, on at least 2 occasions separated by more than 2 months, normal spirometric values, a methacholine PC<sub>20</sub> >16 mg/ml, and a sputum eosinophilia (>3% non-squamous cell). The median (range) sputum eosinophil count one week before the bronchoscopy was 11.3 (3.5-68)%. Normal subjects were asymptomatic and had no evidence of variable airflow obstruction or airway hyperresponsiveness. All subjects were current non-smokers with a past smoking history of less than 10 pack years. None of the subjects had taken inhaled or oral corticosteroids for at least six weeks prior to the study and managed their asthma

with short-acting  $\beta_2$ -agonist taken as required. The study was approved by the Leicestershire and Southampton Ethics Committee and all patients gave their written informed consent. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

### **Protocol and clinical measurements**

Subjects attended on two occasions. At the first visit they had spirometry, allergen skin prick tests, a methacholine inhalation test followed on recovery by sputum induction in those subjects at the Leicester site. Spirometry was performed using a dry bellows spirometer (Vitalograph, Buckingham, UK) with the FEV<sub>1</sub> recorded as the best of successive readings within 100ml. Allergen skin prick tests were performed to *Dermatophagoides pteronyssinus*, cat fur, grass pollen and *Aspergillus fumigatus* solutions with normal saline and histamine controls (Bencard, Nottinghamshire, UK). A positive response to an allergen on the skin prick tests was recorded in the presence of a weal >2mm more than the negative control. The methacholine challenge was performed using the tidal breathing method (Juniper, Cockcroft, & Hargreave 1994) with doubling concentrations of methacholine (0.03 to 128mg/ml) nebulised via a Wright nebuliser. We extended the dose range to include high doses in order to fully explore the dose response relationship. Sputum was induced and processed as previously described (Pavord et al 1997).

At the second visit bronchial mucosal biopsies were taken under local anaesthesia from the right middle and lower lobe carinae via an Olympus fibre-optic bronchoscope (Olympus Company, Tokyo, Japan) in line with the most recent BTS guidelines (British Thoracic Society 2001). All subjects received nebulised 2.5mg salbutamol. Lignocaine (1-4%) was used for local anaesthesia and continuous oxygen given via nasal cannulae throughout the procedure. Mucosal biopsies were immediately transferred into ice-cooled acetone containing the protease inhibitors iodoacetamide (20mM) and PMSF (2mM) for fixation, stored at -20°C for 24h, and then processed into the water soluble resin, glycolmethacrylate (GMA) (Polysciences, Northampton, UK) for embedding.

## **Immunohistochemistry**

Two-micrometer sections were cut, floated on 0.2% ammonia solution in water for 1 min and dried at room temperature for 1-4h. The following mouse IgG<sub>1</sub> monoclonal antibodies were used: CD3 (Dako Ltd, High Wycombe, UK), AA1 to mast cell tryptase (Dako Ltd) and EG2 to the cleaved form of eosinophil cationic protein (ECP) (Pharmacia, Milton Keynes, UK). In a subgroup of four subjects with asthma further sequential sections were stained for mast cell tryptase and chymase (Chemicon International, Harrow, UK) for co-localisation. The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993). Briefly, slides were pretreated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide to inhibit endogenous peroxide. After 2X 5min washes in TBS pH 7.6, blocking medium consisting of Dulbecco's MEM, 10% FCS and 1% BSA was applied for 30 min. Sections were then incubated with the primary antibody for 16-20h overnight at room temperature. Bound antibodies were then labelled with biotinylated rabbit anti-mouse Fab fragments (Dako Ltd,) during a 2h incubation, and demonstrated using the streptavidin-biotin-peroxidase detection system (Dako Ltd). Aminoethylcarbazole (AEC) was applied as the chromogen, which gives a red reaction product. Sections were counterstained with Mayers haematoxylin. Appropriate control sections were similarly treated either with the primary mAb omitted or in the presence of an unrelated antibody of the same isotype (IgG<sub>1</sub>, Dako).

## **Assessment and quantification of immunohistochemical staining**

Areas of airway smooth muscle, sub-epithelial mucosa (lamina propria) and epithelium were identified morphologically and the area calculated using a computer analysis system (Scion Image, Scion Corporation, Maryland, UK). We validated our detection of airway smooth muscle by comparing the area of smooth muscle measured in 11 pairs of contiguous sections by morphology and by positive staining for smooth muscle actin (Dako Ltd.). Nucleated immunostained cells present in coded sections were enumerated in the lamina propria, epithelium and airway smooth muscle, and numbers of cells expressed as the number/mm<sup>2</sup> of submucosa, epithelium and smooth muscle. Cells were counted within the smooth muscle bundles but not adjacent to them and were confirmed to be in the substance

of the smooth muscle by serial sections to avoid counting cells within the mucosal tissue that were juxtaposed due to biopsy artefact. Tryptase and chymase positive mast cells within the airway smooth muscle were co-localised using the image analysis system in a subset of four subjects with asthma who had sufficient airway smooth muscle. Basement membrane and lamina reticularis thickness was measured as the mean of 50 observations at 20 $\mu$ m intervals (Sullivan et al 1998).

Two blinded observers examined 20 sections for the presence airway smooth muscle. In all of those that there was agreement that the airway smooth muscle was assessable the area was >0.1mm<sup>2</sup>. Thus, a minimum area of 0.1mm<sup>2</sup> from a biopsy section of airway smooth muscle was considered sufficient to assess cellular infiltration and 2-4 sections at least 10 $\mu$ m apart were assessed for each patient from 1 biopsy in 23 subjects and 2 biopsies in 18 subjects. There were no differences in the number of sections quantified between groups. The area of smooth muscle for each subject was expressed as the mean of all the assessable sections. A single subject with asthma and 2 with eosinophilic bronchitis had a basement membrane length of <1mm so their width is not reported. All counts were performed blind to clinical characteristics and the specimens from the three groups were intermingled during processing and counting.

### **Statistical Analysis**

Subject characteristics were described using descriptive statistics. Cell counts were expressed as medians (ranges). Basement membrane and lamina reticularis thickness approximated to a normal distribution in each subject group, as confirmed by the Kolmogorov-Smirnov Test for normality, and was described as mean (SEM). Comparisons across 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 and by ANOVA and unpaired t-tests for parametric data. Associations between cell numbers and PC<sub>20</sub> methacholine were established using the Spearman Rank correlation. The relationship between the area of airway smooth muscle assessed by morphology and by positive staining for smooth muscle actin was expressed as the intra-class correlation coefficient. A value of p<0.05 was taken as being statistically significant. All tests were two-tailed. This is the first study to enumerate cell counts in airway smooth muscle from bronchial biopsies and no power calculations were performed to estimate the

number of subjects needed to assess differences in the cellular infiltration of airway smooth muscle. From previous findings on the repeatability of submucosal cell counts from bronchial biopsies, it known that 15 subjects per group would be an optimal number (Richmond et al. 1996).

## Results

The individual values for submucosal eosinophil, and mast cell counts as well as basal lamina thickness are illustrated in figure 3.11. The median (range) submucosal eosinophil counts/mm<sup>2</sup> were 2.1 (0-12.4) in normal controls, 9.5 (2.5-75) in asthma and 10 (3.4-114) in eosinophilic bronchitis. There were significant differences between controls and both asthma and eosinophilic bronchitis (95% CI of difference, 3.2, 18.2, p=0.002 and 4.0, 18.7, p=0.002 respectively), but not between the two disease groups.

The intraepithelial median (range) eosinophil counts/mm<sup>2</sup> of epithelium were 0 (0-10) in normal controls 5.7 (0-150) in asthma and 13.4 (0-191) in eosinophilic bronchitis. There were significant differences between controls and both asthma and eosinophilic bronchitis (95% CI of difference, 0-29.2, p=0.009 and 0-25 p=0.002 respectively), but not between the two disease groups (p=0.74). There were no group differences in the median epithelial T-cells (p=0.70), mast cells (p=0.84) or sub-mucosal T-cells (p=0.53) and mast cells (p=0.85) (Table 3.12).

The mean (SEM) basement membrane and lamina reticularis width (µm) was significantly greater in the subjects with asthma 10 (0.5) and eosinophilic bronchitis 10.8 (1.4) than normal controls 6.7 (0.4) (95% CI of the difference 1.9,4.7, p<0.0001 and 95% CI 0.9, 7.2, p=0.017 respectively), but not between the two disease groups (Figure 3.11).

Smooth muscle could be readily identified according to its morphological appearance (Figure 3.12). The intra-class correlation coefficient between the area of smooth muscle measured morphologically and by positive staining for actin was 0.96.

The median (range) area (mm<sup>2</sup>) of smooth muscle assessed per biopsy section was similar between the subjects with asthma 0.3 (0.16-0.97), eosinophilic bronchitis 0.35 (0.1-1.9) and normal controls 0.3 (0.12-0.91) (p=0.61). In the airway smooth muscle the median (range) mast cells per section in subjects with

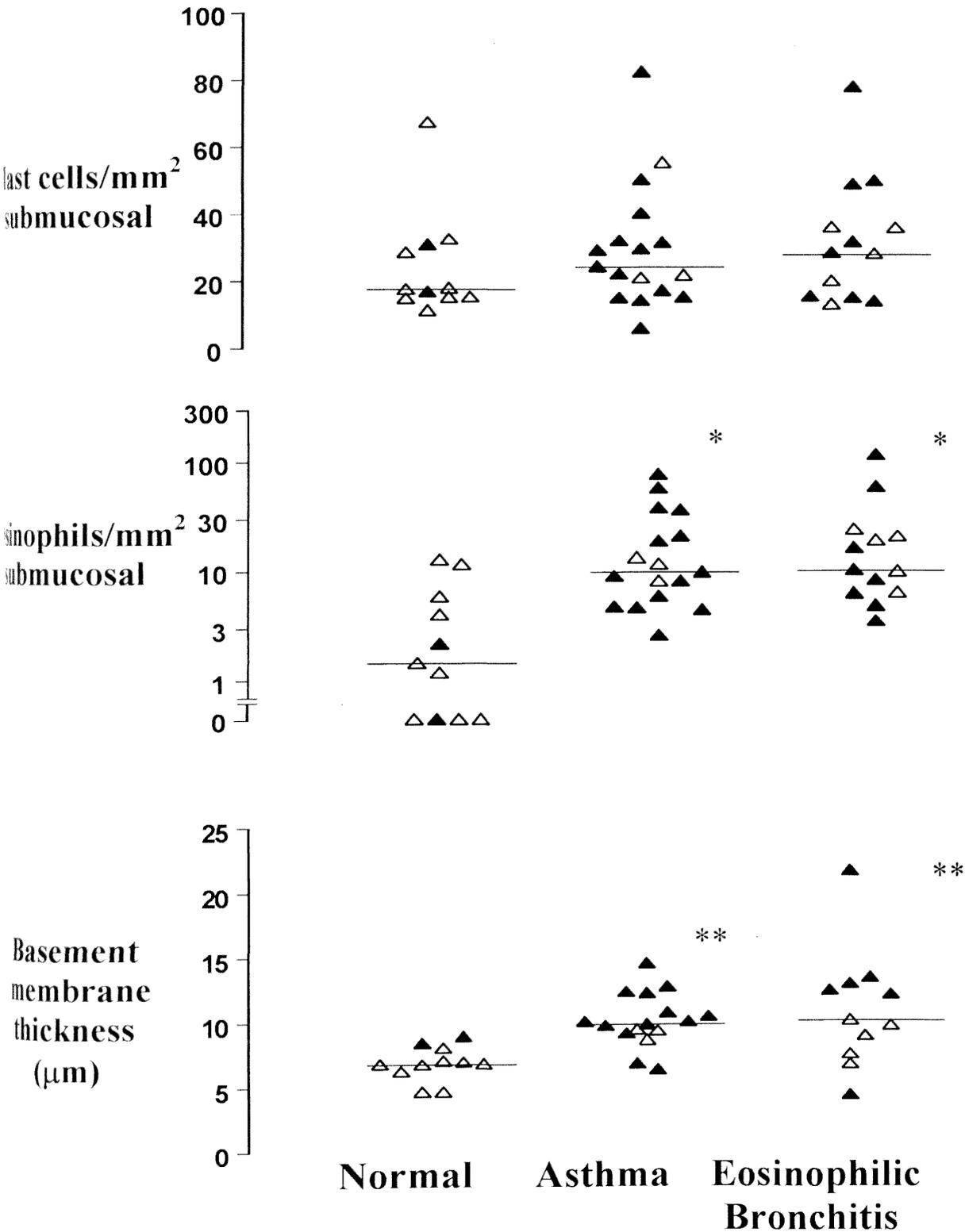
asthma were 2 (0-8), eosinophilic bronchitis 0 (0-2) and normal controls 0 (0-5). The median (range) mast cell count/mm<sup>2</sup> of smooth muscle was significantly higher in the subjects with asthma 5.1 (0-33.3) than in eosinophilic bronchitis 0 (0-4.8) (95% CI of difference 2.5, 6.1; p=0.0001) or normal controls 0 (0-6.4) (0.2, 5.9; p=0.013) (figure 3.13). There was a significant inverse correlation between the number of mast cells infiltrating the bronchial smooth muscle and the methacholine PC<sub>20</sub> in the subjects with asthma ( $r_s=-0.5$ , p=0.03). In a subgroup of 4 subjects with asthma 83% of the airway smooth muscle mast cells were tryptase and chymase positive. Eosinophils were observed in the airway smooth muscle in five subjects with asthma and 2 with eosinophilic bronchitis. T-lymphocytes were only observed in 2 subjects with asthma and 2 normal controls. For all of the parameters described there were no significant differences between atopic and non-atopic individuals within disease groups.

