AN EXPLORATION OF ASTHMA PHENOTYPES

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF MEDICINE
AT THE UNIVERSITY OF LEICESTER
BY

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In memory of Lloyd Weaver.
I. Acknowledgments

I owe a debt of gratitude to Professor Ian Pavord, for my initial appointment, for advice guidance and perceptive comment throughout my research, for his encouragement and confidence in me and for giving me the intellectual and financial freedom to generate and pursue my own hypotheses. In addition I owe thanks to Chris Brightling and Ruth Green for their direction and teaching. Thank you to my colleagues Rosh Siva and Dominick Shaw and thank you to Debbie, Will, Natalie, Fiona, Sue, Maria and Bev whose invaluable contributions I have outlined in the next chapter. I big thank you to all of the patients who participated in my studies and to Asthma UK and University Hospitals of Leicester ho provided the funding. Finally, a special thank you to my wife Alex for her support, constructive criticisms and for our daughters Megan, Aimee and Molly.
II. Statement of work personally performed

**Observational study of the natural history of eosinophilic bronchitis**

Ian Pavord based this study on an original concept. Ian Pavord and I designed the study. I obtained ethical approval, recruited the patients and performed 30% of the physiological measurements myself. Susan McKenna performed the remaining measurements. Natalie Neale performed sputum processing and sputum cell counts by Will Monterio. I did the data analysis.

**Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis**

Christopher Brightling based this study on an original concept and he obtained ethical approval. I recruited 50% of the patients the remainder being recruited by Dr Brightling. Beverley Hargadon and Susan McKenna performed sputum induction. I performed 50% of the bronchoscopies were performed and the remainder were performed by Dr Brightling. Deborah Parker conducted dialysis, spiking recovery and ELISA experiments. Natalie Neale and myself performed cutting and staining of biopsies and Dr Brightling performed blinded counting of stained biopsies. Dr Brightling performed data analysis.
Clinical and pathological features of non-eosinophilic asthma

Ian Pavord based this study on an original concept. I obtained ethical approval for the studies. I recruited 65% of the patients for the bronchoscopy study and all of the patients for the placebo controlled study. I performed 65% of the bronchoscopies and Angela Morgan performed the remainder. Angela Morgan and I performed processing of the BAL and bronchial biopsies equally. Natalie Neale performed blinding of the biopsies and I carried out biopsy counting with quality control by Christopher Brightling. Beverley Hargadon and I performed measurements for the placebo control study equally. Natalie Neale performed sputum processing and cell counts by Will Monterio. I performed the data analysis.

Alveolar nitric oxide concentration in adults with asthma: evidence of distal lung inflammation in refractory asthma

I based this study on an original concept. I designed the studies. I performed 70% of the nitric oxide measurements; Beverley Hargadon performed the remainder. Jeanne Richter wrote the software for regression analysis. I performed 50% of the bronchoscopies, the remainder were performed by Angela Morgan. Angela Morgan and I processed BAL and bronchial wash samples equally. Natalie Neale performed sputum processing and cell counts by Will Monterio. I performed the data analysis.
Evidence of a role for TNF-α in refractory asthma

This study was based on my original concept. I obtained ethical approval and funding. I recruited all of the patients and performed 30% of the physiological measurements the remainder of which were performed by Beverley Hargadon. I performed all of the flow cytometry. Susan McKenna prepared the injections of etanercept/placebo and Dominick Shaw produced the randomisation sequence. I performed the data analysis.
III. Publications arising from this thesis

Original papers


**Reviews**


2. Siva R, **Berry MA** and Pavord ID. Recent insights into the relationship between airway inflammation and asthma. Monaldi Arch Chest Dis. 2003 Oct-Dec; 59(4): 296-9

**Book chapters**


**Abstracts**


3. *Berry MA*, Green RH, Brightling CE, McKenna S, Hargadon B, Wardlaw AJ and Pavord ID. Association between post bronchodilator FEV$_1$ and smoking, atopy, duration of symptoms, inhaled corticosteroid use and
induced sputum cell counts in adults with asthma. Thorax 2003 Dec; 58(Suppl III): iii60


concentration and induced sputum eosinophil counts. Am. J. Respir. Crit. Care Med 2004 Apr; 169(7): A824


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V. Abstract

Classification of patterns of airway function and inflammation is a potentially powerful tool to investigate the complex pathological mechanisms in the asthmatic airway and allow for rational targeting of treatment. Such disease phenotypes include; eosinophilic bronchitis, non eosinophilic asthma and refractory asthma. In this thesis the long term stability of eosinophilic bronchitis has been investigated in a longitudinal study. Induced sputum has been used to investigate a possible difference in expression of IL-13 in asthma and eosinophilic bronchitis. Bronchoscopy has been used to compare the airway immunopathology of eosinophilic and non eosinophilic asthma and the long term stability and response to inhaled corticosteroids in non eosinophilic asthma was studied in a prospective randomised controlled trial. Alveolar nitric oxide concentration has been validated as a measure of distal lung inflammation. Finally the role of TNF-α and response to etanercept has been investigated in patients with refractory asthma. Evolution from eosinophilic bronchitis into asthma was rare, decline in lung function was not increased, although female gender, smoking and prolonged eosinophilic airway inflammation were independent risk factors for an accelerated decline. IL-13 concentrations were elevated in asthma compared to eosinophilic bronchitis, suggesting a role for this cytokine in the development of airway hyperresponsiveness. Non eosinophilic asthma remained stable over the period of investigation and was associated with reduced response to inhaled corticosteroids. Mast cells were present in airway smooth muscle in eosinophilic and non eosinophilic asthma but not normal controls. Subepithelial layer thickening was a feature of eosinophilic but not non eosinophilic asthma. Alveolar nitric oxide was increased in refractory asthma and reduced by oral but not inhaled corticosteroids.
Membrane bound TNF-α and TNF-α receptor 1 and TNF-α converting enzyme were increased on CD14+ve monocytes in refractory asthma and treatment with etanercept improved asthma quality of life and airway hyperresponsiveness.
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1. Introduction

1.1 Asthma, airway inflammation and disease phenotypes

Asthma is a common disease of the airway, which is characterised by symptoms of cough, wheeze and breathlessness which are related to the underlying physiological abnormalities of variable airflow obstruction, airway hyperresponsiveness and fixed airflow obstruction. Associated with these physiological abnormalities is an inflammatory response within the airway. The relationship between airway inflammation and disordered air function and how they interact to cause symptoms remains unclear.

1.1.1 Assessment of airway inflammation

Various methods exist to measure markers of airway inflammation. These include; the measurement of cells and biomarkers found in induced sputum, blood eosinophil count, eosinophilic cationic protein (in blood and sputum), assessment of exhaled gases (including exhaled nitric oxide and carbon monoxide), breath condensate, bronchoalveolar lavage and bronchial biopsy.

Table 1.1.1 (page 3) summarises the feasibility and what is known about the influence on patient outcomes of the various approaches to assessing airway inflammation. Sputum induction has been shown to be easy and safe and sputum differential cell counts and mediator concentrations have been demonstrated to be repeatable and responsive in a variety of clinical situations (Green et al. 2002a; Pavord et al. 1997). Induced sputum analysis has the advantage of providing measurements of the type of airway inflammation (eosinophilic vs. neutrophilic) as
well as its severity. The technique is inexpensive, although it is labour intensive and does require training and experience to obtain reliable results. There are now well-validated methods for induction and processing of induced sputum (see Methods). Induced sputum analysis and measurement of blood eosinophil counts are the only measures to date which have been demonstrated to have a clinically important role in the prevention of severe asthma exacerbations (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a; Horn et al. 1975)

Exhaled nitric oxide has the advantage of being simple to measure and provides an immediate result but it cannot provide information on the nature of the inflammatory response. One important limitation of currently employed non-invasive measurement of airway inflammation is that they are limited to sampling from the proximal airway. Recently however mathematical models have been developed which allow the relative contributions of bronchial and alveolar nitric oxide concentrations to be estimated by measuring exhaled nitric oxide concentration and several different flows. The clinical role of single and flow independent nitric oxide parameters remains to be determined.
Table 1.1.1 Summary of relative merits of different measurement of airway inflammation.

<table>
<thead>
<tr>
<th>Method</th>
<th>Safety and ease of performing technique</th>
<th>Ease of analysing result</th>
<th>Time to result</th>
<th>Cost</th>
<th>Influence on outcome proved</th>
<th>Potential use</th>
</tr>
</thead>
<tbody>
<tr>
<td>induced sputum</td>
<td>+++</td>
<td>++</td>
<td>3-4 hours</td>
<td>moderate</td>
<td>yes</td>
<td>Secondary care</td>
</tr>
<tr>
<td>Blood eosinophil count</td>
<td>++++</td>
<td>++++</td>
<td>30 min</td>
<td>inexpensive</td>
<td>Possible(Horn, Robin, Theodore, &amp; Van Kessel 1975)</td>
<td>Secondary care</td>
</tr>
<tr>
<td>eosinophil cationic protein</td>
<td>++++</td>
<td>++++</td>
<td>3-4 hours</td>
<td>moderate</td>
<td>Not proven conclusively</td>
<td>Research</td>
</tr>
<tr>
<td>exhaled nitric oxide</td>
<td>++++</td>
<td>++++</td>
<td>immediate</td>
<td>expensive</td>
<td>Not proven</td>
<td>Research</td>
</tr>
<tr>
<td>carbon monoxide</td>
<td>+++</td>
<td>+++</td>
<td>immediate</td>
<td>inexpensive</td>
<td>Studies awaited</td>
<td>Research</td>
</tr>
<tr>
<td>breath condensate (hydrocarbons)</td>
<td>++</td>
<td>+</td>
<td>1-2 hours</td>
<td>inexpensive</td>
<td>Studies awaited</td>
<td>Research</td>
</tr>
<tr>
<td>BAL, bronchial wash and biopsy</td>
<td>+</td>
<td>+</td>
<td>2 days</td>
<td>moderate</td>
<td>Not proven</td>
<td>Tertiary care</td>
</tr>
</tbody>
</table>

+ Symbol indicates rating of test for the quality indicated. For example for ease of analysing result + would indicate the most difficult and ++++ the most easy.
1.1.2 Heterogeneity of airway inflammation in asthma

Asthma has been traditionally viewed as a condition where eosinophilic airway inflammation causes airway hyperresponsiveness, which in turn leads to variable airflow obstruction and symptoms. This hypothesis is deeply embedded, to the point where it is incorporated into recent definitions of asthma (2003a). However, cross-sectional and longitudinal studies of airway inflammation using sputum induction in large populations with a diverse range of presentations suggest that this hypothesis requires modification.

The view of the importance of eosinophilic airway inflammation in the pathogenesis of asthma has been heavily influenced by bronchoscopy studies performed over the last 20 years (Djukanovic et al. 1990). These, by necessity, were largely limited to young volunteers with mild disease. The development of a non-invasive technique to assess airway inflammation has made it possible to relate the presence of airway inflammation to objective measures of disordered airway function in larger and more heterogeneous populations than was possible with bronchoscopy studies. In general, these studies have contradicted findings in the earlier bronchoscopy studies in that they have not found a correlation between the sputum eosinophil count and various markers of airway dysfunction (Crimi et al. 1998; Gibson, Simpson, & Saltos 2001; Green et al. 2002b; Jatakanon et al. 1999; Rosi et al. 1999).

One surprising observation has been that a subset of symptomatic asthmatics do not have sputum evidence of eosinophilic airway inflammation (Gibson, Simpson, & Saltos 2001; Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord
Many have a sputum neutrophilia. This sputum profile is evident in corticosteroid-naïve (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b) as well as corticosteroid-treated subjects (Jatakanon, Uasuf, Maziak, Lim, Chung, & Barnes 1999; Wenzel 2003) suggesting it is not always an artefact related to treatment. These findings suggest the presence of a distinct asthma phenotype characterised by a predominantly neutrophilic airway inflammatory response across the range of asthma severity.

An inverse picture to non-eosinophilic asthma is also seen in clinical practice: eosinophilic inflammation in the absence of airway hyperresponsiveness or variable airflow obstruction which presents as an isolated chronic cough is known as eosinophilic bronchitis (Brightling et al. 1999a) and is thought to represent a distinct and stable clinical phenotype. Refractory asthma, which could be defined as asthma that does not respond to inhaled corticosteroids, is thought by many to represent a distinct clinical pattern of disease rather than being uncontrolled “classical” asthma (Wenzel 2003).

Thus cross-sectional studies suggest that to a large extent disordered airway function and eosinophilic airway inflammation appear to be independently regulated, suggesting that the earlier paradigm of a simple causal relationship between them needs to be modified.
1.1.3 The concept and use of asthma phenotypes

Although asthma is a condition which is characterised by variable airflow obstruction, airway hyperresponsiveness, eosinophilic airway inflammation and fixed airflow obstruction, these features can occur to differing degrees in different patients and indeed can occur to differing extent over time within the same patient. Dividing patients into subgroups depending on their predominant pathophysiological abnormality or “phenotype” is a potentially useful tool for examining the underlying pathological basis for a physiological abnormality. An example of this come from work by Brightling et al who, in comparing the immunopathology of eosinophilic bronchitis, a condition characterised by eosinophilic airway inflammation in the absence of airway hyperresponsiveness or fixed or variable airflow obstruction, with “classical” asthma demonstrated that microlocalisation of tryptase positive mast cells to the airway smooth muscle was more closely associated with the presence of airway hyperresponsiveness than any other pathological marker (Brightling et al. 2002a). Another important example of the use of disease phenotype is a study by Green and colleagues who employed a management strategy in difficult to control asthma that targeted patients with increased eosinophilic airway inflammation for treatment with higher dose corticosteroids whilst reducing these treatments in patients without eosinophilic airway inflammation (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). By these methods the authors were able to achieve a two thirds reduction in severe asthma exacerbations compared to standard management, the improved outcome was achieved without any increase in the total amount of corticosteroid used.
1.1.4 Summary

The study of asthma in discrete phenotypes may increase our understanding of the complex pathological mechanisms in the asthmatic airway and allow for more rational targeting of therapeutic interventions. Potential asthma phenotypes identified, which are considered in further detail in the following chapters, include: eosinophilic bronchitis, non-eosinophilic asthma and refractory asthma.
1.2 Eosinophilic bronchitis

1.2.1 History

Gibson et al. (Gibson et al. 1989) first described eosinophilic bronchitis without asthma as a cause of chronic cough in 1989. It is characterised by eosinophilic airway inflammation associated with increased Th2 cytokine expression (Brightling et al. 2002b), in the absence of airway hyperresponsiveness or variable airflow obstruction. Typically the cough responds well to inhaled corticosteroid therapy. Studies where assessment of airway inflammation has been undertaken in patients with chronic cough have shown that eosinophilic bronchitis without asthma may account for up to 10–15% of cases referred for specialist investigation (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a; Camey et al. 1997), although the incidence is likely to depend on the extent to which therapeutic trials of corticosteroids are undertaken prior to referral.

1.2.2 Diagnosis

Isolated chronic cough, traditionally defined as a cough lasting for more than 3 weeks with no overt clinical or radiological evidence of lung disease, is a common reason for referral to a specialist. Several series have shown that a cause of persistent cough can be identified relatively simply in 80–95% of cases by using an “anatomic diagnostic” protocol (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a; Irwin, Corrao, & Pratter 1981; Irwin, Curley, & French 1990; McGarvey et al. 1998). Cough-variant asthma, gastroesophageal reflux, rhinitis with postnasal drip, and eosinophilic bronchitis are the most common causes, although there are often multiple causes (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a). Eosinophilic
bronchitis is defined as a chronic cough in subjects with no symptoms or objective
evidence of variable airflow obstruction, normal airway responsiveness
(provocative concentration of methacholine producing a 20% decrease in FEV1
[PC20] >16 mg/mL) and a sputum eosinophilia (Brightling, Ward, Goh, Wardlaw,
& Pavord 1999a). A similar corticosteroid responsive cough syndrome has been
reported by Fujimura et al. (Fujimura et al. 2000) and has been given the diagnostic
label "atopic cough". This condition has been defined as an isolated chronic cough,
no variable airflow obstruction or airway hyperresponsiveness, and one or more
objective indication of atopy as defined by: blood or sputum eosinophilia, elevated
total or specific IgE, or positive skin tests. Whether eosinophilic bronchitis and
atopic cough represent distinct clinical entities is unclear, although the presence of
BAL eosinophilia in eosinophilic bronchitis (Brightling et al. 2003a) but not atopic
cough (Fujimura, Ogawa, Yasui, & Matsuda 2000), suggests that they might be.

1.2.3 Treatment

Anti-inflammatory treatment with inhaled corticosteroids is the mainstay of therapy
for eosinophilic bronchitis. Patients improve symptomatically and have a significant
fall in their sputum eosinophil count following inhaled corticosteroids (Brightling et
al. 2000b;Gibson et al. 1995). In one study, capsaicin cough sensitivity, which was
moderately increased before treatment (Brightling, Ward, Wardlaw, & Pavord
2000b), improved towards normal following treatment with inhaled budesonide
(400 µg twice daily) and there was a significant positive correlation between the
treatment induced change in cough sensitivity and sputum eosinophil count.
Although there may be subepithelial layer thickening and other changes to suggest airway remodelling in eosinophilic bronchitis (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a), it remains unclear whether therapy for eosinophilic bronchitis should be discontinued when symptoms resolve. The role of other potential therapeutics agents such as antihistamines and leukotriene antagonists needs to be explored (Brightling et al. 2000c).

1.2.4 Pathogenesis

One question of particular interest is whether airway diseases with similar patterns of airway inflammation but different functional abnormalities have underlying differences in their immunopathology. Brightling and colleagues have performed a comparative immunopathological study of eosinophilic bronchitis and asthma which demonstrated that both conditions were associated with a similar degree of sputum, bronchoalveolar lavage fluid, and biopsy eosinophilia and a similar degree of subepithelial layer thickening but highlighted the importance of microlocalisation of mast cells, expressing IL-4 and IL-13 (Brightling et al. 2003b), to the bronchial smooth muscle in asthma but not eosinophilic bronchitis (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a). It is unknown whether the difference in IL-13 expression between these two conditions is reserved to mast cells within the airway smooth muscle bundle or whether there is a more generalised upregulation of IL-13 in asthma compared to eosinophilic bronchitis.

Another intriguing observation is that of increased sputum PGD₂ and histamine concentrations (Brightling, Ward, Woltmann, Bradding, Sheller, Dworski, &
Pavord 2000c) and increased bronchial brushing mast cell numbers (Gibson et al. 1998b) in eosinophilic bronchitis compared to classical asthma.

These findings raise the possibility that microlocalisation of mast cells to the superficial structures, such as sensory nerve endings, may be important in the genesis of cough (figure 1.2.1; page 12). This view is supported by the efficacy of antihistamines in cough associated with asthma (Rafferty et al. 1990) and atopic cough (Fujimura, Ogawa, Yasui, & Matsuda 2000).

1.2.5 Natural History

Although eosinophilic bronchitis responds well to treatment with inhaled corticosteroids in the short term the long-term outcome of the disease is unclear. In a 10 year follow up study of the eight of the patients originally described by Gibson, complete resolution of symptoms and eosinophilic airway inflammation was the commonest outcome but a minority of patients had developed fixed airflow obstruction (Hancox et al. 2001). Brightling has also described a patient with eosinophilic bronchitis who developed fixed airflow obstruction in association with prolonged uncontrolled eosinophilic airway inflammation (Brightling et al. 1999b). Others have speculated that eosinophilic bronchitis is an early stage in the development of an asthma phenotype (Cockcroft 2000) although there is less evidence that evolution to more typical asthma occurs.
Eosinophilic bronchitis

- No mast cell infiltration of smooth muscle
- No airway hyperresponsiveness or variable airflow obstruction

**Mast cell infiltration of smooth muscle**

**Airway hyperresponsiveness and variable airflow obstruction**

**Cough variant asthma**
1.2.6 Conclusions

Eosinophilic bronchitis is a common and treatable cause of chronic cough. The airway inflammation is similar to that seen in asthma although eosinophilic bronchitis is associated with quite different abnormalities of airway function. Recent findings support that these differences might be related to the site of mast cell infiltration of the airways. More definite information on the natural history of eosinophilic bronchitis is necessary to guide best clinical management.
1.3 Interleukin-13 in asthma

1.3.1 Introduction

Interleukin-13 (IL-13) is a proinflammatory Th2 cytokine that has been implicated, primarily from mouse models, as an important mediator in several aspect of the airway immunopathology of asthma (Wills-Karp 2004).

1.3.2 Biology of Interleukin-13

Interleukin-13 is a 17kD glycoprotein encoded within a gene cluster on chromosome 5 (Zurawski & de Vries 1994) which includes interleukin-3, interleukin-4, interleukin-5, interleukin-9 and Granulocyte-Macrophage colony stimulating factor. Th2 polarised CD4+ cells are the predominant source of interleukin-13, although production by Th1 polarised CD4+ and CD8+ cells and a number of non T-Cells including mast cells, basophils and eosinophils (Schmid-Grendelmeier et al. 2002) has been demonstrated.

Interleukin-9 and interleukin-25 have been implicated in the regulation of interleukin-13 since these cytokines induce airway inflammation and airway hyperresponsiveness in transgenic mouse models in a manner not independent of interleukin-13 (Temann, Ray, & Flavell 2002). Mast cells can be induced to produce interleukin-13 in response to adenosine (Ryzhov et al. 2004) and the mast cell mediator histamine enhances interleukin-13 gene expression in Th2 polarised cell lines (Elliott et al. 2001). Endothelin 1 peptides and interleukin-5 have been
demonstrated to have a synergistic effect in stimulating interleukin-13 production by eosinophils (Cui et al. 2004).

Two interleukin-13 receptors have been identified, IL13Rα1 and IL13Rα2 (Caput et al. 1996). While IL13Rα2 has 100-fold greater binding affinity of interleukin-13 than IL-13Rα1 it does not have any intracellular signalling effect and it has therefore been postulated that is a regulatory or decoy receptor (Wills-Karp 2004). The binding of interleukin-13 to the IL-13Rα1 receptor is increased by the presence of the interleukin-4 receptor IL-4Rα and the intracellular effect of interleukin-13 is mediated by signalling through an IL-4Rα1/IL-13Rα1 heterodimer (Zurawski et al. 1995). Association of interleukin-13 with the IL-4Rα1/IL-13Rα1 receptor complex induces activation of signal transducer and activation of transcription 6 (STAT6) and Janus-family kinase (Kuperman et al. 1998).

1.3.3 Interleukin-13 and asthma: evidence from animal experiments

Administration of interleukin-13 to the airways of mice induces airway inflammation with the influx of eosinophils, monocytes/macrophages and lymphocytes, possibly by increasing expression of vascular cell adhesion molecule 1 (VCAM-1) or by increasing local production of chemoattractant molecules (Wills-Karp et al. 1998). This inflammatory response is not as marked as that seen in allergen challenged mice and eosinophilic airway inflammation still occurs in interleukin-13 deficient mice following allergen challenge, suggesting that interleukin-13 is acting alongside other cytokines, such as interleukin-4, in causing allergic airway inflammation.
Interleukin-13 has been particularly implicated in subepithelial layer fibrosis: overexpression of IL-13 in the murine lung leads to a dramatic fibrotic response (Zhu et al. 1999) and administration of soluble IL-13Rα2 immunoglobulin abrogates the fibrotic response to parasitic infection (Chiaramonte et al. 1999). There are several possible mechanisms for this response including; upregulation of arginase I (Zimmermann et al. 2003), activation of transforming growth factor-β (Lee et al. 2001) and stimulation of proliferation of myofibroblasts (Ingram et al. 2003).

Exposure of primary epithelial cells cultured at an air/fluid interface to IL-13 leads to a switch from absorptive to a secretary phenotype (Danahay et al. 2002), increased mucous secretion, reduced ciliary beat frequency (Laoukili et al. 2001) and induces cell proliferation. These changes may indicate and important role for IL-13 for the mucous hypersecretion that is a feature of asthma and asthma exacerbations in particular. Further evidence to support a role for IL-13 in mucous hypersecretion come from the observations that blockade of IL-13 function and the IL-13 signalling pathway prevents these mucous cell changes in mouse models of asthma (Wills-Karp, Luyimbazi, Xu, Schofield, Neben, Karp, & Donaldson 1998).

IL-13 has been demonstrated to increase airway hyperresponsiveness 6 hours after administration (Venkayya et al. 2002), independently of inflammatory cells (Singer, Lefort, & Vargaftig 2002). Although it does not appear to have a direct effect on airway smooth muscle cell contraction, IL-13 increases response to bronchoconstrictor stimuli such as histamine and bradykinin (Shore & Moore
In addition it has been demonstrated that IL-13 reduces smooth muscle relaxation in response to β2-agonists (Laporte et al. 2001).

### 1.3.4 Interleukin-13 in asthma: evidence from human studies

Several studies have demonstrated increased concentration of IL-13 protein and messenger RNA in bronchoalveolar lavage and bronchial biopsy specimens from patients with asthma following allergen challenge (Huang et al. 1995).

A role for IL-13 in human asthma is further supported by the observation that numerous genetic polymorphisms in the IL-13 gene have been associated with asthma (Graves et al. 2000; Heinzmann et al. 2000; van der Pouw Kraan TC et al. 1999).

