THE EFFECTS OF CIGARETTE SMOKE AND OZONE ON THE RESPIRATORY EPITHELIUM

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CONTENTS.

ABSTRACT.

The respiratory epithelium is constantly exposed to oxidants both inhaled and released by inflammatory leucocytes. A balance between oxidants and anti-oxidants is critically important to the maintenance of normal cell function. Cigarette smoke and ozone increase airspace epithelial permeability. In this thesis I have investigated the hypothesis that this phenomenon results from oxidant injury to the respiratory epithelium.

Respiratory epithelial permeability can be studied by the passage of ^{99m}Tc-DTPA from the lungs to blood. A wide range of normal values for the time to 50% clearance (t50) has been quoted in the literature. I compared an uncorrected and 2 background corrected methods of analysis and their repeatability in 7 smokers (S) and 8 non-smokers (NS). No difference in t50 values between the methods was found in S but the uncorrected analysis gave higher values in NS. There was no difference in the repeatability of the methods. Correction with an inter-renal background region of interest was used thereafter as it allows the simple and rapid analysis of any curve.

Fourteen smokers underwent ^{99m}Tc-DTPA lung scans after refraining for 12 hours (chronic smoking, C) and after smoking until 1 hour before study (acute smoking, A). ^{99m}Tc-DTPA clearance was increased in C and further in A. Each smoker also underwent bronchoalveolar lavage (BAL) after either C or A and seven control NS were also studied. Neutrophils in BAL were increased in A. Superoxide release ($O_2^{\cdot -}$) from mixed BAL leucocytes and plasma products of lipid peroxidation were increased in both smoking groups. In BAL fluid (BALF) and epithelial lining fluid (ELF) the latter doubled in C and increased 6 fold in A. Trolox equivalent anti-oxidant capacity (TEAC) decreased in plasma and increased in BALF. Reduced glutathione in the airspaces doubled in C. This increase was abolished in A.

In a further study 15 healthy NS were exposed to filtered air (FA), or ozone 100 or 400ppb for 1 hour, during intermittent exercise. Ozone inhalation increased ELF volume overall. Neutrophils in BAL were increased 6 hours after ozone 400ppb compared to FA. Ozone inhalation

decreased $O_2^{\cdot -}$ release from mixed BAL leucocytes and products of lipid peroxidation in ELF, 1 and 6 hours after 400ppb. *In vitro* exposure of A549 cells to 1000ppb for 1 hour produced focal cell loss, with increased intracellular protein mixed disulphides, export of oxidized glutathione, and cytoskeletal disruption.

In conclusion increased epithelial permeability and neutrophil influx in the airspaces, produced by oxidant inhalation are acute phenomena. In smokers they were associated with increased oxidant stress. In healthy non-smokers, in contrast, acute ozone inhalation was associated with decreased oxidant stress, at least as measured by the extent of lipid peroxidation and oxygen radical release from mixed BAL leucocytes, and in addition anti-oxidant levels were maintained. *In vitro* studies indicated the potential of ozone to increase oxidant stress in the respiratory epithelium and airspaces with profoundly damaging effects.

ABBREVIATIONS.

α_1-AT	α_1-antitrypsin
ABTS	2, 2'-azinobis (3-ethylbenzothrazoline-6-sulphonic acid)
α_1-Pi	α_1-proteinase inhibitor
(A)RDS	(Adult) respiratory distress syndrome
BAL(F)	Bronchoalveolar lavage (fluid)
BLPI	Bronchial leucocyte protease inhibitor
BSA	Bovine serum albumin
COHb	Carboxyhaemoglobin
COPD	Chronic obstructive pulmonary disease
^{51}Cr-EDTA	^{51}chromium labelled ethylenediamine tetra acetate
DTNB	5, 5'-dithionitrobenzoic acid
ELF	Epithelial lining fluid
FA	Filtered air
FBS	Foetal bovine serum
FEV_1	Forced expiratory volume in one second
FITC-D	Fluoroscein isothiocyanate dextran
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
FVC	Forced vital capacity
GBq	Graybecquerels
G(M)-CSF	Granulocyte (macrophage) colony stimulating factor
GP_X/GR	Glutathione peroxidase/reductase
GSD	Geometric standard deviation
GSH/GSSG	Reduced/oxidized glutathione
GST	Glutathione-S-transferase
HIV	Human immunodeficiency virus
HNE	Human neutrophil elastase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
^{113}I-DTPA	^{113}indium labelled diethylenetriamine penta acetate
IL	Interleukin (-1, -6, -8)
ILD/IPF	Interstitial lung disease/Idiopathic pulmonary fibrosis
K_2HPO_4	di-potassium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate

LH	Polyunsaturated lipids
LT	Leukotriene (B_4, D_4)
MBq	Megabecquerels
MDA	Malondialdehyde
MetMb	Metmyoglobin
MMAD	Mass median aerodynamic diameter
$NaBH_4$	Sodium borohydride
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
$NaHCO_3^-$	Sodium bicarbonate
Na_2HPO_4	Anhydrous di-sodium hydrogen phosphate
$NaH_2PO_4.2H_20$	Sodium di-hydrogen orthophosphate dihydrate
$NO/NO_2/NOx$	Nitric oxide/Nitrogen dioxide/Oxides of nitrogen
$OH\cdot$	Hydroxyl radical
$O_2\cdot^-$	Superoxide anion
PBS	Phosphate buffered saline
PCP	*Pneumocystis carinii* pneumonia
PDGF	Platelet derived growth factor (-A, -B)
PMA	Phorbol myristate acetate
ppb/ppm	Parts per billion/million
PrSSG	Protein mixed disulphides
ROI	Reactive oxygen species
RTLF	Respiratory tract lining fluid
SH	Sulphydryl or thiol
SOD	Superoxide dismutase
SSA	Sulphosalicylic acid
t_{50}	Time for lung activity to fall to 50%
^{99m}Tc-DTPA	^{99m}technetium labelled DTPA
TBA(RS)	Thiobarbituric acid (reactive substances)
TCA	Trichloroacetic acid
TEAC	Trolox equivalent anti-oxidant capacity
TLC	Total lung capacity
TNF-α	Tumour necrosis factor-α
TV	Tidal volume
$V_{E/I}$	Expiratory/Inspiratory minute volume
VOC	Volatile organic compounds
2-VP	2-vinyl pyridine

CHAPTER 1. INTRODUCTION.

1.1. THE LUNG AND THE ENVIRONMENT.

Galen said that without health, life is not life. This probably applies more to respiratory disease than to any other system due to its chronic nature resulting in distressing dyspnoea and limitation of exercise tolerance. Respiratory conditions are the single largest cause of spells of certified work incapacity, chronic obstructive pulmonary disease (COPD) having the greatest impact on total working time lost (Lung and Asthma Information Agency, 1992).

The greatest burden of respiratory disease is caused by cigarette smoking with 110 000 related deaths per year in the U.K.. William Foege said at the 8th World Conference on Tobacco OR Health "Of all things that will confuse historians of the next century, certainly the idea of a lethal product, a product of illness and despair, peddled to youngsters for the profit of the peddler, will be the most confusing." Cigarette smoking has been causally linked to mortality from chronic bronchitis (Doll and Peto, 1976). Smoking was uncommon at the beginning of the century, but by the end of the Second World War more than 80% of men and 40% of women smoked. Smoking levels have declined in adults since then and 31% of men and 29% of women currently smoke cigarettes with the highest levels being found in Scotland and the North of England (Lung and Asthma Information Agency, 1993/1). However smoking levels in teenagers aged 11-15 years have increased and between 1988 and 1991 rose from 8-10% and among 15 year olds are already two-thirds of the adult rate with 15% of boys smoking regularly and 25% of girls. Cigarette smoking is increasing in the developing world and between 1970 and 1980 increased 33% in Africa and 24% in Latin America. Between 1960 and 1980 cigarette consumption rose by 400% in India and by 300% in Papua New Guinea (Taylor, 1989).

In addition to the obvious effects of cigarette smoking there is now increasing concern over the effect of environmental pollution on health in general and respiratory disease in particular. Pollution is "the contamination of outdoor or indoor air by one or more natural or man made substances in such a way that the air becomes less acceptable for its

intended use to maintain health" (Tattersfield, 1996). Potential respiratory pollutants include ozone, sulphur dioxide, oxides of nitrogen, acid aerosols and particles less than 10 and 2.5μm which are known as PM_{10} and $PM_{2.5}$ respectively. The respiratory effects of pollution will depend on the geography and climate and the type, nature and burden and perhaps more importantly the combination of pollutants. Classical air pollution was made up of smoke and sulphur dioxide and was associated with the burning of coal and generally occurred in winter. Since the 1960's there has been a decrease in pollution from industrial sources and domestic heating and an increase in pollution from motor vehicles. This has resulted in the new problem of photochemical pollution of which ozone is a constituent. Many pollutants are oxidants and their adverse effects on the respiratory tract may be mediated through "oxidant stress". Cigarette smoke contains 10^{16} free radicals per puff (Janoff *et al.*, 1987) and ozone is the second most powerful oxidant known.

Whilst public health measures aimed at reducing the prevalence of cigarette smoking and at striking a compromise between society and motor vehicles are required, the basic mechanisms of oxidant induced lung injury require continuing investigation, particularly as there is evidence of an increased oxidant burden in many respiratory conditions (Taylor *et al.*, 1986, Cross *et al.*, 1994a).

1.2. THE RESPIRATORY TRACT.

The respiratory tract is divided into the upper and lower respiratory tract which are defined as being above or below the cricoid cartilage. The lower respiratory tract therefore includes the trachea and the bronchi and their divisions. The trachea is lined with a mucous membrane of pseudostratified ciliated columnar epithelium containing goblet cells. The submucosa contains elastic fibres, a capillary plexus and mucous glands. The trachea, main bronchi and lower lobe bronchi are outside the substance of the lung. All other bronchi are situated within the lung. The lower airways are known as bronchi as long as they contain any degree of cartilage after which they become bronchioles. The final branches of these conducting airways are the terminal bronchioles. Subsequent divisions contain increasing numbers of alveoli in their walls and are known as

respiratory bronchioles as they take part in gas exchange. They give off alveolar ducts and alveoli. There are 8-13 subdivisions of bronchi. The smallest bronchi are approximately 1mm in diameter with about 3-4 further subdivisions of bronchioles down to the terminal bronchioles. There are usually 2 subdivisions of respiratory bronchioles each with alveoli arising from its walls with 3-9 divisions into alveolar ducts which are surrounded by alveoli. There are up to 9 generations of alveolar ducts. The total number of alveoli has been estimated to be from $2\text{-}6x10^8$ (Burroughs and Edwards, 1960, Angus and Thurlbeck, 1972, Hansen and Ampaya, 1976), thus giving the airways and airspaces a huge surface area estimated to be $180cm^2$ at the level of the terminal bronchiole and $70\text{-}80m^2$ at the alveolar level (Weibel, 1963).

