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AIRWAYS INFLAMMATION IN ALLERGIC SUBJECTS: RESPONSES TO AIR POLLUTION

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ABSTRACT

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By Dr Joanna Louise Brown

Over the past thirty years there has been a huge increase in the prevalence of allergic disease. The causes of this epidemic remain unclear, but genetic factors alone are unlikely to be able to satisfactorily explain the increase and environmental factors, including air pollution, may be important. The evolution of allergic disease in genetically predisposed individuals and the role that air pollution may play in that process have yet to be evaluated. By studying the baseline characteristics of inflammation in the lower airways of allergic asthmatic and rhinitic individuals, followed by controlled diesel exhaust exposure and re-analysis of the inflammation, the studies presented in this thesis have attempted to shed light on the transition phase of asymptomatic inflammation to clinical asthma and the role that air pollution might play in augmenting this transition.

Epidemiological studies suggest that subjects with allergic rhinitis are more likely to develop asthma compared to healthy controls. There is also considerable experimental data showing that non-asthmatic allergic rhinitics have evidence of asymptomatic lower airways inflammation even when they have not been exposed to allergen. The significance of this inflammation remains unclear but could represent an early stage in the development of clinical asthma. The first study characterises the cellular inflammation in the lower airways of non-asthmatic, asymptomatic allergic rhinitic subjects, comparing them to asthmatic individuals treated with either β2-agonists alone or with and inhaled corticosteroids and healthy controls. As anticipated, asthmatic subjects treated with β2-agonists alone had increased numbers of eosinophils and mast cells compared to healthy subjects, while those asthmatics on inhaled corticosteroids had a statistically significant increase in neutrophils. Subjects with allergic rhinitis had similar inflammatory changes in the lower airways to those detected in the asthmatics treated with β2-agonists alone, but important differences included a smaller increase in effector cells such as eosinophils and an additional increase in the total number of T lymphocytes - specifically in the sub-population of $CD8^+$ T-suppressor lymphocytes. The lack of asthmatic symptoms in the allergic rhinitic subjects may be a dose-dependent effect with insufficient inflammatory cells present to result in bronchoconstriction; or it may be as a result of the different ratio of $CD4^+/CD8^+$ T-cells, with the increase in $CD8^+$ T-suppressor lymphocytes having a protective effect.

The role of air pollution in both the exacerbation of pre-existing asthma and the propagation of new allergic disease has been examined in a number of epidemiological studies around the world. There is good evidence that particulate air pollution, including diesel exhaust, may exacerbate asthma causing an increase in hospital admissions 24-72 hours following the pollution exposure. The mechanisms of this delayed response remain unclear. Previous work has demonstrated that short-term controlled exposures to diesel exhaust induce an acute cellular response in the proximal bronchial tissue with increased numbers of neutrophils, lymphocytes and mast cells in healthy subjects, but not in asthmatic individuals. The second study undertaken in this thesis has demonstrated that the neutrophilic and mast cell inflammation still persists in the lower airways of healthy subjects 18 hours after diesel exhaust exposure in addition to an increase in the numbers of eosinophils. However, subjects with asthma and allergic rhinitis failed to show increased inflammation in the bronchial biopsies, although allergic rhinitics did have an increase in neutrophils in the bronchial wash. There was a reduction in mast cells in the bronchial epithelium of allergic rhinitics, which may reflect increased degranulation, while in the asthmatics treated with β2-agonists alone there was a trend towards a reduction in endothelial expression of VCAM-1. VCAM-1 has previously been shown to be up-regulated in healthy volunteers six-hours post-diesel exhaust exposure and may be correlated with the degree of bronchial inflammation. The significance of the reduction of VCAM-1 expression remains unclear but it is in keeping with the lack of inflammation. A number of hypotheses may be suggested to account for this lack of an inflammatory response to a single diesel exhaust exposure in atopic individuals: the presence of pre-existing low-grade inflammation in the lower airways may be protective; there may be an IL-10-mediated alteration in the inflammatory milieu with a subsequent propagation of allergic response; epithelial damage may be induced subsequently allowing particulates and allergens to penetrate the airways; or apoptosis in the epithelium may be induced leading eventually to an exacerbation of asthmatic symptoms. Further work will need to be performed in order to test these hypotheses.

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LIST OF ABBREVIATIONS

AEC	Amino Ethyl Carbazole
Al	Aluminium
AM	Alveolar Macrophage
BAL	Bronchoalveolar Lavage
BHR	Bronchial Hyperresponsiveness
Ca	Calcium
Cl^-	Chloride
COMEAP	Committee Of the Medical Effects on Air Pollution
COPD	Chronic Obstructive Pulmonary Disease
CV	Coefficient of Variation
DE	Diesel Exhaust
DEP	Diesel Exhaust Particles
Der p 1	Dermatophagoides Pteronyssinus
EGFR	Epidermal Growth Factor Receptors
ELF	Epithelial Lining Fluid
ELISA	Enzyme-Linked Immunosorbent Assay
eNO	Exhaled Nitric Oxide
EPAQS	Expert Panel on Air Quality Standards
Fe	Iron
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GMA	Glycol Methacrylate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GRO-α	Growth-Regulated Oncogene-Alpha
HBEC	Human Bronchial Epithelial Cells
HNEC	Human Nasal Epithelial Cells
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL-	Interleukin-
K^+	Potassium
KT	Kilo tonnes
LFA-1	Lymphocyte Function-Associated Antigen-1
LT	Leukotriene
MAP	Mitogen-Activated Protein
Mg	Magnesium
MIP 1 α	Macrophage Inflammatory Protein I alpha
Mn	Manganese
mRNA	Messenger Ribonucleic Acid
NF-KB	Nuclear Transcription Factor Kappa B
NH^{3+}	Ammonia ion
NHBE	Normal Human Bronchial Epithelial cells
NK-cells	Natural Killer Cells
NO/NO^{3-}	Nitric Oxide
NO_2	Nitrogen Dioxide
OVA	Ovalbumin
PAH	Polycyclic Aromatic Hydrocarbon
PBMC	Peripheral Blood Mononuclear Cells

PC_{20}	Provocation Concentration of inhaled histamine required to reduce the FEV_1 by 20%
PEF	Peak Expiratory Flow
PM	Particulate Matter
PM_{10}	Particles with an aerodynamic diameter equal to or less than 10μM
$PM_{2.5}$	Particles with an aerodynamic diameter equal to or less than 2.5μM
ppm	Parts per million
PSGL-1	P selectin glycoprotein-1
QTL	Quantitative Trait Loci
RANTES	Regulated on Activation, Normal T-Cell Expressed and Secreted
RAST	Radioallergosorbent Test
Raw	Airway Resistance
ROFA	Residual Oil Fly Ash
SABC	Streptavidin-Biotin Complex
SBP	Segmental Bronchial Provocation
SD	Standard Deviation
Si	Silicon
sICAM-1	Soluble Intercellular Adhesion Molecule-1
SO_2	Sulphur Dioxide
SO_4^{2-}	Sulphate ion
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor-beta
T_H2 lymphocytes	T Helper-2 Lymphocytes
TNF- α	Tumour Necrosis Factor-alpha
TSP	Total Suspended Particles
TUNEL	Terminal-deoxynucteotidyl-transferase-mediated Nick End Labelling
VCAM-1	Vascular Cell Adhesion Molecule-1
WHO	World Health Organisation

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PUBLICATIONS

The following publications have arisen from work presented in this thesis:

Reviews

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Book chapters

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Abstracts

1. *A Behndig, **JL Brown**, AJ Frew, *R Helleday, †FJ Kelly, *J Pourazar, *N Stenfors, *T Sandström, *A Blomberg. Diverging airway inflammatory responses to diesel exhaust exposure in healthy and asthmatic subjects with and without inhaled corticosteroid treatment. 12th Annual Meeting. European Respiratory Society. Stockholm, September 2002. *Eur Respir J.* 2002; 20 (38): 220s

2. **JL Brown**, *N Stenfors, *A Behndig, *R Helleday, *H Törnqvist, *A Blomberg, †FJ Kelly, *T Sandström, AJ Frew, SJ Wilson. Assessing the time-course of bronchial inflammation induced by diesel exhaust exposure in allergic asthmatics and rhinitics. American Thoracic Society. 97th International Conference. Atlanta, May 2002. *Am J Resp Crit Care Med.* 2002; 165 (8): A302

3. **JL Brown**, *N Stenfors, *A Behndig, *R Helleday, *H Törnqvist, *A Blomberg, †FJ Kelly, *T Sandström, SJ Wilson, AJ Frew. The cellular constituents of lower airways inflammation in allergic rhinitic subjects compared to asthmatics and healthy. American Academy of Allergy, Asthma and Immunology. 58th Annual Meeting. New York, March 2002. *J Allergy Clin Immun.* 2002; 109 (1): S115

4. **JL Brown**, *N Stenfors, *A Behndig, *R Helleday, *H Törnqvist, *A Blomberg, †FJ Kelly, *T Sandström, AJ Frew, SJ Wilson. Baseline inflammation in the lower airways of allergic rhinitic subjects compared to normal subjects. 11th Annual Meeting. European Respiratory Society. Berlin, September 2001. *Eur Respir J.* 2001; 18 (33): 270s

5. **JL Brown**, *N Stenfors, *A Behndig, *R Helleday, *H Törnqvist, *A Blomberg, †FJ Kelly, *T Sandström, AJ Frew, SJ Wilson. Effects of exposure to diesel exhaust (PM_{10} 100μg/m^3) on rhinitic subjects. American Thoracic Society. 96th International Conference. San Francisco, May 2001. *Am J Resp Crit Care Med.* 2001; 163 (5): A263.

6. *A Behndig, *N Stenfors, *A Blomberg, **JL Brown,** *R Helleday, *J Pourazar, †FJ Kelly, AJ Frew, *T Sandström. Inflammatory airway responses to diesel exhaust exposure in subjects with seasonal allergic rhinitis and healthy subjects. American Thoracic Society. 96th International Conference. San Francisco, May 2001. *Am J Resp Crit Care Med.* 2001; 161 (3): A263.

The following publications have arisen during my DM training that have no direct relevance to this thesis:

1. **J Brown**, AJ Frew: Respiratory section of 'The Oxford Handbook of Clinical and Laboratory Investigation', Edited by Drs D. Provan and A Krentz. Published December 2002. Oxford University Press.

2. **Brown JL,** Frew A J. The efficacy of oromucosal immunotherapy in respiratory allergy. *Clinical and Experimental Allergy.* 2000; 31:1-4.

CHAPTER ONE

Introduction

1.1 EPIDEMIOLOGY OF ALLERGIC DISEASE

1.1.1 Introduction

There can be no doubt that we are currently experiencing a worldwide epidemic of allergic disease (Holgate, S.T. 1999; Weiss, S.T. 2001). The prevalence of allergic asthma and rhinitis has increased considerably over the last thirty years; however the reasons for this remain unclear. Although the risk of allergic disease is clearly inherited, genetic factors alone are not able to satisfactorily explain this sudden increase in allergy. It is generally agreed that environmental factors must be operating, albeit on a genetically susceptible subgroup. Possible explanations include a reduction in exposure to infection during childhood (the hygiene hypothesis) (Abramson, M.J. *et al.* 2000; Anderson, W.J. *et al.* 2001b), changes in intra-uterine nutrition (Howarth, P.H. 1998; Svanes, C. *et al.* 1998), increased exposure to allergens such as house dust mite (Sporik, R. *et al.* 1992), and increasing levels of air pollutants including ozone, sulphur and nitrogen oxides and particulate matter (Wardlaw, A.J. 1995; Koenig, J.Q. 1999). The increases in the volume of road traffic and in air pollution show a parallel rise with the increased prevalence of allergic disorders during this 30-year period (Schrenk, H. *et al.* 1948; von Mutius, E. *et al.* 1992; Wjst, M. *et al.* 1993; Braback, L. *et al.* 1994; Burr, M.L. 1995). Individual pollutants, including diesel exhaust particles (DEP), have been identified in many epidemiological studies as causing adverse health effects (Pope, C.A. *et al.* 1991; Pope, C.A. *et al.* 1992; Dockery, D.W. *et al.* 1993; Pope, C.A. *et al.* 1993; Pope, C.A. *et al.* 1995; Kaiser, J. 1997). Some of these studies demonstrate that an increase in particulate air pollution is followed by exacerbations of asthma and allergic rhinitis. This evidence, and experimental work both *in vitro* and *in vivo*, has led to suggestions that DEP may be instrumental not just in the exacerbation of allergic disease, but also in its initiation and maintenance.

Allergic disease is typically associated with inflammation characterised by a T_H2-type pattern, with increases in $CD4^+$ lymphocytes, eosinophils and their respective cytokines and mediators (Romagnani, S. 2000). In particular, interleukins (IL) -4, -5, -6, -8 and -13, granulocyte-macrophage colony stimulating factor (GM-CSF), and RANTES (regulated on activation, normal T-cell expressed and secreted) appear to be crucial (Gosset, P. *et al.* 1999; Graziano, F.M. *et al.* 1999; Teran, L.M. 2000; Tillie-Leblond, I. *et al.* 2000; Renauld, J.C. 2001), as do tumour necrosis factor (TNF)-α (Baeza-Squiban, A. *et al.* 1999), leukotrienes (LT) -C4, -D4 and -E4 (Adelroth, E. *et al.* 1986; Busse, W.W. 1996; Bisgaard, H. 2000; Leff, A.R. *et al.* 2001) and interferon (IFN)-γ (Vrugt, B. *et al.* 1996; Hamelmann, E. *et al.* 2001; Leong, K.P. *et al.* 2001). Immunoglobulin (Ig) E, mast cells and macrophages also have an important role to play in the allergic response (Flint, K.C. *et al.* 1986; Arm, J.P. *et al.* 1992; Yssel, H. *et al.* 1998).

In order to understand how DEP may influence the incidence and prevalence of allergic disease it is necessary to look at the ways in which DEP can modulate the allergic immune response. In the environment, DEP has been shown to bind pollens and other allergens (Kainka-Stanicke, E. *et al.* 1998). DEP may therefore act as a vector, carrying an increased amount of allergen into the airway; as an adjuvant, enhancing the inflammation that results from the inhalation of allergen; and as an agent that increases the antigenicity of the allergen by modifying its epitopes. Inhaling DEP alone may affect the bronchial epithelium and submucosa and thereby alter the bronchial response to inhaled allergens. DEP contains oxidising metals, which may cause damage to the bronchial epithelium with subsequent changes in epithelial permeability. Mucociliary clearance of particles also appears to be adversely affected by diesel exhaust exposure. This suggests that DEP impairs the ability of the bronchial epithelium and cilia to act as a biological barrier against inhaled substances.

This may allow allergens to remain on the epithelial surface for longer, perhaps allowing them to diffuse into the epithelial layer and increasing the probability of them coming into contact with cells of the immune system (Wolff, R.K. 1986; Bayram, H. *et al.* 1998).

Exposure to DEP alone causes an inflammatory response in the airways, characterised by an increase in neutrophils and inflammatory cytokines (Ohtoshi, T. *et al.* 1998; Nightingale, J.A. *et al.* 2000). This inflammatory reaction may non-specifically sensitise the airways to subsequent allergen exposure, increasing the magnitude of the allergic response.

Not only does allergic disease cause significant morbidity and mortality, it also has important health economic implications. In 1998, asthma in the United States accounted for an estimated 12.7 billion dollars annually in healthcare expenditure (Weiss, K.B. *et al.* 2001). In view of this, understanding the possible causes of allergic disease is clearly an important priority, and may in time lead to its eventual prevention.

1.1.2 Epidemiology of allergic asthma

1.1.2.1 Increasing incidence and prevalence

For many years large epidemiological studies have been reporting an increasing incidence and prevalence of allergic disease (Burr, M.L. *et al.* 1989; Haahtela, T. *et al.* 1990; Poysa, L. *et al.* 1991; Ciprandi, G. *et al.* 1996; Montefort, S. *et al.* 1998; Sly, R.M. 1999). One such study in Sweden analysed the computer records of conscript examinations in 1971 and 1981 with respect to the prevalence of asthma and allergic rhinitis. The material comprised approximately 55,000 18-year-old males in each cohort. During the 10-year period the

prevalence of asthma increased from 1.9 to 2.8% and of allergic rhinitis from 4.4 to 8.4% (Aberg, N. *et al.* 1989).

In recent times the increase in asthma prevalence has been particularly striking. In Finland, the prevalence of physician-diagnosed asthma was found to have increased 3-fold among adolescents from 1977-1991 (Rimpela, A.H. *et al.* 1995), whilst a large New Zealand study of 37,592 people found that the prevalence of asthma symptoms was 25% for six to seven year old children and 30% for thirteen to fourteen year olds (Asher, M.I. *et al.* 2001). Similar results have also been demonstrated in Australia (Robertson, C.F. *et al.* 1998) and the UK (Shamssain, M.H. *et al.* 2001).

Asthma initially appeared to be a disease of the industrialized West, yet there is now clear evidence that it is increasing in the East and in developing countries (Carrasco, E. 1987; Kalyoncu, A.F. *et al.* 1994; Werneck, G. *et al.* 1999; Akcakaya, N. *et al.* 2000; Ozdemir, N. *et al.* 2000). A study of over 4,000 Hong Kong schoolchildren in 1994/5 showed that when compared to previous epidemiological data obtained in 1992, the prevalence rates for asthma had increased by 71%, and asthma severity also showed a similar increasing trend (Leung, R. *et al.* 1997). In Saudi Arabia, the prevalence of asthma in schoolchildren aged between eight- and 16-years increased significantly from 8% in 1986 to 23% in 1995 (Al-Frayh, A.R. *et al.* 2001) whilst in Northeast Thailand and Bangkok there appears to have been a four-fold increase in asthma (Vichyanond, P. *et al.* 1998; Teeratakulpisarn, J. *et al.* 2000). Studies have confirmed that this increase in asthma prevalence is a real phenomenon, and not just a result of improved diagnosis or reporting (Burney, P.G. *et al.* 1990; Ninan, T.K. *et al.* 1992).

1.1.2.2 Relationship to pollution

Air pollution is convincingly associated with many signs of asthma aggravation (Koenig, J. 1999), although a possible causative role remains controversial (Ramadour, M. *et al.* 2000). Isolated case reports of diesel exhaust inducing asthma exist (Wade, J.F. *et al.* 1993), but more convincing causative evidence remains elusive. However, there is some evidence to suggest that traffic-related air pollution may be responsible for an increase in the rate of atopic sensitisation and asthma. In one study, symptoms of wheezing and sensitisation against pollen, house dust mites or cats, and milk or eggs were associated with outdoor nitrogen dioxide (NO_2) in urban areas (Kramer, U. *et al.* 2000). A study of over 5,000 children in Sweden showed that various moderate environmental pollutants may act synergistically to increase bronchial hyperreactivity and allergy, especially in children with a family history of allergy (Andrae, S. *et al.* 1988).

Studies in two German cities have shown that exposure to automobile emissions is related to prevalence of wheezing after controlling for putative confounders such as age, sex, passive smoking and active smoking (Keil, U. *et al.* 1996), but a second German study failed to demonstrate a link between exhaust fumes and allergic asthma (Nicolai, T. 1997). Similarly, in Switzerland, particulate matter (PM_{10}) air pollution was found to have a positive association with chronic cough and nocturnal cough, but no association was found between long-term exposure to air pollution and classic asthmatic and allergic symptoms and illnesses (Braun-Fahrlander, C. *et al.* 1997).

1.1.3 Epidemiology of allergic rhinitis

1.1.3.1 Increasing incidence and prevalence

The increase in asthma prevalence has been paralleled by a rise in allergic rhinitis (Rimpela, A.H. *et al.* 1995; Ciprandi, G. *et al.* 1996; Sly, R.M. 1999). One study in Sweden reported an increase in prevalence from 4.4 to 8.4% during a ten-year period (1971-1981) (Aberg, N. 1989). In the UK during the same period the prevalence of allergic rhinitis in men increased from 10.8 to 19.8 people consulting per 1,000 population, and in women from 10.3 to 19.7 per 1,000 population (Fleming, D.M. *et al.* 1987).

This pattern of increasing disease prevalence and severity is also repeated elsewhere in the world (Goh, D.Y. *et al.* 1996). For example, a study in Bangkok showed almost a three-fold increase during an eight-year period in the prevalence of allergic rhinitis in children (Vichyanond, P. *et al.* 1998).

1.1.3.2 Relationship to pollution

Evidence that an increased incidence of allergic rhinitis may be linked to air pollution comes from a variety of sources (Rusznak, C. *et al.* 1994; Keil, U. *et al.* 1996). In Japan, allergy to cedar pollen is widespread, but there is an increased prevalence in areas with increased road traffic pollution, suggesting that a synergistic relationship may exist (Ishizaki, T. *et al.* 1987). Other studies have demonstrated a positive association between symptoms of allergic rhinitis and outdoor NO_2 levels (Kramer, U. *et al.* 2000). Air pollution also appears to increase sensitisation to aeroallergens such as house dust mite, Cladosporium, orchard grass and birch pollen, with subjects in urban areas showing increased IgE levels compared to those in rural areas (Popp, W. *et al.* 1989).

1.1.4 Allergic rhinitis and the development of asthma

Data from epidemiological studies indicate that nasal symptoms are experienced by as many as 78% of patients with asthma, and that asthma is experienced by as many as 38% of patients with allergic rhinitis (Corren, J. 1997). In the World Health Organisation 'Initiative on Allergic Rhinitis and its effect on Asthma' (ARIA), it is quoted that asthma and rhinitis are common co-morbidities suggesting the concept of 'one airway one disease' (Palma-Carlos, A.G. *et al.* 2001). In terms of pathological processes, the two diseases are certainly similar with increased numbers of eosinophils and their associated proteins and cytokines, mast cell activation and mediator release, up regulation of adhesion molecules, and increased number and activation of T_H2 lymphocytes. Autonomic imbalance also plays a role in both conditions via changes in neural tone to effector tissues and release of neuropeptides (Rowe-Jones, J.M. 1997).

Studies have also identified a temporal relation between the onset of rhinitis and asthma, with rhinitis frequently preceding the development of asthma (Eggleston, P.A. 1988; Linna, O. *et al.* 1992). One study from Sweden showed that almost 40% of subjects with allergic rhinitis developed asthma or lower-airways symptoms over an eleven year period (Danielsson, J. *et al.* 1997), while in the USA, among the participants in a prospective study with a history of both asthma and hay fever, 44.8% developed hay fever first, 34.5% developed asthma first, and 20.7% developed both diseases at the same time (Greisner, W.A. *et al.* 1998). Rhinitis has been identified as an independent risk factor for asthma in both atopic (Plaschke, P.P. *et al.* 2000), and non-atopic subjects with normal IgE levels (Leynaert, B. *et al.* 1999). Patients with allergic rhinitis and no clinical evidence of asthma commonly

exhibit non-specific bronchial hyperresponsiveness, although this in itself does not appear to be a risk factor for developing asthma (Prieto, L. *et al.* 1994).

The shape of the concentration-response curve to methacholine may be important in predicting those allergic rhinitis subjects who will go on to develop asthma: those without evidence of plateau have a degree of diurnal PEF variation similar to that found in patients with mild asthma, suggesting the presence of subclinical inflammation in the lower airways (Prieto, L. *et al.* 1998). Reaction to methacholine challenge also predicts bronchial response to allergen in rhinitics, with increased reactivity to methacholine being positively associated with bronchial hyperresponsiveness to allergen (Bonavia, M. *et al.* 1996). This inflammatory response in non-asthmatic allergic rhinitis patients has also been demonstrated by segmental bronchial provocation (SBP) using grass pollen (Braunstahl, G.J. *et al.* 2000). This resulted in an increase in the number of eosinophils in the bronchial mucosa and in the blood of atopic subjects 24-hours after challenge. There was also evidence of nasal inflammation post-SBP in this group, with an increase in eosinophils in the nasal lamina propria and enhanced expression of IL-5 in the nasal epithelium. Another study has shown that natural pollen exposure was associated with an increase in lymphocyte numbers ($CD4^+$, $CD8^+$, and $CD45RO^+$), eosinophil recruitment, and IL-5 expression in the bronchial mucosa of non-asthmatic subjects with allergic rhinitis (Chakir, J. *et al.* 2000).

Finally, further evidence for the interrelationship between asthma and allergic rhinitis comes from response to treatment; intranasal corticosteroids can prevent the seasonal increases in non-specific bronchial reactivity associated with pollen exposure (Corren, J. *et al.* 1992). Clearly, it would be beneficial to be able to predict which subjects with allergic rhinitis will subsequently develop asthma, and then try to prevent that from occurring.

1.2 PARTICULATE MATTER

1.2.1 Introduction

Particulate matter is generated by the heat combustion processes of motor vehicles. Diesel engines produce up to 100-150 times more particles than petrol engines fitted with catalytic converters (COMEAP 1996) and diesel exhaust is one of the major contributors to particulate air pollution in cities (UNEP 1994; COMEAP 1995)

1.2.2 Classification of particulate matter

Diesel exhaust particulate (DEP) air pollution is a mixture of solid particles and liquid droplets that vary in mass, size and chemical composition depending on the emission sources and meteorological conditions. The levels of particulate matter (PM) air pollution are expressed in relation to the particle size. Particles with an aerodynamic diameter equal to or less than 10μm (PM_{10}) are capable of being inhaled into the airways, and are known to cause adverse health effects (Sydbom, A. *et al.* 2001). Ultrafine particles with an aerodynamic diameter equal to or less than 2.5μm ($PM_{2.5}$), have been shown to cause more lung injury and inflammation than a similar amount of fine particles (Oberdorster, G. *et al.* 1994; Donaldson, K. *et al.* 2001; Oberdorster, G. 2001). Ultrafine particles are capable of penetrating further through the epithelium and vascular walls and are then transported to the lung-associated lymph nodes where they may affect the proliferation of immune cells and antibody production. (Bice, D.E. *et al.* 1985)

In a study that attempted to characterize the emissions from currently used diesel vehicles, the $PM_{2.5}$ emissions comprised approximately 74% of the PM_{10} emission (weight for

weight), and in both fractions carbon was the dominant element. Crustal elements (Fe, Mg, Al, Si, Ca, and Mn) contributed significantly as did the ions Cl^-, NO^{3-}, NH^{3+}, SO_4^{2-}, and K^+ (Gillies, J.A. *et al.* 2001).

Other studies confirm that diesel particulates consist mainly of carbon and have also shown the presence of adsorptively bound organic chemicals including polycyclic aromatic hydrocarbons (PAHs) (Lies, K.H. *et al.* 1986) and other potentially toxic elements (Lipscomb, J.C. *et al.* 1997). The qualitative and quantitative nature of hydrocarbon compounds associated with the particulates may vary with the combustion parameters of the engine and meteorological conditions (Bagnoli, P. *et al.* 1997).

1.2.3 Particulate matter and lung deposition

Epidemiological data suggests that subjects with asthma or chronic obstructive pulmonary disease (COPD) are particularly susceptible to air pollution (Schwartz, J. 1994; Anderson, H.R. *et al.* 1998). One explanation for this may be that the particle deposition rate is increased in these individuals as a result of altered airflow and airway characteristics. In one study comparing particle deposition in the lungs of healthy controls with subjects with varying levels of airway obstruction (smokers, smokers with small airways disease, asthmatics, and patients with chronic obstructive pulmonary disease), a marked increase in particle deposition was seen in all categories of airways disease, but particularly in those with COPD (Kim, C.S. *et al.* 1997). A reduction of 30% in the airway cross-sectional area results in at least a doubling of deposition in the bifurcating airways (Kim, C.S. *et al.* 1998). Histological evidence from post-mortem studies also indicates that accumulation of carbonaceous and mineral dust in the lungs is significantly affected by lung anatomy, with

deposits being greater in first-generation respiratory bronchioles compared to second- and third-generation respiratory bronchioles (Pinkerton, K.E. *et al.* 2000). The greatest retention of particles was in centres of lung acini and this was associated with significant focal wall thickening and remodelling with increases in collagen and interstitial inflammatory cells, including dust-laden macrophages. In consequence, particle burdens could reach threshold limits at local lung regions despite the exposure level occurring within 'acceptable' levels (Kim, C.S. *et al.* 1998). Computer simulations suggest that children are particularly vulnerable due to their breathing pattern - specifically the minute ventilation (the number of breaths per minute multiplied by the tidal volume) (Yu, C.P. *et al.* 1987; Musante, C.J. *et al.* 2000). For an equal exposure, the predicted deposition of particles was approximately twice as high in a 2-year-old child as in the mature adult lung (Yu, C.P. *et al.* 1987).

1.2.4 Particulate toxicity

Diesel exhaust particles consist of a carbon core, onto which chemicals such as acids, (polycyclic) aromatic hydrocarbons (PAH), heavy metals, endotoxins and allergens may be adsorbed. The smaller the particle, the greater their surface area per unit mass, and hence the capacity of smaller particles to carry toxic substances and free radicals increases.

The qualitative and quantitative nature of the hydrocarbon compounds (PAH) associated with the particulates is dependent not only on the combustion parameters of the engine but also on the ambient temperature and climatic conditions (Lies, K.H. *et al.* 1986; Morozzi, G. *et al.* 1997). It is well documented that PAH are mutagenic and may be carcinogenic (Boffetta, P. *et al.* 1997), but there is also evidence that they may increase allergic disease by enhancing IgE production (Takenaka, H. *et al.* 1995; Diaz-Sanchez, D. *et al.* 1997; Tsien, A. *et al.* 1997), and by increasing chemokine secretion. A study using

PBMCs (peripheral blood mononuclear cells), obtained from healthy subjects, showed that secretion of IL-8 and RANTES increased in a dose-dependent manner with increasing concentrations of DEP-PAH, and as a result, the cell supernatants exhibited a significantly enhanced chemotactic activity for neutrophils and eosinophils (Fahy, O. *et al.* 1999).

Other research has concentrated on the effects of the metals present in DEP. Carter *et al* (Carter, J.D. *et al.* 1997) exposed normal human bronchial epithelial (NHBE) cells to residual oil fly ash, (ROFA), which contains large amounts of the transition metals vanadium, nickel and iron, as a model of particulate air pollution. After exposure, the cells were found to produce significant amounts of IL-6, IL-8 and TNF-α. The messenger ribonucleic acid (mRNA) coding for these cytokines was also up regulated. Another study compared the inflammatory response following intratracheal instillation of ultrafine particles of cobalt, nickel, and titanium oxide and found that ultrafine-nickel appeared to be the most injurious to the lung, causing severe and sustained inflammation, cytotoxicity and increased epithelial permeability (Zhang, Q. *et al.* 1998). Other work has shown the ability of particulate matter to generate free radicals as a result of the release of iron (Donaldson, K. *et al.* 1997).

Endotoxin may bind to PM_{10} and has been shown to induce cytokine release from alveolar macrophages (Soukup, J.M. *et al.* 2001) as well as their apoptosis.

There is also evidence to show that grass pollen allergens are able to bind to DEP (Knox, R.B. *et al.* 1997; Schappi, G.F. *et al.* 1999). These DEP may then act as carrier molecules, concentrating and transporting the allergens into the airway and thereby increasing the allergen 'dose' at the bronchial epithelium. The ability of DEP to bind other allergens,

namely Can f 1 (dog), Bet v 1 (birch pollen), Der p 1 (house dust mite) and Fel d 1 (cat), has also been examined (Ormstad, H. *et al.* 1998). The DEP were exposed to either crude allergen extracts or partly purified allergens. Scanning electron microscopy was then used to assess the binding and showed that DEP had the ability to adsorb all four allergens *in vitro*, albeit to a varying extent. As well as increasing the allergen 'dose' at the bronchial epithelium, DEP may alter the way that the allergen is presented to the immune system – for example there is research showing that morphological alterations of airborne allergens may be induced by airborne pollutants, possibly again increasing its antigenicity (Kainka-Stanicke, E. *et al.* 1998).

The toxicity of particulate matter may also be enhanced by the complex interaction with other air pollutants. Ozone and particulate matter have been shown to have a synergistic relationship in rodents (Kleinman, M.T. *et al.* 2000).

1.2.5 Particulate standards

A number of agencies, including the World Health Organization (WHO) and the Expert Panel on Air Quality Standards (EPAQS) of the UK, have recommended air quality guidelines for particulate matter, measured as either PM_{10} or total suspended particles (TSP). Some countries have set additional safety standards for a subdivision of PM_{10}, known as $PM_{2.5}$. This is the ultra-fine component of particulate matter pollution consisting of particles with a diameter ≤ 2.5 μm. As discussed earlier, there is some evidence that this fraction of particulate matter is more pro-inflammatory than PM_{10}, and therefore safe exposure levels should be lower. The air quality standards set for particulate matter vary considerably between countries and health agencies as shown in Table 1.1 (Utell, M.J. *et al.* 1993; UNEP 1994; Guidotti, T.L. 1995; Air Health Strategy 1996; Spurny, K.R. 1996).

Despite short-term downward trends in particulate emissions in the UK during the 1990s, the main source of PM was from heavy goods vehicles and the trends are predicted to reverse in the next millennium. As a result they may approach or surpass recommended exposure levels (Ayres, J.G. 1997).

Table 1.1 Air quality standards for Particulate Matter

WHO	PM_{10}	24 hour average	$70\mu g/m^3$
	TSP	24 hour average	$150\mu g/m^3$
		annual mean	$60\mu g/m^3$
USA	PM_{10}	24 hour average	$150\mu g/m^3$
		annual mean	$50\mu g/m^3$
	$PM_{2.5}$	24 hour average	$25\text{-}85\mu g/m^3$
		annual mean	$15\text{-}30\mu g/m^3$
Canada	PM_{10}	24 hour average	$120\mu g/m^3$
		annual mean	$70\mu g/m^3$
Germany	PM_{10}	annual mean	$75\mu g/m^3$
Europe	PM_{10}	24 hour average	$30\text{-}100\mu g/m^3$
		annual mean	$15\text{-}40\mu g/m^3$
United Kingdom	PM_{10}	24 hour average	$50\mu g/m^3$

(TSP – Total suspended particulates; PM_{10} – Particulate matter $\leq$ 10 μm in diameter)

1.2.6 PM_{10} and mortality

Numerous studies have shown a positive association between daily mortality rates and particulate air pollution, even at PM concentrations below regulatory limits (Pope, C.A. *et al.* 1995; Thurston, G.D. 1996; Peters, A. *et al.* 2000; Samet, J.M. *et al.* 2000). These findings have motivated interest in the shape of the exposure-response curve: there is evidence that PM_{10} and relative risk of death for all causes and cardiorespiratory causes show a linear relation without any indication of a threshold effect (Simpson, R.W. *et al.* 1997; Daniels, M.J. *et al.* 2000). Mortality rates increase on average by 0.7% per 10μg/m^3 increase in PM_{10} concentrations, with greater effects at sites with higher proportions of particulate matter ≤ 2.5 μm in aerodynamic diameter ($PM_{2.5}/PM_{10}$ ratio) (Levy, J.I. *et al.* 2000). There is additional evidence showing that respiratory morbidity is most closely associated with $PM_{2.5}$, the ultra-fine component of PM_{10}, suggesting that it may be the most relevant fraction of particulate matter pollution (Ostro, B. 1995; Anderson, H.R. *et al.* 2001a).

The causes of death attributed to PM_{10} include exacerbations of chronic obstructive pulmonary disease (COPD), pneumonia, heart disease and stroke (Schwartz, J. 1994; Wordley, J. *et al.* 1997), with the very young and the elderly being particularly at risk (Thurston, G.D. 1996; Woodruff, T.J. *et al.* 1997). The UK Committee on the Medical Effects of Air Pollutants (COMEAP) released a statement on the long-term effects of particles on mortality in March 2001. This stated that the relationship between particulates and reduction in mortality appeared to be causal, and that the size of the effect could be substantial. The composition of the particles appears to be important, as does the size of the particles, with $PM_{2.5}$ having the greatest impact upon mortality. The effect of PM_{10} is quantified in Table 1.2, which shows mortality rates and hospital admissions for respiratory diseases per year in urban

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$_2$) is also shown.

Table 1.2 **Numbers of deaths and hospital admissions for respiratory diseases affected per year by PM_{10} and sulphur dioxide in urban areas of Great Britain**
[COMEAP: Report: the Quantification of the Effects of Air Pollution on Health in the United Kingdom.1998]

Pollutant	Health outcomes	GB Urban
PM_{10}	Deaths brought forward (all causes)	8100
	Hospital admissions (respiratory) brought forward and additional	10500
SO_2	Deaths brought forward (all causes)	3500
	Hospital admissions (respiratory) brought forward and additional	3500

1.2.7 PM_{10} and morbidity

PM_{10} has a number of adverse health effects including the exacerbation of pre-existing cardiovascular disease (strokes, myocardial infarction, angina, arrhythmias) (Wordley, J. *et al.* 1997; Schwartz, J. 1999; Wong, T.W. *et al.* 1999; Stieb, D.M. *et al.* 2000; Tolbert, P.E. *et al.* 2000) and respiratory disease (COPD, acute bronchitis, asthma, pneumonia) (Pope, C.A. *et al.* 1993; Dab, W. *et al.* 1996; Gordian, M.E. *et al.* 1996; Sunyer, J. *et al.* 1997; Hajat, S. *et al.* 1999); the promotion of allergic disease (Gavett, S.H. *et al.*

2001); intra-uterine growth retardation (Dejmek, J. *et al.* 2000); and the promotion of carcinogenesis in the lung and bladder (Scheepers, P.T. *et al.* 1992b; Stober, W. *et al.* 1996). In this section, I will be focusing on the effects PM_{10} has on asthma and allergy.

