UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

THE EFFECT OF PARTICULATE MATTER POLLUTION ON BRONCHIAL EPITHELIAL CELL RESPONSES

by

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Thesis for the degree of Doctor of Philosophy

February 2007

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Asthma is increasing in prevalence around the world. It is clinically characterised by shortness of breath, wheeze and cough and pathologically by increased Th2 inflammation, mucus hypersecretion and airway remodelling Epidemiological studies have linked inhalation of particulate matter (PM) with asthma, and although exposure has been shown to lead to acute airway inflammation, there is uncertainty regarding the mechanisms underlying the inflammatory response and the contribution of particulate matter to airway remodelling and asthma chronicity. In this study the effects of diesel exhaust particles (DEP) and residual oil fly ash (ROFA) on bronchial epithelial cell activation are investigated. It is postulated that DEP leads to inflammatory effects on bronchial epithelial cells as measured by the release of IL-8 via release of EGFR ligands and activation of the epidermal growth factor receptor (EGFR). In addition, it is further hypothesised that exposure of bronchial epithelial cells to DEP and ROFA leads to increased mucin gene expression. Recognising the contribution of IL-13 as a key allergic mediator in asthma it is postulated that DEP and IL-13 are synergistic in their effects on bronchial epithelial cells. Bronchial epithelial cells were cultured and exposed to DEP or ROFA. Methylene Blue assay was used to measure cell number, LDH assay to measure cell toxicity, TAQMAN RT-PCR to measure gene expression and ELISA to measure growth factor and cytokine release. In response to DEP > 50μg/ml there was a modest increase in cytotoxicity in H292 cells and primary bronchial epithelial cells (PBEC) but not in 16HBEo$^-$ cells. DEP did not cause increased IL-8 release from H292 or 16HBEo$^-$ cells. Using PBEC there was significantly increased IL-8 gene and protein expression in response to DEP. IL-8 was released via activation of the EGFR. This was demonstrated by neutralisation of the EGFR and involved the shedding of the EGFR ligands Transforming Growth Factor α (TGFα) and Amphiregulin (AR). DEP and ROFA led to enhanced expression of gel forming and membrane bound mucin genes. DEP enhanced the gel forming mucin, MUC2 as well as the membrane bound mucins MUC1 and MUC4. ROFA led to enhanced induction of the gel forming mucins MUC5AC, MUC5B and MUC2 as well as the membrane bound mucin gene; MUC4. The inflammatory effects of DEP were enhanced in an additive fashion in the presence of the Th2 cytokines IL-4 and IL-13, suggesting that these effects of DEP may be more marked in asthmatic airways where Th2 cytokines are over-expressed. DEP activates bronchial epithelial cells through activation of the EGFR. This may contribute to inflammation and remodelling responses that are found in asthma. The increase in mucin gene expression may be a normal protective mechanism but may contribute to the symptoms and pathology of asthma. These changes may, in part, account for the recent epidemiological trends in asthma prevalence.

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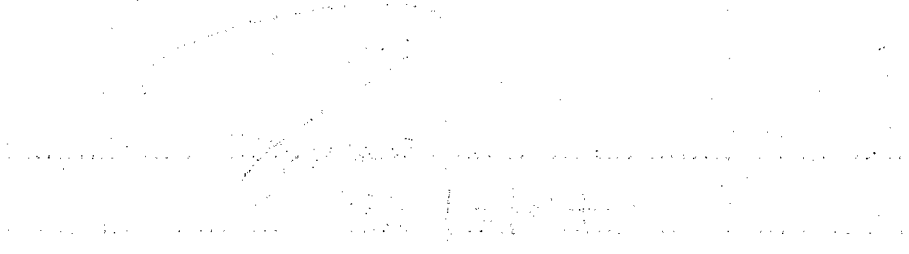
LIST OF ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase domain
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP-1	activator protein-1
AR	amphiregulin
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BEC	bronchial epithelial cells
BEGM	bronchial epithelial growth medium
BHR	bronchial hyperresponsiveness
BPE	bovine pituitary extract
BTC	betacellulin
BW	bronchial wash
CB	carbon black
CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
DE	diesel exhaust
DEP	diesel exhaust particles
DMSO	dimethylsulphoxide
DMTU	dimethylthiourea
DNTPs	deoxynucleoside triphosphates
ECP	eosinophil cationic protein
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
ELISA	enzyme linked immunosorbent assay
EPR	epiregulin
ERK	extracellular signal-regulated protein kinase
ET-1	endothelin-1
FCS	foetal calf serum
FEV-1	Forced expiratory volume in 1 second
FGF	fibroblast growth factor
GINA	Global Initiative for Asthma
GM-CSF	granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptors
Gro	growth related
H_2O_2	hydrogen peroxide
HB-EGF	heparin-binding epithelial growth factor
HDM	house dust mite
ICAM-1	inter cellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IGF	insulin like growth factor
IL	interleukin
IQR	interquartile range
IRS	insulin receptor substrate
ISAAC	international Study of Asthma & Allergies in Childhood
ITS	Insulin Transferin Selenium

JAK	Janus kinase
LDH	lactate dehydrogenase
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
LT	leukotriene
MAPK	mitogen activated protein kinase
MCP	monocyte chemotactic protein
MIP	monocyte inhibitory protein
MPO	myeloperoxidase
MRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NHLBI	national heart lung blood institute
NIST	National Institute of Standards & Technology
NO	nitric oxide
NO_2	nitrogen dioxide,
NRG	neuregulin
NSCLC	non small cell lung cancer
O_2	oxygen
OA	ovalbumin
PAH	polyaromatic hydrocarbons
PBEC	primary bronchial epithelial cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEFR	peak expiratory flow rate
PFT	pulmonary function test
PG	prostaglandin
PI3	phosphoinositide 3-kinase
PKC	phosphokinase-c
PM	particulate matter
PMN	polymorphonuclear cells
RANTES	regulated on activation T cell expressed and secreted
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
ROFA	residual oil fly ash
SD	standard deviation
SE	standard error
SH	Src Homology
sICAM-1	soluble inter cellular adhesion molecule
SO_2	sulphur dioxide
STAT	signal transduction and activation of transcription
TARC	thymus and activation regulated chemokine
TBE	tris-borate-EDTA
TBS	tris buffered saline
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
TSP	total suspended particle
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VGCC	voltage gated calcium channels
VLA	very late antigen
VOC	volatile organic compounds
WHO	world health organisation



ACKNOWLEDGMENTS

The work carried out in this thesis was carried out in the Brook laboratories of the University of Southampton, situated in Southampton General Hospital.

My greatest gratitude and sincere thanks go to Professor Donna Davies, for her expert supervision, ideas, encouragement, sincere interest and concern throughout my research. I feel very much indebted to her for all her time and effort.

I would also like to thank Professor Stephen Holgate and Professor Anthony Frew, as well as Dr Sarah Puddicombe, Dr Audrey Richter, Dr Lynnsey Hamilton, Dr Robert Powell and Dr Christine Boxall, Dr Fabio Buchieri and Dr Kelly Berube for their help during my work leading to this thesis.

PUBLICATIONS ARISING FROM THIS WORK

REVIEW ARTICLES

1) **Parnia S**, Frew A: Chemokines & Atopic Dermatitis J Allergy Clin Immunol 2000 May 150(5) 892 –894

2) Sydbom A, **Parnia S**, Dahlen SV, Sandstrom T, Experimental studies on effects of diesel exhaust emissions. Eur Respir J. 2001 Apr; 17(4) 733-46

3) **Parnia S**, Frew A, Is diesel the cause for the increase in allergic disease? Annals of Allergy Asthma & Immunology 2001 Dec 87 (6 Suppl 3) 18-23

4) **Parnia S**, Brown JL, Frew AJ, The role of pollutants on allergic sensitisation and the development of asthma – Allergy 2002 Dec;57(12):1111-7.

MANUSCRIPTS BEING SUBMITTED

Parnia S, Hamilton L, Puddicome S, Holgate ST, Frew A, Davies D
A mechanistic study of the pro-inflammatory effects of diesel exhaust particles on primary bronchial epithelial cells

Parnia S, Hamilton L, Puddicome S, Holgate ST, Frew A, Davies D
A mechanistic study of the effects of diesel exhaust particles on mucin gene expression in primary bronchial epithelial cells

POSTER PRESENTATIONS

American Thoracic Society Orlando May 2004 – Mucin gene expression in response to DEP exposure in primary bronchial epithelial cells **Parnia S**, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

American Thoracic Society Seattle May 2003 – The role and mechanism of TGFα release from primary bronchial epithelial cells in response to respirable particles **Parnia S**, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

Institute of Health Air Pollution Meeting, Leicester, UK April 2003
The role of respirable particles and TH2 cytokines on inflammatory response from primary bronchial epithelial cells, **Parnia S**, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

European Respiratory Society, Stockholm Sept 2002 – Additive effects of IL-4 and respirable particles on the release of IL-8 from primary bronchial epithelial cells. **Parnia S**, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

American Thoracic Society Atlanta May 2002 – The role of respirable particles on the release of IL-8 from primary bronchial epithelial cells
Parnia S, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

Institute of Health Air Pollution Meeting, Leicester, UK April 2002
The role of respirable particles on the release of IL-8 from primary bronchial epithelial cells, **Parnia S**, Puddicome S, Berube K, Richter A, Holgate ST, Frew A, Davies D

CHAPTER ONE

HEALTH EFFECTS OF PARTICULATE AIR POLLUTION IN ASTHMA

1.1 Air Pollution and Asthma

Asthma is one of the commonest diseases of affluent societies and is increasing in prevalence around the world [Salvi, et al 1999a]. In the UK, this is a particularly worrying occurrence with recent estimates indicating that over 30% of UK school children complain of wheezy episodes [The International Study of Asthma & Allergies in Childhood (ISAAC) 1998]. Although, family studies indicate that genetic factors are important in determining individual susceptibility to this disease, the striking increase in prevalence of asthma over recent decades and the rarity of this disease in less affluent populations has underlined the importance of environmental factors, including air pollution in the causation of this condition.

Although widely perceived to be a new problem, the hazards of air pollution have been recognised since antiquity and one of the first recorded descriptions of air pollution related deaths dates back to the time of the eruption of Vesuvius in AD79. At that time the effects of such air pollution episodes which were naturally occurring were largely confined to the local area. However due to the global impact of modern industrialisation in the last century, the problem of air pollution has become a focus of great concern in these recent times.

In the early 20th century, industrial pollution and domestic coal burning led to the infamous smog's of the Meuse Valley. This was followed by a series of disastrous smog's in the UK and USA in the 1940s and early 1950s which led to clean air legislation, that culminated in a dramatic reduction in the levels of black smoke and sulphur dioxide pollution in our cities. In recent years, however, air pollution has again become an increasing public and political issue due to the rapid growth in world population as well as the rising world-wide migration from rural to urban areas which has brought with it an increased need for transportation and hence an increase in motor vehicle generated air pollutants [Salvi et al 1999a]. This progressive increase in motor vehicle traffic since the 1950s has led to the emergence of new forms of air pollution, particularly "summer smog's" in which fine particulates and ozone accumulate over our cities during periods of high pressure in the summer. During the early 1990s, concern

about the greenhouse gases and volatile organic compounds (VOC) which are produced by petrol engines led a number of European states to reduce the duty on diesel fuel and increase the duty on petrol with a view to encouraging people to buy diesel-powered cars and hence reduce greenhouse emissions.

1.2 Particulate Matter

Particulate matter (PM) is defined as any substance that survives in the atmosphere at standard conditions in the solid or liquid states. In recent years there has been extensive concern regarding PM of smaller sizes and in particular the PM10 category, which are defined as particulate matter with a mass median aerodynamic diameter less than 10μm. In other words all particles passing through a size selective inlet, which allows 50% of 10μm particles. Concern has arisen as particles less than 5-10μm are capable of penetrating the defence mechanisms of the nose and upper air passages and can reach the lower airways that are affected in asthma [Sydbom et al 2001].

There are two major sources of atmospheric particles. The first of these categories are the primary sources, which are emitted directly into the atmosphere and can themselves be divided into two types: manmade and natural sources. The manmade sources include wearing down of roads as well as combustion products generated from traffic. The natural sources include such diverse categories as sea salt, products of forest fires and volcanic eruptions. The second group are the secondary sources that are composed of particles that are formed in the atmosphere; these are usually formed from chemical reactions. Furthermore particulates are further divided based upon their size groups into three different size ranges, or modes [Harrison 1999]. (Figs 1a & 1b):

The coarse mode particulates are usually formed from natural events such as crushing, grinding and abrasion of surfaces or the breaking up of larger particles such as pollen, fungi and also the breaking up of mining materials. It may also contain biological elements such as pollen grains, fungal spores and their fragments. Man-made coarse particles consist mainly of the working of soil and rock from many industrial processes and the wearing down of roads by motor

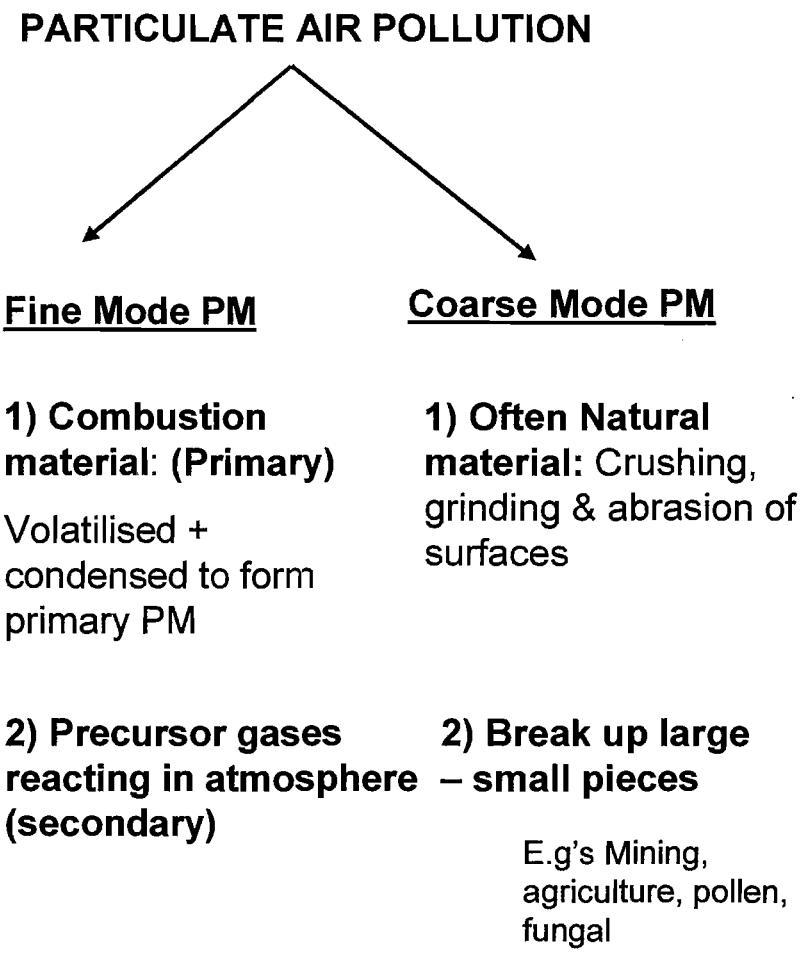


Fig 1(a) The Different Types of Particulate Matter

Traditionally atmospheric particles have been divided into coarse and fine particles, which relate to their sizes, Coarse particles (>1μm) are derived from the breaking up of relatively large particulate pieces which may be natural such as pollen or synthetic (e.g. the wearing down of surfaces such as roads). Fine mode (<1 μm) particles are primarily formed from combustion materials (primary) or from precursor gases that react in the atmosphere (secondary).

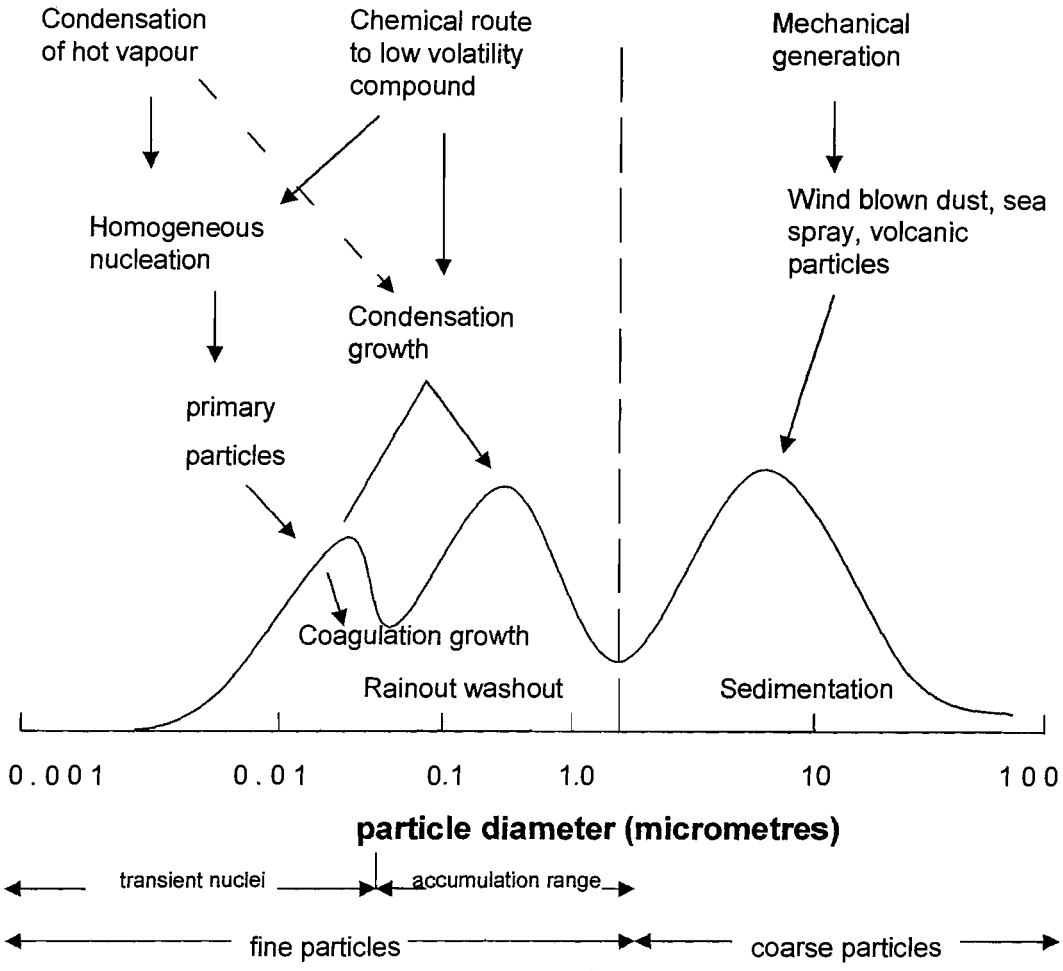


Fig 1(b) The Size Distribution of Particulate Matter

This figure depicts schematically the processes that influence the size distribution and chemical composition of the atmospheric particles. It illustrates the wide range of sizes that are involved in the formation of particles. Aerosols participate through homogeneous and heterogeneous reactions to form particles. Traditionally atmospheric particles have been divided into coarse and fine particles, which reflect the major particles sources, i.e. mechanical (coarse) and combustion plus secondary (fine) . Primary particles that are derived from the breaking down, crushing, grinding & abrasion of surfaces such as sea salt, soil dust, and biological materials have most of their mass in the coarse particle range. Due to their low concentrations and large sizes these do not coagulate together. Gas to particle conversion in the atmosphere takes place when a volatile species reaches a concentration that exceeds its equilibrium vapour pressure or if the temperature is reduced. Another mechanism by which particles are formed is the condensation of several gas species, or nucleation, whereby new particles are formed due to the vapour pressure of the condensing species exceeding the equilibrium vapour pressure of the condensed phase. Once nucleation occurs the new particles, grow by condensation and self coagulation. [Whitby 1978]

vehicles and salts. They also consist of combustion sources (motor vehicles, commercial ovens, utility and industrial boilers), materials handling processes, agriculture and open burning. The fine and ultrafine particles which are the focus of greater health concerns are usually man-made and are derived from combustion material that become volatilised and condense to form primary particulate matter, or precursor gases that react in the atmosphere to form particles [Harrison 1999] (Figs1b). In many major cities of the world diesel exhaust particles comprise the greatest proportion of fine and ultrafine particles [United Nations Environment Program 1994].

Coarse	PM10 (50%< 10μm)
Fine	PM 2.5 (50%<2.5μm)
Ultrafine	PM <0.1μm

Table 1.1: The different sizes of particulate matter

1.3 Diesel Exhaust Particles (DEP)

Optimal combustion of diesel fuel produces water and carbon dioxide, however, in practice there is incomplete combustion leading to the formation of various gases, liquid and solid particles [Sydbom et al 2001]. Compared with petrol engines, diesel engines produce far less carbon monoxide, but give rise to a greater amount of nitrogen oxides and aldehydes, which are particularly prone to cause irritation of the upper respiratory tract. The particles are believed to mediate several of the observed adverse effects. In the 1990's it had been estimated that particulate emissions from diesel engines are over 10 times higher than those emitted from petrol engines of equivalent power running on unleaded petrol, and over 100 times compared with petrol engines fitted with catalytic converters [Nauss et al 1995, Zweidinger et al 1990]. In addition to particles, diesel exhaust contains a complex mixture of gases such as carbon monoxide (CO), nitrogen oxides (NO, NO_2), sulphur dioxide (SO_2), hydrocarbons, formaldehydes, transition metals and carbon particles [Scheepers et al 1992].

In Europe, diesel exhaust is the principal source of fine particulate pollution, whereas in the USA sea spray and agricultural activity are important contributors. Over 80% of diesel exhaust particles are in the ultrafine size range < 0.1 μm, and consist of a carbonaceous core, onto which up to 18,000 different organic compounds may be adsorbed [Kerminen et al 1997, Weisenberger et al 1984]. The dose of particle deposited in the lung depends on both its concentration and its size. Particles below 5 μm have the potential to reach and become deposited in the alveoli, whereas those that are above 5 μm reach the proximal airways and are cleared by the mucociliary escalator [Brain et al 1979, Chow et al 1995]. Previous studies in humans using radioactive particles have demonstrated that particles with a mass median diameter of 2.5 μm undergo 83% total lung deposition, while particles of 11.5 μm have only 31% deposition [Anderson et al 1994].

1.4 Residual Oil Fly Ash

The inorganic residue that remains after incomplete oxidation of carbonaceous materials is termed "fly ash". Fly ash from fossil and waste fuel combustion contributes more than 2.5 $x10^5$ tons annually to the ambient air PM burden in the United States [Costa et al 1997]. Although the ash content of oil used for electric power generation is two to three orders of magnitude less than that of coal, many oil-fired power plants employ few or no particle emission abatement technologies [Fisher et al 1983]. Consequently, fugitive fly ash from the combustion of oil and residual fuel oil contributed 76,000 and 49,000 tons, respectively, to the US national ambient particle burden in 1992 [U.S. Environmental Protection Agency, 1993].

Oil fly ash is frequently in the size category of PM2.5 or less and is principally inorganic. Comparable to ambient air pollution particles, oil fly ash is chemically complex and includes sulphates, silicates, carbon- and nitrogen-containing compounds, contaminants of the fuel, and additives. Metals, including iron, vanadium, and nickel, are present in high concentrations as water-soluble salts in fly ash [Schroeder et al 1987].

Due to its high vanadium concentration the contribution of oil fly ash to total PM is often measured using tropospheric concentrations of vanadium [Schroeder et al 1987]. The concentration of vanadium in ambient air can vary between 25 and 75 ng/m^3 in rural areas, whereas in an urban environment, this value is 60-300 ng/m^3 and can increase 6-fold in the winter [Zelikoff et al 1986]. Although this metal is an essential trace element for humans and certain animals, it occurs sparsely in nature [Byrne et al 1978]. In nature, certain plants such as sugar beets, vines, beech and oak trees can have higher levels of vanadium, the greatest concentrations are found in lower marine animals such as shellfish. Because oil is derived from fossilised marine organisms, vanadium is found in this fuel at a high average concentration and, subsequently, in its fly ash [Byrne et al 1978]. Higher contents of the metal occur in the heavy oils left (i.e., the residual) after the more volatile fractions such as petrol, paraffin, and diesel oil have been distilled, hence the term "residual oil" fly ash (ROFA) [Byrne et al 1978]. In addition to metals, sulphates are also in abundance.

1.5 Health Effects of Particles

Parallel to changes in the pattern of air pollution, the last two decades have witnessed a dramatic increase in the global incidence of allergic diseases such as asthma and rhinitis. This has occurred in too short a space of time to be attributed to genetic changes and it seems likely that environmental factors are responsible [Salvi et al 1999a]. Politicians, pressure groups, and some scientists have proposed that air pollution may be the cause, and there certainly is some evidence to support this.

Many epidemiological studies conducted in different parts of the world have consistently demonstrated an association between ambient levels of air particles and various health outcomes, including mortality, increased exacerbation of asthma, chronic bronchitis, respiratory tract infections, ischaemic heart disease and stroke [Salvi et al 1999a]. In the last six years major concerns have been raised about the effects of fine particulates. In 1993, a seminal paper described consistent associations of fine particulate levels with cardiovascular and

respiratory mortality and morbidity in six US cities [Dockery et al 1993]. Initially this observation was met with some scepticism but the association has subsequently been confirmed in a wide number of settings and in different countries. The ultrafine particles (diameter < 0.05-0.10 μm) are highly reactive and are present in large numbers in the urban environment. They can penetrate through the epithelium and vascular walls and enter the blood stream, and have been reported to produce other systemic effects such as increased carcinogenicity [Sydbom et al 2001], potentiation of autoimmune disorders in mice [Yoshino et al 1999], alterations in blood coagulability and increased cardiovascular disorders [Seaton et al 1995].

Reports from Japan suggest that children living close to roads with heavy traffic are more likely to develop allergies [D'Amato et al 1994]. A higher prevalence of allergy to cedar pollen has been observed in people living close to motorways, as compared with people living in a more rural environment close to a cedar forest and being exposed to the same number of cedar pollens [D´Amato et al 2002]. As DEP make up a significant proportion of airborne particles in most parts of the world, many studies including both human and animal *in vitro* as well as exposure studies have focused on the potential role of these particles in the increased prevalence of worldwide allergy and asthma.

1.6 The Effects of Particulate Matter on Asthma

Asthma is a chronic inflammatory disorder of the airways manifesting as intermittent airflow obstruction that may at times become fixed or progressive [Lange et al 1998]. This condition is characterised by bronchial hyperresponsiveness (BHR), a state in which there is reversible airways obstruction, airway inflammation, and heightened airway responsiveness in response to a variety of stimuli. Clinically, patients' with asthma commonly complain of wheezing, cough and shortness of breath, which are thought to arise from narrowing of the airways. Asthma is characterised by a number of changes in the airway, which include smooth muscle hyperplasia, inflammatory cell infiltration, mucus hypersecretion, mucus plugging, epithelial cell sloughing, and airway remodelling.

For well over 50 years, air pollution has been recognized as capable of precipitating acute respiratory illnesses such as asthma [Atkins 2001]. Ozone, oxides of nitrogen, sulfur dioxide, and particulates (especially those <2.5 μm in diameter) can increase airway responsiveness, a central feature of asthma.

Results of epidemiology studies have been consistent across cultures and with the use of numerous health metrics. Increased rates of mortality and morbidity, including admissions, need for treatment, and activity limitations, have been demonstrated worldwide in relation to exposure to various types of air pollution. A major prospective study showed a 1% increase in admissions for lung disease with every 10-μm increase in ambient concentration of PM10 [Pope 1995]. The same authors later demonstrated a link between long-term exposure to fine particulates and lung cancer and cardiopulmonary mortality [Pope 2002]. However despite this, there is some uncertainty regarding the relationship of air pollution and asthma. Although, some epidemiological studies have demonstrated that increased particulate air pollution may lead to increases in asthma others have suggested that air pollution may not be a major risk factor for the development of asthma, although it may exacerbate asthma in individuals [The International Study of Asthma & Allergies in Childhood (ISAAC) 1998]. In an international study of the prevalence of asthma in 56 countries, asthma prevalence rates tended to be lower in regions such as China and Eastern Europe (geographic areas with some of the world's highest concentrations of ambient particulates and sulfur dioxide) in comparison to nations with lower pollution levels [The International Study of Asthma & Allergies in Childhood (ISAAC) 1998].

Some research has suggested that susceptibity to the adverse effects of particulate pollution may also be related to genetic factors. The most consistently replicated chromosomal regions associated with asthma have been chromosomes 2q, 5q, 6p, 12q, and 13q. Because the formation of reactive oxygen species is a major aspect of the inflammatory process of asthma, genetic aberrations associated with antioxidants such as glutathione S-transferase (GST) may shed light on reasons why some people with asthma seem more at risk of exacerbations as a result of air pollution [Mc Cunney RJ 2005].

Therefore understanding the predisposition of asthmatics to air pollution and the effects of this on their health is complex. Nevertheless there is strong evidence to suggest that exposure to particulates may enhance some of the pathophysiological aspects of asthma for example through enhanced production of IgE. The pathophysiology of asthma can be broadly divided into inflammatory and remodelling components and it has been shown that exposure to particulate matter and in particular DEP and ROFA may contribute to the inflammatory phase of this condition, however the effects of such particulates on the chronic remodelling phase of this condition is not known.

1.7 The Inflammatory Component of Asthma

Atopic asthma is characterised by a specific pattern of inflammation that is largely driven by immunoglobulin (Ig)-E dependent mechanisms. Although many patients with asthma have atopy, in a proportion of patients with asthma there is no evidence of atopy with normal and specific IgE and negative skin tests. This so called 'intrinsic' asthma usually comes on later in life and tends to be more severe than allergic asthma. The pathophysiology is very similar to that of allergic asthma and there is increasing evidence for local IgE production, possibly directed at bacterial or viral antigens [Ying et al 2001].

The relationship between inflammation and clinical symptoms of allergy is not yet clear. There is evidence that the degree of inflammation is related to airway hyperresponsiveness as measured by histamine and methacholine challenge. Increased airway responsiveness is an exaggerated airway narrowing in response to many stimuli and is the defining characteristic of asthma. The degree of airway responsiveness is related to asthma symptoms and the need for treatment. Inflammation of the airways may increase airway responsiveness which thereby allows triggers which would not normally narrow the airways to do so. Inflammation may also lead to an increase in airway responsiveness by activation of airway sensory endings. Although there are episodes of acute inflammation in asthma this is a chronic condition, with inflammation persisting over many years. The mechanisms involved in persistence of inflammation are

not fully understood. Superimposed on this chronic inflammatory state are acute inflammatory episodes which correspond to exacerbations of asthma [Barnes 2003].

The inflammatory features of asthma consist of a dense inflammatory infiltrate in which eosinophils, mast cells, and CD4+ helper T-lymphocytes predominate. Neutrophilic infiltration also arises during asthma exacerbations and in the late response to allergen challenge.

1.7.1 Mast Cells

Mast cells are important in initiating the acute bronchoconstrictor responses to allergen and probably to other indirect stimuli such as exercise and hyperventilation. It is thought that this may take place due to changes in osmolality or thermal changes [Barnes 2003]. Patients with asthma are characterised by a marked increase in mast cell numbers in airway smooth muscle [Brightling et al 2002]. Classically mast cells are activated by allergens through the cross-linking of IgE and as a consequence mast cells become activated and degranulate. Mast cells have long been thought to play a key role in asthma through their release of a variety of mediators, including those that induce airway smooth muscle contraction and bronchoconstriction i.e. histamine, prostaglandin D2 and leukotriene D4. Mast cells are an important early source of IL-4 and other pro-inflammatory cytokines whose secretion may act as a trigger to the induction of subsequent persistent production of IL-4 and IL-5 by lymphocytes [Bradding et al 1992]. Mast cells have also been shown to release factors that are mitogenic for bronchial smooth muscle; their increased numbers within bundles of bronchial smooth muscle of asthmatics may be important in the induction of increased smooth muscle mass [Page et al 2001]. Treatment of asthmatic subjects with steroids results in a decrease in the number of tryptase positive mast cells [Bentley et al 1996]. Furthermore mast cell tryptase appears to play a role in airway remodelling as this mast cell product stimulates human lung fibroblast proliferation [Akers et al 2000].

There are questions about the role of mast cells in more chronic allergic inflammatory events and it seems more probable that other cells, such as macrophages, eosinophils and T–lymphocytes are more important in the chronic inflammatory process, including airway hyperresponsiveness. The importance of IgE in the pathophysiology of asthma has been highlighted by recent clinical studies with humanised anti-IgE antibodies, which inhibit IgE-mediated effects [Fahy et al 2000]. Although anti IgE antibody results in a reduction in circulating IgE to undetectable levels, this treatment results in minimal clinical improvements in patients' with severe steroid dependent asthma. It is now also recognised that mast cells may also release several other mediators that may play a role in the pathophysiology of asthma, including neurotrophins, proinflammatory cytokines, chemokines and growth factors. This has led to a re-evaluation of the role of mast cells [Barnes et al 2003].

1.7.2 Macrophages

Macrophages, which are derived from blood monocytes may traffic into the airways in asthma and may be activated by allergen via low affinity IgE receptors (FCξRII) [Lee et al 1992, Poulter et al 1996]. The enormous immunological repertoire of macrophages allows these cells to produce many different products, including a large variety of cytokines that may orchestrate the inflammatory response. Macrophages have the capacity to initiate a particular type of inflammatory response via the release of a certain pattern of cytokines. Macrophages may both increase and decrease inflammation, depending on the stimulus. Alveolar macrophages normally have a suppressive effect on lymphocyte function, but this may be impaired in asthma after allergen exposure [Spiteri et al 1994]. One anti-inflammatory protein released by macrophages is IL-10 and its secretion is reduced in alveolar macrophages from patients with asthma [John et al 1998]. Macrophages from normal subjects also inhibit the secretion of IL-5 from T-lymphocytes, probably via the release of IL-12, but this is defective in patients with asthma [Tang et al 2001]. Macrophages may therefore play an important anti-inflammatory role, by preventing the development of allergic inflammation. Macrophages may also act as antigen-presenting cells which process allergen for presentation to T-lymphocytes, although alveolar

macrophages are far less effective in this respect than macrophages from other sites, such as the peritoneum [Holt et al 1989].

1.7.3 Dendritic cells

Dendritic cells seem to be the key cells for antigen presentation in asthma [Banchereau et al 2000]. These specialised macrophage like cells have a unique ability to induce a T- lymphocyte mediated immune response and therefore play a critical role in the development of asthma [Banchereau et al 2000]. Dendritic cells in the respiratory tract form a network that is localised to the epithelium and act as very effective antigen-presenting cells [Holt et al 2000]. It is likely that dendritic cells play an important role in the initiation of allergen-induced responses in asthma [Lambrecht et al 2001]. Dendritic cells take up allergens, process them to peptides and migrate to local lymph nodes where they present the allergenic peptides to uncommitted T-lymphocytes and with the aid of co-stimulatory molecules such as B7.1, B7.2 and CD40 they programme the production of allergen-specific T-cells. GM-CSF, which is expressed in abundance by epithelial cells and macrophages in asthma, leads to differentiation of T-helper (Th) 2 cells [Moser et al 2000]. Animal studies have demonstrated that myeloid dendritic cells are critical to the development of Th2 cells and eosinophilia [Lambrecht et al 2000].

1.7.4 Eosinophils

Eosinophil infiltration is a characteristic feature of allergic inflammation. Allergen inhalation results in a marked increase in eosinophils in BAL fluid at the time of the late reaction and there is a correlation between eosinophil counts in peripheral blood or bronchial lavage and airway hyperresponsiveness. Activated eosinophils, are thought to contribute to airway hyperresponsiveness through the release of basic proteins and oxygen free radicals [Gleich Get al 2000, Robinson et al 2002] that also lead to airway epithelial damage [Yukawa et al 1990].

Several mechanisms are involved in the recruitment of eosinophils into the airways [Adamko et al 2002]. Eosinophils are derived from bone marrow

precursors. After allergen challenge eosinophils appear in BAL fluid during the late response and this is associated with a decrease in peripheral eosinophil counts and with the appearance of eosinophil progenitors in the circulation [Woolley et al 1994]. The signal for increased eosinophil production is thought to come from the inflamed airways. Eosinophil recruitment initially involves adhesion of eosinophils to vascular endothelial cells in the airway circulation, their migration into the submucosa and their subsequent activation. The role of individual adhesion molecules, cytokines and mediators has been extensively investigated. Adhesion of eosinophils involves the expression of specific glycoproteins on the surface of eosinophils (integrins) and their expression of such molecules as intercellular adhesion molecule (ICAM)-1 on vascular endothelial cells [Tachimoto et al 2000, Wardlaw et al 1999]. An antibody directed at ICAM-1 markedly inhibits eosinophil accumulation in the airways after allergen exposure and also blocks the accompanying hyperresponsiveness [Wegner CD 1990], although results in other species are less impressive [Sun et al 1994]. However ICAM-1 is not specific for eosinophils and cannot account for the selective recruitment of eosinophils in allergic inflammation. The adhesion molecule very late antigen (VLA)-4 expressed on eosinophils which interacts with vascular cell adhesion molecule (VCAM)-1appears to be more selective for eosinophils [Pilewski et al 1995] and IL-4 also increases the expression of VCAM-1 on endothelial cells [Lamas et al 1988] GM-CSF and IL-5 may be important for the survival of eosinophils in the airways and for 'priming' eosinophils to exhibit enhanced responsiveness.

There are many mediators involved in the migration of eosinophils from the circulation into the airways. The most potent and selective are the chemokines, such as RANTES, eotaxins 1-3 and macrophage chemotactic factor MCP-4 that are expressed on epithelial cells. Once recruited into the airways the eosinophils require the presence of various growth factors of which GMCSF and IL-5 are the most important [Park et al 1998] without which eosinophils undergo apoptosis [Simon et al 2001, De Souza et al 2002].

For many years it was thought that eosinophils played a central role in asthma, however recent studies have raised questions against this. Administration of

antibodies against IL-5 to patients with asthma has been shown to greatly reduce systemic and sputum eosinophilia but has a negligible effect on airflow and airway hyper-responsiveness. Similarly administration of IL-12 which drives differentiation of T cells to a Th1 rather than Th2 state phenotype, has been shown to reduce eosinophil numbers but not airway responsiveness in patients with asthma.

1.7.5 Neutrophils

While considerable attention has focused on eosinophils in allergic disease, there has been much less attention paid to neutrophils. Although neutrophils are not a predominant cell type observed in the airways of patients with mild-to-moderate chronic asthma, they appear to be a more prominent cell type in airways and induced sputum of patients with more severe asthma [Wenzel et al 1997, Jatakanon et al 1999, Gibson et al 2001]. Also in patients who die suddenly of asthma large numbers of neutrophils are found in the airways [Sur et al 1993], although this may reflect the rapid kinetics of neutrophil recruitment compared to eosinophil inflammation. The presence of neutrophils in severe asthma may reflect treatment with high doses of corticosteroids as steroids prolong neutrophil survival by inhibition of apoptosis [Simon et al 2001, Meagher et al 1996, Cox G et al 1995]. However it is possible that neutrophils are actively recruited in severe asthma and the concentrations of IL-8 are increased in induced sputum of these patients [Jatakanon et al 1999]. This in turn may be due to the increased levels of oxidative stress in severe asthma [Montuschi et al 1999]. The fact that patients with even higher degrees of neutrophilic inflammation, such as in COPD and cystic fibrosis, do not have the pronounced airway hyperresponsiveness seen in asthma makes it unlikely that neutrophils are linked to airway responsiveness. However, it is possible that they may be associated with reduced responsiveness to corticosteroids that is found in patients with severe asthma. Neutrophils may also play a role in acute exacerbations of asthma.

1.7.6 T-Lymphocytes

CD4+ T-lymphocytes play a key role in the orchestration of the airway inflammation that characterises asthma through the release of specific patterns of cytokines, resulting in the recruitment and survival of eosinophils and in the maintenance of mast cells. They are now recognised as critical participants in the events leading to asthma [Kay et al 2001]. Histopathological sections from patients with allergic asthma demonstrate peribronchial infiltration of CD4+ T-lymphocytes and bronchoalveolar lavage (BAL) samples from patients with asthma contain increased numbers of activated CD4+ T-lymphocytes after allergen exposure [Bousquet et al 2000]. Although several cell types participate in the airway inflammation seen in asthma, the CD4+ T-lymphocyte appears to be critically important for the events resulting in airway hyperresponsiveness [Barnes 2003].

Antigen presenting cells such as dendritic cells present antigens to T-lymphocytes. CD4+ T-lymphocytes can differentiate toward a T helper type 1 (Th1) or Th2 phenotype [Barnes 2003]. Th2 lymphocytes are important for the host response to helminth infections and are important in the pathogenesis of asthma and other allergic diseases [Wynn 2003]. These cells secrete the cytokines IL-4, IL-5, IL-9, IL-13, and GM-CSF. The naïve immune system is skewed towards a Th2 state. Activated CD4+ cells are found in increased numbers in the airways of people with asthma and are important for the coordination of allergic airway inflammation [Bousquet et al 2000]. Through the release of cytokines they maintain the inflammatory state. They also activate B-lymphocytes through the release of IL-4 that in turn activate mast cells through the release IgE.

1.7.7 Structural cells

Structural cells of the airways, including epithelial cells, endothelial cells, fibroblasts and even airway smooth muscle cells may also be an important source of inflammatory mediators such as cytokines and lipid mediators in asthma. As structural cells outnumber inflammatory cells in the airway, they may

become the major source of mediators driving asthma. Epithelial cells may have a key role in translating inhaled environmental signals into an airway inflammatory response (fig 1c). Inflammatory cells are recruited into the airways by chemokines, which exert some degree of selectivity in the cells that they attract. Eosinophil chemoattractants include eotaxin, IL-5, RANTES and monocyte chemoattractant proteins 3 and 4, whereas neutrophils are attracted mainly by IL-8. These chemokines are produced by inflammatory and structural cells such as airway smooth muscle cells and airway epithelium [Holgate ST et al 2003]. Inflammatory cells bind to adhesion molecules on bronchial vessel endothelium and subsequently undergo a process of transmigration into the airway interstitium [Barnes 2003]. Adhesion molecules that are important in this process include ICAM-1 and VCAM-1, and E-selectin. Airway cells also release survival factors, such as GM-CSF, which extend the life of inflammatory cells at the site of inflammation [Barnes 2003] [fig1.2].

Recent studies have highlighted the importance of two structurally related Th2 cytokines, IL-4 and IL-13, in the events leading to allergen induced BHR [Barnes 2003]. IL-4 is required for the differentiation of T lymphocytes to a Th2 phenotype. Once a Th2 response has been established in the lung, IL-13 appears to be involved in downstream events leading to BHR [Barnes 2003]. The receptor binding domains for IL-13 include IL-13Rα1 and IL-13Rα2 [Wynn 2003]. When expressed alone IL-13Rα1 binds IL-13 with low affinity. However when IL-13Rα1 is co-expressed with IL-4Rα, a high affinity receptor-signalling complex is formed [Aman et al 1996, Hilton et al, 1993]. The second IL-13 binding receptor, IL-13α2, binds IL-13 with high affinity. However structural differences between the cytoplasmic domains of the IL-13 receptors suggests they are functionally distinct. The cytoplasmic region of murine IL-13α2 does not possess an obvious signalling motif and is therefore thought to be a negative inhibitor or a decoy receptor [Wynn 2003]. Thus current evidence suggests that only receptors containing the IL-13α1 subunit are capable of inducing subsequent signals [Lee et al 2001]. Ligation of the IL-13 Rα1/IL-4 Rα receptor complex induces tyrosine phosphorylation of a member of the signal transduction and activation of transcription (STAT) family, STAT-6 which is essential for many of the known biologic functions of IL-13 and IL-4 [Lee et al 2001]. Although other receptor

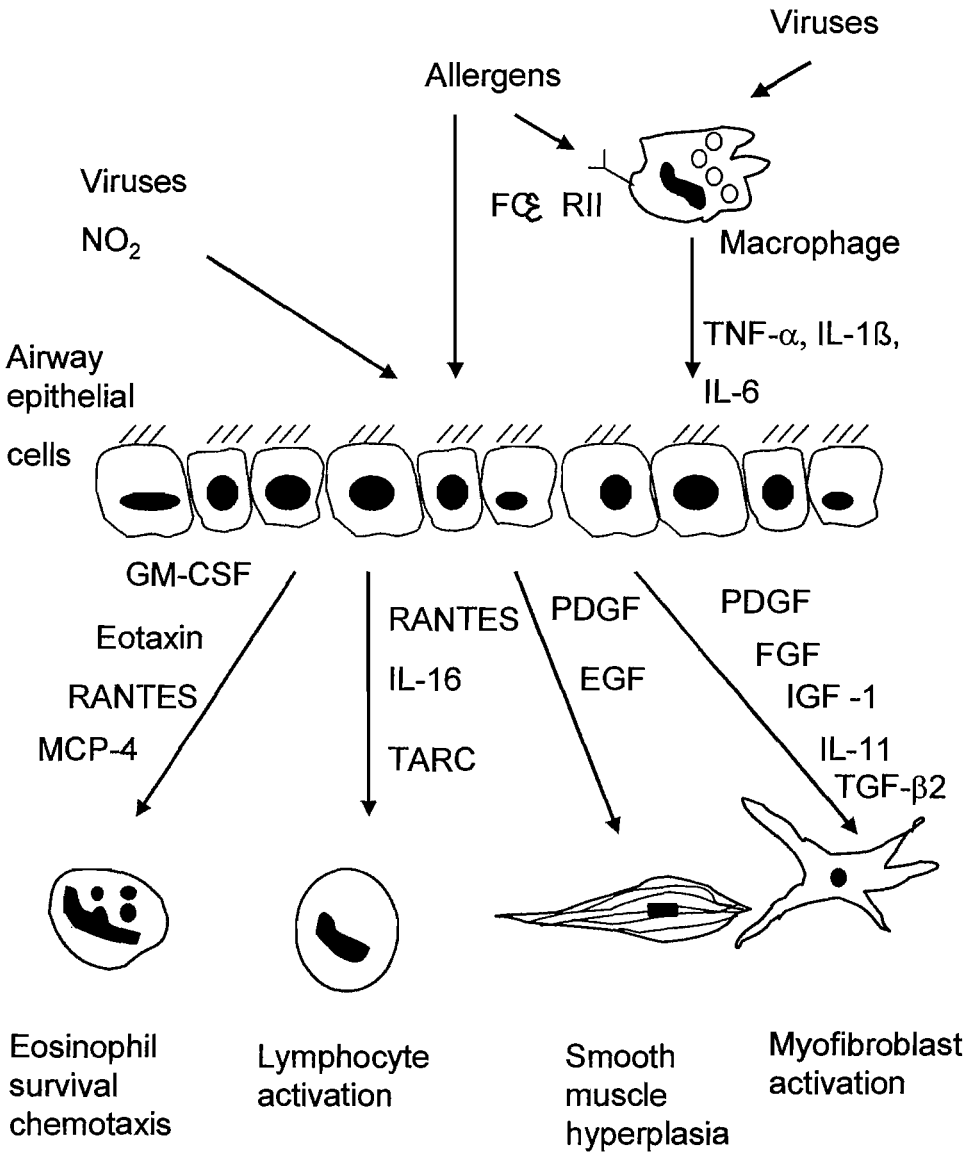


Fig 1(c) Airway epithelial cells and asthma

Airway epithelial cells may play an active role in asthmatic inflammation through the release of many inflammatory mediators, cytokines and growth factors. The diagram above summarises some of the complex interactions of epithelial cells with other inflammatory and structural cells of the airway [Barnes PJ 2003]

O_2 oxygen, NO_2 nitrogen dioxide, TNF= tumour necrosis factor, IL= interleukin, GM-CSF= granulocyte macrophage colony stimulating factor, RANTES =regulated on activation T cell expressed and secreted, MCP= monocyte chemotactic protein, TARC= thymus and activation regulated chemokine PDGF= platelet derived growth factor EGF= epithelial growth factor, FGF= fibroblast growth factor, IGF= insulin like growth factor. ,

signalling pathways, including insulin receptor substrate (IRS)-1 and (IRS)-2 [Jiang et al 2000b] have been shown to be activated by IL-13, they have not been shown to be involved in IL-13 mediated development of allergic asthma [Lee et al 2001]. Mice lacking STAT-6 [Kuperman et al 1998] and mice lacking STAT-6 activation by IL-13, such as mice treated with an IL-13R antagonist [Grunig et al 1998, Wills-Karp et al 1998], are protected from many of the phenotypical features of allergic asthma, suggesting that this pathway is essential to the contribution of IL-13 to asthma. Sustained inhibition of IL-13 throughout the period of allergen challenge reduces airway inflammation and mucus overproduction and abrogates BHR [Jiang et al 2000, Kuperman et al 1998]. Repeated administration of IL-13 to the airways of naïve mice induces airway inflammation, mucus production, and BHR [Jiang et al 2000,Kuperman et al 1998]. Overexpression of IL-13 in the airways of transgenic mice also results in inflammation and mucus overproduction and leads to marked fibrosis, airway remodelling, and increased airway resistance [Eisen et al 1998].

Therefore in summary asthma is a complex disease, involving the interaction of inflammatory cells together with structural cells, through the release of cytokines and growth factors (fig 1d and 1e)

1.8 The effects of DEP in the inflammatory response in asthma

Numerous studies have demonstrated a role for PM including DEP and ROFA in promoting inflammatory responses in asthma. It has been shown that DEP may lead to inflammation and the allergic state through three broad mechanisms. Firstly DEP may act as a carrier for the transport of allergens into the airways by adsorbing allergenic proteins onto their surface. Secondly DEP may act as an adjuvant in switching B cells to produce specific IgE and thirdly DEP may lead to an alteration in a number of downstream immunological mechanisms, leading to increased allergic and asthmatic disorders (fig 1f).

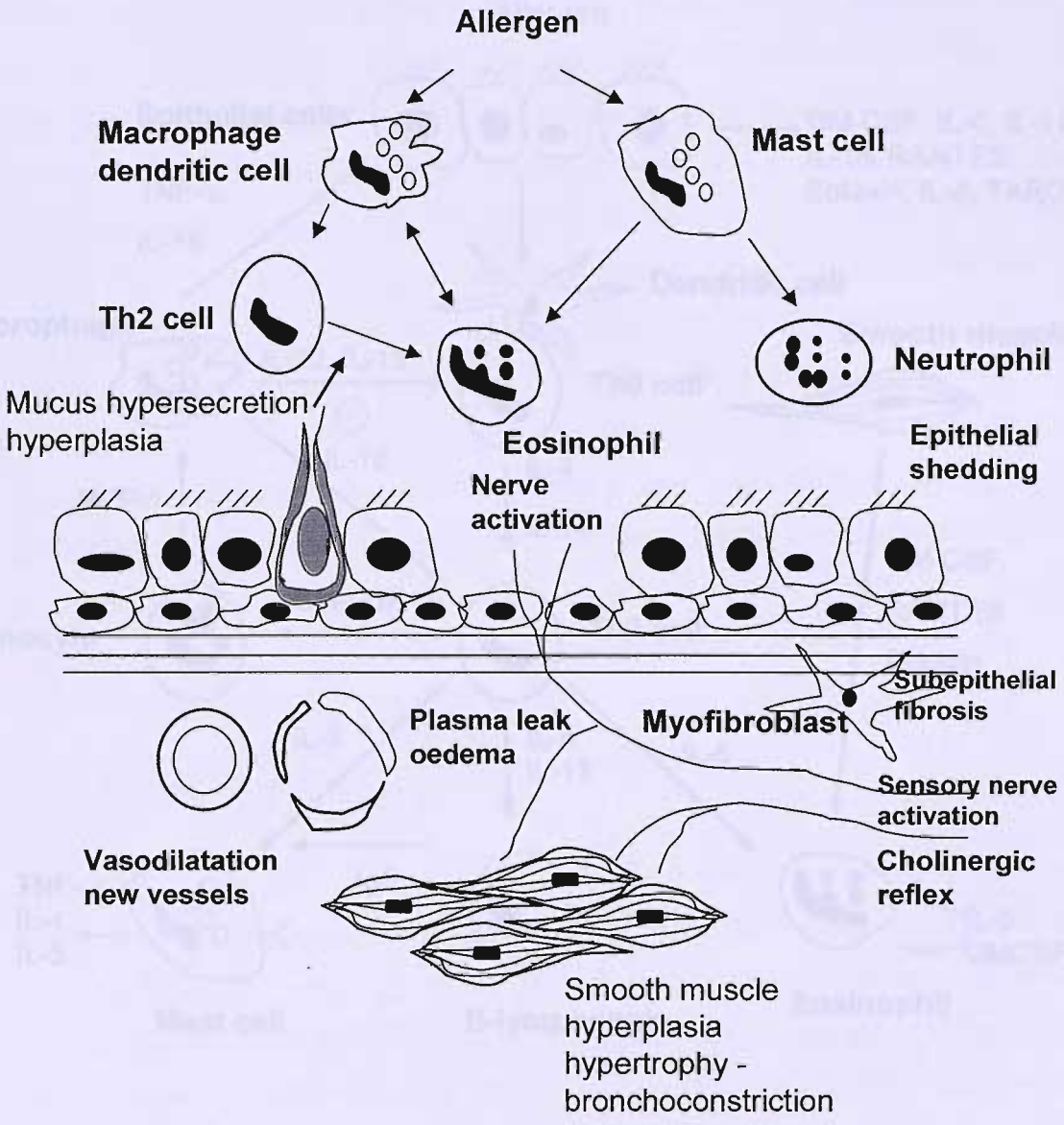


Fig 1(d) The interaction of inflammatory cells and structural cells in the pathophysiology of asthma remodelling

The pathophysiology of asthma is a complex process and involves the interaction of many inflammatory and structural cells that lead to acute and chronic inflammatory effects on the airway [Barnes PJ 2003]

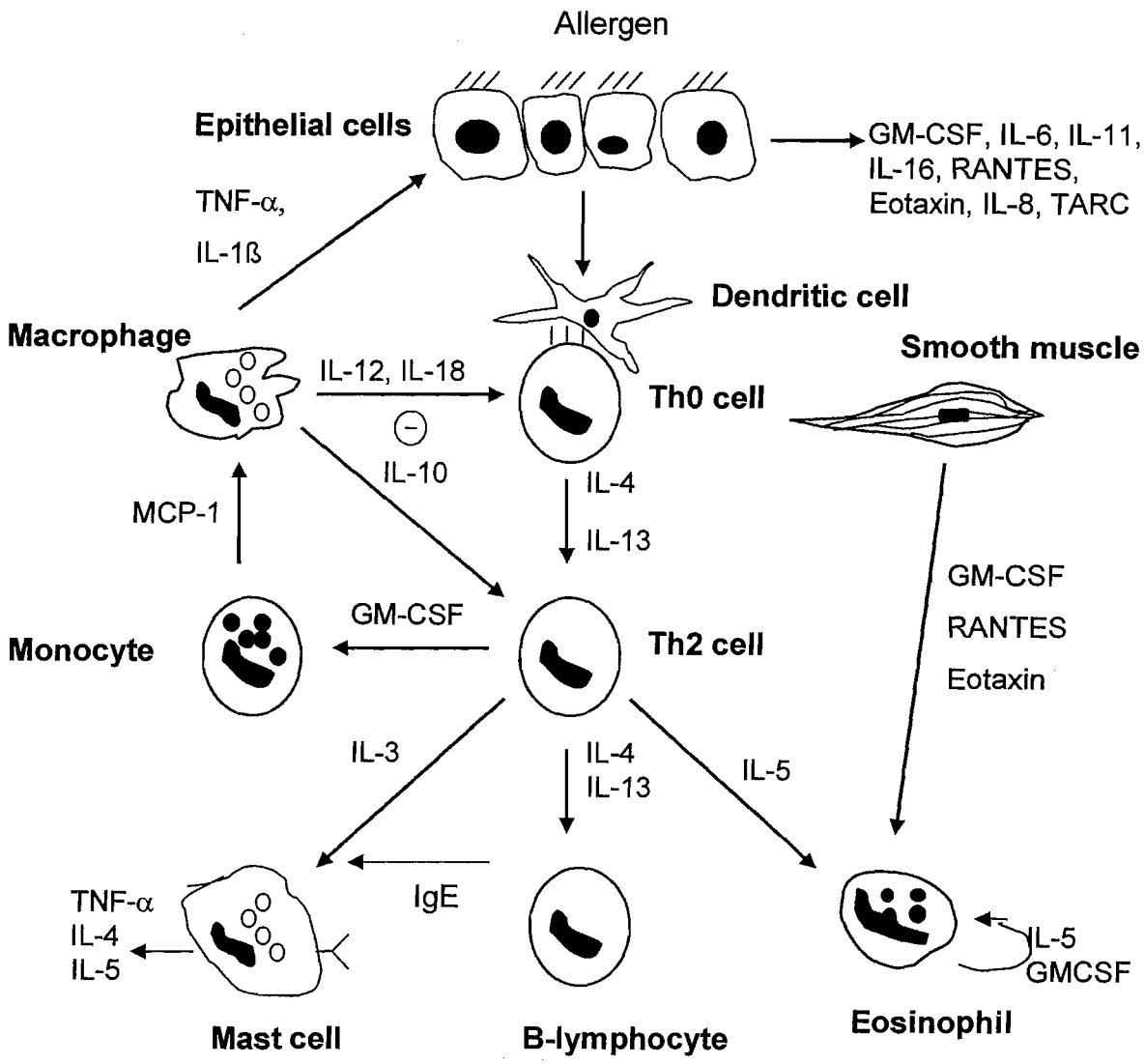


Fig 1(e) The cytokine network in asthma

Many inflammatory cytokines are released from inflammatory and structural cells in the airway and orchestrate and perpetuate the inflammatory response

TNF =tumour necrosis factor, IL= interleukin, GM-CSF=granulocyte macrophage colony stimulating factor RANTES =regulated on activation T cell expressed and secreted, MCP= monocyte chemotactic protein, TARC =thymus and activation regulated chemokine PDGF =platelet derived growth factor EGF= epithelial growth factor FGF= fibroblast growth factor IG insulin like growth factor [Barnes PJ 2003].