The bronchial walls contain 3 layers, the mucosa, submucosa and fibrocartilaginous layer which includes the smooth muscle. The mucosa consists of pseudostratified squamous epithelium on an elastic lamina, thinning distally and then merging with the alveolar epithelium. Most of the mucosa is covered with groups of ciliated cells interspersed with non-ciliated cells. There are 8 different cell types in the mucosa: the ciliated cell, the goblet cell, the Clara cell, the serous cell, basal cells, intermediate cells, brush cells and the endocrine or APUD (amine precursor uptake and decarboxylation) cell. The submucosa is external to the basement membrane and contains elastic fibres, mucous glands, smooth muscle, nerves and lymphatics. The fibrocartilaginous layer continues from the trachea to the smallest bronchus. As the cartilage becomes sparser the fibrous layer thins and merges with the connective tissue of the lung and the fibrous framework around the alveolar ducts. The fibrous layer is pierced by nerves, blood vessels and mucous glands. Mast cells are found in the submucosa and mucosa. Mucus is present over the bronchial epithelium as a continuous sol layer, in which the cilia beat, and a gel layer which is propelled over the surface.

The acinus is that part of the lung distal to the terminal bronchioles and consists of respiratory bronchioles and their alveoli, alveolar ducts, terminal alveolar sacs and alveoli. Alveolar sacs are hemispherical structures the walls of which are composed of alveoli. There are approximately 25 000 acini in the human lung. The walls of the

respiratory bronchioles contain some muscle bundles diminishing peripherally with some muscle fibres in alveolar ducts which are also supported by collagen fibres, reticulin and elastin. The epithelium of the respiratory bronchioles away from the alveolated portions is cuboidal and contains the same cells as the more proximal airways with ciliated and Clara cells being prominent.

The main functions of the respiratory tract epithelium are firstly to act as a barrier against the diffusion of large molecules such as albumin into the airways and airspaces and against inhaled material for example cigarette smoke, pollutants such as ozone, bacteria, viruses and allergens, secondly to maintain the composition and volume of the respiratory tract lining fluids (RTLF's) and thirdly mucociliary clearance. These have been described as the "target functions" of respiratory tract epithelial cells. However an increasing role for airway and airspace epithelial cells as "effector cells" has been described in the regulation of airway reflexes and the maintenance of bronchodilatation and in immunological and inflammatory responses including the production of nitric oxide (NO) and endothelin-1, the expression of major histocompatibility class II antigen expression and the production of arachidonic acid metabolites, chemotactic agents and chemotactic factor inactivators and cytokines, for example granulocyte macrophage and granulocyte-colony stimulating factors (GM-CSF, G-CSF), interleukin-1 (IL-1), IL-6, IL-8, platelet derived growth factor-A (PDGF-A) and PDGF-B (Dye and Adler, 1996).

The alveolar epithelium is a monolayer composed mainly of the very thin type I pneumocyte and the more cuboidal type II pneumocyte (Figure 1.1). Each respiratory bronchiole is accompanied on one side by a muscular pulmonary artery which gives off arteriolar branches which in turn supply the alveolar capillaries. Each alveolus is surrounded by a network of capillaries whose wall is one endothelial cell thick. The alveolar capillary membrane thus consists of a single layer of alveolar epithelial cells, their basement membrane, a thin interstitial space normally between 5 and 10μm in diameter which may contain small amounts of collagen and elastin, unmyelinated nerves and nerve endings and occasional macrophages, the capillary basement membrane and the capillary endothelial cells (Corrin, 1981). In many areas the

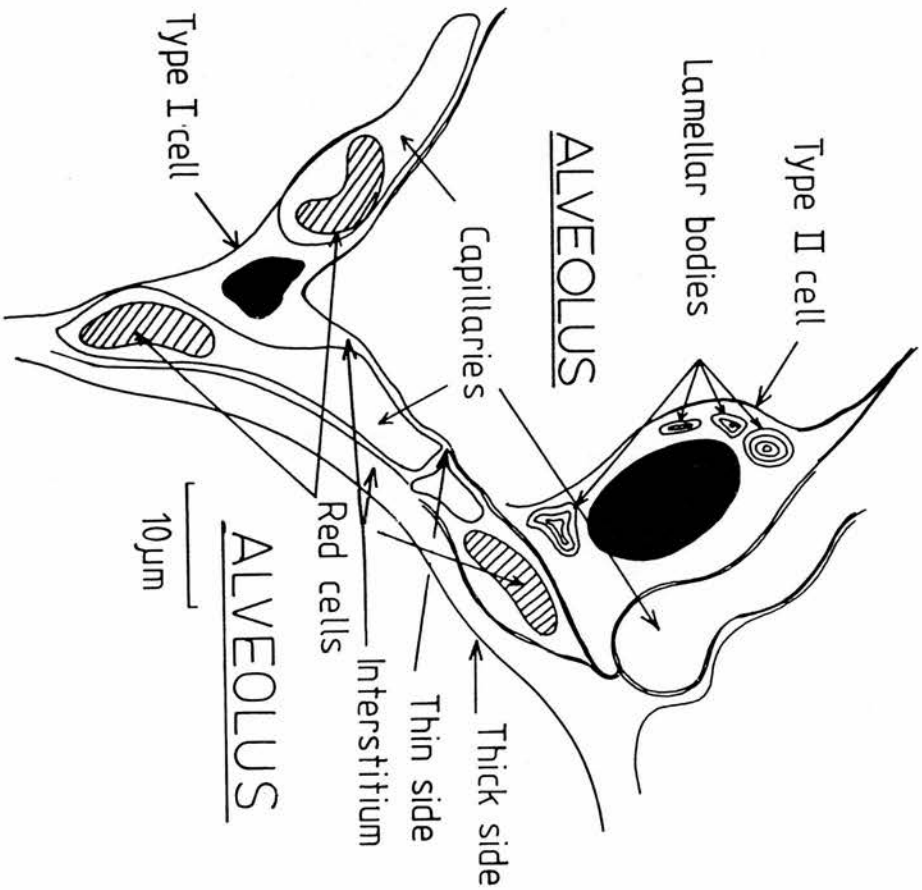


Figure 1.1. Diagrammatic representation of the alveolar capillary membrane.

basement membranes become one eliminating the interstitium and minimizing the distance for gas to diffuse from the alveolus to the erythrocyte to about 0.2μm at its narrowest. The alveolar capillaries join together to form venules, alongside the respiratory bronchioles, which join to form pulmonary veins which run in the interlobular septa.

Ventilation of alveoli may occur through collateral pathways as well as directly down the bronchioles (Menkes and Traystman, 1977). Small openings 10-15μm in size in alveolar walls, known as pores of Kohn (Cordingley, 1972), permit the passage of gas between adjacent alveoli and appear to become more numerous and larger throughout life (Macklem, 1971). In addition short epithelium-lined bronchiole-alveolar communications about 30μm in diameter (Lambert, 1957) allow gas to pass from one bronchiole to alveoli of a neighbouring acinus. Larger diameter interbronchiolar communications may occur between adjacent respiratory bronchioles (Martin, 1966) and collateral ventilation probably also takes place at the level of alveolar ducts (Boyden, 1971).

Type I pneumocytes are specialized for the diffusion of gas from alveolus to capillary. They are fewer in number than type II cells but their surface area is much greater and so they cover the majority of the alveolar surface. They contain endocytic vesicles which transport fluid, small molecules such as large molecular weight proteins and dust particles (Corrin, 1969). Type I cells are connected to each other by tight junctions. They are derived from type II cells and do not undergo further division. Type II cells have microvilli on their apical surface and tend to be grouped in the corners of alveoli. They are rich in mitochondria, endoplasmic reticulum and lamellar bodies which are secreted into the alveolar lumen as surfactant. In acute alveolar injury type II cell proliferation occurs with intermediate cells seen (Bowden, 1981). Endothelial cells line the alveolar capillaries, are the most numerous cells of the alveolar capillary membrane and are not connected by tight junctions thus allowing small molecules and fluid to escape.

Alveolar macrophages are the main phagocytic cell of the acinus and are derived from bone marrow promonocyte stem cells which enter the circulation as monocytes and reach the lung by diapedesis through the

capillary endothelium (Brody *et al.*, 1975). They can penetrate the alveolar epithelium, probably mainly between type I and type II cells and come to lie in the surfactant layer. They contain lysosomal bodies, phagosomes containing particulate matter and whorled structures probably of ingested surfactant. They secrete lysozyme, proteases, complement components, plasmin inhibitors, alpha2 macroglobulin and chemotactic factors. They also release oxidants and play an important immunological role. Surfactant lines the inner surface of the alveolar epithelium and consists mainly of dipalmitoyl lecithin and dipalmitoyl phosphatidylethanolamine, combined with proteins. Surfactant is positively charged and attaches to the negatively charged epithelium. It decreases the surface tension of the alveolar wall during expiration and thus reduces the work of inflation and also acts as a waterproofing material (Hills, 1981, Hills, 1982). Surfactant greatly increases the pressure required for fluid to leak from the interstitium into alveoli. In addition any fluid which does leak tends to break up into droplets because of the hydrophobic nature of surfactant and these will collect in the angles and corners of alveoli where the surface tension generated may force them back into the interstitium (Hills, 1982). Parasympathetic nerves from the vagus carry afferent messages from stretch receptors within the lung, from juxtacapillary J receptors and from bronchial irritant receptors (Seaton *et al.*, 1989). Efferent parasympathetic fibres mediate bronchoconstriction, bronchial gland secretion and probably pulmonary vasodilatation (Nadel and Barnes, 1984). In addition the peptidergic or non-adrenergic, non-cholinergic system exists. Its mediators are probably peptides of which 2 seem particularly important, vasoactive intestinal peptide and substance P (Barnes, 1984).

1.3. MACROPHAGES.

Alveolar macrophages are the major phagocytic cell in the airspaces and are part of the reticuloendothelial system. They are derived from circulating monocytes, bone marrow being the source of their common precursors. These cells are involved in numerous homeostatic, immunologic and inflammatory processes. Mature monocytes migrate into tissues and body cavities where they differentiate into macrophages. The majority of tissue macrophages are derived from monocytes.

However there is evidence that a small percentage of macrophages derive from local division of non-resident mononuclear phagocytes that have arrived in tissues from the bone marrow before completion of cell division. The percentage of alveolar macrophages synthesising DNA is increased 10 times in inflammatory states and in smokers, from 0.5% under normal conditions (Bitterman *et al.*, 1984, Fels and Cohn, 1986) when the macrophage population within tissues remains constant being regularly renewed by the influx of monocytes (Van Furth, 1992).

Macrophages have diverse biological roles (Auger and Ross, 1992). In their phagocytic capacity they provide an immediate defence against foreign elements such as bacteria, viruses, fungi and protozoa prior to leucocyte recruitment. Through phagocytosis and the production of oxygen radicals and proteases, alveolar macrophages can eliminate most particulates and micro-organisms from the distal airways and keep the alveoli sterile. Phagocytosis involves the binding of a particle to the surface of the phagocyte and subsequent engulfment of the bound particle. Fc receptors and complement receptors enable macrophages to recognise and phagocytose opsonised micro-organisms. Opsonisation of bacteria with complement and/or IgG permits macrophage recognition and subsequent phagocytosis of invading micro-organisms. Phagocytosis can also occur without opsonisation if the micro-organism expresses surface molecules that are directly recognised by the macrophage e.g. carbohydrate residues. During phagocytosis alveolar macrophages and other phagocytes release oxygen radicals and enzymes either into the phagosome or the external environment. These are essential for bacterial killing by phagocytes. Alveolar macrophages do not release as many oxygen radicals as neutrophils but they outnumber them by up to 100 fold or more in the airspaces (Greening and Lowrie, 1983). The site of residence of the macrophage, the state of cell maturation and its state of activation all govern the production of oxygen free radicals by macrophages. Alveolar macrophages release more oxygen free radicals than peritoneal macrophages, and peripheral blood monocytes, as they mature, have a reduced capacity to release oxygen free radicals (Drath and Karnovsky, 1975, Nakagawara *et al.*, 1981). Macrophages also assist in cell-mediated immune responses against pathogens, producing immunomodulatory cytokines and regulating immunological responses.