Exposure to increases in PM_{10} has been associated with exacerbations of asthma in both children (Pope, C.A. *et al.* 1991; Pope, C.A *et al.* 1992; Rennick, G.J. *et al.* 1992; Peters, A. *et al.* 1997) and adults (Whittemore, A.S. *et al.* 1980; Schwartz, J. *et al.* 1993; Walters, S. *et al.* 1993; Sheppard, L. *et al.* 1999). It has been shown that children experience reductions in peak expiratory flow (PEF) and increased respiratory symptoms after increases in relatively low ambient PM_{10} concentrations, and that children with diagnosed asthma are more susceptible to these effects than other children (Vedal, S. *et al.* 1998). A study examining the effect of daily variations in the size of particulate matter (10nm to 10μm) from road traffic pollution, and the PEF of children with asthmatic symptoms observed significant associations between PM_{10} and declines in morning PEF (Pekkanen, J. *et al.* 1997). Children with bronchial hyperresponsiveness (BHR) and relatively high serum concentrations of total IgE (>60 kU/L) are also susceptible to air pollution with the prevalence of lower respiratory symptoms increasing significantly by between 32% and 139% for each 100μg/m^3 increase in particulate matter (Boezen, H.M. *et al.* 1999). Asthmatic children may be particularly sensitive to ultrafine particles with one study showing FEV_1 and FVC dropping an average of 34 and 37ml respectively for each increase in $PM_{2.5}$ of 20 μg/m^3 (Koenig, J.Q. *et al.* 1993; Norris, G. *et al.* 1999).

In adults, studies have shown an association between particulate air pollution and increase in the use of asthma medications, (Whittemore, A.S. *et al.* 1980; Dockery, D.W. *et al.* 1994) and in-hospital attendance for asthma attacks (Schwartz, J. *et al.* 1993; Delfino, R.J.

et al. 1994; Lipsett, M. *et al.* 1997; Atkinson, R.W. *et al.* 1999). The very young and the elderly appear to be particularly sensitive to particulate air pollution with particles shown to have a significant association with hospital admissions for asthma in the 0-14 and 65+ age groups (Anderson, H.R. *et al.* 1998; Ostro, B.D. *et al.* 1999; Anderson, H.R. *et al.* 2001a). A small increase in ambient levels of PM_{10} air pollution can have a significant effect on such vulnerable groups, and the number of subjects with a clinically relevant reduction in lung function is quantitatively important (Kunzli, N. *et al.* 2000).

Hay fever has been found to be more common in urban areas than in rural areas and is also exacerbated by air pollution (Aberg, N. 1989; Kramer, U. *et al.* 2000; Hajat, S. *et al.* 2001). In Germany, a large survey of over 2,000 schoolchildren found that both wheezing and allergic rhinitis were associated with traffic density, even when the data were controlled for potential confounders such as active and passive smoking (Weiland, S.K. *et al.* 1994). In Japan, where diesel-powered cars are very popular, epidemiological studies have shown that living within 200 metres of a main road increases the risk of developing allergy to cedar pollen (Ishizaki, T. *et al.* 1987). This data suggests that air pollution may have a role in increasing the risk of an individual becoming sensitised to aeroallergens, a theory that gains further credence by a study that examined the allergic sensitisation of 362 male subjects living in either a heavily polluted area, or in a rural area (Popp, W. *et al.* 1989). Using RAST to house dust mite, Cladosporium, orchard grass and birch pollen, they found that total IgE and specific IgE levels were significantly higher in the urban population compared to the rural population.

1.2.8 PM_{10} levels worldwide

Generally, three major sources of PM_{10} are recognised: namely vehicular emissions, industrial emissions, and soil re-suspension. Of these, vehicular emissions contribute the greatest percentage in urban areas and are of most concern in terms of health effects. One study in Massachusetts, USA found that $PM_{2.5}$ concentrations were greater during morning rush hours and on weekdays (Levy, J.I. *et al.* 2001). These levels were also significantly higher with closer proximity to the bus terminal and on roads reported to have heavy bus traffic.

While concerns about particulate matter air pollution have long been recognised in the industrialized West, it is only recently being appreciated and tackled in developing countries. Many of these countries experienced a rapid increase in the volume of vehicular traffic over recent years coupled with rapid industrialization. Lack of monitoring facilities and less stringent legislative controls on ambient air pollution limits have led to exposure levels being much greater (Schwela, D. 1996). Out of 20 megacities of the world (a megacity being defined as a city with a population of ≥10 million), 12 were found to have levels of particulate matter that exceeded WHO guidelines by a factor of more than two (UNEP 1994). Most of these cities had annual average concentrations of suspended particulate matter in the range of 200-600 $\mu g/m^3$ with peak particulate concentrations of >1000 $\mu g/m^3$ at several sites. Levels of particulate matter measured in homes in Delhi are on average 500 $\mu g/m^3$, and children commuting to school are exposed to PM levels of around 960 $\mu g/m^3$ daily (Kaiser, J. 1997). This compares with a mean personal PM_{10} concentration of 105 $\mu g/m^3$ in schoolchildren in the Netherlands (Janssen, N.A. *et al.* 1997) and a daily $PM_{2.5}$ personal exposure concentration ranging from 2.4 to 47.8 $\mu g/m^3$ (overall individual study mean of 12.9 $\mu g/m^3$) in an elderly

population in USA (Williams, R. *et al.* 2000). These high levels of exposure in developing countries are clearly a cause for concern and may result in an epidemic of pollution-related cardiorespiratory disease.

1.2.9 PM_{10} levels in the United Kingdom

The annual means of hourly PM_{10} concentration in UK cities generally lie between 10–45 $\mu g/m^3$ with peak daily averages of 70-160 $\mu g/m^3$, although hourly means have exceeded 300-400 $\mu g/m^3$ in certain cities (QUARG 1993) (Table 1.3). Predictions suggest that annual emissions of particulate matter in the UK may decrease to below 60 kT over the next decade, but a progressive increase is predicted thereafter (Ayres, J.G. 1997).

Table 1.3: Hourly average PM_{10} concentration in major cities in the UK, 1994 (QUARG 1993)

Site	**Annual mean** ($\mu g/m^3$)	**98th Percentile** ($\mu g/m^3$)	**Maximum in 1 hour** ($\mu g/m^3$)
Belfast	26	66	490
Bristol	24	59	612
Birmingham- central	23	55	311
Birmingham- east	21	50	319
Cardiff	34	76	564
Edinburgh	20	41	307
Hull	26	56	264
Leeds	26	64	310
Leicester	21	50	203
Liverpool	25	68	257
London	27	56	307
Newcastle	26	60	297
Southampton	23	48	291

1.3 DIESEL EXHAUST

1.3.1 Introduction

Diesel exhaust is the single most common source of particulate matter in urban environments. In London, 90% of PM_{10} is derived from diesel exhaust (COMEAP 1995), whilst in Los Angeles it is 40% (Glovsky, M.M. *et al.* 1997).

1.3.2 Composition of diesel exhaust

Emissions from diesel engines are as a result of complete and incomplete combustion of fuel and lubricating oil, and consist of a complex mixture of particulates and gases. Diesel engines produce up to 100-150 times more particles per mile than petrol engines fitted with catalytic converters (COMEAP 1996) and diesel exhaust is one of the major contributors to particulate air pollution in cities (UNEP 1994; COMEAP 1995).

DEP air pollution is a mixture of solid particles and liquid droplets that vary in mass, size and chemical composition depending on the sources and meteorological conditions. The levels of particulate matter (PM) air pollution are expressed in relation to the particle size. Particles with an aerodynamic diameter equal to or less than 10μm (PM_{10}), are capable of being inhaled into the airways, and are known to cause adverse health effects. Ultrafine particles with an aerodynamic diameter equal to or less than 2.5μm ($PM_{2.5}$), have been shown to penetrate further through the epithelium and vascular walls. They may then be transported to the lung-associated lymph nodes where they may affect the proliferation of immune cells and antibody production (Bice, D.E *et al.* 1985).

DEP consist of a carbon core, onto which chemicals such as acids, (polycyclic) aromatic organic compounds, heavy metals, endotoxins and allergens may be adsorbed (Yamaki, N. *et al.* 1986). The quantity of soot particles and the particle-associated organics emitted from the tail pipe of a diesel-powered vehicle depend primarily on the engine type and combustion conditions but also on fuel properties (Scheepers, P.T. *et al.* 1992a). The most important fuel parameters are: polycyclic aromatic hydrocarbons (PAH) content, 90% distillation point, final boiling point, specific heat, aromatic content, density, and sulphur content (Westerholm, R. *et al.* 1994). After emission, the properties of the particles may change because of atmospheric processes such as ageing and resuspension, or the particle-associated organics may be subject to (photo)chemical conversions. This can make the toxicological evaluation of the health hazards of diesel engine emissions difficult (Scheepers, P.T. *et al.* 1992b).

The vapour phase of DE contains typical combustion gases – carbon monoxide, carbon dioxide, nitric oxide and ammonia, as well as low molecular hydrocarbons including aldehydes, organic acids, monocyclic and polycyclic aromatic compounds and their derivatives (QUARG 1996).

1.3.3 Diesel exhaust exposure levels

Working or living near busy arterial roads may result in high levels of diesel exhaust exposure (Jamriska, M. *et al.* 1999). Occupational exposure to diesel exhaust may result in very high levels, particularly if the exposure takes place in a poorly ventilated environment. Personal sampling techniques were used to evaluate fire-fighter exposure to particulates from diesel engine emissions in New York, Boston and Los Angeles (Froines, J.R. *et al.* 1987).

During an eight-hour shift, the resulting exposure levels of total airborne particulates from diesel exhaust were 170 to 480 μ/m^3. Railroad workers, lorry drivers and ferry operatives have also been found to have high DE exposure levels (Woskie, S.R. *et al.* 1988).

1.4 HEALTH EFFECTS OF DIESEL EXHAUST

Assessing the health effects of diesel exhaust is difficult because it is a complex mixture of particulate and gaseous compounds. There is no single marker of DE exposure and instead the health effects are usually calculated as single components, for example particulate matter, nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and carbon monoxide (CO).

Much of the research on adverse effects of diesel exhaust, both *in vivo* and *in vitro*, has been conducted in animals (Weisenberger, B.L. 1984). Questions remain concerning the relevance of exposure levels and whether findings in such models can be extrapolated into humans. It is therefore imperative to further assess acute and chronic effects of diesel exhaust in mechanistic studies with careful consideration of exposure levels. Whenever possible and ethically justified, studies should be carried out in humans (Utell, M.J. *et al.* 2000; Sydbom, A. *et al.* 2001).

1.4.1 Diesel exhaust and mortality

Whilst evidence exists linking particulate air pollution and increased mortality rates (Dockery, D.W. *et al.* 1993), finding similar evidence for diesel exhaust *per se* is difficult. DE makes up the vast majority of particulate matter exposure in urban areas, but extrapolating this to increased mortality proves difficult. Indirect evidence comes from studies using murine

models that suggest that high dose chronic exposure to DE is associated with an increase in mortality (Health Effects Institute 1995). Studies of occupational groups having high exposure levels to diesel exhaust indicate a 20-60% greater risk of lung cancer compared to an unexposed group (Scheepers, P.T. *et al.* 1992b).

1.4.2 Diesel exhaust and morbidity

Acute effects of diesel exhaust exposure include irritation of the nose and eyes, lung function changes, respiratory changes, headache, fatigue and nausea (Rudell, B. *et al.* 1994; Rudell, B. *et al.* 1996). Chronic exposures are associated with cough, sputum production, wheeze (Gamble, J. *et al.* 1987) and also lung function decrements (Ulfvarson, U. *et al.* 1991).

In addition to symptoms, exposure studies in healthy humans have documented a number of profound inflammatory changes in the airways (Salvi, S.S. *et al.* 1997; Rudell, B. *et al.* 1999; Salvi, S.S. *et al.* 1999a; Salvi, S.S. *et al.* 2000). Notably these can be found before changes in pulmonary function can be detected. It is likely that such effects may be even more detrimental in asthmatics and other subjects with compromised pulmonary function. There are also observations supporting the hypothesis that diesel exhaust is one important factor contributing to the allergy pandemic (Frew, A.J. *et al.* 1997; Salvi, S.S. *et al.* 1999b).

1.4.2.1 Changes in symptomatology and lung function tests

Living in urban, as compared with rural areas, is associated with an increased prevalence of respiratory symptoms including chest tightness and breathlessness, exercise-induced coughing attacks and prolonged cough (Braback, L. *et al.* 1994; Oosterlee, A. *et al.* 1996). Similar symptoms have been positively correlated with particulate exposure levels (Dockery, D.W. *et al.* 1989) and exposure to truck traffic (Duhme, H. *et al.* 1996).

Human exposure studies report that the most prominent symptoms during exposure to diesel exhaust are irritation of the eyes and nose and an unpleasant smell (Rudell, B. *et al.* 1996). Both airway resistance (Raw) and specific airway resistance (SRaw) have been shown to increase significantly during diesel exhaust exposures and there is epidemiological evidence that diesel exhaust exposure may also cause acute and chronic lung function impairment with decreases in FEV_1, vital capacity, residual volume and total lung capacity (Dahlqvist, M. 1995; Jammes, Y. *et al.* 1998). High occupational levels of exposure to diesel exhaust (such as workers on a roll-on-roll-off ferry) are associated with a statistically significant temporary reduction in spirometry (Ulfvarson, U. *et al.* 1987). This impairment may be reduced by the equipping of trucks with specially designed microfilters mounted on the exhaust pipes to remove the particulate fraction (Ulfvarson, U. *et al.* 1990).

1.4.2.2 Diesel exhaust and non-specific host defences

Epidemiological data suggests that the incidence of pneumonia and chest infection is increased with increases in particulate matter, NO_2 and SO_2, which are all constituents of diesel exhaust (Schwartz, J. 1994; Sandström, T. 1995). This effect has been confirmed experimentally with acute and subacute exposures of diesel exhaust causing an increased

mortality in mice following exposure to aerolised β-haemolytic streptococci (Campbell, K.I. *et al.* 1981). Repeated exposures in human volunteers to NO_2, one of the constituents of diesel exhaust, resulted in decreased amounts of lavaged alveolar macrophages, B-cells, and natural killer (NK)-cells, while the T-helper-inducer/cytotoxic-suppressor cell ratio was also altered (Sandström, T. *et al.* 1992). Lymphocyte numbers in peripheral blood were also reduced after exposure. These results suggest that repeated exposure with NO_2 adversely affects the immune defence and could contribute directly or indirectly to the increased susceptibility to airway infections.

An increased susceptibility to respiratory infections may also be caused by damage to the ciliated cells and impaired mucociliary clearance, or by reduced alveolar macrophage phagocytosis. There is some evidence that diesel exhaust promotes epithelial damage with subsequent changes in epithelial permeability (Murphy, S.A. *et al.* 1998) and the cilia of the trachea and large airways may also be adversely affected (Ishinishi, N. *et al.* 1988). Mucociliary clearance of particles may therefore be reduced after both acute and chronic exposures to diesel exhaust (Wolff, R.K. 1986).

Diesel exhaust has also been shown in animal studies to depress the phagocytic activity of alveolar macrophages (Castranova, V. *et al.* 1985) and to reduce the clearance of particle-laden macrophages from the lung in a dose-dependent manner (Griffis, L.C. *et al.* 1983; Lee, P.S. *et al.* 1983; Chan, T.L. *et al.* 1984; Heinrich, U. *et al.* 1986). In monocytes and macrophages, phagocytosis is associated with an increase in oxygen (O_2) consumption and superoxide anion (O_2^-) generation, which is known as the 'respiratory burst'. O_2^- is the precursor of highly reactive, oxygen-derived free radicals that are used to kill potential

pathogens. Experimental work *in vitro* suggests that airborne particulate matter has a toxic effect that induces the disintegration of the monocyte or macrophage plasma membrane (Fabiani, R. *et al.* 1997). This causes cytosolic factors (proteins and co-enzymes) necessary for O_2^- production to leak from the cells, thereby reducing superoxide generation and impairing the defence against bacteria and other pathogens. Diesel exhaust may also cause alveolar destruction in a concentration- and duration-dependent manner (Nagai, A. *et al.* 1996).

1.4.2.3 Diesel exhaust and allergic immune response

Allergy can be considered a T_H2 disease, driven by cytokines IL-4, -5, -10 and -13 and associated with an excessive production of IgE antibody to allergens (Bellanti, J.A. 1998). Epidemiological data suggests that allergen exposure in association with air pollution leads to sensitisation and an increase in total and specific IgE. This has been demonstrated experimentally using a murine model. IgE production, in mice immunized with intraperitoneally injected ovalbumin (OVA), was enhanced when the OVA was mixed with DEP (Muranaka, M. *et al.* 1986). This enhanced IgE response with DEP was also seen when the researchers injected Japanese cedar pollen. Japanese cedar pollen in association with pyrene extracted from DEP (Suzuki, T. *et al.* 1993) and polyaromatic hydrocarbons (PAHs) from DEP (Takenaka, H. *et al.* 1995) has a similar effect on IgE production suggesting that the chemical composition of DEP may be important. The ability of DEP to act as an adjuvant in the ragweed allergen response has been examined by nasal provocation in ragweed allergic subjects using 0.3 mg DEP, ragweed allergen, or both. Although allergen and DEP each enhanced ragweed-specific IgE, DEP plus allergen promoted a 16-times greater production of antigen-specific IgE (Diaz-Sanchez, D. *et al.* 1997).

Intranasal or intratracheal challenge of DEP with allergen has an adjuvant effect on IgE production in mice (Muranaka, M. *et al.* 1986; Takafuji, S. *et al.* 1987), and rats (Steerenberg, P.A. *et al.* 1999), and researchers were also able to demonstrate the presence of DEP-loaded macrophages in association with pollen in the alveoli (Steerenberg, P.A. *et al.* 1999). Direct evidence for DEP-enhanced local production of IgE comes from a study where DEP challenge was performed in human volunteers and then nasal lavage carried out (Diaz-Sanchez, D. *et al.* 1994). The results showed an increase in the number of IgE- secreting cells in lavage fluid and a concomitant increase in c-epsilon mRNA production in the lavage cells. Additionally, DEP altered the relative amounts of five different epsilon mRNAs generated by alternative splicing (mRNAs that code for different IgE proteins). These results show that DEP exposure *in vivo* causes both quantitative and qualitative changes in local IgE production. *In vivo* IgE isotype switching (from c-mu to c-epsilon) has also been demonstrated after combined intranasal DEP plus ragweed challenge (Fujieda, S. *et al.* 1998).

DEP exposure is also able to lead to primary sensitisation of humans by driving a *de novo* mucosal IgE response to a neoantigen (Diaz-Sanchez, D. *et al.* 1999). In this study, ten atopic subjects were given an initial nasal immunization with a neoantigen, keyhole limpet hemocyanin (KLH), followed by 2 biweekly nasal challenges with KLH. Identical nasal KLH immunization was also performed on 15 different atopic subjects, but DEPs were administered 24-hours before each KLH exposure. Exposure to KLH alone led to the generation of an anti-KLH IgG and IgA humoral response but no anti-KLH IgE appeared in any subjects. In contrast, when challenged with KLH preceded by DEPs, nine of the 15 subjects produced anti-KLH-specific IgE and had significantly increased IL-4 levels in nasal lavage fluid. These studies demonstrate that DEPs can act as mucosal adjuvants to a *de novo* IgE response and may increase allergic sensitisation.

IL-4 is a key cytokine in the allergic response. Research data suggests that pyrene, a major compound of diesel exhaust particles, is able to enhance basal transcription of the human and mouse IL-4 promoter thus inducing the production of IL-4 (Bommel, H. *et al.* 2000). Nasal challenge with allergen plus DEP causes a significant increase in the expression of mRNA for T_H2-type cytokines (including IL-4, -5, -10 and -13 (Diaz-Sanchez, D. 1997) and *in vitro* work also suggests that DEP are able to skew the immune response toward the T_H2 side (van Zijverden, M. *et al.* 2000). IL-4 is produced by mast cells and *in vivo* work has demonstrated that mast cell activation by DEPs plus allergen will also affect cause mast cell degranulation with histamine release (Diaz-Sanchez, D. *et al.* 2000) and may augment the effects of histamine leading to a subsequent increase in allergic symptoms (Kobayashi, T. *et al.* 1997). Other work suggests that repeated exposure to diesel exhaust may, in time, cause the down-regulation of histamine receptors in the trachea (Lall, S.B. *et al.* 1997). IL-4 production in mediastinal lymph node cells has been shown to be doubled in mice intratracheally instilled with DEP and antigen (Fujimaki, H. *et al.* 1994), and residual oil fly ash (ROFA - a transition metal-rich emission source PM sample) plus antigen can increase the levels of IL-4 in the bronchoalveolar lavage (BAL) eight-fold, compared to antigen alone (Gavett, S.H. *et al.* 1999).

DEP with antigen has also been shown to markedly increase interleukin-5 protein levels in lung tissue and BAL supernatants in mice, compared with either antigen or DEP alone (Takano, H. *et al.* 1997) and also causes an eosinophilia (Takano, H. *et al.* 1998), and neutrophilia in the bronchial submucosa (Miyabara, Y. *et al.* 1998).

1.4.2.4 Diesel exhaust and cellular inflammation

Particulate matter may induce cellular inflammation in the airways through the effects of potent pro-inflammatory cytokines including IL-6, -8, RANTES and GM-CSF. Human monocytes, nasal epithelial cells and bronchial epithelial cells exposed to particulates produce significantly enhanced levels of IL-6 and IL-8 (Steerenberg, P.A. *et al.* 1998; Monn, C. *et al.* 1999; Terada, N. *et al.* 1999), and may also release mediators which induce a systemic inflammatory response that includes stimulation of the bone marrow (Mukae, H. *et al.* 2000).

Human exposure studies in asthmatics show that DE is associated with a significant increase in airway hyperresponsiveness and also induces a significant increase in sputum levels of IL-6 (Nordenhall, C. *et al.* 2001). Freshly generated diesel exhaust (DE) introduced into an *in vitro* cell exposure system stimulated human epithelial cells to induce IL-8 mRNA and also increased IL-8 release (Abe, S. *et al.* 2000). Exposure to DE in healthy subjects similarly causes increases in IL-6, IL-8 and also a polymorphonuclear neutrophilia (Blomberg, A. *et al.* 1999; Nightingale, J.A. *et al.* 2000; Nordenhall, C. *et al.* 2000). In one such study, in which healthy subjects were exposed to one hour of diluted diesel exhaust, DE caused an increase in neutrophils in airway lavage and was also found to be able to induce a migration of alveolar macrophages into the airspaces, together with reduction in $CD3^+$ and $CD25^+$ cells (Rudell, B. *et al.* 1999).

The mechanisms by which DE may increase cytokine production and release have been studied *in vitro*. Suspended DEP (1-50 μg/ml) were found to increase the levels of IL-8 mRNA, largely due to an increase in transcriptional rate (Takizawa, H. *et al.* 1999). This increase was associated with the activation of NF-kappa B, which has been shown to activate

transcription of a great variety of genes encoding immunologically relevant proteins thereby increasing the expression of inflammatory cytokines *in vitro* (Baeuerle, P.A. *et al.* 1994).

Another important process may be the activation of p38 mitogen-activated protein (MAP) kinase. One study showed that DEP induced IL-8 and RANTES production along with the threonine and tyrosine phosphorylation of p38 MAP kinase, indicating the activation of p38 MAP kinase (Hashimoto, S. *et al.* 2000). Inhibiting p38 MAP kinase activation also resulted in the inhibition of the production of IL-8 and RANTES.

It is possible that metals present in an air pollution particle may induce the synthesis and expression of the inflammatory cytokines IL-8, IL-6, and TNF-α. To test this hypothesis, normal human bronchial epithelial (NHBE) cells were exposed to ROFA containing the transition metals vanadium, nickel, and iron (Carter, J.D. *et al.* 1997). NHBE cells exposed to ROFA produced significant amounts of IL-8, IL-6, and TNF-α, as well as mRNAs coding for these cytokines. Cytokine production was inhibited by the inclusion of either the metal chelator deferoxamine or the free radical scavenger dimethylthiourea. Subsequent work has also demonstrated that the acidic, soluble components of ROFA initiate cytokine release in NHBE cells through activation of both capsaicin- and pH-sensitive irritant receptors (Veronesi, B. *et al.* 1999).

As well as increasing interleukins, DEP have also been shown to significantly increase the release of GM-CSF (Ohtoshi, T. *et al.* 1998) and soluble intercellular adhesion molecule-1 (sICAM-1) in NHBE (Bayram, H. *et al.* 1998). NHBE of asthmatic patients have been shown to release significantly greater amounts of IL-8, GM-CSF and sICAM-1 than

HBEC of non-asthmatic subjects (Marini, M. *et al.* 1992), and this increases further with the addition of DEP (Devalia, J.L. *et al.* 1999), which may at least in part explain the increased sensitivity of the airways of asthmatics to air pollutants such as DEP. These findings have not always been reproduced in human exposure studies in asthmatics (Frew, A.J. *et al.* 2001), but healthy subjects examined six- hours after DE exposure, show a significant increase in neutrophils and B lymphocytes in airway lavage, along with increases in histamine and fibronectin (Salvi, S.S. *et al.* 1999a). The bronchial biopsies in this study also showed a significant increase in neutrophils, mast cells, $CD4^+$ and $CD8^+$ T lymphocytes along with up-regulation of the endothelial adhesion molecules ICAM-1 and VCAM-1, with increases in the numbers of LFA-1^+ cells in the bronchial tissue. A marked systemic response was also elicited with significant increases in neutrophils and platelets in the peripheral blood following DE exposure.

1.4.2.5 Diesel exhaust and generation of reactive oxygen species

Inflammation and cytokine release following DE exposure may be as a result of oxidative stress and the generation of reactive oxygen species in macrophages (Casillas, A.M. *et al.* 1999; Hetland, R.B. *et al.* 2001) and other cells (Hitzfeld, B. *et al.* 1997). The free radical activity of PM_{10} has been confirmed *in vivo* by a decrease in reduced glutathione levels in the BAL fluid six-hours after DEP exposure in rats, and confirmed *in vitro* by its ability to deplete supercoiled plasmid DNA, an effect which could be reversed by mannitol, a specific hydroxyl radical scavenger (Li, X.Y. *et al.* 1996). These effects may be abrogated by antioxidants and in particular, antioxidants in the epithelial lining fluid (ELF) of the respiratory tract (Kelly, F.J. *et al.* 1996). As even mild asthmatics have reduced antioxidants in the ELF (Kelly, F.J. *et al.* 1999), this may make them particularly vulnerable to the effects of DE.

To determine if diesel exhaust (DE) exposure modifies the antioxidant defence network within the respiratory tract lining fluids, fifteen healthy, non-smoking, asymptomatic subjects were exposed to diluted diesel exhaust (300μg/m^3 particulates, 1.6 ppm nitrogen dioxide) for one hour (Blomberg, A. *et al.* 1998). Bronchoscopy was performed six hours after the end of DE exposure and ascorbic acid, uric acid and reduced glutathione concentrations were determined in nasal, bronchial, bronchoalveolar lavage and plasma samples. Nasal lavage ascorbic acid concentration increased 10-fold during DE exposure but there was no significant effect on other nasal lavage chemicals, nor plasma, bronchial wash, or bronchoalveolar lavage antioxidant concentrations. The physiological response to acute DE exposure is an increase in the level of ascorbic acid in the nasal cavity. This response appears to be sufficient to prevent further oxidant stress in the respiratory tract of normal individuals.

There is some evidence that it is the transition metals in PM that promote the formation of reactive oxygen species and subsequent lung injury and inflammation, as these effects may be inhibited by deferoxamine, a chelator of transition metals (Monn, C. *et al.* 1999; Gavett, S.H. *et al.* 2001; Prahalad, A.K. *et al.* 2001). It would appear that particle type, size, and surface area are all important factors when considering particle-antioxidant interactions in the airways (Zielinski, H. *et al.* 1999).

1.5 LOWER AIRWAY INFLAMMATION IN ALLERGIC RHINITIC SUBJECTS

Allergic rhinitis is a common chronic condition that is characterized by inflammation of the nasal mucosa. Although allergic rhinitis is a condition with upper respiratory symptoms, there is a growing body of evidence to suggest that allergic rhinitis may be linked to the development of systemic allergic manifestations that include allergic asthma. This is discussed further in section 1.1.4.

1.5.1 Bronchial hyperresponsiveness

Bronchial hyperresponsiveness (BHR) may be used as a surrogate marker for inflammation in the lower airways. It is one of the main features of allergic asthma, but has also been demonstrated to a lesser extent in non-asthmatic allergic rhinitics (Stevens, W.J. *et al.* 1980; Gutierrez, V. *et al.* 1998), suggesting an overlap between both diseases. This suggests that IgE-dependent inflammation may occur in the lower airway that can increase bronchial hyperresponsiveness without at the same time precipitating obvious obstruction. Allergic rhinitic subjects may demonstrate an increase in BHR in response to allergen (Ahmed, T. *et al.* 1981; Boulet, L.P. *et al.* 1995) although this is less than that of asthmatics (Witteman, A.M. *et al.* 1997). Intranasal administration of steroids may reduce lower airways BHR, suggesting that the latter may be due to mediator or chemotactic factors (Aubier, M. *et al.* 1992).

1.5.2 Sputum induction studies

Sputum induction studies may be used to examine lower airway inflammation. Allergic rhinitic subjects with BHR have been shown to have increased numbers of eosinophils in the sputum compared to both allergic rhinitic (AR) subjects without BHR, and with healthy controls (Gutierrez, V. *et al.* 1998). A second study has confirmed these findings although it also showed that sputum eosinophilia failed to be significantly associated with methacholine responsiveness and concluded that bronchial eosinophilia alone is insufficient to cause asthmatic symptoms (Polosa, R. *et al.* 2000). In another study, researchers examined 31 mild asthmatics and 15 AR patients, sensitised to Dermatophagoides pteronyssinus (Der p 1) (Alvarez, M.J. *et al.* 2000b). After exposure to Der p 1, AR patients developed blood and sputum eosinophilia which was similar to that observed among the asthmatics, although the AR subjects had a lower degree of bronchial sensitivity to allergen. The BHR remained unchanged in the AR group, in contrast to the asthmatic group, again implying that sputum eosinophilia and BHR are unrelated. This theory remains controversial, as other studies have demonstrated a positive correlation between sputum eosinophilia and BHR (Foresi, A. *et al.* 1997).

1.5.3 Exhaled nitric oxide levels

Exhaled nitric oxide (eNO) has been proposed as a potential indirect marker of lower airway inflammation in asthma. A number of studies have investigated the existence of lower airways inflammation in allergic rhinitis by measuring eNO (Martin, U. *et al.* 1996; Henriksen, A.H. *et al.* 1999). In one such study, eNO measurements were performed in and out of pollen season in 32 patients with symptomatic and asymptomatic seasonal allergic

rhinitis and in 80 healthy volunteers (Gratziou, C. *et al.* 2001). Exhaled NO was significantly elevated in patients with symptomatic and asymptomatic seasonal allergic rhinitis, both in and out of pollen season, as compared to healthy volunteers. There was a higher increase in eNO from both the upper and lower airways during the allergen exposure season, in patients with symptoms, and in patients with bronchial hyperreactivity. The increased exhaled NO in symptomatic patients was reduced only by inhaled steroids and not by nedocromil. These findings suggest that lower airways inflammation exists in both symptomatic and asymptomatic patients with seasonal allergic rhinitis, and persists out of the pollen season.

1.5.4 Histology of allergic rhinitis compared to asthma

In humans, allergic rhinitis is characterised by an eosinophilic inflammation in the upper airway and thickening of the nasal mucosa (Lim, M.C. *et al.* 1995). Similar findings are reported in the lower airways of asthmatics (Dunnill, M.S. *et al.* 1969; Hogg, J.C. 1997). The upper and lower airways are lined by the same respiratory epithelium although some anatomical differences are evident. For example, the nose has a capacitance vessel network while the lower airways possess smooth muscle. However, both are responsive to neurohumoral influences (Rowe-Jones, J.M. 1997). It is perhaps not surprising that they respond to airborne allergens in a very similar manner.

The similarities in the cellular inflammation of allergic rhinitis compared to asthma may be as a result of the release of the same pro-inflammatory chemokines. When human nasal epithelial cells (HNECs) from atopic individuals were cultured (Calderon, M.A. *et al.* 1997), these cells released significantly greater amounts of IL-8, GM-CSF, TNF-α, and RANTES than HNECs from non-atopic individuals. Other work has also shown that the

inflammatory process in the nose is orchestrated by IL-4 and -5 (White, M.V. *et al.* 1992; Naclerio, R. *et al.* 1997). These pro-inflammatory cytokines are the same as those cultured from human bronchial epithelial cells from asthmatic subjects, and suggest that the inflammatory process in the two diseases is essentially the same. One theory suggests that subjects with allergic asthma differ from subjects with rhinitis only in their capacity to release more mediators into the airways on antigen challenge (Lam, S. *et al.* 1991).

1.5.5 Inflammation in bronchial biopsies of non-asthmatic atopics

A limited amount of experimental work has been done to elucidate the nature and extent of cellular inflammation in the lower airways of allergic rhinitic subjects. One such study took bronchial biopsies from allergic rhinitic subjects both in and out of the pollen season (Chakir, J. *et al.* 2000). This study showed that natural pollen exposure was associated with an increase in lymphocyte numbers, eosinophil recruitment, and IL-5 expression in the bronchial mucosa of non-asthmatic subjects with allergic rhinitis.

A second study compared the lower airways inflammation in bronchial biopsies from atopic asthmatics, atopic non-asthmatics (including subjects with allergic rhinitis or who were skin prick positive), and normals (Djukanovic, R. *et al.* 1992). The numbers of mast cells were no different between the three groups, but electron microscopy showed that mast cell degranulation, although less marked in atopic non-asthmatics, was a feature of atopy in general. The numbers of eosinophils were greatest in the asthmatics, low or absent in the normals and intermediate in the atopic non-asthmatics. In both atopic groups, eosinophils showed ultrastructural features of degranulation. Measurements of subepithelial basement

membrane thickness showed that the collagen layer was thickest in the asthmatics, intermediate in the atopic non-asthmatics and thinnest in the normals. A similar study showed no significant differences between non-asthmatic atopics and healthy controls, although asthmatic subjects had increased levels of $CD25^+$ lymphocytes and activated ($EG2^+$) eosinophils in the airways (Bradley, B.L. *et al.* 1991).

1.6 AIMS OF THIS STUDY

This study had two main aims: firstly to identify the cellular constituents of inflammation in the lower airways of allergic rhinitic subjects and compare this to asthmatics and healthy controls; and secondly to examine the inflammatory response to diesel exhaust 18-hours after exposure in the same groups of atopic and non-atopic subjects.

1.6.1 Inflammation in the lower airways of allergic rhinitics

My hypothesis was that allergic rhinitic subjects would exhibit lower airway inflammation that was similar to that of asthmatics, but would differ either in extent and/or cellular profile and would therefore be asymptomatic. Healthy controls would not show any evidence of inflammation. To test this hypothesis bronchial biopsies were collected from allergic rhinitic, asthmatic and healthy subjects, and processed for immunohistochemistry in order to compare the inflammatory reaction.

1.6.2 Late inflammatory response to diesel exhaust exposure

I hypothesised that the acute inflammatory reaction evident in healthy subjects six-hours after DE exposure would have been further augmented in the asthmatic subjects, making them predisposed to exacerbations of asthma; that the allergic rhinitic subjects might show a similar, but less marked, potentiation of inflammation; while in the healthy controls resolution of the initial inflammatory response may have occurred. Bronchial biopsies were collected from subjects 18-hours after a two-hour exposure to DE. The biopsies were processed for immunohistochemistry and the inflammatory profile compared to the baseline and between subject groups.