1.8.1 DEP as a carrier

Some allergens, such as the major grass pollen Lolium perenne-1 (Lol p1) allergen, have been shown to specifically bind to DEP [Knox et al 1997]. In an *in vitro* study of allergen adsorption to particles such as indoor-suspended particles (SPM) and DEP it was found that the cat allergen (Fel d1), dog allergen (Can f1) and birch pollen allergen (Bet v1) were all found on the surface of SPM whereas house dust mite allergen (Der p1) was not. However, all four allergens were found to be absorbed to DEP [Ormstad et al 1996]. A chemical characterisation of the coating of Birch pollen grains collected during pollen season in the north of Stockholm has shown that the greatest portion (80%) consisted of n-alkanes and n-alkenes, methylketones, ethers, alcohols and amino alcohol were identified [Henricsson et al 1996]. The role of DEP as a carrier of allergens might be a possible mechanism for triggering of asthma attacks and one theoretical explanation for the increase in asthma prevalence [Knox et al 1997].

1.8.2 DEP as an adjuvant for IgE

DEP has been shown to induce IgE production. This has also been mimicked by polyaromatic hydrocarbons (PAH) extracted from DEP which have been shown to enhance IgE production from purified human B-cells stimulated with IL-4 [Takenaka et al, 1995]. In this study PAH-DEP did not induce IgE production in unstimulated B-cells, indicating that it only enhances ongoing IgE production. Phenanthrene, a major polyaromatic hydrocarbon and an important component of DEP, has shown the same effect on IgE production in a human B cell line [Tsien A et al, 1997].

DEP has also been shown to enhance total IgE and cedar pollen specific IgE production in mice exposed either intraperitoneally or intranasally to ovalbumin (OA) mixed with DEP. [Muranaka M et al, 1986, Maejima K et al, 1997].

In order to elucidate which part of the DEP particle, the carbon core or the adsorbed organic substance is responsible for the effect, mice were immunised

four times with either OA, OA with DEP or OA with carbon black (CB). Specific IgE for OA was then analysed. Both DEP and CB showed an adjuvant activity for specific IgE production after intra-nasal instillation, indicating that they were both responsible for the effect [Nilsen et al, 1997]. There have also been other studies which have demonstrated this effect in mice [Lovik et al, 1997].

A difference in antibody response between acute and chronic exposure to DEP was seen in a study in which, after combined intra-tracheal inoculations of DEP and antigen into mice, increased airway inflammation and antigen specific IgG1 only, were seen after 6 weeks [Takano et al, 1997]. However, following a longer exposure (9 weeks), antigen specific IgE was also increased.

In another study, mice were injected intra-tracheally with OA alone or together with DEP [Ichinose et al, 1997]. Significant adjuvant activity of IgE production by DEP (50 μg) was not observed. However, the degree of eosinophilic inflammation in the airway corresponded to specific IgG1 production. In another study the combination of sensitisation to allergen together with diesel exposure led to enhanced infiltration of eosinophils and neutrophils and also an increment of goblet cells, together with enhanced airway resistance and IL-5 and IgG1 production, but not IgE production. Diesel exhaust alone did not induce pathological changes in this study [Miyabara et al, 1998a]. Other studies carried out with higher doses of DEP (300 μg), under similar conditions have demonstrated an increase in specific IgE [Fujimaki et al, 1994] indicating that this response may be dose and/or route dependent.

In man, diesel particles have been shown to potentiate nasal IgE production four days after challenge with DEP [Diaz-Sanchez et al 1994]. There was also an increase in the number of IgE secreting cells in lavage but no increase in IgA secreting cells.

Nasal challenge with a combination of DEP and allergen together has been shown to induce higher ragweed-specific IgE and IgG4 responses compared with DEP alone but with similar total IgE levels [Diaz-Sanchez et al, 1997]. There was

also a change in the cytokine pattern favouring allergic sensitisation. The authors proposed that the synergism between DEP and natural allergens is a key feature in increasing allergen induced respiratory allergic disease.

1.8.3 DEP and allergic inflammation/downstream mediator effects

In addition to the immunological changes brought about in the airways by particulate pollution, patients with asthma have been found to be more adversely affected than the normal population to inhalation of air pollution components [Hobbs et al 1991, Rusznak et al, 1994] and in particular acid aerosols [US Environmental Protection Agency, 1996]. Various studies have shown that overall lung deposition is increased in patients with obstructed airways and abnormal geometry . A 30% reduction in airway cross sectional area results in a deposition increase in the bifurcating airways by more than 100% [Kim et al, 1998]

DEP have been shown to influence the function of both structural and inflammatory cells. *In vitro*, DEP has been shown to induce eosinophil degranulation and adhesiveness to epithelial cells without changing the eosinophil survival rate. These results indicate that DEP may play a significant role in the promotion of nasal hypersensitivity induced by enhanced eosinophil infiltration and degranulation [Terada et al, 1997].

In another study the effect of DEP-PAH was investigated on the mRNA expression and release of IL8, MCP-1 and RANTES by primary bronchial macrophages obtained from healthy subjects. The production of protein in supernatants was assessed by ELISA, and mRNA production by semi-quantitative RT-PCR. There was a dose dependent increase in the secretion of IL8 and RANTES in response to increasing DEP-PAH (range 0.5ng to 50ng/ml). However there was a significant dose dependent inhibition in MCP-1 secretion. The mRNA production coding for IL-8, RANTES, and MCP-1 showed parallel variations to the production of the correspondent proteins. These results suggested that DEP-PAH can modulate chemokine pathways at the transcriptional level [Fahy et al, 1999].

The cytotoxicity of DEP, their phagocytosis, and the resulting immune response have been studied in human bronchial and nasal epithelial cell cultures. DEP exposure led to a time and dose-dependent membrane damage. Transmission electron microscopy showed that DEP underwent endocytosis by epithelial cells and translocated through the epithelial cell sheet. Flow cytometric measurements allowed establishment of the time and dose dependency of this phagocytosis and its non specificity with different particles (DEP, carbon black, and latex were tested). DEP led to a time-dependent increase in IL-8, GMCSF, and IL-1beta release. This inflammatory response occurred later than phagocytosis and it appeared to depend on the extent of adsorbed compounds, as carbon black had no effect on cytokine release. [Boland et al, 1999]

The epithelial cells of asthmatic subjects seem to be more sensitive to DEP than those of non-atopic non-asthmatics. When cultured human bronchial cells were exposed to DEP for 24 hours, there was significant attenuation in the ciliary beat frequency (CBF) in both groups, in response to increasing DEP in both groups, with maximal changes seen at 100 μg/ml. However the asthmatic cell cultures constitutively released significantly greater amounts of IL8, GMCSF, and sICAM-1 and were the only cultures to release RANTES. In response to10 μg/ml of DEP exposure there was a significant increase in the release of IL-8, GMCSF, and sICAM-1 in asthmatic cells. However, exposure to doses of 50 and 100 μg/ml led to a decrease in the release of IL-8 and RANTES. In contrast only these higher concentrations of DEP led to a significant increase in the release of IL-8 and GMCSF in the non-asthmatic cells [Bayram et al, 1998]. In another study the cytokine response to DEP was shown to be synergistic with the response to TNFα [Steerenberg et al, 1998]. These effects may be mediated through the transcription factor NF-kB. Using electrophoretic mobility shift assay DEP has been shown to increase binding to the specific motif of NF-kB but not of transcription factor AP-1. [Takizawa et al, 1999]

Another possible harmful effect of DEP may be through the complement system [Kanemitsu et al, 1998]. Pre-treatment of human serum with DEP extracts (500 -

2500 μg/ml) demonstrated a dose dependent reduction in haemolytic activity of up to 20%. There was activation of the alternative complement pathway, resulting in a decrease in complement haemolytic activity.

Other mechanisms involved in asthma development after DEP exposure may include up regulation of histamine receptor gene expression. [Terada et al 1999] or increased penetration of allergen across the respiratory mucosa [Gorski et al, 1992].

1.8.4 *In vivo* Studies of Inflammatory Responses to DEP Exposure

There have been a number of *in vivo* studies carried out in animals that have demonstrated inflammation in response to particulate exposure. Although, highly relevant it must also be noted that due to physiological differences in animal organs such as the lung in relation to humans, there are differences in the dispersion of particulates in the lungs across species. This has led to the observation that in many animal studies, exposure to doses of particulates far greater than that normally encountered by humans are required to elicit a physiological response. Thus there is a limitation in studying such animal models in relation to the physiological responses observed in humans.

In a study using mice exposed to DEP by intra-tracheal installation, an increase in epithelial eosinophils, lymphocytes and goblet cells together with IgG and IgE and the pro-allergic cytokines IL-2, IL-4, IL-5 and GM-CSF, but not IFN-γ were seen [Takano et al, 1997]. The combination of DEP and OA has also been seen to increase the bronchoconstriction response to inhaled acetylcholine [Takano et al, 1998a]. Furthermore, daily inhalation of DEP may enhance the allergen response, possibly by increased local expression of proinflammatory cytokines such as IL-5 and GM-CSF [Takano et al 1998b]. In one study, increased IgE production was also seen in mice after intra-nasal administration of suspended particulate matter (SPM) together with OA at 3 weekly intervals until 21 weeks [Takafuji et al, 1989].

1- DEP as a carrier	Allergens such as grass pollen (Lol p1) , cat allergen (Fel d1) , dog allergen (Can f1), birch pollen (Bet v1) and house dust mite (Der p1) may adsorb onto DEP and be carried into the airways
2- DEP as an adjuvant for IgE	Diesel particles induce allergen specific IgE production in rodents and humans
3- DEP and downstream mediator effects	***In vitro* studies:** ↑ Eosinophil degranulation/adhesiveness ↑ Macrophage: IL-8, RANTES MCP-1 ↑ Epithelial: IL-8, GMCSF, IL-1β, ICAM -1 ↑ Epithelial endocytosis of DEP ↓ Ciliary beat frequency ↓ Histamine gene expression ↑ **Asthma epithelium**: IL-8, GM-CSF and ICAM-1 ***In vivo* studies:** ***Mice***: ↑ epithelial eosinophils, lymphocytes, mast cells, goblet cells, IgE, IgG, IL-2, IL-4, IL-5 GMCSF ***Healthy humans***: ↑ neutrophils, mast cells, CD3+, CD4+, CD8+ T lymphocytes, IL-4, IL-5, IL-8, GRO-α, GMCSF, ICAM-1, VCAM-1, methyl histamine ***Asthmatic humans***: no neutrophilic response, ↑ higher baseline eosinophil response, ↑ IL-10

Fig 1(f) Summary of some of the effects of DEP on asthma and allergy

To study the effect of DEP on allergen-induced bronchial hyper-responsiveness, mice were sensitised to ovalbumin (OA) and then exposed to diesel exhaust (3,000 $\mu g/m^3$) [Miyabara et al, 1998b]. Three weeks after DEP exposure, they were challenged with OA. Diesel exhaust exposure combined with antigen challenge induced airway hyper-responsiveness and airway inflammation, including increased eosinophils and mast cells in the lung tissue. DEP exposure alone also increased airway hyper-responsiveness but there was no eosinophil infiltration. In a similar set up, diesel inhalations (3,000 $\mu g/m^3$) in combination with OA sensitisation increased the number of goblet cells in lung tissue, caused increased respiratory resistance and increased immune response measured as specific IgE, IgG1 and IL-5 in the lung tissue [Miyabara et al, 1998c].
A rhinitis model in guinea pigs was used to study short-term effects of diesel exhaust (3 hr exposure at 1,000 and 3,200 $\mu g/m^3$) [Kobayashi et al, 1997]. Following diesel exposure alone, there was no induction of sneezing, nasal secretion or congestion. However, DEP augmented the sneezing and nasal secretion induced by histamine, but had no significant effect on histamine-induced nasal congestion, suggesting that acute exposure to high levels of diesel exhaust may cause nasal mucosal hyperresponsiveness, but no overt symptoms of rhinitis.

Healthy, non-smoking human volunteers have been exposed to DEP by intranasal instillation and cytokines in nasal lavage were estimated after 18h by an indirect approach (mRNA) [Diaz-Sanchez et al, 1996] Before challenge, most subjects had detectable mRNA levels of only a few cytokines (IF-γ, IL-2 and IL-13) while after challenge with 0.3 mg of DEP, these three and a number of additional cytokines (IL-4, IL-5, IL-6 and IL-10) were seen in increasing levels. Increase in such nasal cytokine expression after DEP exposure could again contribute to enhanced local IgE production.

To assess the effects of diesel exhaust on human airways, a series of studies have been conducted in which volunteer human subjects have been exposed to diluted diesel exhaust (DE) at concentrations, of 300 $\mu g/m^3$, comparable to those found in occupational settings such as bus garages and North sea ferries [Salvi

et al, 1999c]. A carefully validated system has been used to maintain a specific relationship between the particulate and the gaseous components and to obtain particles of the same size and chemical properties throughout the exposure series [Rudell et al, 1990, 1994]. During the exposure, subjects performed moderate exercise and rested for alternating 15-minute periods. Six hours after the diesel exposure the subjects underwent fibreoptic bronchoscopy and samples were obtained for mediator and histological analysis. The results showed an increase in neutrophils, mast cells, CD3+, CD4+, and CD8+ T-lymphocytes in the airway mucosa along with the upregulation of the adhesion molecules ICAM-1 and VCAM-1 in the vascular endothelium. In addition, increased numbers of cells expressing LFA-1 (the ligand for ICAM-1) were found. Immunohistochemical staining for cytokines has shown enhanced expression of IL-8 and GRO α (Growth related oncogene α) in the normal airway mucosa following exposure to diesel exhaust (DE). In contrast to the inflammatory response seen in the airways, lung function parameters were unaffected following exposure to DE, indicating that lung function tests alone cannot be used to exclude adverse diesel exhaust associated airway responses. However, a neutrophilic response was observed in peripheral blood, in BAL, and in bronchial biopsies. Mast cell activation appeared to have occurred in that there were increased amounts of methyl-histamine in the BAL fluid and increased numbers of stainable mast cells in the bronchial submucosa. Increased numbers of T lymphocytes were observed in the epithelium and submucosa after diesel as compared with the control air challenges. Consistent with this recruitment of lymphocytes and neutrophils, there was marked upregulation of the adhesion molecule ICAM-1 but interestingly there was no obvious difference in the proportion of endothelial cells expressing P-selectin or E-selectin [Salvi et al 1999c].

The most marked change in cytokine expression was seen in IL-8 mRNA, which was significantly upregulated in the biopsies obtained after diesel exposure [Salvi et al, 2000]. IL-8 is a known chemoattractant and activator for neutrophils and this may thus explain the neutrophilic response observed in blood, BAL and tissue compartments. Increased amounts of IL-5 mRNA were also found after

diesel exposure. This is interesting given the reported ability of diesel to act as an adjuvant for IgE production.

Thus, diesel exhaust exposure induces a marked neutrophilic response in normal human airways and peripheral blood, which might go some way to explain the morbidity reported in epidemiological studies of particulate pollution. An associated increase in blood platelet counts could also be implicated in the cardiovascular risk.

However, in a second study comparing healthy with asthmatic subjects, it was found that there was baseline eosinophilic airways inflammation in the asthmatic airways, but exposure to DE induced neither an acute neutrophil influx nor any worsening of the pre-existent eosinophilic inflammation. There was also an unexpected, upregulation of IL-10 production in the bronchial epithelium in the asthmatic group [Stenfors et al 2004]. Based on the known properties of IL-10 [Thompson-Snipes et al, 1991, Seitz et al 1995, Jeannin et al, 1998, Van Scott et al 2000], this upregulation may prevent neutrophilic inflammation while simultaneously altering the airways biology towards a more allergic phenotype.

1.9 The effects of ROFA in the inflammatory responses in asthma

ROFA has been shown to lead to significant lung injury in experimental animal models [Pritchard et al, 1996]. Although, the exact mechanism of lung injury after exposure to ROFA is not understood, injury is thought to arise either by metal-catalysed oxidant generation or by metal ion dysregulation of phosphotyrosine metabolism, or possibly a combination of the two mechanisms [Ghio et al, 1999]. These events are thought to result in phosphorylation-dependent cell signalling and activation of specific transcription factors such as nuclear factor kappa B (NFκB) and AP-1, leading to increased expression and release of pro-inflammatory cytokines.

Although, respiratory epithelial cells provide a protective barrier to inhaled substances, they also play a critical role on lung function, which in part takes

place due to their production of inflammatory mediators. After exposure to ROFA, epithelial cells take up this substance. The particle then generates oxygen-based free radicals, which present an oxidative stress to the cell [Stringer et al, 1998, Jiang et al, 2000]. Exposure to the ash results in increased epithelial permeability, cell detachment, and a lytic injury with release of lactate dehydrogenase [Dye et al, 1997]. The antioxidant dimethylthiourea (DMTU) has a protective effect on cell permeability following exposure to ROFA [Dye et al, 1997, 1999], which supports the finding that the cytotoxic effects of ROFA are mediated by an oxidant mechanism.

Incubation of respiratory epithelial cells with ROFA leads to phosphorylation-dependent signalling reactions that may be modulated by specific redox changes [Samet et al, 1997]. Interestingly, redox-active vanadium compounds can reproduce these events, whereas catalytically active iron and nickel compounds have no effect [Samet et al, 1997]. One transcription factor known to be associated with oxidant responses is NFκB. NFκB is normally sequestered in the cytoplasm as an inactive multiunit complex bound by the inhibitory protein IκB. Various stimuli can activate NFkB which cause phosphorylation of IkB, which is followed by its ubiquitination and subsequent degradation. IkB proteins are phosphorylated by a IkB kinase complex consisting of IKKα, IKKβ, and IKKγ. This phosphorylation results in the exposure of the nuclear localization signals (NLS) on the NF-kappaB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NFκB binds to promoter and enhancer regions of a multitude of genes involved in the inflammatory response, including cytokines, chemokines, and growth factors [Delfino et al,1999]. It is thought that these genes initiate, amplify, and coordinate the inflammatory response. ROFA-induced activation of NFκB is blocked by metal chelators and free radical scavengers, suggesting that this activation is dependent on the generation of oxidants [Quay et al, 1998].

In one study, respiratory epithelial cells exposed to either ROFA or vanadium, but not iron or nickel, showed increased messenger RNA (mRNA) and protein expression of numerous cytokines, including interleukin IL-6, IL-8, and tumour necrosis factor [Carter et al, 1997]. In addition, ROFA leads to the induction of

prostaglandin H synthase 2 expression, as well as enhanced secretion of prostaglandins E2 and F2 from normal human airway epithelial cells [Samet et al, 1996]. As with NFκB activation, deferoxamine and an antioxidant diminish the release of inflammatory mediators induced by ROFA in these cells [Carter et al, 1997].

1.9.1 Exposure studies of the effects of ROFA in animal studies

Instillation of ROFA in rat lungs has been shown to lead to activation of mitogen-activated protein kinases [Silbajoris et al, 2000]. Furthermore, mRNA and protein expression of mediators of inflammation and fibrosis are also elevated in these tissues following ROFA instillation [Su et al, 1995]. In addition, exposure to the emission source particles results in a dose-dependent influx of inflammatory cells [Pritchard et al, 1996, Dreher et al, 1997], which are typically neutrophilic, with a peak influx of cells occurring at 18-24 hrs after exposure. ROFA also causes cell toxicity as evidenced by detachment of ciliated and mucus cells from the epithelial lining of the terminal bronchioles. The influx of inflammatory cells following exposure to ROFA persists for 96 hours with a slow process of resolution [Pritchard et al, 1996]. Instillations of larger amounts of oil fly ash (500 μg or greater) may induce a rapid onset of non-cardiogenic pulmonary oedema [Watkinson et al, 1998].

Animals exposed to vanadium-containing compounds demonstrate neutrophilic inflammatory injury of the bronchi and the distal lung accompanied by significant airflow limitation [Knecht et al, 1985], suggesting that transition metals play a key role in mediating the injury seen after ROFA instillation.

In animals inflammatory lung injury after ROFA administration is accompanied by airway hyperreactivity [Knecht et al, 1992] and an increase in susceptibility to infections [Pritchard et al, 1996]. The metal composition of the ash appears critical to the development of airway hyperreactivity, as assessed by acetylcholine challenge [Pritchard et al, 1996]. In addition, there are effects of ROFA on sensitisation to allergens in animal models of pulmonary allergy, with

significant elevations in eosinophils, IL-10, antigen specific immunoglobulin E, and associated immediate bronchoconstriction responses to antigen challenge [Gavett et al 1997, 1999, Hamada et al, 1999, Lambert et al, 1999]. This effect can be abrogated by DMTU pre-treatment [Lambert et al, 2000], suggesting that the oxidative stress presented by metals present in ROFA is responsible for the airway hyperreactivity and sensitisation to allergens [Hamada et al, 1999].

There are also effects of ROFA exposure on heart function, with a bradycardic response in healthy animals that persists up to 48 hr after instillation [Watkinson et al, 1998]. ROFA may also lead to an elevation in plasma concentrations of fibrinogen in exposed rats [Gardner et al, 2000].

1.9.2 Human lung Injury following exposure to ROFA

There is far less known about the effects of exposure to ROFA in humans. Exposure to oil fly ash has occurred predominantly after occupational exposures of workers engaged in the maintenance of oil-fired boilers in power generating stations [Wyers et al 1946, Williams et al 1952. Browne et al 1955, Sjoberg et al 1955, Lees et al, 1980, Hauser et al, 1995, 1996] leading to what has clinically been termed "boilermakers' bronchitis" or "vanadium bronchitis." Individuals exposed to high concentrations of ash have described eye irritation, sore throat, hoarseness, cough, shortness of breath, wheezing, and, infrequently, symptoms of pneumonitis. Clinically they may demonstrate signs of rhinitis, conjunctivitis, and wheezing. Within 24 hr of exposure to ROFA, dose-dependent decreases in pulmonary function tests have been observed, including diminished vital capacity, forced expiratory volume in 1 sec, and forced expiratory flows [Hauser et al, 1995, 1996]. Bronchoscopic examination shows bronchitis with erythema and discharge in ROFA-exposed individuals. Symptoms and signs subside, and pulmonary function decrements can resolve, within a few days or weeks of cessation of the exposure [Lees et al, 1980].

As a result of the comparable clinical presentations, physiology, and pathology of injury after ROFA and vanadium exposures, it has been suggested that this

metal is a major component responsible for toxicity of this air pollution particle in humans. Vanadium pentoxide (V_2O_5) is widely used as a catalyst for a variety of reactions and in the production of high-strength steel alloys. The first report of a human exposure to vanadium was in 1911 [Dutton 1911]. "Vanadiumism" was defined as a chronic intoxication with the principal evidence of toxicity observed in the lungs, kidneys, and gastrointestinal tract [Dutton et al 1911]. Exposures occur during the mining, separation, and use of V_2O_5 in the steel and chemical industries. Exposure occurs mostly via inhalation, and vanadium is excreted in the urine, with a smaller amount in the faeces. Vanadium dust causes symptoms of respiratory tract irritation with conjunctivitis, sneezing, rhinorrhea, sore throat, and chest tightness [Dutton 1911, Kiviluoto et al, 1979, 1980]. The cough is prominent and characteristically dry and paroxysmal [Dutton 1911]. Examination of vanadium-exposed individuals reveals a greenish discoloration of the tongue, wheezing, and crepitations [Kiviluoto et al, 1979, 1980]. An increase in the inflammatory cells in nasal smears and biopsies from the nasal mucosa accompanies symptoms of respiratory tract irritation. There can be concurrent changes in pulmonary function indices associated with vanadium exposure [Kiviluoto et al, 1979, 1980]. Vanadium workers are more susceptible to tuberculosis and can rapidly succumb to this disease [Dutton 1911]. At high exposure levels, the lungs become highly congested and show a marked destruction of the alveolar epithelium [Dutton 1911]. At high vanadium exposures, haemorrhages are frequent and severe, even causing death [Kiviluoto et al 1979, 1980]. Workers who died from vanadium exposure showed congested lungs with destruction of the alveolar epithelium [Kiviluoto et al 1979, 1980].

1.9.3 Relevance to Injury after ROFA

At the cellular level and in animal models, most investigations support the postulate that transition metals found in ROFA (especially vanadium) participate in chemical reactions to produce reactive species [MacNee et al 1999]. This is associated with tyrosine phosphorylation, intracellular signalling leading to NFκB translocation and activation of other transcription factors, induction of inflammatory mediator expression, and inflammatory lung injury. Cardiac and

systemic effects result from a dissemination of components of ROFA to extrapulmonary tissues, reflexes, or hypoxemia. It is also evident that vanadium in ROFA accounts for the greatest portion of this effect. Vanadium as a contributor to the toxicity of air pollution particles may be specific to ROFA. In support of a potential role of vanadium in the biologic effect of ambient air PM, epidemiological studies have shown a correlation between vanadium levels in the air and the incidence of mortality from bronchitis and pneumonia in British cities [Stocks et al 1960]. However, an extrapolation of the body of investigation on ROFA to the health effects of ambient air PM is complicated by the fact that ambient air PM collected from numerous environments has very small amounts of vanadium. Despite this limitation, data continue to accumulate, suggesting that ambient air and other emission source particles follow a comparable mechanism of action as ROFA, involving phosphorylation reactions [Shukla et al, 2000], transcription factor activation [Shukla et al, 2000], mediator release [Salvi et al, 2000], and inflammatory injury [Ghio et al, 2000].

1.9.4 The toxicology of ultrafine particles: the role of oxidative stress and the limitations of *in vitro* cell culture systems

There is a substantial body of evidence to support the contention that ultrafine particles have extra toxicity and inflammogenicity compared to fine respirable particles of the same material when delivered at the same mass dose. This has now been shown for a range of different materials of generally low toxicity, such as carbon black (CB) and titanium dioxide. It is not fully understood why a large number of particles or a large surface area leads to inflammation, but surface area seems to be the metric that drives inflammation in vivo caused by low toxicity particles [22]. The particle surface may be a source of reactive oxygen species and this has been demonstrated in vitro for ultrafine and fine CB (Fig. 3) [23]. In this experiment fine or ultrafine particles were incubated with a compound that undergoes activation to a fluorescent state when it is oxidised. Ultrafine CB, but not CB, caused a dose dependent increase in fluorescence indicative of oxidation. Furthernore, it has been demonstrated that this effect is not mediated by transition metals nor any other soluble agent [24], but is some consequence of the high surface area interacting with the biological system.

1.10 Airway Remodelling – a pathological hallmark of asthma

The acute and chronic allergic inflammatory responses that take place in asthma have several effects on the target cells of the respiratory tract, resulting in the characteristic changes associated with this condition. Important advances have recently been made in understanding these changes, although their precise role in producing clinical symptoms is often not clear. There is considerable current interest in the structural changes that occur in the airways of patients with asthma that are termed airway remodelling. It is believed that these changes underlie the irreversible changes in airway function that occur in some patients with asthma [Lange et al 1998, Ulrik et al 1994].

Although atopy is a strong risk factor for the development of asthma in children and young adults only 10% of atopics develop chronic asthma and in adults, asthma often develops in the absence of atopy. Therefore some have proposed that rather than IgE-mediated inflammation (a hallmark of atopy) being the initiator of disordered airway function, the epithelium itself may be abnormal and it is this that predisposes the individual with asthma toward local allergen sensitisation, the injurious effects of respiratory viruses and air pollutants as well as the development of airway remodelling. [Holgate 1998].

1.10.1 Epithelial Damage, Injury and Remodelling in Asthma

One of the features of asthma is epithelial damage as evidenced by separation of columnar cells from their basal attachments [Montefort et al 1992] a process that does not occur in other airway pathologies such as chronic obstructive pulmonary disease (COPD), even though both diseases are associated with increased inflammatory cell infiltration. Epithelial damage in asthma has also been shown to correlate with BHR [Chetta et al 1996].

Under normal circumstances, the bronchial epithelium acts as a physical barrier to protect the internal milieu of the conducting airways. In asthma, the epithelium

appears fragile with shedding of columnar cells and there is evidence of abnormalities in the responses of asthmatic epithelial cells to environmental stimuli [Bayram et al, 1997] and oxidants [Bucchieri et al 2002]. Thus, the sensitivity of the epithelial barrier to the action of different components of the inhaled environment such as particulate matter may play a key role in determining the asthmatic phenotype.

Epithelial cells appear to be an important source of mediators in allergic inflammation (fig 1c). Release of mediators in allergic inflammation may be stimulated by various stimuli, resulting in an increased inflammatory response. Epithelial cells may also release growth factors that stimulate structural changes in the airways including fibrosis, angiogenesis and proliferation of airway smooth muscle. These responses may be an attempt to repair the damage caused by chronic inflammation (fig 1c).

In asthmatic airways, there is pathological thickening and increased density of the sub-basement membrane (SBM) collagen layer, an increase in smooth muscle layer, and in microvascular and neural networks. The deposition of interstitial (repair) collagens in the lamina reticularis and an associated increase in the number of myofibroblasts is a characteristic feature of asthma, irrespective of aetiology. There is also goblet cell hyperplasia and metaplasia which leads to increased mucin hypersecretion. In addition it is thought that this may also account for the incomplete therapeutic efficacy of corticosteroids, which is evidenced by the persistence of bronchial hyperresponsiveness (BHR), and the progressive decline in pulmonary function over time that occurs in those asthmatic individuals with more chronic and severe disease [Lange et al 1998].

Recent evidence has suggested that epithelial damage and repair responses may orchestrate airway remodelling in asthma. It is proposed that the damaged epithelium leads to activation of myofibroblasts that lie directly under the epithelial layer in the lamina reticularis. These cells release extracellular matrix (ECM) components and growth factors such as transforming growth factors,

(TGF-β), platelet derived growth factor, (PDGF), fibroblast growth factor, (FGF), GM-CSF and endothelin 1 (ET-1) (fig1c). This view is supported by *in vitro* studies carried out on epithelial monolayers in which mechanical injury has been shown to result in increased release of fibroproliferative and profibrogenic growth factors including ET-1 and TGF-β2 [Tschumperlin et al 2003], basic fibroblast growth factor (FGF), insulin like growth factor (IGF-1), PDGF, [Zhang et al 1999]. Exposure to dusts has also been shown to lead to the release of pro-fibrogenic growth factors such as HB-EGF [Zhang et al 2001], PDGF-A, PDGF-B, TGF-*α*, TGF-*β* and procollagen expression [Churg et al 1999] [Dai et al 1998, 2003]. Furthermore, slowing epithelial repair with an EGFR selective inhibitor augments release of TGF-β2, which plays a key role in promoting transformation of fibroblasts into myofibroblasts that are the primary effector cells in tissue fibrosis [Puddicombe et al 2000b].

As well as structural damage, there is activation or 'stress' of the bronchial epithelium in asthma. This is demonstrated by widespread activation of nuclear transcription factor-κB [Hart et al, 1998, Wilson et al 1999], activator proteins (e.g. activator protein-1) [Demoly et al, 1992] and signal transducer and activation of transcription (STAT)-1 [Sampath et al 1999], as well as increased expression of heat shock proteins [Bertorelli et al 1998] and the cyclin-dependent kinase inhibitor, p21waf [Puddicombe et al 2003]. The altered epithelium becomes an important source of autacoid mediators, chemokines and growth factors [Chung et al 1999] that sustain ongoing inflammation and may thus contribute to remodelling responses.

There is increasing evidence that eosinophils may also contribute to the pathophysiology of airway remodelling by regulating the deposition of extracellular matrix proteins. In severe asthmatics thickening of the subepithelial basement membrane has been shown to be associated with increased bronchial mucosal eosinophil numbers [Wenzel et al 1999]. Tissue eosinophilia and eosinophil degranulation are also associated with several fibrotic syndromes [Todd et al, 1991, Wong et al, 1993, Noguchi et al, 1992] and eosinophils are the source of several pro-fibrogenic molecules such as TGF-*α* [Wong et al, 1990],

TGF-β. [Wong et al, 1991] VEGF [Hoshino et al, 2001], matrix mettaloproteinase-9 [Okada et al, 1997] tissue inhibitor of metalloproteinase-1 [Levi-Schaffer et al, 1999] and IL-13 [Schmid-Grendelmeier et al, 2002]. TGF-β is a potent regulator of fibroblast/myofibroblast function with the production of several extracellular matrix proteins including collagens, proteoglycans and tenascin [Powell et al 1999, Paakko et al 2000].

In one recent study, it was shown that the infusion of anti-IL-5 to asthmatics caused a significant reduction in airway eosinophils and a reduction in tenascin, lumican, and pro-collagen III in the reticular basement membrane compared with placebo. In this study eosinophil TGF-β1 mRNA and protein levels were also reduced in response to treatment with anti IL-5 suggesting that eosinophils may play a role in remodelling responses [Flood-Page et al 2003].

1.10.2 Airway Remodelling: Goblet Cell Hyperplasia and Mucus hypersecretion

One of the major features of airway remodelling that leads to disease morbidity and can also contribute to cases of fatal asthma is goblet cell hyperplasia and mucus hypersecretion.

Mucins are large complex molecules that are present at the interface between many epithelia and their extracellular environments. The cellular sources of mucin (MUC) glycoproteins (mucins) are goblet cells in the surface epithelium and mucus cells in submucosal glands. Mucins are the major constituents of airway mucus and contribute significantly to its viscoelastic and adhesive properties. Other important constituents of airway mucus include plasma derived proteins, especially albumin and products of cell death such as DNA and actin. An overlying mucus layer protects the airway epithelium and traps particulates for mucociliary clearance. These molecules are thus an important component of host defence, but they also represent an important cause of airway obstruction when secreted in excess. In chronic airway diseases such as asthma and chronic bronchitis, submucosal glands hypertrophy, and there is goblet cell hyperplasia in

the airway surface epithelium as well as metaplasia in the distal airways. As part of the overall disease process, these cellular changes often result in mucus hypersecretion and/or the production of mucus with altered physical properties, which in turn may increase resistance to air flow and impair mucociliary clearance. Fatal asthma for example is nearly always associated with airway occlusion from mucus plugs. In addition, excess sputum production is a common symptom in patients with asthma, especially during asthma exacerbations [Barnes 2003].

Mucins consist of a peptide backbone and numerous oligosaccharide side chains that are expressed in two major forms: the membrane-bound mucins and the secreted mucins. In the airways, MUC1 and MUC4 are the predominant membrane-bound mucins that are present on epithelial cell surfaces; MUC5AC, MUC5B and MUC2 are the predominant secreted mucins that contribute to the mucus gel. Several inflammatory mediators increase expression of the secreted mucin genes, MUC5AC and MUC2. Although the role of MUC1 and MUC4 in the airway is not known, it is thought that they may function as receptors or receptor ligands and activate intracellular signalling cascades affecting epithelial functions. It is thought that these mucins may have important functions as activators of intracellular signalling cascades [Yamamoto et al, 1997 Pandey et al, 1995 Carraway et al, 2000].

Recent work has demonstrated a role for the epidermal growth factor receptor (EGFR) in mucin synthesis following stimulation with allergens, Th2 cytokines, cigarette smoke, mechanical wounding and bacterial products. Experimental studies on the EGFR and mucins have been performed in cultured epithelial cells *in vitro* and in rodents *in vivo*. It has been demonstrated that EGFR expression and activation in the airway epithelium is responsible for mucus hypersecretion [Takeyama et al 1999]. In this study, H292 cells were demonstrated to express the EGFR and the tracheobronchial/gastric mucin MUC5AC constitutively. Treatment of these cells with TGF-α, an EGFR ligand increased MUC5AC gene and protein expression. This effect was selectively blocked by EGFR tyrosine kinase inhibitors, indicating EGFR activation [Takeyama et al 1999]. In the same study the administration of EGF and TGF-α (EGFR ligands) on rat airway

epithelium *in vivo* had no effect on goblet cell density, however this was thought to be due to the fact that the EGFR is rarely expressed in the airways of pathogen free rats. Instillation of TNF-α in rat airways resulted in EGFR expression in the airway epithelium but did not cause goblet cell metaplasia. When this was followed by administration of TGF-α this led to marked goblet cell metaplasia that was inhibited by a tyrosine kinase inhibitor suggesting a role for the EGFR in this process [Takeyama et al 1999].

1.10.3 EGFR signalling

The epidermal growth factor receptor (EGFR) serves a key role as a primary regulator of epithelial function, by transducing extracellular signals from its activating ligands into intracellular signalling cascades that lead to various phenotypic changes [Lemmon et al 1994] (fig 1h). Over expression of the EGFR is a feature of asthma and is associated with airway remodelling [Puddicombe et al 2000b] and in particular goblet cell hyperplasia and mucus hypersecretion [Takeyama et al 2001b].

The EGFR is a member of the c-ErbB family of tyrosine kinase receptors and consists of four receptors; EGFR (HER-1, ErbB-1), the orphan receptor HER-2 (ErbB-2/neu), the kinase domain impaired HER 3 (ErbB-3) and HER-4, (Erb B4). All four members are single transmembrane proteins composed of an extracellular region, a single alpha helical transmembrane region and an intracellular region. The extracellular region contains four domains, of which domains 2 and 4 are cysteine rich and domains 1 and 3 are for ligand binding. The intracellular region is subdivided into three main domains. The juxtamembrane domain is primarily used to anchor the transmembrane domain, the tyrosine kinase domain and a long carboxyl terminus tail which contains five-six tyrosine autophosphorylation sites [Wells 1999, Earp et al 1995].

Numerous growth factors bind to the ErbB family. These include EGF, Heparin binding EGF like factor (HB-EGF), transforming growth factor alpha (TGF-α), amphiregulin (AR), epiregulin, (EPR), betacellulin (BTC) and the neuregulins

(NRGs). All contain a homology domain defined by six characteristically spaced cysteine residues which form three intramolecular disulfide linkages required for ligand binding and ErbB receptor activation [Riese et al 1998]. The ligands have been subdivided into four groups based on which receptors they bind. EGF, TGF-α and AR bind the EGFR exclusively, whilst BTC, HB-EGF and EPR bind to both EGFR and ErbB4. NRGs 1 & 2 bind to ErbB3 and ErbB4, and NRGs 3&4 to ErbB4 [Riese et al 1998].

The EGFR ligands are synthesized as transmembrane precursors. The membrane-associated component is cleaved by ADAM metalloproteinases generating an active soluble form [Blobel et al 2000] [Merlos-Suarez et al 2001]. Therefore the membrane-bound and soluble forms exhibit differing potencies leading to juxtacrine, autocrine, and paracrine mechanisms. The effects of EGFR ligands are not identical, responses being dependent both on properties of the ligand and the surface of the target cell. Binding on to the EGFR results in the formation of activated dimeric receptor complexes comprising either homodimers (EGFR2) or heterodimers involving other members of the c-ErbB family [Prenzel et al 2001]. Heterodimeric combinations modulate receptor function with diverse effects on intracellular signalling and cellular responses [Prenzel et al 2001]. Bronchial epithelial cells co-express several members of the c-ErbB Family [Polosa et al 1999] and heterodimerisation may control cellular functions within the bronchial epithelial cells.

The process of homo/heterodimerisation leads to subsequent autophosphorylation on specific tyrosine residues in the receptor carboxyl tail in the intracellular component of the EGFR, which propagates the signal from the cell surface into the cell [Prenzel et al 2001].

EGFR kinase activation triggers downstream signalling of a number of different pathways including those involving phospholipase C-gamma (PLC-γ), and its downstream calcium and protein kinase C (PKC) pathways, PI3 Kinase, and activation of the RAS-MAPK pathway. With regard to the latter, the phosphorylated tyrosine residues on the receptors intracellular tail provide docking sites for proteins containing src homology 2 (SH2) domains and protein

tyrosine binding (PTB) domains. These proteins are either enzymes activated by tyrosine phosphorylation such as PLC-γ or, alternatively are adapter proteins serving to link the receptor with downstream signalling, examples of which include Shc and Grb2 [Hackel et al, 1999].

EGFR signalling leads to phosphorylation of adapter proteins including Shc (p46 and p52 isoforms) and Grb2 which form a complex and lead to ERK activation by converting Ras.GDP to Ras.GTP. This in turn activates the ERK-MAPK cascade, resulting in translocation of the activated MAPKs to the nucleus which lead to altered gene expression [Widmann et al 1999, Brunet et al 1997].

As well as ligand dependent activation of the EGFR, there are also several ligand independent activation mechanisms which may also result in initiation of cell signalling. Stimulation with stressful stimuli such as hydrogen peroxide (H_2O_2) and ultraviolet light causes EGFR tyrosine phosphorylation, recruitment of Shc and subsequent signalling. The mechanism for this activation is not fully elucidated but is thought to involve the redox regulation of tyrosine phosphatases, whereby the oxidant stress acts directly or indirectly to prevent dephosphorylation of the receptor. This may occur via oxidation of a catalytic SH group or by irreversible alkylation of essential cysteine residues on the tyrosine phosphatases [Zwick et al 1999].

Another activating mechanism for the EGFR is via, G protein coupled receptors (GPCRs). These are a large group of cell surface receptors that are capable of transactivating the EGFR [Leserer et al 2000, Ramirez et al 1995]. They consist of seven transmembrane domains, an extracellular amino terminal domain and an intracellular carboxyl terminal tail. GPCRs can be activated by a large variety of stimuli including endothelin-1 (ET-1), Lysophosphatidic acid (LPA), and thrombin. Activation of the GPCRs results in activation of intracellular signalling pathways by certain effector molecules such as adenylate cyclase which regulates cAMP levels. Activation of the GPCRs are thought to activate the EGFR through an unknown mechanism(s) involving Ca^{2+} and SRC kinase. In rat cells it has been shown that the EGFR and Erb-2 are rapidly phosphorylated in

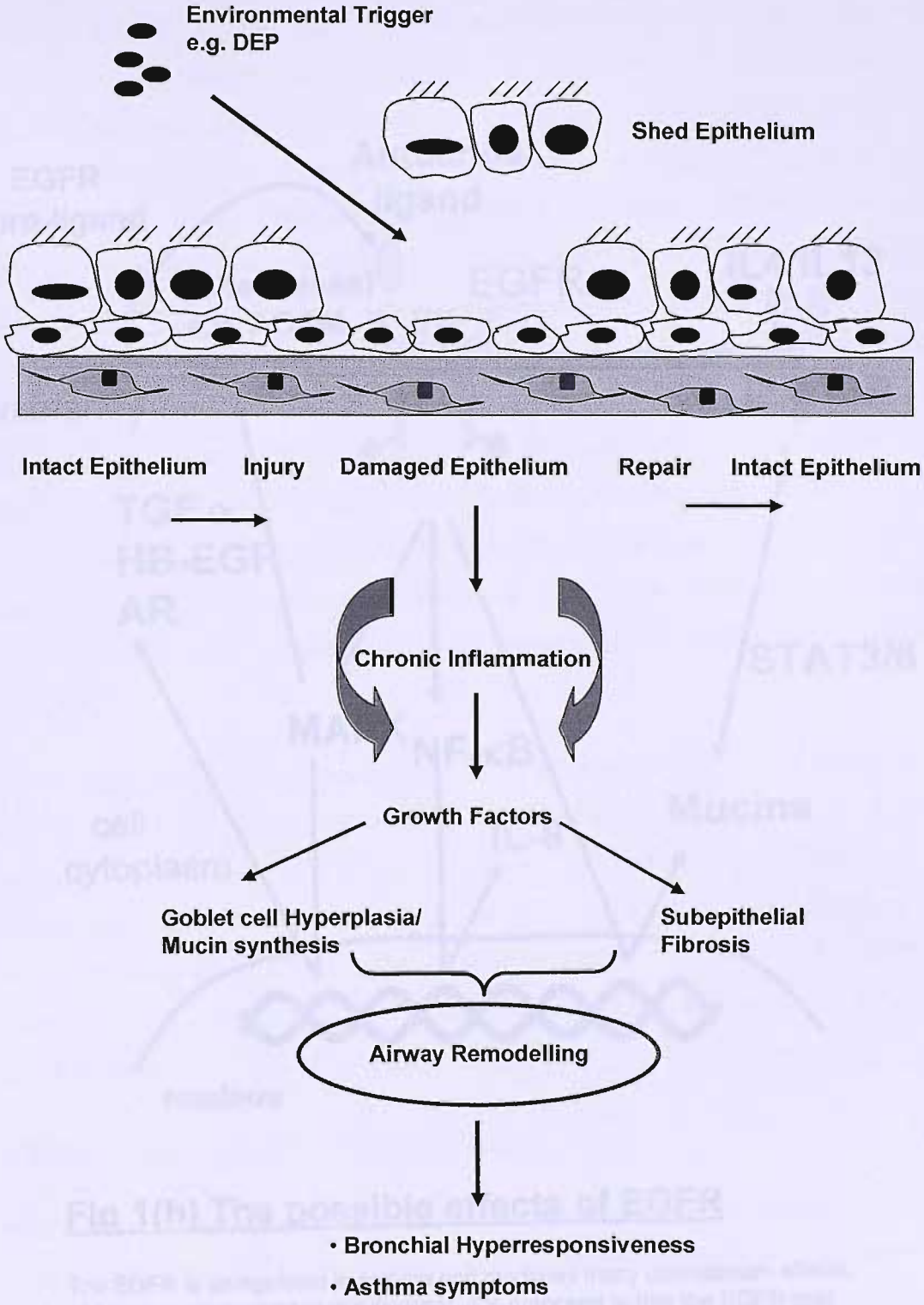


Fig 1(g) The pathogenesis of asthma and the potential effects of DEP

Environmental insults such as DEP at the mucosal surface cause epithelial damage resulting in mediator release which promotes influx of inflammatory cells. In a susceptible individual this leads to continuous cycles of epithelial damage and chronic inflammation. The injury repair responses are thought to cause activation of the epithelial-mesenchymal trophic unit which drives airway remodelling. These remodelling and chronic inflammatory responses combine to cause asthma.

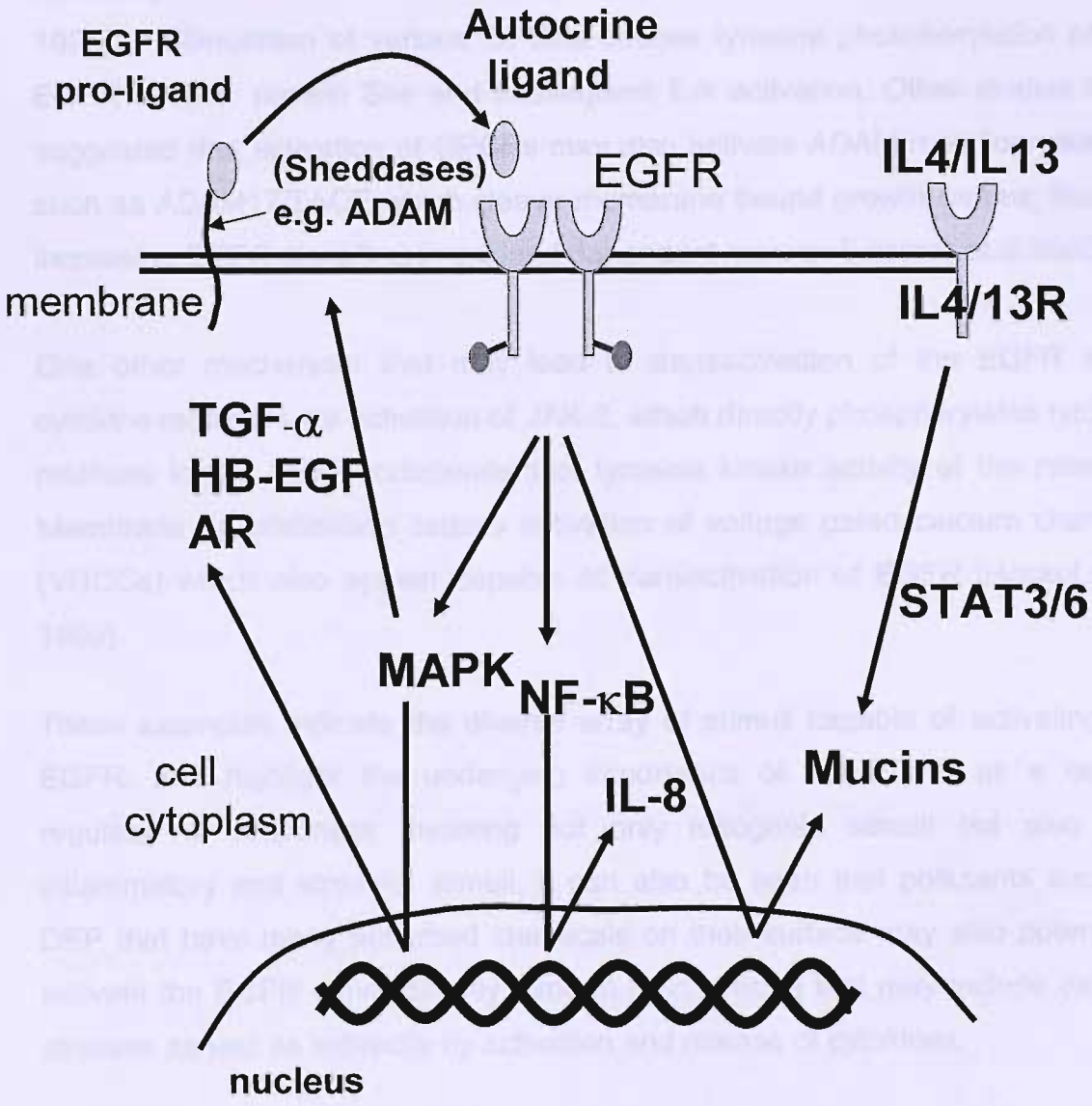


Fig 1(h) The possible effects of EGFR

The EGFR is upregulated in asthma and mediates many downstream effects, which are summarised in this diagram. It is proposed to that the EGFR may become activated following exposure to an environmental trigger such as DEP and then mediate some of the chronic effects that take place in asthmatic airways.

TGF =transforming growth factor, IL= interleukin, AR= Amphiregulin, HB-EGF=Heparin binding epidermal growth factor, MAPK= Mitogen activated protein kinase, R=receptor, STAT= signal transducer and activator of transcription 6

response to ET-1, LPA and thrombin stimulation leading to ERK activation. Moreover this response was blocked with the EGFR inhibitor tyrphostin AG1478, indicating a need for EGFR in GPCR mediated mitogenic signalling [Daub et al 1996]. Stimulation of various GPCRs causes tyrosine phosphorylation of the EGFR adapter protein Shc and subsequent Erk activation. Other studies have suggested that activation of GPCRs may also activate ADAM metalloproteases, such as ADAM17/TACE which cleave membrane bound growth factors, thereby increasing EGFR signalling in a ligand dependent manner [Leserer et al 2000].

One other mechanism that may lead to transactivation of the EGFR is by cytokine receptors via activation of JAK-2, which directly phosphorylates tyrosine residues in the EGFR independent of tyrosine kinase activity of the receptor. Membrane depolarisation causes activation of voltage gated calcium channels (VGCCs) which also appear capable of transactivation of EGFR [Hackel et al 1999].

These examples indicate the diverse array of stimuli capable of activating the EGFR, and highlight the underlying importance of the EGFR as a central regulator of responses involving not only mitogenic stimuli but also pro-inflammatory and stressful stimuli. It can also be seen that pollutants such as DEP that have many adsorbed chemicals on their surface may also potentially activate the EGFR either directly through mechanisms that may include oxidant stresses as well as indirectly by activation and release of cytokines.

1.10.4 Allergen induced mucin synthesis

EGFR expression is sparse in the airway epithelium of healthy humans; however, in conducting airways of mild and moderate asthmatics and in nasal polyps the density of goblet cells is increased and EGFR expression is upregulated. In a study of asthmatics it was seen that EGFR and mucin MUC5AC gene and protein expression were increased and correlated positively with the degree of goblet cell metaplasia present, suggesting a causal relationship [Takeyama et al 2001a].

In rats, sensitisation with ovalbumin (OVA) has been shown to induce EGFR expression and goblet cell metaplasia. This process can be blocked by a selective EGFR inhibitor [Takeyama et al 1999]. The Th2-cytokine IL-13 has also been implicated in OVA induced goblet cell metaplasia. Instillation of IL-13 into the airways of rodents has been shown to induce marked goblet cell metaplasia *in vivo*. Although unclear of the mechanism, EGFR inhibition *in vivo* has been shown to prevent IL-13 induced goblet cell metaplasia [Shim et al 2001]. However *in vitro* studies carried out in H292 cells have shown that IL-13 has little or no effect on mucin gene transcription, suggesting that IL-13 activates the EGFR through an indirect mechanism *in vivo*. In a study of rodents instillation of IL-13 into the airways induced the expression of an IL-8 like neutrophil chemoattractant followed by neutrophil recruitment [Shim et al 2001]. An IL-8 blocking antibody inhibited both IL-13 induced neutrophil recruitment and mucus cell differentiation, establishing that neutrophils and EGFR activation are required for IL-13 induced goblet cell metaplasia [Shim et al 2001].

1.10.5 Mucins, neutrophilic inflammation, cigarette smoke and COPD

Exposure of rats to cigarette smoke has been shown to induce mucus hypersecretion [Coles et al 1979]. Peripheral airways of smokers contain an increased number of goblet cells and inflammatory cells when compared with non-smokers [Maestrelli et al 2001]. EGFR expression is increased in the metaplastic bronchial epithelium of smokers. In one study of the effects of smoke exposure and the EGFR system, it was demonstrated that human neutrophils activated by IL-8, N-formyl methionyl-leucyl-phenylalanine or TNF-α increased EGFR tyrosine phosphorylation and activation of p44/42 MAPK as well as upregulation of MUC5AC expression at both the gene and protein levels in human NCI-H292 cells. These effects were prevented by antioxidants. Thus, reactive oxygen species produced by neutrophils have been shown to activate the EGFR in the absence of a ligand (ligand independent transactivation). Similarly goblet cell metaplasia induced by cigarette smoke has also been shown to involve EGFR activation in H292 cells and in rat airways [Takeyama et al

2001b]. The effects of smoke were prevented completely by EGFR tyrosine kinase inhibitors and partially by antioxidants, suggesting that cigarette smoke causes EGFR activation and mucin production at least in part by oxidative stress. In another study using bronchial epithelial cells it was demonstrated that secretion of IL-8 in response to cigarette smoke is dependent on EGFR activation through autocrine production of EGFR ligands particularly TGF-α [Richter et al 2002].

1.10.6 Mucins and wounding of airway epithelium

Healing in damaged tissues requires proliferation and migration of regenerating cell types. In scrape-wounded monolayers of airway epithelial cells, it has been demonstrated that EGF accelerates cellular repair and a selective EGFR inhibitor impairs cellular repair. Other studies including orotracheal intubation in horses and mechanical denudation of tracheal epithelium in hamsters have demonstrated that mechanical irritation of airways results in goblet cell metaplasia [Takeyama et al 2000]. In one study examining the role of the EGFR pathway on mucin response to mechanical irritation, instillation of agarose plugs into rat airways resulted in striking goblet cell production. This process was inhibited by EGFR inhibitors implicating the EGFR in this response. Neutrophils were seen in epithelium adjacent to the agarose plugs and cyclophosphamide prevented agarose induced neutrophil recruitment and goblet cell metaplasia, Infiltrating neutrophils produce TNF-α and an anti TNF-α antibody prevented agarose induced goblet cell production. These findings implicate neutrophils and TNF-α in the EGFR cascade that results in goblet cell metaplasia from airway wounding [Lee et al 2000].

1.10.7 Mucins and bacterial Infection

Airway infections, especially by Gram-negative bacteria are believed to play an important role in mucus hypersecretion and deterioration in respiratory illnesses such as cystic fibrosis and in COPD. Gram-negative bacterial endotoxin increases epithelial mucosubstances [Harkema et al 1992], which results in goblet cell metaplasia in rat nose [Shimizu et al 1996] and increased mucin synthesis in the rat lower airways [Steiger et al 1995]. It has also been reported

that selective EGFR tyrosine kinase inhibitors prevent Pseudomonas Aeroginosa induced mucin synthesis in human airway epithelial cells, implicating an EGFR cascade in the mucin synthesis [Kohri et al 2002]. Thus, a cellular cascade initiated by the activation of the EGFR is implicated in the epithelial goblet cell response to stimulation with bacteria. Mucin transcription in response to gram-positive bacteria has been shown to be mediated through activation of EGFR. Basbaum and colleagues have reported that the mechanism of EGFR activation in response to bacterial lipoteichoic acid involves cleavage of the transmembrane ligand HB-EGF by ADAM 10, whereas EGFR activation in response tobacco smoke involves cleavage of amphiregulin by ADAM 17 [Basbaum et al 2002].