Phagocytosed antigen is processed and presented to lymphocytes in conjunction with Class I or II molecules of the major histocompatibility complex (Unanue and Allen, 1987) thereby stimulating antigen-specific T cells.

Macrophages are a source of many important secretory substances including cytokines and enzymes, cytokine and enzyme inhibitors, growth factors, hormones, complement components, coagulation factors and extracellular matrix proteins. Many of these secretory products are induced by inflammatory or infectious stimuli and have roles in host defence against tumours, secretory products inhibiting tumour cell growth and leading to lysis of neoplastic cells (Adams and Hamilton, 1992).

Tissue macrophages are central to initiating the acute phase response. Macrophages that have migrated to sites of inflammation have a large and diverse potential for further development. Upon activation (Adams and Hamilton, 1984, Paulnock, 1992), resident macrophages acquire increased capabilities for killing of micro-organisms and tumour cells. Activated macrophages also display maximal secretion of inflammatory mediators such as tumour necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2), IL-1 and IL-6 which cause a secondary release of cytokines including chemotactic factors for neutrophils e.g. IL-8 and monocytes e.g. monocyte chemoattractant protein. During an inflammatory reaction, there is increased recruitment of peripheral blood monocytes to the perturbed site where they differentiate into macrophages. The transition from monocyte to macrophage occurs over approximately 48 hours and involves considerable increase in size, the number of cytoplasmic organelles and surface molecules involved in effector function thereby enhancing the potential capabilities of the differentiated cell.

Different types of macrophages occur in the lungs (Brain, 1988). The alveolar macrophage is the best characterised and most extensively studied and can be differentiated from the interstitial macrophage. It has a lobulated nucleus and a vacuolated cytoplasm containing numerous mitochondria and electron-dense secondary lysosomes (Pratt et al, 1971). Alveolar macrophages are heterogeneous in morphology and function

varying in size from 12 to 40μm (Cohen and Cline, 1971). Pulmonary vascular macrophages have also been reported in humans adhering tightly to the endothelial wall (Dehring and Wismar, 1989). Macrophages are also present in large and small airways, some of which are alveolar that have been carried up by the mucociliary escalator, and some of which are resident cells of the airways, adhering tightly to the epithelial cells. Membrane receptor expression and cell functions, such as phagocytosis and mediator release, have been shown to vary among these different subpopulations (Sibille and Reynolds, 1990). The distribution of subpopulations differs between different disease states and each subpopulation can respond differently to different stimuli.

Macrophages from smokers are larger (up to 50μm) and contain more pigment indicating intracytoplasmic inclusions (Niewoehner *et al.*, 1974, Marcy and Merrill, 1987). They release increased amounts of oxygen metabolites, such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) (Hoidal *et al.*, 1981). Release can be stimulated by several activators including lectins, phorbol esters, N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP), colchicine, arachidonic acid and its metabolites, immune complexes, opsonised zymosan, γ interferon and TNF-α. The glutathione redox system is required for the respiratory burst and membrane phospholipids are involved (Sibille and Reynolds, 1990). Alveolar macrophages are a rich source of the anti-oxidant glutathione (Horton *et al.*, 1987).

Proteolytic activity is a major function of alveolar macrophages. They release a number of enzymes including lysozyme, particularly in smokers, a serine elastase which is probably of neutrophil origin, a metalloenzyme elastase, cathepsin L, collagenase (fibroblast like and type V or gelatinase), β-glucuronidase, acid hydrolases, plasminogen activator and angiotensin converting enzyme (McGowan *et al.*, 1983, Sibille and Reynolds, 1990). They also release a number of anti-proteases including α_1-antitrypsin, α_2-macroglobulin, plasminogen activator inhibitor and collagenase inhibitor (Sibille and Reynolds, 1990).

Alveolar macrophages can have both pro-inflammatory activities through the release of activators in the airspaces and also anti-

inflammatory activities through the release of different inhibitory mediators. The balance between activators and inhibitors and the mechanisms regulating their release is very important in determining the outcome of lung injury.

1.4. NEUTROPHILS.

In most types of acute inflammation, in the lung as in other tissues, the neutrophil predominates for the first 6-24 hours. The mature neutrophil has a segmented nucleus with 2-5 lobes and several hundred cytoplasmic granules containing principally lysozyme and lactoferrin with other larger lysosomal granules containing myeloperoxidase, lysozyme, acid hydrolases and a variety of other bacteriocidal proteins. The earliest recognisable precursor is the myeloblast and with increasing maturation there is increasing condensation and lobulation of the nucleus.

After their release from the bone marrow, neutrophils circulate in the bloodstream with a half life of several hours. Within the bloodstream there is a circulating and a marginated pool of approximately equal size. The lung is a major reservoir for the marginated neutrophils. However true margination in postcapillary venules may not occur in the lungs, the "marginated" pool existing as sequestered cells in the pulmonary capillaries (MacNee and Selby, 1993). It is presumed that all neutrophils eventually leave the circulation either entering normal tissues at sites of inflammation and not returning or being taken up as effete cells by the reticuloendothelial system (Niewoehner, 1988). Cells are thought to leave the bloodstream from the marginated compartment but can exchange freely between the circulating and marginated pools during their brief stay in the blood. Neutrophils migrate from the bloodstream by squeezing between endothelial cells and penetrating the basement membrane. During an acute inflammatory response there are alterations in local blood flow and vascular permeability which facilitate the passage of neutrophils into the area of inflammation.

The major function of neutrophils is phagocytosis, killing and digestion of micro-organisms. Phagocytosis by neutrophils is greatly enhanced when bacteria are coated with immunoglobulin or the C3b component of

complement. The marginated pool can be rapidly recruited to sites of inflammation in response to chemotactic factors. As with macrophages, phagocytosis is associated with the respiratory burst through the activation of a membrane-associated reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase with the generation of oxygen free radicals which are involved in bacterial killing. Neutrophils also release various amounts of oxygen metabolites, including $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$, depending on the stimulus and on its concentration. The majority of neutrophil enzymes are stored in the azurophilic granules. These include acid hydrolases, myeloperoxidase, lysozyme, and neutral serine proteases (Sibille and Reynolds, 1990). Among the neutral serine proteases, neutrophil elastase represents quantitatively the major granule component. It can degrade elastin, type I, II, III, and IV collagen, fibrinogen, fibronectin, and proteoglycans. In addition, elastase as well as the other neutrophil proteases (cathepsin G and collagenase) can activate and inactivate C3 and C5a, thereby being involved in regulating the inflammatory reaction.

As noted above all of these species can damage the lungs. Interactions between bacteria, antibodies, neutrophils, and substances released by the phagocytes themselves augment the inflammatory response and activate the complement, kinin, coagulation, and fibrinolytic cascades. As a result, specific chemotactic substances are generated which actively attract more neutrophils to the site. Phagocytes are attracted to inflamed sites along a "diffusion gradient" of inflammatory mediators and tissue breakdown products. These events are closely followed by a marked increase in marrow neutrophil production.

It has been reported in normal rabbit lungs that only about one alveolus in 100 contains a neutrophil (Cohen *et al.*, 1979). Their half-time for disappearance from the bloodstream is 6 to 7 hours (Flenley *et al.*, 1986). As mentioned above sequestration of neutrophils occurs within the pulmonary microvasculature. The average neutrophil has a mean diameter of 7μm (range 5-8μm), very similar to that of an erythrocyte but larger than the pulmonary capillary lumen which has a mean diameter of 5μm (range 1-16μm) (MacNee and Selby, 1993). The deformability of the neutrophil when negotiating the pulmonary microvasculature is

therefore an important factor in its transit. Sequestration of circulating neutrophils in the pulmonary microvasculature is increased by acute cigarette smoking (MacNee *et al.*, 1989) due to decreased cell deformability (Selby *et al.*, 1991, Drost *et al.*, 1993). Neutrophils accumulate in the airspaces of cigarette smokers, the absolute number being consistently raised (Hunninghake and Crystal, 1983, Nagai *et al.*, 1988, McCrea *et al.*, 1994). As mentioned above this may be mediated through the release of chemotactic factors such as C5a, leukotriene B_4 (LTB_4) (Sibille and Reynolds, 1990) and IL-8 (Kunkel *et al.*, 1991).

1.5. MEASUREMENT OF EPITHELIAL PERMEABILITY.

The alveolar-capillary membrane not only facilitates the exchange of gas but also acts as a barrier against the diffusion of large molecules such as albumin into the airspaces and against inhaled material for example cigarette smoke, pollutants such as ozone, bacteria, viruses and allergens. The tight junctions between type I cells are critical to this barrier function. This barrier can be impaired, for example in fibrosing alveolitis or alveolar oedema and by both cigarette smoke and ozone, producing increased respiratory epithelial permeability (Jones *et al.*, 1980, Kehrl *et al.*, 1987). The alveolar epithelium is thought to be approximately 10 times less permeable than the pulmonary capillary endothelium. It is suggested that the pores in the alveolar epithelium are about 0.8-1.0nm in radius whereas those on the capillary endothelium are about 4.0-8.0nm (Taylor and Gaar, 1970).

The study of gas exchange is not a specific test of barrier function because of the dominant effect of regional distribution of ventilation-perfusion matching on gas exchange. The carbon monoxide diffusing capacity is dominated by the magnitude of the surface area of the alveoli and by the kinetics of the binding of carbon monoxide to haemoglobin and is not particularly sensitive to changes in the functional properties of the barrier itself (Jones *et al.*, 1983). Carbon monoxide, like other gases, readily penetrates cellular membranes and is therefore insensitive to changes in intercellular tight junctions. Closing volume has been used as an indirect index of increased lung water which, if pulmonary vascular and colloid osmotic pressures are normal, may reflect severely abnormal barrier

function but it is of limited value in the study of subtle impairment (Jones *et al.*, 1983).

The simplest technique for the evaluation of the alveolar-capillary barrier is to instill solutions containing indicators into the airways and to measure them in the blood or alveolar fluid (Chinard *et al.*, 1962). Enna instilled drugs into the lungs of rats and after a certain time determined drug concentrations in excised lungs thus calculating the half time of absorption from the lung (Enna and Schanker, 1972). Iodinated polyvinylpyrrolidone has been injected intravenously and recovered by endobronchial washing (Valimaki *et al.*, 1974). Radiolabelled tracer molecules have been instilled into an isolated, saline filled lung segment and both the saline and blood sampled at intervals (Nelson *et al.*, 1978) although concerns have been expressed that instillation of saline into alveoli may itself produce epithelial permeability.