Repeatability of cell counts and reticular lamina thickening are shown in Appendix II.

**Table 3.12** Median (range) cells/ mm<sup>2</sup> submucosa and epithelium

	Eosinophilic Bronchitis	Asthma	Normal
<b>Submucosal</b>			
T-lymphocytes (CD3+)	41.6 (8.9-145)	47.7 (21.6-121.7)	53.3 (8.9-255)
Mast cells (AA1+)	28.1 (12.8-77.8)	23.9 (5.5-82.1)	17.2 (10.9-66.9)
<b>Intra-epithelial</b>			
T-lymphocytes (CD3+)	19.6 (0-257)	43.8 (0-150)	20.6 (0-140)
Mast cells (AA1+)	14.3 (0-125)	16.7 (0-125)	10.5 (0-150)

**Figure 3.11** Submucosal cell counts/mm<sup>2</sup> for mast cells (AA1) and eosinophils (EG2) and basement membrane width in subjects with asthma, eosinophilic bronchitis and normal controls. \*p=0.001 (Kruskal-Wallis test), \*\*p=0.002 (ANOVA), closed triangles atopic subjects, open triangles non-atopic

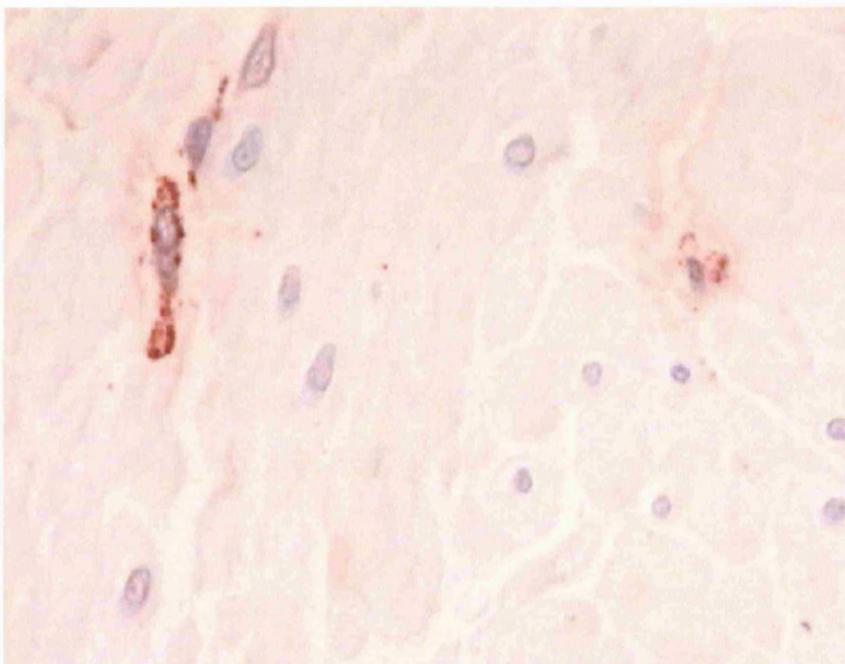


**Figure 3.12** Mast cells in bronchial smooth muscle (a) x100, (b) X400  
Arrows highlight mast cells. Figures from two patients  
with asthma

A



B





## **Discussion**

This study has, for the first time, quantified inflammatory cell infiltration of the airway smooth muscle in patients with asthma. We have demonstrated a striking difference between the number of mast cells within the airway smooth muscle in this disease compared to that found in both normal subjects and patients with eosinophilic bronchitis. This observation has major implications for our understanding of the pathogenesis of asthma and the pathophysiological role that mast cells have in this disease.

The potency of our observation derives from the comparison with a unique disease control group. Eosinophilic bronchitis is informative as it has many of the features of asthma, but is characterised by the persistent absence of variable airflow obstruction and airway hyperresponsiveness (Brightling & Pavord 2000). It therefore follows that any difference in pathology between the two conditions is likely to give important clues to the features which are essential to the asthmatic phenotype. We report here that several of the traditional characteristics of the immunopathology of asthma, including a submucosal and epithelial eosinophilia and thickening of the basement membrane and lamina reticularis thickening are also features of eosinophilic bronchitis and are therefore unlikely to be critical factors causing airway hyperresponsiveness or variable airflow obstruction. Indeed the only difference that we have observed in our detailed comparison of the two conditions was increased mast cells within the airway smooth muscle in asthma.

The hypothesis that mast cells are localised within the airway smooth muscle and that mast cell smooth muscle cell interactions are important in asthma is entirely plausible. Airway smooth muscle has significant secretory capacity suggesting that it has the ability to promote migration of mature mast cells or their progenitors and provide the correct microenvironment to maintain differentiation, activation and survival (Page et al. 2001). Several mast cell products have the potential to adversely effect smooth muscle growth and function and their microlocalisation within the smooth muscle would be predicted to facilitate this interaction. For example, the mast cell derived autacoid mediators histamine, prostaglandin D<sub>2</sub> and leukotriene C<sub>4</sub> are potent airway smooth muscle spasmogens, while the mast cell specific serine protease tryptase could potentially induce bronchoconstriction, airway remodelling and

airway hyperresponsiveness through a variety of mechanisms (Page et al 2001) (Tam & Caughey 1990) (Clark et al. 1995) (Hauck et al. 1999). In a subgroup of subjects with asthma we observed that the airway smooth muscle mast cells were predominately tryptase and chymase positive suggesting that in addition to tryptase, the protease chymase may play a role in the mast cell-smooth muscle cell interaction.

The view that mast cell infiltration into airway smooth muscle is functionally important finds support from our observation that the number of smooth muscle mast cells in asthma was inversely correlated with the degree of airway hyperresponsiveness. Our contention that mast infiltration of airway smooth muscle is more important than eosinophilic mucosal airway inflammation in the development of disordered airway physiology in asthma is consistent with observations from studies with humanised monoclonal antibodies. These have shown that anti-IgE therapy has a variety of beneficial effects in atopic asthma (Busse et al 2001) whereas anti-interleukin (IL)-5, which markedly reduces the sputum eosinophil count has no effect on either airway responsiveness or the response to inhaled allergen (Leckie et al 2000).

The importance of the mast cell in asthma has been questioned by the relatively poor clinical benefit of “mast cell stabilising” drugs such as sodium cromoglycate, nedocromil sodium and the  $\beta_2$ -adrenoceptor agonists. However, both cromoglycate and nedocromil sodium have weak effects on mediator release from lung mast cells and exhibit tachyphylaxis (Okayama et al 1992) and chronic administration of  $\beta_2$ -adrenoceptor agonists, in contrast to attenuating mast cell secretion, can lead to enhanced release of mediators (Swystun et al 2000) (Cho, Hartleroad, & Oh 2001). Furthermore, in asthma the predominant mechanism of mast cell degranulation appears to be piecemeal (Djukanovic et al 1992a), which may well occur through a mechanism with distinct intracellular signalling pathways that are simply not susceptible to current therapies.

We did not find increased numbers of intraepithelial mast cells in asthma or eosinophilic bronchitis. Some studies (Pesci et al. 1993) (Laitinen et al 1993a) but not others (Jeffery et al 1989) (Djukanovic et al 1990b) have reported increased intraepithelial mast cells in asthma. The likely explanation for these conflicting results is that the assessment of airway epithelium from bronchial

biopsies is confounded by variability in epithelial integrity which may reflect a real disease effect or artefact (Ordonez et al 2000). Bronchial brushings or bronchial washings are alternative methods to assess cellular infiltration of the superficial airway. Using these techniques the number of mast cells have been consistently reported to be increased in both asthma (Gibson et al. 1993) (Wardlaw et al 1988) and eosinophilic bronchitis (Gibson et al 1998) suggesting that these methods may be more reliable in the assessment of epithelial mast cells.

Most inflammatory mediators are rapidly inactivated once they leave the cell so that they act across distances of only a few microns. Microlocalization is therefore a fundamental organising principle of inflammatory responses, which has been relatively ignored in previous studies of the immunopathology of asthma. This is in part because of the poor morphology of thick frozen sections and the small amounts of tissue obtained by fiberoptic bronchoscopy. Using GMA embedding and ultrathin sections excellent morphology has been obtained. The identification of smooth muscle was validated using actin staining and was found to be reliable. Although relatively small amounts of smooth muscle were present we believe our data are robust. No difference in the amount of smooth muscle was seen between the three groups so that this does not represent a source of bias. The slides were counted blind and crucially any bias that might result from recognition of pathological changes of asthma is negated by the similar morphology of asthma and eosinophilic bronchitis. In order to obtain sufficient assessable tissue we had to recruit subjects from two centres. However, the decision to recruit from two centres was made *a priori* and all the measurements were performed at one centre and were similar between the two centres so we do not believe this was an important confounding factor.

Our confidence that the increase in airway smooth muscle mast cell numbers in asthma reported here are real is increased by the findings from lung resection and post mortem studies. *Ex-vivo* bronchial rings that demonstrated contractile responses to allergen contained more mast cells within the smooth muscle than those that were unresponsive (Ammit et al 1997) and the number of mast cells within the airway smooth muscle was similar to our findings in subjects with asthma. In fatal asthma mast cells have been reported to be increased throughout the airway smooth muscle bundles compared to control tissue from lung

resection (Koshino et al. 1993). Interestingly in both of these studies the mast cell was the predominant inflammatory cell localised to the airway smooth muscle with a striking paucity of eosinophils and T-lymphocytes (Ammit et al 1997) (Koshino et al 1993), supporting our suggestion that there is a selective recruitment of mast cells. There is clearly a need for more studies examining larger airway samples, perhaps from surgical resections or post-mortem tissue, although the difficulty in ensuring accurate clinical and physiological phenotyping of these subjects would present problems. This approach could be used to investigate whether increased numbers of airway smooth muscle mast cells are an exclusive feature of asthma or are present in other conditions associated with airway hyperresponsiveness such as chronic obstructive pulmonary disease.

In summary our findings suggest that a key factor in the development of the variable airflow obstruction and airway hyperresponsiveness observed in asthma is the microlocalisation of mast cells within the airway smooth muscle bundle. This would be predicted to facilitate specific interactions between the two cell types, which may be an obligatory requirement for the development of the disordered airway physiology. If this proves correct, then specific targeting of the mast cell-smooth muscle interaction may provide a novel approach to the effective treatment for asthma.

## **3.2 Airway eosinophilia a marker of response to corticosteroids**

### **3.2.1 Airway inflammation, airway responsiveness and cough before and after inhaled budesonide in patients with eosinophilic bronchitis**

#### **Abstract**

Eosinophilic bronchitis is a common cause of chronic cough, characterized by sputum eosinophilia similar to that seen in asthma, but unlike asthma the patients have no objective evidence of variable airflow obstruction or airway hyperresponsiveness. We have tested the hypothesis that in eosinophilic bronchitis the inflammation is mainly localized in the upper airway and assessed the response to inhaled corticosteroids in this condition.

In an open study we measured the lower (provocative concentration causing a 20% fall in forced expiratory volume in one second (PC<sub>20</sub>)) and upper (PC<sub>25</sub> MIF<sub>50</sub>) airway responsiveness to histamine, lower and upper airway inflammation using induced sputum and nasal lavage, in 11 patients with eosinophilic bronchitis. We assessed changes in these measures and in cough reflex sensitivity to capsaicin and cough severity after 400 µg of inhaled budesonide for 4 weeks.