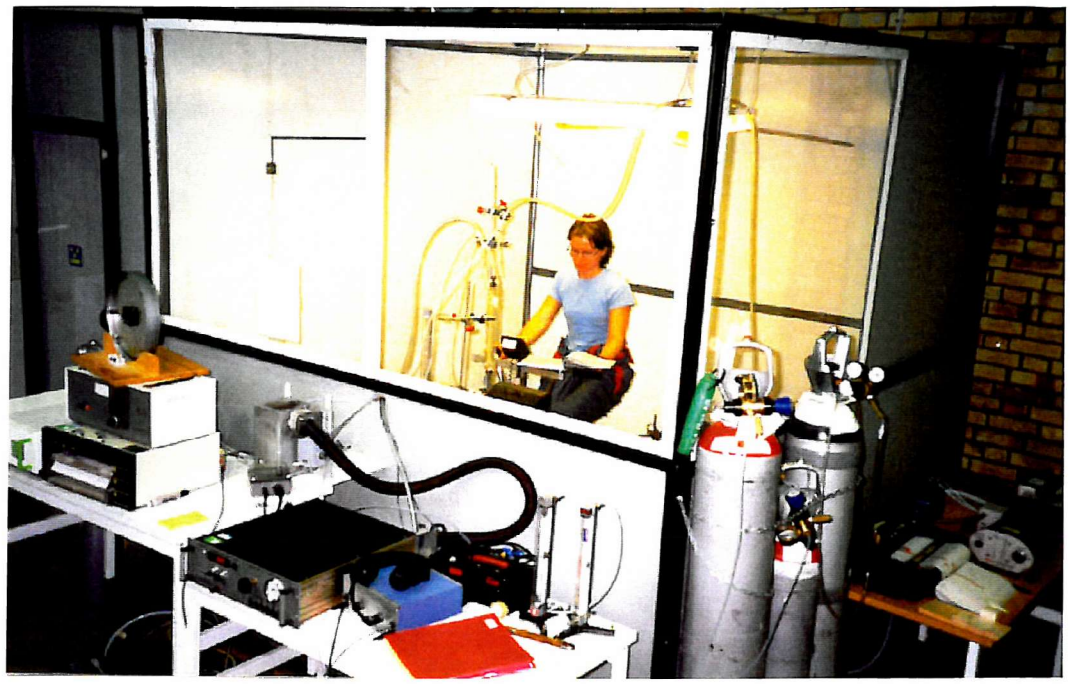
CHAPTER TWO

Materials and Methods

2.1 DIESEL EXPOSURE CHAMBER

Exposures were performed at the Medical Division of the National Institute for Working Life in Umeå, Sweden, in an exposure chamber designed and developed by Rudell *et al* (Rudell, B. *et al.* 1994) (Fig. 2.1). This allows a pre-determined concentration of diluted diesel exhaust to be maintained within the chamber throughout the exposure time. A small entrance lobby attached to the exposure chamber allows the entry and exit of subjects with minimal alteration to the concentration of diluted diesel exhaust within. The chamber itself is constructed with a large observation window and has a radio-communication link installed to allow constant audio-visual contact between the subject and the experiment supervisor. The inlet for diesel exhaust is located in the middle of the back wall, 45cm above the floor. The outlet is in the opposite wall and the air in the chamber is changed every three minutes. Using a shunt tube and an adjustable valve, the diesel exhaust is diluted by mixing with fresh, filtered air and then fed into the chamber at a constant flow rate. The tubing is preheated to 200°C to prevent condensation of the exhaust occurring. Further adjustment to the concentration of the diluted DE may be made by varying the diameter of a diaphragm in the shunt and/or adjusting the valve opening in the shunt tube. The temperature and humidity in the chamber are kept constant throughout the exposure time at 20°C and 50% respectively. The chamber contains an exercise bike, a chair for rest periods, monitoring equipment and water for the subject to drink if thirsty.

Fig 2.1: Diesel exposure chamber. Subject on bicycle ergometer within exposure chamber. Note the monitoring equipment located outside the chamber.



2.1.1 Diesel exhaust - production and monitoring (Fig 2.2)

Diesel exhaust was generated from an idling Volvo diesel engine located in an adjacent room. The engine used was a TD1F Inter-cooler; a 6 cylinder 4 stroke injection turbo charged diesel engine Model 1990. The diesel fuel used was OK Promil 1 (OK Petroleum, Stockholm, Sweden) and its composition was as follows: cetane number 51, aromatics 25% vol, polycyclic aromatic hydrocarbons (PAHs) 0.5% vol, sulphur 0.06% weight, carbon 86.4% weight, hydrogen 13.5% weight, nitrogen <0.02% weight, oxygen <0.1% weight. The 10% volume boiling point was 200°C, 50% volume boiling point was 282°C and 95% volume boiling point was 355°C. Predetermined levels of particulates and NO/NO_2 could be achieved within the chamber within 5-7 minutes and then remained at a steady state throughout the exposure period.

Monitoring established that each subject was exposed to the following constituents of diesel exhaust:

-PM_{10} 100 $\mu g/m^3$

-NO_2 0.4 ppm

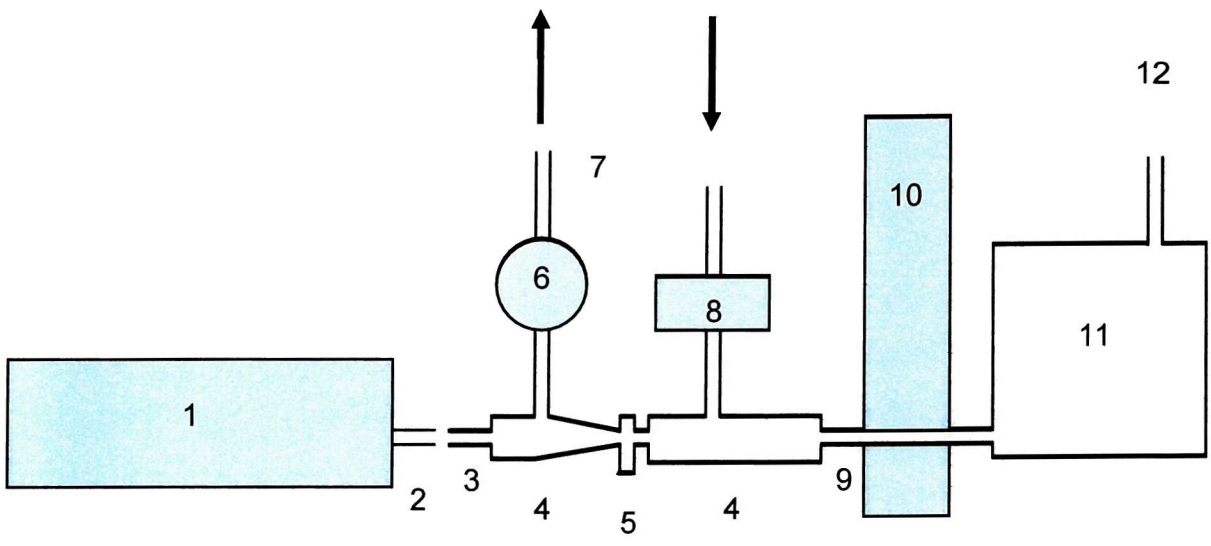
-NO 1.2 ppm

-CO 8.7 ppm

-Total hydrocarbons 1.09 ppm

Continuous analysis of carbon monoxide (CO) was monitored by a Miran 1-A (Foxboro Co., East Bridgewater, Massachussets, USA); nitrogen oxides (NO and NO_2) by a chemiluminescence instrument (CSI 1600 oxides of nitrogen analyser, Columbia, Scientific Industries Corporation, Austin, Texas, USA); total hydrocarbons with an FID instrument, model 3-300 (J.U.M. Engineering, Munich, Germany) with a heated prefilter and calibrated with propane; the number and mass of hydrocarbons by a condensation particle laser counter (Model 3022, TSI Inc., St Paul, Minnesota, USA).

FIG 2.2: SCHEMATIC REPRESENTATION OF THE DIESEL EXHAUST EXPOSURE CHAMBER.



1. Diesel engine
2. Exhaust pipe
3. Flexible metallic tube with an inflatable sleeve
4. Metallic shunt / Dilutor
5. Adjustable diaphragm
6. Valve – outlet for undiluted diesel exhaust
7. Tube for shunting undiluted diesel exhaust
8. Fan for dilution with air
9. Tube for diluted diesel exhaust and inlet into exposure chamber
10. Wall between diesel engine and experimental hall
11. Exposure chamber
12. Outlet / Waste fan

2.1.2 Exposure protocol

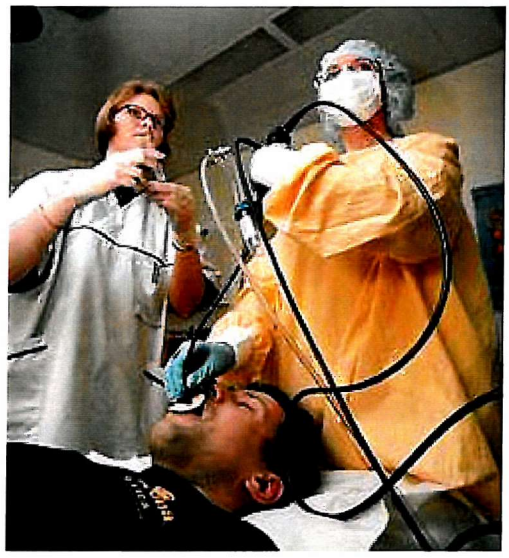
The exposures were supervised by a physician or research nurse and a technical expert. Subjects were randomly exposed to air and diesel exhaust (PM_{10} 100 $\mu g/m^3$) for two-hours on two separate occasions in a single-blinded manner. At least two weeks separated the two exposures. During each exposure the subject alternated rest with gentle exercise on a bicycle ergometer (V_E = 20 L/min/m^2 body surface) in 15 minutes intervals. Every 30 minutes the asthmatic subjects completed a symptom questionnaire to assess their level of discomfort. Asthmatic subjects were encouraged to use a short-acting β2-agonist inhaler if required and pre- and post lung function tests were recorded in this group.

2.2 FIBREOPTIC BRONCHOSCOPY (Fig 2.3)

Fibreoptic bronchoscopies were performed 18-hours post-exposure at the Department of Respiratory Medicine and Allergy at the University Hospital, Umeå, Sweden. Subjects were premedicated with 1.0 mg of atropine given subcutaneously 30 minutes before the procedure. Topical local anaesthetic (lignocaine) was administered to the pharynx, larynx and upper trachea. Most subjects tolerated the procedure without intravenous sedation, but in a few intravenous propofol was used. A flexible fibreoptic video bronchoscope (Olympus BFIT, Tokyo, Japan) was inserted into the airways via a mouthpiece. Three endobronchial biopsies were taken for immunohistochemistry using fenestrated forceps (Olympus FB-21C, Tokyo, Japan). The biopsies were obtained either from the anterior aspect of the main carina and the subcarina of the 3rd - 4th generation airways on the right side, or from the posterior aspect of the main carina and the corresponding subcarina on the left side. The biopsy sites were reversed to avoid artefact in the second bronchoscopy. Bronchial wash (BW) with 2 x

20ml 0.9% saline and bronchoalveolar lavage (BAL) with 3 x 60ml 0.9% saline was also performed after the tip of the bronchoscope was carefully wedged into the lingua or middle lobe bronchus. The samples were collected into a siliconized container placed in iced water. The material obtained from the first bronchial wash was analysed for cell numbers while the second was stored for future analysis of antioxidants and gene transcripts. The bronchial lavage fluid was pooled and analysed for cell numbers.

FIG 2.3: SUBJECT UNDERGOING FIBREOPTIC BRONCHOSCOPY IN UMEÅ, SWEDEN



2.3 Analysis of Bronchial Wash and Bronchoalveolar Fluid

The BW and BAL samples recovered into the siliconized containers were filtered through a nylon filter (pore diameter 100μm, Syntab AB, Malmo, Sweden) and centrifuged at 400g for 15 minutes. The cell pellets were separated from the supernatants and resuspended in PBS at 10^6 cells/ml. The total number of cells was counted in a Burker chamber. Centrifuged specimens with 5 x 10^4 cells/slide were prepared using a cytospin 3® (Shandon Southern Instruments Inc., Sewikly, PA, USA) at 1000 rpm for 5 minutes and differential counts were measured after staining with May-Grunwald Giemsa, counting 400 cells per slide.

2.4 Immunohistochemistry

2.4.1 Principles

Immunohistochemistry is the method by which specific tissue antigens expressed on the surface of cells *in situ* are identified with labelled antibodies. The technique employed in this research was the streptavidin biotin-peroxidase detection system. The tissue is first incubated with a primary antibody. It is then incubated with a secondary antibody covalently linked to biotin, a vitamin with low molecular weight. Avidin is a large glycoprotein that has a high affinity for biotin, each avidin molecule being able to bind up to four molecules of biotin. Preformed streptavidin-biotin complex conjugated to peroxidase is therefore added and links to the biotinylated antibody. This process is shown schematically in Fig 2.4. The peroxidase converts amino ethyl carbazole (AEC), to a coloured (red) reaction product at the end of the antigen:antibody interaction.

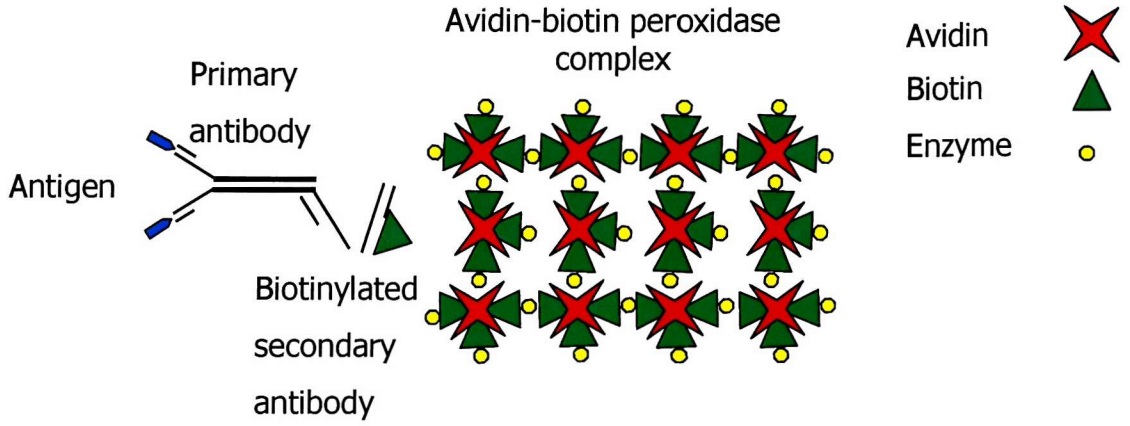


FIGURE 2.4: PRINCIPLES OF IMMUNOHISTOCHEMISTRY

2.4.2 Advantages of Glycol Methacrylate (GMA) resin technique

This technique was first developed by our department in 1993 (Britten, K.M. *et al.* 1993). It has a number of advantages over both frozen sections and paraffin embedding techniques:

- Sections may be cut much thinner (1-2 μm) thereby allowing adjacent sections to be compared for the identification of adhesion molecules
- Tissue morphology is preserved allowing the identification of immunoreactive cells in context
- Antigen sites remain available for immunostaining and do not require prior antigen retrieval

2.4.3 Materials for GMA embedding and tissue staining

- JB4 embedding kit (Park Scientific, Cat No. 0226)
  Contains solution A and B and benzoyl peroxide (GMA resin)

- Acetone (Merck, Cat No. 10003 4Q)
  For tissue fixation and processing

- Phenylmethylsulfonyl fluoride (Sigma, Cat No. P-7626)
- Iodacetamide (Sigma, Cat No. I-6125)
  Protease inhibitors

- Foetal calf serum and bovine serum albumin (Sigma) Blocking media
- Methyl Benzoate (Merck, Cat No. 29214)
  For processing

- 0.1% Sodium azide
- 0.3% Hydrogen peroxide
  Peroxidase inhibitors

- Tris Buffered Saline (TBS) (Merck)
  Mix sodium chloride (80g) + Tris (6.05g) + 1M HCl (38 ml) in 1L distilled water. Adjust pH to 7.65 and add to 9L distilled water to give a final pH of 7.6

- Tris/HCl Buffer (Merck)
  Mix 0.2M Tris (12ml), 0.1M HCl (19ml) and distilled water (19ml), and adjust pH to 7.6

- Biotinylated labelled second stage antibodies (Table 2.1)
- Streptavidin-biotin-peroxidase complex

- Mayer's Haematoxylin
- Toluidine Blue

- Aminoethyl carbazole (AEC)
  Mix AEC (0.4% prepared in dimethyl formamide) (1ml) with 30% hydrogen peroxide (15µl) and acetate buffer (14ml)

Table 2.1: Names and sources of antibodies used in Immunohistochemistry

Antibody	Marker	Cells	Source
AA1	Tryptase	Mast cells	DAKO, High Wycombe, UK
EG2	Cationic protein	Eosinophils	DAKO, High Wycombe, UK
NOE	Neutrophil elastase	Neutrophils	DAKO, High Wycombe, UK
CD3	CD3	T lymphocytes	DAKO, High Wycombe, UK
CD4	CD4	Helper T cells	BD Biosciences
CD8	CD8	Suppressor T cells	DAKO, High Wycombe, UK
CD25	CD25	Activated T cells	DAKO, High Wycombe, UK
CD68	CD68	Macrophages	DAKO, High Wycombe, UK
E selectin	E selectin	Endothelial cells	Cymbus
P selectin	P selectin	Endothelial cells	Immunotech, UK
ICAM-1	ICAM-1	Endothelial cells	Biosource
VCAM-1	VCAM-1	Endothelial cells	Serotec, UK
EN4	Endothelium	Panendothelial marker	Sanbio, UK

2.4.4 Methods

2.4.4.a. GMA embedding and processing

The endobronchial biopsies (measuring approximately 0.5-1mm diameter) were carefully extracted from the forceps and immediately placed in ice-cold acetone containing protease inhibitors (phenylmethyl-sulphonyl fluoride 2nM and iodoacetamide 2nM). The samples were then fixed overnight at –20°C. The next day the samples were put in acetone at room temperature for 15 minutes and then in methyl benzoate for a further 15 minutes. The tissue was then immersed in glycol methacrylate (GMA) monomer (Polysciences, Northampton, UK) plus methyl benzoate at 4°C for seven hours, during which time the GMA solution was changed three times. The tissue was finally embedded in GMA resin (prepared by mixing GMA monomer, N.N-dimethylaniline in PEG 400 and benzoyl peroxide) in flat-bottomed capsules. The biopsy was placed at the bottom of the capsule and the resin poured in carefully to ensure that there were no air bubbles. The capsule lid was closed and the resin was left to polymerise overnight at 4°C.

The blocks of GMA resin were then stored in airtight containers at –20°C until used for immunostaining. The samples were cut into 2μm thin sections using a microtome. The sections were floated onto ammonia water (1:500), picked onto 0.01% poly-L-lysine glass slides and allowed to dry at room temperature for one hour. Toluidine blue staining was performed to assess the best biopsies for immunostaining. Suitable biopsies had to have an analysable area (excluding muscle, glands and crush artefact) of >0.5mm^2 (Sullivan, P. *et al.* 1998). Once cut, sections were either stained that day or were stored wrapped in aluminium foil at –20°C for a maximum of two weeks in order to preserve tissue antigenicity.

2.4.4.b. Immunostaining

Sections were stained using the streptavidin-biotin peroxidase detection system.

Day 1

1. Remove blocking media from fridge.
2. Remove slides from freezer and place on tray
3. Prepare sodium azide solution (0.1% 5µl sodium azide + 50µl 30% H_2O_2).
4. Cover each biopsy specimen with sodium azide (approximately 150µl per slide) and incubate for 30 minutes.
5. Prepare primary antibody dilutions. The optimum dilution is determined by titration.
6. Wash slides with TBS – 3 times for 5 minutes each.
7. Drain slides and cover each biopsy specimen with blocking media (approximately 150µl per slide) and incubate for 30 minutes.
8. Drain slides and apply primary antibody dilutions. Cover with cover slips ensuring there are no trapped air bubbles.
9. Incubate overnight at room temperature.

Day 2

1. Prepare stage 2 antibodies (Biotin anti-mouse) in TBS at 1:300 dilution.
2. Wash slides with TBS – 3 times for 5 minutes each.
3. Drain slides and apply stage 2 antibodies. Incubate for 2 hours at room temperature.
4. Prepare stage 3 antibodies (Streptavidin + Biotin in horse radish peroxidase) in Tris HCl at 1:200 dilution.
5. Wash slides with TBS – 3 times for 5 minutes each.
6. Drain slides and apply stage 3 antibodies. Incubate for 2 hours at room temperature.
7. Wash slides with TBS – 3 times for 5 minutes each.
8. Drain slides and apply 150µl per slide AEC. Incubate for 30 minutes at room temperature.
9. Rinse slides with TBS and wash in running water for 5 minutes.
10. Counterstain with Mayer's Haematoxylin and blue stain for 30 seconds.
11. Wash slides in running water for 5 minutes.
12. Drain and dry slides. Apply crystal mount and bake in oven at 80°C for 10 minutes. Cool slides and mount in DPX.

2.4.5 Quantification of cells and adhesion molecules

Stained inflammatory cells were counted for each section. The epithelium and submucosa were counted separately and areas of damage, mucosal glands, blood vessels, muscle and folded tissue were excluded. The area of the submucosa and length of epithelium counted were measured using computed-assisted image analysis and the results expressed as cells/mm^2 and cells/mm respectively.

With the adhesion molecules, the number of blood vessels stained with a specific monoclonal antibody at standard dilution was compared to the number of vessels stained using the pan-endothelium marker EN4 (endothelium clone 4) in consecutive sections (2- μm apart). The number of positively stained blood vessels was then expressed as a percentage of the total vessel population.

Staining and counting of the biopsies was performed with the technician blinded to the subject group and the exposure. Photographs were obtained of each biopsy at magnification x5 using a camera attached to the microscope.

2.4.6 Tests for repeatability of counting and image analysis

Validation of immunohistochemistry quantification was performed by repeated counting and image analysis on two sets of immunostained slides from two randomly chosen biopsies. The counter was unaware of the previous scores for each biopsy.

Table 2.2: Data showing repeatability of counting on biopsy 1

Monoclonal antibody	Standard Deviation	Mean	Coefficient of Variation (%)
E selectin	3.51	32.50	10.81
P selectin	2.50	48.75	5.13
ICAM-1	7.93	87.25	9.09
VCAM-1	0.50	8.750	5.71
EN4	6.19	70.75	8.74
AA1 submucosa	1.85	18.75	9.86
AA1 epithelium	0	1	0
EG2 submucosa	0	0	0
EG2 epithelium	0	0	0
NOE submucosa	6.70	73.75	8.16
NOE epithelium	0.25	0.875	28.57
CD3 submucosa	3.70	45.50	8.13
CD3 epithelium	1.83	23.00	7.94
CD4 submucosa	2.06	25.75	8.00
CD4 epithelium	0.50	2.75	18.18
CD8 submucosa	1.94	20.75	9.33
CD8 epithelium	1.26	13.75	9.15
CD25 submucosa	0.29	3.25	8.88
CD25 epithelium	0	0	0
CD68 submucosa	0.48	9.38	5.11
CD68 epithelium	0	0	0

Table 2.3: Data showing repeatability of computerised image analysis on biopsy 1

Parameter	Standard Deviation	Mean	Coefficient of Variation (%)
Epithelium (cells/mm)	0.23	2.72	8.37
Submucosa (cells/mm^2)	0.06	1.10	5.04

Table 2.4: Data showing repeatability of counting on biopsy 2

Monoclonal antibody	Standard Deviation	Mean	Coefficient of Variation (%)
E selectin	2.22	22.75	9.75
P selectin	2.63	24.75	10.63
ICAM-1	4.80	95.5	5.02
VCAM-1	0.50	6.75	7.40
EN4	4.65	100.25	4.63
AA1 submucosa	1.66	35.25	4.70
AA1 epithelium	0.25	1.38	18.18
EG2 submucosa	0	0	0
EG2 epithelium	0	0	0
NOE submucosa	7.62	120.13	6.34
NOE epithelium	0.25	10.88	2.30
CD3 submucosa	3.03	66.00	4.59
CD3 epithelium	3.38	31.88	10.59
CD4 submucosa	1.84	30.88	5.97
CD4 epithelium	0.25	2.13	11.76
CD8 submucosa	0.85	30.88	2.77
CD8 epithelium	1.92	26.50	7.22
CD25 submucosa	0	0	0
CD25 epithelium	0	0	0
CD68 submucosa	1.26	14.25	8.83
CD68 epithelium	0.25	0.88	28.57

Table 2.5: Data showing repeatability of computerised image analysis on biopsy 2

Parameter	Standard Deviation	Mean	Coefficient of Variation (%)
Epithelium (cells/mm)	0.30	3.84	7.76
Submucosa (cells/mm^2)	0.03	1.75	1.89

Coefficient of variation is an expression of intra-operator variability and is defined as the ratio of the standard deviation and mean, expressed as a percentage. A coefficient of variation less than 10% suggests that the degree of intra-observer variability is acceptable.

In the results above, most counting of cells shows no evidence of significant intra-observer variability. A number of high coefficient of variation scores are associated with very small actual cell numbers and the variability suggested is disproportionate to that seen in actual practice. Such anomalies are of less significance when comparing the change in cell numbers rather than actual cell numbers. Other causes of a high coefficient of variation score include observer fatigue, errors in the movement of the graticule and high levels of background staining.

2.4.7 Subject methods

Four groups of subjects were recruited to the study: asthmatics treated with β2-agonists alone, asthmatics treated with β2-agonists and inhaled corticosteroids (ICS), allergic rhinitics and healthy controls. Recruitment was largely achieved by advertising in the local newspapers and by posters at the University of Umeå. The profiles of the subjects are shown in Table 2.6. Exclusion criteria included smoking or a history of smoking, airway infection within six weeks prior to or during the study, current use of any medications (other than inhaled corticosteroids and short-term β2–agonists for the asthmatics) and current vitamin C or E supplementation. Pre-study examination of the subjects included taking a medical history and performing a clinical examination, skin prick testing, lung function testing, 12-lead ECG, exercise testing and basal blood tests (including full blood count, electrolytes and coagulation parameters).

Table 2.6: Characteristics of the subjects in each study group

Subject group	Healthy controls	Allergic Rhinitis	Allergic Asthma β2-agonist	Allergic Asthma ICS
Number in group	21	13	16	16
Mean age in years (Range)	24 (21-29)	25 (22-34)	24 (18-32)	26 (19-41)
Gender (Male:Female)	12:9	6:7	8:8	8:8
FEV_1 (% predicted)	98 ± 15	97 ± 15	99 ± 15	99 ± 15
Geometrical mean of PC_{20} (mg/ml)	Not assessed	13.8	Not assessed	Not assessed
Skin prick test to pollens	Negative	Positive	Positive	Positive

The inclusion criteria for the subjects in each of the three groups was as follows:

- Asthmatics
    - Atopic subjects diagnosed with moderate asthma treated with inhaled corticosteroids (dose 200-1200μg per day) and short-acting β2-agonists OR atopic subjects diagnosed with mild asthma treated with inhaled short-acting β2-agonists alone
    - Positive skin prick test
    - FEV_1>80% predicted
- Allergic Rhinitics
    - Atopic subjects diagnosed seasonal allergic rhinitis and requiring medical treatment only during the pollen season
    - Positive skin prick test to birch or grass pollen
    - FEV_1>80% predicted
    - Methacholine test PC_{20}>8mg/ml
- Healthy controls
    - No history of atopy including seasonal allergic rhinitis and asthma
    - Negative skin prick test to birch or grass pollen
    - FEV_1>80% predicted

2.4.7.a. Methacholine challenge

The subjects with allergic rhinitis underwent a methacholine challenge in order to exclude subjects with previously undiagnosed asthma. The procedure was explained to the subject and written consent obtained. The baseline FEV_1 was measured and the subject was then asked to breathe in a nebulised aerosol of methacholine of increasing concentrations. The FEV_1 was measured after each dose and the % fall in FEV_1 from baseline was plotted

against the dose of inhaled methacholine on a logarithmic scale. A dose response curve was constructed and the provocation concentration (PC) of inhaled histamine required to reduce the FEV_1 by 20% (PC_{20}) was derived by linear extrapolation. A diagnosis of asthma was suggested by PC_{20}<8 mg/ml. The subject remained in the respiratory department for 30 minutes following the procedure to observe any delayed reactions.

2.4.7.b. Skin prick testing

All subjects were investigated with skin prick testing to common aeroallergens including birch and grass pollens. The testing was carried out on the inner forearm with the arm coded with a marker pen for the allergens to be tested. A drop of the allergen solution was placed by each code and the skin was then pricked through the drop using the tip of a lancet. The reactions were read at 15 minutes and compared to a positive (histamine) and negative (saline) control.

2.4.7.c. Lung function tests

All subjects were investigated with baseline spirometry including FEV_1, FVC and peak flow. At least three acceptable tracings were obtained and each tracing was examined to ensure the patient had made adequate effort and the results were reproducible with no artefacts. The asthmatic subjects also had respiratory function testing pre- and post- each exposure.

2.4.8 Statistical methods

All data was analysed using SPSS Version 10.0 for Windows statistical package. Symptom scores were obtained for both air and diesel exhaust exposures. The differences between these scores were analysed using the Mann-Whitney U test. P values < 0.05 were considered to be statistically significant.

FEV_1 results were analysed by paired t test before and after each separate exposure. PEF results were analysed using repeated measures ANOVA and Dunnett post test. Missing values were handled with the 'last observation carried forward' technique. P values < 0.05 were considered significant.

The bronchial wash and lavage data from the asthmatic and allergic rhinitic subjects was compared to the healthy controls using the Mann-Whitney U test. The beseline bronchial biopsies (obtained after air exposure) were analysed using the Kruskall Wallis non-parametric ANOVA test. If this indicated a significant difference between the four groups a second statistical test, the Mann-Whitney U test was used to ascertain between which groups the difference existed. Bronchial biopsies obtained after diesel exhaust exposure were compared to the baseline biopsies using the Wilcoxon rank test. P values $<$ 0.05 were considered significant.

CHAPTER THREE

Lower airway cellular inflammation in allergic rhinitic subjects compared to allergic asthmatics and healthy controls

3.1 INTRODUCTION

The incidence of allergy is increasing at a rate greater than can be explained by genetic factors alone. Understanding the mechanisms by which atopy develops and subsequently leads to the manifestations of allergic disease may help to elucidate the reasons for this increase.

It has been shown that allergic rhinitis may precede the development of asthma by months or even years in certain atopic individuals. In addition, there is evidence that subjects with allergic rhinitis may have inflammation in the lower airways without clinical symptoms or bronchial hyperresponsiveness (as measured by a methacholine provocation challenge). Although this lower airways inflammation has been detected indirectly by the examination of induced sputum and by the measurement of exhaled nitric oxide, relatively little work has been done on its cellular characterization by endobronchial biopsy. At this stage, it is not known if asymptomatic inflammation in the lower airways of subjects with allergic rhinitis subsequently progresses and becomes clinical asthma. However, identifying the cellular constituents of the inflammation may be the first step in understanding this process and thereby the development of asthma.

3.2 AIM OF THE STUDY

My hypothesis was that allergic rhinitic subjects would exhibit lower airway inflammation that was similar to that of asthmatics, but would differ either in extent and/or cellular profile and would therefore be asymptomatic. Healthy controls would not show any evidence of inflammation.

3.3 STUDY DESIGN

The subjects and methods used in this study are detailed in Chapter 2. All subjects underwent a fibreoptic bronchoscopy with collection of BW, BAL and endobronchial biopsies. The wash and lavage samples were filtered and the cells resuspended in PBS before being counted. The biopsies were processed into GMA resin and stained for immunohistochemistry cell markers. All the samples were randomly coded and therefore blinded to the examiner. Inter-observer variability was avoided by having only one individual perform the cell counting. The total number of subjects in each group was: 16 asthmatics treated with β2-agonists alone, 16 asthmatics treated with β2-agonists and inhaled corticosteroids, 13 allergic rhinitics and 21 healthy controls.

3.4 STATISTICAL ANALYSIS

The BW and BAL data from the atopic subjects was compared to the healthy controls using the Mann-Whitney U test. Bronchial biopsy samples from all four groups were analysed using the Kruskall Wallis non-parametric ANOVA test. If this indicated a significant difference between the groups a further statistical test, the Mann-Whitney U test was performed to determine between which two groups the difference existed. Statistical analysis was performed using SPSS Version 10.0 for Windows. A p value <0.05 was considered statistically significant.

3.5 RESULTS

3.5.1 Cellular inflammation in the lower airways

Asthmatic subjects treated with β2-agonists alone had statistically significant elevated numbers of eosinophils in the bronchial wash ($p=0.001$) and submucosa ($p=0.032$) compared to healthy controls. In addition, they also had elevated numbers of mast cells in the bronchial wash ($p<0.001$) and in the epithelium ($p<0.001$) compared to healthy controls.

Asthmatic subjects treated with inhaled corticosteroids had no evidence of an eosinophilia or increased mast cells in the bronchial biopsies. However, the percentage of mast cells in the bronchial wash was increased compared to the healthy controls ($p=0.003$). This group also showed increased numbers of neutrophils in the submucosa compared to healthy controls ($p=0.009$). This result was still significant when compared to the numbers of neutrophils in the asthmatics treated with β2-agonists alone ($p=0.023$). In addition, the difference between mast cell numbers in the epithelium was significant between the two groups of asthmatics ($p<0.001$) with the steroid group having less that the group treated with β2-agonists alone. Asthmatic subjects treated with inhaled corticosteroids also had significantly reduced expression of endothelial VCAM-1 compared to healthy subjects ($p=0.013$).

Subjects with allergic rhinitis had statistically significant elevated numbers of eosinophils in the bronchial submucosa compared to healthy controls ($p=0.022$). They also had elevated levels of mast cells ($p=0.022$), $CD3^+$ lymphocytes ($p=0.029$) and $CD8^+$

lymphocytes (p=0.022) in the epithelium compared to healthy controls. The increased numbers of epithelial lymphocytes in the allergic rhinitic subjects remained significant when compared to both groups of asthmatics: the number of $CD3^+$ lymphocytes in allergic rhinitics compared to asthmatics treated with β2-agonists alone (p=0.045) and compared to asthmatics treated with inhaled corticosteroids (p=0.001); and the number of $CD8^+$ lymphocytes in allergic rhinitics compared to asthmatics treated with β2-agonists alone (p=0.036) and compared to asthmatics treated with inhaled corticosteroids (p=0.001).

There were no differences detected between the four groups in the bronchoalveolar lavage fluid.

Mast cells AA1

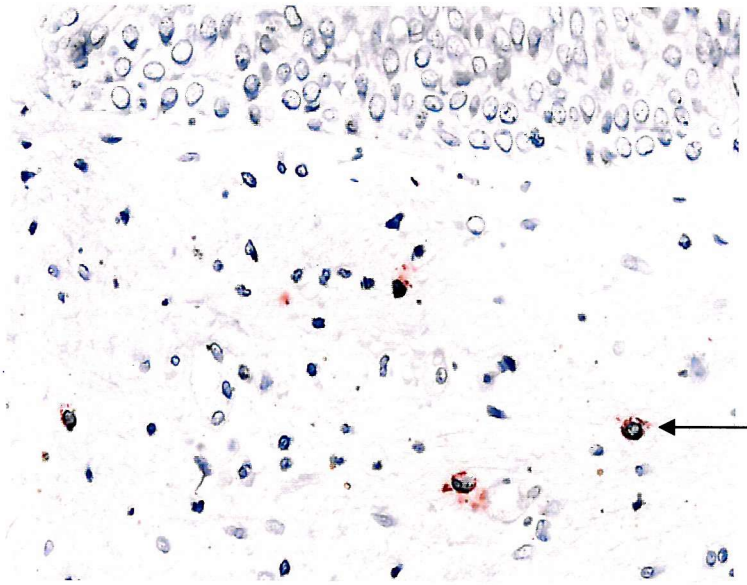


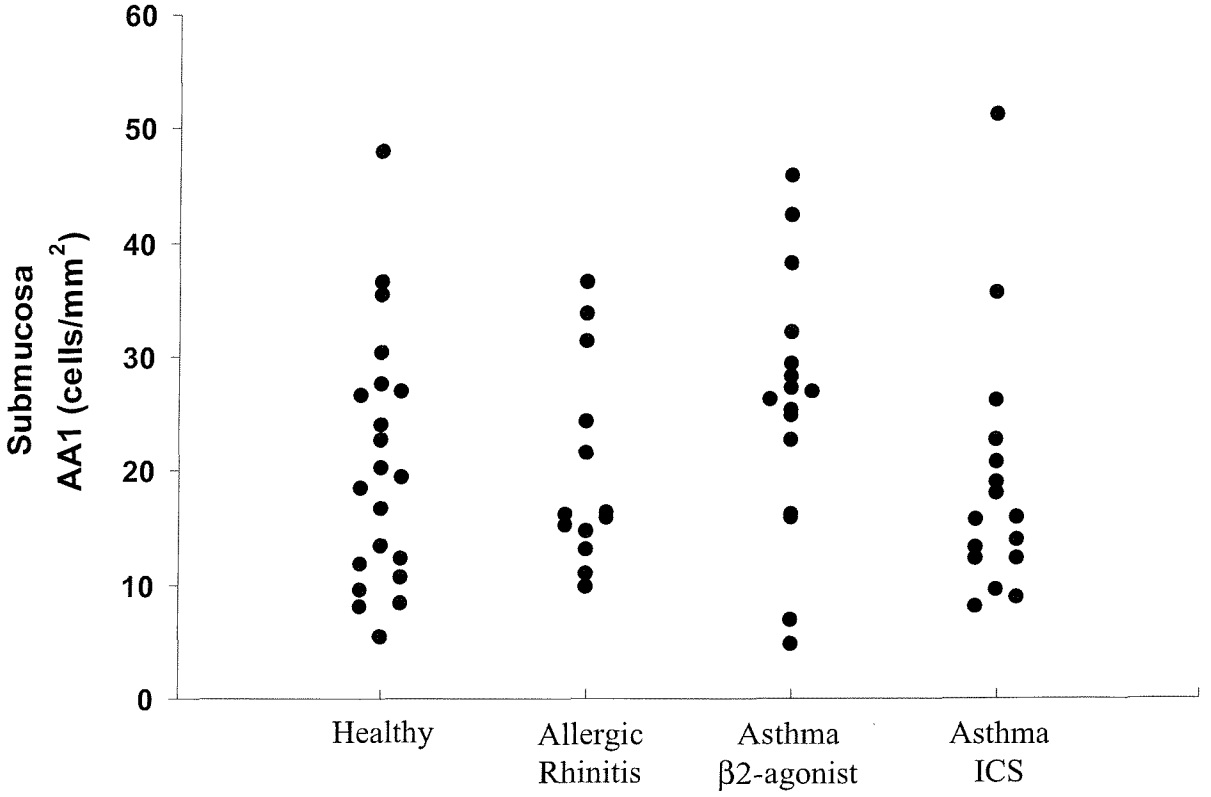
Fig 3.1: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against AA1 (tryptase).