Early studies noticed that pulmonary oedema fluid obtained by deep tracheal suction from ventilated patients with pulmonary oedema and the sepsis syndrome had a protein content similar to that of plasma (Katz *et al.*, 1972, Robin *et al.*, 1972). Although simple this technique as a measure of barrier function did not achieve widespread clinical use because the results are not always pathognomic, sequential oedema liquid protein changes were not understood and oedema liquid often cannot be sampled. The rate of appearance of radiolabelled albumin and other tracers injected intravenously has been measured in suctioned pulmonary secretions and bronchoalveolar lavage (BAL) (Sibbald *et al.*, 1981). In particular ^{131}I-human serum albumin has been used in the adult respiratory distress syndrome (ARDS) (Anderson *et al.*, 1979) and the albumin clearance calculated using the formula:

$$\text{Albumin clearance into the bronchial tree (mls hr}^{-1}) = \frac{A \times B}{C}$$

where; $A = [^{131}I]$ in bronchial aspirate (counts ml^{-1})
B = bronchial aspirate volume (mls hr^{-1})
C = plasma $[^{131}I]$ (counts ml^{-1})

This crude index was able nonetheless to detect a difference between patients with left ventricular failure and those with sepsis and ARDS. It assumes that the suctioned fluid sampled is representative of alveolar fluid rather than inflammatory secretions from the bronchial circulation.

Studies employing multiple tracers instilled by aerosol have compared the rate of appearance in serial blood samples of ^{51}chromium labelled ethylenediamine tetra acetate (^{51}Cr-EDTA) and ^{99m}technetium labelled diethylenetriamine pentacetate (^{99m}Tc-DTPA) which are hydrophilic with that of the lipid soluble ^{125}I-antipyrine which is rapidly removed from the lung. The chelates are very poorly diffusible across the normal cell membrane and an accelerated clearance relative to antipyrine indicates damage to the epithelial barrier. The index of permeability is expressed as the ratio of the area under the chelate curve to that under the antipyrine curve in the first 5 minutes after inhalation by bolus or aerosol (Jones *et al.*, 1978, Huchon *et al.*, 1981). The drawbacks of this technique are the need for 2 isotopes and the long half life of ^{125}I therefore a search was made for a single tracer technique.

The requirement for a tracer molecule to study epithelial barrier function is that it should be chemically inert, electrically neutral, hydrophilic and highly stable and that it does not bind to specific sites on the cell membrane nor is it actively transported across cell barriers. The labelled chelates ^{51}Cr-EDTA, ^{99m}Tc-DTPA and ^{113}indium labelled DTPA (^{113}In-DTPA) fulfill most of these requirements and have been used widely in humans having, in particular, high *in vivo* stability (Jones *et al.*, 1983, Hunter *et al.* 1990). In addition they approximate the molecular radius of the pores in the alveolar epithelium and thus their passage will be a measurement of the barrier function of the epithelium rather than the capillary endothelium. The technique used should be easily applicable and minimally invasive. γ-emitters are preferred as they can be quickly and easily measured.

The reference tracer was abandoned and ^{99m}Tc-DTPA increasingly used as the radioaerosol although there are no data about whether it is transferred by simple diffusion, facilitated diffusion, active transport or vesicular transport although it is assumed to be the first of these. It is

removed predominantly via the pulmonary circulation and <1% via the lymphatics even in injured lungs when removal by the latter route is increased (Rizk *et al.*, 1984, Staub *et al.*, 1990). ^{99m}Tc-DTPA has a molecular weight of 492 daltons with a radius of 0.6nm. An external scintillation counter was initially used to measure the rate of disappearance of the labelled chelate from the lung (Jones *et al.*, 1980). The rate of increase in activity in the leg following an intravenous bolus of ^{99m}Tc-DTPA was measured with a second scintillation counter and was used to correct for the increasing contribution from recirculating background activity with time during a study. Other groups have used only the first 7 or 10 minutes of the lung clearance curve extrapolating the data beyond this, avoiding the portion of the curve affected by recirculation and thus the need to use an intravenous correction (Rinderknecht *et al.*, 1980). Respiratory epithelial permeability may be expressed as the time for 50% of the initial lung activity to disappear (t50) or as the decline in activity min^{-1} (k) derived from t50 = 0.693/k and arbitrarily expressed as a percentage (k100) (Barrowcliffe and Jones, 1987).

The site of deposition is an important factor. It is generally accepted that to minimize tracheobronchial deposition there should be few particles greater than 2μm in diameter. The optimal range for alveolar deposition is between 0.3 and 2μm (Chan and Lippman, 1980). The permeability of the conducting airways is less than that of the terminal airways, when mucociliary clearance is taken into account, this slow clearance perhaps being due to binding to mucus (Barrowcliffe *et al.*, 1987). The clearance rate is not influenced to any extent by variations in pulmonary blood flow (Rizk *et al.*, 1984). Raised lung volume and positive end expiratory pressure both increase ^{99m}Tc-DTPA lung clearance (Nolop, Maxwell *et al.*, 1986, Nolop, Braude *et al,*, 1987). Clearance is also increased in the upper zones at least in the upright posture (Mason *et al.*, 1983, O'Doherty *et al.*, 1985a, Dusser *et al.*, 1986). Although cigarette smoking increases the lung clearance of ^{99m}Tc-DTPA (Jones *et al.*, 1980) it is not affected by the presence of COPD (Huchon *et al.*, 1984) although the deposition of the isotope is more central (Groth *et al.*, 1990). It is important that the isotope remains stable during the study and in this respect no accumulation in the thyroid should be observed, this being the site for uptake of free pertechnetate (Barrowcliffe and Jones, 1987). It has been shown with the

more tightly bound ^{113m}In-DTPA, in comparison with ^{99m}Tc-DTPA, that although some oxidative dissociation of the ^{99m}Tc-DTPA complex may occur in a smoker's lungs it is not sufficient to explain the greater clearance in smokers (Nolop *et al.*, 1987). It is worth noting that clearance is accelerated in smoking even although mucociliary clearance is slowed (Lourenco *et al.*, 1971). ^{99m}Tc-DTPA lung clearance curves, derived from studies of over 20 minutes, may be biphasic requiring 2 exponential equations to fit all the data points. Biexponential ^{99m}Tc-DTPA curves are almost certainly abnormal. Even the very fast clearance times seen in healthy smokers are always monoexponential (Hunter *et al.*, 1990). Biexponential curves are usually seen in acute lung injury (Barrowcliffe and Jones, 1989).

In order to compare results among laboratories it has been recommended that the following factors are reported (Staub *et al.*, 1990):

1. The size of inhaled aerosol as mass median aerodynamic diameter (MMAD) and its geometric standard deviation (GSD).
2. Ventilatory pattern used (tidal volume [TV] and ventilatory rate) during the delivery of the inhaled ^{99m}Tc-DTPA and the time required to deliver the aerosol.
3. How the clearance was calculated from the recorded data, including manner of selection of the initial data points and duration of counting.
4. Description of the data processing used to select the regions used for counting.
5. Verification that the method of preparing ^{99m}Tc-DTPA and its subsequent delivery did not cause dissociation of the ^{99m}Tc label from the DTPA.
6. The method used, if any, to correct for accumulation of background counts during measurement of clearance.

The main problem with the use of ^{99m}Tc-DTPA is its sensitivity, as seen in healthy smokers, and changes in its clearance with changes in lung volume. Staub and colleagues have suggested that molecules intermediate in size between DTPA and albumin should be explored with the goal of finding an agent more specific for lung injury and not influenced by the above factors. However finding a better radioactive

tracer has proved difficult because of incomplete binding, intrapulmonary retention of the label and very slow clearance of larger molecules such as albumin and transferrin (Staub *et al.*, 1990).

The main technique for the investigation of pulmonary endothelial barrier function is pulmonary lymph flow but this is not accessible in the investigation of human patients or subjects (Jones *et al.*, 1983). Other methods have been used for example multiple arterial sampling after an intravenous injection of a range of low molecular weight tracers, allowing calculation of their transit time and distribution volume during a single pass through the pulmonary vasculature, the results being dependent on the distribution of pulmonary blood flow (Chinard *et al.*, 1962). The analysis of lung uptake of labelled urea can provide a useful measure of increased capillary permeability (Brigham *et al.*, 1979). Other workers have studied the movement of radiolabelled protein from blood to interstitium, for example injecting radiolabelled albumin and using a gamma camera to calculate the ratio of activity over the lungs to that over the heart (Sugarman *et al.*, 1982). Alternatively in the protein accumulation index, indium labelled transferrin (molecular weight 76 000) and technetium labelled red blood cells are injected, the latter as an intravascular marker to correct for changes in blood volume in the fixed field of view of a single scintillation detector over the lung (Gorin *et al.*, 1980). A modification of this technique uses a second scintillation counter over the heart (Basran *et al.*, 1985). ^{113m}In-transferrin is a diffusible marker and the counts over the lung field provide a measure of circulating and extravascular labelled transferrin. To correct for the normal clearance of the radiotracer from the circulation and for decay the lung counts are divided by those from the cardiac detector which represents the blood pool. If this indium ratio remains constant then no accumulation of protein has occurred in the lungs but if extravasation into the lungs at a rate greater than lymphatic removal occurs, the ratio increases with time. To correct for possible changes in thoracic blood volume beneath the detector, the indium ratio is divided by an identical ratio for technetium to give the lung transferrin index, the rate of change of which provides the protein accumulation index. This index is unaffected by smoking (Braude *et al.*, 1986) but is increased in ARDS (Rocker *et al.*, 1987) even as soon as the criteria develop although there is

overlap with the normal range (Braude *et al.*, 1986). The protein accumulation index is theoretically most sensitive in the first few minutes after the intravascular injection of the tracer, as at early times the influence of interstitial liquid volume and of losses in lymph or otherwise are negligible. There is some doubt whether ^{113m}In-transferrin is the best tracer as there are transferrin receptors on cells particularly in inflamed tissue. Protein transport across endothelium must be considered although there is little evidence that such pathways play any significant role.

1.6. OXIDANTS AND ANTI-OXIDANTS.

An oxidant anti-oxidant imbalance in favour of oxidants or "oxidative stress" may occur in a number of conditions affecting the lungs for example oxygen toxicity, lung cancer, ischaemia-reperfusion injury and lung transplantation, mineral dust pneumoconiosis, bleomycin and paraquat toxicity, idiopathic pulmonary fibrosis (IPF), infant and adult RDS, bronchopulmonary dysplasia, cystic fibrosis, immune complex mediated lung injury, human immunodeficiency virus (HIV) associated lung disease, asthma and acid aspiration (Cross *et al.*, 1994a). The oxidant anti-oxidant balance is critically important to the maintenance of normal cell function. An imbalance has also been proposed in the lungs in COPD (Taylor *et al.*, 1986).

The lungs are constantly exposed to inhaled oxidants, for example cigarette smoke, ozone, nitrogen dioxide and sulphur dioxide, and those released from inflammatory leucocytes. Cigarette smoke is the main aetiological agent in COPD and contains 10^{16} free radicals per puff (Janoff *et al.*, 1987). The elevated numbers of inflammatory cells in the parenchyma and airspaces of the lungs in smokers are primed to release increased amounts of oxidant species (Hoidal *et al.*, 1981). This may also be true of those sequestered in the pulmonary microvasculature (Brown *et al.*, 1995). Phagocytes, that is macrophages and neutrophils, produce not only $OH\cdot$, $O_2\cdot^-$ and H_2O_2, as mentioned earlier, but also hypochlorous acid (HOCl) and other oxidative products (Cross *et al.*, 1994b). A further powerful oxidant species, peroxynitrite, may be produced by the reaction of $O_2\cdot^-$ and NO when levels of both are increased. Under normal

conditions NO acts as an anti-oxidant by quenching $O_2^{\cdot -}$ (Cross *et al.*, 1994a). Transition metal ions or oxygen radicals and thiols may also interact producing reactive and potentially toxic thiyl or oxysulphur radicals (Munday, 1989, Huston *et al.*, 1992, Korge and Campbell, 1993). Oxidant actions may also be mediated by products of reactions between inhaled toxins and the RTLF's (Cross *et al.*, 1994b).