1.11 Summary

Although asthma has a strong genetic component, the asthmatic phenotype is thought to arise as a consequence of interaction with various environmental factors, including chronic exposure to air pollution, which in turn may activate cellular signalling pathways that lead directly or indirectly to the pathological changes that are characteristic of asthma. As increased exposure to air pollutants in the past two decades has paralleled the increases in asthma prevalence, understanding the mechanistic interactions that take place between the airway epithelium and common air pollutants such as diesel exhaust particles may provide new insights into understanding the gene-environment interactions that may lead to the asthmatic phenotype and in so doing may also be help identify novel treatment modalities for this disease.

1.12 Aim and objectives

As described earlier in this chapter studies have demonstrated that PM and in particular DEP lead to acute airway inflammation. In addition, little is known about the mechanisms leading to an inflammatory response by particulates such as DEP on airway epithelial cells as well as their possible contribution to the chronic pathological features of asthma. Recognising the effects of the EGFR as a mediator of epithelial function and activation linked to cigarette smoke as well as oxidants, it is hypothesised that DEP cause epithelial injury and promote inflammation through the activation of the EGFR and shedding of EGFR ligands. Further recognising the role of the EGFR in promoting mucin secretion, it is also hypothesised that DEP activate mucin responses in bronchial epithelial cells and that the effects of DEP on epithelial cells are heightened in asthmatics when compared to healthy subjects.

The aim of this study is to thus examine the mechanism leading to an acute inflammatory response by DEP in airway epithelial cells in healthy subjects and asthmatics, as well as the ability of DEP to lead to mucin expression. Specifically, it is proposed to (1) analyse the *in vitro* inflammatory effects of DEP as determined through epithelial cell injury, epithelial cell number, as well as the expression of IL-8 gene and protein release in bronchial epithelial cells from normal and asthmatic subjects (2) Analyse the role of the EGFR in the acute inflammatory responses of airway epithelial cells to DEP by studying gene expression and release of EGFR ligands and their effects on the release of IL-8 as a marker of inflammation. (3) Analyse the effects of DEP on the expression of secretory as well as membrane bound mucins; (4) Using an *in vitro* model of an asthmatic airway (using Th2 cytokines), determine whether DEP act in synergy with Th2 cytokines, especially IL-4 and IL-13 to promote IL-8 gene expression and release, as well as EGFR ligands, and mucin gene expression, thus determining whether the effects of DEP are possibly augmented in asthmatics (4) Determine some of the possible mechanisms by which DEP exert their effects on airway epithelial cells. Thus the effects of DEP together with ROFA and carbon black are compared to determine the contribution of organic adhered compounds on the surface of DEP with a transition metal rich particulate such as ROFA.

CHAPTER TWO

MATERIALS AND METHODS

2.1.1 DEP Extraction – University of Cardiff

The experiments carried out in this study were performed using DEP obtained from a number of sources (table 1.1). Japanese DEP stocks were donated as a gift by Dr Riccardo Polosa (University of Southampton), DEP from the USA was purchased from the NIST (National Institute of Standards & Technology, Gaithersburg, MD, USA). In addition collaboration was formed with Dr Kelly BeruBe at the Department of Biomedical Sciences at the University of Cardiff who supplied 'freshly frozen' and 'aged' DEP (see below). Emission particles from a diesel tractor were collected on a Teflon filter, (Omega Speciality Instrument co, Houston USA). The tractor exhaust was placed in close proximity to the filter, which enabled the particles to be collected (fig 2.1). These filters were accurately weighed before and after DEP collection to allow calculation of the final DEP weight. Each filter collected approximately 20-25 mg of DEP. Following collection the DEP was stored at room temperature for 'aged' DEP and used at various time points. For 'fresh frozen' DEP, following collection the DEP was immediately stored at –20°C and sent to Southampton on dry ice. It was then stored in a –20°C freezer until it was used on the epithelial cells

In order to extract the DEP from the filters, each filter was placed in a 20ml Universal container before being soaked in 100 μl of 70% methanol. Approximately 20ml of appropriate culture medium was then added to the Universal container. This was then vortexed for 2 minutes before being placed in a sonicate water bath for 3 minutes. Through this method most of the DEP was extracted off the filter. The filter was then left to dry and then re-weighed. The difference between the weighed values before and after the extraction was used to indicate the final amount of DEP in the solution. The final concentration of DEP in solution was made up to 1mg/ml. In order to measure the weight of salts from the culture medium remaining on the filter after the drying process, a filter without DEP was placed in culture medium and left to dry. The difference in the weight of the filter before and after immersion in the culture medium was measured and found to be negligible.

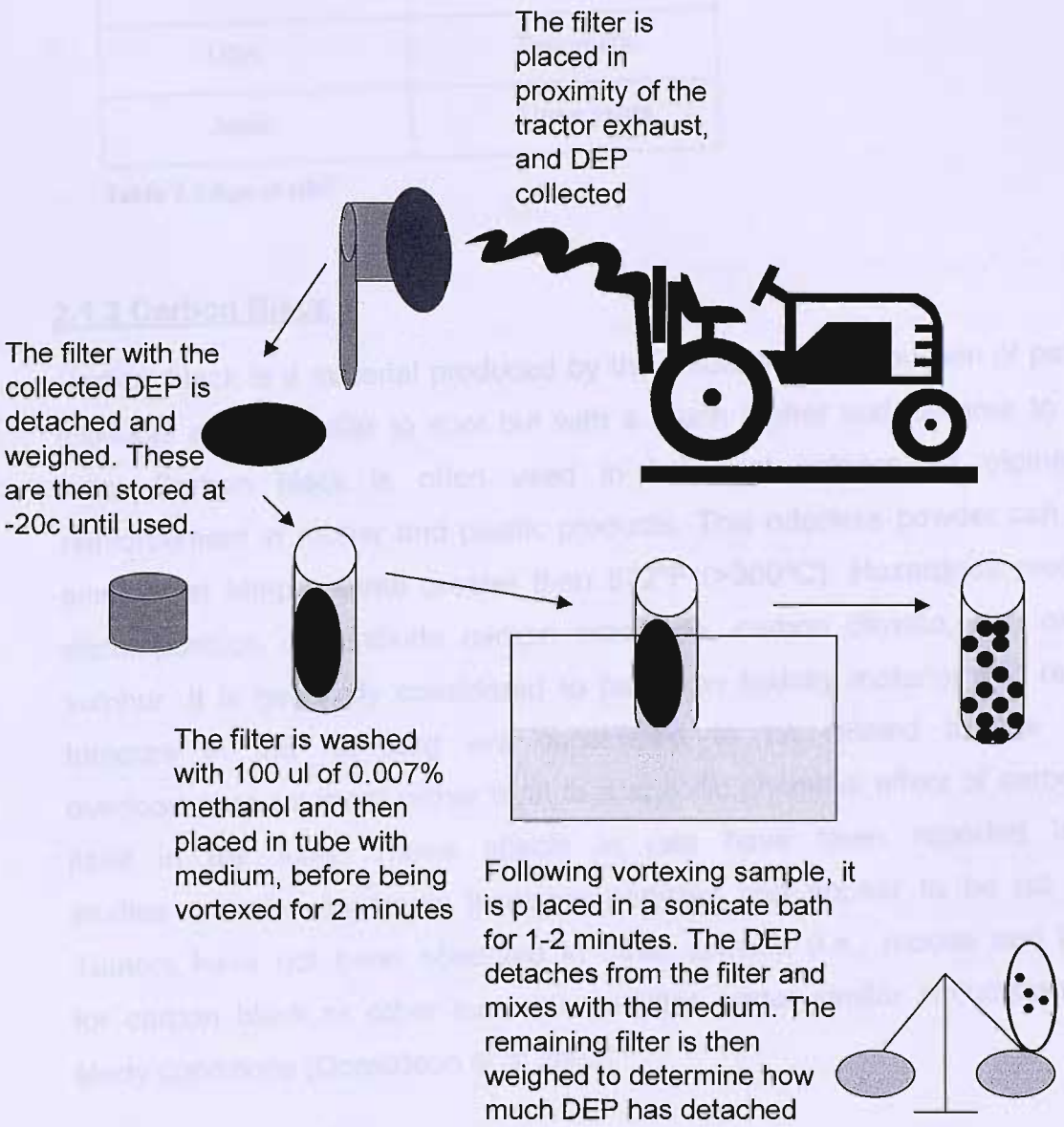


Fig 2a) Collecting Welsh DEP onto a filter

Source	Age
Wales	Three months
USA	Two years
Japan	Three years

Table 2.1 Age of DEP

2.1.2 Carbon Black

Carbon black is a material produced by the incomplete combustion of petroleum products and is similar to soot but with a much higher surface area to volume ratio. Carbon black is often used in industrial settings as pigment and reinforcement in rubber and plastic products. This odorless powder can burn or smolder at temperatures greater than 572°F (>300°C). Hazardous products of decomposition can include carbon monoxide, carbon dioxide, and oxides of sulphur. It is generally considered to be a low toxicity material and reports of tumours in the rat lung are considered to be related to the "particle overload phenomenon" rather than to a specific chemical effect of carbon black itself in the lung. These effects in rats have been reported in many studies on other inorganic insoluble particles and appear to be rat specific. Tumors have not been observed in other species (i.e., mouse and hamster) for carbon black or other insoluble particles under similar circumstances and study conditions [Donaldson et al 2003].

Although during *in vivo* exposure studies the size distribution of DEP is very important as particle size determines the degree of particle deposition in the smaller airways of the lung and hence surface area acting on the lung, it is not possible to determine the actual size of the individual particles with any great accuracy during *in vitro* cell culture studies. This is a limitation imposed by the fact that DEP are hydrophobic and hence they aggregate in cell culture medium into large clumps (approximately 1mm in diameter). Thus, with this limitation in

mind, particle dosage was determined by weight before being dissolved in solution.

2.2 Cell Culture

2.2.1 Human Subjects

Primary bronchial epithelial cells were grown from bronchial brushings obtained from human volunteers [Hurd et al 1991]. Subjects were normal non-atopic controls and atopic asthmatic subjects with disease of varying severity and duration as defined by the NHLBI/WHO GINA asthma guidelines [WHO/NHLBI 1995]. Each subject completed a standard asthma, atopy and quality of life questionnaire; lung function tests (PEF monitoring, spirometry and methacholine PC20), and skin testing to 13 common allergens, total IgE and RAST. The study was approved by the Southampton Joint University and Hospital Ethics Committee. Written informed consent was obtained from the subjects. Primary cultures were established from ten asthmatic volunteers (six males, four females), mean age 34.5 years (range 20–56) and mean forced expiratory volume of 1 s (FEV_1) 72.7% (range 63–83%) predicted. These subjects were using salbutamol only. There were also ten non-asthmatic control subjects (five males, five females), mean age 34.7 years (range 22–54) and mean FEV_1 105.7% (range 95–120%) predicted.

2.2.2 Bronchoscopy

Fibreoptic bronchoscopy was performed by Dr M Steele and Dr M Thornber in the Department of Medical Specialties at the University of Southampton. Subjects were pre-medicated with nebulised salbutamol (2.5mg) and intravenous atropine (0.6 mg), and midazolam (0-5mg intravenously) to achieve moderate sedation. Lignocaine (2-4%) was used for local anaesthesia and continuous oxygen given via a nasal cannulae. A flexible fibreoptic bronchoscope (Olympus) was inserted through a mouthpiece with the patient in the supine position. During

each bronchoscopy bronchial brushings were taken which provided the samples for culture *in vitro*.

2.2.3 Brushed bronchial epithelial cells

The bronchial brushings were placed in 5ml of sterile PBS. Two brushings were used per each 5ml aliquot. These were then detached by use of vigorous agitation. The brush was then rinsed in a fresh vial of PBS before being returned for further brushings. Between four and six brushings were used for each cell culture.

2.2.4 Primary Human Epithelial Cell Cultures

Bronchial epithelial cell brushings were obtained by fibreoptic bronchoscopy according to standard guidelines [Hurd et al 1991]. These bronchial epithelial cells were brushed from the airways of normal or mild asthmatic subjects and established in culture by Dr Sarah Puddicombe over a three-week period in Bronchial Epithelial Growth Medium (BEGM) (Bio Whittaker). In summary, the cells were harvested into sterile PBS to which was added an equivalent volume of 10% foetal calf serum (FCS). The cells were spun for 5 minutes at 150g. Care was taken to ensure that all the cells were pelleted. The cells were re-suspended in a total volume of 3ml complete BEGM (prepared by taking 50ml of BEBM, and adding: 2 ml of Bovine Pituitary Extract (BPE) (13mg/ml), 0.5ml of hydrocortisone (0.5mg/ml), 0.5ml hEGF (0.5μgml), 0.5ml epinephrine (0.5mg/ml), 0.5ml transferrin (10mg/ml), 0.5ml insulin (5mg/ml), 0.5ml triiodothyronine (6.5μg/ml), 0.5ml retinoic acid (0.1μg/ml), 0.5ml GA-1000 (Gentamicin sulphate and amphotericin B (1μl/ml). This complete medium was used within one week of preparation.

The cells were then put into a T25 flask (P0) and to this an extra 1μl GA-100 antibiotic/ml of medium was added. These were cultured in an incubator

overnight at 37°C with 5% CO_2 in air. The medium was removed and the unattached cells transferred to a fresh flask without spinning. 1μl of extra GA-100 antibiotic/ml of medium was added again together with 1ml of fresh BEGM. These were incubated overnight (P0, 2f). This was repeated until there were 4 small flasks. The flasks were fed daily for the first 5 days to maintain antibiotic concentrations.

When the cells were 50-80% confluent they were passaged into a T75 flask. The medium was changed every two days using fresh BEGM until the cells reached confluence. They were then trypsinised and plated at 0.75 x 10^5/well in a 24 well plate for the exposure studies. These cultures usually took up to one week to reach 90% confluence after this point. The cultures were then rendered quiescent using BEBM plus Insulin Transferin Selenium at x1 concentration (ITS). Thus using a 100x concentration of ITS, 500μl was used for every 50mls BEBM) and 1% BSA (500μl of 100mg/ml for every 50mls BEBM). 24 hours later the cells were then stimulated using the appropriate conditions according to each experiment.

Cells at passages 2 or 3 were used for experiments. All culture techniques were carried out in a Microflow class II biological safety cabinet following standard disinfection protocols.

2.3 Immortalised Cell Lines

H292 and 16HBE14o$^-$ cell lines were chosen as they are known to produce reproducible results in experiments involving epithelial cell cultures and can be used to determine mechanistic pathways involving epithelial cells. These cell lines had been extensively used both in our laboratory and others in order to investigate the effects of various markers of environmental insults on the airway epithelium such as cigarette smoke and oxidants. It was thus decided to use these cell lines in order to investigate the effects of particulate pollution. The H292 and 16HBE14o$^-$ cell lines were obtained from Dr A. Richter and Dr S.H

Leir (University of Southampton) respectively from previously stored frozen stocks in liquid nitrogen. H292 cells are a mucoepidermoid cell line derived from a mucoepidermoid lymph node metastasis from a 32 year old woman and had been purchased from ATCC (American Type Culture Collection). 16 HBE14o$^-$ cells had been a kind gift to the department from Dr Gruenert. This is a T-antigen transformed epithelial cell line derived from PBEC that has been shown to retain differentiated epithelial morphology and functions [Cozens et al 1994].

2.3.1 H292 cell culture

H292 cells were cultured in T75 flasks in RPMI containing 10% FBS (foetal bovine serum), 1% Penicillin/Streptomycin and 1% Glutamine. They were cultured in an incubator at 37°C with 5% CO_2 and air. The medium was changed every two days, until the cells had reached 80-100% confluence at which point they were trypsinised according to the protocol below. The cells were then counted according to the protocol below and plated onto a 24 well plate (Nunc or Co-Star) at a density of $1x10^5$ cells/ml for the exposure studies. The remainder of the cells from the T75 flask were used to maintain a stock culture with a split ratio of 1:100. The cells in 24 well culture trays were cultured in an incubator at 37°C and the medium was changed every two days until the cells reached 90% confluence. At this point they were serum starved using ultraculture together with 1% Penicillin/Streptomycin and 1% Glutamine. 24 hours later the cells were stimulated using the appropriate conditions according to each experiment.

2.3.2 16HBEo$^-$ cell culture

16HBEo$^-$ cells were cultured as above except the medium was MEM (Minimum Essential Medium) containing 10% FBS (foetal bovine serum), 1% Penicillin/Streptomycin and 1% Glutamine. For exposure studies the cells plated in the 24 well Co-Star/NUNC plates were at a density of $1x10^5$ cells/ml as described for H292 cells.

2.3.3 Trypsinisation of confluent cell monolayers

Confluent cell monolayers were passaged by addition of trypsin-EDTA concentrate. A 10x trypsin-EDTA concentrate was diluted to 1x working solution by addition of Hanks balanced salt solution (HBSS) (GibcoRel) which contained no magnesium or calcium salts, and was stored at –20°C until use. Prior to trypsinisation a 1x trypsin-EDTA aliquote was defrosted and warmed to 37°C. The medium from the flask was removed and the cells were 'washed' with an appropriate amount of the x1 trypsin-EDTA (1ml for a T75 flask). This was allowed to stand for 1 minute in the incubator, before being removed. Following this an appropriate amount of the x1 tryspin-EDTA concentrate was added (e.g. 3mls for a T75 flask) and the cells were then re-placed in the incubator for approximately 5 minutes. During this time the cells were removed and the flask manually 'tapped' on the side to aid the detachment of the cells. The cells were observed under a light microscope at regular intervals until they were seen to have detached and were floating in the trypsin-EDTA solution. At this point a minimum of an equivalent volume of appropriate medium such as RPMI was added to the detached cells to neutralise the effects of the trypsin-EDTA solution.

2.3.4 Cryogenic storage and regeneration of cell stocks

To create a stock of cell lines, cells were stored as frozen aliquots in liquid nitrogen. After trypsinisation cells were spun at 150g for 5 minutes in a sigma 3-10 desktop centrifuge and the chilled cell pellet dislodged, and re-suspended in 1 ml of pre-cooled culture medium containing 10% (v/v) of the cryoprotectant dimethylsulphoxide (DMSO) (Sigma). One ml aliquots of the cell suspension were placed in cryotubes, frozen at –80°C for 24 hours followed by long term storage in liquid nitrogen vapour. To regenerate a frozen stock, a cryotube was removed from liquid nitrogen and small aliquots of pre-warmed (37°C) culture medium added. The cell suspension was transferred to an $75cm^3$ flask containing 30mls culture medium and left in the Heraeus incubator (37°C/5% CO_2) for 5

hours to allow cells to adhere to the base of the flask. After this time the medium was replaced with 12 ml of fresh culture medium to remove any remaining traces of DMSO, and cells were cultured as described above.

2.3.5 Cell Counting

On passage, the cell suspension was spun at 150g for 5 minutes and the cell pellet re-suspended in 10mls HBSS (to remove serum which inhibits dye uptake). Cell clumps were carefully disrupted via aspiration using a 2.5 gauge needle attached to a 10ml syringe to yield a single cell suspension. The number of viable cells present in the suspension was determined using the Trypan Blue exclusion technique. 10μl of single cell suspension was mixed with 10 μl of 0.4% (w/v) Trypan Blue. 10μl of this solution was placed in each of the two chambers of a Nebauer Haemocytometer and the number of viable cells present (cells that were not blue) in each chamber were counted. This technique works on the principle that cells do not allow the blue dye to penetrate inside the cell, unless the cell membrane has been damaged. The number of cells/ml was taken by calculating the average no. of cells counted in the two chambers and then multiplying this by x 10^4.

2.4 Exposures

2.4.1 Plating and Treatment of Cells in Culture Plates

24 well plates of epithelial cells were prepared as described in section 2.6. Once 90% confluent, cells were starved in UltraCulture (GibcoRel) for 24 hours before treatment with the appropriate stimulus. Primary bronchial epithelial cells were starved in BEBM containing 1% ITS liquid media supplement (Sigma) and 1% BSA.

Most stimuli were prepared at least one hour in advance, pre-warmed and equilibrated to 37°C/5% CO_2 in the Heraeus incubator. The exception to this were TNF-α, IL-4, and IL-13 which were added to the pre-warmed medium 5 minutes prior to use. Stimuli for primary cells were prepared in the same manner as above using BEBM containing 1% ITS and 1% BSA.

Stimulant	Supplier	Concentrations Used
TNFα	Peprotech	10 ng/ml
Diesel Exhaust Particles	NIST/University of Cardiff	10, 30, 50,100,200 μg/ml,
ROFA	University Edinburgh	50, 200μg/ml
Carbon Black M120	University of Cardiff	200 μg/ml
IL-4	Peprotech	20ng/ml
IL-13	Peprotech	20ng/ml

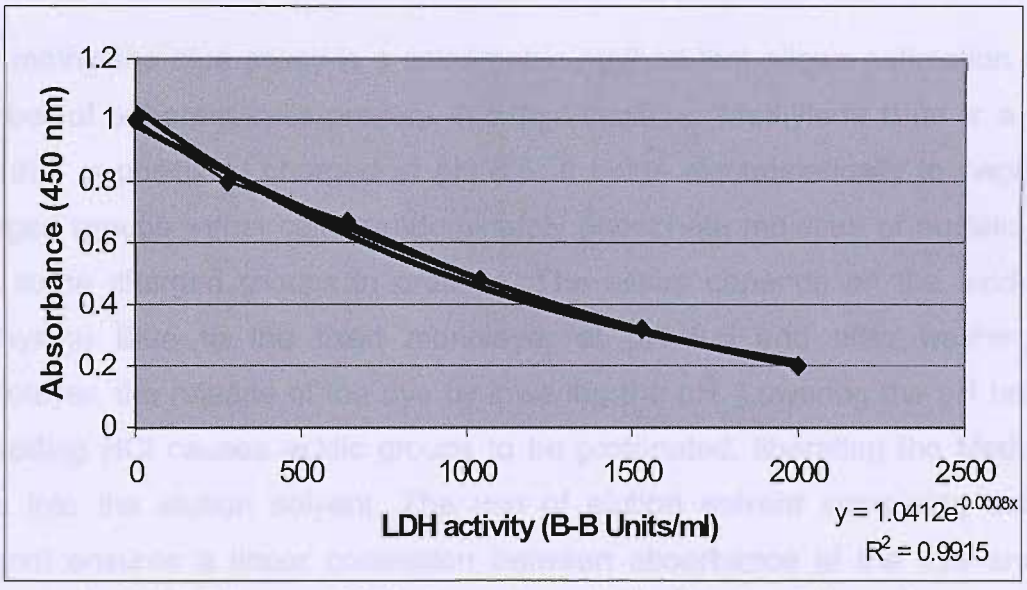
Table 2.2: Stimulants and Concentrations used

After the cells were treated for the appropriate length of time, medium was removed clarified by centrifugation and the supernatant aliquoted so that 100 μl were removed and stored above 4°C for LDH analysis and the rest were stored frozen at –80 °C for subsequent analysis by ELISA. The cell pellet was processed according to its intended use: 1) Fixed in formol saline for determination of cell number. 2) Lysed using 1ml of 1% Triton x100 lysis solution for analysis of total cellular LDH. 3) Solubilized into Trizol for extraction of RNA.

2.5 Lactate Dehydrogenase Assay

LDH was assayed using a kit (Sigma catalogue no. 500) according to the manufacturers instructions. This depends on a colorimetric method for the detection of lactate dehydrogenase (LDH) activity and its ability to catalyse the following reversible reaction: Pyruvic Acid + NADH ←----→ Lactic Acid + NAD.

The reaction equilibrium strongly favours reduction of pyruvate to lactate at a rate proportional to the amount of LDH. Pyruvic acid reacts with 2, 4 – dinitrophenylhydrazine to form an intensely coloured 'hyrazone', which has peak absorbance over the broad wavelength range of 400-550nm. Lactic acid, NAD and NADH do not absorb significantly in this range. Therefore by using pyruvate as substrate, it is possible to accurately measure changes resulting from the conversion of pyruvic acid to lactic acid due to LDH activity. In this assay the amount of pyruvate remaining after incubation is inversely proportional to the amount of LDH activity in the sample. This particular assay measures the total LDH and not specific isoenzymes. A calibration curve for serum LDH was constructed using known amounts of pyruvate, which were known to correspond to a specific amount of LDH activity as measured in BB units/ml. This calibration curve was used to calculate the BB value/ml for a given absorbance at 450nm. These values were then expressed as a percentage compared with values derived from a confluent well of cells from a 24 well plate which had been lysed



using 1ml of 1% Triton x100 detergent in distilled water. The values obtained from this were taken to correspond to total LDH content, and the rest of the values derived from each experiment was expressed as a percentage of this value. The total cell lysis procedure was repeated for each experiment.

Figure 2.1: This shows a calibration curve for LDH activity and absorbance at 450nm. Absorbance of 1.0 was equal to no LDH activity, while absorbance at 0.2 was equal to 2000 B-B units)

I vial of NADH was made up in 1ml of sodium pyruvate (both supplied in the kit), which gives 0.75mM sodium pyruvate and 1.28μM NADH. This solution is unstable and was used immediately.

The assay was performed by placing 100μl of substrate solution into 1 ml disposable cuvettes. 10μl of assay sample (cell supernatant) was added to each cuvette and the solution incubated at 37°C for 30 minutes. After this time, 100μl of colour reagent was (Sigma – part of kit catalogue no 500) added with mixing and the cuvettes were incubated at room temperature for 20 minutes. The reaction was stopped with 1ml 0.4 M NaOH.

Absorbance was read at 450 nm using a spectrophotometer, with the cuvette and all reagents minus LDH as a blank.

2.6 Methylene Blue Assay

The methylene blue assay is a colorimetric method that allows estimation of the number of adherent cells present in a microculture. Methylene Blue is a basic dye that is positively charged at pH 8.5. It binds electrostatically to negatively charged groups within cells, predominately phosphate moieties of nucleic acids and some charged groups in proteins. The assay depends on the binding of Methylene Blue to the fixed monolayer at pH 8.5 and after washing the monolayer, the release of the dye by lowering the pH. Lowering the pH below 2 by adding HCl causes acidic groups to be protonated, liberating the Methylene Blue into the elution solvent. The use of elution solvent containing acidified ethanol ensures a linear correlation between absorbance of the dye and cell number, and enables the assay to be carried out in 96-well plates measuring absorbance with an automated vertical light path microplate photometer. The assay is rapid, reproducible and easy to perform.

At the end of each experiment the medium was removed from the cells which were then fixed with 500μl/well/10% Formol Saline (9g NaCl in 100ml 40%

Formaldehyde and 900ml of Water) for at least 60 minutes. After fixation the plates washed in running water and then blotted dry.

The wells were then stained with 250µl of 1% w/v Methylene Blue in 0.01M borate buffer (3.82g Disodiumtetraborate together with 1% Distilled water) for 30 minutes. The wells were then washed with 0.01M Borate Buffer or running water until no more blue dye was detected in the washing solution, and were then blot dried.

The Methylene Blue in the wells was then eluted using 200µl/well 1:1 (Volume/Volume) ETOH and 0.1M HCL for each of the wells in the 24 well plate.

In the case of cell cultures, the eluted methylene blue solution was then diluted to an appropriate level (typically 1:20) in 1:1 (V/V) ETOH and 0.1M HCL and made into a final volume of 100µl in 24 wells of a 96 well plate before being read at A630 using an ELISA plate reader.

Fig 2.2 Standard Curve: Methylene Blue

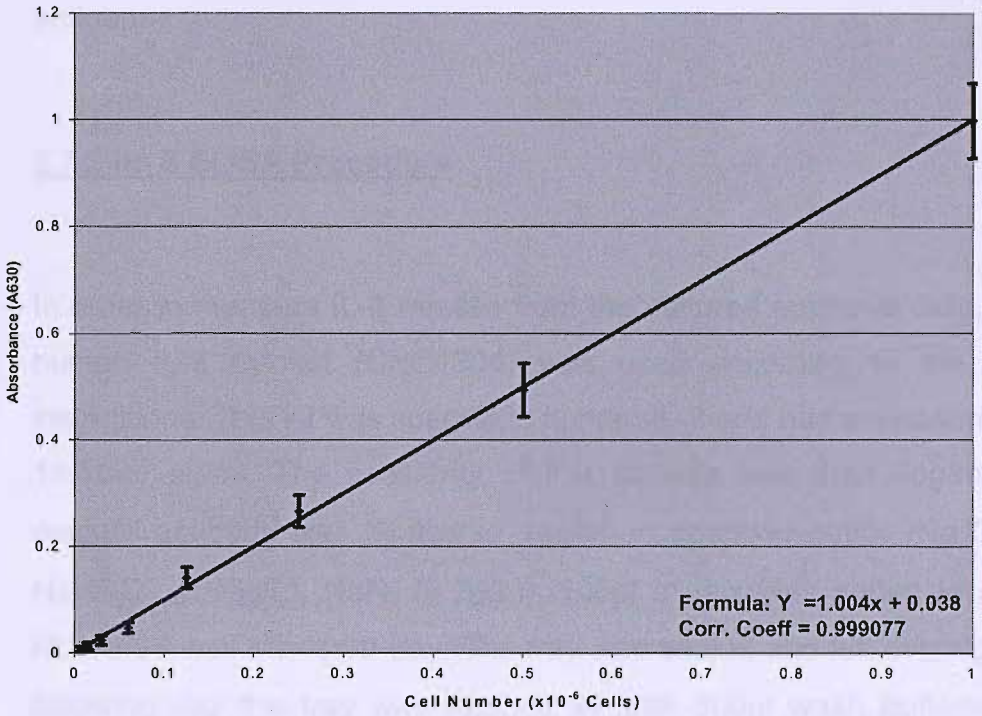


Figure 2.2: This shows a standard curve showing the relationship between cell number and absorbance at 630nm. Absorbance of 1.0 was equivalent to 106 cells/ml. This curve was constructed by using a concentration of 1.0-6 cells/ml (measured by Trypan blue method), which was then diluted 1:2 successively in a 96 well plate to give concentrations of 0.5 x 106, 0.25 x 106 etc. These were then treated with methylene blue and the absorbance measured.

2.7 Enzyme Linked Immunosorbent Assay (ELISA)

2.7.1 Background on ELISA

The enzyme linked immunosorbent assay is a commonly used method for the detection of a wide range of proteins. Wells of microtitre plates are coated with a specific antibody and residual excess binding sites are blocked prior to addition of test samples. If any of the antigen is present in the sample it will bind to the antibody. A second antibody which is often conjugated with an enzyme is added as a 'developing antibody'. The enzyme converts it's substrate resulting in a colour change which is detected spectrophotometrically. The intensity is proportional to the concentration of protein/antibody present. The test is

quantitative as the intensity of the reaction of the sample/medium is compared with a standard curve plotted using a standard with known concentration of antigen.

2.7.2 IL- 8 ELISA Procedure

In order to measure IL-8 release from the cultured epithelial cells, the Biosource human IL-8 cytoset (CHC1304) was used according to the manufacturers instructions. This kit was specific to human IL-8 and had a measurement range of 16-1000 pg/ml. The sensitivity of this kit was less than 5pg/ml. In brief, the capture antibody was diluted to 1μg/ml in coupling buffer (Na_2CO_3, (1.58g/L), $NaHCO_3$ (2.93g/L), NaN_3 (0.2g/L)). 100μl of this was added to each well of a NUNC 96 well Maxisorb tray. The tray was sealed and left overnight at 4°C. The following day the tray was washed x4 with 300μl wash buffer/well (PBS with 0.5ml/L of Tween 20, pH 7.4). Blocking solution (NaCl, (8.0g), Na2HPO4.2H2O, (1.42g), KH2PO4, (0.2g), KCL, (0.2g), BSA fraction V, (5.0g), pH 7.4 in 100 ml dH20) was stored frozen as 5ml aliquots and diluted 1:10 to make a working blocking buffer. 300μl of diluted blocking buffer was added to each well, sealed and left on a rotashaker for 2 hours at room temperature. The plates were then washed x4 with wash buffer, followed by addition of IL-8 standard curve and samples to the wells. The IL-8 standard was prepared by dissolving the stock vial (32ng lyophilised IL-8) in 320 μl of assay buffer (x1 blocking buffer + 0.1% Tween- 20) to give 0.1μg/ml stock. A doubling dilution curve was prepared from 1ng/ml to 15.625 pg/ml. (10μl of 0.1μg/ml stock diluted in 1 ml assay buffer to give 1ng/ml. Serial dilutions were then completed across 6 tubes) 100μl of each standard dilution was added in duplicate to the 96 well tray. Sample medium was added to each well, diluted to a final volume so that the cytokine release was within the linear range of the standard curve.

Following removal of the supernatants and washing of the wells with wash buffer x4, 50μl of biotinylated antibody (diluted to 0.1μg/ml in assay buffer) was then

added to each well. The plate was sealed, placed on a rotashaker at room temperature and left for 2 hours.

The plate was washed x 4 in wash buffer and 100µl of streptavidine-HRP conjugate (diluted 1:2500 in assay buffer) was added to each plate, and incubated for 30 minutes at room temperature.

After a further x4 washes in wash buffer, 100µl of chromagen solution (12 mls chromogen solution (13.6g sodium acetate.$3H_2O$ in 1 litre distilled water, pH to 6.0 with citric acid, and autoclaved), 200µl of TMB (30mg tetra-methyl benzidine in 5 ml DMSO), and 1.2µl 30% H_2O_2 (0.3mls H_2O_2 in 3 mls H_2O) was added to each well, the plate wrapped in foil, placed on the rotashaker and left for 10 minutes. After this the addition of 50µl of 2M H_2SO4 caused the chromagen solution to change from blue to yellow. The absorbance values were read on a microplate spectrophotometer at 450 nm with a 630 nm reference filter.

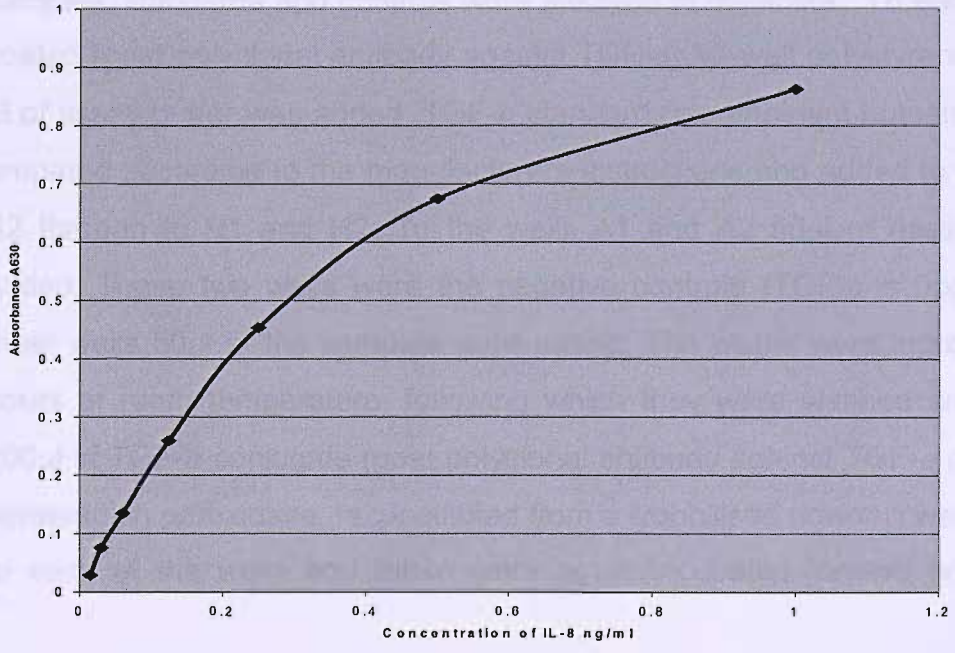


Fig 2.3: This shows a standard curve showing the relationship between IL-8 concentration (ng/ml) and absorbance at 630nm. This curve was constructed using a known concentration of IL-8 according to the manufacturers instructions.

2.8 ELISA for TGFα Assay

In order to perform accurate measurements of TGF-α release from the cultured epithelial cells a commercial TGF-α ELISA kit (Oncogene Research Products, Boston, Mass, USA) was used. This assay employs a quantitative sandwich enzyme immunoassay technique using a polyclonal antibody specific for TGF-*α*, which has already been pre-coated onto 96 well trays and allows measurements of TGF-α in the range 0.55pg/ml-7.10 pg/ml. It is specific for human TGF-α. Standards and samples are pipetted into the wells and any TGF-*α* present is bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-*α* is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TGF-*α* bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

This assay was carried out according to the manufacturers instructions. In brief, the reagents and samples were brought to room temperature before use. All samples, standards and controls were assayed in duplicate. To each of the pre-coated (goat polyclonal antibody against TGF-*α*) 96 well polystyrene plates, 100 μl of assay buffer was added. TGF-*α* standard (recombinant human TGF-*α*) was prepared according to the manufacturers instructions and added to wells B1 and B2 through to H1 and H2. To the wells A1 and A2 50μl of assay buffer was added. These two wells were the negative controls (TGF-*α* = 0pg/ml). To the other wells 50μl of the samples were added. The plates were incubated for two hours at room temperature, following which they were washed and blot dried. 200μl of TGF-*α* conjugate (goat polyclonal antibody against TGF-*α* conjugated to horseradish peroxidase, reconstituted from a lyophilised powder) was then added to each of the wells and these were again incubated for two hours at room

temperature, before being washed. 200μl of substrate solution which was made up from a mixture of chromagen (tetramethylbenzadine) and hydrogen peroxide was then added to the wells and these were incubated for 30 minutes at room temperature. 50μl of stop solution (2N sulphuric acid) was added and the plates were tapped gently to ensure uniform change in colour. Using a microplate reader set at 450nm, with a reference filter correction at 540nm the absorbance of each well was measured.

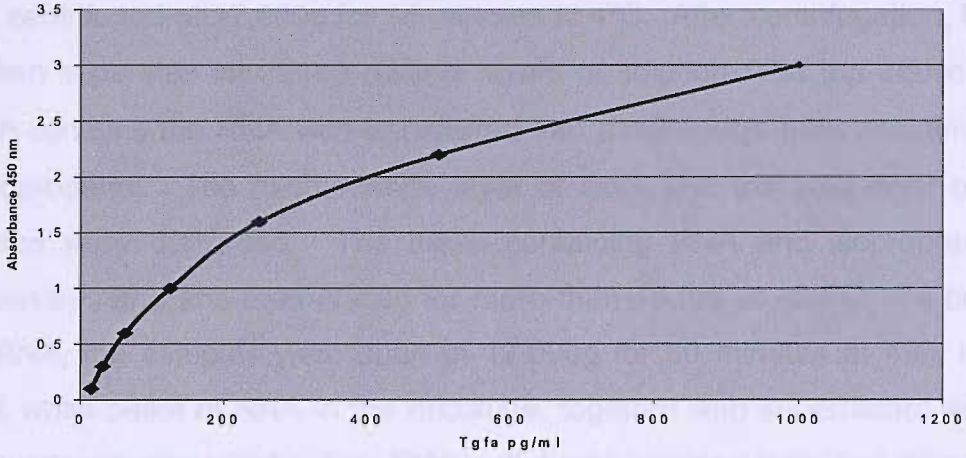


Fig 2.4: This shows a standard curve showing the relationship between TGF-α concentration (pg/ml) and absorbance at 450nm. This curve was constructed using a known concentration of TGF-α according to the manufacturers instructions.

2.9 RNA extraction and analysis

The following work was carried out in a designated area in the laboratory to prevent DNA contamination. In order to further protect against DNA contamination all reagents and tubes used were sterile as well as DNase and RNase free. RNase and DNase free MilliQ quality distilled water (Millipore) and Barrier tips were used throughout the procedures.

2.9.1 RNA Extraction

Epithelial cell cultures were serum starved for 24 hours and then treated with the appropriate stimulus as described in section 2.2. After the supernatant had been removed 0.25ml of Trizol (Invitrogen), a solution of phenol and guanidine-isothiocyanate was added to each of the 24 wells of epithelial cell cultures. This

solution was left on the cells for approximately 5-10 minutes at room temperature to allow the cell components to dissolve and the RNA to be released, before being pipetted up and down and then being transferred to a 1.5ml microfuge tube. These samples were either immediately used for RNA extraction or stored at –80°C until the RNA was extracted.

To each 0.25ml of Trizol, 50μl of chloroform was added following which each tube was shaken vigourously, incubated at room temperature for 5 minutes and then centrifuged at 12,000g for 15 minutes at 4°C. After centrifugation, the Trizol solution separates into three distinct layers of solution. The top aqueous layer, which contains the RNA was transferred into a microfuge tube containing 500μl of isopropanol. The middle white layer of DNA and the pink layer of protein solution were discarded. The tubes containing RNA and isopropranol were shaken by hand and then placed for more than 16 hrs at –20°C. To precipitate the RNA, the samples were spun at 12,000g for 30 minutes at 4°C, leaving a small white pellet of RNA in the micofuge, together with supernatant which was removed and discarded. The RNA pellet was washed with 1ml 75% ethanol. This solution was then spun at 7,500g for 5 minutes at 4°C. The ethanol was carefully removed using an appropriate pipette and the tube was pulsed again before the final traces of liquid were also removed. The remaining pellet was air dried for approximately 10 minutes, which led it to become transparent.

To remove contaminating genomic DNA, the total RNA was treated with DNase solution. This was to ensure that the only DNA present after the reverse transcriptase reaction should be that corresponding to cDNA obtained from the RNA and not contaminating genomic DNA. In order to DNAse treat the following protocol was used:

A master mix was prepared using reagents in the following proportions: 1μl RQ1 DNase1 (Promega), 2μl 10x DNase buffer (Ambion, Austin, USA) and 17μl dH2O per tube of RNA. 20 μl of this mastermix was added to each tube, before being incubated at 37°C for 1 hour. At the end of the incubation period, the tubes were removed and 5μl of neutralisation buffer (Ambion, Austin, USA) was added to

each tube to stop the action of the enzyme. RNA was quantified using agarose gel quantification technique as described below in section 2.9 (ii).

2.9.2 RNA Quantification

The following procedure was carried out in a designated area of the laboratory to reduce the risk of contamination.

The gel tray, tank and combs were washed using RNAse free solution. Once dry, masking tape was stuck to the sides of the gel tray, to prevent leakage of agarose. The agarose solution was made by adding 1.0 g agarose to 130 ml of 1x Tris-Borate-EDTA (TBE) buffer. This solution was then heated in a 750w microwave, until the agarose had visibly dissolved into the TBE buffer. 30µl of ethidium bromide (a solution that chelates into RNA and thus allows visualization of RNA under UV light) was added to the agarose solution. Care was taken to avoid contact with ethidium bromide solution (by using gloves) or spillage on any on the work areas. The gel was then poured into the tray and the appropriate number of combs to the number of samples were inserted. Once the gel was set, the masking tape was removed and the gel placed into the gel apparatus (Biorad), The tray and agarose gel was then covered with sufficient Tris-Borate-EDTA (TBE) solution to cover the gel by at least 5mm.

Either in a separate tube or on an aluminum foil strip 2µl of the sample RNA was mixed with 8µl of RNA loading buffer (30% glycerol, 0.5% Bromophenol Blue, 5mM EDTA and 20mM Tris, pH7.5). This mixture was then loaded onto the gel and the gel was run at 80mAmps (constant) for 30 minutes. The bands were visualised under UV light using the GeneSnap protocol on the Gene Genius Bioimaging system (Syngene). The presence of two clear bands of ribosomal rRNA, (18S and 28S) band, with no smearing confirmed the integrity of the RNA
In order to allow RNA quantification and quality control a standard curve of RNA of known concentration was also run on an agarose gel. Standard RNA was prepared using epithelial RNA that had been quantified and diluted to 0.2µg/µl. The standard curve was prepared by adding 0µl (blank), 1µl (0.2µg), 2µl (0.4µg),

3μl (0.6μg) and 4μl (0.8μg) of standard RNA to RNA loading buffer to give a final volume of 10μl in individual wells.

The RNA was quantified using the GeneTools programme by assigning a track for each individual sample and reading the pixel intensity of bands for both the 18S and 28S bands together. A standard curve was constructed using the standard pixel intensities and sample RNA values were calculated from the gradient and y-intercept.

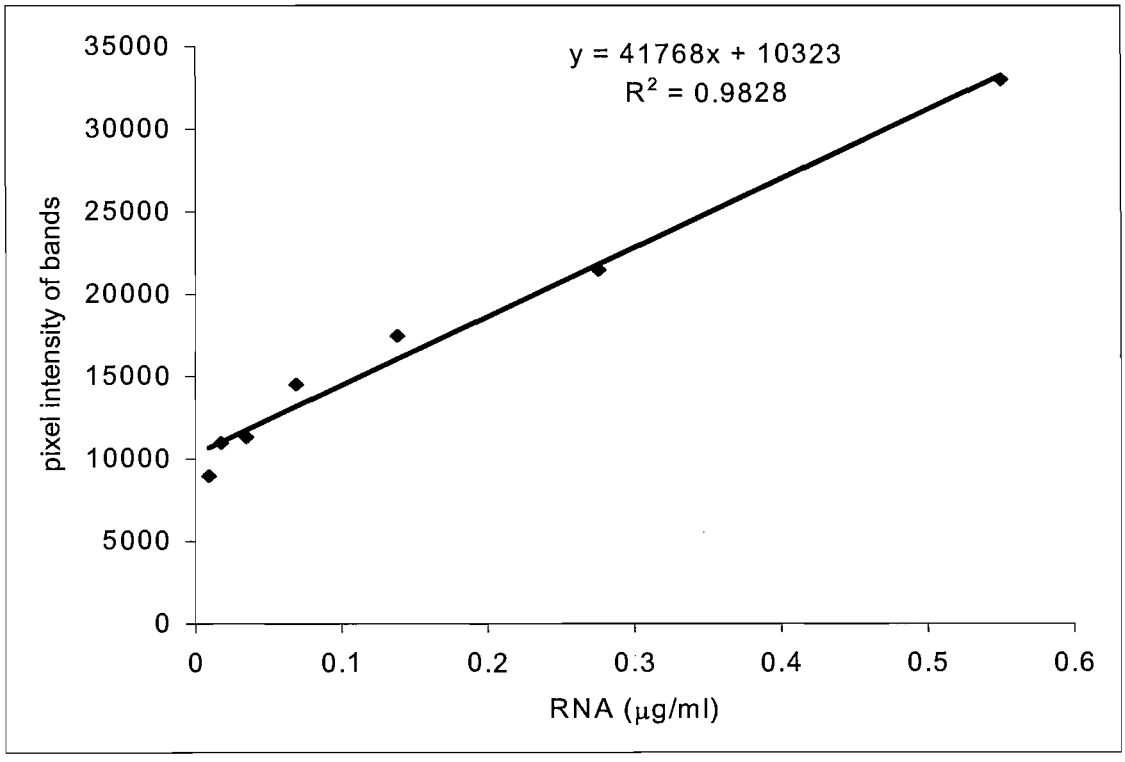


Fig 2.5: This shows a standard curve showing the relationship between RNA (µg/ml) and pixel intensity of bands. This curve was constructed using a known concentration of RNA.

2.9.3 Reverse transcriptase (RT) reaction

In order to carry out Taqman PCR, cDNA is needed. Thus RNA was reverse transcribed to create cDNA for this purpose. This was carried out in sterile 200µl microtubes in the following manner:

Following quantification of RNA, 1µg of RNA was incubated with 1µl random hexamer primers (3µg/ml) (MWG Biotech) and 1µl dNTP (10mM) (Invitrogen). The final volume was made up to 8µl with distilled water. A negative RT control and negative template control were included (no RNA template or no RT enzyme). The RNA samples were incubated for 5 minutes at 85°C to denature the RNA and ensure that it was single stranded. Following this, the tubes were removed from the machine and placed on ice, while a RT master mix was made up for all the tubes. The volume for 1 tube was: 0.5µl M-MLV RT (Promega) enzyme, 4µl 5x RT buffer (Promega) and 5.5µl dH2O. This was multiplied by the

number of tubes.10μl of this RT master mix was added to each well and the final volume of 20μl was incubated for 1 hour at 42°C. The enzyme reaction was then neutalised by a 10 minute incubation at 85°C. The RT samples were diluted 1:10 in DNase free water and then stored at - 20°C.

2.9.4 Taqman Real Time Quantitative PCR

Taqman probes are oligonucleotides of approximately 25 bases that are complementary to a target sequence of cDNA. They also contain a fluorogenic reporter dye FAM (6-carboxyfluorescein) and a quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine). During the annealing stage of PCR the probe binds to a specific region of interest between the forward and reverse primers. During the PCR cycle, Taq-polymerase binds and extends the cDNA. When in contact with the probe the Taq-polymerase enzyme cleaves the FAM from the probe, through 3' exonuclease activity, which causes an increase in fluorescent intensity of the reporter dye due to its separation from the quencher. Therefore during Taqman – PCR, the amount of cDNA of interest is proportional to the rate at which the fluorescence reaches the threshold (CT value). Therefore a value obtained at a lower cycle indicates relatively higher amounts of cDNA.

During the Taqman PCR the samples were controlled using an internal 18S ribosomal RNA standard (this is an endogenous control), which was also run with each sample. Therefore in each tube analysed, results were obtained for the target probe and the 18S rRNA probe. This was later used to normalise the samples during the relative quantification of gene expression.

2.9.5 Taqman Reaction

The Taqman reaction was carried out using 5μl of cDNA template, which was added to 19μl Taqman Master mix and 1μl target primer/probe mix. Taqman Master mix consisted of the following per (19μl) tube.

2.5μl 10x real time PCR buffer (no $MgCl_2$), 5μl $MgCl_2$ (6.6mM), 2μl dNTP (0.26mM), 0.125μl Hot Goldstar Taq enzyme (0.625units), 9μl dH2O and 0.375μl 20x ribosomal 18S rRNA primers and probe mix. The buffer, $MgCl_2$, dNTP and enzyme were purchased from Eurogentec (Seraing, Belgium). The 18S rRNA primer and probe mix was purchased from Applied Biosystems (Foster City, U.S.A.).

2.9.6 Primers and probes

The forward and reverse primers and probes were purchased from MWG biotech or Oswell and optimised according to the Perkin-Elmer protocol. Primers and probes were designed using Primer Express (Perkin-Elmer Biosystems, Warrington, UK) by Dr R. Powell. The primers were designed to span two exons on the gene of interest on the cDNA, such that the primers and probes cannot recognise any genomic DNA, only cDNA transcribed from the mRNA. 22.5μl of forward primers and 22.5μl reverse primers were added to an appropriate volume of probe to give a final concentration of 6.25pmol/μl. The primer/probe mix was made up to 100μl with dH_2O. 1μl of this stock gives a final concentration of 250nM probe and 900nM forward and reverse primers.

Target Gene	Forward	Reverse	Probe(5' FAM & 3'TAMRA)
AR	gtggtgctgtccttgatac	gcttcccagagtaggtgtcattg	trccaatccagcagcataatggcctga
HB-EGF	gatctggacctttgagagtcactt	tcccgtgctcctccttgtt	agccacacaagcactggccacacca
TGF-α	ctagttggttctgggctttgatct	tggttttgggcatttgagtca	ttccaacctgcccagtcacagaagg
IL-8	aaggaaccatctcactgtgtgtaaac	ttagcactccttggcaaaactg	ctgccaagagagccacggccag

Table 2.3: Primers and probes used for semiquantitative RT-PCR

2.9.7 C-DNA amplification

The Taq polymerase was activated on heating to 95°C for 10 minutes. The amplification then consisted of 40 cycles of denaturation at 95°C for 15 seconds followed by an annealing/extension phase for 1 minute at 60°C.

2.9.8 Analysis of Taqman PCR data: The Delta Delta CT Method

The computer software plotted an emission intensity verses time (cycle number) graph. The computer programme automatically set a threshold for both the FAM and VIC graphs.

Optimisation of primers and probe concentration was carried out by Dr Robert Powell (University of Southampton) using a known concentration of cDNA. cDNA (5ng/ml) mix was added to a fresh eppendorf container and serial 10-fold dilutions (with RNase free water) were conducted to produce a dilution series ranging from 5ng/μl – 5pg/μl. Primers were supplied at 100 μM. 35 μl of sense and antisense primer were diluted 1:10 (in RNase free water). In order to determine the optimum concentration ratio, different sense to antisense primer concentration ratios were investigated and representative Taqman traces were obtained for each of these different ratios (e.g. 50nM:50nM, 300nM:300nM, and 900nM:900nM). Once the primers had been tested and shown to be efficient, then the Delta Delta CT Method was be used to quantify gene expression.

The Delta Delta CT method relies on the use of an internal 18S rRNA control for the target gene being tested. This allows relative comparison of the expression of a specific gene for different stimuli. Using this method, for every given stimulus, the amount of target gene (e.g. IL-8) expression is normalised using internal 18S rRNA. Normalised values are thus obtained for all conditions (e.g. serum free medium, diesel 50, diesel 100 etc) in any given experiment which can then be compared to give an indication of relative gene expression. This can then be done by comparing the normalised values to a particular reference (e.g. serum free medium) to give a relative change in gene expression in response to the stimulus (e.g. diesel 50 or diesel 100).

The principle behind this technique is summarised below. Using a known concentration of cDNA a dilution series of cDNA is initially used to set up a standard curve. The corresponding CT values for the various concentrations of the standard curve are obtained from the amplification plots using Taqman PCR.

In order to determine the relative amount of gene expressed the calculations shown below were carried out.

When expressed as a log value, the standard curves exhibit linear proportionality and can be represented by the general equation: y=mx +c, where in this case:

y=CT value
m=gradient of the standard line
c= ordinal intercept,

Knowing the original amount of cDNA (e.g 25ng), using the above equation and the CT values the log cDNA and thus the antilog cDNA value for both the 18S rRNA and tested primer (e.g. IL-8) can be determined. The value for the tested primer is then normalised to the 18S rRNA, which controls or the quality of the RNA across different samples. The antilog value gives the relative dilution of the specific sample tested compared with the initial measured sample. This is first measured for the 18S rRNA (e.g. 0.7 dilution of 25 ng cDNA), therefore giving a specific amount of cDNA (e.g if the initial amount of undiluted cDNA was 25 ng, then 0.7 of 25ng=(0.7x25ng=17.5 ng cDNA). The same steps are then repeated to determine the amount of cDNA (ng) for the primers being tested (e.g. IL-8). Once the calculations are made then a relative dilution value is obtained For example if there is 0.03 dilution of undiluted sample, this gives 0.03x25ng=0.75ng cDNA of the tested primer. Thus normalised amount of tested primer (e.g. IL-8) to 18S rRNA= cDNA (ng) for tested primer (e.g. IL-8)/ cDNA (ng) for 18S rRNA = 0.7/17.5=0.04.

y-c/m=x

The following formula was used: x=2^(-T19), where x was the difference between the Fam and Vic values for each template, which had itself been controlled to a designated gene (usually baseline gene) activity. This thus provided quantification of the relative induction of gene activity compared to a particular gene (usually baseline)

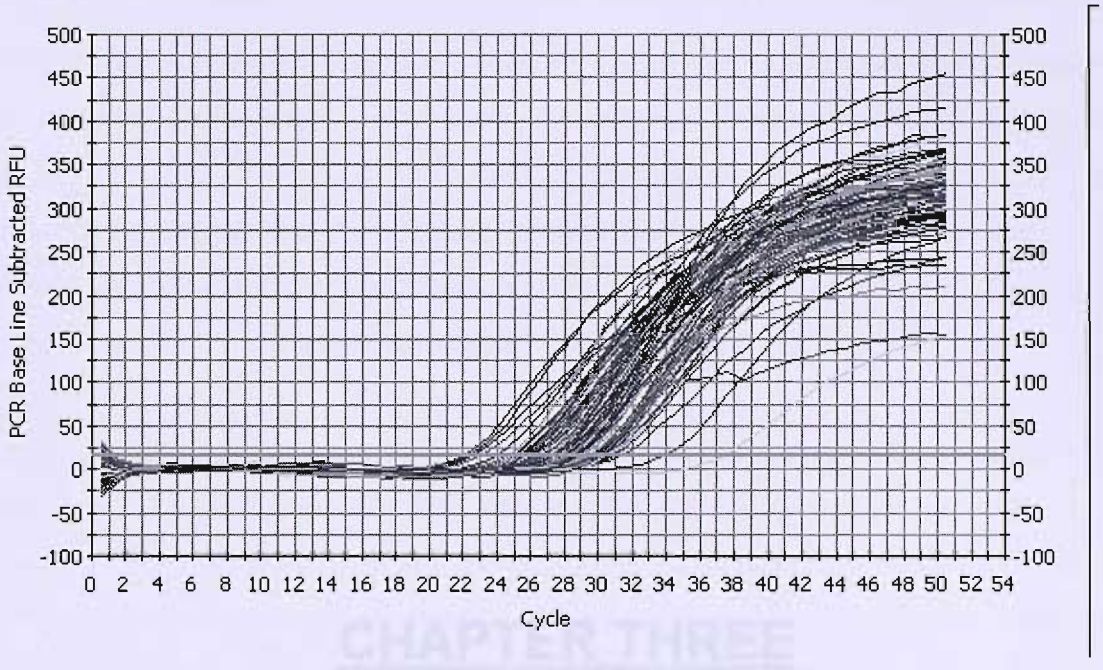


Figure 2.6 shows a typical Taqman PCR trace. The CT value is determined from the point at which the curve rises above baseline. This is then used to calculate gene expression.