A nasal eosinophilia was present in only three patients with one having upper airway hyperresponsiveness. Following treatment with inhaled corticosteroids the geometric mean sputum eosinophil count decreased from 12.8% to 2.9% (mean difference 4.4-fold, 95% confidence interval (CI) 2.14-10.02), the mean±sem cough visual analogue score on a 100 mm scale decreased from 27.2±6.6 mm to 12.6±5.7 mm (mean difference 14.6, 95% CI 9.1-20.1) and the cough sensitivity assessed as the capsaicin concentration required to cause two coughs (C<sub>2</sub>) and five coughs (C<sub>5</sub>) improved (C<sub>2</sub> mean difference 0.75 doubling concentrations, 95% CI 0.36-1.1; C<sub>5</sub> mean difference 1.3 doubling concentration, 95% CI 0.6-2.1). There was a significant positive correlation between the fold change in sputum eosinophil count and doubling dose change in C<sub>5</sub> after inhaled budesonide (r=0.61).

In conclusion upper airway inflammation is not prominent in eosinophilic bronchitis and that inhaled budesonide improves the sputum eosinophilia, cough severity and sensitivity suggesting a causal link between the inflammation and cough.

## **Introduction**

Eosinophilic bronchitis is a condition presenting with chronic cough and characterized by sputum eosinophilia like asthma, but unlike asthma the patients have no objective evidence of variable airflow obstruction or airway hyperresponsiveness (Gibson et al 1995) (Gibson et al 1989b). We (Brightling et al 1999b) and others (Carney et al 1997) have shown that eosinophilic bronchitis is the cause of cough in 10-20% of patients presenting to a respiratory specialist. Patients with eosinophilic bronchitis commonly have upper airway symptoms (Brightling et al 1999b) (Gibson et al 1995), so one possibility is that the inflammation is mainly localized to the upper airway. There is some evidence that the cough and sputum eosinophilia in patients with eosinophilic bronchitis improves with inhaled corticosteroids (Gibson et al 1995), arguing against predominant upper airway inflammation. However, no studies have examined the effect of inhaled corticosteroids on objective markers of cough sensitivity and it is not known whether the beneficial effects of inhaled corticosteroids are mediated through improvement in upper airway inflammation.

We looked for direct and functional evidence of upper airway inflammation by measuring nasal lavage eosinophil count (Bascom et al. 1988) (Bucca et al 1995a) and extrathoracic airway responsiveness to histamine (Bucca et al 1995a) (Bucca et al. 1995b) (Bucca et al. 1991). We repeated the measures after 4 weeks treatment with inhaled budesonide and related changes in inflammation to objective changes in cough sensitivity.

## **Methods**

### **Subjects**

Eleven subjects were recruited from outpatient clinics after presenting with an isolated chronic cough lasting >3 weeks from a group of 20 patients identified

with eosinophilic bronchitis. Four subjects had previously been involved in a study investigating the prevalence of eosinophilic bronchitis, but the measurements in this study are independent of the previous report (Brightling et al 1999b). Subjects had a cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal peak expiratory flow (PEF) variability (maximum within day amplitude percentage mean <20% over 2 weeks), a methacholine provocative concentration of histamine causing a 20% fall in forced expiratory volume in one second (FEV<sub>1</sub>; PC<sub>20</sub>) >16 mg·mL<sup>-1</sup>, a normal chest radiograph and a sputum eosinophilia >3% nonsquamous cells (our normal range 0-1.9%). Subject's details at the time of diagnosis are illustrated in table 3.13. The median time from diagnosis to entry into the study was 2 months. None had taken oral or inhaled corticosteroids for at least 1 month before entry into the study. The subjects gave full informed consent to participate in the study. The protocol was approved by the Leicestershire Health Authority ethics committee. Some subjects with eosinophilic bronchitis participated in more than one study see Appendix I.

## Measurements

Allergen skin sensitivity was measured by skin-prick testing to *Dermatophagoides pteronyssinus*, cat fur, grass pollen and *Aspergillus fumigatus* solutions, with normal saline and histamine controls (Bencard, Newark, Nottinghamshire, UK) (Methods 2.1.1). Cough severity was rated on a 100 mm horizontal visual analogue scale (VAS) with 0 being no cough and 100 being worst cough ever (2.1.10). Cough sensitivity was assessed using the capsaicin cough challenge (Choudry & Fuller 1992) (O'Connell et al 1994) (Methods 2.1.7). Subjects capsaicin (0.5-500 µM) *via* a nebulizer attached to a breath-activated dosimeter delivering 8 mL. The number of coughs in response to each concentration was counted and recorded. The challenge was stopped when the concentration elicited 5 coughs or the highest dose of capsaicin was reached.

Airway responsiveness was measured using the tidal breathing method (Juniper, Cockcroft, & Hargreave 1994). Doubling concentrations of histamine (0.03-16 mg·mL<sup>-1</sup>) were nebulized via a Wright nebulizer (a gift from Fisons, Leicestershire, UK) (Methods 2.1.3). Histamine was used in preference to

methacholine as upper airway hyperresponsiveness has only been demonstrated using histamine. Patients inhaled through a mouthpiece during tidal breathing for 2 min. FEV<sub>1</sub> and maximal mid-inspiratory flow (MIF<sub>50</sub>) was measured 30 and 90 s after each inhalation using a rolling seal spirometer. The challenge was stopped either after a >20% fall in FEV<sub>1</sub> or a maximal dose of histamine (16 mg·mL<sup>-1</sup>) was reached.

Sputum was induced and processed as previously described (Pavord et al 1997; Pizzichini et al 1996a) (Methods 2.14, 2.2.1.2). Nasal lavage was obtained as described by Bucca *et al* (Bucca et al 1995a) (Methods 2.1.6). Due to the nasal lavage being less cellular than the sputum a differential cell count of >100 cells was derived. An experienced observer blind to clinical characteristics performed cell counts.

### Study design

Subjects attended the respiratory function laboratory on four occasions. At the first visit the duration of cough, its severity assessed by VAS and the presence or absence of rhinitis defined as upper airway symptoms: coryza, post-nasal drip and pain with tenderness over the paranasal sinuses together with evidence of nasal or pharyngeal mucosal inflammation were recorded. Cough reflex sensitivity was assessed by a capsaicin challenge. In the same week at the same time of day standard histamine challenge test was performed incorporating both FEV<sub>1</sub> and inspiratory flow volume loop measurements (MIF<sub>50</sub>). Induced sputum and a nasal wash were collected after recovery from the challenge. Subjects were then started on budesonide 400 µg inhaled *via* a turbohaler (AstraZeneca, Herfordshire, UK) twice daily for 4-weeks. The tests were repeated in the same sequence at the same time of day at two further visits at the end of treatment 24 h after the last dose of budesonide.

### Analysis

Intrathoracic and extrathoracic responsiveness were expressed as the PC<sub>20</sub> and >25% reduction in MIF<sub>50</sub> (PC<sub>25</sub>) respectively. Intra- and extrathoracic hyperresponsiveness were defined as PC<sub>20</sub> and PC<sub>25</sub> <8 mg·mL<sup>-1</sup>. Histamine PC<sub>20</sub>, PC<sub>25</sub>, concentration required to cause two coughs (C<sub>2</sub>) and five coughs (C<sub>5</sub>)

were calculated by linear interpolation of log dose response curve and were described as geometric means. Cough paroxysms of more than five coughs were not possible to quantify so a censored value of 10 coughs was assigned. Sputum eosinophil count and total cell count were log normally distributed and described as geometric mean (log sem). Other sputum cell characteristics are described as median (interquartile range). Change in sputum eosinophil count was expressed as fold differences with 95% confidence intervals (CI) and change in C<sub>2</sub> and C<sub>5</sub> in doubling doses. The significance of changes was assessed using Student's paired t-test or Mann-Whitney U-test and correlation performed by Pearson's correlation coefficient. The number of subjects with eosinophilic bronchitis selected was determined by the number available and willing to participate in the study. No power calculation was performed.

## **Results**

The geometric mean sputum eosinophil count did not significantly change from diagnosis, 13.2%, to entry into the study, 12.8%, (mean difference 1.03-fold, 95% CI 0.6-1.7; p=0.8). There was no significant difference in any other sputum cell count. The characteristics of the patients at the time of diagnosis are shown in table 3.13 and at baseline in table 3.14.

**Table 3.13** Characteristics of subjects at diagnosis

<b>Number of subjects</b>	<b>11</b>
Age*	51(24)
Male	6
FEV <sub>1</sub> % pred <sup>+</sup>	105±8
FEV <sub>1</sub> /FVC % <sup>+</sup>	82±1.6
Methacholine PC <sub>20</sub> <16 mg·mL <sup>-1</sup>	0
Atopy	3
Rhinitis	7
Cough duration in months*	25 (30)
Current smokers	0
Pack-year history >5	0
<b>Sputum characteristics</b>	
Eosinophil count % <sup>#</sup>	13.2 (0.12)
Neutrophil count %*	52 (37)
Macrophage count %*	30 (30)
Epithelial count %*	3 (3)
Lymphocyte count %*	0 (1)
Total cell count ×10 <sup>6</sup> ·mL <sup>-1</sup> of sputum <sup>#</sup>	2.05 (0.07)
Viability %*	78 (23)
Squamous cells %*	6 (5)

FEV<sub>1</sub>: forced expiratory volume in one second; FVC: forced vital capacity; PC<sub>20</sub>: provocative concentration of histamine causing a 20% in FEV<sub>1</sub>. \*: median (interquartile range); <sup>+</sup>: mean±sem; <sup>#</sup>: geometric mean (log sem).

**Table 3.14** Patient characteristics before and after inhaled budesonide

	<b>Pre-inhaled steroids</b>	<b>Post-inhaled steroids</b>
FEV <sub>1</sub> % pred <sup>+</sup>	103±7	110±9
FEV <sub>1</sub> /FVC <sup>+</sup>	80.5±1.5	79.9±1.2
Histamine PC <sub>20</sub> <16 mg·mL <sup>-1</sup>	0	0
Histamine PC <sub>25</sub> <16 mg·mL <sup>-1</sup>	2	2
C <sub>2</sub> (Capsaicin concentration μM) <sup>#</sup>	3.24 (0.04)	5.48 (0.07)*
C <sub>5</sub> (Capsaicin concentration μM) <sup>#</sup>	14.1 (0.10)	34.4 (0.11)*
Visual analogue scale mm <sup>+</sup>	27.7±6.6	12.6±5.7*
<b>Sputum characteristics</b>		
Eosinophil count % <sup>#</sup>	12.8 (0.13)	2.9 (0.20)*
Neutrophil count % <sup>**</sup>	62 (38)	52 (40)
Macrophage count % <sup>**</sup>	25 (20)	40 (31)
Lymphocyte count % <sup>**</sup>	0 (1)	0 (1)
Epithelial count % <sup>**</sup>	1 (1)	2 (4)
Squamous cells % <sup>**</sup>	8 (10)	5 (8)
Viability cells % <sup>**</sup>	75 (11)	72 (49)
Total cell count ×10 <sup>6</sup> ·mL sputum <sup>#</sup>	2.2 (0.10)	1.9 (0.09)