Direct measurement of free radical activity is technically difficult. Most investigators rely on indirect measurements, for example, the extent of lipid peroxidation, overall anti-oxidant capacity or evidence of oxidative damage to proteins and DNA. Oxygen radicals generated close to a cell membrane oxidise membrane phospholipids resulting in lipid peroxidation, a process which may continue in a chain reaction. Several methods exist for the determination of the extent of lipid peroxidation in biological samples although none are specific (Petruska *et al.*, 1990). They include measurement of lipid hydroperoxides by titration with iodine release, stimulation of PGH synthase activity or luminol-enhanced chemiluminescence. Other techniques rely on the measurement of products formed during lipid peroxidation. Conjugated dienes, which absorb ultraviolet (UV) light in the range 230-235nm are formed immediately following initiation of lipid peroxidation. Using gas-liquid chromatography, expiration of ethane and pentane, gases commonly generated during lipid peroxidation, may be quantified *in vivo*. Another technique involves the measurement of fluorescent products which are produced when dialdehydes, one of many types of products of lipid peroxidation, react with amino groups of proteins, phospholipids or nucleic acids to yield Schiff bases. The most popular method of lipid peroxide detection is the thiobarbituric acid reactive substances (TBARS) test. Malondialdehyde (MDA) is produced by free radical attack on polyunsaturated fatty acids in cell membranes. It can be complexed with 2 molecules of thiobarbituric acid (TBA) to yield an adduct with maximum absorption at 532nm. The MDA-TBA adduct can be measured fluorometrically (Yagi, 1976) or by high pressure liquid chromatography (HPLC) (Petruska *et al.*, 1990).

Anti-oxidants in the RTLF's, mucus and epithelial lining fluid (ELF), provide the first line of defence against oxidant stress. They include

mucin, ascorbic acid, the lipid soluble α-tocopherol, reduced glutathione (GSH) and the enzymes of its redox cycle for example glutathione reductase (GR) and glutathione peroxidase (GP_X), uric acid, the metal binding proteins caeruloplasmin, transferrin, and lactoferrin, the anti-oxidant enzymes superoxide dismutase (SOD) and catalase, sacrificial proteins particularly albumin and unsaturated lipids (Cross *et al.*, 1994b). The anti-oxidants present in greatest molar concentrations in ELF are GSH (100-400μM), ascorbic acid (100μM), uric acid (90μM) and albumin-thiol (albumin-SH) (70μM) (Cantin *et al.*, 1987, Cross *et al.*, 1994b).

The thiol anti-oxidant GSH is one of the most important anti-oxidants in the lungs. It is a tripeptide, γ-glutamyl-cysteinyl-glycine. It is present in approximately 100 fold greater concentrations in ELF than in plasma (Cantin *et al.*, 1987) and is also an important intracellular anti-oxidant in lung cells. Both intra- and extracellular GSH are depleted by oxidants. The source of GSH in ELF is thought to be the epithelial cell but this remains uncertain (Heffner and Repine, 1989). Macrophages also contain high levels of intracellular GSH (Horton *et al.*, 1987). Thiols such as N-acetyl cysteine and GSH are able to scavenge $OH\cdot$, H_2O_2 and HOCl (Aruoma *et al.*, 1989). Other thiol anti-oxidants include protein-SH groups, for example in albumin, and mucin (Cross *et al.*, 1994a). Albumin contributes 43% of the Trolox equivalent anti-oxidant capacity (TEAC) in plasma (Miller *et al.*, 1993) and has 1 SH group per molecule. It may form albumin-thiyl radicals or albumin-SH group conjugates with electrophiles present in cigarette smoke (Rahman and MacNee, 1996).

Ascorbic acid is an important water soluble extracellular anti-oxidant, sparing other anti-oxidants, such as α-tocopherol, through its recycling, and by scavenging toxic free radical products (Frei *et al.*, 1990). Uric acid is secreted by the same upper airway epithelial cells that secrete mucin and α-tocopherol and by type II alveolar epithelial cells along with surfactant (Peden *et al.*, 1993, Rustow *et al.*, 1993). It is a major low molecular weight anti-oxidant present in the lining fluids of the upper respiratory tract (Peden *et al.*, 1993). It appears to be the most important scavenger of ozone in plasma contributing 33% of the TEAC and may also be important in the respiratory tract (Cross *et al.*, 1992).

There are technical difficulties in studying anti-oxidants in ELF and in epithelial cells. Both are normally obtained by BAL. This technique involves the instillation of an aliquot or aliquots of warmed normal saline into a segment of the lung followed by its aspiration. Approximately 50-60% is normally recovered. It results in the dilution of ELF by a factor which may not only be uncertain but also variable, both from BAL to BAL and between operators and with regard to the different anti-oxidants under study (European Society of Pneumology Task Group on BAL, 1989). A correction is therefore necessary for this dilution, the most satisfactory being the albumin (Ward *et al.*, 1993) and the urea methods (Rennard *et al.*, 1986). Certain anti-oxidants are found in greater concentration in ELF than in plasma most notably GSH and ascorbic acid. The reason for this apparent concentration of GSH in ELF compared to plasma is not known (Cantin *et al.*, 1987). Uric acid and albumin-SH levels are lower in ELF than in plasma (Cross *et al.*, 1994b).

It has been proposed that certain oxidants, ozone for example, may not penetrate the surface layer of mucus or ELF (Pryor, 1992). Nonetheless plasma anti-oxidants are also important in protecting the lungs. Oxidant injury to the respiratory tract results in neurogenic inflammation, involving endothelial permeability and epithelial "leak", resulting in the transudation of plasma anti-oxidants into the interstitium and then into mucus and ELF (Persson *et al.*, 1991, Cross *et al.*, 1994b) perhaps increasing the total anti-oxidant capacity of the RTLF's. This transudation may be the result of direct activation or injury of the cells of the respiratory epithelium or may result from the activation of neurohumoral mediator pathways.

The effectiveness of anti-oxidant protection in RTLF will depend on the type of anti-oxidants present, their concentration and the depth and surface area of the lining layer. Protection of both constituents of the lining fluid itself and the underlying epithelial cells and interstitium presumably results (Cross *et al.*, 1994b).

1.7. SMOKING AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE.

Smoking is the most important cause of chronic bronchitis and emphysema or COPD (U.S. Department of Health and Human Services, 1984). Having been introduced to the Old World by Sir Walter Raleigh, the health effects of smoking were recognised by King James VI of Scotland and I of England. It is 200 years since Samuel Johnson's lungs became the first recorded case of emphysema and Charles Badham coined the term "chronic bronchitis". In 1834 Laennec made the observation that "in emphysema the air makes its escape from the air cells much slower than in a healthy state of the organ. This seems to indicate either more difficult communication between air contained in the air cells and that of the bronchi, or else diminished elasticity of the air cells themselves... both causes conspire to produce the effect in question" (Laennec, 1846). In 1870 Sigmund reported that heavy smokers suffered "affections" of the nose, mouth and throat more frequently than non-smokers and in a more virulent fashion. In 1897, Mendelssohn reported the incidence of "affections" of the respiratory tract to be 60% greater in smokers than in non-smokers, as well as somewhat greater in those who inhaled compared with smokers who did not inhale. Since then the incidence of COPD has increased dramatically and recently particularly in women.

The most important evidence linking smoking and mortality from chronic bronchitis comes from the study of doctors which found that the death rate was significantly higher in cigarette smokers than in non-smokers and increased with the amount smoked (Doll and Peto, 1976). Chronic bronchitis is defined by "the production of sputum on most days during at least 3 consecutive months in 2 successive years" (Medical Research Council, 1965). Emphysema is defined by the National Heart Lung and Blood Institute in the U.S. as "a condition of the lung characterized by abnormal, permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by the destruction of their walls, and without obvious fibrosis" (Snider *et al.*, 1985). COPD is defined as "abnormal values in tests of expiratory flow, which have not changed substantially over several months and which are not related to specific causes of airflow obstruction" (American Thoracic Society, 1987). Approximately 80-90% of deaths from COPD are attributable to smoking

(U.S. Department of Health and Human Services, 1984). In the late 1980s, in the U.K., the prevalence of chronic bronchitis was 15-20% in middle aged men and about 8% for middle aged women (Strachan, 1995). In 1986 71 099 people died as a result of COPD in the U.S., a 33% increase from 1979. The mortality was 1.8 fold greater in men than women and 2.8 fold greater in whites than blacks. The incidence of chronic bronchitis in 1980 was 36.1 per 1000 and emphysema 11.4 per 1000 (Stratton and McCabe, 1990).

In 1963, Laurell and Eriksson observed that subjects whose serum was deficient in α_1-antitrypsin (α_1-AT) [α_1-proteinase inhibitor (α_1-Pi)] were more likely to develop emphysema than normal subjects (Laurell and Eriksson, 1963). Gross subsequently showed that papain could produce emphysema when instilled into the trachea of experimental animals (Gross *et al.*, 1979). From these original observations the protease-anti-protease theory of the pathogenesis of emphysema developed which in its simplified form states that cigarette smoke attracts alveolar macrophages to accumulate around the terminal bronchioles, the site of development of centrilobular emphysema (Niewoehner *et al.*, 1974). They release chemotactic factors which attract neutrophils to this site from the circulation (Hunninghake *et al.*, 1980). The neutrophils then release human neutrophil elastase (HNE) which can attack the lung interstitial matrix particularly elastin. HNE binds covalently to α_1-AT and is inactivated, thus forming a very effective defence for the lung against attack by HNE. Disturbance of this protease-anti-protease balance is thought to be important in the pathogenesis of emphysema (Idell and Cohen, 1983). This can be caused by cigarette smoke and reactive oxygen intermediates released from neutrophils which can oxidize the critical methionine residue at position 358 in α_1-AT, which is the active site for interaction with HNE, producing inactivation of α_1-AT (Gadek *et al.*, 1979, Carp *et al.*, 1982). The evidence that this occurs *in vivo* in humans is controversial (Gadek *et al.*, 1979). However elevated levels of elastin derived peptides have been found in the blood of smokers, levels being more elevated in those with severe airflow limitation suggesting the presence of an enhanced elastase burden (Kucich *et al.*, 1985). Other antiproteases may also have a protective role in emphysema for example anti-leucoprotease (Kramps *et al.*, 1988). Human alveolar macrophages

also attack elastin. They can bind human neutrophil elastase and incorporate it intracellularly with subsequent release of the enzyme in an active form (McGowan *et al.*, 1983). They also secrete a cysteine protease and may also secrete a metal dependent proteinase (Flenley 1986). The total number of inflammatory cells and the number of neutrophils in the airspaces of smokers are increased (Hunninghake and Crystal, 1983, Martin *et al.*, 1985, Koyama *et al.*, 1991). They show increased oxidative metabolic responses (Hoidal *et al.*, 1981) and enhanced cytotoxic potential (Davis *et al.*, 1988). Long-term cigarette smoking also increases the number of neutrophils in the peripheral circulation and in lung parenchyma (Niewoehner, 1988, McGowan and Hunninghake, 1989). Acute cigarette smoking has been shown to cause the sequestration of neutrophils to the pulmonary vasculature (MacNee *et al.*, 1989). Once sequestered their subsequent migration into the airspaces may be mediated through the release of chemotactic factors such as C5a, LTB_4 (Sibille *et al.*, 1990) and IL-8 (Kunkel *et al.*, 1991).