2.10 Statistics

For all experiments using H292 and 16HBE cell lines and primary bronchial epithelial cells a non-parametric test using a Kruskal Wallis test followed by a Wilcoxon Signed Rank Test or Mann Whitmey U Test was performed. SPSS 10 was used for all statistical analysis.

CHAPTER THREE

VALIDATION OF EXPERIMENTAL SYSTEMS

Characterisation of the response of bronchial epithelial cells to diesel exhaust particles

3.1 Introduction

Despite much research into the acute effects of DEP exposure, many questions still remain concerning the relevance of exposure levels and whether exposure to air pollutants such as DEP may lead to the development of chronic asthma in genetically susceptible humans. It is also not understood whether the health effects of diesel exhaust particles are due to the adsorbed compounds on the surface of DEP, the carbon core or combination of the two.

DEP are not a single fixed particle type but rather a particle whose composition and hence biological activity may vary depending on many factors including the fuel source, other environmental factors including pollutants and allergens as well as time elapsed since combustion [Sjogren et al 1996, Ullman 1989, van Zijverden 2001]]. Thus, the composition of DEP may vary considerably when examined from different sources depending on factors such as the fuel source, engine type, duty cycle, operating conditions such as the load and speed, lubricating oil used and whether emission control systems are used. In one recent study it was demonstrated that responses of cultured airway epithelial cells treated with diesel exhaust varies with engine load [Madden et al 2003]. In addition once diesel emissions leave the exhaust pipe, they are also affected by a complex set of processes including atmospheric transport and chemical transformation [Cohen et al, 1999, Winer et al 1995]. These interactions affect their toxic, mutagenic and carcinogenic activity. There is also a difference between 'fresh' DEP, which have not undergone atmospheric transformation and 'aged' DEP particles, which have lost some of the materials adhered onto their surface such as volatile organic compounds.

One of the main areas of concern are the many volatile organic compounds which adhere to the surface of DEP, and are rapidly lost after diesel exhaust is released into the atmosphere. It is currently not understood whether the health effects of diesel exhaust particles are only due to the volatile organic compounds, or whether adsorbed substances on the surface of DEP, the carbon core or a combination of these factors also contribute. Therefore, in this first part of the study, the effects of several sources (Welsh, Japanese, USA) and ages of DEP

('fresh frozen' vs. 'aged') were studied on bronchial epithelial cells to elucidate the potential mechanism leading to the observed effects of DEP.

Another area of variation during *in vitro* studies is the cell type used for study. Commonly, tumour derived or virally transformed human bronchial epithelial cell lines are used. These cells are considered more robust and technically easier to use than primary bronchial epithelial cells (PBEC), which are obtained from human volunteers, and are considered to lead to more reproducible results than experiments using PBEC. Although, PBEC are more likely to reflect human responses to a given stimulus than tumour derived or virally transformed cells, they are obtained from different individuals and this often leads to a greater degree of variation in measured responses.

The aim of this chapter is thus to investigate and validate the optimal experimental conditions required for the study of the mechanistic effects of DEP on bronchial epithelial cells. Therefore, in these series of experiments the activities of several sources and 'ages' of DEP on different bronchial epithelial cell types are investigated. In particular it is aimed to study:

1) The effects of DEP from three different sources and two different 'ages' on bronchial epithelial cell number, and cell viability.
2) The effects of DEP on pro-inflammatory responses as measured by the release of IL-8 from bronchial epithelial cells.
3) To compare the effects of DEP with ROFA on cell number, viability and pro-inflammatory responses.

3.1.1 Types of particles used in the studies

a) Diesel Exhaust Particles (DEP)

As described in chapter two, DEP from three different sources (Japanese, Welsh, USA) were obtained and their effects on human respiratory epithelial cells were studied. As discussed above and in chapter one the composition of diesel exhaust particles vary depending on many factors including the fuel type and atmospheric conditions where the particles have been generated. Thus in this chapter DEP from different sources were studied to determine their effects.

b) M120 – Carbon Black

M120 (carbon black), a neutral carbon particle without any transition metal, or volatile organic constituents was used as a negative particulate control in these experiments. This would allow determination of whether the observed effects of the DEP were due to the carbon core of the particulates or the adsorbed substances.

c) Residual Oil Fly Ash (ROFA)

Residual Oil Fly ash (ROFA) is an industrial pollutant that contains metals, acids, and other materials complexed to a particulate core. A majority of the *in vitro* and animal model investigations support the postulate that transition metals present in ROFA (especially vanadium) participate in chemical reactions to produce reactive oxygen species [MacNee et al 1999]. This is associated with tyrosine phosphorylation, nuclear factor kappa B and other transcription factor activation, induction of inflammatory mediator expression, and inflammatory lung injury. The properties of this emission source particle make it particularly useful as a positive particulate control for ambient air particulate matter in studies of biologic effects and therefore, in this chapter this particle type is used largely as a positive control for the study of the effects of DEP on bronchial epithelial cells.

3.1.2 Methanol control used in the studies

As described in section 2.1 in order to ensure that DEP is removed from the collection filters a concentration of 0.007% methanol was used during the removal stage of DEP from the filters. In the following experiments separate controls were used using this concentration of methanol compared with serum free medium. In all the experiments this concentration of methanol led to similar results as serum free medium.

3.2 Results

3.2.1 The Effect of DEP on Epithelial Cell Number in H292 cells

The quantity of cytokines measured in biological assays is dependent on active production from the cells and is therefore related to final cell number. Therefore, during *in vitro* studies any reduction in cell number will alter the absolute quantity of cytokines measured. Exposure to particulates may alter overall cell number by affecting cell proliferation or by causing cytotoxicity and/or apoptosis. The aim of these experiments was i) to determine the effect of different sources of DEP on cell number and ii) to determine whether any observed effects are mediated by the carbon core or adsorbed compounds on the surface of DEP and iii) to determine the most suitable source and concentration of particle that will not significantly alter cell number for further study of the effects of DEP on bronchial epithelial cells.

'Aged' DEP (collected greater than 6 months previously and stored under room conditions) from three sources, Wales, USA and Japan as well as 'fresh frozen' Welsh DEP were studied following 24 and 48 hrs exposure. Fresh frozen Welsh DEP was studied to determine the effect of time on the biological effects of DEP. This type of DEP was frozen following collection and then stored at –20˚C and used within two weeks. The effect of this type of particle was used in comparison with 'aged' particles, as an indicator of the effects of time. Methylene blue, a colorimetric assay allowing estimation of the number of adherent cells present in

microculture was used to determine final cell number in H292 Cells. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon test (SPSS software).

Initial studies were performed using NCI H292 cells, which are an established cell line frequently used for studies of bronchial epithelial cell function.

Untreated H292 cell number increased x 2.0 from baseline in the first 24 hours (fig 3.1a). In the second 24-hour period this was reduced to x1.5 (from 2.0 at 24 hours to 3.0 at 48 hours of baseline) of the 24-hour levels (fig 3.1), suggesting that possibly due to contact inhibition, increased cell confluence has an inhibitory effect on the rate of H292 cell proliferation.

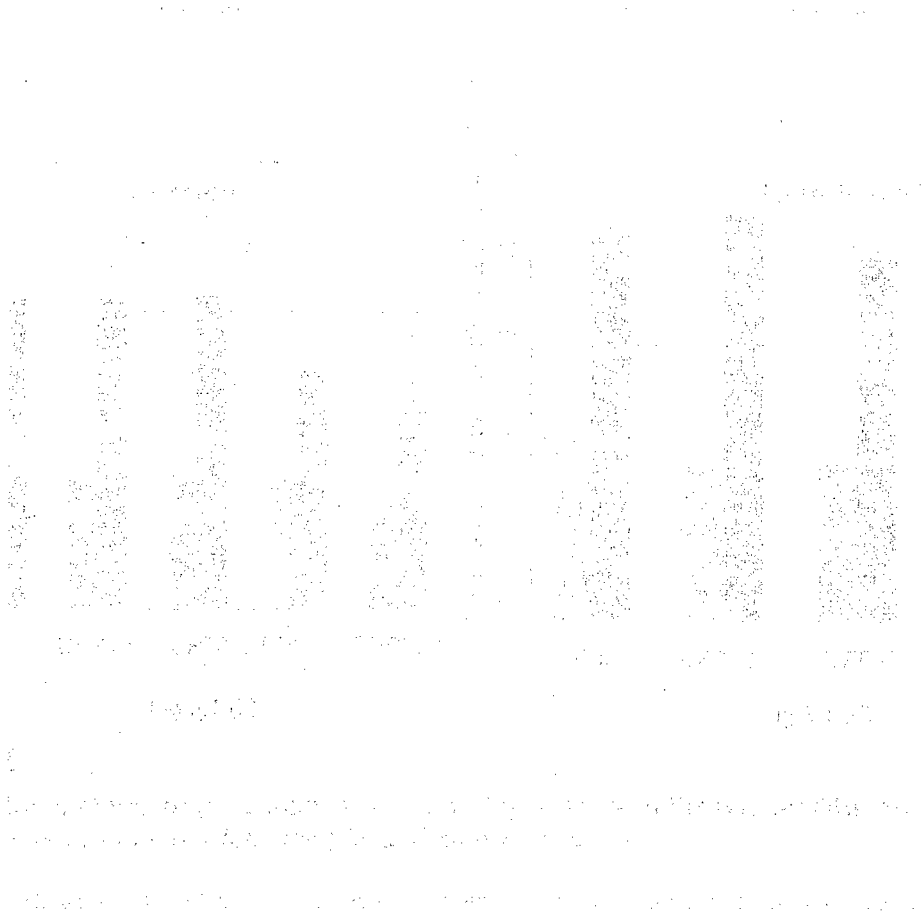
In response to Welsh DEP, there was no significant suppression of cell number at 24 hrs with DEP concentration from 10-200μg/ml (fig 3.1a). Following 48 hours, exposure to Welsh DEP 10μg/ml led to a significant increase in cell number from 310 ± 27% baseline to 383 ± 43% baseline n=8 (p=0.006) (fig 3.1a). It is possible that this could have taken place as a defence mechanism to counteract the potentially toxic effects of the particles on the cell lines by increasing proliferation. At concentrations of DEP 50 μg/ml and above there was no significant reduction in cell number (fig 3.1a).

As with Welsh DEP, 24 hour and 48 hour exposure to Japanese or USA DEP 10-200μg/ml did not lead to a significant reduction in cell number (n=8) (fig 3.1b and 3.1c).

In order to study the effects of 'aging' on the ability of DEP to affect H292 cell number, Welsh DEP, collected and fresh frozen at –20° C was used on cell cultures at two weeks. Results obtained from this two-week-old frozen Welsh DEP were very similar to the 'Aged' Welsh DEP. 24 & 48 hour exposure to two-

week-old frozen Welsh DEP did not lead to a significant reduction in cell number at 10- 200μg/ml (n=8) (fig 3.1d).

Therefore in summary, at 24 and 48 hours DEP did not lead to a statistically significant reduction in cell number at 10-200μg/ml. This effect was similar for DEP tested from all four sources. These data would also suggest that DEP in the concentration range 50-200μg/ml is suitable for investigating the effects of DEP on epithelial cells, as they do not alter cell number. However, these data does not indicate whether DEP alters cell viability, which could also alter final cell responses.



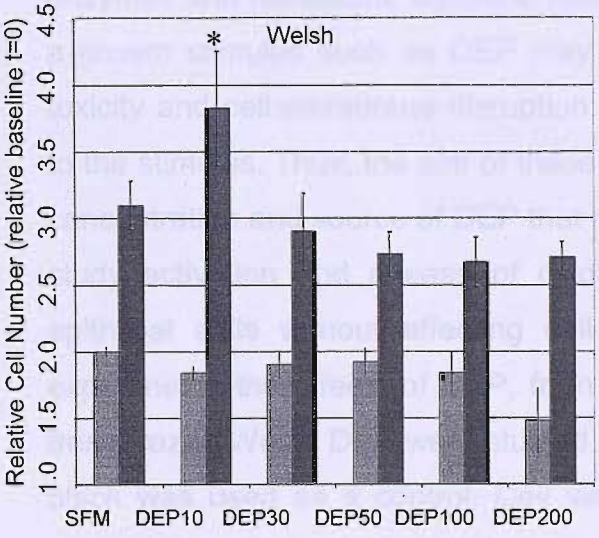


Fig 3.1 (a)

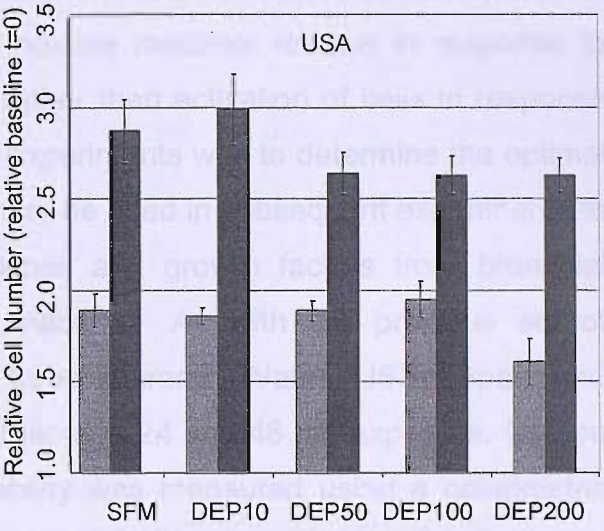


Fig 3.1 (c)

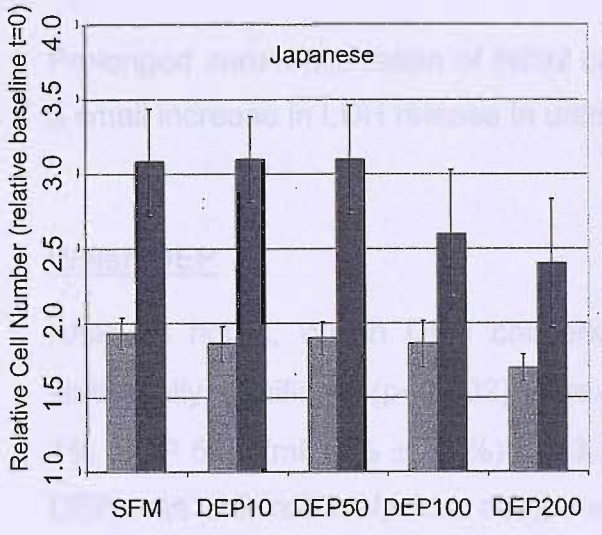


Fig 3.1 (b)

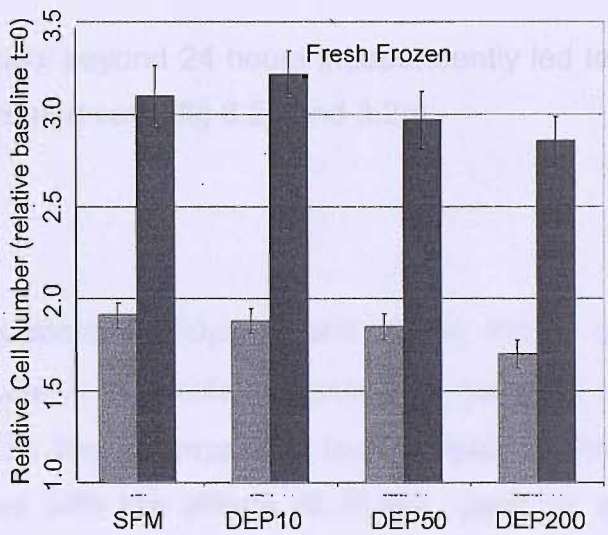


Fig 3.1 (d)

Fig3.1

The Effect of Welsh (a), Japanese (b), USA (c) and 'Fresh Frozen' (d) DEP on H292 epithelial cell number following 24 and 48 hrs exposure

H292 cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh, Japanese & USA DEP for 24 hours. Cell number was measured using methylene blue and corrected relative to cell number at t=0 (baseline). Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

Key: SFM=serum free medium, DEP = diesel exhaust particles (μg/ml)

* P=0.006 (data not significant unless indicated) (n=8)

24 hour

48 hour

3.2.2 The effect of DEP on H292 epithelial cell viability

Toxic stimuli often lead to cell damage and disruption to the cell membrane which may lead to a reduction in final cell number as well as the release of intracellular enzymes and mediators. Cytokine release in response to toxic concentrations of a known stimulus such as DEP may indicate mediator release in response to toxicity and cell membrane disruption rather than activation of cells in response to the stimulus. Thus, the aim of these experiments was to determine the optimal concentration and source of DEP that may be used in subsequent experiments to study activation and release of cytokines and growth factors from bronchial epithelial cells without affecting cell viability. As with the previous set of experiments the effects of DEP, from three sources, (Wales, USA, Japan) and fresh frozen Welsh DEP were studied following 24 and 48 hrs exposure. Carbon black was used as a control. Cell viability was measured using a colorimetric method to estimate lactate dehydrogenase (LDH) release from cells. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon test (SPSS software).

Prolonged serum starvation of H292 cells beyond 24 hours independently led to a small increase in LDH release in untreated cells (fig 3.2b and 3.2c).

Welsh DEP

After 24 hours, Welsh DEP concentrations of 50μg/ml and above led to a statistically significant ($p<0.002$) increase in LDH release (untreated cells 5% ± 1%, DEP 50μg/ml 10% ± 1.2%) (fig 3.2). The maximal LDH level measured with DEP was still relatively low compared with the effects of ROFA, used as a positive particulate control (DEP 200μg/ml = 10% ± 1.6%, ROFA 200μg/ml = 77% ± 2%, n=10(data not shown)). At 48 hours, there was a significant increase (13%) in LDH at concentrations of DEP 10μg/ml -200μg/ml ($p<0.02$), n=10), although again these are relatively low (fig 3.2).

Japanese DEP

At 24 hrs Japanese DEP also led to a dose dependent increase in LDH release from 3% ± 0.3% to 12% ± 2% (DEP 100μg/ml) and 22.5% ± 4% (DEP 200μg/ml) ($p<0.001$), n=10 (fig3.2b). This effect was also observed at 48hrs with DEP concentration 100μg/ml and above leading to significant toxicity ($p<0.001$), n=10) (fig 3.2b). With doses of DEP 200μg/ml there was an increase in toxicity from 22.5% ± 4 % at 24 hours up to 35% ± 8% at 48 hours (fig 3.2b). However there was comparatively greater toxicity at 24hrs than at 48hrs. At 24 hrs there was a x7 increase in LDH levels associated with 200μg/ml DEP compared with untreated cells, whereas at 48hrs this was reduced to x3.5 (200μg/ml), indicating that the greatest toxic effects take place in the first 24hrs and that much of the extra cell toxicity beyond 24 hrs occurs as a result of prolonged culture of confluent H292 cells at 48 hours rather than a specific effect of the DEP.

USA DEP

At 24 hours, there was significant H292 cell toxicity associated with the higher concentrations of USA DEP from 4% ± 0.8%, to 11% ± 0.8% (100μg/ml), and 16% ± 1.6 % (200μg/ml) ($p<0.001$), n=10 (fig 3.2c). This effect continued at 48hrs with toxicity increasing to 21% ± 1.2 % (DEP200μg/ml) ($p<0.001$) n=10) (fig 3.2c). As with previous experiments prolonged cell culture at 48 hrs itself led to a modest but significant toxicity (x2) in untreated cells compared to 24hrs.

Fresh Frozen DEP

As with the experiments carried out with other types of DEP, the higher concentrations of 'fresh frozen' DEP led to significant LDH release, 5% ± 0.6% (baseline) versus, 11% ± 0.5 % at 100μg/ml and 16% ± 1% at 200μg/ml ($p<0.001$) (n=10, fig 3.2d). This was approximately equal to the effect seen with 'aged' Welsh DEP. Following 48 hours of exposure to 'fresh frozen' Welsh DEP there was also increased toxicity at concentrations greater than 50μg/ml,

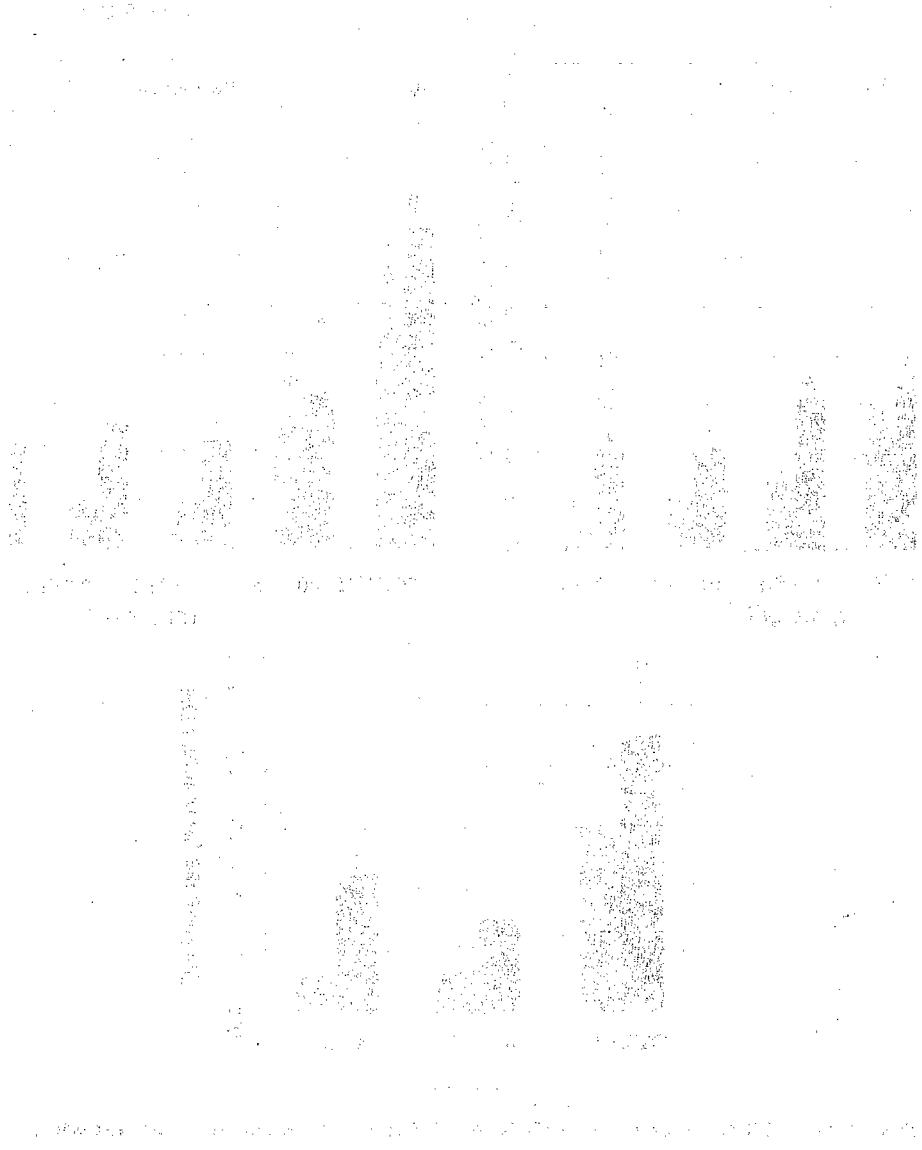
(DEP50μg/ml = 15.5 ± 0.5, DEP100μg/ml = 17 ± 1.0, DEP200μg/ml= 21% ± 1.5%, ($p<0.001$) (fig 3.8) however there was also an increase in baseline toxicity from 5% ± 0.6%, at 24 hours to 10% ± 0.6% at 48 hours (fig 3.8), which was similar to 'aged' particles, indicating that at this time point the increased toxicity was largely due to the cells being cultured for longer periods.

This study shows that prolonged culture of H292 cells beyond 24hrs leads to a modest increase in toxicity in H292 cells independent of any other stimulus, and that DEP have further cytotoxic effects on H292 cells. Depending on their source the three 'aged' DEP tested had a differential effect (Japan> USA> Wales) on LDH release from H292 cells. Japanese DEP (maximal toxicity 35% at 48 hrs) was far more toxic than DEP from Wales and the USA, which had relatively small effects on cell toxicity.

Fresh frozen Welsh DEP led to similar results to 'aged' Welsh DEP, suggesting that DEP collected and fresh frozen at –20°C has similar results to those stored under room conditions for many years. It may probably be that under these conditions, volatile organic compounds found on DEP may have been lost prior to the freezing of the DEP samples as they were being collected or even possibly even lost after they were frozen at –20°C. At any rate, the data obtained from this series of experiments does not demonstrate increased toxicity in association with this type of 'fresh' DEP. An improved method to test the effects of 'fresh' versus 'aged' DEP, which lay outside the limitations of the facilities available in our laboratory, would be needed to further investigate this type of DEP. As carbon black did not lead to an increase in LDH release compared to untreated cells in H292 cells at 24 or 48hrs (figs 3.2e), these results indicate that the toxic effects of DEP on H292 bronchial epithelial cells are due to the adsorbed compounds on their surface rather than the carbon core.

These experiments have demonstrated that the majority of the toxic effects of DEP on H292 cells take place in the first 24 hours following exposure and that DEP at concentrations greater than 50μg/ml has a toxic effect on H292 cells. The toxic effects of DEP also depend on the conditions under which DEP has been

collected. The Japanese DEP that was used in these experiments had been collected following the combustion of diesel that was relatively rich in lead compared to DEP collected from Wales and the USA. In these experiments Welsh DEP was least cytotoxic, and therefore this type of DEP was selected for future experiments into the effects of DEP on bronchial epithelial cells.



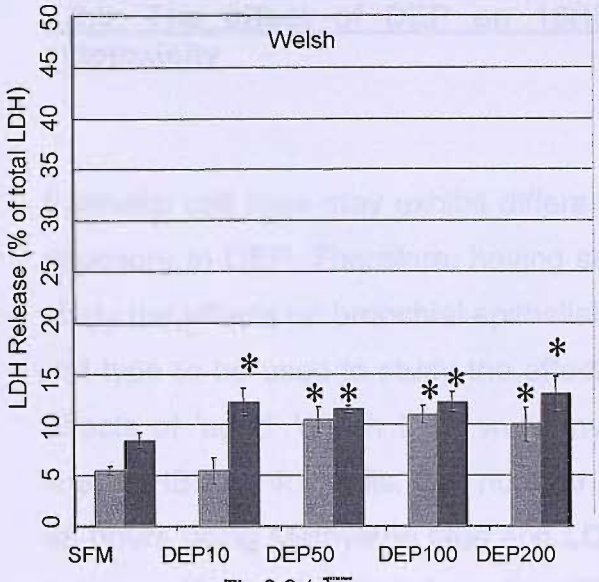


Fig 3.2 (a)

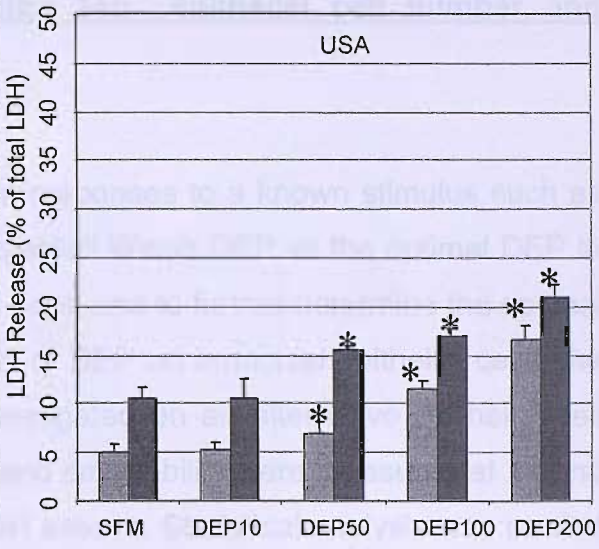


Fig 3.2 (c)

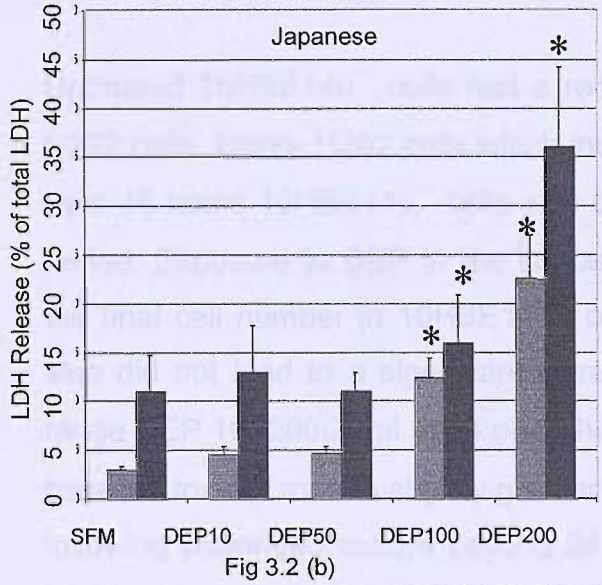


Fig 3.2 (b)

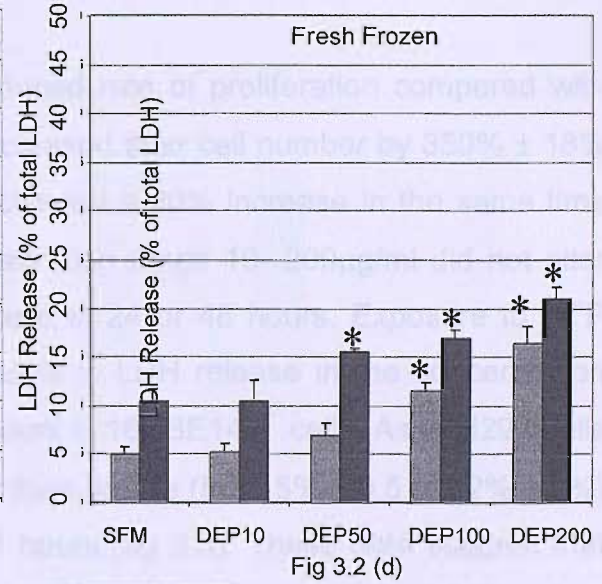


Fig 3.2 (d)

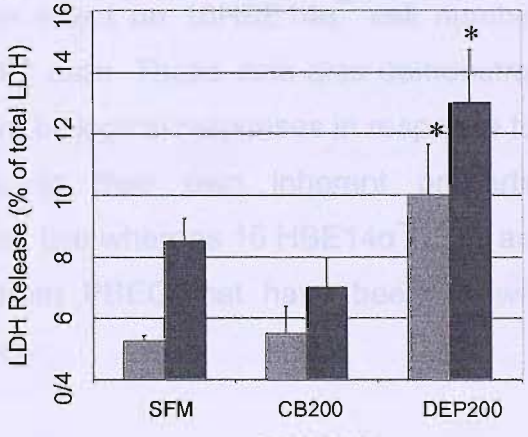


Fig 3.2 (e)

Fig 3.2

The Effect of Welsh (a), Japanese (b), USA (c) and Fresh Frozen (d) DEP and carbon black (e) on H292 cell viability following 24 & 48 hrs exposure

H292 cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh, Japanese & USA DEP for 24 hours. LDH release was measured and expressed relative to total LDH release. Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

24 hour

48 hour

Key: SFM=serum free medium, CB = carbon black (μg/ml) DEP = diesel exhaust particles (μg/ml) * P<0.002 (data not significant unless indicated) (n=10)

3.2.3: The effect of DEP on 16HBEo$^-$14o$^-$ epithelial cell number and cytotoxicity

Epithelial cell lines may exhibit different responses to a known stimulus such as exposure to DEP. Therefore, having selected Welsh DEP as the optimal DEP to study the effects on bronchial epithelial cells, and to further determine the optimal cell type to be used to study the effects of DEP on bronchial epithelial cells, the effects of 'aged' Welsh DEP were investigated on an alternative epithelial cell line; 16HBEo$^-$14o$^-$ cells. Cell number and cell viability were measured at 24 and 48 hours using Methylene blue and LDH assays. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon test (SPSS software).

Untreated 16HBE14o$^-$ cells had a reduced rate of proliferation compared with H292 cells. Unlike H292 cells which increased their cell number by 350% ± 18% over 48 hours 16HBE14o$^-$ cells only showed a 20% increase in the same time period. Exposure to DEP in the concentration range 10- 200μg/ml did not alter the final cell number in 16HBE14o$^-$ cells at 24 or 48 hours. Exposure to DEP also did not lead to a significant increase in LDH release in the concentration range DEP 10- 200μg/ml at 24 or 48 hours in 16HBE14o$^-$ cells. As in H292 cells baseline toxicity rose to slightly greater than double (from 5% ± 0.5 to 12% ± 3%) following prolonged culture beyond 24 hours (fig 3.3). These data suggest that DEP has a smaller effect on 16HBE14o$^-$ cell number and cell viability when compared with H292 cells. These data also demonstrate that different cell lines may exhibit different biological responses in response to a given stimulus. This is likely to be due to their own inherent properties. H292 cells are a mucoepidermoid cell line whereas 16 HBE14o$^-$ cells are a transformed epithelial cell line derived from PBEC that have been shown to retain differentiated epithelial morphology.

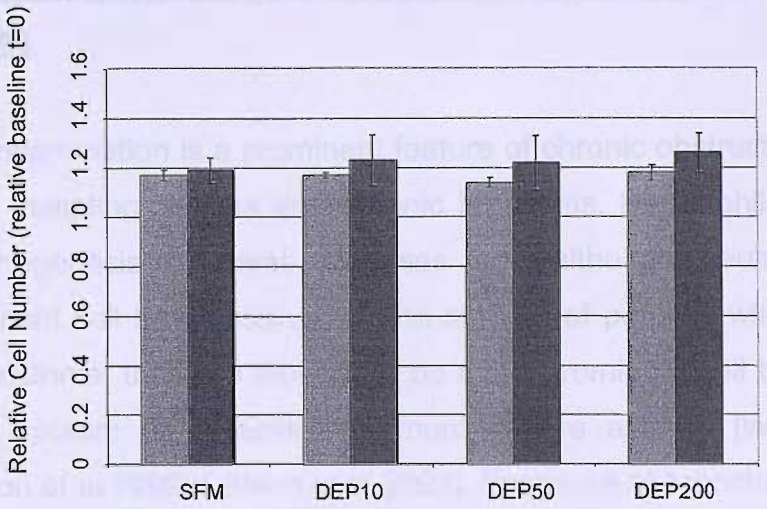


Fig 3.3 (a)

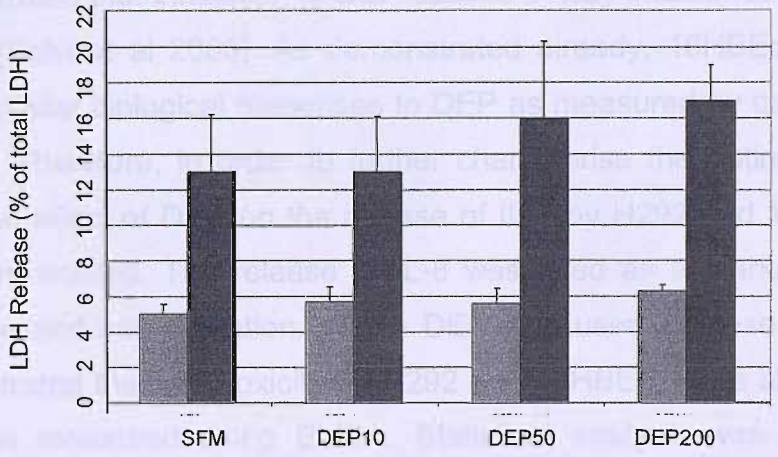


Fig 3.3 (b)

Fig 3.3

The Effect of Welsh DEP on 16HBE cell number (a) and viability (b) following 24 & 48 hrs exposure

16HBE cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. Cell number was expressed relative to cell number at t=0 (baseline0. LDH release was measured and expressed relative to total LDH release. Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

Key: SFM=serum free medium, DEP = diesel exhaust particles (μg/ml)

(n=8). (data not significant unless indicated).

24 hour

48 hour

3.2.4: The Effect of DEP on IL-8 Release from H292 and 16HBEo$^-$ Epithelial Cell Lines

Airway inflammation is a prominent feature of chronic obstructive diseases of the airways, including asthma and chronic bronchitis. Neutrophils are implicated in the pathogenesis of these diseases and although neutrophils are not a predominant cell type observed in the airways of patients with mild-to-moderate chronic asthma, they are thought to be more prominent cell type in airways and induced sputum of patients with more severe asthma [Wenzel et al 1997, Jatakanon et al 1999, Gibson et al 2001]. Exposure of bronchial epithelial cells to DEP has been shown to lead to the release of pro-inflammatory cytokines including IL-8, a potent neutrophil chemoattractant. Human *in vivo* studies have demonstrated that inhalation of DEP causes airway inflammation and the release of IL-8 [Salvi et al 2000]. As demonstrated already, 16HBEo$^-$ and H292 cells exhibit similar biological responses to DEP as measured by cell number and cell viability. Therefore, in order to further characterise the optimal cell type to be used, the effect of DEP on the release of IL-8 by H292 and 16HBEo$^-$ epithelial cells was studied. The release of IL-8 was used as a marker of inflammatory response and cell activation. Welsh DEP was used in these experiments as it demonstrated the least toxicity on H292 and 16HBEo$^-$ cells at 24 and 48 hours. IL-8 was measured using ELISA. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon test (SPSS software).

DEP did not lead to significantly increased release of IL-8 from either H292 or 16HBEo$^-$ cells compared with serum free medium at 24 or 48 hrs. In contrast, stimulation with TNFα led to a doubling of IL-8 release from these epithelial cells in relation to serum free medium (H292 24hr untreated cells = 5000 pg/ml $\pm$ 500 pg/ml, TNF-α = 9300 pg/ml $\pm$ 1400, $P<0.05$) (fig 3.4). Similar effects were also seen at 48 hours and with 16HBEo$^-$ cells, although 16HBEo$^-$ cells released almost x10 less IL-8 than H292 cells. Exposure to either Japanese or USA DEP at similar concentrations up to 200μg/ml also did not lead to statistically significant increased release of IL-8 at 24 or 48 hrs (data not shown).

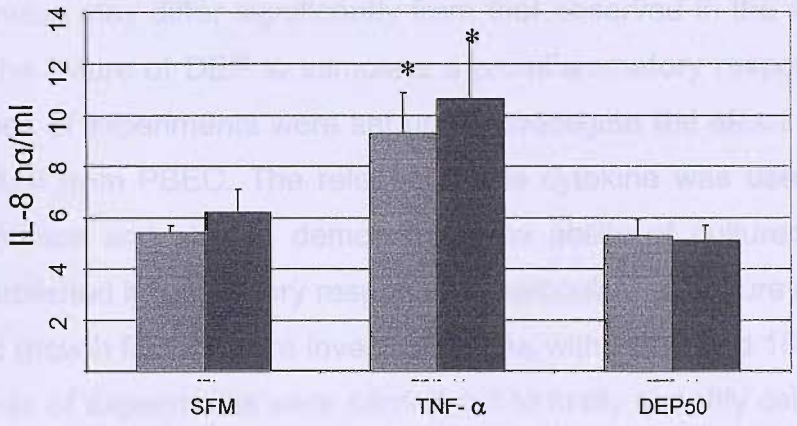


Fig 3.4 (a)

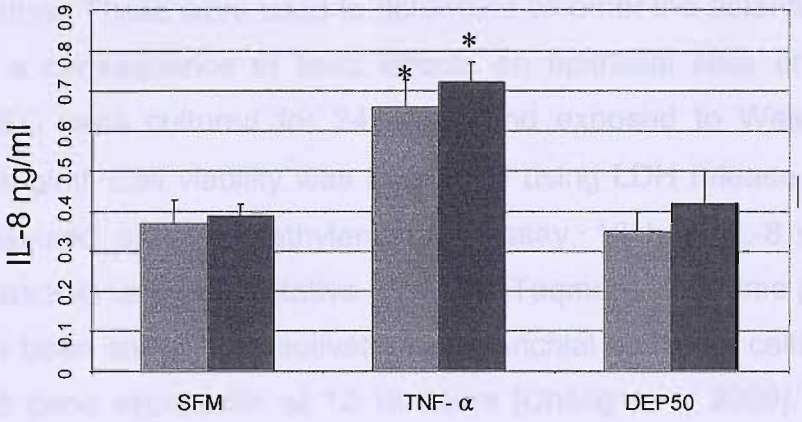


Fig 3.4 (b)

Fig 3.4

IL-8 Release From H292 (a) & 16HBE (b) Cells Following 24 & 48 hr Exposure to DEP

H292 and 16HBE cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. IL-8 release was measured using ELISA. Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

Key: SFM=serum free medium, TNF α = Tumour Necrosis Factor α, DEP = diesel exhaust particles (μg/ml)

* P<0.02 (data not significant unless indicated) (n=8)

24 hour

48 hour

3.2.5: The Effect of DEP On Primary Bronchial Epithelial Cell Viability, Cell Number, IL-8 Induction and Release

The responses of bronchial epithelial cell lines, H292 and 16HBEo$^-$ to a given stimulus may differ significantly from that observed in the *in vivo* setting. In view of the failure of DEP to stimulate a proinflammatory response in the cell lines a series of experiments were set up to investigate the effect of DEP in the release of IL-8 from PBEC. The release of this cytokine was used as a marker of cell activation and also to demonstrate the ability of cultured cells to develop an established inflammatory response to particulate exposure before other cytokines and growth factors were investigated. As with H292 and 16HBEo$^-$ cells, an initial series of experiments were carried out to firstly quantify cell viability and final cell number. These were used to determine whether the potential release of IL-8 may be a consequence of toxic effects on epithelial cells or activation of PBEC. PBEC were cultured for 24 hours and exposed to Welsh DEP 50, 100 and 200μg/ml. Cell viability was measured using LDH release and cell number was measured using a methylene blue assay. 18 hour IL-8 gene expression was measured using quantitative RT-PCR (Taqman). This time point was chosen as it has been shown that activation of bronchial epithelial cells leads to augmented IL-8 gene expression at 12-18 hours [Chang et al 2000]. 24 hour IL-8 release measured using ELISA. Statistical analysis was carried out using Kruskal Wallis test followed by a Wilcoxon Signed Ranked test.

PBEC were exposed to increasing concentrations of DEP up to 200μg/ml (24hrs). Exposure of PBEC to DEP for 24hrs elicited a dose dependent increase in LDH reaching significance at concentrations greater than 50μg/ml (untreated cells median=4% (IQR 3.8-4.2, DEP 100μg/ml median = 12% (IQR 11-14), DEP 200μg/ml median= 21% (IQR21-25), $p<0.05\%$, n=10) (fig 3.5). As with the cell lines, DEP up to 200μg/ml (24hrs) did not lead to significant changes in cell number compared to baseline (fig 3.5), which suggests cell proliferation in response to lysis. Based upon these data a dose of 50μg/ml DEP was used to further test the effects of DEP on IL-8 release.

As it was demonstrated that the concentration of DEP used to study the effects of this particulate on primary bronchial epithelial cell lines does not alter final cell number, cytokine release was not corrected for final cell number. Following 18 hours exposure, DEP 50μg/ml and 200 μg/ml led to significant IL-8 gene expression (DEP50μg/ml median=3.5 (IQR 2.3-14.0) p=0.01 and DEP200μg/ml median = 5.0 (IQR 3.0-10.0) p=0.005 n=10, basal expression = 1.3 (IQR 0.5-2.0) (fig 3.6). This response was equivalent to 80% ±10% of IL-8 gene expression obtained with TNF-α 10ng/ml (data not shown). IL-8 release at 24 hours was significantly enhanced in response to DEP 50μg/ml (median basal release = 2.2ng/ml (IQR 1.0-7.0) median DEP50μg/ml = 6.8ng/ml (IQR 6.0-9.0) ng/ml, and median DEP200μg/ml = 20ng/ml (IQR 9.0-35.0) n=10 P<0.001, (fig 3.6). This response was equivalent to 64% of IL-8 release obtained with TNFα 10ng/ml. The results of these experiments indicate that DEP leads to increased IL-8 release from PBEC by activation of IL-8 gene expression. Carbon black did not lead to an increase in IL-8 release (fig 3.6) or significant induction of IL-8 gene expression (data not shown), suggesting that DEP have an inflammatory effect as measured by the release of IL-8 that is different to carbon black. Furthermore PBEC are more sensitive to the inflammatory effects of DEP as measured by the release of IL-8 than H292 or 16HBEo$^-$ cells, a result that is consistent with published reports of the effects of DEP on IL-8 release from PBEC [Bayram et al 1998, Boland et al 1999]. Therefore these experiments have indicated that amongst the cell types tested, PBEC are the optimal bronchial epithelial cell type to be used to further study the effects of DEP on epithelial cell growth factor and cytokine responses. In addition, they have also confirmed that DEP at a concentration of 50μg/ml does not alter cell viability significantly.

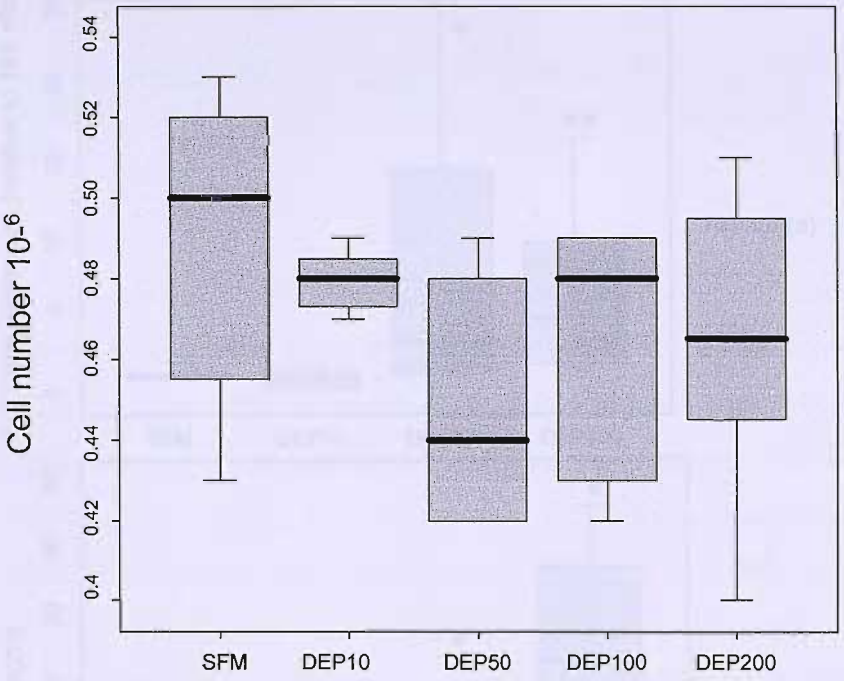


Fig 3.5 (a)

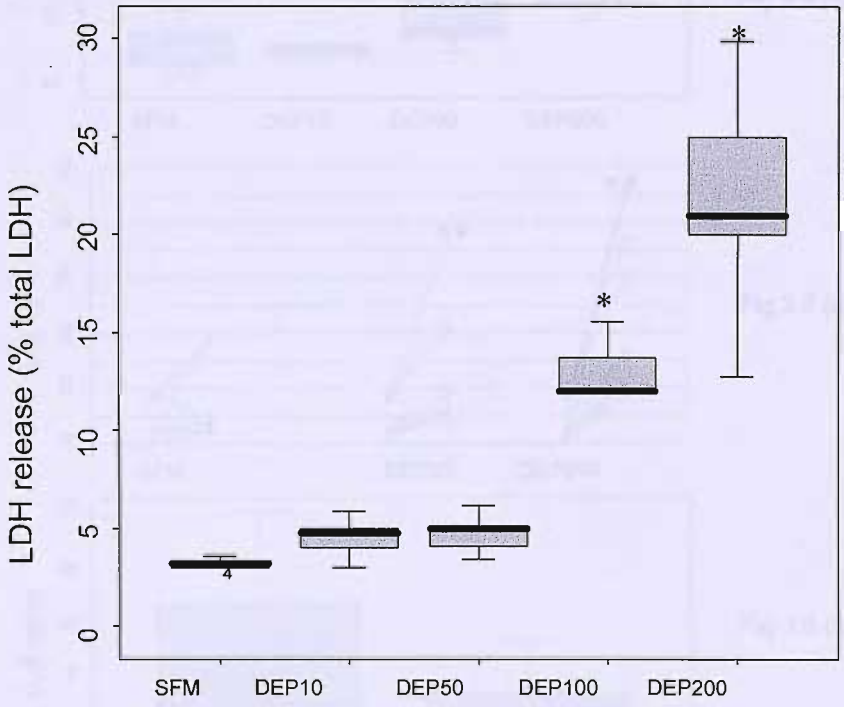


Fig 3.5 (b)

Fig 3.5

The Effect of Welsh DEP on PBEC number (a) and viability (b) following 24 hrs exposure

PBEC were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. Cell number was measured as relative increase from t=0. LDH release was measured and expressed relative to total LDH release. Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

Key: SFM=serum free medium, DEP = diesel exhaust particles (μg/ml)

*P <0.001 (n=10). (data not significant unless indicated).

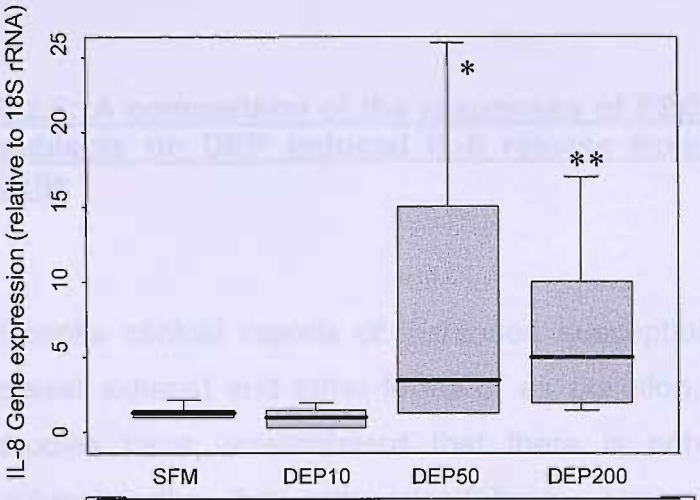


Fig 3.6 (a)

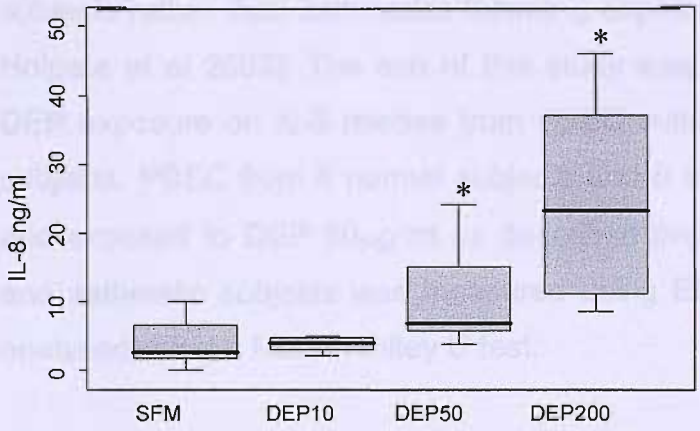


Fig 3.6 (b)

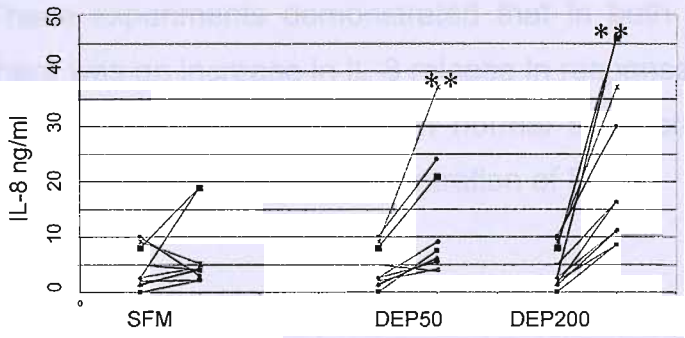


Fig 3.6 (b)(i)

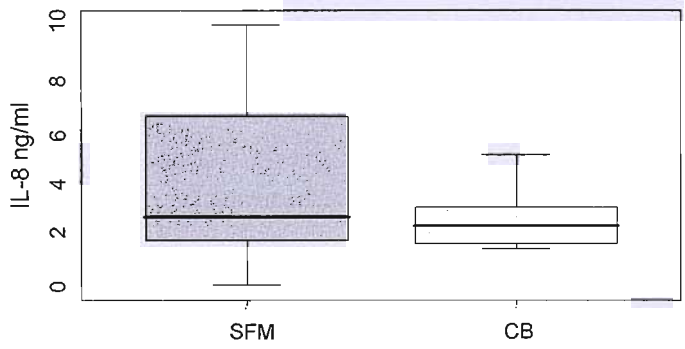


Fig 3.6 (c)

Fig 3.6

The Effect of Welsh DEP on PBEC IL-8 gene expression at 18 hrs (a) and median IL-8 release (b) as well as individual responses following 24 hours exposure

PBEC cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. IL-8 release was measured using ELISA. Samples were compared with serum free medium. Statistical analysis was carried out using Kruskal Wallis followed by Wilcoxon signed rank test.

Key: SFM=serum free medium, DEP = diesel exhaust particles (μg/ml) CB= carbon black(μg/ml)
* $p<0.01$ ** $p<0.005$ (n=10)

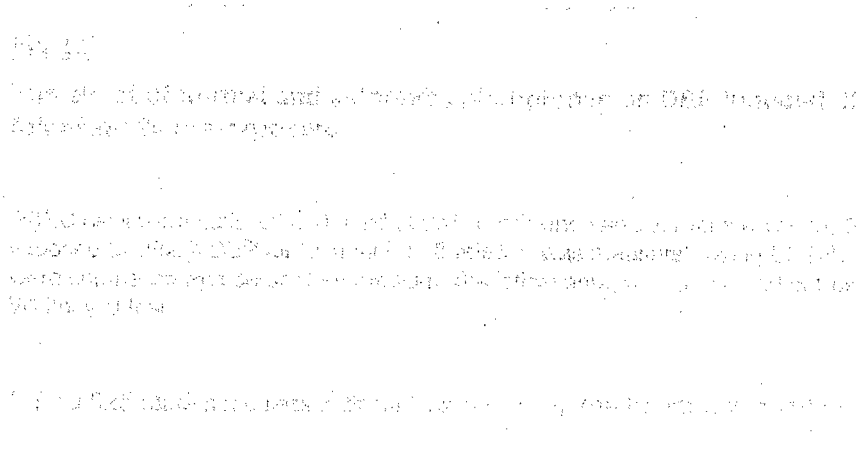
Fig 3.6 (b(i) demonstrates the individual responses to DEP

Fig 3.6 (c) demonstrates the response to carbon black

3.2.6: A comparison of the responses of PBEC from normal and asthmatic subjects on DEP induced IL-8 release from primary bronchial epithelial cells

Despite clinical reports of increased susceptibility of patients with asthma to diesel exhaust and other forms of air pollution, a number of human exposure studies have demonstrated that there is enhanced IL-8 release in normal subjects rather than asthmatics following exposure to DEP [Stenfors et al 2004, Holgate et al 2003]. The aim of this study was thus to establish the effects of DEP exposure on IL-8 release from PBEC cultured from asthmatic and normal subjects. PBEC from 8 normal subjects and 8 asthmatic subjects were cultured and exposed to DEP 50μg/ml as described previously. IL-8 release for normal and asthmatic subjects was measured using ELISA. The data was statistically analysed using a Mann Whitey U test.

These experiments demonstrated that in both normal and asthmatic subjects there was an increase in IL-8 release in response to DEP (fig 3.7). This data has demonstrated that PBEC from normal and asthmatic subjects release IL-8 in response to a non-toxic concentration of DEP.