### 1.3.5 Summary

Interleukin-13 is a proinflammatory Th2 cytokine which has been found in increased concentration in the airways of patients with asthma and is implicated in many of pathological processes occurring in the asthmatic airway, including; airway inflammation, subepithelial collagen layer thickening, mucous hypersecretion and airway hyperresponsiveness. Contrasting interleukin-13 expression in asthma and eosinophilic bronchitis may yield important information as to the role of this cytokine in the clinical and physiological manifestations of these airways diseases.
1.4 Non eosinophilic asthma

1.4.1 History

Clinicians have long regarded asthma as a heterogeneous disease (Aas 1981; Rackemann 1921) although detailed clinicopathological studies have tended to emphasise the similarities in the underlying airway pathology and disordered function between patients (Humbert et al. 1996; Humbert et al. 1999). Airway inflammation in asthma has usually been assessed invasively using bronchoscopic techniques and consequently studies are usually confined to a small population of young adults with mild atopic asthma. Whether the findings can be applied to a wider, more heterogeneous population analogous to that seen in clinical practice is unclear. The recent development of induced sputum techniques has allowed airway inflammation to be assessed non-invasively and has provided the opportunity to study a more diverse range of patients. Using this technique a number of groups have identified a subset of adults who have clear physiological evidence of asthma but no induced sputum evidence of eosinophilic airway inflammation (Gibson, Simpson, & Saltos 2001; Pavord, Brightling, Woltmann, & Wardlaw 1999; Wenzel et al. 1999).

1.4.2 Definition

Various cut-off points for the presence of a sputum eosinophilia have been suggested ranging from 1.9 to 5%. While 1.9% represents 2 standard deviation above the mean of sputum eosinophil counts in a normal population, 3% has been more closely associated with clinical outcomes in asthma (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). In order to
take into account the temporal and treatment effects, a useful definition of non
eosinophilic asthma might be: a patient with clear evidence of airway
hyperresponsiveness and/or variable airflow obstruction and the absence of a
sputum eosinophilia on two separate occasions at least one month apart, while the
patient is symptomatic but not taking inhaled or oral corticosteroids. Other
diagnoses such as bronchiectasis or chronic obstructive pulmonary disease, which
are associated with airway hyperresponsiveness and asthma-like symptoms, should
be excluded before a firm diagnosis could be made.

1.4.3 Prevalence of non eosinophilic asthma

Green et al have shown that 25% of corticosteroid naïve symptomatic asthmatics
presenting to an adult respiratory clinic have a normal sputum eosinophil count
(Green, Brightling, Woltman, Parker, Wardlaw, & Pavord 2002b) and other
investigators have reported a frequency as high as 50% of non-eosinophilic,
neutrophilic asthma in subjects with refractory asthma (Wenzel, Schwartz,
Langmack, Halliday, Trudeau, Gibbs, & Chu 1999), subjects studied during an
asthma exacerbation (Fahy et al. 1995) and in subjects taking high doses of inhaled
corticosteroids (Gibson, Simpson, & Saltos 2001).

One difficulty in determining the true incidence of non eosinophilic asthma is that
the diagnosis is often made on a single observation and in a disease characterised by
variability we cannot be sure that the distinctive phenotype remains stable, although
in a study by Green et al the phenotype did appear to remain stable over the period
of one year (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw,
& Pavord 2002a). It has often been assumed that the absence of eosinophils and
perhaps the presence of airway neutrophilia in these subjects is artefact, related to treatment with high dose inhaled on oral corticosteroids or that the phenotype is only seen in more severe disease. However, the demonstration of the same profile of airway inflammation in subjects who have mild disease and who are corticosteroid naïve (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b) suggests that this asthma phenotype is present throughout the spectrum of asthma severity.

1.4.4 Clinical features

Subjects with non-eosinophilic asthma from the studies by Pavord and colleagues and Green et al tended to be female, non-atopic and have adult onset disease with onset of symptoms around the menopause (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b; Pavord, Brightling, Woltmann, & Wardlaw 1999). In another study however these differences in baseline characteristics were not seen (Godon et al. 2002).

1.4.5 Immunopathology and possible mechanisms

There have been no specific investigations into the immunopathology of non eosinophilic asthma. However, in a bronchoscopy study of patients with refractory asthma Wenzel et al (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999) identified a subgroup that had predominantly neutrophilic airway inflammation with absence of eosinophils and normal basement membrane thickness. These findings support the concept that non-eosinophilic, neutrophilic asthma is a pathologically distinct entity, although again the patients studied were
taking high doses of inhaled steroids so it is not clear to what extent these findings reflect the effects of treatment.

This observation of a neutrophilic inflammatory response, as well as elevated interleukin-8 concentrations (Gibson, Simpson, & Saltos 2001), has led some authors to speculate that inflammatory stimuli such as environmental endotoxin, viral infection or ozone are responsible for activation of the innate immune system and consequent release of proinflammatory cytokines and chemokines leading to predominantly neutrophilic airway inflammation in patients with non eosinophilic asthma (Douwes et al. 2002).

1.4.6 Response to treatment

Studies have linked baseline eosinophilic inflammation to response to oral and inhaled corticosteroids (Brown 1958;Meijer et al. 2002;Pavord, Brightling, Woltmann, & Wardlaw 1999) and one of the controversial issues relating to non eosinophilic asthma is the suggestion that these patients respond less well to corticosteroids (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b). In a 1 year study investigating the clinical role of measuring sputum eosinophil counts, patients without a sputum eosinophilia who had their anti-inflammatory treatment reduced did not suffer any adverse effects (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). However Godon et al found that patients without a sputum eosinophilia responded equally well to inhaled corticosteroids as those with a sputum eosinophilia (Godon, Boulet, Malo, Cartier, & Lemiere 2002). None of these studies was placebo controlled and this latter study, which specifically recruited patients during a period of instability, may have
been particularly affected by regression to the mean bias. Additionally these studies categorised patients according to a single measurement of sputum eosinophil count which may, by not taking into account temporal fluctuations in disease, have led to some misclassification of patients.

1.4.7 Summary

There is a pressing need to establish whether non-eosinophilic asthma represents distinctive phenotype, which is present in subjects with mild disease, who are not receiving corticosteroids, and to identify whether there are pathological features in common with classical eosinophilic asthma. There is also a need for prospective, randomised controlled trials of inhaled corticosteroids in patients with non eosinophilic asthma to determine whether this phenotype genuinely has a corticosteroid unresponsive pathology.
1.5 Refractory asthma

1.5.1 What is severe or therapy resistant asthma?

Asthma is a syndrome characterised by symptoms of variable airflow obstruction, fixed airflow obstruction and recurrent exacerbations which may be related to the underlying physiological and pathological abnormalities of airway hyperresponsiveness, remodelling and eosinophilic airway inflammation respectively. These features may be present to varying degrees in any patient and may also vary over time in the same patient. Definitions of asthma severity have been based on the prominence of any one or more of these features: a methacholine PC_{20} less than 0.1mg/ml (Woolcock 1986), an FEV_{1} <60% predicted (Global initiative for asthma 1995) or a persistent sputum eosinophil count >3% (ten Brinke et al. 2004). These definitions do not take account of any pharmacological intervention and a more encompassing definition would take into account a failure to control symptomatic outcomes of asthma or the use of unusually high amounts of medications to do so (Cockcroft & Swystun 1996). A useful published definition for refractory asthma is that given by the American Thoracic Society workshop on refractory asthma (ATS 2000), in which account is taken of the amount of medication required to achieve control, the degree of symptoms and/or exacerbations and previous history of near fatal asthma (Appendix I) It should be noted that this definition is based on practice in America where less inhaled corticosteroid is typically used than in Britain.
1.5.2 Size of the problem

Asthma affects a large number of patients in developed countries: refractory asthma occurs in 1-11% of these patients (ATS 2000) and so affects a considerable number of patients. The economic burden of refractory asthma is amplified by the increased morbidity suffered and consequent additional use of medication, loss of days working, unscheduled clinic attendances and hospital admissions. The combination of these factors creates a considerable health resource burden and a patient with refractory asthma will consume on average six times more health care resources than a patient with mild to moderate disease (Serra-Batilles et al. 1998). Both the American Thoracic Society and the European Respiratory Society have identified research into mechanisms of refractory asthma as priorities (ATS 2000; Chung et al. 1999).

1.5.3 Clinical features

Although patients with refractory asthma may present with a variety of clinical manifestations, two main clinical patterns of airway dysfunction have been described: stable day to day disease control with dramatic exacerbations and erratic day to day symptom control (Chan et al. 1998). Patients with refractory asthma have a high incidence of psychosocial issues and co-morbid conditions and are more likely to have had previous assessment by a respiratory specialist (Heaney et al. 2003). Other factors which have been associated with more severe asthma or near fatal asthma include previous admissions to intensive care units (de Klerk et al. 2002), high medication use (Campbell et al. 1996), obesity (Akerman, Calacanis, &
Madsen 2004), cigarette smoking (Thomson, Chaudhuri, & Livingston 2004) and environmental exposure to endotoxin (Michel et al. 1996).

1.5.4 Physiological features

The presence of asthma has been demonstrated to be an important determinant of the rate of decline in lung function (Lange et al. 1998) and the observation that some patients with asthma have persistent airflow limitation despite treatment with bronchodilators and oral corticosteroids (Brown, Greville, & Finucane 1984) led to the hypothesis that the airways in asthma undergo structural remodelling. Consequently low post bronchodilator FEV₁ and FEV₁/FVC are common, although not universal, features of refractory asthma. The observation that patients with adult onset asthma have on average a lower post bronchodilator FEV₁ than patients with childhood onset disease suggests that in addition to the presence of asthma there are other factors which determine the rate of decline in lung function.

Airway hyperresponsiveness is more marked in refractory asthma with a lower dose of methacholine causing a 20% fall in FEV₁ (Cockcroft et al. 1992), a larger response to methacholine (Hargreave et al. 1986) and a failure of plateaux in response to methacholine challenge (Gulsvik & Vale 1986). Loss of lung elastic recoil and hyperinflation are other physiological abnormalities which have been associated with refractory asthma or the risk of a near fatal event (Gelb et al. 2004).
1.5.5 Radiological features

High resolution computerised tomography of the thorax has highlighted a number of structural differences between the appearance of the lungs in refractory asthma and mild asthma, including: emphysema (Vignola et al. 2004), bronchiectasis (Paganin et al. 1996) and airway wall thickening. In a study examining HR-CT appearances in asthma the prominence of centrilobular structures, thought to be a marker of small airway disease, was positively associated with risk of near fatal asthma [ref Lee 2004]. Some of the changes found on HR-CT in refractory asthma reflect those found in other conditions such as chronic obstructive pulmonary disease and obliterative bronchiolitis and in one study there were no features that discriminated between refractory asthma and obliterative bronchiolitis (Jensen et al. 2002).

1.5.6 Pathological features

The pathology of refractory asthma has been studied in several ways: post mortem studies of asthma deaths, bronchial and transbronchial biopsy studies and non-invasive measurement of airway inflammation. Post mortem studies have suggested that a distal inflammatory process with structural changes is present in patients who suffered asthma death (Huber & Koessler 1922), although these types of study are difficult to control and it is not possible to determine whether these changes are peculiar to refractory asthma. Hypertrophy of the airway smooth muscle does appear to be selectively associated with severe asthma (Benayoun et al. 2003) which may be mediated by TGF-β stimulation (Minshall et al. 1997) or other factors such as platelet activating factor, interleukin-4 or interleukin-13 (Redington 2000).
There is evidence to suggest that alteration of mucus gland size and/or number is a feature of severe asthma and asthma death: mucus plugging and the occupation of a higher percentage of lumen by mucus glands have been demonstrated in post mortem studies of asthma deaths (Aikawa et al. 1992) and increased mucus gland size was a specific feature of severe asthma in a bronchoscopy study (Benayoun, Druilhe, Dombret, Aubier, & Pretolani 2003). Large airway infiltration by eosinophils is another feature that has been described in patients who suffered asthma related death compared to patients with asthma who suffered an unrelated death (Synek et al. 1996).

Subepithelial fibrosis is caused by deposition of collagen and fibronectin in the lamina reticularis giving the appearance of basement membrane thickening on light microscopy. However electron microscopy has revealed that the lamina rara and lamina densa, the true basement membrane, are normal in these tissues and the term basement membrane thickening has been replaced with subepithelial fibrosis (Elias et al. 1999). Subepithelial fibrosis has been suggested as a possible important feature of airway remodelling in asthma and has been shown to correlate with airway remodelling in cartilaginous, but not membranous, airways (James et al. 2002). However, the presence of subepithelial fibrosis in mild asthma (Benayoun, Druilhe, Dombret, Aubier, & Pretolani 2003) as well as rhinitis (Chakir et al. 1996) and eosinophilic bronchitis (Brightling, Symon, Birring, Bradding, Wardlaw, & Pavord 2003a) together with the absence of subepithelial fibrosis in refractory asthma without eosinophilia (Wenzel, Schwartz, Langmack, Halliday, Trudeau,
Gibbs, & Chu 1999) suggest that it may be more a feature of mucosal eosinophilia than airway remodelling.

A combination of increased smooth muscle mass, glandular hypertrophy, inflammatory infiltration, fibrotic deposition and mucosal oedema lead to a thickening of all of the components of the airway wall. This leads to a multifactorial change in the dynamic effects of smooth muscle contraction, with enhance luminal effacement and reduced parenchymal tethering leading to a destabilisation of the forces which maintain airway calibre and hence to airway collapse (Elias, Zhu, Chupp, & Homer 1999). Thus it seems likely that airway remodelling in its broadest sense, rather that subepithelial fibrosis specifically, is associated with refractory asthma.

1.5.7 Airway inflammation

Granulocyte infiltration of the airway mucosa does not appear to be a specific feature of refractory asthma (Benayoun, Druilhe, Dombret, Aubier, & Pretolani 2003), indeed studies have emphasised the heterogeneity of the in inflammatory response in terms of eosinophilic and neutrophilic airway inflammation (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999). It is possible this is due to the presence of one or more inflammatory phenotype of refractory asthma, with refractory eosinophilic asthma representing a more classical disease but with impaired response to inhaled corticosteroids, and refractory non-eosinophilic asthma representing a separate, corticosteroid insensitive condition (Pavord, Brightling, Woltmann, & Wardlaw 1999).
1.5.8 Corticosteroid responsiveness

Diagnosing refractory asthma is dependent on assessment of treatment concordance and the clinician being satisfied that this is good or perfect. Even where there is good patient compliance with therapy there appears to a spectrum of response to inhaled corticosteroids. There have been several proposed mechanisms for this including: the presence of relatively steroid insensitive neutrophilic airway inflammation (Brown 1958), increased expression of the non functional glucocorticoid receptor beta splice variant (Leung et al. 1997) and a prominence of a more distal inflammatory process (Carroll et al. 1993) – a site which may not be accessed by inhaled corticosteroids.

1.5.9 Management of refractory asthma

The majority of patients with eosinophilic refractory asthma who comply with therapy can be controlled with oral corticosteroids; although there may be a subgroup who require parenteral corticosteroids (ten Brinke, Zwinderman, Sterk, Rabe, & Bel 2004). The use of these medications is limited by side effects, particularly osteoporosis, hypertension, hyperlipidaemia and hyperglycaemia. Other immunomodulatory drugs including methotrexate (Shiner et al. 1990), gold (Nierop et al. 1992) and cyclosporin A (Alexander, Barnes, & Kay 1992) have been shown to reduce the amount of oral corticosteroid to gain control of asthma although all of these agent have been associated with idiosyncratic toxicity, particularly affecting the liver and kidney.
Therapeutic strategies targeting individual cytokines such as interleukin-5, interleukin-13, immunoglobulin-E and tumour necrosis factor alpha have shown some promise as new therapies in refractory asthma (Barnes 2001).

1.5.10 Summary

Refractory asthma is an important clinical conditional with wide social-economic implications; it has a variety of clinical manifestations and a heterogeneous immunopathological basis. Investigation into the mechanisms and novel treatments for refractory asthma are a research priority.
1.6 Tumour necrosis factor alpha and refractory asthma

1.6.1 History

A link between exposure to infectious organisms and necrosis of tumours has been established since the late 19th century (Coley 1883) and the presence of a serum factor responsible for this observation was postulated following experiments in the 1960’s in which serum from mice inoculated with bacterial lipopolysaccharide was demonstrated to have the ability to damage cultured sarcoma cell lines (O'Malley, Achinstein, & Shear 1988). In the 1970’s the convergence of investigations into septic shock syndrome, cachexia and tumour necrosis led to the characterisation of a protein named cachectin/TNF which later was determined to be two highly homologous proteins which were named TNF-α, produced by macrophages and monocytes and TNF-β or lymphotoxin which is produced by lymphocytes (Warren, Ward, & Johnson 1988). There followed an intense period of investigation into the biological actions of tumour necrosis factor and, although a role as a chemotherapeutic agent in cancer medicine has been prevented by side effects, much of great biological importance has been learnt about this pleiotropic, proinflammatory cytokine which plays a key role in the innate immune response and diseases associated with dysregulated innate immunity.

1.6.2 Innate immunity

Innate immunity provides immediate host defence against invading organisms prior to activation of the adaptive immune system (Medzhitov & Janeway, Jr. 2000). A key element of the innate immune system is the activation of membrane bound pattern recognition molecules, which detect common bacterial cell surface products
such as polysaccharide, carbohydrates and lipopolysaccharides, which leads to increased cellular production of proinflammatory cytokines including interleukins 1, 6 and 12 and TNF-α, primarily by macrophages.

1.6.3 Biology of TNF-α

TNF-α is initially produced as a 26kD membrane anchored precursor protein (Kriegler et al. 1988) which is subsequently cleaved, principally by TNF-α converting enzyme (TACE) (Zheng et al. 2004), to release the 17kD free protein (Davis et al. 1987). These proteins form into biologically active homotrimers (Smith & Baglioni 1987) which act on the ubiquitously expressed TNF-α receptors 1 and 2 (p55 and p75 or TNFRi and TNFRii) (Brockhaus et al. 1990). The receptor-ligand interaction causes intracellular signalling without internalisation of the complex which leads to phosphorylation of NF-κB to activate the p50-p65 subunit which interacts with the DNA chromatin structure to increase transcription of pro-inflammatory genes, such as IL-8, IL-6 and TNF-α itself (Barnes & Karin 1997), which leads to inflammatory responses such as neutrophil activation and recruitment (figure 1.6.1; page 33).
Figure 1.6.1 Schematic representation of the biology of TNF-α.
As well as causing increased secretion of inflammatory cytokines, TNF-α has direct systemic effects which are highly dose dependent: chronic low dose exposure causes anorexia, protein catabolism, insulin resistance and subendocardial inflammation, whereas acute exposure to high doses causes fever, shock, adult respiratory distress syndrome and acute tubular necrosis (Tracey & Cerami 1994). Response to TNF-α activation is balanced by shedding of the extracellular domain of the TNF-α receptors which occurs in response to stimulation by TNF-α (Lantz et al. 1990), lipopolysaccarides (Leeuwenberg, Dentener, & Buurman 1994), mitogens and interleukin-10 (Leeuwenberg, Jeunhomme, & Buurman 1994). Humans with a mutation of the TNF-α receptor 1 gene express a non sheddable TNF-α receptor 1 and suffer from and auto-inflammatory periodic syndrome known as TNF-α receptor 1 associated periodic syndromes (TRAPS) which are characterised by fever and severe localised inflammation (McDermott et al. 1999). Mice “knocked in” to express the non sheddable TNF-α receptor 1 have enhanced macrophage activation and increased release of effector molecules in response to Toll like receptor stimulation (Xanthoulea et al. 2004). In addition these mice suffered from an auto inflammatory condition with evidence of inflammatory arthritis.

1.6.4 TNF-α in inflammatory conditions

An autoimmune inflammatory arthritis mediated by TNF-α is known to occur in humans: rheumatoid arthritis is a common destructive arthropathy in which TNF-α is produced by macrophages and monocytes in response to activation by CD4+ T cells. TNF-α is measurable in increased concentration in the synovial fluid and in the serum (Choy & Panayi 2001). Antagonism of the TNF-α either by treatment
with recombinant soluble receptors or neutralising antibodies in patients with rheumatoid disease leads to improvement in disease activity scores (Olsen & Stein 2004). Similarly positive results are seen in treatment of other conditions which are mediated by TNF-α, including Crohn’s disease and Bechet’s disease.

1.6.5 A potential role for TNF-α in asthma

The possibility that TNF-α contributes the dysregulated inflammatory response seen in the asthmatic airway is raised by the findings of increased TNF-α mRNA (Ying et al. 1991) and protein (Bradding et al. 1994) in the airway of patients with asthma. Moreover, the administration of inhaled recombinant TNF-α to normal subjects leads to the development of airway hyperresponsiveness and an airway neutrophilia (Thomas, Yates, & Barnes 1995) and administration of TNF-α to patients with asthma leads to an increase in airway hyperresponsiveness as measured by a reduction in methacholine PC\textsubscript{20} (Thomas & Heywood 2002). The mechanism behind this observation has not been fully elucidated: it could represent a direct effect of TNF-α on airway smooth muscle (Adner et al. 2002) or be mediated by the release of the cysteinyl-leukotrienes LTC\textsubscript{4} and LTD\textsubscript{4} (Huber, Beutler, & Keppler 1988). The local release of mediators from mast cells localised to the airway smooth muscle has recently been suggested to be important in the pathogenesis of airway hyperresponsiveness and bronchoconstriction in asthma (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a). TNF-α induces histamine release from human mast cells directly (van Overveld et al. 1991) and participates in a positive autocrine loop that potentiates human mast cell cytokine secretion (Coward et al. 2002) and it is possible that TNF-α is involved in mast cell/smooth
muscle interaction and that this is particularly important in the development of airway hyper-responsiveness.

TNF-α has a number of other actions which may be relevant to asthma: it is chemoattractant for neutrophils and monocytes (Onda, Nagakura, & Iikura 1987) it increases the cytotoxic effect of eosinophils on endothelial cells (Slungaard et al. 1990), it is involved in activation and cytokine release by T-Cells (Scheurich et al. 1987) and it increases epithelial expression of adhesion molecules such as ICAM-1 and V-CAM-1 (Lassalle et al. 1991) which play an important role in the conduction of T-Cells to the lung and in the subsequent development of airway hyper-responsiveness (Walter et al. 2002).

1.6.6 TNF-α in refractory asthma

In addition to its relevance to asthma in general, TNF-α has a number of properties that might be relevant to refractory asthma, including: recruitment of neutrophils (Thomas, Yates, & Barnes 1995), induction of glucocorticoid resistance (Franchimont et al. 1999), myocyte proliferation (Amrani et al. 1996) and stimulation of fibroblast growth and maturation to myofibroblasts by promoting TGF-β expression (Desmouliere et al. 1993; Sullivan et al. 2005). Further support for a possible role for TNF-α in refractory asthma comes from the observation that a number of factors associated with TNF-α up-regulation including obesity (Hotamisligil et al. 1995), smoking (Churg et al. 2002) and endotoxin exposure (O'Malley, Achinstein, & Shear 1988) are also associated with more severe or corticosteroid resistant asthma (Michel et al. 1991; Chaudhuri et al. 2003; Michel et al. 1991). This raises the possibility that the coexistence of a high TNF-α state with asthmatic
airway inflammation may be important in the pathogenesis of refractory asthma. Interest in the role of TNF-α in refractory asthma has been increased by the findings of an uncontrolled study showing that treatment with the recombinant soluble TNF-α receptor etanercept caused a marked improvement in airway hyperresponsiveness (Babu, Davies, & Holgate 2004).

1.6.7 Summary

TNF-α is a pleiotropic proinflammatory cytokine for which there is circumstantial evidence for a role in asthma and refractory asthma in particular. Its effects are regulated both by local concentrations and cell surface receptor dynamics and it has recently become possible to modify TNF-α activity using humanised monoclonal antibodies or recombinant soluble TNF-α receptors (Olsen & Stein 2004). Investigation into the possibility of TNF-α axis up-regulation in refractory asthma and placebo controlled trials of TNF-α antagonism in this group of patients are required to investigate the potential role of these therapeutic strategies in refractory asthma.
2. Original hypotheses

1. Eosinophilic bronchitis is a stable disease phenotype.

2. There is an accelerated decline in FEV\textsubscript{1} in patients with eosinophilic bronchitis.

3. There is a difference in expression of interleukin-13 between asthma and eosinophilic bronchitis.

4. Non-eosinophilic asthma and eosinophilic asthma represent distinct pathological phenotypes.

5. There is a difference in response to inhaled corticosteroids in eosinophilic and non-eosinophilic asthma.

6. Alveolar nitric oxide is a practical and valid tool for measuring distal airway inflammation in patients with asthma.

7. Alveolar nitric oxide concentration is elevated in patients with refractory asthma.

8. Oral but not inhaled corticosteroids reduce alveolar nitric oxide concentration.

9. There is increased activity of the TNF-\(\alpha\) axis in patients with refractory asthma.
10. Treatment of patients with refractory asthma with the soluble TNF-α receptor etanercept leads to an improvement in asthma quality of life and airway hyperresponsiveness.
3. Methods

3.1 Clinical methods

3.1.1 Allergen sensitisation

Atopy was assessed either by specific IgE or skin prick tests to Dermatophagoides pteronyssinus, cat fur, grass pollen and Aspergillus fumigatus solutions with normal saline and histamine controls (Alk-Abello, Berkshire, UK). A positive response to an allergen on the skin prick test was recorded in the presence of a weal $>2\text{mm}$ more than the negative control.