1.8. OZONE.

Urban air pollution was a major problem in the U.K. until after the turning point of the infamous London fog of December 1952 (Ministry of Health, 1954). Such pollution resulted mainly from the burning of coal for domestic and industrial purposes. The Clean Air Act of 1956 was introduced following this and since then there has been a major decrease in the levels of smoke and sulphur dioxide to acceptable levels.

Ozone or O_3 is the main constituent of photochemical pollution. It is otherwise found in the "ozone layer" in the stratosphere between 20 and 50km above the surface of the Earth. Its name is derived from the Greek "ozein", which means "to smell", since it has a characteristic odour which defies description.

The structure of ozone is represented as a resonance hybrid of 2 equivalent Lewis formulae (Figure 1.2). It is commonly prepared by allowing a low temperature electric discharge or UV radiation to pass through O_2:

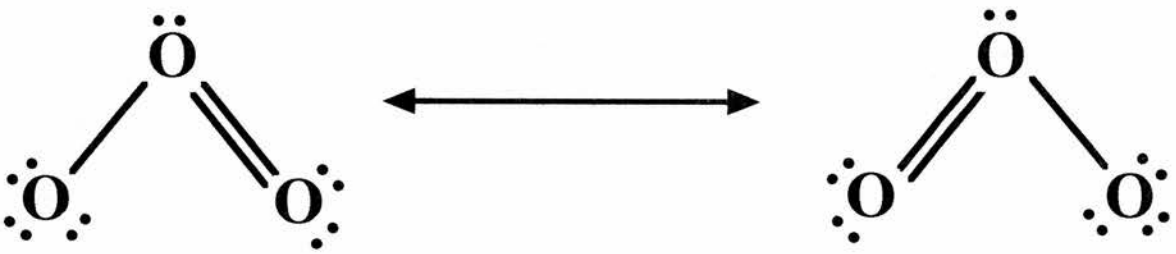


Figure 1.2. The structure of ozone is represented as a resonance hybrid of 2 equivalent Lewis formulae.

$3O_2\,(g) \longrightarrow 2O_3\,(g)$

At room temperature ozone tends to decompose on standing as follows:

$2O_3\,(g) \longrightarrow 3O_2\,(g)$

Ozone is the most important trace constituent in the stratosphere, which is a stable cloudless region, with little vertical circulation and is formed thus:

$O_2 \longrightarrow$ UV radiation $\longrightarrow 2O$ (i)

$O + O_2 + M \longrightarrow O_3 + M$ (ii)

M is a third body such as an oxygen, nitrogen or other molecule present in the atmosphere which must be present to take up part of the energy otherwise the ozone molecule would probably decompose rapidly. By doubling (ii) and adding it to (i) the overall reaction is obtained:

$3O_2 \longrightarrow 2O_3$

Because this reaction is initiated by radiant energy it is called a photochemical reaction. Most of the ozone is produced over the tropics in the upper stratosphere between 25 and 35km. However due to movement the highest concentrations are found over the polar regions at about 15km. Ozone in this layer protects us from the harmful effects of UV light and is therefore extremely desirable. The ozone molecules absorb UV radiation thus:

$O_3 \longrightarrow$ UV radiation $\longrightarrow O_2 + O$

Almost all of the O atoms formed then recombine, to form ozone, as above in (ii). In recent years the ozone content of the ozone layer has decreased over the South Pole producing a "hole" which has been extending alarmingly.

Ozone is also found at ground level in the troposphere where the background level is normally 25-35 parts per billion (ppb). This is only 2-3 fold less than the level which is generally accepted to be associated with adverse health effects. Eighty per cent of ground level ozone is formed in the troposhere and 20% originates in the stratosphere (Hough and Derwent, 1990). In the U.S., standards recommend that ground level ozone does not exceed 0.12 parts per million (ppm) for 1 hour on more than 1 occasion in each year. This standard is exceeded for 60% of the U.S. population. In California the standard is tighter, the level being 0.10ppm. The World Health Organisation recommend that the ozone level should not exceed 76-100ppb for 1 hour or 50-60ppb for 8 hours (World Health Organisation, 1987). This is exceeded by all the rural monitoring sites in the U.K. each year except remote sites in ozone poor years. Ground level ozone tends to be lower in urban areas than in the suburban and rural areas adjacent, due to destruction by NO emitted by motor vehicles and other combustion sources. In Los Angeles ozone levels reach 300-400ppb and in 1977 and 1978 there were 20-30 days and 60-75 days respectively where the level was greater than 200ppb. The highest levels in the U.K. were recorded during the long hot summer of 1976, 258ppb being recorded in Oxfordshire (United Kingdom Photochemical Oxidants Review Group, 1987) and, in 1990, 161ppb at Lullington Heath.

Ozone production in the troposphere depends on volatile organic compounds (VOC's), sunlight and low wind speed. VOC's react with oxides of nitrogen (NO_x) as follows:

$$N_2 + O_2 \longrightarrow 2NO$$

$$2NO + O_2 \longrightarrow 2NO_2$$

$$NO_2 \longrightarrow \text{UV radiation} \longrightarrow NO + O \text{ (Photochemical decomposition)}$$

The atomic oxygen formed is extremely reactive:

$$O + O_2 + M \longrightarrow O_3 + M$$

After ozone is formed it can react with more NO to produce NO_2:

$$NO + O_3 \longrightarrow NO_2 + O_2$$

For every molecule of NO_2 delivered to the atmosphere a molecule of ozone may be formed. The overall reaction is given below:

$$NO_X + VOC \xrightarrow{UV} O_3$$

VOC's are derived from solvent use and industrial processes (51%) and motor vehicles (37%) and NO_X from motor vehicles also (48%) and power stations (29%). There are no direct emission sources of ozone.

Normal subjects show a wide range of sensitivities to ozone, the effects being increased by exercise and the duration of exposure, at least up to several hours (Horstman *et al.*, 1990). In healthy subjects and in those with pre-existing disease environmental levels of ozone may produce symptoms and a decrease in lung function which is reversible and short lived. There is no threshold for the effects of ozone.

There has been a doubling in ozone concentrations since 1900 although measurements did not generally began until 1971. There is now a network of monitoring stations in the U.K., established in 1987 following a review of U.K. ozone data by the Department of the Environment's Photochemical Oxidants Review Group, co-ordinated by Warren Spring Laboratory (United Kingdom Photochemical Oxidants Review Group, 1987). There has been no general increase in ozone levels since 1971 (Lung and Asthma Information Agency, 1993/5).

The main sink for ozone is dry deposition at the ground particularly on leafy foliage. This explains why the highest levels in Scotland are found on the tops of the Cairngorm mountains where foliage is sparse and heather plentiful. The highest levels in the U.K. occur between April and September, each "ozone episode" lasting on average between 1 and 5 hours with gaps between of 20 hours. Ozone build up is cumulative, over episodes which are generally of 1-5 days duration, with levels falling at night as air cools and falls to the level of the foliage. Levels are highest on

the South Coast there being 5-10 times the number of hours where the level exceeds 60ppb than in the North of Scotland. There are 100-200 hours each year where the level exceeds this and 10-50 hours annually where it exceeds 100ppb (United Kingdom Photochemical Oxidants Review Group, 1987). Levels tend to be highest in the mid to late afternoon.

Approximately 40% of ozone is taken up in the nasopharynx and 60% in the lower respiratory tract (Gerrity and Weister, 1987). There is essentially complete absorption at the beginning of the proximal alveolar region (Hu *et al.*, 1992). In rats prolonged ozone exposure produces a loss of cilia from ciliated cells and spreading of Clara cells on the basement membrane surface with a decrease in luminal surface area (Barry *et al.*, 1988). An increase in the volume of type I and type II cells occurs and this is related to total exposure (Chang *et al*,. 1991).

Ozone may cause cough, shortness of breath and chest pain on inspiration. The dose is a product of the ozone concentration, duration of exposure and minute ventilation, the most important being the concentration. Ozone causes certain changes in lung function. Forced expiratory volume in one second (FEV_1), vital capacity (VC) and total lung capacity (TLC) are reduced with no change in residual volume or functional residual capacity. The fall in VC is usually almost as great as the fall in FEV_1. There is a close relationship between ozone dose and both FEV_1 and VC especially if any fall is expressed as a percentage. An increase in airway resistance and specific airway resistance occurs. There is no change in static or dynamic lung compliance but lung volume is reduced at any given static lung recoil pressure. Maximum static lung recoil pressure is reduced. There is no change in maximum inspiratory or expiratory mouth pressures. Respiratory rate (RR) increases and TV decreases. Respiratory epithelial permeability to ^{99m}Tc-DTPA increases (Kehrl *et al.*, 1987). Exercise capacity decreases and bronchial responsiveness increases (Schelegle and Adams, 1986, Hazucha, 1987, Hazucha *et al.*, 1989). Most studies have shown an increase in the response to allergen following ozone exposure (Yanai *et al.*, 1990, Molfino *et al.*, 1991). The effects of ozone on lung function generally resolve after 24 hours (Folinsbee and Hazucha, 1989, McDonnell *et al.*, 1985). With

continued exposure the effects are maximal at 2 days and minimal at 5 days with adaptation lasting 7-20 days (Horvath *et al.*, 1981).

The mechanism of the effects of ozone on lung function is not well understood. The reduction in VC and TLC may be due to involuntary inhibition of full inspiration by stimulation of non-myelinated C-fibres possibly by prostaglandins released during airway inflammation (Hazucha *et al.*, 1989). Although bronchoconstriction occurs it is much less than is seen with conventional bronchoconstrictor agents relative to symptoms and the fall in VC. There is a wide variability in response to ozone between subjects (Horstman *et al.*, 1990, Weinmann *et al.*, 1995). The change in FEV_1 is not related to baseline FEV_1 nor to bronchial responsiveness (Folinsbee *et al.*, 1988). The effects may be greater in young women than young men (Messineo and Adams, 1990, Aris *et al.*, 1995).

Smokers may be less responsive to ozone (Shephard *et al.*, 1983, Kagawa 1984). One chamber study has suggested that asthmatics may be more responsive (Kreit *et al*,. 1989). In patients with COPD no changes in FEV_1 or VC have been seen following exposure of up to 200ppb for 2 hours but the total ozone dose received is low in such subjects due to their limited exercise ability (Solic *et al.*, 1982, Linn *et al.*, 1983). The elderly may be less responsive (Drechsler-Parks *et al.*, 1987) and black adults more responsive than white adults. Whilst it has been said that the changes in lung function are no different in "at risk" groups it has been concluded by the Advisory Group on the Medical Aspects of Air Pollution Episodes that there are relatively few studies comparing at risk groups with most being poorly controlled or with insufficient numbers (Department of Health, 1991).