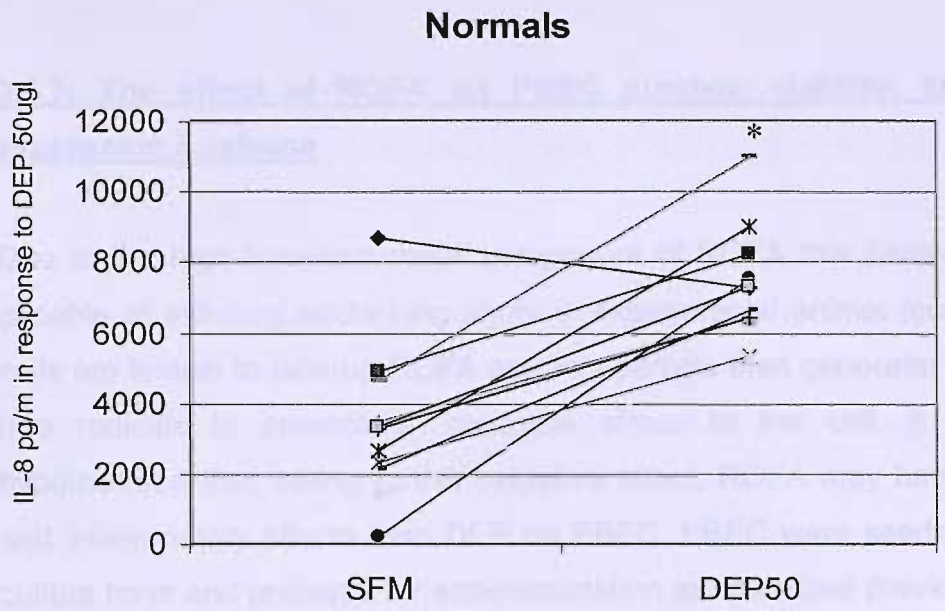


Fig 3.7 (a)

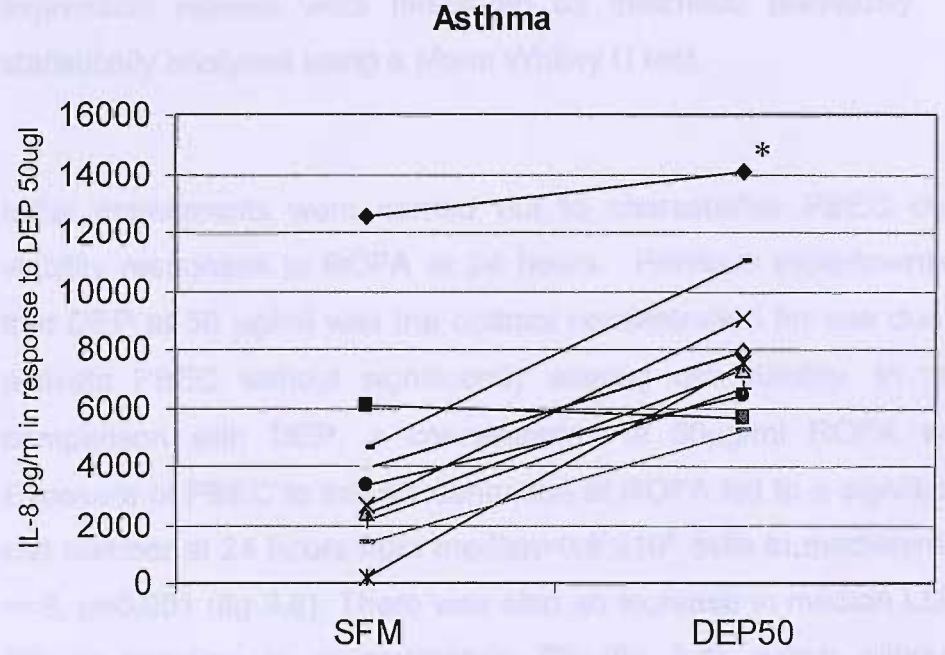


Fig 3.7 (b)

Fig 3.7

The effect of normal and asthmatic phenotypes on DEP induced IL-8 release following 24 hrs exposure

PBEC cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. IL-8 release was measured using ELISA. Samples were compared with serum free medium. Statistical analysis was carried out using Mann Whitney U test.

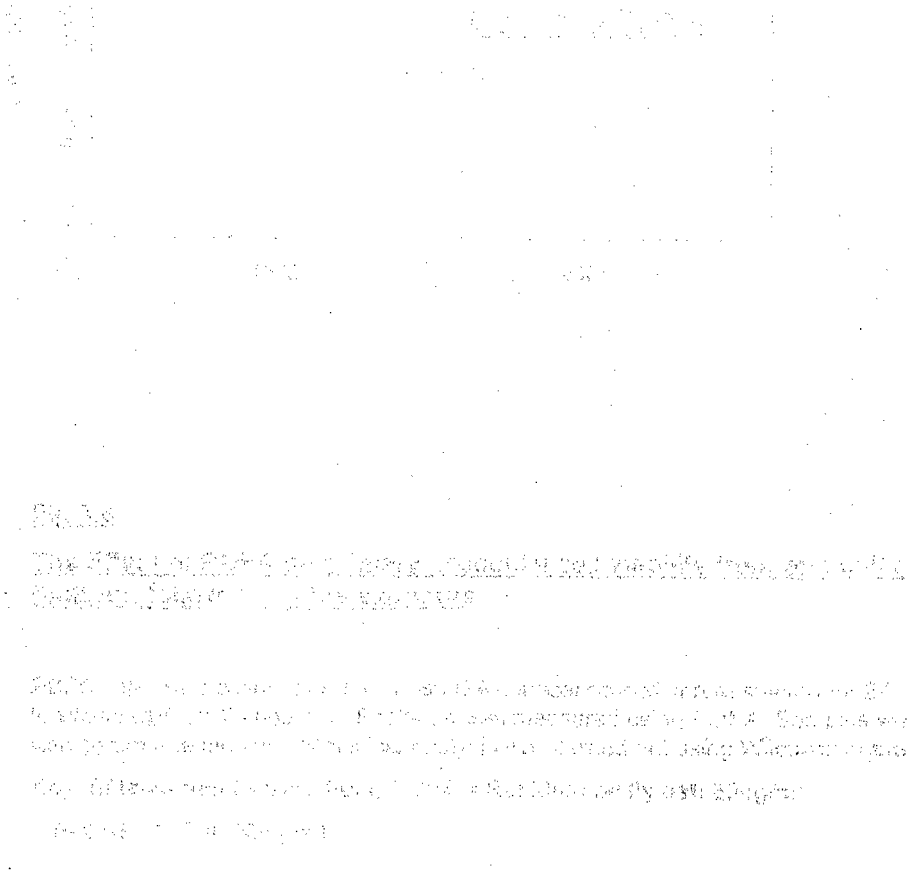
* P=0.025 (data not unless indicated significant u). (n=10 normal, n=10 asthmatics).

3.2.7: The effect of ROFA on PBEC number, viability, and IL-8 gene expression & release

Due to the high transition metal component of ROFA this particulate matter is capable of inducing acute lung injury in experimental animal models. Epithelial cells are known to take up ROFA and this particle then generates oxygen-based free radicals to present an oxidative stress to the cell. It was therefore hypothesised that, owing to this oxidative effect, ROFA may have greater toxic and inflammatory effects than DEP on PBEC. PBEC were seeded onto 24-well culture trays and prepared for experimentation as described previously. The cells were exposed to BEBM ± ROFA. Cell viability, final cell number, IL-8 gene expression release were measured as described previously. The data was statistically analysed using a Mann Whitey U test.

Initial experiments were carried out to characterise PBEC cell number and viability responses to ROFA at 24 hours. Previous experiments had indicated that DEP at 50 μg/ml was the optimal concentration for use due to its ability to activate PBEC without significantly altering cell viability. In order to enable comparison with DEP, a concentration of 50μg/ml ROFA was also used. Exposure of PBEC to this concentration of ROFA led to a significant reduction in cell number at 24 hours from median=0.5 $x10^6$ cells to median=0.42 x 10^6 cells, n=8, p=0.001 (fig 3.8). There was also an increase in median LDH release from 3% at baseline to approximately 7% (fig 3.8), which although statistically significant ($p<0.05$) was still relatively low. This effect of ROFA (7%) on PBEC viability was almost similar to the effect of DEP 50μg/ml (5%) on PBEC (fig 3.5). As these two particles have different properties, the observation that ROFA alters final cell number, whereas DEP doesn't may indicate that the different properties of these two particulates has an effect on the proliferative ability of cells. Although not tested using a specific proliferation assay the strong oxidative effect of ROFA on cells may make them less able to proliferate, whereas owing to its reduced oxidative capacity, DEP may allow cells to increase proliferation and hence compensate for any cell loss.

Exposure of PBEC to ROFA 50μg/ml led to a highly significant (x 17) increase in IL-8 gene expression at 18 hours (n=8, p=0.005, fig 3.9). There was also a significant and approximately equal (x17) increase in IL-8 release in supernatants at 24 hours (baseline=2 ng/ml (IQR 1-5), ROFA = 35 ng/ml IQR (20-50) n=8, p=0.01, fig 3.23)). Experiments carried previously had demonstrated that DEP also leads to an increase in IL-8 gene expression and protein release, however the effects of ROFA were greater than DEP 50μg/ml (DEP 50 induced median IL-8 gene expression = 3.5 (IQR 2.3-14.0) p=0.01 (fig 3.6)). This would suggest that the components of ROFA such as transition metals lead to greater inflammatory effects than DEP, which is relatively less rich in transition metals.



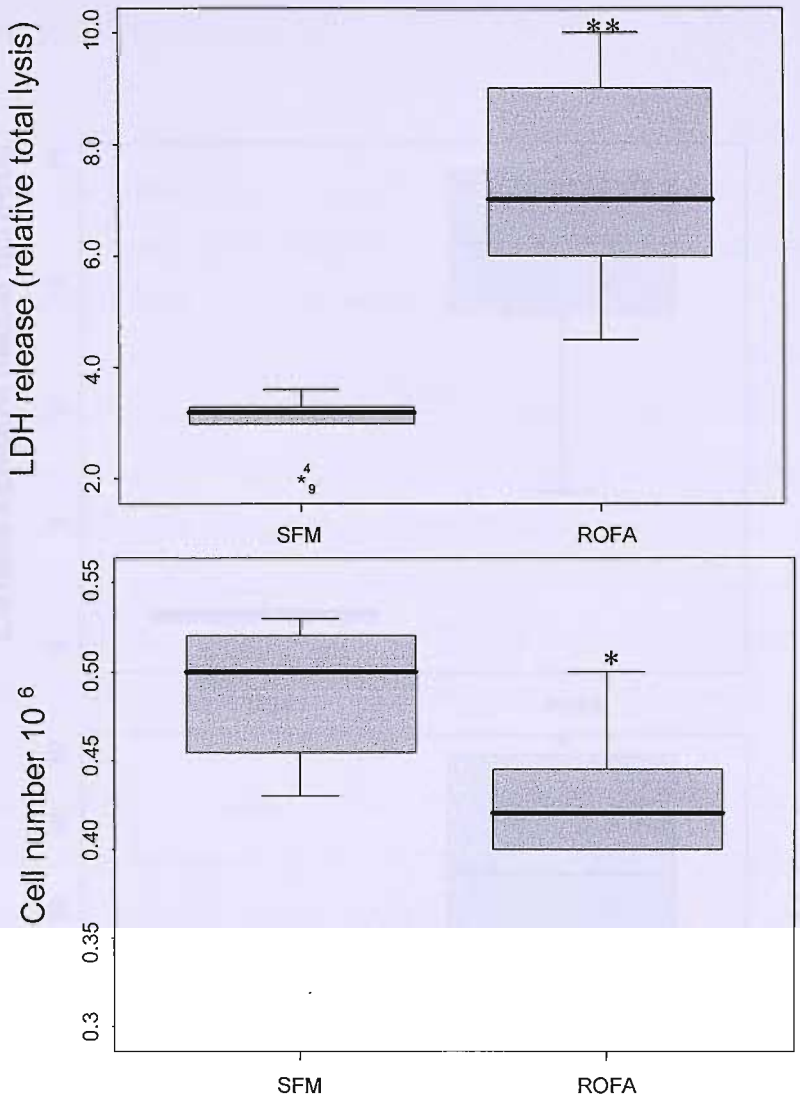


Fig 3.8

The Effect of ROFA on primary bronchial cell viability (top) and cell number (bottom) following 24 hrs exposure

PBEC cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. IL-8 release was measured using ELISA. Samples were compared with serum free medium. Statistical analysis was carried out using Wilcoxon signed rank test.

Key: SFM=serum free medium, ROFA = Residual oil fly ash 50ug/ml

*** P=0.01 ** P=0.005 (n=8)**

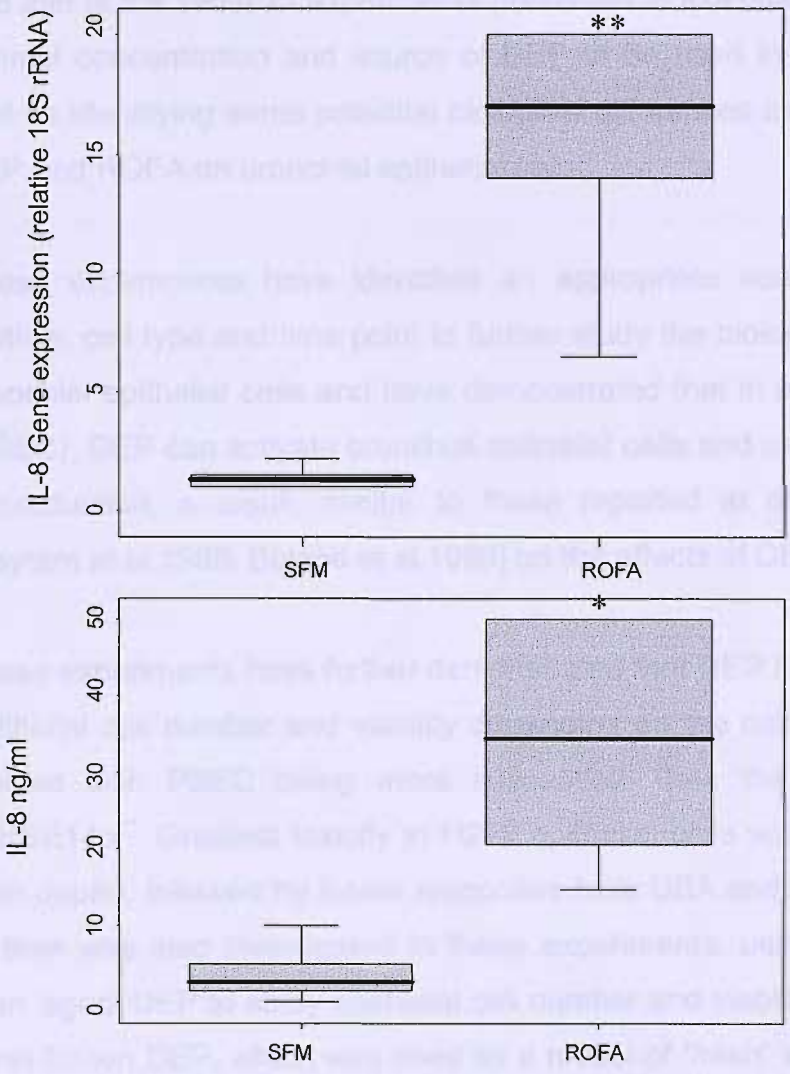


Fig 3.9

The Effect of ROFA on primary bronchial cell IL-8 gene expression (top) (18hrs) & release (bottom) following 24 hrs exposure

PBEC cells were cultured and used at 95% confluence and serum starved for 24 hours exposed to Welsh DEP for 24 hours. Cell number was expressed relative to cell number at t=0 (baseline0. LDH release was measured and expressed relative to total LDH release. IL-8 was measured using ELISA. Statistical analysis was carried out using Wilcoxon signed rank test. Samples were compared with serum free medium.

Key: SFM=serum free medium, ROFA = Residual oil fly ash 50ug/ml

*** P=0.01 ** P=0.005 n=8**

3.3 Discussion

The aim of the series of experiments presented in this chapter was to identify the optimal concentration and source of DEP to be used in future experiments as well as identifying some potential biological differences as regards the effects of DEP and ROFA on bronchial epithelial cells.

These experiments have identified an appropriate source of diesel exhaust particle, cell type and time point to further study the biological effects of DEP on bronchial epithelial cells and have demonstrated that in an appropriate cell type (PBEC), DEP can activate bronchial epithelial cells and cause upregulation of IL-8 production, a result, similar to those reported in other published studies [Bayram et al 1998, Boland et al 1999] on the effects of DEP.

These experiments have further demonstrated that DEP have different effects on epithelial cell number and viability depending on the cell type and DEP source studied with PBEC being more susceptible than the cell lines H292 and 16HBE14o^{-}. Greatest toxicity in H292 epithelial cells was associated with DEP from Japan, followed by lesser responses from USA and then Wales. The effect of time was also investigated in these experiments, using 'fresh frozen' rather than 'aged' DEP to study epithelial cell number and viability. Using two-week old fresh frozen DEP, which was used as a model of 'fresh' Welsh DEP, there were similar results to those observed with the equivalent 'aged' Welsh DEP. Although this may be truly due to the fact that there are no differences in action between the two types of DEP, an alternative and more likely explanation is that this may be due to the limitations of collecting DEP and the fact that many of the volatile organic compounds (VOCs) including PAH evaporate very quickly after the DEP is released into the atmosphere. Therefore in the time that particles are collected onto a filter and placed in a container there may have already been significant loss of the VOCs even before they were frozen at - 20°C. As it has been shown that the composition of DEP varies depending on the time elapsed following combustion [Cohen et al 1999], the latter explanation is more likely to account for the observed effect. In addition, following combustion, and the loss of the gaseous phase of diesel exhaust and the VOCs including the PAHs, the

remaining carbon core together with the adsorbed chemicals remain in the atmosphere as respirable particles that may migrate and settle in different regions due to the effects of wind and other atmospheric conditions [Cohen et al 1999]. Although, some human exposure to DEP will be to freshly generated diesel exhaust, such as in traffic, the majority of exposure to PM and thus DEP is of the 'aged' particles that remain in the atmosphere. Therefore, although the 'aged' type of DEP tested in these series of experiments are unlikely to accurately represent the effects of the 'fresh' type of DEP inhaled by people in traffic, it is nevertheless a good model to study exposure to 'aged' DEP, which is the majority of the type of DEP that people are exposed to in urban conditions. An alternative method that could have been used to study the difference in 'fresh' and 'aged' DEP would have been to test the effects of known concentrations of freshly generated DEP on cell cultures in an *in vitro* exposure chamber.

Carbon black did not lead to increased toxicity in H292, 16HBE14o$^-$ cells or PBEC and thus it was concluded that the different composition of DEP and carbon black accounts for this finding.

Although, exposure to 'aged' Welsh DEP in 16HBE14o$^-$ and PBEC did not lead to a significant alteration in cell viability, they did lead to some increase in toxicity in H292 cells. In addition, although there was no activation of H292 and 16HBE14o$^-$ cells in response to DEP as measured by IL-8 release, exposure of PBEC did lead to a dose dependent increase in IL-8 release, which matched the *in vivo* situation [Salvi et al 2000] more closely. Thus, these data highlight some of the difficulties in choosing an appropriate cell type for *in vitro* studies. Consequently, although there is a role for the use of bronchial epithelial cell lines in the *in vitro* investigation of the responses of a given stimulus, there are limitations with the extrapolation of the data to the *in vivo* situation. In many *in vitro* studies, cell lines are preferred to PBEC, as cell lines are generally less demanding than primary cells. One reason is that there are less limitations with obtaining an adequate supply of cells needed for investigating the mechanistic effects of a given stimulus such as DEP, thus making them an attractive cell source to PBEC. In addition to limitations of supply, another benefit of using cell lines is that less variation would be expected in the results, whereas in PBEC

greater variation would be expected due to the natural variation observed in response between individuals that is a hallmark of *in vivo* studies. Nevertheless, despite the difficulties and potential limitations with the use of PBEC, these experiments have indicated that the optimal cell type to be used for further experiments into the effects of DEP on bronchial epithelial cells is PBEC, and thus in future experiments this cell will be used.

Finally, in these experiments the release of IL-8 was investigated as a marker of epithelial cell activation, as DEP have been shown to lead to the release of this cytokine in response to activation of PBEC in published studies [Bayram et al 1998, Boland et al 1999]. IL-8 is largely considered to be a potent neutrophil chemotactic factor and may be released in response to a wide variety of stimuli, including exposure to PM, pro-inflammatory cytokines, microbes and their products, as well as environmental changes such as hypoxia, reperfusion, and hyperoxia [Mukaida et l 2003]. However, there is also evidence, indicating that IL-8 may have a wide range of additional actions on various other cell types, including lymphocytes, monocytes, endothelial cells, and fibroblasts [Mukaida et al 2003]. Thus, in addition to its acute neutrophilic pro-inflammatory effects, it is thought that IL-8 may also have a crucial role in various pathological conditions such as chronic inflammation and fibrosis [Mukaida et al 2003]. Thus, although the release of IL-8 has been demonstrated to take place in response to DEP, the potential mechanisms leading to the release of this cytokine and other potential downstream effects on chronic pulmonary changes have are not fully understood and make up the focus of further studies in this thesis.

The studies in this chapter have also indicated that exposure of PBEC from subjects with both asthmatic and normal phenotypes to DEP leads to an increase in IL-8 release. It was further demonstrated that in addition to DEP, ROFA also activate PBEC and cause upregulation of IL-8 production. However, this effect was greater than with DEP. Whereas with DEP there was approximately x3 increase, with ROFA there was x17 increase in both IL-8 gene expression and protein release compared to untreated cells. Interestingly, despite this pro-inflammatory effect, ROFA 50μg/ml had only a marginally higher toxic effect than

DEP 50μg/ml on PBEC but did lead to a significant reduction in cell number unlike DEP. Therefore it can be concluded that the higher transition metal component of ROFA leads to augmented inflammatory effects on PBEC when compared with DEP. In addition, CB did not lead to higher release of IL-8 compared with unstimulated cells. Thus the data presented in this chapter suggest that the chemical component of DEP, rather than the particle itself is responsible for cell activation. The results of these studies further indicate that Welsh DEP at a concentration 50μg/ml is a suitable source to study the effects of DEP on bronchial epithelial cells, while PBEC rather than H292 or 16HBEo^{-} cells are the optimal cell type for further study. Finally, as regards identifying an appropriate time point, the effect of prolonged cell culture in cell lines up to 48hrs led to a doubling of baseline toxicity, which although still relatively low indicate that exposure of bronchial cells beyond 24hrs has an undesirable effect on cell viability and cell number irrespective of any additional stimuli such as DEP, thus indicating 24 hour culture to be a more suitable time point for further study.

Therefore in summary, experiments carried out in this chapter have demonstrated that Welsh DEP at a concentration of 50 μg/ml is most appropriate for use in further experiments. Furthermore, it was also determined that of the three different epithelial cell types, PBEC were the most suitable cell type for the study of the effects of DEP. However despite the observed effects of DEP on PBEC, ROFA is a more potent ambient particle at inducing pro-inflammatory effects on PBEC.

CHAPTER FOUR

A MECHANISTIC STUDY OF THE PRO-INFLAMMATORY EFFECTS OF DEP & ROFA ON PRIMARY BRONCHIAL EPITHELIAL CELLS

4.1.1 Introduction

Having demonstrated that DEP and ROFA have an inflammatory effect on PBEC, as evidenced by the release of IL-8, a series of studies were set up to investigate the mechanism of release of this cytokine from PBEC.

In asthma, damage to the bronchial epithelium leads to the release of pro-inflammatory mediators, which can propagate ongoing airways inflammation in response to other mediators and cytokines such as the TH-2 cytokines IL-4 and IL-13 [Lordan et al 2002]. There is also increased expression of the EGFR [Amishima et al 1998]. Activation of this receptor can elicit a range of responses including, inflammation, [Monick et al 2005], proliferation and induction of differentiation [Downward et al 1984, Schreiber et al 1983], as well as goblet cell hyperplasia and mucus expression [Takeyama et al 1999], thus potentially leading to phenotypical changes that are characteristic of chronic asthma.

Recent studies using cigarette smoke extract (CSE) have demonstrated that secretion of IL-8 is dependent on EGFR activation and that exogenous EGF can also induce IL-8 production in primary bronchial epithelial cells [Richter et al 2002]. Bronchial epithelial cells produce ligands for the EGFR, which include transforming growth factor α (TGFα)-, heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AR) [Polosa et al 1999]. These growth factors are produced as transmembrane precursor molecules whose processing and release (ectodomain shedding) is a highly regulated process involving metalloproteinases, and functionally contribute to epithelial maintenance and repair [Massague et al 1993]. HB-EGF shedding has been associated with transactivation of the EGFR by G-protein coupled receptors [Prenzel et al 1999].

Thus, it was hypothesized that IL-8 release from PBEC in response to DEP is mediated by EGFR activation through increased shedding of the EGFR ligands TGFα, AR and HB-EGF (fig 4a).

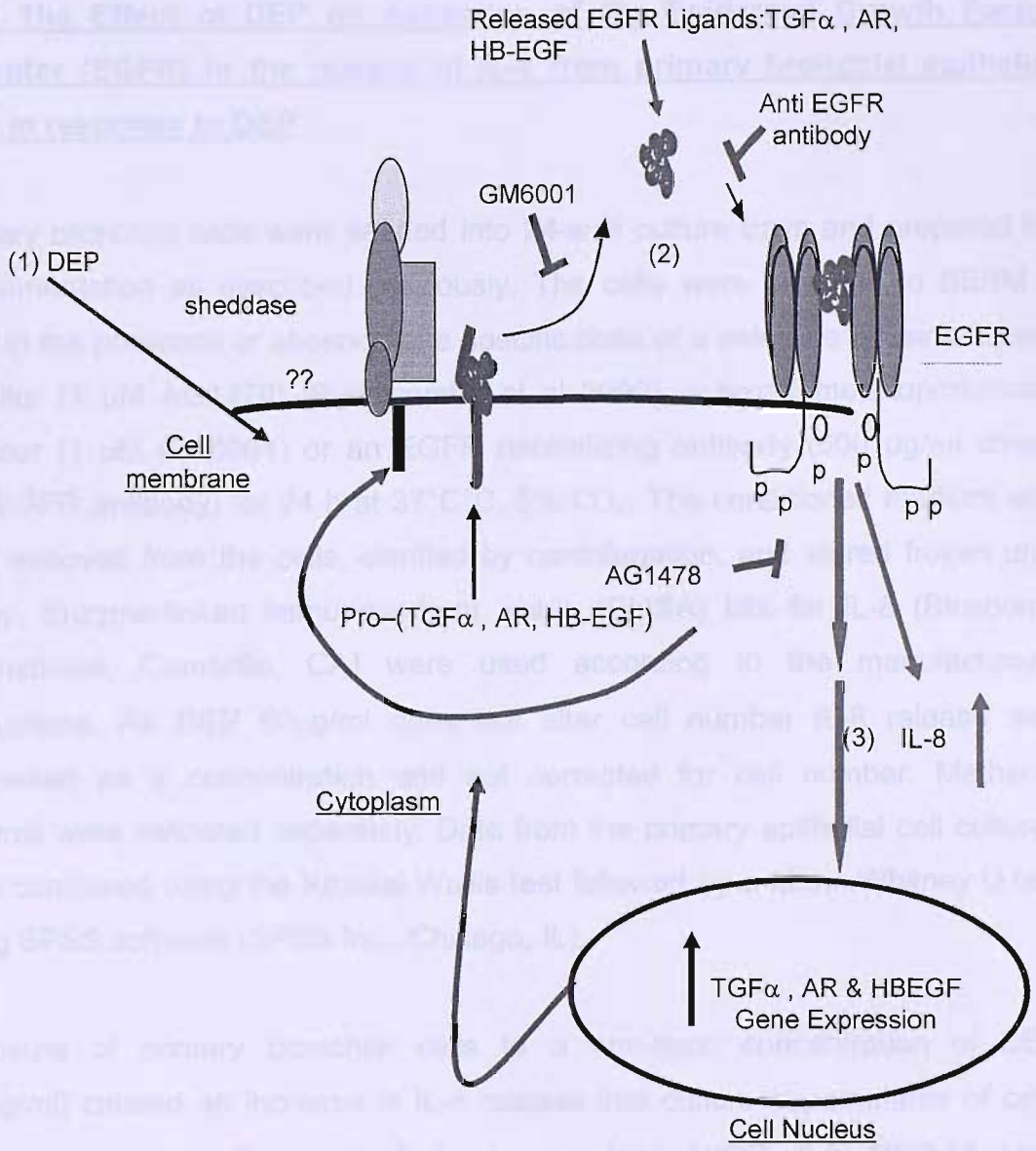


Fig 4a) The mechanism for DEP mediated IL-8 release

It is proposed that DEP activates sheddases (ADAM) on the cell membrane (1) which in turn release membrane bound pro- EGFR ligands (pro-TGFα, AR and HB-EGF) (2). The shed ligand then binds to the EGFR and activates the receptor leading to downstream effects including release of IL-8 and heightened gene expression for EGFR ligands (3).

RED (Inhibitors): GM6001 = broad inhibitor of metalloproteinase activity, Anti EGFR antibody, AG1478= inhibitor of tyrosine phosphorylation

4.2 Results

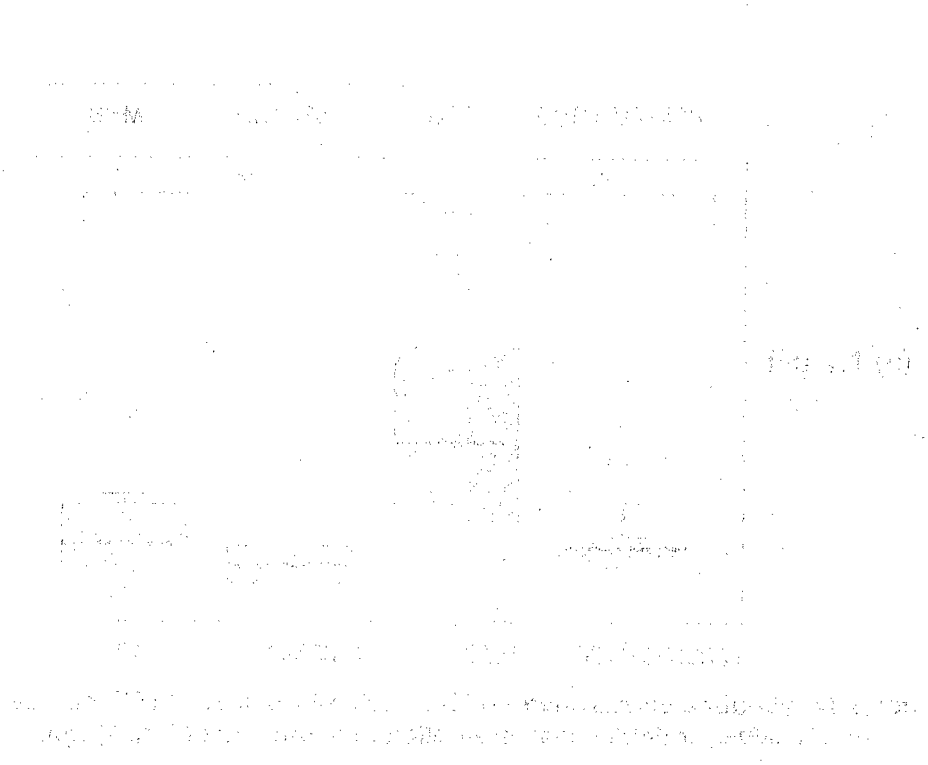
4.2.1 The Effect of DEP on Activation of the Epidermal Growth Factor Receptor (EGFR) in the release of IL-8 from primary bronchial epithelial cells in response to DEP

Primary bronchial cells were seeded into 24-well culture trays and prepared for experimentation as described previously. The cells were exposed to BEBM ± DEP in the presence or absence of a specific dose of a selective tyrosine kinase inhibitor (1 μM AG1478) [Puddicombe et al 2000], a broad metalloproteinase inhibitor (1 μM GM6001) or an EGFR neutralizing antibody (500 μg/ml sheep anti-EGFR antibody) for 24 h at 37°C°C, 5% CO_2. The conditioned medium was then removed from the cells, clarified by centrifugation, and stored frozen until assay. Enzyme-linked immunosorbent assay (ELISA) kits for IL-8 (Biosource International, Camarillo, CA) were used according to the manufacturer's instructions. As DEP 50μg/ml does not alter cell number IL-8 release was expressed as a concentration and not corrected for cell number. Methanol controls were validated separately. Data from the primary epithelial cell cultures were compared using the Kruskal Wallis test followed by a Mann Whitney U test using SPSS software (SPSS Inc., Chicago, IL).

Exposure of primary bronchial cells to a non-toxic concentration of DEP (50μg/ml) caused an increase in IL-8 release into culture supernatants of cells (fig 4.1) (untreated cells: median IL-8 release = 4ng/ml (IQR= 2-8), DEP 50μg/ml: median IL-8 release = 15 ng/ml (IQR= 9-23), n=10, p=0.02), which was significantly reduced after the cells were pre-treated with the EGFR-specific tyrosine kinase inhibitor, AG1478 (DEP 50μg/ml: median IL-8 release = 15 ng/ml (IQR= 9-23); DEP+AG1478 =3 ng/ml (IQR=1-5), n=10, $P < 0.01$) (fig 4.1) Application of neutralizing antibodies for the EGFR also completely inhibited DEP induced IL-8 release (DEP 50μg/ml: median IL-8 release = 15ng/ml (IQR= 9-23); DEP+ anti-EGFR antibody = 8ng/ml (IQR=7.5-8.5), n=10, $P < 0.01$) (fig 4.1) indicating that activation of the EGFR had taken place through binding to the receptor. Binding to the EGFR takes place through the shedding of EGFR

ligands and therefore in order to investigate whether activation of the EGFR took place due to the shedding and release of EGFR ligands, PBEC were pre-treated with the broad metalloproteinase inhibitor (GM6001). This led to a significant reduction in IL-8 levels in supernatant (DEP 50μg/ml: median IL-8 release = 15ng/ml, DEP+GM6001= 5.5ng/ml (IQR = 3.5-6.5) n=10 $p<0.05$) (fig 4.1) suggesting that the observed release of IL-8 was due to shedding of membrane bound EGFR agonists (fig 4.1).

Therefore, these data suggest that the release of IL-8 from PBEC in response to DEP, is mediated by the EGFR and involves shedding of EGFR ligands from the surface membrane as well as binding of these ligands to the EGFR, which in turn lead to phosphorylation of tyrosine residues and activation of the EGFR.



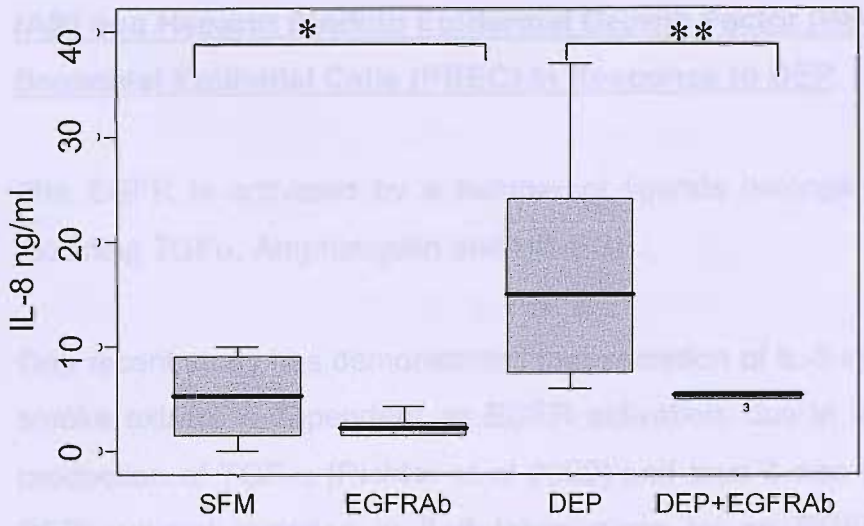


Fig 4.1 (a)

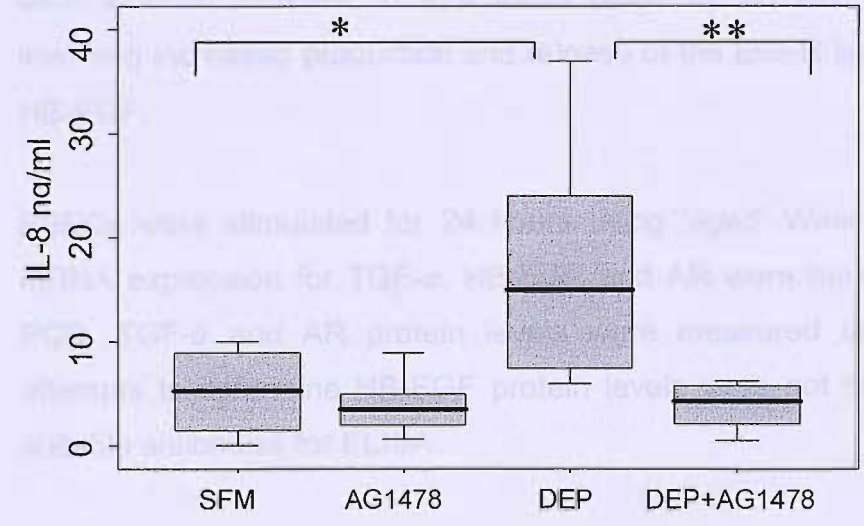


Fig 4.1 (b)

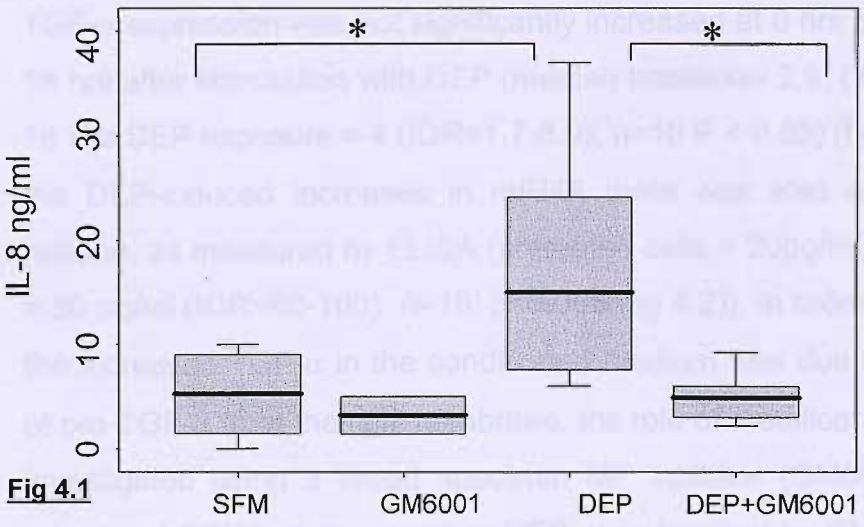


Fig 4.1 (c)

Fig 4.1

IL-8 release from PBEC treated with DEP & EGFR neutralising antibody (a), tyrosine kinase inhibitor (Ag1478) (b), broad metalloproteinase inhibitor (GM6001) (c)

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP for 24 hours. IL-8 release was compared with SFM and DEP+inhibitors. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles 50μg/ml , AG1478 = tyrosine kinase inhibitor, EGFRAb = EGFR neutralising antibody, GM6001 = metalloproteinase inhibitor

*** P<0.05 ** P<0.01 (n=10).**

4.2.2 The Release of Transforming Growth Factor-α (TGF-α), Amphiregulin (AR) and Heparin Binding Epidermal Growth Factor (HB-EGF) From Primary Bronchial Epithelial Cells (PBEC) in Response to DEP

The EGFR is activated by a number of ligands belonging to the EGF family, including TGFα, Amphiregulin and HB-EGF.

One recent study has demonstrated that secretion of IL-8 in response to cigarette smoke extract is dependent on EGFR activation, due in large part to autocrine production of TGF-α [Richter et al 2002] and thus it was hypothesised that the DEP induced increase in IL-8 takes place by an EGFR mediated pathway involving increased production and release of the EGFR ligands, TGF-α, AR and HB-EGF.

PBECs were stimulated for 24 hours using 'aged' Welsh DEP. The levels of mRNA expression for TGF-*α*, HB-EGF and AR were measured by quantitative PCR. TGF-*α* and AR protein levels were measured using ELISA, however attempts to determine HB-EGF protein levels were not made due to a lack of suitable antibodies for ELISA.

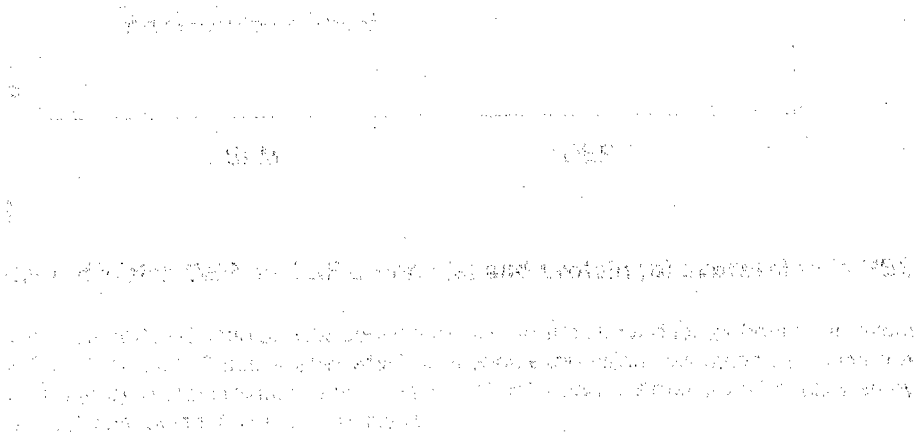
TGF-α expression was not significantly increased at 6 hrs but did increase 2-fold, 18 hrs after stimulation with DEP (median baseline= 2.2, (IQR= 1.8–3.1), median 18 Hrs DEP exposure = 4 (IQR=1.7-6.0), n=10 $P < 0.05$) (fig 4.2). Consistent with the DEP-induced increases in mRNA there was also an increase in TGF-α release, as measured by ELISA (untreated cells = 20pg/ml, (IQR=15-20), DEP50 = 80 pg/ml (IQR=60-100), n=10, $p<0.005$ (fig 4.2)). In order to determine whether the increased TGF-α in the conditioned medium was due to increased shedding of pro-TGF-α from the cell membrane, the role of metalloproteinase's (MPs) were investigated using a broad spectrum MP inhibitor (GM6001). The augmented release of TGF-α in response to DEP, was blocked by GM6001 (TGFα release: DEP alone= 65 pg/ml (IQR= 50 – 100), DEP+ GM6001 = 10 pg/ml (IQR= 0 – 20) (n=10, $P < 0.005$) (fig 4.3), indicating that the increased TGF-α measured is due to shedding of pro-TGF-α from the epithelial cell membrane.

Binding of shed TGF-α to the EGFR was investigated using a specific EGFR neutralising antibody to block binding onto the receptor. This led to significant augmentation of TGF-α levels in the supernatant (TGF-α release: DEP alone= 65ng/ml (IQR= 50 – 100), DEP+ Neutralising antibody = 200ng/ml (IQR= 180 – 220) (n=10, $P < 0.01$) (fig 4.3). These data suggest that the EGFR-neutralizing antibody prevents ligand binding and internalisation of the receptor-bound ligand whereas in the absence of the antibody, TGF-α is rapidly utilised by the cells. Following this, the activation of the receptor was investigated using an inhibitor of downstream EGFR tyrosine kinase activity (AG1478). Inhibition of downstream EGFR phosphorylation using AG1478 led to a highly significant reduction in TGFα levels (DEP alone= 65ng/ml (IQR= 50 – 100), DEP+ AG1478 = 20ng/ml (IQR= 10 – 30) (n=10, $P < 0.005$) (fig 4.3), indicating that following binding onto the EGFR, TGFα leads to activation of downstream pathways that may lead to IL-8 release and is also needed for activation of itself in an autocrine manner. Therefore, exposure of PBEC to DEP leads to the release of TGFα, which may account for the mechanism by which IL-8 is increased in response to DEP.

AR mRNA expression was not significantly increased at 6 or 18 hrs. However, despite a lack of mRNA response there was enhanced secretion of AR into the culture medium at 24 hours (untreated cells = 500 pg/ml, (IQR=0.4-0.8), DEP50 = 700 pg/ml (IQR=600-900), n=10, $p<0.01$) (fig 4.4), suggesting that the increased AR in culture medium is, mainly due to increased shedding of this growth factor into the culture medium rather than being due to increased production. In order to investigate epithelial shedding of AR the broad spectrum MP inhibitor (GM6001) was used. The increase in AR release in response to DEP, was blocked by GM6001 (DEP alone= 700pg/ml (IQR= 600 – 900), DEP+ GM6001 = 300 pg/ml (IQR= 200 – 450) (n=10, $P < 0.05$) (fig 4.5) indicating that the increased AR in supernatant is due to increased shedding from the membrane. Following this, the binding of AR to the EGFR was studied to determine the potential contribution of AR to activation of the EGFR. This was carried out using a specific EGFR neutralizing antibody to block binding onto the receptor, as well as an inhibitor of downstream tyrosine kinase activity (AG1478).

Inhibition of the EGFR using a specific antibody led to a mild but statistically significant reduction in AR levels in the supernatant (AR release: DEP alone= 700pg/ml (IQR= 600 – 1000), DEP+ Neutralising antibody = 600pg/ml (IQR= 500 – 700) (n=10, $P < 0.05$) (fig 4.5). This indicates that there was relatively little binding of AR to the EGFR, suggesting that following release of AR and TGF-α from the membrane by DEP, TGF-α may bind more preferentially to the EGFR compared to AR. Inhibition of downstream EGFR phosphorylation using AG1478 also led to a highly significant reduction in AR levels (DEP alone= 700pg/ml (IQR= 600 – 1000), DEP+ AG1478 = 200pg/ml (IQR= 180 – 400) (n=10, $P < 0.01$) (fig 4.5), indicating that activation of the EGFR is needed for AR release. HBEGF mRNA levels increased after 6hrs, and returned back to baseline by 18hrs, although these also did not achieve statistical significance (fig 4.6).

These data suggest that secretion of IL-8 in response to DEP is dependent on EGFR activation and that of the three EGFR ligands examined TGF-α is likely to make the most substantial contribution to this response.



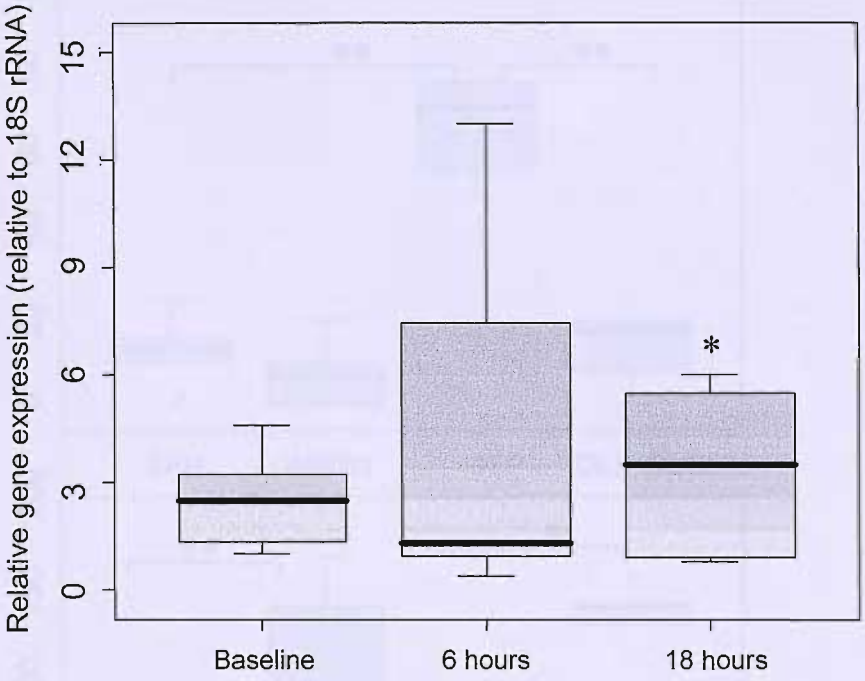


Fig 4.2 (a)

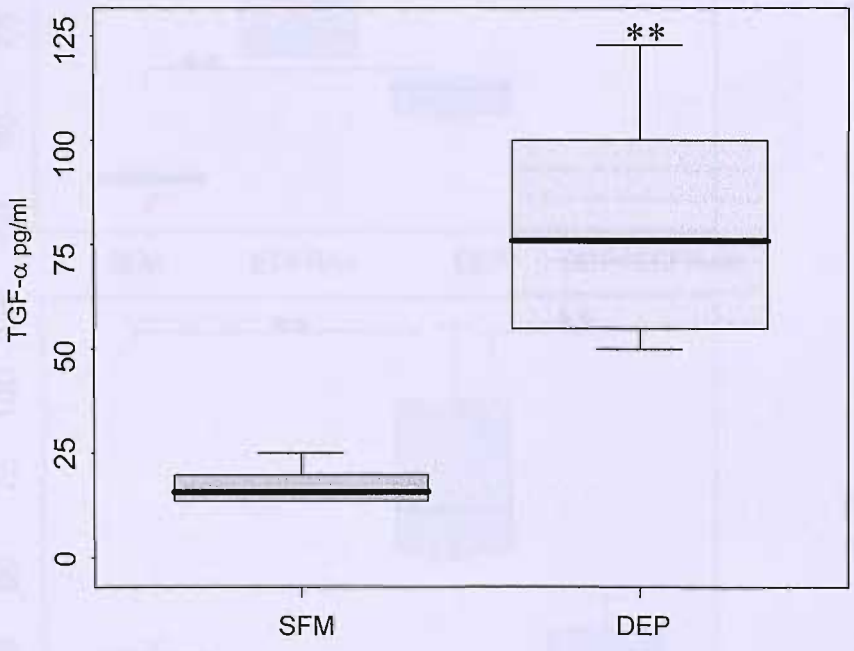


Fig 4.2 (b)

Fig 4.2

The Effect of Welsh DEP on TGF-α gene (a) and protein (b) expression in PBEC

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP for 6 and 18 hours, after which was gene expression was measured compared with baseline (t=0). Protein release was measured after 24 hours. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key:SFM=serum free medium, Baseline = unstimulated (t=0 hrs), DEP = diesel exhaust particles 50μg/ml,

*** P<0.05 ** P<0.005 (n=10)**

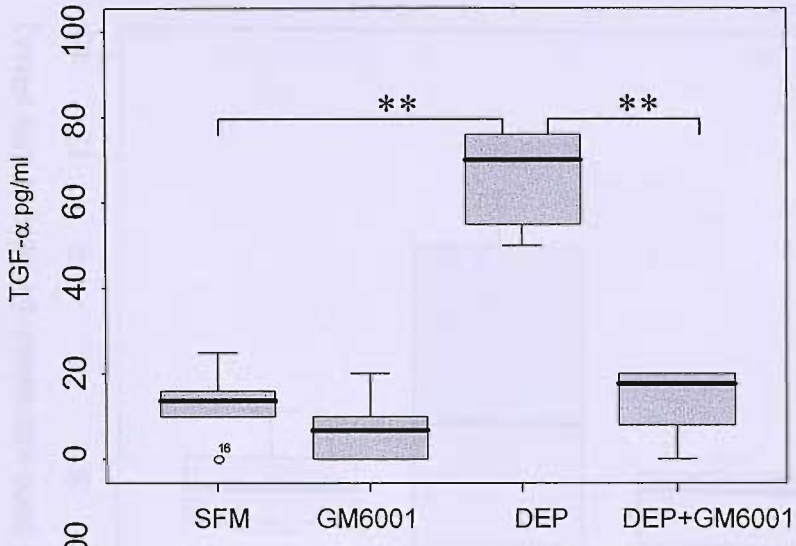


Fig 4.3 (a)

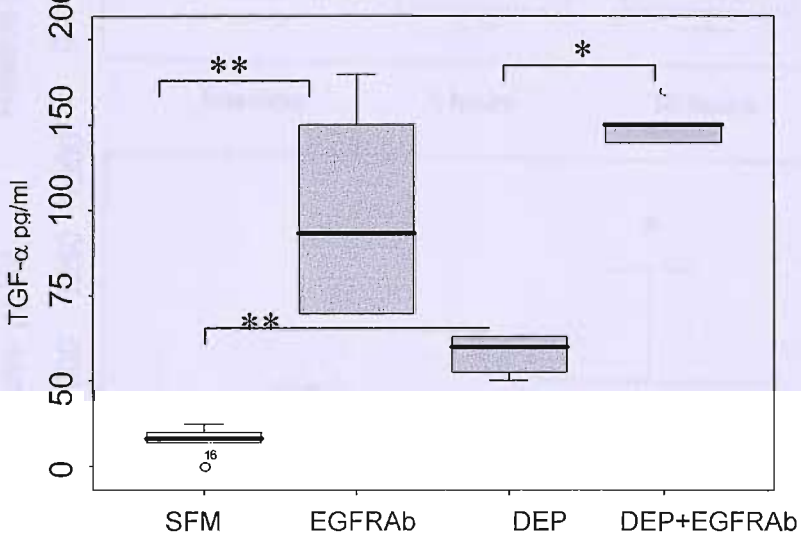


Fig 4.3 (b)

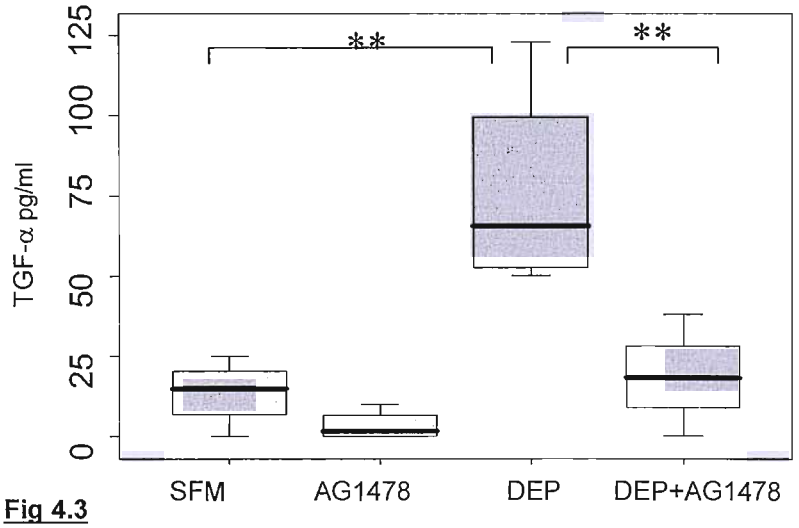


Fig 4.3 (c)

Fig 4.3

The Effect of Welsh DEP on TGF-α release from PBEC treated with a GM6001 (broad metalloproteinase inhibitor) (a) an EGFR neutralising antibody (b) and a tyrosine kinase inhibitor (Ag1478) (c)

PBEC were cultured and used at 95% confluence, serum starved for 24 hours and exposed to Welsh DEP for 24 hours, after which TGF -α release was compared with SFM and DEP+inhibitor. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key:SFM=serum free medium, DEP = diesel exhaust particles 50μg/ml, **AG1478 = tyrosine kinase inhibitor, EGFRAb = EGFR neutralising antibody, GM6001 = metalloproteinase inhibitor** * P<0.01 ** P<0.005 n=(10)

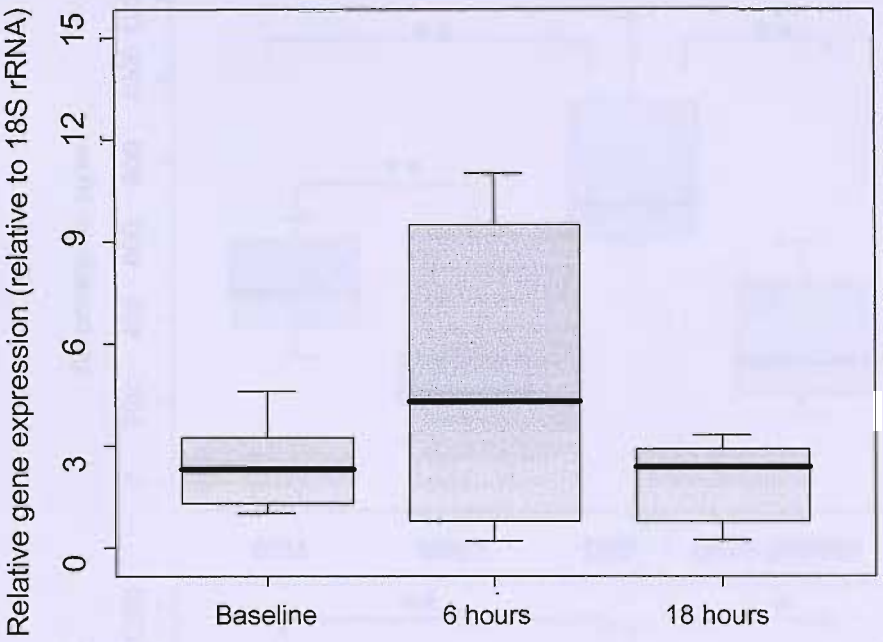


Fig 4.4 (a)

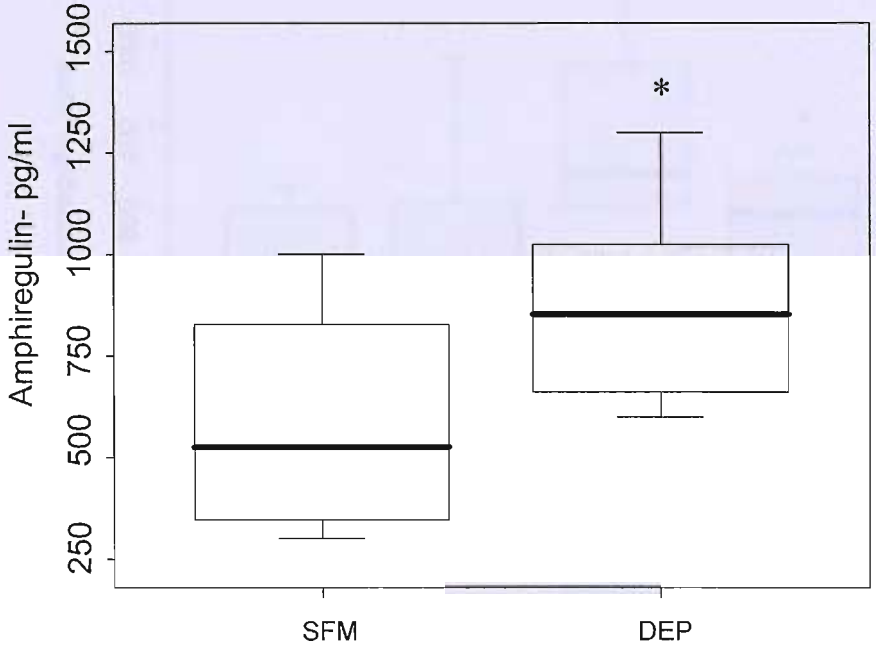


Fig 4.4 (b)