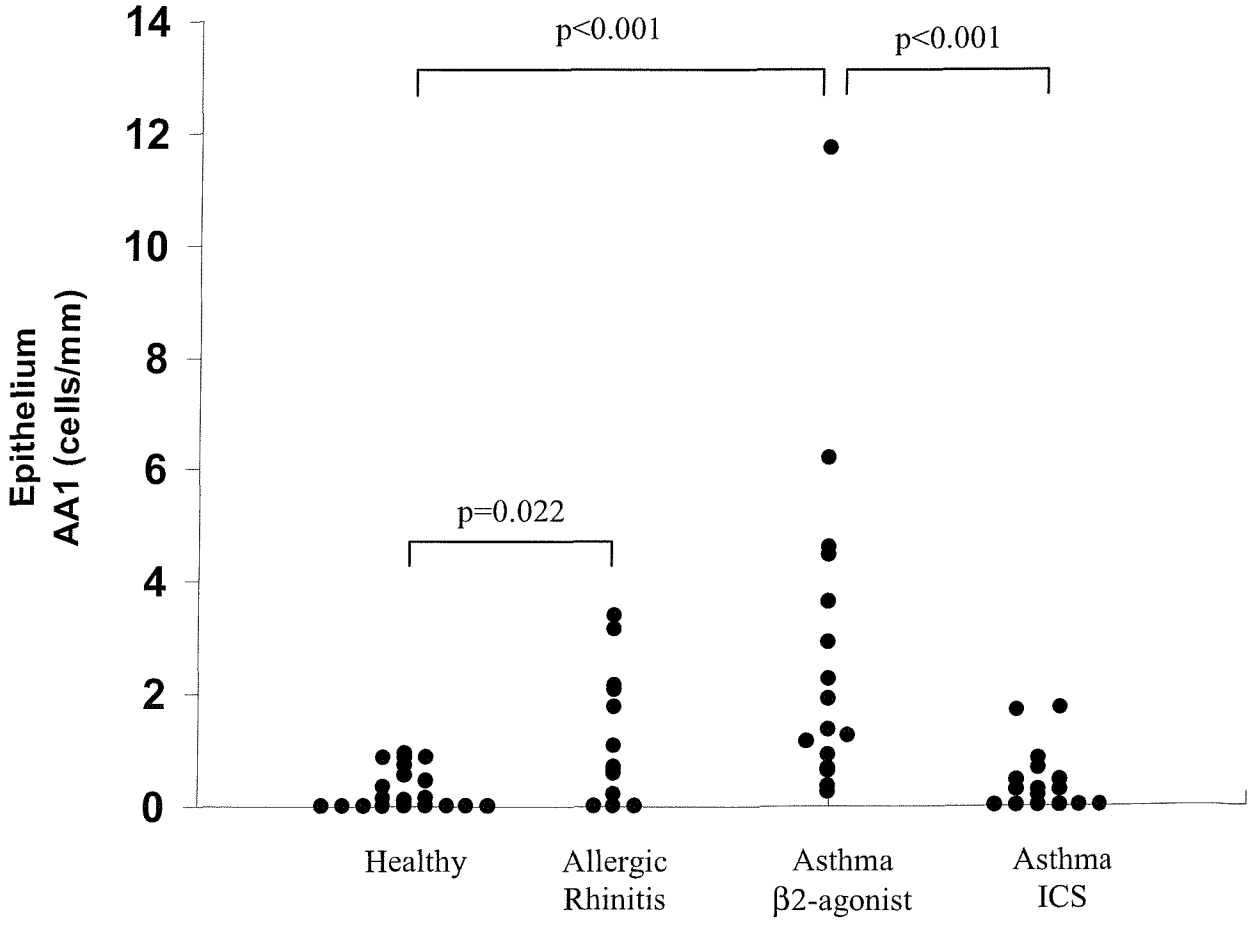
There were no statistically significant differences between the four subject groups in the numbers of mast cells observed in the submucosa or in the BAL fluid.

In the epithelium, the Kruskall Wallis test showed that significant differences existed between the four groups ($p<0.01$). Subsequent analysis with the Mann-Whitney U test showed that mast cells were significantly elevated in the allergic rhinitic subjects ($p=0.002$) and in the asthmatic subjects treated with β2-agonist alone ($p<0.001$) compared to the healthy controls. The difference in the number of mast cells also reached statistical significance between the two sub-groups of asthmatic subjects, with those treated with β2-agonist alone having greater numbers that those treated with inhaled corticosteroids ($p<0.001$).

In the bronchial wash, the percentage of mast cells was significantly increased in both asthmatic groups ($p<0.001$ and $p=0.003$ respectively) compared to the healthy controls.

GRAPHS 3.1A AND 3.1B: MAST CELL NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





Eosinophils EG2

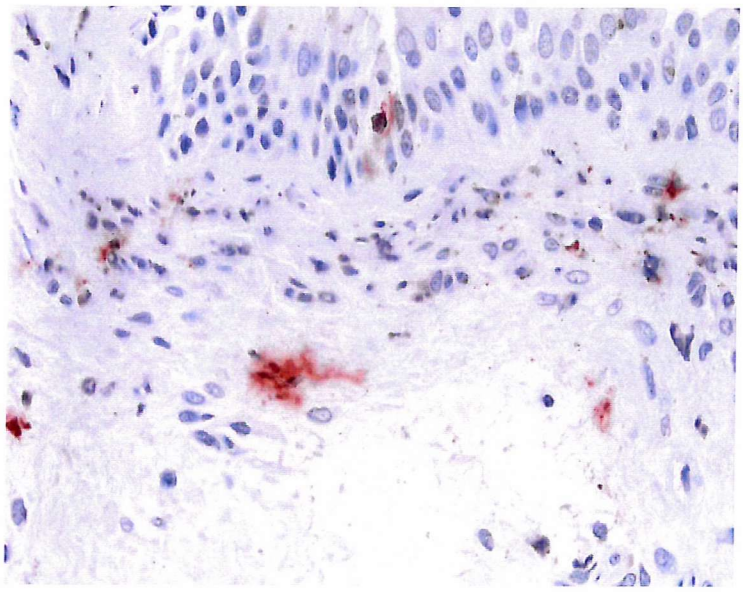


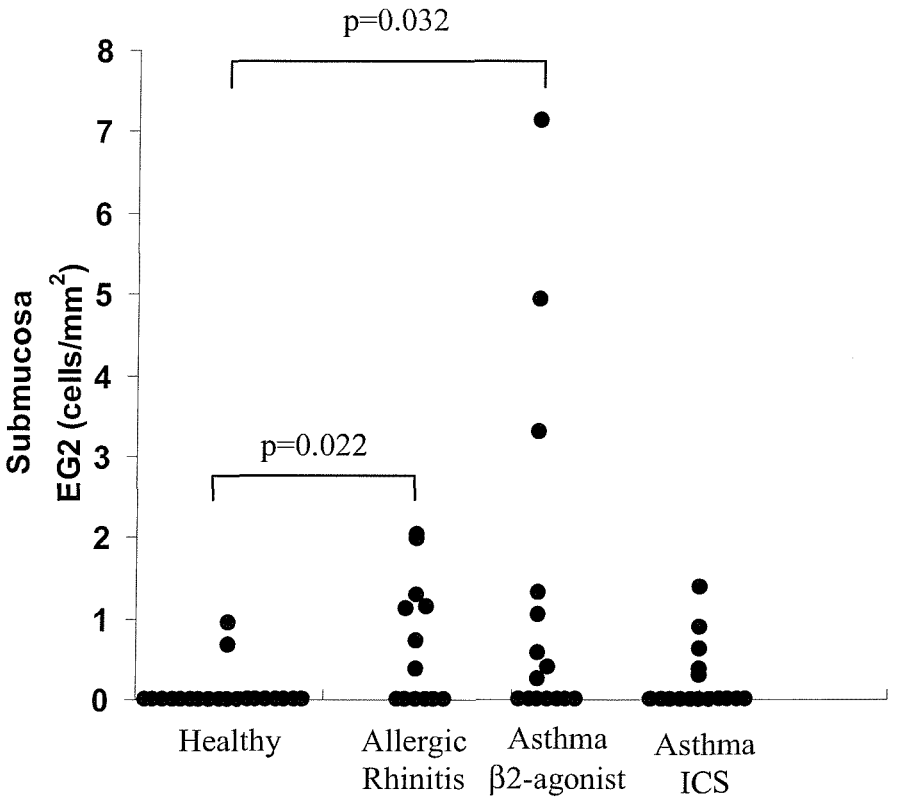
Fig 3.2: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibody directed against eosinophil cationic protein (EG2).

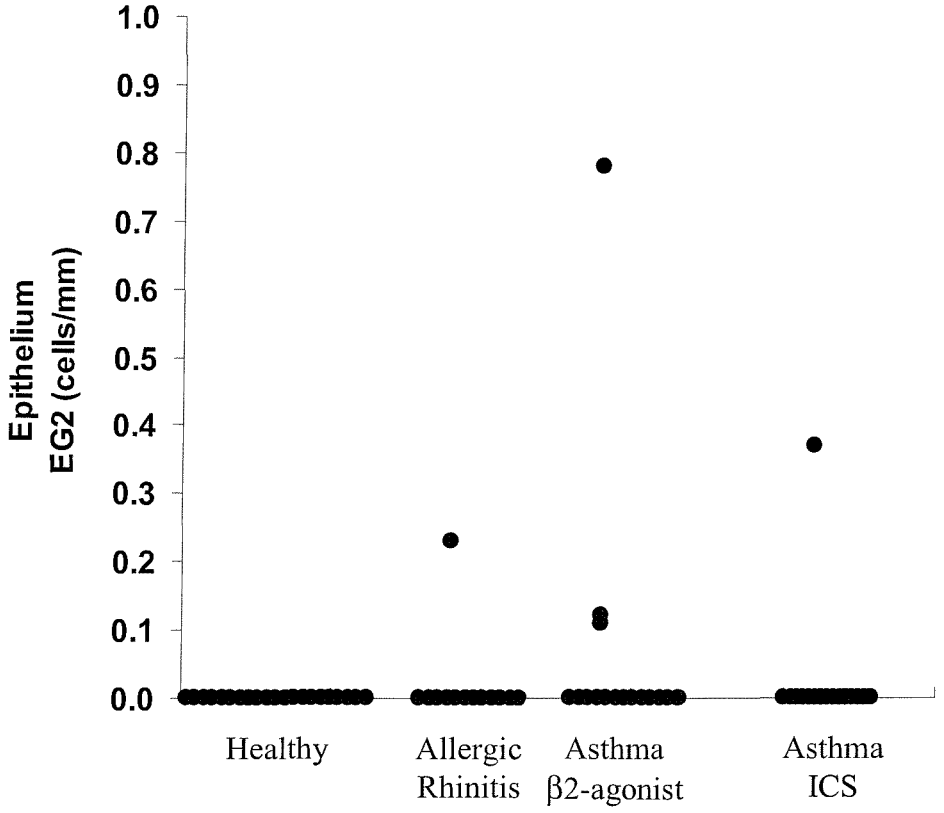
In the bronchial submucosa, the Kruskall Wallis test detected a significant difference in the numbers of eosinophils between the four groups ($p=0.013$). Both allergic rhinitic subjects ($p=0.022$) and asthmatics treated with β2-agonists alone ($p=0.032$) had significantly increased numbers of eosinophils compared to healthy controls. Asthmatics treated with inhaled corticosteroids did not demonstrate any evidence of an eosinophilia.

In the epithelium, the Kruskall Wallis test did not indicate the presence of statistically significant differences between the four groups. The numbers of eosinophils detected in the epithelium were very low in virtually all the subjects making statistical analysis unrewarding.

In the bronchial wash, asthmatics treated with β2-agonists alone ($p=0.02$) had a significantly increased percentage of eosinophils compared to healthy controls. There were no significant differences in the BAL fluid.

GRAPHS 3.2A AND 3.2B: EOSINOPHIL NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





Neutrophils NOE

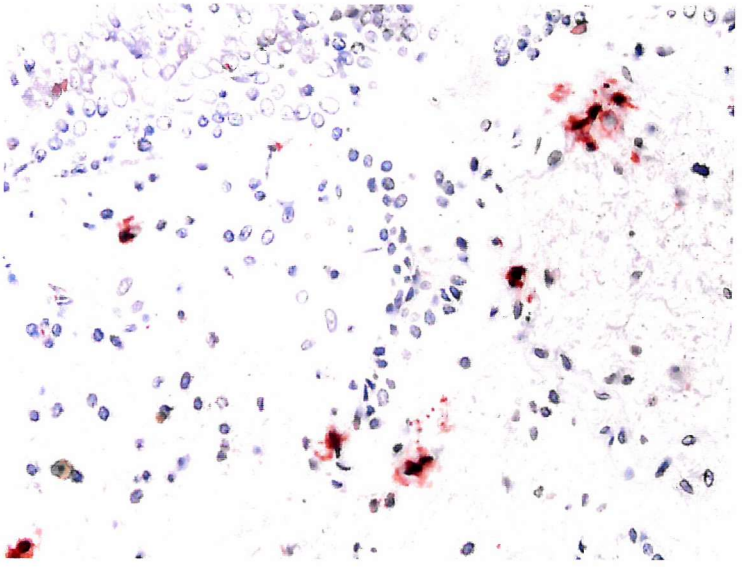


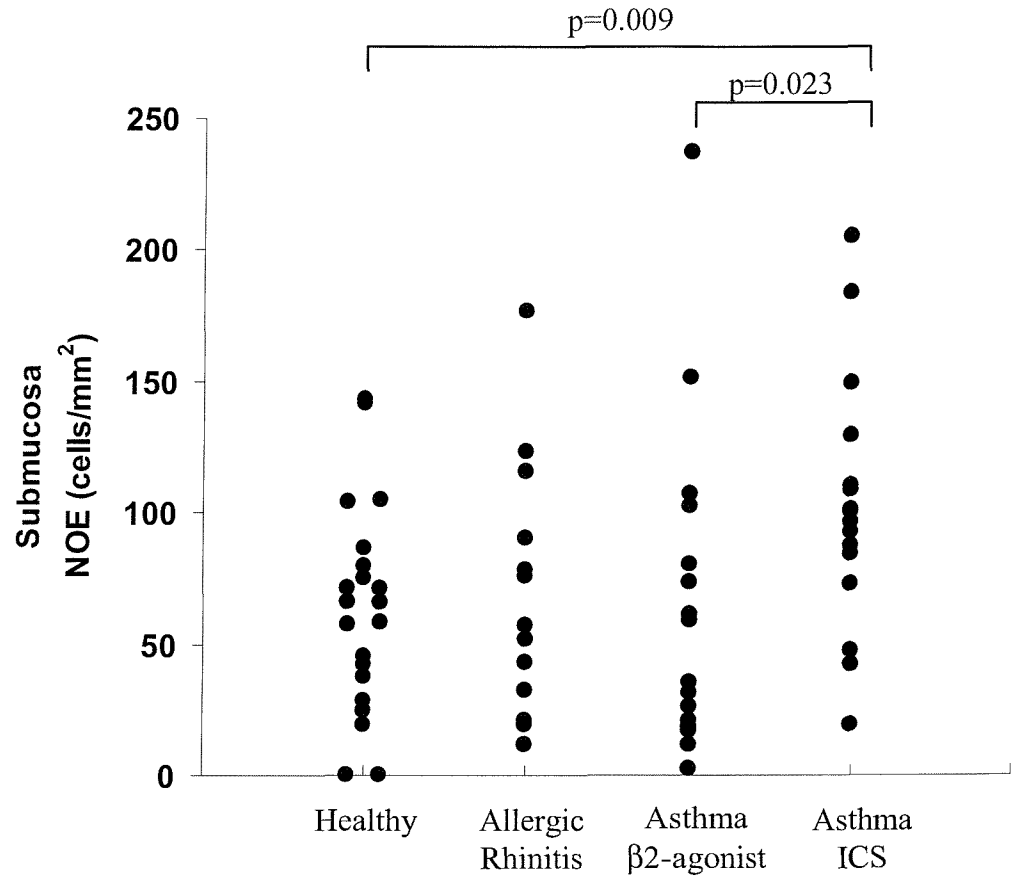
Fig 3.3: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against NOE.

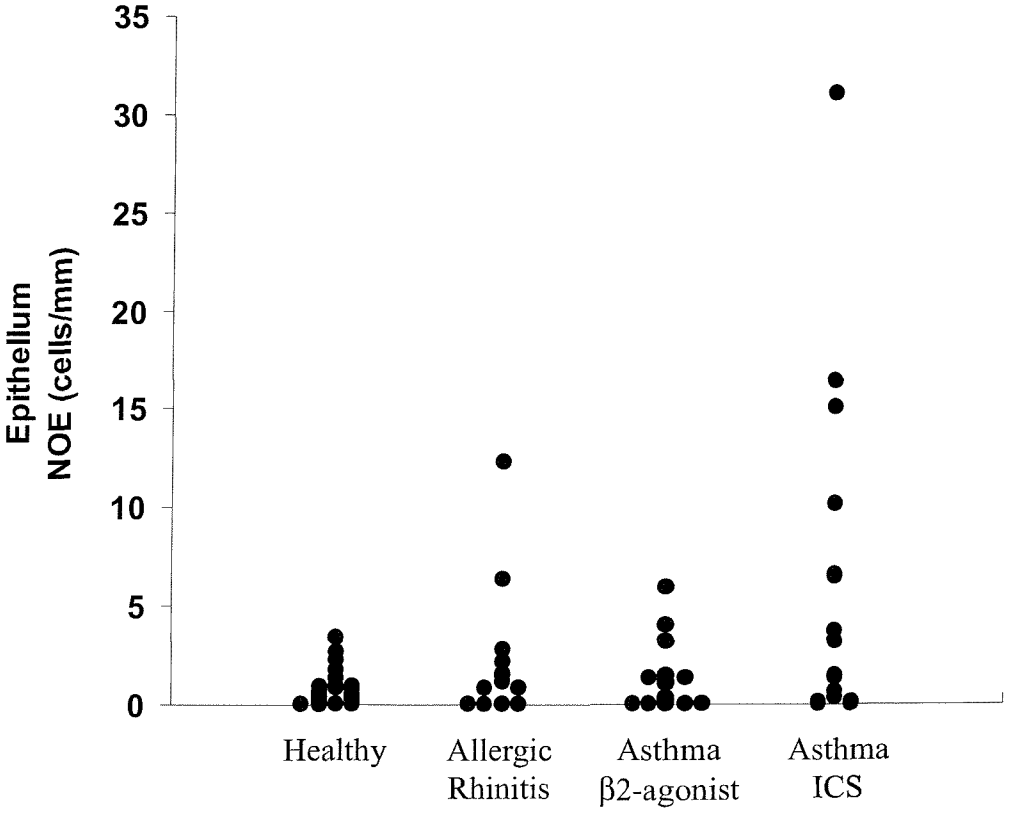
The Kruskall Wallis analysis of the numbers of neutrophils in the submucosa revealed that a significant difference existed between the four groups ($p=0.047$). Further analysis with the Mann-Whitney U test showed that the asthmatics treated with inhaled corticosteroids had a significantly increased number of neutrophils compared to the healthy controls ($p=0.009$). The increase in neutrophils in the asthmatics treated with inhaled corticosteroids was still significant when compared to the asthmatics treated with β2-agonist alone ($p=0.023$).

There were no significant differences in the number of neutrophils in the epithelium detected between the four groups, although there is a trend for the numbers to be greater in the asthmatics treated with inhaled corticosteroids.

There were no significant differences detected in the bronchial wash or lavage samples between the four groups.

GRAPHS 3.3A AND 3.3B: NEUTROPHIL NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





Total Lymphocytes $CD3^+$

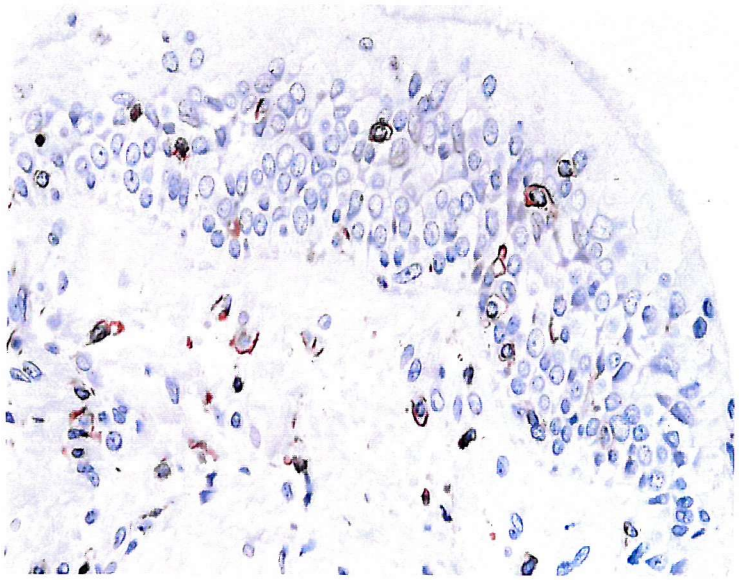
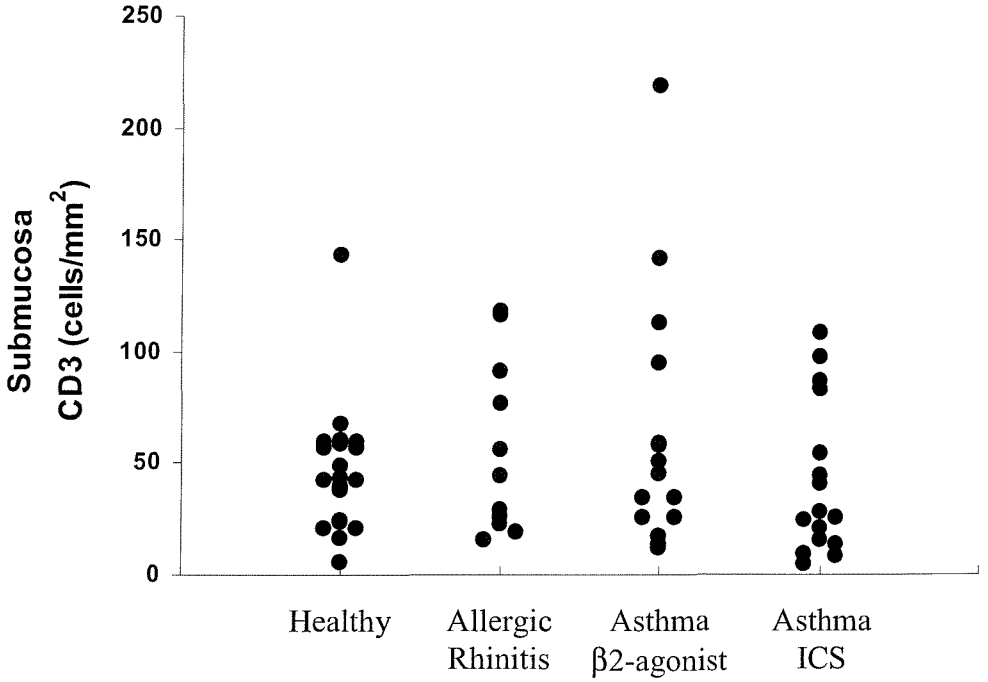


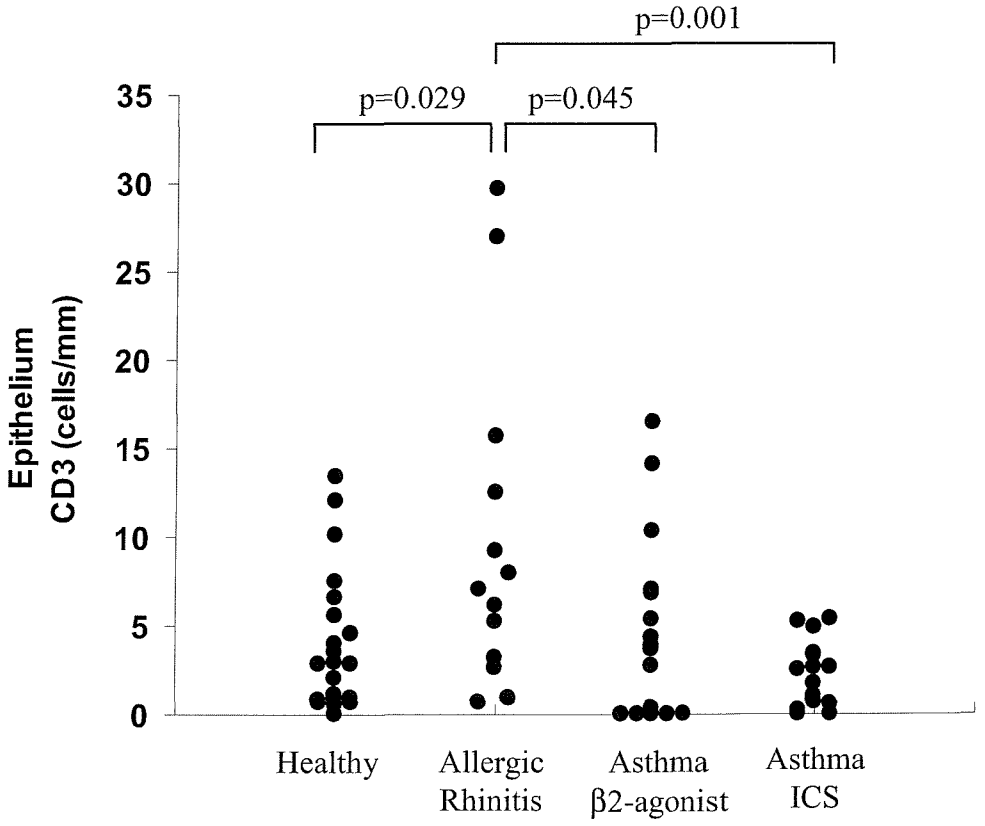
Fig 3.4: Human bronchial submucosa and underlying epithelium immunostained using monoclonal antibodies directed against $CD3^+$ cells.

There were no significant differences detected between the four groups in terms of the numbers of $CD3^+$ lymphocytes in the bronchial submucosa, bronchial wash or lavage.

In the epithelium, the Kruskall Wallis test indicated that a statistically significant difference was present ($p=0.019$). Further analysis with the Mann-Whitney U test showed that the allergic rhinitic subjects had elevated numbers of $CD3^+$ lymphocytes compared to healthy controls ($p=0.029$). The increase in the $CD3^+$ lymphocytes in the allergic rhinitics was also significant when compared to both groups of asthmatics ($p=0.045$ and $p=0.001$ respectively).

GRAPHS 3.4A AND 3.4B: TOTAL LYMPHOCYTE CELL NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





T helper lymphocytes $CD4^+$

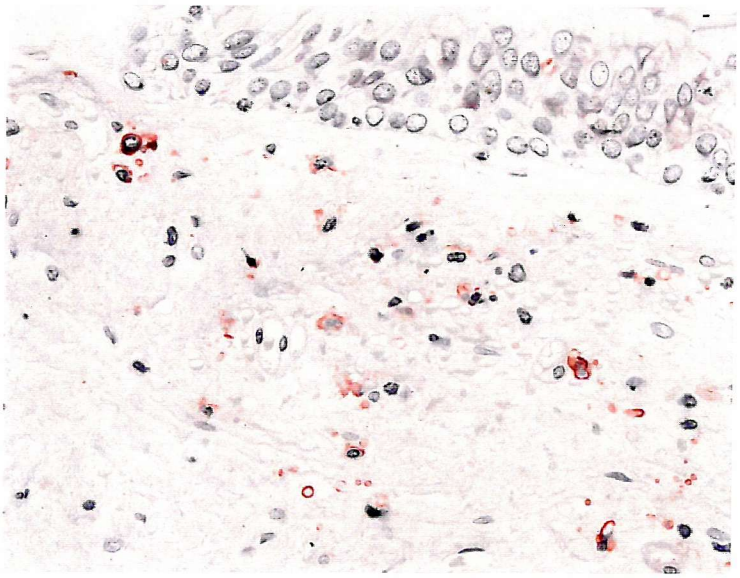
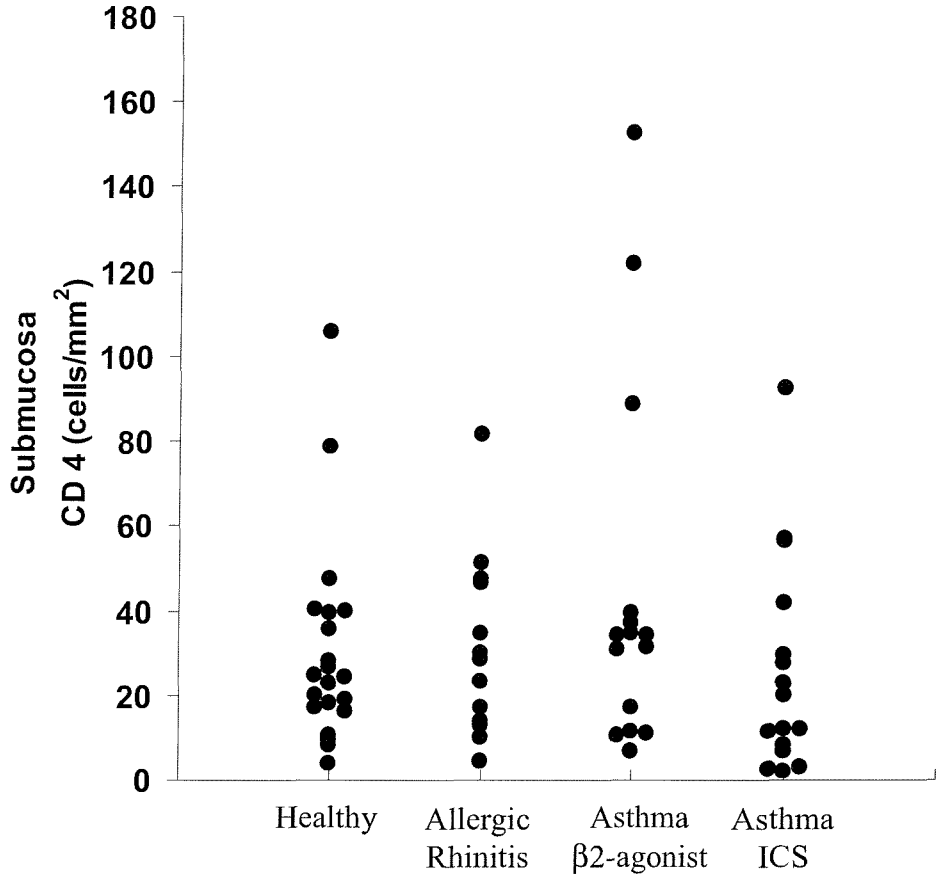
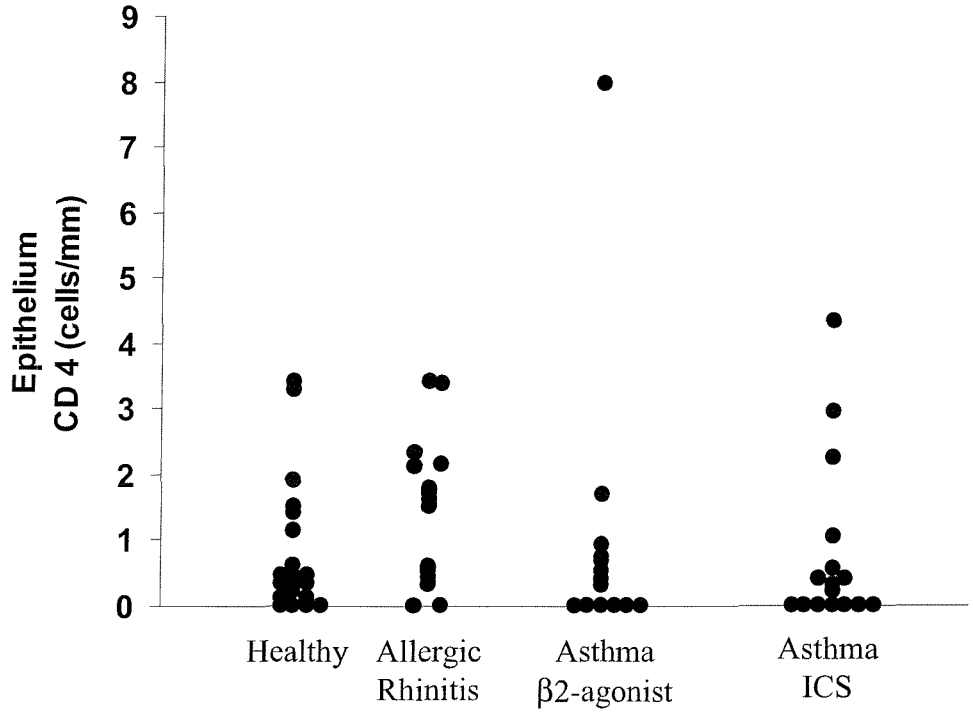


Fig 3.5: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against $CD4^+$ cells.

There were no statistically significant differences in the numbers of $CD4^+$ lymphocytes in either the bronchial submucosa or epithelium between the four groups. In addition, there were no significant differences detected in the bronchial wash or lavage samples between the four groups.

GRAPHS 3.5A AND 3.5B: $CD4^+$ LYMPHOCYTE NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





T suppressor lymphocytes $CD8^+$

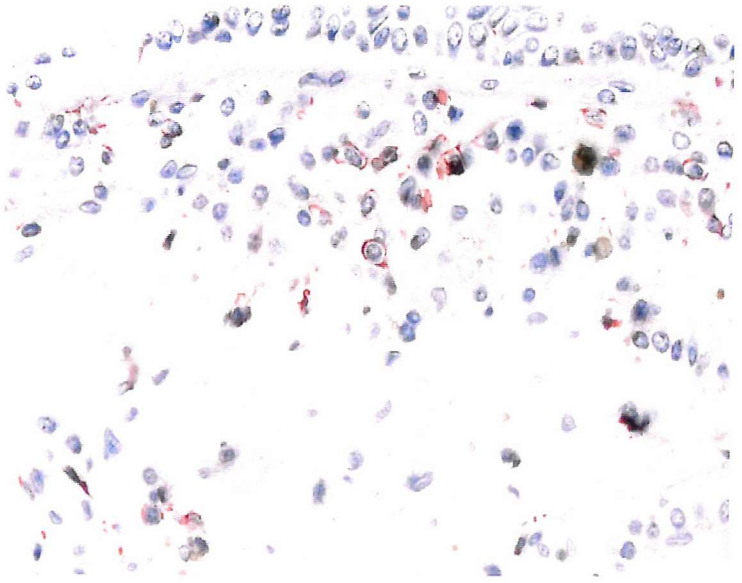
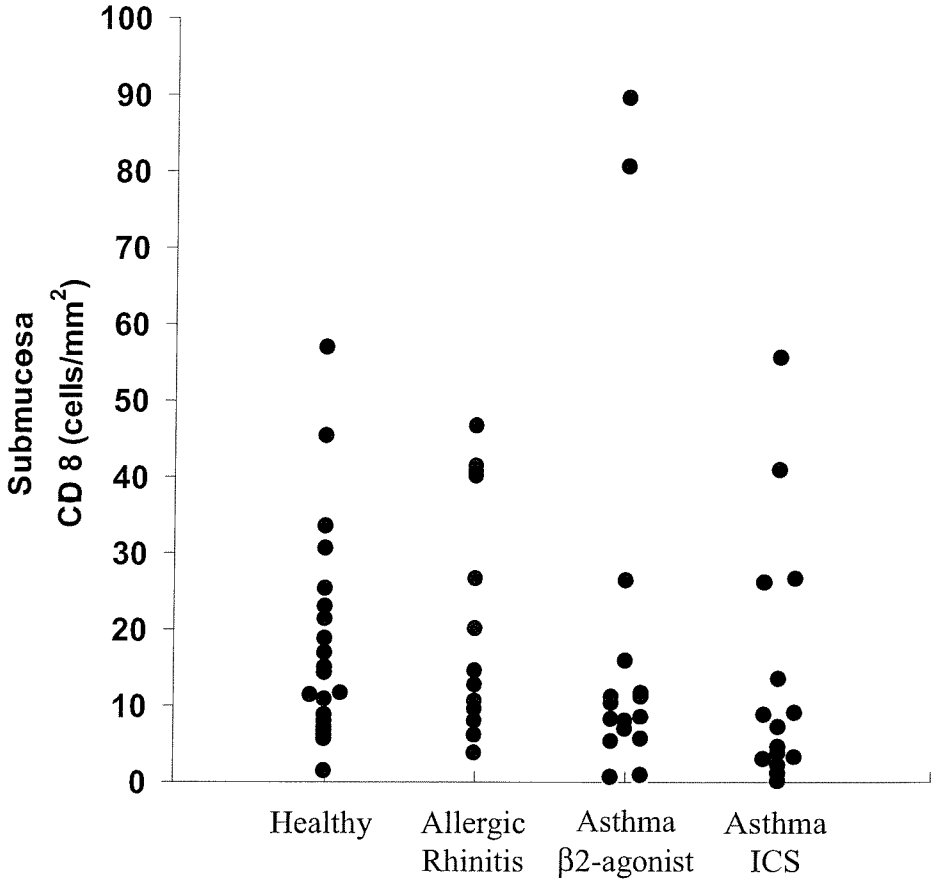


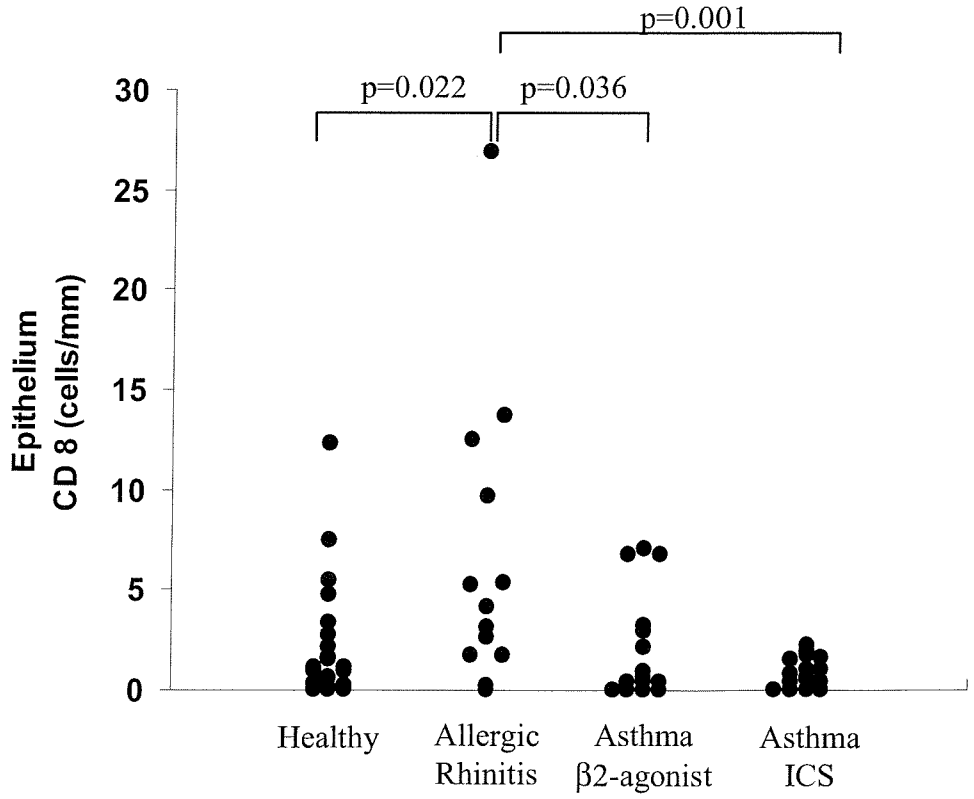
Fig 3.6: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against $CD8^+$ cells.