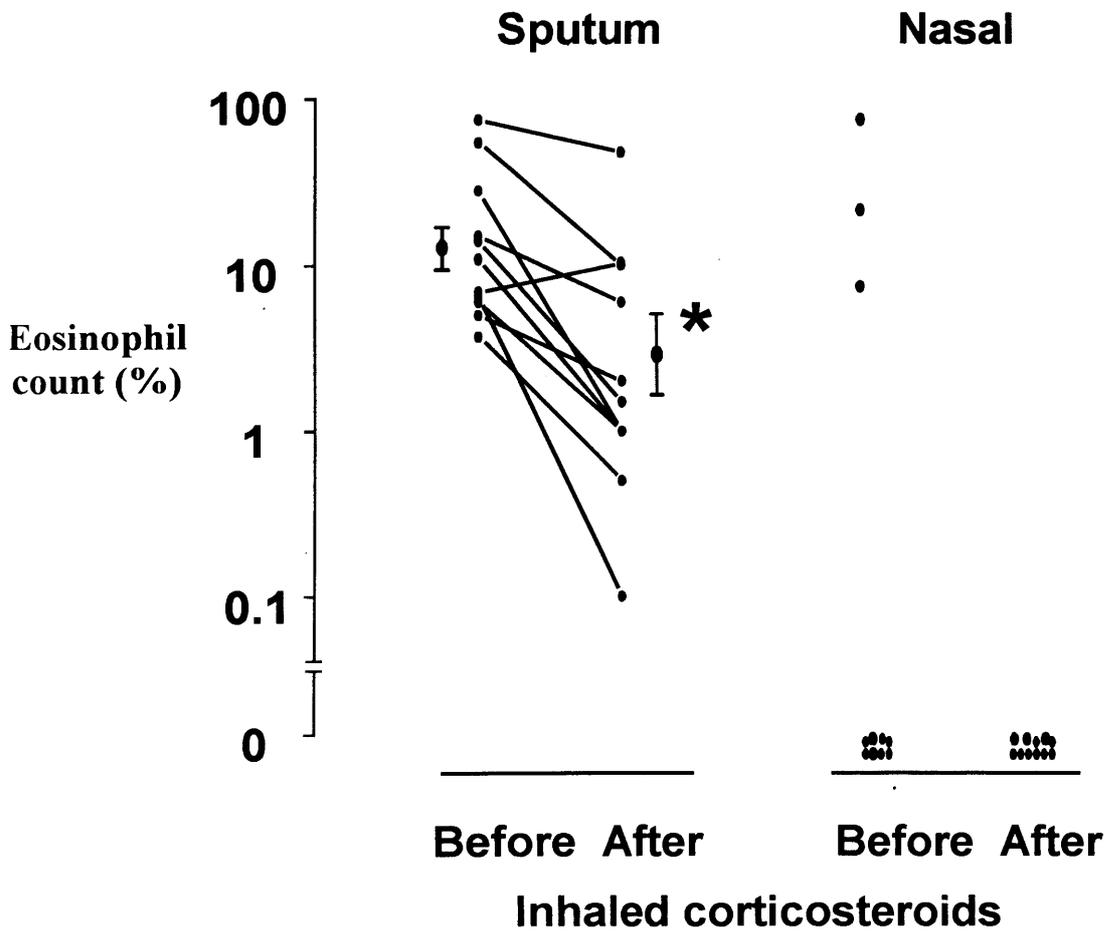
FEV<sub>1</sub>: forced expiratory volume in one second; FVC: forced vital capacity; PC<sub>20</sub>: provocative concentration of histamine causing a 20% in FEV<sub>1</sub>. PC<sub>25</sub>: provocative concentration causing a >25% reduction in maximal mid-expiratory flow; C<sub>2</sub>: capsaicin concentration required to cause two coughs; C<sub>5</sub>: capsaicin concentration required to cause five coughs. <sup>+</sup>: mean±sem; <sup>#</sup>: geometric mean (log sem); \*: p<0.05; \*\*: median (interquartile range)

Eosinophils were present in the nasal lavage of three patients, two of whom had a greater nasal than sputum percentage eosinophil count. This eosinophilic rhinitis improved after inhaled budesonide (Figure 3.14). Extrathoracic airway responsiveness was demonstrated in one of these patients. One further patient had extrathoracic hyperresponsiveness, but had nasal lavage evidence of a neutrophilic rhinitis. All of the subjects had normal lower airway responsiveness before and after inhaled budesonide with no significant change in the slope of the dose-response curve.

The changes after treatment with inhaled budesonide are summarized in table 3.14. Following treatment, the sputum eosinophil count decreased significantly from 12.8% to 2.9% (mean difference 4.4-fold, 95% CI 2.14-10.02;  $p < 0.01$ ; figure 3.14). The mean cough VAS was significantly reduced from 27.2 mm to 12.6 mm following treatment (mean difference 14.6 mm; 95% CI 20.1-9.1;  $p < 0.01$ ).  $C_2$  increased from 3.3  $\mu\text{M}$  to 5.5  $\mu\text{M}$  and  $C_5$  from 14.1  $\mu\text{M}$  to 34.4  $\mu\text{M}$  after treatment ( $C_2$  mean difference 0.75 doubling concentrations, 95% CI 0.36-1.1;  $p < 0.01$ ;  $C_5$  mean difference 1.3 doubling concentrations, 95% CI 0.6-2.1;  $p < 0.01$  figure 3.15). There was a significant correlation between the fold change in eosinophil count and the doubling concentration change in  $C_5$  after inhaled steroids ( $r = 0.61$ ;  $p < 0.05$ ; figure 3.16).

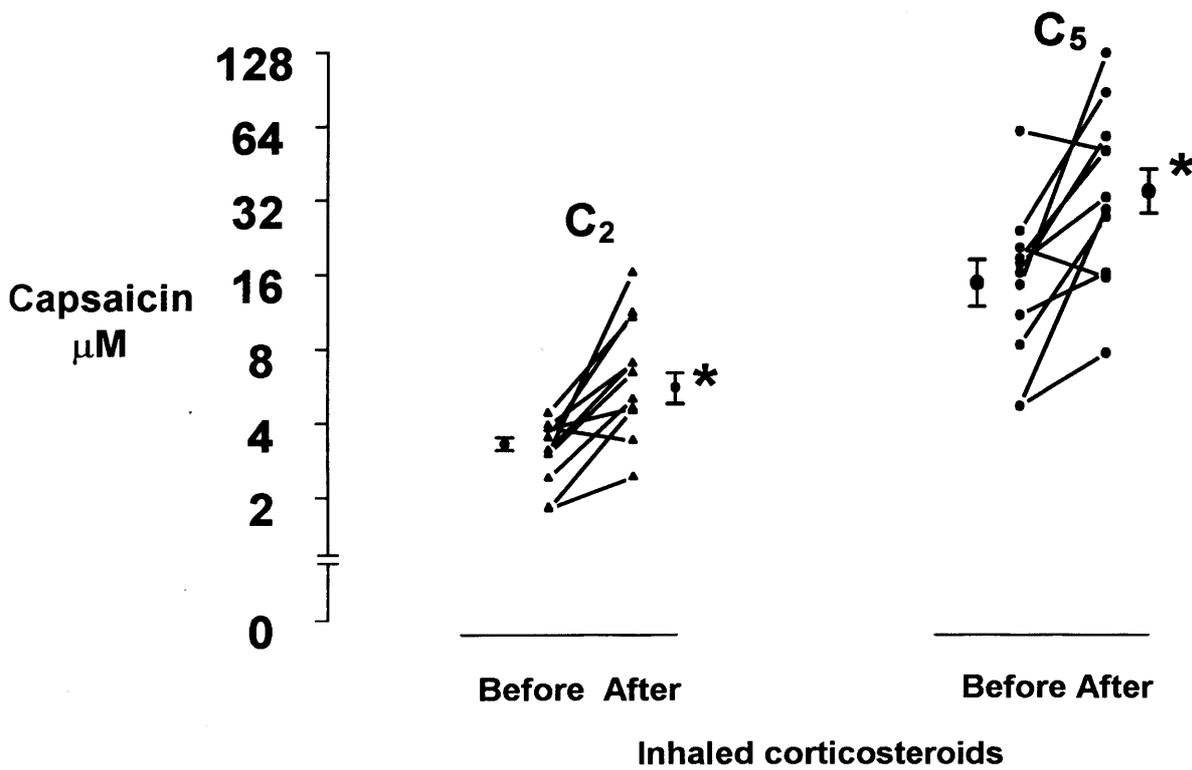
**Figure 3.14** Sputum and nasal lavage eosinophil count before and after inhaled budesonide with sputum.

Individual values and geometric mean±sem are given. Normal range for sputum eosinophil count is 0-1%. \*  $p < 0.05$ .



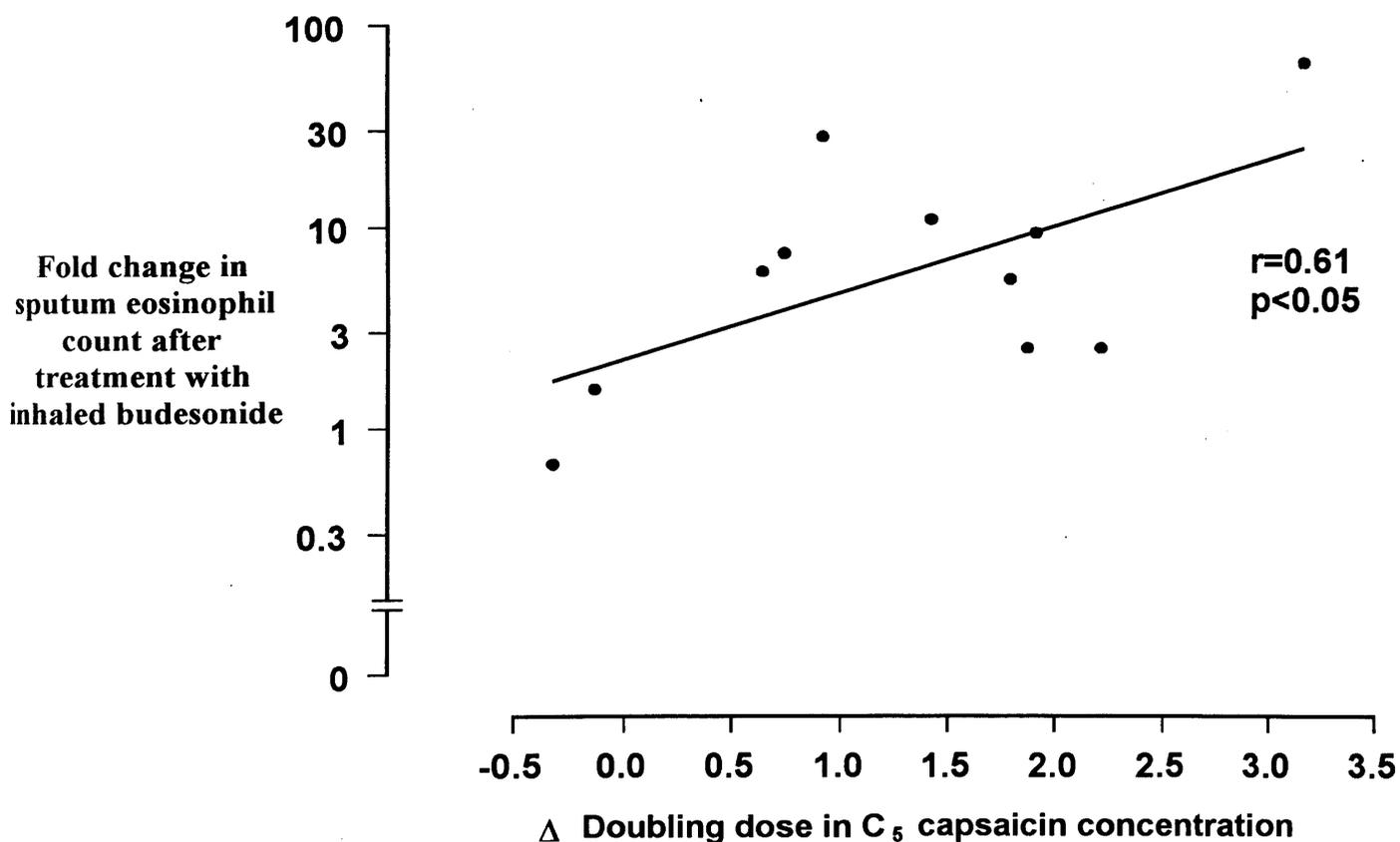
**Figure 3.15** Capsaicin concentration causing two coughs and five coughs before and after inhaled budesonide.

Individual values and geometric mean $\pm$ sem are given. \* $p < 0.05$ .



**Figure 3.16** Relationship between fold decrease in sputum eosinophil count after treatment with inhaled budesonide and cough sensitivity assessed as the doubling fold increase in capsaicin concentration required to cause five coughs.

Regression line is shown  $r=0.61$ ,  $p<0.05$ .



## Discussion

This study has shown that upper airway inflammation and hyperresponsiveness are not prominent features of eosinophilic bronchitis. We have confirmed previous findings (Gibson et al 1995) that inhaled corticosteroids decrease the sputum eosinophil count and improve cough. We have extended these findings by showing objective evidence of improvement in capsaicin cough sensitivity following treatment.

Like other studies (Gibson et al 1995) evaluating the effect of treatment in patients with eosinophilic bronchitis, this study was uncontrolled. Eosinophilic bronchitis is a recently recognized condition, which although a common cause of chronic cough (Brightling et al 1999b) (Carney et al 1997), is not sufficiently prevalent to easily recruit for placebo controlled studies. Furthermore, the main focus of the current study was not the efficacy of treatment but the relative effects on upper and lower airway inflammation. We doubt whether the improvement in cough severity and sensitivity coupled with the marked decrease in sputum eosinophilia after treatment was due to regression towards the mean because the sputum eosinophil count did not significantly change from diagnosis at least 1 month before entry into the study. Laboratory assessments were performed blind to patient's clinical characteristics and treatment so results are unlikely to have been biased.