3.1.2 Spirometry

Spirometry was performed with a Compact Vitalograph spirometer (Vitalograph, Buckinghamshire, UK). Bronchodilator reversibility was assessed 15 minutes after administration of $200\mu\text{g}$ salbutamol administered via a volumatic spacer device. FEV$_1$ was recorded as the better of two successive readings within 100ml. Spirometers were calibrated daily.

3.1.3 Airway hyperresponsiveness

Using the standard tidal breathing method the concentration of methacholine causing as 20% fall in FEV$_1$ was recorded as the PC$_{20}\text{FEV}_1$ (Juniper, Cockcroft, & Hargreave 1994). In brief, following measurement of the baseline FEV$_1$ subjects inhaled saline followed by doubling concentrations of methacholine from
0.03mg/ml to 16mg/ml via a Wright’s nebuliser with a flow of 0.13ml/min driven by dry compressed air. Subjects were instructed in tidal breathing which was continued for 2 minutes with a nose clip. The FEV\textsubscript{1} was measured 30 and 90s after the dose was administered. If the FEV\textsubscript{1} falls less than 20% the procedure was repeated with the next highest concentration. If the FEV\textsubscript{1} fell more than 20% from the baseline (or the highest concentration had been given), no further methacholine was given. Methacholine PC\textsubscript{20}FEV\textsubscript{1} concentration was calculated by linear interpolation of the log dose response curve. The output of the Wright’s nebuliser was monitored monthly.

3.1.4 Sputum induction

Instructions for patients

Prior to commencing the induction the procedure was fully explained to the patient with emphasis on the following:

i) Instruction on spitting out saliva generated during the inhalation into a “discard” vessel.

ii) Instruction to blow their nose and rinse their mouth, swallowing the water, prior to trying to expectorate sputum.

iii) Instruction was given on how to expectorate effectively.

iv) A reminder not to swallow sputum.

v) Guidance on posture: sitting straight up during the nebulisation and leaning forward during expectoration.
Protocol

Subjects were pre-treated with inhaled salbutamol 200μg 10 to 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 with an output of 0.9ml/min and a median mass diameter of 5.5μm (Medix, Harlow, UK). Tidal breathing was employed, taking slightly deeper breaths every minute. After each inhalation patients blew their noses and rinsed their mouths, to minimise nasal contamination, before expectorating sputum into a sterile pot. FEV₁ was measured after each inhalation. If the FEV₁ fell by more than 10% but less than 20% the same concentration of saline was administered for the subsequent inhalation. If the FEV₁ fell by more than 20% of the best post bronchodilator value, or if significant symptoms occurred, the procedure was discontinued and a further dose of inhaled or nebulised salbutamol was given. The nebuliser was calibrated by the same individual on a monthly basis.

Safety procedures during sputum induction

The usual laboratory resuscitation apparatus plus nebulised salbutamol was readily available during the induction. A doctor was immediately available if the test was being performed by a nurse of technician.

3.1.5 Measurement of exhaled nitric oxide concentration

Exhaled nitric oxide concentration was measured using an online chemiluminescence analyser (NIOX; Aerocrine, Stockholm, Sweden). Nitric oxide free air was inhaled via a nitric oxide “scrubber” to tidal volume and then exhaled at
a constant pressure (>10cmH₂O) for 10 seconds and flow was controlled by a standard valve at either 10, 30, 50, 100 or 200ml/sec. The exhaled nitric oxide concentration was recorded a mean of 3 recording of the plateau phase. The chemiluminescence analyser was calibrated biweekly according to the manufacturer’s instructions.

3.1.6 Symptom visual analogue scores
Symptom scores were recorded using 100mm visual analogue scales fixed at both ends by no symptom and the worst symptoms ever for each of the symptoms of cough, wheeze and breathlessness (appendix 1).

3.1.7 Asthma quality of life questionnaire
Health status was assessed using the asthma quality of life questionnaire (Juniper et al. 1992). This consists of 32 questions measuring four domains; symptoms, limitation of activities, emotional dysfunction and response to environmental stimuli. The limitation of activities domain was examined by the subject specifying five frequent activities in which they experienced asthma symptoms and scoring the degree of limitation of each 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.
3.1.8 Peak flow variability

The maximum peak expiratory flow (PEF) amplitude % mean was derived from the maximum within day variability observed over a 14 day period as the ratio of the difference between the highest and lowest PEF and the mean PEF.

3.2 Laboratory methods

3.2.1 Protocol for sputum processing

Sputum free from salivary contamination was selected and weighed. To the selected sputum four times the weight of 0.1% dithiothrietol (DTT; Sigma, Poole, UK) was added. The sputum was dispersed by gentle aspiration into a Pasteur pipette, vortexed for 15s and then rocked on a bench spiromix for 15 minutes. After addition of an equal volume of Dulbecco’s phosphate buffered saline (D-PBS; Sigma, Poole, UK) the sputum suspension was filtered through 48µm gauze and centrifuged at 790g for 10 minutes. The sputum supernatant was removed and stored at -80°C for later analysis. The cell pellet was suspended in 90µl of D-PBS. 10µl was removed and total cell count, squamous contamination and cell viability was assessed by the tryphan blue exclusion method. The cell suspension was adjusted with D-PBS to 0.5x10^6 cells/ml and cytospins were prepared from 75µl aliquots at 18.1g for 6 minutes using a Shandon III cytocentrifuge (Shandon, UK). The cytospins were stained in neat Romanowski stain for five minutes and fixed in dilute stain for 25 minutes.
Romanowski stain preparation:

1.5g Azure-B-thiocyanate in DMSO was dissolved at 37°C and 0.5g Eosin was dissolved in 300ml methanol at room temperature. The Azure blue solution was slowly added to the Eosin and stored away from light.

Dilute Romanowski stain:

62ml 10mM HEPES buffer pH 7.2
3.5ml DMSO
4.6ml Romanowski stain

Differential cell counts

A sputum differential cell count was obtained by counting >400 non-squamous cells on a Romanowski stained cytospin. Cell counts were performed blinded to the clinical status of the patient and, where applicable, to the randomisation group. A proportion of slides were recounted without reference to the original results to assess repeatability.
4. Studies

4.1 Eosinophilic bronchitis

4.1.1 Observational study of the natural history of eosinophilic bronchitis

Abstract

BACKGROUND
Eosinophilic bronchitis is an important cause of chronic cough. Treatment with inhaled corticosteroids is associated with a short-term improvement in cough and reduced sputum eosinophil count but the longer-term outcome is uncertain.

OBJECTIVE
To determine the long term outcome in patients diagnosed with and treated for eosinophilic bronchitis.

METHODS
We have performed a longitudinal study of symptoms, eosinophilic airway inflammation, spirometry and airway hyperresponsiveness in all patients diagnosed with eosinophilic bronchitis over seven years.

RESULTS
We identified 52 patients with EB and longitudinal data of greater than one year (mean 3.1 years) was available in 32 patients, all of whom were treated with inhaled steroids. Three (9%) patients developed symptoms consistent with asthma and a methacholine PC_{20}<8mg/ml on one or more occasion. Five (16%) patients developed fixed airflow obstruction defined by a persistent post bronchodilator
FEV1/FVC <70%. One (3%) patient had complete resolution of symptoms and eosinophilic airway inflammation off treatment. The remaining patients had ongoing eosinophilic airway inflammation and/or continuing symptoms. Multiple linear regression identified smoking, female gender and area under the curve of sputum eosinophil count over time as the most important predictors of decline in FEV1.

CONCLUSIONS

The most common outcome in eosinophilic bronchitis is continuing disease and complete resolution is rare. Asthma and fixed airflow obstruction developed in relatively few patients. The most important factors associated with a more rapid decline in FEV1 were female gender, smoking and prolonged eosinophilic airway inflammation.
Introduction

Eosinophilic bronchitis in the absence of asthma was originally described by Gibson et al (Gibson, Dolovich, Denburg, Ramsdale, & Hargreave 1989) and has subsequently been recognised as an important cause of chronic cough (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a), being present in 10-15% of patients referred for specialist investigation. Asthma and eosinophilic bronchitis are associated with a similar Th2 cytokine driven airway inflammation (Brightling, Symon, Birring, Bradding, Pavord, & Wardlaw 2002b; Brightling, Symon, Birring, Bradding, Wardlaw, & Pavord 2003a); however airway hyperresponsiveness and variable airflow obstruction, which are the defining features of asthma, are not present in eosinophilic bronchitis.

Eosinophilic bronchitis responds well to treatment with inhaled corticosteroids in the short term, with a reduction in symptoms and fall in sputum eosinophil count (Brightling, Ward, Wardlaw, & Pavord 2000b), however the long term outcome of the disease is unclear. In a 10 year follow up study of the eight patients originally described by Gibson, complete resolution of symptoms and eosinophilic airway inflammation was the commonest outcome but a minority of patients had developed fixed airflow obstruction (Hancox, Leigh, Kelly, & Hargreave 2001). Brightling has also described a patient with eosinophilic bronchitis who developed fixed airflow obstruction in association with prolonged uncontrolled eosinophilic airway inflammation (Brightling, Woltmann, Wardlaw, & Pavord 1999b). Others have speculated that eosinophilic bronchitis is an early stage in the development of an asthma phenotype (Cockcroft 2000) although there is less evidence that evolution to more typical asthma occurs.
More definite information on the natural history of eosinophilic bronchitis is necessary to guide best clinical management. The aim of this prospective observational study was to investigate the outcome in patients with eosinophilic bronchitis who were diagnosed in our institution over 7 years. We have investigated annual decline in FEV₁ and have attempted to identify variables that might be associated with this.

Methods

SUBJECTS

We studied all patients diagnosed with eosinophilic bronchitis at Glenfield Hospital between December 1996 and June 2003. These represented 10% of the patients referred for specialist investigation of chronic cough. Eosinophilic bronchitis was defined as a cough for greater than 2 months, a sputum eosinophil count >3%, normal FEV₁ and FEV₁/FVC ratio (>80% predicted and >70% respectively), an increase of less than 15% in FEV₁ 20 minutes following inhalation of 200μg of salbutamol, within single day peak flow amplitude as percent of mean of less than 20% with twice daily measurements over 2 weeks and methacholine PC₂₀>16mg/ml. The study was approved by the Leicestershire and Rutland research ethics committee and all patients gave written informed consent before inclusion.

MEASUREMENTS

FEV₁ and forced vital capacity (FVC) were measured using a rolling seal spirometer (vitalograph®, UK), which is calibrated daily. Methacholine Challenge
Test was performed using the standard tidal breathing method (Juniper, Cockcroft, & Hargreave 1994) and the concentration of methacholine causing a 20% fall in FEV₁ recorded as the methacholine PC₂₀. Induced sputum was obtained using 3, 4 and 5% saline administered via an ultrasonic nebuliser and processed as previously described (Pavord, Pizzichini, Pizzichini, & Hargreave 1997). Sputum eosinophil and neutrophil differential counts were expressed as a percentage of total non-squamous cells. Symptom visual analogue scores were assessed using a 100mm scale fixed at both ends by no symptoms and worst symptoms ever (Brightling et al. 2001).

PROTOCOL
We measured FEV₁, FVC, visual analogue score, sputum differential cell count and methacholine PC₂₀ annually. If, at the end of the study period, patients had no significant symptoms and their sputum eosinophil count was less than our normal range (1.9%) (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b) then an assessment of symptoms, airway responsiveness and sputum induction was repeated after withholding inhaled corticosteroid treatment for 2 months.

ANALYSIS
Patients were assigned four different outcomes: Continuing disease, defined as evidence of significant ongoing symptom of cough and/or a sputum eosinophil count of more than 3% on or off treatment or after a 2 month withdrawal of budesonide. Disease remission, defined as no significant symptoms and no evidence of eosinophilic airway inflammation after a 2-month withdrawal of budesonide. Asthma was defined as symptoms other than cough that were consistent with
asthma and a methacholine PC\textsubscript{20} of less than 8mg/ml at any point during follow up.

Fixed airflow obstruction was defined as a FEV\textsubscript{1}/FVC ratio of less than 70% following inhalation of 200\textmu g of salbutamol, which persisted throughout the subsequent observation period. All available data on FEV\textsubscript{1} was used in a linear regression analysis to calculate the annual change in post bronchodilator FEV\textsubscript{1}, which was then adjusted for height by dividing by height in metres squared (Lange, Parner, Vestbo, Schnohr, & Jensen 1998). Patients were classified as to whether they were likely to have had significant occupational dust exposure during their employment according to previous criteria (Scott, Johnson, & Britton 1990). Area under the curve for sputum eosinophil count over time was calculated using the trapezium method (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). Multiple independent linear regression was used to identify factors associated with rate of decline in FEV\textsubscript{1}; gender and age were entered into the model as factors which are known to be associated with rate of decline in FEV\textsubscript{1}. Sputum eosinophil counts were Log transformed prior to analysis, as they assume a Log normal distribution, and sputum neutrophil counts were corrected for age (Woodruff et al. 2001). All statistical tests were performed using SPSS 10 for Windows.

Results

We identified 52 patients (29 Male) with eosinophilic bronchitis. Patient characteristics are given in table 4.1.1.1 (page 58). Longitudinal data of greater than one year was obtained in 32 patients. The diagnosis was made less than one year ago in nine patients, six patients declined inclusion in the longitudinal study and five patients were lost to follow up. The mean duration of follow up was 3.1 years.
(range 1 to 6 years) and the mean number of observations of spirometry, PC_{20} and sputum eosinophil count were; 4.8, 2.6 and 3.2 respectively. There was no significant correlation between baseline cough visual analogue score and sputum eosinophil count \( (r=-0.148, p=0.43) \). All patients received treatment with inhaled budesonide 200 to 400μg twice daily from the time of diagnosis. 20 (63%) of patients had a reduction in cough visual analogue score that was outside the 95% range for repeat measures (15mm) following treatment and overall there was a mean improvement of 16mm (95% C.I. 7, 25; \( p=0.002 \)). Complete resolution of symptoms occurred in 6 (18%). patients. There was a significant improvement in sputum eosinophil count from baseline (9.8%) to first measurement following treatment with inhaled corticosteroids (1.5%, mean fold difference 6.4, 95% C.I. 2.9, 13.8; \( p<0.001 \)). 2 patients had sufficient ongoing symptoms and eosinophilic airway inflammation to justify the addition or oral corticosteroids, which led to resolution of symptoms and eosinophilic airway inflammation in both cases. No patients received treatment with long acting β_{2}-agonists.

Three (9%) patients developed symptoms other than cough that were consistent with asthma and a PC_{20}<8mg/ml at some point during follow up. Five (16%) patients developed a persistent post bronchodilator FEV_{1}/FVC <70%. One (3%) patient had complete resolution of symptoms and eosinophilic airway inflammation off treatment. Of the 23 (72%) remaining patients 13 had ongoing symptoms and a sputum eosinophil count >3% on one or more occasion, 7 had ongoing otherwise unexplained cough and a persistent sputum eosinophil count <3% (of which 3 were
cigarette smokers) and 3 had no symptoms and a sputum eosinophil count on one or more occasion of >3% (Figure 4.1.1.1; page 53).

**Figure 4.1.1.1 Outcome in eosinophilic bronchitis**

The mean height corrected change in FEV₁ for the group was -19ml/year (95% CI; 9.8, 28.6). The mean height corrected change in FEV₁ was -18ml/year in patients who did not develop fixed airflow obstruction and -26ml/year in those that did (mean difference 8ml, 95% C.I. -16, 34; p=0.5). Multiple linear regression identified smoking (B=-30.7; S.E. 9.0; p=0.004), female gender (B=-17.3; S.E. 7.1; p=0.028) and area under the curve of sputum eosinophil count over time (B=-3.0; S.E. 1.1; p=0.013; model statistics; R²=0.713, p=0.009) as the most important predictors of decline in FEV₁.
Discussion

This is the largest longitudinal study of outcome in eosinophilic bronchitis. We identified 52 patients over 7 years representing 10% of patients seen in our institution with chronic cough over this time. As reported before (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a), subjects with eosinophilic bronchitis were predominantly middle aged, non atopic and non smokers. In our series there was a small excess of male patients in contrast to other cough syndromes where female preponderance is common (Morice & Kasterlik 2004). Exposure to occupational dust was no more common than would be expected in the general population (Scott, Johnson, & Britton 1990). The most common outcome was continuing symptoms and eosinophilic airway inflammation, although a significant minority of patients developed fixed airflow obstruction. Few patients developed symptoms and objective evidence of asthma.

Our finding that ongoing disease is the commonest outcome contrasts with the findings of Hancox et al, who in a 10 year follow up of 8 patients found that complete resolution of symptoms and eosinophilic airway inflammation was the most common outcome (Hancox, Leigh, Kelly, & Hargreave 2001). Some of the difference may be as a result of the shorter follow up period in our study. However our results are consistent with the outcome in atopic cough, a condition with many features in common with eosinophilic bronchitis (Fujimura et al. 2003). In a longitudinal study of outcome in atopic cough only one out of a series of 63 patients with atopic cough went on to develop symptoms and objective evidence of asthma.
We have gone further than these earlier studies by estimating annual decline in FEV₁. This was not significantly different from that expected in a healthy age matched non smoking population (Lange, Parner, Vestbo, Schnohr, & Jensen 1998). Our entry criteria may have biased our findings since we only recruited patients with normal spirometry values and it is possible that the population may have a reduced rate of decline in FEV₁ (i.e. the healthy smoker phenomenon). All of our patients were taking inhaled budesonide throughout the study period so we are unable to exclude the possibility that untreated individuals have a more rapid decline in FEV₁. The fact that a persistently elevated sputum eosinophil count was related to decline in FEV₁ would be consistent with this. Our findings that female gender and as well as prolonged eosinophilic inflammation was associated with a more rapid decline in FEV₁ are consistent with our earlier observation that non smokers with fixed airflow obstruction are predominantly female and commonly have a sputum eosinophilia (Birring et al. 2002). Smoking was unusual in our population, perhaps reflecting referral bias since smokers with cough are unlikely to be referred for specialist opinion. However we have shown that smoking also has an independent effect on decline in FEV₁ suggesting that smokers with eosinophilic bronchitis might be particularly likely to develop COPD. We (Brightling et al. 2000a) and others (Fabbri et al. 2003) have shown that up to 40% of smokers or ex-smokers with moderate to severe COPD who are not taking inhaled steroids have a sputum eosinophilia supporting the view that the presence of eosinophilic bronchitis might be linked to the development of fixed airflow obstruction in some smokers.

Opinion on the nature of eosinophilic bronchitis ranges from the condition being part of an asthma spectrum to it being a clinically and pathologically distinct
condition (Brightling, Ward, Goh, Wardlaw, & Pavord 1999a; Cockcroft 2000; McGarvey & Morice 2003). Our demonstration that in most patients the clinical expression of disease is unchanged over up to 7 years and that the development of features of asthma was unusual is more consistent with the latter view; although we recognise that the outcome might have been different in untreated patients. Inhaled corticosteroid treatment was associated with improvement in cough and reduction in eosinophilic airway inflammation in most patients although complete resolution of these features was uncommon. Persistent cough was present in 23 of the patients in our series, the majority of whom had ongoing eosinophilic airway inflammation. Treatment concordance was not formally assessed in our study and we cannot discount the possibility that persistent cough was due to poor treatment concordance; another possibility is the presence of relative inhaled corticosteroid resistance - the complete response to oral corticosteroids in two cases would support this. An interesting observation was that some of our patients had persistent cough but no evidence of eosinophilic airway inflammation. In some cases this may have reflected the presence of other triggers for cough such as smoking or rhinitis. However this observation suggests that cough might not be directly causally associated with eosinophilic airway inflammation. The lack of association between cough symptoms and sputum eosinophil counts at baseline is consistent with this view. A plausible explanation for the persistent cough is the presence of activated mast cells in the superficial airways, since studies have shown that sputum mast cell mediator concentrations are high (Birring et al. 2004; Brightling, Ward, Woltmann, Bradding, Sheller, Dworski, & Pavord 2000c) and bronchial brushing mast cell numbers are increased in eosinophilic bronchitis (Gibson et al. 1998a). The pathological implications for the observation that some
patients who have eosinophilic bronchitis subsequently develop asthma merits further investigation, specifically as to whether smooth muscle mast cell infiltration develops alongside this change in phenotype.

In summary we have shown that eosinophilic bronchitis is usually a stable disease phenotype, which remains clinically distinct from asthma. A subgroup of patients with treated eosinophilic bronchitis may have a more rapid decline in FEV₁. Smoking, female gender and prolonged sputum eosinophilia are probably associated with this outcome. Further work is necessary to determine whether more widespread investigation of airway inflammation and more aggressive anti-inflammatory treatment will lead to a reduction in the development of fixed airflow obstruction.
Table 4.1.1.1 Demographic data at diagnosis of all patients identified with eosinophilic bronchitis.

<table>
<thead>
<tr>
<th></th>
<th>Cross sectional data available</th>
<th>Longitudinal data available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male)</td>
<td>52 (29)</td>
<td>32 (19)</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>53 (17-83)</td>
<td>54 (17-80)</td>
</tr>
<tr>
<td>FEV₁ (% of predicted)</td>
<td>99.2 (17.5)</td>
<td>101 (17.1)</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>79.2 (7.5)</td>
<td>79.5 (6.6)</td>
</tr>
<tr>
<td>Sputum eosinophil count (%)**</td>
<td>8.23 (0.23)</td>
<td>9.8 (0.32)</td>
</tr>
<tr>
<td>Sputum neutrophil count (%)</td>
<td>53 (22)</td>
<td>55 (21)</td>
</tr>
<tr>
<td>PC₂₀ (mg/ml)</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>IgE (ku/l)**</td>
<td>46 (1.2)</td>
<td>43 (1.0)</td>
</tr>
<tr>
<td>Blood eosinophil count (x10⁹/l)</td>
<td>0.31 (0.25)</td>
<td>0.35 (0.29)</td>
</tr>
<tr>
<td>Atopic (%)</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Potential occupational dust exposure (%)</td>
<td>44</td>
<td>48</td>
</tr>
<tr>
<td>Cough visual analogue score (mm)</td>
<td>39 (28)</td>
<td>36 (21)</td>
</tr>
</tbody>
</table>

Values are given as mean(standard deviation) except where marked * mean(range) and ** geometric mean(log standard deviation). FEV₁ – forced expiratory volume in one second. FVC – forced vital capacity. PC₂₀ – provocative concentration of methacholine causing a 20% drop in FEV₁. IgE – Immunoglobulin E.
4.1.2 Sputum and bronchial submucosal interleukin-13 expression in asthma and eosinophilic bronchitis

Abstract

BACKGROUND
Non-asthmatic eosinophilic bronchitis is a condition characterised by the presence of eosinophilic airway inflammation in the absence of airflow obstruction or airway hyperresponsiveness. In asthma the Th2-type cytokine interleukin (IL)-13 has been implicated in the development of airway inflammation and hyperresponsiveness. Whether the expression of IL-13 is different between these two conditions is unknown.

OBJECTIVE
We sought to investigate whether IL-13 expression is increased in asthma compared to eosinophilic bronchitis.

METHODS
Sputum samples from subjects with mild asthma (n=30), eosinophilic bronchitis (n=15) and normal controls (n=16) were dialysed for 18 hours against PBS using a 10Kda dialyser and IL-13 concentration was measured by ELISA. In a subgroup of these patients IL-13 protein expression in bronchial biopsies was assessed by immunohistochemistry.
RESULTS

The concentration of sputum IL-13 was higher in mild asthma than normal controls (p=0.03) and eosinophilic bronchitis (p=0.03). The median (IQR) number of IL-13+ cells/mm² submucosa was significantly higher in asthma 4 (8) than eosinophilic bronchitis 1.7 (1.9) and normal controls 0.5 (1.1) (p=0.004). 83% of the cells expressing IL-13 in the submucosa were eosinophils and 8% were mast cells. The median (IQR) proportion of eosinophils that expressed IL-13 was higher in the subjects with asthma 16 (10)\% than those with eosinophilic bronchitis 7 (3)\% (p=0.02).

CONCLUSION

The increased expression of IL-13 in asthma compared with eosinophilic bronchitis supports the concept that IL-13 may play a critical role in the pathophysiology of asthma.
Introduction

Asthma is characterised by the presence of variable airflow obstruction, airway hyperresponsiveness, and an airway inflammatory response usually characterised by eosinophilic airway inflammation (Wardlaw et al. 2000). There is increasing evidence that the development and maintenance of airway inflammation in asthma is due to the production of Th2 cytokines such as interleukin (IL)-4, IL-5 and IL-13 (Robinson 2000).