Fig 4.4

The Effect of Welsh DEP on Amphiregulin gene (a) and protein (b) expression in PBEC

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP for 6 and 18 hours, after which gene expression was measured and was compared with baseline(t=0). Protein release was measured after 24 hours and was compared with serum free medium. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key:SFM=serum free medium, Baseline = unstimulated (t=0 hrs) **DEP = diesel exhaust particles 50μg/ml, * P<0.01 (n=10)**

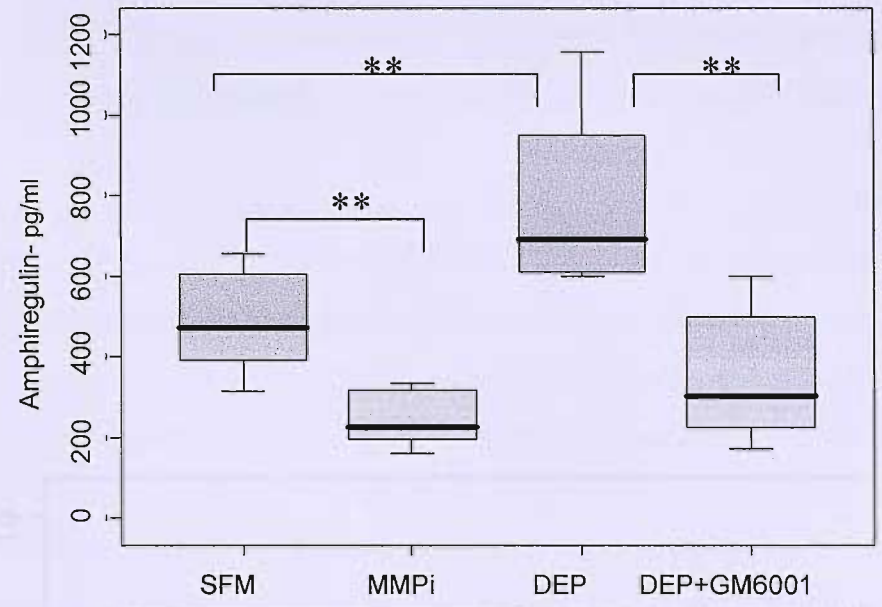


Fig 4.5 (a)

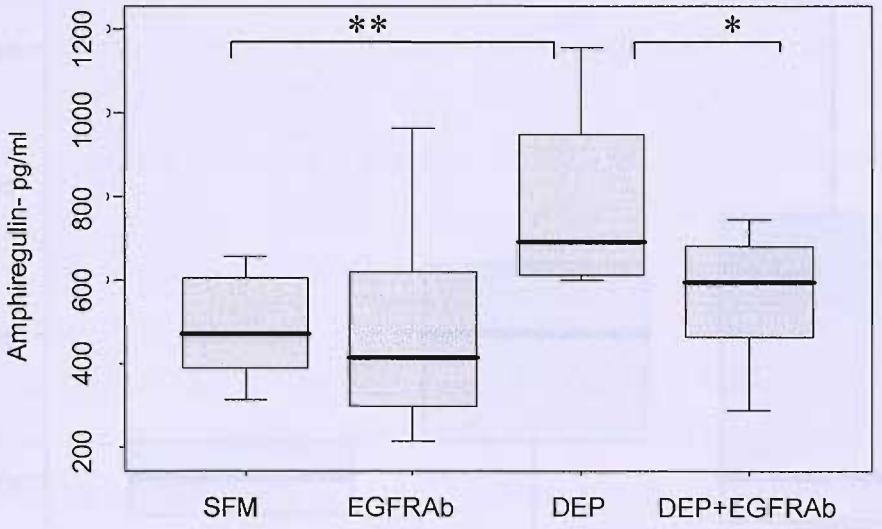


Fig 4.5 (b)

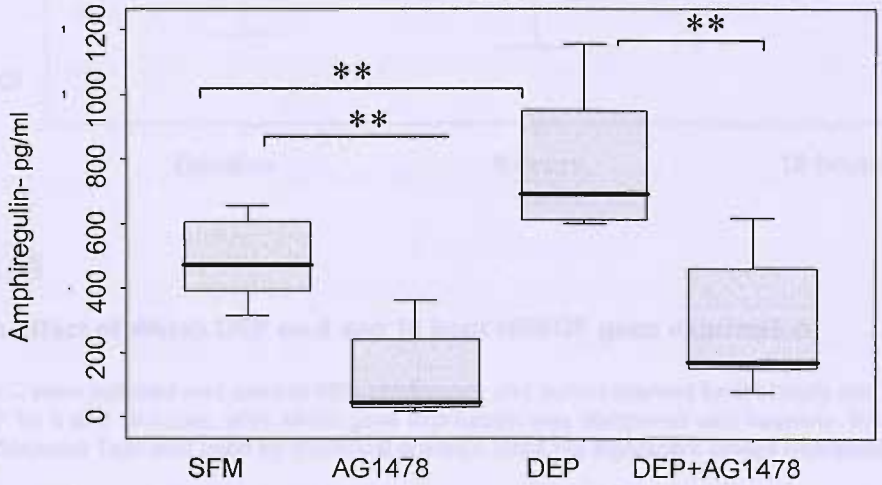


Fig 4.5 (c)

Fig 4.5

The Effect of Welsh DEP on Amphiregulin release from PBEC treated with a broad metalloproteinase inhibitor (GM6001) (a) an EGFR neutralising antibody (b) and a tyrosine kinase inhibitor (Ag1478) (c)

PBEC were used at 95% confluence, serum starved and exposed to Welsh DEP for 24 hours. Amphiregulin release was compared with SFM and DEP+inhibitors. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis. Key:SFM=serum free medium, DEP = diesel exhaust particles 50μg/ml, AG1478 = tyrosine kinase inhibitor, EGFRAb = EGFR neutralising antibody, GM6001= metalloproteinase inhibitor
* P<0.05 ** P<0.01 n=(10)

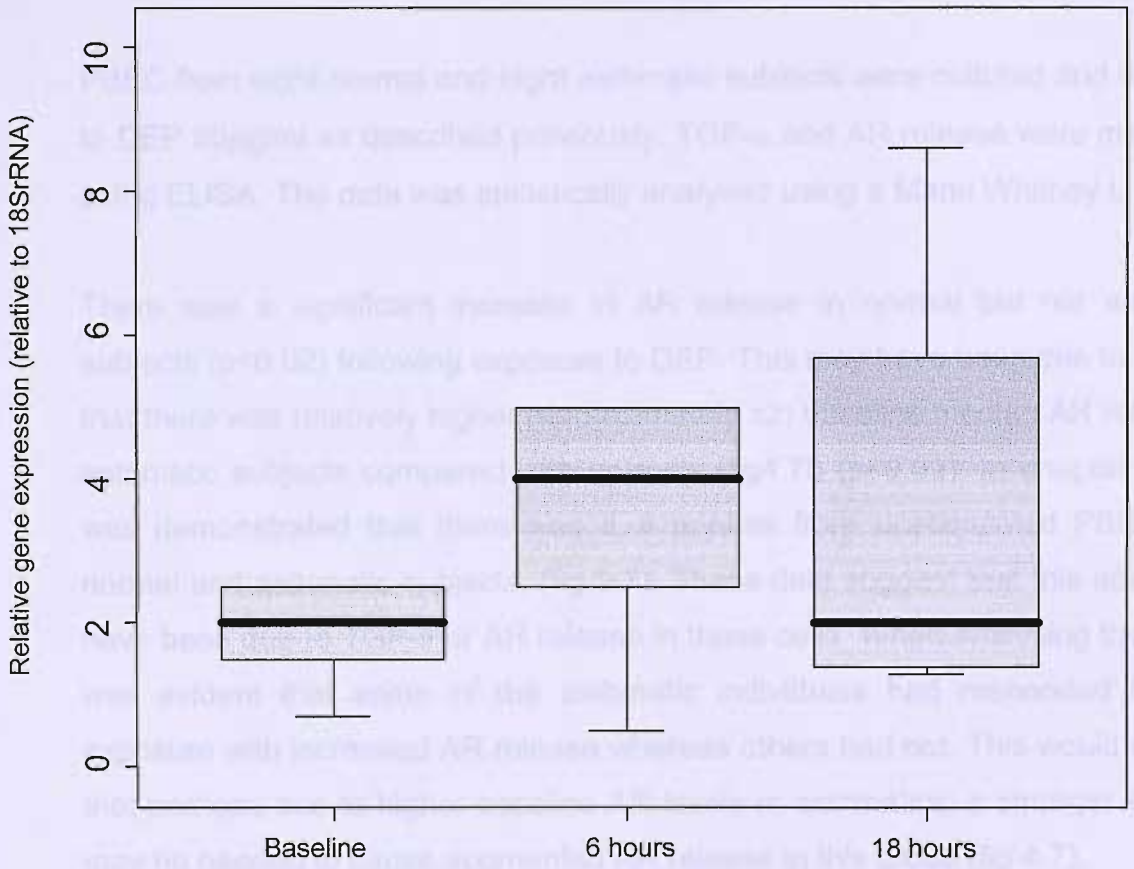


Fig 4.6

The Effect of Welsh DEP on 6 and 18 hour HBEGF gene expression

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP for 6 and 18 hours, after which gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis (data not significant unless indicated).

Key: baseline= unstimulated (t=0 hrs), DEP = diesel exhaust particles, (n=10).

4.2.3 The Effect of Normal and Asthmatic Phenotypes on DEP Induced TGF α and Amphiregulin Release From Primary Bronchial Epithelial Cells

Having determined the effect of DEP on the release of TGF-α and AR from PBEC in the previous study, it was hypothesised that the release of these growth factors would be heightened in asthmatics compared with normal subjects. Therefore, the aim of this study was to establish the effects of DEP on TGF-α and AR release from PBEC cultured from asthmatic and normal subjects.

PBEC from eight normal and eight asthmatic subjects were cultured and exposed to DEP 50μg/ml as described previously. TGF-α and AR release were measured using ELISA. The data was statistically analysed using a Mann Whitney U test.

There was a significant increase in AR release in normal but not asthmatic subjects ($p<0.02$) following exposure to DEP. This may have been due to the fact that there was relatively higher (approximately x2) baseline median AR release in asthmatic subjects compared with normals (fig4.7i) ($p<0.02$). In chapter three it was demonstrated that there was IL-8 release from unstimulated PBEC from normal and asthmatic subjects, (fig 3.7). These data suggest that this effect may have been due to TGF-*α* or AR release in these cells. When analysing the data it was evident that some of the asthmatic individuals had responded to DEP exposure with increased AR release whereas others had not. This would suggest that perhaps due to higher baseline AR levels in asthmatics, a stronger stimulus may be needed to cause augmented AR release in this group (fig 4.7).

There was a significant increase in TGF-*α* release following exposure to DEP in both the normal subjects and the asthmatic subjects (fig 4.7) ($p<0.02$), although there was no difference the two groups at baseline or following stimulation (fig 4.7). Due to the unavailability of a suitable HB-EGF antibody it was not possible to test the release of this growth factor in asthmatics and normals. These data suggest that at baseline, AR secretion is higher in asthmatics than normals and there is no difference in AR and TGF-*α* release between asthmatic and normal subjects.

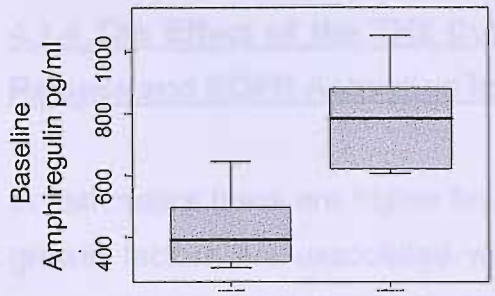
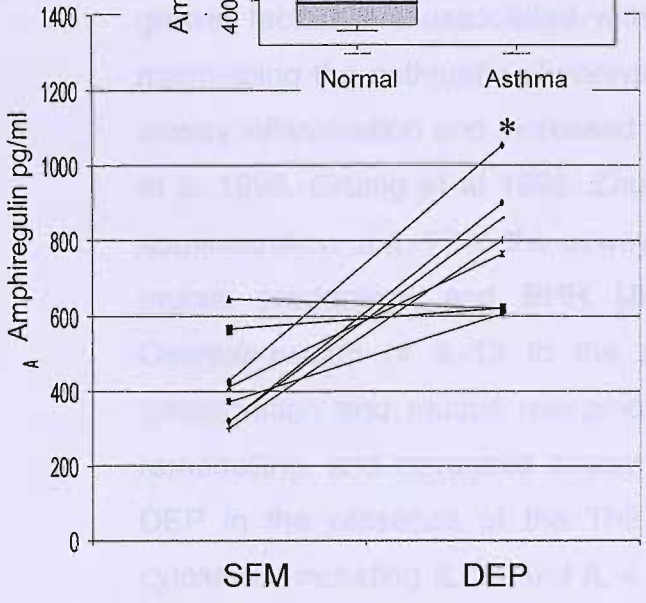
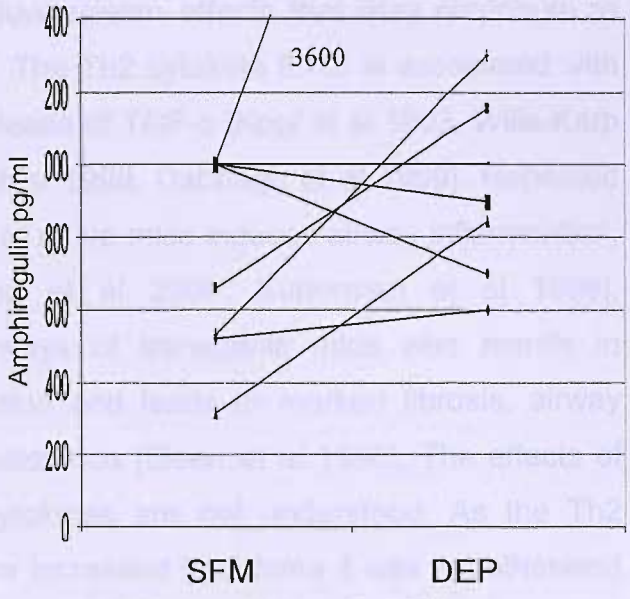


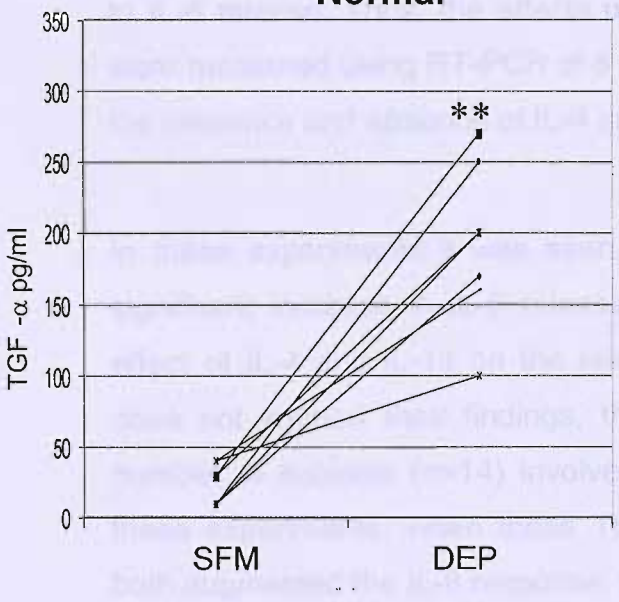
Fig 4.7i



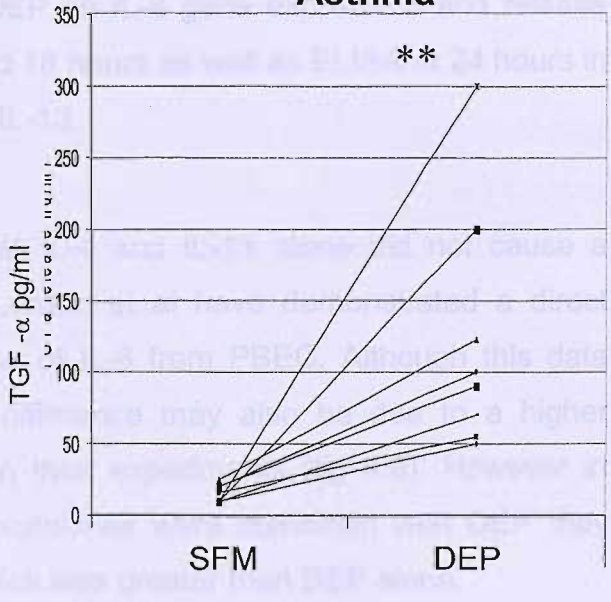
(a) Amphiregulin Release: Normal



(b) Amphiregulin Release: Asthma



(c) TGF-α Release: Normal



(d) TGF-α Release: Asthma

Fig 4.7

The effect of normal and asthmatic phenotypes on DEP induced 24 hour amphiregulin and TGF-α release from PBEC

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP for 24 hours, after which TGF -α release was measured. Wilcoxon Test was used for statistical analysis.
Key:SFM=serum free medium, DEP = diesel exhaust particles 50μg/ml,
*** P<0.05 ** P<0.02 (n=8)**

4.2.4 The Effect of the TH2 Cytokines IL-4 and IL-13 on DEP Induced IL-8 Release and EGFR Activation in PBEC

In asthmatics there are higher levels of the Th2 cytokines, IL-4 and IL-13. These growth factors are associated with downstream effects that may contribute to maintaining the asthmatic phenotype. The Th2 cytokine IL-13 is associated with airway inflammation and increased release of TGF-α [Kopf et al 1993, Wills-Karp et al 1998, Grunig et al 1998, Zhu et al 1999, Dabbagh et al 1999]. Repeated administration of IL-13 to the airways of naïve mice induces airway inflammation, mucus production, and BHR [Jiang et al 2000, Kuperman et al 1998]. Overexpression of IL-13 in the airways of transgenic mice also results in inflammation and mucus overproduction and leads to marked fibrosis, airway remodelling, and increased airway resistance [Eisen et al 1998]. The effects of DEP in the presence of the Th2 cytokines are not understood. As the Th2 cytokines, including IL-13 and IL-4 are increased in asthma it was hypothesised that DEP may act synergistically with the TH2 cytokines, IL-4 and IL-13 in leading to IL-8 release. Thus, the effects of DEP on IL-8 gene expression and release were measured using RT-PCR at 6 and 18 hours as well as ELISA at 24 hours in the presence and absence of IL-4 and IL-13.

In these experiments it was seen that IL-4 and IL-13 alone did not cause a significant increase in IL-8 release. Lordan et al have demonstrated a direct effect of IL-4 and IL-13 on the release of IL-8 from PBEC. Although this data does not support their findings, the difference may also be due to a higher number of subjects (n=14) involved in their experiments (fig 4.8). However in these experiments, when these Th2 cytokines were combined with DEP they both augmented the IL-8 response, which was greater than DEP alone.

The addition of IL-4 to DEP caused an increase in median IL-8 release (IL-4 alone=6 ng/ml, DEP alone=8.5 ng/ml and DEP+ IL-4 =15 ng/ml n=8, ($p<0.05$)) (fig 4.8). The addition of IL-13 to DEP also caused an increase in median IL-8 response (IL-13 alone = 8 ng/ml, DEP alone = 11 ng/ml, DEP+IL-13 = 20 ng/ml (n=6, $p<0.05$, fig 4.8).

Having demonstrated that the TH2 cytokines IL-4 and IL-13 have an additive effect on IL-8 release, it was hypothesised that these cytokines may also lead to augmented EGFR ligand release. Therefore gene expression for TGF-α, AR and HBEGF as well as TGF-α and AR protein levels in the presence and absence of IL-4 and IL-13 were measured.

Consistent with findings in the literature, IL-4 did not cause an increase in TGF-α gene or protein expression, (fig 4.9), whereas IL-13 led to an increase in TGF-α protein levels [Lordan et al 2002, Booth et al 2001] (fig 4.10). Both IL-4 and IL-13 caused an increase in amphiregulin protein but not gene expression (fig 4.11 and 4.12). Neither IL-4 nor IL-13 affected HB-EGF gene expression (fig 4.13 and 4.14). The combination of DEP with IL-4 or IL-13 did not affect TGF-α or amphiregulin gene expression (at 6 or 18 hours), or protein expression from PBEC compared to DEP alone at 24 hours (figs 4.9-4.12). Due to a lack of suitable antibodies for HBEGF, protein levels for this growth factor were not determined by ELISA.

These data suggest that the Th2 cytokines IL-4 and IL-13 have an additive effect in combination with DEP on IL-8 release. However, although DEP itself caused an increase in EGFR ligand expression, this effect was not increased in combination with the TH2 cytokines, IL-4 or IL-13.

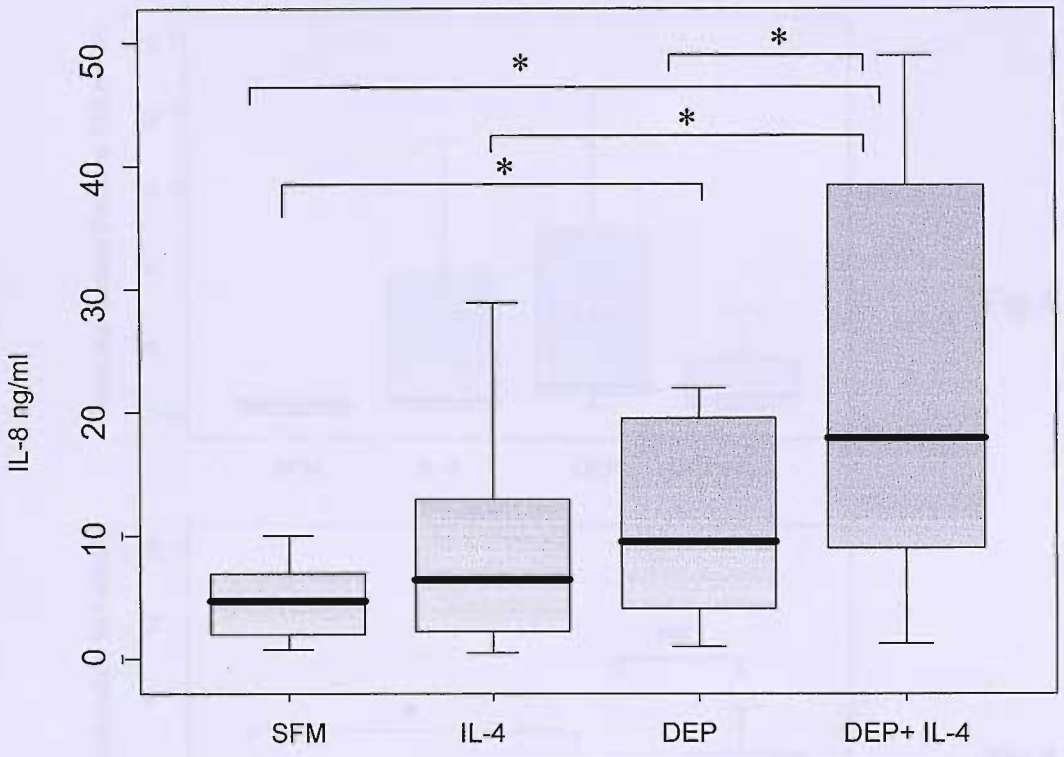


Fig 4.8 (a)

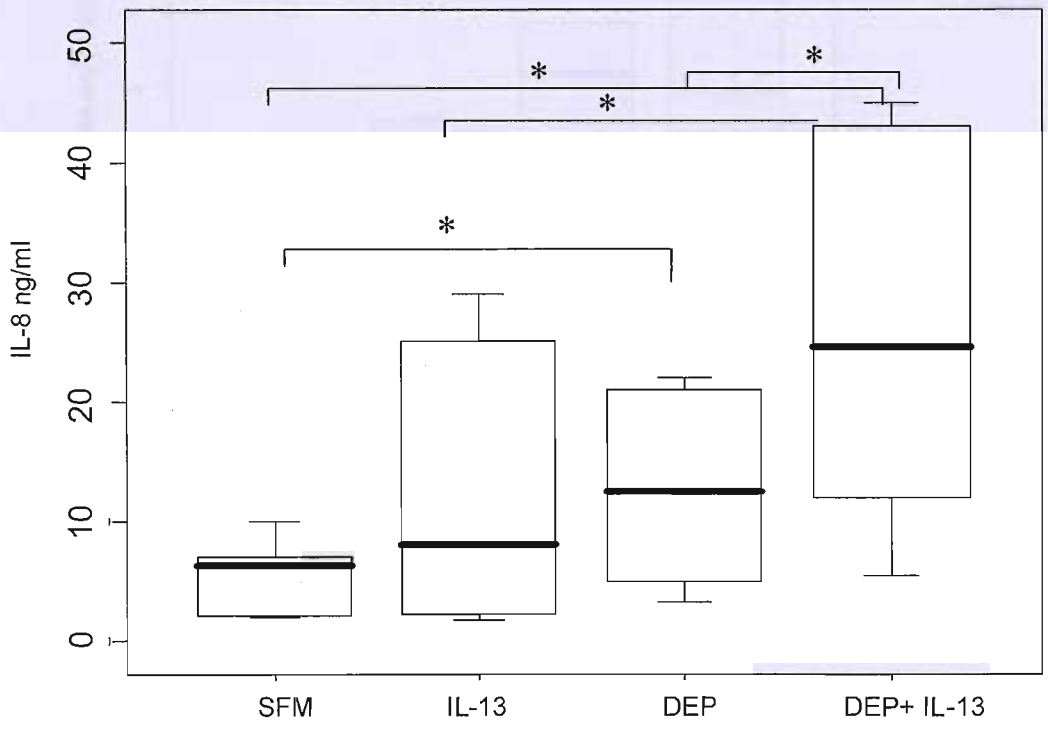


Fig 4.8 (b)

Fig 4.8

The effect of IL-4 (a) and IL-13 (b) on DEP induced IL-8 release from PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with or without IL4 or Il13 for 24 hours, after which IL8 release was measured. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles,
* $P<0.05$ (n=6)

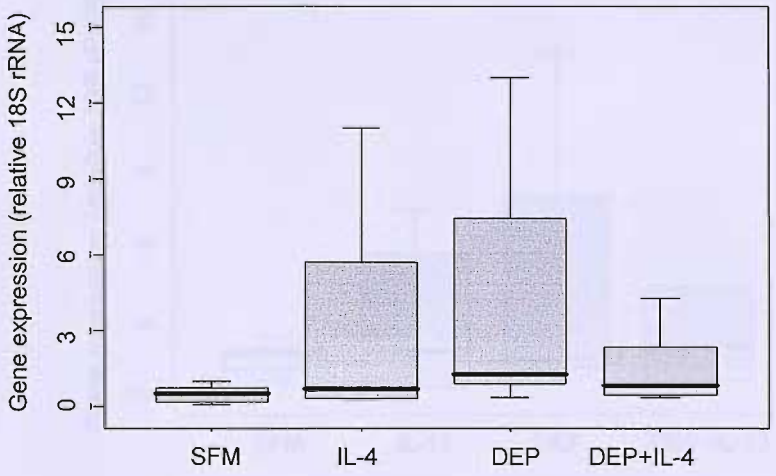


Fig 4.9 (a)

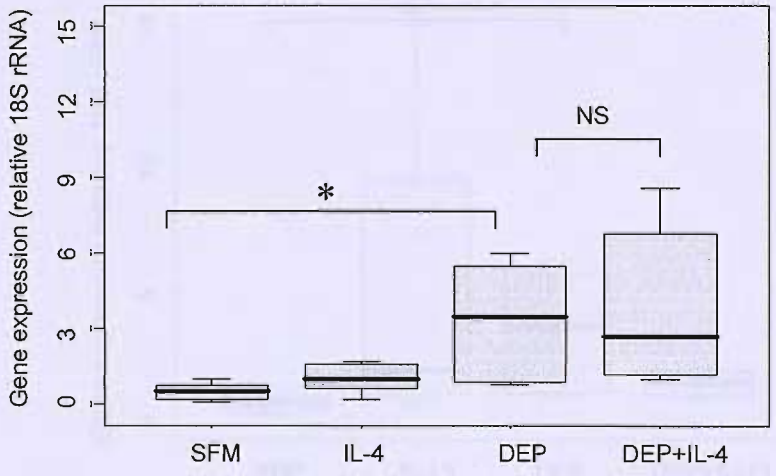


Fig 4.9 (b)

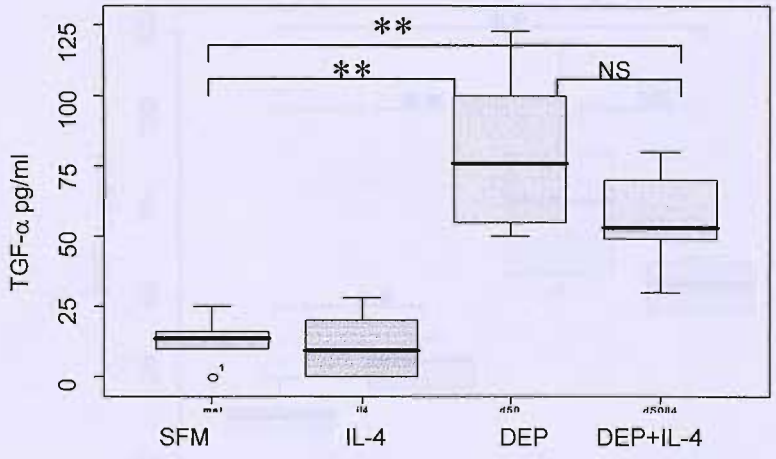


Fig 4.9 (c)

Fig 4.9

The effect of IL-4 on 6 hr (a) & 18 hr (b) DEP induced TGF-α gene expression and 24 hour protein release (c) in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with or without Il4 or Il13 for 24 hours, after which TGF - α gene expression was measured at 6 and 18 hours. Protein release was measured. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.
Key: SFM=serum free medium, DEP = diesel exhaust particles
* P<0.05 ** P<0.01 (n=8)

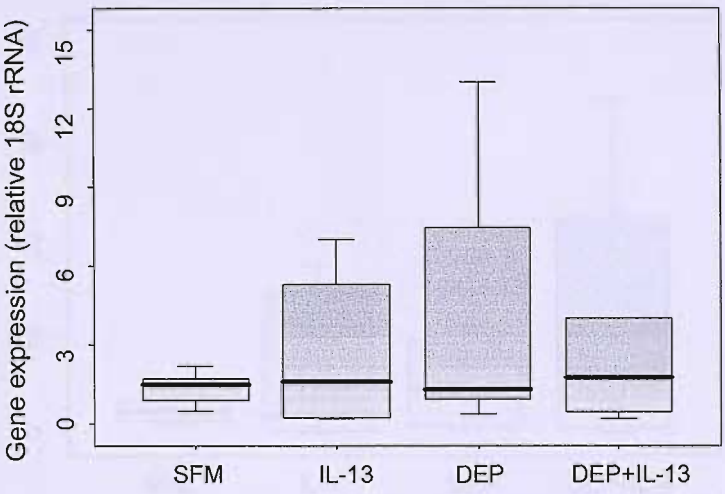


Fig 4.10 (a)

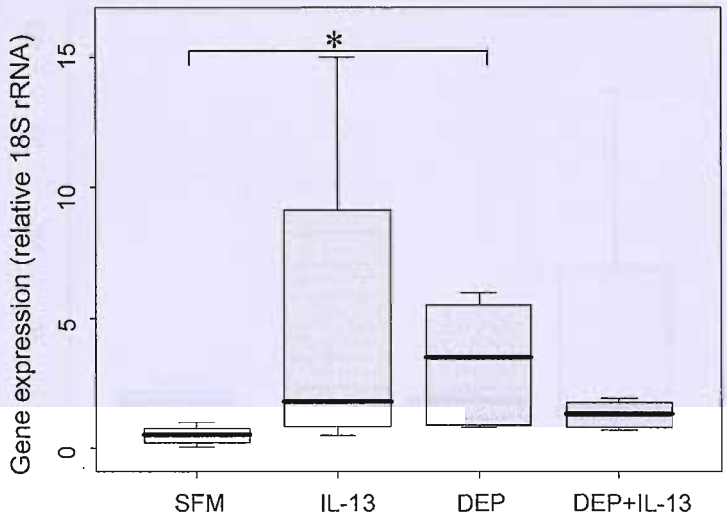


Fig 4.10 (b)

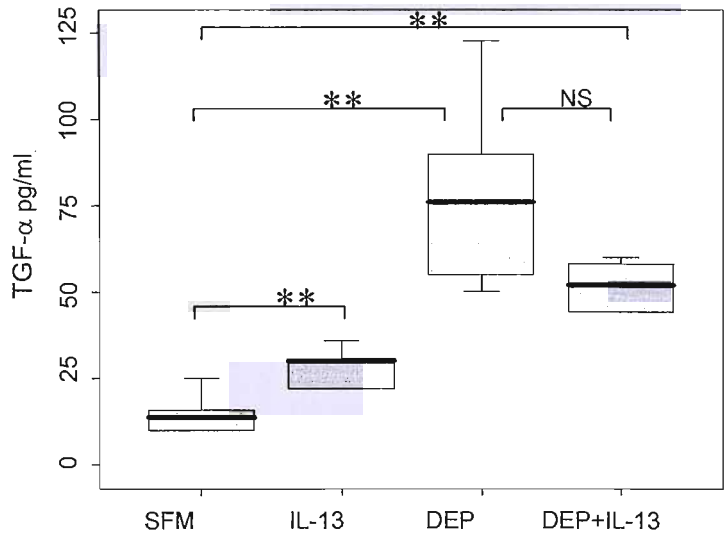


Fig 4.10 (c)

Fig 4.10

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced TGF-α gene expression and 24 hour protein release (c) in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which gene expression was measured.protein release was measured at 24 hours Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles

* P<0.05 ** P<0.01 (n=8)

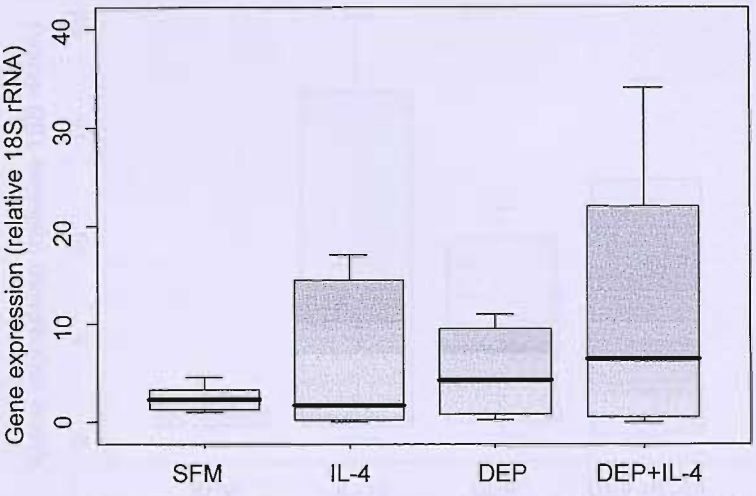


Fig 4.11 (a)

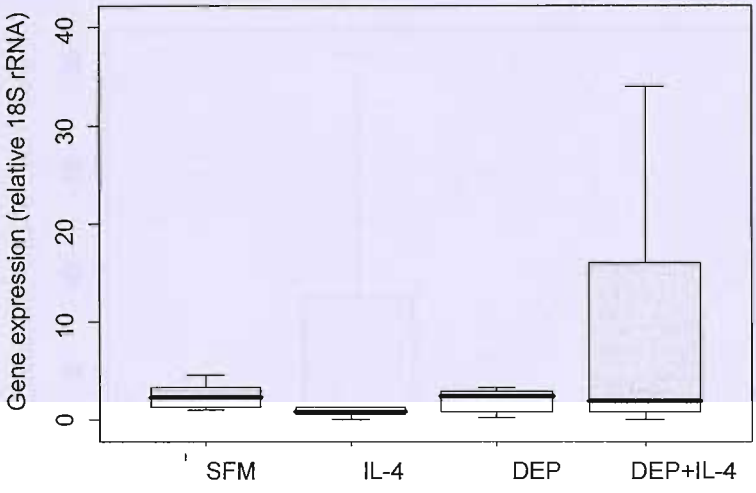


Fig 4.11 (b)

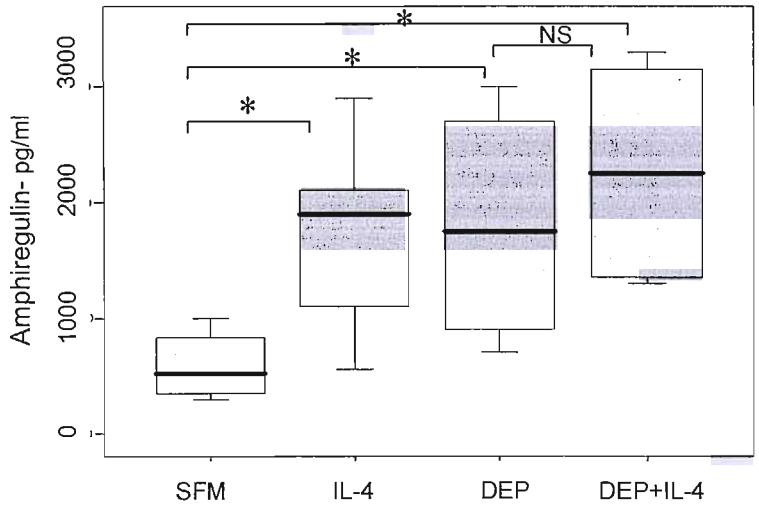


Fig 4.11 (c)

Fig 4.11

The effect of IL-4 on 6 hr (a) & 18 hr (b) DEP induced AR gene expression and 24 hour protein release (c) in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL4 for 6 and 18 hours, after which gene expression was measured.protein release was measured at 24 hours Kruskal wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles * P<0.001 (n=8)

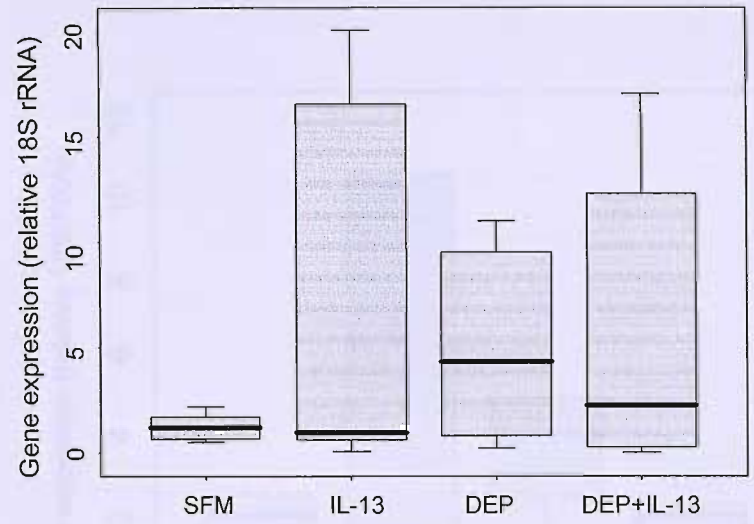


Fig 4.12 (a)

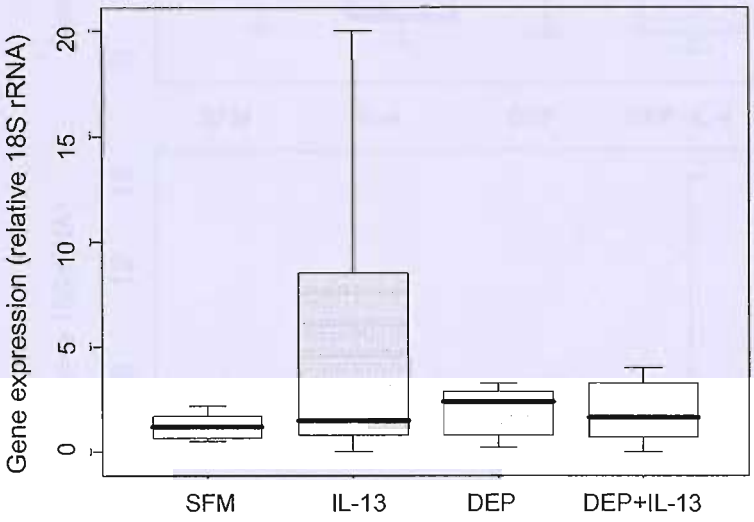


Fig 4.12 (b)

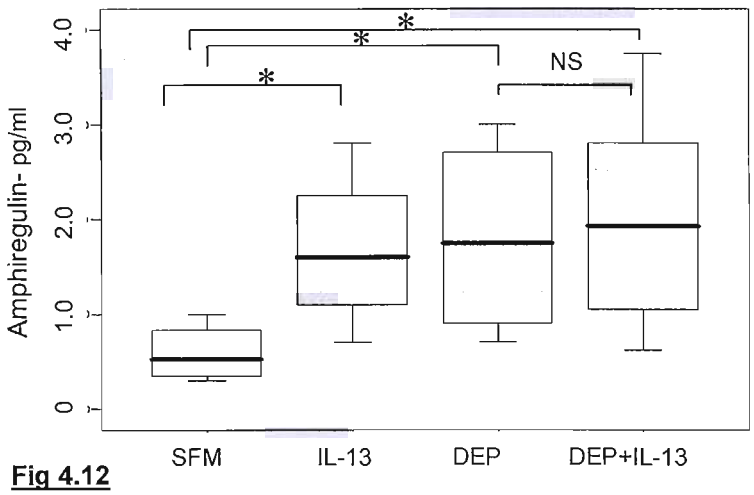


Fig 4.12 (c)

Fig 4.12

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced AR gene expression and 24 hour protein release (c) in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL-13 for 6 and 18 hours, after which gene expression was measured.protein release was measured at 24 hours Kruskal Wallis by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles * P<0.001 (n=8)

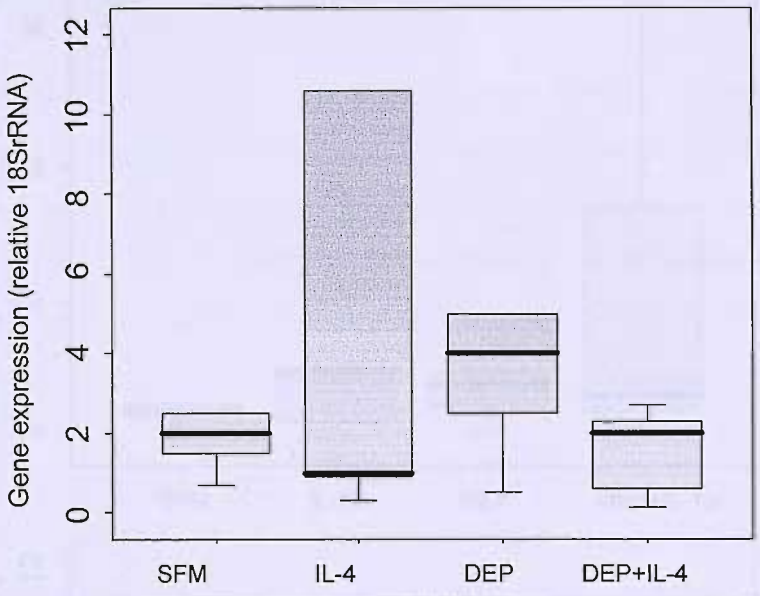


Fig 4.13 (a)

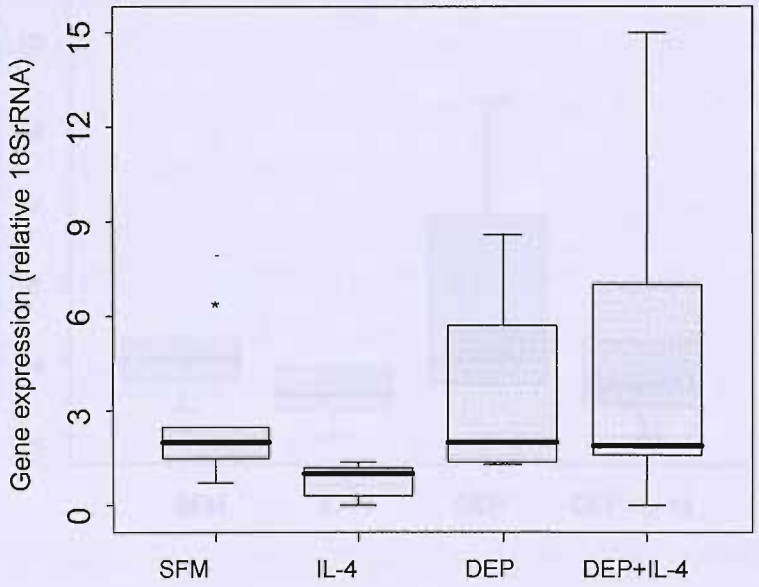


Fig 4.13 (b)

Fig 4.13

The effect of IL-4 on 6 hr (a) & 18 hr (b) DEP induced HB-EGF gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL-4 for 6 and 18 hours, after which gene expression was measured. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles (n=8)

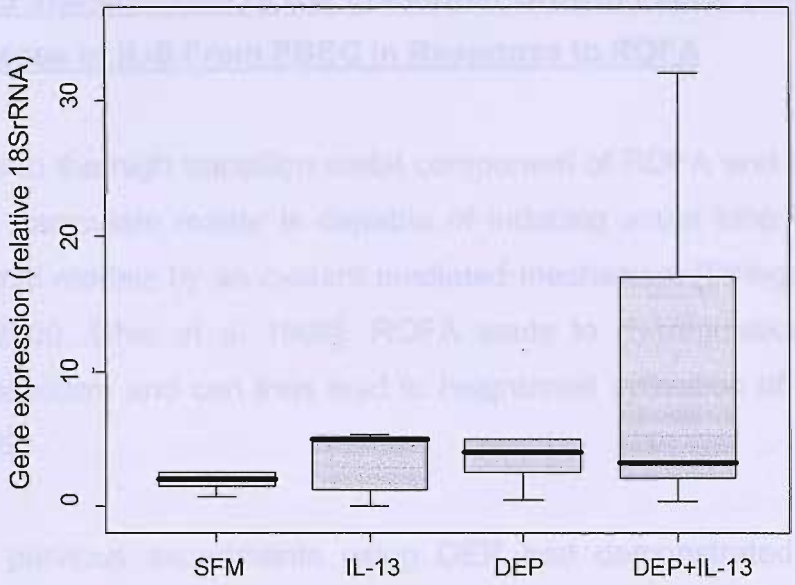


Fig 4.14 (a)

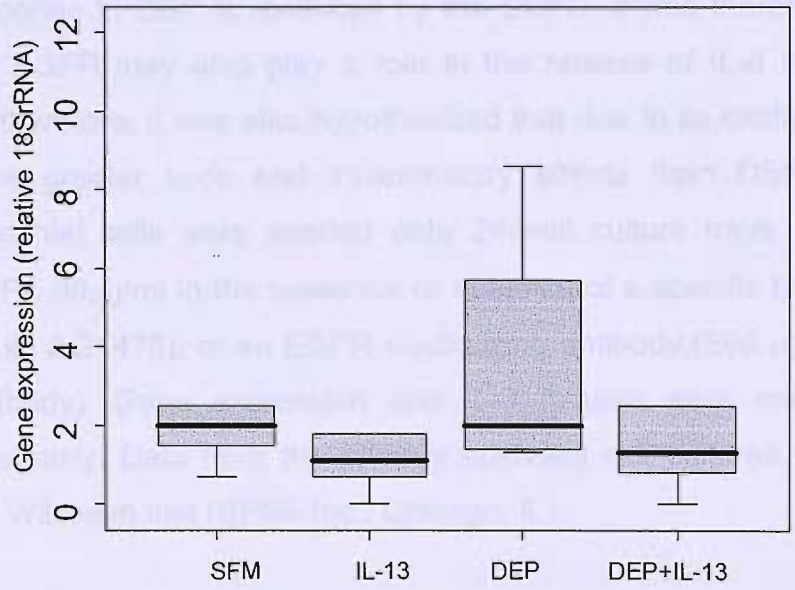


Fig 4.14 (a)

Fig 4.14

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced HB-EGF gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL-4 for 6 and 18 hours, after which gene expression was measured. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, DEP = diesel exhaust particles (n=8)

4.2.5 The Function of the Epidermal Growth Factor Receptor (EGFR) in the Release of IL-8 From PBEC in Response to ROFA

Due to the high transition metal component of ROFA and in particular vanadium this particulate matter is capable of inducing acute lung injury in experimental animal models by an oxidant mediated mechanism [Stringer et al 1998, Jiang et al 2000, Ghio et al 1999]. ROFA leads to dysregulation of phosphotyrosine metabolism and can thus lead to heightened activation of the EGFR [Ghio et al 1999].

As previous experiments using DEP had demonstrated that IL-8 release in response to DEP is mediated by the EGFR, it was therefore hypothesised that the EGFR may also play a role in the release of IL-8 in response to ROFA. Furthermore, it was also hypothesised that due to its oxidative effect ROFA may have greater toxic and inflammatory effects than DEP on PBEC. Primary bronchial cells were seeded onto 24-well culture trays, exposed to BEBM ± ROFA 50μg/ml in the presence or absence of a specific tyrosine kinase inhibitor (1 μM AG1478), or an EGFR neutralizing antibody (500 μg/ml sheep anti-EGFR antibody). Gene expression and IL-8 release were measured as described previously. Data from the primary epithelial cell cultures were compared using the Wilcoxon test (SPSS Inc., Chicago, IL).

As in previous experiments, ROFA led to increased release of IL-8 from PBEC (fig 4.15) The addition of EGFR neutralising antibody led to a significant reduction in IL-8 levels (ROFA: IL-8 release = 9 ng/ml (IQR=9-17ng/ml, ROFA + Floss: IL-8 release = 4ng/ml (IQR=3-7ng/ml, n=5, $p<0.02$) (fig 4.15), suggesting that the release of IL-8 is dependent on ligand binding to the EGFR. Interestingly, inhibition of the downstream effects of the EGFR using a specific tyrosine kinase inhibitor (AG1478) did not lead to a significant change in IL-8 release in response to ROFA. This observed lack of inhibition of IL-8 release may however be due to the strong oxidant potential of this particulate, which leads to dysregulation of phosphotyrosine metabolism of the EGFR, this in turn inhibits the return of the

EGFR to its resting state following phosphorylation, which can maintain the EGFR in an activated state.

As the effects of ROFA on IL8 release were shown to be dependent on EGFR binding, it was thus further hypothesised that this effect may be mediated by the release of an EGFR ligand. Therefore the effect of ROFA on TGFα, amphiregulin and HBEGF expression were investigated.

TGF-α gene expression was not significantly increased at 6 hrs but did increase 3-fold 18hrs after stimulation with ROFA (median baseline= 1.2, (IQR= 0.8–2.1), median ROFA after 18hr exposure = 3 (IQR=0.8-25), n=8 $P < 0.05$) (fig 4.16). There was also enhanced secretion of TGF-α into the culture medium (untreated cells = 0.02ng/ml (IQR=0.01-0.03), ROFA = 0.67 ng/ml (IQR=0.65-0.8), n=8, $p<0.005$ (fig 4.16)). Amphiregulin mRNA expression was also significantly increased at 6 hrs (untreated cells median gene expression= 1.2 (IQR 0.5-2.0), ROFA 6hrs = 4.2 (IQR 0.5-9.0), n=8, $p<0.05$, (fig 4.17)). There was also enhanced secretion of AR into the culture medium at 24 hours (untreated cells = 0.5ng/ml, (IQR=0.35-0.8), ROFA = 0.8 ng/ml (IQR=0.7-1.3), n=8, $p<0.003$) (fig 4.17). As with AR, HBEGF mRNA levels increased after 6hrs, (baseline= 2, (IQR= 1.5–2.5), 6 hrs ROFA exposure = 5 (IQR=3-5), n=8 $P = 0.003$) (fig 4.18). These results suggest that exposure of PBEC to ROFA leads to induction of gene expression for TGFα, AR and HBEGF, as well as increased release of TGF-α and AR in culture medium.