The effect of a histamine challenge on sputum cell counts is unclear, but the study design was such that the relationship between the challenge and sputum induction was the same before and after treatment so any effect of histamine on cell counts would not have biased the results. Bronchial provocation with methacholine has been shown to have no effect on sputum cell characteristics (Confalonieri et al 1998) and histamine has been reported to result in no alteration in bronchial biopsy morphology or cellular infiltration (Soderberg et al. 1993). However, 24 h after a histamine challenge there is an increase in the lymphocyte, mast cell and neutrophil count with no reported change in the eosinophil count in bronchoalveolar lavage (Soderberg et al. 1989). In the current study the sputum induction was performed immediately after a histamine challenge. It is unlikely that histamine would have an immediate effect on the cell characteristics of sputum and in support of this there was no significant

difference in the cellular characteristics of the subjects at diagnosis after methacholine compared with the study baseline after histamine.

We chose to test their hypothesis that eosinophilic bronchitis is predominantly due to upper airway inflammation by directly assessing inflammation using nasal wash differential cell count and looking for functional evidence by measuring upper airway responsiveness. Bucca *et al* (Bucca et al 1995a) have shown that a fall in inspiratory flow following a histamine challenge, known as extrathoracic hyperresponsiveness, corresponded with increased oedema, erythema and hypersecretion of the pharyngolaryngeal mucosa seen on laryngoscopy was associated with upper airway inflammation such as rhinosinusitis and pharyngitis. Allergic rhinitis is typically associated with a nasal wash eosinophilia (Bascom et al 1988). Upper airway hyperresponsiveness (demonstrated by a dose-related decrease in MIF<sub>50</sub> during a histamine challenge) (Bucca et al 1995a) (Bucca et al 1995b) (Bucca et al 1991) has been shown to be associated with upper airway inflammation. Neither nasal wash eosinophilia nor upper airway hyperresponsiveness were prominent in the current patients, in spite of upper airway symptoms being common, arguing against our hypothesis. It is possible that inhaled corticosteroids improve upper airway inflammation and the effects of budesonide may have been mediated through this mechanism in some patients. We also cannot exclude the possibility that the eosinophilic airway inflammation is confined to another part of the upper airway, not directly sampled by nasal lavage. However, overall we felt that the absence of predominant upper airway inflammation coupled with the presence of a sputum eosinophilia, which decreased with inhaled budesonide directed at the lower airway, is more in keeping with lower airway inflammation. A lower airway inflammation in eosinophilic bronchitis is further supported by recent bronchoscopy evidence of lower airway inflammation (Gibson et al 1998) (Brightling et al 1999a) (Studies 3.1.3, 3.1.5, 3.1.6).

It was found that the capsaicin cough sensitivity was similar to previously reported asthmatics with chronic cough and more than asthmatics without chronic cough, patients with cough due to upper airway inflammation, postnasal drip and healthy control subjects (Choudry & Fuller 1992). Typically patients with eosinophilic bronchitis have a cough productive of scanty sputum in the morning (Brightling et al 1999b) in keeping with a bronchitic component to their

cough. The current findings suggest that heightened cough sensitivity might also contribute. Following inhaled corticosteroids the cough sensitivity improved and the degree of this improvement significantly correlated with the change in the sputum eosinophil count suggesting that eosinophilic airway inflammation is causally associated with enhanced cough reflex sensitivity.

In conclusion, it has been shown that upper airway inflammation and upper airway hyperresponsiveness are not prominent in eosinophilic bronchitis. This study has confirmed that inhaled corticosteroids improve cough severity, sensitivity and reduce the sputum eosinophil count and have shown that this is associated with a significant decrease in cough sensitivity. These findings support the presence of a lower airway inflammation in eosinophilic bronchitis. Further work is required to establish why an apparently similar airway inflammation is associated with such different abnormalities of airway function in eosinophilic bronchitis and asthma.

### **3.2.2 Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial**

#### **Abstract**

Some patients with chronic obstructive pulmonary disease (COPD) respond to corticosteroid therapy. Whether these patients have different airway pathology from other COPD patients is unclear. We tested the hypothesis that response to prednisolone is related to the presence of eosinophilic airway inflammation.

We did a randomised, double-blind, crossover trial. Patients who had COPD treated with bronchodilators only were assigned placebo and 30 mg prednisolone daily for 2 weeks each, in a random order, separated by a 4-week washout period. Before and after each treatment period, we assessed patients with spirometry, symptom scores, the chronic respiratory disease questionnaire (CRQ), incremental shuttle walk test, and induced sputum. Analysis was done by intention to treat.

Eighty-three patients were recruited, of whom 67 were randomised. The geometric mean sputum eosinophil count fell significantly after prednisolone (from 2.4% to 0.4%; mean difference six-fold [95% CI 3.1-11.4]) but not after placebo. Other sputum cell counts did not change. After stratification into tertiles by baseline eosinophil count, postbronchodilator forced expiratory volume in 1 s (FEV<sub>1</sub>) and total scores on the CRQ improved progressively after prednisolone from the lowest to the highest eosinophilic tertile, compared with placebo. The mean change in postbronchodilator FEV<sub>1</sub>, total CRQ score, and shuttle walk distance with prednisolone compared with placebo in the highest tertile was 0.19 L (0.06-0.32), 0.62 (0.31-0.93), and 20 m (5-35), respectively.

Our findings suggest that eosinophilic airway inflammation contributes to airflow obstruction and symptoms in some patients with COPD and that the short-term effects of prednisolone are due to modification of this feature of the inflammatory response. The possibility that sputum eosinophilia identifies a subgroup of patients who particularly respond to long-term treatment with inhaled corticosteroids should be investigated.

## **Introduction**

The role of corticosteroids in stable chronic obstructive pulmonary disease (COPD) is uncertain (Boushey 1999). Guidelines reflect the perception that a subgroup of patients respond to this treatment (British Thoracic Society 1997), although identification of characteristics associated with a positive response to short-term or long-term treatment with corticosteroids has been difficult (Calverley 1999). Corticosteroids effectively modify eosinophilic airway inflammation in asthma, (Pavord et al. 1999b) (Keatings et al 1997b) but there is less evidence that they effect the neutrophilic inflammation that predominates in COPD (Keatings et al 1997b). One possibility is that corticosteroids are effective in COPD patients who have eosinophilic airway inflammation.

Early reports suggested that sputum eosinophilia could predict clinical benefit from corticosteroids (Shim, Stover, & Williams, Jr. 1978), but the methods of sputum assessment were crude and the findings were not consistent (Clifton 1978). Over the past 10 years, important advances have been made in the technique of sputum induction and analysis and methods are now reliable, valid, (Pizzichini et al 1996a) responsive, and safe (Hunter et al 1999). Pizzichini and colleagues (Pizzichini et al 1998) used these methods to show, in a small, single-blind study, that patients with sputum eosinophilia had a greater improvement in forced expiratory volume in 1 s ( $FEV_1$ ) and health status after a short course of prednisone than those without. We did a double-blind placebo-controlled crossover study to define the sputum characteristics of patients with COPD and to investigate the relation between the sputum eosinophil count and the response to 2 weeks of treatment with prednisolone.

## **Methods**

### **Patients**

We recruited, from respiratory clinics, patients who had symptoms of chronic airflow obstruction, postbronchodilator  $FEV_1$  of less than 70% predicted, and an  $FEV_1$ /forced vital capacity (FVC) ratio of less than 70%. We enrolled those who had no substantial improvement in  $FEV_1$  after taking 2.5 mg nebulised salbutamol (<15% or, if  $FEV_1$  <1.2 L, <200 mL improvement). We excluded patients if they had a clinical diagnosis of asthma, a history of childhood respiratory disorders, variability in symptoms not associated with infections, a

history of acute wheeze, breathlessness, or deterioration associated with allergens, or an exacerbation within 6 weeks of trial entry. Patients taking regular oral corticosteroids were excluded. In patients taking inhaled corticosteroids, these drugs were discontinued for at least 1 month before randomisation. We withdrew patients from the study if they had a moderate exacerbation requiring inhaled corticosteroids or antibiotics, a severe exacerbation needing oral corticosteroids, or a severe intercurrent illness. The study was approved by the local research ethics committee and all patients gave written informed consent.

### **Study design**

Patients were randomly assigned, by random numbers, prednisolone 30 mg daily and placebo for 2 weeks each in a random order (Figure 3.17). The two agents were administered as single white capsules. These were prepared by the Royal Hallamshire Hospital Pharmacy Department, Sheffield, UK. Glenfield Hospital Pharmacy Department did the randomisation, distributed the study agents, and held the trial codes, which was disclosed after the study. The two treatment periods were preceded by a 4-week run-in period and separated by a 4-week washout period, since shorter washout times have been associated with a carryover effect (Tweeddale, Alexander, & McHardy 1987). Patients attended on four occasions before, and 4-6 h after the last dose of study medication, at the same time of day on each occasion more than 6 h after their last dose of bronchodilator, and 24 h after the last dose of long-acting  $\beta$ 2-agonists. At each visit we assessed patients by spirometry before and 15 min after 2.5 mg nebulised salbutamol, the chronic respiratory disease questionnaire (CRQ) for health status, a 100 mm visual analogue scale for symptom scores (no symptom to the worst symptom ever), an incremental shuttle walk test, and sputum induction for differential cell count. The timing of events was kept constant in relation to the nebulised salbutamol.

We recorded details of smoking status, normal treatment, and atopic and childhood respiratory history. We recorded symptom scores for dyspnoea, cough, sputum production, and wheeze. Spirometry was done with a Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Salbutamol was administered via a Flaem Nuova Type II nebuliser (Deva Medical, Runcorn, Cheshire) with a median particle size of 2  $\mu$ m and the patient breathing tidally

(Methods 2.1.2). We recorded FEV<sub>1</sub> as the better of two successive readings within 100 mL. Lung-function tests were done with a benchmark (P K Morgan, Chatham, UK) and lung volumes assessed by the helium dilution method. We took venous blood samples to measure peripheral blood eosinophil count, total IgE and radioallergosorbent tests to *Dermatophagoides pteronyssinus*, cat fur, and grass pollen (2.1.1).

We assessed health status with the CRQ, which consists of 20 questions in four domains: dyspnoea, fatigue, emotions, and mastery (Guyatt et al 1987). A seven-point Likert scale was used for each question and the total score and each domain score was recorded out of seven, with a minimum important difference of 0.5 (Methods 2.1.8). The incremental shuttle walk test was done according to a standard protocol (Singh et al 1992), which has been shown to be repeatable after one practice walk. All patients had a practice walk before entry into the study. We recorded the total distance of the completed shuttles (Methods 2.1.9).

Sputum was induced and processed as previously described (Pavord et al 1997) (Pizzichini et al 1996a) (Methods 2.1.4, 2.2.1.2).

We measured neutrophil elastase, IL-8 and eosinophilic cationic protein (ECP) in the cell-free supernatant (Methods 2.2.2.1, 2.2.2.2, 2.2.2.3).

