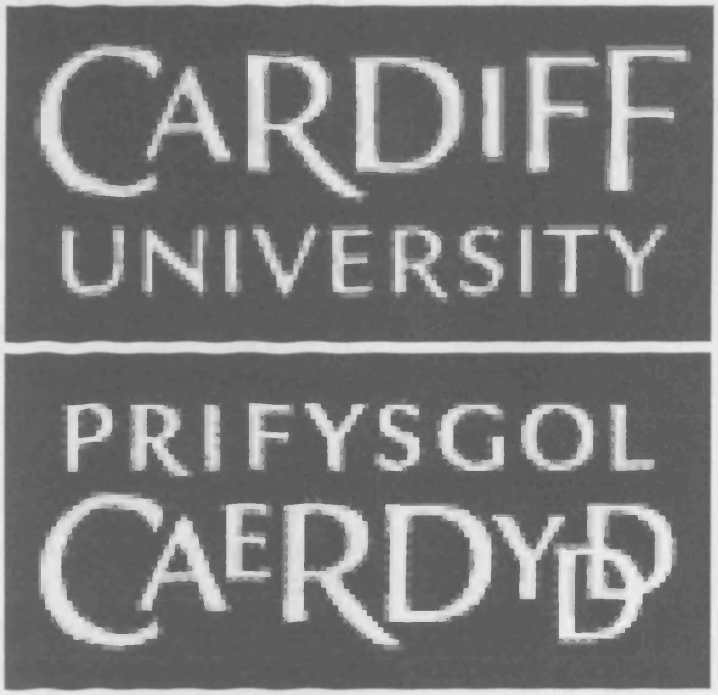
Toxicological assessment of the pulmonary response to air pollution particles

By

Helen Wise



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Cardiff School of Biosciences,
Cardiff University,
PO Box 911,
Cardiff CF1 3US

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
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AE	Attachment efficiency
cDNA	Complementary deoxyribonucleic acid
CD_{50}	Mass required to detach 50 % of cells as compared with untreated controls = 100 %
CD_{20}	Mass required to detach 20 % of cells as compared with untreated controls = 100 %
DEP	Diesel exhaust particles
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EM	Electron microscopy
Hepes	N-2-hydroxyethypiperazine-N'-2-ethanesulphonic acid
HPLC	High Performance Liquid Chromatography
HRP	Horse radish peroxidase
kb	Kilobase ladder
LFC	Lung free cells
LP	Lung parenchyma
MMLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
NADPH	β-nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NF-kB	Nuclear factor kappa B protein
PBS	Phosphate buffered saline
PM_{10}	Particulate matter less than 10 microns
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
SSC	Saline-sodium citrate buffer
TBE	Tris-borate-ethylenediaminetetracetic acid
TBS	Tris buffered saline

Publications

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Reynolds, L., Jones, T. Wise, H., and Richards RJ. (2003). "Toxicity of airborne dust generated by open cast coal mining." Mineralogical magazine **67**(2): 139-150.

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There is strong epidemiological evidence of association between PM_{10} (particulate matter with an aerodynamic diameter less than or equal to 10 microns) and adverse health outcomes including death and increased hospital admissions for cardio-pulmonary conditions. Ambient PM_{10} surrogates such as diesel exhaust particles (DEP), a common component of UK PM_{10}, have been shown to induce lung inflammation in both humans and rodents. To date, few studies have reported on the toxicological response of UK PM_{10} in experimental animals.

This thesis summarises the characterisation of the pulmonary toxicity of Cardiff urban PM_{10}. Firstly, the pulmonary toxicological responses in male Sprague Dawley rats following the intratracheal instillation of Cardiff urban PM_{10} were examined. A mild, but significant, change in lung permeability was observed in the lung post instillation of a high (10 mg) dose of the whole PM_{10} as adjudged by increases in lung to body weight ratio and total acellular lavage protein. Such effects were less marked following instillation of a water-soluble fraction (80% of the total mass) but histological examination showed that lung capillaries were swollen in size with this treatment. Secondly, expression profiling of PM exposed lung tissue identified distinct genes differentially expressed as a consequence of exposure to a high dose (10 mg) of whole PM and equivalent dose of water soluble component of PM. Such changes were linked to different histopathological events within the lung. In conclusion, conventional toxicological, histological and toxicogenomic studies have indicated that Cardiff PM_{10} exhibits low bioreactivity in the form of mild permeability changes.

In vitro toxicological assessment of the bio reactivity of the PM sample using primary cultures of epithelial type II and alveolar macrophage cells were used to identify the relative contribution of the different fractions (whole, washed "durable" fraction and water soluble component) to the overall PM toxicity. Correlations with total metal content and particle size were identified in the different cell types. Cellular analysis using advanced fluorescence labelling and microscopy identified the ability of the PM to induce subtle changes in cell and nuclear morphology that may result in increased cell death and apoptosis even at low particulate doses. Expression profiling of the PM exposed cells proved difficult and realised limited information regarding the cellular responses to the toxicant. This approach failed to identify the same gene changes reported in the lung tissue profiling, illustrating the importance of the different cell types within the lung to orchestrate a pulmonary response.

In conclusion, the use of toxicogenomics and gene expression arrays in toxicological is still relatively novel approach to the assessment of lung injury. The study addresses initial problems and offers some possible solutions regarding the use of macro arrays and expression profiling in toxicology research. Expression arrays offer considerable scope as a research tool and in future will provide a powerful insight into gene changes on a global scale.

Chapter 1.0

General Introduction

1.0 General Introduction

1.1 The Lungs

The primary function of the lung is the exchange of respiratory gases, it is also responsible for the synthesis and turnover of connective tissue matrix. The nature of the lung, with its extensive surface area and immense capillary network renders the lung highly susceptible to both blood and air borne toxicants and is known to be a specific target for and accumulate many toxins and drugs including the anti-neoplastic cancer drug, bleomycin and the herbicide paraquat. Thus the lung is at the frontline of defence to many air- and blood- borne toxins and has an important role in detoxification to prevent tissue and systemic toxicity. The lung is responsible for the synthesis and turnover of protective epithelial lining fluid, including surfactant and contributing to the repertoire of enzymes providing front line defence from inhaled foreign objects.

The lungs (Figure 1.1) consist of five lobes, the left lung has one oblique fissure dividing into superior and inferior lobes whilst the right lung has two fissures (oblique and horizontal) dividing into superior, middle and inferior lobes. The trachea, a cartilage supported tube connects the bronchi within the lungs to the nasal and buccal cavity, each bronchus divides repeatedly to form a tree of airways, the bronchioles, which terminate with alveoli, the respiratory surface of the lungs.

1.2 The Alveoli

1.2.1 The architecture of the alveoli

The role of the alveolus is the exchange of respiratory gases. The alveolus therefore contains a thin cellular barrier called the epithelium physiologically adapted for optimal gas exchange (Figure 1.2). There are five main cell types within the alveoli: alveolar macrophages, endothelial cells of the pulmonary capillaries, epithelial type I and II cells and fibroblasts of the interstitium.

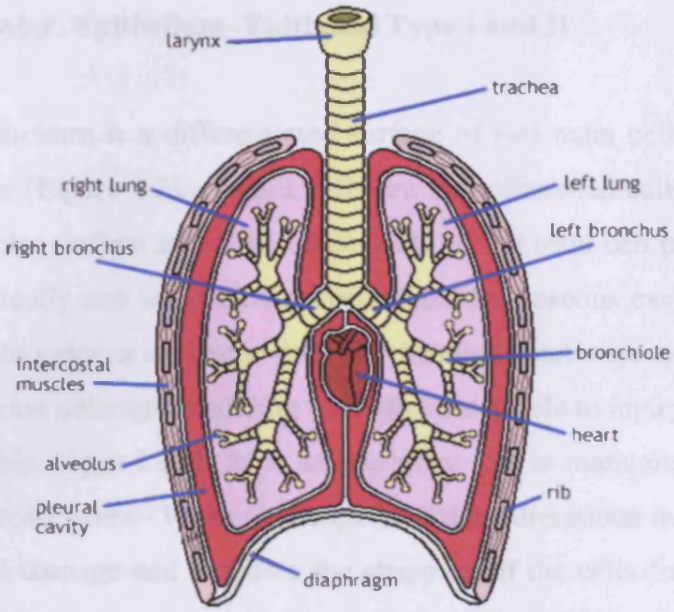


Figure 1.1: A diagram illustrating the human respiratory system. Source: Encarta 2000.

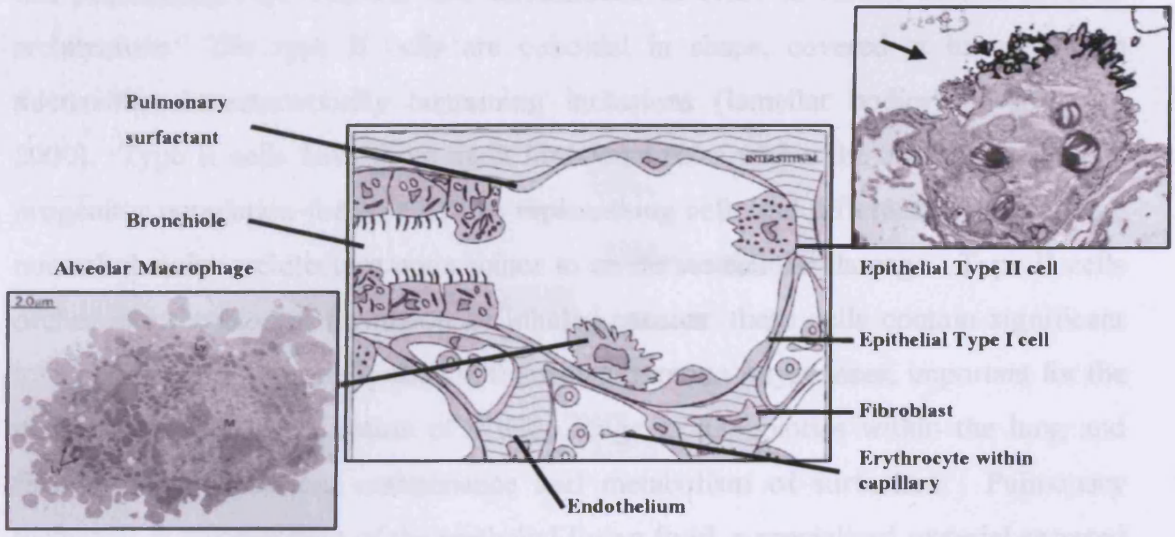


Figure 1.2: Representation of the alveoli illustrating the five main cell types within the lung: macrophages, epithelial type II and type I cells, fibroblasts and endothelial cells. (Hoet and Nemery., 1993). Pictures inset are EM images of alveolar macrophages and epithelial type II cells (courtesy of Mr. D Dinsdale, 2000).

1.2.2 The Alveolar Epithelium- Epithelial Type I and II

The alveolar epithelium is a differentiated surface of two main cell types: epithelial type I and II cells (Figure 1.2). Type I cells are large flattened cells, covering up to 90% of the alveolar surface area, constituting 8% of the total cell population. They are both metabolically and anatomically specialised for gaseous exchange, providing a slender barrier to gaseous exchange with low metabolic activity, reducing their own oxygen consumption although rendering the cells susceptible to injury upon inhalation of foreign materials. Type I cells have an important role in maintaining the blood air barrier at the alveolar level. When challenged, surface alterations including blebbing may result in cell damage and facilitate the stripping of the cells from the basement membrane.

Upon injury and damage to the type I cell, epithelial type II cells (or stem cells within this population) will replicate and differentiate in order to restore normal alveolar architecture. The type II cells are cuboidal in shape, covered at its apex with microvilli, characteristically containing inclusions (lamellar bodies) (Castranova, 2000). Type II cells have three main functional roles within the alveoli, firstly as a progenitor population for type I cells, replenishing cells and differentiating to restore normal alveolar architecture upon minor to moderate cellular damage. Type II cells orchestrate the biotransformation of inhaled species: these cells contain significant levels of enzymes especially the P450 dependant mono-oxygenases, important for the metabolism and detoxification of a wide range of xenobiotics within the lung and finally in the secretion, maintenance and metabolism of surfactant. Pulmonary surfactant is a component of the epithelial lining fluid, a specialised material secreted and metabolised by epithelial type II cells serving to reduce the surface tension at the air water interface within the alveolus, providing stability of the airways and preventing collapse of the alveoli. Surfactant comprises of dipalmitoyl phosphatidylcholine (50%), unsaturated phosphatidylcholines (25%), phosphatidylglycerol (5-10%), cholesterol (5%) and protein (8-10%). The surfactant phospholipids are transported and processed within the lamellar bodies of the type II cells and by exocytosis, a process that has been found to be stimulated upon inhalation exposure to many toxicants (Dobbs et al, 1978). Type II cell dysfunction or

failure of the immature type II cells to produce sufficient surfactant culminates as respiratory distress syndrome, common in premature babies.

Type II cells contain well-developed Golgi apparatus, indicative of their high metabolic activity. Their role as the progenitor population is forefront in the repair and restructuring of the alveolus. The epithelial cell is the first cell to replicate and differentiate following epithelial damage, a consequence of exposures and local inflammation. It has also been postulated that the replaced cell population becomes less susceptible to subsequent exposure (Mason et al., 1977). Hyperplasia of the type II cell population has been traced in both lung injury and the developing lung (Quinlan et al., 1995). Upon injury or damage to the type I cell population competent type II cells will replicate and differentiate becoming flattened and gaining the type I cell phenotype. However, upon sustained or severe damage to the epithelium may result in uncontrolled cell division (hyperplasia) of the type II cells, resulting in thickening of the epithelium.

1.2.3 The Alveolar Macrophage (AM)

Alveolar macrophages are derived from bone marrow and form part of the mononuclear phagocytic system of the body. AM's represent a heterogeneous population of scavenger cells with the primary function to phagocytosis and clearance of cellular debris (from normal cell turnover), foreign particles and micro-organisms within the lung (Sibille and Reynolds, 1990). Metchnikoff (1881) was amongst the first to recognise the importance of these phagocytic cells "[They] engulf the *tubercule bacilli* readily but perish shortly after or become engulfed themselves by other mononuclear phagocytes". Alveolar macrophages are distinct from their pulmonary interstitial counterparts in that they have no ability to replicate and have poor antigen processing and presentation capabilities. Free alveolar macrophage cells can be easily isolated from the lung, from the bronchial-alveolar lavage and are morphologically distinct from neutrophils and other alveolar cells, with large rounded nuclei. Upon challenge *in vivo,* these cells are responsible for the phagocytosis of particles, inciting the further recruitment of other phagocytic cells and initiation of inflammation within the alveolus.

1.2.4 Endothelial Cells and Interstitial cells

The endothelial cells are squamous flattened cells bound to the basement membrane and make up the capillary walls surrounding the alveolus. Interstitial cells encompass a multitude of cell types including smooth muscle and fibroblast cells, producing connective tissue proteins including elastin and collagen. Inappropriate activation of these cells by inhaled xenobiotics can result in excess deposition of these products attributing to the lung pathogenesis. Cell to cell communication, directly via cell contacts or indirectly via soluble mediators, is an important mechanism to control and regulate the cell activities and cell cycle in the alveolus. Overlying Type II epithelial cells repress fibroblast cell cycle and division (Shannon et al., 1987), loss of this repression may result in the proliferation and activation of the underlying fibroblasts, or the improper deposition of cell products such as collagen within the alveoli to the detriment of lung flexibility and reducing the efficiency of gaseous exchange within effected tissue.

1.2.5 Clara Cells of the Upper Airways

Clara cells are non-ciliated secretory cells within the bronchioles. These cells contain a variety of enzymes including P450 mono-oxygenases, central to the detoxification of xenobiotics, in addition, they serve as progenitor cells for new Clara cells and ciliated cells of the airway (Oreffo et al., 1990).

1.3 Particulate Air Pollution

1.3.1 PM_{10} and Health Effects: An Overview

The earliest documented links of adverse health effects with air pollution were made in medieval England. In 1733, a prominent scientist of the day, John Arbuthnof, stated, *"city air was not as friendly as that of the country- for its replete with sulphorous streams of fuel."* Episodes of severe air pollution have arisen since the industrial revolution, the London smog's of the 1950's with PM_{10} levels exceeding

6000 $\mu g/m^3$ (24 hr average) recorded resulted in the implementation of the Clean Acts from 1956. These acts have successfully improved air quality, but the increase in traffic has resulted in the re-emergence of air pollution episodes within many of our towns and cities exceeding the government and European implemented quality standards (50 $\mu g/m^3$ 24 hr averages) (POST Technical Report, 82, 1996).

Air pollution monitoring has reported a decline in the levels of pollution, measurable by ambient levels of PM_{10}, since the notable pollution episode in London (1952) but health effects are still being reported. These adverse effects may not arise as a result of the general level of pollution, but may be attributed to a more specific aspect of air pollution (Frampton et al., 1999). Whilst levels of larger particles and exhaust gases have decreased, there has been an increase in the prevalence of smaller sized particles that make up very little, by mass, of collected samples, but contribute a highly significant proportion, by number and surface area, of samples (Pooley, *personal communication* 2000).

Several countrywide networks funded by Department of the Environment/Local Authority currently monitor UK PM_{10} levels. The Automated Urban Network measures the hourly concentrations of a range of pollutants including particles, established in 1992, it now comprises of more than 29 urban sites. Respirable particles (PM_{10}) are measured by mass (μg per m^3). Annual levels are usually in the range 20-30$\mu g/m^3$, with the highest UK levels recorded (62 $\mu g/ m^3$) during a pollution episode in October 1994 (POST Technical Report, 82, 1996). The national 24 hour standard for air pollution (50 $\mu g/m^3$) set by E.P.A.Q.S. (Expert Panel on Air Quality Standards) has been exceeded at most of these sites since monitoring was initiated. A number of new UK EPAQS policies have been designed to reduce vehicle exhaust emissions by up to 39% by 2010.

1.3.2 PM_{10}

Particulate matter is a generic term used to describe airborne particles with a wide range of chemical and physical characteristics (POST Report 82, 1996). Particulate air pollution is a suspended mixture of solid and liquid particles varying in size,

composition, origin and effects (Dockery and Pope, 1994). The size and composition of particles are influenced by source. Coarse particles (~7 microns in aerodynamic diameter) are predominately from mechanical processes, erosion, re-suspended soils and sand whilst finer particles, contributing 80-95% of the total number of particles, result from controlled combustion processes including traffic and some industrial processes.

Major elements of particulate matter in the U.K. include sulphates, nitrates, chloride, sodium, ammonium, acids, elemental carbon particles, organic carbon compounds, metals, crustal and biogenic material (Harrison, 2000). Metals may contribute up to 5% of a typical PM sample (Harrison, 1995) and are postulated to be highly influential towards the overall toxicity of a PM sample (Adamson et al., 1999 and 2000. Gavett et al., 1997). The metals present in ambient particulate pollution may derive from both natural and anthropogenic sources and levels of several transition and heavy metals including copper, lead, vanadium and zinc are commonly measured in PM samples. Secondary pollution arises as a result of interactions and chemical reactions between pollutant moieties within the atmosphere and includes gases and organics that may associate or accentuate particle toxicity.

In vivo studies investigating the toxicity of urban PM_{10} in animal models have used diesel exhaust particles (DEP) as a surrogate for urban PM_{10} (Figure 1.3). DEP contributes up to 90% of urban particulate matter and was considered to well represent the highly heterogeneous, physicochemical mixture (Bérubé *et al.*, 1999).

Characterisation of DEP reveals that it consists of distinct types, aggregates of carbon spherules, contained within an organic coating and as liquid drops: a hydrated sulphate nucleus encompassed by a mainly organic coating (Harrison, 1995). Characterisation studies of the DEP particles conclude that the particles consist of three morphological types: (1) individual particles or spherulites, with a mean diameter of 30nm; (2) small aggregates of spherulites in the form of chains or clusters and (3) large agglomerated spherules 1-2μm in diameter (Bérubé *et al.*, 1999).

A typical South Wales PM_{10} sample (Figure 1.3) is a heterogenous mixture of carbon soot, diesel exhaust particles, salts and minerals, smelter particles consisting of chiefly iron particles and anthropogenic particles.

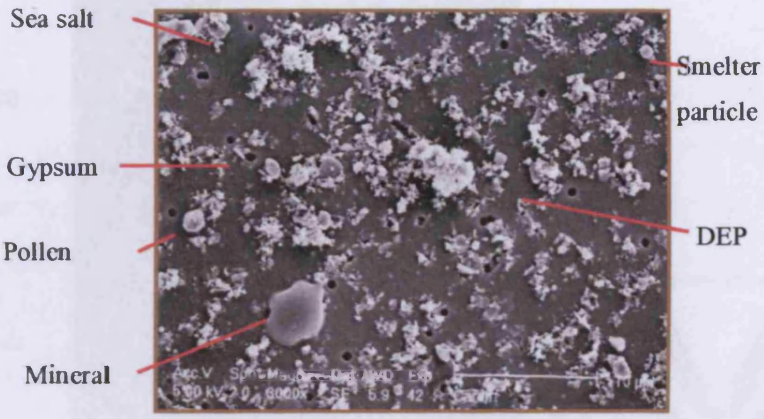


Figure 1.3: SEM image of a typical Cardiff (South Wales, urban) PM_{10} sample on a polycarbonate filter. The sample may contain up to 80% (by number of DEP) and smelter particles blown in from nearby steel works at Port Talbot (up to 6%).

1.3.3 South Wales PM_{10}

A high volume cascade impact collector (Harvard) has been used to collect samples of PM_{10} from around the country (Moreno, 2003)(Figure 1.4). This sampling technique enables sufficient sample to be collected to allow full chemical, morphological characterisation and assessment of the reactivity of the PM both *in vivo* and *in vitro*. This analysis of the bio-reactivity of both in cell free toxicology (Greenwell et al., 2003), cellular toxicity *in vitro* and *in vivo* with a well characterised sample provides the potential to attribute the toxicity with chemical or morphological characteristics of the ambient sample. The collector samples air at 1100 litres/min^{-1} through three polyurethane foam (PUF) filters within the collecting head. Particles bigger than 10 μm (insects, fragments of plant or hairs) impact onto the first PUF substrate, the air then moving to the next step by passing through a 10 μm inlet with the 10-2.5 μm particles, the air then progresses through to the 2.5 μm inlet, depositing 2-5-0.1 μm in a third PUF (Moreno et al., 2003).

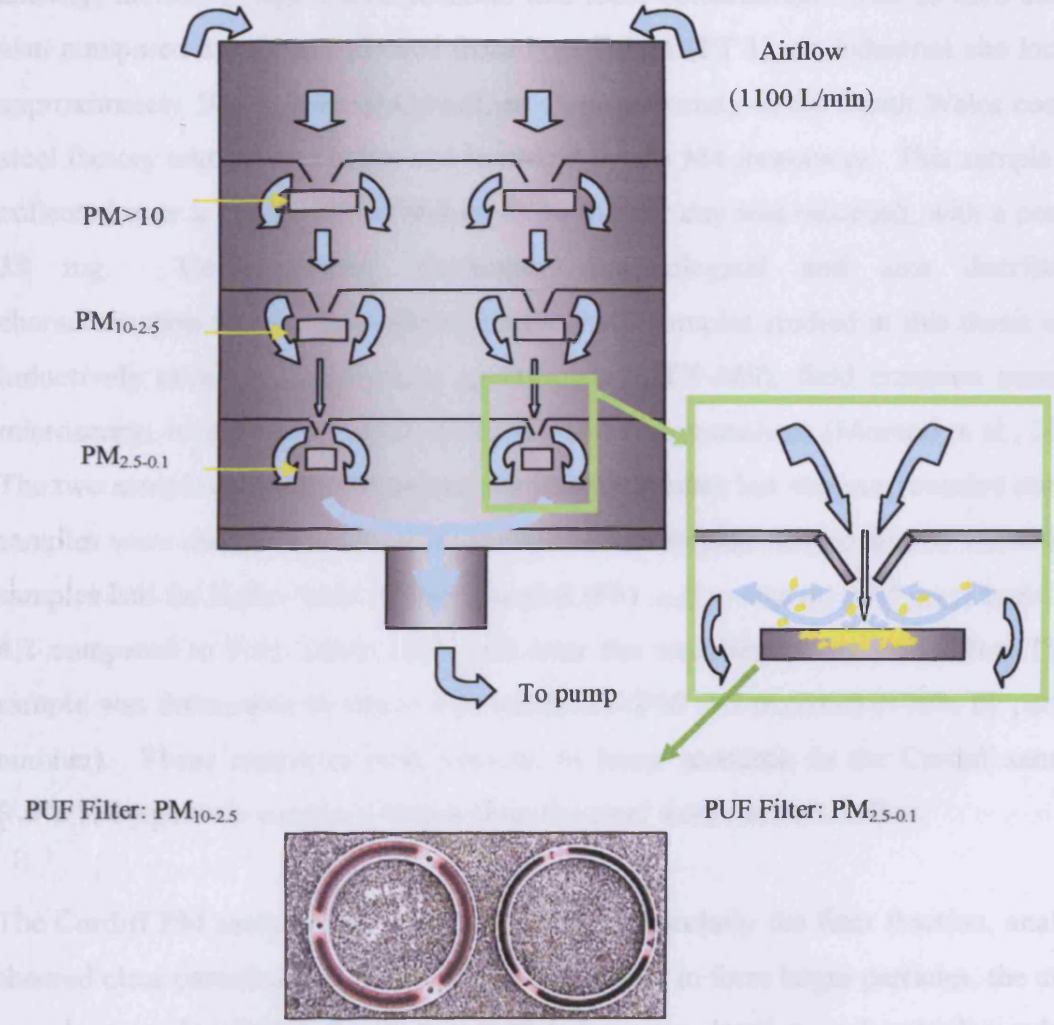


Figure 1.4: A diagram illustrating the mechanisms of the Harvard high volume cascade elutriator PM collector used for the larger scale collection of $PM_{10-0.1}$ for all PM samples described in this thesis. (Moreno et al., 2003)

The studies described in this thesis concentrate on the bio-reactivity of two main collection sites, Cardiff and Port Talbot. The Cardiff collection site is located within the civic centre; approximately 500 metres form the city centre. It is positioned in close proximity to several major routes including Park Place (100 m, NE), the main A470 (250 m, SW) and the main route through Cardiff (A48, 1600 m). The main Cardiff sample (CF 2) used for the studies described in this thesis concerns a collection period of 43 days (January 2001- March 2001) when a daily average of 38 mg was recorded. The Cardiff location represented an urban location close to the

South Wales coast (approximately 3.5 km), with high volume levels of local human activity, including high traffic volumes and local construction. The *in vitro* studies also compared a sample collected from Port Talbot (PT 1), an industrial site located approximately 50 km west of Cardiff, in close proximity to the South Wales coast, a steel factory and power station and bordered by the M4 motorway. This sample was collected over a 25-day period and up to 30 mg per day was recorded, with a peak of 38 mg. Comprehensive elemental, morphological and size distribution characterisation has been completed on the PM_{10} samples studied in this thesis using inductively coupled plasma mass spectrometry (ICP-MS), field emission scanning microscopy, scanning electron microscopy and image analysis (Moreno et al., 2003). The two sample sites showed several common attributes but analysis revealed that the samples were distinct in both chemical and morphological compositions. The Cardiff samples had far higher ratio of finer fraction ($PM_{<2.5}$) to coarser ($PM_{10-2.5}$) (ratio 2.8-4.2 compared to Port Talbot ratio 1-2) over the sampling period. The Port Talbot sample was dominated by dense iron spherules (PM<2.5 microns) (>30% by particle number). These spherules were present, in lesser amounts, in the Cardiff samples (<6% % by particle number), blown from the steel works at Port Talbot.

The Cardiff PM samples were heterogeneous, particularly the finer fraction, analysis showed clear particles and finer particles nucleating to form larger particles, the urban sample was dominated by soot particles from combustion and vehicles exhaust emissions. Elemental analysis showed that the finer fraction and coarser fraction had a slightly different composition. The coarser fraction comprised chlorides (26%, chiefly coastal origin), sulphates (23%), elemental and organic carbon compounds (20%) and other compounds (1%- nitrates, silicates, iron containing moieties, carbonates). The finer fraction contained fewer chlorides (20%), more sulphates (35%) and more nitrates (21%) by particle number. Compounds common to samples from both locations included elemental and organic compounds, including biological compounds such as pollen, bacterial and fungal spores. The chlorides are chiefly sodium chlorides or ammonium chlorides, wind blown from the sea or from salt treatment of nearby roads. Iron (II) spherules dominate the Port Talbot samples (>30% by mass of samples collected); much of the *in vitro* bio-reactivity of the samples is attributed to this transition metal (Greenwell et al., 2003). The spherules

are condensates of iron released during the high temperature smelting procedures at the nearby steel works. Nitrogen containing compounds chiefly comprised of irregularly shaped nitrate and sodium nitrates. Silicates include minerals such as quartz, kaolinite and feldspars, from erosion of local geological features and local activities such as quarrying and construction.

1.3.4 PM_{10} Health Effects

Particulate matter of less than ten microns in aerodynamic diameter (PM_{10}) is considered as respirable and will penetrate into the trachea and larger airways of the lung; finer particles ($PM_{<2.5}$) will enter the smaller airways and alveoli

Increases of ambient levels of particulate matter are correlated with increased incidences of cardiovascular and pulmonary related deaths, increased hospital admissions and exacerbation of lung conditions (Anderson *et al.*, 1991). Many epidemiology studies conclude that 1% of the population are at a higher risk of mortality as a result of a 10 $\mu g/m^3$ increase in ambient PM_{10} levels (Seaton *et al.*, 1995), an expected 1.4% increase in cardiovascular deaths.

Personal exposure to ambient PM_{10} is reflected in both indoor and outdoor PM_{10} levels. Individuals spend op to 90% of their day within an indoor environment. Indoor pollution levels are heavily dictated by the conditions outside in addition to indoor activities (Janssen et al., 1998). Thus PM_{10} exposure comprises a significant risk to health.

In the UK there appears a straight-line relationship between exposure and response (health effects), up to levels of approximately 200 $\mu g/m^3$ (POST Report 82, 1996). Hospital admissions for asthma related conditions increase by 2 % and by 1 % for all respiratory conditions for every 10 $\mu g/m^3$ increase (Dockery and Pope, 1994).

Summaries of UK based studies are illustrated in Table 1.1. In 1991, small increases in mortality (10%), hospital admission rates (4 %)- especially amongst young children and elderly or those with pre-existing cardiovascular conditions- were observed in

central London and correlated with increases up to 3.4 times the monthly average levels of total suspended particles (referred to as black smoke) (Anderson et al., 1995).

Table 1.1: Summary of health effects attributed to rise in PM_{10} levels in the UK. Adapted from POST Report 82 (1996). Based on data from both time series studies and panel studies. Conclusions drawn from such analyses show a significant link between particulate levels and short-term adverse health effects.

Health Effect	% Change ($10\mu g/m^3$ increase)
Mortality	
Total	0.7 - 1.6 %
Respiratory	1.5 - 3.7 %
Cardio-vascular	0.8 - 1.8 %
Hospital Admissions	
Asthma	1.9 - 2.1 %
Respiratory conditions	0.8 - 3.4 %
Exacerbation of Asthma	
Attacks	1.1 - 11.5 %

Epidemiological studies on school children during air pollution episodes reported a repression in lung function during and directly after episodes, normal lung function was restored up to two weeks following the improvement in air conditions. Although such effects are transient, increases in air particulates have been associated with other complications, a multitude of independent studies have shown a coherence of effects across a range of related health outcomes (Dockery and Pope, 1994). Particles promote the premature death, hastening the onset of death in compromised and susceptible individuals and increases susceptibility to infectious conditions (Seaton, 1995).

A number of studies have also correlated the changes in ambient PM_{10} levels with alterations in lung function. Scarlett (1995) reported a decrease in lung function in symptomatic and asymptomatic school children with increases in ambient pollution levels, this change has also been noted in adults. It has been postulated that small shifts in lung function could be detrimental to those in a borderline state of health to

elicit a clinically important response (Scarlett et al., 1995). The impact on short-term health and respiratory effects have been well described, longer-term health impacts may include increased mortality. Data from a wide range of studies indicate that increased incidences of non-neoplastic cardio-pulmonary disease and asthma may be associated with long-term exposure to particles (IEH (2000) *Airborne Particles:* Exposure in the home and health effects).

Frampton (1999) studied the effect of a short term closure of a local steel mill in the Utah Valley (1986-1989) and the subsequent changes in air pollution to evaluate the contribution of the metals to observed health effects in the nearby population. Pope (1999) reported that during the 12-month shut down, local levels of PM_{10} were reduced and this decline was accompanied by a decrease in reported health effects. These findings were supported by both instillation studies in the rat (Dye et al., 2001) and *in vitro* studies using primary rodent epithelial cells (Pagan et al., 2003). These studies correlated increases in pulmonary inflammation, cellular stress and toxicity with the increased metal content of the ambient PM samples, specifically copper, vanadium and zinc (Dye et al., 2001).

Frampton (1999) concluded that respiratory health effects were influenced by changes in particle source and composition as well as exposure composition. The toxicity of the suspended particles is influenced by many factors including bulk chemical and trace element composition, acid content and particle size distribution (Harrison, 1995). Particulate matter is an anthropogenic mixture of chemicals and the composition varies according to source, locality (local activities etc), climatic factors and seasonal influences. Particles typically contain high levels of elemental carbon, sulphates, nitrates (oxidation products of sulphur- and nitrogen- oxides), ammonium and associated organic material. The trace element composition of PM samples is often influential on its overall toxicity. Increased levels of bioavailable transition metals such as zinc, lead and iron, increase toxicity of samples in animal models (Richards *et al.*, 1989. Adamson *et al.*, 2000). *In vivo* studies (Rice et al., 2001) instilled equi-molar doses of a variety of metal sulphates into the rat and concluded that inflammation and lung responses were distinct for different metals. Therefore, chemical and metal composition must be taken into consideration, in addition to

particle size, when ascertaining the overall toxicity of pollutant or toxicant samples. Particulate size influences the behaviour of the aerosol, smaller particles are more likely to be deposited within the respiratory tract and remain airborne for longer, more likely to be transported further from their source than larger particles. In animal studies, fine and ultrafine particles have been found to provoke different magnitude of response in the lung, than larger particles. Oberdorster (1992) compared the biological effects of ultrafine (0.001-0.1μm), fine (0.1-2μm) and coarse (2-10μm) titanum dioxide particles *in vivo*. It was concluded that the more severe response elicited by the smaller particles arose as a result of increased surface area and an increased particle number per unit mass of the ultrafine particles.

1.4 The Lung Response to Inhaled Particles

1.4.1 Particle Deposition within the Respiratory Tract

Particle deposition patterns within the lung have been modelled using radiolabelled particles to estimate the physical behaviour of particles in a simple anatomical model of the lung. Age, sex, health and personal activity levels are all instrumental in dictating the deposition of particles within the lung in addition to changes in deposition efficiency with different particle sizes. Higher deposition efficiencies of fine particles (<0.5 μm diameter) were found in the smaller airways to terminal bronchioles and alveolar regions compared to coarser components (>1 μm), these efficiencies increased in younger children for the same environmental exposure (MRC (2000).

The lung has three main means of defence: mechanical, phagocytic and immune response. Nasal hair and impaction filters coarser particles (PM>10 microns in diameter) and foreign objects from the inhaled air. The nasopharynx serves to humidify passing air. The trachea and bronchi are lined with ciliated epithelium containing mucosal cells, responsible for secretion of a mucoid material lining the airways. Large particles enter the mouth and nose becoming deposited within these larger airways and are engulfed within the mucus secretion. Underlying ciliated cells beat in unison, pushing the overlying material into the upper regions, until it can be removed, either by coughing or swallowing (the mucocillary escalator). A key to the

success of this method of clearance is the efficient production of peri-ciliary fluid, the maintenance of appropriate physiochemical properties of the mucus and integrity of the ciliated epithelium (MRC 2000). Lung or disease conditions that compromise the functioning of respiratory cilia such as cystic fibrosis will impede the clearance of particles in this manner. Particles within the smaller airways may also be removed from the lung via a macrophage-mediated clearance. Scavenging alveolar macrophages ingest the particulates or cellular debris and either digest the material or become particle laden and more rounded and removed from the airspaces via the mucocillary escalator. Failure to remove the particles or their associated chemicals may result in interaction with the respiratory epithelium. The chief targets for the particles would appear to be the epithelial cells, pulmonary alveolar macrophages and inflammatory cells. Particles are also removed from the lung tissue over a longer period of time involves the lymphatic drainage. Particles have been traced to the lymph nodes following exposure to inhaled particles. Lee (2003) reported cytoxicity within the lymph nodes themselves following lung instillation of crystalline silica, resulting in significant cellular changes both within the lung and pulmonary lymph nodes.

1.4.2 Particle Interactions within the Respiratory Airways

The fate of a particle deposited into the respiratory system is dependant on its solubility and ability of the lung to clear the particles from the airways. The bronchio-alveolar lavage fluid (BALF) is the first physical interface encountered by inspired particulate matter (Zielinski *et al., 1999*) and contains the antioxidant defences of the lung. The mucous and lining fluids sequester up to 35% of a delivered dose of PM into the lung. The BALF contains a plethora of antioxidants, such as ascorbic acid (AA), glutathione (GSH), super oxide dismutase and uric acid (UA), which sequester particles and associated compounds within the lung (Kelly and Richards, 1999). The BALF also contains surfactant, a lipid and protein rich solution that has an important role in the smaller airways of the lung, reducing surface tension of the airways and pulmonary alveoli. However, persistent particles may not be impeded by the BALF, the fluid may even serve to facilitate the release of potent

chemical entities, such as bioavailable metals and free radicals, to invoke further challenge to the lung.

Particle deposition results in an immediate increase in surfactant and mucoidal production. Bioreactive particles may be able to penetrate the protective BALF and interact with the underlying epithelial cells. Alveolar macrophage (AM) cells will phagocytose the particles, and remove them from the respiratory tract. Particle laden macrophages become rounded and less mobile and may be degraded or removed from the lung via the mucocillary escalator or via the lymphatic system. Morrow (1988) showed that particles themselves might exert toxicity independent of any chemical interaction- the "particle effect" (Oberdorster, 1991). Pulmonary toxicity was achieved by decreasing the lung clearance of the particles with doses that resulted in as little as 6% of the alveolar macrophages becoming laden with engulfed material served to compromise of AM mediated pulmonary clearance. Macrophage mediated pulmonary clearance was significantly halted when 60% AM were loaded with particles. Exposure and alveolar macrophage mediated phagocytosis of extremely toxic particles, such as quartz, may result in toxicity to these cells by the internalised particles, further impairing pulmonary clearance. The decrease in particle clearance results in increased lung retention, increased exposure of the particles to the alveolar cells, increased epithelial cell mediated uptake of particles, increased interstitialisation and subsequent interaction with fibroblasts, culminating in lung inflammation, neutrophil influx and *pulmonary oedema*, measurable by biochemical and histological methods.

Alveolar macrophages are the primary source of pro-inflammatory mediators, and cytokines (including IL-8, TNF-α and IL-1) (Becker, 1996. Donaldson et al., 2003). The simultaneous release of chemotactic factors and signal molecules from activated (phagocytosing) macrophages attract more phagocytes to the alveolar surface, influencing epithelial permeability hence allowing cells from the blood to enter the lung tissue, protecting the lung from inhaled particles and simultaneously promoting further particle ingestion. With increased lung burden and overload at higher or longer exposures to suspended particles, large scale activation of AM's results in excess release of these factors (reactive oxygen species, free radicals, proteases and

growth mediators) and may result in AM cell lysis, spewing the cell contents and re releasing particles into the alveolar region. All of these have the potential to contribute to chronic lung injury, influencing neighbouring cells and if transported into the blood stream to initiate systemic effects (Khandoga et al., 2003). These effects are summarised in Figure 1.5.

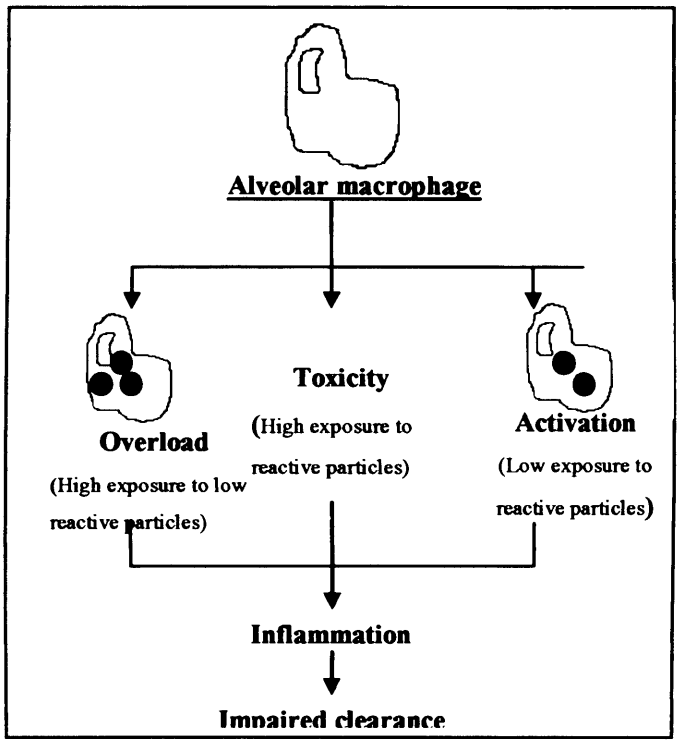


Figure 1.5: Summary of the interactions of particles with alveolar macrophages. Low reactive particles include titanium oxide and reactive particles e.g. quartz. Adapted from IEH (2000) *Airborne Particles:* Exposure in the home and health effects.

Fine particles enter the interstitium more readily than larger sized particles (Richards, *personal communication*, 2003), eliciting a greater magnitude of inflammatory responses, a consequence of the larger surface areas of the particles. The smaller particles appear to remain undetected by AM's longer and induce local chemotactic factors (to induce migration of AM's) poorer than larger equivalent particles and so remain as free particles able to migrate into the lung cells and across the epithelium (Oberdorster, 1992). The longevity of the particles at the respiratory epithelium, the greater the possibility of damage to the underlying cells. Alveolar cells such as

pulmonary macrophages, epithelial cells, fibroblasts and endothelial cells have the potential to release chemical signals into the airways such as tumour necrosis factors (TNF) and interleukin's (IL), propagating the inflammatory response with the area. Macrophage phagocytosis is accompanied by the release of chemotactic mediators that may affect neighbouring cells or increase the permeability of the endothelial cells lining the blood capillaries, assisting neutrophil migration from the circulation and interstitium into the local area. Increases in the epithelial permeability facilitates the interstitisation of particles into the lung tissue and compromises epithelial integrity. Loss of the epithelial barrier integrity results in an influx of plasma protein into the alveolar space, *pulmonary oedema*. Reactive oxygen species (ROS) either in association with the particles themselves, produced from reactions with components in the inhaled PM (e.g. transition metal) or released from the lung cells may be released into the alveolar space. Mammalian cells contain an elaborate repertoire of enzymatic and non-enzymic antioxidants to scavenge ROS, under conditions of oxidant stress, the normal balance of ROS and antioxidant defences may be lost, leading to increased risk of oxidant mediated lung injury to the cells within the alveoli and their cellular components (cellular membrane and DNA).

A significant percentage of the any instilled dusts will be retained within the lung tissues. Oberdorster (1992) reported that 15% of ultrafine titanium oxide was retained, indicating the fraction that had left the alveolar surface and been taken up by the macrophages and into the interstitium. Ultrafine particles enter the interstitium more readily than the equivalent larger particles therefore exert greater pulmonary toxicity

It is possible that interstitialisation and subsequent uptake by and activation of interstitial macrophages creates a chemotactic gradient in the lungs shifting the influx of inflammatory cells into the interstitium rather than the alveolar space, decreasing the respective signs of inflammation in the alveolar space. Thus, reduced numbers of neutrophils are counted in the alveolar space in response to instillation of ultrafine particles compared to an equivalent dose of larger particles (Donaldson et al., 1998). Damage to interstitial cells including the fibroblasts and disruption to the cell communication between epithelial: interstitial communications. Disruption to cellular

communications may result in loss of control of cell cycles, inappropriate cell replication and activation, deposition of cell products within the alveolar space and culminating, in extreme cases, remodelling of the alveolar regions and pulmonary fibrosis. The epithelial and pulmonary responses to lung injury is summarised in Figure 1.6.

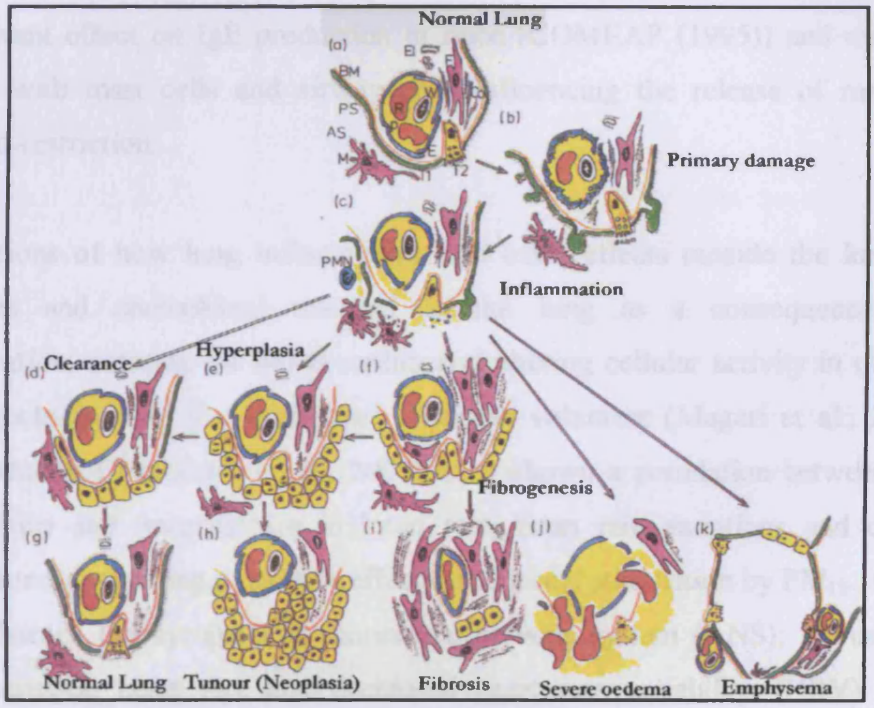


Figure 1.6: Diagram summarising alveolar cell damage and repair. BM (Basement Membrane), F (fibroblasts), Co (collagen), PS (surfactant), AS (alveolar space), E (endothelial cell), M (macrophage), T1 (Type 1 cell), T2 (type II cell) and PMN (polymorphonuclear leuocyte). Source: Richards (1991).

1.4.3 Local and Systemic Effects of Air Pollution Particles

Particles deposited within the respiratory tract provoke local inflammation, trigger allergic reactions in sensitised individuals and may exhibit cytotoxic effect to the lung epithelium resulting in cellular damage and pulmonary changes. Adverse effects may be either immediate (respiratory) or longer term (systemic effects).

Significant exposure to particulates or inhaled toxicants has been shown to elicit lung injury and inflammation, characterised by the compromisation of gaseous exchange and epithelial destruction leading to cell influx, surfactant dysfunction and pulmonary

oedema. Inflammation is considered to be a response to the myriad of mediators, oxidants, growth factors and reactive oxygen species (ROS) generated at the lung surface in response to exposure to particles, toxicants or micro-organisms (Leikauf 2002).

Several studies have linked the increase in asthma symptoms and induction of asthma with ambient particles, some particles such as acidic particles of diesel exhaust have an adjuvant effect on IgE production in mice (COMEAP (1995)) and may interact directly with mast cells and airway cells influencing the release of mediators of bronchio-restriction.

Speculations of how lung inflammation can exert effects outside the lung include cytokines and chemokines released in the lung as a consequence of lung inflammation, entering the blood stream and altering cellular activity in other major organs including the liver and heart. Human volunteer (Magari et al., 2002) and animal studies (Watkinson et al., 2001) have shown a correlation between ambient PM_{10} levels and irregularities in heart rate, heart rate variations and core body temperatures suggesting a putative effect on neuronal stimulation by PM_{10}. Cytokines may influence the sympathetic autonomic nervous system (ANS); increased vasal tone, increased heart rate and decreased heart rate variability (HRV) has been observed in the elderly during pollution episodes (Dockery, *personal communication*) potentially leading to increased incidences of cardiovascular attacks and mortality. Cytokines from sites of lung inflammation, may pass into the blood resulting in systemic oxidative state and/or enter the liver resulting in protein changes influencing fibrinogen levels, other cytokines and cell mediators. Increased blood coaguability induced by IL-6 expression, increased haemostasis, increased platelet and neutrophil levels and increased fibrinogen levels could contribute to an increased risk of cardiovascular complications (Seaton et al., 1999. Khandoga et al., 2003). Studies following the occupational exposure and heart rate variability in boilermakers (Magari et al., 2002) suggest an association between exposure to PM (sub 2 microns), particularly airborne metals and significant irregularities in cardiac autonomic function. Seaton (1995) hypothesised that particle deposition within the lungs of the elderly, very young or those with cardio pulmonary disease was increased compared

to those healthy general population. This increased exposure would therefore carry an increased and potentially exacerbated risk in changes in inflammation, changes in blood coagulation and plasma viscosity that would pre-dispose to acute cardiovascular events. An alternative mechanism by which inhalation of PM may result in greater systemic effects relate to the normal balance in antioxidant defences, Rahman (1996) recorded severe imbalances in oxidant-antioxidant in the favour of oxidant stress in the plasma of smokers, chronic asthmatics and acute COPD patients, with evidence of lipid peroxidation. A model of effects induced by diesel exhaust/PM_{10} in rats is illustrated in figure 1.7.

Longer-term exposure of particles, particularly diesel exhaust particles has been linked to increased incidences of cancer, attributed to surface associated PAHs and nitro-PAH's. Bronchioalveolar tumours (adenomatous and carcinomatous lesions-type II cell hyperplasia and metaplasia) and squamous cell tumours have been reported in rats following high exposure to some particles (Morrow, 1996). Driscoll (1996) proposed that such neoplasia might be a secondary consequence of the chronic inflammation process, requiring prolonged inflammation and arising as a consequence of oxidant mediated genotoxicity.

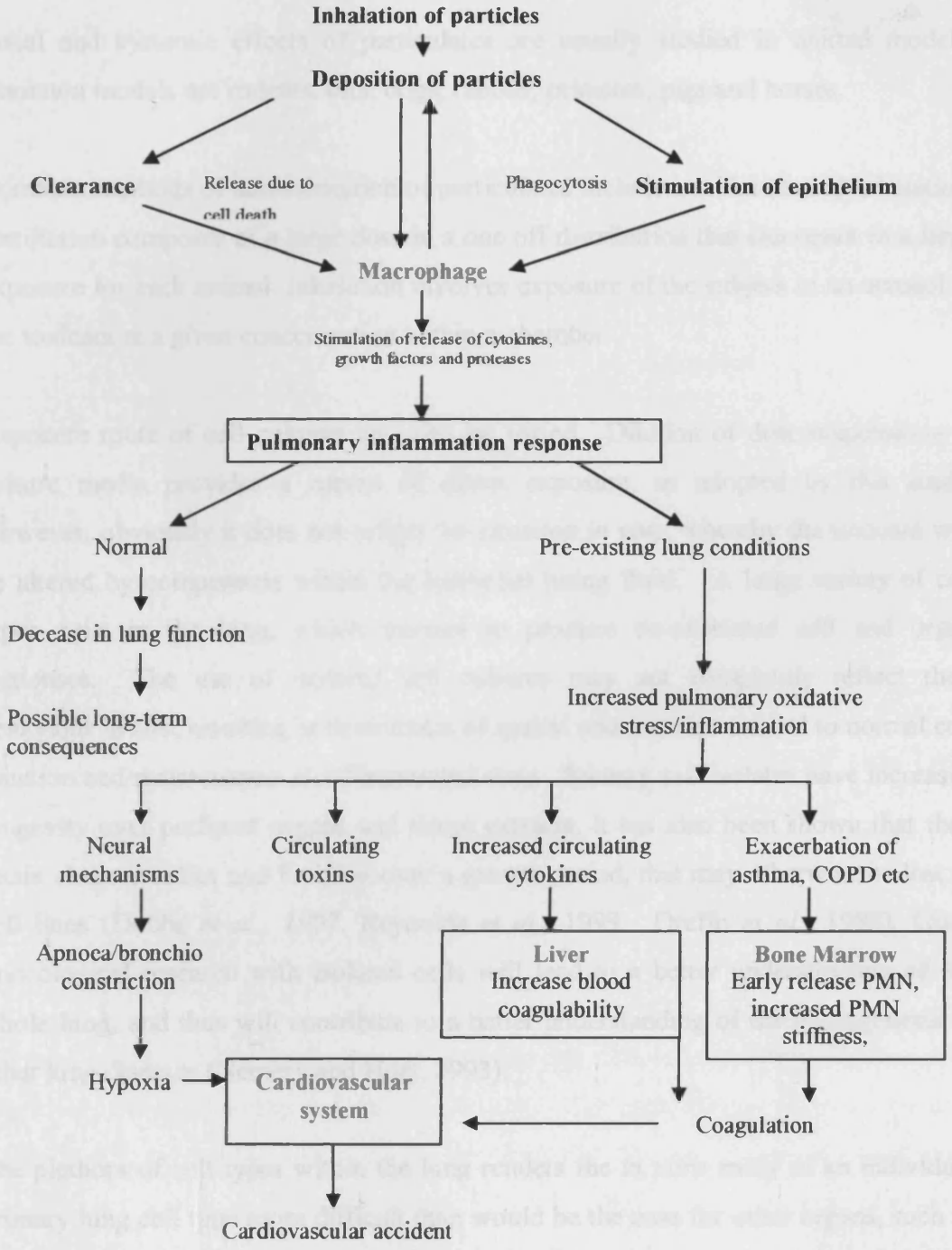


Figure 1.7: Possible mechanisms for the propagation of local and systemic effects of particle inhalation: Source: IEH (2000) *Airborne Particles:* Exposure in the home and health effects.

1.5 Studying the Effects of Air Pollution

Local and systemic effects of particulates are usually studied in animal models. Common models are rodents, cats, dogs, rabbits, primates, pigs and horses.

Common methods of administration of particulates include instillation and inhalation. Instillation composes of a large dose in a one off distribution that can result in a large exposure for each animal. Inhalation involves exposure of the subject to an aerosol of the toxicant at a given concentration within a chamber.

Exposure route of cell cultures can also be varied. Dilution of dust suspensions in culture media provides a means of direct exposure, as adopted by this study. However, obviously it does not reflect the situation *in vivo*, whereby the toxicant will be altered by components within the epithelial lining fluid. A large variety of cell types exist in the lung, which interact to produce co-ordinated cell and organ responses. The use of isolated cell cultures may not completely reflect their behaviour in *situ*, resulting in destruction of spatial relationships critical to normal cell function and maintenance of differentiated state. Primary cell isolates have increased longevity over perfused organs and tissue extracts, it has also been shown that they retain characteristics and function over a greater period, that may otherwise be lost in cell lines (Dobbs *et al., 1997.* Reynolds *et al.,* 1999. Oreffo *et al.,* 1988). Good toxicological research with isolated cells will lead to a better understanding of the whole lung, and thus will contribute to a better understanding of the pathogenesis of other lung diseases (Nemery and Hoet, 1993).

The plethora of cell types within the lung renders the *in vitro* study of an individual primary lung cell type more difficult than would be the case for other organs, such as the liver. Standardised, efficient isolation methods are required to produce functionally viable homogenous type II cell cultures, in order to produce reproducible, reliable results from any subsequent study. Epithelial type II cells have been isolated from the lung by several different methods (Table 1.2). Great emphasis has been placed on isolating and culturing of the cells to maximise the sustained expression of type II cell characteristics and phenotypes *in vitro* (Oreffo, 1987, Reynolds, 2001).