The public health effects of ozone have been studied. There appears to be no association between symptoms and the ambient ozone level. It has been concluded that pollution, of which ozone is a part, may produce a small increase in asthma morbidity and utilisation of medical services. An individual contribution for ozone cannot be identified. In public health studies asthmatics appear to show the same sensitivity to ozone as non-asthmatics. The Advisory Group states that it seems unlikely that ozone explains the epidemic pattern of asthma observed in various parts

of the world (Department of Health, 1991). They also state that it seems unlikely that ozone can explain the seasonal periodicity of asthma although a modifying effect is not ruled out. Peak ozone levels occur in May, June and July (Bower *et al.*, 1991). Asthma mortality is highest in August and hospital admissions in September (Khot and Burn, 1984, Khot *et al.*, 1984). The prevalence and severity of asthma have increased in the U.K. in the past 20-30 years (Burr, 1987) but there has been a long term reduction in ozone levels at 3 sites in south east England (Bower *et al.*, 1991). In the summer of 1976 in the U.K. when ozone levels were increased markedly for several weeks there was no increase in asthma related mortality (Khot and Burn, 1984, Khot *et al.*, 1984). Supporting evidence comes from Ontario where between 1974 and 1983 there was an increase in asthma admissions but no clear trend in ozone levels (Bates and Sizto, 1987). The Advisory Group comments that there is a general lack of data on trends in ozone with which to test the hypothesis that it is associated with the increase in asthma (Department of Health, 1991).

1.9. AIMS.

The aim of this thesis is to investigate the effects of cigarette smoke and ozone on the respiratory epithelium. Initial studies compared 3 methods of ^{99m}Tc-DTPA clearance from the lungs to blood as a measure of airspace epithelial permeability and assessed their repeatability. Both cigarette smoke and ozone have been shown to increase airspace epithelial permeability (Jones *et al.*, 1980, Kehrl *et al.*, 1987). The effect of cigarette smoking occurs within 3 days of starting smoking (Minty *et al.*, 1984) and is rapidly reversible on stopping (Minty *et al.*, 1981, Mason *et al.*, 1983). Controlled studies of the acute effects of cigarette smoking on airspace epithelial permeability have not previously been performed however. Cigarette smoke contains 10^{16} free radicals per puff (Janoff *et al.*, 1987) and thus ozone inhalation was used as a model for acute oxidant effects on the normal respiratory tract. Using a background corrected method of ^{99m}Tc-DTPA lung clearance, employing an inter-renal region of interest, further studies of airspace epithelial permeability were performed in chronic cigarette smokers following acute smoking and in normal volunteers following exposure to ozone at environmentally relevant levels. Neutrophils accumulate in the airspaces of cigarette

smokers (Hunninghake and Crystal, 1983, Nagai *et al.*, 1988, McCrea *et al.*, 1994) and increased levels of the anti-oxidant GSH are found in their ELF (Cantin *et al.*, 1987). Ozone also produces an influx of neutrophils into the airspaces of normal non-smokers (Koren *et al.*, 1989, Devlin *et al.*, 1991, Koren *et al.*, 1991, Schelegle *et al.*, 1991). BAL was therefore performed to investigate the effect of acute cigarette smoking in chronic smokers and ozone exposure in healthy volunteers on the percentage and number of neutrophils and oxidant status in the airspaces. Previous studies have suggested a role for the cytoskeleton in maintaining the integrity of the respiratory epithelium (Bhalla *et al.*, 1990, Yu *et al.*, 1994). *In vitro* studies of ozone exposure on the integrity of the respiratory epithelium, oxidant status and the cytoskeleton were thus performed.

CHAPTER 2. MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Reagents.

5-sulphosalicylic acid, cytochrome c, triethanolamine, 5, 5'-dithionitrobenzoic acid, glutathione reductase, nicotinamide-adenine dinucleotide phosphate reduced form, glutathione reduced form and oxidised form, bovine serum albumin, phorbol myristate acetate, superoxide dismutase, hydrogen peroxide, thiobarbituric acid, 1, 1, 3, 3,-tetramethoxypropane, myoglobin type III, potassium ferricyanide, 2, 2'-azinobis (3-ethylbenzothrazoline-6-sulphonic acid), trichloroacetic acid and EDTA-Na_2 were obtained from the Sigma Chemical Co., Poole, England.

Dulbecco's phosphate buffered saline was obtained from Gibco BRL, Paisley, Scotland and neutralised to pH 7.4. Eagle's minimal essential medium, foetal bovine serum, L-glutamine, sodium bicarbonate, trypsin-EDTA 10%, 10mm Nunc tissue culture treated inserts and Nunc single chamber glass slides were also from Gibco.

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbansaeure), 2-vinyl pyridine and sodium borohydride were supplied by Hoffman-la Roche, Aldrich Chemical Co., Dorset, England.

Potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, sodium di-hydrogen orthophosphate dihydrate, di-sodium hydrogen phosphate anhydrous, acetonitrile, dextrose, phosphoric acid, 60% perchloric acid, n-butanol and conical tubes were supplied by BDH, Dorset, England.

^{99m}Tc-DTPA was prepared from sodium pertechnetate injection obtained from an Amertec II generator (Code MCC20) and a Pentetate II kit (Code N108) both supplied by Amersham International, Aylesbury, England. Sodium ^{125}iodide was also from Amersham.

Cyclimorph, atropine, lignocaine, normal saline and medical grade O_2 and air were obtained from the hospital pharmacy. Medical grade 95% air

and 5% CO_2 was obtained from the British Oxygen Company, Glasgow, Scotland. Benzylpenicillin was supplied by Britannia Pharmaceuticals Ltd., Redhill, Surrey, England and streptomycin by Evans Medical Ltd., Horsham, West Sussex, England. Diff Quik was supplied by Merz Dade, Switzerland. Purafil was supplied by the Purafil Corporation, Sutton Coldfield, England. The A549 cells are a type II alveolar epithelial adenocarcinoma cell line (ECACC No. 86012804) and are maintained in continuous culture in the Rayne laboratory. 20% normal rabbit serum was supplied by the Scottish Antibody Production Unit, Lanarkshire, Scotland. Rhodamine-conjugated phalloidin was obtained from Cambridge Bioscience, Cambridge, England. The PD-10 Sephadex column G-25M was obtained from Pharmacia Biotech Ltd., Milton Keynes, England. 96 well microtitre plates were supplied by Greiner, Dursley, England. 75ml tissue culture flasks were obtained from Falcon, NJ, U.S.A.. 30mm plastic petri dishes were obtained from Becton Dickinson, Plymouth, England. 0.2μm membranes were supplied by Millipore, Milton Keynes, U.K.. Dialysis tubing was obtained from Medicell Int Ltd., Liverpool, England.

2.2. SPIROMETRY.

FEV_1 and forced vital capacity (FVC) were both performed on a dry spirometer supplied by Vitalograph Ltd., Buckingham, England. The best of 3 attempts was recorded and reproducibility maintained by ensuring that there was no more than 100mls between the best 2 of each.

2.3. CARBOXYHAEMOGLOBIN.

Ten mls of venous blood was taken into a lithium heparin tube for carboxyhaemoglobin (COHb) measurement on an IL 282 Co-oximeter, Instrumentation Laboratory, Lexington, Massachusets, U.S.A.. Samples were stored at -20^{o}C and were analysed in duplicate, the mean of the 2 measurements being taken. Care was taken to ensure that when samples were stored before analysis that only a minimal amount of air remained in the tube to prevent deterioration of the sample. On testing this there was no change in measurements on samples tested daily on up to 5 consecutive days.

2.4. ^{99m}Tc-DTPA LUNG CLEARANCE.

2.4.1. ^{99m}Tc-DTPA administration and data collection.

Except for an initial sham study each subject inhaled 1200 megabequerels (MBq) of nebulised ^{99m}Tc-DTPA from an Ultravent nebuliser (Mallinkrodt Medical Ltd., Petten, Holland) at a radioactive concentration of 1.8 graybequerels (GBq)/2ml (Figure 2.1). The MMAD (±SD) of the particles generated is 0.59±0.04μm with a GSD of 1.79±0.14μm as measured by a seven stage cascade impactor (Smith *et al.*, 1992). A flow of 12 litres per minute O_2 was used to generate the aerosol, which subjects inhaled for 2 minutes, while supine and wearing a noseclip, during normal tidal breathing to prevent proximal deposition through turbulent air flow (Brain and Valberg, 1979). RR was counted during the 2 minute inhalation period. Subjects were then imaged supine using a Siemens gamma camera positioned posteriorly with a 140 kilo electron volt (keV) low energy, all purpose collimator (Siemens plc., Bracknell, England) linked to a Bartec computer (Bartec Medical Systems Ltd., Farnborough, England) and Unix Sun workstation (Sun Microsystems Inc., Camberley, England) with Micas System V software (Nodecrest Ltd., Byfleet, England). Counts were acquired in 30 second time frames for 30 minutes at a resolution of 128x128. An intravenous injection of 20MBq ^{99m}Tc-DTPA at a concentration of 50MBq/2.5ml was given at 20 minutes to correct for background activity.

2.4.2. Image analysis.

A typical lung ventilation image is shown in Figure 2.2. A region of interest was drawn with a cursor around each lung field (Figure 2.3) at peak activity, approximately 2 pixels within the outermost lung contour avoiding the mediastinum. No activity was seen to accumulate in the region of the thyroid gland. In initial studies both corrected and uncorrected analyses were performed. A background region of interest (ROI) was drawn over the inter-renal area (Langford *et al.*, 1986) and over each shoulder excluding lung tissue (O'Doherty *et al.*, 1985b), following the intravenous correction (Figure 2.3). Each ROI was normalised for area and a correction made for ^{99m}Tc decay. A semi logarithmic plot of time versus activity was then made for all ROI's (Figure 2.4). Uncorrected