There were no statistically significant differences in the numbers of $CD8^+$ lymphocytes between the four groups in the bronchial submucosa, the bronchial wash or lavage samples.

In the epithelium, the Kruskall Wallis test was p=0.015 indicating the existence of statistically significant differences. The subjects with allergic rhinitis were found to have elevated levels of $CD8^+$ lymphocytes when compared to healthy controls (p=0.022), asthmatics treated with β2-agonists alone (p=0.036), and asthmatics treated with inhaled corticosteroids (p=0.001).

GRAPHS 3.6A AND 3.6B: $CD8^+$ LYMPHOCYTE NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





Activated lymphocytes $CD25^+$

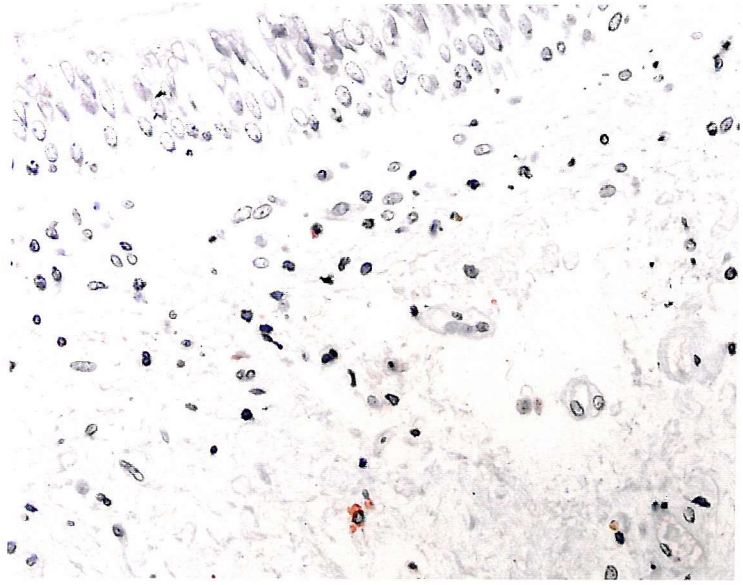
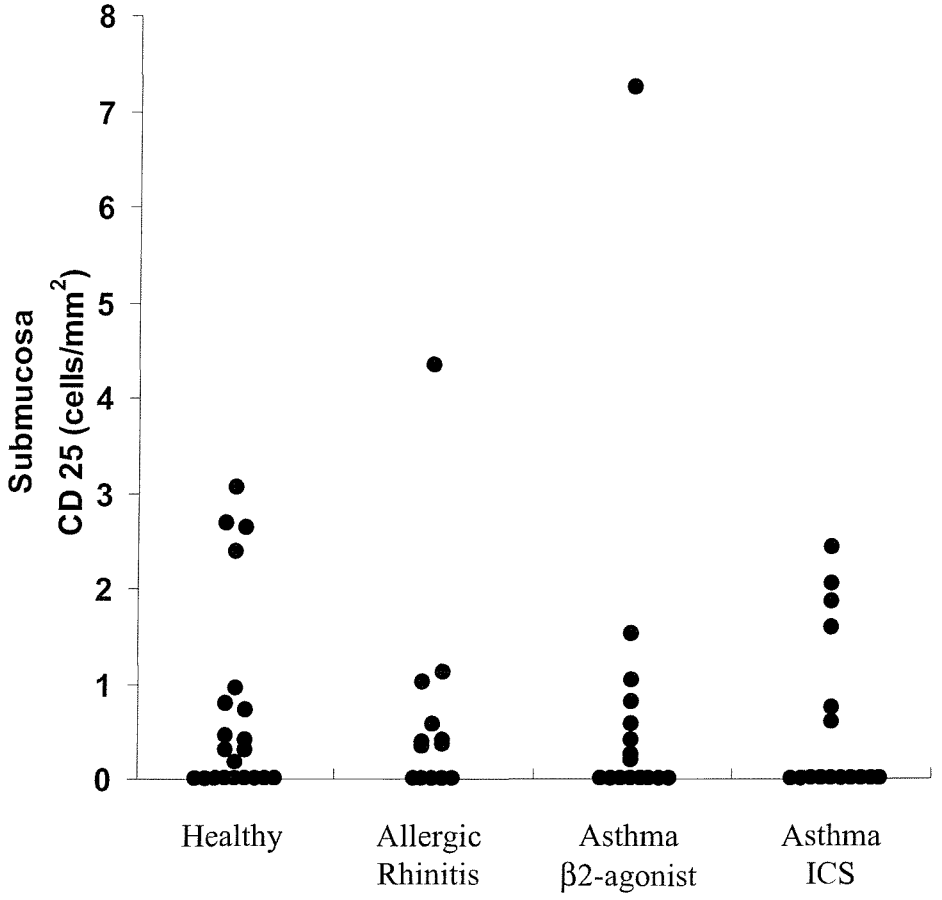
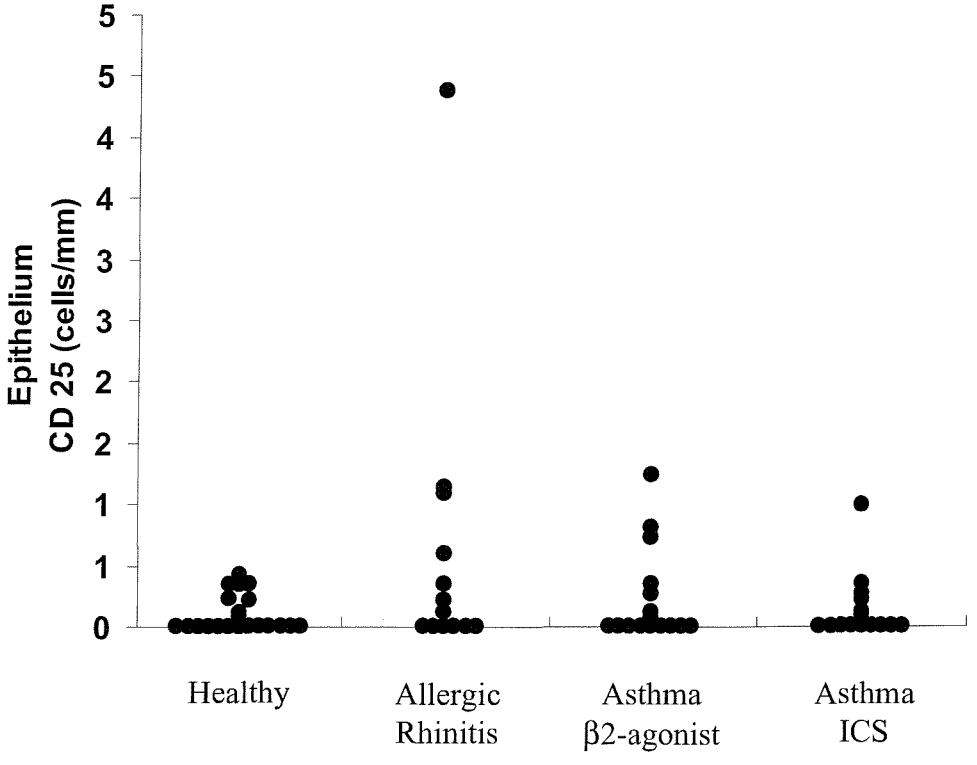


Fig 3.7: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against $CD25^+$ cells.

There were no statistically significant differences in the numbers of $CD25^+$ lymphocytes in either the bronchial submucosa or epithelium between the four groups. In addition, there were no significant differences detected in the bronchial wash or lavage samples between the four groups.

GRAPHS 3.7A AND 3.7B: $CD25^+$ LYMPHOCYTE NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





Macrophages $CD68^+$

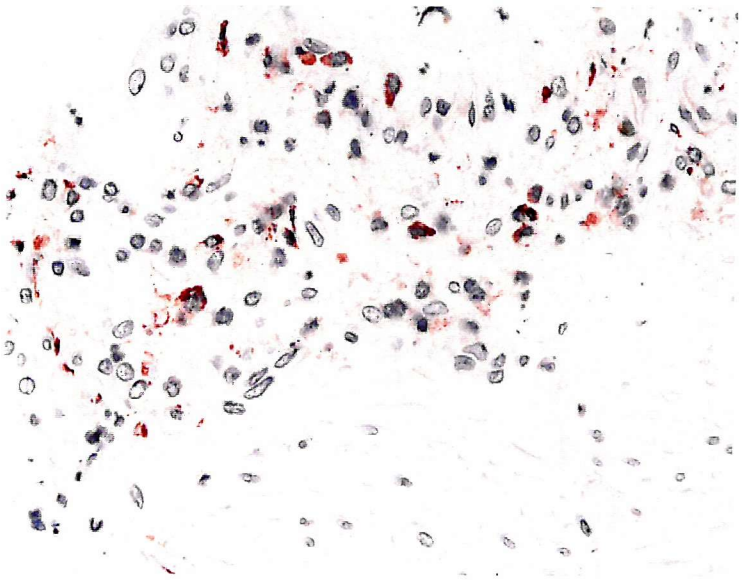
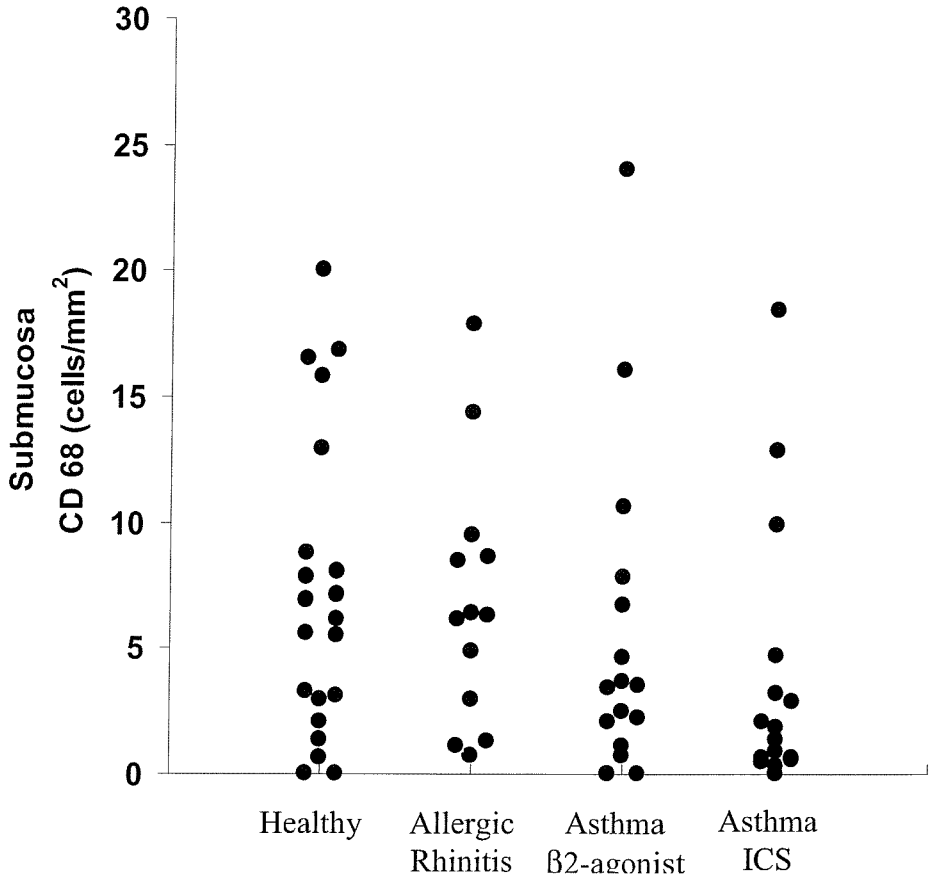
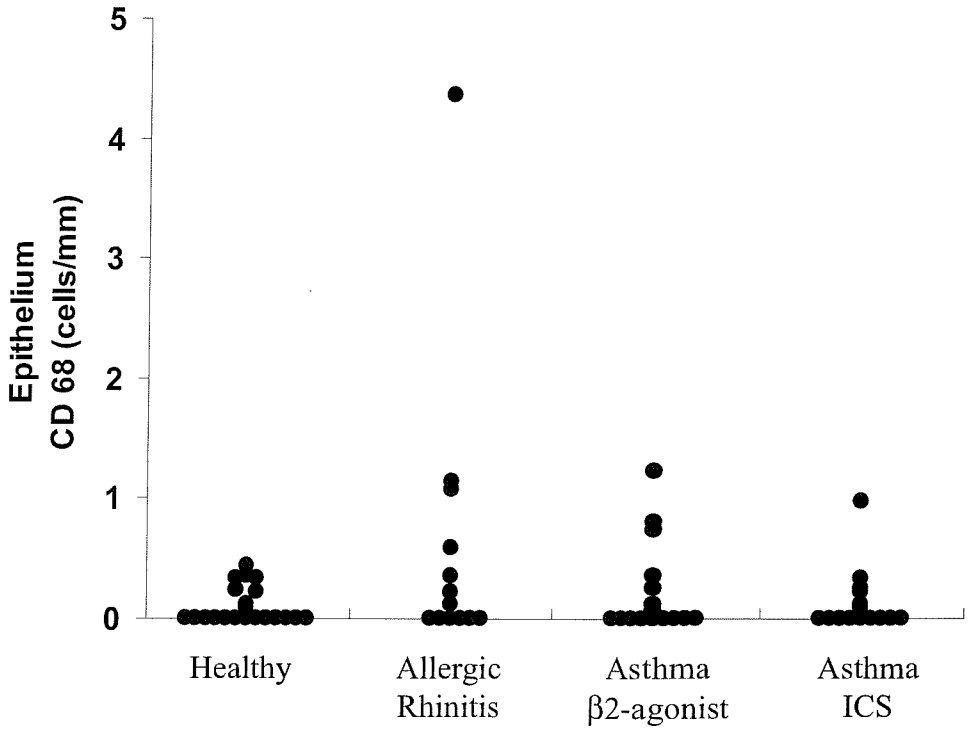


Fig 3.8: Human bronchial epithelium and underlying submucosa immunostained using monoclonal antibodies directed against $CD68^+$ cells.

There were no statistically significant differences in the numbers of $CD68^+$ lymphocytes in either the bronchial submucosa or epithelium between the four groups. In addition, there were no significant differences detected in the bronchial wash or lavage samples between the four groups.

GRAPHS 3.8A AND 3.8B: MACROPHAGE NUMBERS IN THE BRONCHIAL SUBMUCOSA AND EPITHELIUM OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS





3.5.2. Endothelial adhesion molecule expression

In the bronchial submucosa there was constitutive expression of the endothelial adhesion molecules, E selectin, P selectin, ICAM-1 and VCAM-1. No significant differences were detected between the four groups for the endothelial adhesion markers E selectin, P selectin and ICAM-1.

The Kruskall Wallis test indicated a significant difference existed between the four groups in the expression of VCAM-1 ($p<0.001$). Allergic rhinitic subjects had an increased endothelial expression of VCAM-1 compared to healthy controls ($p=0.003$) and both groups of asthmatics ($p=0.001$ and $p<0.001$ respectively). The asthmatic subjects treated with inhaled corticosteroids also had significantly decreased expression of endothelial VCAM-1 compared to the healthy controls ($p=0.013$).

E selectin

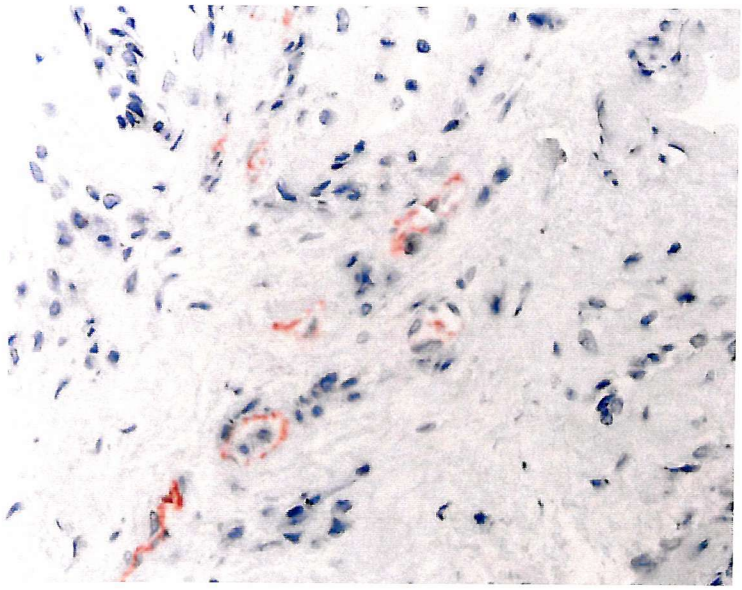
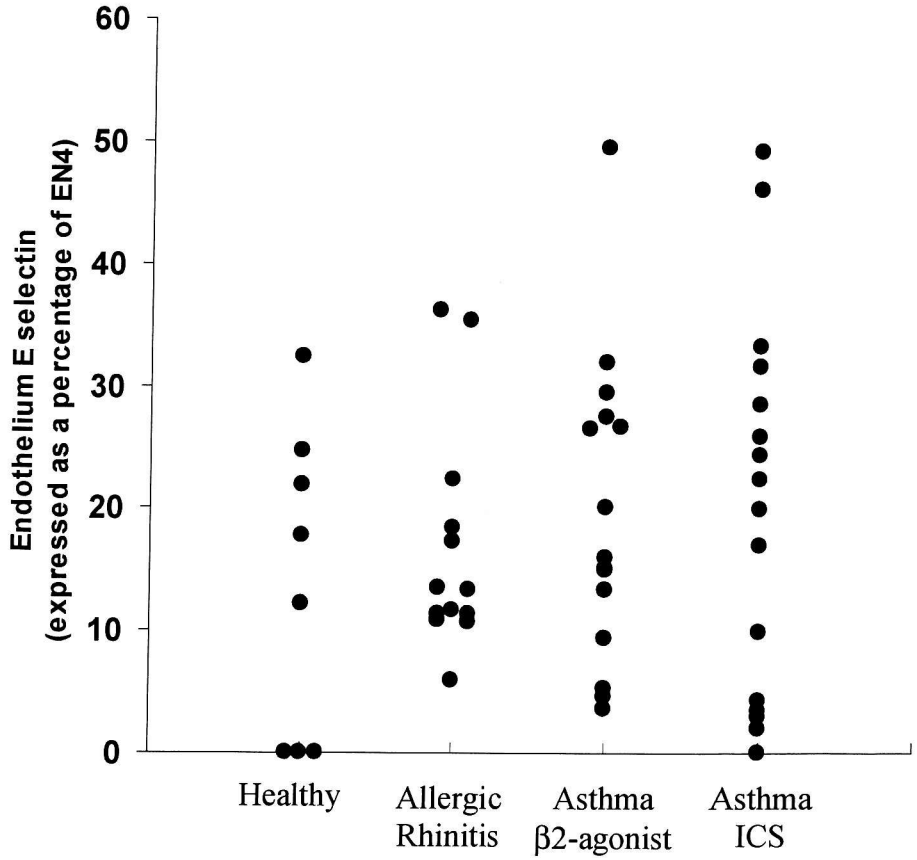


Fig 3.9: Human bronchial submucosa immunostained using monoclonal antibodies directed against E selectin.

Graph 3.9 showing the quantification of E selectin (expressed as a percentage of EN4) in the bronchial submucosa taken from healthy, allergic rhinitic and asthmatic subjects.
There were no significant differences between the four groups.

GRAPH 3.9: E SELECTIN IN THE BRONCHIAL SUBMUCOSA OF HEALTHY CONTROLS AND ALLERGIC SUBJECTS



P Selectin

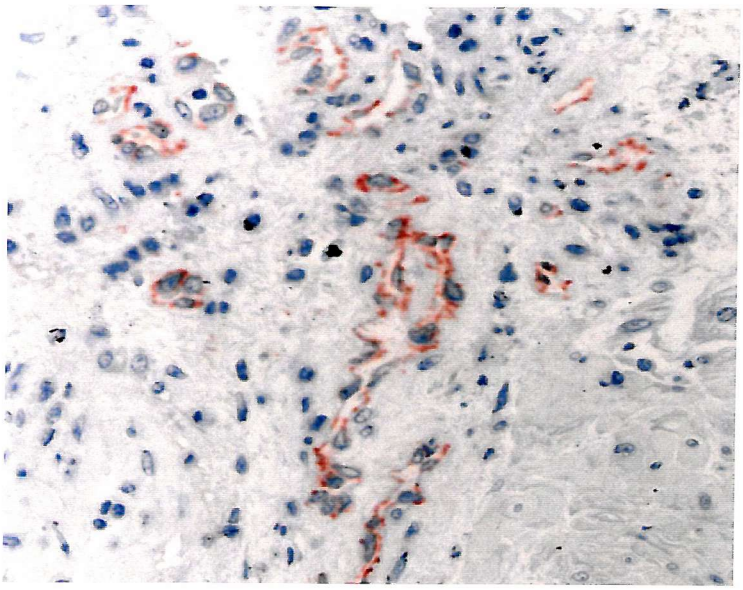
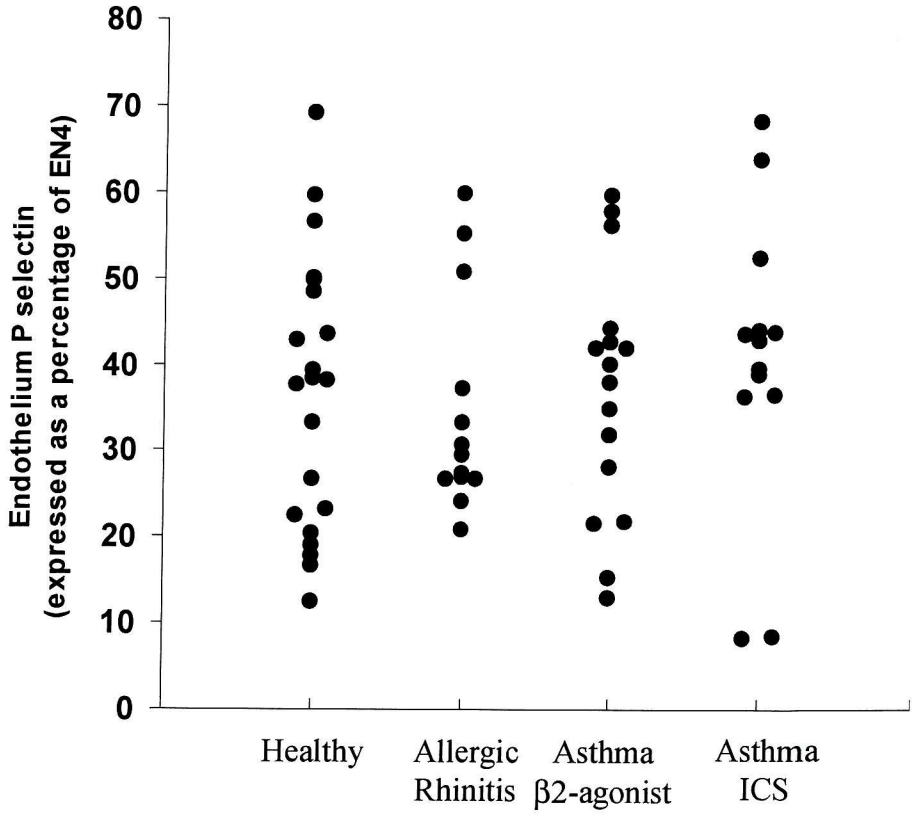


Fig 3.10: Human bronchial submucosa immunostained using monoclonal antibodies directed against P selectin.

Graph 3.10 showing the quantification of P selectin (expressed as a percentage of EN4) in the bronchial submucosa taken from healthy, allergic rhinitic and asthmatic subjects. There were no significant differences between the four groups

GRAPH 3.10: P SELECTIN IN THE BRONCHIAL SUBMUCOSA OF HEALTHY CONTROLS AND ALLERGIC SUBJECTS



ICAM-1

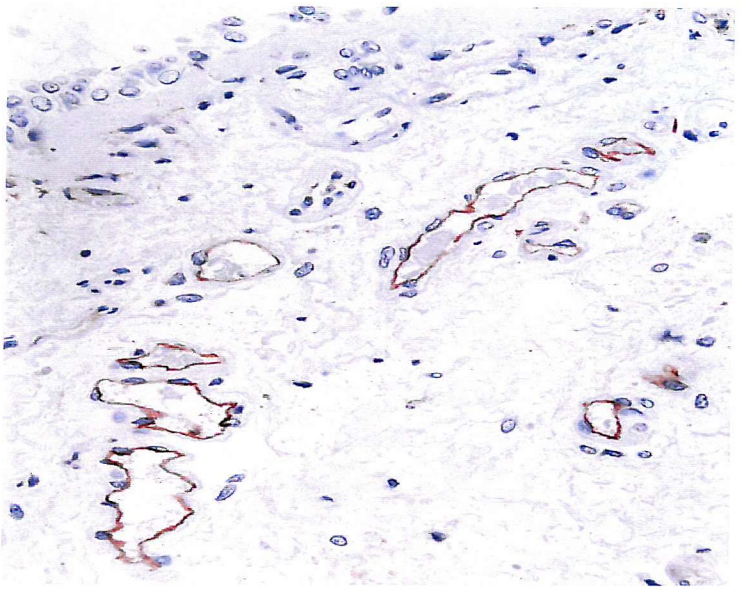
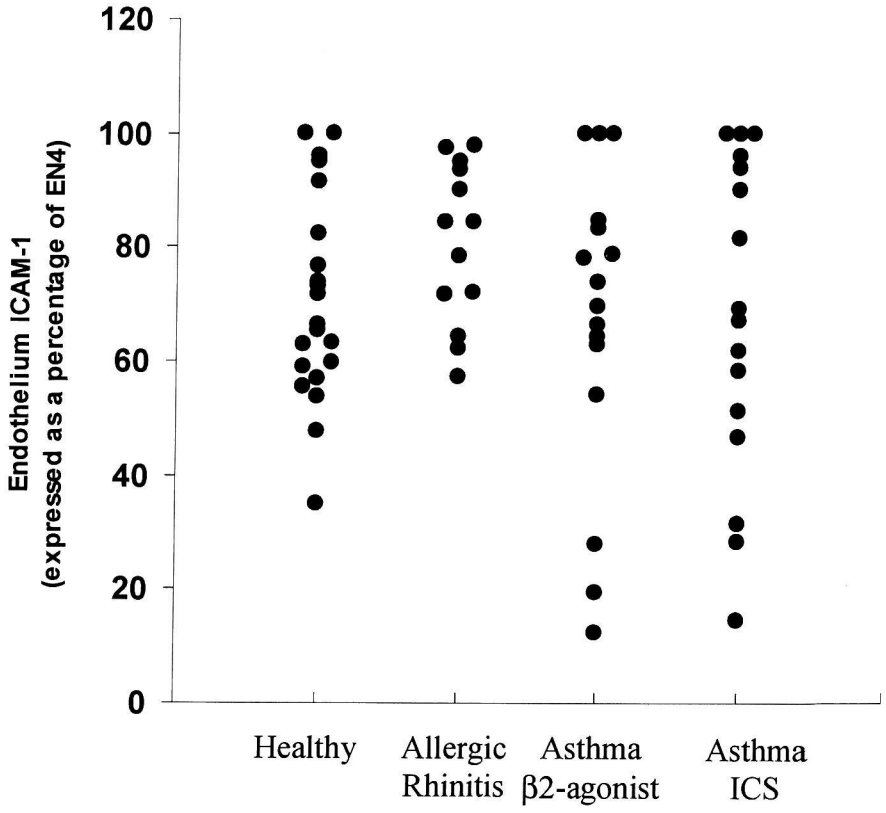


Fig 3.11: Human bronchial submucosa immunostained using monoclonal antibodies directed against ICAM-1.

Graph 3.11 showing the quantification of ICAM-1 (expressed as a percentage of EN4) in the bronchial submucosa taken from healthy, allergic rhinitic and asthmatic subjects.
There were no significant differences between the four groups.

GRAPH 3.11: ICAM-1 IN THE BRONCHIAL SUBMUCOSA OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS



VCAM-1

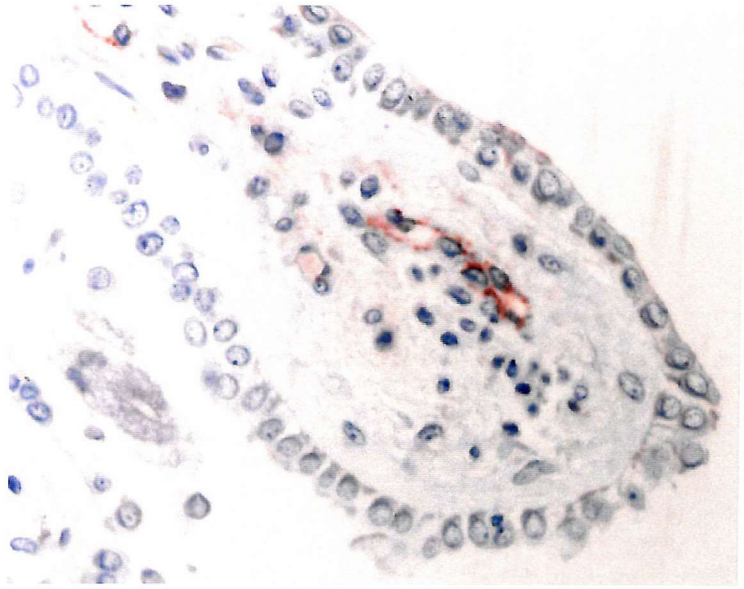
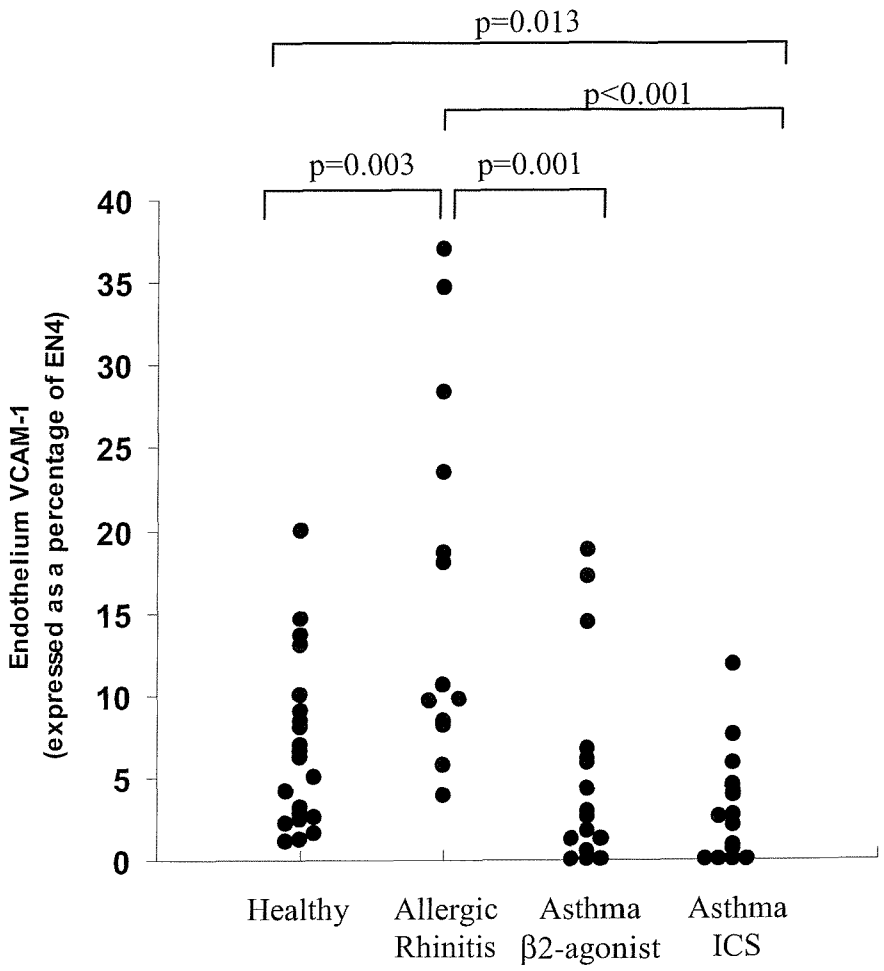


Fig 3.12: Human bronchial submucosa immunostained using monoclonal antibodies directed against VCAM-1.

See graph 3.12 overleaf showing the quantification of VCAM-1 (expressed as a percentage of EN4) in the bronchial submucosa taken from healthy, allergic rhinitic and asthmatic subjects.

Kruskall Wallis analysis of the four groups revealed a significant difference between them ($p<0.001$). The endothelial expression of VCAM-1 was significantly increased in allergic rhinitic subjects compared to both groups of asthmatic subjects ($p=0.001$ and $p<0.001$ respectively) and healthy controls ($p=0.003$). In the asthmatic group treated with inhaled corticosteroids, the endothelial expression of VCAM-1 was significantly reduced compared to healthy controls ($p=0.013$).

GRAPH 3.12: VCAM-1 IN THE BRONCHIAL SUBMUCOSA OF THE HEALTHY CONTROLS AND ALLERGIC SUBJECTS



3.5.3 Eosinophil and Neutrophil inflammation

While it is undoubtedly true that most patients with asthma have evidence of an eosinophilic inflammation in the lower airways, it is clear from the data already presented that this is not universally the case. In both groups of asthmatics, those treated with β2-agonists alone and those treated with inhaled corticosteroids, there were individuals who did not have any evidence of an eosinophilic inflammation. While it may be argued that in the asthmatics treated with β2-agonists alone the disease is too mild to cause an increase in the eosinophils in the lower airways, in the asthmatics treated with inhaled corticosteroids the steroids may have attenuated the allergic inflammation and instead induced a neutrophilic response.

Alternatively, there is evidence that a subgroup of severe asthmatic patients exists who do not have an increase in eosinophils in the lower airways but may instead have a neutrophilic inflammation (Sur, S. *et al.* 1993; Wenzel, S.E. *et al.* 1999). In view of this, I have further analysed my data looking at the relationship between eosinophils and neutrophils in the submucosa in both groups of asthmatic subjects and in the rhinitic subjects. The numbers of eosinophils in the epithelium of all the subjects was too few to analyse with any confidence. Given that the subjects recruited into this study were not severe asthmatics, I was interested to see if there was any correlation between the numbers of neutrophils and eosinophils in any of the three groups. I anticipated that there would be no correlation in the allergic rhinitic and asthmatics treated with β2-agonists alone, but in the asthmatics treated with inhaled corticosteroids I hypothesised that a negative correlation would exist with the corticosteroids reducing eosinophil numbers and increasing neutrophil numbers. In all cases, no correlation was seen between the numbers of eosinophils and neutrophils.