Any study of isolated type II cell cultures requires the consistent isolation of high purity populations. Type II cell isolation has been developed over the last 20 years and in a variety of methods adopted. The isolation methods adopted and subsequent culturing can introduce great variability within studies.

Table 1.2: Historical development of Epithelial type II isolation

Research group	**Date**	**Method adopted**
Kikkawa & Yoneda	1974	Crude trypsin/Ficoll gradient
Greenleaf et al.	1979	Trypsin/centrifugal elutriation
Dobbs et al.	1980 & 1986	Elastase/Trypsin Centrifugal Elutriation/Panning with IgG
Leary et al.	1982	Phosphine-3R Probe on rabbit type II cells/Laser flow cytometry
Finkelstein & Shapiro	1982	Low elastase/Trypsin
Skillrud and Martin	1984	Elastase/Percoll gradient
Richards et al.	1987	Trypsin/Percoll gradient

Primary epithelial type II cells can be maintained in culture but will readily lose their characteristics of differentiated function *in vitro*. The meaningfulness of any *in vitro* study on type II cell cultures is highly dependant on the similarities in morphology and function of the cell culture compared to *in vivo*. Culturing of the cells is of great importance and requires mimicking the cell environment *in vivo*, this has been achieved through culturing on biological substrata such as ECM at high plating densities (Reynolds, 2000) or using cell feeder layers to mimic cell to cell contacts between type II cells and fibroblasts (Shannon 1987).

1.6 Toxicogenomics and Gene Expression Studies

1.6.1 Introduction to Toxicogenomics

Toxicology investigates the harm that chemicals may cause to humans. Toxicogenomics is an extension of this science, reflecting recent advances in our knowledge of the genome and technology evolved around this. Described by the National centre for Toxicogenomics as "*an important complementary investigation, not replacement for existing strategies, aimed to significantly increase and improve our understanding of basic biological responses to environmental stressors and toxicants*" (NCT statement, 2000). Specifically, toxicogenomics may be defined as the study of the relationship between structure and activity of the genome and adverse biological effects of exogenous agents (Aardema et al., 2002), concerned with the characterisation of the genetic responses of cells and tissues to variety of toxicant and chemical insults.

Toxicogenomics is one of the families of emerging global disciplines, termed "-omic" technologies. Other strategies encompass the study of alternate intracellular bio molecules as an insight into cellular events: DNA (genomics), RNA (transcriptiomics), proteins (proteomics) and cellular metabolites (metabonomics).

1.6.2 The Basis of Toxicogenomics

Toxicity is often preceded by, and results in, an alteration of gene expression and therefore toxicogenomics may serve as a more sensitive, characteristic and measurable endpoint than more conventional techniques. The challenge for the toxicologist is to define, under given experimental conditions, the specific expression profile elicited by a given toxicant (Nuwaysir, 1999). It is hoped that toxicogenomics may provide an opportunity for scientific advance into the categorization of toxicants according to their mode of action. Toxicogenomics has been employed to assess and characterise changes in transcriptional and signal responses as a consequence of a variety of stresses, providing an insight into mechanisms of toxicity. Fingerprints of

specific gene changes will provide the necessary information to design predictive toxicity, defining mechanisms with the potential to predict toxic responses. Identification of biomarkers that may serve as early sensitive indicators of potential toxicity following personal exposure before any clinical manifestations.

The gene array system is used to produce expression profiles or expression "signatures" from samples promoting the identification of key genes involved in the response, recovery and toxicity. The system has great potential, a powerful tool yielding large amounts of information on the snap shot of a sample's expression profile, providing a differential gene display with information on genes identically, similarly or distinctly expressed in the lung (Leikauf *et al.*, 2000). This could potentially assist in hazard assessment of chemicals without the reliance on larger scale animal studies and lead to a greater insight into putative mechanisms of toxicity. The generation of a comprehensive data bank of gene profiles for a range of chemicals and toxicants could facilitate their classification based on similarities in key obligatory genetic processes leading to the toxic effect.

The gene array technology could produce toxicant profiles that may serve to further our understanding of the mechanism of toxicity or the signalling pathways leading up to the onset of toxicity. They have the potential to be more sensitive, assessing relative changes at lower dose levels than those required to exert effects in animal studies, importantly, reduction in doses used may reflect more of the real life exposure to toxicant. Currently animal studies may use higher doses of exposure to ascertain the nature of the toxic effect and extrapolate any conclusions back to real life exposure. Alternatively, the use of human cell systems and arrays may further reduce the need to extrapolate between species. Two main types of gene array are oligonucleotide arrays (containing short oligo sequences bound to the solid surface) and cDNA gene arrays. Arrays may be either macroarrays, cDNA sequences fixed to a flexible membrane such as nitro-cellulose or nylon, or microarrays (biochips), using a solid surface such as glass. Micro arrays have a greater capacity than macroarrays.

However, the generation of large amounts of data lends to be easily misinterpreted. Investigators must decipher between sensitive changes in gene expression that may

not be correlated to the toxicant at all, merely an adaptive or inconsequential change not related to the experimental conditions, alternatively, the changes may reflect part of a defence mechanism that may or may not contribute to cell toxicity.

Macro or microarrays have been successfully incorporated in studies examining key gene changes and early responses to specific toxicants and to generate more global impression of changes in genes within cell pathways. The gene array has been employed as a means to generate hypotheses: fishing for clues which may be consequently supported by alternative methods, or as mechanistic assessments of toxicity, using designed arrays of relevant genes to examine perturbations on known gene pathways.

There are technical difficulties that must be overcome prior to successful analysis of any data. The array reflects changes only at the RNA level; changes at the protein level are not taken into consideration. The generation of large volumes of data can be overwhelming and it is important to consider the physiological relevance of any results. However, arrays have been successfully used to profile genes e.g. for down stream of tumour suppresser genes in lung cancer invasion (Kaminiski *et al., 2000*) and have proven to be a simple and accurate technique, comparable with the conventional southern blot.

1.6.3 Limitations and Requirements in Array Data Handling

Nadon (2002) outlines features of array experiments that may influence the validity of such experimental conclusions if not correctly addressed within the experimental design and data analysis, a goal shared by the MIAME guidelines (Brazma et al., 2000). A common aim of all array experiments is the identification of differentially expressed genes between two or more different scenarios, which may be attributed to true biological basis rather than random error. Suitable experimental design, including the use of standardised array protocols and adequate number of replicates of array experiments (a minimum of 3-4 replicates per treatment group has been recommended- Nadon, 2002) will minimise the systemic errors and reduce random variations in gene expression.

Data transformation to stabilise the variance within the array data and to correct the data to a more normal distribution is required. Speed (2000) recommended the use of log transformation, although log transformation of raw expression data background adjusted) serves to eliminate significant numbers of extremely low expressed/non expressed genes (adjusted expression is close to zero). Rocke and Durbin (2001) examined the effect of such a transformation on the variance amongst replicates and concluded that using log transformation (background adjusted expression $+c$)- whereby c is any constant positive integer, serves to provide greater data stabilisation for all highly expressed or lower expressed genes. Other approaches have incorporated more detailed and advanced mathematic algorithms to establish transformation calculations (Hawkins, 2001). Kroll (2002) describes and evaluates a variety of scaling factors for normalisation within array experiments. The paper, primarily assessing a methodology to determine the quality of array data sets, establishes the use of an array mean expression, array trimmed mean and median expression values as potential scaling factors. The study concludes that using their data sets; a trimmed mean normalisation-scaling factor provided "reasonable normalisation result", the use of a mean expression value was highly biased toward extremely expressed genes (sensitive to saturation and high background noise). All values were explored in the development of the procedures to analyse array data (chapter 3).

Laboratory based validation is a necessity, providing an independent experimental verification of gene expression levels preferably within the same samples/tissue source used in the initial array experiments (Chuaqui et al., 2002). Da Silva (2003) adopted the Atlas nylon arrays (Chapter 3 and 6) to assess the pulmonary responses following exposure to the pulmonary toxin, ricin. The paper outlines the array procedure and subsequent validation of results using RT-PCR. Normalisation of the array data using trimmed mean scaling factors, as explored in chapter 3, in conjunction with transformation and statistical evaluation provided promising results and led to the identification of candidate genes in the pulmonary toxicity observed following inhalation exposure to ricin in mice. The study links genomic, histological

and physiological effects arising as a consequence of the ricin exposure and contributes to the understanding of the ricin intoxication process (Da Silva, 2003).

1.7 Aims and Objectives

The overall aim of the study is to evaluate the toxicity of well-characterised PM_{10} collected from the South Wales conurbation with a variety of model test systems.

Firstly, changes induced in lung permeability and inflammation were examined following the instillation of PM_{10} into healthy rats (Chapter 2). Assessment of the changes were made utilising quantitative biochemical and cellular techniques of lung lavage samples coupled with more subjective histopathological assessment. Gene profiling was then carried out on selected lung tissue samples to identify important molecular changes brought about by PM_{10} following the pollutant-induced increased permeability and inflammation (Chapter 3). This work was carried out using commercially available macroarrays and required development of the best methods to interpret the mass of bioinformatic data.

Following this, attention was given to two important target cells for PM_{10} in the lung, namely the alveolar macrophage and epithelial type II cell. The cells were isolated, exposed *in vitro* to PM_{10} and the toxicity assessed utilising a semi-quantitative detachment assay and nuclear changes associated with cell apoptosis (Chapter 4). In the latter regard, such studies were carried out utilising a new experimental tool, the IN Cell Analyser™ 1000 in conjunction with Amersham Biosciences, UK. Once the *in vitro* assays provided a dose that caused stress to the cells, but not overt cell death, a final gene expression study to identify candidate genes for PM_{10} was undertaken (Chapter 5).

It was anticipated that this series of investigations could better identify the importance of durable particles and the role of soluble metals in producing mild pulmonary changes or stress in important lung target cells.

Chapter 2.0

In vivo characterisation of the pulmonary toxicity induced by the instillation of Cardiff PM_{10} into the rat

2.0 *In vivo* characterisation of the pulmonary toxicity induced by instillation of Cardiff PM10 into the rat

2.1 Introduction

The respiratory surface comprises approximately 75 m^2, over 40 times greater than the exterior body surface area. The respiratory surface constitutes the primary defence against inhaled foreign bodies and xenobiotics, harbouring an extensive myriad of defence mechanisms and strategies to facilitate the detection and detoxification of these moieties. A healthy adult inhales approximately 20 m^3 of air per day (Baeza-Squiban et al., 1999), national guidelines for air quality have enforced a 24 hr standard of 50 $\mu g/m^3$ for PM_{10} at this level there is a potential exposure of up to 1 mg per day, a significant and possible threat to human health.

Epidemiological studies have reported strong associations between ambient PM_{10} (particulate air pollution with a mean aerodynamic diameter less than or equal to 10 microns) and several adverse health outcomes, including death and increased hospital admissions for cardiopulmonary and respiratory conditions (Anderson et al., 1995, Pope et al., 1995, Schwartz et al., 2001).

There have been few studies whereby animals have been exposed to ambient PM_{10} for toxicological investigation. Studies by Watkinson (2001) investigated the extra-pulmonary responses of rodents to ambient pollutants. The studies provided support for human epidemiological evidence by showing decreases in important indices of cardiac and thermo-regulatory functions. The same study also reported increases in blood pressure, decreases in heart rate and core body temperature, increased incidences of arrhythmia and lethality following both instillation and inhalation exposure of particulate matter and co-exposure of PM and ozone in rats, mice and guinea pigs.

The instillation of PM surrogates such as diesel exhaust particles into male rats has indicated that these particles induce transient but early changes in pulmonary oedema and inflammation (Murphy et al., 1998). Studies by Adamson et al (1999) examined the toxicity of doses (inhalation exposure to 54 $\mu g/m^3$ over a four-hour period) of an

atmospheric dust sample collected from Ottawa (EHC-93). The studies involved the instillation of a fractionated sample (mean diameter <1μm) into mice, and reported that inflammatory responses, epithelial necrosis and damage were evident within a week post-instillation. Investigations (Adamson et al, 1997, 1999 and 2000) showed that this bioreactivity could be attributed, in part, to the soluble fraction (15% by mass) of the EHC-93 sample and specifically the bioavailable (water soluble) metal content including zinc and copper salts that were found at high levels in this fraction.

The investigations described above were possible in that large amounts of a respirable fraction of PM could be obtained from processing ambient matter collected by huge roof filter traps. Recent improvement in sampling ambient particulate matter has facilitated the collection of increased yields of PM_{10} by means of high volume air collectors. The particles are collected as $PM_{10\text{-}2.5}$ or $PM_{2.5\text{-}0.1}$ fractions and are directly trapped by inert (polyurethane foam) filters that do not contribute to the bioreactivity of the PM. Such collections better represent the mixture of ambient particulate pollution than many of the surrogate dusts previously used. By means of such high volume samplers the collection and morphological and chemical analysis has recently been completed on PM_{10} samples from the South Wales conurbation (Moreno et al., 2003). These workers have shown that the levels of detectable transition metals and zinc were present in both soluble and insoluble fractions and were highly dependent on source and weather conditions during sampling. In contrast to the Canadian sample, the south Wales, UK soluble fraction contributed up to 70% by mass of the $PM_{10\text{-}0.1}$. Further *in vitro* toxicological studies showed that most of the oxidative potential of the UK PM samples was found in the water soluble fraction of PM_{10} (Greenwell et al., 2002). The contribution of individual and mixtures of these soluble metals, including zinc and selected transition metals, to the oxidative capabilities of this PM fraction is still under investigation. Ascertaining the contribution of the various components of PM_{10} is an important step towards deciphering the mechanisms involved leading up to observed lung inflammation and systemic health effects.

Use of the superior, high volume collectors enables sufficient PM samples for *in vivo* studies and the aim of the present investigation was to examine the short-term pulmonary responses to fully chemically and morphologically characterised urban PM samples. Cardiff PM_{10} has been fully characterised within our laboratory, using Field

Emission Scanning Electron Microscopy and Image Analysis (morphological analysis) in addition to EPXMA and Inductively coupled plasma mass spectrometry (ICP-MS) for chemical composition (Moreno et al, 2003). Conventional toxicological methods were used to assess pulmonary oedema and inflammatory cellular influx at the alveolar surfaces following instillation of three different fractions of the PM_{10}, the whole PM_{10}, the water soluble component and the insoluble "durable" particles that remain after washing the whole PM_{10}. The water soluble fraction comprises of metals and organic compounds, and has been found to contribute up to 70% by mass of the particles (Moreno et al., 2003). Durable particles have greater longevity at the epithelial surface and the carbonaceous material, devoid of adsorbed compounds and reactive species have been found to induce inflammation (Oberdorster et al., 1990). In the present study, a dose of PM or a derived fraction (0-10 mg/animal, n=5) was introduced by a single intracheal instillation into healthy male Sprague Dawley rats. Animals were sacrificed at 3 days post-instillation, to ascertain the extent of pulmonary oedema and existence of inflammation, and at 6 weeks, to examine if any inflammation persisted or was just an early transient event. Pulmonary oedema and inflammatory events were measured at each time point using changes in lung to body weight ratio, changes in total acellular lavage protein and total and differential large free cell counts. Lung parenchyma to body weight ratio and total lavage protein are indicators of pulmonary oedema, a consequence of a loss of integrity to the pulmonary epithelium accompanied by an influx of cellular and plasma protein into the alveolar space. Free cell counts and differential staining were incorporated to establish the magnitude of inflammatory and phagocytic cellular influx into the alveolar regions. This semi quantitative assessment of lung damage was correlated with lung histology to assess changes of lung and lymph node architecture arising from PM_{10} instillation.

This part of the work programme was completed in conjunction with Dr Lucy Reynolds and was funded under the NERC urgent programme.

2.2 Materials

Male (200-250g) *Sprague Dawley* Rats (pathogen free, CD-1) were purchased from Charles River Ltd, Margate, Kent. Euthatal and Halothane was purchased from Rhone Merieux, Harlow, Essex. Streptomycin/penicillin (P 0906 was purchased from Sigma, Poole, Dorset. The sterile Luer cannulae (200/300/050) were obtained from Portex, Hythe, Kent. Bradford Reagent (B6916.), Evans Blue (E2129) and Tween-20 (P7949) were purchased from Sigma, Poole, Dorset. HRP Conjugate Sheep anti mouse Secondary Antibody (NA 931) was purchased from Amersham UK. rT_{140} was kindly donated by Dr Mary McElroy (Rayne Laboratories, University of Edinburgh). Immobilon-P membrane (P15552) was supplied by Millipore. Lambs Quick stain was purchased from Raymond A Lamb (Lamb/034-K)

2.3 Methods

2.3.1 Preparation of Instillate

$PM_{10\text{-}2.5}$ and $PM_{2.5\text{-}0.1}$ were collected on polyurethane foam (PUF) filters using the High Volume Cascade Impact Illutriator (Moreno et al, 2003). In brief, air is sucked through the collection head at a rate of 1100 L/min. This system provides a larger collection of dusts than other methods (TEOM, Partisol, Negretti) providing sufficient sample for *in vitro* and *in vivo* studies. The PM samples were removed from the filter using a standardised protocol (Moreno et al, *2003*), enabling the consistent preparation of fractions between other studies (Greenwell et al., 2003. Moreno et al., 2003). The PM used in this study (see section 2.3.1) was collected near Cardiff city centre over a 43-day period in January-March 2001, termed CF 2, the same sample was also used for tissue and cell toxicogenomic assessment and *in vitro* cellular toxicity studies in this thesis (Chapter 3-5).

Pre-weighed PUF filters containing both the 10-2.5 micron and 2.5-0.1 micron fractions of PM_{10} were halved and each portion placed in 5 ml double distilled and autoclaved water. The samples were loaded onto a Vortex (Vortex Genie 2, Scientific Industries) for one hour at medium speed and then sonicated gently (1 minute) to

remove the dust particles. The PUF filters were removed from the solution (designated wash 1) and transferred to a fresh volume of water (5 ml, double distilled) and underwent a second period of vortexing (30 minutes) and sonication (1 minute). The solution was stored overnight (4°C) and then was vortexed (3 hours, medium speed) once again and sonicated (1 minute) ensuring that the filters were well submerged in the water.

The PUF was removed and water squeezed from it. The solution was transferred to a fresh (weighed) tube (designated wash two) and both the first and second wash were freeze dried to leave the collected PM. This was weighed and the percentage recovery calculated.

The $PM_{10\text{-}2.5}$ and $PM_{<2.5}$ fractions were pooled and re-suspended in sterile water to a stock concentration of 20 mg/ml. A volume of this stock was removed and centrifuged (1,000 x g, 2 hours). The supernatant containing the water soluble fraction at a concentration equivalent to 20 mg/ml of PM_{10} was stored. The durable washed particles were re-suspended to the equivalent concentration. Freshly collected and processed samples were used for the experiments to reduce the possible effect of ageing on the toxicity observed (Murphy et al., 1999. Johnston et al., 2000). Whole PM_{10} and the equivalent doses of washed particles and water soluble component fractions were prepared for 5 mg, 10 mg and 20 mg and saline sham-treated control (n=5) (0.15 M NaCl). Each animal was instilled with 0.5 ml of solution.

2.3.2 Instillation and Sacrifice

Male Sprague Dawley rats were acclimatised within the animal holding facility for one week prior to instillation. The animals were kept on wire-bottom cages with pelleted food and tap water *ad libitum*. The animals were lightly anaesthetised with Halothane before receiving a single intratracheal instillation of PM suspended in 0.5 ml of saline (0.15 M). Sham-treated controls received saline only. Animals (n=5) were instilled with doses of 10 mg, 5 mg, 2.5 mg of each whole PM sample and the equivalent doses of washed "durable" particles and water soluble fraction of Cardiff PM (CF 2). Animals (n=5) from each exposure group were sacrificed at 3 days post

instillation and 6 week post instillation. A further set of animals (n=3) exposed to 10 mg whole PM_{10} and water soluble components (3 days) and 10 mg whole PM (6 weeks) in addition to respective sham-treated saline controls, were instilled for histological examination. All animals were carefully monitored post instillation and throughout the recovery time. Body weights and general health of each animal was carefully recorded in accordance with Home Office guidelines. With all treatments, there was no significant alteration in body weights or any adverse effects on the well being activity of any animal.

All animals were sacrificed by anaesthesia with Halothane and then administered a lethal intraperitoneal injection (1-2 ml) of Euthatal. Each animal was subsequently weighed and cardio-respiratory death confirmed prior to dissection.

The fur was washed with ethanol (70 % v/v) and the ventral surface skin removed. The peritoneal cavity was opened by midline incision and the major dorsal blood vessels cut. A tracheotomy was performed and a Luer Cannula, attached to a 20 ml syringe, securely tied into place in the trachea. The diaphragm was subsequently opened and the ventral portion of the rib cage and thymus removed.

A Luer cannula attached to a gravity feed of sterile saline was then fed into the pulmonary artery and the right atrium was cut upon expansion to allow fluid to exit. The lungs were perfused via artificial ventilation with 8-10 ml of air by means of the syringe attached to the luer cannula of the trachea, until the pulmonary circulation was clear of all blood to produce white parenchyma lung tissue (usually 8-10 ventilations).

The heart was removed and the lungs and trachea dissected free from the carcass. The oesophagus and any fatty tissue were dissected from the lungs and trachea. Any mucoidal material or blood clots on the exterior of the tissue were removed by means of absorbent tissue and the lung and trachea weighed. Digital photographs of the gross anatomy of the lung were taken at this stage to detect any overt damage and deposition of particulate material.

The lungs were lavaged (five to eight times) with 6-8 mls of saline (0.15 M). The resultant lavage fluid was pooled in a centrifuge tube and maintained on ice. Lavage

was centrifuged (300 X *g*, 15 minutes) and the cell pellet re-suspended in saline (0.15 M, usually 3 mls) to enable the large free cell count (LFC) to be estimated using a haemocytometer. Aliquots of the supernatant lavage fluid were frozen in liquid nitrogen and stored for protein analysis.

The remaining lung parenchyma was cut from the trachea, dried and snap frozen in liquid nitrogen in preparation for genetic studies (see chapter 3) and stored below minus 70°C until use. The trachea weight was subtracted from the initial lung and tracheal weight to give an approximation of lung parenchyma weight for each animal. This was used to calculate the lung parenchyma to rat body weight ratio, used as an estimation of pulmonary oedema within the animals.

Lavage free cells were diluted to 200,000 cells/ml in saline (0.15 M). Cytospin preparations were prepared from these samples. Slides and filters were sealed into cytospin chambers and the diluted LFC suspension (0.5 ml) added prior to centrifuging (8,000 X *g*, 5 minutes). Slides were removed and left to air dry and stored at room temperature. Slides were fixed and subsequently stained with a general-purpose nuclei stain and finally mounted. In brief, a three step fixing and staining procedure as carried out in accordance to manufacturers protocol (Raymond A Lamb Ltd). The Lamb Quick stain kit uses three solutions, fixative to stop metabolic processes, an acid dye with affinity for basic cell components and a basic dye, with affinity for acidic cell components, staining cells on the cytospin - a Romanowsky type effect. Each slide was dipped in fix solution (4 seconds), followed by both dyes in turn (4 seconds), with a final rinse in water before being left to air-dry at room temperature. The slides were then mounted using DPX mount and analysed. This stain facilitates the differentiation of cell types by highlighting characteristic features of cells within the section.

Neutrophils were identified based on differences in size and nuclei structure from macrophage cells. Neutrophils are smaller and their nuclei more lobular in appearance, in contrast to the larger and rounder nuclei of the macrophage. Increases in total cell numbers (macrophages and neutrophils) are indicative of an inflammatory response. An increase in the proportion of neutrophils is usually considered as a more acute or progressive inflammation (Richards et al., 1999).

2.3.3 Preparation of Lung tissue for Histological Assessment

Animals exposed to 10 mg whole, 5 mg whole and 10 mg water soluble fractions of Cardiff PM (3 days) and 10 mg whole PM (6 weeks) alongside sham-treated controls at each time point (n=3) were sacrificed as outlined previously. Non-perfused lungs were dissected free from the lung tissue and lymph tissue removed. The lung tissue was immediately fixed in 10 % v/v formal saline introduced via a tracheal cannula. The whole lung preparation was then stored in 10 % v/v formal saline for at least 2-3 days to ensure complete fixation. The equivalent lobe from each lung was dissected free and paraffin embedded in preparation for slide preparation. The sections were fixed onto glass slides and stained using Hematoxylin and Eosin (H and E), a general-purpose stain universally used for routine histological examination of tissue sections. Hematoxylin acts as a basic stain and binds nucleic acids, which appear blue in the nucleus and cytoplasm. Eosin is an acid aniline dye that stains the more basic proteins of the cytoplasm pink, nuclei stain blue-black, red blood cells appear orange to red shades and fibrin deposits appear deep pink to red. Slides from each animal were also stained with Trichrome Massons. This stain is commonly used to assess the deposition of collagen in tissue sections. Cell nuclei appear blue to black, muscle, red blood cells and fibrin appear red and connective tissue stain blue-green using this stain.

Lung sections were subsequently analysed for morphological changes arising as a result of the instillation.

2.3.4 Conventional Toxicological Assessment

Total protein was measured in the lavage fluid using a standard Bradford™ assay, using a series of diluted bovine serum albumin (BSA) standards. Briefly, a sample of lavage fluid (100 μl, n=3) was mixed with Bradford™ reagent (Sigma, 50 μl), alongside BSA standards diluted (0-20 μg/ml) total protein used on a 96 well plate. The plate was incubated (5 minutes, room temperature (RT)) and absorbance recorded (595 nm).

$rT1_{40}$ analysis was completed on lung lavage fluid supernatant. $rT1_{40}$ is a monoclonal antibody marker for an apical surface protein of rat type I cells, a potential target for PM_{10}. Thus increases in $rT1_{40}$ in the lavage fluid would be expected if the type I cells were compromised or damaged following PM administration (Dobbs et al., 1988). Lung lavage was diluted in pH 8.2 tris buffered saline (TBS - 15.5 mM Tris and 0.15 M NaCl) to a final concentration of 2 μg protein/100 μL TBS, lung tissue to 1 μg protein/100 μL TBS. The lavage samples (100 μl) were applied to Immobilon - P membrane (Millipore) using a dot-blot apparatus (Biorad). The membranes were then blocked (2hrs, RT) with 5 % w/v non-fat milk in TBS. They were subsequently incubated with monoclonal antibody against $rT1_{40}$ (1:100 diluted in blocking buffer) (30 minutes), washed with TBS containing 0.05 % v/v Tween-20 (TBS-Tween) (30 minutes) and incubated with sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000 dilution, Amersham, 30 minutes, Dobbs et al., 1988). Blots were washed again with TBS - Tween and then developed utilising enhanced chemiluminescence reagents (ECL, Amersham). Blots were exposed to film (Amersham Hyperfilm ECL) for 30 minutes. In all cases a control blot was performed in which primary antibody was not added. Semi-quantitative analysis of results was achieved by reading the membrane on a plate reader at 540 nm (McElroy et al., 1995).

Adult rat lung homogenate of known protein concentrations was used to standardise the $rT1_{40}$ assay. For data analysis 1 μg of $rT1_{40}$ was equivalent to the $rT1_{40}$ concentration of 1 μg (wet weight) of adult rat lung homogenate.

2.3.5 Expression of results and Statistical Analysis

Data for each group (n=5) was tested for normality using the Anderson Darling statistical test for normality. Non-parametric data was analysed using a Mann-Whitney statistical test. Parametric data was analysed using a two-sample t-test. A significant difference between the group data was taken when $p<0.05$.

2.4 Results

2.4.1 Collection and Preparation of the PM samples

The Cardiff $PM_{10-0.1}$ sample was collected as part of a comparative study described by Moreno (2003). A total of 697.05 mg was collected in the Cardiff location, over a 43-day period between January 2001 and March 2001, a daily average of 38 mg per day. Weather conditions and wind direction were recorded during the collection period and were described as variable, with wet conditions dominating the first 15 days of sampling, followed by a period of colder, drier and less humid conditions. The $PM_{2.5-0.1}/PM_{10-2.5}$ ratio during these collections was high (2.8-4.2) as expected with a PM sample containing a high contribution of traffic related pollution. PUF filters were changed every 4-11 days depending on collection rates. $PM_{10-2.5}$ and $PM_{2.5-0.1}$ fractions were recombined after removal from the filters with respect to the average recorded $PM_{2.5-0.1}/PM_{10-2.5}$ ratio to provide the $PM_{10-0.1}$ sample for instillation.

2.4.2 *In vivo* Toxicity of PM

2.4.2.1 Summary of *in vivo* Responses to Cardiff PM Instillation

A summary of the mean data recorded for each group is shown in Table 2.1. Changes in lung to body weight ratio, total non-specific lavage protein and large free cell counts have been calculated as group averages and SEM calculated (n=5). A high degree of animal-to-animal variation was observed in the response to the different PM fractions by each parameter with the exception of lung to body weight ratio.

Table 2.1: Summary of group mean (n=5) and Standard Error of mean (SEM) for changes in lung to body weight ratio, total non specific protein in the lavage and rT_{140} reactivity of the lavage at 3 days and 6 week post instillation.

Time point	Fraction	Dose (mg)	Lung/body weight ratio		Total Lavage Protein (μg per animal)		rT140 reactivity	
			Mean	SEM	Mean	SEM	Mean	SEM
3 days	Control	0	0.0044	0.0001	2041	307	1511	272
	Whole PM	2.5	0.0045	0.0001	2030	266	663	68
	Whole PM	5	0.0048	0.0001	3358	328	608	68
	Whole PM	10	0.0053	0.0002	5044	785	1360	375
	Washed	2.5	0.0045	0.0001	1934	149	350	45
	Washed	5	0.0047	0.0000	3927	316	809	171
	Washed	10	0.0047	0.0002	3682	231	431	36
	Soluble	2.5	0.0041	0.0000	1639	52	488	42
	Soluble	5	0.0044	0.0003	2076	286	527	50
	Soluble	10	0.0046	0.0003	4304	662	745	139
6 weeks	Control	0	0.0034	0.0001	2445	43	320	50
	Whole PM	2.5	0.0036	0.0001	2869	281	1006	200
	Whole PM	5	0.0033	0.0001	3059	403	365	54
	Whole PM	10	0.0031	0.0001	2982	135	557	123
	Washed	2.5	0.0028	0.0001	2481	193	770	145
	Washed	5	0.0031	0.0001	3199	403	763	167
	Washed	10	0.003	0.0002	3307	277	554	73
	Soluble	2.5	0.0031	0.0002	1974	0.1581	542	63
	Soluble	5	0.0033	0.0001	2.250	0.2259	1010	184
	Soluble	10	0.0031	0.0001	2721	0.2119	351	50

2.4.2.2 Changes in Lung to Body Weight Ratio

The lung to body weight ratio is a parameter examining the extent of pulmonary oedema within the lung. Increases in lung parenchyma weight are accompanied by loss of integrity of the barrier function of the epithelium, permitting the influx of plasma protein and water accumulation into the airways. A dose dependant increase in lung to body weight ratio was observed at 3 days post instillation (Figure 2.1a). Statistical significance was only observed at the highest dose of the whole Cardiff PM_{10} fraction (10 mg, $p=0.02$), a 25% increase from sham-treated control and indicative of pulmonary oedema. At six weeks, this effect was diminished and no significant ($p<0.05$) differences from sham-treated controls were observed with any of the samples (Figure 2.1b).

2.4.2.3 Changes in Total Non-specific Lavage Protein

Total non-specific protein from the cell free lung lavage is a more sensitive measure of alveolar oedema than lung to body weight ratio. Instillation of the PM and other toxicants result in cellular damage and compromises the barrier function, resulting an increase in plasma protein and cellular debris within the alveolar space.

Dose dependant increases in total protein levels were observed for both water soluble and whole PM preparations at 3 days, this trend was also apparent in all whole, washed and water soluble fraction at six weeks. Approximately a two-fold increase in total protein was observed following treatment with as little as 5 mg whole PM or the equivalent dose of 10 mg water soluble fraction. Less pronounced increases were observed at six weeks. These results suggest the presence of pulmonary damage at both time points in all fractions of PM examined. The 10 mg dose or equivalent water soluble dose induced significant oedema at 3 days, as did 5 mg doses of whole and washed particles (durable fraction). However, instillation of the 2.5 mg and 5 mg of water soluble fraction failed to result in any increase in protein levels from the sham-treated controls. At six weeks, only 5 mg and 10 mg doses of the particle containing fractions resulted in significant increases in lavage total protein. In contrast the highest dose of the water soluble fraction had no effect at 6 weeks whereas the lowest

dose (2.5 mg equivalent dose) produced a significant drop in lavage acellular protein compared with sham-treated rats. These results are illustrated in figure 2.2a-b.

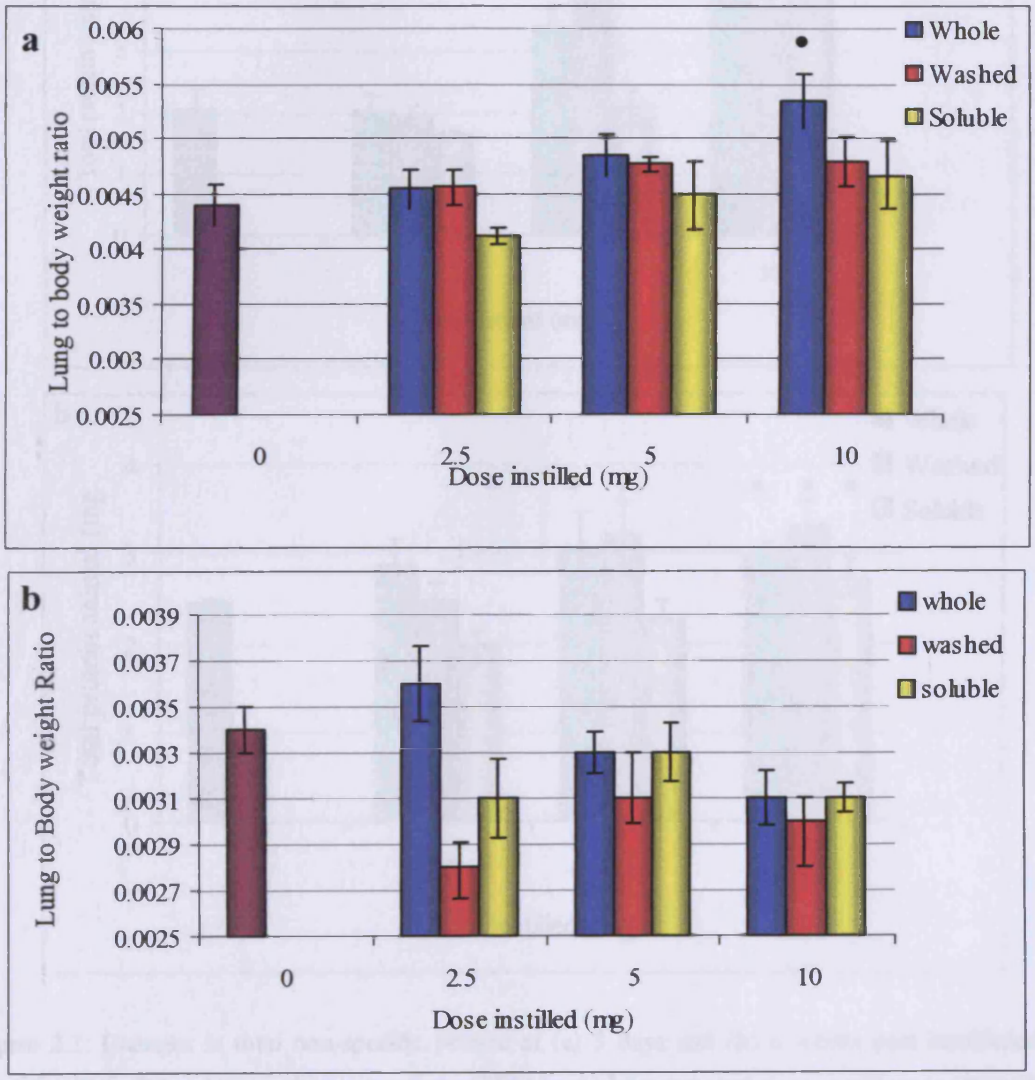


Figure 2.1: Changes in the lung to body weight ratio following instillation of the increasing Cardiff (CF 2) PM fractions at (a) 3 days and (b) 6 weeks post instillation. Values shown are group mean (n=5) and vertical bars representing SEM of each group.• denotes statistical significance ($p<0.05$).

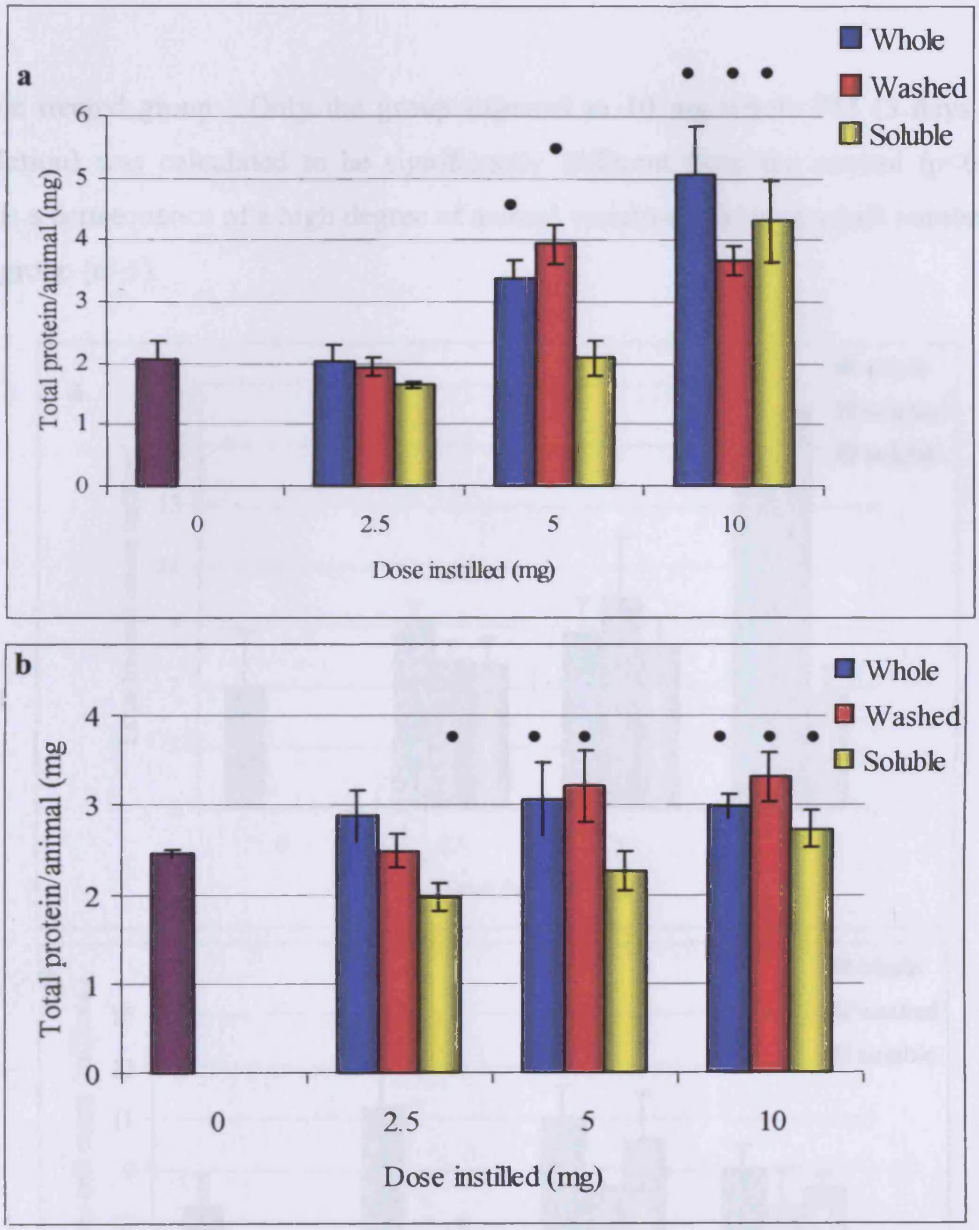


Figure 2.2: Changes in total non-specific protein at (a) 3 days and (b) 6 weeks post instillation of Cardiff (CF 2) PM_{10}. Values shown are mean ±SEM (n=5) • denotes statistical significance ($P<0.05$)

2.4.2.3 Large Free Cell Numbers and Differential Counts

The large free cell (LFC) counts for whole, washed and water soluble instilled animals have been summarised previously (Table 1). There was a dose dependant trend at 3 days post instillation, with cell numbers of each treated group exceeding that of the sham-treated group with exception to animals exposed to the water soluble fraction (figure 2.3). At the earlier time point of 3 days, an increase of almost two fold was observed in free cells numbers at the highest dose, 10 mg (washed "durable" particles and whole PM). The increases were less apparent for all doses of water

soluble treated group. Only the group exposed to 10 mg whole PM (3 days post instillation) was calculated to be significantly different from the control ($p<0.05$). This is a consequence of a high degree of animal variation related to small numbers in each group (n=5).

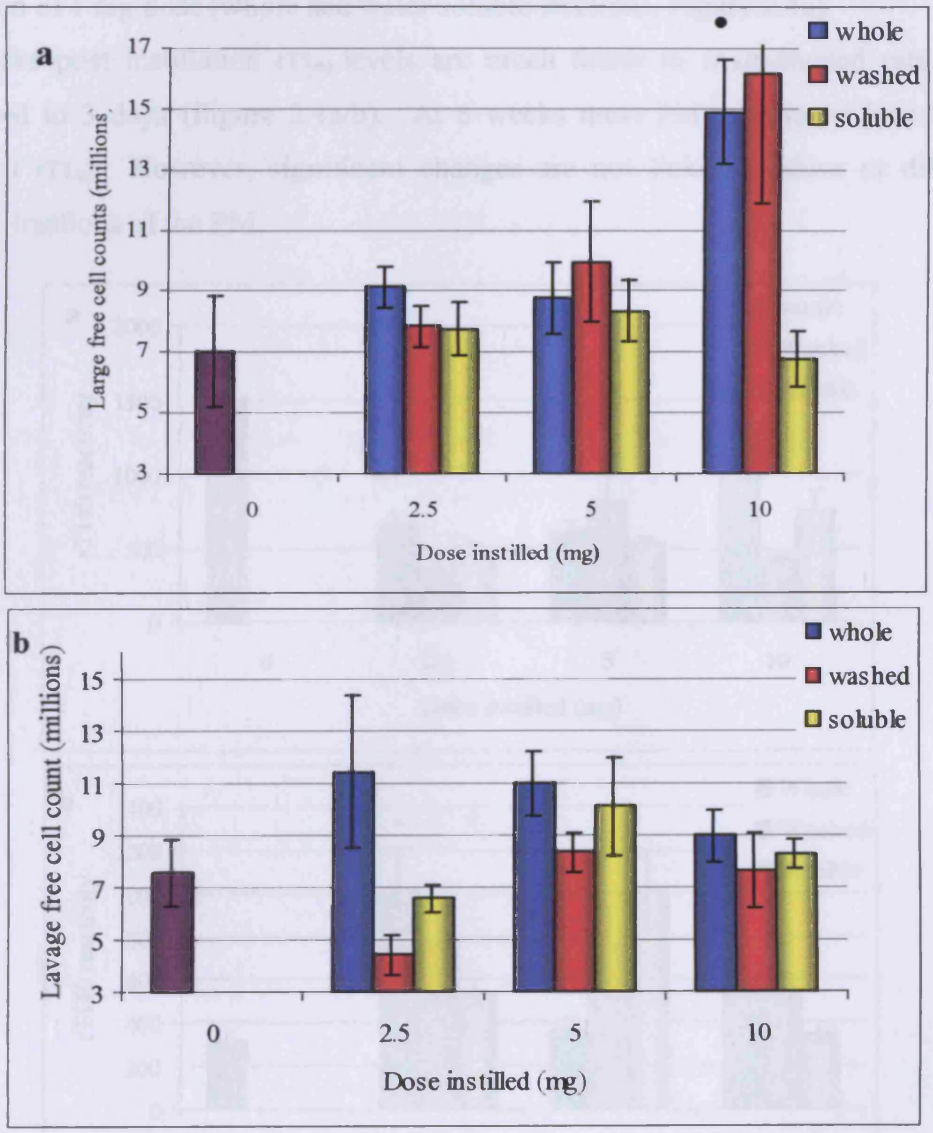


Figure 2.3: Large free cell counts changes in response to whole PM, washed durable particles and water soluble fraction at (a) 3 days and (b) six weeks post instillation. Data shows group mean values (n=5) ± SEM (vertical bars). • denotes statistical significance from control at the appropriate time ($P<0.05$).

Cytospin preparations of the lavage cells and cellular staining to differentiate alveolar macrophages and neutrophils showed that there was no significant presence of any neutrophils in any of the doses or fractions at either time point, indicating only a macrophage response at the lung surface.

2.4.2.5 Changes in an Epithelial Type I Cell Marker in the Pulmonary Lavage

The apical epithelial type I cell marker, $rT1_{40}$, was quantified in the lavage fluid from all animals (Figure 2.4). Levels of $rT1_{40}$ are high (1400) in sham-treated animals and significantly lowered following most PM sample treatments at 3 days with the exception of 1 mg dose (whole and water soluble fractions, Figure 2.4a)

Six weeks post instillation $rT1_{40}$ levels are much lower in sham-treated rats (300) compared to 3 days (Figure 2.4a/b). At 6 weeks most PM samples induce higher levels of $rT1_{40}$. However, significant changes are not linked to dose or different specific fractions of the PM.

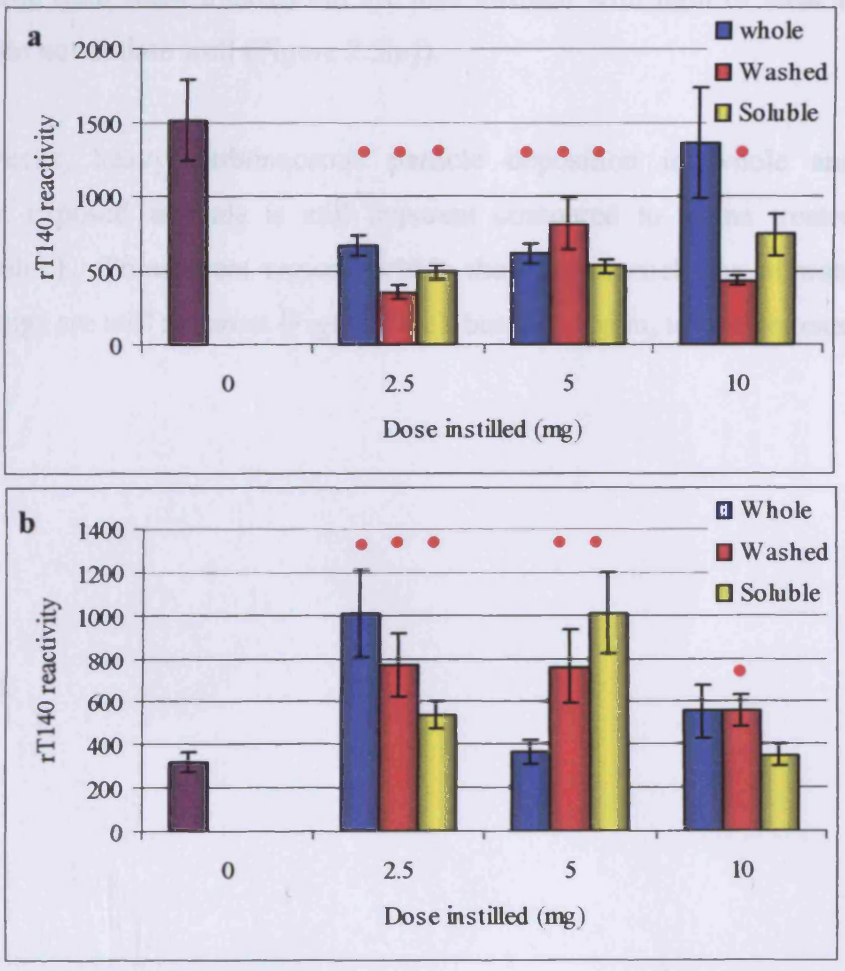


Figure 2.4: A graph showing the decrease in $rT1_{40}$ reactivity in lavage fluid collected from (a) 3 day and (b) 6 week post instillation. Reactivity of $rT1_{40}$ relative to reactivity in 1μg lung tissue. Values shown are group mean (n=5)±SEM. • denotes a statistical significance of $p<0.01$.

2.4.3.2.6 **Gross Anatomy of the Lungs**

Digital images of the whole lungs following dissection are shown in figure 2.5 and 2.6. Lungs from sham-treated rats deflate very readily and consist of perfectly white parenchyma lobes (Figure 2.5a). In contrast, animals treated with whole PM (Figure 2.5b-d) are mostly swollen and at 3 days there are clear regions of black particle (probably mostly from diesel exhaust) deposition throughout the parenchyma. The heaviest deposits of these particles are chiefly in the main bronchial/bronchiole branches (Figure 2.5d and g). Lungs treated with water soluble fraction of PM_{10} remain white (like sham-treated) but are also swollen with light or clear translucent areas that do not deflate well (Figure 2.5h-j).

At six weeks, heavy carbonaceous particle deposition in whole and washed component exposed animals is still apparent compared to saline treated animals (Figure 2.6b-c). Translucent regions within the lung parenchyma of water soluble exposed lungs are still apparent (Figure 2.6d), but once again, to a lesser extent than at 3 days.

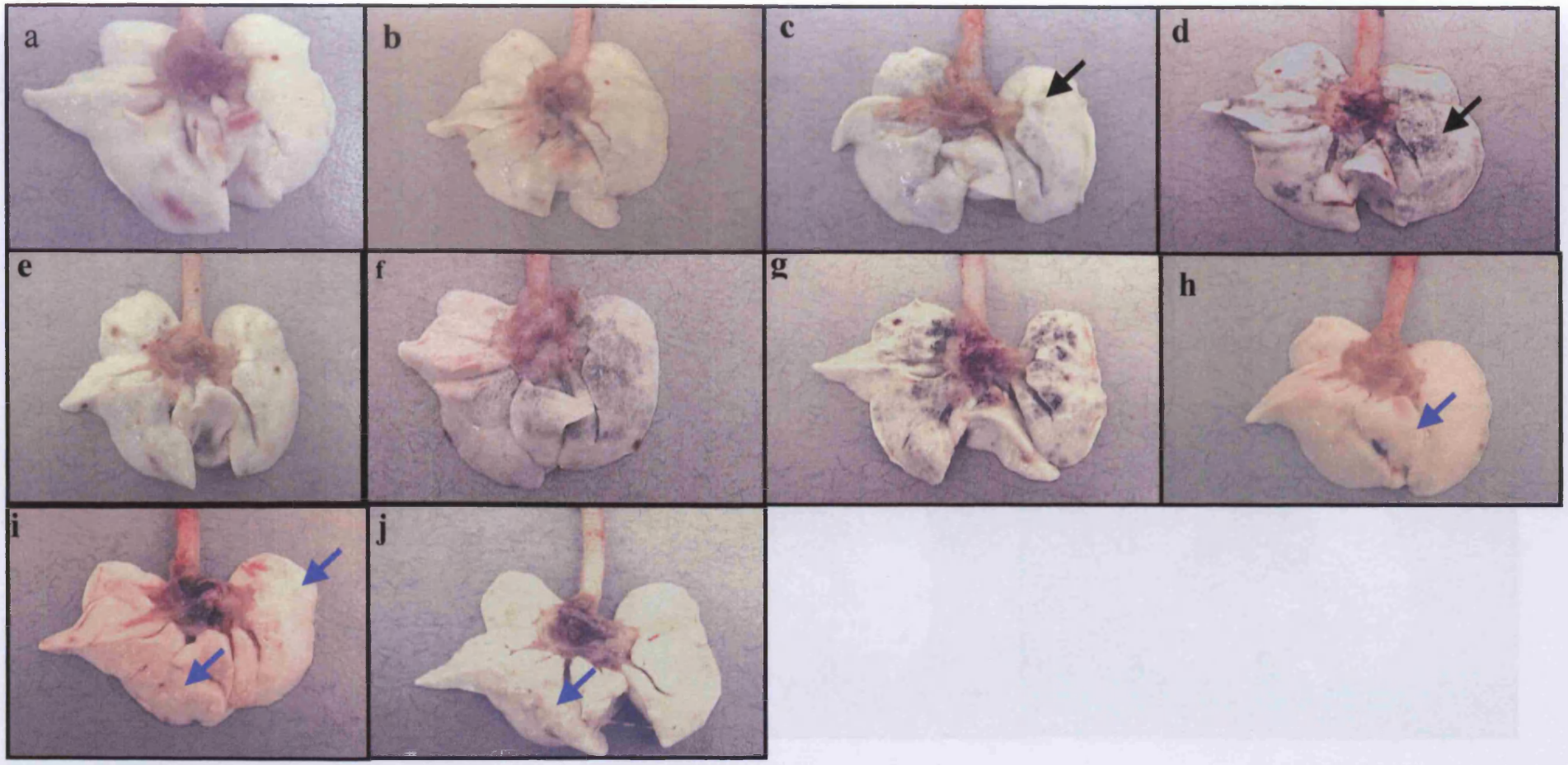


Figure 2.5: Gross anatomy of the lungs at 3 days post exposure of (a) sham-treated rats (b-d) whole treated PM_{10} 2.5 mg, 5 mg and 10mg respectively (e-g) equivalent doses of washed durable particles and (h-j) equivalent doses of water soluble component of PM_{10}. Black arrows indicate regions of particle deposition in whole and washed PM exposed lungs. Blue arrows indicate swollen regions of lung tissue.

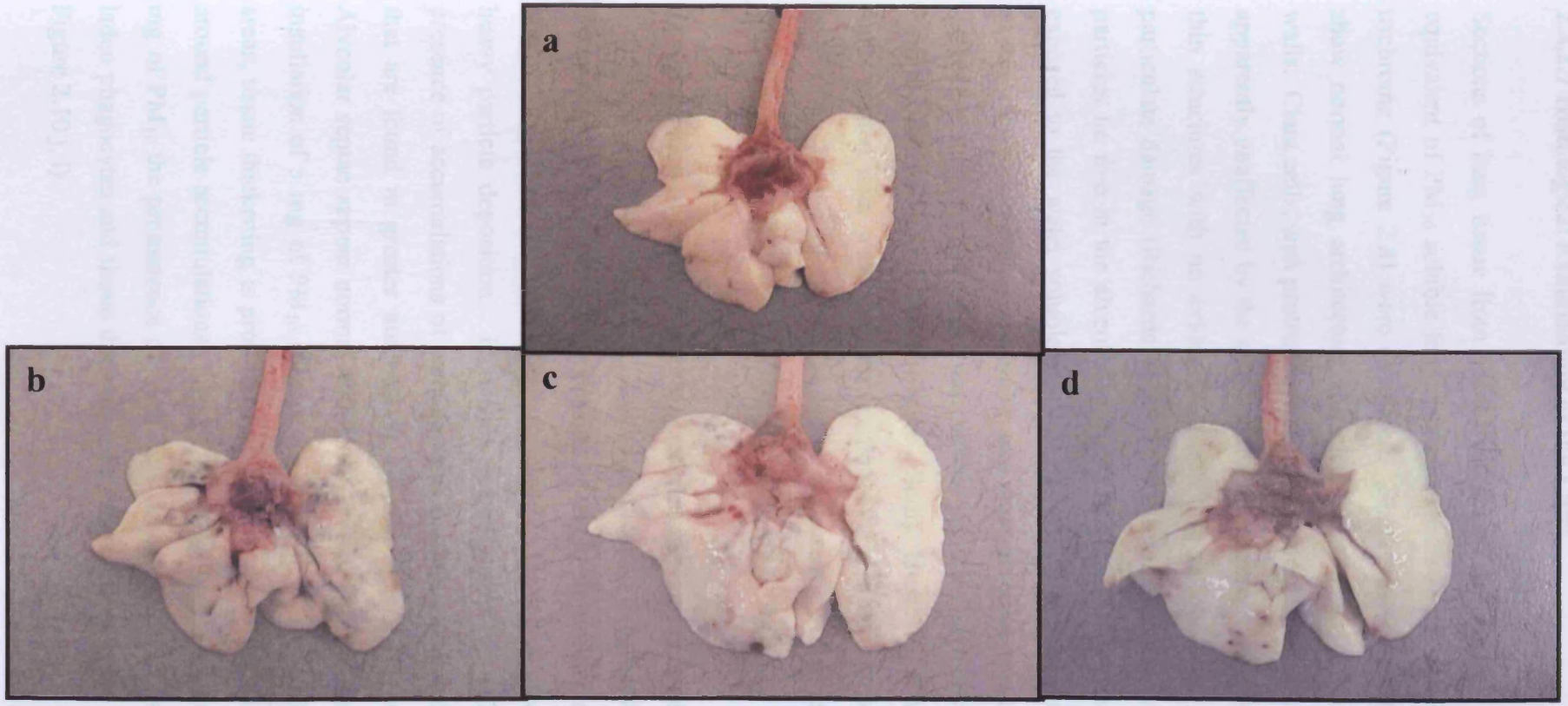


Figure 2.6: Gross anatomy of the lung at 6 weeks following exposure to a) sham-treated saline animals (b) 10 mg whole PM (c) 10 mg equivalent dose of washed durable particles and (d) the equivalent dose of water soluble component.