Eosinophilic bronchitis, like asthma, is characterised by eosinophilic airway inflammation, but unlike asthma there is no airway hyperresponsiveness or bronchoconstriction. The airway immunopathology of asthma and eosinophilic bronchitis is almost identical (Brightling, Symon, Birring, Bradding, Pavord, & Wardlaw 2002b; Brightling, Symon, Birring, Bradding, Wardlaw, & Pavord 2003a; Gibson, Zlatic, Scott, Sewell, Woolley, & Saltos 1998b). However, one fundamental difference between asthma and eosinophilic bronchitis is that in asthma mast cells, expressing IL-4 and IL-13, infiltrate the airway smooth muscle (Brightling, Symon, Holgate, Wardlaw, Pavord, & Bradding 2003b). It is unknown whether this difference in IL-13 expression between these two conditions is reserved to mast cells within the airway smooth muscle bundle or perhaps there is a more generalised upregulation of IL-13 in asthma compared to eosinophilic bronchitis.

We hypothesised that there is differential expression of IL-13 in asthma and eosinophilic bronchitis. To test our hypotheses we measured the sputum IL-13
concentration and the number of bronchial submucosal cells that express IL-13 in subjects with asthma, eosinophilic bronchitis and normal controls.

Methods

SUBJECTS
Patients with eosinophilic bronchitis, asthma and healthy volunteers were recruited from respiratory clinics and from staff at Glenfield Hospital. Subjects with asthma (n=30) gave a history of asthma symptoms 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}<8mg/ml); 2) >15% improvement in FEV_{1} 10 minutes after 200mg inhaled salbutamol or 3) PEF (>20% maximum within day amplitude from twice daily PEF measurements over 14 days). The subjects with eosinophilic bronchitis (n=15) had an isolated cough, no symptoms or objective evidence of variable airflow obstruction, a methacholine PC_{20}>16mg/ml and a sputum eosinophilia (>3% non-squamous cell). None of the subjects with asthma or eosinophilic bronchitis had been treated with corticosteroids for at least 6-weeks prior to recruitment. The healthy subjects (n=16) gave no history of respiratory diseases, had normal spirometry and normal airway responsiveness (PC_{20}>16mg/ml). None of the subjects had smoked except for five subjects with asthma all with <5 pack year history. Ten subjects with eosinophilic bronchitis, mild asthma and normal controls had been included in an earlier study (Campbell et al. 2001), but were re-characterised for this study. The protocol was approved by the Leicestershire ethics committee.
Subjects had spirometry, allergen skin prick tests to *Dermatophagoides pteronyssinus*, cat fur, grass pollen and *Aspergillus fumigatus*, a methacholine inhalation test (Juniper, Cockcroft, & Hargreave 1994) followed on recovery by sputum induction (Pavord, Pizzichini, Pizzichini, & Hargreave 1997).

One-week later, a subgroup of patients with mild asthma (n=8), eosinophilic bronchitis (n=7) and normal controls (n=7) underwent bronchoscopy (British Thoracic Society 2001). Bronchial biopsies were taken from the right middle and lower lobe carinae. Mucosal biopsies were processed into glycolmethacrylate (GMA) (Polysciences, Northampton, UK).

**IMMUNOHISTOCHEMISTRY**

The technique of immunostaining applied to GMA embedded tissue has been described previously (Britten, Howarth, & Roche 1993). The mouse monoclonal antibodies used were: CD3 for T-lymphocytes (Dako, High Wycombe, UK), AA1 to mast cell tryptase (Dako), major basic protein (MBP) for eosinophils (Caltag-Medsystems, Silverstone, UK) and IL-13 (R&D, Abingdon, UK). Sequential sections were stained for IL-13 and tryptase or MBP to assess co-localisation as described previously (Brightling, Symon, Holgate, Wardlaw, Pavord, & Bradding 2003b).

**VALIDATION OF IL-13 ELISA**

*Effect of DTT on Recovery of IL-13 standard*

IL-13 was measured by ELISA (Caltag-Medsystems and R&D). The effect of DTT on the recovery of IL-13 was first assessed by comparing the concentrations of a
standard curve generated with IL-13 reconstituted with or without DTT (0.045% final concentration) (n=2).

Spiking before removal of DTT by ultrafiltration

The standards were reconstituted in phosphate buffered saline with and without DTT (0.045%), frozen at −80°C overnight defrosted the following day before analysis to mimic the sputum processing protocol. The standards were dialysed for 18 hours against PBS using a 10Kda dialyser (Sigma, Poole, Dorset, UK) and then measured by ELISA. Recovery of standards spiked with DTT were compared to standards without DTT (n=2).

Spiking of DTT processed sputum sample

Sputum supernatants processed with DTT from eight subjects were divided into an aliquot spiked or not spiked with exogenous IL-13 of a known concentration. The samples and standards were dialysed as described above and the recovery of spiked IL-13 was determined.

IL-13 ELISA

Sputum IL-13 was measured by ELISA (Caltag-Medsystems). The inter-assay and intra-assay variability was between 5-10%. The limit of detection was 10pg/g of sputum for IL-13.

ANALYSIS

Sputum supernatants with undetectable levels of cytokine were assigned the concentration of zero. Non-parametric data were compared across the groups by Kruskal-Wallis test and between groups by Mann-Whitney test. IL-13 was not
measurable in over half of the subjects in each group thus differences between groups were also compared by chi-squared test. Significance was accepted at the level of 95%.

Results

The mucolytic dithiothreitol (DTT) affected recovery of IL-13 using both commercial ELISA kits (Caltag-Medsystems and R&D). The percentage recovery (coefficient of variation) of standards spiked with DTT compared to standards without DTT was similar with both assays 38% (14%) and 36 (29%) for the Caltag-Medsystems and R&D ELISA respectively. The Caltag-Medsytems ELISA was more sensitive and therefore was further validated. The recovery of IL-13 standard after dialysis was 90% (13% coefficient of variation) confirming that DTT added to the standard did not markedly affect IL-13 recovery after dialysis. The recovery of the exogenous IL-13 spike added to sputum samples was 95% (6.5% coefficient of variation) demonstrating that our assay was valid. Therefore sputum IL-13 concentration was measured in all of the samples and standards after dialysis.

The clinical characteristics and sputum cell counts were as shown (table 4.1.2.1; page 70). The sputum eosinophil count was higher in the subjects with eosinophilic bronchitis and asthma compared to the normal controls (p<0.001). The individual values for induced sputum IL-13 concentration were as shown (figure 4.1.2.1; page 72). The sputum IL-13 concentrations were significantly different comparing across the groups (p=0.014). In the subjects with corticosteroid naïve asthma the concentration of sputum IL-13 was higher than normal controls (p=0.03) and subjects with eosinophilic bronchitis (p=0.03). Similarly, the number of subjects
with measurable sputum IL-13 was higher in those subjects with asthma (13/30) compared to eosinophilic bronchitis (1/15) and normal controls (2/16) (p=0.011). There were no significant differences in age or gender between the subjects with measurable sputum IL-13 versus those with immeasurable sputum IL-13 and in the asthmatics there was no difference in atopic status or airway hyperresponsiveness between subjects with and without measurable sputum IL-13.

The number of IL-13+ cells/mm² of submucosa was significantly higher in the subjects with asthma than in normal controls (p=0.003), or subjects with eosinophilic bronchitis (p=0.03) (table 4.1.2.2; page 71). The median (IQR) proportion of IL-13+ cells that were co-localised to mast cells was 8 (38)% and to eosinophils was 83 (29)% (figure 4.1.2.2; page 73). There was a similar increase in the number of submucosal eosinophils in the subjects with asthma and eosinophilic bronchitis, which was increased, compared to the normal controls (p=0.008), but there was no difference in the number of submucosal T-cells or mast cells between groups (table 4.1.2.2; page 73). Therefore the median (IQR) proportion of eosinophils that expressed IL-13 was higher in the subjects with asthma 16 (10)% than those with eosinophilic bronchitis 7 (3)% (p=0.02).

Discussion

This is the first study to fully validate the measurement of IL-13 in induced sputum. In addition this study has made two important and novel observations. Firstly, we have shown that the expression of IL-13 protein in the bronchial submucosa and the sputum concentration of IL-13 were increased in asthma compared to eosinophilic
bronchitis supporting the concept that IL-13 plays a critical role in the pathophysiology of asthma. Secondly, we identified that most of the cells in the bronchial submucosa that expressed IL-13 protein were eosinophils. The number of eosinophils in the submucosa was similar in asthma and eosinophilic bronchitis suggesting that expression of IL-13 by eosinophils is different between these two conditions.

The validity of the measurement of any cytokine in induced sputum samples is dependent upon a robust assay. The recovery of some cytokines, such as IL-5, has been improved by the addition of protease inhibitors (Kelly et al. 2001). The use of DTT as a mucolytic to aid dispersion of the sputum sample has proved particularly problematic in the measurement of some cytokines and chemokines. A recent study reported that the recovery of CCL11 was markedly affected by DTT and good recovery was only achieved by omitting DTT in the processing of the sample (Hadjicharalambous et al. 2004). We found that the recovery of IL-13 was also affected by the addition of the mucolytic DTT. This problem was overcome by dialysing the samples to remove the DTT. We are confident that our IL-13 assay was valid as the recovery of exogenous spiked IL-13 was almost 100%. Whether dialysis of sputum supernatant may improve the recovery of other cytokines and mediators affected by the addition of DTT needs to be further explored.

In asthma IL-13 is elevated in BAL, bronchial biopsies and sputum (Huang, Xiao, Kleine-Tebbe, Paciotti, Marsh, Lichtenstein, & Liu 1995; Humbert et al. 1997; Komai-Koma et al. 2001). In addition, IL-13, but not IL-4, has been shown to attenuate relaxation to b-agonists and augment contractility to acetylcholine.
suggesting that one of the main effects of IL-13 may be the induction of airway hyperresponsiveness (Grunstein et al. 2002; Laporte, Moore, Baraldo, Jouvin, Church, Schwartzman, Panettieri, Jr., Kinet, & Shore 2001). In support of this view we found that the sputum IL-13 concentration and IL-13 expression in the bronchial submucosa was increased in asthma compared to eosinophilic bronchitis, a condition with a similar immunopathology to asthma, but with normal airway responsiveness (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a).

One important question is why is there differential expression of IL-13 in asthma and eosinophilic bronchitis? Several cell types have been described as important sources of IL-13 including T-lymphocytes (Huang, Xiao, Kleine-Tebbe, Paciotti, Marsh, Lichtenstein, & Liu 1995; Humbert, Durham, Kimmitt, Powell, Assoufi, Pfister, Menz, Kay, & Corrigan 1997; Kotsimbos, Ernst, & Hamid 1996), mast cells (Burd et al. 1995), eosinophils (Schmid-Grendelmeier, Altznauer, Fischer, Bizer, Straumann, Menz, Blaser, Wuthrich, & Simon 2002) and basophils (Li, Sim, & Alam 1996). We cannot be certain of the cellular source of IL-13 in the sputum, however we found that most of the cells that expressed IL-13 protein in the bronchial submucosa were eosinophils with a smaller proportion that were mast cells. There was a similar degree of eosinophilic inflammation in asthma and eosinophilic bronchitis. Thus, the proportion of eosinophils that expressed IL-13 was increased in asthma compared to eosinophilic bronchitis, suggesting that there is a fundamental difference in the production of IL-13 by airway eosinophils between these two conditions. Further work is required to investigate this difference in eosinophil function between eosinophilic bronchitis and asthma.
In summary, the increased expression of IL-13 in asthma compared with eosinophilic bronchitis supports the hypothesis that IL-13 plays a role in the pathophysiology of asthma. Specific therapies directed at IL-13 may offer additional benefit in the treatment of asthma.
### Table 4.1.2.1 Subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Eosinophilic Bronchitis</th>
<th>Mild Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>16</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Age*</td>
<td>38.1 (5.6)</td>
<td>53.5 (2.4)</td>
<td>54.8 (4.7)</td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Atopy</td>
<td>2</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>PC_{20}FEV_{1} (mg/ml)^A</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>1.75 (0.23-7.4)</td>
</tr>
<tr>
<td>FEV₁ % predicted^#</td>
<td>107.1 (4.8)</td>
<td>101 (3.1)</td>
<td>84.1 (4.2)</td>
</tr>
<tr>
<td>Bronchodilator reversibility (%)^#</td>
<td>1.3 (0.9)</td>
<td>0.8 (0.6)</td>
<td>5.3 (2.4)</td>
</tr>
<tr>
<td>FEV₁/FVC %^#</td>
<td>84.3 (2.3)</td>
<td>81.7 (1.1)</td>
<td>71.9 (2.2)</td>
</tr>
<tr>
<td>Total cell count (x10^6/g sputum)^#</td>
<td>1.7 (0.4)</td>
<td>3.1 (0.9)</td>
<td>2.5 (0.7)</td>
</tr>
<tr>
<td>Eosinophil %^A</td>
<td>0.4 (0-2.5)</td>
<td>12.8 (3-68)</td>
<td>4.2 (0-40)</td>
</tr>
<tr>
<td>Neutrophil %^#</td>
<td>32 (4.3)</td>
<td>50.6 (5.9)</td>
<td>60.7 (5.3)</td>
</tr>
<tr>
<td>Macrophage %^#</td>
<td>62.4 (4.2)</td>
<td>25.2 (4.9)</td>
<td>26.9 (4.3)</td>
</tr>
<tr>
<td>Lymphocyte %^#</td>
<td>2.7 (0.9)</td>
<td>0.5 (0.2)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>Epithelial cells %^#</td>
<td>3.7 (1.5)</td>
<td>1.8 (0.8)</td>
<td>3.1 (0.8)</td>
</tr>
<tr>
<td>Squamous cell contamination %^#</td>
<td>4.6 (1.7)</td>
<td>5.7 (1.6)</td>
<td>8.8 (1.7)</td>
</tr>
<tr>
<td>Viability %^#</td>
<td>63.4 (6.5)</td>
<td>73.1 (3.6)</td>
<td>64.6 (3.9)</td>
</tr>
</tbody>
</table>

^median(range)
^ mean(SEM)
Table 4.1.2.2 The median (IQR) number of eosinophils (MBP+), mast cells (tryptase+), T-lymphocytes (CD3+) and IL-13+cells/mm² submucosa in subjects with asthma, eosinophilic bronchitis or normal controls. *p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>Eosinophilic Bronchitis (n=7)</th>
<th>Normal (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP+cells/mm²</td>
<td>20 (23)*</td>
<td>28 (34)*</td>
<td>7 (10)</td>
</tr>
<tr>
<td>Tryptase+cells/mm²</td>
<td>21 (12)</td>
<td>26 (13)</td>
<td>22 (14)</td>
</tr>
<tr>
<td>CD3+cells/mm²</td>
<td>42 (59)</td>
<td>41 (24)</td>
<td>59 (110)</td>
</tr>
<tr>
<td>IL-13+cells/mm²</td>
<td>4 (8)*</td>
<td>1.7 (1.9)</td>
<td>0.5 (1.1)</td>
</tr>
</tbody>
</table>
Figure 4.1.2.1 Sputum IL-13 concentration for each subject with eosinophilic bronchitis, normal controls or asthma. Value for p represents result of Chi-squared test. IL-13 – interleukin-13.
**Figure 4.1.2.2 a)** Photomicrograph of IL-13+ cells highlighted by the arrows (x400) co-localised with MBP+ eosinophils on the corresponding sequential section (b).

4.2.1 Clinical and pathological features of non-eosinophilic asthma: a distinct asthma phenotype characterised by inhaled corticosteroid resistance.

**Abstract**

Introduction

Non-eosinophilic asthma is a newly recognised clinical phenotype since the introduction of inhaled corticosteroid therapy. Few studies have investigated the underlying airway biopathology and there are no data from clinical trials investigating the effect of inhaled corticosteroids.

Methods

All patients with asthma were investigated for the following variables:

- Levels of IL-13 and other cytokines
- Bronchial Provocation Tests (BPT) using methacholine
- Bronchoalveolar Lavage (BAL)
- Transtracheal biopsies

Flow amplitude was measured at the peak of each day of BPT, 7 days of each day for 2 weeks. Day-to-day variability was monitored using the mean of the first 3 days of each week.

Results

A significant correlation was observed between IL-13 levels and airway eosinophilia. This finding suggests a role for IL-13 in the pathogenesis of non-eosinophilic asthma.
4.2 Non eosinophilic asthma

4.2.1 Clinical and pathological features of non-eosinophilic asthma: a distinct asthma phenotype characterised by inhaled corticosteroid resistance

Abstract

INTRODUCTION
Non-eosinophilic asthma has been identified as a potentially important clinical phenotype since there is some evidence that it is resistant to inhaled corticosteroid therapy. No studies have investigated the underlying airway immunopathology and there are no data from placebo controlled studies examining the effect of inhaled corticosteroids.

METHODS
All patients with asthma were symptomatic and had one or more of the following markers of variable airflow obstruction: methacholine PC\textsubscript{20}<8mgml\textsuperscript{-1}, increase in FEV\textsubscript{1} of 15% or greater following inhalation of 200\textmu g of salbutamol and/or peak flow amplitude as percent of mean over 14 days of >20%. Endobronchial biopsies were taken from 11 patients with non-eosinophilic asthma, 12 patients with eosinophilic asthma and 10 normal control subjects. The patients with non eosinophilic asthma and 6 patients with eosinophilic asthma entered a randomised, double blinded, placebo controlled cross over study of inhaled mometasone 400\textmu g once daily for 8 weeks.
RESULTS

Patients with eosinophilic asthma had a median 23 bronchial submucosal cells positive for major basic protein per mm$^2$ which was higher than both normal controls (0 cells/mm$^2$, $p=0.043$) and patients with non-eosinophilic asthma (4.4 cells/mm$^2$, $p=0.016$). Submucosal mast cells numbers were not different between the groups. However, airway smooth muscle mast cell numbers were higher in eosinophilic asthma (8 cells/mm$^2$) and non-eosinophilic asthma (9 cells/mm$^2$) compared to normal controls (0 cells/mm$^2$, $p=0.016$). There were no significant differences in the number of submucosal cells positive for neutrophil elastase. The subepithelial collagen layer thickness was 10.3μm in patients with eosinophilic asthma compared to 5.8μm in non-eosinophilic asthma and 5.1μm in normal controls ($p=0.002$). 8 weeks treatment with inhaled mometasone led to a net 5.5 doubling dose improvement in methacholine $PC_{20}$ in patients with eosinophilic asthma and a 0.5 doubling dose improvement in the non-eosinophilic asthma group (mean difference 5.1 doubling doses, 95% C.I. 1.1, 9.1; $p=0.018$). There was a net 1.0 point improvement in Juniper asthma quality of life following treatment with inhaled mometasone compared to placebo in the eosinophilic asthma group and a 0.2 improvement in the non-eosinophilic asthma group (mean difference 0.9, 95% C.I. 0.27, 1.43; $p=0.008$).

CONCLUSIONS

Non-eosinophilic asthma represents a pathologically and clinically distinct disease phenotype, which is characterised by absence of eosinophilic airway inflammation in sputum and bronchial biopsies, normal subepithelial collagen layer thickness and resistance to the effect of short-term treatment with inhaled corticosteroids.
Introduction

Clinicians have long regarded asthma as a heterogeneous disease (Aas 1981; Rackemann 1921) although detailed clinicopathological studies have tended to emphasise the similarities in the underlying airway pathology and disordered function between patients (Humbert, Durham, Ying, Kimmitt, Barkans, Assoufi, Pfister, Menz, Robinson, Kay, & Corrigan 1996; Humbert, Menz, Ying, Corrigan, Robinson, & Duram 1999). The recent development of safe, non invasive induced sputum techniques has provided the opportunity to study airway inflammation in a diverse range of patients. Using this technique we and a number of other groups have identified a subset of adults who have clear physiological evidence of asthma but no induced sputum evidence of eosinophilic airway inflammation (Gibson, Simpson, & Saltos 2001; Pavord, Brightling, Woltmann, & Wardlaw 1999; Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999). This asthma phenotype is potentially clinically important since several uncontrolled studies have suggested that it is associated with corticosteroid resistance (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b; Meijer, Postma, Kauffman, Arends, Koeter, & Kerstjens 2002; Pavord, Brightling, Woltmann, & Wardlaw 1999).

Non eosinophilic asthma is present in 25% of patients presenting to an adult respiratory clinic with symptomatic asthma (Green, Brightling, Woltmann, Parker, Wardlaw, & Pavord 2002b). Other investigators have reported that up to 50% of patients with refractory asthma (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999), patients studied during an asthma exacerbation (Fahy, Kim, Liu, & Boushey 1995) and patients taking high doses of inhaled corticosteroids (Gibson, Simpson, & Saltos 2001) do not have a sputum eosinophilia. In a recent
longitudinal study of patients with severe asthma we noted a subset of patients who had stable non eosinophilic disease over 12 months. Moreover there is evidence that non eosinophilic asthma is present in untreated symptomatic patients. These observations suggest that this asthma phenotype is not solely explained by the effects of corticosteroids therapy and that it is a stable phenotype in some patients.

There have been no investigations into the immunopathology of non-eosinophilic asthma in patients who are naïve to corticosteroid treatment. Several sputum studies have noted that a sputum neutrophilia is often present. Wenzel et al have used bronchial biopsies to characterise the underlying airway immunopathology of patients with refractory asthma (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999), in which the presence of a subgroup that have a predominant neutrophilic airway inflammation was identified, absence of eosinophils and normal basement membrane thickness were the main features. These findings support the concept that non-eosinophilic, neutrophilic asthma is a pathologically distinct entity, although the patients studied were taking high doses of inhaled steroids so it is not clear to what extent these findings reflect the effects of treatment.

The aim of this study is to compare the immunopathology of eosinophilic and non eosinophilic asthma with normal controls and compare the response to the inhaled corticosteroid mometasone in a prospective randomised, placebo controlled trial in eosinophilic and non eosinophilic asthma.
Methods

SUBJECTS

Subjects were recruited from Glenfield Hospital clinics and by local paper advertisement. All subjects with asthma had symptoms consistent with asthma and at least one of the following objective measures of airway hyperresponsiveness and/or variable airflow obstruction: methacholine \( \text{PC}_{20} < 8 \text{mg/ml}^{-1} \), increase in \( \text{FEV}_1 \) of 15% or greater following inhalation of 200\( \mu \)g of salbutamol and/or peak flow amplitude as percent of mean over 14 days of >20%. Patients with eosinophilic asthma were recruited in two separate groups, one for the bronchoscopy study and one for the placebo controlled study. Normal control subjects had no respiratory symptoms, normal spirometry values and a methacholine \( \text{PC}_{20} > 16 \text{mg/ml} \).

Non eosinophilic was diagnosed in patients with asthma who had a sputum eosinophil count below 1.9%, 2 standard deviation above the mean for a normal population, on two occasions separated by one month while still symptomatic and not receiving inhaled or oral corticosteroids. All patients with non eosinophilic asthma had high resolution computerised tomography to exclude sub clinical bronchiectasis and we excluded patients who were felt to be symptomatic because of uncontrolled co-morbid conditions such as rhinitis and gastro-oesophageal reflux disease.

None of the patients had ever smoked, had a respiratory tract infection within six weeks of recruitment or received inhaled or oral corticosteroids for 3 months before entering the study. This study was approved by the Leicestershire and Rutland ethics committee and all patients provided informed written consent.
MEASUREMENTS

Single flow NO was recorded at 50mlsec\(^{-1}\) as previously described (Kharitonov, Alving, & Barnes 1997) and alveolar nitric oxide concentration was derived from measurements at 10, 30, 50, 100 and 200mlsec\(^{-1}\). Spirometry was measured using a rolling seal spirometer (Vitalograph, UK). The methacholine concentration causing a 20% fall in FEV\(_1\) (PC\(_{20}\)) was measured using the tidal breathing method as previously described (Juniper, Cockcroft, & Hargrave 1994). Symptom visual analogue score was measured using three 100mm scales representing cough, wheeze and breathlessness (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). Asthma quality of life was measured using the Juniper asthma quality of life score (Juniper & et al 1993). Sputum induction was performed and samples processed as previously described (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). Atopy was determined by a response of 2mm greater than negative control to skin prick testing to common aeroallergens.

Sputum IL-8, Cyseinyl-leukotriene and Histamine were measured using standard ELISA kits (BD Pharmagen, Immunotech and Cayman chemicals respectively) and eosinophilic cationic protein (ECP) was measured using a fluorescence immunoassay (UniCAP test, Pharmacia, Uppsala, Sweden). These assays have been previously validated for use in sputum supernatants (Birring, Parker, Brightling, Bradding, Wardlaw, & Pavord 2004).
The BAL and bronchial wash aspirates were filtered through 48µm gauze and diluted to a cell concentration of 0.5x10⁶ cells per/ml for cytospins and 5x10⁶ cells per/ml for flow cytometry. Cytospins were made with 75µl of aspirate and stained with Romanowski stain prior to counting by a person blind to the subjects details. Eosinophil counts were given as a percentage of at least 400 inflammatory cells counted.

Flow cytometry was used to measure CD4 and CD8 on the cells surface of CD3 positive lymphocytes. Interleukin-4 and interferon gamma production was stimulated with PMA and calcium ionophore for 4 hours in the presence of a secretion inhibitor. After permiabilisation intracellular staining for IL-4 and IFN-gamma was performed and expression measured in CD3+ve lymphocytes (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a).

Bronchial biopsies were processed and embedded in glycol methacrylate following which 2µm sections were cut and immunostaining performed for major basic protein, neutrophil elastase and tryptase followed by counter staining with haemotoxylin as previously described (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord 2002a). Biopsies were counted by an individual blinded to the patients clinical status (Mike Berry) who recorded the number of cells/mm² positive for major basic protein, neutrophil elastase and tryptase in the submucosa, the number of tryptase positive cells/mm² in the airway smooth muscle and the
subepithelial collagen layer thickness, which was recorded as the mean of 50 measurements taken over a distance of at least 1 mm.