FIG 3.13: EOSINOPHILS AND NEUTROPHILS IN THE SUBMUCOSA OF ALLERGIC RHINITIC SUBJECTS

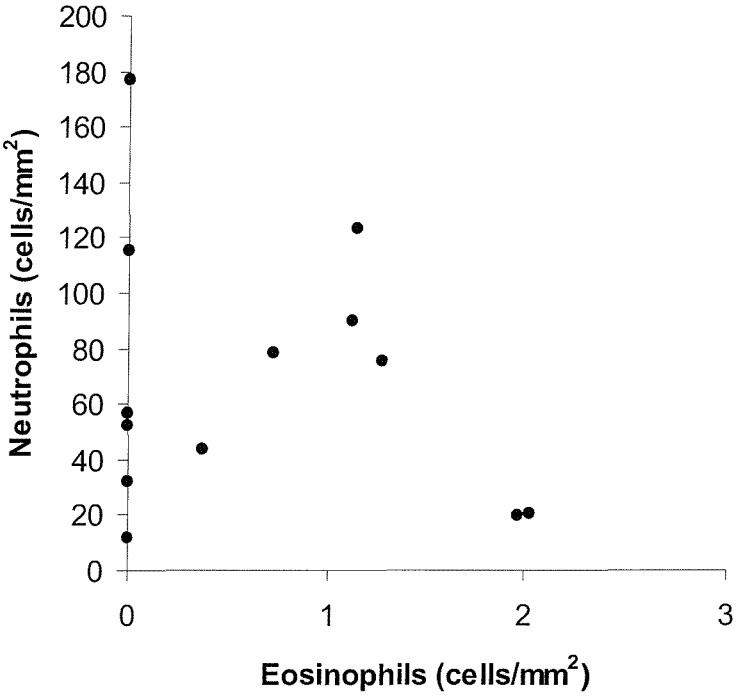
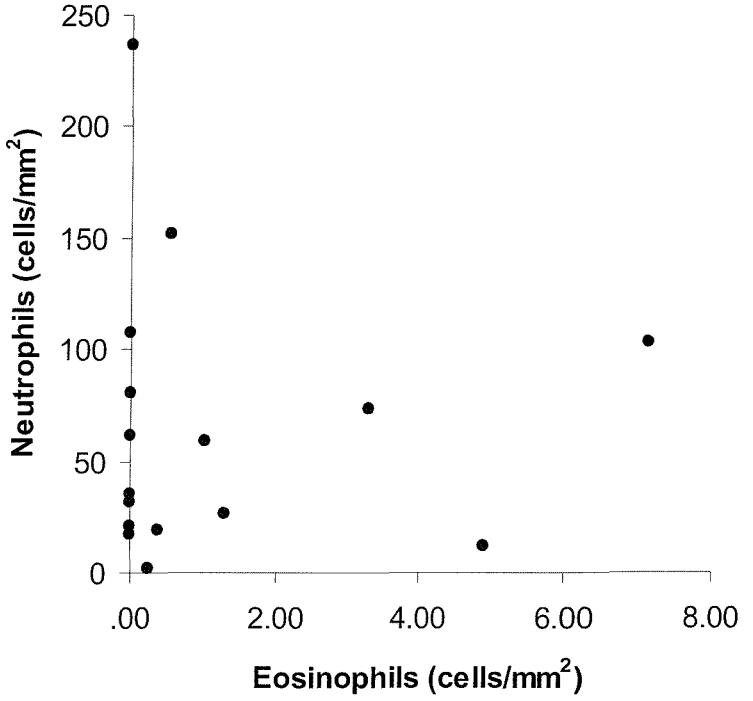


FIG 3.14: EOSINOPHILS AND NEUTROPHILS IN THE SUBMUCOSA OF ASTHMATICS TREATED WITH B2-AGONISTS ALONE



3.6 DISCUSSION

Asthma has been characterised as a chronic inflammatory airway disease defined by reversible airway obstruction and non-specific airway hyper-responsiveness. In this study, the bronchial biopsies and bronchial wash samples taken from asthmatic subjects treated with β2-agonists alone, demonstrated elevated levels of eosinophils and mast cells, a result which is consistent with the cellular inflammation described in previous studies as a feature of allergic asthma (Azzawi, M. *et al.* 1990; Djukanovic, R. *et al.* 1990; Poston, R.N. *et al.* 1992). Interestingly, the asthmatics treated with inhaled corticosteroids did not show an increased number of eosinophils and mast cells in the biopsies, presumably because the steroids reduced the allergic inflammatory response in the lower airways. This raises the question: should all asthmatics be treated with inhaled corticosteroids given that even those with clinically very mild disease (treated with β2-agonist only) have evidence of significant lower airways inflammation? It is not known whether this inflammation causes subsequent progression in the severity of asthma and further work will have to be done to see if those individuals with most inflammation develop moderate/severe asthma symptoms in the future.

Eosinophils and mast cells were also significantly increased in the subjects with allergic rhinitis, suggesting that they are a feature of atopy in general. The degree of cellular inflammation may dictate the expression of the asthmatic state, with the asthmatic subject having a sufficient inflammatory 'load' to cause clinical symptoms, while the subject with allergic rhinitis remains asthma-symptom free due to a low-grade level of bronchial inflammation only. The asthmatic individuals showed an increase in the percentage of mast cells and eosinophils in the bronchial wash. The presence of these cells in the airway lumen may also be significant in the development of symptomatic disease. As before, it is not known

whether the low-grade inflammation seen in the allergic rhinitics may precede the development of asthma, nor whether treatment with inhaled corticosteroids might prevent such a progression from occurring. Further work will need to be performed in this area in order to clarify those issues.

The mast cell has been shown to provoke an asthmatic response to allergen (Djukanovic, R. *et al.* 1990) and several studies have demonstrated that mast cell numbers are increased in BAL fluid obtained from asthmatics (Kirby, J. *et al.* 1987; Wardlaw, A. *et al.* 1988). Mast cell numbers have also been shown to be elevated, compared to healthy controls, in bronchial biopsies obtained from asthmatics (Beasley, R. *et al.* 1989). In most studies of allergic disease, the mast cell is usually located in the bronchial epithelium, at the interface of the internal and external environment within the lung, where it may respond to allergens and other exogenous stimuli (Bingham, C.O. *et al.* 2000). This is reflected in the results presented in this thesis: no statistically significant differences were detected between healthy, asthmatic (β2-agonist only) and allergic rhinitic subjects in the mast cell population in the submucosa; but a clear gradient existed between the three groups for mast cell numbers in the bronchial epithelium, with asthmatics (β2-agonist only) having the most mast cells (mean 2.74, median 1.64), healthy controls having the least (mean 0.29, median 0.12), and the subjects with allergic rhinitis having intermediate numbers of mast cells (mean 1.21, median 0.7). In addition the percentage of mast cells was increased in the bronchial wash taken from the asthmatic subjects (mean 2.05, median 0.88 for the asthmatics treated with β2-agonists only; and mean 1.03, median 0.5 for the asthmatics treated with ICS) compared to the healthy controls (mean 0.17, median 0).

The activation of mast cells leads to the release of mediators that contribute to the early phase of asthmatic inflammation, and mast-cell-derived products may also contribute to the late-phase asthmatic response (Bingham, C.O. *et al.* 2000). The cytokines interleukin (IL)-4, -5, -6, and tumour necrosis factor-alpha (TNF-α), which are considered to play a pivotal role in asthmatic inflammation, have been shown to be produced by mast cells (Bradding, P. *et al.* 1994), and it has also been demonstrated that bronchoalveolar mast cells will release histamine in response to IgE-dependent challenge (Flint, K.C. *et al.* 1986). The increase in mast cells in the bronchial epithelium of allergic rhinitic subjects is therefore a cause for concern in as much as it provides the necessary mechanisms for the induction of asthma in such individuals and may contribute to the perpetuation of the inflammatory response.

The eosinophil is considered a major effector cell of allergic disease as a result of its ability to produce inflammatory mediators such as leukotrienes, major basic protein and eosinophil peroxidase (Djukanovic, R. *et al.* 1990). Increased eosinophil numbers in the bronchial tree are found in post-mortem studies of patients dying from status asthmaticus (Arm, J.P. *et al.* 1992) and also in less severe asthmatic disease (Beasley, R. *et al.* 1993). The finding that eosinophilia is present in the lower airways of both asthmatic and atopic non-asthmatic individuals was described by Howarth *et al* (Howarth, P.H. *et al.* 1991), who showed that while eosinophil numbers were highest in the asthmatics, they were also significantly increased in allergic rhinitics compared to healthy controls. The results of my study show a similar pattern with increased numbers of eosinophils in the bronchial submucosa of asthmatics treated with β2-agonists only (mean 1.18, median 0.13), and allergic rhinitic subjects (mean 0.67, median 0.38), compared to healthy controls (mean 0.8, median 0). The asthmatics treated with β2-agonists only also showed an increased percentage of

eosinophils in the bronchial wash (mean 1.11, median 1.0) compared to healthy controls (mean 0.29, median 0.2).

Sputum eosinophilia has been associated with asthma severity (Louis, R. *et al.* 2000), and in allergic rhinitic subjects, has been shown to be significantly associated with adenosine hyperresponsiveness, although not methacholine hyperresponsiveness (Polosa, R. *et al.* 2000). Allergen challenge in both asthmatics and allergic rhinitics results in a similar increase in sputum eosinophilia (Alvarez, M.J. *et al.* 2000b) suggesting that the difference between asthmatics and allergic rhinitics may lie only in the quantitative bronchial response to allergen inhalation. The results from my study show that there are some asthmatic subjects who do not have an increase in eosinophils at baseline. No significant differences were detected between the numbers of eosinophils in the allergic rhinitic subjects, the asthmatics treated with β2-agonists alone or the asthmatics treated with inhaled corticosteroids. However, given that the numbers in each group were small, this may account for the failure to achieve statistical significance. The other possible explanation is that the disease process in the asthmatics treated with β2-agonists alone was too mild to differentiate them from the allergic rhinitic subjects, while the increase in eosinophils was attenuated in the asthmatics treated with inhaled corticosteroids.

As discussed previously, there is also a body of evidence suggesting that a neutrophilic inflammation may be clinically significant in those asthmatic patients who do not have clear signs of an eosinophilic infiltration of the lower airways (Sur, S. *et al.* 1993; Sampson, A.P. 2000). The eosinophilic and neutrophilic inflammation in both groups of asthmatic subjects in this study appeared to be totally independent of each other. This is a

surprising result: one could hypothesise that the inflammation would be positively correlated with the increase in cytokines, in particular IL-8, from the eosinophils resulting in a neutrophilia as well; or one might have anticipated a negative correlation with the neutrophilic inflammation representing asthma that was well controlled on inhaled corticosteroids, or more controversially, a different phenotype of asthma more commonly seen at the severe end of the disease spectrum. The fact that the two cell populations do not appear to be related to each other in any way suggests that independent mechanisms for their recruitment and retention may be at work.

This study also shows a variation in the numbers of macrophages in bronchial epithelium although this failed to achieve statistical significance. Although some individuals from each of the four groups did not have any macrophages present in the epithelium, in some atopic individuals there was a marked increase compared to the healthy controls. Macrophages can bind allergen-specific IgE molecules and subsequent contact with the specific allergen induces the extra-cellular release of a variety of proinflammatory cytokines, including IL-1, IL-6, GM-CSF and TNF-α (Joseph, M. *et al.* 1981). Macrophage-derived TNF-α amplifies the inflammatory response by its capacity to enhance eosinophil cytotoxicity (Kay, A.B. 1989), while GM-CSF influences eosinophil function and survival (Arm, J.P. *et al.* 1992). The degree to which macrophages release such cytokines may influence the development of allergic asthma.

Lymphocytes are also implicated in the development of asthma with T_H2-cell activation and production of cytokines playing an important role in the development and maintenance of allergic disease (Leong, K.P. *et al.* 2001). In this study, allergic rhinitics had a

significantly elevated total lymphocyte population ($CD3^+$) in the bronchial epithelium (mean 9.83, median 7.09), compared to healthy controls (mean 3.97, median 2.84). This was greater than that seen in both groups of asthmatics, and largely consisted of $CD8^+$ T-suppressor lymphocytes. It would appear that the lymphocyte inflammation is attenuated by treatment with inhaled corticosteroids as there is a trend for this group of asthmatics to have a lower number of both $CD3^+$ (mean 2.18, median 2.09) and $CD8^+$ lymphocytes (mean 0.86, median 0.71) compared to the asthmatics treated with β2-agonist alone ($CD3^+$ mean 4.47, median 3.26; $CD8^+$ mean 2.0, median 0.61), although this failed to reach statistical significance.

However, it is surprising that the subjects with allergic rhinitis have an increased lymphocytic inflammation compared to the asthmatics treated with β2-agonists. This may represent a protective phenotype in the allergic rhinitic subjects, protecting them from the development of asthma. There was a trend for $CD4^+$ T-helper lymphocytes to be elevated in the bronchial epithelium in the subjects with allergic rhinitis (mean 1.52, median 1.71) compared to the healthy controls (mean 0.79, median 0.4), although this failed to reach statistical significance. Asthmatic subjects treated with β2-agonists alone also showed a slight increase (mean 0.94, median 0.4). $CD4^+$ T-helper lymphocytes are primarily involved in the induction and maintenance of the allergic inflammation responsible for asthma, and the evidence of their presence in both symptomatic and asymptomatic individuals again raises the possibility that the development of asthma may be dependent upon the quantity of cells recruited. Activated lymphocytes ($CD25^+$) have previously been shown to be increased in the lower airways of allergic asthmatics (Azzawi, M. *et al.* 1990) and although this study did not find a significant difference, a trend in this direction can be detected.

An important step in the recruitment of leucocytes to an inflammatory focus is margination to the vascular endothelium. This process is promoted by cell adhesion molecules, including E selectin, P selectin, ICAM-1 and VCAM-1. ICAM-1 has previously been shown to be up-regulated on vascular endothelium in allergic inflammation (Arm, J.P. *et al.* 1992) and after allergen challenge, and the intensity and extent of both VCAM-1 and ICAM-1 expression has been shown to correlate significantly with the number of eosinophils in bronchial tissue (Bentley, A.M. *et al.* 1993). In this study, no significant differences were found between both groups of asthmatics, allergic rhinitics and healthy controls for E selectin, P selectin and ICAM-1. There was a significant increase in the amount of the cell adhesion molecule VCAM-1 detected in allergic rhinitic subjects (mean 16.61, median 10.61), compared to healthy controls (mean 6.79, median 6.17) and both groups of asthmatics (mean 5.2, median 2.71 and mean 3.16, median 2.6 respectively). Eosinophils have been shown to bind to VCAM-1 adhesion molecules (Walsh, G.M. *et al.* 1996) and may explain the mechanism whereby eosinophils are increased in the bronchial epithelium of the allergic rhinitic subjects.

The reason for the failure to detect increased adhesion molecule expression in the allergic asthmatic individuals remains unclear. In the asthmatics treated with β2-agonists alone, it may reflect the presence of a more chronic inflammatory process, with subsequent down-regulation of acutely increased adhesion molecule expression. In the asthmatics treated with inhaled corticosteroids, previous research has shown that glucocorticoids significantly reduce surface expression of VCAM-1 expression on BEAS-2B bronchial epithelial cells *in vitro* (Atsuta, J. *et al.* 1999). Several commonly used inhaled glucocorticoids were employed in the study and all inhibited VCAM-1 expression in a dose-dependent manner. These results

suggest that suppression of epithelial VCAM-1 expression in the second group of asthmatics is due to their treatment with inhaled corticosteroids.

3.7 CONCLUSIONS

In conclusion, this study demonstrates the presence of inflammatory changes in the bronchial airways of non-asthmatic allergic rhinitic subjects. These inflammatory changes are similar to those of detected in the lower airways of allergic asthmatics, but differ in terms of the number of inflammatory cells, which is less in the allergic rhinitics, and in the presence of increased numbers of $CD8^+$ T-suppressor lymphocytes. The lack of asthmatic symptoms in the allergic rhinitic subjects may be a dose-dependent effect; with insufficient inflammatory cells present to engender bronchoconstriction or it may be that a different ratio of $CD4^+/CD8^+$ T-cell interaction is required.

CHAPTER FOUR

Late inflammatory response in the lower airways of atopic and healthy subjects following short-term exposure to diesel exhaust

4.1 INTRODUCTION

Diesel exhaust is a complex mixture of gases and particulates and forms a major component of air pollution in major cities and industrialised areas. Epidemiological studies have demonstrated that diesel exhaust has a negative impact on respiratory and cardiovascular health, and human and animal exposure studies are beginning to provide greater understanding into the mechanisms by which diesel exhaust exerts its biological effects. We have previously shown that the exposure of healthy subjects to relatively high levels of diesel exhaust fumes (300$\mu g/m^3$ for two hours) induces an acute inflammatory reaction in the lower airways that is evident six-hours post-exposure. Epidemiological data suggests that diesel exhaust exposure may exacerbate pre-existing lung disease with a lag phase of 24-72 hours. It is therefore possible that this acute inflammation is potentiated in asthmatic individuals, resulting in the clinical manifestations of an acute asthma attack 24-72 hours later. No studies have yet addressed the time course of the inflammatory responses in the lower airways to diesel exhaust, nor compared those responses in atopic individuals with healthy controls.

4.2 AIM OF THE STUDY

The objective of this study was to test the hypothesis that 18-hours after DE exposure, the acute inflammation evident at six-hours post exposure, would have been further augmented in the asthmatic subjects, making them predisposed to exacerbations of asthma; that the allergic rhinitic subjects might show a similar but less marked potentiation of inflammation; while in the healthy controls resolution of the acute inflammatory response may have occurred.

4.3 STUDY DESIGN

The subjects and methods used in this study are detailed in Chapter 2. Each subject was randomly exposed to air or diesel exhaust (100μg/m^3) on two distinct occasions separated by three weeks. The exposures were carried out in a single-blinded fashion in a specially designed exposure chamber in Umeå, Sweden. During each two-hour exposure the subjects alternated rest with exercise on a bicycle ergometer (V_E= 20l/min/m^2 body surface) in 15-minute intervals. Every 30 minutes the asthmatic subjects completed a symptom questionnaire to assess their level of discomfort. Asthmatic subjects were encouraged to use a short-acting β2-agonist inhaler if required and pre- and post lung function tests were recorded in this group.

Eighteen hours after each exposure the subjects underwent a fibreoptic bronchoscopy with collection of BW, BAL and endobronchial biopsies. The wash and lavage samples were filtered and the cells resuspended in PBS before being counted. The biopsies were processed into GMA resin and stained for immunohistochemistry cell markers. All the samples were

randomly coded and therefore blinded to the examiner. Inter-observer variability was avoided by having only one individual perform the cell counting. The total number of subjects in each group was: 32 asthmatics (16 treated with β2-agonists alone and 16 treated with β2-agonists and inhaled corticosteroids), 13 allergic rhinitics and 21 healthy controls.

4.4 STATISTICAL ANALYSIS

All data were analysed using SPSS Version 10.0 for Windows statistical package. Symptom scores were obtained from the asthmatic subjects for both the air and diesel exhaust exposures. The differences between these scores were analysed using the Mann-Whitney U test. A p value <0.05 was considered statistically significant.

Lung function measurements (PEF and FEV_1) were obtained from the asthmatic subjects immediately before and after each exposure on day 0. Additional measurements of PEF were obtained during the next 48-hours. The FEV_1 results were ananlysed by paired t test before and after each separate exposure. The PEF results were analysed using repeated measures ANOVA and Dunnett post test. Missing values were handled with the 'last observation carried forward' method. A p value <0.05 was considered statistically significant.

The BW and BAL data from the allergic subjects was compared to the healthy control subjects using the Mann-Whitney U test. A p value <0.05 was considered statistically significant.

Bronchial biopsy data obtained post-air and post-diesel exhaust exposures were analysed by Wilcoxon rank test. A p value <0.05 was considered statistically significant.

4.5 RESULTS

4.5.1 Symptom Data

Symptom scores were obtained from each asthmatic subject before each exposure to air or diesel exhaust and every thirty minutes during the exposure. The Borg symptom scale was used (Borg, G.A. 1982) and information gathered concerning the following symptoms:

- Breathing difficulties
- Chest tightness
- Cough
- Dizziness
- Eye irritation
- Nose irritation
- Headache
- Unpleasant smell
- Unpleasant taste
- Sore throat
- Nausea
- Fatigue

Data was analysed using the Mann Whitney test. The only significant differences between the two exposures were in smell and taste, with higher scores during the diesel exhaust exposure.

Table 4.1: Significant Results from the Symptom Data in the Asthmatic Subjects comparing Air and Diesel Exhaust Exposures

	Smell	**Taste**
Asthma β2-agonist	p=0.07	NS
Asthma ICS	p=0.004	p=0.024

4.5.2 Lung Function Measurements

Lung function measurements (PEF and FEV_1) were obtained from the asthmatic subjects immediately before and after each exposure on day 0. Subjects were provided with a peak flow meter and instructed in its use in order to obtain PEF readings at the following time points:

- the evening after the exposure (day 0) - E0
- the following morning (day 1) - M1
- the afternoon (day 1) - A1
- the evening (day 1) - E1
- the following morning (day 2) - M2

The FEV_1 results were ananlysed by paired t test before and after each separate exposure. The PEF results were analysed using repeated measures ANOVA and Dunnett post test. Missing values were handled with the 'last observation carried forward' method.

There was a significant reduction in FEV_1 after diesel exhaust exposure in the asthmatics treated with β2-agonists alone ($p= 0.03$) as shown in table 4.2. All other FEV_1 measurements failed to reach statistical significance. One might predict that the asthmatics treated with β2-agonists alone would be more sensitive to the effects of diesel exhaust given that they had higher levels of bronchial inflammatory cells at baseline, compared to asthmatics treated with ICS.

The PEF readings were not significantly reduced immediately after diesel exhaust exposure in either of the asthmatic groups. However, the subsequent PEF readings taken the morning, afternoon and evening after diesel exhaust exposure did show a significant decrement compared to baseline in both asthmatic groups (Tables 4.3 and 4.4). This effect was of similar magnitude in both the asthmatics treated with β2-agonists alone and those treated with ICS. However, a similar reduction in PEF was also measured in both asthmatic groups at the same time-points following exposure to air. The role of diesel exhaust in causing the decrement in lung function is therefore uncertain. These findings may reflect the variability in lung function seen in asthmatic patients *per se*. There may also be an element of recording error as the asthmatic subjects recorded their own peak flows durong the post-exposure period.

Table 4.2: FEV_1 (L/min) in the Asthmatic Subjects before and after Air and Diesel Exhaust Exposures

	Asthma β2- agonists		**Asthma ICS**	
	Pre exposure	Post exposure	Pre exposure	Post exposure
Air	4.19 (0.23)	4.14 (0.22)	3.88 (0.19)	3.90 (0.18)
Diesel	4.15 (0.21)	4.07 (0.21)*	3.89 (0.19)	3.89 (0.19)

* indicates a significant result (p=0.03)

The figure in brackets is the SEM= Standard Error of the Mean

Table 4.3: PEF (L) readings in the Asthmatics treated with β2-agonists alone pre- and post -Diesel Exhaust Exposures

	PEF readings						
	Pre 0	**Post 0**	**E0**	**M1**	**A1**	**E1**	**M2**
Air	516 (27.0)	514 (27.4)	517 (26.6)	495 (24.6)	473 (26.4)*	464 (24.3)*	473 (24.8)*
Diesel	528 (22.7)	512 (23.2)	513 (23.1)	492 (20.4)*	486 (26.9)*	495 (24.6)*	467 (23.8)*

Table 4.4: PEF readings in the Asthmatics treated with ICS pre- and post-Diesel Exhaust Exposures

	PEF readings						
	Pre 0	**Post 0**	**E0**	**M1**	**A1**	**E1**	**M2**
Air	508 (26.5)	508 (28.2)	506 (26.8)	498 (26.8)	486 (26.1)	472 (21.5)*	484 (26.1)*
Diesel	517 (29.0)	503 (26.3)	504 (26.6)	485 (23.7)*	466 (22.5)*	468 (23.4)*	478 (22.3)*

* indicates a significant result compared to the pre-exposure PEF ($p<0.05$)

The figure in brackets is the SEM= Standard Error of the Mean

Pre 0 = PEF taken immediately pre-exposure on day 0

Post 0 = PEF taken immediately post-exposure on day 0

E0 = PEF taken the evening after exposure on day 0

M1 = PEF taken the morning after exposure on day 1

A1 = PEF taken the afternoon after exposure on day 1

E1 = PEF taken the evening after exposure on day 1

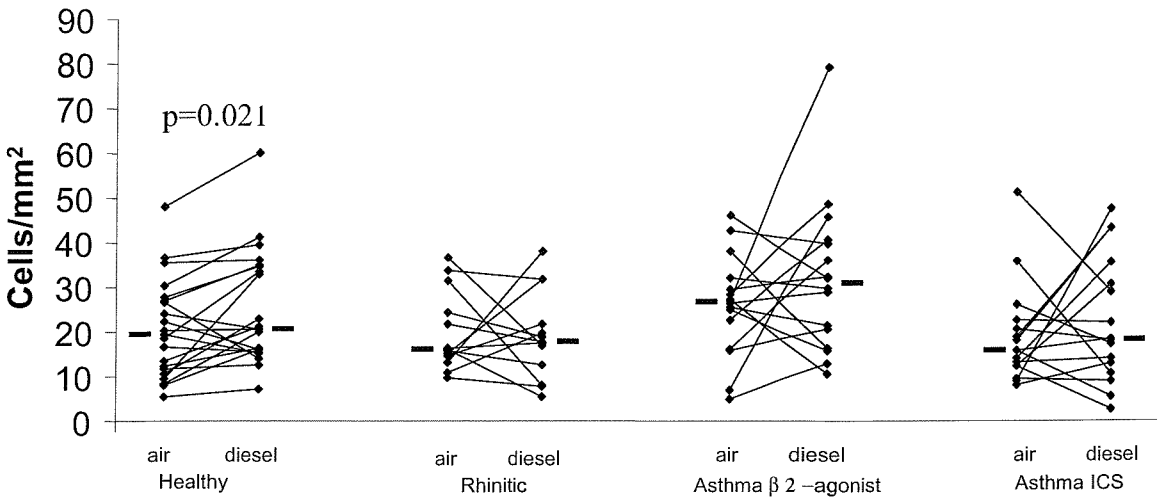
M2 = PEF taken the morning after exposure on day 2

4.5.3 Cellular inflammation in the lower airways

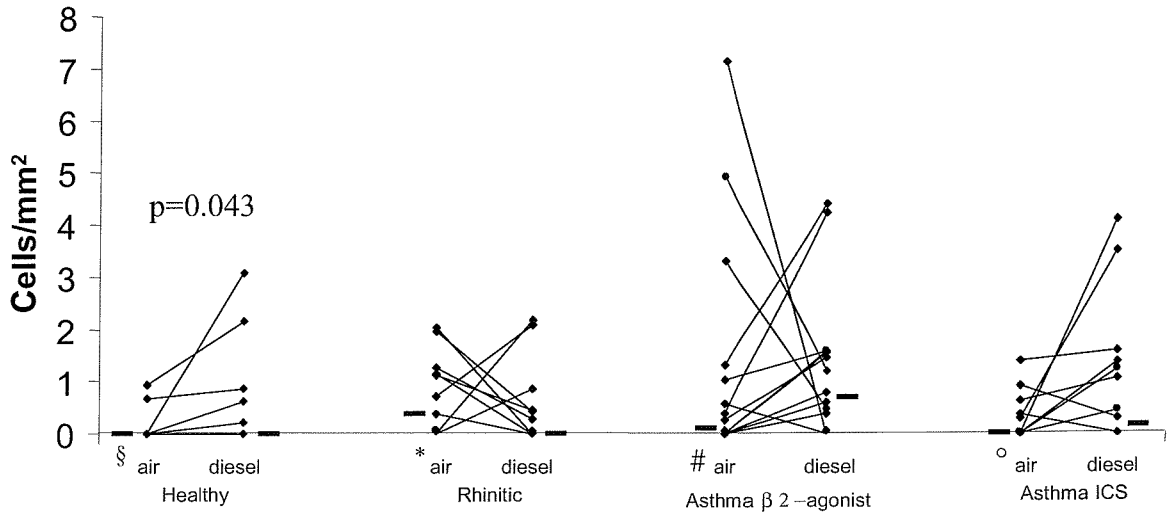
In healthy controls there was a statistically significant increase in mast cells (p=0.021), eosinophils (p=0.043) and neutrophils (p=0.016) in the bronchial submucosa 18-hours post diesel exhaust exposure (Graphs 4.1-4.3). The increase in the neutrophils in the submucosa was associated with a significant increase in the proportion of neutrophils in the BW (p=0.024). There was also a significant reduction in the proportion of macrophages in the BW (p= 0.033).

In the subjects with allergic rhinitis, the number of mast cells in the bronchial epithelium was significantly reduced post diesel exhaust exposure (p=0.026) (Graph 4.4). Like the healthy subjects, allergic rhinitics also demonstrated a significant increase in the proportion of neutrophils in the BW (p=0.04), and a significant reduction in the proportion of macrophages in the BW (p= 0.02). In addition, the proportion of eosinophils was reduced in the BAL (p= 0.03).

Asthmatic individuals did not have any evidence of statistically significant cellular inflammation in the lower airways post diesel exhaust exposure.



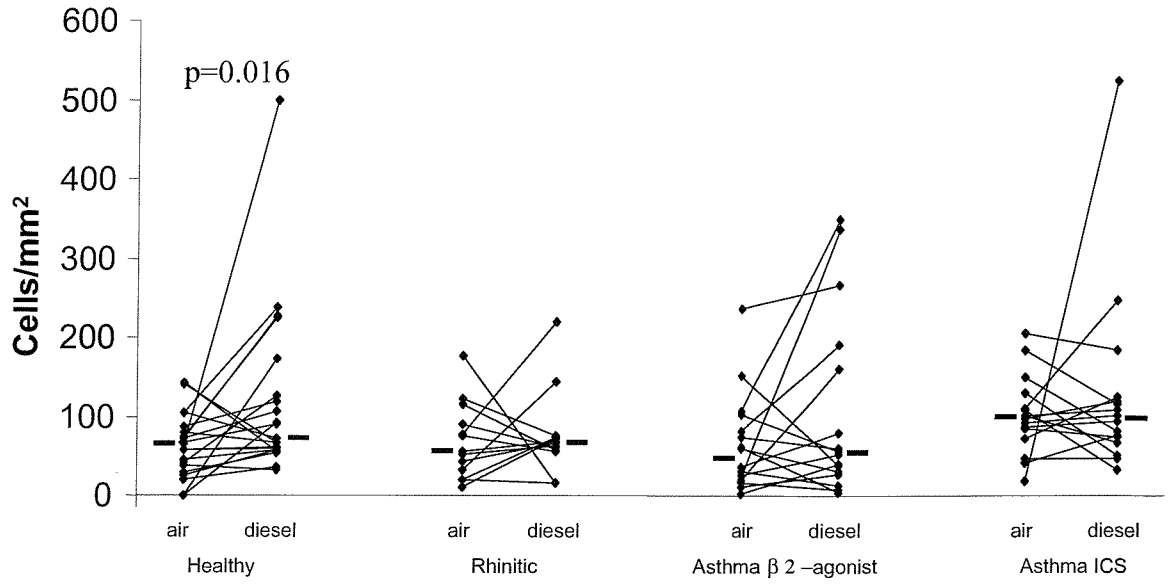
GRAPH 4.1: MAST CELLS IN THE BRONCHIAL SUBMUCOSA 18-HOURS POST DIESEL EXHAUST EXPOSURE



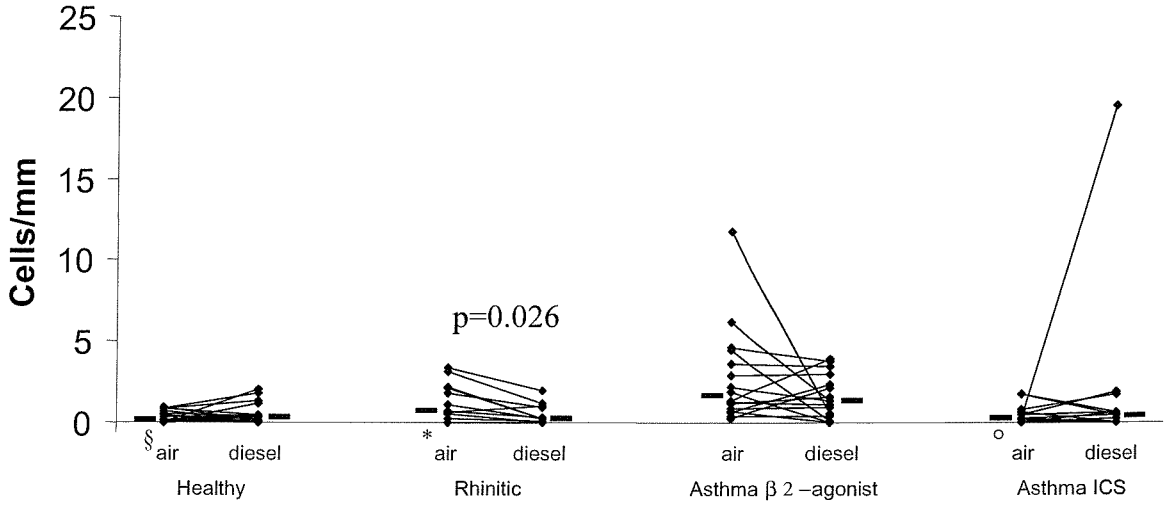
GRAPH 4.2: EOSINOPHILS IN THE BRONCHIAL SUBMUCOSA 18-HOURS POST DIESEL EXHAUST EXPOSURE.

The symbols represent the number of individuals (n) with zero eosinophils/mm^2 pre- and post-diesel exhaust exposure.

§ (n=19); * (n=6); # (n=8); ° (n=11).



GRAPH 4.3: NEUTROPHILS IN THE BRONCHIAL SUBMUCOSA 18-HOURS POST DIESEL EXHAUST EXPOSURE



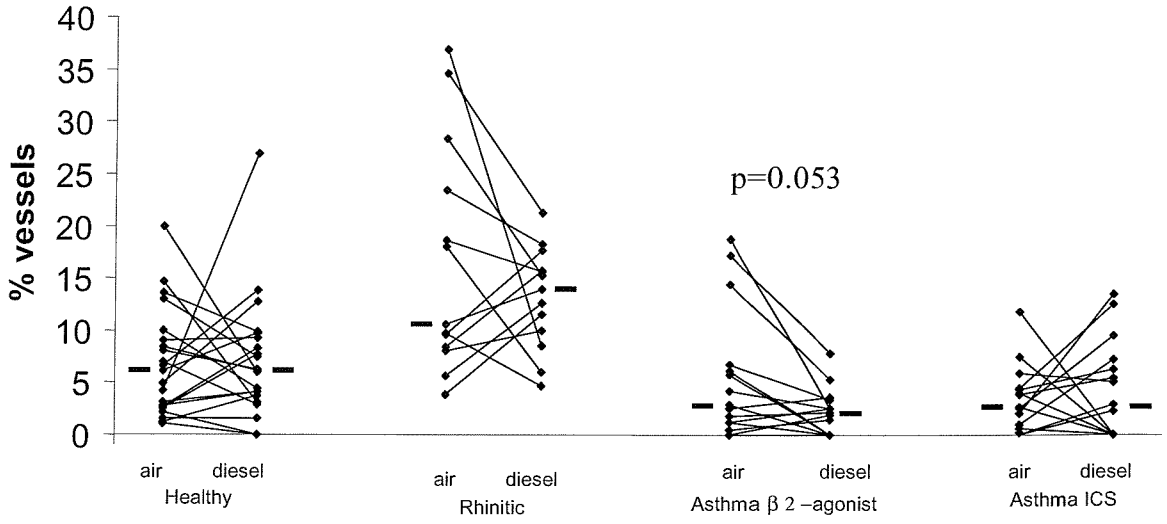
GRAPH 4.4: MAST CELLS IN THE BRONCHIAL EPITHELIUM 18-HOURS POST DIESEL EXHAUST EXPOSURE.

The symbols represent the number of individuals (n) with zero eosinophils/mm pre- and post-diesel exhaust exposure.

§ (n=9); * (n=3); ° (n=6).

4.5.4 Endothelial adhesion molecule expression

BW, BAL and biopsy specimens from asthmatic subjects treated with β2-agonists alone had no change in the number of inflammatory cells present 18-hours post diesel exhaust exposure, but did show a reduction in the endothelial expression of VCAM-1 which just failed to reach statistical significance (p=0.053)(Graph 4.5).



GRAPH 4.5: ENDOTHELIAL EXPRESSION OF VCAM-1 18-HOURS POST DIESEL EXHAUST EXPOSURE

4.6 DISCUSSION

In this study, subjects were exposed to diluted diesel exhaust and then underwent bronchoscopy and endobronchial biopsy 18-hours later. Healthy subjects exhibited a persistent mast cell, eosinophilic and neutrophilic inflammation in the bronchial submucosa, and also had an increased proportion of neutrophils in the BW. Macrophage numbers were proportionately decreased in the BW. Allergic rhinitics showed a decrease in mast cells in the epithelium, but the same pattern as the healthy subjects in the bronchial wash, with an increase in the proportion of neutrophils and a decrease in macrophages. In addition, the allergic rhinitics had a reduced proportion of eosinophils in the BAL fluid. The asthmatics treated with β2-agonists alone had a decrease in the endothelial expression of VCAM-1.

The exposure protocol used in this study has been designed and validated in order to provide a consistent composition of the key elements of diesel exhaust (Rudell, B. *et al.* 1994). The concentration of diesel exhaust used in this study is comparable to the levels experienced in many of our major cities in the UK and is frequently exceeded in air pollution episodes.

It has previously been shown that diesel exhaust exposure can induce an increase in mast cells, neutrophils and $CD4^+$ and $CD8^+$ lymphocytes in the lower airways of healthy subjects six-hours post-exposure (Salvi, S.S. *et al.* 1997). In the study presented in this thesis, the healthy subjects had evidence of an increase in mast cells, eosinophils and neutrophils 18-hours post exposure, but no evidence of an increase in T lymphocytes.