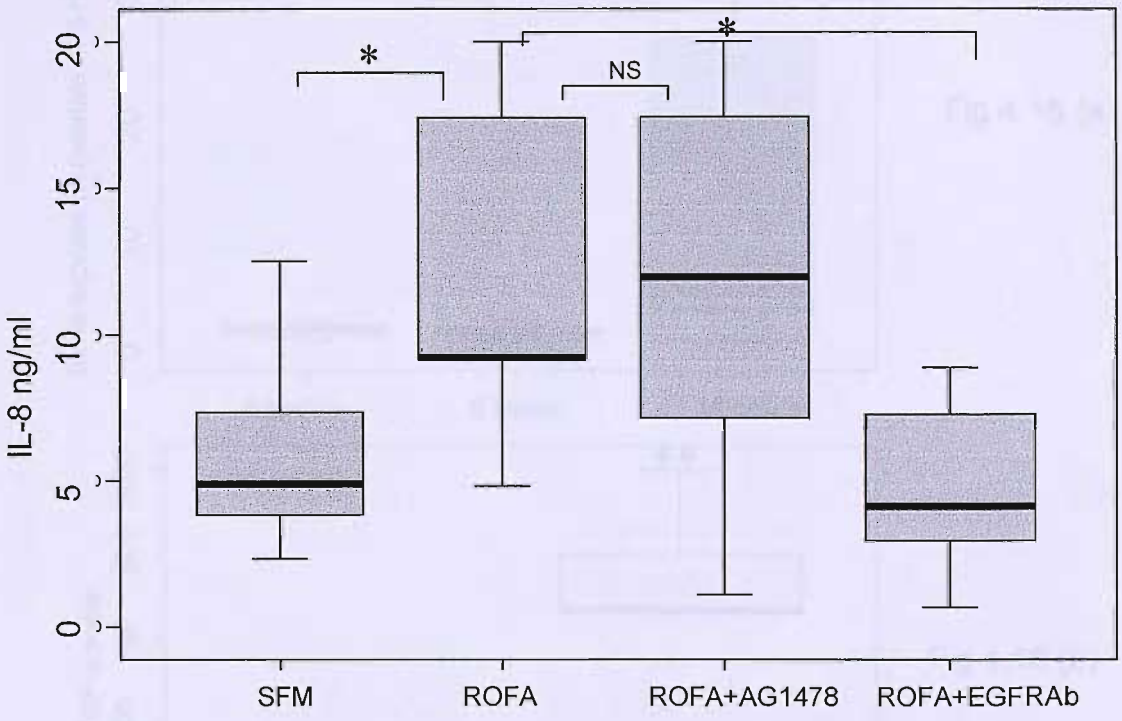


Fig 4.15

The Effect of ROFA on IL-8 release from PBEC treated with a tyrosine kinase inhibitor (Ag1478) and an EGFR neutralising antibody (b)

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to ROFA for 24 hours, after which IL-8 release was measured. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, ROFA = Residual Oil Fly Ash 50μg/ml , AG1478 = tyrosine kinase inhibitor, EGFRAb = EGFR neutralising antibody,
*** P<0.02 (n=8).** (Data courtesy of Dr L Hamilton March 2004)

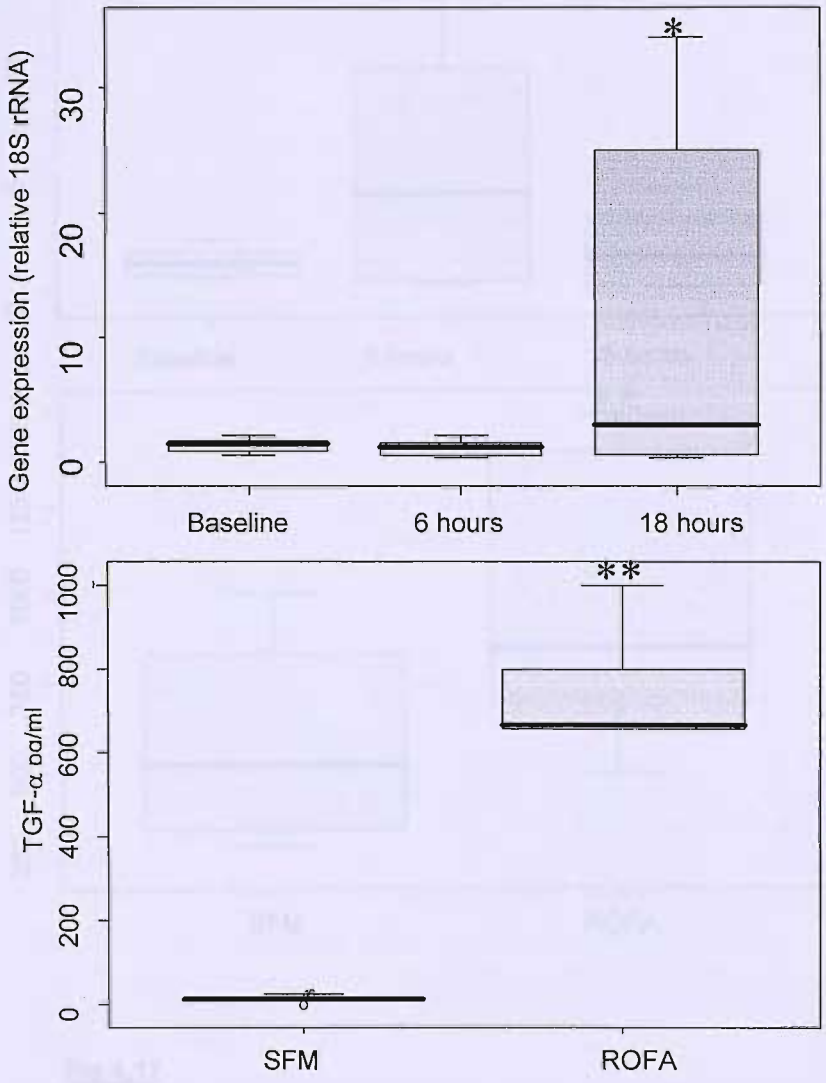


Fig 4.16 (a)

Fig 4.16 (b)

Fig 4.16

The Effect of ROFA on TGF- α 6 and 18 hr gene expression (a) and 24 hour release (b) from PBEC

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to ROFA for 24 hours, after which TGF- α gene expression was measured at 6 and 18 hours. Protein release was measured at 24 hours. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, Baseline = unstimulated (t=0 hrs) **ROFA = Residual Oil Fly Ash 50μg/ml ,**
*** P<0.05 ** P<0.005 (n=8).**

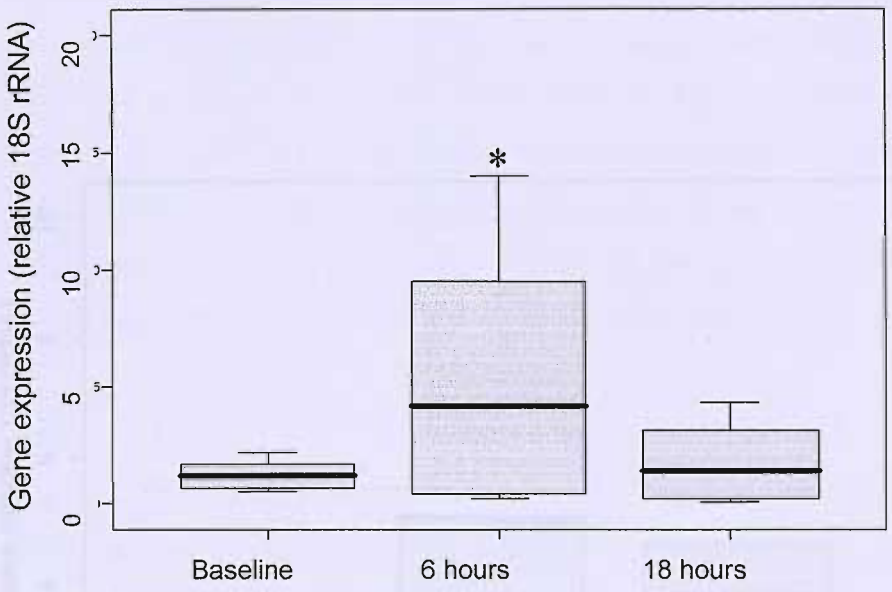


Fig 4.17 (a)

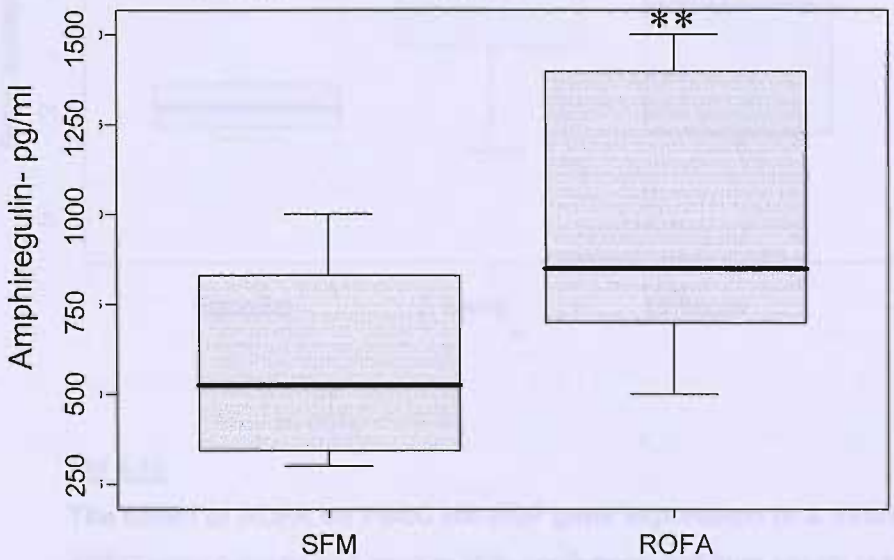


Fig 4.17 (a)

Fig 4.17

The Effect of ROFA on AR 6 and 18 hr gene expression (a) and 24 hour release (b) from PBEC

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to ROFA for 24 hours, after which amphiregulin gene expression was measured at 6 and 18 hours. Protein release was measured at 24 hours. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: SFM=serum free medium, Baseline = unstimulated (t=0 hrs) **ROFA = Residual Oil Fly Ash 50μg/ml ,**
*** P<0.05 ** P<0.003 (n=8).**

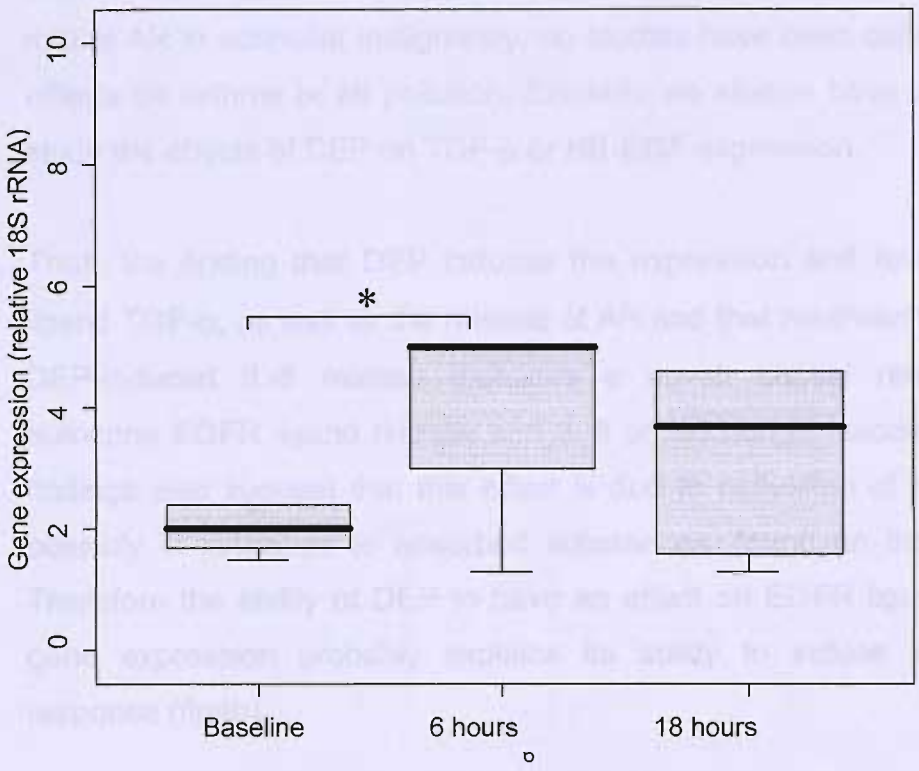


Fig 4.18

The Effect of ROFA on PBEC HB-EGF gene expression (6 & 18hrs)

PBEC were cultured and used at 95% confluence and then serum starved for 24 hours. The cells were then exposed to ROFA for a further 6, 18 hours after which HBEGF gene expression was measured (n=8). Kruskall Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: Baseline = gene expression at t=0, ROFA = Residual oil fly ash 50ug/ml

* = P = 0.003

4.3 Discussion

Although, the production of IL-8 by bronchial epithelial cells in response to DEP is well recognized, no studies had previously explored the EGFR as a mediator of DEP-induced IL-8 release. The effects of DEP on TGF-α, AR and HB-EGF expression from PBEC had also not been investigated. Despite knowledge of the role of AR in epithelial malignancy, no studies have been carried out to study its effects on asthma or air pollution. Similarly, no studies have been carried out to study the effects of DEP on TGF-α or HB-EGF expression.

Thus, the finding that DEP induces the expression and release of the EGFR ligand TGF-α, as well as the release of AR and that neutralizing antibodies block DEP-induced IL-8 release indicates a novel, causal relationship between autocrine EGFR ligand release and IL-8 production in response to DEP. These findings also suggest that this effect is due to activation of metalloproteinases, possibly in response to adsorbed substances, found on the surface of DEP. Therefore the ability of DEP to have an effect on EGFR ligand processing and gene expression probably explains its ability to induce a pro-inflammatory response (fig4b).

The observation that AG1478 blocked DEP-induced IL-8 release more efficiently than EGFR neutralizing antibody, suggests that stimulation of EGFR phosphorylation may also occur by an alternative, ligand-independent mechanism such as oxidant-mediated effects which has previously been reported et al Zwick 1999], as well as by direct autocrine ligand binding. This is a possibility with DEP, which is known to have oxidant effects due to adsorbed substances found on its surface.

These studies are the first to indicate a direct effect of DEP on EGFR ligand expression. Based on the observation that the DEP mediated increased transcription of TGF-α mRNA preceded IL-8 release at 24 hrs, it is possible that this ligand may be a primary mediator of the DEP-induced responses. As there was an increase in IL-8 mRNA at 18hours, alternatively it may be that IL-8 release is also mediated through another separate pathway, such as activation of

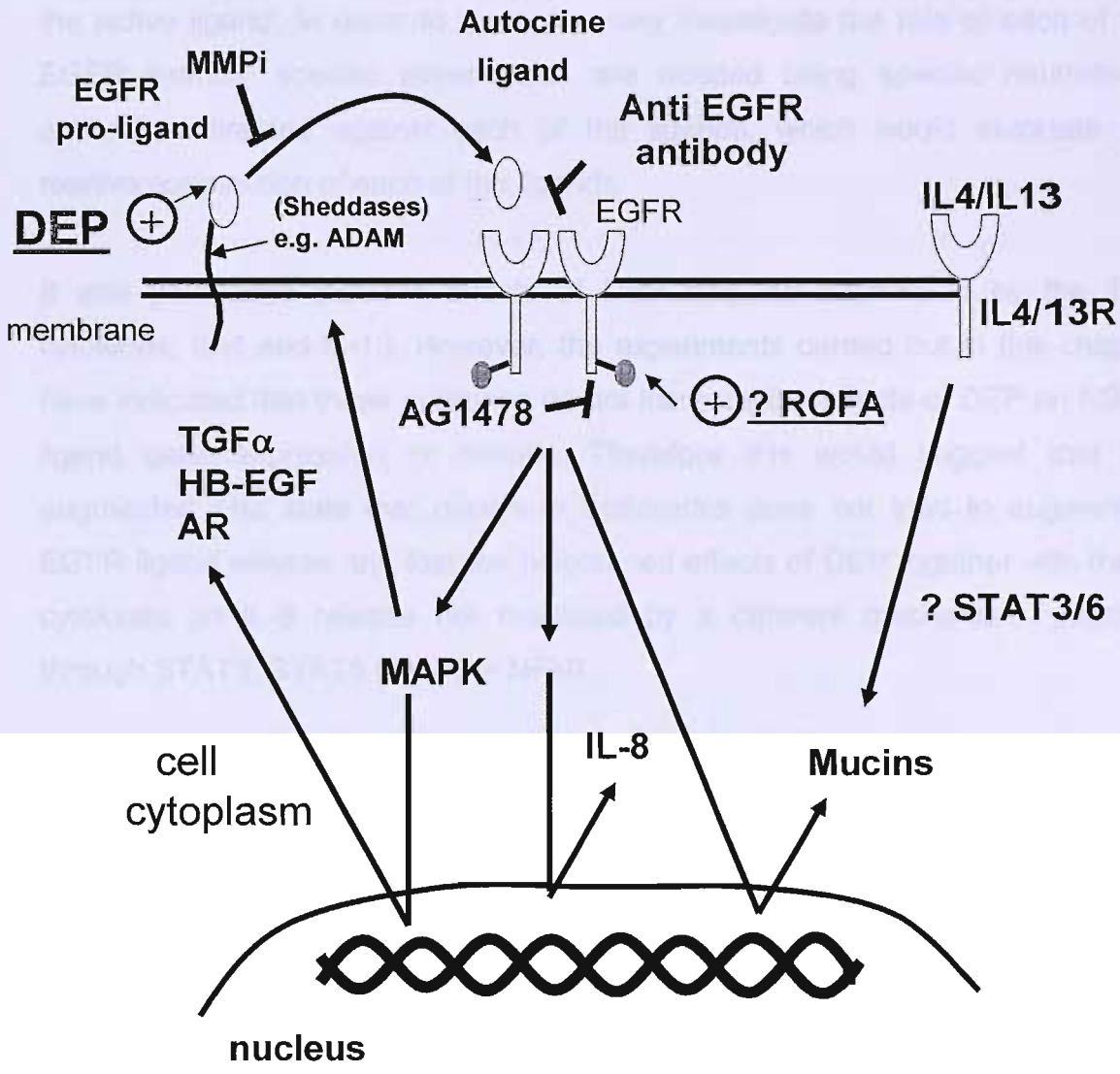


Fig 4b) The effect of DEP and ROFA on the release of EGFR ligands

DEP activates the EGFR by the release of EGFR ligands. ROFA has a heightened effect compared with DEP possibly due to its effects on the dephosphorylation of

INHIBITORS (in red):
MMPi = broad inhibitor of metalloproteinase activity, Anti EGFR antibody, AG1478= inhibitor of tyrosine phosphorylation

NFkB. Although there was also augmented AR release in response to DEP, however, because blocking antibodies to the EGFR caused a preferential accumulation of TGF-α in the culture medium, it was concluded that this may be the active ligand. In order to more precisely investigate the role of each of the EGFR ligands, specific experiments are needed using specific neutralising antibodies directed against each of the ligands, which would elucidate the relative contribution of each of the ligands.

It was postulated that the effects of DEP may be augmented by the TH2 cytokines, IL-4 and IL-13. However, the experiments carried out in this chapter have indicated that these cytokines do not increase the effects of DEP on EGFR ligand gene expression or release. Therefore this would suggest that the augmented Th2 state that occurs in asthmatics does not lead to augmented EGFR ligand release and that the heightened effects of DEP together with these cytokines on IL-8 release are mediated by a different mechanism, possibly through STAT3, STAT6 (fig4b) or NFkB.

ROFA has a number of physical and chemical properties (discussed further in chapters one and three), which differentiate it from DEP and may lead to alternative responses on EGFR activation. In brief, ROFA is a principally inorganic particulate with a high concentration of transition metals and in particular vanadium, whereas DEP is an organic particulate with a lower transition metal content. It was demonstrated that the release of IL-8 in response to ROFA is dependent on the EGFR and that ROFA leads to increased gene expression for amphiregulin, TGF-α and HB-EGF as well as the release of amphiregulin and TGF-α in PBEC. The release of amphiregulin was similar in magnitude to that observed with DEP, whereas ROFA led to an approximate x10 increase in TGF-α, compared with DEP. Although it was demonstrated that IL-8 release in response to ROFA is dependent on the EGFR, surprisingly, inhibition of EGFR kinase activity using AG1478 did not effect IL-8 release. It is postulated that this effect may be due to an inhibitory effect of ROFA on the de-phosphorylation of the tyrosine kinase component of the EGFR to resting state following phosphorylation. Therefore, ROFA and DEP both lead to an increase in

the release of the EGFR ligands TGF-α and amphiregulin, however ROFA has an augmented effect on the release of TGF-α as well as the induction of gene expression for amphiregulin and HBEGF, which would suggest that this effect is mediated by the components of ROFA such as transition metals. (fig4b).

Ligand-independent transactivation of the EGFR has been shown to take place in response to reactive oxygen species produced by neutrophils [Takeyma et al 2000]. Activation of the EGFR has been shown to lead to goblet cell production as well as excess mucus secretion [Takeyama et al 2001a]. Although not studied with DEP, studies with cigarette smoke extract have shown that, antioxidants only partially inhibit epithelial cell mucin synthesis [Takeyama et al 2001a], suggesting that other mechanisms also contribute to this response. As TGF-α has been shown to be involved in the process of goblet cell differentiation [Booth et al 2001], these studies suggest that the ability of DEP to induce expression and release of TGF-α provides a direct mechanism whereby DEP may affect epithelial function, which may contribute to mucus hypersecretion that is a prominent feature of asthma and leads to both significant disease morbidity and mortality.

EGFR ligands are involved in a number of effects that could also potentially lead to the effects seen in chronic asthma. TGF-*α* is associated with mucin hypersecretion and pulmonary fibrosis as well as branching morphogenesis during lung development [Baughman et al 1999, Korfhagen et al 1994 Hardie et al 2001, Le Cras et al 2004]. Amphiregulin (AR) is an epidermal growth factor (EGF)-related peptide that can bind to heparin and operates exclusively through the EGFR. Studies have shown that this peptide is expressed in PBEC [Polosa et al 1999, Asano et al 1997]. Increased levels of this growth factor are associated with malignancy in the breast [Ma et al 2001]. HB-EGF, another EGFR ligand, has been shown to mediate mucin transcription [Basbaum et al 2002] and fibroblast proliferation [Nolan et al 2004]. This growth factor has been shown to be upregulated by vanadium, through an oxidant dependent mechanism, as well as cigarette smoke extract [Ingram et al 2003].

In conclusion, in this chapter it has been demonstrated that DEP utilizes the EGFR to stimulate IL-8 release through shedding of autocrine EGFR ligands. The ability of DEP to induce expression and release of ligands for the EGFR suggests that it may have other direct effects on epithelial function. For example, release of EGFR ligands may have other paracrine effects on underlying mesenchymal cells linked to airway remodelling or on epithelial function such as goblet cell hyperplasia.

CHAPTER FIVE

A STUDY OF THE EFFECTS OF DEP & ROFA ON MUCIN GENE EXPRESSION IN PRIMARY BRONCHIAL EPITHELIAL CELLS

5.1 Introduction

In the previous chapter, it was demonstrated that DEP activates the EGFR to stimulate IL-8 release through shedding of EGFR ligands that act in an autocrine fashion. Among other things activation of this receptor has been demonstrated to regulate mucin synthesis [Takeyama et al 2000, 2001].

The major function of mucus is to protect the lung through mucociliary clearance against foreign particles and chemicals entering the lung. Mucins are large, heavily glycosylated glycoproteins that are expressed in two major forms: the membrane-bound mucins and the gel forming mucins [Davies et al 2002, Dekker et al 2002]. In the airways, MUC1 and MUC4 are the predominant membrane-bound mucins that are present on epithelial cell surfaces; MUC5AC, MUC5B and MUC2 are the predominant gel forming mucins that contribute to the mucus gel [Davies et al 2002, Dekker et al 2002].

Evidence for the regulatory role of EGFR in mucin production has come from studies on human cell lines as well as animal studies. As described in chapter one, H292 cells express the EGFR constituently and treatment of these cells with the EGFR ligand, TGFα, leads to an increase in MUC5AC gene and protein expression [Takeyama et al 1999]. EGFR tyrosine kinase inhibitors selectively block this effect [Takeyama et al 1999]. The inflammatory cytokine, TNFα together with TGFα can lead to goblet cell metaplasia, suggesting that this effect is augmented in the presence of inflammatory cytokines. This can be inhibited by selective EGFR tyrosine kinase inhibitors [Takeyama et al 1999] (fig5a).

Healthy individuals have few goblet cells in their airways, but in patients' with hypersecretory diseases, including asthma, goblet-cell upregulation results in mucus hypersecretion, airway plugging, and in some cases death. Multiple stimuli produce mucus hypersecretion via EGFR expression and activation, as well as goblet-cell metaplasia. Cigarette smoke has been shown to lead to goblet cell metaplasia by activation of EGFR. Further evidence has also demonstrated

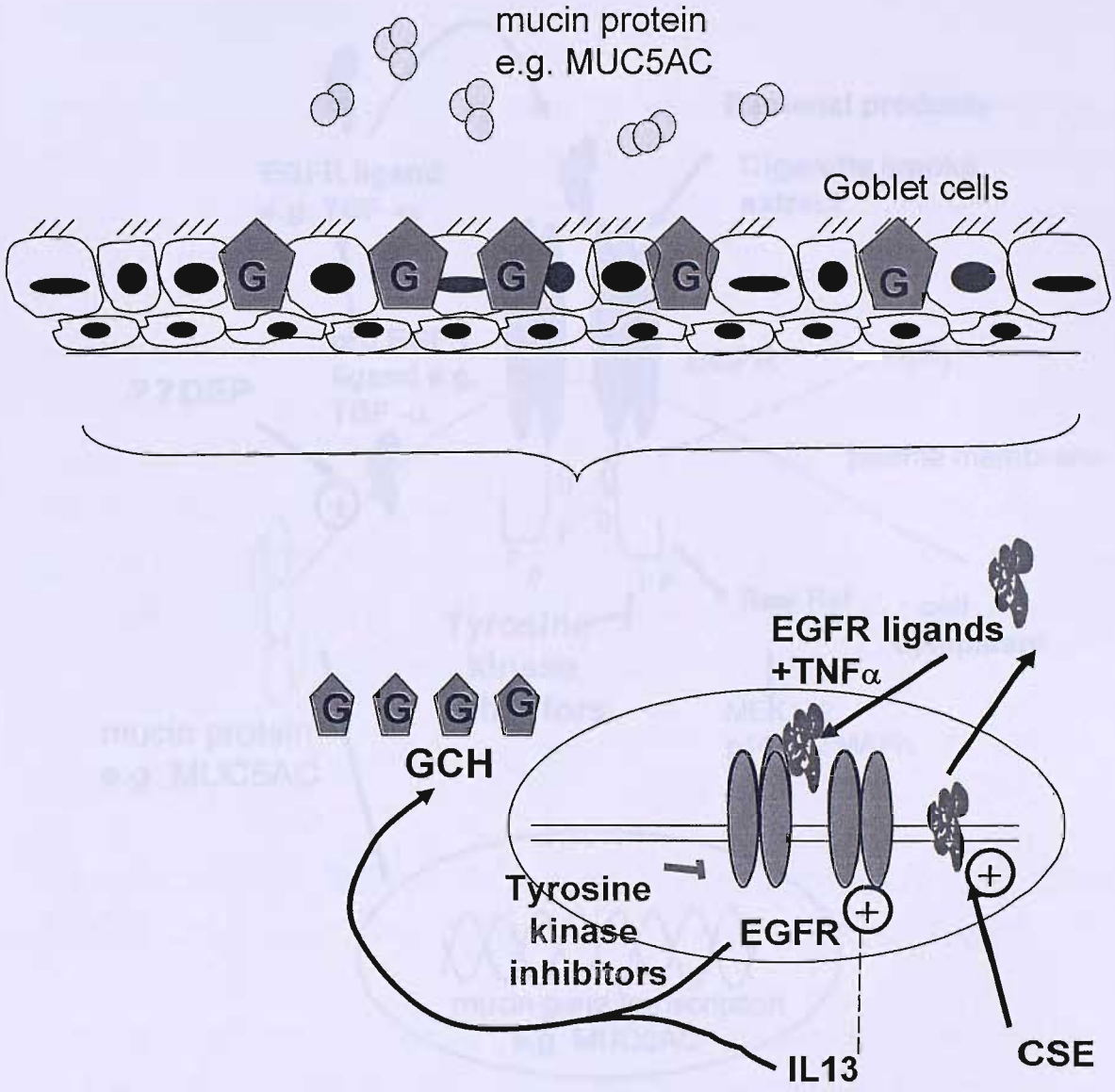


Fig 5a

The mechanism of EGFR activation and goblet cell hyperplasia.

Activation of the EGFR with specific ligands such as TGF-α in the presence of an inflammatory mediator (TNFα) has been show to lead to goblet cell hyperplasia (GCH) . This effect is inhibited using specific tyrosine kinase inhibitors. Goblet cells release secretory mucins such as MUC5AC. Cigarette smoke extract (CSE) has been shown to lead to GCH by a direct mechanism on the EGFR whereas IL 13 leads to GCH through indirect activation of the EGFR.

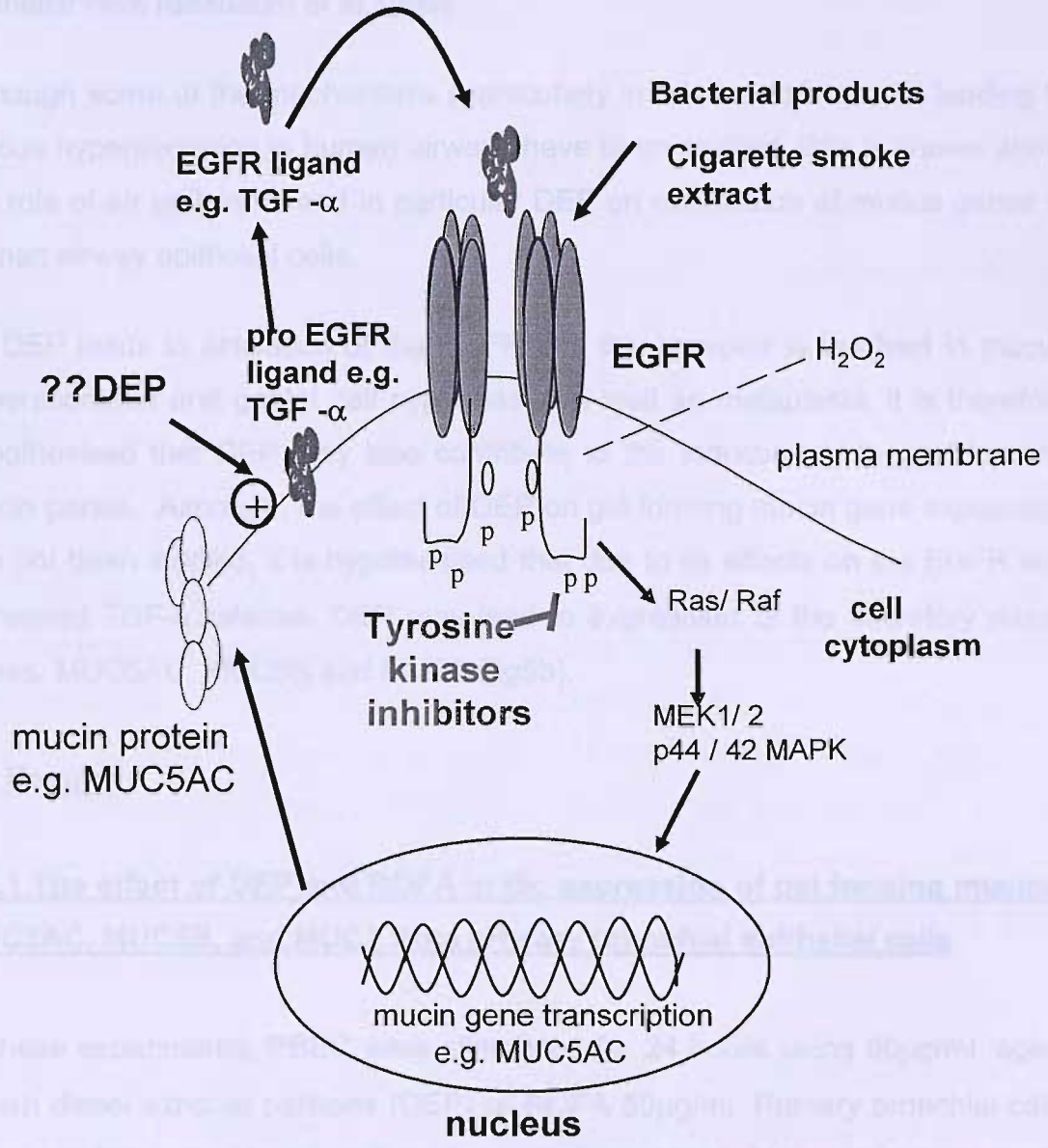


Fig 5b

The mechanism of mucin expression

Activation of the EGFR induces mucin synthesis. Ligand binding to the EGFR triggers receptor dimerization, leading to phosphorylation of tyrosine residues in the intracellular domain of the receptors. This activation process can also occur independently of ligand binding in response to stimuli such as oxidative stress (e.g. by H_2O_2) . The downstream signalling cascade results in mucin transcription and mucin synthesis. This effect can be inhibited with specific tyrosine kinase inhibitors. It has been shown that cigarette smoke extract and bacterial proteins (pseudomonas aeroginosa) lead to mucin synthesis by activation of the EGFR. It is thus postulated that due to its effects on EGFR ligand shedding and activation of the EGFR DEP also leads to mucin expression.

MAPK = mitogen activated protein kinase, MEK MAPK kinase

that ROFA can induce the production of MUC5AC gene and protein in bronchial epithelial cells [Basbaum et al 1999].

Although some of the mechanisms (particularly in relation to bacteria) leading to mucus hypersecretion in human airways have been studied, little is known about the role of air pollutants and in particular DEP on expression of mucus genes in human airway epithelial cells.

As DEP leads to activation of the EGFR and this receptor is involved in mucus hypersecretion and goblet cell hyperplasia as well as metaplasia, it is therefore hypothesised that DEP may also contribute to the induction of the gel forming mucin genes. Although, the effect of DEP on gel forming mucin gene expression has not been studied, it is hypothesised that due to its effects on the EGFR and increased TGF-α release, DEP may lead to expression of the secretory mucin genes, MUC5AC, MUC5B and MUC2 (fig5b).

5.2 Results

5.2.1 The effect of DEP and ROFA in the expression of gel forming mucins, MUC5AC, MUC5B, and MUC2 from primary bronchial epithelial cells

In these experiments, PBEC were stimulated for 24 hours using 50μg/ml 'aged' Welsh diesel exhaust particles (DEP) or ROFA 50μg/ml. Primary bronchial cells were seeded into 24-well culture trays and prepared for experimentation as described previously and then exposed to BEBM ± DEP. Gene expression was measured using Taqman RT-PCR. Data from the primary epithelial cell cultures were compared using the Wilcoxon test, respectively, using SPSS software (SPSS Inc., Chicago, IL, USA).

Consistent with previous reports, stimulation of PBEC with ROFA led to a significant increase in MUC5AC gene expression at 18 hours (MUC5AC gene expression in PBEC (median baseline=0.7 (IQR=0.5-15), ROFA (18hrs) = 236.0 (IQR =40-410), n=8, $p<0.05$)) (fig 5.1a). There was no significant increase in

MUC5AC gene expression following either 6 or 18-hour exposure of PBEC to DEP (fig 5.1b).

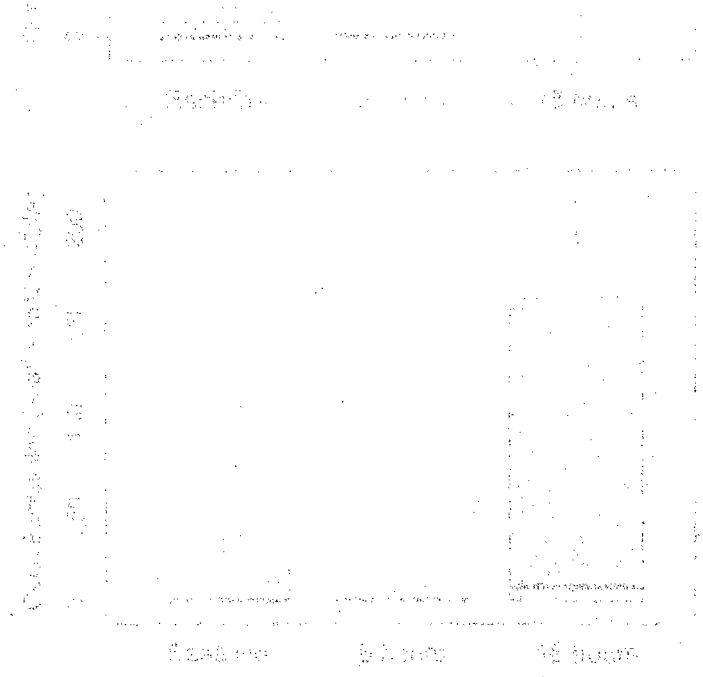
As well as MUC5AC, ROFA also caused a significant increase in MUC5B gene expression at 18 hours (median baseline=1.4 (IQR=1-10), ROFA (18hrs) = 50.0 (IQR =25-85), n=8, p=0.001)) (fig 5.2a). Unlike ROFA, DEP did not cause increase in MUC5B gene expression (fig5.2b). Despite the lack of a statistically significant increase in median induction of MUC5AC and MUC5B gene expression at 18 hours in response to DEP, there was significant variability in the responses as demonstrated by the large interquartile range for DEP induced MUC5AC (0-150) and MUC5B (0-60) gene expression. This suggests that perhaps DEP had caused cells from some individuals to respond by increased gene expression for MUC5AC and MUC5B, whereas others had not responded. This observation would suggest that DEP is less potent than ROFA and therefore the concentration of DEP used in these experiments may have been too low to cause all subsets of individuals to respond to this stimulus. Thus, it is feasible that at higher concentrations of DEP more individuals may respond. A dose response curve may indicate whether this observation is simply due to an insufficient concentration of DEP or that DEP is unable to stimulate gene expression for MUC5AC and MUC5B.

In these experiments, there was also an increase in MUC2 gene expression at 6 and 18 hours in response to ROFA exposure (median baseline=0.9 (IQR=0.7-1.2), ROFA (6hrs) = 5 (IQR= 1.0-9.0) ROFA (18hrs) = 33 (IQR = 7.0-65) (fig 5.3a). Similarly there was a significant (but less prominent than ROFA) increase in MUC2 gene expression at 18 hours following exposure to DEP (fig 5.3b). At 18 hours, baseline median MUC2 gene expression in response to DEP was increased by approximately x4 (baseline median = 1.0 (IQR= 0 -1.8), 18 hour median = 4 (IQR=3.8-8), n=8, p=0.001) (fig 5.3b).

The different responses of PBEC to ROFA and DEP as measured through the release of the gel forming mucins suggests that either these particles exert their effects through different mechanisms, or that they share the same mechanism but at least in the case of MUC5AC and MUC5B the concentration of DEP may

not have been potent enough to stimulate their expression. In the case of MUC2, as both DEP and ROFA stimulated its expression, this would suggest that these two particles act through similar mechanisms.

The increase in gene expression for these mucins by PBEC may act as a protective mechanism against the effects of ROFA and DEP.



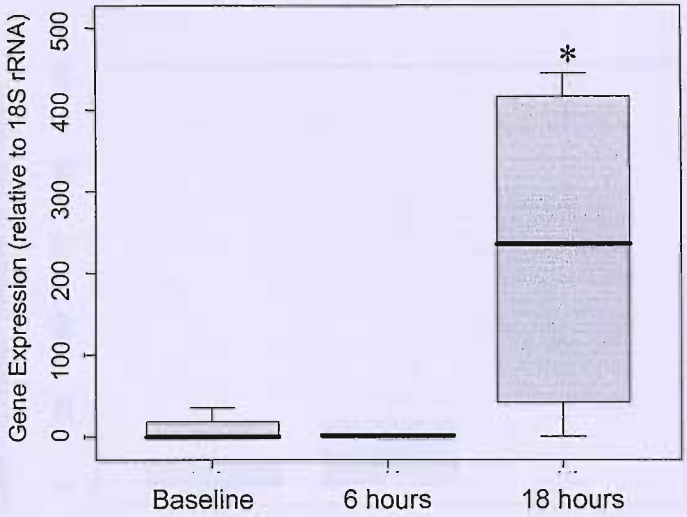


Fig 5.1 (a)

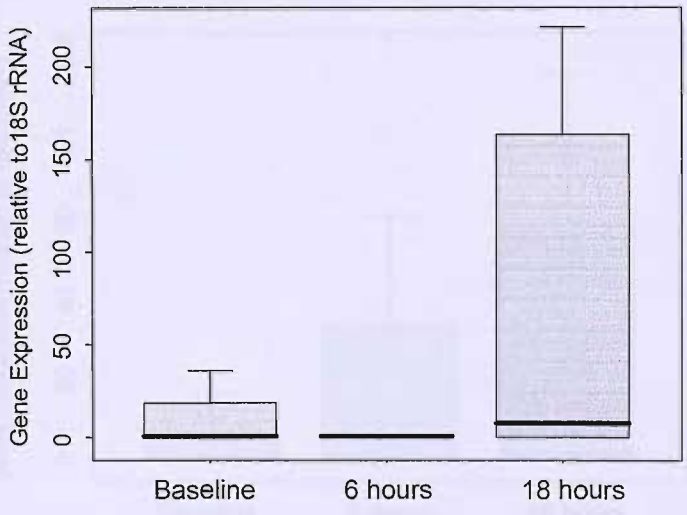


Fig 5.1 (b)

Fig 5.1

The effect of ROFA 50μg/ml (a) and DEP 50μg/ml (b) on MUC5AC gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP or ROFA for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: baseline=serum free medium, (t=0)
*** P < 0.05 n=8**

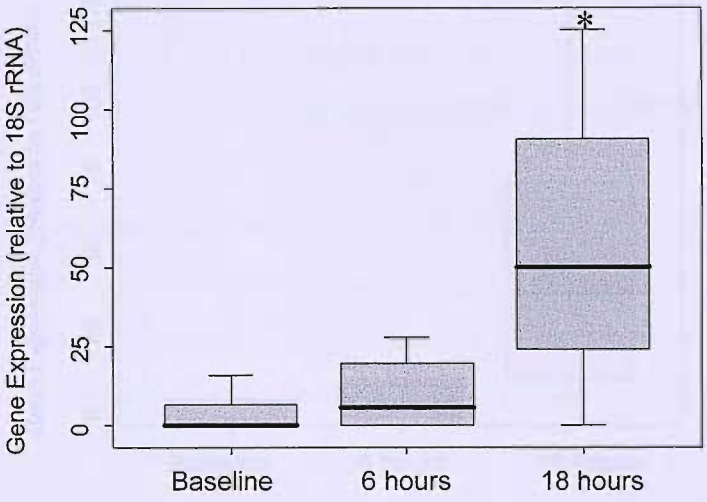


Fig 5.2 (a)

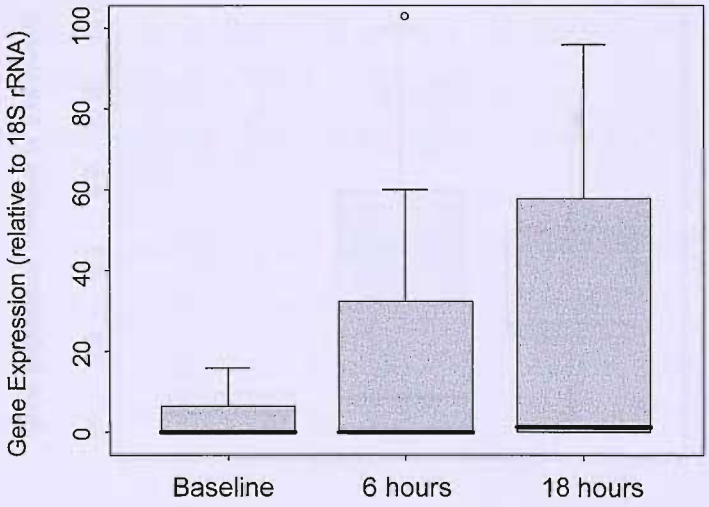


Fig 5.2 (b)

Fig 5.2

The effect of ROFA 50μg/ml (a) and DEP 50μg/ml (b) on MUC5B gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP or ROFA for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: baseline=serum free medium (t=0)
*** P = 0.001 n=8**

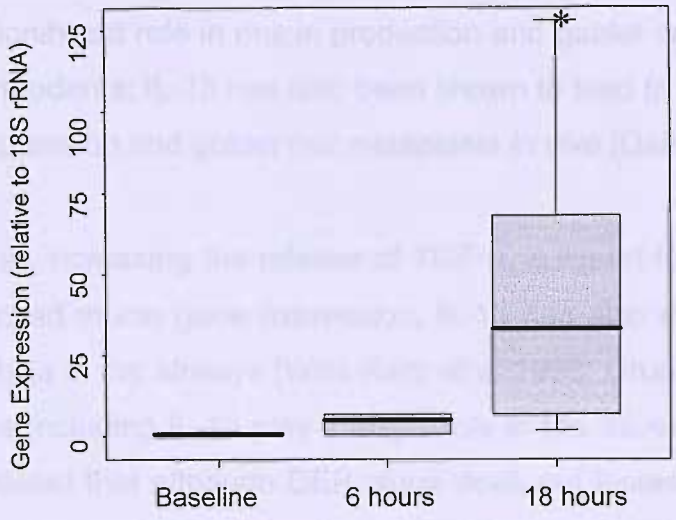


Fig 5.3 (a)

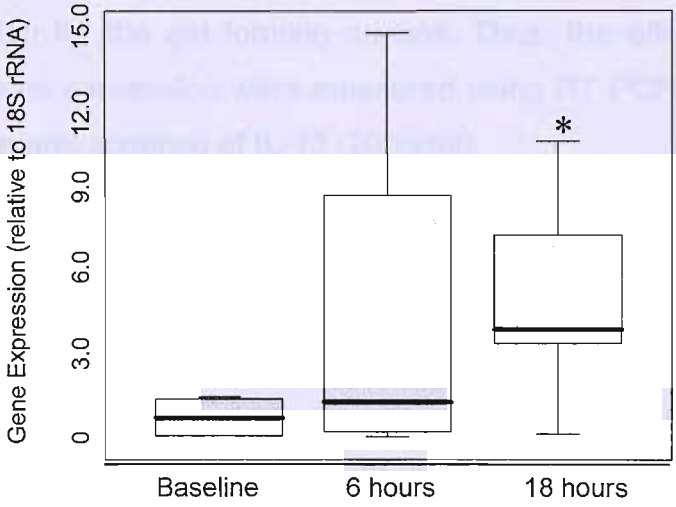


Fig 5.3 (b)

Fig 5.3

The effect of ROFA 50μg/ml (a) and DEP 50μg/ml (b) on MUC2 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP or ROFA for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: baseline=serum free medium, t=0
*** P = 0.001 n=8**

5.2.2 The effect of IL-13 on DEP induced expression of gel forming mucins (MUC5AC, MUC5B, MUC2) from PBEC

As discussed in detail in chapter one, the Th2 cytokine IL-13 has been shown to play a significant role in mucin production and goblet cell hyperplasia [Shim et al 2001]. In rodents, IL-13 has also been shown to lead to the induction of MUC5AC gene expression and goblet cell metaplasia *in vivo* [Dabbagh et al 1999].

As well as increasing the release of TGF-α, a ligand for the EGFR that is linked to enhanced mucin gene expression, IL-13 can also enhance inflammation and eosinophilia in the airways [Wills Karp et al 1998, Grunig et al 1998]. As the Th2 cytokines including IL-13 play a major role in the development of asthma, it was hypothesised that although DEP alone does not increase MUC 5AC or MUC5B gene expression, the combination of DEP with IL-13 may lead to enhanced gene expression for the gel forming mucins. Thus, the effects of DEP on secretory mucin gene expression were measured using RT-PCR at 6 and 18 hours in the presence and absence of IL-13 (20ng/ml).

IL-13 led to a significant yet transient increase in MUC5AC gene expression at 6 hours, which returned to baseline by 18 hours (fig 5.4). DEP and IL-13 together also caused a significant increase in MUC5AC gene expression in PBEC (median baseline=1.0 (IQR=0.5-25), IL-13 alone (6hrs) = 60 (IQR=40-250), DEP+ IL-13 (6hrs) = 125 (IQR =60-250), n=8, $p<0.01$)) (fig 5.4). However, while the median increase in MUC5AC gene expression in response to the combination of IL-13 together with DEP was almost double that of IL-13 alone this was not statistically significant.

In these experiments, IL-13 also led to a significant yet transient increase in MUC5B at 6 hours n=8, $p<0.05$) (fig 5.5) However, the combination of IL-13 together with DEP, did not lead to a significant increase in MUC5B gene expression at 6 or 18 hours (fig 5.5).

The addition of IL-13 or DEP to PBEC did not lead to an increase in MUC2 gene expression at 6 hours. At 18 hours there was an increase in MUC2 gene expression in response to DEP and IL-13. There was however no additional increase in MUC2 gene expression in response to the addition of IL-13 to DEP (fig 5.6).

As expected, based on other studies in the literature, IL-13 increased gene expression for MUC5AC. Although DEP did not increase MUC5AC gene expression alone, in combination with IL-13 there was a x125 increase in median gene expression for this mucin. Although there was higher median levels than IL-13 alone (IL-13=60) this was not statistically significant, suggesting again that either IL-13 together with DEP has not additive effects or that a larger number of experiments or higher concentration of DEP are needed to lead to a statistically significant response. Further experiments using higher concentrations of DEP and larger numbers of individuals may allow a clearer understanding of the interactions of IL-13 and DEP.

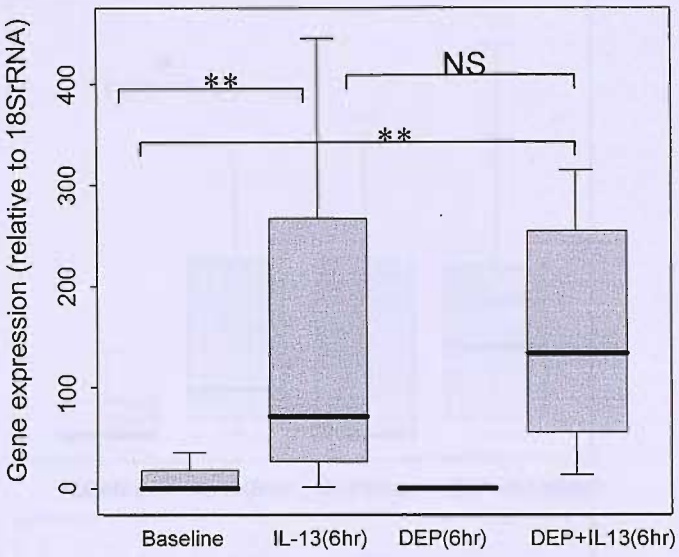


Fig 5.4 (a)

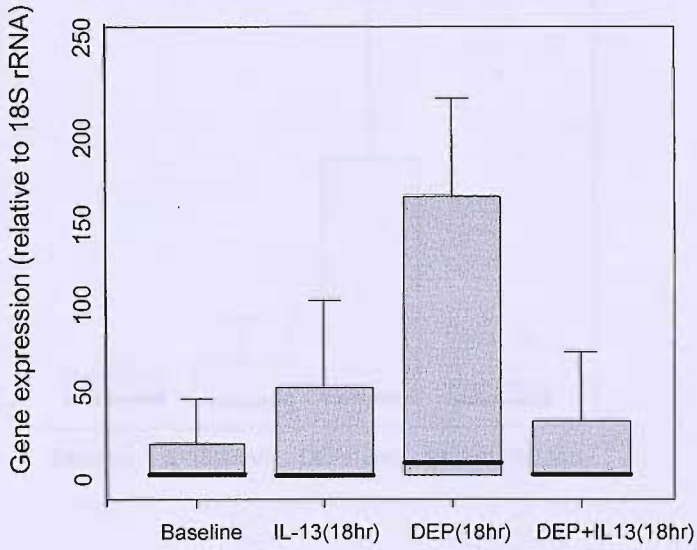


Fig 5.4 (b)

Fig 5.4

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced MUC5AC gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: DEP(6hr) = diesel exhaust particles 50 at 6 hrs, dep5018 = diesel exhaust particles at 18 hrs, depIL13= diesel exhaust particles + IL-13
**** P<0.01 n=8**

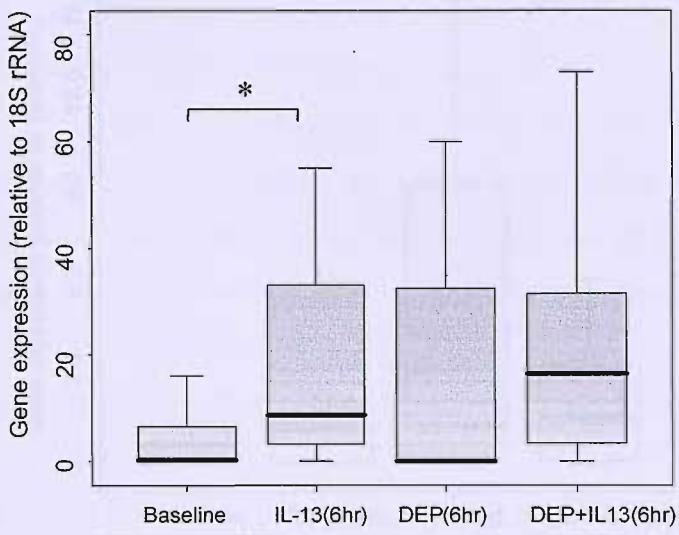


Fig 5.5 (a)

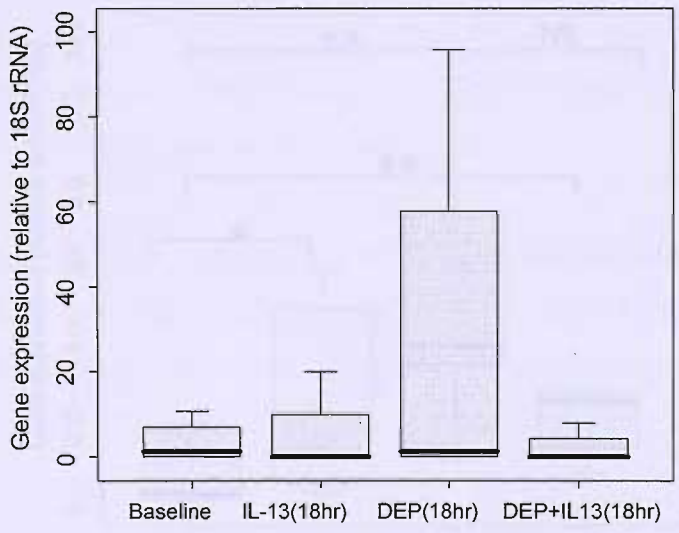


Fig 5.5 (b)

Fig 5.5

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced MUC5B gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: DEP(6hr) = diesel exhaust particles 50 at 6 hrs, dep5018 = diesel exhaust particles at 18 hrs, depIL13= diesel exhaust particles + IL-13

*** P<0.05 n=8**

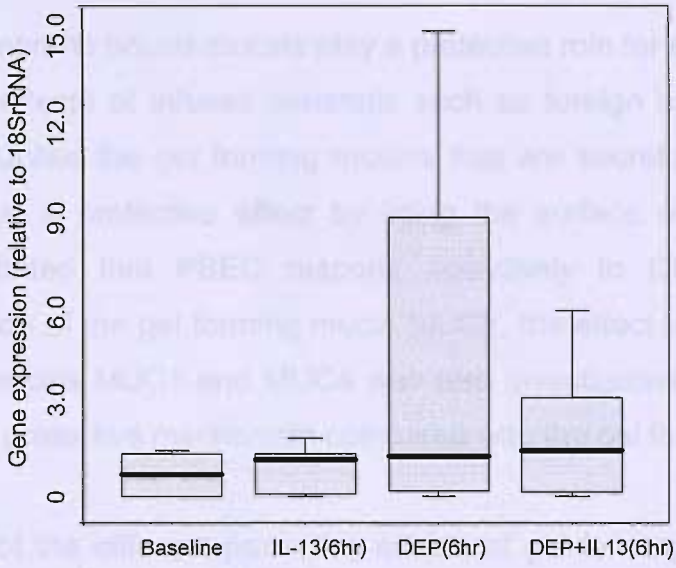


Fig 5.6 (a)

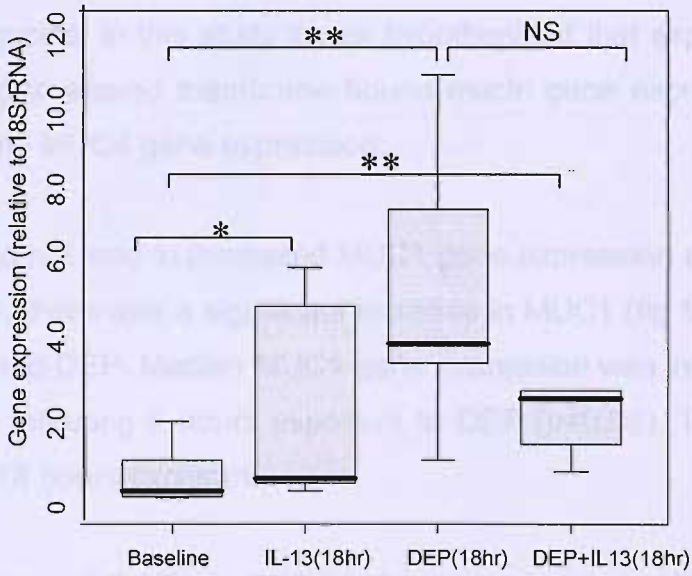


Fig 5.6 (b)

Fig 5.6

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced MUC2 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: DEP = diesel exhaust particles at 6 hrs, IL13= interleukin-13

*** P<0.05 ** P<0.002 n=8**

5.2.3 The effect of DEP on the expression of membrane bound mucins, MUC1 and MUC4 from primary bronchial epithelial cells

The membrane bound mucins play a protective role for epithelial cells against the harmful effects of inhaled materials such as foreign organisms and particulate matter. Unlike the gel forming mucins that are secreted by the epithelial cells, they have a protective effect by lining the surface of epithelial cells. Having demonstrated that PBEC respond selectively to DEP by increasing gene expression of the gel forming mucin MUC2, the effect of DEP on the membrane bound mucins MUC1 and MUC4 was also investigated as this would suggest a different protective mechanism compared with the gel forming mucins.

In view of the different protective effects of gel forming mucins and membrane bound mucins, in this study it was hypothesised that exposure of PBEC to DEP may lead to altered membrane bound mucin gene expression as measured by MUC1 and MUC4 gene expression.

ROFA did not lead to increased MUC1 gene expression at 6 or 18 hours (fig 5.7). However, there was a significant increase in MUC1 (fig 5.7b) gene expression in response to DEP. Median MUC1 gene expression was increased x2 compared to baseline following 6 hours exposure to DEP ($p<0.01$). This increased further to x3 after 18 hours exposure.

The addition of ROFA to PBEC led to an increase in MUC4 gene expression at 18 hours (baseline median = 1 (IQR = 0.2 –1.5), ROFA (18hrs) = 5.5 (IQR=1.0-16) (fig 5.8). In response to DEP, median MUC4 gene expression also increased to x3 at 6 hours ($p<0.05$) and by 18 hours had reduced to approximately x2 (fig 5.8).

These experiments further suggest that PBEC respond differently to ROFA and DEP, implying that these particles act differently on these cells. The response to DEP was predominantly to increase the membrane anchored mucins MUC1 and MUC4, whereas ROFA markedly increased the gel forming mucins as well as MUC4 but not MUC1.