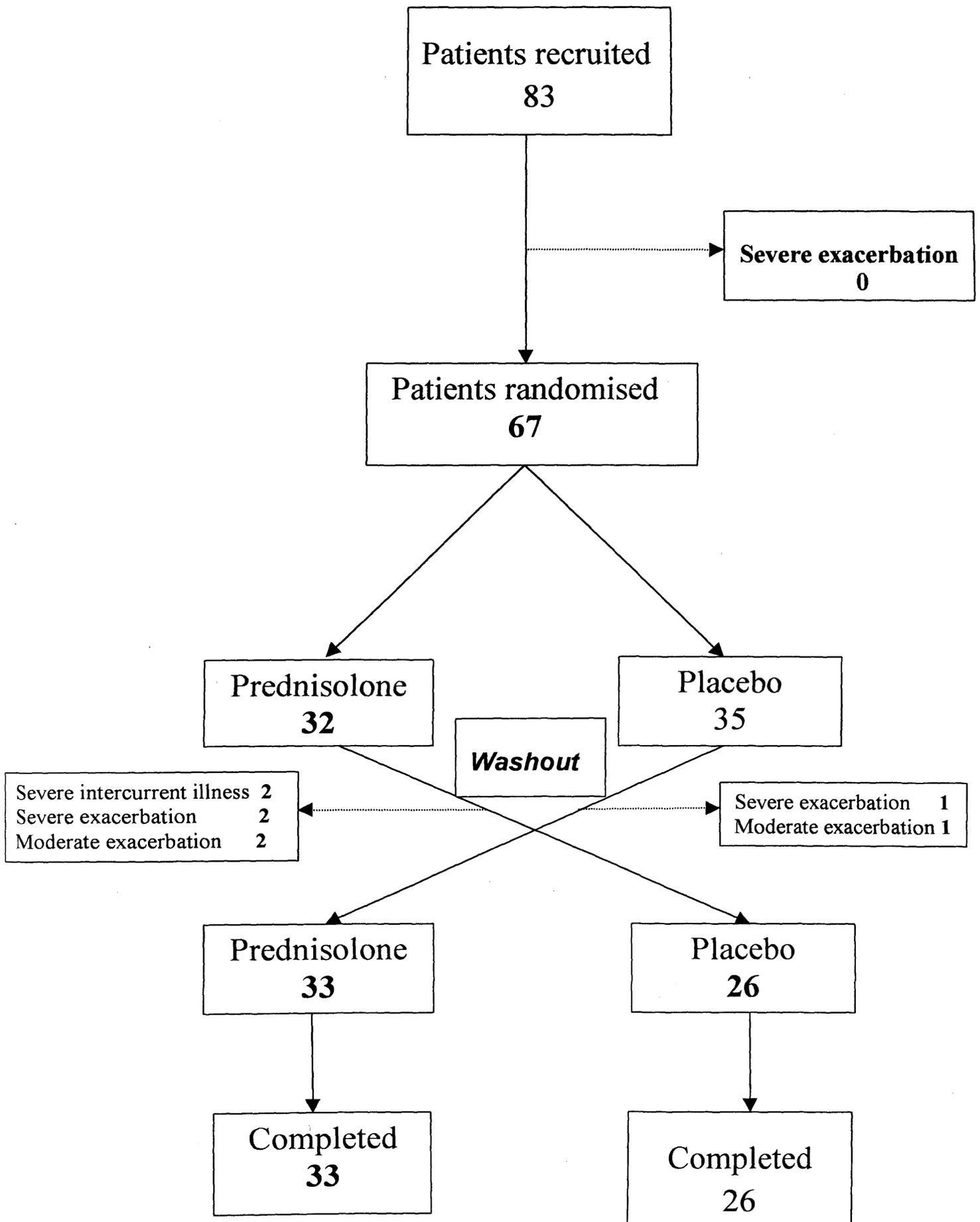
The primary outcomes were change in postbronchodilator FEV<sub>1</sub>, total CRQ score, and shuttle walk distance after prednisolone compared with placebo. The secondary outcome measures were changes in the individual domains of chronic respiratory disease questionnaire, symptom scores, and sputum characteristics.

### **Statistical analysis**

To assess the association between sputum eosinophil count and primary outcomes, we stratified patients into tertiles by baseline sputum eosinophil count. Baseline data were derived from measurements taken before the first treatment phase. The study had more than 80% power at the 5% level to detect a 150 mL difference in the change in FEV<sub>1</sub> within tertiles, assuming a within-patient SD of 100 mL (Tweeddale, Alexander, & McHardy 1987). A sample size of eight patients in each tertile would have been sufficient to achieve this power. If, however, we allowed for the possibility of a carry-over effect and a 10% dropout rate before completion of the first treatment phase, a sample size of 20 patients in each tertile was required.

We analysed all data with the Minitab statistical package for Windows (version 11). Spirometry, shuttle walk distance, total and domain CRQ scores, and symptom scores are expressed as mean (SE). Eosinophil count, total cell count, and mediator concentrations were log normally distributed and are described as geometric mean (log SE) with fold change (95% CI). We report other baseline sputum differential cell counts as mean (SE) and change after treatment for these as mean (95% CI). Cell viability and squamous-cell contamination are expressed as median (range). Improvement in the primary and secondary outcome variables after prednisolone compared with placebo are reported as paired mean differences (95% CI). We compared differences in patients' characteristics between tertiles by ANOVA, with the Student-Newman-Keuls procedure to correct for multiple comparisons. The significance of the trend between tertiles in the change in primary and secondary outcome variables was analysed by linear regression. All p values are two-tailed. Analyses were done by intention to treat. Patients who withdrew in the washout phase were assigned a net change of zero for the second treatment phase.

**Figure 3.17** Trial Profile



## Results

83 patients were recruited, of whom 67 were randomised (Figure 3.17). Seven of 45 patients taking inhaled corticosteroids at recruitment were excluded before randomisation because they developed moderate exacerbations in the run-in period. The baseline characteristics of the remaining 38 patients did not differ significantly from the corticosteroid-naïve patients. Eight patients withdrew during the washout period (Figure 3.17). Treatment period or order did not influence values before treatment or the changes in the primary outcome variables.

The geometric mean sputum eosinophil count significantly decreased after treatment with prednisolone from 2.4% to 0.4% (mean difference six-fold [95% CI 3.1-11.4],  $p < 0.0001$ ) but not after placebo (Table 3.15). Treatment-associated change did not differ between treatments for other sputum cell counts. 29 (43%) patients had a baseline eosinophil differential count higher than the normal range in our laboratory ( $>3\%$ ). The mean paired difference between prednisolone and placebo treatment for the change in primary outcomes for the whole group were: postbronchodilator FEV<sub>1</sub> 0.07 L (95% CI 0.01-0.14,  $p=0.02$ ), total score on CRQ 0.32 (0.17-0.47,  $p=0.0001$ ), and shuttle walk distance 12 m (3-21,  $p=0.01$ ). After stratification into tertiles by the baseline sputum eosinophil count, patients were well matched for age, sex, smoking history, and atopy, and did not differ in baseline spirometry, lung function, or total score on CRQ (Table 3.16).

Mean difference between prednisolone and placebo increased progressively from the lowest to the highest eosinophilic tertile for change in postbronchodilator FEV<sub>1</sub> ( $p=0.003$ ) and total score for CRQ ( $p=0.02$ ), but not for shuttle walk distance ( $p=0.56$ , Figure 3.18). The mean change in postbronchodilator FEV<sub>1</sub>, CRQ scores, and shuttle walk distance with prednisolone compared with placebo in the highest tertile were 0.19 L (0.06-0.32,  $p=0.005$ ), 0.62 (0.31-0.93,  $p=0.0005$ ), and 20 m (5-35,  $p=0.013$ ), respectively. The change in secondary outcome measures showed a similar progressive improvement from least to most eosinophilic tertile. The trend was significant for the chronic respiratory disease scores for dyspnoea ( $p=0.005$ ), fatigue ( $p=0.04$ ), and mastery domains ( $p=0.01$ ), and the symptoms of wheeze ( $p=0.02$ ), sputum production ( $p=0.04$ ), and breathlessness ( $p=0.01$ , Table 3.17). The decrease in eosinophilic cationic protein in response to prednisolone was

significant for the highest eosinophilic tertile (Table 3.17). The concentration of elastase and interleukin 8 did not differ between the tertiles or in response to prednisolone (Tables 3.16 and 3.17). The two-week repeatability of the outcome measures assessed in the placebo arm is shown in Appendix III.

**Table 3.15** Mean (SE) outcome measures before and after treatment

	<b>Before prednisolone</b>	<b>After prednisolone</b>	<b>Before placebo</b>	<b>After placebo</b>
FEV <sub>1</sub> (L)	1.03 (0.05)	1.09 (0.05)	1.09 (0.05)	1.08 (0.05)
Postbronchodilator FEV <sub>1</sub> (L)	1.10 (0.05)	1.15 (0.05)	1.15 (0.06)	1.14 (0.06)
CRQ total score	3.98 (0.13)	4.34 (0.14)	4.03 (0.14)	4.08 (0.14)
Shuttle (m)	217 (13)	230 (14)	213 (12)	214 (12)
TCC (10 <sup>6</sup> cells/g sputum)*	2.79 (0.05)	2.37 (0.04)	2.66 (0.05)	2.63 (0.05)
Eosinophil count (%)*	2.35 (0.09)	0.39 (0.14)†	2.18 (0.09)	2.08 (0.13)
Neutrophils (%)	67.9 (3.2)	70.4 (2.7)	73.7 (2.5)	69.0 (2.7)
Macrophages (%)	21.5 (2.3)	25.4 (2.7)	22.1 (2.5)	22.7 (2.4)
Bronchial epithelial cells (%)	1.17 (0.2)	1.37 (0.23)	0.99 (0.16)	1.47 (0.35)
Lymphocytes (%)	0.40 (0.07)	0.44 (0.15)	0.35 (0.05)	0.71 (0.25)
Squamous cells (%)‡	1.85 (0.51)	3.15 (0.49)	2.6 (0.40)	2.2 (0.51)
Viability (%)‡	76 (28-95)	67 (20-86)	73 (23-91)	71 (18-89)
ECP ng/mL sputum*	1454 (0.08)	801 (0.08)	1149 (0.09)	1236 (0.09)
Elastase µg/mL*	53.2 (0.12)	31 (0.12)	42.5 (0.11)	36.5 (0.11)
Interleukin 8 (ng/mL)*	117 (0.1)	105 (0.1)	112 (0.08)	97 (0.1)

TCC=total cell count; ECP=eosinophilic cationic protein; CRQ=chronic respiratory disease questionnaire.\*Geometric mean (log SE). †p<0.01. ‡Median (range).

**Table 3.16** Baseline characteristics after stratification into tertiles by baseline eosinophil counts

	Baseline eosinophil count tertile		
	<1.3 (n=22)	1.3-4.5 (n=23)	>4.5 (n=22)
<b>Patients' characteristics</b>			
Male/female	13 (59%)	13 (57%)	15 (68%)
Mean (range) age (years)	68 (42-82)	66 (49-83)	64 (47-78)
Current smoker	6	6	5
Ex-smokers	14	17	15
Mean (SE) number of pack-years	33 (4.1)	35 (2.5)	37 (4.3)
<b>Test outcomes (mean [SE])</b>			
Atopic by positive RAST	7	7	6
IgE (KU/L)	110 (40)	145 (52)	108 (31)
FBC Eo ( $\times 10^9/L$ )	0.12 (0.02)	0.23 (0.04)	0.17 (0.02)
Shuttle walk distance (m)	176 (21.1)*	247 (22.5)	246 (23.3)
Total CRQ score	3.92 (0.25)	4.04 (0.19)	4.08 (0.25)
FEV <sub>1</sub> (L)	1.15 (0.09)	1.11 (0.09)	0.96 (0.08)
Postbronchodilator FEV <sub>1</sub> (L)	1.19 (0.14)	1.15 (0.09)	1.01 (0.08)
FEV <sub>1</sub> % predicted	44.3 (2.6)	43.6 (3.5)	37.8 (3.0)
FVC (L)	2.30 (0.14)	2.25 (0.11)	2.32 (0.17)
Total lung capacity % predicted	93 (4.7)	96 (3.6)	97 (3.6)
Corrected carbon monoxide transfer	89 (7.6)	91 (7.7)	84 (8.5)
Coefficient (KCO) % predicted			
<b>Sputum characteristics†</b>			
Eosinophils (%)	0.5 (0.08)	2.5 (0.02)	12.3 (0.07)
Neutrophils (%)	79.4 (4.0)	77.4 (2.5)	59.1 (5.3)*
Total cell count ( $10^6$ cells/g sputum)	2.69 (0.1)	2.74 (0.08)	2.88 (0.08)
ECP (ng/mL)	1183 (0.13)	869 (0.13)	3162 (0.12)*
Elastase ( $\mu\text{g/mL}$ )	78 (0.19)	27 (0.25)	48 (0.17)
Interleukin 8 (ng/mL)	97 (0.18)	98 (0.14)	106 (0.17)

**CRQ domain scores (mean [SE])**

Dyspnoea	3.54 (0.24)	3.42 (0.24)	3.36 (0.26)
Fatigue	3.36 (0.30)	3.82 (0.26)	3.75 (0.26)
Emotion	4.12 (0.26)	4.53 (0.25)	4.66 (0.30)
Mastery	4.64 (0.28)	4.4 (0.25)	4.56 (0.32)

**Symptom scores (mean [SE])**

Cough VAS (mm)	47.3 (6.1)	26.1 (3.5)*	36.3 (5.6)
Sputum VAS (mm)	36.4 (5.6)	19.9 (2.5)*	30.6 (5.2)
Dyspnoea VAS (mm)	50.1 (5.0)	43.8 (4.7)	54.2 (5.3)
Wheeze VAS (mm)	43.0 (6.3)	26.7 (5.6)	31.3 (5.9)

**Sputum characteristics‡**

Lymphocytes (%)	0.3 (0.09)	0.3 (0.07)	0.6 (0.13)*
Macrophages (%)	19.7 (4.2)	22.6 (3.9)	21.3 (3.5)
Bronchial epithelial (%)	0.9 (0.4)	1.0 (0.2)	1.3 (0.3)
Squamous cells (%)	3.4 (0.42)	1.1 (0.51)	2.6 (0.51)
Viability (%)	77 (35-89)	79 (27-95)	68 (23-83)

RAST=radioallergosorbent test. \* $p < 0.05$ . †Values are geometric mean (SE), except neutrophils, which are mean (SE). ‡Values are geometric mean (log SE), except squamous cells and viability, which are median (IQR).