2.4.2.7 Histological Examination of the Lung

Sections of lung tissue from sham-treated rats and animals exposed to the 10 mg equivalent of PM_{10} soluble fraction stained with H and E (Figure 2.7) or Masson's trichrome (Figure 2.8) were compared. Sham-treated rats (Figure 2.7 and 2.8a-d) show normal lung architecture and clear alveolar spaces and thin epithelial lined walls. Clara cells, with protruding heads are normal in the bronchiolar epithelium and apparently unaffected by the saline instillate (Figure 2.7b). Alveolar septa remain as thin structures with no evidence of swelling as observed during some forms of particulate damage (Richards et al., 1999) (Figure 2.7b). Macrophages, devoid of particles lie free in the alveolar spaces (Figure 2.7d). At low magnification, lungs exposed to the water soluble fraction of PM_{10} have similar architecture to sham-treated rats with normal bronchioles and clear alveolar spaces. The absence of any particulate matter confirms that the durable particles have been successfully removed from the water soluble fraction (Figure 2.7e-h). However, at higher magnification the lungs treated with water soluble fraction of PM_{10} have noticeably greater areas of general thickening of the tissue which represent swollen micro-capillaries or the interstitial regions around these capillary areas (Figure 2.7 and 2.8 f-h).

The instillation of 5 mg whole PM_{10} resulted in significant deposition of particles in focal regions of the lung tissue. Low magnification examination of the tissue (Figure 2.9 and 2.10h) shows that large regions of tissue are unaffected by the instillation of material but some regions, particularly close to the major airways have regions with heavy particle deposition. Increasing magnification of such areas highlights the presence of accumulations of carbonaceous particle associated alveolar macrophages that are found in greater numbers in alveolar spaces (Figure 2.9g, h and 2.10h). Alveolar septae appear normal with little evidence of cellular thickening following instillation of 5 mg of PM_{10} (Figure 2.9 f and h, figure 2.10h). However, in focal areas, tissue thickening is prominent with some evidence of cellular influx/increases around particle accumulations (Figure 2.9 and2.10 h). When rats are exposed to 10 mg of PM_{10} the prominence of focal dust deposition, alveolar accumulation of dust laden phagocytes and tissue thickening process is much more marked (Figure 2.9 i-l, Figure 2.10 j, l).

At six weeks post instillation of 10 mg of PM_{10}, some residual particles are apparent in the alveolar regions particularly in individual macrophages (Figure 2.11 and 2.12). However, fewer macrophage accumulations are apparent than at the earlier time point and those that are evident are often within the larger airways (Figure 2.11b). It is also noticeable that the extensive tissue thickening in most interstitial areas are far less than that noted for 3 days exposure, suggesting partial resolution of the early acute effects. This may be related to clearance mechanisms for macrophages that join the mucocillary escalator or are transported via the interstitium to the thoracic lymph nodes.

Histological sections of lymph nodes from rats instilled with 10 mg of whole PM do show the presence of carbonaceous particles by 6 weeks (not shown). However, the cellular architecture and form of the lymph nodes are unchanged from that observed in sham-treated rats.

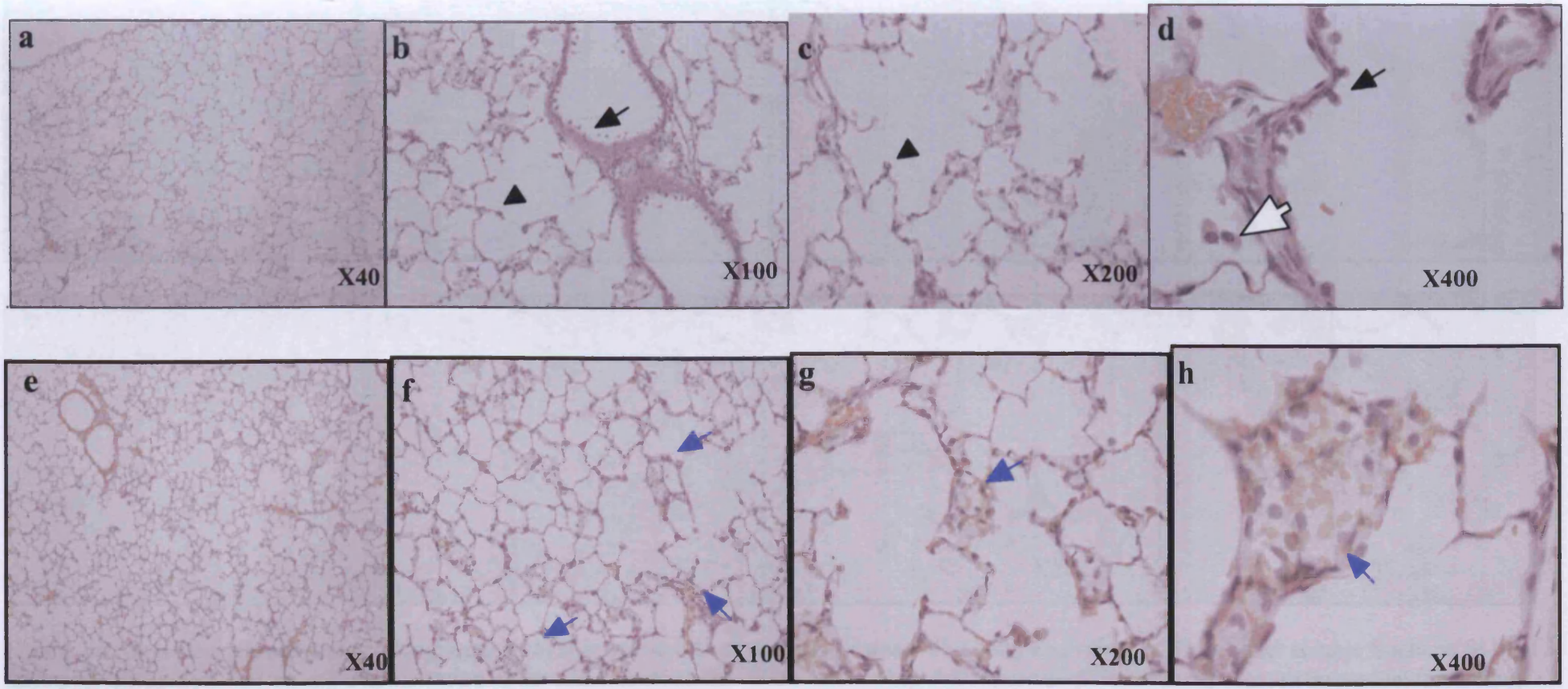


Figure 2.7: H and E staining of sham treated (a-d) and rats instilled with 10 mg equivalent of PM_{10} water soluble fraction (e-h) at 3 days post instillation. Increasing lens magnification is provided. **Black arrow**-protruding heads of Clara cells in the bronchiolar epithelium. **Black arrow heads**- alveolar septum. **White block arrow**- alveolar macrophage. **Blue arrow**- areas of tissue thickening involving micro-capillaries or interstitium.

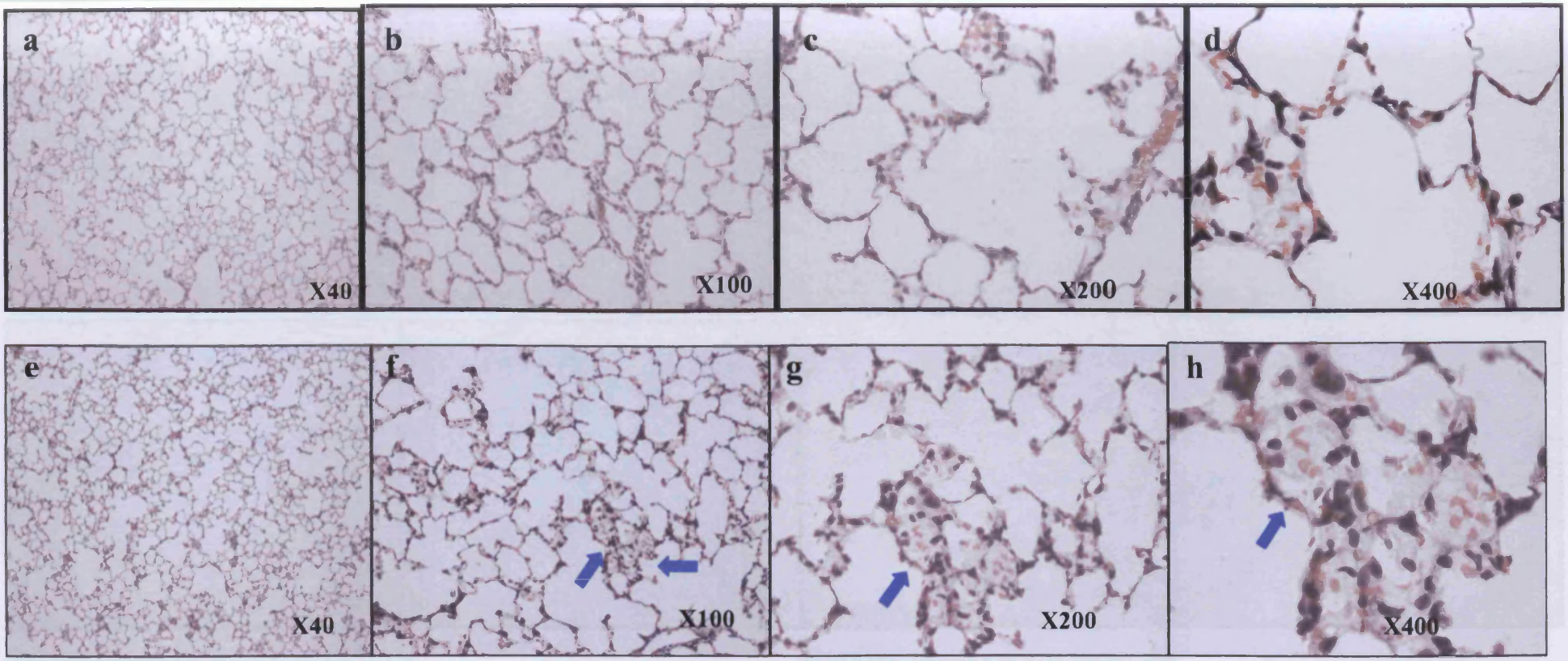


Figure 2.8:Massons trichrome stained lung sections of sham treated (a-d) and rats treated with 10 mg equivalent of PM_{10} water soluble fractions (e-h) at 3 days post instillation. Increasing magnification of the tissue is provided. **Blue arrows** highlight areas of alveolar or capillary or interstitial thickening with prominent collection of red blood cells (stained orange/pink with masons stain).

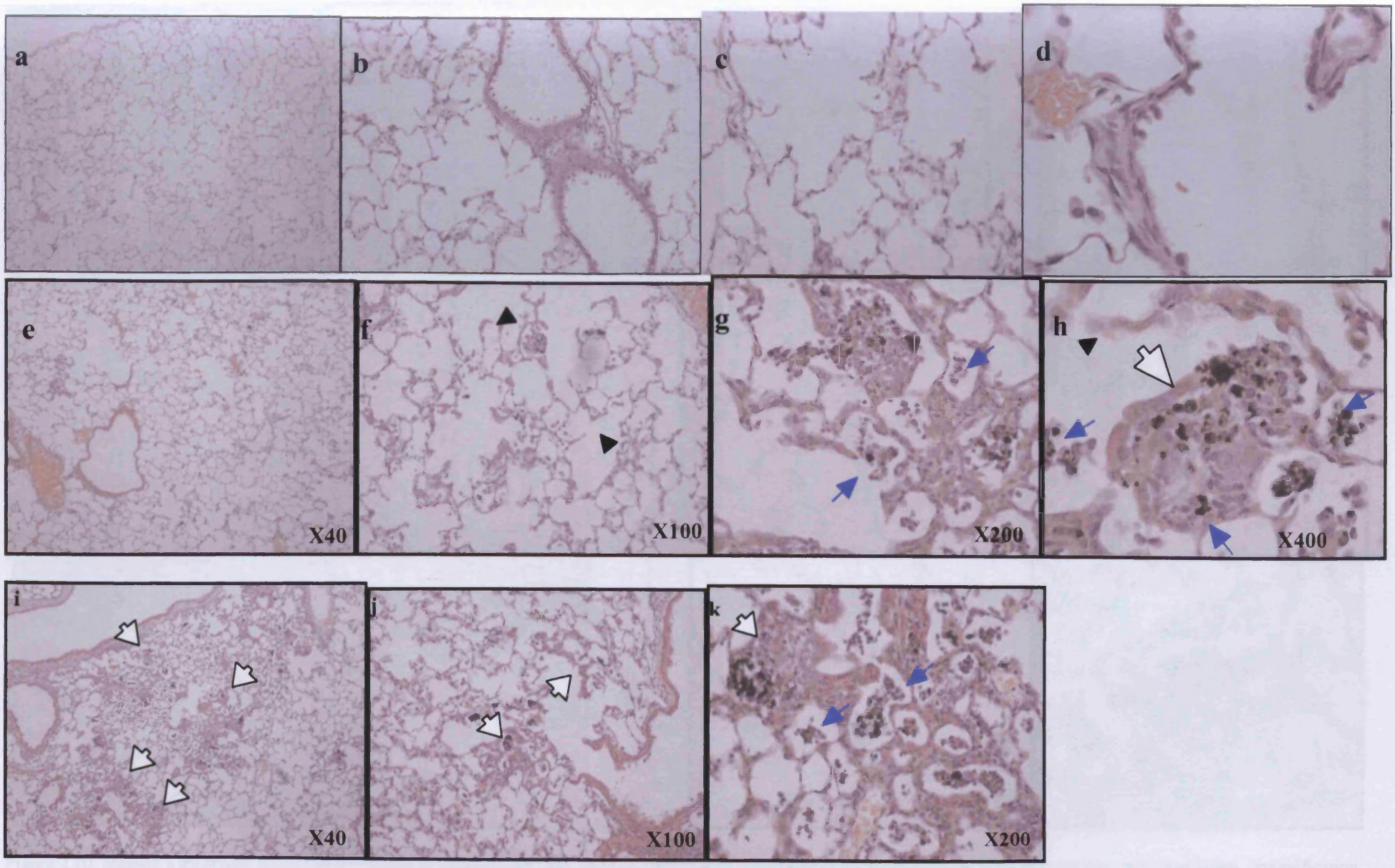


Figure 2.9: H and E stained lung sections of sham treated (a-d) and rats treated with 5 mg (e-h) or 10 mg (I-l) whole PM_{10} at three days post instillation (magnification of images provided). Blue arrow highlights macrophage accumulations containing black carbonaceous material within the alveolar spaces. Black arrowhead indicates alveolar septae. White arrows show regions of thickened tissue around particle aggregations.

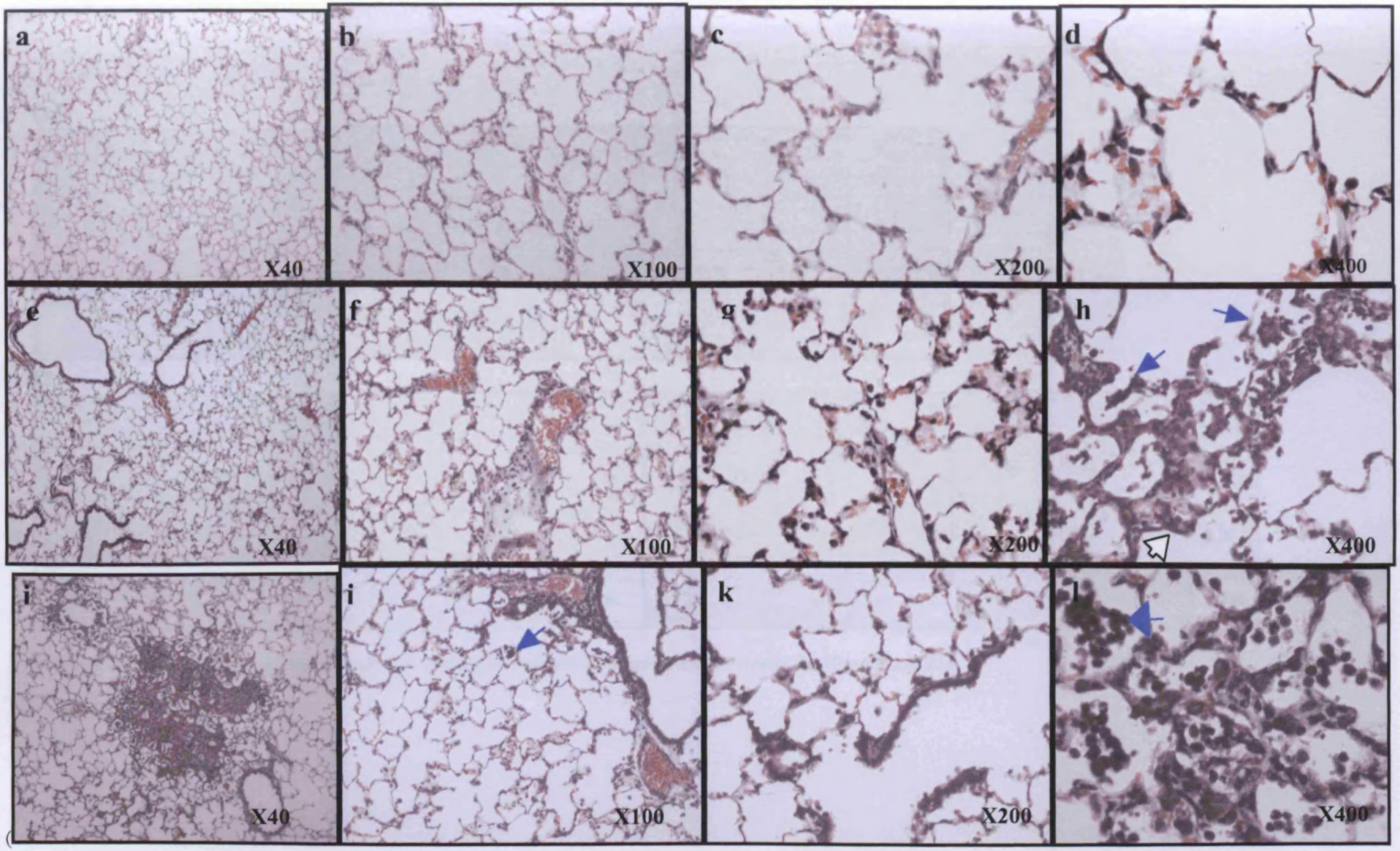


Figure 2.10: Masson's trichrome stained lung sections of sham treated (a-d) and rats treated with 5 mg (e-h) or 10 mg (I-l) whole PM_{10} at three days post instillation (magnification of images provided). Blue arrow highlights macrophage accumulations containing black carbonaceous material within the alveolar spaces. Black arrowhead indicates alveolar septae. White arrows show regions of thickened tissue around particle aggregations.

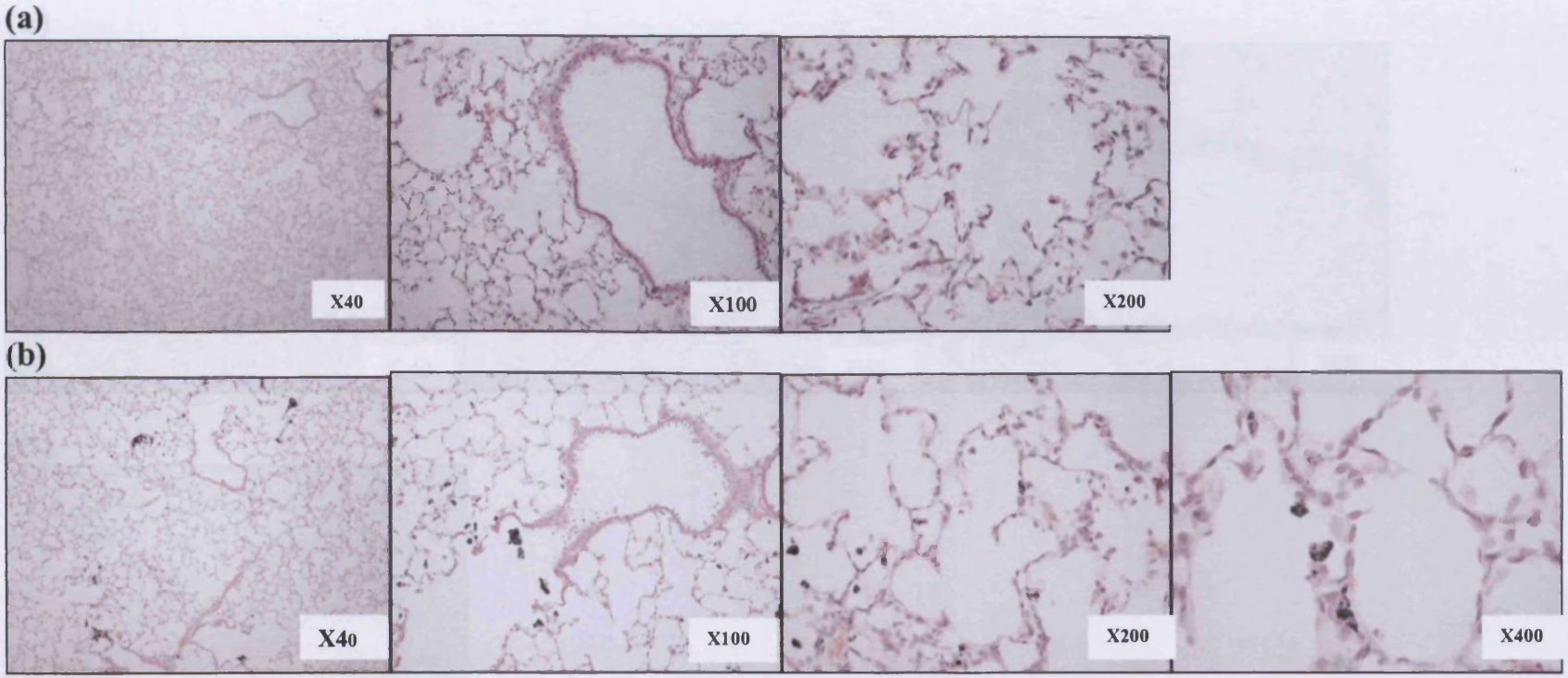


Figure 2.11: Hand E staining of lung sections at 6 weeks post exposure to (a) saline treated control (b) 10 mg whole PM

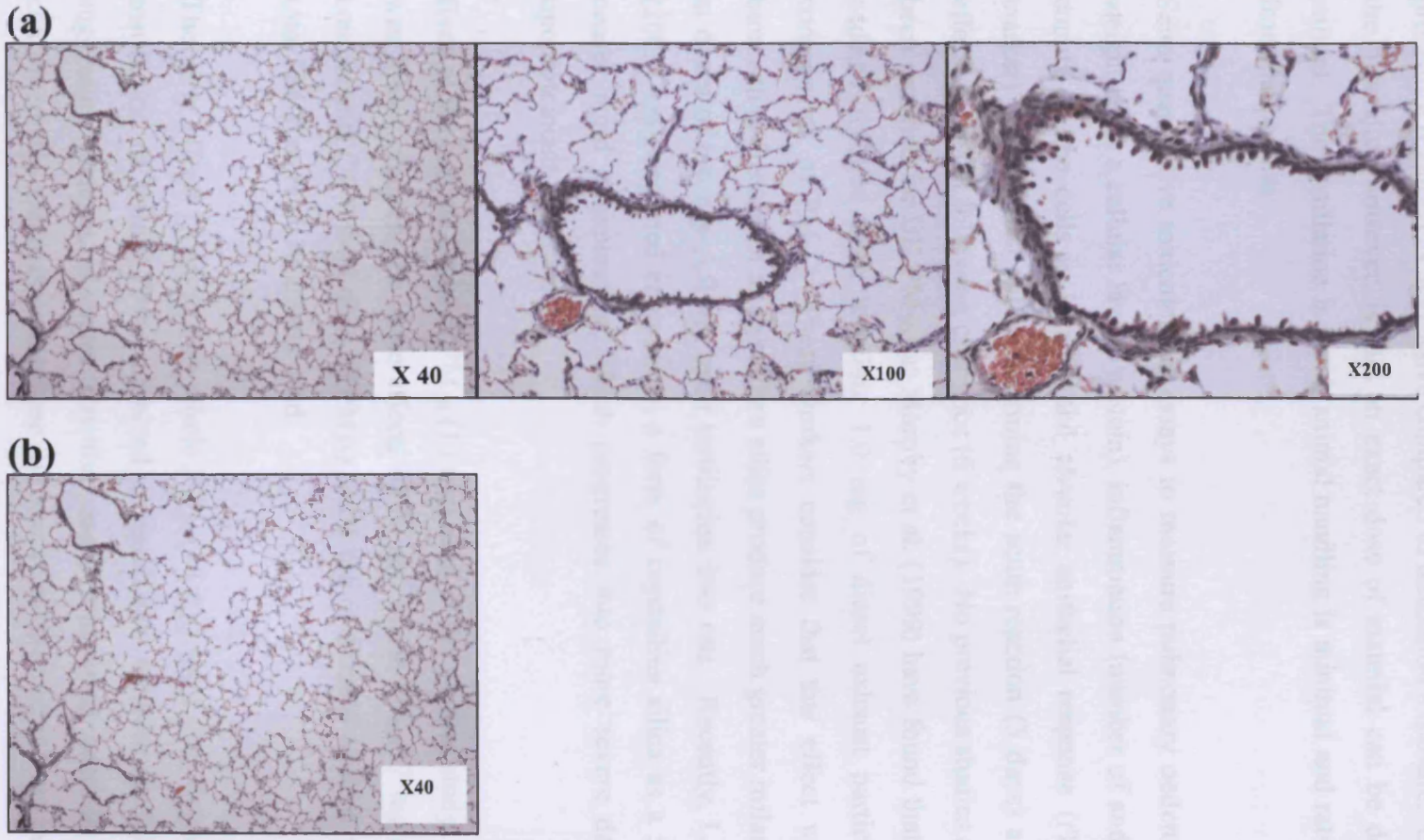


Figure 2.12: Massons Trichrome staining of lung sections at 6 weeks post exposure to a) saline treated control and (b) 10 mg whole PM.

Discussion

A conventional toxicological and histopathological approach has been used to examine the effects of whole PM_{10}, durable particles derived from this sample (30% of the total mass) and the water soluble components of the ambient particulate matter (70% of the mass). PM_{10} and its component parts were instilled into rat lungs, a procedure regarded as aggressive compared to inhalation exposure. One advantage of the procedure however, is that an exact dose of material can be delivered to each animal. The instillation is rapid, animal handling is minimal and rats recover rapidly from the process.

Semi quantitative toxicological assays to measure pulmonary oedema (lung to body weight ratio, a cellular lavage protein), inflammation (number of and differential cell counts of free cells in lavage) and alveolar epithelial response ($rT1_{40}$- type I cell marker) were carried out to determine the acute reaction (3 days) and to see if any effects persisted following clearance (6 weeks). No previous studies of this type have been reported for UK PM_{10} but Murphy et al. (1999) have found that a transient lung oedema resulted from instilling 1.0 mg of diesel exhaust particles, one of the components of PM_{10}. These workers consider that this effect was mild in that bioreactive dusts such as crystalline silica produce much greater inflammatory effects, at doses of 0.6 mg- 1.0 mg upon instillation into rats. Recently, Lee and Richards (2003) have reported cristobalite, a form of crystalline silica as a 5.0 mg instillate causes rapid inflammation, which progresses into more severe damage including lipoproteinosis and fibrosis.

Even at the highest doses of PM_{10} (10 mg) used in the present study, no such damage was found. Indeed, the acute effects of 10 mg whole PM_{10}, 7 mg of water soluble components (70% total mass of PM_{10}) and 3 mg of durable washed particles (30% of total PM_{10}) were all relatively mild.

The early effects (3 days) with whole PM_{10} (10 mg) were dose dependant. A 10 mg instillation of whole PM_{10} produced a significant increase in lung body weight suggesting oedema. A more sensitive assay measuring acellular lavage protein confirmed this oedematous response and was also observed at 5 mg instillation but not

at a lower dose of 2.5 mg. The oedematous effect (lavage protein) was also noted with the durable particles alone at both 5 and 10 mg equivalent instillations. These durable particle preparations only contain 1.5 and 3.0 mg respectively in mass suggesting that the oedematous response may be more closely linked to particles than the soluble components of PM_{10}. Two further aspects of the data emphasise this deduction. Firstly, only the 10 mg soluble (actually 7 mg in mass) of the water soluble component instillate produced a significant increase in acellular lavage protein at 3 days which is only transient (not observed at 6 weeks). Secondly, significant increases in acellular lavage protein were found with both whole PM_{10} and the durable fraction of PM_{10} at the 2 highest doses used after 6 weeks suggesting significant oedema.

Oedema results from damage to the endothelial cells lining the capillaries or the type 1 alveolar epithelial cells lining the alveolar surface. Thus, changes in a marker protein, $rT1_{40}$, for type 1 cells might be detected following alveolar damage or permeability changes. Indeed, following instillation of all PM_{10} fractions, levels of $rT1_{40}$ in lavage were substantially reduced compared to sham-treated rats. There seems to be no simple explanation for this unless some mass destruction of type 1 cells have occurred followed by rapid turnover of this cell membrane marker. Histological examination of the tissue offers no evidence for such an event. Ironically at 6 weeks post instillation when other measurements and histopathological examination suggested that the lung had repaired and was returning to normal architecture, a number of significant increases in lavage $rT1_{40}$ were detected. Again, there is no simple explanation for this observation.

Increases in the free cell counts, specifically of macrophage but not neutrophils, suggest a mild inflammatory response. This effect was only present at day 3 for the highest dose (10 mg) of PM_{10} and resolved by 6 weeks. This correlated very well with the observations of the lung tissue sections. No macrophage response was detected with the water soluble fraction but focal aggregation of dust laden macrophages filled alveolar spaces in animals instilled with whole PM_{10} after 3 days. This latter effect was not detected after 6 weeks with whole PM_{10} when only individual particle laden macrophages were seen. Thus, the effect of a relatively large instilled dose of whole PM_{10} is transient with very little likelihood of disease

progression as noted with other particulates such as crystalline silica (Murphy et al., 1999. Lee and Richards, 2003). Perhaps one reason for this is that much of the mass of the PM_{10} was present as a soluble component, which readily translocates the lung and enters the bloodstream. The durable particles of PM_{10} that remain are mostly diesel exhaust particles previously shown to be of low bioreactivity and thus readily cleared from the lung. One major route of clearance for diesel exhaust particles is via the lymphatic system and in the present study, black carbonaceous materials were found in the otherwise undamaged lymph node 6 weeks after instillation of the PM_{10}. Similar findings for diesel exhaust particles have been reported (Richards et al., 1999).

In summary, instillation of whole PM_{10} at high concentrations in rats produces mild but transient permeability and inflammatory changes. Much of the effects noted may be caused by the durable particle component(s) of the PM_{10} although major mass soluble components are not without effects. The data obtained in this study provides an excellent basis to conduct a toxicogenomic investigation examining changes in specific parenchymal genes associated with mild and transient lung damage, details of which are reported in the following chapter.

Chapter 3.0

Toxicogenomic profiling of Cardiff PM_{10} induced lung injury

3.0 Toxicogenomic profiling of Cardiff PM_{10} induced lung injury

3.1 Introduction

Toxicology is the science of predicting and understanding the adverse effects resulting from the exposure of organisms to chemical/physical or biological agents (Kaminski *et al.,* 2000). In this regard, the use of animal model to provide different endpoints of lung toxicity has proved invaluable. The relevance of animal models to risk assessment for man is under constant debate. This is because no animal model can mimic exactly the cell types, their xenobiotic metabolism or protective systems found in humans. Nevertheless, once the lung is treated with a toxicant the cellular responses and disease development process (see Figure 1.6) is very similar in humans and animals. This makes the continued the use of animal models vital for a better understanding of the mechanism of protection provided by lung cells and how these systems can go wrong resulting in damage endpoints such as oedema, inflammation and fibrosis.

In the previous chapter a quantitative conventional approach showed that inflammatory and oedematous reactions were produced in rodent lungs following the instillation of PM_{10} collected in South Wales conurbation. However, these studies provided no information on the molecular basis of these changes. To understand more of such changes a variety of approaches may be adapted. Firstly, the expression of candidate genes, thought to be important in inflammation or oedema could be examined in the lung tissue by quantitative PCR. Such an approach suffers from selection problems and may miss important links between different gene activities. A second approach is to examine global gene expression using the newly developed commercial macro- or microarray systems. This approach involves generating fluorescent or radiolabelled cDNA transcripts from RNA samples, hybridisation to the target array bound probes and semi-quantification and a bioinformatic comparison of relative gene expression between untreated and toxin treated samples.

To date, there have been no reports in the literature whereby array technology has been employed to examine the effects of PM_{10} on the lung. However, Reynolds and

Richards (2000), using a commercially available macroarray, reported that consistent changes in gene expression concerned with DNA metabolism, cellular repair and detoxification could be detected following instillation of diesel exhaust particles into rat lungs. In the present study it was decided to use a similar macroarray to examine the effects of PM_{10} (with diesel exhaust particles a major numerical component) on the rat lung. The advantages perceived with this array system was the manageable number of genes (1176 genes) and in that the system was commercially available, important findings could potentially be reproducible intra and inter laboratories. The chosen macroarray is relatively inexpensive compared to other microarray systems and if proven useful, could be readily available to a wide spectrum of laboratories.

Thus, the primary objective was to examine changes in lung gene expression following the instillation of whole PM_{10} (two doses: 5 mg and 10 mg) and the 10 mg equivalent dose of the water soluble fraction of ambient Cardiff sample. Lung tissues from animals already treated with these samples (Chapter 2) were selected on the basis that conventional toxicology had shown oedematous and inflammatory changes compared with sham-treated animals. At the commencement of this toxicogenomic study there was no general agreement how to handle and draw reliable conclusions from the huge amount of data generated from macroarrays. In 2000, the MIAME guidelines (minimum information for a microarray experiment) (Speed et al., 2000) had devised and subsequently adopted by many journals. The chief aims of these guidelines were to standardise the recording and presentation of experimental data to facilitate the critical assessment by third parties and the determination of reproducibility of the study. For this purpose, a more simplistic analysis approach using basic computer packages were favoured, allowing array data to be evaluated in an easy step by step manner over large complex array analysis packages.

Thus, during the course of the study, methods had to be developed (alongside colleagues) to distinguish consistent small changes in gene expression with appropriate stringency for the production of meaningful gene lists for further investigation. Once gene lists reflecting gene changes induced by PM_{10} were established it was necessary to check the quality of the array data. Therefore the expression of selected lung genes were further examined by qPCR for comparison with the findings from the array data.

3.2 Materials

3.2.1 Materials and Suppliers

RNeasy™ Mini kit (74104) containing all appropriate buffers, solutions and vials and Deoxyribonuclease I, QIA™ shredder (79654) purchased from Qiagen. β-Mercaptoethanol (436022 A) was obtained from BDH Biochemical Laboratory Supplies, Poole . Ultra-pure (HPLC) Grade Ethanol (95% v/v) (CAS 64-17-5) was bought from Fisher Life Sciences.

Tri-reagent ™ solution (T-9424), Isopropanol (>99% v/v) (I 9516), Chloroform (>99% v/v, containing no iso-amyl alcohol or additives) (C 2432), Agarose (Type 1A)(A 0169), 20X TBE* (T 4415), Ethidium Bromide (10mg/ml) (E 1510) and Loading Dye (G 2526) were all purchased from Sigma – Aldrich.

The nylon Atlas™ Rat 1.2 array system (7854-1) was purchased from BD Biosciences in kit form. Each kit containing: Cot-1 DNA (1 mg/ml), 10 X Termination mix (0.1 M EDTA, 1 mg/ml $MgCl_2$, pH8.0), Reaction buffer (250 mM Tris-HCl), 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 10 X dNTP mix (5 mM of each dNTP except dATP), DTT (100 mM), 10X CDS Primer mix, Powerscript MMLV Reverse Transcriptase and nucleospin™ extraction/purification columns and wash solutions.

[α-^{32}P]-d.ATP (10 μCi/mmol, Cat No. PB 10204) was purchased from Amersham, UK. Sheared Salmon Testes DNA (D7656, 10 mg/ml) was bought from Sigma. Atlas™ Image 2.0 and Atlas™ Navigator (Clontech) were used to interpret the array data. 20% w/v SDS solution (ultra pure) (EC 874) and 20% w/v SSC Solution (ultra pure) (EC-873) were purchased from National Diagnostics. The Kodak Phospho-imager screen (Kodak 118 8077) was purchased from Biorad.

Oligo DT Random hexamers were purchased from Gibco (100 pmoles/μl). dNTPs (each at 100 mM, U13300), Moloney Murine leukaemia virus Reverse Transcriptase (MMLV-RT) and appropriate buffer, RNase inhibitor, 100bp DNA ladder (G2102), pGEM-T vector system (A3600), DNA Wizard SV Mini prep kits (A1340), Ampicillin (1 mg/ml), IPTG (1.2 g diluted to 0.1 M in water, V3955), X-Gal (50 mg/ml, V3941) were all purchased from Promega. Relevant gene primer sequences,

Gene Elute Agarose spin columns (56500) were purchased from Sigma. BD Advantage™ 2 PCR kit (639207) was purchased from BD Biosciences and contained 50x Polymerase Mix (Taq DNA Polymerase and Taqstart antibody (1.1 μg/μl) in 10x Storage buffer (50% v/v Glycerol, 15 mM Tris-HCl, 75 mM KCl, 0.05% v/v EDTA, pH 8.0). 10x Advantage™ 2 PCR buffer (400 mM Tricine KOH, 150 mM KOAc, 35 mM $Mg(OAc)_2$, 37.5 μg/ml BSA, 0.05% v/v Tween-20 and 0.05% v/v Noridet P40). PCR Sybr green Quantitect kit (204243) was purchased from Qiagen. Thermofast®96 well detection pates (AB1100) and cap strips (AB0866) were purchased from Abgene, UK.

3.2.2 Stock Solutions for the Macroarray procedure

♦ Master Mix:

Single Reaction (per reaction): 2 μl Reaction buffer (250 mM Tris-HCl), 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 1 μl 10 X dNTP mix (5 mM of each dNTP except dATP), 0.5 μl DTT (100 mM) and 3.5 μl [α-P32].dATP (10 μCi/mmol).

Double reaction* (per reaction, used instead of single rxn if sample RNA (2 μg) volume exceeds 2 μl): 4 μl Reaction buffer (250 mM Tris-HCl), 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 2μl 10 X dNTP mix (5 mM of each dNTP except dATP), 1 μl DTT (100 mM) and 5 μl [α-^{32}P]-d.ATP (10 μCi/mmol).

♦ Luria-Bertani (LB)- agar plates and LB liquid media containing antibiotic were made up fresh as required.

3.3 Methods

3.3.1 Safety Implications

Radio-isotope (^{32}P): All radioactive work was carried out in a designated radiation area and all radioactive samples stored and waste disposed of in compliance with local guidelines.

Competent *E. Coli* (JM109): Low risk genetic modification with the disabled vector and cells was carried out. All equipment, waste and surfaces were sterilised and disposed of in accordance with GM guidelines.

3.3.2 Selection of PM Exposed and Sham-treated control animals for Toxicogenomic Analysis

The toxicogenomic analysis was completed on selected rat lung tissue from the instillation study (Chapter 2). Rats were selected from sham-treated, water soluble (10 mg) and whole PM exposed groups (5 mg and 10 mg) at three days post instillation.

Conventional parameters used to assess the pulmonary responses of individual rats showed animal-to-animal variations. Animals were therefore selected based on their individual responses differing from sham-treated controls. Changes in lung to body weight ratio, lung lavage acellular protein and increases in large free cell counts were used to ascertain three animals from each treatment group most severely affected from the treatment.

3.3.3 RNA Extraction

Two methods of RNA extraction from lung tissue were explored, RNeasy™ (Qiagen) and Tri-reagent ™ (Sigma).

Total RNA was isolated from lung tissue using RNeasy Mini kit, Qiagen. The technique provides a fast and simple procedure to isolate RNA from a small amount of starting material. The system combines the selective binding properties of a silica gel based membrane which under high salt conditions, binds long RNA molecules and serves to wash and then elute a purified product suitable for a range of subsequent techniques (supplier's manual).

In summary, lavaged whole lung tissue isolated from the rat was snap frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. The tissue cells were lysed and homogenated in the appropriate volume of denaturing guanidine isothiocynate (GITC) containing buffers, which acts to immediately denature any

RNases, thus preventing any initial denaturation of the RNA. Samples were either stored at -80°C in the GITC-buffer or used immediately. Samples were disrupted by either centrifuging (>8,000 x g, 2 minutes) in a QIA shredder column or passing the lysate (at least five times) through a 20 G needle fitted to a syringe. This serves to reduce viscosity of the lysates, shearing genomic DNA and high molecular weight cellular components. All insoluble material and high molecular weight DNA were removed by centrifugation (12,000 x g, 3 minutes) and the supernatant retained in a fresh tube. An equal volume of ethanol (70 % v/v) was added, to provide appropriate conditions for the binding of the RNA. The entire sample was loaded, in successive aliquots, to the RNeasy mini column and centrifuged in a bench top centrifuge (>8,000 x g, 15 seconds). The elutant was discarded and deoxyribonuclease suspended in provided buffer (Qiagen) was incubated (RT, 20 minutes) in the column then eluted by centrifugation (>8,000 x g, 30 s). The column was rinsed with wash buffers provided with the kit, RW-1 (700 μl, spin >8,000 x g, 15 seconds). An optional on-column DNase treatment was completed at this stage. An aliquot of DNase (Qiagen) suspended in ultra pure water (70 μl) was applied to each sample column and incubated (RT, 15 minutes). The enzyme was denatured and eluted from the column by centrifugation (spin >8,000 x g, 15 seconds) and washed twice with RW1 wash buffer (350 μl, spin >8,000 x g, 15 seconds) and twice with the ethanol-containing buffer, RPE, (500 μl, spin >8,000 x g, 15 seconds) in order to remove all contaminants. The column was centrifuged (>8,000 x g 1 minute) to eliminate any residual traces of ethanol from the RPE buffer that would interfere and contaminate the RNA. The RNeasy column was transferred to a new, sterile Eppendorf and RNase / DNase free water was applied to the column (20-30 μl). The column was left to equilibrate on ice for a few minutes before being spun in a bench centrifuge (1 minute, >8,000 x g) to elute the RNA.

Total RNA from lung tissue was isolated using Tri-reagent™ as outlined in the Sigma Technical Bulletin (MB 205). This technique provides a quick and convenient one step procedure based on an extraction outlined by Chominski and Saachi (1987), allowing the simultaneous extraction of RNA, DNA and protein.

Tri-reagent™ (1 ml), a mono-phase solution of guanidine thiocyanate (to denature RNA degrading enzymes) and phenol was added to the ground lung tissue (~1 mg). The tissue was lysed by thoroughly mixing the suspension with a pipette to form a

homogenous lysate. All insoluble material and high molecular weight DNA were removed by centrifugation (12,000 x g, 10 minutes at 4°C) and the supernatant retained in a fresh tube. Chloroform (0.2 ml per ml of Tri-regent™) was added and mixed vigorously before centrifugation (12,000 x g, 15 minutes, 4°C). This resulted in a three-layered solution containing a red organic phase (protein), an interphase (DNA) and colourless upper aqueous phase (RNA). The latter was carefully removed and isopropanol (0.5 ml per ml of Tri-regent™ used) was added, the mixed sample was left to stand (-20°C, 30 minutes) and subsequently centrifuged (12,000 x g, 10 minutes, 4°C) to precipitate the RNA. The pellet was washed in 75 % v/v ethanol (1 ml) and a pellet reformed by centrifugation. The pellet was air dried to remove all residual ethanol before being resuspended in RNase/DNase free water (20-30 μl).

3.3.4 Quantifying the RNA and Checking its Integrity

The purity and yield of all RNA samples were assessed regardless of extraction procedure using the GeneQuant™ (Pharmacia). Purity was established by measuring the 260:280 nm ratio. The ratio for RNA suitable for subsequent gene analysis should be between 1.8-2.1. A ratio outside this range indicates contamination in the sample. The yield of the sample was deduced from the absorbency of the sample at 260 nm.

A 1-2 μl specimen of the RNA sample was size fractionated on a 1 % w/v agarose gel (0.5 g/50ml 0.5 % v/v TBE with 1 μl o f 10 mg/ml Ethidium bromide) in 0.5 % v/v TBE (with <1% v/v 10 mg/ml Ethidium Bromide). Each well was loaded with 1-2 μl RNA in water, 2 μl loading dye, to a total of 10 μl in high purity RNase/DNase free water. The gelS were subjected to electrophoresis (30 minutes, 80 volts) and viewed under UV using the Syngene™ Gel documentation System.

3.3.5 RT-PCR

Reverse Transcription-PCR was completed on early preparations of DNase treated RNA samples isolated using the Qiagen RNeasy™, with the aim to confirm complete removal of all functional DNase enzyme that would be detrimental to subsequent array procedures.

Reverse Transcription (RT) was completed on total RNA. Random hexamers (200 pmoles) and target RNA was mixed up to a final volume of 11.5 μl in HPLC water and incubated at 70°C (10 minutes) then quick chilled on ice (5 minutes). The preparation was made up to a final volume of 20 μl with dNTPs (500 μM of each), 1X reaction buffer (10 mM Tris-HCl, 15 mM KCl, 0.6 mM $MgCl_2$, 10 mM dithioreitol, pH 8.3), RNasin Ribonuclease inhibitor (20 units, Promega) and Moloney Murine Leukaemia Virus (MMLV) RNase H- Reverse Transcriptase (200 units, Promega). The solution was incubated 42°C (1 hour) followed by 90°C (30 s), made up to 100 μl with tissue culture grade water and used immediately. A sample of total RNA was made up with the same RT mixture but the volume of MMLV-RT was replaced with water (no RT).

PCR was completed on 10 μl of cDNA (with RT and no RT cDNA samples) using GAPDH forward and reverse primers. The primers and optimised PCR conditions are shown below (S.Irvine, personal communication, 2001).

GAPDH Forward Primer 5'**CCCTTCATTGACCTCAACTACATGG**-3'
GAPDH Reverse Primer 5'**AGTCTTCTGGGTGGCAGTGTGATGG**-3'

The PCR mixture consisted of the following: 10 μl cDNA, 0.5 μl forward and reverse primers (100 μM each), 1.5 mM $MgCl_2$, 5μl 10 X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8 at 25°C, 0.1% v/v Tween-20), 0.5 μl of each dNTP (10 mM) and 1.25 units of Taq Polymerase and made up to a final volume of 50 μl in HPLC grade water.

The PCR cycle employed a denaturing step at 95°C (5 minutes) to denature the cDNA, annealing (55°C, 1 minute), extension (72°C, 2 minute) and melting (94°C, 30 seconds) with a final long extension on the final cycle (72°C, 10 minutes). PCR was continued for 32 cycles and the cDNA cooled and stored at 4°C.

The resultant products were electrophoresed on a 1.5 % w/v agarose gel (80 volts, 30 minutes).

3.3.6 Macroarray Preparation

3.3.6.1 Preparation of the Membranes

The macroarray membranes were washed with deionised water and were each placed in a hybaid tube. Salmon Sperm (0.5 mg/membrane) was denatured (95°C, 5 minutes) and placed on ice. The salmon sperm was added to hybridisation solution (5 ml per membrane) and added to each membrane and left to prehybridise (68°C, 30 minutes, with constant agitation).

3.3.6.2 Preparation of ^{32}P-labelled first strand cDNA Probe

Isolated, genomic free RNA (2 μg) was mixed with 10X CDS Primer mix (1-2 μl), and placed on ice. The master mix stock (Materials) and the RNA/Primers were preheated (70°C, 2 minutes and then 48°C, 2 minutes). MMLV Reverse Transcriptase (1 μl per reaction) was added to the cooled (48°C) master mix and heated (48°C) for a further 2 minutes alongside the RNA. Master mix (8 μl single reaction or 14 μl double reaction) was added to the RNA immediately incubated at 48°C (25 minutes). The reaction was terminated using 10 X termination mix (1 μl) and placed on ice. The labelled cDNA probe was purified from unincorporated ^{32}P-labelled nuclides and small cDNA fragments through a nucleospin column supplied with the array membranes The reaction mixture was diluted to a final volume of 200 μl in binding (NT-2) solution and applied to the column. The solution was centrifuged (>10,000 x g, 1 minute) and elutant, containing unincorporated radionucleotides, discarded. The column was washed in an ethanol buffer (NT 3, 400 μl) and centrifuged at each step. The radioactivity of each elutant was assessed to ensure that all unbound dNTPS were removed. The purified probe was subsequently eluted in Eluting buffer (NT-E, 100 μl) incubated (1 minute, room temperature) in the column before centrifugation (2 minutes, 10 000 x g). 10X Denaturing solution (11 μl, 1 M NaOH,10 mM EDTA) was added and the solution placed at 68°C (20 minutes) to allow full denaturation of the cDNA probe. C_ot-1 DNA (5 μl) and 2X Neutralisation solution (111 μl, 27.6 g $NaH_2PO_4 \bullet H_2O$ in 200 ml H_2O (pH 7.0, adjusted with 10 M NaOH) were added and placed at 68°C for a further 10 minutes.

3.3.6.3 Hybridisation of the Probe to the Array

The denatured radiolabelled probe was added to the hybaid bottle containing the membrane and hybridised overnight (68°C) with continuous turning.

The membranes were washed in wash solution 1 (0.1% v/v SSC, 0.5% v/v SDS) and the more stringent wash solution 2 (0.01% v/v SSC, 0.25% v/v SDS) as appropriate until the radioactive counts dropped to 10-20 c.p.m. (usually 2 washes of 10-20 minutes each in wash solution 1 was sufficient). The membranes were removed, wrapped well and placed under a phosphor-imager screen (Kodak) within a cassette and left (room temperature) for 5-7 days depending on the final radioactivity on the membrane.

The exposed phosphorimager screen was scanned (50 μm sensitivity) and the resultant image analysed using the Atlas Image 2.01a (BD Biosciences). The software facilitated the identification of differentially expressed genes in compared arrays.

3.3.6.4 Stripping the Membranes

The array membrane was stripped of the cDNA probe prior to storage at -20°C. The membranes were boiled vigorously in 0.5 % v/v SDS solution (5-10 minutes) and rinsed (wash solution 1). The membranes were checked for radioactivity prior to storage in a sealed bag at -20°C. The phosphor imager screen was cleaned by exposure to bright light (15 minutes) and stored at room temperature until required

3.3.6.5 Visualisation of the Array

Scanned images of the gene arrays were visualised using Atlas™ image 2.01a (BD Biosciences, UK). This programme assists in alignment of the membrane image with the outline plan of the array. Nylon arrays are versatile and economical way to produce macroarray data. However, with repeated use they can become increasingly distorted, requiring careful alignment and positioning before meaningful conclusions can be made. Image 2.01a is a useful programme, calculating background intensity levels and adjusting spot intensity accordingly. Estimated background intensities were

calculated from each array based on assigning regions of membrane devoid of any target cDNA to adjacent regions of the array containing target sequences and attached probe. It was deemed to be a more accurate approach than assigning a selected region to describe the extent of background noise across a potentially variable array membrane. Finally, the programme was used to complete comparative analyses between individual test/control arrays or used to generate average controls and exposed profiles. To ensure that all array analyses were comprehensive and similar, it was judged that all variables were set as equal. Therefore, the ratio signal threshold was reduced on all aligned arrays to 50% and all genes were reported to an excel worksheet. All analyses were therefore conducted on similar analysis outputs within the excel package.

3.3.7 Data Handling and Bioinformatics

3.3.7.1 Transformation and Normalisation of the Raw Array Data

Data exported from the Atlas programme consisted of several data components including an estimation of local background intensity for each gene, the intensity of each spot corresponding to each gene and a background adjusted intensity for each gene. The lists of background adjusted signal spot intensity for each gene were compared across each exposure and sham-treated lungs.

The data derived directly from the Atlas 2.01a programme has not been normalised or transformed in any way. The distributions of replicated raw expression values (and consequently ratio data) tends to be skewed, thus violating the assumptions of many statistical tests and distorting many *p*-values associated with parametric tests such as ANOVA and t-tests. Log transformation reduces the random error of raw expression data. The first important step was to correct for this using a standard data transformation, to ensure the data is approximately normally distributed to allow normal statistical assumptions to be made. Statistical evaluation of the distribution of the data using an Anderson Darling test ($p>0.05$) confirmed that the raw array data was not normally distributed.

For this purpose, the $\log_{10}$ (adjusted intensity +c) was calculated for each gene intensity spot on each array prior to normalisation. Log-transformed data are generally preferred to ratios of raw signal intensity (Nadon et al., 2002).

All arrays were globally normalised, a normalisation technique involving calculation of all gene intensities to produce a scaling factor and adjusting accordingly rather than to a single or a few housekeeping genes. Alternative approaches with more recent arrays involve spiking the RNA sample with known amounts of foreign transcripts to calculate a scaling factor to correct the relative expression of all other genes. Early analysis had confirmed that variation within the housekeeping genes did occur.
Log_{10} transformation served to correct the data to an approximately normal distribution, thus allowing normal statistical assumptions to be inferred. Normalisation adopted for this study was a two step procedure, reducing heteroscedasticity of the data resulting from random errors and alteration in background noise and allowing more meaningful comparisons between the arrays.

The first step of normalisation involved calculating the median spot intensity within the array as a scaling factor, an estimate of error. The intensity for each spot on the array was then divisible by this value to correct for variations across the array. The second step involved calculating the median spot intensity value of each gene across each of the arrays (control and exposed profiles). The intensity of each spot was then divisible by this new scaling factor for each gene on the array to produce the final normalised data. A variety of different scaling factors were examined, including the mean, median and ranked/trimmed mean. Mean expression values were highly influenced by the extreme behaviour of relatively few genes (i.e. highly expressed/over expressed genes and nominally expressed genes), trimmed means (adjusted expression values from each array or genes across the arrays were ranked and trimmed accordingly) and median values produced only minimal differences to the final data and so the median values were adopted as the scaling factor in all subsequent analyses. This global method uses an estimate of error (the median expression for each array) to control for proportional differences across membranes and assumes that most probes are unaffected by treatment. This was proven to be the case in previous studies by Reynolds (2000), whose experiments using diesel exhaust particles reported that overall 5% of the genes examined were altered by the exposure. If a large proportion of the genes are altered by exposure, the global approach could

serve to mask the effect of differentially expressed genes or, create differential effects where none exist (Nadon et al., 2002).