PROTOCOL

Patients attended for a screening visit at which exhaled nitric oxide concentration was measured prior to skin prick testing, spirometry, methacholine challenge testing and sputum induction. If the patient was asthmatic and the sputum eosinophil count was less than 1.9% they attended a second screening visit after 4 weeks at which these tests were repeated. Bronchoscopy was performed at least 10 days following the screening visit according to American Thoracic Society guidelines. 20 ml of warmed sterile saline solution was instilled into the bronchus intermedius and aspirated; this was analysed as the wash sample. Three sequential samples of 60 ml of warmed sterile saline solution was then instilled into the middle lobe bronchus and then aspirated; the pooled aspirate from these samples was analysed as the BAL. Finally 3 to 6 biopsies were taken from the middle lobe carina.

At least four weeks after bronchoscopy patients with non eosinophilic asthma were entered into a randomised, placebo controlled crossover study of inhaled mometasone; a separate group of patients with eosinophilic asthma entered this study as a comparison group. Placebo or mometasone 400 μg were inhaled once daily via matched twisthaler® devices. Treatment phases were randomly allocated; they lasted for 8 weeks and were separated by a four week wash out phase. All other asthma medications were kept constant during the period of the study.
Flow independent exhaled nitric oxide parameters, spirometry, methacholine PC$_{20}$, induced sputum, symptom visual analogue scores and asthma quality of life were measured at baseline and after 8 weeks of each treatment, 24 hours after the previous treatment.

**ANALYSIS**

Primary outcome measures for the mometasone trial were difference in doubling dose change in methacholine PC$_{20}$ between placebo and mometasone at 8 weeks and difference in change in asthma quality of life score between placebo and mometasone at 8 weeks. Secondary outcome measures were net change in post-bronchodilator FEV$_1$, symptom visual analogue score, exhaled nitric oxide concentration, alveolar nitric oxide concentration, sputum eosinophil count, sputum and neutrophil count. This study was analysed as a mechanistic study and only data from patients who completed both treatment phases was analysed.

Methacholine PC$_{20}$ was calculated by linear interpolation of the change in FEV$_1$/concentration of methacholine, on a log dose response curve, as the inhaled concentration of methacholine causing a 20% reduction in FEV$_1$. Change in methacholine PC$_{20}$ was expressed in doubling dose concentrations. Alveolar NO concentration was calculated for each patient using a non linear model previously described (Berry et al. 2005).

Data was tested for normality of distribution using the Kolmogorov-Smirnov test. Data which was log normally distributed was log transformed prior to analysis. Between group comparisons of three groups were made using one way ANOVA
with Tukey’s post hoc test for individual group comparison when data was normally distributed or Kruskal-Wallis test when it was not. Between two group comparisons were made using either independent t-tests or Mann-Whitney U-test according to distribution. Descriptive statistics are given as mean (standard error) for normally distributed data, geometric mean (log standard error) for log normally distributed data and median (interquartile range) for data that was not normally distributed.

Results

SUBJECT CHARACTERISTICS
Subject baseline clinical characteristics are given in table 4.2.1.1 (page 88). High resolution computerised tomography of the thorax did not reveal any evidence of bronchiectasis in any of the patients with non eosinophilic asthma. Patients with asthma had significantly lower FEV$_1$ as percent of predicted, FEV$_1$/FVC and methacholine PC$_{20}$ and significantly higher β2-reversibility compared to normal controls but there was no difference between eosinophilic and non eosinophilic asthma. No patients classified as non eosinophilic asthma developed a sputum eosinophil count above 1.9% during the period of investigation.

BRONCHOSCOPY STUDY
Patients with non-eosinophilic asthma had lower sputum eosinophil counts, bronchial wash eosinophil counts and BAL eosinophil counts than patients with eosinophilic asthma, table 4.2.1.2 (page 89). There was no significant difference between sputum, bronchial wash and BAL neutrophil counts and no difference was
seen in the percentage of BAL lymphocytes positive for IL-4 or IFN-γ between the groups. There was no difference in blood or BAL lymphocyte CD4/CD8 ratio between the groups.

Patients with eosinophilic asthma had a median (Interquartile range) 23 (29) bronchial submucosal cells positive for major basic protein per mm² which was higher than both normal controls who had 0 cells/mm² (9.4) and patients with eosinophilic asthma who had 4.4 cells/mm² (7.9) (p=0.03, figure 4.2.1.1; page 91). There was no significant difference between the groups in the number of submucosal cells positive for tryptase (13 cells/mm² (5.7) vs. 11 cells/mm² (15) vs. 22 cells/mm² (33); p=0.52) however the number of tryptase positive cells in the airway smooth muscle was elevated in eosinophilic asthma 8 cells/mm² (12) and non eosinophilic asthma 9 cells/mm² (56) compared to normal controls (0 cells/mm² (1.8); p=0.016, Figure 4.2.1.1; page 91). There were no significant differences in the number of submucosal cells positive for neutrophil elastase. The subepithelial collagen layer thickness was 10.3µm (3.1) in patients with eosinophilic asthma compared to 5.8µm (3.0) in non-eosinophilic asthma and 5.1µm (2.1) in normal controls (p=0.002, figure 4.2.1.1; page 91).

**PLACEBO CONTROLLED STUDY**

No patients withdrew from this study after enrolment. Treatment period or order did not influence values before treatment or the change in primary outcome measures. Compared to placebo 8 weeks treatment with inhaled mometasone led to a net 5.5 (1.3) doubling dose improvement in methacholine PC₂₀ in patients with eosinophilic
asthma and a 0.5 (1.3) doubling dose improvement in the non eosinophilic asthma group (mean difference 5.1 doubling doses, 95% C.I. 1.1, 9.1; p=0.018). There was a net 1.0 (0.2) point improvement in Juniper asthma quality of life following treatment with inhaled mometasone compared to placebo in the eosinophilic asthma group and a 0.2 (0.2) improvement in the non eosinophilic asthma group (mean difference 0.9, 95% C.I. 0.27, 1.43; p=0.008, figure 4.2.1.2; page 92).

Secondary outcome measures are given in table 4.2.1.3 (page 90).

Discussion

We describe 12 patients with symptomatic asthma who had distinct sputum and bronchial biopsy pattern of airway inflammation characterised by the absence of eosinophilic airway inflammation and normal basement membrane thickness. In common with previous reports, patients tended to be non-atopic middle aged females; there was some sputum evidence of neutrophilic airway inflammation – which was noted in previous studies – although it was not evident in bronchial biopsies. Importantly airway responsiveness and asthma quality of life did not change significantly with inhaled mometasone when compared to placebo. This was in sharp contrast to findings in patients with asthma and eosinophilic airway inflammation, where inhaled steroids were associated with a marked improvement in these features. Our patients were symptomatic and had clear evidence of variable airflow obstruction suggesting that the absence of eosinophilic airway inflammation does not reflect remission of underlying disease. Asthma is a variable condition and it remains important to establish the extent to which the distinct pathological and clinical features are stable. Several features suggest that they are: firstly, we have
performed 5 observations of airway inflammation using induced sputum and have not identified eosinophilic inflammation on any occasion; secondly there was no evidence of an airway eosinophilia in any compartment of the lung sampled and finally classification of patients based on the sputum eosinophil count identified groups with a differing response to inhaled corticosteroids.

This is the first randomised control trial to specifically compare the response to inhaled corticosteroids in eosinophilic and non eosinophilic asthma. Our findings of a significantly reduced response to inhaled mometasone in patients with non eosinophilic asthma compared to eosinophilic asthma are consistent with some previous uncontrolled studies. In contrast to these findings, Godon et al found a similar response to inhaled corticosteroids in eosinophilic and non eosinophilic asthma. This study was not controlled and recruited patients during a period of uncontrolled asthma and it is possible that the improvement seen in their patients with non eosinophilic asthma reflected regression to the mean. In addition patients in Godon's study were younger and more likely to be atopic than previous descriptions of patients with non eosinophilic asthma, as well as our study sample, and the measurement of sputum eosinophil count at one time point in their patients may have lead to the misclassification of some patients with eosinophilic asthma into the non eosinophilic group.

Our finding of a normal subepithelial collagen layer thickness in our patients with non eosinophilic asthma is consistent with the finding by Wenzel and colleagues in severe asthma. The fact that subepithelial layer thickening is found in patients with rhinitis and eosinophilic bronchitis without asthma and not in patients with non
eosinophilic asthma suggests that this finding may be related to eosinophilic airways disease rather than asthma per se.

Although our study demonstrated a difference in response to inhaled corticosteroids in eosinophilic compared to non eosinophilic asthma we cannot conclude from our sample size that there is no significant response to inhaled mometasone in non eosinophilic asthma.

Our findings suggest that eosinophilic and non eosinophilic asthma represent distinct clinical and pathological phenotypes. The common physiological features of bronchial hyperresponsiveness and variable airflow obstruction may be causally related to the presence of tryptase positive mast cells in the airway smooth muscle. The concept of a causal relationship between eosinophilic airway inflammation and variable airflow obstruction and airway hyperresponsiveness is specifically refuted. We have demonstrated that patients with non eosinophilic asthma have a significantly reduced response to inhaled corticosteroids compared to eosinophilic asthma. Longer, larger studies are required to determine whether there is any clinical significant response to these treatments in non eosinophilic asthma and whether inhaled corticosteroids can be safely withdrawn in patients with non eosinophilic asthma.
Table 4.2.1.1 Subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Eosinophilic asthma</th>
<th>Non eosinophilic asthma</th>
<th>Sig. (ANOVA)</th>
<th>Sig. (Eos. vs. non eos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (Male)</td>
<td>10 (5)</td>
<td>12 (8)</td>
<td>11 (3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>41 (22, 61)</td>
<td>42 (27, 69)</td>
<td>47 (19, 68)</td>
<td>0.65</td>
<td>-</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>105 (3.9)</td>
<td>90.3 (6.2)</td>
<td>88 (4.9)</td>
<td>0.05</td>
<td>0.96</td>
</tr>
<tr>
<td>FEV₁/FVC</td>
<td>82 (1.8)</td>
<td>73.9 (2.9)</td>
<td>69 (5.6)</td>
<td>0.025</td>
<td>0.58</td>
</tr>
<tr>
<td>β2-Reversibility (%)</td>
<td>1.1 (0.9)</td>
<td>9.5 (2.5)</td>
<td>12.7 (3.4)</td>
<td>0.001</td>
<td>0.67</td>
</tr>
<tr>
<td>Methacholine PC₂₀ (mg/ml) †</td>
<td>&gt;16</td>
<td>0.67 (0.2)</td>
<td>0.83 (0.2)</td>
<td>&lt;0.001</td>
<td>0.15</td>
</tr>
<tr>
<td>Atopic (%)</td>
<td>2 (20)</td>
<td>8 (66)</td>
<td>2 (18)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data given as mean (S.E.) except * - mean (range) and † - geometric mean (log S.E.)

FEV₁ - forced expiratory volume in 1 second, FVC - forced vital capacity, β2-Reversibility - percent increase in FEV₁ following inhalation of 200µg of salbutamol, Methacholine PC₂₀ - concentration of inhaled methacholine which causes a 20% fall in FEV₁.
Table 4.2.1.2 Subject inflammatory cell counts.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Eosinophilic asthma</th>
<th>Non eosinophilic asthma</th>
<th>Sig. (ANOVA/KW)</th>
<th>Sig. (Eos. vs. non eos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum eosinophil count (%) †</td>
<td>0.26 (0.1)</td>
<td>4.0 (0.1)</td>
<td>0.65 (0.1)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sputum neutrophil count (%)</td>
<td>31.6 (6.9)</td>
<td>38.3 (7.9)</td>
<td>76.2 (9.0)</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Bronchial wash eosinophil count (%) †</td>
<td>0.6 (0.2)</td>
<td>2.0 (0.2)</td>
<td>0.39 (0.3)</td>
<td>0.017</td>
<td>0.017</td>
</tr>
<tr>
<td>Bronchial wash neutrophil count (%)</td>
<td>27.4 (6.3)</td>
<td>25.1 (5.0)</td>
<td>27.9 (6.7)</td>
<td>0.94</td>
<td>-</td>
</tr>
<tr>
<td>BAL eosinophil count (%) †</td>
<td>0.6 (0.2)</td>
<td>1.4 (0.1)</td>
<td>0.43 (0.1)</td>
<td>0.03</td>
<td>0.029</td>
</tr>
<tr>
<td>BAL neutrophil count (%)</td>
<td>14.7 (4.1)</td>
<td>7.2 (2.4)</td>
<td>8.4 (2.5)</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>BAL IL-4 (% of CD3+ve lymphocytes)</td>
<td>4.1 (1.2)</td>
<td>3.9 (1.3)</td>
<td>7.0 (2.3)</td>
<td>0.36</td>
<td>-</td>
</tr>
<tr>
<td>BAL IFN-γ (% of CD3+ve lymphocytes)</td>
<td>32.3 (9.6)</td>
<td>32.3 (8.6)</td>
<td>33.3 (5.7)</td>
<td>0.99</td>
<td>-</td>
</tr>
<tr>
<td>Submucosal MBP +ve cells (mm²)*</td>
<td>0 (9.4)</td>
<td>23 (29)</td>
<td>4.4 (7.9)</td>
<td>0.03</td>
<td>0.016</td>
</tr>
<tr>
<td>Submucosal NE +ve cells (mm²)*</td>
<td>10 (31)</td>
<td>10 (24)</td>
<td>9 (22)</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>Submucosal tryptase +ve cells (mm²)*</td>
<td>13 (5.7)</td>
<td>11 (15)</td>
<td>22 (33)</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>Smooth muscle tryptase +ve cells (mm²)*</td>
<td>0 (1.8)</td>
<td>8 (12)</td>
<td>9 (56)</td>
<td>0.016</td>
<td>0.2</td>
</tr>
<tr>
<td>Subepithelial collagen layer thickness (µm)*</td>
<td>5.1 (2.1)</td>
<td>10.3 (3.1)</td>
<td>5.8 (3.0)</td>
<td>0.002</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Data given as mean (S.E.) except † - geometric mean (log S.E.) and * - median (interquartile range). BAL - bronchial alveolar lavage, IL-4 - interleukin-4, IFN-γ - interferon gamma, MBP - major basic protein and NE - neutrophil elastase, ANOVA - analysis of variance, KW - Kruskal-Wallis.
Table 4.2.1.3 Subject characteristics before and after 8 weeks treatment with inhaled mometasone and placebo.

<table>
<thead>
<tr>
<th>Week</th>
<th>Placebo</th>
<th>Mometasone</th>
<th>Significance mometasone vs. placebo</th>
<th>Placebo</th>
<th>Mometasone</th>
<th>Significance mometasone vs. placebo</th>
<th>Significance Eos. vs. non eos.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methacholine PC_{20} (mg/ml)†</td>
<td>1.5 (0.2)</td>
<td>0.2 (0.3)</td>
<td>0.5 (0.2)</td>
<td>2.1 (0.3)</td>
<td>0.01</td>
<td>1.4 (0.2)</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td>Asthma QOL score</td>
<td>5.5 (0.3)</td>
<td>5.5 (0.4)</td>
<td>5.6 (0.3)</td>
<td>6.6 (0.2)</td>
<td>0.004</td>
<td>5.0 (0.3)</td>
<td>5.0 (0.3)</td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>94 (7.9)</td>
<td>93 (8.4)</td>
<td>94 (7.3)</td>
<td>94 (7.9)</td>
<td>0.62</td>
<td>86 (3.8)</td>
<td>84 (4.1)</td>
</tr>
<tr>
<td>Symptom VAS (mm)</td>
<td>59 (27)</td>
<td>76 (29)</td>
<td>28 (7.6)</td>
<td>12 (7.1)</td>
<td>0.33</td>
<td>141 (41)</td>
<td>122 (39)</td>
</tr>
<tr>
<td>Exhaled NO (ppb) †</td>
<td>56 (0.1)</td>
<td>79 (0.1)</td>
<td>65 (0.1)</td>
<td>21 (0.1)</td>
<td>0.003</td>
<td>13 (0.1)</td>
<td>15 (0.1)</td>
</tr>
<tr>
<td>Alveolar NO (ppb)</td>
<td>9.2 (1.2)</td>
<td>12 (2.3)</td>
<td>5.7 (1.2)</td>
<td>4.8 (1.3)</td>
<td>0.27</td>
<td>3.2 (0.5)</td>
<td>2.4 (0.6)</td>
</tr>
<tr>
<td>Sputum eosinophils (%) †</td>
<td>7.1 (0.1)</td>
<td>9.9 (0.1)</td>
<td>11 (0.2)</td>
<td>2.3 (0.2)</td>
<td>0.001</td>
<td>0.6 (0.2)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>Sputum neutrophils (%)</td>
<td>26 (5.6)</td>
<td>33 (7.2)</td>
<td>40 (8.9)</td>
<td>45 (11)</td>
<td>0.93</td>
<td>63 (7.7)</td>
<td>67 (7.6)</td>
</tr>
</tbody>
</table>

Data given as mean (standard error) except where marked † - geometric mean (log standard error). FEV₁ - forced expiratory volume in one second, PC_{20} - dose of inhaled methacholine causing a 20% fall in FEV₁, QOL - quality of life, VAS - visual analogue score, NO - nitric oxide.
**Figure 4.2.1.1** Expression of submucosal cells with positive staining for major basic protein, smooth muscle cells for mast cell tryptase (AAl) and subepithelial collagen layer thickness. Values of p on figures refer to Kruskal-Wallis test.

a) $p=0.99$

b) $p=0.016$

c) $p=0.002$

Bars represent medians.

Kruskal Wallis $p=0.03$. 

Bars represent medians.

Kruskal Wallis $p=0.016$. 

Bars represent medians.

Kruskal Wallis $p=0.002$. 

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Figure 4.2.1.2 Methacholine PC$_{20}$ and Juniper asthma quality of life score before and after placebo and mometasone. Values of p refer to result of paired t-tests.

Values of p refer to result of paired t-tests.

Placebo Mometasone

Non eosinophilic asthma

Placebo Mometasone

Eosinophilic asthma

Placebo Mometasone

Non eosinophilic asthma

Eosinophilic asthma

Diamond symbol represents mean/Geometric mean
4.3 Refractory asthma

4.3.1 Alveolar nitric oxide concentration in adults with asthma: evidence of distal lung inflammation in refractory asthma

Abstract

BACKGROUND
Recent studies have suggested that alveolar nitric oxide concentration is a non-invasive test of distal lung inflammation.

METHODS
We have determined whether alveolar nitric oxide concentration can be measured in patients with asthma of varying severity, tested the hypothesis that there is an association between alveolar nitric oxide and broncho-alveolar lavage (BAL) eosinophil count and determined whether refractory asthma is characterised by a raised alveolar nitric oxide concentration. Finally we have assessed the effect of two weeks of prednisolone 30mg once a day on alveolar nitric oxide concentration.

RESULTS
Alveolar nitric oxide concentration was both measurable and repeatable in patients with refractory asthma. We found a positive correlation between alveolar nitric oxide concentration and BAL eosinophil count ($r=0.79, p=0.006$) but not bronchial wash or sputum eosinophil count. Alveolar nitric oxide concentration was increased in patients with refractory asthma (7.1ppb) compared to mild to moderate asthma (3.4ppb) and normal controls (3.4ppb,$p<0.001$) and reduced by treatment with prednisolone.
CONCLUSIONS

Our findings support the hypothesis that alveolar nitric oxide is a measure of distal airway inflammation and are in keeping with the notion that distal lung inflammation is present in refractory asthma.
Introduction

Refractory asthma can be defined as asthma that cannot be controlled satisfactorily with inhaled corticosteroids (ATS 2000). It is present in 5% of a random population of patients with asthma and the majority of patients attending specialist respiratory clinics. Patients with refractory asthma suffer considerable morbidity and consume a large proportion of the health budget attributed to asthma management - on average six times more per patient than mild asthma. The American Thoracic Society(ATS 2000) and the European Respiratory Society (Chung, Godard, Adelroth, Ayres, Barnes, Barnes, Bel, Burney, Chanez, Connett, Corrigan, de Blieck, Fabbri, Holgate, Ind, Joos, Kerstjens, Leuenberger, Lofdahl, McKenzie, Magnussen, Postma, Saetta, Salmeron, & Sterk 1999) have identified research into the mechanisms of refractory asthma as an important priority.

An attractive hypothesis for pathogenesis of refractory asthma is the presence of inflammation in the distal lung, an area that might not be accessed by inhaled corticosteroids. Post mortem and bronchoscopy with transbronchial biopsy studies have shown inflammation in the alveoli and small airways, although these studies were uncontrolled so it is not possible to determine whether this feature is peculiar to refractory asthma (Kraft et al. 1996).

Airway inflammation can be measured non-invasively by measuring the sputum eosinophil count and single flow exhaled NO concentration, although these tests are limited to sampling the proximal airway. However two compartment models of pulmonary NO production have been described (Silkoff et al. 2000; Tsoukias & George 1998) which can be used to calculate the alveolar contribution to exhaled NO
concentration. Alveolar NO concentration is elevated in conditions associated with distal lung inflammation such as pulmonary fibrosis (Lehtimaki et al. 2001a) and chronic obstructive pulmonary disease (Hogman et al. 2002). Unlike bronchial nitric oxide, alveolar nitric oxide production is not reduced by inhaled fluticasone in patients with asthma, suggesting that it may be derived from a site not accessed by inhaled corticosteroid treatment (Lehtimaki et al. 2001b). Other methods that might be useful in the assessment of distal lung inflammation include BAL (Pizzichini et al. 2004) or transbronchial biopsy (Sutherland et al. 2004) although these methods are invasive and their application to patients with refractory asthma is limited. Alveolar nitric oxide concentration has been demonstrated to be associated with BAL eosinophilic cationic protein concentration in children with steroid treated atopic asthma (Mahut et al. 2004) but whether it is possible to measure alveolar nitric oxide is patients with refractory asthma and how alveolar nitric oxide relates to other measurements of distal inflammation is unknown.

We have determined whether it is possible to measure alveolar nitric oxide concentration in patients with refractory asthma and determined repeatability. We have tested the hypothesis that alveolar nitric oxide concentration is associated with distal lung inflammation as reflected by bronchioalveolar lavage (BAL) eosinophil count and that increased alveolar nitric oxide concentration is a feature of refractory asthma. Finally we have investigated the effect of treatment with oral corticosteroids and higher dose inhaled steroids on alveolar nitric oxide concentration.
Methods

SUBJECTS

Consecutive patients attending the difficult asthma clinic at Glenfield Hospital who fulfilled the American Thoracic Society criteria for refractory asthma (ATS 2000) were invited to participate. We also recruited volunteers with mild to moderate asthma and healthy volunteers who had no respiratory symptoms and normal spirometry from our research database and from local advertisement. This study was approved by the Leicestershire and Rutland ethic committee.

All subjects with asthma had symptoms consistent with asthma and at least one of the following objective measures of airway hyperresponsiveness and/or variable airflow obstruction: methacholine $PC_{20} < 8 \text{mgml}^{-1}$, increase in $FEV_1$ of 15% or greater following inhalation of $200 \mu\text{g}$ of salbutamol or peak flow amplitude as percent of mean over 14 days $> 20\%$. Methacholine $PC_{20}$ was performed only if the $FEV_1$ was $>1 \text{ litre}$. Refractory asthma was diagnosed according to ATS criteria with the exception of the dose of inhaled corticosteroid which was increased to $\geq 2000 \mu\text{g}$ beclomethasone equivalent per day to reflect the use of higher doses in UK practice; patients had at least one major criterion and two minor criteria for refractory asthma. Moderate asthma was diagnosed if no major and no more than 1 minor criterion for diagnosing refractory asthma was met and patients were on less than $2000 \mu\text{g}$ of beclomethasone equivalent. Mild asthma was classified as those patients taking $\beta_2$-agonist only with no minor or major criteria for refractory asthma.

Treatment concordance was assessed to be good or perfect in all patients with refractory asthma (ATS 2000) on a 5 point scale ranging from very poor to perfect.
Our assessment was based on measurement of serum prednisolone, cortisol and theophylline concentrations, domiciliary assessment by a consultant pharmacist and by analysis of primary care records of prescription issue and collection. We excluded patients who were felt to be symptomatic because of uncontrolled comorbid conditions such as rhinitis and gastroesophageal reflux disease.

PROTOCOL

Exhaled NO concentration was measured at flows of 10, 30, 50, 100 and 200ml/sec using an online chemiluminescence analyser (NIOX, Aerocrine, Stockholm, Sweden) prior to any other measurements. Spirometry was measured using a rolling seal spirometer (Vitalograph, UK). The methacholine concentration causing a 20% fall in FEV₁ was measured using the tidal breathing method with doubling doses of inhaled methacholine inhaled though a Wright’s nebuliser as previously described (Juniper, Cockcroft, & Hargreave 1994). Asthma control was assessed using the Juniper asthma control score (Juniper et al. 2000). Sputum induction was performed as previously described (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). To assess repeatability 25 randomly selected subjects were invited for a second measurement of alveolar nitric oxide concentration after 2 weeks.