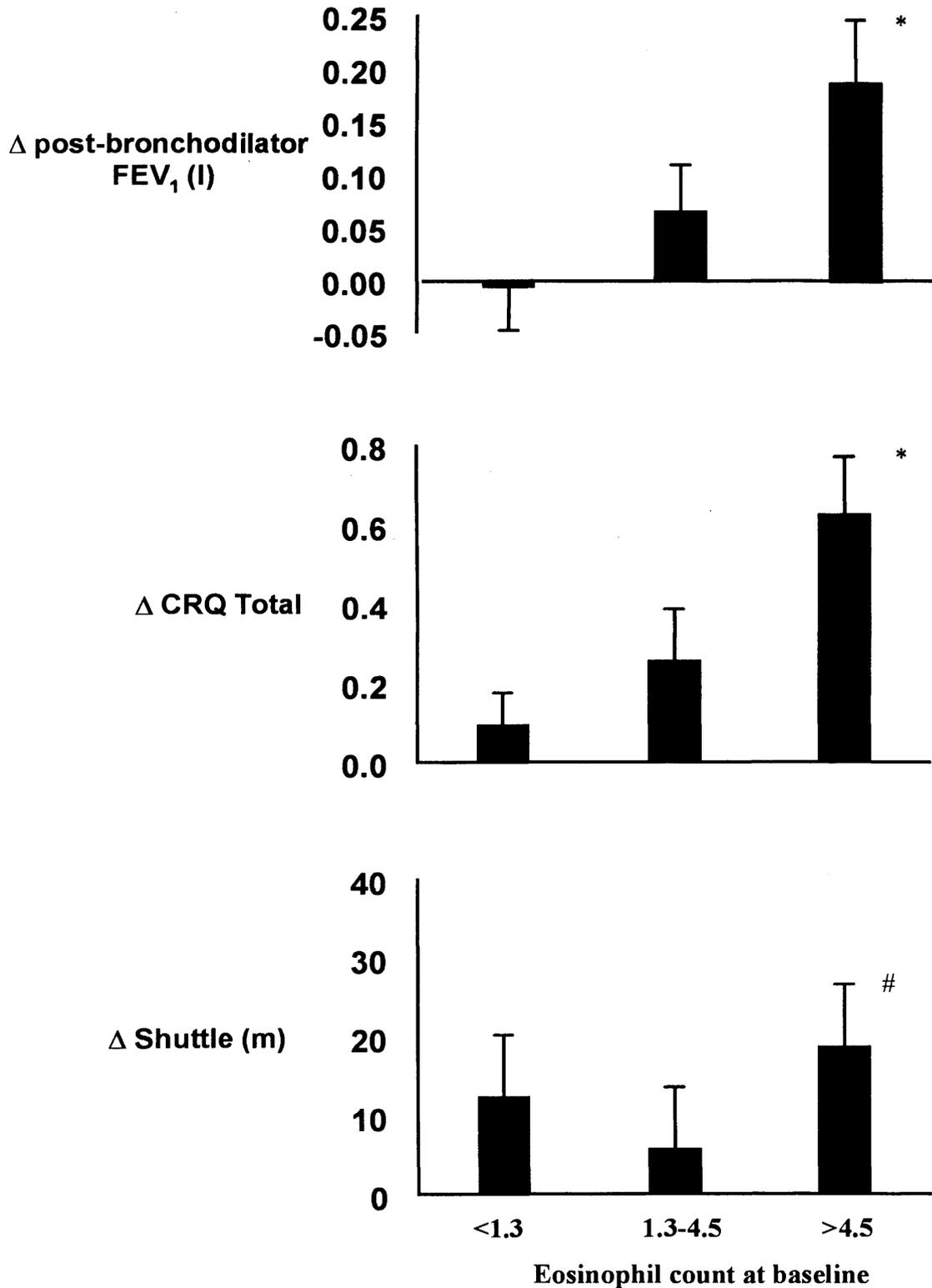
**Table 3.17** Mean (95% CI) change in secondary outcomes after prednisolone treatment compared with placebo

	<b>Eosinophil-count tertile</b>		
	<1.3 (n=22)	1.3-4.5 (23)	>4.5 (22)
<b>CRQ domain*</b>			
Dyspnoea CRQ	0.15 (-0.04 to 0.35)	0.27 (-0.11 to 0.65)	0.93 (0.50 to 1.35)†
Fatigue CRQ	0.09 (-0.28 to 0.46)	0.42 (0.06 to 0.79)‡	0.56 (0.20 to 0.91)†
Emotion CRQ	0.08 (-0.18 to 0.35)	0.12 (-0.15 to 0.39)	0.19 (-0.11 to 0.49)
Mastery CRQ	0.03 (-0.23 to 0.30)	0.20 (-0.202 to 0.59)	0.82 (0.37 to 1.26)†
<b>Symptom scores§</b>			
Cough VAS†	2 (-27 to 31)	41 (9 to 74)‡	53 (-1 to 106)
Sputum VAS†	6 (-15 to 26)	34 (2 to 66)‡	45 (18 to 72)†
Dyspnoea VAS†	5 (-3 to 14)	14 (3 to 27)‡	32 (9 to 55)†
Wheeze VAS†	-1 (-15 to 13)	21 (-1 to 14)	42 (-0 to 85)
<b>Sputum indices  </b>			
Total cell count	1.03 (0.62 to 1.72)	1.38 (0.93 to 2.03)	1.11 (0.74 to 1.65)
Eosinophil count	3.1 (0.9 to 10.6)	5.4 (1.2 to 24.2)†	6.9 (1.3 to 36.4)†
Neutrophil count	-3.0 (-12.5 to 6.5)	-4.5 (-15.2 to 6.2)	-19.3 (-31.8 to -6.8)‡
ECP	1.2 (0.6 to 2.7)	1.5 (0.7 to 3.1)	3.4 (1.4 to 8.1)†
Elastase	1.9 (0.5 to 7.5)	1.2 (0.2 to 6.5)	2.8 (0.5 to 13.8)
Interleukin 8	1.3 (0.3 to 5.6)	0.7 (0.1 to 2.9)	2.1 (0.7 to 6.2)

\*Absolute decrease. †p<0.01. ‡p<0.05. §Percentage decrease. ||Fold decrease except neutrophil count, which is absolute decrease.

**Figure 3.18** Mean (SE) absolute increase in primary outcomes for each tertile after prednisolone compared with placebo

\*p<0.01, #p<0.05



## Discussion

Eosinophilic airway inflammation was common among patients who had stable moderate and severe COPD, and postbronchodilator FEV<sub>1</sub>, health status, and exercise capacity were improved by prednisolone. Greater improvements were seen in patients with higher baseline sputum eosinophil counts than for those with lower counts, and the improvements were associated with striking reductions in sputum eosinophil count and sputum concentration of the activated eosinophil product eosinophilic cationic protein. Cell and molecular markers of neutrophilic inflammation were not affected. These findings strongly suggest that eosinophilic airway inflammation contributes to airflow obstruction and symptoms in some patients with COPD, and the effects of corticosteroids are due to inhibition of this feature of the inflammatory response in COPD.

The mean effect of prednisolone on FEV<sub>1</sub> after bronchodilator use was slight, even in the highest tertile of sputum eosinophil count, which suggests that other factors, such as a permanent structural defect and corticosteroid-unresponsive non-eosinophilic airway inflammation are the main causes of airflow obstruction in COPD. Changes in health status and symptom scores associated with treatment were more obvious, and in most domains of the CRQ surpassed the minimum difference for clinical importance (Guyatt et al 1987). As with FEV<sub>1</sub>, improvement in these variables was related to the sputum eosinophil count, which suggests that it represents an effect on the airways rather than a non-specific corticosteroid effect.

Our findings for FEV<sub>1</sub>, health status, sputum inflammatory cell counts, and sputum mediators are in accord with those of Pizzichini and colleagues (Pizzichini et al 1998). The findings are also consistent with those of Chanez and colleagues (Chanez et al. 1997) who showed that patients with COPD who improved after open-label treatment with prednisolone generally had pathological features of asthma on bronchial biopsy and bronchoalveolar lavage. By contrast, Keatings and colleagues (Keatings et al 1997b) saw no effect on FEV<sub>1</sub> or sputum eosinophil count after 2 weeks of treatment with oral prednisolone in a small single-blind study, although there was no strong sputum evidence of eosinophilic airway inflammation. The effects of inhaled corticosteroids on airway inflammation in COPD are less consistent. In one open study, 2 months of treatment with inhaled beclomethasone dipropionate lowered

the sputum neutrophil count (Confalonieri et al 1998), whereas Keatings and co-workers (Keatings et al 1997b) found no change in sputum differential cell counts after 2 weeks of inhaled budesonide compared with placebo. Whether the effects of inhaled and oral corticosteroids differ is unclear and needs further study.

COPD is associated with sputum and bronchial biopsy evidence of neutrophilic airway inflammation and the extent of this inflammation, as reflected by the induced sputum neutrophil differential count, inversely correlates with FEV<sub>1</sub> and the decline in FEV<sub>1</sub>, which suggests that it is functionally important (Keatings & Barnes 1997) (Di Stefano et al 1998) (Stanescu et al 1996). The patients in our study, who had moderate and severe COPD, had sputum neutrophilia, in keeping with predominant neutrophilic airway inflammation. Little attention has been paid to the presence of eosinophilic airway inflammation in stable COPD, although reports of smaller numbers of patients with COPD of comparable severity have noted similar group mean induced sputum eosinophil counts (Confalonieri et al 1998) (Saetta et al 1994).

The origin of eosinophilic airway inflammation in COPD is unclear, although the presence of an asthmatic component to the fixed-airways obstruction is assumed (Barnes 1998). An asthmatic component was unlikely in our population since we rigorously excluded patients who had variable airflow obstruction and clinical features suggesting asthma. Furthermore features such as a blood eosinophilia, atopy, smoking history, and physiological evidence of emphysema were no more or less common in patients in the highest eosinophilic tertile. It is more likely that smoking (Matsumoto et al. 1998) and other mechanisms that recruit neutrophils into the airway mucosa in COPD cause a degree of eosinophil influx. In some patients this influx might amount to functionally important eosinophilic airway inflammation. Explanation of the high sputum eosinophil counts observed in some of our patients is, however, difficult. One possibility is that eosinophilic COPD starts as eosinophilic bronchitis. This disorder is a common cause of chronic cough in middle age, characterised by a sputum eosinophilia but no symptoms and functional evidence of variable airflow obstruction or airway hyper-responsiveness (Brightling et al 1999b). Although characterised by normal spirometric values at the time of diagnosis, eosinophilic

bronchitis has been associated with an accelerated decline in FEV<sub>1</sub> and the development of COPD (Brightling et al 1999a).

There is currently much interest in the role of long-term inhaled corticosteroid treatment in COPD (Boushey 1999) (Calverley 1999). Investigators, in two large, placebo-controlled trials, have shown a small improvement in FEV<sub>1</sub> in the first 3 months of treatment (Burge et al. 2000) (Pauwels et al. 1999); such improvement was not seen in a third study in which patients with a positive oral corticosteroid response were excluded (Vestbo et al. 2000). Treatment did not modify the subsequent rate of decline in FEV<sub>1</sub> over 3 years in any of those studies, but in one, 500 µg inhaled fluticasone twice daily lowered the exacerbation frequency, especially in patients who had more severe disease (Burge et al 2000). The important remaining question is whether these minor clinical benefits are confined to a definable subgroup of patients. Although we cannot discount that corticosteroids might have additional or different effects when given for long periods or via the inhaled route, our findings raise the possibility that a simple sputum test might allow treatment to be targeted to a population who would benefit especially. Further studies to investigate the role of induced sputum as a predictor of long-term response to inhaled corticosteroids are a priority.

## 4. Conclusions

### 4.1 Summary of findings

This thesis is the first study to investigate in detail the clinical and immunopathological features of eosinophilic bronchitis and to define the relationship between an airway eosinophilia and the response to corticosteroids in eosinophilic bronchitis, asthma and chronic obstructive pulmonary disease (COPD).