Normalised data was subsequently tested for normality once again using the Anderson darling Test for normality on random selection of genes using the statistical package, Minitab ($P>0.05$).

3.3.7.2 Selection of Gene Lists

Several methods of analysis were explored and the following section describes four approaches.

3.3.7.2.1 Pair wise Comparison

The simplest way to identify candidate genes across related experiments is a simple analysis to establish lists of consistently up- or down regulated genes, incorporating a very basic approach. Data from the array profiles were transformed (as outlined previously). Single sham-treated control/PM exposed profiles were normalised together, as previously described and compared in a pair wise manner. Fold changes in gene expressions were compared between different pairs. A two fold ratio change is detectable by the most sensitive alternative methods in greater than 50% of cases, whilst a 5 fold alteration may be detected in more than 90 % of cases (Dr. L Reynolds, BD Biosciences, *personal communication*).

It was considered that retaining the pair-wise comparisons might have been a more sensitive method to detect changes in the animals that may have differing extent of pulmonary responses to the toxicant exposure. It was anticipated that examining only the fold change did not take into consideration any extent of change. Gene A may have an adjusted intensity of 10 under control conditions increasing to 20 under experimental conditions and still undergo a two-fold change. Whereas Gene Y may increase from 1000 to 2000 for the equivalent fold induction. The second gene would therefore be more likely to be detected using conventional techniques. An estimation of extent of fold change could be enforced by applying an arbituary minimum relative difference/change in relative expression of 1000 or 2000 for each gene.

A mean sham-treated profile was calculated from the expression of the replicated profiles (n=3) and then used in comparisons to individual PM exposed arrays. Lists of genes expressed in a minimum of two out of the three comparisons were analysed. The effect of using mean or median control array values was also evaluated (data not shown).

3.3.7.2.2 Expression Range Analysis

This method was developed in collaboration with fellow colleague, Miss Dominique Balharry.

The method uses transformed and normalised data to estimate ranges of gene expression of both sham (saline) treated (n=3) and PM exposed (n=3) lung samples. Genes that have non-overlapping gene expression in both sham-treated and PM exposed profiles were considered (Figure 3.1). A further condition was enforced to further reduce the candidate gene lists. Although a notable difference in relative expression levels of the genes were apparent, this method had no semi quantitative measure of the change. Therefore a minimum fold change in gene expression was used to further select altered genes between control and test arrays.

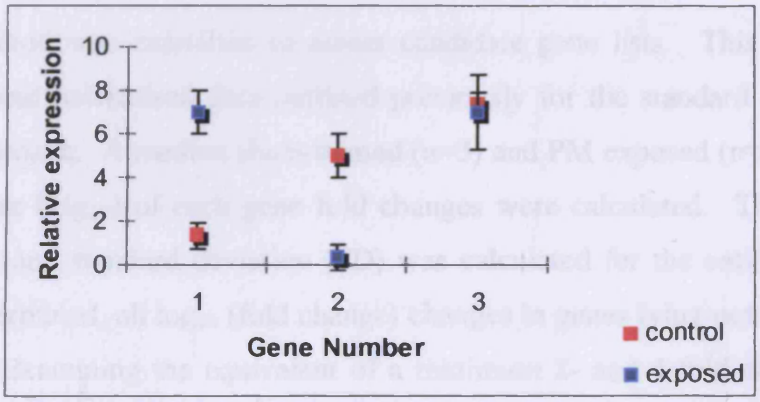


Figure 3.1: A representation of the expression range analysis. A range of expression based on the replicate samples produces an accepted variation in expression. Comparing these ranges of sham-treated and PM exposed profiles to ascertain significantly altered genes. Both gene 1 and 2 would be deemed significantly changed whilst gene 3 would not.

3.3.7.2.3. Statistical Comparison

Sham-treated control (n=3) and PM exposed (n=3) profiles were normalised together as previously described prior to analysis. Lists of candidate genes from this analytical approach were selected on the basis of a combination of two statistical features. Firstly, a student's *t*-test was completed. This tested the unpaired, unequal variance control and exposed profiles and genes were deemed significantly differentially expressed when $p<0.05$. Array profiles of three individual animals were completed for each experimental and control condition, this is a low replicate number for strong statistical inferences and combine with the large degree of animal variation and variability within the array procedure itself, it was considered that for the purpose of such a pilot study it would be justified to extend the statistical significance to $p<0.01$. The *t*-test alone reduced the gene list considerably, however, it made no allowance for the extent of variation of expression of genes amongst individual animals. It was deemed important that a normal range of variation of gene expression was acceptable between control arrays. Therefore, a 2 standard deviation range of expression was calculated and genes with a mean exposed expression outside this area were deemed significant by this parameter. The gene analyses were combined to produce a suitably shortened gene list, proving that this method was stringent enough to obtain candidate gene lists for further analysis.

3.3.7.2.4. Log_{10} fold Change Method

A fourth method was examined to assess candidate gene lists. This method used transformed and normalised data outlined previously for the standard deviation and statistical approach. A median sham-treated (n=3) and PM exposed (n=3) profile and the logarithmic (log_{10}) of each gene fold changes were calculated. The mean log_{10} (fold change) and standard deviation (SD) was calculated for the entire array and 2 SD range determined, all log_{10} (fold change) changes in genes lying outside this range were noted. Examining the equivalent of a minimum 2- and 4-fold change in gene expression further reduced these lists.

3.3.7.3 Analysis Overview

The previous sections contain detailed information regarding data handling and computation of the array data. These steps are simplified in the following flowchart (Figure 3.2)

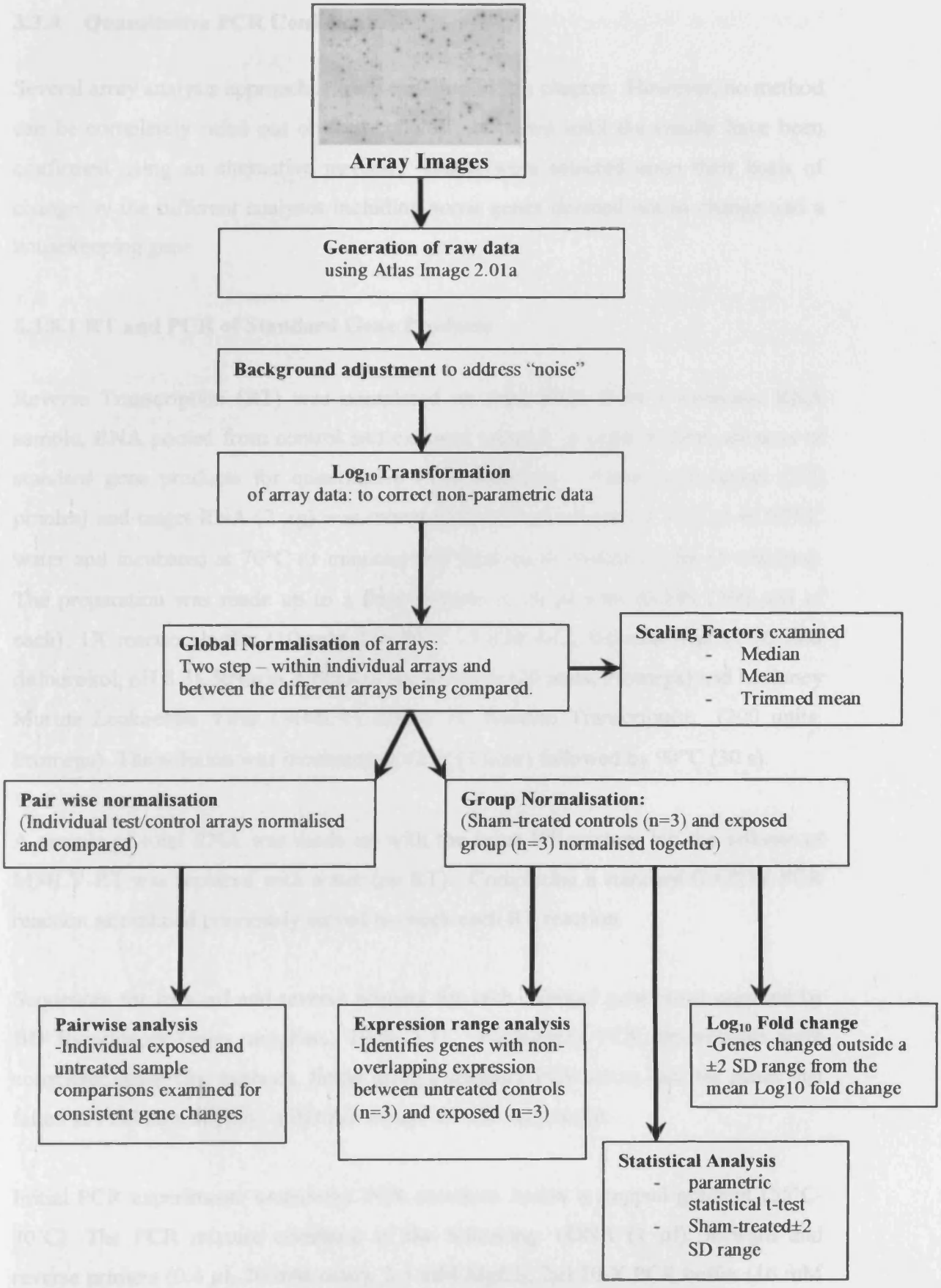


Figure 3.2: A flow chart to summarise each step involved in the analysis of the macroarray and the different analytical approaches used to generate candidate genes in the lung injury resulting from PM_{10} exposure.

3.3.8 Quantitative PCR Confirmation

Several array analysis approaches were explored in this chapter. However, no method can be completely ruled out or firm conclusions drawn until the results have been confirmed using an alternative method. Genes were selected upon their basis of change by the different analyses including some genes deemed not to change and a housekeeping gene.

3.3.8.1 RT and PCR of Standard Gene Products

Reverse Transcription (RT) was completed on total RNA from a reference RNA sample, RNA pooled from control and exposed samples in order to have aliquots of standard gene products for quantitative PCR standards. Random hexamers (200 pmoles) and target RNA (2 μg) was mixed up to a final volume of 11.5 μl in HPLC water and incubated at 70°C (3 minutes) and then quick chilled on ice (5 minutes). The preparation was made up to a final volume of 20 μl with dNTPs (500 μM of each), 1X reaction buffer (10 mM Tris-HCl, 15 mM KCl, 0.6 mM $MgCl_2$, 10 mM dithioreitol, pH 8.3), RNasin Ribonuclease inhibitor (20 units, Promega) and Moloney Murine Leukaemia Virus (MMLV) RNase H- Reverse Transcriptase (200 units, Promega). The solution was incubated at 42°C (1 hour) followed by 90°C (30 s).

A sample of total RNA was made up with the same RT mixture but the volume of MMLV-RT was replaced with water (no RT). Completing a standard GAPDH PCR reaction as outlined previously served to check each RT reaction.

Sequences for forward and reverse primers for each selected gene were supplied by BD Biosciences (array suppliers, Table 3.1). Preliminary PCR experiments were completed using two methods, firstly using a standard PCR mixes and, for genes that failed to PCR successfully, a BD Advantage 2 PCR enzyme kit.

Initial PCR experiments underwent PCR reactions across a stepped gradient (55°C-70°C). The PCR mixture consisted of the following: cDNA (1 μl), forward and reverse primers (0.4 μl, 20 mM each), 2.5 mM $MgCl_2$, 2μl 10 X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8 at 25°C, 0.1% v/v Tween-20) dNTP (0.4 μl of at 10 mM each) and 1.25 units of Taq Polymerase and made up to a final volume of 20

μl in HPLC grade water. Each PCR reaction was accompanied by suitable water blanks, devoid of any cDNA to guard against contamination.

Initial PCR cycles (30) comprised of an initial denaturation (94°C, 2 minutes), cycle denaturation (94°C, 30 seconds), annealing (55-70°C, 1 minute), extension period, (72°C, 2 minutes) and final extension period (72°C, 5 minutes) and left to cool (10°C).

The Advantage PCR system was adopted for difficult genes that failed to amplify under the extensive optimisations using the alternative method. This system involved a patented ready-made PCR buffer containing an optimal mixture of Taq Polymerase and 10x BD advantage™ 2 PCR Buffer (see materials section). In brief, a PCR reaction was made up as follows: 5 X Advantage™2 reaction buffer (5 μl), appropriate forward and reverse primers (1 μl, 20 mM each), dNTP mixture (1 μl) containing each dNTP at a concentration of 10 μM, Taq Polymerase mix (1 μl) and made up to 50 μl in molecular grade water. PCR cycles were completed as outlined in the Advantage™ 2 protocol, involving a two step PCR cycle (30-35 cycles) incorporating an initial denaturation period (95°C, 1 minute), two cycle steps of 95°C (30 seconds) and 68°C (1 minute) followed by a final elongation (68°C, 1 minute).
PCR reactions were size-fractionated on a 1.5% w/v agarose gel (45 minutes, 80-90 volts) alongside a suitable DNA ladder (Promega) and single bands for each gene were excised and then eluted using a gel extraction kit (Sigma) in TE buffer to stabilise the DNA product.

Table 3.1: Selected gene sequences for (1) forward and (2) Reverse primers (BD Biosciences).

Coordinate	Genbank	Gene Name	Fragment length(bp)			PRIMER (bp)	Expected Change in expression
B04m	M59980	potassium voltage gated channel,	193	primer 1	TGATCGTGTCTAACTTCAGTCGGATC	26	Down regulated
				primer 2	CTAACGAAGGCCGGTTCATCCTCC	24	
C02b	X52625	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	239	primer 1	GCCACACCAGGATCTGCTCTTGAC	24	Up regulated
				primer 2	GAGGGACGCCGGGCGTAAGTTC	22	
D09n	L08490	gamma-aminobutyric acid (GABA-A) receptor	234	primer 1	GGACAACACCACTGTCTTCACGAG	24	Down regulated
				primer 2	TTCAGCCGGAGCACTGTCATGGG	23	
D14i	U37101	Colony stimulating factor 3 (granulocyte)	297	primer 1	AGCTGCTGCTGTGGCACAGTGCAC	24	Down regulated
				primer 2	GCAGTTGGCTCAGGCACTTTGTCTG	25	
D12m	D16817	mRNA for metabotropic glutamate receptor mGluR7	281	primer 1	GGATCATTGCCACCATCTTTGTCATG	26	Down regulated
				primer 2	GGGAGCTGTCACCGATTTCTTGCC	24	
G43	V01217	cytoplasmic beta-actin	285	primer 1	CCCTCTGAACCCTAAGGCCAACCG	24	No change
				primer 2	GTGGTGGTGAAGCTGTAGCCACGC	24	
B02k	L31619	Cholinergic receptor, nicotinic, alpha polypeptide 7	228	primer 1	CCCAGATGTCACCTACACAGTAACC	25	Up regulated
				primer 2	ATCAAGGGCACAGAATCAGATGTTGC	26	
B13g	Y13380	Amphiphysin II (AMPH2)	300	primer 1	GCGAACAAGATAGCAGAGAACAATGAC	27	Down regulated
				primer 2	GTTAGTTTGAGCTACAAGATGAGCCTG	27	
C12i	U49729	Bcl2-associated X protein	298	primer 1	TGATTGCTGACGTGGACACGGACTC	25	Up regulated
				primer 2	GCCACAAAGATGGTCACTGTCTGCC	25	
E05a	M22899	Interleukin 2 (IL2)	282	primer 1	TGGACTTACAGGTGCTCCTGAGAGG	25	Up regulated
				primer 2	CTGGCTCATCATCGAATTGGCACTC	25	
E10m	U23443	p21 (CDKN1A)-activated kinase 1	305	primer 1	TGGAGTTCCTGCATTCAAACCAAGTC	26	Down regulated
				primer 2	GGTAGCAATGAGGTACAAGGCTCTC	25	

3.3.8.2 Ligation of PCR products and Transformation

PCR fragments were ligated into pGEM-T vector (Promega) overnight (4°C) using a standard manufacturer's protocol. In brief, a sample of PCR product (3 μl), pGEM-T vector (50 ng), T4 DNA ligase (3 Weiss Units) and 2 x Ligation Buffer (5 μl) (60 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% v/v Polyethylene glycol, pH7.8) was made up to 10 μl with molecular grade water and incubated overnight (4°C, overnight) for optimal transformation. The ligated vector was transformed into competent *E.Coli* (JM109, L2001) and the cells plated onto X-Gal (0.5 mM)/IPTG (80μg/ml) supplemented LB plates containing ampicillin (100 μg/ml) and cultured overnight (16-20 hours, 37°C). Transformed colonies were identified using blue/white colour screening. Only cells containing the pGEM-T vector were ampicillin resistant, whilst cells containing the vector with an insertion of the ligated PCR product were unable to metabolise the IPTG to its blue metabolite. White colonies were sub-cultured overnight and in LB-Ampicillin (100 μg/ml) media.

The plasmids were isolated from the cultured colonies using the DNA Wizard SV Mini-prep system (Promega). In brief, cells were pelleted from the culture media and suspended in the supplied Wizard Plus SV Cell Resuspension buffer (5 mM Tris-HCl, 10 mM EDTA, 100 μg/ml RNase A, pH 7.5). The cells were lysed (Wizard Plus SV Cell Lysis Solution- 0.2 M NaOH, 1% w/v SDS). The solution was neutralised upon addition of Wizard Plus SV Neutralisation Solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, final pH 4.2) and high molecular weight cellular components pelleted by centrifugation (14,000 x g, 10 minutes, room temperature). The supernatants were applied to vacuum filters and successively washed with Column Wash buffer (500 μl- 162.8 mM potassium acetate, 22.6 mM Tris HCl, 0.109 mM EDTA) and dried under vacuum (10 minutes). Residual wash buffer was removed by centrifugation (14000 x g, 2 minutes) prior to the DNA being eluted in molecular grade water (20-40 μl, 14000 x g, 1 minute) and stored at –20°C.

A sample of each mini-prep product was digested with DNA endonucleases, chosen to specifically release the amplified insertion within the pGEM-T vector. Sal I and Nco 1 were selected and used in association with appropriate Promega Buffer. In brief, digestion of each mini-prep sample (10 μl) was completed using the enzymes (2

Weiss units) in Buffer D. The mixture was incubated (37°C, 1.5 h) to allow full digestion of the plasmids. The linearised products were size-fractionated on an agarose gel (1.5% w/v, 85 volts, 45 minutes) and were viewed under UV. Samples with a band corresponding to the expected insert were sent for sequencing (Molecular Biology Sequencing Services, Cardiff University) to identify the correct insert. Mini prep samples containing the correct inserts were accurately quantified and diluted (100 pg/μl - 10 fg/μl) for quantitative PCR (qPCR) standard curves for each gene.

3.3.8.3 q-PCR

The qPCR was accomplished using the ABI Sequence Detection System (ABI PRISM 7700). Initial standard curves of each gene were size-fractionated prior to quantification using samples to confirm successful PCR amplification of the inserts using the SYBR green quantification. Standard curves of the plasmid DNA and inserts (100 pg/μl – 10 fg/μl) diluted in molecular grade water (n=3, total volume used 1 μl) were added to a 2x Quantitect Sybr green PCR buffer (25 μl) (HotStar Taq DNA polymerase in Qiagen Sybr green PCR buffer), forward and reverse primers (concentration used) and made up to 50 μl with water and were appropriately dispensed into qPCR 96 well plates and sealed prior to use.

A 40 cycle PCR was used on the ABI 7700: an initial plate warming (50°C, 2 minutes), DNA polymerase activation (15 minutes, 95°C) followed by 40 cycles of 95°C (30 seconds) and 68°C (1 minute).

The specificity of the PCR products were checked by agarose gel electrophoresis (1.5% w/v agarose gel, 80 volts, 45 minutes) to rule out primer dimer and multi product interference. Once successful PCR amplification had been achieved, fresh cDNA (1 μl) from each experimental and control samples were analysed for each gene alongside the appropriate standard curve as described previously.

Initial data analysis of the qPCR experiments was carried out using the ABI PRISM detection system. The ABI PRISM system records the change in fluorescence of the PCR mixture with increasing cycle numbers and produces a sigmoid-shaped amplification plot. A calculation of a cycle threshold (Ct, a constant fluorescence level) across the series of standard dilutions produces a linear standard curve and

regression equation to estimate the initial concentration of the gene in each experimental sample. An unpaired student t-Test (assuming unequal variance) was conducted to determine statistically significant differences in expression levels between experimental and sham-treated controls.

3.3 Results

3.4.1 Selection of PM-exposed and Sham-treated rats for Toxicogenomic Assessment

Three rats from each group (sham-treated control, 5 mg whole PM, 10 mg whole PM and 10 mg equivalent dose of water soluble component of PM) were selected on the basis of increases in lung to body weight ratio, increases in lavage protein and increases in LFC relative to saline treated (sham) control (Table 3.2).

Table 3.2: Summary table for selected (n=3) animals exposed to whole and water soluble PM and saline treated (controls). Data from conventional toxicological assessment (chapter 2) includes lavage protein and lung to body weight ratio (pulmonary oedema) and large free cell (LFC) within the lung (inflammation)

Experiment Number	PM Fraction	Dose	Lavage protein mg/animal	Ratio $x10^{-3}$	LFC millions
1	Whole	10 mg	4.30	5.1	18.3
4	Whole	10 mg	3.70	5.7	19.6
5	Whole	10 mg	4.10	5.6	13.0
12	Soluble	10 mg	3.46	4.7	9.5
14	Soluble	10 mg	3.03	4.6	8.4
15	Soluble	10 mg	5.16	5.6	5.1
16	Whole	5 mg	3.22	5.0	7.4
17	Whole	5 mg	2.43	5.2	12.3
18	Whole	5 mg	3.27	4.9	10.7
46	Saline	0	1.47	4.2	5.5
48	Saline	0	2.31	4.0	4.8
49	Saline	0	1.91	4.1	7.2

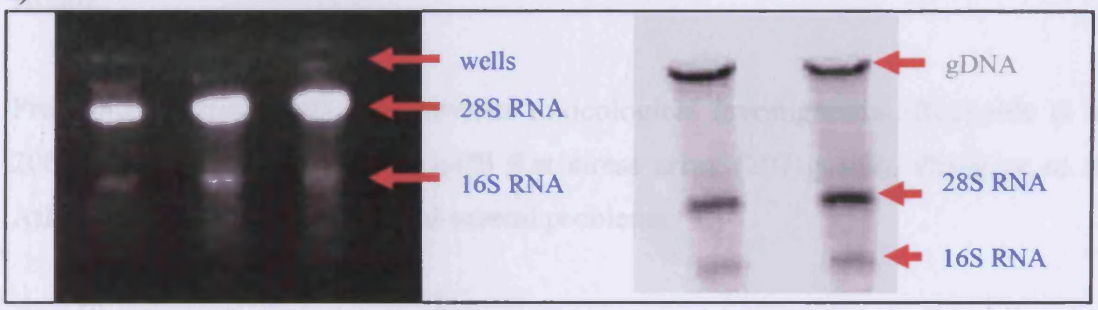
3.4.2 Isolation and Preparation of RNA

RNA isolation from lung tissue in sufficient amount and purity was obtained using the RNeasy ™ protocol (Qiagen). This method incorporated a simple and effective DNA nuclease (DNase) clean up step to be incorporated within the process, eliminating

DNA in the RNA sample with a reduced risk of DNase contamination. RT-PCR was completed on preliminary samples to ensure that the DNase was efficiently removed from the RNA preparations. Contaminating DNA would serve to distort the expression profile of the sample whilst failure to remove DNase from the preparation would prevent probe synthesis during the array procedure.

The RNA was visually analysed to help ascertain that no significant degradation had taken place using the Syngene™ gel doc. System (Figure 3.3a). DNase treatment of all samples was completed during RNA isolation and RT-PCR was completed to ensure genomic DNA and DNase free preparations (figure 3.3b).

a)



b)

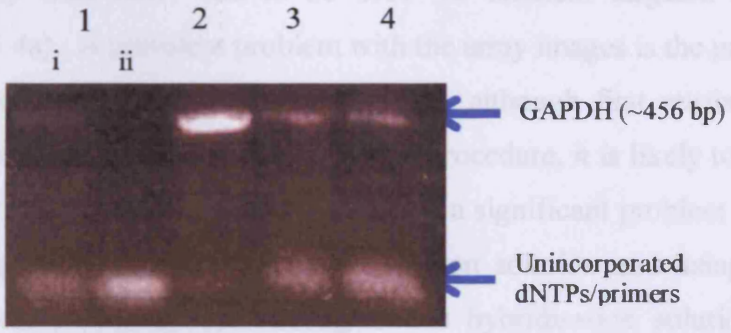


Figure 3.3: a) The sample of isolated RNA size-fractionated on a gel was viewed under UV to check the integrity and can be indicative of genomic DNA contamination (gDNA). The presence of a third band would be indicative of genomic contamination. Smeared bands would indicate RNA degradation. (b) RT PCR products were subjected to electrophoresis on 1.5 % w/v agarose gel visualised using Syngene. Well 1 (i & ii): No RT (DNase treated RNA). Well 2-4:DNase treated RNA (RT+) (n=3). Bands identified on basis of size compared to DNA ladder (not shown)

3.4.3 Generation of Macroarray Profiles

Macroarray profiles from selected animals were completed using the Atlas ™ Rat1.2 nylon macroarray (BD Biosciences). This array contains 1176 cDNA spots including 9 housekeeping genes and negative controls. The genes present on the array may be broadly functionally classified into the following groups: Cell surface antigens, transcription factors and DNA binding proteins, cell cycle regulators, immune system proteins, extra-cellular transporters, oncogenes, tumour suppressor stress response, ion channels and transport, ECM proteins, trafficking proteins, metabolic proteins, apoptosis related proteins, cellular receptors, DNA metabolism proteins and housekeeping genes (GAPDH, tubulin α1, ornithine decarboxylase, cytoplasmic β-Actin).

Preliminary experiments and previous toxicological investigations (Reynolds et al., 2001) had used the smaller Atlas™ Rat stress array (207 genes), changing to the Atlas™ Rat 1.2 array highlighted several problems.

Sufficient high quality total RNA had to be used for efficient targeted reverse transcription (Figure 3.4a). A prevalent problem with the array images is the presence of darkened edges along the membranes (figure 3.4b), although first attributed to inadvertent drying of the membranes during the whole procedure, it is likely to be the result of a blocking problem. Non-specific binding was a significant problem (figure 3.4c), this was minimised by replacing the hybridisation solution and using fresh Salmon sperm. Repeated heating and cooling of the hybridisation solution was detrimental to its effectiveness. However, darkened patches are still visible on the array membrane, although much of this effect on calculating adjusted gene expressions can be eliminated using the Atlas Image 2.01a visualisation package (BD Biosciences) by selecting individual areas adjacent to sets of gene to calculate the background noise. Successful array images have been achieved using DNase treated RNA samples (figure 3.4d)

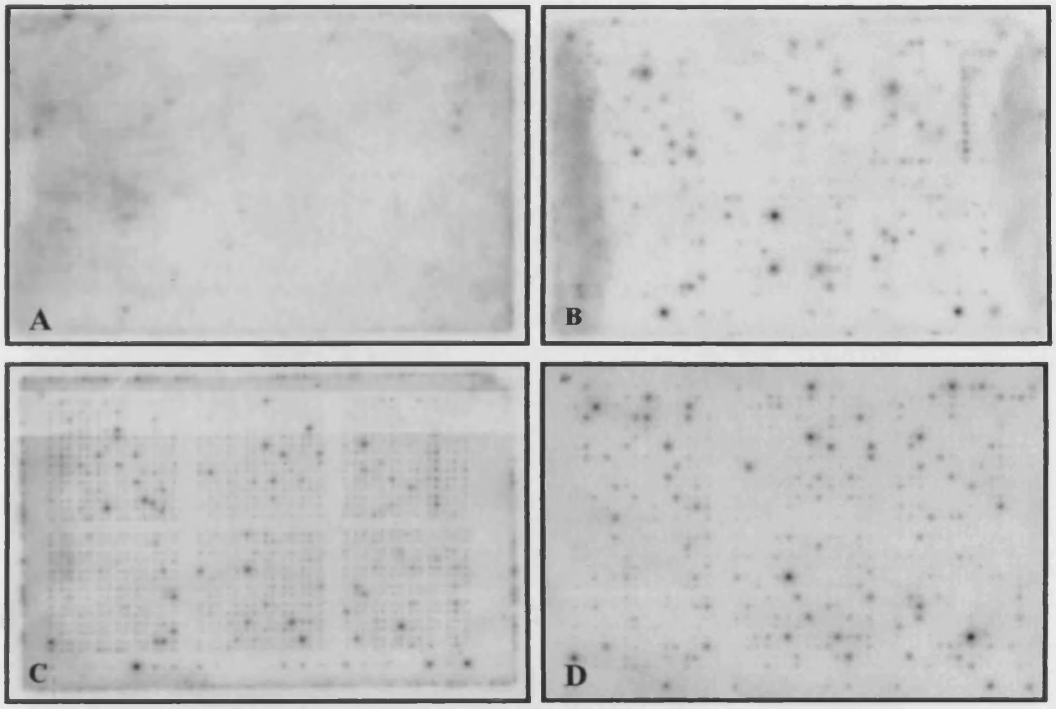


Figure 3.4: Spectrum of images found with the Rat 1.2 macroarray: (A) some spots are present, loss of RNA integrity/failure of RT. (B) Darkened edges, drying of membrane or inadequate blocking. (C) Uniform spots apparent, non-specific binding. (D) A successful array with low background and clear defined gene spots.

The macroarray images from sham-treated, whole PM (5 mg and 10 mg) and water soluble (10 mg equivalent dose) lung tissues are shown in figure 3.5a-d. There was some variability in gene expression across the replicates, a reflection of the differing susceptibility of each animal to the instillate.

3.4.4 Data Transformation and Normalisation

Random genes were selected to test for normality using the Anderson Darling test for normality within the statistical package, Minitab™. Resultant plots are shown for one gene at each stage of transformation and normalisation. The raw expression data of random genes failed to be consistently normally distributed. Log_{10} transformation is commonly adopted to ensure that all data is approximately normal, thus allowing normal statistical assumptions to be made. Transformation and subsequent normalisation resulted in the gene array data to become largely normally distributed (Figure 3.6).

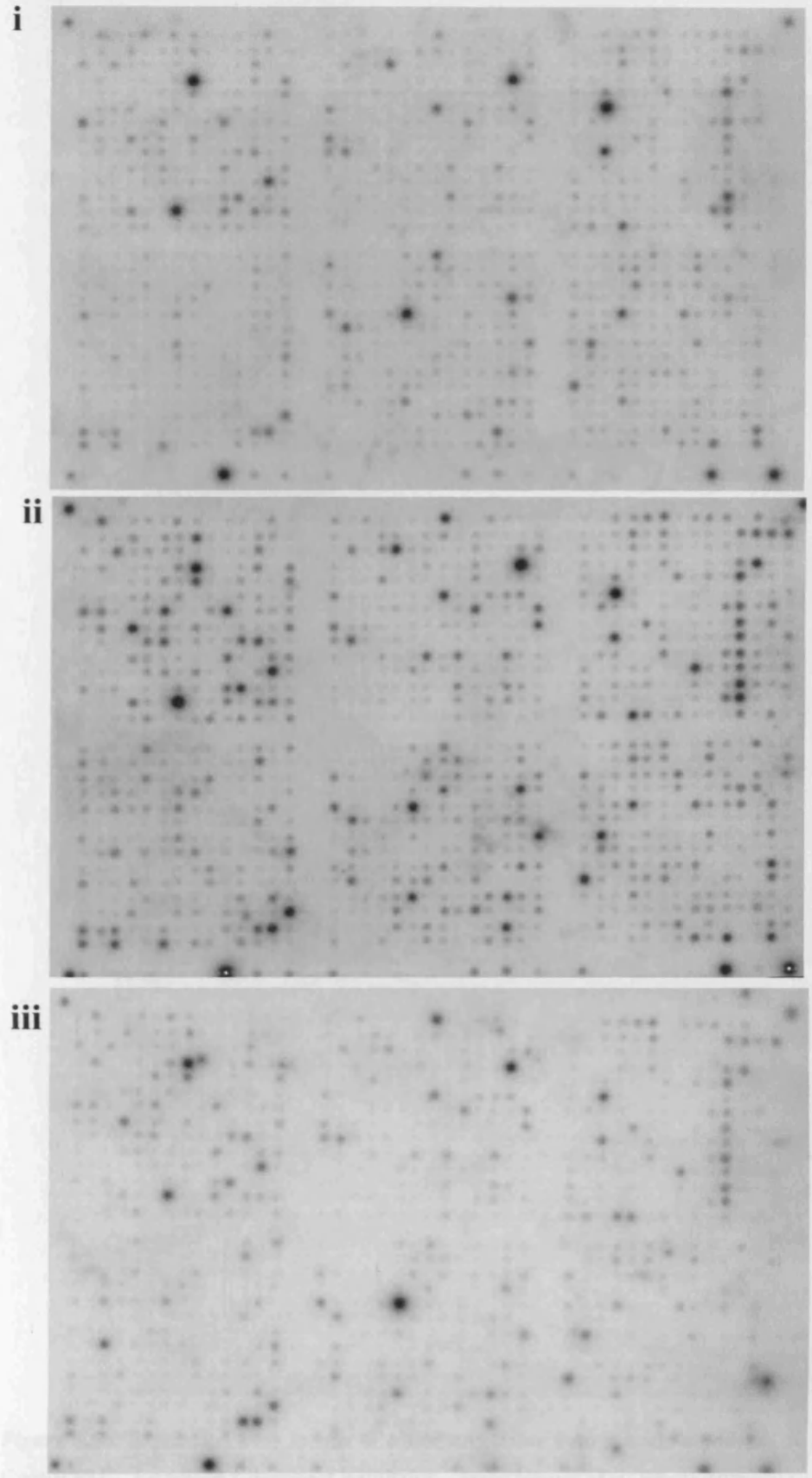


Figure 3.5a: Expression array images of whole lung tissue from animals exposed to saline only (sham-treated). Array images of (i) Control 46, (ii) control 48 and (iii) control 49.

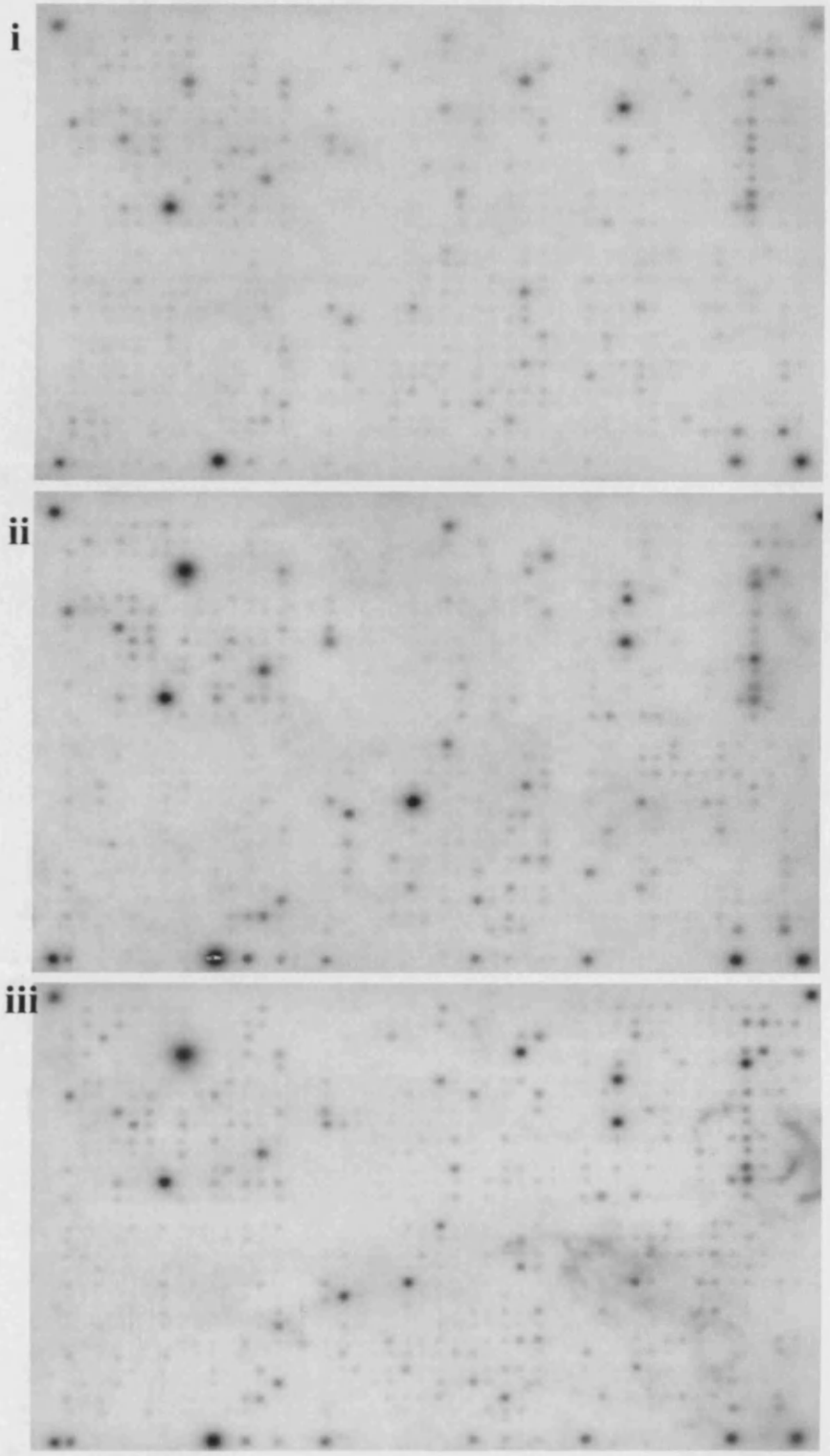


Figure 3.5b: Expression array images of whole lung tissue from animals exposed to 10 mg whole Cardiff PM_{10}, sacrificed 3 days post instillation. Array images of (i) Whole 10 mg (1), (ii) Whole 10 mg (4) and (iii) Whole 10 mg (5).

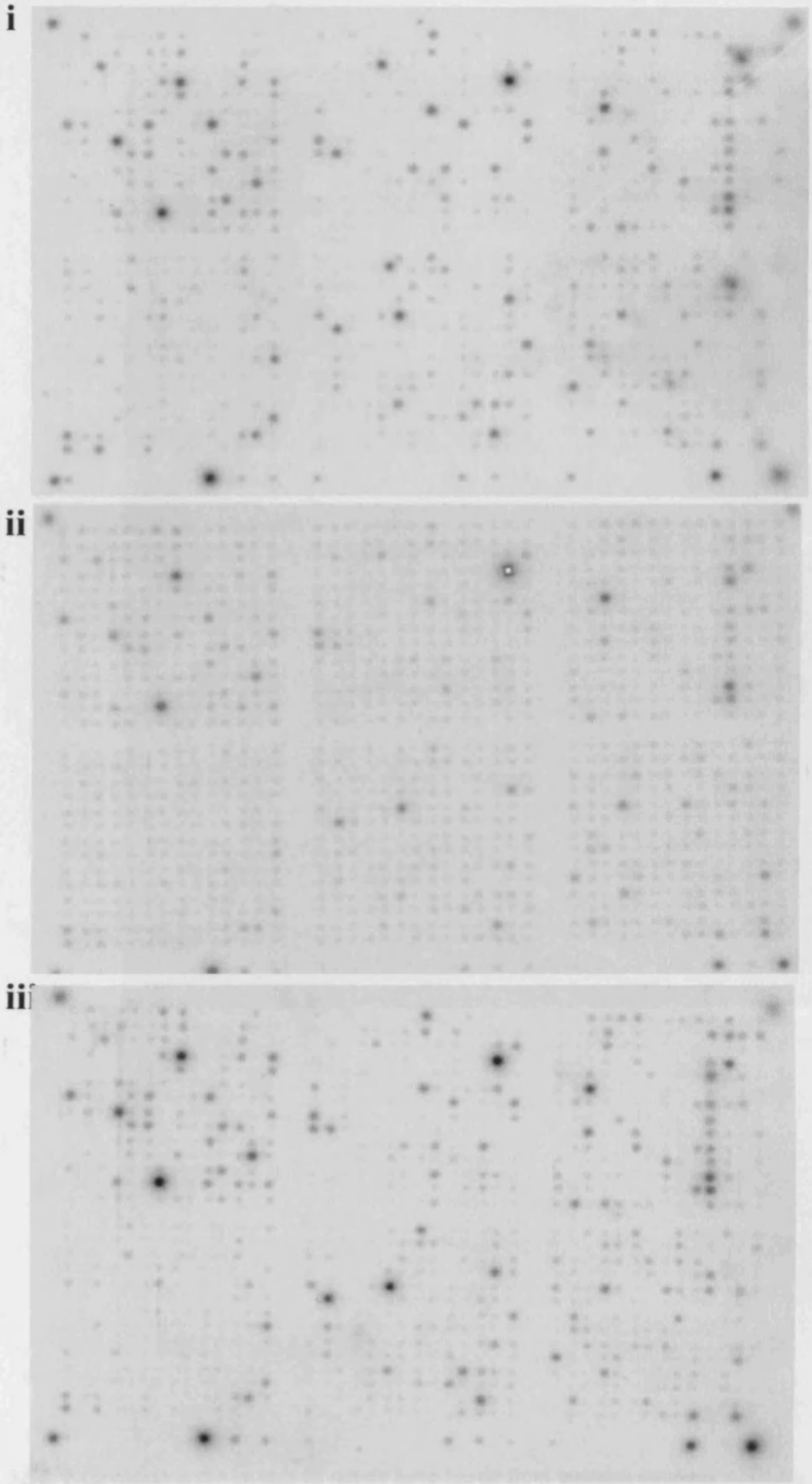


Figure 3.5c: Expression array images of whole lung tissue from animals exposed to 5 mg whole Cardiff PM_{10}, sacrificed 3 days post instillation. Array images of (i) Whole 5 mg (16), (ii) Whole 5 mg (17) and (iii) Whole 5 mg (18).

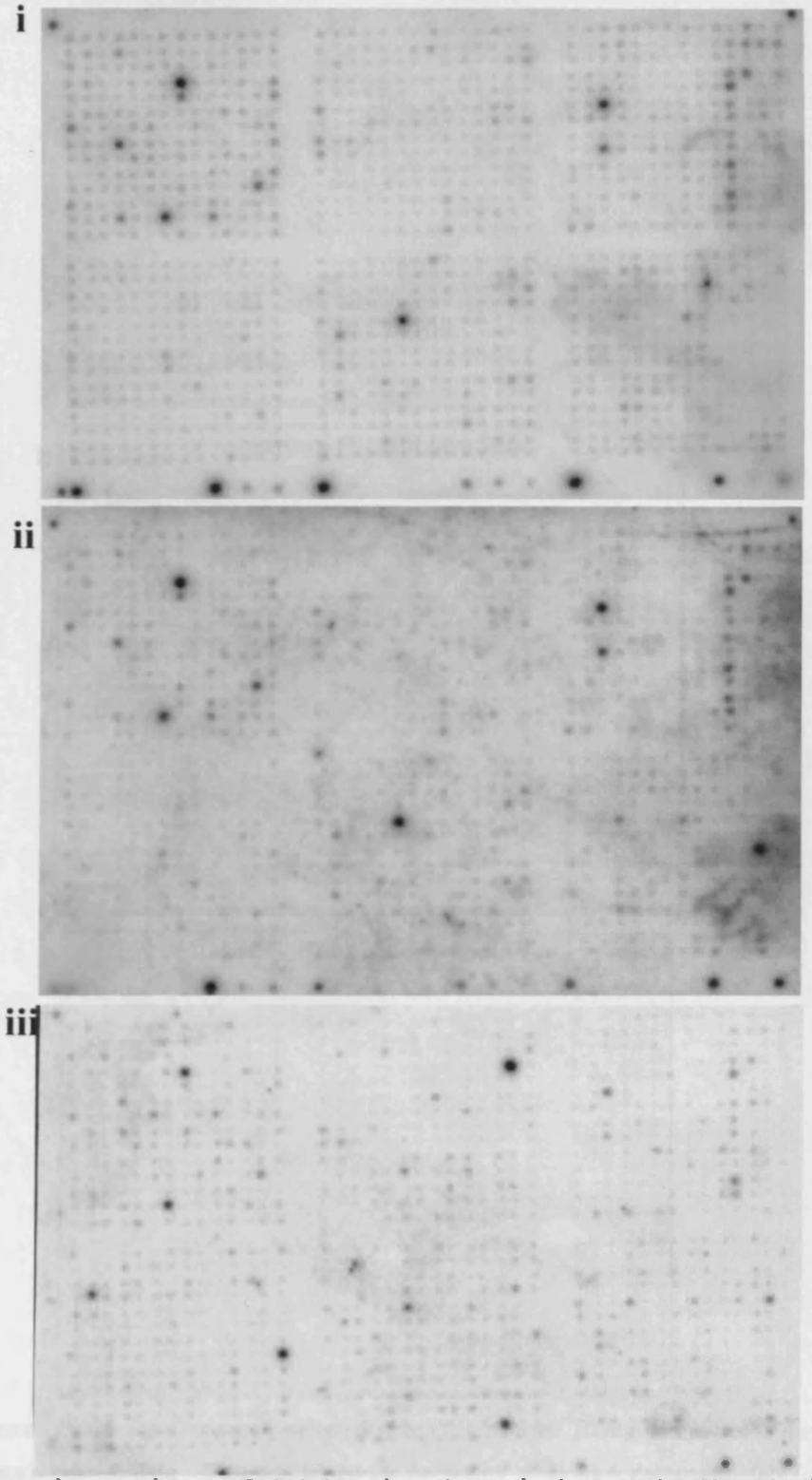


Figure 3.5d: Expression array images of whole lung tissue from animals exposed to 10 mg (equivalent dose) water soluble Cardiff PM_{10}, sacrificed 3 days post instillation. Array images of (i) Soluble 10 mg (12), (ii) Soluble 10 mg (14) and (iii) Soluble 10 mg (15).

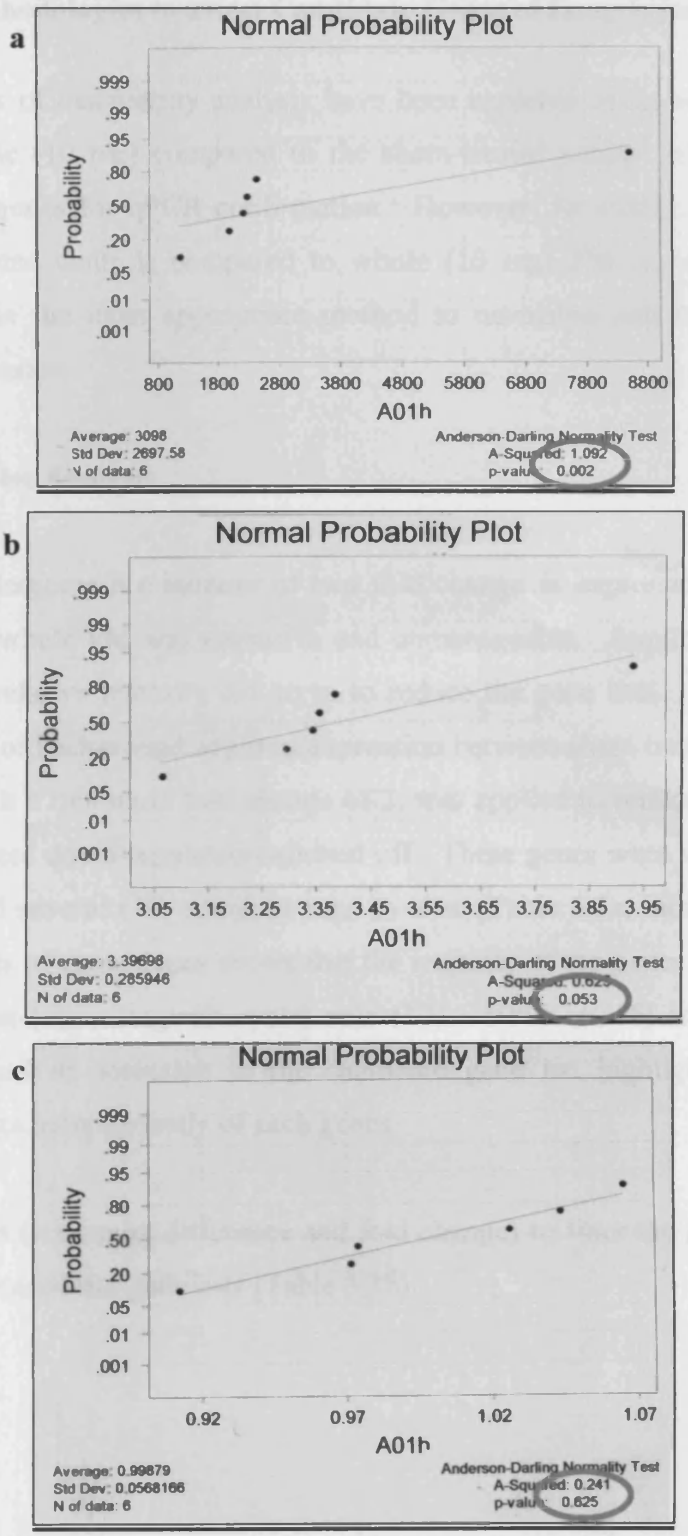


Figure 3.6: Normality plots for a randomly selected gene (A01h) from 10 mg whole treated (n=3) and sham-treated controls (n=3) data. Data was tested for normality as a) Raw expression data (adjusted intensities) ($p<0.05$), b) $\log_{10}$ transformed data ($p=0.05$) and finally c) Two step normalised data (final data) ($P>0.05$).

3.4.5 Different Methodologies to assess Candidate Genes of Lung Injury

The different methods of macroarray analysis have been explored using each whole (10 mg, 5 mg) and water soluble (10 mg) compared to the sham-treated control group (n=3) to enable selection of suitable genes for qPCR confirmation. However, for clarity, only those analyses concerning sham-treated controls compared to whole (10 mg) $PM_{10-0.1}$ exposed samples are described, to ascertain the most appropriate method to normalise and assess the significant changes in gene expression.

3.4.5.1. Pair wise Analysis

The list of genes undergoing a minimum of two fold change in expression profiles following instillation of 10 mg whole PM was extensive and unmanageable. Application of an arbitrary minimum change in relative intensity did serve to reduce the gene lists. Implementation of a minimum difference (of background adjusted expression between sham-treated and PM exposed samples) of 4000, with a minimum fold change of 2, was applied to reduce the gene lists to 25 genes, all of which were down-regulated/switched off. These genes were visually examined on the array profiles, and several (16) could be seen to alter (Table 3.3a, indicated by bold type). Basic function analysis of these genes shows that the majority of the genes have a receptor (15) or cell communication (signalling/cell cycle) role (10). HPRT (G15) is considered to be a housekeeping gene and its inclusion in the candidate gene list highlights the necessity to normalise the array data independently of such genes.

Increasing stringencies (minimum difference and fold change) to filter the gene analyses served to suitably reduce the candidate gene lists (Table 3.3b).

Table 3.3: a) Candidate gene lists and functional analysis of genes shortlisted using pair wise comparison of 10 mg whole PM exposures compared to an average calculated sham-treated profile (n=3).

Whole 10 mg PM exposed rat lung expression profile				Gene	Classification(s)	
Gene code	Difference in gene expression					
	Test 1	Test 4	Test 5			
D02h	-6593	-5509	-4421	atrial natriuretic peptide clearance receptor precursor	Hormone Receptors G Protein-Coupled Receptors Nuclear Receptors	
D03n	-17076	-13604	-13952	glucagon receptor precursor		
D04j	-10769	-7509	-6297	calcium-independent alpha-latrotoxin receptor		
D06m	-6894	-5666	-6894	galanin receptor 2		
D06n	-20462	-15534	-18170	secretin receptor		
D07l	-6631	-4643	-6631	melanocortin receptor 4		
D08h	-10143	-8483	-8947	vitamin D3 receptor		
D08n	-9306	-5610	-9162	glutamate receptor subunit zeta 1 precursor	Neurotransmitter Receptors Ligand-Gated Ion Channels	
D09i	-6996	-6508	-4600	glutamate receptor 4 precursor		
D09j	-6806	-6134	-6806	gastric inhibitory polypeptide receptor precursor		
D10l	-7879	-4707	-7879	glutamate receptor, ionotropic		
D10m	-6103	-4731	-6103	acetylcholine receptor alpha		
D12m	-27873	-15313	-21701	metabotropic glutamate receptor 7 precursor	Other Receptors (by Ligands)	G Protein-Coupled Receptors
D13h	-8660	-5620	-5476	low-density lipoprotein receptor precursor		Endocytosis Proteins
D13k	-12311	-7375	-5619	nociceptin precursor; orphanin FQ	Neuropeptides	
D13l	-7948	-4408	-7112	neuropeptide Y precursor		
D14i	-8751	-7587	-5703	granulocyte colony stimulating factor	Growth Factors, Cytokines and Chemokines	
E01i	-4236	-4236	-4236	fibroblast growth factor 5		
E04n	-5191	-4191	-5191	interleukin 1, beta	Interleukins and Interferons	
E05j	-6279	-4411	-5207	insulin-like growth factor binding protein 3 precursor	Extracellular Transporters and Carrier Proteins	
E05l	-9514	-7238	-9126	insulin-like growth factor-binding protein	Other Extracellular Communication Proteins	
E07n	-6528	-5268	-5840	c-Jun N-terminal kinase 3 (stress-activated)	Intracellular Kinase Network Members and other Stress Response Proteins	
F13h	-7882	-7030	-6070	Ear-3; V-erbA related protein	Oncogenes and Tumor Suppressors and Nuclear Receptors	
F13l	-9054	-8362	-4754	growth factor	Other Cytoskeleton and Motility Proteins	
G15	-10480	4120	-4496	hypoxanthine-guanine phosphoribosyltransferase	Housekeeping Gene and Xenobiotic Metabolism	

Table 3.3 b) Summary of candidate gene lists undergoing increased stringency of filtering to achieve lists of manageable length for continued study. Fold change is the extent of change in relative gene expression between sham-treated control and PM exposed expression profiles. "Difference" in gene expression between the sham-treated and PM exposed

Stringency and parameters of screening for candidate genes		**Number of genes undergoing changes relative to sham-treated control**
Fold change	**Minimum difference**	
2 fold	None applied	329
3 fold		329
4 fold		329
5 fold		25
2 fold	1000 difference	156
	2000 difference	86
	3000 difference	44
	4000 difference	25

Applying the same pair wise comparisons to water soluble PM exposed profiles, a mean calculated control array normalised against each experimental profile in turn, 3 genes were altered by greater than 2 fold in each comparison. These genes were: a ligand-gated ion channel (B02k), a voltage gated ion channel (B04m) and a hormone/nuclear receptor (D08j), the direction of the expression change was inconsistent across the three exposed samples (data not shown).

A list comprising of 22 genes were identified as being undergoing a greater than two fold change in expression (in at least two of the three experimental arrays, minimum of 3,000 change in intensity) following exposure to the water soluble fraction of Cardiff PM. Only one gene increased (10 fold, B02k), the remaining genes were down-regulated or switched off. Basic functional classification of the genes showed that large numbers of the genes were associated with ion and water transporters (10), intracellular kinases and phosphatases (9) and receptors (6), these genes may be strongly correlated with the cellular changes at the lung surface.

3.4.5.2 Expression Range Analysis

The next approach calculated an acceptable range in expression in both sham-treated and PM exposed groups - a result of natural animal variation and differing individual susceptibility towards the instillate. Candidate genes were deemed as genes with non-overlapping range in expression between the sham-treated and PM exposed groups, the lists for all exposure groups were extremely large and an additional parameter examining the fold change in gene expression between mean sham-treated control and mean test (n=3) was incorporated.