The atopic individuals (allergic rhinitic and asthmatic subjects) had no evidence of an inflammatory response in the bronchial biopsies at eighteen-hours post diesel exhaust exposure. This is also in keeping with a previous exposure study that examined the lower airway responses in allergic asthmatic subjects six-hours after exposure to diesel exhaust (Frew, A.J. *et al.* 2001). Although an up-regulation in IL-10 was identified in bronchial epithelium of the asthmatic individuals in that study, exposure to diesel exhaust did not induce an acute neutrophilia or any worsening of the pre-existent eosinophilic inflammation. Terashima *et al* have demonstrated that alveolar macrophages incubated with PM_{10} produce mediators that increase the transit times of neutrophils through the bone marrow and stimulate their release into the peripheral circulation (Terashima, T. *et al.* 1997). Repeated NO_2 exposure in healthy subjects has also been shown to result in a decrease in neutrophil numbers in the bronchial epithelium (Blomberg, A. *et al.* 1999). In the paper by Blomberg *et al*, the bronchial wash revealed a twofold increase in content of neutrophils and a 1.5-fold increase in myeloperoxidase indicative of both migration and activation of neutrophils in the airways. It is therefore conceivable that the lack of inflammation seen in the asthmatic and allergic rhinitic individuals is because the neutrophil population has already re-equilibrated in the bronchial submucosa and epithelium. If this is the case, one would expect that an increase in neutrophils might still be detected in the bronchoalveolar lavage samples. As detailed above, both healthy individuals and subjects with allergic rhinitis showed evidence of a significant increase in the proportion of neutrophils in the bronchial wash ($p=0.033$ and $p=0.04$ respectively), but not the bronchial lavage fluid. The bronchial wash is taken from the proximal airways, as opposed to the distal lavage samples. The neutrophil response was most marked in the proximal portion and could possibly be explained by the view that particles of different sizes may have distinct effects on the airways. Future work will involve measuring the chemokine IL-10 in the bronchial epithelium of the asthmatic subjects to ascertain if it is

upregulated. Based on the known properties of IL-10 (Seitz, M. *et al.* 1995; van Scott, M.R. *et al.* 2000), this upregulation may prevent neutrophilic inflammation while simultaneously promoting an allergic phenotype.

Another possible explanation for the lack of inflammation in the atopic subjects is that the diesel exhaust concentration (100μg/m^3 for two hours) is too low and does not induce an inflammatory response of sufficient amplitude to be detected in airways that already have evidence of inflammation at baseline. Repeating this study with a diesel exhaust concentration of 300μg/m^3 for two hours may provide a stronger signal and allow the increased amplitude of inflammation to be detected. Alternatively, repeated challenges of low dose diesel exhaust would mimic more closely the natural pattern of exposure and may also provide a strong enough signal for inflammation to be detected.

Airway neutrophil infiltration is associated with asthma (Fahy, J.V. *et al.* 1995) chronic obstructive airways disease (Peleman, R. *et al.* 1999) and with other pulmonary diseases such as cystic fibrosis (Nakamura, H. *et al.* 1992), idiopathic pulmonary fibrosis (Watters, L. *et al.* 1987) and acute bronchitis (Sibille, Y. *et al.* 1990). Neutrophils contain inflammatory and toxic mediators that may cause structural and functional damage to the lung (Kay, A.B. 1986). The fact that a neutrophilic inflammation may be induced in healthy subjects by exposure to diesel exhaust is clearly of concern and may explain the epidemiological data linking diesel exhaust and adverse respiratory health.

Mast cells are strongly associated with the development and maintenance of asthma, and being largely located within the epithelial layer of the bronchi they are able to respond

quickly to exogenous stimuli (Bingham, C.O. *et al.* 2000). Tryptase, released from human mast cells, increases the reactivity of bronchi to histamine (Berger, P. *et al.* 1999). This alteration is accompanied by a change in the mast cell distribution within the airway wall, with an increase in mast cell number within the subepithelial tissue, whereas mast cell numbers in the epithelial layer concomitantly decrease. It is possible that the mast cells in the allergic rhinitic subjects trafficked out into the lumen of the airway or into the submucosa; however examination of these two compartments fails to demonstrate an increase. Conceivably, a shift in mast cell population has occurred but the increase in the cell numbers in these two compartments is too small to detect.

Human mast cells have been shown to both store and secrete IL-4 and TNF-α, which regulate cellular inflammation in allergic disease (Bradding, P. *et al.* 1993), and DEPs have been shown experimentally to enhance mast cell degranulation in the presence of allergen, although not on their own (Diaz-Sanchez, D. *et al.* 2000). In this study, we were using monoclonal antibodies directed against mast cell tryptase (AA1) to detect mast cells. It is therefore possible that activated mast cells that had released tryptase were no longer identified in the staining process, and that mast cell numbers have been reduced in the allergic rhinitics as a result of degranulation.

The healthy controls had evidence of a significant increase in the numbers of mast cells and eosinophils in the submucosa post-diesel exhaust exposure. This suggests that diesel exhaust may promote an inflammatory allergic milieu in the lower airways of subjects who are non-atopic. It is possible that concomitant exposure to allergen may result in allergic sensitisation and even in asthma. This is an important finding and may help to explain the

epidemiological evidence suggesting that allergy is more common in industrialized countries and the overall rapid increase in the incidence of allergy during the past four decades.

The reduction in the proportion of macrophages in the BW in both the healthy and allergic rhinitic subjects is of uncertain significance. Castronova *et al* have shown that in rats, diesel exhaust exposure does not appear to affect the viability of macrophages, but may suppress their phagocytic activity (Castronova, V. *et al.* 1985). Other work employing cultured macrophages exposed continuously to a well-defined model of PM, demonstrated a time-dependent expression and release of TNF-α and the induction of apoptosis (Chin, B.Y. *et al.* 1998).

VCAM-1 is expressed on the capillary endothelium and is a leucocyte adhesion marker. Expression of VCAM-1 was significantly reduced in the asthmatics treated with β2-agonists 18-hours post-diesel exhaust exposure. In allergic asthma, expression of VCAM-1 has been shown to be mediated by IL-4 and TNF-α and subsequently leads to the selective migration of eosinophils into the airways (Schleimer, R.P. *et al.* 1992; Fukuda, T. *et al.* 1996; Walsh, G.M. *et al.* 1996; Yamamoto, H. *et al.* 1998). Although the mechanisms and significance of the reduction in VCAM-1 expression in the asthmatics in the diesel exposure study remains unclear, the finding is coherent in terms of the lack of an eosinophilia. In the healthy controls, who did have evidence of an eosinophilia, there is no statistically significant increase in the expression of VCAM-1 although it is clear that some individuals did have a very profound upregulation of VCAM-1.

P selectin is a capillary endothelial and leucocyte adhesion marker that is expressed early in the inflammatory cascade and promotes the tethering of inflammatory cells, particularly T lymphocytes and eosinophils, thus aiding the trafficking of cells into the submucosa. One study showed that the adhesion of human peripheral blood T cells to nasal polyp endothelium could be inhibited by approximately 70% by employing antibodies against P selectin and its counter-receptor P selectin glycoprotein-1 (PSGL-1) (Symon, F.A. *et al.* 1999). A similar study showed almost complete inhibition of eosinophil adhesion by a monoclonal antibody (mAb) against P selectin and by a chimeric molecule consisting of the Fc portion of human IgG and the lectin binding domain of P selectin, which binds to the P selectin ligand on leucocytes (Symon, F.A. *et al.* 1994). In previous studies P selectin levels have been increased in asthmatic individuals compared to healthy controls, although in other work there is no apparent difference in the levels of P selectin between asthmatics of variable severity and healthy controls (Vrugt, B. *et al.* 1996; ten Hacken, N.H. *et al.* 1998).

This study failed to detect any alteration in P selectin expression in neither the healthy controls nor the atopic individuals. Previous work looking at the late (24 hours) response of adhesion molecules in asthmatics challenged with allergen failed to detect any cellular inflammation or alteration in P selectin expression. This suggests that P selectin responses are rapid and quickly revert to the steady state, as does any bronchial cellular inflammation if the stimulus is removed. It is possible that in this study P selectin first increased in response to the diesel challenge and was then restored.

4.7 CONCLUSIONS

A number of epidemiological studies have shown that diesel exhaust has an adverse effect on respiratory health, with those individuals with pre-existing lung disease at greatest risk. Previous human *in vivo* studies have demonstrated that diesel exhaust causes an acute inflammatory reaction in the lower airways of healthy individuals. In this study we used a validated exposure protocol to challenge asthmatic subjects (treated with β2-agonist alone or β2-agonist and inhaled corticosteroids), allergic rhinitic subjects and healthy controls to diesel exhaust and assess the late inflammatory reaction in the lower airways.

Healthy controls showed evidence of persistent cellular inflammation in the lower airways comprising mast cells, eosinophils and neutrophils. This result was in keeping with the increased neutrophils and mast cells observed in the bronchial biopsies of the healthy subjects six-hours after exposure to diesel exhaust. Surprisingly there was no evidence of an upregulation of endothelial and leucocyte adhesion molecule expression, which might be expected to account for the cellular influx.

Allergic rhinitic subjects also had reduced levels of mast cells in the epithelium as detected by immunohistochemical methods using antibodies to tryptase. As discussed previously, this finding may have been as a result of degranulation of the mast cells with subsequent release of the tryptase, or it could have been as a result of migration of mast cells into the bronchial lumen. The reason why allergic rhinitic subjects alone should be affected in this way remains unclear, but the finding may indicate a propensity to be more sensitive to diesel exhaust pollution, as might the increase in the proportion of neutrophils in the BW.

The expression of VCAM-1 in asthmatic individuals treated with β2-agonists alone was reduced 18-hours post-diesel exhaust exposure. This finding is not entirely unexpected given the lack of cellular inflammation but its significance remains unclear. It would appear that the role of VCAM-1 in the process of inflammation and asthma is not yet fully understood.

This study confirms that healthy individuals not only have an acute response to diesel exhaust exposure, but that they continue to demonstrate a significant inflammatory response 18-hours post diesel exhaust exposure. This study is the first to characterize the time course of inflammation in the airways after exposure to diesel exhaust. In contrast, the atopic individuals did not show any evidence in the bronchial biopsies of an inflammatory response 18-hours after diesel exhaust exposure, a result which is consistent with the lack of response seen acutely at six-hours. The only increase in inflammation was an increase in the proportion of neutrophils in the BW of the allergic rhinitic subjects. This leaves the mechanisms for the adverse health effects of diesel exhaust, and in particular the lag-effect seen in terms of exacerbation of pre-existing lung disease, largely unexplained.

CHAPTER FIVE

Summary and Final Discussion

5.1. SUMMARY OF RESULTS

The studies presented in this thesis have demonstrated that:

A) Allergic rhinitic subjects have evidence of an asymptomatic inflammation in the lower airways when biopsied outside of the pollen season. This is broadly similar to the inflammation seen in the lower airways of asthmatic subjects and comprises eosinophils, mast cells, and both $CD3^+$ and $CD8^+$ lymphocytes. Allergic rhinitic subjects also have significantly elevated expression of endothelial VCAM-1 compared to allergic asthmatic and healthy subjects.

B) The inflammatory response 18-hours after controlled exposure to diesel exhaust is different in atopic and healthy subjects. In the healthy controls, there is an increase in mast cells, eosinophils and neutrophils in the bronchial submucosa 18-hours post diesel exhaust exposure. There is an associated increase in the proportion of neutrophils in the BW. This increase in neutrophils was also detected in the BW of the allergic rhinitic subjects. However, the bronchial biopsies from asthmatic and allergic rhinitic individuals did not exhibit any cellular inflammation in either the submucosa or the epithelium. In the bronchial biopsies from subjects with allergic rhinitis, the number of mast cells in the bronchial epithelium was significantly reduced post-diesel exhaust exposure; while in the asthmatic group treated with β2-agonist alone there was a statistically significant reduction in the adhesion molecule VCAM-1.

5.2 DISCUSSION

5.2.1. Inflammation in the lower airways of atopic subjects

There is evidence of an increasing epidemic of allergic disease in the industrialized countries of the world (Holgate, S.T. 1999; Weiss, S.T. 2001). The cause of this increase remains unclear but possible aetiological causes may be suggested by epidemiological studies. Such studies have documented the increased risk of the development of allergic asthma in individuals with pre-existing allergic rhinitis (Corren, J. 1997; Greisner, W.A. *et al.* 2000), and a long-term follow-up study of atopic individuals has shown that about 10% of allergic rhinitic patients develop asthma, as compared with 3.6% of those with no prior history of rhinitis (Danielsson, J. *et al.* 1997). The risk factors associated with the development of asthma in individuals with rhinitis are: a family history of asthma and atopy, indicating a common genetic link (Weiss, S.T. *et al.* 1980; Sherman, C.B. *et al.* 1990); a personal history of atopy as defined by skin test reactivity to common aeroallergens, such that 32% of allergic rhinitic individuals have associated asthma as compared to 14% of non-allergic rhinitic individuals (Wright, A.L. *et al.* 1994); and the presence of bronchial hyperresponsiveness (Laprise, C. *et al.* 1997).

Asymptomatic bronchial hyperresponsiveness is generally thought to be caused by airway inflammation (Barnes, P.J. 1989; Kay, A.B. 1991), and has been shown to precede the development of asthma in both adults and children (Sparrow, D. *et al.* 1987; Hopp, R.J. *et al.* 1990). This asymptomatic inflammation has been measured indirectly by the measurement of exhaled nitric oxide (Henriksen, A.H. *et al.* 1999; Gratziou, C. *et al.* 2001) and directly quantified by the examination of induced sputum (Foresi, A. *et al.* 1997) and bronchial biopsies (Djukanovic, R. *et al.* 1992), both of which identified increased numbers of mast

cells and eosinophils in atopic non-asthmatic subjects who were biopsied outside of the pollen season. It is interesting that airways inflammation has been shown to exist in individuals without evidence of either BHR (Alvarez, M.J. *et al.* 2000a) or asthma (Djukanovic, R. *et al.* 1992). This suggests that while asthma is an inflammatory disorder of the airways, inflammation alone is insufficient to explain the chronic nature of the disease and its progression.

Asymptomatic lower airways inflammation may represent one of the earliest stages in the development of asthma and raises two questions: firstly, are additional triggers required to catalyse the development of symptomatic asthma and secondly, would intervention at this stage alter the risk of progression to asthma? This latter question is clearly important, as asthma is associated with significant morbidity and mortality (Mannino, D.M. *et al.* 1998; Walsh, L.J. *et al.* 1999), as well as representing a substantial burden on health resources (Barnes, P.J. *et al.* 1996).

There is some evidence that early intervention in such individuals may be of benefit in reducing the subsequent progression of asthma. In one such study, forty-four subjects mono-sensitised to *Dermatophagoides pteronyssinus*, with perennial rhinitis and BHR to methacholine, were randomly assigned to receive specific immunotherapy (SIT) or placebo in a double-blind study conducted over a period of two years (Grembiale, R.D. *et al.* 2000). After one year of treatment, a 2.88-fold increase in the provocative dose of methacholine producing a 20% decrease in FEV_1 was recorded in the SIT-treated group, with a further increase to fourfold at the end of year two. At the end of the study, the methacholine $PD_{20}FEV_1$ was within the normal range in 50% of treated subjects. In contrast, no changes in methacholine $PD_{20}FEV_1$ were found in the placebo group throughout the study. Although 9%

of subjects given placebo developed asthma, none of those treated with SIT did. This study suggests that immunotherapy may be an effective prophylactic treatment for rhinitic patients with hyperreactive airways. This may mean that in future, those atopic individuals at risk of asthma are identified and offered preventative treatment with SIT.

In the first study presented in this thesis the bronchial inflammation in the lower airways of allergic rhinitic subjects was characterized and compared with the bronchial inflammation seen in a group of asthmatics. In recognising the cellular processes involved, I hoped to be able to suggest a hypothesis for the induction and maintenance of inflammation in such atopic individuals leading ultimately to the development of asthma. The second study was designed to look at the late inflammatory response to diesel exhaust in the same individuals. It was hoped that the pattern of inflammation would help to elucidate the effects of diesel exhaust in exacerbating pre-existing asthma, and possibly even in promoting the development of asthma in non-asthmatic atopic individuals.

A number of theories have been proposed concerning the additional triggers needed to induce the development of asthma. These include the role of air pollution (Salvi, S.S. *et al.* 1999b), allergen sensitisation (Arshad, S.H. *et al.* 2001) and epithelial damage (Holgate, S.T. *et al.* 1999). Several pathologic changes occur in the airway epithelium in asthma, but the relationship between these changes and the initiation and progression of asthma remains poorly understood (Fahy, J.V. 2001).

One possibility is that changes in the structure and function of the epithelium, induced by environmental exposure to allergens or pollution in genetically susceptible subjects,

represent primary pivotal events that occur early in the pathogenesis of asthma. There is a growing body of evidence suggesting that the epithelium is not merely a passive barrier but actively generates a range of mediators that may play a role in the inflammatory and remodelling responses that occur in the lungs in asthma (Holgate, S.T. 2000a). The theory is that epithelial injury is mediated by exogenous factors such as air pollutants, viruses and allergens as well as by endogenous factors including the release of proteolytic enzymes from mast cells (tryptase, chymase) and eosinophils (MMP-9). Following injury, the normal epithelium should respond with increased proliferation driven by ligands acting on epidermal growth factor receptors (EGFR) or through transactivation of the receptor. The epithelial response to these stimuli in asthma appears to be impaired, despite upregulation of CD44 capable of enhancing presentation of EGF ligands to EGFR. As the epithelium is 'held' in this repair phenotype, it becomes a continuous source of proinflammatory products as well as growth factors that drive airway wall remodelling. For example, the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF), whose principal source in the airways is the epithelium, can prolong eosinophil survival, while levels of transforming growth factor (TGF-β) have been shown to be increased in association with increased fibroblasts in asthmatics (Hoshino, M. *et al.* 1998). Fibroblasts are believed to be involved in the deposition of collagen in the sub-epithelial basement membrane, which is a characteristic feature of the remodelling response in asthma (Chiappara, G. *et al.* 2001). Both myofibroblasts and epithelium are involved in early lung development (epithelial-mesenchymal trophic unit), and the remodelling response observed in asthma may represent a reactivation of this process. The difference between asymptomatic bronchial inflammation in allergic rhinitic subjects and the symptomatic inflammation seen in asthmatics may be that the asthmatics have a primary defect in the epithelium such that it responds abnormally to various stimuli and cannot undergo the normal repair response (Holgate, S.T. *et al.* 1999).

Alternatively, these epithelial changes may occur simply as a consequence of pivotal early events in other systems, such as impaired maturation of the immune system with persistence of a predominant T_H2 subtype of $CD4^+$ cells. *In utero*, T cells of the foetus are primed by common environmental allergens that cross the placenta. As a result, and possibly also to maintain maternal tolerance, the immune response of virtually all new-born infants is dominated by T_H2 cells (Prescott, S. *et al.* 1998). During subsequent development of the normal infant's immune system, a shift towards a T_H1 state occurs. In atopic infants this 'immune deviation' occurs at a significantly slower rate and may not occur at all (Yabuhara, A. *et al.* 1997). As a result, allergen-specific T_H2 cell responses produce T_H2 cytokines that are associated with the expression of atopy and asthma in later life (Robinson, D.S. *et al.* 1992), (Figure 5.1).

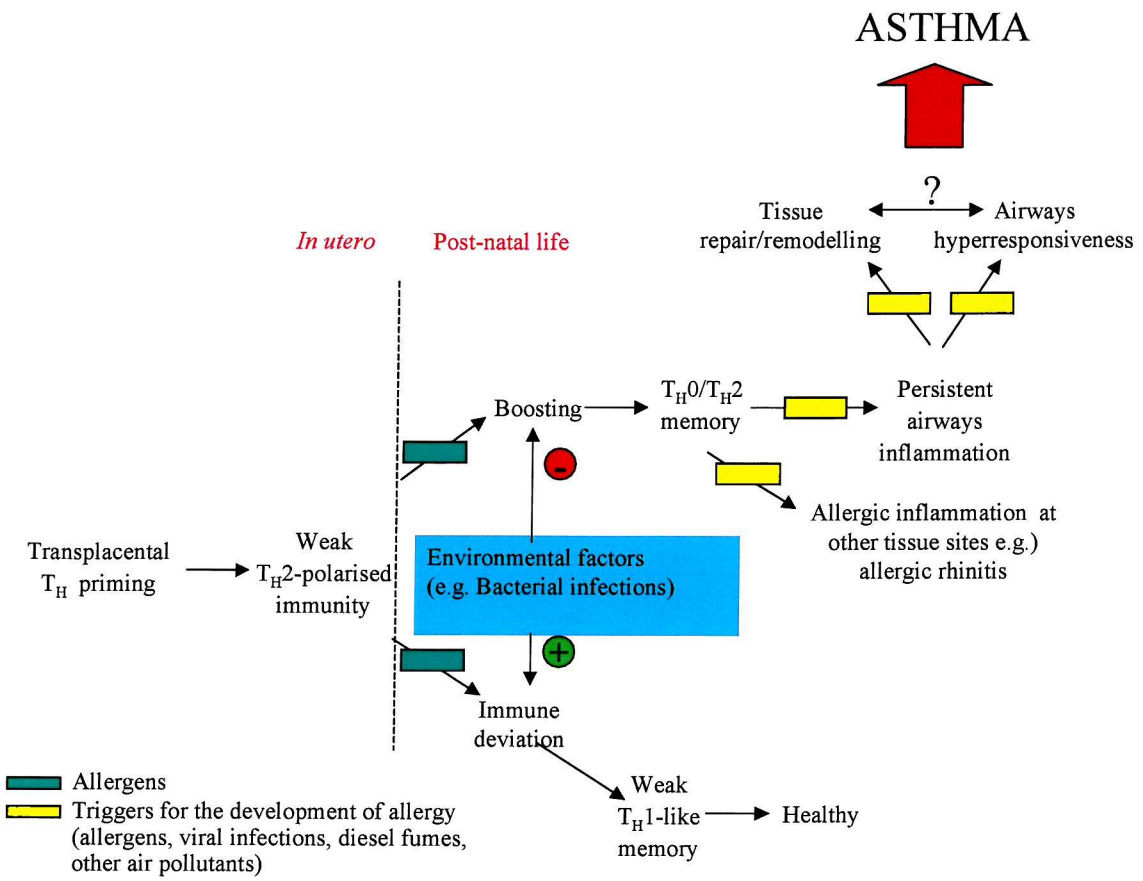


FIGURE 5.1: PROGRESSION OF PRIMARY ALLERGIC SENSITISATION IN EARLY CHILDHOOD TO DEVELOPMENT OF ASTHMA

The diagram shows the development of a weak T_H2-polarised immunity *in utero* as a result of maternal transplacental T_H priming. After birth, the infant is exposed to environmental factors such as bacterial infections. In the healthy child, these factors stimulate immune deviation and lead to the development of a T_H1 phenotype despite continued exposure to allergens. Alternatively, immune deviation may be inhibited due to reduced exposure to environmental factors. This results in an augmented T_H2 memory. Further allergen exposure or exposure to other triggers for the development of allergy, may eventually result in the symptoms of allergic rhinitis and/or asthma.

$CD4^+$ T-helper cells produce cytokines that attract the leucocytes that are ultimately associated with acute and chronic inflammation in the airways. The key role of these lymphocytes has been demonstrated in a number of studies including *in vivo* mouse models. For example, Mishima *et al* have shown that $CD4^+$ T cells that are transferred adoptively from sensitised to naïve animals can induce BHR, which is associated with IL-5 production and eosinophilia (Mishima, H. *et al.* 1998). Of the T_H2-type cytokines, IL-5 is considered to be of particular relevance to asthma pathogenesis by virtue of its pro-eosinophilic properties. IL-5 promotes eosinophil maturation, activation and survival (Lopez, A.F. *et al.* 1988; Rothenburg, M.E. *et al.* 1989; Walsh, G.M. *et al.* 1990), as well as enhancing responsiveness to RANTES (Schweizer, R.C. *et al.* 1994) and eotaxin (Mould, A.W. *et al.* 1997). In the study presented in Chapter 3, the asthmatic subjects treated with β2-agonists alone showed a trend towards increased $CD4^+$ T cells in the epithelium, although this failed to reach statistical significance. Subjects with allergic rhinitis showed an even greater increase in the numbers of $CD4^+$ T cells in the epithelium, and in addition had elevated levels of $CD8^+$ T cells as well. While the pathogenic role of $CD4^+$ T cells in asthma is largely undisputed, the role of $CD8^+$ T cells remains unclear.

Previous work has shown increased numbers of $CD8^+$ and $CD4^+$ T cells in bronchial biopsies from stable asthmatics compared to those from non-asthmatic atopic individuals (Azzawi, M. *et al.* 1990), although another study failed to show any significant difference in T cells between these two groups and healthy controls (Bradley, B.L. *et al.* 1991). One theory suggests that $CD8^+$ cytotoxic (T_C) cells contribute to the inflammatory process in asthma by virtue of having a type-2 cytokine phenotype (T_C2). Evidence to support this theory comes from the observation that after stimulation, human peripheral blood $CD8^+$ lymphocytes were the dominant source of macrophage inflammatory protein 1 alpha (MIP 1 α) and RANTES

(Conlon, K. *et al.* 1995). The chemokines, MIP 1 α and RANTES, are potent regulators of leucocyte trafficking suggesting that $CD8^+$ lymphocyte have a role in recruiting cells to sites of inflammation. $CD8^+$ T cells from patients with asthma may also be an important source of the T_H2-type cytokine IL-4 (Stanciu, L.A. *et al.* 1997). Stanciu *et al*, measured levels of IL-4 and interferon-gamma (IFN-γ) by ELISA in cell lysates and in 20- and 48-hour cultures of concanavalin A-stimulated purified peripheral blood $CD8^+$ T cells in seven patients with mild atopic asthma and seven healthy non-atopic subjects. Resting $CD8^+$ T cells in patients with asthma contained significantly more IL-4 than those of healthy non-atopic subjects, with no difference in intracellular IFN-γ levels. In the healthy control subjects, but not in the patients with asthma, levels of intracellular IL-4 correlated negatively with levels of IFN-γ in resting $CD8^+$ T cells. Stimulation with concanavalin A produced a consistent and significant increase in secretion of IFN-γ, but not IL-4, with no difference between the two groups of subjects. IL-4 is a proinflammatory cytokine, involved in recruitment of eosinophils into the airways (Fukuda, T. *et al.* 1996), the generation of IgE (Yssel, H. *et al.* 1998), differentiation of T_H2 cells (Renauld, J.C. 2001), expression of CD23 (FcεRII, low-affinity receptor for Fc-portion of IgE) (Park, H.J. *et al.* 1997), and up-regulation of MHC-II on IL-4-responsive cell types (Pai, R.K. *et al.* 2002). In this way, it would appear that $CD8^+$ T cells are pro-inflammatory and implicated in the development of asthma.

In the light of this data, one would expect that asthmatic individuals would have significantly greater numbers of $CD8^+$ T cells than either subjects with allergic rhinitis or healthy controls. In fact, the data presented in this thesis demonstrated that allergic rhinitic subjects had increased numbers of $CD8^+$ T cells compared to both healthy and asthmatic subjects. While this may represent a stage of early inflammation leading to the development of symptomatic asthma, an alternative explanation is that the increased levels of $CD8^+$ T cells

are in some way protective. Evidence for this theory comes from work performed by Huang *et al* (Huang, T.J. *et al.* 1999). This group used a Brown-Norway rat model of asthma. The animals were ovalbumin (OVA)-sensitised and subsequent exposure to OVA induced BHR and cellular inflammation comprising eosinophils, $CD4^+$ T cells and $CD8^+$ T cells. When a monoclonal antibody was used to deplete $CD8^+$ T cells, subsequent OVA exposure resulted in significantly reduced levels of $CD8^+$ T cells, but increased levels of eosinophils and BHR. In these animals, the T_H1 cytokine expression (IFN-γ and IL-2) was significantly reduced, while T_H2 cytokine expression was unchanged. This suggests that $CD8^+$ T cells have a protective role in allergen-induced BHR and eosinophilic inflammation, probably through activation of the T_H1 cytokine response.

Further work is needed in order to determine whether the increased numbers of $CD8^+$ T cells in the allergic rhinitic subjects in this thesis are pro-inflammatory or protective. Performing in-situ hybridisation to look for mRNA for both T_H1 and T_H2 cytokines could elucidate this further.

A consistent finding in the lower airways of asthmatics has been the increased numbers of eosinophils in both the submucosa and epithelium. In this study we found that allergic rhinitic subjects had numbers of eosinophils in the submucosa that were intermediate between asthmatics treated with β2-agonists alone and healthy controls. There were no significant differences between the atopic individuals and the healthy controls in the number of eosinophils in the epithelium. Given that the epithelium is exposed to inhaled pollens and other antigens, one might expect to see an increase in epithelial eosinophils during acute pollen exposure. My findings may reflect the fact that the atopic subjects were biopsied

outside of the pollen season and therefore the levels of inflammation in both the upper and lower airways were presumably lower than they would have been with allergen exposure.

The potential of the eosinophil to produce inflammatory mediators such as leukotrienes has placed it as a major effector cell of allergic disease. In a study where allergic rhinitic patients were challenged with inhaled allergen, they responded with an eosinophilic inflammation enhancement in the lower airways which was very similar to that observed among allergic asthmatics (Alvarez, M.J. *et al.* 2000b), but was not associated with an increase in BHR, suggesting that BHR is not mediated by eosinophils alone (Alvarez, M.J. *et al.* 2000a). IL-3, IL-5, and GM-CSF are haematopoietic growth factors that increase the survival of eosinophils in culture and enhance certain eosinophil functions, such as mediator generation and toxicity. Alveolar macrophages derived from asthmatic subjects produce two- to three-fold more GM-CSF than do those from normal control subjects (Arm, J.P. *et al.* 1992) and therefore an increase in macrophage-cell numbers may markedly affect the eosinophil response. One might hypothesise that allergic rhinitics would have reduced numbers of macrophages compared to the asthmatic individuals, and that this factor was responsible for their lack of bronchial hyperresponsiveness despite evidence of eosinophilic and mast cell inflammation. However, the subjects with allergic rhinitis had macrophage numbers in both the bronchial submucosa and epithelium that were equivalent to or greater than the asthmatics (although no statistically significant differences were observed between the groups). This hypothesis unfortunately cannot be used to explain the lack of bronchial hyperresponsiveness in the subjects with allergic rhinitis, and suggests that the development of BHR is a complex matter and requires further studying.

Macrophages have also been implicated in airway remodelling, which is an important characteristic of asthma and may explain why airway inflammation alone is not sufficient to

cause asthmatic symptoms. In one such study alveolar macrophages were isolated by bronchoalveolar lavage in 14 control subjects, 14 asthmatics and 14 chronic bronchitics (Vignola, A.M. *et al.* 1996). The spontaneous and lipopolysaccharide- or concanavalin A-induced release of TGF-β and fibronectin was measured by ELISA. TGF-β and fibronectin released by alveolar macrophages possess a fibrogenic potency, which promotes the airway changes associated with remodelling. Alveolar macrophages from asthmatics released greater amounts of TGF-β and fibronectin than those from control subjects, although not as much as those from patients with chronic bronchitis.

In allergic conjunctivitis, researchers have demonstrated that symptoms may occur even in the absence of an eosinophilic inflammation in the conjunctival epithelium and lamina propria. However, an increase in mast cells was apparent suggesting that symptoms may be related to mast cell-mediated changes (Anderson, D.F. *et al.* 1997). In this study, there was no significant difference in the mast cell numbers in the submucosa, but a clear gradient existed in the epithelium, with allergic asthmatics and rhinitics having increased mast cell numbers compared to healthy controls. The role of the mast cell in the experimentally provoked early asthmatic response to inhaled allergen has been firmly established (Djukanovic, R. *et al.* 1990). The degranulation of mast cells results in the release of histamine and tryptase and is associated with the development of bronchoconstriction (Schulman, E.S. 1993). However, there is no clear correlation between mast cell numbers and BHR (Smith, H. 1992). This suggests that the relationship between cellular inflammation and BHR is complex.

Interactions between cell adhesion molecules and their ligands are an integral part of inflammatory processes and may have direct relevance to the pathology of asthma. Previous

work in this area has shown that VCAM-1 may be instrumental in recruiting eosinophils and activated T-lymphocytes into the airways of asthmatic subjects (ten Hacken, N.H. *et al.* 1998). Other work has suggested that after allergen challenge, the extent of both VCAM-1 and ICAM-1 expression in asthmatics may be increased and correlates significantly with the number of eosinophils (Bentley, A.M. *et al.* 1993). In work by Gosset *et al*, low expression of ICAM-1 was observed in healthy subjects, while E selectin and VCAM-1 were not expressed at all. In contrast, patients with allergic asthma had a significant increase of ICAM-1 expression on epithelium and endothelium, and E selectin and VCAM-1 were over-expressed on endothelium (Gosset, P. *et al.* 1995). In these patients, adhesion molecule expression on endothelium was correlated with both eosinophilia and neutrophilia. In this current study, allergic rhinitic subjects had increased staining for VCAM-1 compared to both asthmatic and healthy subjects. Although eosinophil and neutrophil numbers were increased in the allergic rhinitic subjects compared to healthy controls, they were not as high as those in the asthmatic subjects. This suggests that a complex pattern of regulation exists for ICAM-1, E selectin, and VCAM-1 *in vivo*, where they may reflect the degree of ongoing inflammation.

In order for airways inflammation to become clinically recognised as asthma, both BHR and bronchospasm must be present. Given that the same cells are present in both asymptomatic bronchial inflammation and in clinically recognised asthma, it would appear that inflammation alone is insufficient to cause asthma. Instead, symptoms are likely to result from a complex interaction of cellular inflammation and remodelling. This may imply that an additional susceptibility is present in those individuals who do progress from allergic rhinitis alone to allergic rhinitis and asthma. This susceptibility may be genetic (Bleecker, E.R. 1998) or related to the burden of allergen exposure or other environmental triggers such as air pollution (Rusznak, C. *et al.* 1997).

5.2.2. Inflammatory mechanisms of particulate matter

There is clear evidence that air pollution may exacerbate pre-existing lung disease, but there is no data showing its direct implication in the development of asthma *per se*. The mechanisms by which diesel exhaust may exacerbate asthma remain unclear, but epidemiological evidence suggests that both acute and lag effects are likely with symptoms developing up to 24-72 hours after exposure (Pope, C.A. *et al.* 1991). In healthy adults, diesel exhaust exposure studies have been shown to cause an acute inflammatory response in the lower airways (Salvi, S.S. *et al.* 1999a). After short-term exposure to 300μg/m^3 of diesel exhaust there was a significant increase in neutrophils and B-lymphocytes in airway lavage, along with increases in histamine and fibronectin. The bronchial biopsies obtained six-hours after DE exposure showed a significant increase in neutrophils, mast cells, $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes, and also an upregulation of the endothelial adhesion molecules ICAM-1 and VCAM-1. This response appears to be mediated by airway epithelial cells and alveolar macrophages, as these are the first cells to encounter the inhaled pollutants. Cytokines released by these cells then recruit other cells into the airways from the blood circulation and bone marrow (Salvi, S.S. *et al.* 2000). Interestingly, the acute inflammation was not associated with any significant changes in standard lung function tests measured immediately after DE exposure.

In asthmatic individuals, acute exposure to diesel exhaust failed to demonstrate an inflammatory reaction (Frew, A.J. *et al.* 2001). In this study, 25 healthy subjects and 15 subjects with mild asthma were exposed to DE (PM_{10} concentration 108μg/m^3) for two hours. The healthy subjects developed increased airway resistance (R_{aw}), which is a more sensitive marker for airflow limitation than forced expiratory variables, after DE and there was a significant increase in neutrophils and lymphocytes in airway lavage. Consistent with the

neutrophilia, there were significant increases in IL-6 and IL-8 in the bronchial wash, and increased IL-8 mRNA gene transcripts in the bronchial biopsies. The asthmatic group had a significantly greater R_{aw} before exposures compared to the healthy controls. DE exposure resulted in a further increase that was proportionate to the increase in R_{aw} induced by DE in healthy subjects. However, in the asthmatic individuals, this increase in R_{aw} was not associated with a neutrophilia, or an increase in IL-6 and IL-8. In addition, DE did not exacerbate the asthmatics pre-existing eosinophilic airways inflammation. Immunostaining for epithelial cytokines and chemokines showed no increase in GM-CSF, Gro-α, RANTES, TNF-α or NF-κB. Staining for IL-10 did reveal a four-fold increase in the bronchial epithelium in the asthmatic group. There is evidence to suggest that IL-10 may prevent neutrophilic inflammation while simultaneously altering the airway biology towards a more allergic phenotype (Ishizaki, T. *et al.* 1987; Thompson-Snipes, L. *et al.* 1991; Seitz, M. *et al.* 1995; Jeannin, P. *et al.* 1998; van Scott, M.R. *et al.* 2000).

As a result of this study, a number of questions emerged: is the time-course of inflammation different in healthy and asthmatic subjects, so that the apparent lack of response in the asthmatics could be ascribed to the time they were sampled; given the lag effect of clinical response to particulate air pollution demonstrated by epidemiological studies, would sampling the asthmatic individuals 12-24 hours after diesel exhaust exposure show an inflammatory response; alternatively, did asthmatics respond in a fundamentally different way to diesel exhaust exposure compared to healthy controls; and given the lack of cellular inflammation, what was the mechanism for the increase in R_{aw} post-diesel exhaust exposure in the asthmatic individuals?