Patients with refractory asthma, who on clinical grounds required an increase in anti-inflammatory medication, defined as a Juniper asthma control score >1.57 and/or a sputum eosinophil count >3%, had either oral corticosteroid treatment initiated or a doubling of their inhaled corticosteroid and were invited for repeat measurement of alveolar nitric oxide concentration after at least 2 weeks.
All subjects with mild to moderate asthma who were not taking inhaled corticosteroid underwent fibre-optic bronchoscopy and bronchoalveolar lavage at least 2 weeks after the initial assessment. 20ml of warmed sterile saline solution was instilled into the bronchus intermedius and aspirated this was analysed as the wash sample. Three sequential samples of 60ml of warmed sterile saline solution were then instilled into the middle lobe bronchus and then aspirated; the pooled aspirate from these samples was analysed as the BAL. The aspirates were filtered through 48μm gauze and diluted to a cell concentration of $0.5 \times 10^6$ cells per/ml. Cytospins were made with 75μl of aspirate and stained with Romanowski stain prior to counting by a person blind to the subjects details. Eosinophil counts were given as a percentage of at least 400 inflammatory cells counted.

**ANALYSIS**

Alveolar NO concentration was calculated for each patient using a non linear model described by Silkoff et al (Silkoff, Sylvester, Zamel, & Permutt 2000). Briefly exhaled nitric oxide and exhalation flow were used to resolve the following non-linear equation $C_E = C_W \cdot (1 - \exp(-D_{NO}/V)) + C_{ALV} \cdot \exp(-D_{NO}/V)$ where $C_E$ is the exhaled concentration of nitric oxide, $C_W$ is the airway wall concentration of nitric oxide, $D_{NO}$ is the diffusion constant for nitric oxide, $V$ is the exhalation flow and $C_{ALV}$ in the alveolar nitric oxide concentration. Maximal bronchial NO output was calculated using a linear method described by Tsoukias and George (Tsoukias & George 1998) where nitric oxide output is plotted against exhalation flow and the intercept recorded as the maximal bronchial NO output. Repeatability was assessed using intraclass correlation coefficient and standard deviation of within subject standard deviation.
Kolmogorov-Smirnov tests of normality were performed to test the distribution of data prior to analysis. Alveolar nitric oxide concentrations were normally distributed and between groups comparisons were made using one way ANOVA and independent sample t-tests. Alveolar nitric oxide concentration before and after oral steroid or double dose of inhaled steroids were compared with a paired t-test. Total IgE, exhaled NO concentration, maximal bronchial NO output and sputum and BAL eosinophil counts were found to be Log normally distributed and were therefore Log transformed prior to analysis, a value 0.1 was assigned to measurements of 0. Correlation was assessed using Pearson’s product moment correlation co-efficient. All statistical analyses were performed using SPSS 10 for windows and non-linear regression analyses were performed using NLREG (Phillip H. Sherrod, www.nlreg.com) commands called from Microsoft Access.

Results

Patient details are given in table 4.3.1.1 (page 107). Patients with refractory asthma had significantly lower forced expiratory volume in on second (FEV\textsubscript{1}) as percent of predicted (mean 62\% vs. 94.2\%, mean difference 32.2\%; 95\% confidence intervals; 24.2, 40.2; p<0.001) and FEV\textsubscript{1}/FVC ratio (mean 69\% vs. 78\%; mean difference 9.0; 95\% confidence interval of the difference 3.6, 12.5; p<0.01) than patients with mild to moderate asthma. There were no significant differences between sputum eosinophil counts, single flow nitric oxide concentration maximal bronchial NO output or other measured variables between the asthma groups (table 4.3.1.1; page 107).
Alveolar NO concentration measurements were possible in all patients and controls and were repeatable (intraclass correlation coefficient 0.95, within subject standard deviation 0.25).

There was a positive correlation between BAL eosinophil counts and alveolar nitric oxide concentration in patients with mild to moderate asthma (r=0.79, p=0.006; figure 4.3.1.1; page 109) but the association between alveolar NO and bronchial wash eosinophil counts in this group was not significant (r=0.6, p=0.06; figure 4.3.1.1; page 109). In contrast there was a positive association between single flow nitric oxide and bronchial wash eosinophil count (r=0.69, p=0.027; figure 4.3.1.1; page 109) but no association between single flow NO and BAL eosinophil count (r=0.25, p=0.49; figure 4.3.1.1; page 109). Similarly bronchial NO output was positively correlated with bronchial wash eosinophil count (r=0.71, p=0.022) but not BAL eosinophil count (r=0.1, p=0.76). Sputum eosinophil count was positively correlated with single flow nitric oxide (r=0.7, p=0.02) and bronchial NO (r=0.82, p=0.004) but not alveolar nitric oxide (r=0.2, p=0.6).

The mean (S.E.M.) alveolar NO concentration was 7.1ppb (0.70) in the refractory asthma group, 3.4ppb (0.46) in the mild to moderate asthma group and 3.4ppb (0.38) in the normal controls (p<0.001). There were significant differences in alveolar NO concentration between refractory asthma and normal controls (difference 3.7ppb, 95% confidence intervals of the difference 1.59, 5.83; p=0.001) and mild to moderate asthma (difference 3.7ppb, 95% confidence intervals of the difference 2.0, 5.4; p<0.001) but not between normal controls and mild to moderate asthma (figure 4.3.1.2; page 110).
Within the mild to moderate asthma group there was no difference in alveolar NO concentration between the steroid naïve and inhaled steroid treated group (3.6ppb vs. 3.4ppb, p=0.82). Within the refractory asthma group there was no significant difference in the concentration of alveolar NO in subjects receiving oral corticosteroids and those who were not. There was no relationship between alveolar nitric oxide and the dose or type of inhaled corticosteroid used, no significant difference in alveolar NO concentration between eosinophilic and non-eosinophilic asthma and no relationship with Juniper asthma control score. There was no difference in alveolar nitric oxide concentration between groups within the refractory asthma group defined according to the most prominent secondary feature of refractory asthma. There was a weak negative correlation between alveolar NO concentration and post bronchodilator FEV₁ percent predicted (r=-0.28, p=0.017).

The baseline concentration of alveolar nitric oxide was not significantly different in the patients who were started on oral corticosteroids (mean 7.9ppb) than those who received a doubling dose of their inhaled corticosteroid (mean 7.5ppb, mean difference 0.7, 95% C.I. -3.9, 2.5; p=0.65). Other baseline characteristics were similar (table 4.3.1.2; page 108). Treatment with oral corticosteroids led to a significant reduction in alveolar nitric oxide concentration (7.9ppb to 3.6ppb, mean difference 4.3, 95% C.I. 1.3, 7.4; p=0.008) whereas a doubling of the dose of inhaled corticosteroids did not (7.5ppb to 8.0ppb, mean difference -0.6, 95% C.I. -3.2, 2; p=0.62; figure 4.3.1.3; page 111). The difference in change in alveolar nitric oxide concentration between two treatment groups was significant (mean difference 5.0, 95% C.I. 1.0, 5.0; p=0.017).
Discussion

We found that measurement of alveolar NO concentration was feasible in a range of patients with asthma, including refractory asthma, and that results are repeatable over 2 weeks. In patients with mild to moderate asthma who were corticosteroid naïve, alveolar nitric oxide concentration correlated more closely with BAL than bronchial wash eosinophil cell counts whereas the reverse was true with single flow NO concentration. Bronchial NO output, a measure of NO flux, was also related to sputum and bronchial wash eosinophil counts but not BAL cells. Increased alveolar NO was seen in patients with refractory asthma who were already receiving high dose inhaled steroids. Finally a two week course of oral prednisolone but not a one month course of double in dose of inhaled corticosteroids reduced alveolar NO concentration in patients who required a step up in their treatment. These findings support our hypothesis that alveolar NO is a measure of inflammation in the distal lung and are consistent with the view that refractory asthma is associated with distal lung inflammation. Our study suggests that inflammation in this site responds to oral but not inhaled corticosteroids.

Bronchoscopy and BAL is a relatively invasive procedure, so we were limited to studying the relationship between alveolar NO and BAL eosinophils in subjects with mild asthma. Alveolar NO concentration were normal in this population and there was limited between patient variability in alveolar NO concentration. Despite these limitations we found a strong positive correlation between alveolar NO and BAL eosinophil count but not bronchial wash eosinophils. The opposite was true for single flow nitric oxide and bronchial NO output. A previous study has highlighted the weak relationship between BAL and sputum cell counts and have suggested that this is due
to sampling of different compartments, with BAL accessing the distal lung and sputum the proximal airway (Pizzichini, Pizzichini, Kidney, Efthimiadis, Hussack, Popov, Cox, Dolovich, O'Byrne, & Hargreave 2004). Our finding of a strong positive correlation between BAL eosinophils and alveolar NO may therefore be consistent with the view that elevated alveolar NO reflects eosinophilic inflammation in the distal lung.

We have shown that alveolar NO concentration is higher in patients with refractory asthma than in both normal controls and patients with mild to moderate asthma. There was no difference in proximal airway inflammation assessed by sputum eosinophil count, single flow nitric oxide concentration or bronchial NO output, implying that the difference in alveolar NO concentration is due to the difference in the site rather than the intensity of the lower airway response. Nocturnal wheeze was reported by a similar proportion of patients in all asthma groups so our findings are unlikely to be due to differences in the prevalence of nocturnal asthma, a condition that has been associated with high alveolar NO concentrations (Lehtimaki et al. 2002). Similarly there was no relationship between asthma control, assessed using the Juniper asthma control score, at the time of sampling and alveolar nitric oxide concentration, suggesting that distal airway inflammation occurs in patients who require high doses of inhaled steroids or oral steroids to achieve control of their symptoms as well as patients with uncontrolled asthma.

The presence of inflammation in the distal lung suggests a possible mechanism for inhaled corticosteroid resistance, the characteristic feature of refractory asthma, since the distal lung might not be accessed by inhaled corticosteroids which preferentially
deposit in the larger airways (Martin 2002). The concept that distal lung inflammation is not modified by inhaled corticosteroids is supported by the presence of high alveolar NO in patients who were taking high dose inhaled corticosteroids and by an earlier study, in mild to moderate asthma, showing that inhaled fluticasone does not reduce alveolar NO (Lehtimaki, Kankaanranta, Saarelainen, Turjanmaa, & Moilanen 2001b). Further support for this view and evidence that even elevated alveolar nitric oxide concentrations cannot be modified by inhaled corticosteroids comes from our observation that a double dose of inhaled corticosteroid did not reduce alveolar nitric oxide concentration. By contrast, oral prednisolone significantly reduced alveolar NO. The clear implication of this finding is that alveolar nitric oxide concentration reflects inflammation in a site that can be accessed by systemic but not inhaled corticosteroids.

An important limitation of our investigation of the effect of oral and inhaled corticosteroids on alveolar nitric oxide concentration is that patients were not randomised to the different treatments and the interventions were not placebo controlled. However patients who received prednisolone or higher dose inhaled corticosteroids had similar pre-treatment characteristics so our findings are unlikely to be biased by differences in severity of disease. We also doubt that the reduction in alveolar nitric oxide concentration seen with prednisolone was due to regression to the mean, since a similar reduction was not seen with higher dose inhaled steroids. Nevertheless we recognise that further randomised placebo controlled studies are required to investigate the effects of oral and inhaled corticosteroids on alveolar nitric oxide concentration before our findings can be regarded as definitive.
There is increasing recognition that refractory asthma is a heterogeneous disease (ATS 2000)(2). Studies have highlighted the presence of non-eosinophilic pathology in some patients (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999). Others have identified fixed airflow obstruction (ten Brinke et al. 2001) or recurrent exacerbation (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a) as important phenotypes. Implicit in the definition is the concept that asthma control cannot be achieved with inhaled corticosteroids or that high dose treatment is required (ATS 2000)(2). In our population increased alveolar nitric oxide concentration appeared to be a feature of patients with different criteria for a diagnosis of refractory asthma, perhaps consistent with the view that distal lung inflammation is a common feature in different types of refractory asthma. However we recognise that the power of our study to identify a difference is limited and further work is required to investigate this important question. One intriguing observation was the significant correlation between post bronchodilator FEV₁ and alveolar nitric oxide concentration, suggesting that distal lung inflammation might be involved in the development of fixed airflow obstruction in asthma.

In conclusion our preliminary study suggests that alveolar nitric oxide is a potentially useful technique for investigating the role of distal lung inflammation in asthma and other airway diseases. Our findings are consistent with the presence of distal lung inflammation in refractory asthma and suggest that eosinophilic inflammation in this site might be associated with inhaled but not systemic corticosteroid resistance.
### Table 4.3.1.1 Clinical characteristics of study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild to Moderate asthma</th>
<th>Refractory asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (Male)</td>
<td>13(6)</td>
<td>25(12)</td>
<td>27(14)</td>
</tr>
<tr>
<td>Atopic (%)</td>
<td>3 (23)</td>
<td>11 (44)</td>
<td>14 (52)</td>
</tr>
<tr>
<td>Total IgE ***</td>
<td>16.2 (0.6)</td>
<td>172(1.1)</td>
<td>354(0.6)</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>38(23-49)</td>
<td>42(18-72)</td>
<td>44(21-69)</td>
</tr>
<tr>
<td>Age at onset of symptoms (years)*</td>
<td>n/a</td>
<td>14(1-36)</td>
<td>16(1-58)</td>
</tr>
<tr>
<td>Nocturnal symptoms (number)</td>
<td>0</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>FEV1 % of predicted</td>
<td>96.6(5.9)</td>
<td>94.2(7.4)</td>
<td>62(21.1)</td>
</tr>
<tr>
<td>FEV1/FVC(%)</td>
<td>79.6 (7.8)</td>
<td>78.0 (6.7)</td>
<td>69.0 (13)</td>
</tr>
<tr>
<td>Single flow nitric oxide concentration (ppb, 50ml/sec)***</td>
<td>16.5 (0.18)</td>
<td>24.6 (0.31)</td>
<td>31.7 (0.28)</td>
</tr>
<tr>
<td>Maximal bronchial NO output (nl/min)***</td>
<td>39.3 (0.2)</td>
<td>54.3 (0.4)</td>
<td>64.3 (0.4)</td>
</tr>
<tr>
<td>Sputum eosinophil count %**</td>
<td>0 (0-1.3)</td>
<td>6.3 (0-65.8)</td>
<td>7.8 (0-53.3)</td>
</tr>
<tr>
<td>Sputum neutrophil count %</td>
<td>57.3 (24.9)</td>
<td>54.9 (25.5)</td>
<td>62.0 (27.2)</td>
</tr>
<tr>
<td>Total sputum cell count (x10⁶)***</td>
<td>0.4 (0.9)</td>
<td>1.5 (0.8)</td>
<td>1.8 (0.5)</td>
</tr>
<tr>
<td>PC_{20} (mg/ml)***</td>
<td>&gt;16</td>
<td>0.4(0.6)</td>
<td>0.4(0.7)</td>
</tr>
<tr>
<td>Number on oral steroids</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are given as mean(standard deviation) except where marked * mean(range), **median(range) and *** geometric mean(log standard deviation).
Table 4.3.1.2 Clinical and physiological characteristics before and after inhaled or oral corticosteroids.

<table>
<thead>
<tr>
<th></th>
<th>Oral corticosteroids</th>
<th>Inhaled corticosteroids</th>
<th>Difference</th>
<th>Sig.</th>
<th>Difference</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td></td>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Alveolar NO (ppb)</td>
<td>7.9(1.4)</td>
<td>3.58(0.5)</td>
<td>4.3(1.3, 7.4)</td>
<td>0.008</td>
<td>7.48(1.6)</td>
<td>8.0(1.9)</td>
</tr>
<tr>
<td>Single flow NO (ppb)</td>
<td>32.4(0.1)*</td>
<td>17.8(0.1)*</td>
<td>1.8(0.8, 4.4)**</td>
<td>0.16</td>
<td>41.6(0.1)*</td>
<td>41.3(0.1)*</td>
</tr>
<tr>
<td>Sputum eosinophil count (%)</td>
<td>8.5(0.2)*</td>
<td>0.6(0.2)*</td>
<td>15.5(4.7, 50)**</td>
<td>0.001</td>
<td>8.9(0.1)*</td>
<td>1.0(0.2)*</td>
</tr>
<tr>
<td>Juniper asthma control score</td>
<td>2.8(0.6)</td>
<td>1.6(0.2)</td>
<td>1.2(0.01, 2.3)</td>
<td>0.036</td>
<td>2.6(0.3)</td>
<td>1.8(0.5)</td>
</tr>
<tr>
<td>Post bronchodilator FEV₁ (% of predicted)</td>
<td>64(8.6)</td>
<td>78(5.4)</td>
<td>14(2, 26)</td>
<td>0.027</td>
<td>73(5.2)</td>
<td>77(4.8)</td>
</tr>
<tr>
<td>FEV₁/FVC ratio (%)</td>
<td>74(2.8)</td>
<td>76(3.0)</td>
<td>2.2(0.4, 4.8)</td>
<td>0.086</td>
<td>71(4)</td>
<td>72(2.4)</td>
</tr>
</tbody>
</table>

Data given as mean(standard error) except where marked * geometric mean(log standard error). Differences given as difference (95% confidence intervals) except where marked ** fold difference (95% confidence intervals).
Figure 4.3.1.1 Relationship between alveolar nitric oxide and bronchoalveolar lavage (BAL) eosinophil count (a) and bronchial wash eosinophil count (b) and the relationship between single flow nitric oxide concentration and BAL eosinophil count (c) and bronchial wash eosinophil count (d). Values of r and p refer to Pearson’s product moment correlation coefficient.
Figure 4.3.1.2 Alveolar nitric oxide concentration in normal controls and patients with mild to moderate asthma and refractory asthma. Values of p refer to Tukey's post hoc test.
Figure 4.3.1.3 Alveolar nitric oxide concentration before and after treatment with inhaled or oral corticosteroids. Values of $p$ refer to paired t-tests.

- Double dose inhaled steroids: $n=10$, $p=0.034$.
- Oral steroids: $n=11$, $p=0.002$.
**4.3.2 Evidence for a role of TNF-α in refractory asthma**

Abstract

**BACKGROUND**

TNF-α has been proposed as an important mediator in the genesis of refractory asthma. The development of TNF-α antagonists has made it feasible to investigate the role of TNF-α in refractory asthma.

**METHODS**

We have measured markers of TNF-α activity on peripheral blood monocytes in 10 patients with refractory asthma, 10 patients with mild to moderate asthma and 10 normal controls and have performed a pilot study of the effects of treatment with the soluble TNF-α receptor etanercept 25mg twice weekly in a placebo controlled double blind cross over study in the patients with refractory asthma.

**RESULTS**

We found a significantly increased membrane TNF-α, TNF-α receptor 1 and TNF-α converting enzyme cell surface density on peripheral blood monocytes in patients with refractory asthma compared to patients with mild to moderate asthma and normal controls. When compared to placebo, 10 weeks treatment with etanercept was associated with a 3.5 (95% C.I. 0.06, 7.0; p=0.046) doubling dose increase in methacholine PC_{20}, 0.85 point (95% C.I. 0.16, 1.54; p=0.02) increase in asthma quality of life score and a 320ml (95% C.I 8, 550; p=0.013) improvement in post bronchodilator FEV₁. There was a significant reduction in sputum supernatant histamine concentration with etanercept.
compared to placebo (-22ngml$^{-1}$ vs. 4 ngml$^{-1}$, mean difference 26ngml$^{-1}$, 95% C.I. 5, 48; $p=0.022$) but no changes in other markers of airway inflammation.

CONCLUSIONS
Patients with refractory asthma have evidence of up regulation of the TNF-α axis. Treatment with etanercept leads to improvement in airway hyperresponsiveness, asthma quality of life and FEV$_1$ at 10 weeks. The findings of this pilot study are based on a small number of patients and require confirmation in larger studies but they do provide supportive evidence of a role for TNF-α in refractory asthma.
Introduction

An important minority of patients with asthma have persistent symptoms, abnormal airway function, airway inflammation and/or exacerbations despite treatment with inhaled corticosteroid therapy (ATS 2000). Patients with refractory asthma suffer considerable morbidity and mortality and consume a disproportionate amount of the health resource burden attributed to asthma (Serra-Batllés, Plaza, Morejon, Comella, & Brugues 1998). Treatment options are limited and the American Thoracic Society has identified research into the mechanisms and treatment of refractory asthma as a priority (ATS 2000).

The airway pathology of refractory asthma differs from mild to moderate asthma in that there is a more heterogeneous pattern of inflammatory response (Wenzel, Schwartz, Langmack, Halliday, Trudeau, Gibbs, & Chu 1999), with greater involvement of neutrophils (2003b), involvement of the distal lung (Berry, Hargadon, Morgan, Shelley, Richter, Shaw, Green, Brightling, Wardlaw, & Pavord 2005) and increased airway remodelling (Busse et al. 1999). TNF-α is a pleiotropic proinflammatory cytokine expressed in increased amounts by mast cells (Bradding, Roberts, Britten, Montefort, Djukanovic, Mueller, Heusser, Howarth, & Holgate 1994) and present in increased concentration in bronchioalveolar fluid from the asthmatic airway (Broide et al. 1992). It has a number of properties that might be relevant to refractory asthma including: induction of airway hyperresponsiveness in man (Thomas, Yates, & Barnes 1995) and laboratory animals (Kips, Tavernier, & Pauwels 1992); recruitment of neutrophils (Thomas, Yates, & Barnes 1995); induction of glucocorticoid resistance (Franchimont,
Martens, Hagelstein, Louis, Chrousos, Belaiche, & Geenen 1999); and stimulation of fibroblast growth and maturation to myofibroblasts by promoting TGF-β expression (Desmouliere, Geinoz, Gabbiani, & Gabbiani 1993; Sullivan, Ferris, Pociask, & Brody 2005). Interest in the role of TNF-α in refractory asthma has been increased by the findings of increased concentration of TNF-α in bronchoalveolar lavage fluid from patients with more severe asthma (Broide, Lotz, Cuomo, Coburn, Federman, & Wasserman 1992) and by an uncontrolled study showing that treatment of patients with refractory asthma with the recombinant soluble TNF-α receptor etanercept caused a marked improvement in airway hyperresponsiveness (Babu et al. 2002).

The biological activity of TNF-α is mediated by the 26kD transmembrane precursor protein (Kriegler, Perez, DeFay, Albert, & Lu 1988) (membrane TNF-α) as well as the cleavage product, 17kD free TNF-α (Davis, Narachi, Alton, & Arakawa 1987). This cleavage is principally mediated by TNF-α converting enzyme (TACE) (Zheng, Saftig, Hartmann, & Blobel 2004) and the free TNF-α subsequently form highly active homotrimers (Smith & Baglioni 1987) which interact with two distinct TNF-α receptors on the cell surface (Brockhaus, Schoenfeld, Schlaeger, Hunziker, Lesslauer, & Loetscher 1990). The effect of TNF-α activation is reduced by cleavage of the receptor from the cell surface to become soluble TNF-α receptors (Lantz, Malik, Slevin, & Olsson 1990). Bioactivity of TNF-α is therefore likely to be reflected by increases in membrane TNF-α, TACE, free TNF-α, cell surface receptors and soluble receptors. We have used peripheral blood mononuclear cells to test the hypothesis that there is up-regulation of the TNF-α axis in refractory asthma by measuring membrane TNF-α, TNF-α receptors, and TACE
cell surface densities. We have sought further evidence of a role for TNF-α by performing a randomised, double blind, placebo controlled, cross over pilot study of the effects of treatment with etanercept on airway hyperresponsiveness and asthma quality of life in patients with refractory asthma.

Methods

SUBJECTS

Ten normal controls, ten patients with mild to moderate asthma and ten patients with refractory asthma volunteered for this study. All subjects with asthma had clinical features consistent with asthma and at least one of the following objective measures of airway hyperresponsiveness and/or variable airflow obstruction: methacholine provocative concentration required to cause a 20% fall in FEV₁ \( (PC_{20}) < 8 \text{mgml}^{-1} \); increase in FEV₁ of 15% or greater following inhalation of 200μg of albuterol; and/or peak flow amplitude as percent of mean over 14 days of >20%.

Patients with refractory asthma were recruited from the difficult asthma clinic at Glenfield Hospital. All had attended for at least a year and were considered to have the best control that could be achieved and to have stable treatment requirements. They all had refractory asthma diagnosed according to the ATS workshop on refractory asthma criteria (ATS 2000) with the dose of inhaled corticosteroids modified to >2000μg beclomethasone equivalent per day to reflect European practice (6). Patients had at least one major and two minor criteria for refractory asthma. Treatment concordance was assessed to be good to perfect in all patients with refractory asthma (ATS 2000; Berry,
Hargadon, Morgan, Shelley, Richter, Shaw, Green, Brightling, Wardlaw, & Pavord 2005). Our assessment was based on measurement of serum prednisolone, cortisol and theophylline concentrations, domiciliary assessment by a consultant pharmacist and by analysis of primary care records of prescription issue and collection. We excluded patients who were felt to be symptomatic because of uncontrolled co-morbid conditions such as rhinitis and gastro-esophageal reflux disease. Patients were excluded if they had a recent contact with a patient with pulmonary tuberculosis, if they had a personal history of tuberculosis, if there were any radiological features suggestive of current or previous tuberculosis or if they had a grade III or IV HEAF test.