A list of 162 genes were identified as potential candidate genes (greater than 2 fold change) following instillation of 10 mg whole PM_{10} sample, compared to 44 genes following the equivalent dose of the water soluble fraction. These 44 genes from the water soluble candidate gene list were all present in the whole PM exposed lists. These 44 genes comprise of surface antigens (3), cell cycle controllers including oncogenes and tumour suppressors (2), cell (energy, lipid, carbohydrate) metabolism (8), ion channels and transporters (5), trafficking and targeting (1), growth factors and cytokines (4), hormone and neurotransmitter receptors (12), signalling/communicators (7) and proteases (2) (data not shown). The genes all follow the same trends with exception of B03k, the ligand gated ion channel/hormone receptor (acetylcholine receptor subunit), which undergoes a 2.6 fold decrease in response to water soluble and 2.8 fold increase following whole PM exposure.

The numbers of genes generated using a 2 fold change in gene expression to select significant genes as the cut off were large, increasing this 2 fold change reduced lists to a more manageable lengths. The effect of adding these parameters to the filtering of candidate genes on gene list numbers is illustrated in Table 3.4.

Implementing a minimum of 4 fold changes reduced the lists substantially, 30 genes (10 mg whole PM) and 3 genes (10 mg water soluble). The three genes common to both lists are B09c- symporter/antiporter (17 fold increase in whole treated, 36 fold increase in water soluble), B14b– trafficking and targeting proteins (5.5 fold increase following both exposures) and E11k- adaptor protein tyrosine phosphatase (4.2 fold and 4.8 fold decreases respectively).

Table 3.4 Summary of candidate gene list numbers with increasing stringency (implementing a higher minimum fold change required to select the genes) following the instillation of whole Cardiff PM (10 mg). * Number of genes undergoing changes relative to sham-treated control

Fold change	Number of genes*	Up regulated genes	Down regulated genes
2 fold	162	97	65
3 fold	54	34	20
4 fold	30	27	13
5 fold	18	12	6

3.4.5.3 Statistical Analysis

Macroarray profiles of tissue from sham-treated (saline instilled), whole PM and water soluble PM exposed rats (n=3) showed a high degree of variation in gene expression within each treatment group, a reflection of the different animals susceptibility to the instillate. Calculating an acceptable range in relative expression in sham-treated controls would allow for this natural variance and facilitate the identification of genes expressed outside these ranges as a consequence of toxicant exposure. A mean (n=4) and 2 standard deviation range of relative expression of each gene was calculated from the sham-treated controls and compared to the PM exposed expression profiles.

Analysis of the whole PM (10 mg) profiles showed that 94 genes were designated as expressed outside a 2 SD range of the mean control array with a fold change of at least 2 fold (induced or down-regulated).

Using the students t-test and a minimum of two fold change in gene expression of mean control (sham-treated) and test (10 mg whole PM) array profiles, seven genes were deemed significantly altered ($p<0.05$) (9 genes $p<0.1$). All of these genes were expressed outside a 2 standard deviation range ("normal" variation) in control profiles (Table 3.5a). The conventional toxicology reported mild pulmonary oedema and increase macrophage accumulation within the focal regions of the lung. Interestingly,

examining the known functions of the candidate genes, they include a range of cytokines, growth factors, hormones and interleukins (6) and receptor proteins (3). Table 3.5b illustrates the effect of increasing the stringency of filtering the gene lists on candidate genes.

Table 3.5: a) Candidate genes proposed to be involved in the pathogenicity of whole PM_{10} (10 mg) instilled into healthy rats. Tabulated data showing resultant relative expressions of both sham-treated controls (n=3) and whole PM exposed (n=3), fold changes, *p*-value from student t-test of the data and the candidate gene names/gene functions. Purple text indicates genes expressed outside a sham-treated control mean± 2 SD (n=3) in PM exposed (10 mg whole) expression profiles.
b) Summary table of number of genes in candidate lists with increasing stringency of both statistical methods applied to expression profiles (sham-treated controls and whole PM exposed (10 mg).

a)

Atlas Gene code	Fold change	P value t-test	Gene name	Functional Classification(s)
D04a	Off	<0.01	calcitonin receptor precursor	Hormone Receptors G Protein-Coupled Receptors
D06a	-4	0.075	serotonin receptor	
D06k	Off	<0.01	cannabinoid receptor 1	
D14e	-13	0.026	glial cell line-derived neurotrophic factor precursor	Growth Factors, Cytokines and Chemokines
E01h	Off	0.001	fibroblast growth factor 9	
E01i	Off	0.000	fibroblast growth factor 5	
E03b	2.5	0.079	thyroliberin precursor	Hormones
E05a	4.8	0.025	interleukin-2 (IL-2)	Interleukins and Interferons
E10m	Off	<0.01	PAK-alpha serine/threonine kinase	Intracellular Kinase Network Members

b)

Gene selection	Stringency	Number of genes
Sham-treated mean ± 2 SD	2 fold change	94
	3 fold change	39
	4 fold change	8
Student t-test	P<0.1	9
	P<0.05	7
Combination	2 fold/p<0.1	4

3. 4.5.4. Log_{10}(fold change) Analysis

The Log_{10} fold change analysis method failed to produce manageable candidate gene lists. There was also less flexibility in the stringency level to assess the gene lists and most reported genes were either completely switched on or off. The stringency of the analysis could be increased by two parameters: increasing the fold change and increasing the acceptable range in fold change (ie. 2SD is less stringent than a 3 SD). The effect of altering these parameters is shown in Table 3.6. A total of 177 genes had a log_{10} (fold change) outside a 2SD range of the mean fold change that would seriously alter the mean value), produced significant candidate gene lists and a minimum fold change of 2. Of these, 150 genes were down regulated/ switched off and 27 were up regulated/switched on following treatment.

Table 3.6: Summary of candidate gene lists obtained using increasing stringencies of log_{10} (fold change) analysis method in lung tissue exposed to whole Cardiff (CF 2) PM (10 mg). * Number of genes undergoing change relative to sham-treated controls

Fold change SD range	Fold change	Number of genes*	Expression change
2 Standard Deviation	No limit applied	177	23 switched on 137 switched off 4 up regulated 13 down regulated
	2 fold	177	
	20 fold	173	
3 Standard Deviation	No limit applied	176	15 up/switched on 161 down regulated/off

Examining the median profiles of control (sham-treated) and water soluble genes by the same parameters (2 SD, minimum two fold change) resulted in list of 100 genes. However these lists showed a reverse trend, 98 genes being up regulated or switched on compared to only 2 down regulated or switched off. Of these genes, 43 genes were altered by a minimum of four fold (2 down regulated or switched off).

Comparing the whole and water soluble effects judged by this method of analysis, 45 genes were common to both candidate gene lists (2 SD range, minimum of a 2 fold change). These 45 genes comprised trafficking and targeting proteins (9), cell surface antigens (1), metabolism related (10), ion channels, transporters and diffusers (16), growth factors/transcription factors including chemokines and cytokines (4), extracellular matrix proteins (1) and hormones (4) in addition to one housekeeping gene, polyubiquitin, a molecular tag to identify damaged molecules and label them for destruction. Eleven of these genes do not present the same increase/decrease in soluble treated or whole PM exposed profiles.

3.4.6 Quantitative PCR Confirmation

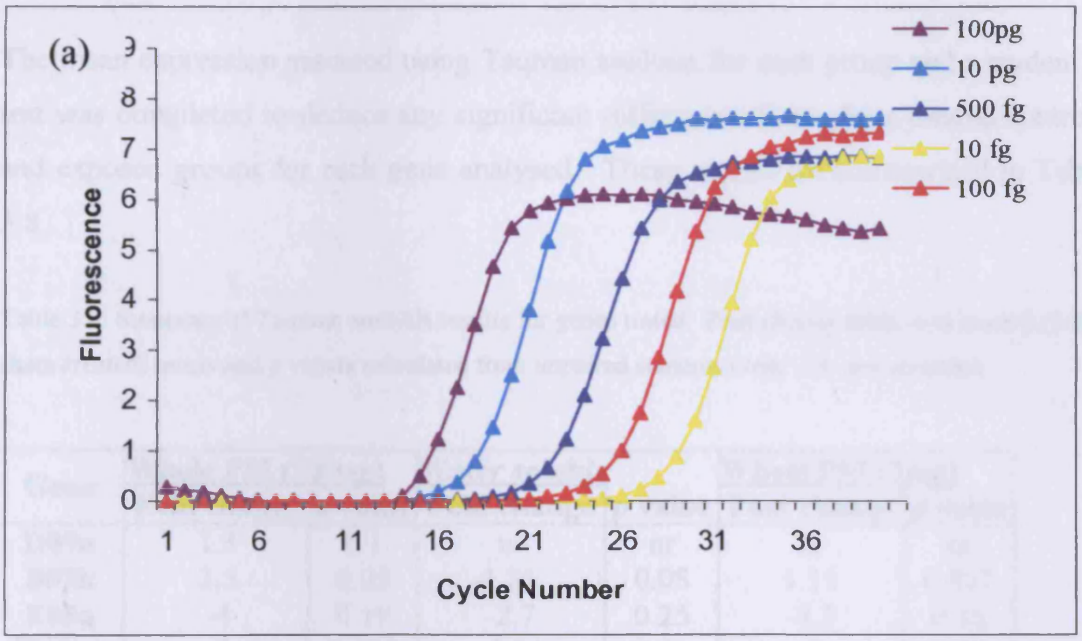
Ten genes were selected from the different candidate gene lists. No genes were common to all lists from the different methods of analysis and so it was of considerable importance to establish the best way to analyse the data that could be confirmed using other methods. Ideally, genes that showed significant up regulation and down regulation, in addition to those genes that displayed no significant change were selected. Details of each selected gene and the analytical method that shortlisted them are summarised in Table 3.7. An additional gene, the housekeeping gene, β-actin (G43), was also assessed.

Two genes failed to be successfully analysed using qPCR. One gene (C12i) failed to be amplified during PCR and a second gene (B13g) failed to be inserted or transformed successfully into competent cells using either pGEM-T or $PGLA_2$ basic (blunt ended) vectors into competent *E.Coli* (JM109) cells. The remaining eight genes were successfully analysed.

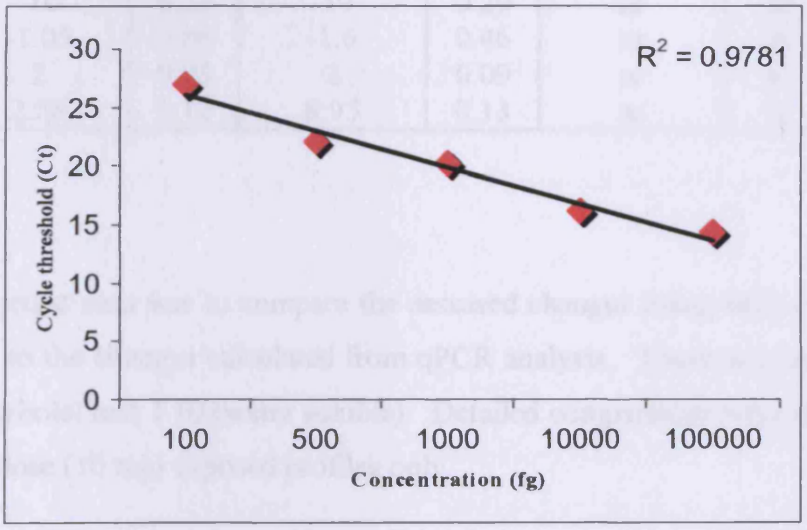
Each PCR product from the gene primers were ligated into pGEM-T and transformed into competent *E.Coli* (JM109) cells. Incorporated DNA inserts corresponding to the expected lengths were sequenced to confirm the correct identity. Quantitative PCR standard curves were calculated for each PCR product (Figure 3.7a-c).

Table 3.7: Summary of the selected genes and predicted behaviour based on the different analysis method adopted.

Atlas™ Gene Code	Gene Name	Fold change	Experiment	Analysis method
B04m	Voltage gated Potassium channel	Down regulated (>-3 fold change)	Water soluble PM (10 mg)	Pair wise comparison
C02b	Carbohydrate metabolism protein	Up regulated (switched on in all 3 arrays)		
D09n	GABA receptor	Switched off (whole treated) Down regulated (water soluble)	Whole PM (10 mg) Water soluble	Pair wise comparison (mean and median control v individual exposed
D12m	Glutamate receptor	Down regulated	Whole PM exposed analyses	Pair wise comparison
E04n	Interleukin 1β	Switched off		
B13g	Amphiphysin II	3 fold decrease		Statistical method Expression range analysis
B02k	Acetylcholine receptor	11 fold increase (P=0.02)	Water soluble exposed	
C12i	Clusterin	4 fold increase (P=0.07)		
E05a	Interleukin 2	5 fold increase (P=0.012)	Whole PM exposed	
E10m	Serine/threonine kinase	Switched off (P<0.01)		Log_{10} fold change analysis



(b)



(c)

Sample	Cycle threshold	Estimated Concentration
Sham-treated Control	19	1160 fg
Whole PM (10 mg)	25	11.5 fg
Whole PM (5 mg)	22	380 fg
Water Soluble	20	159 fg

Figure 3.7: An example of Taqman analysis data. Data shown are expressed as mean value (n=3) for a single rat tissue sample. Each tissue sample was analysed using qPCR in triplicate, tissue samples from each animal analysed in the macroarray experiment were also analysed using qPCR (n=3). a) Sigmoid shaped standard curve of fluorescence of Taqman PCR analysis for a series of standard dilutions for gene E05a. b) Standard curve and regression analysis r^2 value for E05a. c) An example of the Ct values calculated for experimental samples.

The mean expression assessed using Taqman analysis for each group and a student t-test was completed to deduce any significant differences from sham-treated controls and exposed groups for each gene analysed. These results are summarised in Table 3.8.

Table 3.8: Summary of Taqman analysis results for genes tested. Fold change relative to control (3 day sham-treated) levels and *p* values calculated from unpaired students t-test. Nr- not recorded

Gene	Whole PM (10 mg)		Water soluble		Whole PM (5mg)	
	Fold change	*p* value	Fold change	*p* value	Fold change	*p* value
D09n	1.3	0.1	nr	nr	nr	nr
B02k	2.5	0.05	1.81	0.08	1.11	0.827
E05a	-4	0.19	-2.7	0.25	-8.3	0.16
E10m	-2	0.135	-1.01	0.91	-2	0.15
C02b	-10	0.28	-10	0.29	nr	nr
B04m	-1.05	0.86	-1.6	0.46	nr	nr
E04n	2	0.08	2	0.09	nr	nr
G43	2.58	0.12	8.95	0.13	nr	nr

The next important step was to compare the assessed changes using each method of array analysis to the changes calculated from qPCR analysis. These are summarised in Table 3.9 (whole) and 3.10 (water soluble). Detailed comparisons were completed on the higher dose (10 mg) exposed profiles only.

The more consistently matched method of analysis appears to be the statistical approach. This method would be improved with increasing the replicate numbers of the arrays, either from using increased animal numbers, or using multiple arrays from the same animal tissue sample and calculating mean animal expression levels and then comparing mean values as described. Increasing replicate numbers increases the strength of statistical assumptions conferred by the calculations.

It can therefore be concluded that a combination of calculating a range (2 SD) of normal variation in expression and simple statistical analysis was most appropriate to identify changes in gene expression induced by PM_{10}. This analytical approach allowed a higher degree of control of stringency to filter insignificant changes in the

genes. This may be important when examining pulmonary toxicity that is highly variable not only between animals but within the tissue. Histological examination of the lung tissue (Chapter 2) confirms that only focal regions of issue are affected even at the higher doses of whole PM exposure. This poses a significant problem, selection of the affected tissue for expression profiling and confirmation with qPCR using the same RNA would be the best way to ensure that important candidate genes are consistently involved in the lung pathology. However, different preparations of RNA from each sample had to be used as degradation of the original samples examined by the arrays prevented subsequent qPCR analysis on the same samples.

3.4.7 Generating Final Candidate Gene Lists

Quantitative PCR has identified that the statistical approach was more successful at producing candidate genes that may be confirmed using alternative methods. Therefore the following section comprises, in full, the candidate genes produced as a result of this approach.

3.4.7.1 10 mg Whole PM-exposure

As described previously, using a combination of the students t-test and a minimum of two fold change in gene expression produces a candidate gene list of seven genes ($p<0.05$) or 9 genes ($p<0.1$). All of these genes were expressed outside a 2 standard deviation range ("normal" variation) in sham-treated expression profiles (Figure 3.8).

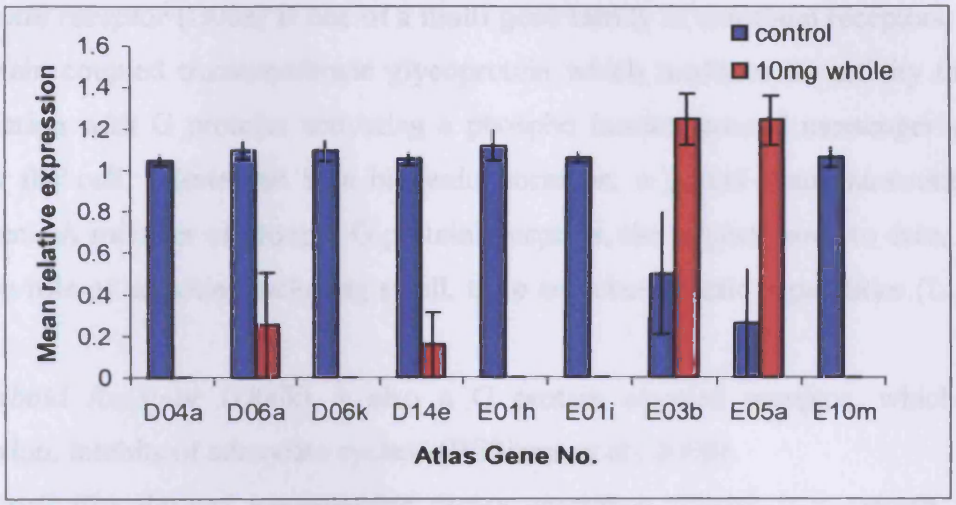
Table 3.9: A summary comparing the changes of gene expression judged by the various array analysis method and Taqman confirmation in response to **whole PM** (10 mg) exposure. Expected trends and p values (italics) are included where appropriate in addition to stringency of array analysis.

Gene	Taqman Analysis	Array Analysis				
		Pair wise	**Statistics**	**Expression range**	**Log_{10} fold change**	**Conclusions**
B02k	+2.5 *(0.05)*	Min +8 in 2/3	+10 fold *(p=0.2)*	Not present	Switched on (3SD)	Statistical trend
B04m	-1.05 *(0.88)*	Down regulate (>3,000 difference)	-3 fold *(p=0.19)*	-3.41 fold	Switched off (3SD)	Trend matched
C02b	-10 *(0.28)*	Up regulated	+2.4 fold *(p=0.1)*	+21 fold	Switched on (3SD)	No correlation
D09n	+1.3 *(0.1)*	Switched off (all 3)	-2.0 fold *(p=0.269)*	Not present	Down regulated (-50) 3SD	No correlation
E04n	+2 *(0.08)*	Switched off (>3,000)	-3 fold *(p=0.16)*	-2.05 fold	Switched off (3SD)	No correlation
E05a	-4 *(0.19)*	Little change (+1.1 fold)	+4.5 *(p=0.025)*	Not present	Not present	No correlation
E10m	-2 *(0.135)*	Switched off (>1000 difference)	Switched off *(p<0.01)*	-5 fold	Switched off (3SD	Trend matched
G43	+2.28 *(0.12)*	+1.1-1.3 fold changes	+1.1 fold *(p=0.04)*	+2 fold	Not present	Trend matched

Table 3.10: A summary comparing the changes of gene expression judged by the various array analysis method and Taqman confirmation in response to **water soluble** PM (10 mg) exposure. Expected trends and p values (italics) are included where appropriate in addition to stringency of array analysis.

Gene	Taqman Analysis	Array analysis				Conclusion
		Pair wise	Statistical	Expression range	Log_{10} fold change	
B02k	+1.8 *(p=0.08)*	+10 in 2/3 min difference 750	Outside 2SD range +17.6 *(p<0.01)*	Not present	Switched on (2SD)	Trend matched
B04m	+1.6 *(p=0.48)*	Switched off In 3/3 arrays	Not outside 2 SD range -2.5 *(p=0.38)*	Not present	Not present	No correlation
C02b	-10.0 *(p=0.29)*	+1.24 change in all 3 arrays min. diff 400	Not outside 2 SD range +2.7 (p<0.1)	Not present	Switched on (2 SD)	No correlation
D09n	nr	Min –2 in 2/3 arrays	Outside 2SD range -1.5 *(p=0.2)*	Not present	Not present	nr
E04n	-2.0 *(p=0.09)*	Switched off (2/3 arrays)- Min 500 difference	Not outside 2 SD range +1.0 *(p=0.78)*	-2.6	Not present	Pair wise Expression range
E05a	+2.7 *(p=0.25)*	+1.2 to 1.5 in 2/3 arrays min difference 400	Not outside 2 SD range +2.6 *(p=0.067)*	Not present	Not present	Statistical
E10m	+1.1 *(p=1)*	No change-+1-1.3 fold change	Not outside 2 SD range +1.1 *(p=0.13)*	Not present	Not present	Statistical
G43	+8.9*(p=0.13)*	+1.00 (no change)	+1.0 (no change) *(p≈1)*	Not present	Not present	No correlation

a)



b)

Atlas Gene code	Mean control	Control SD	Mean test	Fold Change	t-test P value	Gene	Functional Classification
D04a	1.045	0.046	0	Off	2.47E-05	Calcitonin receptor precursor	Hormone Receptors G Protein-Coupled Receptors
D06a	1.097	0.086	0.252	-4	0.0749	Serotonin receptor	
D06k	1.093	0.093	0	Off	0.0001	Cannabinoid receptor 1, neuronal	
D14e	1.053	0.053	0.155	-7.5	0.0260	Glial cell line-derived neurotrophic factor precursor	Growth Factors, Cytokines and Chemokines
E01h	1.118	0.145	0	Off	0.0006	Fibroblast growth factor 9 (FGF9);	
E01i	1.061	0.045	0	Off	2.23E-05	Fibroblast growth factor 5 (FGF5);	
E03b	0.500	0.584	1.246	2.5	0.0789	Thyrotropin-releasing hormone precursor	Hormones
E05a	0.259	0.519	1.241	4.8	0.0251	Interleukin -2 (IL-2)	Interleukins and Interferons
E10m	1.064	0.090	0	Off	0.0001	PAK-alpha serine/threonine kinase	Intracellular Kinase Network Members

Figure 3.8: Candidate genes proposed to be involved in the pathogenicity of whole PM_{10} (10 mg) instilled into healthy rats. a) Graphical representation of the genes. Values are mean control (n=4) and exposed (n=3) and SEM variations. b) Tabulated data showing resultant relative expressions of both conditions, fold changes, *p*-value from student t-test of the data and c) the candidate gene names and gene functions.

The nine genes deemed significant following exposure to whole PM (10 mg) comprise hormone receptors (3), growth factors and cytokines (3), hormones (1), interleukin (1) and kinase network proteins (1).

Calcitonin receptor precursor (D04a) is a G-protein coupled, transmembrane glycoprotein and a signalling receptor for calcitonin. It is a GTP binding protein, a member of group 2 of G protein coupled receptors, biogenic amine receptors, whose activity is mediated by G proteins and upon stimulation, activates adenylate cyclase.

Serotonin receptor (D06a) is one of a multi gene family of serotonin receptors, also a G-protein coupled transmembrane glycoprotein which mediates its activity through association with G proteins activating a phospho inositol second messenger system within the cell. Serotonin is a biogenic hormone, a potent neurotransmitter and mitogen. A member of group 1 G protein receptors, the largest group to date, with a diverse role of activities including smell, taste and chemotactic capabilities (Li et al., 2004).

Cannaboid Receptor (D06k) is also a G protein coupled receptor, which upon activation, inhibits of adenylate cyclase (Williams et al., 2003).

Glial cell line derived neurotrophic factor precursor (D14e) is a growth factor, capable of acting as a signal molecule stimulating cell growth and proliferation. Increased levels of this glycoprotein has been shown to enhance survival and differentiation of dopaminergic neurons but is reported to undergo a 7-fold decrease following exposure to PM_{10}. A member of the TGF-β family, it serves as an anti-apoptotic signal molecule, involved in neurogenesis and signal transduction (Blesch et al., 2003).

Fibroblast Growth Factor 9 (E01h) a potent growth factor with strong mitogen and heparin binding capabilities, it has a positive role in cellular growth, development and repair, stimulating proliferation in FGF receptor containing cells. It is a member of the heparin binding growth factor family and has been implicated in the regeneration of (glial) tissue post injury and in the stimulation of glial cell tumours (Miyamoto et al., 1993).

Fibroblast Growth factor 5 precursor (E01i) is a proto-oncogene, a member of the heparin binding growth factor family, it serves as a mitogen and potent signalling molecule (Hattori et al., 1996).

Thyrotrophin releasing hormone (TRH) (E03b) has been implicated as being important in causing the stress-induced alterations in the motor function of cells within the gastrointestinal tract (Huo et al., 2004). This gene has been reported to undergo a 2.5 fold up regulation relative to sham-treated controls ($p<0.08$) in these experiments.

Interleukin-2 (E05a) displays a potent ability to stimulate the proliferation of many cell types including epithelial cells in the gut and activated lymphocytes, natural killer cells, monocytes and B cells (Cao et al., 2002). This interleukin is strongly associated with natural killer cells, although there is no evidence to suggest the presence of such

cells following the instillation of whole PM_{10}. This cytokine is crucial to the regulation of the immune response and the relative expression has been reported to increase as a direct consequence of exposure of the lung to whole PM.

p21 activated kinase (E10m) is a serine threonine kinase; upon activation it is hypothesised to target the GTPase effector, a link to the JNK MAP kinase pathway (Ke et al., 2004). It was reported to undergo a significant decrease in expression following instillation of whole ambient particulates, a trend confirmed by Taqman analysis.

Models of the molecular and cellular events leading up to lung injury following exposure to diesel exhaust particles (DEP) and ambient PM conclude that complex interactions between the pulmonary cells manifest in the dysfunction of normal lung clearance and repair mechanisms at the lung surface (Oberdorster 1990, 1992. Donaldson et al., 1998).

Retention studies by Oberdorster (1992) highlighted that the phagocytosis of particulates, irrespective of their chemical composition, by AMs, was accompanied by the release of a myriad of mediators resulting in epithelial damage (proteases and oxidants), growth factors and chemotactic factors. The release of such factors would serve to produce bioreactive chemical moieties that may result in further damage to the epithelium and cells within the alveolar region, signal molecules to promote the replication and repair mechanisms and molecules to produce a chemotactic gradient, encouraging the recruitment of activated phagocytic cells into the alveolar space to facilitate clearance of the particles. The lung response following the instillation of 10 mg whole Cardiff (urban) $PM_{10\text{-}0.1}$ was a chiefly macrophage mediated response. Histology sections showed that particles in close association with alveolar macrophages remaining in the alveolar spaces and very little evidence of particles crossing the epithelium (interstitialisation), in agreement with other assessments revealing a relatively mild and short lived pulmonary oedema and pulmonary damage. At 3 days post instillation, slight damage to the epithelium was reported, a possible consequence of particle/chemical-cell interaction or the effect of inflammatory signalling molecules produced by the activated AMs. Cross talk between the cells within the alveoli via signalling molecules may serve to trigger cellular events that may protect neighbouring cells from damage and the onset of apoptosis resulting from PM interactions and promote the replication of epithelial cells to replace damaged

cells and restore epithelial integrity. Indeed examples of such genes, including anti apoptotic are present in the candidate gene lists. Some of the candidate genes (E10m) have been linked with the MAP Kinase pathways indicates the potential of direct effect of signalling molecules derived from activated macrophages and damaged epithelial cells on neighbouring cells to promote the transcription and hence expression of immediate early genes and transcription factors.

3.4.7.2 5 mg Whole PM-exposure: A Dose Dependant Effect?

Analysis of the macroarray data from tissue exposed to the lower dose (5 mg) of whole PM_{10} concluded that no genes were viewed as significant if the 2SD range, t-test significance ($p<0.05$ or 0.1) and minimum 2 fold change were all simultaneously applied to the gene profiles. Examination of each parameter alone did produce some candidate gene lists and so basic analyses on these lists were undertaken. These extremely low stringency examinations were insufficient to produce a final candidate gene list as produced for the higher (10 mg) dose, but did serve to assess the profiles for any indication of dose dependant changes in gene expression. A total of 8 genes, all down regulated, were expressed outside the 2 SD range of normal variation (control) and greater than a two-fold change between the mean test and control (Table 3.10a). A total of 25 genes were significantly altered ($p<0.05$) but none of these genes were expressed outside of the normal control range. No genes were altered outside the 2SD range and were significant as judged by the student t test. Genes undergoing the most dramatic changes (15 highest up regulation and 15 highest down regulation) are shown in Table 3.10b. This list was compiled to assess whether the most dramatically altered genes showed any correlation to those genes differentially expressed following exposure to either whole (10 mg) PM or equivalent dose of water soluble and includes gated ion channels (4), transporter proteins (7), intracellular kinases (3) and xenobiotic/lipid metabolism proteins (2) which are all common functions to genes altered following the other instillates.

The lists of significantly altered genes following 5 mg and 10 mg whole PM_{10} exposure were compared to examine a potential dose dependant effect. None of the 9 genes (10 mg whole PM gene list) were present in the candidate gene list following instillation of 5 mg whole PM. Each gene from the list of nine genes taken following 10 mg exposure were traced on the 5 mg expression profiles, no gene showed any

significant difference ($p>0.5$, fold changes 0.91 – 1.1). It is likely that the considerable variation in response to the 5 mg dose has masked any effects at the genetic level.

Table 3.10a: Candidate gene lists of 5 mg whole exposed animals at three days post exposure. Eight candidate genes expressed outside a 2 SD range of normal expression, altered greater than two fold.

a)

Atlas Gene List	Mean Sham-treated Control	SD	Mean PM exposed (5 mg)	Fold Change	Gene Name
B02d	1.0917	0.191	***0.3714***	*-3*	glucose transporter protein
B04j	1.0413	0.111	***0.4918***	*-2*	sodium channel protein 6 (SCP6)
B11d	1.0147	0.033	***0.3788***	*-3*	Aquaporin (pancreas and liver; AQP 8)
B13g	1.0087	0.016	***0.3361***	*-3*	amphiphysin II
B13h	1.0718	0.130	***0.4342***	*-2.5*	intrinsic factor precursor
B14f	1.0309	0.029	***0.3301***	*-3*	fatty acid-binding protein
D08m	1.0709	0.117	***0.3482***	*-3*	NOR-1; member of thyroid/steroid receptor super family
F01n	1.1791	0.256	***0.35931***	*-3*	inositol polyphosphate 4-phosphatase type II alpha

Table 3.10b: Candidate gene lists of 5 mg whole exposed animals at three days post exposure – the top 15 up regulated and down regulated genes.

Gene List	2SD range	*P*-value (T test)	Fold Change	Gene	Functional Classification	
B01c	N	0.530	-2.0	cytochrome P450 VII (CYP7)	Complex Lipid Metabolism	Xenobiotic Metabolism
B02d	Y	0.185	-2.9	glucose transporter protein	Facilitated Diffusion Proteins	
B03d	Y	0.289	-2.4	acetylcholine receptor gamma	Ligand-Gated Ion Channels	
B04i	Y	0.380	-2.1	sodium channel protein 6 (SCP6)	Voltage-Gated Ion Channels	
B11d	Y	0.235	-2.7	aquaporin (AQP 8)	Other Membrane Channels and Transporters	
B12h	N	0.349	-2.9	myelin proteolipid protein (PLP);	Extracellular Matrix Proteins	
B13g	Y	0.183	-3.0	amphiphysin II (AMPH2)	Endocytosis Proteins	
B13h	Y	0.278	-2.5	Intrinsic factor precursor (INF; IF);		
B14f	Y	0.167	-3.1	fatty acid-binding protein	Other Trafficking and Targeting Proteins	
D08m	Y	0.169	-3.1	NOR-1;thyroid/steroid receptor superfamily	Hormone Receptors	Nuclear Receptors
E07g	N	0.482	-2.1	cAMP-dependent protein kinase catalytic subunit	Intracellular Kinase Network Members	
E07k	N	0.522	-2.0	Jak3 tyrosine-protein kinase		
E07l	N	0.533	-2.0	stress-activated protein kinase gamma (SAPK-gamma)	Intracellular Kinase Network Members	Other Stress Response Proteins
F01n	Y	0.137	-3.3	inositol polyphosphate 4-phosphatase type IIα type IIβ	Phospholipases and Phosphoinositol Kinases	
F02n	Y	0.177	-2.2	adenylyl cyclase type VI (ADCY6)	Adenylate/Guanylate Cyclases and Diesterases	
A01e	N	0.280	2.5	kidney band 3 anion exchange protein	Cell Surface Antigens	
A03e	N	0.424	2.4	B7.1		
A04e	N	0.199	2.0	urokinase receptor		
A12a	N	0.455	2.3	transforming growth factor alpha (TGFa)	Growth Factors, Cytokines and Chemokines	
B03e	N	0.536	2.0	acetylcholine receptor delta	Ligand-Gated Ion Channels	
B05e	N	0.484	2.1	skeletal muscle sodium channel protein alpha subunit (SCN4A)	Voltage-Gated Ion Channels	
B07m	N	0.225	2.0	Na+/Cl- betaine/GABA transporter	Symporters and Antiporters	
B07n	N	0.525	2.0	excitatory amino acid transporter 3		
B10n	N	0.241	2.6	synaptic vesicle amine transporter	Other Membrane Channels and Transporters	
B13n	N	0.227	2.0	secretogranin II precursor	Other Trafficking and Targeting Proteins	
C02b	N	0.347	3.0	cytosolic hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase);	Simple Carbohydrate Metabolism	
C05g	N	0.176	3.0	bile-salt-activated lipase precursor (BAL)	Metabolism of Cofactors, Vitamins and Related Substances	
C07h	N	0.217	2.8	cytochrome P450 2C22 (CYP2C22)	Complex Lipid Metabolism	Xenobiotic Metabolism
E04d	N	0.527	2.0	preprolactin (Prl)	Hormones	
F01b	N	0.124	2.3	guanine nucleotide release/exchange factor	Intracellular Adaptors and Receptor-Associated Proteins	

3.4.7.3 10 mg Water Soluble PM-exposure

Analysis of the water soluble exposed profiles compared to sham-treated controls, produces 42 genes whose expression has changed by a minimum of two fold. Of these genes, 5 genes were deemed significantly altered ($p<0.05$) by a students t-test (Figure 3.10a) whilst a further 13 genes were significant to the 90% level (all up regulate, Figure 3.10b).

One hundred and sixty nine genes were deemed to have a mean exposed relative expression outside that of a 2 SD "normal" variation range of control expression, 7 of which have a greater than two fold change in expression (B01k, B02k, B03k, B03a, B07n, D08j, E03a).

Application of both a 2 SD range and t-test ($p<0.05$) stringency, reduce the candidate gene list to just three genes. *Neuronal Acetylcholine receptor protein-α* (B02k) forms part of a pentameric transmembrane protein ion channel. Upon ligand binding, this subunit undergoes extensive conformational changes resulting in opening of the gated ion channel (Oshikawa et al., 2003). Array analysis reported a significant fold increase (17 fold, $p<0.01$) and the trend was confirmed by Taqman analysis (1.8 fold increase). The second gene, a *nicotinic acetylcholine receptor α2 subunit* (B03k) was reported to undergo a significant increase in expression compared to sham-treated controls. This is an interleukin/interferon gated ion channel associated receptor that acts, upon stimulation to open the associated potassium channel into the cell and serves to acts as an inhibitory factor to adenylate cyclase activity (Khirougss et al., 2004). Finally, the up regulated *excitatory amino acid transporter 3* (B07n), a solute transporter that may act as a gradient driven symporter or antiporter (Foguet et al., 1992). Reducing the stringency of the t-test ($p<0.1$) as summarised in figure 3.10b incorporates an additional 15 genes, all up regulated. These genes include a wide range of membrane channels (aquaporin) including gated ion channels (calcium and potassium ion channels), targeting and transporter proteins (epiphorphin, solute transporters and P-glycoprotein) and signalling molecules (interleukin 6 and prolactin). All such genes could be hypothesised to be involved in the oedematous response suggested by conventional toxicological examination.

Expression of the 18 candidate genes deemed significant following exposure to the water soluble component (70% by mass of Cardiff PM) were assessed in the whole PM exposed profiles. Of the 18 genes, 1 gene was found to be expressed outside a mean sham ± 2SD range (B02k, p=0.21) whilst three genes were significantly altered ($p<0.1$) from sham-treated controls (B06i, C02b, E05h). This slight correlation indicates that there are mild similarities in the lung response to whole and water soluble only fractions of the same PM sample, as would be expected from a whole PM sample composed of 70% by mass water soluble component. However, the large effect of the particle fraction may have masked the changes elicited by the water soluble fraction. Each of the four genes identified (Table 3.12) undergoes a similar directional fold change compared to sham-treated controls.

Figure 3.11: Candidate genes proposed to be involved in the pathogenicity of water soluble PM_{10} (10 mg) instillation into healthy rats. a) Graphical representation of the genes deemed significantly altered relative to saline treated controls ($p<0.05$). Values are mean control (n=4) and exposed (n=3) and SEM variations. b) Tabulated data showing resultant relative expressions of the 18 genes significantly altered ($P<0.1$, min. 2 fold change), fold changes, *p*-value from student t-test of the data and the candidate gene names and functions for each gene are given. Red type indicates the 3 genes undergoing a minimum 2 fold change in expression, relative expression in exposed samples outside a sham-treated mean ± 2 SD range and $p<0.05$.

a

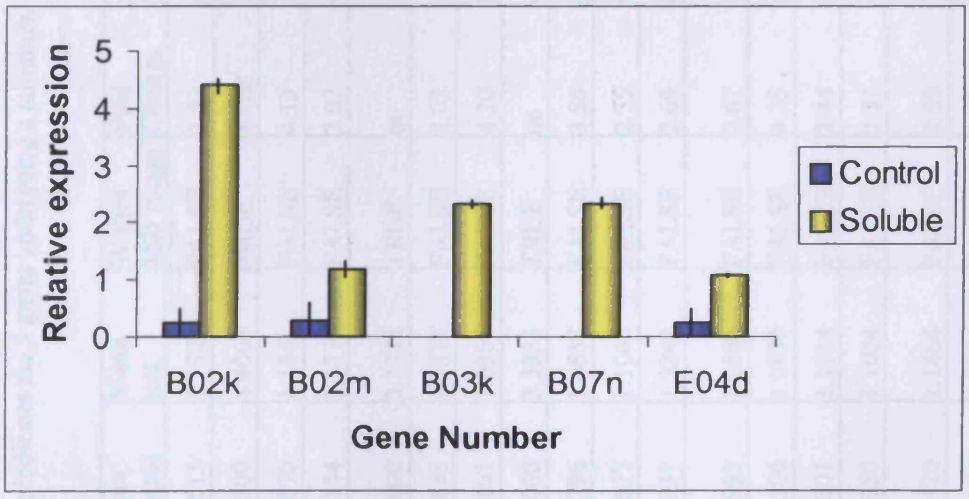


Table 3.11: Candidate genes proposed to be involved in the pathogenicity of water soluble PM_{10} (10 mg). Tabulated data showing resultant relative expressions of the 18 genes significantly altered (P<0.1, min. 2 fold change), fold changes, *p*-value from student t-test of the data and the candidate gene names and functions for each gene are given. Red type indicates the 3 genes undergoing a minimum 2 fold change in expression, relative expression in exposed samples outside a sham-treated mean ± 2 SD range and p<0.05.

Gene code	Mean control	Mean test	Within 2SD range	Fold Change	*P* value T-test	Gene	nction	
B01n	0.4613	1.1928	FALSE	2.59	0.0702	multidrug resistance protein 2	Drug-Resistance Proteins	ABC Transporters
B02k	0.2500	4.4063	TRUE	17.63	0.0001	neuronal acetylcholine receptor protein α7	Ligand-Gated Ion Channels	
B02m	0.2875	1.1863	FALSE	4.13	0.0452	cyclic nucleotide-gated channel		
B03j	0.6184	1.2770	FALSE	2.07	0.0634	purinergic receptor P2X3, ligand-gated ion channel		
B03k	0.0000	2.3333	TRUE	on	0.0009	neuronal nicotinic acetylcholine receptor alpha 2 subunit	Ligand-Gated Ion Channels	Hormone Receptors
B06i	0.5635	1.1377	FALSE	2.02	0.0648	potassium channel, voltage gated	Voltage-Gated Ion Channels	
B06n	0.4001	1.6805	FALSE	4.20	0.0592	calcium channel, beta subunit,		
B07n	0.0000	2.3333	TRUE	on	0.0013	excitatory amino acid transporter 3	Symporters and Antiporters	
B08m	0.3796	1.4658	FALSE	3.86	0.0622	sodium-potassium-chloride cotransporter		
B12b	0.4677	1.1000	FALSE	2.35	0.0999	water channel	Other Membrane Channels and Transporters	
B12n	0.4931	1.3242	FALSE	2.69	0.0588	syntaxin 2 (STX2)	Exocytosis Proteins	
C02b	0.3952	1.0554	FALSE	2.67	0.0730	cytosolic hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase)	Simple Carbohydrate Metabolism	
E04d	0.2500	1.0875	FALSE	4.35	0.0423	preprolactin (Prl)	Hormones	
E05b	0.4801	1.1714	FALSE	2.44	0.0871	interleukin 6 (IL-6)	Interleukins and Interferons	
E05h	0.4650	1.1024	FALSE	2.37	0.0981	leptin precursor; obesity factor	Other Extracellular Communication Proteins	
E05n	0.4420	1.1464	FALSE	2.59	0.0674	cocaine/amphetamine-induced rat transcript		
E06h	0.4674	1.3074	FALSE	2.80	0.0507	secretogranin 3		
F01b	0.5273	1.2341	FALSE	2.34	0.0999	guanine nucleotide release/exchange factor (GNRP)	Intracellular Adaptors	

Table 3.12 Assessment of the water soluble candidate genes in 10 mg whole PM exposed samples. Either the student t–test or 2 SD range filtering. The data showed that changes in only one of the four genes were similarly expressed.

Atlas Gene Number	Change reported water soluble profile	Outside a sham mean ± 2SD range	P value (T-test)	Fold change
B02k	17	Yes	0.21	10
B06i	2	No	0.1	1.8
C02b	2.6		0.1	2.4
E05h	2.3		0.1	2.3

3.5 Discussion

The primary aim of this study was to examine changes in lung gene expression following the instillation of whole PM_{10} (two doses: 5 mg and 10 mg) and the 10 mg equivalent dose of the water soluble fraction of ambient Cardiff sample. A chief concern of this experimental strategy was to develop a standardised method of data handling, normalisation and transformation within our research group that was capable of yielding meaningful candidate gene lists. The instillation of the PM toxicant produces a highly variable response in healthy rats. Lung tissue samples from experimental animals exposed to Cardiff PM_{10} were selected based on a heightened response based on conventional assessments (Chapter 2). Expression profiles of each animal were generated and compared to produce candidate gene lists. Different animals were used in each analysis; there was no replication of the same sample or indeed, the same RNA. This was perhaps was a failing, increasing the replicates of array profiles in this manner whilst still retaining low numbers of experimental animals would have increased the reliance of statistical approaches used and addressed issues relating to the reproducibility of the array. However, conversely use of the same RNA or tissue sample fails to take into account the different level of effect the exposure has had on individual animals.

The variation between animal responses has provided a challenge for this experiment. The wide variation in responses as well as differing severity of a conventional toxicological endpoint meant that a uniform effect across the whole was impossible. The use of equivalent lobes of tissues from each animal was implemented to

standardise tissue selection between samples, but it did not ensure that each tissue sampled was equally affected by the PM instillate. In this respect, subsequent studies with another pulmonary toxin (bleomycin) inducing a more homogenous level of effect across the whole lung has been successfully used to assess the data handling (Dominique Balharry, *personal communication,* 2003). In the bleomycin investigation the homogenous effect of the toxicant on lung tissue coupled with the greater animal numbers (n=9 sham, n=9 exposed) led to very consistent agreement between six candidate genes derived from the macroarray and qPCR.

Log_{10} transformations stabilise the variance of the data expressed at high levels, however, their performance on data expressed at low or near background levels is less effective (Durbin et al., 2002). Background adjustment of the raw expression data could result in adjusted signal intensities of less than 0, in genes with very low expression. In this case, all negative values must be treated as 0 or cause such signal intensities to be disregarded, which was not advisable and subsequent log calculations produced negative values, unsuitable for further analyses of the data. It was preferable to incorporate a stabilising transformation that incorporates all signal intensities, irrespective whether the intensity is high or near to background levels. The introduction of the addition of a constant value (*c)* to each gene background adjusted-intensity reduced the number of spots with intensities close to background level, preventing the elimination of these genes from subsequent analyses (Durbin et al., 2002). The value for the present study was taken as 1. Therefore for all cases when background adjusted expression intensities tended to 0, the $\log_{10}(0+1)$ was calculated and assigned as 0 rather than $\log_{10}(0)$. Durbin and colleagues claim that it would also be appropriate to use non-background adjusted spot intensities (i.e. the raw spot intensity) instead of the addition of a constant value (c). However, because of the extent of variation between background noise between different arrays and even, in some cases, between regions of arrays, it was considered better to work from adjusted data.

Proper normalisation of array data was of great importance when considering the sources and extent of variations in detection and visualisation of expression changes. Major sources of fluctuations expected in an array experiment include probe, target and array preparation, hybridisation process, background over shining and effects

resulting from image processing. This list incorporates mRNA preparation and transcription efficiency, labelling efficiencies, systematic variations, hybridisation parameters and image analysis (Schuchard et al., 2000).

Commercially available arrays were purchased to ensure that all arrays and reagent were of the highest possible standards. It was noted however, that background noise, radiolabel incorporation and probe hybridisation was variable between batches of arrays purchased and that efficiencies generally declined with repeated use. The background noise, also noted amongst other Atlas™ array users, was generally higher than found with other commercially available arrays and could, mask differentially expressed genes with low expression levels. Reduction of background noise is vital to the improvement of accuracy of macroarray data experiments (Yang et al., 2001)

Four approaches to analysis of the gene profiles were explored and outlined in this chapter. Simple pair wise comparisons using a mean calculated control profile was adopted as the first method. This method was initially favoured because it retained simple control/test comparisons, however, it failed to take into account of any normal range of expression that may be observed between different animals. Indeed, this method produced large gene lists that had to be reduced by application of arbitrary values attempting to give more weight to a simple fold change in gene expression. The methods were then developed in an attempt to address this "normal range" in gene expression in sham-treated control and PM exposed profiles. The expression range analysis, evaluated the normal range of expression of the genes in both control and exposed samples, calculated a minimum and maximum error in each gene for both sham-treated and exposed samples (n=3) to identify candidate gene with non overlapping range in expression. Candidate gene lists devised from this approach showed a large degree of similarity between water soluble and whole PM exposed profiles, which was to be expected from a sample that is 70% water soluble (Moreno et al, 2003). The third method, the statistical approach was introduced to address variation in expression between similarly treated animals. Global normalisation of all test and control profiles together was a necessary for the comparison of gene expression across the arrays allowing simple statistical evaluation of the data. This approach assessed the similarity in range of control and exposed expression (statistical tests). A fourth approach was $\log_{10}$ fold change analysis method. This calculated an

alternative range in fold changes across the arrays rather than relative expression itself. Calculating the $\log_{10}$ ratio of mean control and test profiles and calculating a 2 SD range in $\log_{10}$ ratio from the mean. This method failed to produce short candidate gene lists.

The most successful method was the statistical approach. Even with the extremely focal regions of PM affected tissue and the low replicate numbers, changes in expression of candidate genes, some confirmed by qPCR, were detectable following instillation of a low bioreactive PM sample. The comparison of the different fractions indicate that some genes are similarly affected between water soluble and whole PM exposed samples, but these changes fall below the stringency of the filtering adopted for the final candidate gene lists. With increased replicate numbers, increasing the number of animals and replicates from the same tissue sample, it may be possible to examine the expression profiles using a lower stringency to identify markers characteristic to the exposure in animals with differing susceptibility to PM exposure.

Basic functional analysis of the candidate genes following both exposure to water soluble fraction and whole PM, include genes with known cellular functions consistent with the events at the lung surface confirmed by the conventional toxicological study (Chapter 2) and by previous published models of lung injury. This is an encouraging result and strengthens the use of macroarray technology to examine the effect of such toxicants on a larger cross section of genes and molecular pathways rather than several genes in isolation to gain further insights into the molecular basis for many of the observed lung and systemic effects post such exposures.

The change of patterns and numbers of gene changes observed between the two instillates (whole and water soluble PM fraction) could be a reflection of the different airway responses towards the different fractions. The durable particles present within the full fraction encourage macrophage invasion of the alveolar space to clear the foreign materials. This cell response is rarely observed in the absence of the particles in the water soluble fraction. The water soluble fraction of PM_{10} has been shown to pass through the lung tissue and instigate systemic changes within hours of instillation (Whittaker, 2002). The soluble fraction would cause immediate damage and leave the

airways extremely quickly. Therefore, by 3 days, pulmonary events would be chiefly concerned with repair and restoration of airway architecture. In comparison, the whole PM_{10} fraction (30% -by number-of durable particles) has greater longevity at the epithelial surface and by 3 days, pulmonary damage and clearance are likely to be continuing.

The histological analysis of the water soluble fraction of PM exposed tissue displays very different airway responses compared to those following the instillation of whole dust samples. At high magnification, the lungs treated with water soluble fraction of PM_{10} have noticeably greater areas of general thickening of the tissue which represent swollen micro-capillaries or the interstitial regions around capillary areas. The candidate genes identified using toxicogenomic analysis all display a similar function within the cell, many serve as ion channels and transporters that may serve a common role to restore the normal fluid balance within these tissues. Calcium has been strongly implicated in the stress response of pulmonary cells and the presence of calcium channels in candidate genes may be part of the recovery of these cells.

The candidate genes identified from the macroarray profiling do correlate with the cellular events observed at the lung surface, characterised by both histology and biochemical markers. The down regulation of an anti-apoptosis factor (D14e) and induction of proliferative factors (E01h and E05a) in response to the instillation of whole PM_{10} (10 mg) may constitute part of the process of lung repair following the injury, promoting the removal of damaged cells and their replacement. The dominance of significant changes in the increased expression of ion channels (B02k, B03k and D07n) may represent a lung response to the fluid imbalance observed in the histology, arising as a consequence of exposure to water soluble component of PM_{10}.

In toxicological terms the Atlas™ macroarray system provides a useful preliminary screen to identify important candidate genes expressed during lung damage. However, accurate quantification and confirmation of these gene changes will always require the use of other techniques i.e. qPCR. The Atlas™ array system was successful, however the macroarray procedure required large amounts of total RNA and the success rate to achieve suitable array images for the study was not always reliable.

Chapter 4.0

Establishing the toxicity of PM_{10} to primary lung cell cultures

4.0 Establishing the toxicity of PM_{10} to primary lung cell cultures

4.1 Introduction

The high degree of cell heterogeneity of the lung renders the *in vitro* study of cellular and biochemical features more difficult than other organs (Nemery and Hoet, 1993). There are in excess of 40 different cell types within the lung, the alveolar epithelial type II cell, alveolar macrophage and the non-ciliated bronchiolar epithelial cell (Clara cell) have received the greatest attention in toxicological studies due to their pivotal roles in the transformation, detoxification and clearance of foreign materials and toxicants from the lung. The use of primary cells in culture has provided an important insight into the mechanisms of toxicity of many pulmonary toxicants. These assays incorporate an array of molecular and cellular endpoints including cell death or more subtle symptoms of cellular stress and dysfunction including secretion of cellular signalling molecules and oxidative capabilities.

The use of isolated cell cultures may not completely reflect their behaviour in *situ*, resulting in destruction of spatial relationships between different cell types critical to normal cell function and maintenance of differentiated states. However, primary cell isolates have increased longevity over perfused organs and tissue extracts, it has also been shown that they retain characteristics and function over a greater period that may otherwise be lost in cell lines (Dobbs et al., 1997. Reynolds *et al.*, 1999. Oreffo *et al.*, 1988). Good toxicological research with isolated cells will lead to a better understanding of the whole lung, and thus will contribute to a better understanding of the pathogenesis of other lung diseases (Nemery and Hoet, 1993).

One objective for this thesis was to provide toxicogenomic profiling of primary lung cells (epithelial type II and alveolar macrophage cells) following treatment with particulate matter (PM_{10}) collected from ambient air in the south Wales conurbation. The emphasis of this work was to improve the development of *in vitro* toxicity assays (thus reducing animal experiments) and provide detailed information at the molecular level of mechanisms by which PM_{10} may damage individual lung cells. In order to achieve these objectives, a number of steps were necessary. The first step involved

the isolation and culture of the primary lung cells and the second required the determination of the toxicity of the PM samples under investigation. These steps form the basis of this chapter. The purpose of the work outlined in this chapter was to establish the doses of PM_{10} that produced low toxicity but could result in measurable genetic changes in the target cells in subsequent studies (chapter 6). There are a number of ways to assess toxicity in cell culture but cell attachment and detachment assays for both type II (Reynolds et al., 1999. Murphy et al., 1999) and alveolar macrophage cells (Richards, *unpublished*) have already been used in our laboratory. The PM toxicity was assessed by its ability to impair cellular function and viability to attach to the culture substrata and has been judged to provide a subtle response by the cells. These studies established CD_{50} values (the total dose, by mass, required to damage or detach 50% of the cells in a monolayer cell culture), which allowed the comparison of differential toxicity of particles derived from variable sources and sites.

The following chapter outlines the isolation and culture of two lung cells, the alveolar macrophage and alveolar epithelial type II cell, from healthy rats and characterisation of the cell cultures by phase contrast microscopy, observing cell shape and features, such as the presence of lamellar bodies, to identify the cell types *in vitro*.

Of specific interest in the present study was a comparison of an urban PM_{10} (Cardiff, CF 2) and an industrial sample (Port Talbot, PT 1). The former sample, used in the previous *in vivo* toxicity assessment (Chapters 2 and 3), is dominated by traffic particulates whereas the industrial sample contains significant numbers (>30%) of iron spherules derived from a local steel works. Thus, a toxicity study with primary lung cells should identify how such cells respond to these different samples.

Current research on air pollution toxicity has heavily implicated the water soluble fraction of PM_{10} in the observed toxic effects (Adamson et al., 1999 and 2001, Dye et al., 2001, Greenwell et al., 2003). Therefore in the present study, primary cell cultures received a suspension of whole PM_{10} (particles plus soluble fraction), the equivalent doses of washed PM (containing solely the washed durable fraction) and water soluble fraction of PM (durable particles removed by centrifugation) from both Cardiff and Port Talbot samples. In summary, this study investigates the toxicity of two characterised PM samples from two distinct sources within the South Wales

conurbation towards primary alveolar macrophages and epithelial type II cells maintained *in vitro*.