Eosinophilic bronchitis was identified as a common cause of chronic cough (Brightling et al 1999b), which responds well to inhaled corticosteroids (Brightling et al 2000a). In addition, we demonstrated that a sputum eosinophilia predicts a good response to corticosteroids in asthma and COPD.

The different association between airway inflammation and dysfunction in asthma and eosinophilic bronchitis was not found to be due to localisation of the inflammatory process in the upper airway in eosinophilic bronchitis (Brightling et al 2000a) or differences in the state of activation of the inflammatory process as assessed by induced sputum inflammatory mediator concentrations (Brightling et al 2000b).

Eosinophilic bronchitis was characterised by sputum, bronchial wash and bronchoalveolar lavage (BAL) eosinophilia and bronchial submucosal evidence of eosinophilic airway inflammation, increased Th2 cytokine expression and basement membrane thickening to the same degree as those with asthma. In asthma and eosinophilic bronchitis there was increased constitutive intracellular expression of IL-4 from BAL T-cells. We quantified the inflammatory cell infiltration of the airway smooth muscle and found a striking increase in the number of mast cells within the airway smooth muscle in asthma compared to eosinophilic bronchitis and normal subjects.

Thus, the immunopathology of eosinophilic bronchitis is very similar to asthma demonstrating dissociation between eosinophilic airway inflammation, structural changes of the airway, Th2 cytokine expression and the abnormalities in airway physiology that characterise asthma. Our findings suggest that in asthma the microlocalisation of mast cells within the airway smooth muscle is a key factor

in the development of variable airflow obstruction and airway hyperresponsiveness. Thus, specific targeting of the mast cell-smooth muscle interaction may provide a novel approach to the effective treatment for asthma.

## **4.2 Techniques developed and problems encountered**

A number of techniques, which were new to our laboratory, were developed and validated as part of this thesis. The techniques included: capsaicin cough challenge, nasal lavage, analysis of chemokine receptors and intracellular cytokines by three colour flow cytometry, elastase spectrofluorimetric assay and immunohistochemistry using glycomethacrylate resin.

The only technical problem encountered for which insufficient time was available to overcome was the development of in-situ hybridisation. Probes for both IL-4 and IFN- $\gamma$  were produced, but a satisfactory positive control for IL-4 was not identified. Thus, this technique could not be adequately validated. However, we developed a method for the quantification of intracellular expression of cytokines from bronchoalveolar lavage (BAL) T-cells. This enabled us to measure the cytokine protein within CD4 and CD8 BAL T-cells rather than mRNA expression, which may be of more functional significance.

## **4.3 Criticisms**

Some potential criticisms of the work presented in this thesis are addressed in the discussion of each chapter. However, some more general issues deserve particular attention. The studies that compared the immunopathology of eosinophilic bronchitis and asthma (Chapters 3.1.2, 3.1.3, 3.1.4, 3.1.5) were all cross-sectional studies. Some of the subjects had been assessed on several occasions and anectodally they appeared to have a consistent phenotype, but no formal longitudinal studies were performed as part of this thesis. Similarly, subjects with eosinophilic bronchitis were only investigated in one uncontrolled interventional study (Chapter 3.2.1). As of yet there are no published reports of controlled treatment trials for patients with eosinophilic bronchitis. These are important areas of future study as outlined below.

In some, but not all of the studies the number of subjects required were based on power calculations. The number of subjects needed to observe a clinically significant change in lung function following oral corticosteroids in subjects with COPD was carefully calculated to have 80% power at the 5% level (Chapter 3.2.2). In contrast no formal estimate of the sample size to observe an effect on symptoms or airway inflammation was made for the study on subjects with eosinophilic bronchitis treated with corticosteroids (Chapter 3.2.1). This would be important in the planning of any future interventional studies. The number of subjects chosen for the immunopathological comparison of eosinophilic bronchitis and asthma (Chapter 3.1.3, 3.1.4) was based on published power calculations that suggest the optimal number of subjects is approximately 15 in each group (Richmond et al. 1996). However, this sample size is based on the variability of submucosal counts and no data has been published on cell counts within the airway smooth muscle. Future interventional studies looking at treatment effects on mast cells in airway smooth muscle will need to take into account the variability of these cell counts.

All of the subjects with asthma that underwent bronchoscopy had mild asthma with few symptoms and were treated with short-acting bronchodilators alone. Thus, our subjects were a selective group and may not be representative of asthma in the general population. This criticism is particularly pertinent for the study investigating inflammatory cells within the airway smooth muscle as only subjects with assessable airway smooth muscle were included selecting a subgroup with possibly a distinct immunopathology. Although this may be true the submucosal cell counts from those subjects with assessable smooth muscle were similar to those without airway smooth muscle. Furthermore, it would seem likely that subjects with more severe disease may have more inflammatory cells in their bronchial biopsies suggesting that the increase in mast cells in the airway smooth muscle may have been underestimated. It is important that future studies include subjects with asthma that encompass the whole spectrum of disease severity.

### 4.3 Future studies

This thesis has not attempted to explore the possible aetiology of eosinophilic bronchitis. Only a limited number of aeroallergens were included in the assessment of atopy. Thus, the proportion of subjects with eosinophilic bronchitis with atopy may be underestimated. To further investigate the role of atopy in eosinophilic bronchitis a more exhaustive panel of allergens would need to be used. Local IgE production could be investigated by measuring the expression of IgE heavy chain in the bronchial biopsies. The possibility that viral infections may be important in the development of eosinophilic bronchitis would be difficult to confirm. However, the presence of latent viruses could be studied from bronchial biopsies.

There is a clear need to define the natural history of eosinophilic bronchitis. It is unknown how often eosinophilic bronchitis progresses onto asthma or COPD or whether the condition is usually benign and self-limiting. The long-term response to inhaled corticosteroids together with the effects of other therapies such as antihistamines or leukotriene inhibitors needs to be addressed. Similarly, how subjects with eosinophilic bronchitis respond clinically and pathologically to controlled respiratory infections or allergen challenge would be informative.

This thesis has provided further evidence questioning the importance of eosinophilic airway inflammation and other important immunopathological features of asthma in the development of variable airflow obstruction and airway hyperresponsiveness. The only immunopathological characteristic, which distinguished eosinophilic bronchitis from asthma, was the presence of mast cells within airway smooth muscle of patients with asthma but not those with eosinophilic bronchitis or normal subjects. Therefore this suggests that the recruitment of mast cells to the airway smooth muscle is a critical factor in the development of the asthma. It is possible that the release of mast cell chemoattractants and growth factors by airway smooth muscle leads to mast cell recruitment, and subsequently, mast cell infiltration, adhesion and activation in response to both inhaled allergen and airway smooth muscle proinflammatory mediators such as chemokines and cytokines propagates disordered airway smooth muscle function in asthma. This may in turn enhance mast cell

responsiveness to immunological and non-immunological stimuli. Thus, a positive feedback loop between mast cell activation and disordered airway smooth muscle physiology, leading to amplification of the airway inflammatory response and bronchial hyperresponsiveness. If this is true, then inhibition of mast cell infiltration into, and adhesion to the airway smooth muscle in asthma will attenuate the disease. Future projects will need to investigate mast cell chemotaxis and adhesion and the functional consequences of the mast cell-airway smooth muscle interaction on mast cell/airway smooth muscle function.

Although eosinophilic airway inflammation may be dissociated from disordered airway physiology in asthma, the presence of a sputum eosinophilia predicts a good response to corticosteroids in eosinophilic bronchitis, asthma and COPD. Whether the eosinophil is a by-stander or important effector cell in the development of disease is still undecided, but a sputum eosinophil count is a good biomarker for corticosteroid responsive disease. Thus, future studies need to address whether directing treatment towards normalising the sputum eosinophil count in airway diseases alters exacerbation rates or disease progression such as airway re-modelling.

We and others have begun to answer these important questions, which I anticipate will further inform our understanding of the basic mechanisms of airway diseases and have significant impact on our clinical practice.

**Appendix I Clinical characteristics of subjects with eosinophilic bronchitis and listing of which studies they participated in. (Patients were re-characterised for each study, values given are for the first study)**

	Age	Gender	Total IgE (KU/l)	Atopic	3.1.1	3.1.2	3.1.3	3.1.4	3.1.5	3.2.1
1 SH	28	M	51	Y			X	X	X	
2 KK	30	F	266	Y	X	X	X	X	X	X
3 MP	45	M	27	N			X	X	X	
4 SB	51	M	98	Y			X	X	X	
5 TT	50	M	91	Y			X	X		
6 JR	68	F	42	N			X	X		
7 AH	49	F	41	N			X	X	X	X
8 MT	46	M	225	Y			X	X	X	
9 JS	62	F	30	N	X	X	X	X	X	X
10 JC	54	M	89	Y			X	X	X	
11 YD	42	M	63	Y			X	X	X	
12 EE	62	M	107	Y		X	X	X		X
13 HP	43	F	34	Y	X	X	X	X	X	X
14 RJ	43	F	68	N			X	X	X	
15 JJ	33	M	124	Y			X	X	X	
16 RP	68	M	68	N			X	X	X	
17 PG	50	F	NK	N	X	X				X
18 IH	64	F	NK	Y	X	X				X
19GH	77	F	NK	N	X					
20MS	67	F	NK	N	X					
21PW	43	M	NK	N	X					
22 AR	62	M	NK	N	X					
23 HJ	63	M	NK	Y	X					
24MH	29	F	NK	N	X					
25PM	50	M	61	Y	X	X				X
26 RR	36	M	NK	N						X
27 BP	68	M	NK	Y						X
28 SS	63	M	NK	N		X				X

## Appendix II Repeatability of bronchial biopsy measures

(see 3.1.3, 3.1.4, 3.1.5)

### Between observer repeatability of cell and cytokine counts in bronchial biopsy and basement membrane thickness.

	Number of repeat measurements	Correlation
Mast cells (AA1)	18	0.86 (Spearman)
Eosinophils (EG2)	12	0.95 (Spearman)
T-lymphocytes (CD3)	17	0.96 (Spearman)
IL-4 (3H4)	41	0.78 (Spearman)
Basement membrane thickness	12	0.79 (Pearson)

### Within biopsy repeatability of bronchial biopsy cell and cytokine counts.

	Number of repeat measurements	Intraclass correlation
Mast cells (AA1)	42	0.86
T-lymphocytes (CD3)	42	0.64
Eosinophils (EG2)	42	0.95
IL-4 (3H4)	41	0.67

### Between biopsy repeatability of bronchial biopsy cell and cytokine counts.

	Number of repeat measurements	Intraclass correlation
Mast cells (AA1)	11	0.61
T-lymphocytes (CD3)	10	0.48
Eosinophils (EG2)	10	0.94
IL-4 (3H4)	10	0.42

**Appendix III Within subject 2-week repeatability of outcome measures in COPD measured from placebo arm (see 3.2.2)**

	Visit 1	Visit 2	Within subject SD	ICC
Neutrophil (%)	74 (2.5)	69 (2.7)	17.8	0.57
Eosinophil (%)*	2.2 (0.09)	2.1 (0.13)	0.66	0.63
Macrophage (%)	22 (2.5)	23 (2.4)	16.4	0.54
Lymphocyte (%)	0.3 (0.05)	0.7 (0.25)	1.95	0.15
Epithelial cells (%)	1.0 (0.16)	1.5 (0.35)	2.8	0.32
TCC x10 <sup>6</sup> /g sputum*	2.7 (0.04)	2.6 (0.05)	0.31	0.54
ECP ng/g*	1149 (0.09)	1236 (0.09)	0.48	0.70
IL-8 ng/g*	112 (0.08)	97 (0.1)	0.62	0.71
Elastase µg/ml*	42.5 (0.11)	36.5 (0.11)	1.07	0.4
CRQ: Total	4.03 (0.14)	4.08 (0.14)	0.18	0.95
CRQ: Dyspnoea	3.52 (0.16)	3.44 (0.17)	0.33	0.91
CRQ: Fatigue	3.72 (0.17)	3.77 (0.16)	0.33	0.90
CRQ: Emotion	4.46 (0.17)	4.6 (0.17)	0.22	0.96
CRQ: Mastery	4.61 (0.18)	4.59 (0.19)	0.26	0.94
VAS: Dyspnoea (mm)	48.2 (3.2)	48.0 (3.2)	4.82	0.94
VAS: Wheeze (mm)	32.1 (3.6)	31.8 (3.5)	8.62	0.87
VAS: Cough (mm)	32.4 (4.6)	33.5 (3.4)	7.75	0.87
VAS: Sputum (mm)	26.8 (3.0)	27.5 (3.1)	4.82	0.94

\*Geometric mean (log SEM), ^ Median (IQR), # Mean (SEM)

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