The study described in this thesis was designed to shed light on some of those questions. It was postulated that the cellular inflammatory response after diesel exhaust exposure would be greater in the lower airways of asthmatic subjects compared to healthy subjects at 18-hours, and that this inflammation would be characterised by both neutrophils and lymphocytes. In other words, the asthmatics might respond at 18-hours post-diesel exhaust exposure in the same way that healthy controls responded at six-hours. This theory would help to explain the delayed clinical sensitivity of asthmatics to diesel exhaust exposure as described by numerous epidemiological studies which clearly demonstrate a lag period between exposure and asthma exacerbations (Schwartz, J. *et al.* 1993; Chew, F.T. *et al.* 1999).

An alternative explanation might be that it is not the time frame of response that is different in asthmatics compared to healthy controls, but that the asthmatics respond in a completely different way to diesel exhaust exposure compared to healthy individuals. By including non-asthmatic allergic rhinitic individuals in the study we also hoped to be able to offer an explanation for the differences observed between the responses of asthmatic and healthy individuals: whether the difference was a feature of atopy in general, or whether it was as a result of the altered airway biology of the asthmatic.

The results show a persisting neutrophilia 18-hours post-diesel exhaust exposure (PM_{10} concentration 100μg/m^3) in the healthy subjects. This result is in keeping with the neutrophilia seen previously in healthy individuals six-hours after diesel exhaust exposure (PM_{10} concentration 108 and 300μg/m^3) (Salvi, S.S. *et al.* 1999a; Frew, A.J. *et al.* 2001). In addition, healthy individuals also had an increase in the numbers of mast cells and eosinophils in the bronchial submucosa 18-hours post-diesel exhaust exposure. This finding may be

important in explaining the observed ability of diesel exhaust exposure to promote respiratory allergy (Miyamoto, T. 1997; D'Amato, G. *et al.* 2000; Ring, J. *et al.* 2001; Salvi, S.S. 2001).

However, there is a relative lack of response in the asthmatic and allergic rhinitic individuals 18-hours post exposure to diesel exhaust. As discussed earlier, acute exposure studies to diesel exhaust have not demonstrated an inflammatory reaction in asthmatic individuals at six-hours post exposure (Frew, A.J. *et al.* 2001). There are a number of possible explanations for the apparent lack of response in atopic individuals to diesel exhaust exposure. These may be summarised as: a lack of response secondary to an insufficient 'dose' of diesel exhaust; the timing of the sampling at 18 hours 'misses' the inflammation; there is a neutrophilic inflammation but it is associated with increased neutrophil apoptosis and therefore no overall increase in neutrophils is identified; or finally, that the atopic individuals respond to diesel exhaust exposure in a different way to the healthy individuals. I will now discuss each of these hypotheses in some detail.

The 'dose' of diesel exhaust (PM_{10} concentration 100μg/m^3) inhaled in this study was relatively low compared to the peak levels encountered during air pollution episodes in major cities. For example, in one study the peak levels of average PM_{10} concentration in one hour measured in Bristol and Cardiff were 612 and 564μg/m^3 respectively (QUARG 1993). Hence, it is feasible that the inflammatory response was not triggered in the asthmatic subjects because the trigger provided by a PM_{10} concentration of 100μg/m^3 was too weak. However, in a previous diesel exhaust exposure study, also using a PM_{10} concentration of 100μg/m^3, healthy subjects responded with a neutrophilic and lymphocytic inflammation, whereas asthmatic subjects did not (Frew, A.J. *et al.* 2001). Given the clinical sensitivity of asthmatic

individuals to particulate air pollution, as evidenced by studies showing that it provokes exacerbation of asthma and increases hospital attendance (Whittemore, A.S. *et al.* 1980; Schwartz, J. *et al.* 1993; Walters, S. *et al.* 1993; Sheppard, L. *et al.* 1999), one would expect that asthmatic subjects would show cellular inflammation at the same, if not lower, 'doses' of diesel exhaust compared to the healthy subjects. This makes the hypothesis that too low a 'dose' of diesel exhaust was employed seem unlikely although it remains a possibility given that there was no alteration in respiratory function tests performed over the 18-hour study period.

A second explanation for the lack of an inflammatory response, also relating to the 'dose' of diesel exhaust employed, might be that repeated exposures over a series of days are necessary to induce inflammation in asthmatic subjects. In this hypothesis, the single 'dose' of diesel exhaust (PM_{10} concentration $100\mu g/m^3$) may be adequate, but only if exposure is repeated over a number of days. This would more accurately mirror the exposure an individual receives in the real world. The asthmatic individuals may show virtually no response initially, but after repeated exposures, the diesel exhaust exposures have a cumulative effect resulting in a cascade of inflammation that eventually triggers an exacerbation of small airways disease. As in the first argument with regard to 'doses' of diesel exhaust, the fact that healthy individuals were able to mount an inflammatory response immediately to low dose diesel exhaust exposure (Frew, A.J. *et al.* 2001), suggests that asthmatics, with their increased clinical sensitivity, should not need repeated doses in order to do the same. It is true however, that trying to recreate the exposures to ambient air pollution that occur in individuals walking across a city, within a laboratory setting are virtually impossible. Not only do individuals receive cumulative doses on subsequent days, but they also inhale complex combinations of pollutants, such as ozone, particulates, nitrogen dioxide

and sulphur dioxide. There is evidence that these chemicals act synergistically to promote increased levels of airway inflammation, which is correlated with increased asthma morbidity (Delfino, R.J. *et al.* 1994; Vincent, R. *et al.* 1997; Korrick, S.A. *et al.* 1998; Hajat, S. *et al.* 2001). The exposure study described in this thesis emphasises the difficulty in studying the effects of diesel exhaust pollution and in designing experimental models that recreate the conditions encountered in the environment.

An alternative explanation for the lack of response seen in the asthmatic subjects at 18-hours post-diesel exhaust exposure is that the biopsies were taken either too early, or too late. In order for this hypothesis to be true, the sampling of the airways at six-hours post exposure in the previous study (Frew, A.J. *et al.* 2001), and 18-hours post exposure in this study would have to have missed both the acute and late phases of inflammation in the asthmatic subjects. These time points were selected partly for practical reasons, allowing for both exposure and biopsy to take place during normal working hours, but also based on the knowledge that some epidemiological studies have shown a lag effect of 24-48 hours between exposure and symptoms. It may be that the inflammatory response in atopic individuals has a bimodal distribution with an initial acute phase response that has resolved by six hours, before a second late inflammatory response that develops after 18 hours. There is no experimental data available to dispute this theory but common sense suggests that it is unlikely that the inflammation would have resolved completely at both time points. This is supported by the fact that healthy individuals showed a neutrophilic response, which was present acutely at six hours and persisted at 18 hours. Ethical reasons prevent the repeated sampling of individuals and so we are left with 'snap shots' of the inflammatory response in the lower airways, which may be difficult to interpret.

A third hypothesis for the lack of a neutrophil response in asthmatic subjects is that there is more neutrophil apoptosis in atopic individuals. Experimental evidence already exists showing that particulate matter from diesel exhaust is able to induce apoptosis in pulmonary macrophages (Chin, B.Y. *et al.* 1998) and in bronchial epithelial cells via a mitochondrial pathway (Nel, A.E. *et al.* 2001). The study by Chin *et al*, demonstrated that cultured macrophages exposed continuously to a well-defined model of PM (benzo [α] pyrene adsorbed on carbon black) exhibited a time-dependent expression and release of the cytokine tumour necrosis factor-alpha (TNF-α), which evoked a TNF-α dependent apoptosis. Further evidence to support this hypothesis comes from Le Prieur *et al*, who used precision-cut rat lung slices in organotypic culture placed in a biphasic air/liquid system and exposed them to diesel exhaust for six hours. Using the histochemical TUNEL (Terminal-deoxynucteotidyl-transferase-mediated Nick End Labelling) method, apoptotic cells were detected more frequently in slices exposed to diesel exhaust than in control slices. Cytokine production (TNF-α, IL-1β) in the culture medium was measured using an ELISA (Enzyme-Linked Immunosorbent Assay) technique and levels of TNF-α were found to correlate with apoptosis.

However, no experimental evidence is currently available to substantiate the theory that particulate matter from diesel exhaust may cause apoptosis in neutrophils, nor that atopic individuals would be particularly prone to this. Common sense suggests that it would be statistically unlikely for the asthmatics in both this study and the previous study (Frew, A.J. *et al.* 2001), to have neutrophilic inflammation and apoptosis so evenly matched that no change in cell numbers is seen after diesel exhaust exposure at both six hours and 18 hours.

This leaves us with the fourth hypothesis, that asthmatic and allergic rhinitic individuals respond in a fundamentally different way to diesel exhaust exposure compared with healthy subjects. In the study looking at the acute response of asthmatics to diesel exhaust exposure, after six hours there was a statistically significant reduction in bronchial wash eosinophils (Frew, A.J. *et al.* 2001). This was accompanied by a reduction in eosinophils in the bronchial biopsies that failed to reach statistical significance. These findings were accompanied by an increase in IL-10, which as described earlier may reduce neutrophilia, whilst promoting an allergic milieu. It is possible that the clinical sensitivity of asthmatics to diesel exhaust is mediated via IL-10 and further work will need to be performed in order to ascertain whether this is present in the samples at 18 hours.

Other possibilities include the induction of epithelial damage by diesel exhaust, possibly mediated by increased epithelial apoptosis. As described previously, the epithelium is now believed to play an important role in the generation of mediators, which then play a role in the inflammatory and remodelling responses that characterize asthma (Holgate, S.T. 2000a). Further imaging of the epithelium with electron microscopy, in both asthmatics and healthy controls, is required in order to test this hypothesis. Future work will also involve an attempt to detect up-regulation of chemokines and cytokines in the asthmatic group.

It is conceivable that the asthmatics studied in this thesis were 'protected' by the low-grade inflammation already present in the airways. This theory would also explain the lack of response seen in the allergic rhinitic subjects who also had baseline inflammatory changes. Perhaps the inflammation leads to increased mucus protection such that diesel exhaust particles fail to penetrate the epithelium and therefore do not produce an inflammatory response. In more severe asthmatics, the mucus layer may already be breached, with

epithelium disruption and remodelling occurring as a consequence of the asthmatic process. Such individuals would therefore be highly susceptible to diesel exhaust exposure as the particles could easily penetrate the disrupted epithelium.

In the study presented in this thesis, the only finding in the asthmatic subjects at 18-hours post-diesel exhaust exposure was a statistically significant reduction in the expression of the adhesion molecule VCAM-1. As discussed previously, VCAM-1 is associated with the recruitment of eosinophils, and a reduction in expression of VCAM-1 is reflected in the absence of an eosinophilic inflammation in these individuals. There is little evidence to suggest what the significance of such a result might be, nor whether it may be detrimental either acutely or over a period of time. Further work needs to be done in this area to establish if it is a consistent finding and to elucidate its significance. Inhaled corticosteroids appear to cause down-regulation of the expression of VCAM-1 at baseline, as has been demonstrated *in vitro* in a previous study (Atsuta, J. *et al.* 1999), and may also prevent any fluctuation in VCAM-1 expression in response to diesel exhaust exposure.

Interestingly, the bronchial biopsies from the allergic rhinitic subjects do not show the neutrophilic inflammation associated with diesel exhaust exposure in healthy individuals, but instead more closely resemble the two asthmatic groups in their lack of response. This study showed a significant reduction in mast cells in the epithelium of allergic rhinitic subjects 18-hours after diesel exhaust exposure. This finding may be explained by an increase in apoptosis in the mast cells. Mast cells are the principal initial effector cells in the modulation of allergic inflammation. They arise from $CD34^{+}$ pluripotent stem cells, circulate through the blood as committed but undifferentiated cells, and migrate into tissues where they mature in

the presence of Kit-ligand (stem cell factor) and other locally produced cytokines (Nilsson, G. *et al.* 1996). Mast cells undergo apoptosis when stem cell factor is not available.

An alternative explanation may be that the mast cell numbers have been reduced secondary to degranulation, with replacement in the allergic rhinitic individuals being slower than that in asthmatic subjects. In previous experimental work characterizing the cellular inflammation of asthmatics in bronchial biopsies, the number of mast cells was not significantly different in the epithelium or submucosa between asthmatic and healthy subjects (Djukanovic, R. *et al.* 1990). However, electron microscopy showed morphologic features of mast cell and eosinophil degranulation in the asthmatics, which was not detected by staining. This suggests that relying on immunohistochemical analysis alone may underestimate the actual numbers of mast cells in the tissue, particularly if they have degranulated. Future work may be to examine the bronchial tissue obtained from both asthmatic and allergic rhinitic subjects pre- and post diesel exhaust exposure in order to quantify their numbers more accurately.

When activated through Fc epsilon RI, mast cells release and generate a wide variety of cytokines including IL-4 and IL-5, as well as histamine, neutral proteases and heparin sulphate, prostaglandins and cysteinyl leukotrienes (Nilsson, G. *et al.* 1996; Holgate, S.T. 2000b). These chemical mediators attract inflammatory cells that infiltrate the airway wall as a result of increased vascular permeability (Kaliner, M. 1989). Mast cells are therefore of key importance in controlling the response to diesel exhaust.

5.2.2.1 Particle deposition

The key-determining factors in the dose of particulates deposited in the lung are: the exposure concentration, the duration of exposure and the breathing rate and pattern of the individual. This study used a dose of PM_{10} 100μmg/m^3, which is considered to be a relatively low exposure concentration, compared to those levels experienced during heavy traffic flow in major cities in the UK (QUARG 1993). The inflammatory response to diesel exhaust is known to follow a linear dose-response curve. It is therefore likely that a more significant inflammatory response to the diesel exhaust would have identified if we had used a higher concentration. Ethically, it was felt appropriate to limit the diesel exhaust exposure to the lowest levels that were likely to induce inflammation.

Particle deposition may also be affected by such variables as the type of engine they were generated from and the composition of diesel fuel (Lloyd, A.C. *et al.* 2001), and the breathing pattern and respiratory tract anatomy of the individual inhaling them. It appears from deposition models that increased doses of particulates are deposited in adults with abnormal lung anatomy (such as chronic obstructive pulmonary disease or asthma) (Kim, C.S. *et al.* 1997), and in children due to their particular breathing pattern (Yu, C.P. *et al.* 1987; Musante, C.J. *et al.* 2000). The average diameter of diesel exhaust particles is between 50-100nm making them easily respirable and capable of being deposited in both the connecting airways and the alveoli. As minute ventilation increases, the fraction of particulates deposited in the alveoli also increases.

5.2.2.2 Particle clearance

Particle clearance depends upon the existence of an intact, fully functioning ciliated epithelium. The particles are first trapped in mucus and then propelled by the mucociliary clearance mechanisms into the throat and finally swallowed, or alternatively phagocytosed by alveolar macrophages (AM). Inhaling large quantities of particles may over-burden clearance mechanisms (Lippmann, M. *et al.* 1980; Wolff, R.K. *et al.* 1989) and in particular AM-mediated lung clearance (Oberdorster, G. 1995). This may mean that particles are present within the lungs for a greater period of time, allowing an inflammatory response to occur and resulting in damage to the bronchial epithelium which augments the process of remodelling (Holgate, S.T. 1999).

The study presented in this thesis employed a relatively low-dose ($100\mu g/m^3$) of diesel exhaust exposure for a total period of two hours. This may not be a sufficiently high dose to impair the clearance mechanisms and therefore results in less inflammation than studies using higher doses ($300\mu g/m^3$) of diesel exhaust (Salvi, S.S. *et al.* 1999b). Clearance does not appear to be only related to a single exposure dose, but is also adversely related to cumulative exposure doses (Griffis, L.C. *et al* 1983; Cheng, Y.S. *et al.* 1984). Performing repeated exposure studies with low dose diesel exhaust over a series of days may show an enhanced inflammatory response as a consequence of impaired clearance and would more accurately mirror the environmental exposure individuals are subjected to in everyday life.

The subjects recruited into these studies lived in Northern Sweden, which has extremely low levels of ambient pollution. It is possible that acute exposure to diesel exhaust may not produce the same effects in individuals who live in an area with high ambient levels

of pollution. Also, the subjects employed in this study were young adults with a mean age of 25 years. It is likely that subjects with severe asthma or COPD would mount an increased inflammatory response; however the ethical implications of exposing such vulnerable individuals are obviously greater.

5.2.2.3 Particle toxicity

Particle size has been shown to be an important determinant of toxicity with ultrafine particles (<2.5 μm in diameter) capable of evoking a greater inflammatory response than larger diesel particles (Oberdorster, G. *et al.* 1994; Li, X.Y. *et al.* 1997). This relates to the surface area of the particle, with smaller particles having an increased volume to surface area ratio and therefore able to carry an increased amount of substances adsorbed onto the surface. These substances include transition metals capable of generating oxygen radicals (Carter, J.D. *et al.* 1997; Monn, C. *et al.* 1997), which are then able to stimulate the release of other inflammatory mediators. It is not known what proportion of the particles in this study were ultrafine particles, but increasing this proportion by filtering techniques may result in an increased inflammatory response at 18-hours.

5.2.2.4 Diesel exhaust exposure

As discussed previously, this study used a low dose of diesel exhaust – 100μg/m^3 – as compared to previous exposure work using 300 μg/m^3. This followed a desire to reproduce the levels of diesel exposure that were encountered in ambient air. In the UK, annual levels of PM_{10} in metropolitan areas generally range from 10-45 μg/m^3, with maximum daily averages of 70-150 μg/m^3 (QUARG 1996). Reducing the level of diesel exhaust exposure may have reduced the amplitude of the inflammatory response making the signal more difficult to detect

at 18-hours. Previous epidemiological studies have indicated that there is no threshold level at which PM effects disappear, but presumably at lower levels of PM concentration, only the individuals with severe asthma are affected. The individuals included in this study were mild/moderate asthmatics well controlled on either a β2-agonist alone or on a β2-agonist and inhaled corticosteroid, and may not represent the group of asthmatics who are particularly vulnerable to diesel exhaust exposure. For every $10\mu g/m^3$ increase in PM concentrations, epidemiological studies have shown a 3% increase in asthma attacks, a 3.4% increase in emergency room visits for asthma, a 2.9% increase in bronchodilator use, and a 0.15% decrease in FEV_1 (Goldsmith, C.A. *et al.* 1999). It would be reasonable to expect that such dramatic clinical manifestations of asthma would also be accompanied by an increase in lower airways inflammation and this again suggests that the concentration of diesel exhaust used in this study may have been too low as no changes in clinical parameters were observed, or that the asthmatics with relatively mild disease respond in a totally different way to diesel exhaust exposure compared with asthmatics with more severe disease.

5.2.2.5 Diesel exhaust promotes the development of allergic asthma

Diesel exhaust particles may promote the development of the allergic state in the following ways; by acting as transport molecules adsorbing allergens; by acting as adjuvants in the production of allergen-specific IgE; by causing damage to the airway epithelium thus initiating the process of airway remodelling and inflammation that characterises asthma; and by manipulating the T_H1/T_H2 axis. These mechanisms can be used to postulate an explanation for the rapid increase in allergic disease in industrialised countries: an increase that has occurred at too rapid a pace to be ascribed to genetic factors alone.

These four mechanisms by which DEP may promote allergy have been discussed previously in this thesis. To summarise these processes briefly; allergenic material may be concentrated by binding to the larger diesel exhaust particulates, thus resulting in an increased 'dose' being delivered to the airways (Knox, R.B. *et al.* 1997); the binding of pollen or other inhaled allergens to DEP may modulate the allergenic epitopes and thereby increase their allergenicity (Kainka-Stanicke, E. *et al.* 1998); DEP has been shown to have a direct enhancing effect on allergen-specific IgE production (Steerenberg, P.A. *et al.* 1999); and DEP causes an inflammatory response in the airways which may then non-specifically sensitise the airways to subsequent allergen exposure or may initiate the process of remodelling. The role that DEP plays in modulating the T lymphocyte immune response is complicated and is probably best explained diagrammatically. The manifestation of allergic disease can be viewed as two interconnecting axes: the balance of T_H1 and T_H2 differentiation is shown on the X-axis and the spectrum of clinical and subclinical disease on the Y-axis (Fig 5.2). Pro-inflammatory cytokines such as TNF-α and IL-12 drive the system towards the manifestation of disease, whereas inhibitory-cytokines, IL-10 and TGF-β, suppress inflammatory responses and result in a subclinical response. Diesel exhaust has been shown to increase levels of IL-12 secreted from alveolar macrophages (Saito, Y. *et al.* 2002), and in the presence of allergen to increase the production of T_H2 cytokines (Diaz-Sanchez, D. *et al.* 1997; Tsien, A. *et al.* 1997; Ohtoshi, T. *et al.* 1998; Boland, S. *et al.* 1999; Terada, N. *et al.* 1999; Takizawa, H. *et al.* 2000). In this way, diesel exhaust drives individuals towards a T_H2 differentiation and in the presence of TNF-α and IL-12, this cellular inflammation may result in symptomatic allergic disease. This has the effect of increasing the population who are susceptible to the development of allergic disease. Viewed another way, it could be considered to reveal individuals who have a genetic

predisposition to allergy at a lower threshold than would previously have been the case. This may explain the rapid increase seen in allergic disease over the past 50 years.

There is evidence that certain individuals are more sensitive to diesel exhaust than others, although research attempting to characterise candidate genes, which might be used to identify sensitive individuals, is still in its infancy. Ohtsuka *et al* (Ohtsuka, Y. *et al.* 2000) have identified QTLs (Quantitative Trait Loci) on chromosomes 11 and 17, which are associated with immune dysfunction and result in an increased response to particulates in mouse models. Further studies in human subjects are required to identify candidate genes by linkage analysis, and to verify susceptibility genes.

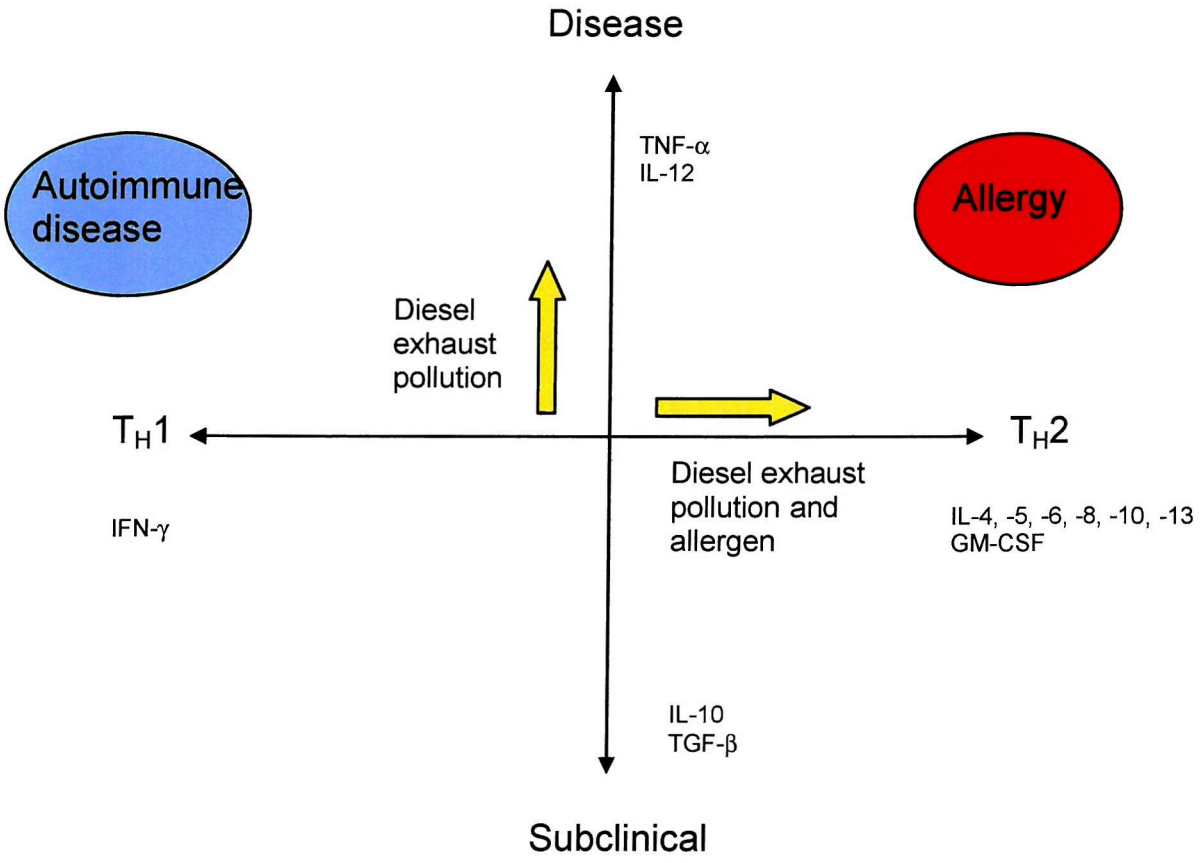


FIGURE 5.2: SCHEMATIC REPRESENTATION OF THE ROLE OF DIESEL EXHAUST POLLUTION IN DRIVING THE DEVELOPMENT OF THE ALLERGIC STATE

The X-axis represents the spectrum of immune response with T_H1 and T_H2 at each end of the spectrum. The Y-axis represents the manifestation of the immune reaction with an exaggerated response resulting in disease at one extreme and a subclinical response at the other. Diesel exhaust pollution in the presence of allergen increases T_H2 cytokines thus driving the immune response towards a T_H2 phenotype. Diesel exhaust also increases the production of TNF-α and IL-12, which may lead to the clinical manifestation of disease, in this case asthma.

Allergic- and pollutant-induced inflammation interact: allergic inflammation modifies the response to pollutants, whilst pollutant exposure modifies allergen response. This produces an escalation of inflammation eventually reaching the threshold for clinical disease. In addition, there is experimental evidence that diesel exhaust alone promotes atopy in that it increases antigen-specific IgE (Fujieda, S. *et al.* 1998).

In more recent years the levels of diesel exhaust exposure have been falling in industrialised countries, while at the same time, exposure levels of ultrafine particles and polyaromatic compounds have been increasing. Ultrafine particles have been shown to be the most chemically active fraction of particulate matter producing an enhanced inflammatory response compared to PM_{10}. Polyaromatic compounds have also been shown to induce airways inflammation and to enhance ongoing IgE production (Tsien, A. *et al.* 1997) but did not demonstrate that the polyaromatic compounds could initiate IgE production. There is considerable evidence that DE particulates are capable of increasing IgE production to allergens and that it may also play a role in promoting sensitisation to neo-allergens (Diaz-Sanchez, D. *et al.* 1994; Diaz-Sanchez, D. 1997; Diaz-Sanchez, D. *et al.* 1999). This evidence supports the theory that diesel pollution is increasing the susceptibility of a population to develop allergic disease.

5.3 FUTURE DIRECTIONS

The work presented in this thesis offers new information about the time-course of the inflammatory response to diesel exhaust. In order to try to understand why asthmatics are clinically more susceptible to diesel exhaust pollution than healthy individuals, further exposure studies may need to be performed with sampling intervals at times other than six- and 18-hours post exposure. Repeated exposures over a series of days would recreate the exposure pattern more commonly seen in the 'real world' and would offer insight into inflammatory response. Exposure to different combinations of pollutants, such as diesel exhaust and ozone, would also help to examine possible additive or synergistic effects. In addition, it would be useful to examine the combined effects of diesel exhaust and allergen exposure in atopic individuals to see if the diesel exhaust augments the response to the allergen.

Studies of diesel exhaust exposure in subjects with chronic obstructive pulmonary disease are also necessary to identify the process that makes them particularly vulnerable to air pollution. However, as such individuals often have co-morbidities such as ischaemic heart disease, a rigorous selection process would have to be employed to ensure that such studies did not exacerbate other diseases.

This study has demonstrated certain cellular responses to diesel exhaust exposure. In order to understand the chemical signalling that has stimulated this response, and hence to identify key cells in orchestrating the process, PCR studies will be performed to look at cytokine expression. In the atopic individuals IL-10 will be measured to see if this cytokine has been up regulated thereby inhibiting a neutrophilic response. In addition, further staining,

such as staining for EGF ligands and markers of apoptosis, will be performed on the bronchial biopsies to ascertain if there is activation of remodelling processes or other forms of epithelial damage in the atopic subjects.

APPENDIX

Summary of Data

SUMMARY OF DATA

Group / Marker	Healthy n=21		Allergic Rhinitis n=13		Asthma β2-agonist n=16		Asthma ICS n=16	
	air	**diesel**	**air**	**diesel**	**air**	**diesel**	**air**	**diesel**
Phase I: Inflammatory Cells – Submucosa								
AA1	19.4* (11.7-26.9)	20.6* (15.4-34.6)	16.1 (14.6-24.4)	17.8 (12.6-21.7)	26.6 (21.0-30.0)	31.0 (19.5-39.9)	15.7 (12.3-21.1)	18.0 (12.4-31.9)
EG2	0†* (0-0)	0* (0-0)	0.4† (0-1.2)	0 (0-0.5)	0.1† (0-1.1)	0.7 (0-1.6)	0 (0-.3)	0.1 (0-1.3)
NOE	65.8†* (37.2-79.6)	71.8* (58.4-126.8)	56.7 (32.0-89.9)	66.8 (57.9-73.4)	47.2† (20.1-85.9)	53.9 (29.1-167.7)	98.2† (81.1-115)	98.0 (71.9-119.8)
CD3+	42.7 (24.6-58.3)	40.0 (34.8-46.9)	28.7 (24.8-76.1)	39.1 (26.0-62.1)	39.7 (23.1-67.3)	56.5 (38.5-77.7)	26.4 (14.9-60.8)	26.5 (15.1-61.3)
CD4+	24.4 (16.9-39.2)	20.2 (10.4-32.4)	28.6 (13.7-46.4)	29.3 (17.0-59.0)	34.1 (14.4-38.1)	26.0 (16.0-46.7)	16.0 (7.8-32.5)	11.3 (6.1-23.1)
CD8+	14.3 (8.0-22.8)	11.9 (9.5-21.9)	14.5 (9.4-39.9)	19.9 (5.1-30.6)	9.3 (6.6-12.6)	15.1 (5.6-25.9)	5.9 (2.8-16.6)	6.8 (4.6-10.7)
CD25+	0.3 (0-0.8)	0 (0-0.3)	0.4 (0-0.6)	0 (0-0.9)	0.1 (0-0.6)	0.6 (0-1.8)	0 (0-0.9)	0 (0-0.7)
CD68+	6.1 (2.9-8.8)	5.3 (4.0-7.3)	6.3 (3.0-8.6)	3.9 (3.0-5.3)	3.4 (1.8-7.0)	3.5 (0.1-8.6)	1.6 (0.6-3.6)	2.2 (0.6-5.5)
Phase II: Adhesion Molecules								
E selectin	21.9 (17.7-24.6)	35.6 (32.7-42.6)	13.4 (11.4-18.4)	14.7 (13.7-22.0)	15.6 (9.4-26.9)	16.4 (12.1-28.4)	21.2 (4.0-29.2)	9.4 (6.6-25.6)
P selectin	37.9 (22.3-48.2)	42.3 (38.1-50.6)	29.4 (26.5-37.0)	38.7 (29.1-44.6)	38.9 (26.4-42.8)	37.6 (25.5-41.6)	39.2 (17.2-43.8)	17.8 (12.4-47.9)
ICAM-1	66.4 (59.1-82.1)	68.3 (64.1-98.4)	84.3 (71.7-93.4)	93.2 (78.1-98.9)	71.7 (60.8-83.7)	77.8 (62.3-88.5)	68.3 (50.2-94.3)	87.1 (54.7-100)
VCAM-1	6.2† (2.6-9.0)	2.9 (0-4.1)	10.6† (8.4-23.4)	13.9 (10.0-15.8)	2.7†* (1.0-6.3)	2.1* (0-2.8)	2.6† (0.5-4.4)	2.6 (0-6.5)

Group / Marker	Healthy n=21		Allergic Rhinitis n=13		Asthma β2-agonist n=16		Asthma ICS n=16	
	air	**diesel**	**air**	**diesel**	**air**	**diesel**	**air**	**diesel**
Phase III: Inflammatory Cells - Epithelium								
AA1	0.1† (0-0.5)	0.3 (0-0.4)	0.7†* (0.2-2.1)	0.3* (0.1-1.0)	1.64† (0.2-3.7)	0.7 (0.2-1.9)	0.3 (0-0.5)	0.4 (0-0.7)
EG2	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0† (0-.0)	0 (0-0)
NOE	0.6 (0.3-0.9)	0.8 (0.4-1.7)	1.2 (0-2.2)	1.7 (0.9-3.3)	0.7 (0-1.4)	0.9 (0.3-1.9)	2.3 (0.2-7.5)	1.1 (0.3-2.3)
CD3+	2.8† (0.9-5.6)	2.7 (0.7-6.3)	7.1† (3.2-12.5)	4.6 (3.0-9.5)	3.3† (0-6.9)	2.5 (1.1-4.9)	2.1† (0.7-3.3)	0.8 (0.7-2.5)
CD4+	0.4 (0.1-1.2)	0.4 (0-0.7)	1.7 (0.4-2.2)	0.8 (0.5-1.3)	0.4 (0-0.8)	0.2 (0-0.9)	0.3 (0-0.7)	0 (0-0.3)
CD8+	1.1† (0.3-2.7)	0.8 (0.5-2.4)	4.1† (1.7-9.7)	2.8 (1.0-4.9)	0.6† (0.3-3.0)	1.1 (0.2-3.2)	0.7† (0.3-1.6)	0 (0-1.0)
CD25+	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
CD68+	0 (0-0.2)	0.1 (0-0.3)	0.1 (0-0.6)	0.2 (0-0.5)	0 (0-0.3)	0 (0-0.4)	0 (0-0.1)	0 (0-0.2)

Data are given as medians and interquartile range (Q_1-Q_3). The adhesion molecules ICAM-1, VCAM-1 and selectins are expressed as a percentage of EN4 positive vessels. Other parameters are given as number/mm^2. Significant differences within each group are marked with *. Significant differences between groups are marked with †.

	Healthy n=21		Allergic Rhinitis n=13		Asthma β2-agonist n=16		Asthma ICS n=16	
	air	**diesel**	**air**	**diesel**	**air**	**diesel**	**air**	**diesel**
Phase IV: Inflammatory Cells – Bronchial Wash (BW) and Bronchoalveolar Lavage (BAL) Fluid								
BW cell/ml x 10^4	9 (6-10)	9.5 (7-12)	12.4 (8-16)	9.8 (8-16)	7.6 (6-10.0)	10.0 (5-11)	8.7 (7-12)	9.8 (6-14)
BAL cell/ml x 10^4	10.6 (9-13)	11.6 (8-15)	7.9 (7-13)	10.0 (8-12)	9.9 (9-13)	10.6 (9-14)	12.0 (7-15)	12.2 (9-14)
BW-Macrophage %	85.9 (82-88)	80.4* (67-86)	83.0 (78-86)	77.5* (71-81)	89.2 (83-93)	89.2 (82-93)	87.3 (82-92)	83.0 (78-92)
BAL-Macrophage %	87.9 (83-92)	87.0 (84-90)	85.2 (83-93)	85.3 (84-90)	88.6 (86-93)	89.7 (86-92)	89.8 (87-93)	92.6 (86-94)
BW-Neutrophil %	11.1 (9-15)	17.6* (12-30)	13.0 (11-20)	19.8* (12-25)	7.8 (5-13)	7.0 (5-11)	9.4 (6-16)	12.2 (6-19)
BAL-Neutrophil %	0.8 (0.6-1.4)	1.0 (0.6-1.6)	1.5 (0.6-2.2)	1.4 (0.8-3.0)	1.1 (0.4-1.8)	1.0 (0.4-2.2)	0.8 (0.4-1.3)	0.6 (0.4-1.5)
BW-Lymphocyte %	3.0 (1.2-4.6)	2.2 (1.2-4.9)	3.1 (2-4)	2.9 (1-5)	1.7 (0.7-4)	2.2 (1-4)	1.9 (1-3)	1.2 (1-3)
BAL-Lymphocyte %	9.9 (7-14)	10.4 (8-15)	12.8 (5-15)	12.0 (6-14)	7.4 (5-11)	8.0 (6-12)	9.0 (6-12)	6.1 (5-13)
BW-Eosinophils %	0.2 (0-0.4)	0.2 (0-0.4)	0.2 (0-0.6)	0.2 (0-0.4)	1.0† (0.5-1.5)	0.8 (0.4-1.8)	0.2 (0.05-0.8)	0.5 (0-1.2)
BAL-Eosinophils %	0.2 (0-0.4)	0.2 (0-0.4)	0.4 (0-0.8)	0.2* (0-0.4)	0.7 (0-1.4)	0.4* (0.2-1.4)	0.1 (0-1.1)	0.1 (0-0.8)
BW-Mast cells %	0 (0-0.25)	0 (0-0.25)	0.25 (0-0.8)	0 (0-0.4)	0.88† (0.2-2.2)	1.75 (0.3-2.5)	0.5† (0.2-0.8)	0.38 (0-0.5)
BAL-Mast cells %	0 (0-1.1)	0 (0-0.7)	0.25 (0-0.75)	0.25 (0.2-0.6)	0.38 (0-1.2)	1.25* (0-2.8)	0.62 (0-1.2)	0.5 (0-0.9)

Data is given as medians and interquartile range (Q_1-Q_3)

Significant differences within each group are marked with *. Significant differences between a group and the healthy control are marked with †.

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