4.2 Materials

4.2.1 Material Suppliers

Male (200-250g) *Sprague Dawley* Rats (pathogen free) were purchased from Charles River Ltd, Margate, Kent. Euthatal and Halothane was purchased from Rhone Merieux, Harlow, Essex. Trypsin (Type 1, T - 8003), DNase (DN - 25), Streptomycin/Penicillin (P - 0906), Evans Blue (E-2129), Tween 20 (P-7949) and Percoll (P 1644) were all purchased from Sigma, Poole, Dorset.
DCCM-1 (050 – 010 - 1) was ordered from Biological Industries, distributed by Q.B.S. Ltd, 12 Pembroke Avenue, Denny IND Centre, Waterbeach, Cambridge, CBB5 9PB. L-Glutamine (25030 - 024), Gentamycin (15750 - 045), Anti – PPLO (15220 - 049), 10 X PBS (with calcium and Magnesium, 14080-048, without 14200-067), Foetal Calf Serum (10109 - 163) and Ultroser G (091 - 25950H) were purchased from Gibco BRC, Life Technologies Ltd., High Wycombe, Bucks. The plastic Petri dishes (2050200) were ordered from Triple Red and the EHS Sarcoma derived gel plates (ECM) (E - TCMT - F) from Biological Industries. The sterile Luer cannulae (200/300/050) were obtained from Portex, Hythe, Kent.

4.3 Methods

4.3.1 Type II Cell Isolation

Alveolar macrophages and epithelial type II cells were isolated using a method outlined by Oreffo (1987), using a trypsin digestion and Percoll gradient selection. In brief, rats (usually 200-300 g) were anaesthetised with Halothane and then administered a lethal intraperitoneal injection (1-2 ml) of Euthatal. The rat was subsequently weighed and cardio-respiratory death confirmed prior to dissection.
The fur was washed with ethanol (70 % v/v) and the ventral surface skin removed. The peritoneal cavity was opened by midline incision and the major dorsal blood vessels cut.

A tracheotomy was performed and a Luer Cannula, attached to a 20 ml syringe, securely tied into place in the trachea. The diaphragm was subsequently opened and the ventral portion of the rib cage and thymus removed. A Luer cannula attached to a gravity feed of sterile saline was then fed into the pulmonary artery and the right atrium was cut upon expansion to allow fluid to exit.

The lungs were perfused via artificial ventilation with 8-10 ml of air by means of the syringe attached to the Luer Cannula of the trachea. Ventilations (usually 8-10) were continued until the pulmonary circulation was clear of all blood to produce white parenchymal lung tissue.

The heart was removed and the lungs and trachea dissected free from the carcass. The oesophagus and any fatty tissue were dissected from the lungs and trachea. Any mucoidal material or blood clots on the exterior of the tissue were removed by means of absorbent tissue and the lung and trachea weighed.

The lungs were lavaged (five to eight times) with 6-8 mls of 0.15 M saline. The resultant lavage fluid was pooled in a centrifuge tube and maintained on ice. The large free cell count (LFC) was obtained by counting a sample of the cells using a haemocytometer. The large free cell population comprises of monocytes including macrophages, in healthy animals these cells consist of up to 95 % alveolar macrophage cells.

The lung was digested as outlined by Oreffo (1987). Briefly, this involved attaching the lung and cannula to a 20 ml syringe and filling the intact lung with 0.25% w/v Trypsin solution in Solution B (0.133 M NaCl, 5.2 mM KCl, 2.59 mM Na_2HPO_4, 10.3 mM Hepes and 1.0 mg/ml Glucose, 1.29 mM $MgCl_2$ and 1.89 mM $CaCl_2$) (6-8 ml). The solution was subsequently discarded and the lungs refilled with fresh trypsin and suspended in saline at 37°C for approximately 30 minutes or until the lungs looked mottled and translucent. The digested lung was removed from the saline and the trachea, bronchi and any other contaminating tissues were dissected free from the lung parenchyma and weighed, allowing calculation of the total lung parenchymal weight. Foetal calf serum (FCS) (1 ml) was added to the lung parenchyma, to stop the action of the trypsin. The tissue was diced and made up to 25 ml with DNase I

solution (250 μg/ml (7500 U/100 ml) in Solution A (0.133 M NaCl, 5.2 mM KCl, 2.59 mM Na_2HPO_4, 10.3 mM Hepes and 1.0 mg/ml Glucose, pH 7.4). The suspension was inverted (4 minutes) to encourage the full dissipation of the cell aggregates. The cell suspension was filtered through three filters, surgical gauze, 150 μm mesh and finally a 30 μm mesh, to remove all tissue clumps. This resulted in a mixed cell suspension termed the *primary digest*. The volume and cell content of the suspension were recorded. The cell count provides an estimate of the number of type II cells isolated (approximately 30% type II, Oreffo et al., 1987).

The primary cell isolate was mixed well using a pasteur pipette and applied to a freshly made, cold (4°C) discontinous Percoll gradient to segregate the cells based on their density, the type II cells are lipid rich and have lower density than contaminating red blood cells and macrophages. Each Percoll gradient comprises of 10 ml of light gradient (1 ml Solution C (1.33 M NaCl, 52 mM KCl, 25.9 mM Na_2HPO_4, 103 mM Hepes and 10 mg/ml Glucose, pH 7.4), 50 μl FCS, 2.7 ml Percoll made up to 10 mls with distilled water) containing a few drops of phenol red to allow the two layers to be distinguished, on top of the same volume of heavy gradient (1 ml solution C, 50 μl FCS, 6.5 ml Percoll made up to 10 mls with distilled water). The preparation was centrifuged in a RC – 3B (swing-out head) centrifuge (20 minutes, 250 x g, 4°C).

After centrifugation, distinct layers were visible in the Percoll gradient. The Type II rich layer was recovered in a fresh, sterile 50 ml centrifuge tube and the volume made up to 40 mls with DNase II solution (50 μg/ ml or 2000 U/200 ml, 0.01% v/v gentamycin, 0.05% v/v anti-PPLO in Solution A) to wash the cells. The cells were recovered as a pellet, by centrifugation as before and the resultant supernatant discarded. The cell pellet was re-suspended in 5 ml of sterile filtered DCCM-1 media containing 1% v/v penicillin and streptomycin and 2% v/v L-glutamine and was transferred to a petridish for incubation at 37° C for 1.5 hour. This procedure, referred to as differential attachment, separates the cells based on their ability to attach to the surface of the petri dish. Contaminating fibroblasts and macrophages will readily attach to the plastic surface whilst type II cells will not adhere as readily, resulting in an enriched supernatant of type II cell population by the end of the incubation.

4.3.2 Type II Cell Culture and Characterisation

The type II cell-enriched supernatant was removed from the petri dish and the final cell count of the purified type II cell population was calculated. The cells were plated at 250,000-350,000 cells/96 well (ECM coated) plate (equivalent to 6500-9000 cells/mm^2) in DCCM-1 containing 1% v/v penicillin and streptomycin, 2% v/v L-glutamine and 2% v/v ultroser G, a serum substitute that facilitates the attachment and spreading of the type II epithelial cells. The cells were incubated at 37° C, in 5 % carbon dioxide (95% air) and the cultures washed (Solution A containing 1% v/v penicillin/streptomycin) and media replaced as required.

Pulmonary epithelial type II cells were typically cultured until a confluent population consisting of well spread cells showing the characteristic features of type II cells. These were obtained, when plated at 250,000-350,000 cells/96 well, at between 24 and 48 hours in culture.

A nitroblue tetrazolium/ nicotinamide adenine dinucleotide phosphate (NBT/NADPH) stain of type II epithelial cells was completed on preliminary cultures to ascertain purity. The stain is specific to NADPH reductase, abundant in the type II epithelial cell and Clara cells. Cells attached to the culture substrata or cytospin preparations were washed in PBS (no calcium or magnesium) and bathed in formalsaline (10 seconds) and washed well. Filter sterile NBT stain solution (10 mg NBT, 10 mg NADPH (reduced) in 10 mls PBS) was added to the cells and incubated (37°C, 20 minutes) and washed off in PBS (1% v/v penicillin/streptomycin). Type II cells were stained dark blue in close association with the lamellae present in the cytoplasm, whilst Clara cells, containing greater amounts of the enzyme had a dark blue cytoplasm and a white nuclear region. The most likely staining reaction is for NADPH-dependent P450 reductase located in the microsomal fraction of Clara and Type II cells.

4.3.3 Attachment Efficiency and Purity Data from Cell Cultures

The attachment efficiency of the type II cell population was calculated using phase contrast photography. Photographs are considered more accurate than counting the cells by eye through the microscope. The type II cells were identified as those cells containing four or more lamellar bodies. Mature type II cells may contain up to 50 lamellar bodies but will lose these characteristic features and upon culture *in vitro* become elongated (type I-like morphology). The attachment efficiency was based on the total number of cells within a given area of the well relative to control (untreated) populations.

4.3.4 PM_{10} Collection and Preparation for Cell Studies

Coarse ($PM_{10-2.5}$) and fine ($PM_{2.5-0.1}$) PM fractions collected on PUF (polyurethane foam) filters on the high volume cascade elutriator collector (Moreno et al., 2003) and removed from the collection substrate using standardised methods. The two fractions were recombined to produce a single whole $PM_{10-0.1}$ suspension and the washed and water soluble fractions were prepared as previously described (Chapter 2). $PM_{10-0.1}$ suspensions were diluted to a stock concentration of 4 mg/ml or equivalent in DCCM-1 media containing antibiotic (1% v/v penicillin/streptomycin). Washed particles contribute 30% by mass (70% water soluble) to the Cardiff sample (Moreno et al., 2003), equivalent doses refer to the mass of washed particle or water soluble component found in a 4 mg/ml preparation of whole PM. The equivalent dose was used in all studies for these two fractions.

4.3.5 *In vitro* CD_{50} Experiment: Attachment assay

Two approaches to *in vitro* toxicity assessment were examined, firstly the attachment assay, examining the effect of increasing doses of each PM preparation on the attachment of cells to the substrata. The purpose of the attachment assay is to determine the amount of particle/toxicant required to prevent the attachment of 50% of the cell population and has been successfully used previously (Jones et al., 1993, Richards et al., 1990, Davis et al., 1988). Freshly isolated type II epithelial cells were plated at 250,000 cells (6500 cells/mm^2) in DCCM media (100 μl) (1% v/v

penicillin/streptomycin, 4% v/v L-Glutamine, 4% v/v Ultroser-G) into an equal volume of media (containing 1% v/v Penicillin/Streptomycin) containing the appropriate doses of PM_{10}: 0 μg (control), 12.5 μg, 25 μg, 50μg, 100 μg and 200 μg (equivalent to 0.325 – 5.2 $\mu g/mm^2$). Cells were cultured for 24 - 48 hours (until the control population was confluent and well spread). This method produced highly variable results, particularly following exposure to the fractions containing particles (whole and washed). Such variability could arise as a result of physical interference of the particles with the cells, hindering their attachment irrespective of their biological reactivity. The cell detachment assay, assessing the dose or equivalent dose of PM required to cause cells to detach from the culture substrata, was adopted for the subsequent study.

4.3.6 *In vitro* CD_{50} Experiment: Detachment Assay

The detachment assay is used to determine the amount of particle/toxicant required to detach 50% of the cell population from a culture of confluent cells. Dust applied in fresh media to a confluent population result in a mild toxicity causing an alteration in the cell adherence properties. In effect such a study may better represent the exposure of cells to PM as they are attached in situ in the lung to a basement membrane.

Type II epithelial cells were isolated and cultured at a density of 250,000 cells/well in a 96 well ECM-coated plate. The cells were cultured until they were well spread (24 - 48 hours). The DCCM-1 media was then removed and the cells were washed in solution A (without calcium or magnesium). Freshly prepared $PM_{10\text{-}0.1}$ samples in media or the media alone (control) (DCCM-1 media : 1% v/v penicillin/streptomycin, 2% v/v L-Glutamine and 2% v/v Ultroser G) to a maximal volume of 200 μl were added to the cells (0 μg-200 μg/well). The cells were incubated for 24 hours (36°C, 5% CO_2/95 % air) after which time they were washed in PBS to remove unattached and damaged cells. The remaining attached cells were examined using phase contrast microscopy and photographed.

4.3.7 Alveolar Macrophage Detachment assays

Preliminary experiments were set up to investigate the optimum plating density and culture times for the primary alveolar macrophages.

Alveolar macrophages were isolated from the lung lavage as previously described. Cells (> 90% purity) were plated at densities ranging from 50,000-150,000 cells per 96 well on plastic wells in DCCM-1 media (1% v/v penicillin/streptomycin and 2 % v/v L-Glutamine). The cells were cultured up to 24 hours (36°C, 5 % CO_2/95 % air) and washed and harvested to determine the minimum time required for maximal cell attachment. The optimal exposure time for PM in subsequent assays was completed using Port Talbot (Port Talbot 1) PM_{10} sample.

The CD_{50} was established using two methods: (i) total non-specific protein, measured by the Bradford Assay (Bradford, 1976) and (ii) using cell counts from photographs taken whilst under phase contrast microscopy.

4.3.8 Changes in Cell and Nuclear Morphology in Response to PM_{10} Exposure: IN Cell Analyser™ 1000

During the past few years, rapid progress has been made in identifying morphological and biochemical apoptotic criteria as well as some of the molecules responsible for the regulation and execution of apoptosis. Morphological characteristics of apoptosis include cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation; these are the final stages of the complex highly regulated process. Early, biochemical indicators of apoptosis are characterised by plasma membrane changes in phospholipid orientation, and mitochondrial changes. The following investigation utilised the IN Cell Analyser™ 1000 (Amersham) and was completed in conjunction with Dr S. Murphy (Amersham Biosciences, Cardiff) to identify subtle changes in cell and nuclear morphology following *in vitro* exposure to PM_{10}. The IN Cell Analyser 1000 (Amersham) is a line scanning confocal imaging system performing live and fixed cellular assays. It allows the simultaneous analysis of several endpoints using 3-colour imaging combined with x40 Nikon objective lens.

The IN Cell analyser system has been applied to this study to identify whether the plasma membrane alterations at the cell surface typically observed in the initial stages of apoptosis such as the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer and external surface of the cell. Annexin V was used as a sensitive probe for PS and can be used to identify apoptotic cells in culture. Another stain used to identify cellular changes was the Hoechst stain, a DNA intercalating dye that was been used to characterise the nuclear changes that occur during apoptosis. MitoTracker (Deep Red) probes, cell permeant mitochondrion-selective dyes accumulate in active mitochondria and were used to identify alterations in mitochondrial morphology and organelle functioning associated with cellular stress and pathology.

Epithelial type II cells were isolated, cultured until confluent and subsequently exposed to Cardiff PM_{10} (CF 2)- whole and water soluble fractions only (0-200 μg – whole PM, 0- 400 μg water soluble) as described previously (section 4.3.4). Cells were exposed to PM suspended in DCCM-1 media (1% v/v penicillin/streptomycin, 2% v/v ultroser G, 2% v/v L glutamine) for 24 hours before media and PM were removed and cells washed (section 4.3.6). Cells were stained with Annexin V (Molecular probes) diluted in Krebs ringer Bicarbonate (Gibco) to a final concentration of 16μl Annexin per ml of buffer and applied (100 μl to each well). The cells were incubated (20 minutes, 37°C) before the apoptotic marker solution removed and cells washed (1x PBS). The cells were fixed using 4% v/v paraformaldehyde solution and stored (4°C). Fixed cells were probed with MitoTracker (Deep Red 633 (25 μM) and Hoechst solution (25 μM) in a final volume of 100 μL per well (10 min at 37 °C, 5 % CO_2) and rinsed in PBS. Cells were imaged on the IN Cell Analyser 3000 at 37 °C using the following filter sets: Hoechst Ex: 352 nm, Em: 455 nm; MitoTracker (Deep Red 633 Ex: 644 nm; Em: 665 nm. The images were analysed using the granularity algorithm of the IN Cell Analyser™ 1000 in both the nuclear and cytoplasmic compartments. In brief, the granularity algorithm is used to analyse both nuclear and cytoplasmic signals. The algorithm works by drawing a bounding box around the biology of interest then counting how many granules of the specified dimensions are in each box. In each case, the nuclear channel is used to identify each cell as an object of interest and the bounding box may be expanded from

the nuclear size upwards. A granule size is then chosen to best represent the biology and the punctuated patterns are quantified within the bounding boxes.

4.4 Results

4.4.1 Cell Isolation and Primary Cell Culture

During the course of this thesis a total of 32 separate isolations of 104 individual rats were undertaken (Table 4.1). There are two useful guides to general health of the animals indicating that the animal and thus the lungs were free of infection. Healthy animals maintain a lung to body weight ratio between 0.0030 and 0.0045. This range is dependent on the body weight in that higher ratios are found in younger animals. With the standard lavage technique employed the LFC lies between 3-5 million cells in animals free of infection or any inflammatory process occurring at the lung surface. As can be seen from the data and in Table 4.1, most type II cell isolations were carried out on healthy rats and realised approximately 10 million purified type II cells/animal.

NBT staining of cell preparations sampled from various stages of the type II cell isolation procedure shows an increase in type II cell purity (data not shown). Epithelial cells (including type II cells) are enriched with NADPH enzyme and will appear as darkened cells whereas alveolar macrophages show only a slight background reaction with the NBT stain (Figure 4.1). Comprehensive staining of samples following each step of the isolation procedure has been completed (Oreffo et al., 1988).

Table 4.1: Type II cell isolations (n=104 rats) in this study. The mean values are shown ± Standard deviation for type II cell isolations. The LP: body weight ratio and final cell counts of both type II and Large Free Cell (macrophage) are included. The average type II cell yield per animal is approximately **9.4**±4.7 million and mean (non-treated) macrophage yield per animal is **3.4** ± 1.6 million. Nr- not recorded.

Isolation No.	**No. Animals**	**Body Weight (g)**	**LP/Body mass Ratio ($x10^{-3}$)**	**Type II cells (millions)**	**LFC count (millions)**
1	2	229±20	2.814	13.5±1.0	3.8±0.8
2	2	252±3	2.812	13.4	5.2
3	2	303±10	3.519	8.0±4	2.1±0.7
4	2	329±38	3.30±0.1	4	2.1
5	2	391±86	3.65±0.94	14±3.5	2.9±0.7
6	2	426±29	3.03±0.77	7.5±1.9	1.9±0.1
7	2	424±19	3.28±0.31	16.3±5.2	3.1±0.95
8	2	316±64	2.64±0.09	14±0.5	2±0.1
9	2	445±288	3.48±1.58	12.7±4	3.3±1.5
10	2	464±70	3.28±0.37	14.9±4.4	4.4±0.9
11	2	344±4	4.24±0.89	8.5±5.3	3.1±0.2
12	6	298±18	3.80±0.59	10±3.4	4.5±1.0
13	2	370±10	3.55±0.32	6.6±3.2	5.7±0.04
14	5	427±45	3.04±0.06	14.2±3.2	5.6
15	2	473±16	3.03±0.06	9.4±3.6	6±2.8
16	2	495±42	4.32±0.03	16±5.6	6.9±1.5
17	3	399±32	2.5±3.23	8.4±0.3	2.4±0.4
18	4	408±55	3.7±0.052	nr	2.2±1.4
19	2	428±11	3.33±0.07	6.3±1.2	6.5±2.4
20	2	466±1	3.22±0.03	nr	4.0 ±0.1
21	2	534±43	2.6	nr	5.4±0.8
22	4	129±10	6.2±0.99	12±3.4	2.2±0.5
23	4	315±31	3.9±0.06	12.2±2.2	3.38±0.84
24	4	301±32	4.0±0.012	11.4±2.9	2.77±0.83
25	6	388±30	3.34±0.03	12.8±2.5	1.96±0.76
26	4	267±29	4.58±0.11	3.7±1.3	2.21±0.63
27	3	313±24	4.02±0.04	13.4±3.0	2.49±1.27
28	3	295±15	3.76±0.36	13.2±0.7	2.5±1.27
29	6	261±45	4.79±0.25	4.6	2.23±0.41
30	6	295±26	4.46±0.75	6.8±1.7	1.13±0.46
31	6	202±7	4.78±0.12	7.4±1.49	3.85±0.33
32	6	242±14	4.24±0.59	6.7±1.6	3.09±0.8

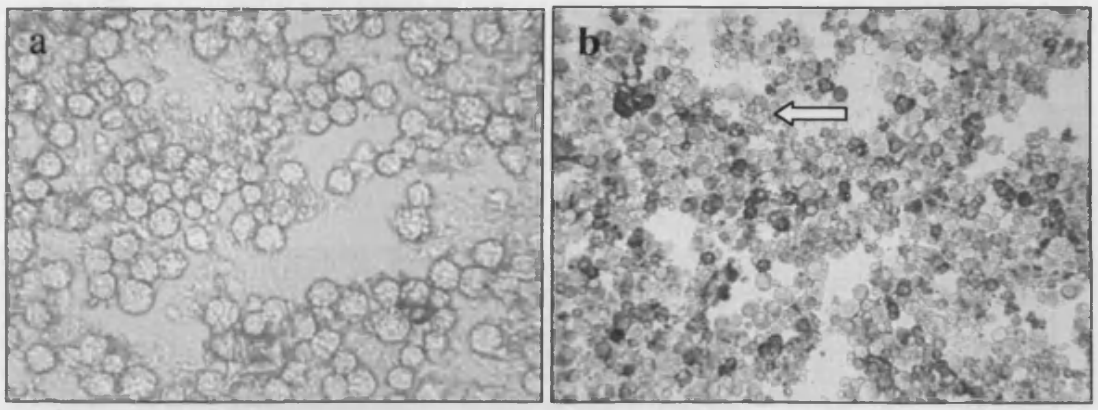


Figure 4.1: Images of (a) background NBT staining cells from lavage (magnification x250) and (b) cytospin of final preparation of primary type II cell isolates prior to culturing (magnification X400) White arrow indicates heavily stained cells.

Type II cells are morphologically distinguishable using phase contrast microscopy, they are characteristically rounded in shape and contain multiple (≥4) lamellar bodies. The photographs (Figure 4.2) record a loss of the characteristic rounded type II cell shape to an elongated morphology, accompanied with a gradual loss of the lamellar bodies upon culture. This differentiation is a major role of the type II's cell population; to replace damaged type I cells. These cells were removed at various times in culture and tested for reactivity to a type I cell marker – $rT1_{40}$ (Dobbs et al., 1988). The cells showed no reactivity up to day five in culture (data not shown). Reactivity towards $rT1_{40}$, the apical surface protein characteristic to type I cells would be gained upon differentiation to a type I morphology, at approximately day 6-7 *in vitro* (Reynolds, 2000).

Epithelial type II cells were cultured in DCCM-1 cell culture media (1 % v/v antibiotics, 2 % v/v Ultroser G attachment protein, 2 % v/v L-Glutamine) and plated on extracellular matrix (37°C, 5% CO_2). Cells were typically confluent at 24-48 h under these conditions (Figure 4.2 a and b) and were characteristically well spread across the culture substrata and rounded in appearance containing lamellar bodies, visible as speckles in the phase contrast photographs. At high plating densities (250-350,000 cells/38mm^2 equivalent to 6500- 9200 cells/ mm^2) these cells retained the type II morphology until day 6 in culture (Figure 4.2 c-f). At lower densities or beyond this time, the cells progressively lost their rounded appearance, becoming elongated as they differentiated to a type I like morphology (Figure 4.2 g-h).

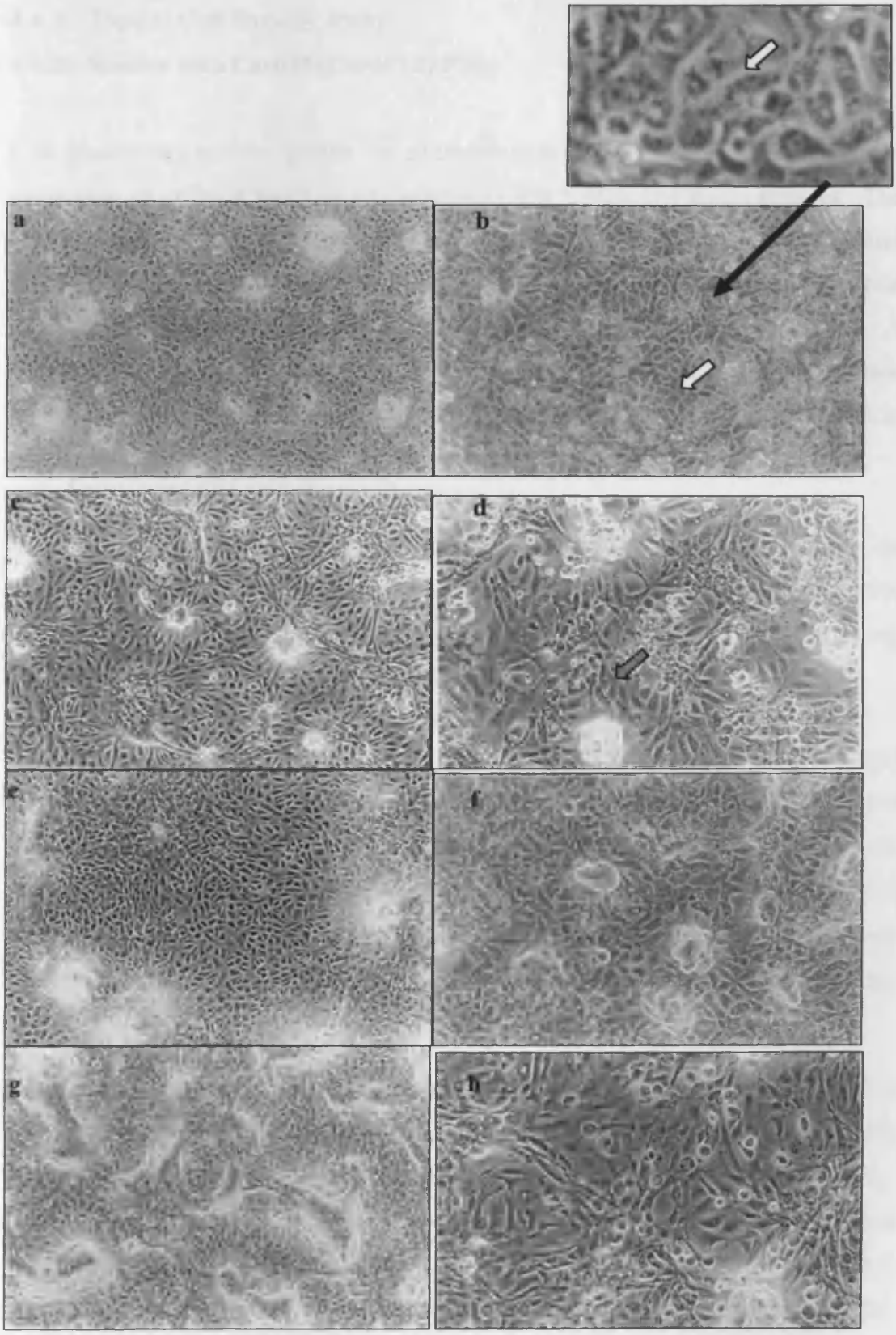


Figure 4.2 Primary rat epithelial type II cells in culture (a & b) at 48h (x100, x200 magnification) (c & d) at 72h (x100, x200 magnification) (e & f) at 96h (x10, x20 magnification) and (g & h) at 120h (x100, x200 magnification). White arrows indicate the distinctive type II cell with defined lamellar bodies (black specks) well spread over the culture substrata, progressively losing the characteristic morphology to a more elongated shape upon culture (red arrow).

4.4.2 Type II Cell Toxicity Assays

4.4.2.1 Studies with Cardiff (Cardiff 2) PM_{10}

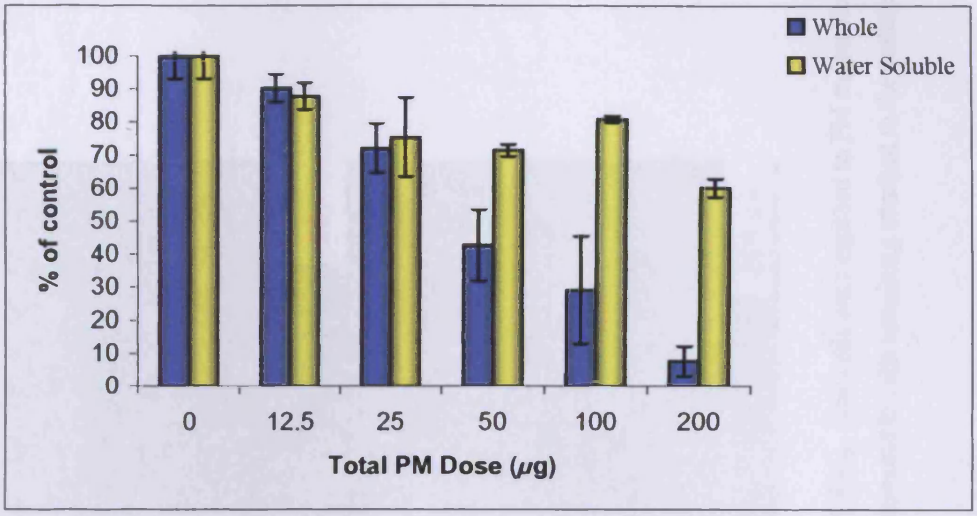
Cell detachment toxicity studies on epithelial type II cell isolates were completed using both an urban (Cardiff 2) and industrial (Port Talbot 1) $PM_{10-0.1}$ samples. The cells remaining attached following PM treatment, expressed as percentage of control (untreated) cultures (n=3) shows that the toxicity present upon exposure to the whole PM sample is retained within the fraction containing the particles (Figure 4.3). A maximum detachment of 90% of the cells was recorded at the highest dose of whole PM examined (200 μg/96 well) compared to just 40% detachment with exposure to the equivalent dose of the water soluble constituent.

Regression analysis was performed on Minitab™ to calculate the dose (or its equivalent) required to cause the detachment of 50% of the cells (CD_{50}) compared to untreated control populations (Figure 4.3b). The CD_{50} for Cardiff PM_{10} is 81 μg (whole PM_{10}) but much higher (385 μg) for the water soluble fraction.

A second urban (Cardiff) sample (Chapter 2, CF 2), collected during a 43 day period (January 2001-March 2001) was also assessed using this method. The cell responses to each of the three fractions, whole $PM_{10-0.1}$and equivalent doses of washed particles and water soluble fraction were assessed. Phase contrast photographs (Figure 4.4) were used to record the loss of type II cell attachment following exposure to whole (Figure 4.4), washed durable particles (Figure 4.5) and water soluble component (Figure 4.6).

As noted with CF 1, the effects of CF 2 are similar in that the durable particles need to be present to exert the greatest effect on cell detachment. The water soluble component is considerably less toxic than either the washed PM_{10} or whole PM_{10}. Graphical representation of the data is summarised in Figure 4.7. The whole and washed samples (CD_{50} 102 μg and 100 μg respectively) have over twice the relative toxicity than that following exposure to the water soluble component alone (CD_{50} 223μg). A statistical difference between exposed and control (untreated) cultures was observed beyond a 50 μg/well (whole), 100 μg/well (washed) and 200 μg/well (water soluble) exposure.

a



b)

PM fraction (CF 1)	r^2	CD_{50} Equivalent mass (μg/well)	CD_{50} Actual mass (μg/well)
Whole Cardiff	0.85	80.6 μg	80.6 μg
Water Soluble Cardiff	0.66	270μg	385 μg

Figure 4.3: Toxicity of PM_{10} (CF 1) in a detachment assay of primary alveolar type II cells. A) Graph showing progressive detachment of cells exposed to increasing doses (or equivalent doses of PM) of a summer urban particulate sample. b) Regression analysis summary showing the calculated equivalent doses (and actual dose) required to result in the detachment of 50% of the cell culture compared to untreated control (n=3). All masses given in μg per 96 well.

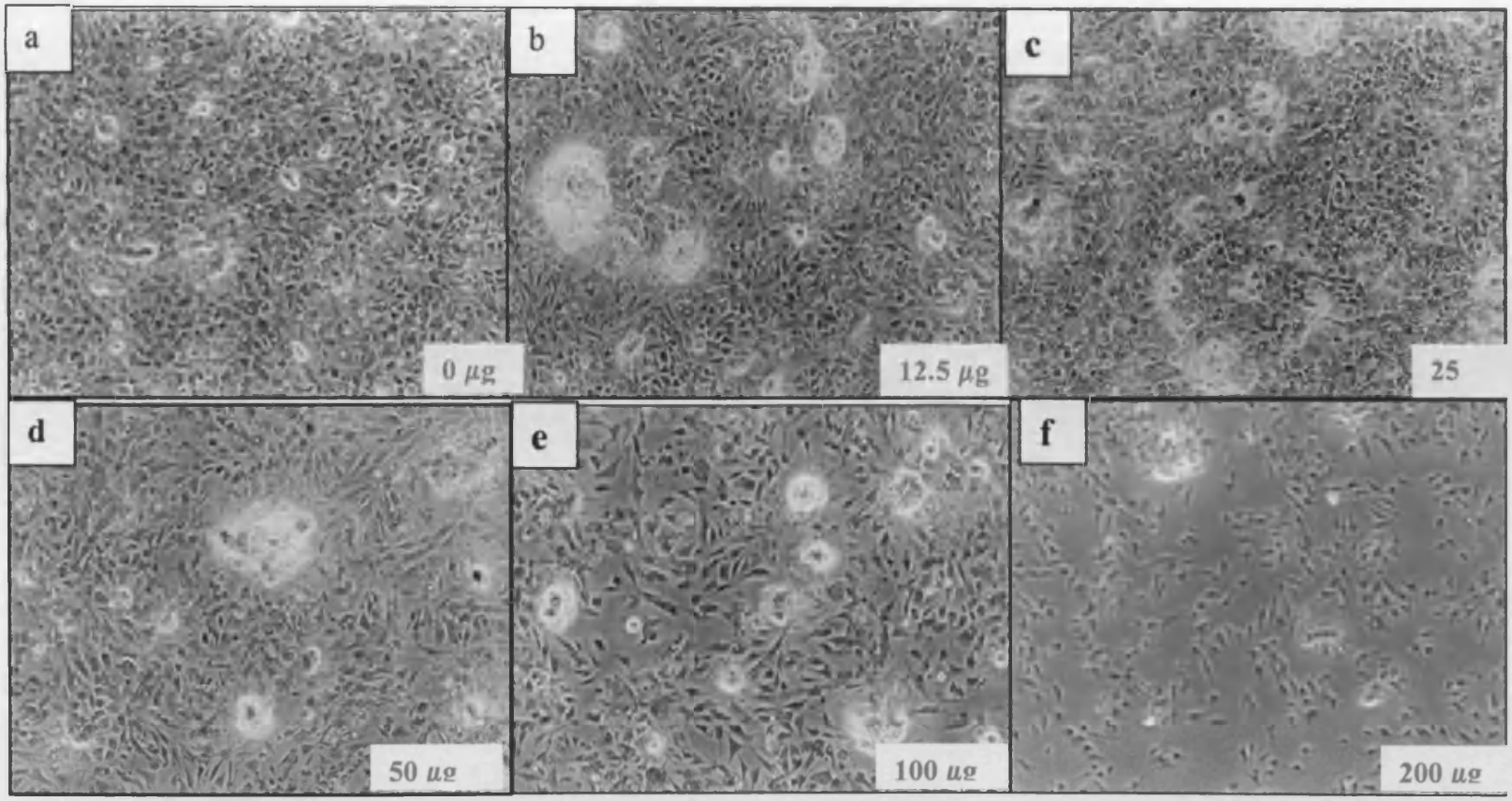


Figure 4.4: Phase contrast images (x100 magnification) of epithelial type II cells exposed to whole Cardiff (urban, CF 2) PM_{10}. The cells were exposed to PM suspensions (100µl). Red type indicates the mass of total equivalent delivered dose (µg/well). The images show a dose dependant decrease in cells remaining attached to the culture substrata following 24 hour exposure to the water soluble PM (0 – 200 µg/well).

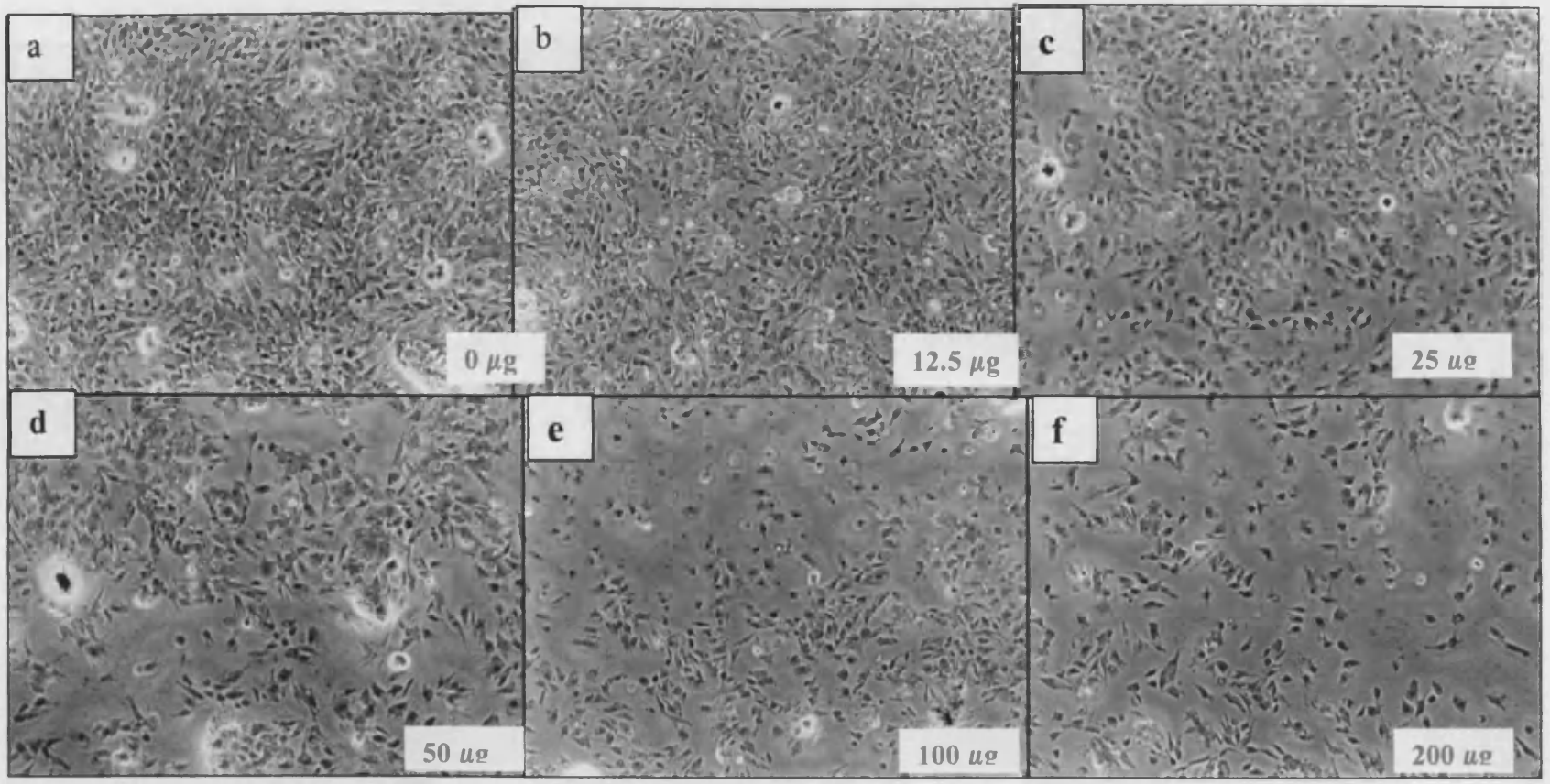


Figure 4.5: Phase contrast images (x100 magnification) of epithelial type II cells exposed to fractions of washed Cardiff (urban, CF 2) PM_{10}. The cells were exposed to PM suspensions (100µl). Red type indicates the mass of total delivered dose (µg/well). The images show a dose dependant decrease in cells remaining attached to the culture substrata following 24 hour exposure to the washed PM (0 – 200 µg/well) suspended in DCCM-1 cell media

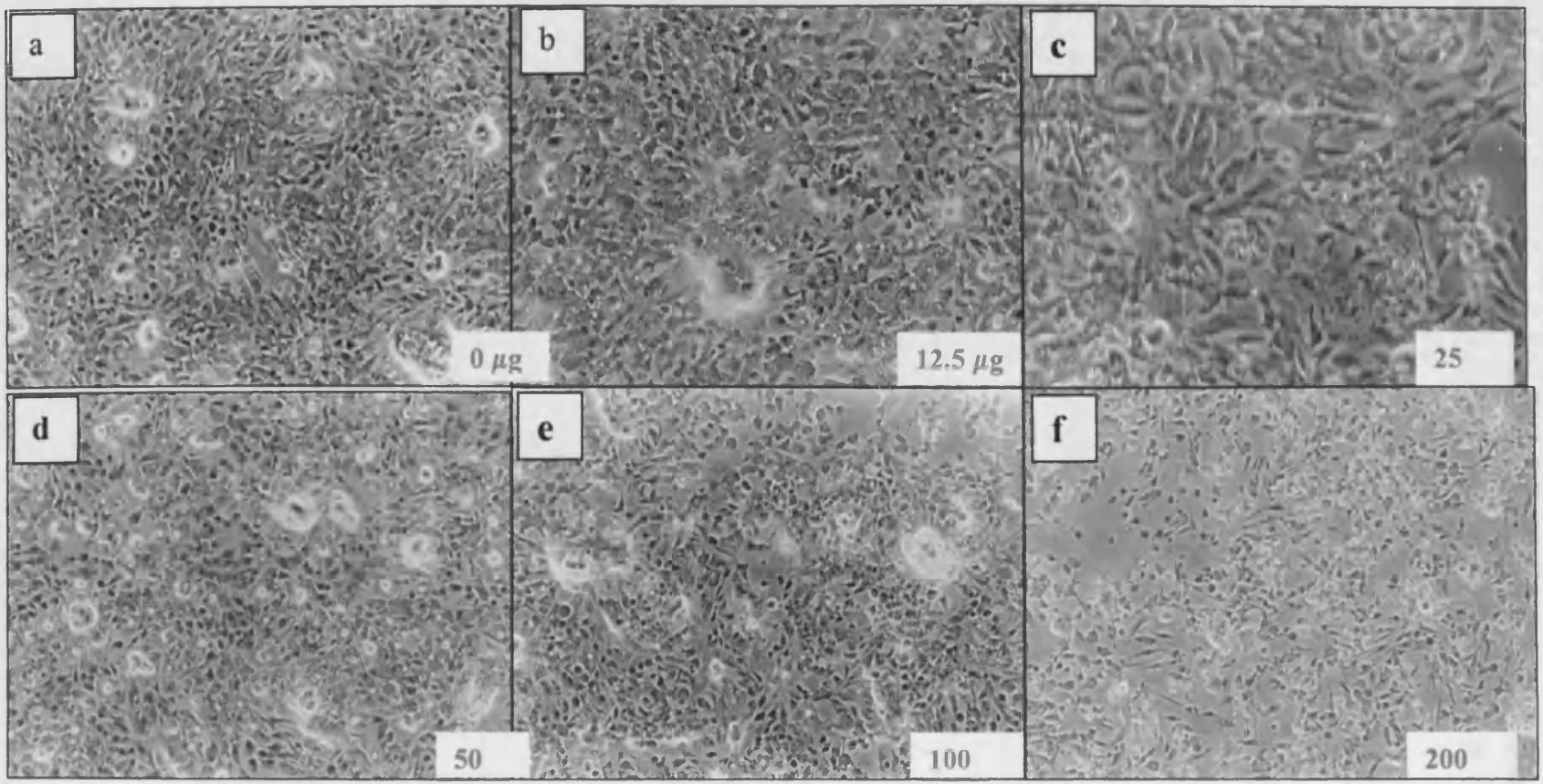
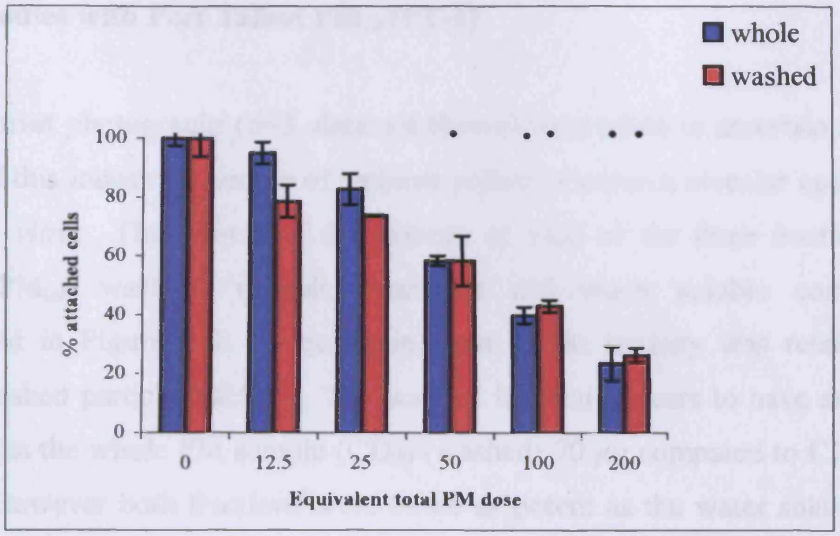
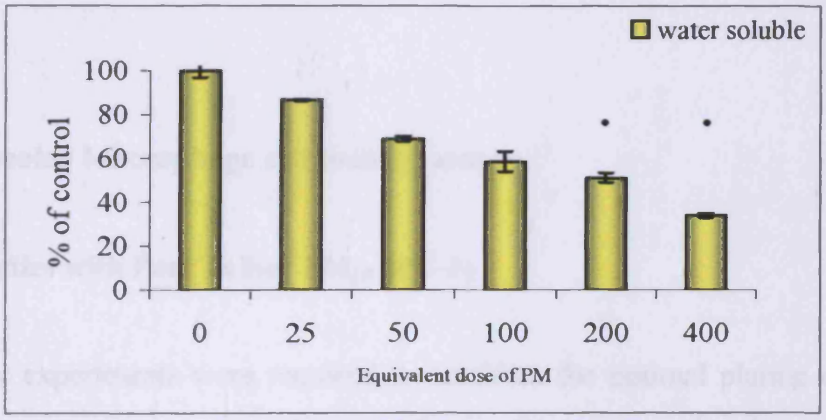


Figure 4.6: Phase contrast images (x100 magnification) of epithelial type II cells exposed to water soluble fraction of Cardiff (urban, CF 2) PM_{10}. The cells were exposed to PM suspensions (100µl). Red type indicates the mass of total equivalent delivered dose (µg/well). The images show a dose dependant decrease in cells remaining attached to the culture substrata following 24 hour exposure to the water soluble PM (0 – 400 µg/well) suspended in DCCM-1 cell media

a)



b)



c)

PM Fraction (CF 2)	r^2	CD_{50} equivalent mass in μg/well	CD_{50} Actual dose in μg/well
Cardiff Whole	0.87	102 μg	102.0 μg
Cardiff Washed	0.86	100.5 μg	30.15 μg
Cardiff Soluble	0.81	223 μg	156.1 μg

Figure 4.7: Toxicity of PM_{10} (CF2) in a detachment assay of primary alveolar type II cells. a+b) Graphical representation of dose of PM_{10} and cells remaining attached and (c) regression analysis summary showing the CD_{50} and actual dose, n=3 for each dose/sample, vertical bars are SD from the mean * denotes statistical change. All masses given in μg per 96 well.

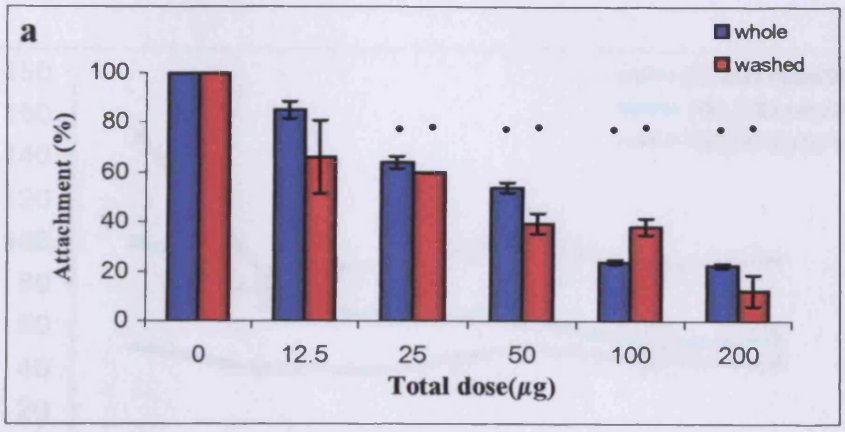
4.4.2.2 Studies with Port Talbot PM_{10} (PT-1)

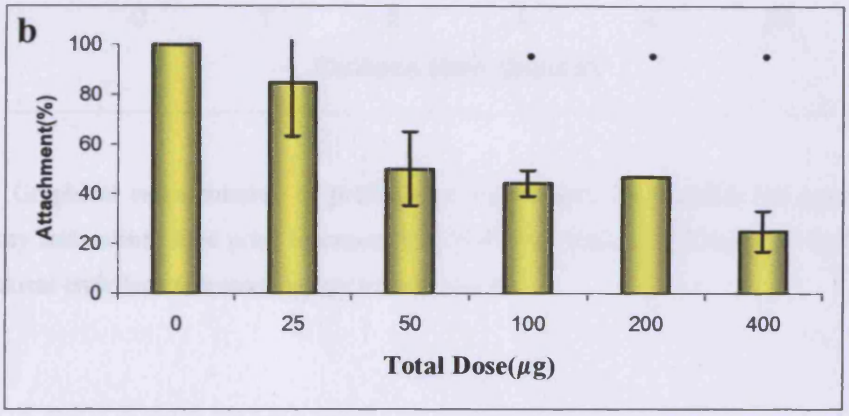
Phase contrast photographs (n=3, data not shown) were taken to ascertain the relative toxicity of this industrial sample of ambient pollution towards alveolar epithelial type II cells *in vitro*. The effects of the toxicity of each of the three fractions, whole ambient PM_{10}, washed "durable" particles and water soluble component is summarised in Figure 4.8. Once again most of the toxicity was retained in the durable/washed particle fraction. The washed fraction appears to have an increased toxicity than the whole PM sample (CD_{50} (washed) 70 μg compared to CD_{50} (whole) 82 μg). However both fractions were twice as potent as the water soluble fraction (164 μg).

4.4.3 Alveolar Macrophage cell toxicity assays

4.4.3.1 Studies with Port Talbot PM_{10} (PT-1)

Preliminary experiments were required to establish the optimal plating density and culture time prior to exposure (Figure 4.9) for the primary alveolar macrophages. Culturing the primary cells at 150,000 cells/96 well (4000 cells/mm^2) for a period of 4 hours prior to exposure resulted in the greatest attachment of the cells to the plastic culture substrata (Figure 4.9).





c

PM Fraction (PT-1)	r^2	CD_{50} equivalent mass in μg/well	CD_{50} Actual dose in μg/well
Whole	0.76	82.0 μg	82.0 μg
Washed	0.76	70.0 μg	21.0 μg
Water soluble	0.65	164.5 μg	115.2 μg

Figure 4.8: Toxicity of PM_{10} (CF 2) in a detachment assay of primary alveolar type II cells. (a and b) Graphical representation of dose of PM_{10} and cells remaining attached following PM exposure, n=3 for each dose/sample, vertical bars are SD from the mean of whole (blue) and washed (red) preparations and b) water soluble (yellow) fractions of Port Talbot PM_{10}, an industrial ambient particulate solution. • denotes statistical change ($p<0.05$). All masses given in μg per 96 well. (c) Regression analysis summary showing the CD_{50} (equivalent dose of whole PM) and actual dose (μg).

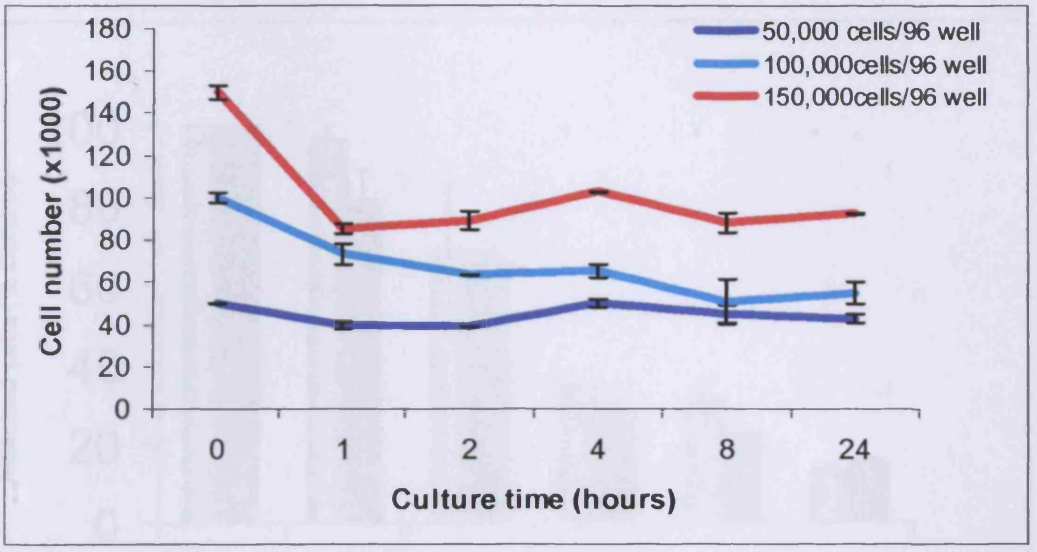


Figure 4.9: Graphical representation of preliminary experiments to establish the most appropriate plating density and culture time prior to exposure to PM. Attachment efficiency (n=2) for each time point and vertical error bars represents range from the mean.

Having established the optimum time for the culture time for maximal alveolar macrophage attachment, it was necessary to ascertain the most suitable PM_{10} exposure time for this cell type. Cells were cultured and subsequently exposed for 4 and 20 hours. There were little marked differences between the toxicity observed at the two exposure times and so a 4-hour exposure was adopted for all assessments (Figure 4.10).

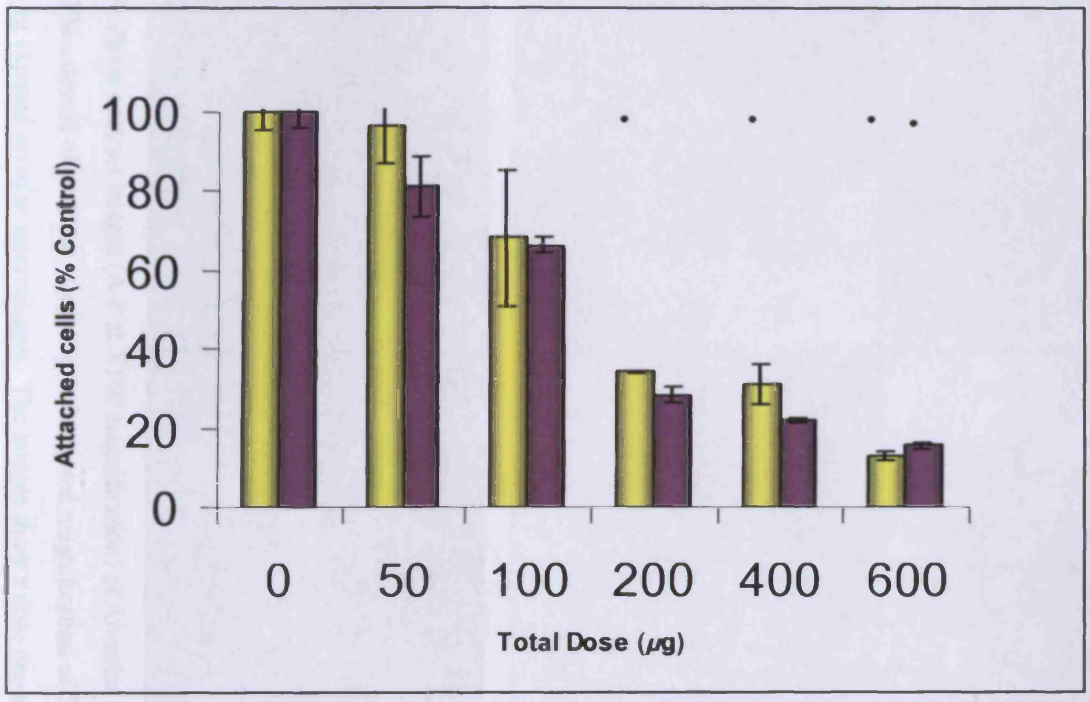


Figure 4.10: Graphical representation of preliminary experiments to establish the most appropriate exposure time of primary alveolar macrophages to whole PM (PT 1). Attachment efficiency (n=4) for each time point and vertical error bars represents standard deviation from the mean for each dose following a 4 hour (yellow) and 20 hour (red) exposure.