Moderate asthma was diagnosed if no major and no more than 1 minor criterion for diagnosing refractory asthma were met (ATS 2000; Berry, Hargadon, Morgan, Shelley, Richter, Shaw, Green, Brightling, Wardlaw, & Pavord 2005) and patients were taking only as required short acting β2-agonists and less than 2000µg of beclomethasone equivalent per day. Mild asthma was classified as those patients taking β2-agonist only with no minor or major criteria for refractory asthma. All patients classified as having mild to moderate asthma met the GINA (2000) criteria for intermittent or mild persistent asthma; all patients with refractory asthma met the GINA classification for severe persistent asthma.

Normal control subjects had no respiratory symptoms, normal spirometry values and a methacholine PC20 >16mg/ml. All controls and subjects with asthma were non smokers with a smoking history of less than 5 pack years and none reported a lower respiratory
tract infections in the 3 months prior to the study. All subject provided written informed consent to participate in the study. The protocol was approved by the Leicestershire and Rutland regional ethics committee.

MEASUREMENTS

Peripheral blood mononuclear cells were separated from 20ml of peripheral blood treated with 500units of heparin using a density gradient method (Histopaque 1077, Sigma, UK). Cells were counted and suspended in phosphate buffered saline with 0.5% bovine serum albumin at a concentration of 5x10^6 cells per millilitre. 0.5x10^6 cells were transferred to 5ml tubes and non-specific binding was blocked by adding 10% foetal calf serum (FCS) for 15 minutes. Fluorochrome labelled antibodies (CD14-PE, TNFrl FITC, TNFr2 FITC, TACE FITC and membrane TNF-FITC; R&D, UK) or an equal concentration of isotype matched control (Mouse IgG1-PE, Mouse IgG2A FITC and Mouse IgG1-FITC; R&D, UK) were then added to each sample in the presence of 10% FCS and incubated for 30 minutes on ice. Fluorescence was measured using a laser flow cytometer (FACScan, Becton Dickinson, USA). The geometric mean fluorescence for membrane TNF-α, TNFrl and TACE were calculated for CD14 positive cells using Cellquest (Becton Dickinson, USA).

Single flow NO was recorded at 50mlsec^{-1} as previously described (Kharitonov, Alving, & Barnes 1997) and alveolar nitric oxide concentration was derived from measurements at 10, 30, 50, 100 and 200mlsec^{-1}. FEV₁, forced vital capacity (FVC) and forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) were measured using a rolling
seal spirometer (Vitalograph, UK). The methacholine concentration causing a 20% fall in 
FEV₁ (PC₂₀) was measured, if the FEV₁ was greater than 1 litre, using the tidal breathing 
method with a maximum inhaled concentration of 16 mg/ml as previously described 
(Juniper, Cockcroft, & Hargrave 1994). Symptom visual analogue score was measured 
using three 100mm scales representing cough, wheeze and breathlessness (Green, 
Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & Pavord 2002a). Asthma 
quality of life was measured using the Juniper asthma quality of life score (Juniper & et 
al 1993). Sputum induction was performed and samples processed as previously 
described (Green, Brightling, McKenna, Hargadon, Parker, Bradding, Wardlaw, & 
Pavord 2002a). Sputum IL-8, Cysteiny1-leukotriene (LTC₄, D₄ & E₄) and Histamine were 
measured using standard ELISA kits (BD Pharmagen, Immunotech and Cayman 
chemicals respectively) and eosinophilic cationic protein (ECP) was measured using a 
fluorescence immunoassay (UniCAP test, Pharmacia, Uppsala, Sweden). These assays 
have been previously validated for use in sputum supernatants (Birring, Parker, 
Brightling, Bradding, Wardlaw, & Pavord 2004). The sensitivity levels of the assay were 
2, 13x10⁻³, 0.8x10⁻³, 50x10⁻³ ng/ml for ECP, cysteinyl leukotrienes, IL-8 and histamine. 
The intra-assay coefficient of variability was 5-10% and the interassay coefficient of 
variability 3-15% across a range of concentrations of mediators measured.

PROTOCOL

All assessments were performed at the same time of day, at least six hours after the last 
dose of short acting β₂-agonist, 24 hours after long acting β₂-agonist and theophylline 
and 72 hours after ipratropium. Other treatments were continued uninterrupted.
Demographic details were collected and FEV₁ before and 20 minutes after 200μg of inhaled albuterol was assessed 48 hours before subjects attended for the following assessments in the following order: exhaled nitric oxide measurements; 20ml blood drawn from the anticubital fossa; measurement of methacholine PC₂₀; sputum induction and symptom score assessment. Patients with refractory asthma then entered a randomised, placebo controlled, double blind cross over study comparing the effect of etanercept and placebo.

Placebo (1ml of 0.9% saline) or etanercept (25mg in 1ml) was administered subcutaneously twice weekly. Injections were identical in appearance; they were prepared by an unblinded investigator who had no other role in the study and were administered in the clinic by a blinded investigator. The order of treatment was determined using a randomisation sequence prepared from a random number generator. The code was stored in a sealed envelope in a locked refrigerator. Preparation and storage of the study drug was overseen by the Glenfield Hospital Pharmacy, who also held a copy of the code in case of emergency. The dose was chosen as this regime was used in an earlier uncontrolled study (Babu, Arshad, Bell, Mccloughlin, Howarth, Chauhan, & Holgate 2002). Treatment phases lasted 10 weeks and were separated by a four week wash out phase. The wash out phase was chosen because the half life of etanercept is 70 hours and clinical experience in rheumatoid arthritis suggested that symptoms return within one month (information on manufacturer’s datasheet). All other asthma medications were kept constant during the period of the study.
Flow independent exhaled nitric oxide parameters, methacholine PC$_{20}$, induced sputum, symptom visual analogue scores and asthma quality of life were measured at baseline and after 5 and 10 weeks of each treatment, 3 days after the previous injection. Spirometry, bronchodilator reversibility and single flow nitric oxide concentration were measured weekly; the post bronchodilator FEV$_1$, FVC and FEF$_{25-75}$ obtained two days before baseline, week 5 and week 10 visits were taken as the baseline, week 5 and week 10 values. The protocol with timings of measurements is summarised in figure 4.3.2.1 (page 135).

ANALYSIS
Membrane TNF-α, TACE and TNF-α receptor 1 and TNF-α receptor 2 fluorescence were calculated as fold increase in fluorescence above isotype matched control (figure 4.3.2.2; page 136). Between groups comparisons were made using one way analysis of variance (ANOVA) with Tukey’s post hoc test for the three individual group differences.

Primary outcome measures for the etanercept trial were difference in doubling dose change in methacholine PC$_{20}$ between placebo and etanercept at 10 weeks and difference in change in asthma quality of life score between placebo and etanercept at 10 weeks.

Between and within group differences conformed to normality as assessed by the Kolmogorov-Smirnov test; they were compared using paired t-tests. Analysis was on intention to treat basis: patients who withdrew during a treatment phase for asthma related reasons were assigned a value equal to the worst net outcome for that treatment phase; patients who withdrew for non asthma reasons were assigned the last recorded
spirometric values and for other measures a value equal to their 5 week assessment if they had completed 5 weeks treatment or no change if they had not. Patients who withdrew during the washout phase were assigned baseline data from the previous treatment phase and no net change for the second treatment phase. We also performed a post hoc analysis of the net change in the population who completed both phases of the study (per protocol population). An earlier uncontrolled study estimated a treatment effect on methacholine airway responsiveness of 4 doubling doses (Babu, Arshad, Bell, Mccloughlin, Howarth, Chauhan, & Holgate 2002). Our study had a 90% power to detect a 2 doubling dose change in methacholine PC$_{20}$ assuming a within subject standard deviation of 1 doubling dose (Juniper, Cockcroft, & Hargreave 1994).

Secondary outcome measures were net change in post-bronchodilator FEV$_1$, forced expiratory flow between 25 and 75% of FVC (FEF$_{25-75}$), FVC, symptom visual analogue score, exhaled nitric oxide concentration, alveolar nitric oxide concentration, sputum eosinophil count, sputum neutrophil count sputum supernatant IL-8, eosinophilic cationic protein (ECP), histamine and cysteiny1 leukotriene concentrations and sputum total cell count.

Methacholine PC$_{20}$ was calculated by linear interpolation of the change in FEV$_1$/concentration of methacholine as the inhaled concentration of methacholine causing a 20% reduction in FEV$_1$. If the FEV$_1$ did not fall by >20% following challenge with 16mg/ml a value of 32mg/ml (twice the highest measurable dose) was assigned and a fall of >20% in FEV$_1$ following 0.9% saline was assigned a value of 0.015mg/ml (half
the lowest measurable dose). Change in methacholine PC\textsubscript{20} was expressed in doubling concentrations. Alveolar NO concentration was calculated for each patient using a non linear model previously described (Berry, Hargadon, Morgan, Shelley, Richter, Shaw, Green, Brightling, Wardlaw, & Pavord 2005). Multiple independent linear regression was used to explore the relationship between baseline peripheral blood monocyte membrane TNF expression and net change in methacholine PC\textsubscript{20} and Juniper asthma quality of life.

Results

PATIENT CHARACTERISTICS

Baseline characteristics are shown in table 4.3.2.1 (page 132). Individual data on treatment, criteria for diagnosis of refractory asthma and randomisation sequence is presented in table 4.3.2.2 (page 133). Patients with refractory asthma had significantly lower post bronchodilator FEV\textsubscript{1} and FEV\textsubscript{1}/FVC compared to normal controls and patients with mild to moderate asthma. There was a trend towards increased sputum neutrophil count in refractory asthma (p=0.10). Alveolar nitric oxide was significantly higher in patients with refractory asthma than in those with mild to moderate asthma and normal controls (Table 4.3.2.1; page 132).

TNF EXPRESSION ON MONOCYTES

Mean (SEM) fold increase in fluorescence from control of membrane TNF-\alpha was 8.9(0.9) in refractory asthma, 3.8(0.7) in normal controls (mean difference 5.1, 95% C.I. 2.5, 7.7; p<0.001; figure 4.3.2.2; page 136) and 3.3(0.4) in mild to moderate asthma (mean difference 5.5, 95% C.I. 2.7, 8.4; p<0.001). There were also significant between group
differences, with significantly higher values in refractory asthma compared to the other
groups, in expression of TNF-α receptor 1 (p<0.001) and TNF-α converting enzyme
(p<0.001) but not TNF-α receptor 2 expression (p=0.48, table 4.3.2.1; page 132).

RANDOMISED CONTROLLED TRIAL

One patient withdrew for personal reasons during the washout phase after etanercept
treatment due to a change in employment and one patient withdrew from week 4 of the
second treatment phase (etanercept) due the death of a relative and the development of a
cough productive of sputum associated with repeated isolation of *Haemophilus influenzae*
developed a course of oral antibiotics. Data from this case was analysed as an asthma related
withdrawal. There was no other missing data. Otherwise treatment was well tolerated
with no significant injection site reactions. Treatment period or order did not influence
values before treatment or the change in primary outcome measures (Table 4.3.2.3; page
134 and Figure 4.3.2.3; page 137).

PRIMARY OUTCOMES

There was a significant reduction in fold difference in fluorescence of peripheral blood
monocyte cells for membrane TNF-α over isotype control of -6.9 (2.6) following
treatment with etanercept compared to -0.1 (0.6) following placebo (mean difference 6.8,
95% C.I. 0.5, 13.1; p=0.037). Etanercept treatment was associated with a progressive
improvement in methacholine PC_{20} which was 2.3 doubling doses of methacholine at 10
weeks compared to -1.2 doubling doses after 10 weeks treatment with placebo (mean
difference 3.5 doubling doses, 95% C.I. 0.07, 7.0; p=0.046; figure 4.3.2.3; page 137). The
Juniper asthma quality of life improved by 0.85 points with etanercept compared to -0.02 points with placebo (mean difference 0.86, 95% C.I. 0.16, 1.54; p = 0.02; figure 4.3.2.3; page 137). Both net change in PC_{20} and asthma quality of life with etanercept treatment compared to placebo were independently associated with baseline peripheral blood monocyte membrane TNF-α expression (Adjusted R squared 0.73, p = 0.004) and change in membrane TNF expression from baseline (Adjusted R squared 0.56, p = 0.023). The net change in PC_{20} and asthma quality of life remained significant when analysis was restricted to the per protocol population (p = 0.035 and 0.04 respectively).

SECONDARY OUTCOMES

Post bronchodilator FEV\textsubscript{1}, FVC and FEF\textsubscript{25-75}, total symptom scores and quality of life scores, exhaled and alveolar nitric oxide concentration, sputum eosinophil, neutrophil and total cell counts and sputum supernatant histamine concentrations before and after treatment with etanercept and placebo are shown in table 4.3.2.3 (page 134). The change in FEV\textsubscript{1} over the 10 weeks of etanercept and placebo treatment is given in figure 4.3.2.3 (page 137) Individual symptoms scores, quality of life domains scores and sputum IL-8, ECP and cysteinyl leukotriene concentrations are shown in the table 4.3.2.4 (page 138). There was a significant improvement in post bronchodilator FEV\textsubscript{1} after treatment with etanercept which became significant compared to placebo by week 6 and was 320ml (95% C.I. 80ml, 550ml p = 0.013) by week 10 (table 4.3.2.3; page 134 and figure 4.3.2.3; page 137). Total symptom visual analogue score at 10 weeks improved by 49mm after treatment with etanercept compared to 9mm with placebo (mean difference 40mm, 95% C.I. 7, 73; p = 0.012). There were no significant differences in single flow nitric oxide concentration, alveolar nitric oxide concentration, sputum total or differential cell counts.
or sputum ECP, IL-8 or cysteinyl-leukotriene concentration (table 4.3.2.4; page 138).
Sputum histamine concentration reduced from 36.1 (12) to 14.0 ng ml\(^{-1}\) (11.7) with etanercept and increased from 37.0 (11.9) to 41.3 ng ml\(^{-1}\) (11.5) with placebo (mean difference in change in histamine concentration 26 ng ml\(^{-1}\), 95% C.I. 5, 48; p=0.022).

Discussion

We have demonstrated that patients with refractory asthma have up regulation of the TNF-\(\alpha\) axis as evidenced increased membrane bound TNF-\(\alpha\), TNF receptor 1 and TACE cell surface density on peripheral blood monocytes. Antagonism of TNF-\(\alpha\) with 10 weeks of etanercept led to a significant reduction in monocyte membrane TNF-\(\alpha\) expression and improvement in methacholine PC\(_{20}\) asthma quality of life, FEV\(_1\) and symptom scores compared to placebo. Baseline peripheral blood monocyte membrane TNF-\(\alpha\) and the extent to which it was reduced by etanercept treatment were both independently associated with the net improvement in both primary outcome measures.

We chose to assess TNF-\(\alpha\) activity by measuring membrane bound TNF-\(\alpha\), cell surface receptor and TACE expression on peripheral blood monocytes as monocytes/macrophages are an important source of TNF-\(\alpha\) and the technique is non invasive and suitable for repeated measurements. Membrane TNF-\(\alpha\) was used to assess the response to treatment since there is evidence that it is more closely associated with biological activity (Armstrong et al. 2000) and clinical outcome in septic shock (Pellegrini et al. 1996) than other markers. Our choice of assay was influenced by a previous study reporting difficulties measuring free TNF-\(\alpha\) in sputum supernatant

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(Gamble et al. 2003) and by our desire to make assessments after etanercept treatment which meant that invasive methods could not be used and assay of free TNF-α or TNF-α receptors in sputum or serum were not applicable since these measures are likely to be affected by etanercept. The extent to which measurements made in peripheral blood monocytes relate to up regulation of the TNF-α in the airway is unclear, although our demonstration of a relationship between membrane TNF-α expression and the response to etanercept, and an earlier study showing that regulation of TNF-α production by peripheral blood monocyte and alveolar macrophages is similar in asthma (Hallsworth et al. 1994), suggests that the effect seen on peripheral blood monocytes are relevant to those in more biologically relevant sites. More work is required to determine the main source of TNF-α in the airway in refractory asthma and how this relates to markers of TNF-α activity on peripheral blood mononuclear cells.

Potential explanations for the increased TNF-α activity on peripheral blood mononuclear cells include the co-existence of asthma with other inflammatory conditions associated with increased TNF-α activity and genetic differences in the TNF-α gene or genes associated with regulation of TNF-α production. It is unlikely to reflect the effects of corticosteroid treatment since in vitro studies show that corticosteroids reduce TNF-α production by monocytes (Waage & Bakke 1988). An effect of other treatments cannot be excluded although there is no strong biological rational to support such an effect. Ours was a small study so we are not in a position to systematically investigate factors which may be involved in up regulation of TNF-α activity or between patient differences in the degree of TNF-α up regulation. Further case control studies should investigate these
possibilities. The relevance of increased cell surface density of TNF-α receptor 1 but not 2 in patients with refractory asthma is unclear, although the findings are consistent with a previous study suggesting that reduced shedding of the TNF-α receptor 1 from the cell surface is associated with airway hyperresponsiveness (Halasz et al. 2002).

The beneficial effects of antagonism of TNF-α with etanercept on markers of asthma control supports the view that TNF-α contributes to the pathogenesis of refractory asthma. This was a pilot study involving small numbers of patients and the results could have been compromised by missing data or the cross over design. Thus the findings cannot be regarded as definitive and they need to be confirmed in larger studies. However, there are a number of factors which increase confidence that the effects seen are real and important. Firstly, the beneficial effects were evident in both intention to treat and per protocol analyses and the former were significant despite conservative handling of missing data. Secondly, the effects of etanercept were evident across multiple outcome measures and most were significant both within and between treatments. Finally, the findings of our pilot study were similar to those of an earlier uncontrolled study, with a particularly large effect on markers of airway function (Babu, Arshad, Bell, Mccloughlin, Howarth, Chauhan, & Holgate 2002). Our study adds to this earlier work by showing that the improvement in airway function seen with etanercept treatment was associated with a significant improvement in asthma quality of life and reduction in symptoms. Exhaled nitric oxide, alveolar nitric oxide and sputum markers of neutrophilic or eosinophilic airway inflammation were not altered by etanercept treatment, suggesting that the beneficial effect of TNF-α blockade is primarily mediated via improvement in
airway smooth muscle function. There is evidence that TNF-α increases human airway smooth muscle responsiveness in vitro by a direct effect on TNF-α receptor 1 (Amrani, Chen, & Panettieri, Jr., 2000) and inhaled TNF-α increases airway hyperresponsiveness in normal controls (Thomas, Yates, & Barnes, 1995) and patients with asthma (Thomas & Heywood, 2002). The local release of mediators from mast cells present in the airway smooth muscle has recently been suggested to be important in the pathogenesis of airway hyperresponsiveness and bronchoconstriction in asthma (Brightling, Bradding, Symon, Holgate, Wardlaw, & Pavord, 2002a). TNF-α is a product of airway mast cells (Bradding, Roberts, Britten, Montefort, Djukanovic, Mueller, Heusser, Howarth, & Holgate, 1994), induces histamine release from human mast cells directly (van Overveld, Jorens, Rampart, de Backer, & Vermieire, 1991) and participates in a positive autocrine loop that potentiates human mast cell cytokine secretion (Coward, Okayama, Sagara, Wilson, Holgate, & Church, 2002); it is possible that inhibition of these effects are important the beneficial effect of etanercept treatment on airway function. This concept is supported by the reduction in sputum histamine concentration seen with etanercept treatment in our study.

Our study suggests that the beneficial effects of etanercept may be confined to patients with refractory asthma since increased markers of TNF-α activity on peripheral blood monocytes were not seen in patients with mild to moderate asthma. For this reason, and because treatment with an expensive and potentially toxic drug could not be justified clinically, we did not study the effects of etanercept in patients with mild to moderate asthma. The view that systemic dysregulation of the TNF-α axis is peculiar to refractory
asthma is supported by a study showing that the TNF-α production by peripheral blood mononuclear cells in response to lipopolysaccharide and other stimuli is increased in patients with severe asthma but not in those whose asthma was controlled on low dose inhaled corticosteroids (Waserman et al. 2000). In addition, there is preliminary evidence that two weeks treatment with etanercept has no effect on airway responsiveness or the bronchoconstrictor and inflammatory response to endobronchial allergen challenge in subjects with mild atopic asthma (Rouhani et al. 2005). The findings of this study are limited by the short duration of treatment and small sample size and further work in less severe asthma is required before we can conclude with confidence that the beneficial effects of etanercept are limited to refractory asthma.

Our motive for studying the effects of etanercept was to investigate the hypothesis that TNF-α contributes to the pathophysiology of refractory asthma. We did not set out to conduct a clinical trial which could be used to direct treatment. We chose methacholine airway responsiveness as a primary outcome since there is evidence particularly implicating TNF-α in the pathogenesis of airway hyperresponsiveness (Amrani, Chen, & Panettieri, Jr. 2000; Kips, Tavernier, & Pauwels 1992; Thomas & Heywood 2002; Thomas, Yates, & Barnes 1995) and because earlier experience with etanercept treatment in refractory asthma suggested a large effect on this measure (Babu, Arshad, Bell, Mccloughlin, Howarth, Chauhan, & Holgate 2002). Our study was powered to detect a smaller change in methacholine responsiveness to that seen in this earlier uncontrolled study. The study design and sample size were not appropriate to show an effect of etanercept treatment on other clinically relevant asthma outcomes such as exacerbation...
frequency, to investigate between patient differences in treatment response or to provide meaningful data on the adverse effects of treatment. The significance of the persistent isolation of *H. Influenzae* in one of our patients is uncertain but it does raise the possibility of increased risk of infection or impaired clearance of bacteria with etanercept treatment. The findings of our study provide a strong basis for the larger and longer studies that are needed to investigate the effect of etanercept on long term outcomes in refractory asthma, and to evaluate whether treatment is associated with any significant adverse effects.
Table 4.3.2.1 Baseline clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (N= 10)</th>
<th>Patients with Mild-to-Moderate Asthma (N=10)</th>
<th>Patients with Refractory Asthma (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (no. of subjects)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>38</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>Range</td>
<td>23–49</td>
<td>18–72</td>
<td>25–59</td>
</tr>
<tr>
<td>Age at onset of symptoms (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>—</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Range</td>
<td>—</td>
<td>1–36</td>
<td>1–58</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liters</td>
<td>3.8±0.4</td>
<td>3.7±0.9</td>
<td>2.4±0.7†</td>
</tr>
<tr>
<td>% of predicted value</td>
<td>97±18</td>
<td>94±23</td>
<td>62±21†</td>
</tr>
<tr>
<td>FEV₁/FVC ratio (%)</td>
<td>80±25</td>
<td>78±21</td>
<td>65±17†</td>
</tr>
<tr>
<td>Nitric oxide — ppb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exhaled</td>
<td>16.5±0.2</td>
<td>34.2±0.3</td>
<td>41.8±0.2†</td>
</tr>
<tr>
<td>Alveolar</td>
<td>3.4±1.2</td>
<td>3.4±1.6</td>
<td>10.2±15†</td>
</tr>
<tr>
<td>Sputum measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.3±0.2</td>
<td>4.1±0.7</td>
<td>5.6±0.8†</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>57±25</td>
<td>55±26</td>
<td>62±27</td>
</tr>
<tr>
<td>Total cells (×10⁷/mg)</td>
<td>0.6±0.2</td>
<td>0.9±0.4</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Plasma IgE (IU/ml)</td>
<td>16±0.6</td>
<td>172±1.1</td>
<td>77±0.9</td>
</tr>
<tr>
<td>PC₂₀ (mg of methacholine/ml)</td>
<td>&gt;16</td>
<td>0.4±0.6</td>
<td>0.14±0.1</td>
</tr>
<tr>
<td>Continuous oral corticosteroids (no. of subjects)</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>TNF-α receptor 1 — ratio</td>
<td>2.3±0.3</td>
<td>3.1±0.2</td>
<td>5.5±0.6¶</td>
</tr>
<tr>
<td>TNF-α-converting enzyme — ratio</td>
<td>2.8±0.4</td>
<td>3.5±0.5</td>
<td>6.5±0.5¶</td>
</tr>
<tr>
<td>TNF-α receptor 2 — ratio</td>
<td>3.9±0.3</td>
<td>3.9±0.4</td>
<td>3.5±0.2</td>
</tr>
</tbody>
</table>

* Unless otherwise noted, plus–minus values are means ±SD. FEV₁ denotes post-bronchodilator forced expiratory volume in one second, FVC post-bronchodilator forced vital capacity, and PC₂₀ concentration of inhaled methacholine required to induce a 20 percent decrease in FEV₁.
† P<0.05 for the comparison among the groups by analysis of variance.
‡ Plus–minus values are geometric means ±log SD.
§ Values are the ratio of fluorescence as compared with that for the isotype-matched control.
¶ P<0.001 for the comparison among the groups by analysis of variance.
Table 4.3.2.2 Baseline characteristics of patients with refractory asthma.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Age at Onset</th>
<th>Atopy</th>
<th>Plasma IgE</th>
<th>Inhaled Fluticasone Dose</th>
<th>Systemic Prednisolone Dose</th>
<th>Long-Acting β₂-Agonist</th>
<th>Other Treatment</th>
<th>ATS Criteria*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1†</td>
<td>58/M</td>
<td>58</td>
<td>No</td>
<td>19</td>
<td>1000</td>
<td>20</td>
<td>Salmeterol</td>
<td></td>
<td>1, 2, 3, 4, 6</td>
</tr>
<tr>
<td>2‡</td>
<td>25/F</td>
<td>6</td>
<td>Yes</td>
<td>99</td>
<td>2000</td>
<td>—</td>
<td>Salmeterol</td>
<td>Aminophylline, 200 mg twice daily</td>
<td>2, 3, 4, 5, 6, 7, 8</td>
</tr>
<tr>
<td>3†</td>
<td>57/F</td>
<td>27</td>
<td>No</td>
<td>32</td>
<td>500</td>
<td>10</td>
<td>Salmeterol</td>
<td>Montelukast, 10 mg/day orally</td>
<td>1, 3, 4, 5, 6, 7, 9</td>
</tr>
<tr>
<td>4‡</td>
<td>40/F</td>
<td>12</td>
<td>No</td>
<td>75</td>
<td>1000</td>
<td>30</td>
<td>Formoterol</td>
<td>Ipratropium</td>
<td>1, 2, 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td>5†</td>
<td>30/F</td>
<td>2</td>
<td>Yes</td>
<td>4298</td>
<td>2000</td>
<td>—</td>
<td>Salmeterol</td>
<td></td>
<td>1, 2, 3, 4, 5, 6, 7, 9</td>
</tr>
<tr>
<td>6‡</td>
<td>59/F</td>
<td>19</td>
<td>Yes</td>
<td>11</td>
<td>2000</td>
<td>—</td>
<td>Salmeterol</td>
<td>Aminophylline, 300 mg twice daily</td>
<td>2, 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td>7‡</td>
<td>33/M</td>
<td>2</td>
<td>Yes</td>
<td>2066</td>
<td>2000</td>
<td>15</td>
<td>Salmeterol</td>
<td>Montelukast, 10 mg/day orally</td>
<td>1, 2, 3, 4, 5, 6, 7, 9</td>
</tr>
<tr>
<td>8‡</td>
<td>49/M</td>
<td>2</td>
<td>Yes</td>
<td>131</td>
<td>2000</td>
<td>—</td>
<td>Salmeterol</td>
<td>Montelukast, 10 mg/day orally</td>
<td>2, 3, 5, 6, 7</td>
</tr>
<tr>
<td>9‡</td>
<td>47/F</td>
<td>29</td>
<td>Yes</td>
<td>18</td>
<td>1000</td>
<td>20</td>
<td>Salmeterol</td>
<td>Aminophylline, 375 mg twice daily</td>
<td>1, 2, 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td>10‡</td>
<td>50/M</td>
<td>1</td>
<td>No</td>
<td>7</td>
<td>500</td>
<td>7.5</td>
<td>Salmeterol</td>
<td></td>
<td>1, 3, 4, 5, 6, 8</td>
</tr>
</tbody>
</table>

* The American Thoracic Society (ATS) criteria for refractory asthma are as follows. One or both of the following major criteria: 1, continuous treatment (more than 50 percent of the year) with oral corticosteroids; 2, required treatment with high-dose inhaled corticosteroids; and two or more of the following minor criteria: 3, the need for daily “reliever” medication; 4, the presence of symptoms requiring daily treatment; 5, persistent airflow obstruction (defined by a forced expiratory volume in one second [FEV₁] value that is less than 80 percent of the predicted value); 6, a history of one or more urgent care visits for asthma; 7, the need for three or more bursts of oral corticosteroids per year; 8, prompt deterioration in clinical condition after a reduction in the corticosteroid dose by less than 25 percent; and 9, a history of near-fatal asthma.