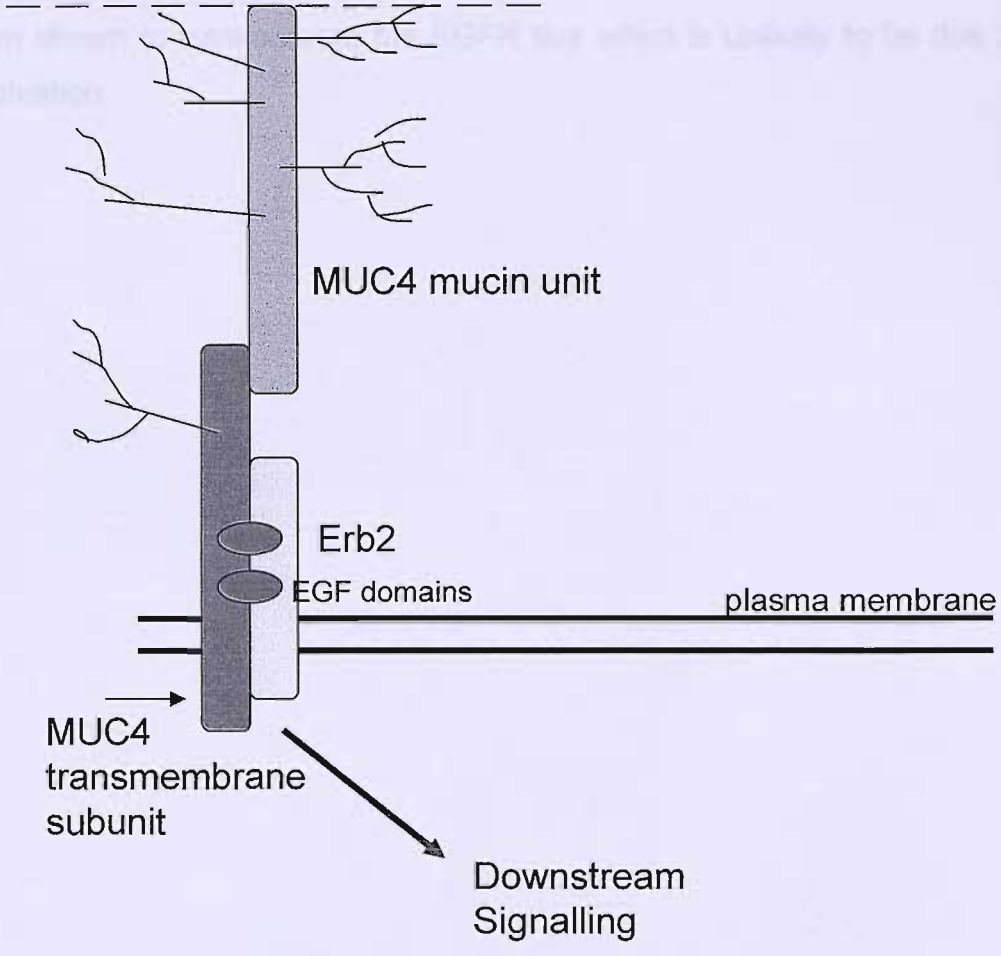


Fig 5C

The structure of MUC4 and its complex with Erb2 receptor

The structure of MUC4 with its transmembrane subunit, its two EGF binding domains and the mucin subunit. The mucin subunit has been truncated for ease of representation. MUC4 is an Erb2 ligand and activates this receptor which then in turn leads to downstream effects

This data suggests that exposure of PBEC to DEP leads to a largely selective increase in membrane bound mucins. The lack of induction of MUC1 gene expression in response to ROFA suggests that at least for MUC1 the mechanism of gene expression may not be oxidant mediated. As oxidants such as ROFA have been shown to transactivate the EGFR this effect is unlikely to be due to EGFR activation.

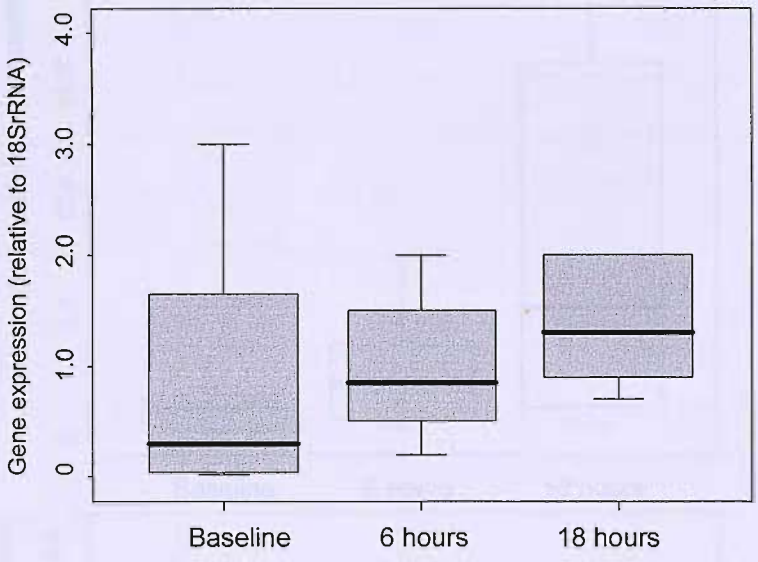


Fig 5.7 (a)

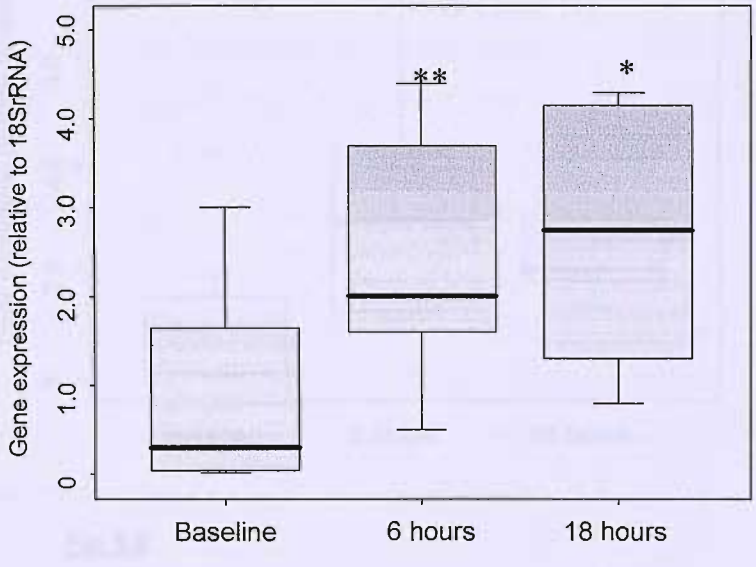


Fig 5.7 (b)

Fig 5.7

The effect of ROFA (a) and DEP (b) on MUC1 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP or ROFA for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: baseline=serum free medium (t=0),

*** P<0.05 ** P<0.01 n=8**

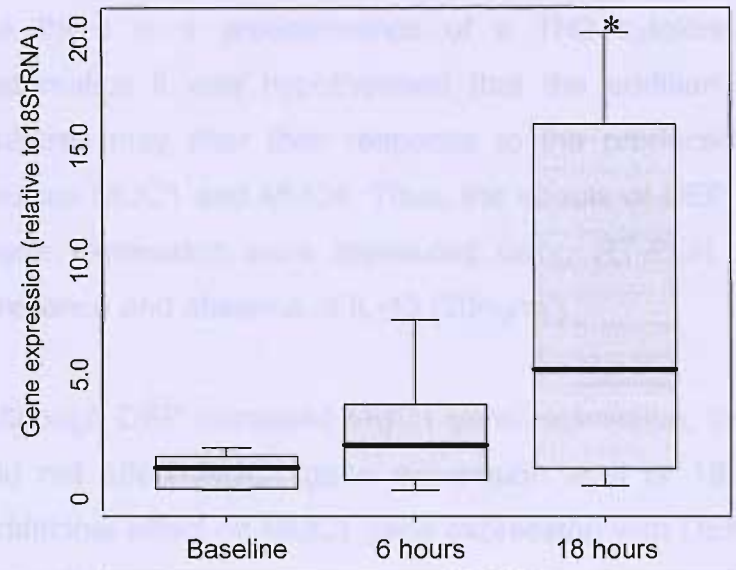


Fig 5.8 (a)

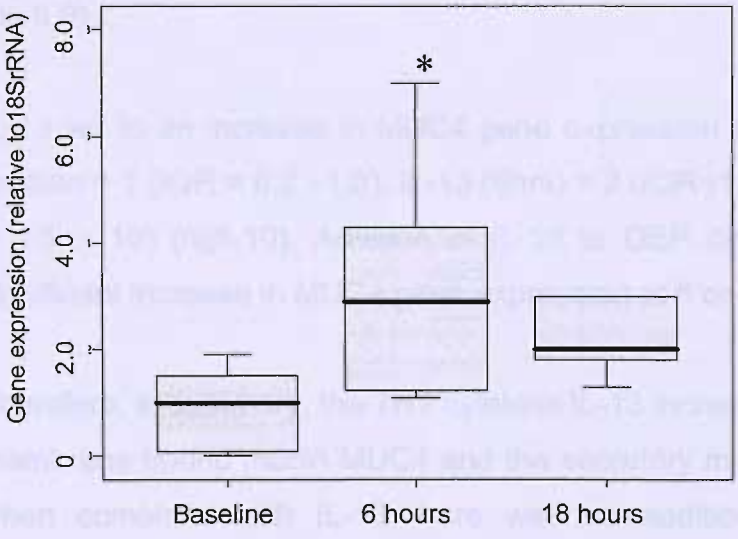


Fig 5.8 (b)

Fig 5.8

The effect of ROFA (a) and DEP (b) on MUC4 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP or ROFA for 6 and 18 hours, after which was gene expression was measured. Gene expression was compared with baseline. Kruskal Wallis followed by Wilcoxon Test was used for statistical analysis.

*** P<0.05 n=8**

Key: baseline=serum free medium (t=0),

5.2.4 The effect of the IL-13 on DEP induced expression of membrane bound mucins MUC1 and MUC4 from PBEC

As there is a predominance of a TH2 cytokine profile including IL-13 in asthmatics it was hypothesised that the addition of IL-13 to DEP in PBEC cultures may alter their response to the production of the membrane bound mucins MUC1 and MUC4. Thus, the effects of DEP on membrane bound mucin gene expression were measured using RT-PCR at 6 and 18 hours in the presence and absence of IL-13 (20ng/ml).

Although DEP increased MUC1 gene expression, the addition of IL-13 to PBEC did not affect MUC1 gene expression at 6 or 18 hours. There was also no additional effect on MUC1 gene expression with DEP when combined with IL-13 (fig 5.9)

IL-13 led to an increase in MUC4 gene expression at 6 and 18 hours (baseline median = 1 (IQR = 0.2 –1.8), IL-13 (6hrs) = 2 (IQR=1.8-5), IL-13 (18hrs) = 2 (IQR = 1.5 – 10) (fig5.10). Addition of IL-13 to DEP did not lead to a statistically significant increase in MUC4 gene expression at 6 or 18 hours.

Therefore, in summary, the TH2 cytokine IL-13 increases gene expression for the membrane bound mucin MUC4 and the secretory mucin MUC5AC in PBEC, but when combined with IL-13 there was no additional effect on mucin gene expression.

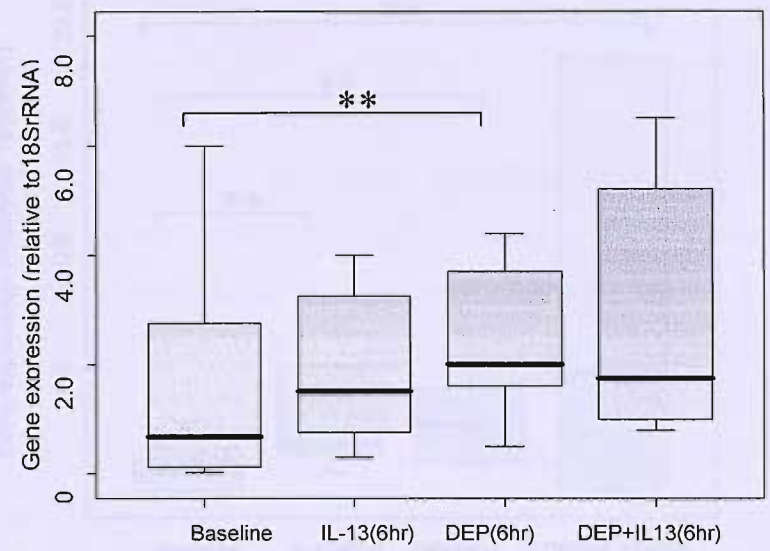


Fig 5.9 (a)

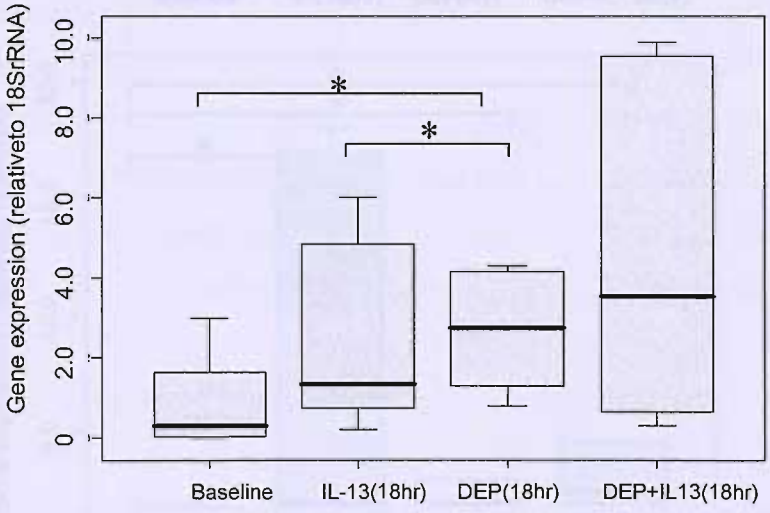


Fig 5.9 (b)

Fig 5.9

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced MUC1 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which was gene expression was measured. Kruskall Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: DEP(6hr) = diesel exhaust particles 50 at 6 hrs, dep5018 = diesel exhaust particles at 18 hrs, depIL13= diesel exhaust particles + IL-13

*** P<0.05 ** P<0.01 n=8**

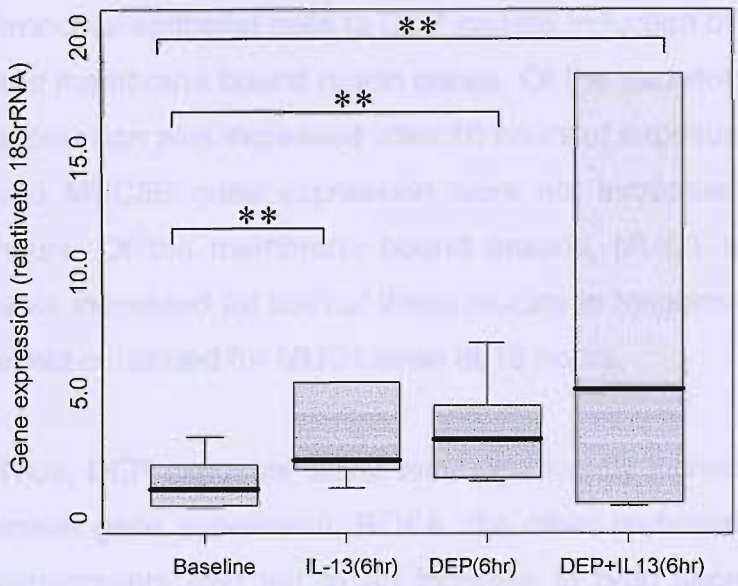


Fig 5.10 (a)

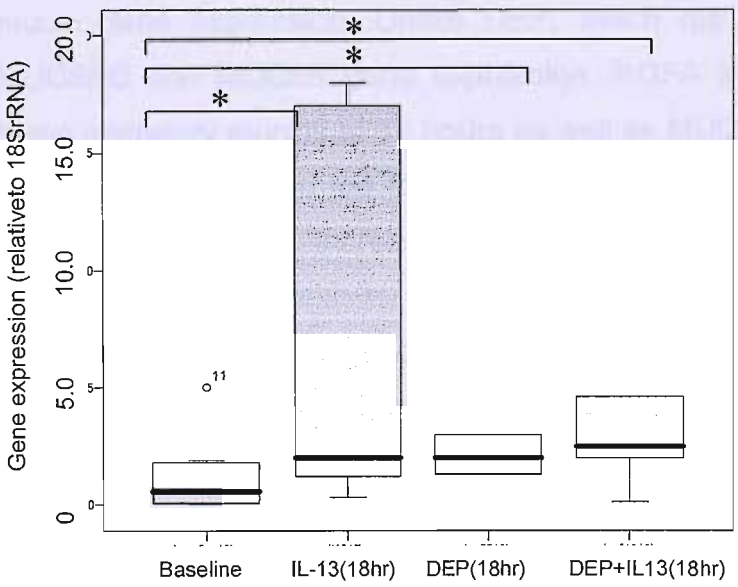


Fig 5.10 (b)

Fig 5.10

The effect of IL-13 on 6 hr (a) & 18 hr (b) DEP induced MUC4 gene expression in PBEC.

PBEC were cultured and used at 95% confluence and serum starved for 24 hours and exposed to Welsh DEP with o without IL13 for 6 and 18 hours, after which was gene expression was measured.Kruskall Wallis followed by Wilcoxon Test was used for statistical analysis.

Key: DEP(6hr) = diesel exhaust particles 50 at 6 hrs, dep5018 = diesel exhaust particles at 18 hrs, depIL13= diesel exhaust particles + IL-13
*** P<0.05 ** P<0.01 n=8**

5.3 Discussion

Experiments in this chapter have demonstrated that exposure of primary bronchial epithelial cells to DEP causes induction of expression of both secretory and membrane bound mucin genes. Of the secretory mucins tested, MUC2 gene expression was increased after 18 hours of exposure to DEP, whereas MUC5AC and MUC5B gene expression were not increased above baseline at 6 or 18 hours. Of the membrane bound mucins, MUC1 and MUC4, gene expression were increased for both of these mucins in response to DEP by 6 hours, and this effect continued for MUC1 even at 18 hours.

Thus, DEP exposure alone may significantly increase MUC2, MUC1 and MUC4 mucin gene expression. ROFA, the other particulate examined in this series of experiments also led to an increase in both secretory and membrane bound mucin gene expression. Unlike DEP, which did not lead to an increase in MUC5AC and MUC5B gene expression, ROFA led to an increase in both of these secretory mucins at 18 hours as well as MUC2 at both 6 and 18 hours. As with DEP, ROFA also led to an increase in MUC4 gene expression but unlike DEP, it did not lead to increased MUC1 gene expression.

The effects of DEP were also tested in the presence of IL-13. This cytokine alone caused increased gene expression for MUC5AC, MUC5B, MUC2 and MUC4 but there was no statistically significant additive effect when this was combined with DEP.

Although these experiments have demonstrated a clear response to DEP and ROFA in terms of the induction of mucin gene expression, they have not demonstrated whether these responses are followed by the release of mucins. Therefore these experiments have highlighted a need to perform further experiments to define the release of mucin protein products in response to DEP and ROFA. These can be done both in the *in vitro* setting on epithelial cell cultures as well as in the *in vivo* setting using human exposures and immunohistochemical staining on human biopsies. It would also be possible to

characterise these effects more clearly by using induced sputum and assessing the changes in mucin profile following exposure to DEP in an exposure chamber.

ROFA and DEP caused a differential response as regards mucin gene expression. This observation may be due to the fact that they have different compositions. ROFA is an inorganic particulate that is rich in transition metals, in particular vanadium, whereas DEP is a predominantly organic particle with a smaller concentration of transition metals adsorbed onto its surface. The effect of vanadium on the EGFR is to lead to inhibition of the dephosphorylation of this receptor by inhibition of phosphotyrosine phosphatases. This effect may lead to a stronger activation of the EGFR than DEP which has less transition metals. Maintaining the EGFR in a phosphorylated state leads to a sustained activation response of the receptor. This effect may also account for the observation that ROFA has a stronger pro-inflammatory response as measured by the release of IL-8 and also leads to greater MUC5AC gene expression than DEP. It would be of interest to determine the extent of phosphorylation of the EGFR and to compare the effects of DEP in the presence and absence of vanadium in cell lysates using western blotting.

An alternative explanation for the different effects of ROFA and DEP may be due to their oxidant effects. This could be tested using PBEC pre-treated with antioxidants such as glutathione, which would inhibit the strong oxidant effects of ROFA brought about due to its high transition metal content. It would also be possible to elucidate the mechanism of action of ROFA and DEP on mucin gene responses. Further experiments to determine the effects of vanadium as the potential factor leading to the different observed effects of ROFA and DEP, would be to add this transition metal to DEP and observe the effects of DEP in combination with vanadium on PBEC mucin gene expression.

During these experiments it was also noted that the effects of DEP on mucin gene expression were transient, whereas ROFA had a more sustained response, which may also in part be due to the stronger oxidant induced effects of ROFA.

Mucus hypersecretion is a problem in many chronic pulmonary disorders and is associated with cigarette smoking, nasal polyps, orotracheal intubation-induced injury and bacterial infections. Neutrophils and their products are implicated in cystic fibrosis, COPD, and acute severe asthma.

The EGFR is involved in epithelial differentiation into mucin-containing goblet cells by specific inflammatory mediators [Takeyama et al 1999]. In the preceding chapter it was demonstrated that DEP exposure leads to activation of the EGFR, and shedding of EGFR ligands, which may account for the effects of DEP on increased mucin gene expression. In addition, DEP leads to increased IL-8 release, which is a potent neutrophil chemoattractant. Goblet-cell metaplasia is thought to arise from the interaction of multiple cells including epithelial cells, together with eosinophils, and neutrophils [Shim et al 2001]. Furthermore, in asthmatics, there is a skew towards a TH2 cytokine profile including IL-13, which is a potent factor in inducing differentiation of primary epithelial cells into goblet cells, and enhanced MUC5AC release [Fahy et al 2002]. Thus, although DEP alone, did not lead to increased MUC5AC gene expression, in combination with IL-13 there was an increased effect on MUC5AC gene expression which did not reach statistical significance. This may have been due to the fact that the combination of DEP and IL-13 does not cause increased effects on MUC5AC expression, or alternatively that a larger concentration of DEP and larger numbers of subjects are needed. This is an important area for study as the combination of DEP exposure and higher baseline IL-13 levels found in asthmatic airways may potentially contribute to goblet cell hyperplasia, and airway remodelling in asthmatics exposed to DEP, thus leading to greater disease morbidity and mortality.

From a defence point of view, the augmented mucin gene response of epithelial cells in response to particulates may play a normal protective response to clear the particulates (gel forming mucins) and protect the epithelial cell surface (membrane bound mucins). However, it is possible that over-stimulation of these responses may result in increased morbidity due to effects on airflow dynamics and the development of infections. These suggestions would fit with results from epidemiological studies that have indicated an increase in respiratory (including

asthma) disease morbidity and mortality in response to raised levels of PM, which may be due to increased mucus hypersecretion, a well-known cause of disease morbidity. ROFA has a greater toxic effect and is likely to be a more potent activator of the EGFR, and therefore causes increased gel forming mucin gene expression. This observation may suggest a greater protective response to aid the clearance of these particles. Since mucins contribute to clearance of particulates, the observation that DEP leads to less potent mucin activation in comparison to ROFA may suggest that after long term *in vivo* exposure to these particles, DEP may remain in the airways for longer periods as it may not be cleared as effectively.

The data derived from these experiments suggest that the increased MUC1 gene expression in response to DEP is not likely to be due to increased activation of the EGFR as this mucin was not increased in response to ROFA. To confirm the lack of a role for the EGFR in response to ROFA and DEP on MUC1 responses cells could be pre-treated using specific EGFR inhibitors such as AG1478 and EGFR neutralising antibody. Alternative explanations for the effects of DEP on MUC1 gene expression may be due to a mechanisms involving NFkB. This hypothesis could be examined using corticosteroids in cell culture as an inhibitor of the action of NFkB.

MUC1 and MUC4 are membrane-bound mucins that are abundantly present on epithelial cell surfaces [Davies et al 2002, Dekker et al 2002]. Although, the role of these mucins had not been studied in relation to asthma or exposure to air pollution including DEP, they are thought to play a significant role in lung cancer and cell signalling [Schroeder et al 2001, Carraway et al 2000 & 2002]. MUC1 is over expressed and differentially glycosylated by adenocarcinomas (including lung), and is believed to contribute to invasive and metastatic potential by contributing to cell surface adhesion properties [Awaya et al 2004, Nassar et al 2004]. MUC1 and MUC4 mucins are also thought to function as receptors or receptor ligands and activate intracellular signalling cascades affecting epithelial functions [Schroeder et al 2001, Carraway et al 2000 & 2002, 2003, Lillehoj EP et al 2004]. MUC4, has recently been identified as a ligand for ErbB2, the major heterodimerization partner of the epidermal growth factor receptor [Carraway et

al 2003] (fig5c). In mouse mammary gland cells, MUC1 has also been demonstrated to have downstream signalling effects through the EGFR and activation of MAP kinases [Schroeder et al 2001]. Therefore exposure to particulate pollutants may augment cell signalling indirectly through the activation of MUC1 and MUC4.

The major limitation with this work lies in the fact that the results are focused on gene expression data. Release of mucin protein in response to DEP has not been examined. Under clinical conditions, measured release of mucins in response to airway pollutants such as DEP and ROFA would provide a more accurate understanding of the health effects of these particulates. These studies did not address the potential differences between differentiated and undifferentiated cells. Experiments carried out in this chapter on the effects of DEP and ROFA on undifferentiated PBEC monolayers may model the effects of these particles on basal cells, such as occurs on areas of denuded epithelium in moderate to severe asthma. Normal airways are characterised by differentiated epithelial cells with basal and columnar cells and thus interactions of particles on these differentiated cells may lead to different responses. IL-13 has been shown to enhance the expression of MUC5AC which is itself thought to be an important factor in the differentiation of basal cells to goblet cells. Further experiments to determine the effects of DEP and ROFA in differentiated cells can be carried out using cell cultures on the air liquid interface. These experiments have demonstrated novel pathways that reveal original insights into the potential effects of DEP on human epithelial cells, and thus their possible effects on chronic pulmonary disease morbidity and mortality. These data further demonstrate that in addition to the causes outlined above, DEP exposure may account for a novel mechanism to explain mucus production, and thus may in part account for the evidence that has arisen from the large numbers of epidemiological studies that have implicated exposure to DEP in increased pulmonary disease morbidity and mortality, particularly in association with asthma and COPD. Therefore in summary experiments in this chapter have highlighted a novel area of research into the potential adverse health effects of DEP linked to mucus production.

CHAPTER SIX

FINAL DISCUSSION

6.1 Summary of novel findings

DEP have a differential effect on epithelial cell number and viability in H292 and 16HBE cell lines as well as in PBEC, depending on their source and hence chemical composition. Exposure of PBEC from both asthmatic and normal subjects to DEP leads to an increase in IL-8 release. This effect was enhanced in the presence of a Th2 rich environment. ROFA also leads to the release of IL-8 but to a greater extent than DEP on a weight basis. The toxicity associated with PM such as DEP and ROFA as well as their effects on IL-8 release are due to adsorbed materials rather than the carbon core. IL-8 release in response to DEP takes place via the shedding of EGFR ligands AR and TGF-α and autocrine binding to the EGFR, which then activates the induction of IL-8 gene expression and release of IL-8 protein. ROFA leads to increased gene expression for amphiregulin, TGF-α and HB-EGF as well as the release of amphiregulin and TGF-α in PBEC. Exposure of PBEC to DEP and ROFA leads to altered responses in both secretory and membrane bound mucin gene expression. DEP predominantly increased expression of the membrane bound mucin genes whereas ROFA increased both secretory and membrane bound mucin gene expression.

6.2 Implications of findings

6.2.1 DEP as a marker of the effects of particulate matter on bronchial epithelial cells

As the barrier between the external environment and the internal milieu, the bronchial epithelium is exposed to viruses, inhaled pollutants and allergens that have the potential to cause tissue injury directly by cytotoxicity or proteolysis and indirectly by involving immune and inflammatory cells. In asthma, it has been proposed that the products of eosinophils and mast cells as well as allergens and oxidants are major causes of epithelial damage. [Bayram et al 1997, Bucchieri et al 2002]. Oxidants increase epithelial permeability by damaging tight junctions and this may account for the actions of DEP and to a larger extent ROFA. In

addition to their direct effects on epithelial structure, it may be that DEP and ROFA could lead to altered cell function through induction of a stress response via receptor-mediated processes or by generation of reactive oxygen especially in the case of ROFA with activation of pro-inflammatory transcription factors [Ghio et al 2002].

Epidemiological evidence has suggested a relationship between concentrations of ambient air particulate matter and adverse health effects. Many different sources contribute to the particulate fraction of ambient air pollution, including DEP and ROFA and therefore in these studies these particles were used as markers of the actions of particulate matter on bronchial epithelial cells. It has been shown that DEP induces similar effects to particulate matter collected from a busy European city, although its effects may be of a greater magnitude than PM [Baulig et al 2003]

A difficulty with the study of particulates such as DEP is that they exhibit great variation as discussed in chapters 2 and 3. The studies conducted in this thesis have characterised some of the responses of bronchial epithelial cells to 'aged' particles. However, they have not characterised the response of PBEC to 'freshly' generated DEP which also contains the volatile organic components. An attempt was made during these studies to investigate the effects of time on PBEC responses to DEP using 'fresh frozen' rather than 'aged' DEP. In these experiments similar results to those observed with the equivalent 'aged' DEP were observed. The most likely reason for this is that in the time that particles are collected onto a filter and placed in a container there may have already been significant loss of the volatile organic compounds even before they are frozen to -20°C. Although some human exposure to DEP will be to freshly generated diesel exhaust, such as in traffic, these studies have not addressed the effects of such exposure on bronchial epithelial cells. However, the majority of human exposure to ambient PM and in particular DEP is to the aged variety that remains in the atmosphere and is transported by alterations in atmospheric conditions. Therefore, although from a scientific point of view there is the possibility of obtaining different results depending on diesel source as well as engine characteristics, the use of aged DEP in various studies has consistently

demonstrated a pro-inflammatory effect both *in vitro* and *in vivo*. This particulate type is also the type that is most relevant in terms of human exposure and therefore its use in the study of the biological effects of this particulate can still be supported.

An improved method to test the effects of freshly generated DEP on epithelial cells is through the development of sterile bronchial epithelial cell exposure chambers that unfortunately were not available in our laboratories. In these studies cells would be directly exposed to known concentrations of DEP in the air and the effects of this type of exposure would be compared to the effects of exposure to aged DEP.

It is possible that during collection and storage of PM for study, other inflammatory compounds such as allergens and lipopolysacharide (LPS) present in the atmosphere may attach onto the surface of DEP. Therefore the potential binding of biologically active organic substances such as LPS or allergens to particulate matter such as carbon black and DEP should also be investigated when studying the biological effects of respirable particles in an *in vitro* or *in vivo* setting. This can be done using specific monoclonal antibodies (e.g. LPS monoclonal antibody, Cell Sciences, MA, USA) and ELISA.

There may be considerable differences in responses between PBEC derived from subjects with normal, mild/ moderate and severe asthma when exposed to an environmental particulate such as DEP. Although the primary aim of the work carried out in this thesis was to identify the mechanistic effects of DEP on bronchial epithelial cells, an attempt was also made to identify potential differences between response from asthmatic and normal subjects. The studies carried out on the effects of DEP on mucin gene expression were limited to asthmatic subjects, and therefore the effects on normal subjects were not identified.

6.2.2 The Effects of DEP and ROFA on Cytotoxic & Inflammatory Mechanisms in Asthma

In these studies it was demonstrated that DEP leads to epithelial injury as well as activation of the EGFR and release of IL-8, whereas in higher concentrations it leads to significant epithelial cell toxicity. Recent published studies have shown that DEP may also lead to epithelial shedding, a characteristic feature of asthma by altering human airway epithelial cell adhesion and repair mechanisms. In a study using a bronchial epithelial cell line it was demonstrated that DEP caused alteration on the actin cytoskeleton and the extracellular matrix thus leading to potential shedding of epithelial cells [Doornaert et al 2003]. Therefore, inhalation of potentially large concentrations of DEP may lead to significant epithelial cell toxicity and shedding in humans and may contribute to the reports of chronic asthma. In addition, following chronic inhalation, particulates may remain in the airways and therefore, it may be that by remaining in the airways for prolonged periods of time, the damage caused by DEP will be heightened and lead to further cell shedding. This process may be exaggerated in severe asthmatics, where there are more denuded areas of epithelium together with a loss of ciliary activity.

IL-8 is a potent neutrophil chemotactic factor and may be released in response to a wide variety of stimuli, including exposure to PM, pro-inflammatory cytokines, microbes and their products, as well as environmental changes such as hypoxia, reperfusion, and hyperoxia. As well as causing the release of IL-8, DEP may interact with cytokines in other ways. A recent study has demonstrated that DEP also bind IL-8 and thus can induce a more sustained inflammatory response by further directing the movement of neutrophils [Seagrave et al 2004]. Neutrophil products can augment airway inflammation and thus heighten asthma severity. There is also evidence, indicating that IL-8 may have a wide range of additional actions on various other cell types, including lymphocytes, monocytes, endothelial cells, and fibroblasts. Thus, in addition to its acute neutrophilic pro-inflammatory effects, it is thought that IL-8 may also have a crucial role in various pathological conditions such as chronic inflammation and fibrosis.

Areas of epithelial damage are associated with upregulation of the EGFR, which may play a role as a repair mechanism in aiding the replacement of shed epithelium in asthmatics. Normally, this effect would be expected to lead to augmented IL-8 release in asthmatics as the EGFR activates the release of this cytokine and may account for the increase in neutrophils seen in asthmatic airways. In this thesis it was demonstrated that DEP exposure leads to increased IL-8 release in PBEC. When comparing the release of this cytokine in asthmatics and normals it was observed that cells derived from normal subjects express higher baseline levels of IL-8 compared with asthmatics, although there was an increase in IL-8 in response to DEP in both groups. An *in vitro* study by Devalia and colleagues has demonstrated heightened IL-8 release in cells derived from asthmatic subjects compared with normal subjects [Devalia et al 2001]. However a number of *in vivo* exposure studies of normal and asthmatic subjects to DEP has demonstrated that there is heightened expression of IL-8 in bronchial biopsies bronchial wash, bronchoalveolar lavage and induced sputum derived from normal subjects but not in asthmatics [Nordenhall 2001, Stenfors et al 2004, Salvi et al 2000, Holgate et al 2003]. These human *in vivo* studies have investigated the effects of DEP exposure on IL-8 release in asthmatics at 6 hours post exposure and found that this cytokine had not increased in the asthmatic group. The investigators did not study the release of IL-8 beyond 6 hours and so this lack of IL-8 response may simply indicate that they had not studied this cytokine at the appropriate time point. Parallel to the increase in IL-8 exposure of normal subjects but not asthmatics in response to DEP, it has been shown that there is airway neutrophilia in normal subjects following exposure to DEP [Holgate et al 2003].

In this thesis the release of IL-8 was demonstrated to take place due to activation of the EGFR in response to DEP. This particulate led to shedding and release of EGFR ligands, amphiregulin, TGFα and HB-EGF. During the final part of work carried out in this thesis an independent study carried out by a French group also found similar results and demonstrated that DEP activate the release of amphiregulin in a bronchial epithelial cell line [Blanchet et al 2004] further confirming the findings in this thesis. Further work carried out in the department has demonstrated that of the three ligands tested amphiregulin contributes the

most to activation of the EGFR. Thus DEP may mediate a pro-inflammatory effect as well as potential downstream effects through the EGFR, indicating that DEP can activate the release of pro-inflammatory cytokines. This may explain the lack of response in some patients with asthma to corticosteroids which inhibit NFkB dependent mediator release. This finding may have significant implications for the development of newer forms of treatment for asthma that are discussed below.

DEP may also lead to development of asthma and the allergic state by directing Th2 cytokine release. In human nasal epithelial cells, the presence of allergen together with DEP challenge caused a significant increase in the expression of mRNA for TH0 and TH2 type cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) with a pronounced inhibitory effect on IFN-gamma gene expression, suggesting that DEP can enhance B-cell differentiation, and by initiating and elevating IgE production, may play an important role in the increased incidence of allergic airway disease [Diaz Sanchez et al 1997]. DEP has also been shown to stimulate the production of the eosinophil chemoattractant eotaxin by normal human peripheral airway epithelial cells as well as by the bronchial epithelial cell line BET-1A. DEP showed an additive effect on IL-13 stimulated eotaxin expression [Takizawa et al 2003]. In addition, exposure of lung macrophages to DEP leads to a reduction in IL-12 that may partly explain the presumed adjuvant effect of DEP in atopy by altering the Th1/Th2 balance in favour of a Th2 state [Nilsen et al 2003]. Pyrene, one of the major compounds of DEP can induce the transcription of IL-4 messenger RNA and expression of IL-4 protein in primary human T cells [Bommel et al 2000]. Human basophils, exposed *in vitro* to DEP extract, express IL-4 in both allergic and non-allergic subjects. Therefore the effects of DEP were studied in the presence of IL-13 and IL-4 in this thesis. It was demonstrated that these cytokines had an additive effect on the release of IL-8 from PBEC.

Asthma is a complex disease characterised by interactions between structural and inflammatory cells such as epithelial cells, neutrophils, T-cells, basophils and eosinophils. Having studied the effects of DEP on epithelial cell monocultures, further work resulting from this thesis would be to investigate the effects of DEP

on integrated culture systems involving epithelial cells together with other inflammatory cells such as neutrophils, basophils and T cells in normal and asthmatic subjects.

6.2.3 The effects of DEP on mucin expression in asthma

Activation of the EGFR has been shown to be associated with mucin expression and goblet cell hyperplasia and thus in this thesis the effects of DEP on mucin expression were studied. Mucins play a significant defensive role for epithelial cells against the harmful effects of inhaled materials such as foreign organisms, allergens and particulate matter. In addition to a providing a protective layer these molecules provide lubrication, prevent dehydration, and offer protection from proteolysis to epithelial surfaces. As microbial challenge is a frequent problem on the epithelial surface, mucins protect against attack by inhibiting microbial access to the epithelial surface as well as leading to bacterial binding onto mucin carbohydrates at the cell surface [Vimal et al, 2000, Lillehoj et al 2002].

Mucins are abundant on most epithelial surfaces and are concentrated at the apical surface consistent with their function as a protective barrier with adhesion-modulating properties. However in many illnesses such as asthma, COPD and cystic fibrosis, excess mucus secretion is associated with significant morbidity. Therefore, as with many protective physiological processes such as inflammation, when in excess, mucus secretion may lead to harm. Many stimuli can lead to mucus hypersecretion including chronic exposure to allergens and chronic colonisation of the airways with infective organisms. Although the effects of cigarette smoke on mucin expression in epithelial cells have been explored to some extent [Takeyama et al 2001, Shao et al 2004] the effects of particulate matter particularly DEP has remained largely unexplored.

Although needing to be confirmed with protein data, the discovery, as part of work carried out in this thesis, that exposure to DEP can lead to increased mucus gene expression in PBEC is a novel finding that may contribute to the increased morbidity and mortality associated with exposure to PM.

The effects of mucins on respiratory morbidity and mortality have traditionally been explained in the context of their mechanical effects on the airways. Increased mucin production in obstructive airway diseases such as asthma and COPD may lead to significant respiratory distress, failure and even death due to mechanical obstruction to airflow and plugging of the airways. However in addition to their mechanical effects, recent studies have suggested that particularly MUC1 and MUC4 can have significant roles in signal transduction and cell signalling, which may lead to modulation of chronic inflammation, remodelling and metastatic spread [Schroeder et al 2001, Carraway et al 2000 & 2002, Rahn et al 2004, Hayashi et al 2001]. In this thesis, it was demonstrated that DEP caused augmented gene expression for MUC1 and MUC4 whereas ROFA cause increased MUC4 but not MUC1 gene expression. However due to their effects on the membrane bound mucins these particles may have effects on downstream cell signalling.

MUC1 is a large, transmembrane mucin glycoprotein that is expressed on the apical surface of most simple epithelial surfaces, including mammary gland, reproductive tract, kidney, bladder and lung. It has been demonstrated that inflammatory stimuli such as TNF-α, IL-1β, and IL-6 lead to augmented expression of this mucin [Li et al 2003]. This protein contains three domains: a short cytoplasmic and transmembrane domain as well as a large extracellular domain. In addition to providing a role in protecting the epithelial surface, this molecule has also been shown to have significant downstream effects. A series of recent studies have shown that the highly conserved cytoplasmic tail of MUC1 interacts specifically with a series of important signal transducing molecules including, β–catenin [Yamamoto et al 1997], as well as other signalling molecules including Grb2/Sos [Pandey et al, 1995]. MUC1 has also been shown to interact with cell receptors. Activation of EGFR with EGF has been shown to induce tyrosine phosphorylation of the MUC1 cytoplasmic tail [Schroeder et al 2001, Li et al 2001] as well as activation of ERK1/2 [Schroeder et al 2001]. Also, EGF mediated activation of ERK 1/2 is drastically enhanced in the presence of high levels of MUC1 in the mouse mammary gland [Schroeder et al 2001]. Thus potential stimuli, including growth factors or cytokines directly or through activation of their receptors may affect MUC1 stability, localisation at the cell

surface, or phosphorylation state. MUC1 may also enhance the activity of cell receptors such as EGFR.

Other work has demonstrated that as well as modulating cell signalling, direct interactions with the MUC1 ectodomain, e.g. by microbes or selectins, also could trigger signalling events. In a recent study Pseudomonas Aeruginosa was shown to activate MAP kinase by phosphorylation of MUC1 [Lillehoj et al 2004].

ICAM-1 is the only known direct ligand of the MUC1 extracellular domain. The MUC1 peptide core has been shown to mediate adhesion of tumour cells to adjacent cells via binding to intercellular adhesion molecule-1 (ICAM-1). In breast cancer cells contact with ICAM-1 has been shown to initiate a calcium-based signal. It is thought that this involves a Src family kinase, phosphoinositol 3-kinase (PI3 Kinase), or phospholipase C [Rahn et al 2004].

The MUC1 molecule can also be proteolytically released from the surface of epithelial cells by a number of sheddases. In a human uterine epithelial cell line it was shown that activation of TACE/ADAM-17 by the action of TNF-α led to shedding of this molecule [Thathiah et al 2004]. Further characterisation of the proteolytic activity mediating MUC1 release indicated that MUC1 shedding is also accelerated by the tyrosine phosphatase inhibitor pervanadate. During work carried out in this thesis it was observed that ROFA and DEP had different effects on the expression of MUC1. Studies have shown that vanadate the predominant transition metal on ROFA leads to shedding of MUC1 [Thathiah et al 2004] into soluble form which may account for the finding that ROFA does not lead to increased gene expression, as it may instead lead to increased shedding of membrane bound MUC1 rather than activation of gene expression for this molecule. It would thus be very interesting to measure soluble MUC1 following exposure to this particulate.

ROFA could also lead to the shedding of MUC1 in a different way. In one study pervanadate, was shown to stimulate MUC1 shedding in TACE/ADAM-17 deficient cells, indicating activation of a metalloproteinase activity distinct from TACE/ADAM-17 [Thathiah et al 2004].

DEP was also shown to activate the EGFR by direct and indirect effects including augmented shedding of the EGFR ligand TGF-α. Activation of ADAM 17 leads to shedding of TGFα as well as MUC1. Therefore DEP may activate EGFR mediated downstream signaling pathways by both direct activation of the receptor through ligand shedding, as well as shedding of MUC1. Human exposure studies to DEP have demonstrated increased ICAM1 expression in bronchial biopsies following DEP exposure [Salvi et al 1999]. ICAM1 is a ligand for MUC1 and can also lead to activation of signalling pathways, thus the interaction of DEP together with MUC1 may further lead to augmented downstream effects. As DEP leads to shedding of TGFα this particulate is also likely to lead to shedding of MUC1 molecule. Even though the effects of soluble MUC1 are not understood at present it is likely that it will have paracrine signalling and adhesion effects on epithelial cells.

Human exposure studies have also demonstrated that exposure to DEP leads to augmented IL-10 release in asthmatic subjects compared with normal subjects [Stenfors et al 2004]. Although the authors of this study were unable to explain the mechanism leading to the release of this cytokine, it is possible that it is mediated by the effects of MUC1. Recently it has been demonstrated that MUC1 leads to augmented release of IL-10 in dendritic cells [Monti et al 2004]. Thus it is possible that exposure to DEP leads to increased MUC1 which in turn may lead to increased IL-10 release in asthmatics.

The membrane mucin MUC4, also called sialomucin complex (SMC) is a glycoprotein heterodimer composed of mucin and transmembrane subunits. The transmembrane component contains two EGF-like domains and acts as an intramembrane ligand for the receptor tyrosine kinase, ErB2, the major heterodimerization partner of the epidermal growth factor receptor. [Carraway et al 2000]. It is produced by multiple epithelial cells in both membrane and soluble forms [Carraway et al 2002] Recently it has been shown that inflammatory stimuli lead to increased expression of this mucin. In one study neutrophil elastase, an inflammatory protease was shown to increase MUC4 expression in normal human bronchial epithelial cells [Fischer et al 2002]. In oesophageal cells MUC4

expression was shown to increase in response to bile acids suggesting that inflammatory processes lead to increased expression for this mucin. Interestingly it has been proposed that TGFβ may cause increased expression of this mucin in pancreatric cells. Thus it may be that through the activation of inflammatory processes DEP also activates expression of MUC4, which would normally function as a steric protector for the epithelial surface, however this mucin may also act as a signal transducer by binding onto and activating ErB2. As epithelial cell injury has been shown to lead to augmented release of TGFβ it may be that DEP also activate the expression of this mucin through injury to epithelial cells.

Exposure of undifferentiated cells to IL-13 and ROFA but not DEP, led to augmented MUC5AC gene expression above baseline levels, suggesting that although these cells can be induced to express more MUC5AC and MUC5B, DEP is not capable of causing this effect in undifferentiated cells. Therefore these findings would suggest that DEP and ROFA may either exhibit different mechanisms of action as regards MUC5AC gene expression, or that DEP is less potent and therefore an effect might have been expected to take place at higher concentrations of this particle.

Normal human tracheobronchial epithelial cells grown in the air-liquid interface have been shown to differentiate into a mucociliary epithelium over a two-three week period and express increasing mRNA levels of the airway mucin genes MUC5AC and MUC5B as the cultures age, whereas they express relatively low levels of MUC2 throughout which does not increase with time. MUC5AC gene and protein expression have been shown to increase in response to inflammatory stimuli [Gray et al 2003, Matt et al 2003] During work carried out in this thesis, DEP caused increased MUC2 gene expression, the predominant secretory mucin that is expressed in undifferentiated cells, however in differentiated cells it may also lead to increased gene expression for the secretory mucins MUC5AC and MUC5B. Thus it is possible that the expression of MUC5AC in undifferentiated cells can be induced with more potent stimuli whereas less potent stimuli may also induce this effect in differentiated cells.

Thus, DEP exposure alone may significantly increase MUC2, MUC1 and MUC4 mucin gene expression, whereas ROFA leads to an increase in MUC5AC, MUC5B, MUC2 and MUC4 gene expression.

As work carried out in this thesis has demonstrated that exposure of PBEC to DEP leads to increased gene expression for both secretory and membrane bound mucins, future work should be directed to the investigation of the release of mucin proteins as measured by immunofluorescent staining or western blot on cell lysates, and the role of the EGFR in mediating these responses. Thus inhibitors such as AG1478 and anti EGFR antibodies could be used to determine the mechanism of mucin production in PBEC obtained from both normal and asthmatic subjects exposed to DEP and ROFA. This would help to characterise the mucin response to an environmental stimulus such as DEP and lead to the development of further treatment for the management of mucin hypersecretion in respiratory disorders such as asthma.

As mucins have a protective role on epithelial cells, it is possible that following their expression they may also alter the responses of these cells to particulates such as DEP and ROFA. It would thus be interesting to study the effects of PM on differentiated epithelial cells cultured at the air liquid interface which have already expressed mucins on their surface. Future work could also be directed at identifying mucin gene expression changes in animal models of *in vivo* exposure, although there are differences in the distribution of particulates in animal lungs than humans. A more appropriate system may be to study the effects of PM on humans following *in vivo* exposure as measured through induced sputum, bronchial wash and bronchial biopsies. Should future studies demonstrate that DEP also leads to increased mucin protein expression both in airway cells and in sputum, this would potentially have significant consequences for understanding how exposure to an environmental factor such as DEP may lead to the development of asthmatic features or the worsening of this condition in those already affected by it.

The EGFR mediates MUC5AC expression and bacterial extracts have been shown to activate MUC5AC expression by activation of the EGFR [Song et al 2001, Kohri et al 2002]. Activation of the EGFR may also partly explain the increased susceptibility of asthmatics to DEP exposure. Thus shedding of EGFR ligands in response to DEP may account for the effects of DEP on increased mucin gene expression. Expression of membrane bound mucins may augment these effects as MUC1 can heighten the activity of the EGFR and EGFR ligands can lead to signalling through MUC1. MUC4 also is a ligand for ErB2 and may lead to downstream effects by activating this receptor. DEP could also lead to altered mucin gene expression by a second indirect mechanism involving IL-8 release, a potent neutrophil chemoattractant leading to neutrophil infiltration which together with other inflammatory cells may also lead to goblet cell metaplasia. Therefore there is a necessity to study the effects of DEP on mucin expression particularly the secretory mucins MUC5AC and MUC5B in differentiated cells.

These downstream signalling effects may in part also explain the increased disease morbidity and mortality associated with DEP. These findings demonstrate a newly explored area of understanding into the interactions of various stimuli and mucins.

6.2.4 The effects of DEP on airway remodelling

While human exposure studies have implicated air pollutants in the acute inflammatory response, questions remain about the chronic effects of air pollutants on the lung. When assessed by high-resolution computed tomography (HRCT), patients with chronic, severe asthma have thicker airways compared to those of normal subjects [Awadh et al 1990]. This is due to the re-modelling process that takes place in the airways of asthmatics. While traditionally it has been thought that airway inflammation precedes, and is responsible for, airways remodelling, a study in children found that children who subsequently developed asthma had airway eosinophilia and increased epithelial SBM collagen thickening present up to 4 years before asthma was clinically diagnosed [Warner et al 1998]. In rats, DEP exposure has been found to cause increased collagen

deposition in the airways during lung development [Mauderly et al 1987]. Together, these data suggest that the period during foetal lung development and/or early life may be critical for the interaction of environmental factors with a susceptible genotype. Thus, remodelling of the airways in asthma may actually precede or occur in parallel with inflammation. It is therefore imperative to develop an understanding of the long-term effects of environmental factors such as air pollutants, including DEP in the chronic changes in asthma, if newer insights are to be gained in the treatment of chronic and severe asthma.

One of the characteristic chronic features of asthma is airway remodelling. This is characterised by thickening and increased density of the sub basement membrane (SBM) collagen layer, deposition of interstitial collagens, smooth muscle hyperplasia, increased microvascular networks, goblet cell hyperplasia as well as mucus hypersecretion. TGF-α has been shown to be associated with airway fibrosis in mice. Transgenic mice overexpressing TGF-α have been shown to develop emphysema and fibrosis during postnatal alveologenesis [Hardie et al 2001]. In one study in transgenic mice, human TGFα mRNA was expressed in pulmonary epithelial cells in the lungs of transgenic mice. Adult mice bearing the TGF-α transgene developed severe pulmonary fibrosis. Fibrotic lesions were observed in peribronchial, peribronchiolar, and perivascular regions, as well as subjacent to pleural surfaces. Peripheral fibrotic regions consisted of thickened pleura associated with extensive collagen deposition. Alveolar architecture was disrupted in the transgenic mice with loss of alveoli in the lung parenchyma [Korfhagen et al 1994].

The pathognomonic feature of asthma is sub-basement fibrosis which has been shown to correlate with disease severity, and chronicity. Myofibroblasts are the effector cells responsible for the deposition of matrix proteins. In asthma the number and activity of these cells are increased. These cells continuously interact with the overlying epithelial cells in a dynamic manner through the release of cytokines and growth factors. Therefore as exposure of epithelial cells to DEP augments the release of this growth factor in epithelial cells it is possible that this growth factor may contribute to the subbasement fibrosis that occurs in

asthma by activating myofibroblasts. It may be that exposure to this air pollutant may contribute to these histological changes which may predate the occurrence of asthma symptoms. Moreover, DEP could also contribute to this process through epithelial cell damage. The EGFR is over expressed in areas of epithelial cell damage and this could lead to increased signalling following exposure to DEP and injury to epithelial cells. In view of the central role that EGFR-regulated pathways have in coordinating signals that control bronchial epithelial cell behaviour, it is likely that the high level of EGFR expression in asthma has widespread consequences on the epithelial phenotype, irrespective of its cause.

Therefore more detailed mechanistic studies are needed including epithelial, fibroblast co-cultures at the air liquid interface to further determine the interactions of the epithelial-mesenchymal trophic unit in response to DEP exposure and their potential role in leading to subbasement membrane fibrosis. Such studies will undoubtedly determine potential methods and modalities to treat asthma and reduce the progression from acute asthma to a chronic state characterised by histological and pathophysiological to the airway structure.

The discovery of the mechanism(s) by which an environmental stimulus such as DEP could lead to sub-epithelial fibrosis in genetically susceptible individuals would potentially enable the targeting of specific treatment aimed at possibly reversing the remodelling process. Further research should be directed at the study of epithelial/myofibroblast co-cultures at the air liquid interface. Using ELISA the release of pro-fibrogenic growth factors such as bFGF (FGF-2), insulin-like growth factor I (IGF-I), platelet-derived growth factor BB (PDGF-BB), TGF-β, and endothelin 1 (ET-1) would be measured. Activation of myofibroblasts can be measured using expression of alpha smooth muscle actin (α-SMA). Further research to characterise the effects of DEP on remodelling responses could be carried out in animal models and also following human *in vivo* exposure studies, in which bronchial biopsies could be stained for the release of pro-fibrogenic growth factors using immunohistochemistry.

6.2.5 Targeting the EGFR as a new form of treatment in asthma

Although effective treatment exists in the acute phase of asthma, there is a significant need to develop newer forms of treatment for asthma to potentially reverse the long-term chronic effects of this condition. The treatment of asthma is currently largely limited to the use of corticosteroids and β2-agonists, which have been present since the 1960s' and 1970s'. In the 1990's leukotriene inhibitors such as Montelukast, which are also effective in certain subtypes of asthma, such as exercise induced asthma and aspirin sensitivity have also been introduced. Last year there was also the introduction of anti IgE treatment in asthma, however despite this there is still a significant need for newer forms of treatment for asthma. Corticosteroids are the mainstay of the pharmacological treatment of asthma. Although glucocorticoids are the most effective drugs currently available for asthma treatment, a few asthmatic patients fail to respond well to these compounds, with a small percentage showing complete resistance [Adcock et al 2003]. Understanding the mechanisms leading to this lack of response is very important particularly as this subgroup of patients take up 50% of asthma [Adcock et al 2003].

As it has been demonstrated that DEP and ROFA can lead to epithelial cell inflammation by activation of the EGFR, this may explain some of the lack of response to corticosteroids as these drugs do not inhibit EGFR modulated pathways [Puddicombe et al 2000]. It would therefore be appropriate to identify further novel and innovative treatment modalities. The evidence that DEP as well as cigarette smoke activate the EGFR, point to a potentially new and novel area for the treatment of asthma. There are indeed some [Nadel et al 2001] who support the use of this form of treatment in the management of asthma and in fact in recent years specific anti EGFR treatment has been licensed in the treatment of various head and neck cancers as well as non-small cell lung cancer (NSCLC). In cancer cells, aberrant signalling through the epidermal growth factor receptor (EGFR) activates pathways that stimulate many of the properties associated with neoplasia, including proliferation, migration, stromal invasion, tumour angiogenesis, and resistance to cell death-inducing signals. Because of

the frequency of abnormalities in receptor signalling in human cancers, the EGFR is an attractive target for therapeutic development.

Monoclonal antibodies and small molecule tyrosine kinase inhibitors are the two classes of agents that are the furthest advanced in clinical development. Although pharmacologic and mechanistic differences exist between the two classes, the results of pre-clinical studies suggest that both inhibit proliferation, have little normal tissue toxicity, and are additive/synergistic with standard therapies. The results from early clinical trials have indicated that both classes of agents are well tolerated and have anti tumour activity. Although the results of such trials in cancer are encouraging, the fact that the EGFR mediates reactions that ultimately result in cell growth, differentiation, proliferation, and death, inhibition of this receptor may still prove to lead to undesirable side effects. This is particularly the case as asthma is largely a non-fatal disease and therefore unlike patients with NSCLC who would be expected to have a reduced life span those with asthma would by enlarge be expected to live a near normal life span. As the EGFR has important regulatory effects on epithelial cell mitogenesis, the inhibition of this receptor could potentially have significant long-term downstream effects that need to be considered and studied in detail before its use in asthma can be supported. It has been shown in many studies as well as in those carried out in this thesis that DEP have cytototoxic effects on epithelial cells and therefore inhibition of the EGFR would potentially have significant downstream effects on epithelial repair processes. Thus, carcinogenic particles such as DEP could remain on the airway surface for longer periods and also penetrate deeper into the airway and potentially lead to illnesses such as lung cancer. Although, in patients who have little hope of survival such as those with NSCLC, it may be ethically acceptable to be exposed to such possible side effects, that may be more difficult to justify in patients with asthma who would be expected to live a normal life span. Nevertheless inhibition of EGFR and its tyrosine kinase activity represents a logical targeted approach in asthma and may provide a new area of treatment. It would however be wise to review the side effect profile in patients with NSCLC and other cancers treated with these new anti EGFR agents over a longer period of time before exposing patients with asthma to these novel treatment modalities.

6.3 Summary

Work carried out in this thesis has demonstrated novel mechanistic findings in relation to the action of DEP and ROFA, which have been used as surrogate markers for the action of PM as well as markers of the action of an environmental factor in causing asthma on bronchial epithelial cells. It was demonstrated that DEP and ROFA have significant pro-inflammatory effects on bronchial epithelial cells, which at least in the case of DEP, are mediated through the EGFR and involve the shedding of the EGFR ligands Transforming Growth Factor α (TGFα) and Amphiregulin (AR) and are augmented in the presence of Th2 cytokines. Exposure of bronchial epithelial cells to DEP and ROFA leads to enhanced expression of gel forming and membrane bound mucin genes, which could contribute to disease morbidity and mortality in asthma.

The studies described have also identified potential new areas of treatment for asthma, which need further research based upon the EGFR and its down stream effects on epithelial cells which are known to have a key modulatory role in maintaining the asthmatic phenotype.

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