Regression analysis (r^2=0.8261) estimates the CD_{50} of PT1 towards alveolar macrophages as 275.8 μg. Statistical analysis (student t-test) showed that a significant difference ($p<0.05$) in toxicity was found following 200 μg/well (whole) and beyond, whilst the highest equivalent dose (600 μg/well) was required for a significant toxicity with the water soluble fraction (data not shown).

4.4.3.2 Studies with Cardiff PM_{10} (CF 2)

Alveolar macrophages were exposed to whole and water soluble fractions of Cardiff PM_{10} (CF 2) fractions. Phase contrast photographs (Figure 4.11) were used to establish the CD_{50} dose of the urban sample.

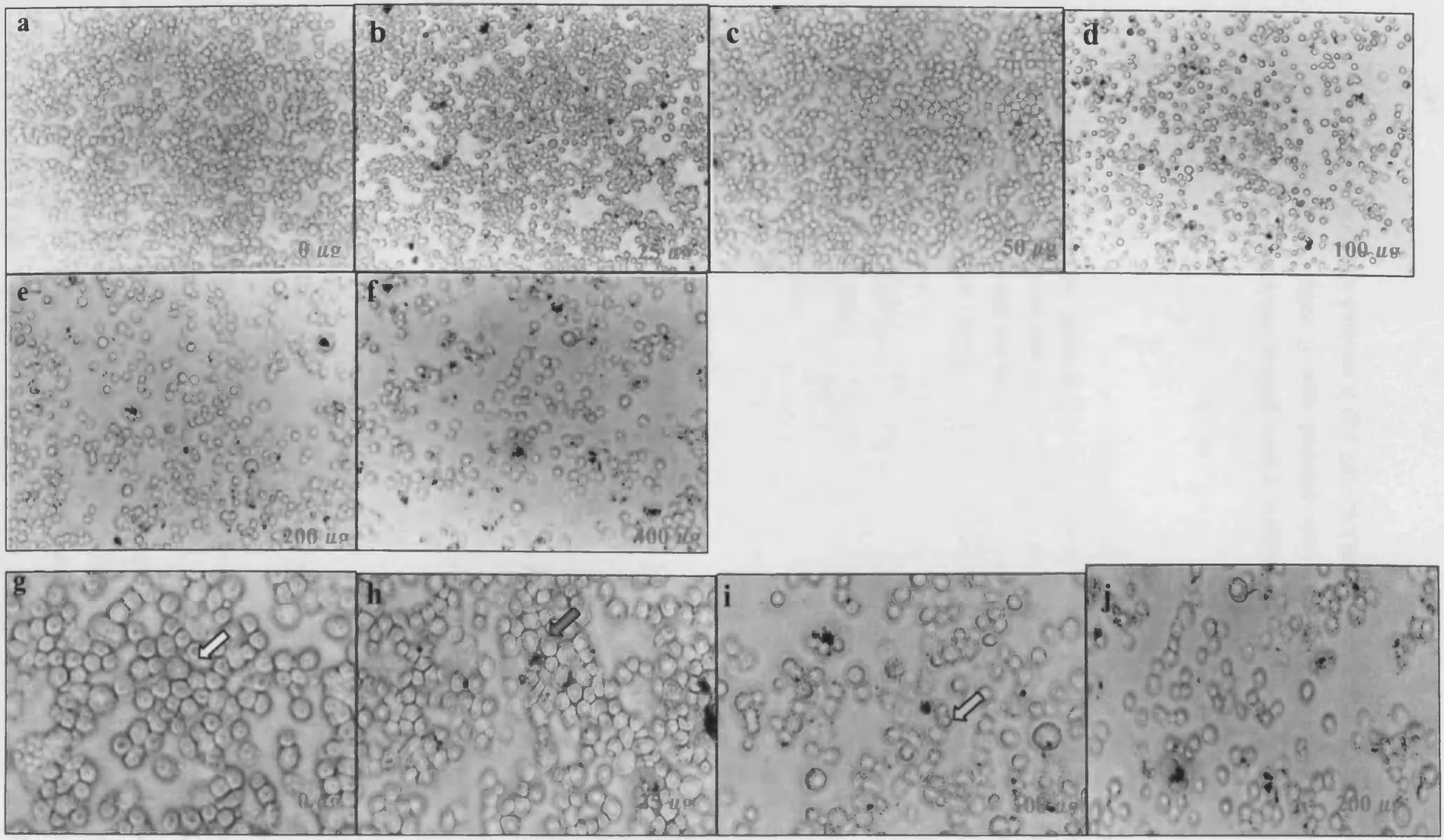
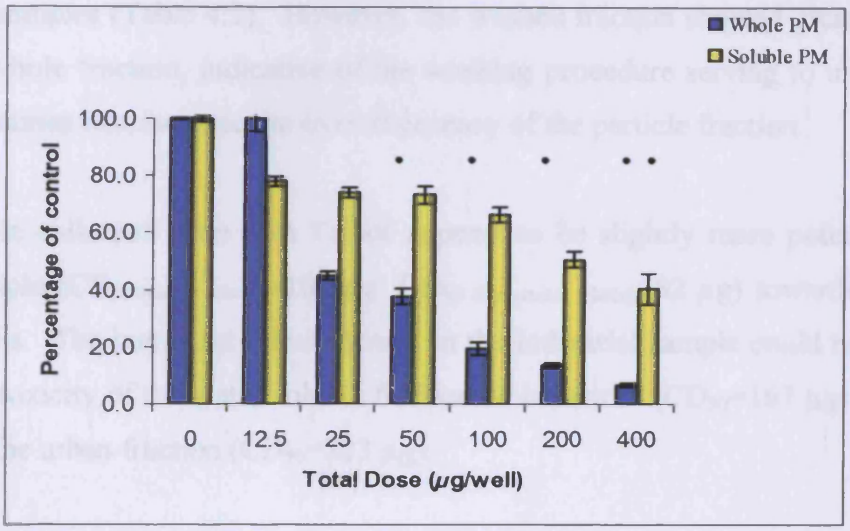


Figure 4.11: Phase contrast images (A-F at X100 magnification) of Alveolar macrophage cultures exposed to a) 0, b) 25µg c) 50 µg d) 100 µg e) 200 µg f) 400 µg of whole fraction of Cardiff PM_{10} devoid of particles. (g-j) shows increased magnification of the dosed primary cells: g) well rounded control cells h) AM's exposed to 25µg i) 100 µg exposed AM's and j) 200 µg exposed alveolar macrophages. The images show a dose dependant decrease in cells remaining attached to the culture substrata following 4 hour exposure to the dust suspended in cell media. White arrows show well-rounded, healthy attached cells from untreated cultures. Red arrows indicate cells accumulating around dust particles even at low doses (25 µg) and yellow arrows highlight regions of particles visible around the cell periphery.

Once again, the toxicity of the whole (particle containing) PM_{10} sample is considerably greater than the water soluble fraction alone (Figure 4.12). Less than 10% cells remained attached at the highest dose of whole PM_{10} (400 μg) compared to approximately 40% equivalent dose of water soluble component. A statistical difference ($p<0.05$), judged by the students t test, was found following exposure to 50 μg/well whole PM ($p<0.03$) and above and only at the highest dose of water soluble exposure (400 μg/well, $p<0.03$).



PM fraction (PT 1)	r^2	CD_{50} equivalent mass in μg/well	CD_{50} Actual dose in μg/well
Whole fraction	0.53	50.3 μg	92.9 μg
Water Soluble	0.79	271.0 μg	387.1 μg

Figure 4.12: Toxicity of PM_{10} (CF 2) in a detachment assay of primary alveolar macrophages. a) Graphical representation of dose of PM_{10} and cells remaining attached following exposure to whole blue) and water soluble (yellow) components of CF 2 PM_{10}, n=3 for each dose/sample, vertical bars are SD from the mean ● denotes statistical change ($p<0.05$). (b) Regression analysis summary showing the CD_{50} and actual dose, all masses given in μg per 96 well.

4.4.4 Comparison of CD_{50} values

The different fractions of the urban sample (whole, washed and water soluble) display differences in relative toxicity towards cultured type II cells (Table 4.2). The reactivity of the whole CF 2 sample (CD_{50}=102 μg) is retained chiefly in the durable

(washed) particles (CD_{50}=100 μg), both of these fractions are twice as potent at inducing cell detachment as the water soluble fraction (CD_{50}=223 μg). This strongly implicates the particles themselves or a factor in close association with them as dominating the observed toxicity in this assay.

Similarly, the epithelial cells exposed to the water soluble fraction of the industrial sample, Port Talbot (PT 1), were less effected than those exposed to either the washed or whole samples (Table 4.2). However, the washed fraction showed greater toxicity than the whole fraction, indicative of the washing procedure serving to unmask bio-reactive entities that increase the overall potency of the particle fraction.

The sample collected from Port Talbot appears to be slightly more potent than the urban sample ($CD_{50\ (urban\ whole)}$=100 μg, $CD_{50\ (industrial,\ whole)}$=82 μg) towards epithelial type II cells. The increased metal content in the industrial sample could result in the increased toxicity of the water soluble fraction of industrial (CD_{50}=167 μg) compared to that of the urban fraction (CD_{50}=223 μg).

There is a less clear-cut pattern of toxicity of the PM samples towards the primary alveolar macrophages. The whole urban sample (CD_{50}= 93 μg) is more reactive than the water soluble fraction (CD_{50}=271 μg) but only a negligible difference between the two fractions in the industrial sample (CD_{50} (whole)= 275 μg, CD_{50} (soluble)=271 μg).

Table 4.2: Summary table illustrating the relative toxicity of urban (Cardiff) and industrial (Port Talbot) PM_{10} and their associated fractions towards epithelial type II and alveolar macrophages *in vitro*. CD given in total mass of PM (equivalent mass and actual mass) applied to cells (μg/well).

PM_{10} source	Cell Type	Fraction	CD_{50} equivalent mass	CD_{50} actual mass
Cardiff (urban) (CF 2)	Type II	Whole	102 μg	102 μg
		Washed	100.5 μg	30.15 μg
		Water Soluble	223.0 μg	156.1 μg
	AM	Whole	92.9 μg	92.9 μg
		Water Soluble	271.0 μg	189.7 μg
Port Talbot (Industrial) (PT 1)	Type II	Whole	82.0 μg	82 μg
		Washed	70.0 μg	21.0 μg
		Water Soluble	164.5μg	115.2 μg
	AM	Whole	275.8μg	275.8 μg
		Water Soluble	271.4 μg	189.7 μg

Some chemical composition analysis has been completed on the two main samples investigated (Table 4.3, Moreno, *unpublished*). Characterisation of each PM sample included elemental analysis, the levels of six metal elements with known toxic effects are summarised in Table 4.3.

Table 4.3: Elemental analysis of the urban (CF 2) and industrial (PT 1) PM samples of six selected metals. Characterisation completed by Dr. T Moreno (2003, *unpublished*). Concentration of each metal given in μg/g of PM.

Element (μg/g PM)	**Urban Cardiff (CF 2) PM_{10}**		**Industrial Port Talbot (PT 1) PM_{10}**	
	Whole	**Water soluble**	**Whole**	**Water soluble**
Vanadium	1227	454	425	111
Manganese	1288	710	5148	1118
Iron	55174	3378	156059	4309
Copper	565	335	38	138
Zinc	25262	4480	17278	4892
Lead	3979	548	1669	409
Total metal	**87495 μg/g**	**9905 μg/g**	**180617 μg/g**	**10977 μg/g**

Correlating the CD_{50} and metal content (μg/g PM) for each sample towards both cell types (Figure 4.13) show that there is a relationship between toxicity and metal content. Comparing actual doses (Table 4.3) in order of decreasing toxicity:
PT 1(whole)>CF 2 (whole)>PT 1 (wash)>PT 1(soluble)>CF 2 (soluble)>CF 2 (wash)-
this is in strong agreement with total metal content of the PM samples. No similar trend is apparent for alveolar macrophage (Figure 4.14).

Regression analysis was performed between the total metal content and toxicity of the sample (CD_{50}) of epithelial type II cells (Figure 4.13) and the primary alveolar macrophages (Figure 4.14). Type II cells toxicity correlated better with total metal content (r^2=0.76, p=0.128) compared to macrophage toxicity (r^2= 1, p>0.9). In particular, increases in type II cell toxicity corresponded with increased iron (r^2=0.68, p<0.15) and zinc (r^2=0.69, p<0.15). In contrast alveolar macrophage toxicity showed weak correlation with iron, but was linked best with changes in vanadium (r^2=0.89, p<0.05), copper (r^2=0.74, p<0.15), and lead (r^2=0.87, p<0.06). Although generally weak correlations, these patterns do indicate trends that levels of a small number of metals or chemicals may be highly influential to the overall toxicity of the PM sample, a finding also concluded by Whittaker (2003).

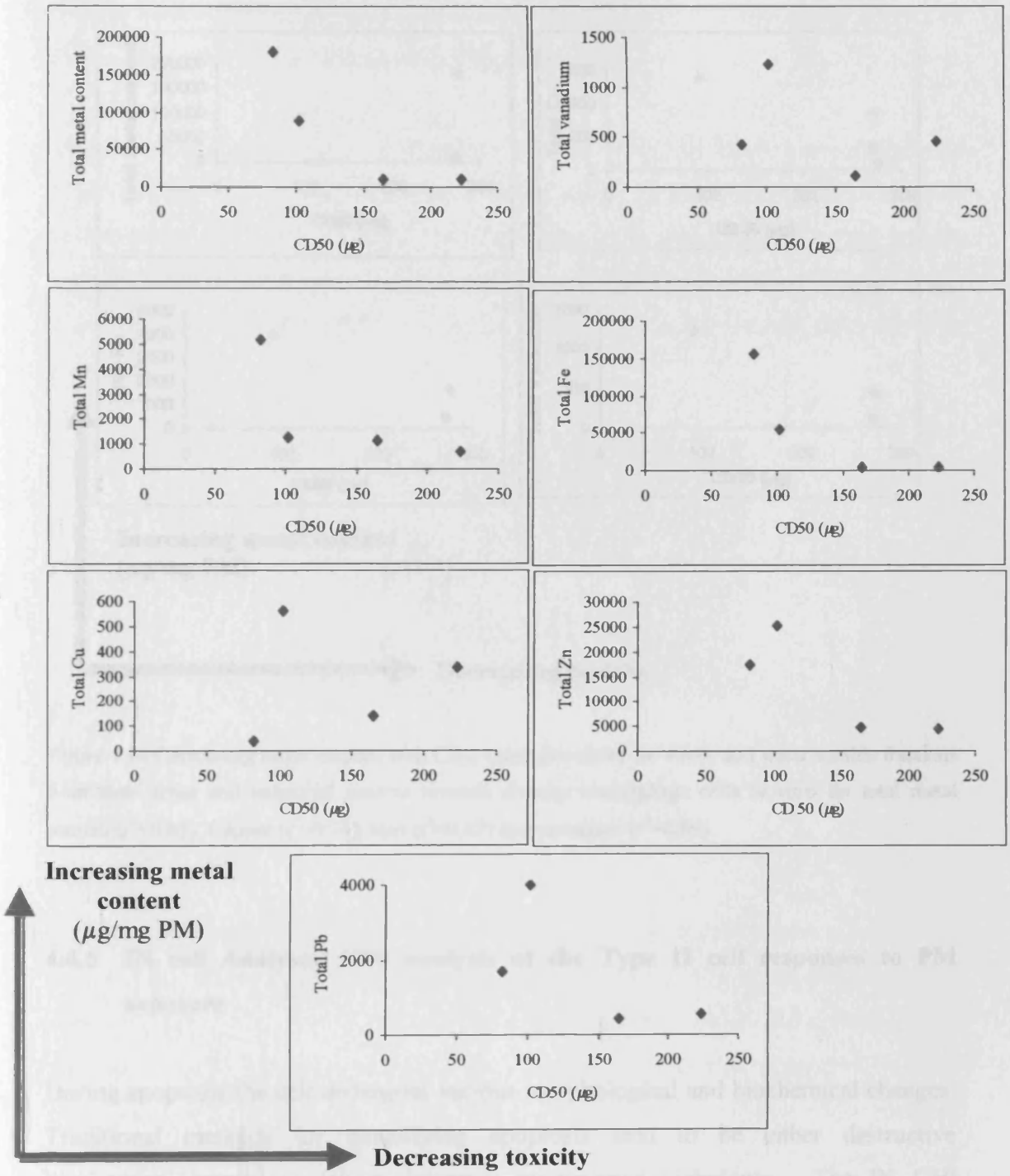


Figure 4.13:Correlating metal content with CD_{50} value calculated for whole and water soluble fractions from both urban and industrial sources towards epithelial type II cells.

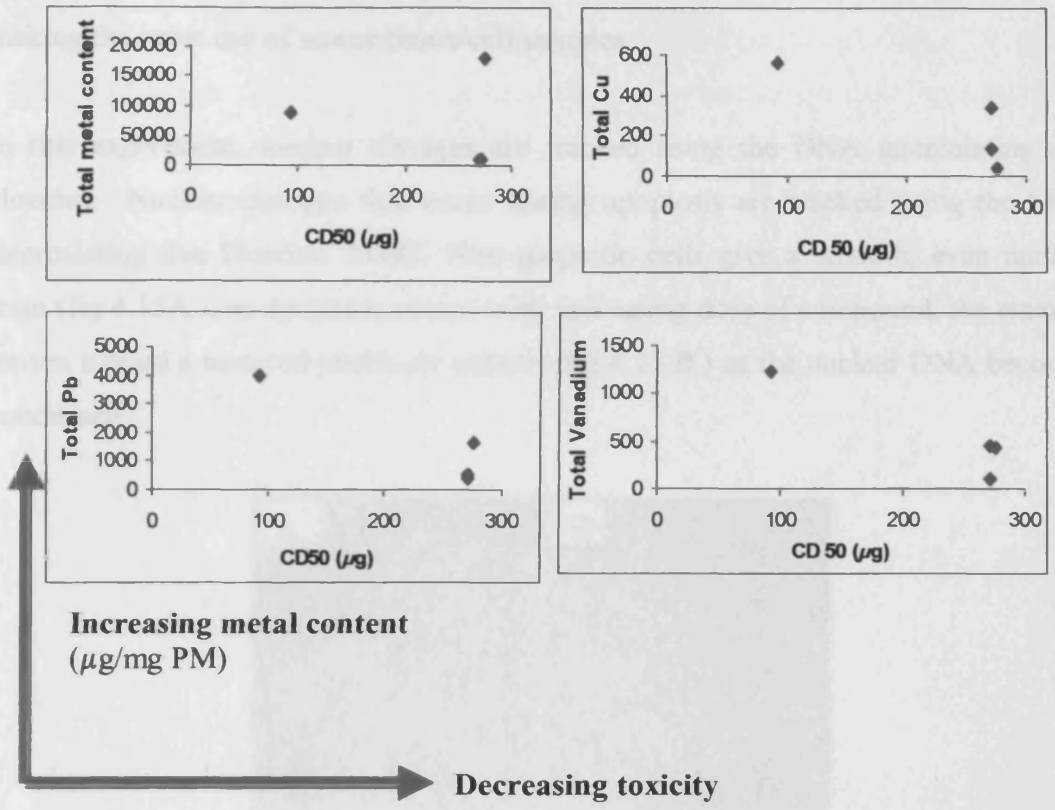


Figure 4.14:Correlating metal content with CD_{50} value calculated for whole and water soluble fractions from both urban and industrial sources towards alveolar macrophage cells *in vitro* for total metal content (r^2<0.05), Copper (r^2=0.74), lead (r^2=0.87) and vanadium (r^2=0.89).

4.4.5 IN cell Analyser 1000 analysis of the Type II cell responses to PM exposure

During apoptosis the cell undergoes various morphological and biochemical changes. Traditional methods for quantifying apoptosis tend to be either destructive biochemical assays, or labour intensive microscopic techniques. The IN Cell Analyser™ 1000 combines non-destructive methods of quantitation of morphological and biochemical changes using specifically targeted fluorescent dyes in multiplex high throughput with real-time analysis. In this manner several different endpoints can be monitored on a cell-by-cell basis or by using a population average simultaneously. The multiplex nature of the data acquisition increases the accuracy of the information

as it is derived from the same population of cells. This has the added benefit of making the most use of scarce tissue/cell samples.

In this experiment, nuclear changes are tracked using the DNA intercalating dye Hoechst. Nuclear changes that occur during apoptosis are tracked using the DNA intercalating dye Hoechst 33342. Non-apoptotic cells give a smooth, even nuclear stain (fig 4.15A.), as apoptosis occurs with increasing dose of compound, the staining moves toward a textured punctuate pattern (fig 4.15.B.) as the nuclear DNA becomes condensed.

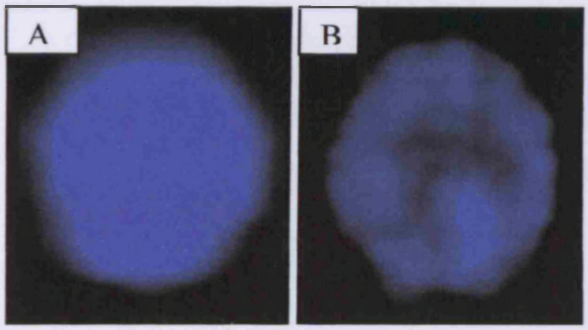


Figure 4.15. A: Control nucleus (Murine hepatocyte) showing smooth staining pattern following Hoechst 33342 staining. B: Hoechst stained nuclear condensation observed following exposure to an apoptosis-inducing agent for 4 hours (Dr S. Murphy, *in press* 2004)

Hoechst and annexin staining of whole PM exposed type II cells reveal a decrease in total cell nuclei fluorescence upon increasing exposure to whole PM (Figure 4.16a) corresponding to a reduction in intensity upon condensation of the cellular nuclei associated with the onset of apoptosis and a simultaneous dose dependent increase ($P<0.05$) in annexin staining, corresponding to an increased incidence of the onset of apoptosis in exposed cultures (Figure 4.16b). The images of control and exposed cell cultures (Figure 4.16c) illustrate the increased incidence of positive annexin stain, although less apparent is the change in appearance of the nuclei, which change from a smooth stained pattern to one similar to that shown previously (Figure 4.15b) indicating the onset on nuclei condensation in effected cells. Regression analysis on the decrease in cellular fluorescence (Hoechst's stain) predicts a CD_{50} value of 99.4 μg, approximately equivalent to the CD_{50} using the cell detachment assay using equivalent PM sample (Cardiff 2)(102 μg). Similar regression analysis on cell data

following exposure to water soluble component of Cardiff PM (Figure 4.17) to the same type II cell cultures estimate a CD_{50} value of 132.7 μg (actual dose =189.6 μg). This is below the value predicted by the cell detachment assays (223 μg equivalent dose of whole PM, 318 μg actual dose, Table 4.2). These assays confirm the cell detachment conclusion that PM exposure does result in cellular changes including detachment and cell death.

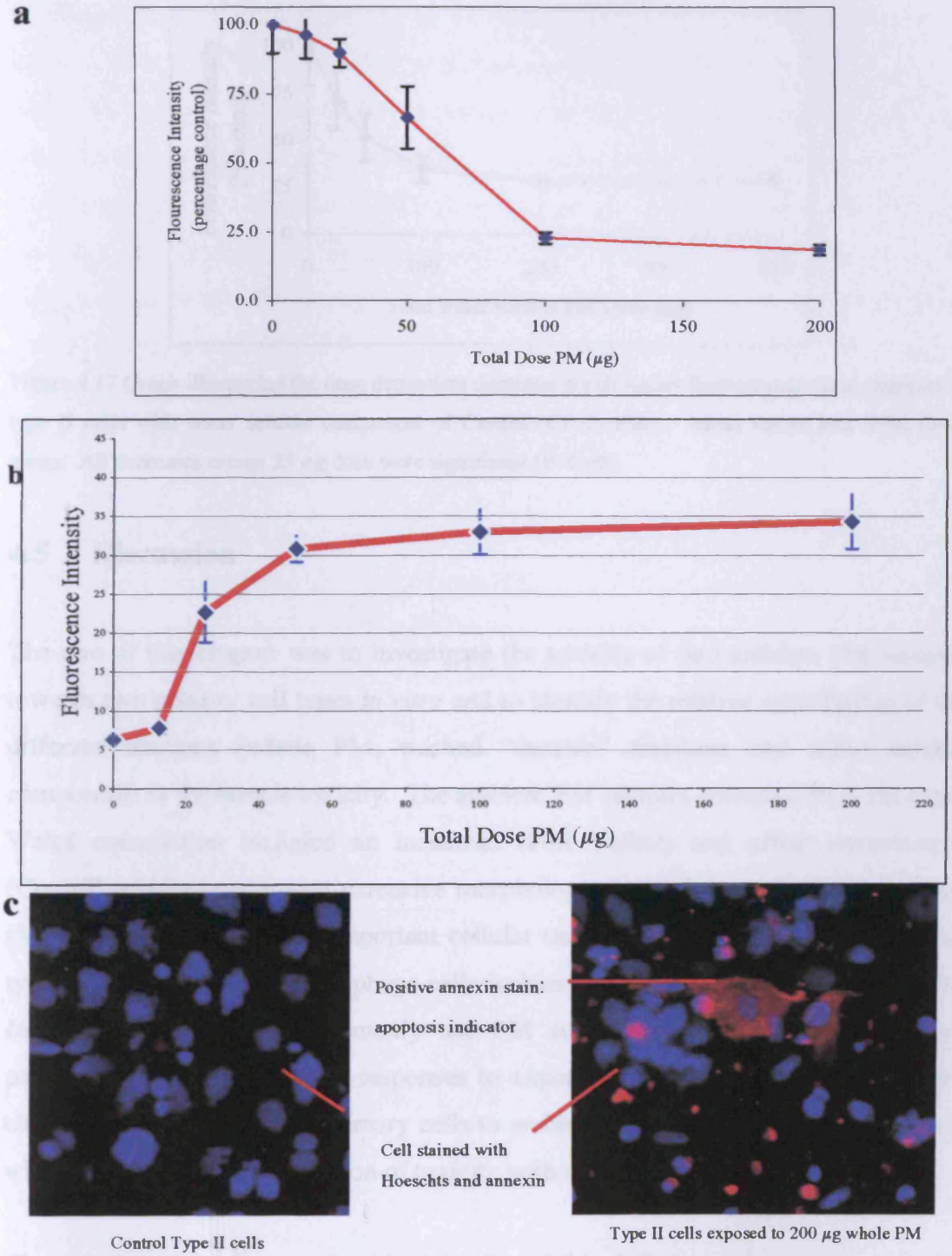


Figure 4.16:(a) Dose response decrease in cells remaining fluorescent upon exposure to whole PM (CF 2) (b) Dose dependent increase in annexin staining upon exposure of type II cell cultures to whole PM_{10}. Mean value and SEM range shown (n=6). All data points are significantly different from control ($p<0.05$). (c) Images of cells (control and culture exposed to 200 μg whole PM). Cell cultures stained with Hoechst's (blue) and annexin (red) showing increases expression of the apoptotic signal (PS on the outer surface of the cell) following PM exposure accompanied by changes in nuclear morphology.

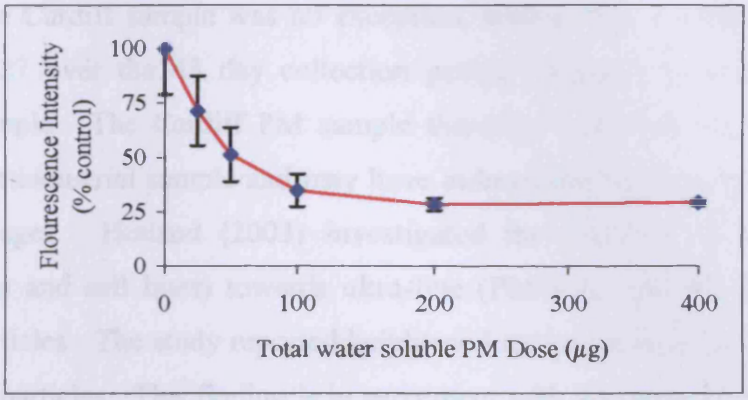


Figure 4.17 Graph illustrating the dose dependant decrease in cell nuclei fluorescence upon exposure of type II cells with water soluble component of Cardiff (CF 2) PM_{10}. Mean values and SEM (n=6) given. All decreases except 25 μg dose were significant ($P<0.05$).

4.5 Discussion

The aim of this chapter was to investigate the toxicity of two ambient PM samples towards two primary cell types *in vitro* and to identify the relative contribution of the different fractions (whole PM, washed "durable" fractions and water soluble component) to the sample toxicity. The ambient PM samples collected from the south Wales conurbation included an industrial (Port Talbot) and urban environment (Cardiff) and had undergone extensive morphological and chemical characterisation (Moreno et al, 2003). Two important cellular targets within the lung: the epithelial type II cell and alveolar macrophage cells isolated from the rat were exposed to PM. *In vitro* toxicity studies commonly use PM surrogates including diesel exhaust particles to assess the cellular responses to exposure. However, these experiments characterise the response of primary cells to ambient samples, full characterisation of which has allowed the correlation of toxicity with metal content.

The alveolar macrophage and epithelial cells exhibited distinct susceptibility towards the urban and industrial PM samples. Type II epithelial cells showed greater sensitivity towards the industrial sample (PT 1) compared to urban sample (CF 2), in direct contrast to primary alveolar macrophages. Such differences in susceptibility may be attributed to the differences in particle size distribution between the two samples. A typical urban sample may contain up to 80 % by number of road related pollution (including diesel exhaust particles) and is highly influenced by the finer

fraction. The Cardiff sample was no exception, with a $PM_{2.5}$ to PM_{10} ratio ranged from 2.84-4.27 over the 43 day collection period compared to 1.08-1.94 for the industrial sample. The Cardiff PM sample therefore contained more particles per mass than the industrial sample and may have induced the heightened response from the macrophages. Hetland (2003) investigated the response of epithelial cells (primary cells and cell lines) towards ultra-fine (PM<0.1), fine ($PM_{2.5}$) and coarse ($PM_{2.5-10}$) particles. The study reported heightened responses in primary cell cultures to the coarse particles. This finding is in agreement with the present study, in that the coarse particle dominated industrial sample displayed greater toxicity towards the epithelial cells than the fine particle dominated urban sample.

The relative contributions of the different fractions of the ambient PM_{10} samples were assessed in both PM samples. It was found that most of the bioreactivity of the whole PM samples towards both type II and alveolar macrophage cells were retained in the durable particles, indicating that chemical or physical characteristics retained by the particles themselves confer significant toxicity in this system. This is in agreement with studies by Imrich (2000), who reported strong correlations of AM cytokine release and concentrated ambient air particles, specifically the insoluble fraction and failed to find any correlation with any metal component within the insoluble particles including iron, manganese and vanadium.

Correlating metal content and cellular toxicity identified distinct trends between the two cell types. Strong correlations with total metal content, iron and zinc and type II cells were apparent. Such a finding corroborates the heightened cellular responses with the industrial sample that contained over three times the level of iron than that of the Cardiff sample. Alveolar macrophage toxicity was particle dominated rather than dependent on metal content. Therefore, poor correlation with total metal content and macrophage toxicity was observed, but associations could be made between 3 metals (copper, vanadium and lead). These findings collectively indicate distinct mechanisms of toxicity and cellular targets of different metals within the lung.

Most of the data from the present study strongly implicated the importance of the durable (washed particle) component in the toxicity endpoint measured. This correlates with the work reported in this thesis (Chapter 2) of the histopathological

responses observed with instilled PM_{10}. However, *in vitro*, cell free assays have indicated that the water soluble fraction of PM, containing bioavailable metal components represent the most damaging fraction probably by oxidative processes (Greenwell et al., 2002). In truth, both the durable (which may still contain metal components) and the bioavailable metals in the water soluble fraction can provide toxicity. In this context it is noteworthy that the alveolar macrophages being phagocytic are most sensitive to the PM sample with an increased proportion of finer particles (indicated by an increased PM_{10} to $PM_{2.5}$ ratio). The type II cell, which is not noted for engulfing particles, displays sensitivity towards the metal components of the PM and is most sensitive to the PM with a larger metal component (Port Talbot). Also worthy of note is the differences in between cell free assays, cellular *in vitro* studies and animal instillation investigations. In the cell free assays the PM toxicant has direct access to DNA (and causes scission). This may or may not occur in cell systems, which require the uptake of the (metal) toxicant, processing in the cytoplasm and potential transport to the nucleus in enable interaction with the DNA. The situation is far more complex *in vivo* in that instilled bioavailable metals may react with antioxidants in the epithelial lining fluid and then, being water soluble, rapidly enter the bloodstream (e.g. vanadium reaches the heart via the lung circulation in less than 15 minutes post exposure, Dreher et al., 2002). Such complexity is yet to be achieved *in vitro* where bioavailable metals are not cleared and thus remain in contact all the time with target cells.

Some pilot studies using the IN Cell Analyser™ 1000 identified changes in patterns observed in the nucleus following the exposure of primary cultures of epithelial type II cells exposed to ambient PM_{10}. The results were derived from the same population and hence gave statistically robust results especially given the variable nature of primary cell cultures. The multiplex nature of this equipment could in future studies substantially reduce the quantity of primary cells and compound required for toxicological assay.

Nuclear condensation typical of late stage apoptosis was clearly visible and quantifiable at all concentrations of PM and equivalent water soluble fractions adopted in this assay. These assays illustrate that apoptosis has been induced in epithelial type II cell cultures following exposure to whole and water soluble fractions

of Cardiff PM. Thus, the multiplex assays agreed with the results from the cell detachment assay and show that the whole PM sample is more reactive than the water soluble component alone.

In summary, the work in this chapter has defined the susceptibility of primary lung cells *in vitro* to whole PM and its constituent durable particles and bioavailable metal containing water soluble fraction. The study has established doses at which toxicity can be observed and implicated apoptosis as a possible mechanism in this process. It would be useful if such cultures could be employed in expression profiling the PM-induced changes in target lung cells and such an investigation is reported in the following chapter (Chapter 5).

Chapter 5.0

Preliminary Genetic analysis of primary cells

5.0 Preliminary Genetic analysis of cells

5.1 Introduction

The use of animals as models for investigation has provided an insight into toxicity. However, the extent that such models resemble humans, or the physiological relevance of the extensive exposures involved, has often been debated. The use of large number of animals over a prolonged time scale has been hampered by both ethical and economical concerns. The development of modern techniques that may serve to reduce reliance of such studies on animals has been encouraged. It has been increasingly apparent that in order to gain insights into biological mechanisms of toxicity, it is necessary to ascertain whether the toxin has specific cellular targets *in vivo* and to characterise the responses of these cells to exposure (Nemery et al., 1993). Establishing *in vitro* techniques in toxicology for this purpose using cell lines, primary cells from experimental animals and human cells has clarified many cell responses and has increased the use of human cells, addressing the extrapolation problems of experimental animal data to the human situation.

The nature of the initial interactions between particles and the lung will determine the subsequent pulmonary effects. Lung cells at the forefront of these interactions are the alveolar macrophages and the pulmonary epithelial cells. Phagocytosis during the macrophage mediated lung clearance will result in the release of inflammatory mediators, inducing further activation of phagocytes and influx of inflammatory cells. Overwhelming this particle clearance mechanism will result in the release of excess inflammatory mediators, which may become detrimental to the pulmonary epithelium. These inflammatory mediators may further compromise the efficiency of macrophage mediated clearance (Oberdorster et al., 1992) resulting in increased particle longevity at the respiratory surface and increased interactions with the epithelium (increasing the risk of direct toxic effects and uptake by the epithelial cells). Monn (2002) reported that exposure of alveolar macrophages to PM extracts results in decreased cell viability and compromises phagocytic and immune capabilities to subsequent inert particle exposure.

Previous chapters in this thesis have established that the Cardiff (urban) PM_{10} sample provokes a mild response *in vivo* in healthy rats and exposure to PM *in vitro* results in moderate toxic effects towards homogenous primary lung cells. A large range of studies have investigated the *in vitro* responses of pulmonary cell lines including macrophage and epithelial cell lines, towards toxic compounds with well documented responses *in vivo*. These studies have identified changes in gene and protein expression of a selected range of inflammatory mediators and cell responses following exposure, commonly interleukins and NFκB, antioxidant capabilities in the cells and the effect on DNA integrity in cells (Comet Assay- Knaapen et al., 2002. Plasmid assay-Greenwell et al., 2002) as a measure of bioreactivity. *In vitro* studies have revealed a complex series of gene expression changes associated with PM or PM surrogate exposure, inextricably linked with particle size (surface area) (Stone et al., 1998) and metal content (Wilson et al., 2002) of the samples. Stone (1998) used the macrophage cell line MM6 (Monomac 6) in exposure studies with characterised particles (carbon black) and found differential responses of the cells to the fine and ultra-fine fractions of this PM surrogate. The release of calcium was found to be a consistent cellular event, within minutes of exposure and has been postulated as a mechanism to drive the stress and inflammatory response in these cell types.

Consistent changes in early (3 days) gene expression were detectable in whole lung tissue samples following instillation of the well-characterised ambient sample (Chapter 2) and candidate genes involved in the pulmonary responses to different PM fractions have been previously described (Chapter 3). The present chapter describes the novel application of expression profiling of homogenous primary cells following exposure to low doses of well-characterised ambient $PM_{10-0.1}$ samples. The chief aim of this study was to apply the macroarray technology to *in vitro* toxicological assessment using cultured primary cells and to assess the system as a suitable model for future screening of pulmonary toxicants. Previous gene profiling of lung tissue (Chapter 3) identified key changes in gene expression associated with PM exposure. Expression profiling of primary lung cells will establish whether similar expression changes can be linked to one or more cell types within the lung.

An earlier chapter (Chapter 4) identified the doses of whole PM_{10} (Cardiff and Port Talbot) required to cause the detachment of 50% (CD_{50}) of a primary culture of alveolar macrophages and epithelial type II cells *in vitro*. Experiments run in

conjunction with Amersham Biosciences (Dr Murphy, 2003) (chapter 4) confirmed that low-level exposure to whole PM_{10} induced cellular changes indicative of apoptosis within the primary cell cultures. Thus the present study used a low-level exposure (CD_{20}) to whole PM_{10} to identify whether these cellular changes resulted in consistent genetic changes associated with exposure.

5.2 Materials

5.2.1 Materials and Suppliers

RNeasy™ Mini kit (74104) containing all appropriate buffers, solutions and vials and Deoxyribonuclease I, QIA™ shredder (79654) purchased from Qiagen.
β-Mercaptoethanol (436022 A) was obtained from BDH Biochemical Laboratory Supplies, Poole. Ultra-pure (HPLC) Grade Ethanol (95% v/v) (CAS 64-17-5) was bought from Fisher Life Sciences.

Tri-reagent ™ solution (T-9424), Isopropanol (>99% v/v) (I 9516) and Chloroform (>99% v/v, containing no iso-amyl alcohol or additives) (C 2432) were purchased from Sigma – Aldrich.

QuickPrep® Micro mRNA Purification kit was purchased from Pharmacia Biotech and all appropriate solutions provided.

Agarose (Type 1A)(A 0169), 20X TBE* (T 4415), Ethidium Bromide (10mg/ml) (E 1510) and Loading Dye (G 2526) were all purchased from Sigma – Aldrich.
**Diluted to 0.5% v/v TBE in distilled water, prior to use.*

The nylon Atlas™ Rat 1.2 array system (7854-1) was purchased from BD Biosciences (formerly Clontech). Each kit containing: Cot-1 DNA (1 mg/ml), 10 X Termination mix (0.1 M EDTA, 1 mg/ml $MgCl_2$, pH8.0), Reaction buffer (250 mM Tris-HCl), 375 mM KCl, 15 mM $MgCl_2$, pH 8.3), 10 X dNTP mix (5 mM of each dNTP except dATP), DTT (100 mM), 10X CDS Primer mix, Powerscript MMLV Reverse Transcriptase and nucleospin™ extraction/purification columns and wash solutions.

[α-^{32}P]-d.ATP (10 μCi/mmol, Cat No. PB 10204) was purchased from Amersham, UK. Sheared Salmon Testes DNA (D7656, 10 mg/ml) was bought from Sigma. Atlas™ Image 2.0 and Atlas™ Navigator (BD Biosciences) were used to interpret the array data. 20% w/v SDS solution (ultra pure) (EC 874) and 20% w/v SSC Solution (ultra pure) (EC-873) were purchased from National Diagnostics. The Kodak Phospho-imager screen (Kodak 118 8077) was purchased from Biorad.

5.3 Methods

5.3.1 Cell isolation, Culture and Exposure

Murine alveolar macrophages were isolated from the saline lung lavage of healthy, untreated animals as previously described (Section 4.3.1). Primary epithelial cells were isolated using trypsin digestion and Percoll™ purification from rat lung tissue as described by Oreffo et al., 1987), also described previously (Chapter 4, section 4.3.1). Alveolar macrophages were cultured in DCCM 1 media (2% v/v L Glutamine, 1% v/v Penicillin and streptomycin) on plastic for four hours (37°C, 5% CO_2) prior to exposure. Epithelial cells were cultured until a well-spread, confluent culture was achieved and whole PM applied in fresh DCCM-1 media (2% v/v L-Glutamine, 1% v/v Penicillin and Streptomycin, 2% v/v Ultroser G). Cells were exposed under culture conditions to the total dose of whole PM that caused a total cell detachment of 20% (CD_{20}) of the cell culture (Chapter 4). A dose resulting in the detachment of 20% of a cell culture relative to an untreated culture (CD_{20}) (Chapter 4) was calculated (μg/mm^2 of culture substrata) for each PM sample and applied to cells. A single length of exposure was adopted for each cell type for all gene expression studies based on literature investigating cell toxicity (6 hour – epithelial type II, 4 hour- alveolar macrophage). Cells were removed from the culture substrata and pooled with unattached cells and were recovered by centrifugation from the cell culture media and stored (-80°C) prior to RNA isolation.

Control cells received fresh DCCM-1 media devoid of PM in lieu of exposure and were cultured under the same conditions alongside PM-exposed samples.

5.3.2 RNA Extraction and Macroarray Analysis

Total RNA was isolated and treated to remove any residual DNA contamination using the Qiagen RNeasy™ protocol (Chapter 4). Tri-reagent ™ (Sigma) was also investigated but more efficient collection and DNase treatment was achieved using the Qiagen method.

Expression profiling on the isolated total RNA from exposed and control samples of alveolar macrophage and epithelial cells were completed in accordance with protocols previously described in full (Chapter 3). In brief, radio labelled cDNA probes were purified using column filtration and centrifugation and hybridised to the nylon arrays overnight with constant agitation. Any unbound probe was removed using wash solution 1 (0.1 % v/v SSC, 0.5 % v/v SDS) until the radioactivity counts detectable on the membrane had fallen to 10-15 dps. The membrane was sealed and exposed to a phosphoimage screen (Kodak) and left for 5-7 days depending on the final radioactive counts. The screen was subsequently scanned using a phosphoimager (Kodak) and visualised using Atlas™ image 2.01a (BD Biosciences), as previously described.

To ensure that all array analyses were similar, all variables were set as equal. The ratio signal threshold was reduced on all aligned arrays to 50% and all genes were reported to an excel worksheet, all analyses were therefore conducted on similar analysis outputs within the excel package.

The most appropriate method of gene analysis was investigated in chapter 3. In brief, $\log_{10}$ transformation and normalisation of each control and exposed expression profile was completed to produce normally distributed data, allowing the application of statistical assumptions. The calculation of a mean sham-treated control and ±2 SD range in relative expression of each gene allowed the identification of genes with an expression outside of this range following PM exposure. A parametric students t-test was used to calculate statistically significant changes in gene expression. These indices were used to apply a degree of stringency to filtering the candidate genes involved in the genetic response to the PM_{10}.

5.4 Results

5.4.1 Isolation and Preparation of RNA and Macroarray Procedures

RNA preparations of superior yield and purity were isolated using the RNeasy RNA extraction kit (Qiagen). Type II epithelial cells and macrophages were disrupted and homogenised using both the QIA shredder and needle as outlined in the protocol to optimise RNA yield and RNA was isolated from as little as 6 million cells. RNA was isolated in accordance to the Qiagen technique, initial RT PCR analysis showed that the DNase treatment did reduce the yield of RNA from the preparation but the active enzyme was successfully removed from final preparation.

Production of quality RNA in significant quantity has proven extremely difficult from the primary isolates. There are a number of explanations for this, firstly, the yield and concentration of RNA from both cell types was highly variable and generally very low. This proved problematic because the Atlas™ nylon macroarray required large amounts of total RNA (2 μg) at high concentration (maximum 2-4 μl). Secondly, particularly in PM-exposed cells were collected as a cell pellet prior to lysis. Thus, the cell lysate inevitably contained some particles, which have been shown to incite oxidative damage to DNA (Greenwell et al., 2003). Therefore, some loss of RNA and DNA may occur prior to RNA isolation. Alveolar macrophages also have an extensive array of digestive enzymes and so upon lysis, the activity of these molecules may further degrade and destroy the RNA. Whilst many of the particles were removed by centrifugation of the lysate prior to RNA isolation, it does not however remove any reactive soluble fraction from the preparation. Again oxidative bioavailable metals may be responsible for DNA and RNA losses prior to genetic analysis.

Many of the problems with the RNA and array procedures have been addressed, certainly for whole lung preparations, however, arrays using the primary isolates have proven to be, and still are, significantly less reliable. The low success rate of achieving suitable array images for the exposed primary isolates meant that the procedure was costly, both in terms of cell isolates from animals and PM_{10} sample. Therefore expression profiling was only carried out successfully using the urban (Cardiff, CF 2) PM_{10} sample on epithelial type II cells (n=4) and industrial (PT 1) PM_{10} sample on alveolar macrophages (n=2). The low availability of RNA prevented

confirmation of macroarray data using alternative techniques, and there were considerable differences in gene profiles between cell isolations from different animals.

There was a high incidence of non-specific hybridisation (background noise) on the arrays from primary cell isolates, at first attributed to lower quality starting RNA material and poor probe purification. The background noise became increasingly dependent on the batch of array membranes and reagents used and it became apparent that problems with certain batches of arrays membranes and consumables were a contributory factor.

5.4.2 Compilation of Candidate Genes- Epithelial Type II cell

Expression profiling epithelial type II cells in response to Cardiff (CF 2) PM_{10} exposure (6 hours) were successfully completed (n=4) and compared to sham-treated controls (n=4). Images of each array from epithelial type II cells are included in Figure 5.1 and 5.2.

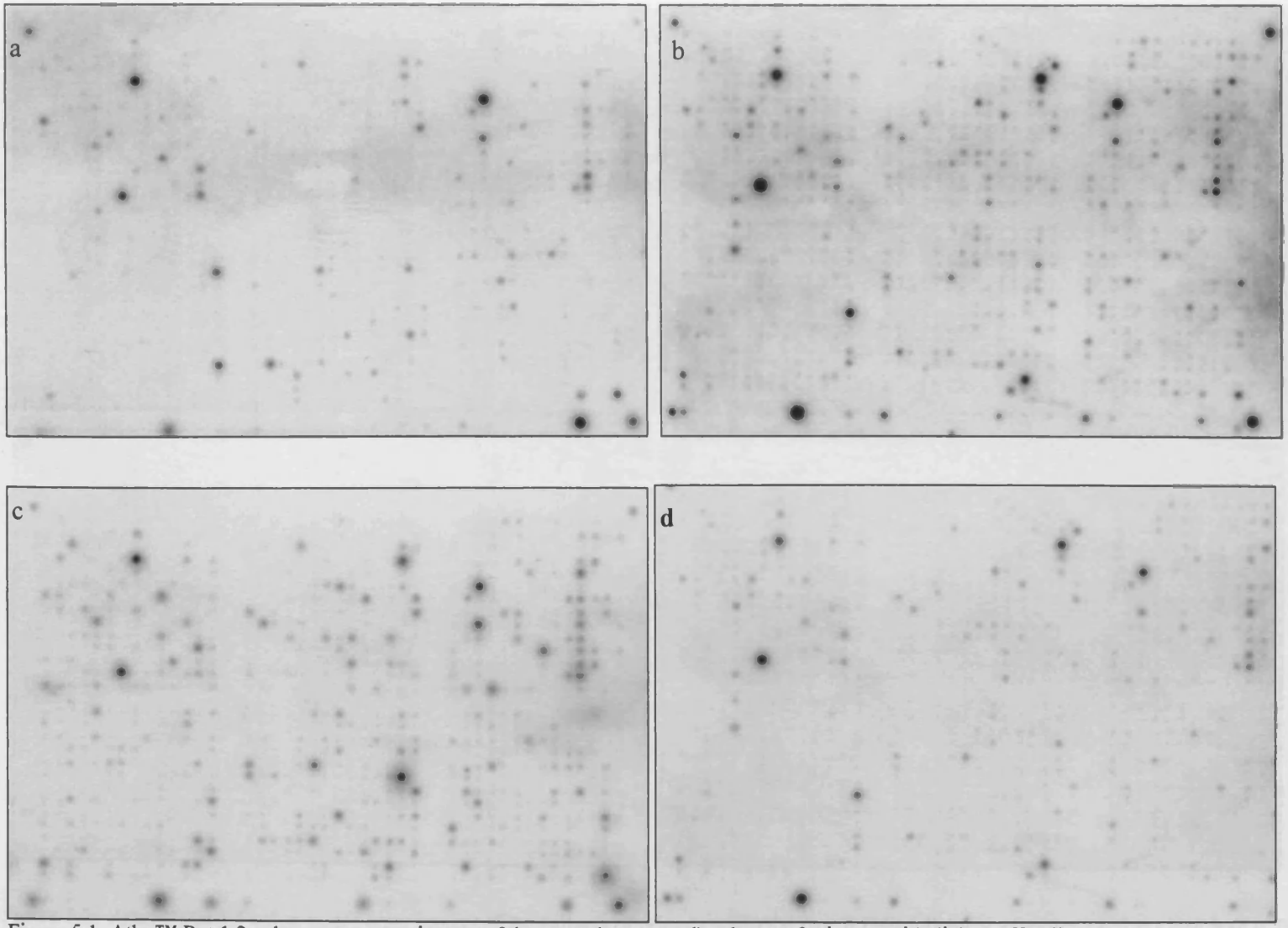


Figure 5.1: Atlas™ Rat 1.2 nylon macro array images of the control (untreated) cultures of primary epithelial type II cells.

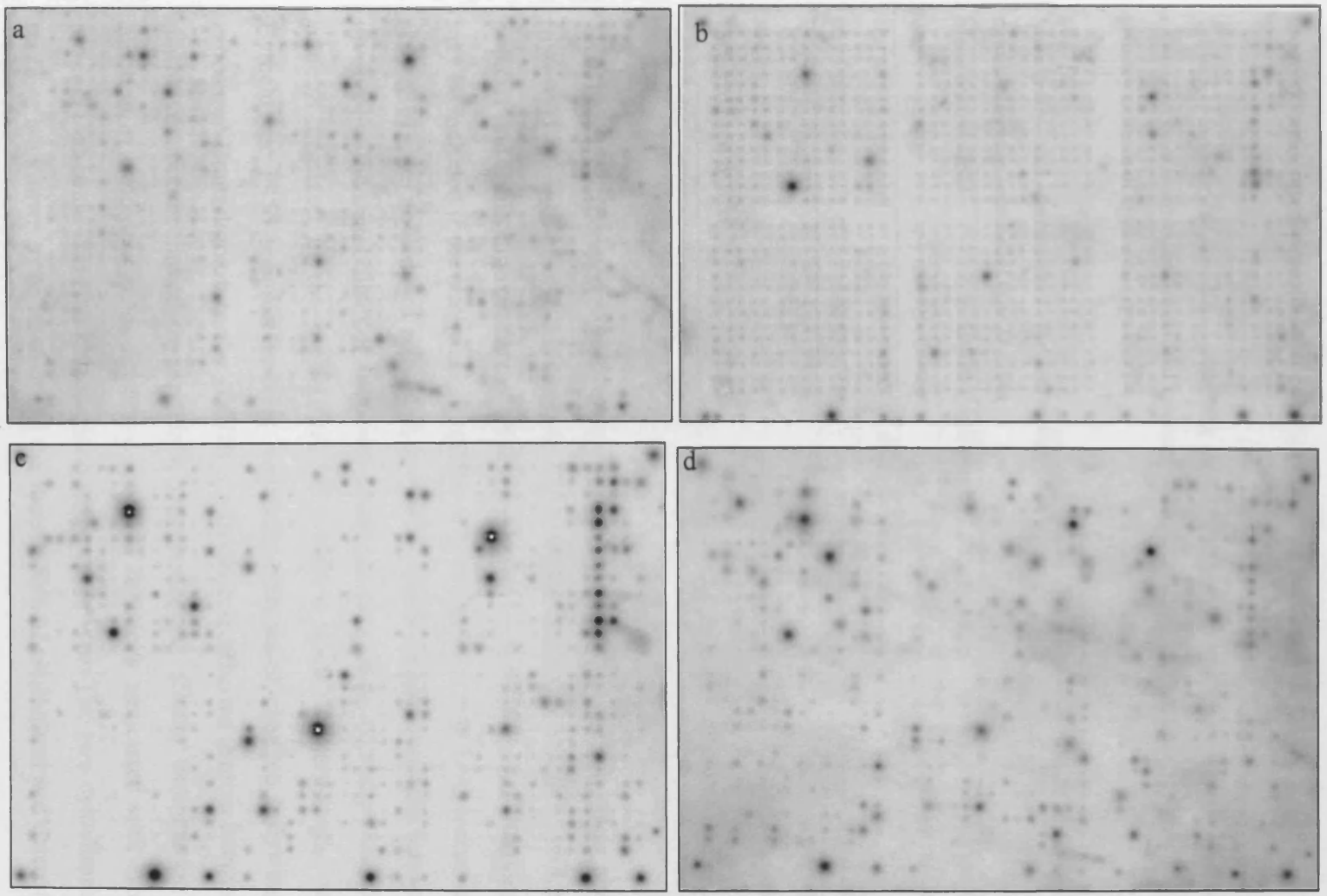


Figure 5.2: Atlas™ Rat 1.2 nylon macro array images of the cultures of primary epithelial type II cells following exposure (6hr) to a low dose (CD_{20}) of Cardiff (CF 2) PM_{10}

Following transformation and normalisation, candidate gene lists were compiled. The analytical approach confirmed by qPCR in chapter 3 was adopted for this experiment. The effect of increasing the stringency and adopting statistical (student t-test) and control±2SD range filtering is summarised in Table 5.1. A total of 12 genes were expressed outside a mean control±2SD following the low level exposure (CD_{20}) to the PM sample. These genes were involved in xenobiotic metabolism (3), transporters and ion channels (2), cytokines and extracellular communication proteins (5) and receptor associated proteins/cell cycle controlling protein (1).

Table 5.1: Summary of gene lists undergoing increased stringency of filtering to achieve a final list of candidate genes involved in the cellular response to an *in vitro* low exposure to Cardiff (CF 2) PM_{10} by primary cultures of epithelial type II cells.