† This patient received etanercept first in the crossover trial.
‡ This patient received placebo first in the crossover trial.
§ This patient received 80 mg of triamcinolone per month.
Table 4.3.2.3 Measurements before, during and after 10 weeks treatment with placebo and etanercept.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wk 0</th>
<th>Wk 5</th>
<th>Wk 10</th>
<th>Within-Group Difference (95% CI)</th>
<th>Wk 0</th>
<th>Wk 5</th>
<th>Wk 10</th>
<th>Within-Group Difference (95% CI)</th>
<th>Between-Group Difference (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence for membrane-bound TNF-a ratio†</td>
<td>8.3±5.1</td>
<td>—</td>
<td>8.2±5.4</td>
<td>-0.1 (-1.3 to 1.1)</td>
<td>11.5±7.2</td>
<td>—</td>
<td>4.6±2.2</td>
<td>-6.9 (-12.8 to -1.0)‡</td>
<td>-6.8 (-13 to -0.5)‡</td>
</tr>
<tr>
<td>Asthma quality-of-life score§</td>
<td>4.2±1.0</td>
<td>4.3±1.0</td>
<td>4.1±1.2</td>
<td>-0.02 (-0.6 to 0.6)</td>
<td>4.1±0.9</td>
<td>4.6±1.0</td>
<td>4.9±1.1</td>
<td>0.84 (0.3 to 1.4)§</td>
<td>0.85 (0.2 to 1.5)§</td>
</tr>
<tr>
<td>PC_{20} (mg of methacholine/ml)**</td>
<td>0.17±0.3</td>
<td>0.08±0.3</td>
<td>0.07±0.2</td>
<td>-1.2 (-1.9 to 0.5)</td>
<td>0.14±0.1</td>
<td>0.21±0.3</td>
<td>0.44±0.4</td>
<td>2.3 (0.6 to 4.0)§§</td>
<td>3.5 (0.07 to 7.0)§§</td>
</tr>
<tr>
<td>FEV_{1} (liters)</td>
<td>2.36±0.9</td>
<td>2.27±0.9</td>
<td>2.22±0.8</td>
<td>-0.14 (-0.3 to 0.03)</td>
<td>2.30±0.8</td>
<td>2.37±0.7</td>
<td>2.48±0.6</td>
<td>0.18 (-0.04 to 0.4)</td>
<td>0.32 (0.08 to 0.55)</td>
</tr>
<tr>
<td>FVC (liters)</td>
<td>3.12±0.9</td>
<td>2.9±0.8</td>
<td>3.12±0.9</td>
<td>0.01 (-0.12 to 0.11)</td>
<td>2.94±1.0</td>
<td>3.08±1.0</td>
<td>3.23±1.1</td>
<td>0.29 (0.05 to 0.52)</td>
<td>0.29 (0.01 to 0.57)§</td>
</tr>
<tr>
<td>FEV_{15-75} (liters/sec)**</td>
<td>1.87±0.7</td>
<td>1.88±0.7</td>
<td>1.87±0.8</td>
<td>0.01 (-0.1 to 0.1)</td>
<td>1.98±0.8</td>
<td>2.08±0.8</td>
<td>2.24±0.8</td>
<td>0.26 (0.02 to 0.5)§</td>
<td>0.27 (-0.1 to 0.6)</td>
</tr>
<tr>
<td>Symptom score (mm)††</td>
<td>146±79</td>
<td>136±72</td>
<td>137±70</td>
<td>-9 (-51 to 34)</td>
<td>125±50</td>
<td>92±44</td>
<td>77±54</td>
<td>-48 (-90 to -7)‡</td>
<td>-39 (-71 to -7)‡</td>
</tr>
<tr>
<td>Exhaled nitric oxide concentration (ppb)**</td>
<td>40.9±0.5</td>
<td>43.3±0.4</td>
<td>46.2±0.4</td>
<td>1.1 (0.8 to 1.6)</td>
<td>44±0.5</td>
<td>32.5±0.4</td>
<td>37.2±0.4</td>
<td>0.84 (0.5 to 1.4)</td>
<td>0.75 (0.5 to 1.0)</td>
</tr>
<tr>
<td>Sputum measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)**</td>
<td>4.3±0.8</td>
<td>2.5±0.9</td>
<td>4.1±0.8</td>
<td>0.95 (0.3 to 3.0)</td>
<td>5.9±0.7</td>
<td>3.9±0.7</td>
<td>4.8±0.9</td>
<td>0.80 (0.4 to 3.64)</td>
<td>0.85 (0.3 to 1.3)</td>
</tr>
<tr>
<td>Neutrophils (%)§</td>
<td>65.4±22.6</td>
<td>68.7±25.5</td>
<td>64.7±31.8</td>
<td>-0.7 (-30 to 28)</td>
<td>55.8±27.2</td>
<td>61.6±23.2</td>
<td>59.8±34.8</td>
<td>3.9 (-5.7 to 13.5)</td>
<td>4.6 (-28 to 38)</td>
</tr>
<tr>
<td>Total cells (&gt; 30^+*/mg)**</td>
<td>1.7±0.1</td>
<td>2.6±0.1</td>
<td>2.4±0.1</td>
<td>1.4 (0.8 to 2.5)</td>
<td>1.2±0.1</td>
<td>1.5±0.1</td>
<td>2.1±0.1</td>
<td>1.7 (1.1 to 2.7)§§</td>
<td>1.2 (0.4 to 2.0)</td>
</tr>
<tr>
<td>Histamine (ng/ml)§</td>
<td>37.0±11.9</td>
<td>—</td>
<td>41.3±11.5</td>
<td>4.3 (-3.9 to 12.5)</td>
<td>36.1±12.0</td>
<td>—</td>
<td>14.0±11.7</td>
<td>-22 (-41.2 to -2.7)§§</td>
<td>-26 (-5 to -48)§§</td>
</tr>
</tbody>
</table>

* Unless otherwise indicated, plus-minus values are means ±SD. CI denotes confidence interval. FEV_{1} post-bronchodilator forced expiratory volume in one second, FVC post-bronchodilator forced vital capacity, FEV_{15-75} the post-bronchodilator forced expiratory flow between 25 percent and 75 percent of FVC, and PC_{20} the concentration of inhaled methacholine required to induce a 20 percent decrease in FEV_{1}.

† Values are the ratio of fluorescence as compared with that for the isotype-matched control.

§ Scores can range from 1 (totally limited) to 7 (not at all limited).

¶ P<0.05.

‖ P<0.01.

** Plus-minus values are geometric means ±log SD. Differences in the PC_{20} are expressed as doubling concentrations. Otherwise, differences are expressed as a ratio.

†† The sum of three 100-mm visual-analogue scales was used; higher scores indicate more severe symptoms.

+++ P=0.01.
Figure 4.3.2.1 Study plan.

Phase 1
Washout
Phase 2

Study week

Measurements of exhaled and alveolar nitric oxide, PC_{20}, cell counts in induced sputum sample, symptom scores, and quality of life at wk 0, 5, 10, 14, 19, 24.
Measurement of membrane-bound TNF-α and sputum supernatants at wk 0, 10, 14, 24.

Placebo or 25 mg of etanercept twice weekly
Crossover
Placebo or 25 mg of etanercept twice weekly

Treatment periods lasted 10 weeks and were separated by a 4-week washout period. The washout phase was chosen because the half-life of etanercept is 70 hours and, according to information provided by the manufacturer, clinical experience in patients with rheumatoid arthritis suggested that symptoms return within one month after treatment is stopped. Assessments were made at the same time of day at the times and in the order listed. Short-acting β2-agonist treatment was withheld for more than 6 hours and treatment with long-acting β2-agonists and ipratropium bromide withheld for more than 12 hours before each visit. PC_{20} denotes the concentration of inhaled methacholine required to induce a 20 percent decrease in the forced expiratory volume in one second (FEV_1), FVC forced vital capacity, and FEF_{25-75} forced expiratory flow between 25 and 75 percent of FVC.
Figure 4.3.2.2 Representative Flow-Cytometric Plots (Panels A and B), Representative Histograms from a Patient with Mild Asthma (Panel C) and a Patient with Refractory Asthma (Panel D), and the Ratios of Membrane-Bound TNF-α in the Three Groups (Panel E).

Panel A shows a representative flow-cytometric plot with forward and side scatter; monocytes are identified on the basis of their typical pattern of size and granularity (R1). Panel B demonstrates further selection of monocytes (shown as R2) on the basis of gating on the cells staining for CD14. Panel C shows a representative histogram from a patient with mild asthma, and Panel D a representative histogram from a patient with refractory asthma. The ratio of fluorescence for membrane TNF-α as compared with that for an isotype-matched control is calculated by dividing the geometric mean fluorescence for membrane TNF-α (FL1) by that of the isotype-matched control. Panel E shows the ratio of fluorescence for membrane TNF-α as compared with that for the isotype-matched control in subjects from each group. Open symbols in Panel E represent patients who received etanercept first in the crossover trial, and closed symbols those who received placebo first. The characteristics of the individual patients are described in Table 2. Diamonds with I bars represent means ±SE.
Figure 4.3.2.3 Concentration of inhaled methacholine causing a 20% decrease PC\textsubscript{20} in forced expiratory volume in one second (FEV\textsubscript{1}) (Panel A) and asthma quality of life (Panel B) before during and after 10 weeks of Etanercept or placebo and the cumulative mean (+SE) change in FEV\textsubscript{1} after the inhalation of 200μg of salbutamol each week during the 10 week treatment trial (Panel C).

Open symbols in Panels A and B represent patients who received etanercept first in the crossover trial, and closed symbols those who received placebo first. In Panel B, scores can range from 1 (totally limited) to 7 (not at all limited). The open diamonds in the placebo group and the closed hexagon in the etanercept group at weeks 5 and 10 are assigned values.
Table 4.3.2.4 Additional subject characteristics before, during and after 10 weeks treatment with etanercept and placebo.

<table>
<thead>
<tr>
<th>Week</th>
<th>Placebo</th>
<th>Etanercept</th>
<th>Within treatment difference (95% C.I.)</th>
<th>Between treatment difference (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>10</td>
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<td>-16 (-32, 1)</td>
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<td>5</td>
<td>10</td>
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<td>-5 (-28, 17)</td>
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<tr>
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<td>5</td>
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<td>-3 (-17, 12)</td>
<td>-9 (-21, 4)</td>
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<tr>
<td>0</td>
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<td>5</td>
<td>10</td>
<td>4 (-15, 23)</td>
<td>24 (-41, -7)*</td>
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</table>

### Visual analogue scores

- **Cough**
  - Placebo: 54 (25)
  - Etanercept: 49 (24)
  - Within treatment: -10 (-24, 4)
  - Between treatment: -16 (-32, 1)

- **Wheeze**
  - Placebo: 39 (37)
  - Etanercept: 38 (31)
  - Within treatment: -3 (-17, 12)
  - Between treatment: -9 (-21, 4)

- **Breathlessness**
  - Placebo: 53 (29)
  - Etanercept: 49 (26)
  - Within treatment: 4 (-15, 23)
  - Between treatment: -24 (-41, -7)*

### Juniper Asthma Quality of Life Score

- **Symptoms**
  - Placebo: 3.8 (1.4)
  - Etanercept: 4.1 (1.3)
  - Within treatment: 0.01 (-0.30, 0.31)
  - Between treatment: 1.13 (0.50, 1.77)**

- **Activities**
  - Placebo: 4.2 (1.2)
  - Etanercept: 4.2 (1.2)
  - Within treatment: 0.01 (-0.21, 0.21)
  - Between treatment: 0.78 (0.18, 1.38)*

- **Emotions**
  - Placebo: 4.4 (1.4)
  - Etanercept: 4.6 (1.5)
  - Within treatment: -0.2 (-0.88, 0.68)
  - Between treatment: 0.72 (0.12, 1.30)*

- **Environment**
  - Placebo: 4.5 (1.4)
  - Etanercept: 4.5 (1.4)
  - Within treatment: 0.02 (-0.07, 0.1)
  - Between treatment: 0.4 (0.02, 0.78)*

### Alveolar NO concentration (ppb)

- Placebo: 10.8 (17)
- Etanercept: 6.6 (3.8)
- Within treatment: -1.6 (-9.2, 6.1)
- Between treatment: -3.8 (-11, 3.1)

### Sputum IL-8 (ng ml\(^{-1}\))

- Placebo: 164.8 (22)
- Etanercept: 143.9 (40)
- Within treatment: -20.9 (-69, 27)
- Between treatment: 153.0 (23)
- Mean difference (95% confidence intervals): 2.5 (-2.3, 7.3)

### Sputum Cys-Lt (ng ml\(^{-1}\))

- Placebo: 9.5 (2.9)
- Etanercept: 8.6 (2.5)
- Within treatment: -0.8 (-5, 3.4)
- Between treatment: 10.2 (1.5)
- Mean difference (95% confidence intervals): 1.0 (-3.0, 4.1)

### Sputum ECP (pg ml\(^{-1}\))

- Placebo: 1.1 (0.2)
- Etanercept: 1.3 (0.2)
- Within treatment: 0.2 (-0.1, 0.4)
- Between treatment: 0.9 (0.2)
- Mean difference (95% confidence intervals): -0.3 (-0.6, 0.1)

Mean (S.D.) measurements from patients before and after placebo and etanercept treatment with within treatment and between treatment differences and 95% confidence intervals. IL-8 – interleukin 8, Cys-Lt – Cysteinyl leukotriene, ECP – eosinophilic cationic protein. Data given as mean (standard deviation) except where marked † geometric mean (log standard deviation). Between group results are given as mean difference (95% confidence intervals) except where marked † where fold difference (95% confidence intervals) are given. * denote p<0.05 and ** p<0.01.
5. Conclusions

5.1 Summary of findings in relation to original hypotheses

**Eosinophilic bronchitis is a stable disease phenotype.**

During the period of our study the majority of patients with eosinophilic bronchitis continued to have cough and/or eosinophilic airway inflammation, the development of asthma and fixed airflow obstruction were relatively unusual. The original series described by Gibson et al were found to have resolved their symptoms over a period of 10 years, so it is possible that we have not followed up our patients for long enough to conclude with certainty that eosinophilic bronchitis remains stable in the very long term. The fact that the airway hyperresponsiveness seen in asthma is thought to be associated with airway smooth muscle mast cell myositis, a feature not present in eosinophilic bronchitis, poses the question as to whether this pathological finding develops in those patients with eosinophilic bronchitis who subsequently develop asthma. Our findings relate only to patients with treated disease.

**There is an accelerated decline in FEV₁ in patients with eosinophilic bronchitis.**

There was no difference in the rate of decline in FEV₁ in our patients with eosinophilic bronchitis compared to what we would have anticipated from previous studies. Smoking, female gender and prolonged eosinophilic airway inflammation were factors associated with a more rapid decline in FEV₁. We did not have a normal control population nor a population with eosinophilic bronchitis who were not treated;
our conclusions are therefore limited to patients with eosinophilic bronchitis who were treated with inhaled corticosteroids.

**There is a difference in expression of interleukin-13 between asthma and eosinophilic bronchitis.**

Interleukin-13 expression in sputum and bronchial submucosal cells appeared to be a feature of asthma rather than eosinophilic bronchitis. These findings support the proposed role of this cytokine in the genesis of airway hyperresponsiveness. There remain some questions about the validation of our ELISA; specifically as to whether it is valid to extrapolate findings of the validation steps performed in 2 patients to a wider ranges of subject. Further work is necessary to validate this method in a broader range airways disease with differing severity.

**Non-eosinophilic asthma and eosinophilic asthma represent distinct pathological phenotypes.**

Non-eosinophilic asthma can be distinguished pathologically from eosinophilic asthma by; the absence of eosinophils in the sputum, bronchial wash, BAL and submucosa and by a normal subepithelial collagen layer thickness. Airway smooth muscle mast cells were present in both eosinophilic and non-eosinophilic asthma.
There is a difference in response to inhaled corticosteroids in eosinophilic and non-eosinophilic asthma.

There is a reduced response to inhaled mometasone in patients with non-eosinophilic compared eosinophilic asthma, with no significant improvement in asthma related quality of life or methacholine PC\textsubscript{20}. Our sample size was not large enough to determine whether there is a small but significant response to inhaled mometasone in patients with non-eosinophilic asthma.

**Alveolar nitric oxide is a practical and valid tool for measuring distal airway inflammation in patients with asthma.**

Measurement of alveolar nitric oxide concentration is feasible and repeatable in a wide range of subjects with asthma. In patients with mild asthma who were not being treated with inhaled corticosteroids, there was a close relationship with BAL eosinophil counts but not sputum or bronchial wash. These data support the use of alveolar nitric oxide as a non-invasive measure of distal airway inflammation.

**Alveolar nitric oxide concentration is elevated in patients with refractory asthma.**

There was a higher mean concentration of alveolar nitric oxide in patients with refractory asthma compared to those with mild to moderate disease and normal controls. There was a great deal of heterogeneity within the refractory asthma groups which was not associated with clinical or physiological markers of disease.
Oral but not inhaled corticosteroids reduce alveolar nitric oxide concentration.

Alveolar nitric oxide concentration was reduced following treatment with oral corticosteroids. This observation is unlikely to represent regression to the mean, as a similar group of patients were treated with higher dose inhaled corticosteroids and did not have a reduction in alveolar nitric oxide concentration. This observation was not however placebo controlled and the treatment allocation was not random, which affects our confidence in the results. Our findings are consistent with the notion that there is distal airway inflammation in refractory asthma and this may be relevant to inhaled corticosteroid resistance, since these drugs are not delivered to this site.

There is increased activity of the TNF-α axis in patients with refractory asthma.

Patients with refractory asthma have increased expression of membrane TNF-α, TNF-α receptor 1 and TNF-α converting enzyme on the cell surface of peripheral blood monocytes compared to patients with mild to moderate asthma and normal controls. How the findings in peripheral blood cells relate to upregulation within the lung of patients with refractory asthma is unknown, as is the relationship between the markers we have measured and soluble TNF-α and receptors. Whether our observations reflect increase TNF-α production, altered cell surface shedding or reduced breakdown of TNF-α merits further investigation.
Treatment of patients with refractory asthma with the soluble TNF-α receptor etanercept leads to an improvement in asthma quality of life and airway hyperresponsiveness.

When compared to placebo, etanercept treatment lead to a significant improvement in asthma related quality of life and improved methacholine responsiveness in patients with refractory asthma. Baseline membrane TNF-α was associated with clinical response. This small, pilot study confirms TNF-α as a potential therapeutic target in refractory asthma and larger, longer trials are merited to determine its effect on more clinically relevant outcomes and its safety profile in this condition.

5.2 Future directions

The comparative use of asthma phenotypes has allowed the identification of features which are more associated with specific physiopathological abnormalities, such as increased IL-13 and airway hyperresponsiveness, upregulated TNF-α and refractory asthma and more distal inflammation and refractory asthma. These observations coupled with the newly available anti-cytokine treatments may offer rational, targeted therapeutic interventions to patients with specific asthma phenotype such as; anti-IL-13 to patients in whom airway hyperresponsiveness is a particular isolated problem.

Our study of etanercept in refractory asthma was small and had limited power to detect many clinically important outcomes. Larger, longer studies are required to clarify whether there are any specific adverse events in asthma, to assess pathological markers of airway remodelling and assess the impact on long term outcomes such as exacerbation rate and decline in lung function.
Clinical trials are also required to assess whether gaining non invasive information on distal lung inflammation by measuring alveolar nitric oxide concentration can be used to improve outcome in asthma.

Since previous studies have suggested that 25% of patients with asthma in primary care have non-eosinophilic asthma, studies in this population are required to determine whether not treating patients with non eosinophilic asthma with inhaled corticosteroids leads to a less favourable outcome.
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Appendix I

Refractory asthma: workshop consensus for typical clinical features

**Major characteristics**

1. Treatment with continuous or near continuous (>50% of year) oral corticosteroids

2. Requirement for treatment with high dose inhaled corticosteroids
   
   (>-1260 micograms/day beclomethasone equivalent)

**Minor characteristics**

1. Requirement for daily treatment with a controller medication

2. Asthma symptoms requiring treatment on a daily or near daily basis

3. Persistent airflow obstruction (FEV₁<80% predicted)

4. One or more urgent care visits per year

5. Three or more oral steroid bursts per year

6. Prompt deterioration with <25% reduction in oral or inhaled corticosteroid dose

7. Near fatal asthma in the past
Appendix II Summary of ethical approval for studies

Observational study of the natural history of eosinophilic bronchitis

**Study title:** Observational study of the natural history of eosinophilic bronchitis.

**Study reference:** 6693

Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis

**Study title:** Characterisation of the clinical and immunopathological features of eosinophilic bronchitis. (As amendment)

**Study reference:** 4977

Clinical and pathological features of non-eosinophilic asthma (Bronchoscopy study)

**Study title:** Mechanisms of T-Lymphocyte recruitment to the lung in asthma. (As amendment)

**Study reference:** 07619

Clinical and pathological features of non-eosinophilic asthma (Mometasone study)
Study title: Response to inhaled corticosteroids in eosinophilic and non-eosinophilic asthma: a randomised controlled trial.

Study reference: 6726

MHRA exception ref: 8000/12698

Alveolar nitric oxide concentration in adults with asthma

Study title: Mechanisms of T-Lymphocyte recruitment to the lung in asthma. (As amendment)

Study reference: 07619

Evidence of a role for TNF-α in refractory asthma

Study title: The efficacy of the soluble ligand binding receptor, etanercept, in severe asthma: a randomised controlled trial.

Study reference: 6993

MHRA exemption ref: 8000/12744

Study reference refers to the reference number assigned by the Leicestershire local research committee. MHRA exemption ref: refers to the reference for confirmation of exemption under the medicines exemption from licences (special cases and miscellaneous provisions) order 1972 for study drugs.