Stringency and parameters for screening for candidate genes (epithelial type II cells)		**Number of genes**	
Mean control±2SD (n=4)	No minimum fold change	111 genes	3 genes up-regulated
	≥ 2 fold change	12 genes	3 genes up regulated
Unpaired t-test only $p \leq 0.05$	No minimum fold change	54 genes	
	≥ 2 fold change	11 genes	All min.4 fold change
Mean control±2SD and Unpaired t-test $p \leq 0.05$	≥ 2 fold change	2 genes 3 genes (if $p \leq 0.1$)	2 genes up regulated 1 gene down regulated

Two genes are deemed significantly altered by both the ± 2SD range and t-test ($p \leq 0.05$), ≥ 2 fold change parameters: Cytochrome P450 (VII) (B01c, switched on, $p=0.05$) and Brain digoxin carrier (B02b, switched on, $p=0.05$). An additional gene may be included if the statistical significance is extended to $p \leq 0.1$: Cytochrome P450 (14) (B01g, x3 fold down regulated, $p=0.09$). Both cytochromes are known to be involved in the metabolism of xenobiotics and can also be functionally classified as a proteins involved in the metabolism of complex lipids. Interestingly, the two Cytochrome P450 subunits undergo opposing directional changes following PM_{10} exposure. Brain digoxin carrier (B01b) is a facilitated diffusion protein integral to the plasma membrane linked to xenobiotic transportation. Closer examination of the function of candidate genes reveals a range of functions associated with each gene product that could contribute to the lung response *in vivo*. The two cytochrome P450 proteins are known to be involved in the metabolism of cholesterol and detoxification

of xenobiotics. B01c (cholesterol P450 1A1) catalyses the rate determining reaction in the conversion of cholesterol to bile acids. In contrast, the down regulated cytochrome P450 51A1 is catalyses the biosynthesis of cholesterol. The third gene, brain digoxin carrier protein is a member of the organic anion transporter proteins *(oatps)* and functionally related to the multi drug resistance protein (B01n, Table 5.2). These gene products are known to be present in barriers such as the blood brain barrier in endothelial cells or epithelia, cells connected by highly regulated tight junctions and regulate the movement of waste products and xenobiotics across the barrier. These polyspecific organic anion transporters are part of the ATP binding cassette (ABC) super-family and are known to function in concert with the detoxification enzymes to protect the organism from potentially harmful compounds. They are known to be expressed, under normal physiological conditions, in cells and tissues that provide a barrier for the body towards toxicant entry, typical of the lung (Hagenbuch et al., 2002). Table 5.2 comprises a list of candidate genes (12) identified as significant (mean control ±2SD range and a minimum 2 fold alteration in expression). A total of eleven genes were identified as significantly altered ($p<0.05$) by a minimum of 2 fold following PM exposure but not outside a 2 SD range of the mean control expression. These genes are shown in Table 5.3. Both lists (Table 5.2 and 5.3) contain genes involved in intracellular signalling and gated ion channels. Although these genes are not significant by both parameters (Mean control ±2SD range, $p<0.05$, minimum 2 fold alteration in expression) they represent an underlying cell response to these low level exposures.

Table 5.2: The candidate genes (12) deemed significant (outside mean control ±2SD) and minimum of two fold change (no minimum statistical significance applied). Negative fold change indicates decrease in expression relative to untreated (control) cultures of primary pulmonary epithelial type II cells. Genes in red type indicate the 3 genes shortlisted (t-test $p \leq 0.1$), minimum 2-fold change and outside mean control ±2SD. TRUE indicates the genes were expressed outside a mean control ±2SD range.

Atlas Gene code	>2SD range	Fold change	T test (p value)	Gene	Function	
B01c	TRUE	on	0.05	cytochrome P450 VII (CYP7)	Xenobiotic metabolism	Complex lipid metabolism
B01g	TRUE	-3	0.09	cytochrome P-450 14	Xenobiotic transporter	
B01n	TRUE	-2	0.15	multidrug resistance protein 2 (MDR2)	Drug resistance	abc transporter
B02b	TRUE	on	0.05	Brain digoxin carrier protein	Facilitated diffusion	Xenobiotic transporter
B02l	TRUE	-2	0.14	sulfonylurea receptor (SUR)	Signalling	
B03l	TRUE	-2	0.15	purinergic receptor	Ligand gated ion channel	
B05e	TRUE	on	0.48	skeletal muscle Na^+ channel protein alpha subunit	Voltage gated ion channel	
E01a	TRUE	-2	0.14	insulin like growth factor II (IGF-II)	Growth factor, cytokine, chemokine	
E02a	TRUE	-2	0.11	neurotrophin 5, trk and trkb activating		
E05a	TRUE	-2	0.15	interleukin-2 (IL-2)	Signalling	
E06c	TRUE	-2	0.15	Neurexophilin 1 (NEUROPHILIN)		
E07a	TRUE	-2	0.14	arrestin D	Intracellular kinase	

Table 5.3: The candidate genes (11) deemed significantly altered ($p \leq 0.05$) by a minimum of 2-fold change relative to untreated controls in primary cultures of epithelial type II cells. Genes in red type indicate the 2 genes shortlisted (t-test $p \leq 0.05$), minimum 2-fold change and outside mean control ±2SD.

Atlas Gene code	>2SD range	Fold change	T test (*p*-value)	Gene	Function
B01c	TRUE	On	0.05	cytochrome P450 VII (CYP7)	Xenobiotic metabolism
B02b	TRUE	On	0.05	BRAIN DIGOXIN CARRIER PROTEIN	Facilitated diffusion and Xenobiotic transporter
B03c	FALSE	4.5	0.03	Cyclic nucleotide-activated channel	Ligand gated ion channel
C02c	FALSE	4.0	0.04	Type 1 hexokinase (HK1)	Metabolism
C03c	FALSE	4.5	0.03	Alcohol dehydrogenase class 1	
F02j	FALSE	4.0	0.04	Ca^{2+}-independent phospholipase A2	Phospholipase
F03j	FALSE	4.5	0.03	Calbindin D28;	Calcium binding protein
F04h	FALSE	4.0	0.04	Putative protein kinase C regulatory protein	Kinase activators, cell signalling
F05i	FALSE	4.0	0.04	Frizzled-1 (FZ-1) dishevelled receptor	Receptor protein
F06l	FALSE	4.0	0.04	Secretogranin V	Trafficking and targeting
F06n	FALSE	4.4	0.04	elastase 2 precursor	Serine protease

5.4.3 Compilation of Candidate Genes- Alveolar Macrophage Cell

Expression profiling alveolar macrophages in response to Port Talbot (PT 1) PM_{10} exposure (4 hours) were successfully completed (n=2) and compared to sham-treated controls (Figure 5.3). Low replicate numbers were a consequence of extremely unreliable isolation of sufficient high quality RNA for array analysis.

There was a high degree of variability in gene expression across both control and PM exposed cell cultures. Despite the low replicate numbers, analysis of the expression profiles did produce concise lists of candidate genes (Table 5.4). The high degree of variability in gene expression across the arrays resulted in no genes being expressed outside a mean control ±2SD range. A total of 15 genes, all up regulated, were deemed to be significantly ($p \leq 0.05$) altered following exposure to PM (4 hours) by a minimum of two fold relative to sham-treated control cultures. These 15 genes (Table 5.5) comprise: ion channels (8), facilitated diffusion proteins (3), xenobiotic transporters and metabolism (3) and transcription factors (1).

Table 5.4: Summary of gene lists undergoing increased stringency of filtering to achieve a final list of candidate genes involved in the cellular response to *in vitro* exposure to Port Talbot (PT 1) PM_{10}.by primary cultures of alveolar macrophage cells.

Stringency and parameters for screening for candidate genes (alveolar macrophage)		**Number of genes**	
Mean control±2SD (n=2)	No minimum fold change	No genes	
	≥ 2 fold change		
Unpaired t-test only $p \leq 0.05$	No minimum fold change	21 genes	15 genes up regulated
	≥ 2 fold change	15 genes	All up regulated

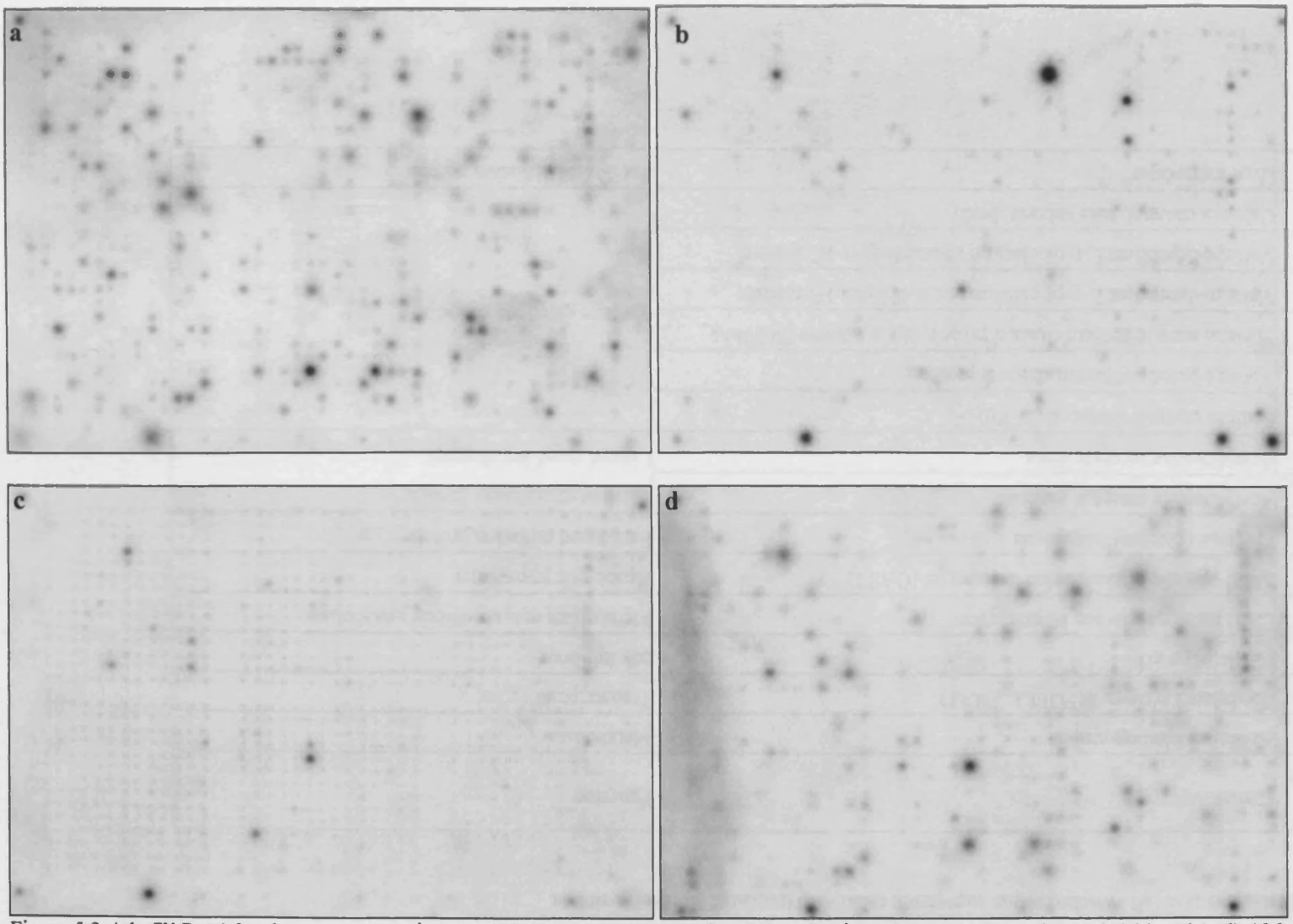


Figure 5.3 Atlas™ Rat 1.2 nylon macro array images of (a-b) control (untreated) cultures of primary alveolar macrophages (AMs) and (c-d) AMs following exposure (4hr) to a low dose (CD_{20}) of Port Talbot (PT 1) PM_{10}

Table 5.5: The candidate genes (15) deemed significant (outside mean control ±2SD) and minimum of two fold change in response to industrial PM_{10} by primary cultures of alveolar macrophages. The fold change indicates increase (all switched on) in expression relative to untreated (control) cultures.

Atlas Gene code	**Fold change**	**T-test (*p* value)**	**Gene**	Function
A10a	on	0.02	Androgen binding protein	Metabolism
A11a	on	0.03	Fos-related antigen 1 (FOSL1; FRA1)	Transcription factor
A11b	on	0.02	P21; cip1; waf1	cdk inhibitor
A14h	on	0.01	Liver carboxylesterase 10 precursor	Metabolism inc. xenobiotic metabolism
B01j	on	0.02	Multi-specific organic anion transporter (OAT1)	Xenobiotic transporter
B02f	on	0.04	Fructose (glucose) transporter	Facilitated diffusion protein
B03d	on	0.01	Acetylcholine receptor gamma	Facilitated diffusion protein
B03e	on	0.02	Acetylcholine receptor delta	Ligand gated ion channel
B04i	on	0.05	Sodium channel protein 6 (SCP6)	Voltage gated ion channel
B05d	on	0.03	Voltage-gated potassium channel protein	
B05e	on	0.01	Skeletal muscle sodium channel protein alpha subunit (SCN4A)	
B05f	on	0.01	Voltage-dependent L-type calcium channel alpha 1D subunit	
B05g	on	0.03	Voltage-dependent L-type calcium channel alpha 1C subunit	
B06n	on	0.02	Calcium channel, beta subunit, brain	
B11e	on	0.01	Urea transporter	Facilitated diffusion protein

5.4.4 Comparison of the Type II and Alveolar Macrophage responses to PM Exposure

There appears to be some correlation between the alveolar macrophage and epithelial type II cell responses. A multi-specific organic anion transporter (*oatp*, B01j) is up-regulated as a consequence of PM exposure in type II cells, a similar gene (B02b) is altered in a likewise manner in alveolar macrophages and may constitute part a general cell response to prevent cellular toxicity. In both candidate gene lists (type II cells and alveolar macrophages) the *oatps* are expressed alongside genes with products with established roles in detoxification (A14h in the alveolar macrophage candidate gene list). These gene products may work in concert to reduce entry of the toxic compound into the cell and to detoxify, preventing any overt cellular toxicity. In agreement with Stone (1998) three candidate genes appear to be voltage gated calcium channels (B05f, B05g, B06n). The role of calcium influx to drive the stress response in these cells has been established with experiments with PM or PM surrogates and the increase, at the RNA level reported by the arrays could form part of either the initial stress response or contribute to restoration of the cellular calcium levels following exposure. The presence of potassium (B05d) and sodium channels (B04i) may contribute to the changes in the electrochemical gradient within the cell required to drive the cellular response following exposure.

5.5 Discussion

The objective for this study was to take a novel approach to identify changes in gene expression following PM exposure to primary cells *in vitro*. Firstly, it was anticipated that different cell types such as the alveolar macrophages would express different genes than those of epithelial type II cells that are largely concerned with surfactant secretion and xenobiotic metabolism. Secondly, it was hoped to determine if gene expression in either cell type could be linked to whole lung gene changes already recorded in the *in vivo* investigations (Chapter 3). Finally, it was intended to link any gene expression induced by PM in primary cells with those already noted by others using cell lines. The use of cell lines offers the chance of greater reproducibility and with a few limitations regarding availability of cells for study, offering more readily available and more efficient RNA isolation for macroarray investigations. However,

as pointed out by Hetland (2003) cell lines may lack the receptors of some primary cells and respond quite differently to toxic substances.

Only limited success in achieving the aims was realised. Mostly this lack of success was due to the failure to consistently isolate sufficient concentrations of total RNA from control and PM exposed cell cultures. In brief, there are several possible explanations. Firstly, there could have been some degradation of the RNA from the cells by the PM, proven to have significant ability to cause oxidative damage to plasmid DNA Greenwell et al., 2003). Secondly, RNA degradation may have been carried out by the cells themselves. The Atlas™ nylon arrays required large amounts of RNA and proved suitable for tissue profiling (Chapter 3) when abundant source of RNA was available. This was not the case in the cell studies and it is therefore possible that commercially available microarray systems, requiring less RNA, would be better employed when RNA is available in only small quantities. The limited success in obtaining gene profiles could also be related to the use of primary cells and there are few, if any, reports in the literature related to their use in toxicogenomics. In contrast, the use of cell lines in many studies has provided a more reliable and low cost source of cells to study, but it has been highlighted that primary cells and many cell lines have different responses to toxic compounds (Hetland et al., 2003). This chapter was a novel approach to assessing the cellular response to characterised PM_{10} and it was designed to incorporate primary cells rather than cell lines.

The expression profiling was completed on cells exposed to a dose that was shown to incite the detachment of 20% of the cell culture of freshly prepared whole PM_{10} sample (CD_{20}). As discussed previously, the exposure to this low dose and short exposure time (4hr alveolar macrophages, 6 hr –epithelial type II cells) was adopted based on previous research investigating the response of cultured cells (macrophages and epithelial cells) to toxins exposed *in vitro* (Li et al., 2000. Hetland et al., 2003. Monn et al., 2003). Previous studies (Chapter 4) had indicated that cell death was occurring at doses used in the CD_{50} experiments. The low dose used for the expression profiling (CD_{20}) was used in an attempt to achieve genetic profiling of the cellular response to a mild exposure rather than the genetic changes resulting in cell death. The candidate genes from studies of both cell types show that changes in cell detoxification/metabolism and transporter proteins may represent the early response to PM_{10}.

Expression profiling of the cells in isolation did not reveal any similar genes found to be expressed or repressed in the whole lung expression profiles (Chapter 3). This may be a consequence of the prominent role of cross talk between the large numbers of different cell types within the lung and which would be absent in a homogenous culture of primary isolates. However, the identification of key candidate genes, in both cell types examined, with known roles in cell signalling, detoxification and transport provided more details in the response of the cell to prevent toxicity following a mild toxicant challenge. Such candidate genes are therefore worthy of further investigation especially if further numbers of experiments provide reproducible data. Adapting the macroarray technology to isolated primary cells *in vitro* has not been easy but represents a start to studies that will become increasingly important in future pollutant research.

Chapter 6.0

General Discussion

6.0 General Discussion

6.1 Study Aims and Objectives

The aim of this study was to evaluate the toxicity of a characterised urban ambient PM_{10} sample. The first investigation examined the biological responses of healthy male rats to a single intracheal instillation of urban PM_{10} (0-10 mg)(Chapter 2). This assessment was carried out using established methods to examine the extent of oedema and inflammation arising as a direct consequence of the instillate. This instillation study also examined the contribution of equivalent doses (0-10 mg) of the different fractions of the ambient PM_{10} (whole PM, washed durable fraction and water soluble fraction) towards the overall toxicity of the sample. The study was then extended to incorporate the novel application of gene profiling lung tissue samples from affected rats, to evaluate whether the observed changes at the lung surface could be attributed to detectable changes in gene expression. This investigation necessitated the development of methods of gene analysis to establish a suitable approach to identify candidate genes for the lung pathology and confirmation of the analysis methods using qPCR (Chapter 3). The study then progressed to examine the toxicity of the PM sample (urban and industrial) towards homogenous cultures of primary type II and alveolar macrophage cells (Chapter 4).

This chapter identified the contribution of the different fractions to the overall toxicity in both cell systems and previous full chemical and morphological characterisation facilitated the correlation of observed toxicity to total metal content, source of sample and particle sizes. A small-scale investigation was then carried out using a new cellular imaging system, completed in conjunction with Amersham Biosciences. This approach used florescence labelling and laser analysis to identify subtle changes in cell and nuclei morphology following exposure to PM levels below those doses deemed cytotoxic in the cell detachment assay. This *in vitro* characterisation of the sample toxicity was extended to toxicogenomic analysis, with limited success. Failure to consistently isolate sufficient high quality RNA in concentrations and quantity required for the array analysis prevented obtaining a high number of

replicates deemed necessary for meaningful analysis. Some analyses have been completed and suggest that although the approach is challenging, this method could produce a useful insight into the cellular mechanisms of defence, toxicity and repair within cells following such an exposure.

6.2 Overview of Results and Conclusions

Histological and biochemical analysis characterised the early (3-day) and longer-term (6 week) effects of instilled PM_{10} in male healthy rats. The subtle dose dependant PM_{10} induced changes in parameters including the lung to body weight ratio and total lung surface protein at 3 days were indicative of a mild pulmonary oedema at this time point. However, this event was transient, with no significant changes in lung to body weight ratio at 6 weeks post instillation. The more sensitive parameter, total lung surface protein indicated some retained effects at 6 weeks following the instillation of both particle-containing fractions (whole and washed particles) at the higher doses (5 mg, 10 mg). A dose dependant increase in total surface protein was observed following exposure to the water soluble fraction, but a significant change was only observed at 10 mg equivalent exposure ($p<0.05$). A statistically significant reduction in total lung surface protein was observed at 6 weeks (2.5 mg only) but no further trend was observed for this parameter. A dose dependent increase in lavage free cell numbers was also observed for both whole and washed PM fractions at 3 days post instillation, although statistically significant changes were detectable only at the highest dose (10 mg whole particles), a consequence of a high degree of variation in different animals within the group (n=5). The fraction devoid of particles, the water soluble fraction, produced no significant changes in free cell number at either 3 days or 6 weeks post instillation. There was no significant recruitment of neutrophils into the lung, a further indicator of a low bioreactive instillate. In conclusion, conventional assessment reported only minimal changes in lung to body weight ratio, total surface protein and lavage free cells, indicating a low response to the instilled PM and PM fractions. The whole PM and washed PM fraction elicited the most response, a low-level response was noted with the soluble fraction, illustrating that the presence of the soluble material had a pulmonary effect *in vivo*. Histological examination confirms this low-level response in the healthy rat whereby only small

focal regions of particle accumulation and a slight epithelial effect was apparent. A chiefly macrophage mediated lung event was detectable following both 5 mg and 10 mg exposure. In contrast, water soluble (10 mg PM equivalent dose) exposed lung tissue presented regions of alveolar, capillary or interstitial thickening, with prominent collections of red blood cells.

Toxicogenomic analysis of selected animals (n=3) from sham-treated controls; whole particle exposed (5 mg, 10 mg) and water soluble (equivalent dose of 10 mg whole PM) produced expression profiles of the lung responses. A critique of the macroarray method is provided later. The array profiling and subsequent analysis produced candidate gene lists that would be suitable for continued studies. The candidate genes following the exposure to whole and water soluble PM fractions were similar in terms of functionality but contained no similar genes, indicating a distinct lung response at 3 days, as judged by histological assessment. A 3 day post instillation had been adopted for the early time point on the basis of previous studies identifying this time as peak for oedema and lung damage in rat models, however, changes in heart rate and gene expression in the heart attributed to soluble metals have been identified within an hour of instillation. The peak of the lung response to the water soluble fraction may occur much sooner than this time point resulting in a stage of repair by the 72 hour time point. In contrast, gene changes as a consequence of on going particle-induced cellular damage may be detected following the instillation of whole and washed fractions. The low replicate number (n=3 for each treatment group) was adopted for this first expression profiling study, this is very low for strong statistical assumptions and increased replicates would be favoured in further studies. The expression profiling of a lower dose (5 mg) whole PM exposed proved unsuccessful, attributed, in part, to the use of different animals for each array producing very different expression profiles within the treatment group. Changes in gene expression for the other treatment groups were confirmed by qPCR, although more comprehensive confirmation of all candidate genes should be completed. The finalised candidate gene lists of each PM fraction exposure could be linked to the observed pathology and warrant further investigation.

The characterisation of the response towards two ambient PM samples: industrial (Port Talbot 1) and urban (Cardiff 2) in cell free assays have been completed by a

former postgraduate within our laboratory (Greenwell et al., 2003). The present study investigated the toxicity within a cell system, and adopted two primary cell types for this purpose; the alveolar macrophage and epithelial type II cell. A simple detachment assay was enlisted to ascertain the relative toxicity of both the ambient PM samples and respective fractions towards the two cell types. The alveolar macrophage and epithelial type II cell were differentially affected by the two PM samples. The Port Talbot sample was more toxic towards the type II cells, whilst the alveolar macrophages were more affected by the urban sample. However, most reactivity of the sample was retained within the durable fraction in each cell type. Characterisation of the samples (Moreno et al., 2003) allowed correlation with metal content. The toxicity of the samples towards the alveolar macrophages was greatest with the finer particle dominated fraction (urban, PM_{10} to $PM_{2.5}$ ratio 2-8-4.2 compared to the same ratio in the Port Talbot sample 1-2) and could be postulated to reflect greater particle numbers per mass to which these cells had been exposed. In contrast, the type II cells were more susceptible to the coarse particle dominated sample (industrial), a finding in agreement with Hetland (2003). Type II cell toxicity correlated well with total metal content of the samples, no such trend was apparent with the alveolar macrophages, however correlations did exist between specific metals with both cell types. Such correlations were generally weak, a reflection of low replicate numbers and sample numbers examined, however the results indicate trends that specific metals could dictate the cytotoxicity of the PM sample in different cell types. Cellular imaging of PM exposed type II cell cultures provided evidence that subtle cellular and nuclear changes were detectable at the doses used in the detachment assays. These results supported the use of a low PM exposure (CD_{20}) for subsequent toxicogenomic profiling, the expression profiling of the cellular events leading up to increased apoptosis and cell death and damage rather than cellular signalling concerned with cell death itself. Limited success with cellular expression profiling was achieved. This may have been a consequence of poor cell and RNA availability associated with primary cells compared to cell lines. However this approach was favoured over the use of cell lines, toxicological studies have highlighted slight differences in cell morphology, biology and responses to toxicant challenge in cell lines compared to primary isolates. The macroarray system adopted for these studies required considerable (2 μg) total RNA. Microarrays, although more expensive, require only a fraction of the RNA and may be more appropriate for

primary cell studies with limited amounts of RNA. The low replicate numbers realised in this study hampers the reliability of the conclusions, but the study is a useful preliminary investigation, addressing the problems with expression profiling of primary cells rather than lung tissue. Candidate gene lists of PM exposed epithelial type II cells (sham-treated control n=4, PM exposed n=4) produced candidate genes involved in the removal and detoxification of molecular species. Functional analysis of these candidate genes indicates genes associated with ion channels and calcium metabolism. Such changes can be linked to events postulated to be taking place within the lung (Donaldson et al., 2001) and known cellular events of stress and repair (Stone et al., 1998).

6.3 Critique of the Methods and Approaches Adopted

Conventional toxicological assessment, in conjunction with histological examination, was utilised to characterise the pulmonary responses to assess the bioreactivity of urban PM (CF 2) in the rat. However, the conventional analysis provided very little evidence of the cellular and molecular events leading up to the endpoints observed and no information regarding the different mode of toxicity between the different fractions, implied by the distinct histology of water soluble and whole PM exposed lung tissue. However, the conventional assessments alongside the toxicogenomic profiling, enabled identification of changes at the lung surface and provided the potential to link such with changes in gene expression.

The Atlas ™ macroarray system was adopted for the expression-profiling studies. This nylon array offered a versatile method that that did not require large amounts of specialist equipment. Previous experiments had used the smaller array from the rat array series (Rat stress array, BD Biosciences, 207 genes) with great success (Reynolds et al., 2001). The larger Rat 1.2 array (1176 genes) was considered superior because of the nature and scope of the genes examined. However, this change introduced significant problems, including background noise and non-specific binding to every cDNA spot. Despite increased measures to identify batches of faulty membranes and consumables, the overall efficiency of successful arrays was poor, particularly during the profiling of PM exposed cells, with very limited starting

material. The macroarray proved generally successful with the tissue profiling, with more abundant high quality RNA, but repeated use of the nylon arrays did result in considerable distortion of the membranes. Alternative commercial microarray systems, including BD Biosciences and Affymetrix, require lower quantities of starting materials and use glass as a substrata rather than nylon. The microarray systems offer the potential to examine genome-wide changes in gene expression but require specialised equipment and are often only suitable for single use.

The design of the study was based around a large *in vivo* experiment involving short-term exposure (3 days and 6 weeks post instillation) of several different doses (0 mg, 2.5 mg, 5 mg and 10 mg) to whole PM, washed PM and water soluble component of PM. Three animals were selected on the basis of heightened pulmonary responses as judged by the conventional assessment. Triplicate expression profiles were considered sufficient, but upon development of the bio informatic analysis, it became apparent that this low number weakened any statistical assumption made. The use of three individual animals for profiling introduced another large degree of variation above that found in repeated tissue samples. It would be advisable, in a case such as this, to introduce more replication, not necessarily extending the number of animals, but in repeating the arrays using the same samples and analysing multiple arrays. Another challenge to the success of the array analyses was the highly heterogeneous level of pulmonary responses achieved following the exposure to the PM and respective fractions. The lung tissue histology resulting from the instillation identified a focal effect, with large regions of the tissue remaining unaffected. Confirmation of the array results using a different section of tissue, which may be either more or less affected than the tissue selected for array analysis, would jeopardise the consistency of such gene changes. This was an unforeseen error in the design of this study.

The cell detachment assay adopted to assess the *in vitro* toxicity of ambient PM samples was relatively non-specific. Further investigations into changes within the cell and nuclear morphology indicated that there were mild cell changes indicative of the onset of apoptosis that may provide a more subtle marker of cell stress than that of detachment, particularly in the epithelial type II cell cultures.

In conclusion, this study has been successful in addressing the primary objective of characterisation of the bioreactivity of an ambient PM sample both *in vivo* and *in vitro*. The Cardiff PM sample used through out the study proved to have low bioreactivity compared to highly potent pulmonary toxicants such as silica used in other studies. The toxicity was retained chiefly in the fractions containing the particles themselves, although the water soluble component, chiefly water soluble metals did incite a low level effect. The toxicogenomic profiling of the whole lung tissue realised distinct lists of candidate genes worthy of further investigation, and highlighted the possibility of different times of effect, if not different mechanism of toxicity by the water soluble and particle containing fractions. None of the candidate genes identified could be attributed to the two cells examined *in vitro,* however. Expression profiling of these cells, although hampered by practical problems, has identified genes with functions that may be linked with known events at the lung surface and illustrated the potential of this technique as a possible approach to investigating the toxicity of PM_{10}. The cell systems were successful to ascertain the *in vitro* toxicity of a characterised industrial and urban PM sample. The study highlighted that differences in particle characteristics and particularly the metal content were linked to overall toxicity.

The use of gene arrays, although introducing several problems, can successfully be used to identify genes that may be otherwise overlooked by other studies that may be important in the cellular and tissue responses towards PM and other pulmonary toxins.

Bibliography

Aardena, M and MacGregor, J. (2002). Mutation Research 499 p13-25. "Toxicology and Genetic in the New Era of "Toxicogenomics": Impact of the "-Omics" Technologies."

Adamson, I, Young, B and Bakowski, L. (1997). Am. J. Physiol Soc. "Enhanced Alveolar Type II Cell Growth on a Pulmonary ECM over Fibroblasts."

Adamson, I, Prieditis, H and Vincent, R. (2000). Toxicology and Applied Pharmacology 166 p111-19. "Zinc Is the Toxic Factor in the Lung Response to an Atmospheric Particulate Sample."

Adamson, I, Prieditis, H and Vincent., R. (1999). Toxicology and Applied Pharmacology 157 p43-50. "Pulmonary Toxicity Is Due to the Soluble Fraction."

Adamson, I, Vincent, R and Bjarnason, S. (1999). Am. J. Respir. Cell Mol. Biol. 20 p1067-72. "Cell Injury and Interstitial Inflammation in Rat Lung after Inhalation of Ozone and Urban Particlulates."

Albelda. E. and Sheppard, M. (2000). Am.J.Cell Mol. Biol. 23 p265-69. "Functional Genomics and Expression Profiling."

Anderson, H, Limb, E, Bland, J, Ponce-de-Leon, A, Strachan, D and Bower, J. (1995). Thorax 50 p1188-93. "Health Effects of an Air Pollution Episode in London, December 1991."

Anderson, H, Ponce-de-Leon, A, Bland, J, Bower, J, Emberlin, J and Strachan., D. (1998). Thorax 53 p842-48. "Air Pollution, Pollens and Daily Admissions for Asthma."

Bassett, D, Eisen, M and Boguski, M. (1999). Nature genetics 21 p51-55. "Gene Expression Informatics- Its All in Your Mine."

Becker, S, Soukup, J and Gallagher, J. (2002). Toxicology In Vitro 16 p209-18. "Differential Particulate Air Pollution Indiced Oxidant Stress in Human Granulocytes, Monocytes and Alveolar Macrophages."

Becker, S, Soukup, J, Gilmore, I and Devlin, R. (1996). Toxicology and Applied Pharmacology 141 p637-48. "Simulation of Human and Rat Alveolar Macrophages by Urban Air Particulates: Effects on Oxidant Radical Generation and Cytokine Production."

Bérubé, K, Jones, T, Williamson, B, Winters, Morgan and Richards, R. (1999). Atmospheric Envronment 33 p1599-613. "Physicochemical Characterisation of Diesel Exhaust Particles : Factors for Assessing Biological Activity."

Blesch, A. and Tuszynski, MH. (2003). J. Comp. Neurology 74 (4) p552-61. "Cellular GDNF Delivery Promotes Growth of Motor and Dorsal Column Sensory Axon after Partial and Complete Spinal Cord Transections and Induces Re-myelination."

Bowtell, A. (1999). Nature genetics 21 p25. "Options Available- from Start to Finish- for Obtaining Expression Data by Microarray."

Bradford, M. (1976). Analytical Biochemistry 72 p248-54. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilising the Principle of Protein-Dye Binding."

Braeza-Squiban, A, Bonvallot, A, Boland M, and Marano, T. (1999). Cell Biology and Toxicology 15 p375-80. "Airborne Particles Evoke an Inflammatory Response in Human Airway Epithelium. Activation of Transcription Factors."

Brazma, A, Hingamp, P, Quackenbush, J, Sherlock, G, Spellman, P, Stoeckert, C, Aach, J, Ansorge, W, Ball, C, Causton, H, Gaasterland, T, Glenisson, P, Holgate, F, Kim, I, Markowitz, V, Matesse, J, Parkinson, H, Robinson, A, Sarkens, U, Schulze-Kremer, S, Stewart, J, Taylor, R, Vilo, K and Vingron, M. (2001). Nature Genetics

29 p365-71. "Minimum Information About Microarray Experiment (MIAME)-Towards Standards for Microarray Data."

Brunekreef, B, Hoek, G, Fischer, P and Spieksma, F. (2000). The Lancet. 355 p1517. "Relation between Airborne Pollen Concentrations and Daily Cardiovascular and Respiratory-Disease Mortality."

Brunekreef, B and Holgate, S. (2002). The Lancet 360 (9341) p1233-42. "Air Pollution and Health."

Cao, C., Xia, Q., Chen, Y., Zhang X. and Shen, Y. (2002). Pflugers Archives 443 (40 p665-42. "Opoid Receptor-mediated Effects of IL-2 on the $[Ca^{2+}]_i$ Transient Contraction in Isolated Ventricular Myocytes of the Rat."

Castranova, C. (2000). Inhalation Toxicology 12 **(53)**p7-14. "From Coal Mine Dust to Quartz, Mechanisms of Toxicity."

Chomzynski, P and Sacchi, N. (1987). Analytical Biochemistry 162 p156-59. "Single Step Method of Rna Isolation by Acid Guanidium Thiocyanate-Phenol-Chloroform Extraction."

Chuaqui, R, Bonner, R, Best, C, Gillespie, J, Flaig, M, Hewitt, S, Philips, J, Krizman, D, Tangrea, M, Ahram, M, Linehan, M, Knezevic, V and Emmert-Buck, M. (2002). Nature Genetics 32 **(509-514)** "Post-Analysis Follow up and Validation of Microarray Experiments."

Costa, D and Dreher, K. (1997). Environmental Health Perspectives 105 **(5)** "Bioavaliable Transition Metals in Particulates Matter Mediate Cardiopulmonary Injury in Healthy and Compromised Animal Models."

DaSilva, L, Cote, D, Roy, C, Martinez, M, Duniho, S, Pitt, L, Downey, T and Dertzbaugh, M. (2003). Toxicon 41 **(7)** p813-22. "Pulmonary Gene Expression Profiling of Inhaled Ricin."

Davies, Oreffo and Richards.R.J. (1988). Chimicaoggi p11-16. "The Toxic Effect of Metal Ions on Rat Alveolar Type II Cells in Primary Culture."

Dobbs, Plan, Maglio, Dumars and Allen. (1997). Am. Phys. Soc pL347. "Maintenance of Differentiated Type Ii Cell Phenotype by Culture with Apical Air Surface."

Dobbs, William and Gonzalez. (1988). Biochimica and Biophysica Acta 970 p146-56. "Monoclonal Antibodies Specific to Apical Surfaces of Rat Alveolar Type I Cells Bind to Surfaces of Cultured but Freshly Isolated Type II Cells."

Dockery, D, and Pope, A. (1995). Annu Rev. Public Health 15 p107. "Acute Respiratory Effects of Particulate Air Pollution."

Dockery, D. (2001). Environmental Health Perspectives 109 **(4)**p483-86. "Epidemiological Evidence If Cardiovascular Effects of Particulate Air Pollution."

Donaldson, K, Li, X and MacNee, W. (1998). J.Aerosol Sci 29 **(5/6)**p553-60. "Ultrafine Particles Mediated Lung Injury."

Donaldson, K, Stone, V, Borm, P, Jimenez, L, Gilmour, P, Schins, R, Knaapen, A, Rahman, I, Faux, S, Brown, D and MacNee, W. (2003). Free Radical biol. Medicine 34 **(11)**p1369-82. "Oxidative Stress and Calcium Signalling in the Adverse Effects of Environmental Particles (Pm10."

Donaldson, K, Stone, V, Seaton, A and McNee, W. (2001). Environmental Health Perspectives 109 **(4)** p523-27. "Ambient Particle Inhalation and the Cardiovascular System: Potential Mechanisms."

Dreher, I. (2000). Inhalation Toxicology 12 **(3)** p45-7. "Particulate Matter Physicochemistry and Toxicology."

Dreher, K, Jaskot, R, Lehman, J, Richards, J, McGee, J, Ghio, A and Costa, D. (1997). Journal of Toxicology and Environmental Health 50 p285-305. "Soluble Transition Metals Mediate Residual Oil Fly Ash Induced Lung Injury."

Driscoll. (1996). Inhalation Toxicology 8 (**1**) p139-53. "Role of Inflammation in Development of Rat Lung Tumors in Response to Chronic Particle Exposure."

Durbin, Hardin, Hawkins and Rocke. (2002). Bioinformatics 18 (**1**)p105-10. " A Variance-Stabilising Transformation for Gene-Expression Microarray Data."

Dye, J, Adler, K, Richards, J and Dreher, K, (1997). Am. J. Respir. Cell Mol. Biol. 17 p625-33. "Epithelial Injury Induced by Exposure to Residual Oil Fly-Ash Particles: Role of Reactive Oxygen Species."

Dye, J., Lehmann, J., et al., (2001). Environmental Health Perspectives. 109 (3): 395-374. "Acute pulmonary toxicity of particulate matter filter extracts in rats: Coherence with epidemiologic studies in Utah Valley residents."

Foguet, M., Hoyer, D., Pardo, L., Paketh, A., Kluxen, F., Kalkman, H., Stuhmer, W. and Lubbert, H. (1992). EMBO J. 11 (9) p 3481-7. "Cloning and Functional Characterisation of the Rat Stomach Fundus Serotonin Receptor."

Frampton, Ghio, Samet, Carson, Carter and Devlin. (1999). American Journal of Physiology 277 pL960-67. "Effects of Aquaous Extracts of PM_{10} Filters from Utah Valley on Human Epithelial Cells."

Gavett, S, Madison, S, Dreher, K, Winsett, D, McGee, J and Costa, D. (1997). Environmental Research 72 p162-72. "Metal and Sulphate Composition of Residual Oil Fly Ash Determines Airway Hyperreactivity and Lung Injury in Rats."

Ghio, A, Stoneheurner, J, Dailey, L and Carter, J. (1999). Inhalation Toxicology 11 p37. "Metals Associated with Both the Water Soluble and Insoluble Fractions of an Ambient Air Pollution Particle Catalyse Oxidative Stress."

Greenwell, L. (2003). Ph.D Thesis._"The bioreactivity and physio-chemical properties of particulate matter."

Greenwell, L, Jones, T and Richards, R. (2002). Environmental Monitoring and Assessment 79 p251-73. "The Collection of PM_{10} for Toxicological Investigation: Comparison between Different Collecting Devices."

Greenwell, L, Moreno, T, Jones, T and Richards, R. (2002). Free Radical biol. Medicine 32 (**9**)p898-905. "Particle Induced Oxidative Damage Is Ameliorated by Pulmonary Antioxidants."

Hagenbach, B, Gao, B and Meier, P. (2002). News Physiol Sci 17 p231-34. "Transpot of Xenobiotics across the Blood-Brain Barrier."

Harrison and Jones. (1995). Science of the total Environment 168 p195-24. "Chemical Composition of Airborne Particles in the Uk Atmosphere."

Harrison and Yin. (2000). Science of the total Environment 249 p85-101. "Particulate Matter in the Atmosphere: Which Particles Are Important for Its Effects on Health?."

Hattoriy, Y., Yamasaki, M. and Itoh, W. (1996). Biochem. Biophys. Acta 1306 (1) p 31-3. "The rat FGF-5 mRNA Variant Generated by Alternative Splicing Encodes a Novel Form of FGF-5."

Henderson, Driscoll, Harkema, Lindenschmidt, Chang, Maples and Barr, (1995). Fundamental and Applied Toxicology 24 p183-97. "A Comparison of the Inflammatory Response of the Lung to Inhaled Versus Instilled Particles in F344 Rats."

Hetland, R, Cassee, F, Refsnes, M, Schwarze, P, Lag, M, Boere, A and Dybing, E, (2003). Toxicology *in vitro* 17 "Release of Inflammatory Cytokines, Cell Toxicity and Apoptosis in Epithelial Lung Cells after Exposure O Ambient Particles of Different Size Fractions."

Houseley, DG and Richards, RJ. (2001). Chapter 5: "Protection against inhaled pollutants and individual sensitivity." The Urban Atmosphere and its effects. Air Pollution Reviews: volume 1 (Imperial College Press). Editors: Peter Brimblecombe & Robert Maynard.

Huo, L., Munzberg, H., Nillni, E. and Bjorback, C. (2004). Endocrinology 145 (5) p 2516-23. "Role of Signal Transducers and Activators in the Regulation of trh GeneExpression."

Imrich, A, Ning, Y and Kobzik, L. (2000). Toxicology and Applied Pharmacology 167 p140-50. "Insoluble Components of Concentrated Air Particles Mediate Alveolar Macrophage Responses in Vitro."

Johnston, I, and Orberdorster, G. (2000). Toxicology and Applied Pharmacology 168 p208-15. "Pulmonary Effects Induced by Ultrafine Ptfe Particles."

Kaminski, Allard, Pittet, Grifiths, Morris, Huang, Sheppard and Heller, (2000). PNAS 97 **(4)** p1778-83. "Global Analysis of Gene Expression in Pulmonary Fibrisis Reveals Distinct Programmes Regulating Lung Inflammation and Fibrosis."

Ke, Y., Wang, L., Pyle, W., de Tombe, P. and Solaro, R. (2004). Circ. Res. 94 (2) p 194-200. "Intracellular Localisation and Functional Effects of p21Activated Kinase-1 in Cardiac Monocytes."

Kelly, F and Richards, RJ. (1999). Air Pollution and Health (Academic Press) ISBN 0-12-352335-4. Chapter 10: "Antioxidant defences in the extracellular compartment of the human lung."

Khandoga, A., Stampfl, A. (2004). Circulation 109 p1320-1325. "Ultrafine particles exert prothrombic but not inflammatory effects on the hepatic microcirculation in healthy mice *in vivo.*"

Knaapen, A, Albrecht, C, Becker, A, Hohr, D, Winzer, A, HAenen, G, Borm, P and Schins, R. (2002). Carcionogenesis 23 **(7)** p1111-20. "DNA Damage in Lung

Epithelial Cells isolated from Rats Exposed to Quartz: Role of Surface Reactivity and Neutrophic Inflammation."

Kodovanti, U, Hausser, R, Christiani, D, Meng, Z, McGee, J, Ledbetter, Richards, J and Costa, D. (1998). Toxicological Sciences 43 p204-12. "Pulmonary Responses to Oil Fly Ash Particles in the Rat Differ by Virtue of Their Specific Soluble Metals."

Koike, E, Hirano, S, Shimojo, N and Kobayashi, T. (2002). Toxicological Sciences 67 p241-46. "Cdna Microarray Analysis of Gene Expression in Rat Alveolar Macrophages in Response to Organic Extract of Diesel Exhaust Particles."

Khiroug, S., Khiroup, L. and Yakel, JL. (2004). Neuroscience124 (4) p817-22. "Rat Nicotiinic ACh Receptor α2β2 Channels."

Lee, SH and Richards RJ (2003). *in press.* Occupational and Environmental Medicine.
"Bioreactivity potential of Montserrat Volcanic Ash.

Li, N, Venkatesan, I, Miguel, A, Kaplan, R, Gujuluva, C, Alam, J and Nel, A. (2000). J. Immunology "Induction of Heme Oxygenase 1 Expression in Macrophage by Diesel Exhaust Particle Chemicals an Quinones Via the Antioxidant Responsive Element."

Li, QH, Nakadate, K., Tanaka-Nakadate, S., Nakatusakas, S., Cui, Y. and Watanake, Y. (2004). J. Comp. Neurol. 469 (1) p128. "Unique expression patterns of 5-HT2A and HT2C receptors in the rat brain."

Lieukhauf, McDowell, Gammon, Wesselkamper, Barchurski, Puga, Wiest, Leikauf and Prows. (2000). Inhalation Toxicology 12 (**3**) p59-73. "Functional Genomics of Particles Induced Lung Injury."

McElroy, M., Pittet, J., Hashimoto, S., Allen, L., Wiener-Kronish, J. & Dobbs, L. (1995) American Journal of Physiology 268, pp. L181-L186

Madden, M, Richards, J, Dailey, L, Hatch, G and Ghio, A. (2000). Toxicology and Applied Pharmacology 168 p140-48. "Effect of Ozone on Diesel Exhaust Particle Toxicity in Rat Lung."

Magari, S, Schwartz, J, Williams, P, Hauser, R, Smith, T and Christiani, D. (2002). Environmental Health Perspectives 110 **(9)** p875(6). "The Association of Particulate Air Metal Concentrations with Heart Rate Variability."

Mason, R, Dobbs, L, Greenleaf, R and Williams, M. (1977). Federation Proceedings 36 **(13)** p2696. "Alveolar Type II Cells."

Miyamoto, M., Naruo, K., Seko,C., Matsumoto, S., Konda, T. and Kurowaka, T. (1993). J. Mol. Cell. Biol. 13 (7) p 4251-9. "Molecular Cloning of a Novel Cytokine cDNA Encoding the Ninth Member of the Fibroblast Growth Factor."

Monn, C and Becker, S, (1999). Toxicol. & Applied Pharmacology 155 **(3)** p245-52. "Cytotoxicity and Induction of Proinflammatory Cytokines from Human Monocytes Exposed to Fine ($PM_{2.5}$) and Coarse Particles (Pm10–2.5) in Outdoor and Indoor Air."

Monn, C, Fendt, R and Koller, T. (2002). Inhalation Toxicology 14 "Ambient PM_{10} Extracts Inhibit Phagocytosis of Defined Inert Model Particles by Alveolar Macrophages."

Monn, C, Naef, R and Koller, T. (2003). Environmental Research 91 **(1)** p35-44. "Reactions of Macrophages Exposed to Particles <10 microns."

Moreno, T, Jones, T and Richards, R. (2003). Science of the Total Environment (*in press*) "Aerosol Particulate Matter from Urban and Industrial Environments: Examples from Cardiff and Port Talbot, South Waes, Uk."

Morrow, P, Haseman, J, Hobbs, C, Driscoll, K, Vu, V and Oberdorster., G. (1996). Fundamental and Applied Toxicology 29 p155-67. "The Maximum Tolerated Dose for Inhalation Bioassays: Toxicity V's Overload."

Murphy, S, Bérubé, K, Pooley, F and Richards., R. (1998). Life Sciences 62 (**19**) p1789-99. "The Response of the Lung Epithelium to Well Characterised Particles."

Murphy, S, Bérubé, K and Richards., R. (1999). Occupational & Environmental Medicine 56 p813-19. "Bioreactivity of Carbon Black and Diesel Exhaust to Primary Clara and Type Ii Epithelial Cell Cultures."

Murphy, S, Dinsdale, D, Hoet, P, Nemery, B and Richards, R. (1999). Methods In Cell Science 21 p31-38. "A Comparative Study of the Isolation of Type Ii Epithelial Cells from Rat, Hamster, Pig and Human Lung Tissue."

Nadon, R and Shoemaker, J. (2002). Trends in genetics 18 (**5**) p265-71. "Statistical Issues with Microarrays: Processing and Analysis."

Nemery, B and Hoet, P. (1993). Toxicology In vitro. 7 (**4**)p359-64. "Use of Isolated Lung Cells in Pulmonary Toxicology."

Nuwaysir. (1999). Molecular Carcinogenesis 24 "Microarrays and Toxicology: The Advent of Toxicogenomics."

Oberdorster, G. (2002). Inhalation Toxcology 14 p29-56. "Toxicokinetics and Effects of Fibrous and Nonfibrous Particles."

Oberdorster, G, Ferin, J, Gelein, R, Soderholm, S and Finkelstein, J. (1992). Environmental Health Perspectives 97 p193-99. "Role of the Alveolar Macrophage in Lung Injury: Studies with Ultrafine Particles."

Oberdorster, G and Yu, C. (1990). J.Aerosol Sci 21 (**1**) pS397-S401. "The Carcinogenic Potential of Inhaled Diesel Exhaust: A Particle Effect?."

Orberdorster, G and Utell, M. (2002). Environmental Health Perspectives 110 (**8**) pA440. "Ultrafine Particles in the Urban Air : To the Respiratory Tract- and Beyond? Is the Central Nervous System yet Another Target for Ultrafine Particles?."

Oreffo, V, Thomas, D and Richards, RJ. (1988). Chimicaoggi p57-61. "Comparative Toxicity Studies on Cultured Alveolar Epithelial Type Ii Cells and Lung Fibroblasts Exposed to Agents That Induce Pulmonary Oedema."

Oshikawo, J., Toyo, Y., Fujita, T., Egawa, M., Kawabe, J., Umemura, S. and Ushikawa, Y. (2003). Am. J. Physiol. Cell Physiol. 285 (3) p C567-74. "Nicotinic ACh Receptor α7 Regulation of cAMP Signal within Lipid Rafts."

Pagan, I., Costa, D., et al. (2003). ." Journal of Toxicology and Environmental Health (Part A) 66 p1087-1112. "Metals mimic airway epithelial injury induced by *in vitro* exposure to Utah Valley ambient particulate matter extracts

Pope, A. (1996). Toxicology 111 p 149-55. "Adverse Health Effects of Air Pollutants in a Nonsmoking Population."

POST Technical report: Fine Particles and Health. (1996) Parliamentary Office of Science and Technology. 82.

Quinlan, T, Bérubé, K, Marsh, J, Janssen, Y, Taishi, P, Leslie, K, Hemenway, D, OShaughnessy, P, VAcek, P and Mossman, B. (1995). American Journal of Pathology 147 (**3**) p728-39. "Patterns of Inflammation, Cell Proliferation and Related Gene Expression in Lung after Inhalation of Chrysotile Asbestos."

Rahman, I. (2002). Biochemical Pharmacology 64 p935-42. "Oxidative Stress , Transcription Factors and Chromatin Remodelling in Lung Inflammation."

Rahman, I, Mulier, B, Gilmour, P, Watchorn, T, Donaldson, K, Jeffrey, P and MacNee, W. (2001). Biochemical Pharmacology 62 p787-94. "Oxidant-Mediated Lung Epithelial Cell Tolerance: The Role of Intracellular Glutathione and Nuclear Facor-Kappa B."

Reynolds, L, Jones, T, Bérubé, K, Wise, H and Richards, R. (2003). Mineralogical magazine 67 (**2**)p139-50. "Toxicity of Airborne Dust Generated by Open Cast Coal Mining."

Reynolds, L, McElroy, M and Richards, R. (1999). IJBCB 31 p951-60. "Density and Substrata Are Important in Lung Type II Cell Transdifferentiation in Vitro."

Reynolds, L, Murphy, S and Richards, R. (2000). Invitro and Molecular Toxicology 13 p173-79. "The Toxicity of Modified and Non Modified Dep on Different Alveolar Lung Epithelial Cell Cultures."

Reynolds, L and Richards, R. (2001). Toxicology 165 p145-52. "Can Toxicogenomics Provide Information on the Bioreactivity of Diesel Exhaust Particles?."

Rice, T, Clarke, R, Godleski, J, Al-Mutairi, E, Jiang, N, Hauser, R and Paulaskis, J. (2001). Toxicol. & Applied Pharmacology 177 p46-53. "Differential Ability of Transition Metals to Induce Pulmonary Inflammation."

Richards, R. (1997). Biologist 44 **(1)** p249. "Small Particles, Big Problems."

Richards, R, Davies, N and Atkins, J. (1987). Lung 165 p143-58. "Isolation, Biochemical Characterisation and Culture of Type Ii Cells of the Rat."

Richards, R, Oreffo, V and Lewis. (1990). Environmental Health Perspectives 85 p119-27. "Clara Cell Cultures from Mouse and Their Reaction to Bronchiolar Toxins."

Schwartz, J. (1994). Environmental Research 64 p26-35. "What Are People Dying of on High Air Pollution Days."

Schwartz, J. (2000). Occupational & Environmental Medicine 57 p692-97. "Daily Deaths Are Associated with Combustion Particles Rather Than So2 in Philadelphia."

Schwartz, J and Neas, L. (1999). Epidemiology 11 **(1)** p6-10. "Fine Particles Are More Strongly Associated Than Coarse Particles with Acute Respiratory Health Effects in Schoolchildren."

Seaton, A. (1996). J R Soc Med 89 p609-07. "Particles in the Air: The Enigma of Urban Air Pollution."

Seaton, A, MacNee, W and Donaldson, K. (1995). The Lancet. 345 "Particulate Air Pollution and Acute Health Effects."

Seaton, A, and Soutar, A. (1999). Thorax 54:1027-1032. "Particulate air pollution and the blood."

Shannon, J, Jennings, S and Nielson, L. (1992). American Physiology Society "Modulation of Alveolar Type Ii Cell Differentiated Function in Vitro."

Squiban, AB, Bonvallot, V, Boland, S and Marano, R. (1999). Toxicology In Vitro 13 p817-22. "Diesel Exhaust Particles Increase NF Kappa B DNA Binding Activity and C Fos Proto Oncogene Expression in Human Bronchial Epithelial Cells."
Stone, V. (1998). Toxicol. Invitro 12 p649-59. "The Role of Oxidative Stress in Prolonged Inhib. Effect of Ultrafine Cb on Epithelial Cell Function."

Stone, V, Brown, D, Watt, N, Wilson, M, Donaldson, K, Ritchies, H and MacNee, W, (2000). Inhalation Toxicology 12 (**3**) p345-51. "Ultrafine Particle Mediated Activation of Macrophages: Intracellular Calcium Signalling and Oxidative Stress."

Watkinson, W, Campen, M, Nolan, J and Costa, D. (2001). Environmental Health Perspectives 109 (**4**)p539. "Cardiovascular and Systemic Responses to Inhaled Pollutants in Rodents: Effects of Ozone and Particulate Matter."

Whittaker, A. (2003). Ph.D Thesis . "Black Smokes: Past and present."

Williams, EJ., Walsh, FS. and Doherty, P. (2003). J. Cell Biol. 160 (4) p 481-6. "The FGF receptor uses the Cannaboid Receptor Signalling System to Couple to an Axonal Growth Response."

Yang, M, Ruan, Q, Eckenrode, S, Wu, S, McIndore, R and She, J. (2001). Physiol Genomics 7 p45-53. "A Statistical Method for Flagging Weak Spots Improves Normalisation and Ratio Estimates in Microarrays."

Zielinski, Berube, K, Mudway, I, Murphy, S, Richards, R and Kelly, F, (1999). Am. Phys. Soc L719 "Modelling the Interactions of Particulates with Epithelial Lining Fluid Antiioxidants."