Figure 2.1. Subject inhaling ^{99m}Tc-DTPA

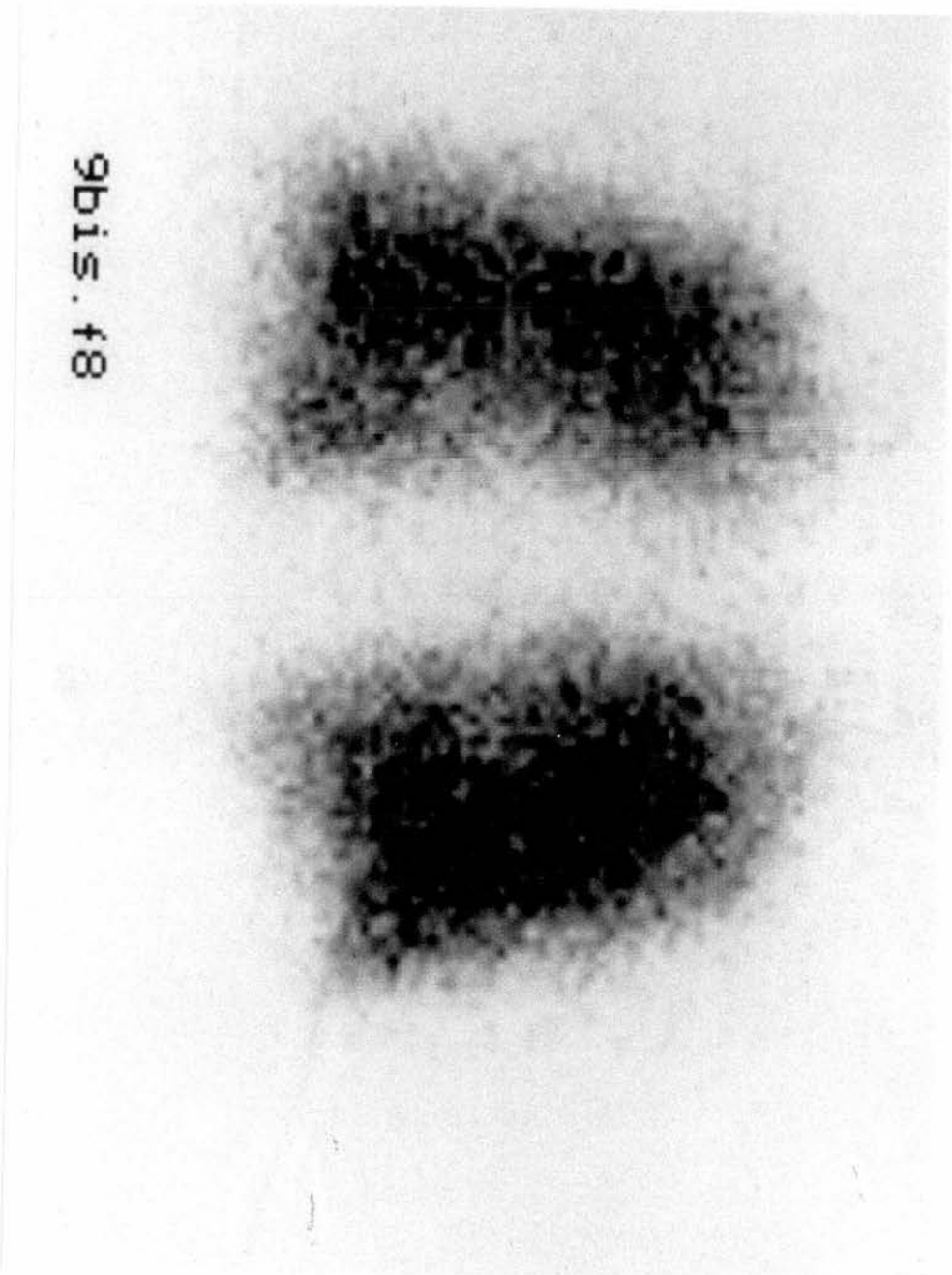


Figure 2.2. Typical lung ventilation image

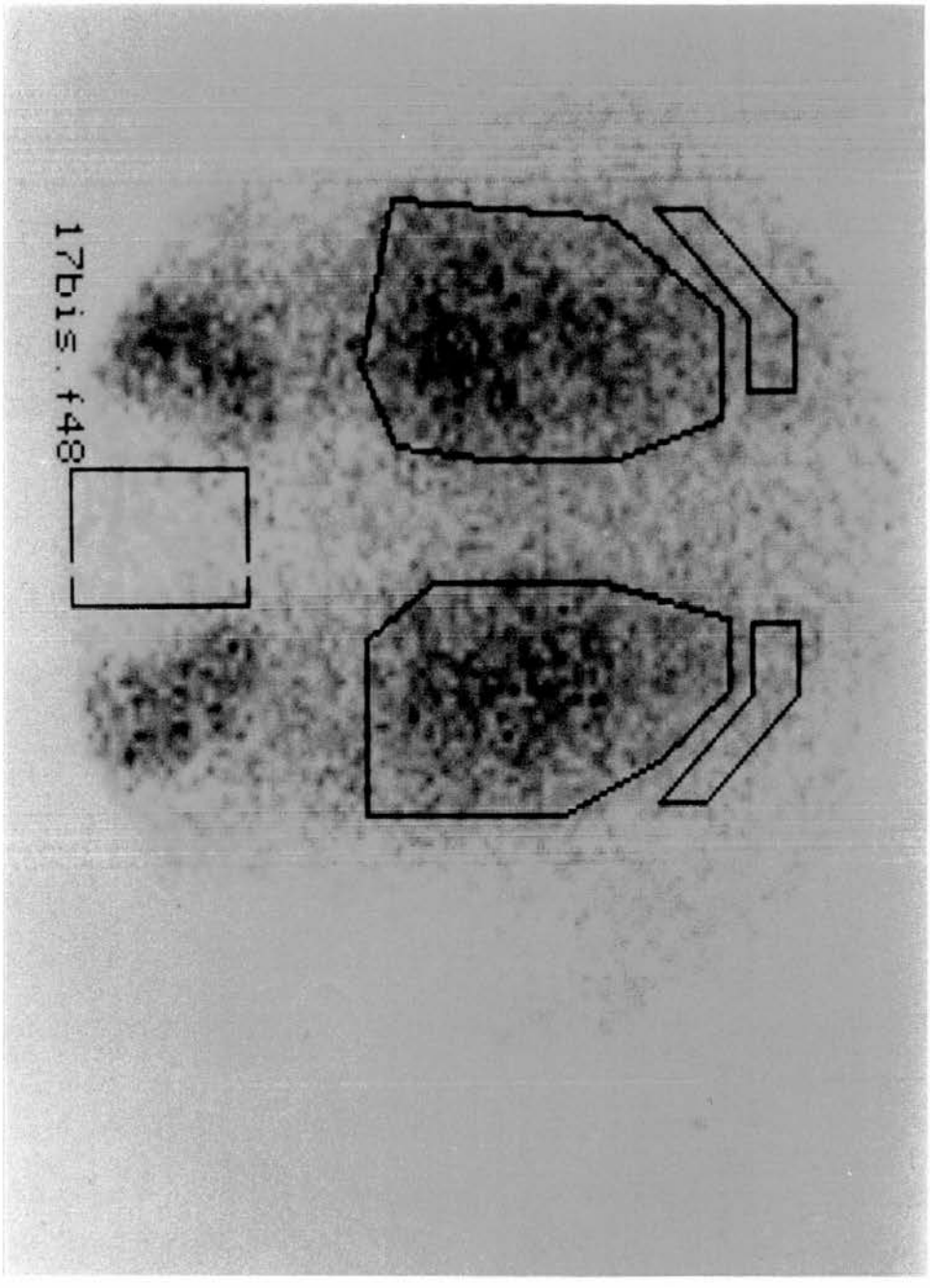
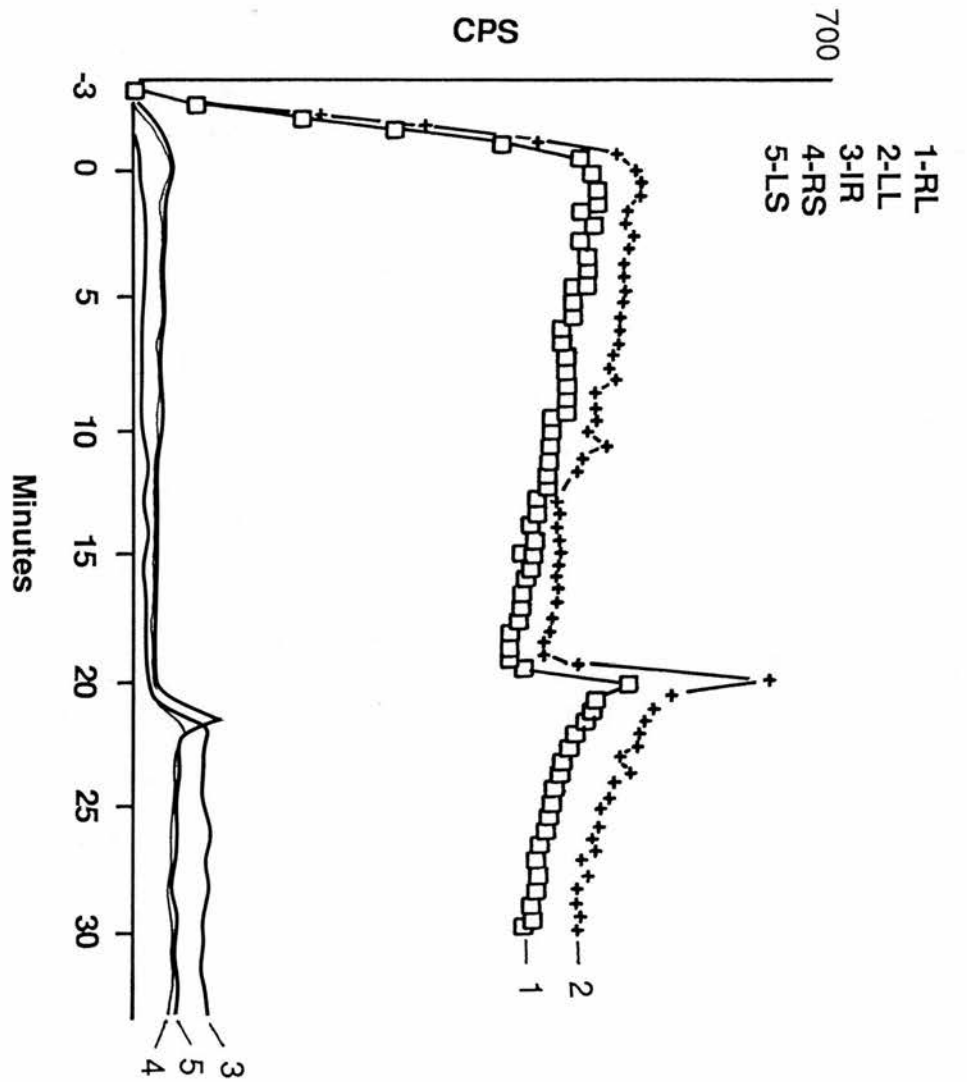


Figure 2.3. Lung and background regions of interest

Figure 2.4. Semi logarithmic plot of time versus activity for all regions of interest. RL-right lung, LL-left lung, IR-inter-renal, RS-right shoulder, LS-left shoulder, CPS-counts per second.


CPS
700
1-RL
2-LL
3-IR
4-RS
5-LS
-3
0
5
10
15
20
25
30
Minutes
1
2
3
4
5

analysis was made on the first 7 minutes after peak activity. This method is used by certain groups as it is assumed that no significant background accumulation of isotope occurs within this period (Rinderknecht *et al.*, 1980, Mason *et al.*, 1983). Other groups believe that a correction factor for recirculating background activity is required (Jones *et al.*, 1980, Minty *et al.*, 1981, O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). This was calculated from the ratio of the increase in counts over each lung field and the increase over the appropriate background ROI's following the intravenous injection. Each point in the background curves was multiplied by the appropriate ratio. The corrected background curves were then subtracted from the uncorrected lung curves. Monoexponential lung clearance was observed and the time for lung activity to fall to 50% of the initial value (t50) was calculated by linear regression analysis of the first 20 minutes of the corrected lung curves.

2.5. BRONCHOSCOPY AND BRONCHOALVEOLAR LAVAGE.

2.5.1. Sedation and preparation.

Patients were sedated with intravenous cyclimorph (5-10mg) immediately prior to the procedure and intravenous atropine (0.6mg) was given. Topical lignocaine was applied to the nasopharynx (4% 0.5mls and 2% 2mls) and to the vocal cords and major airways (2% 6mls).

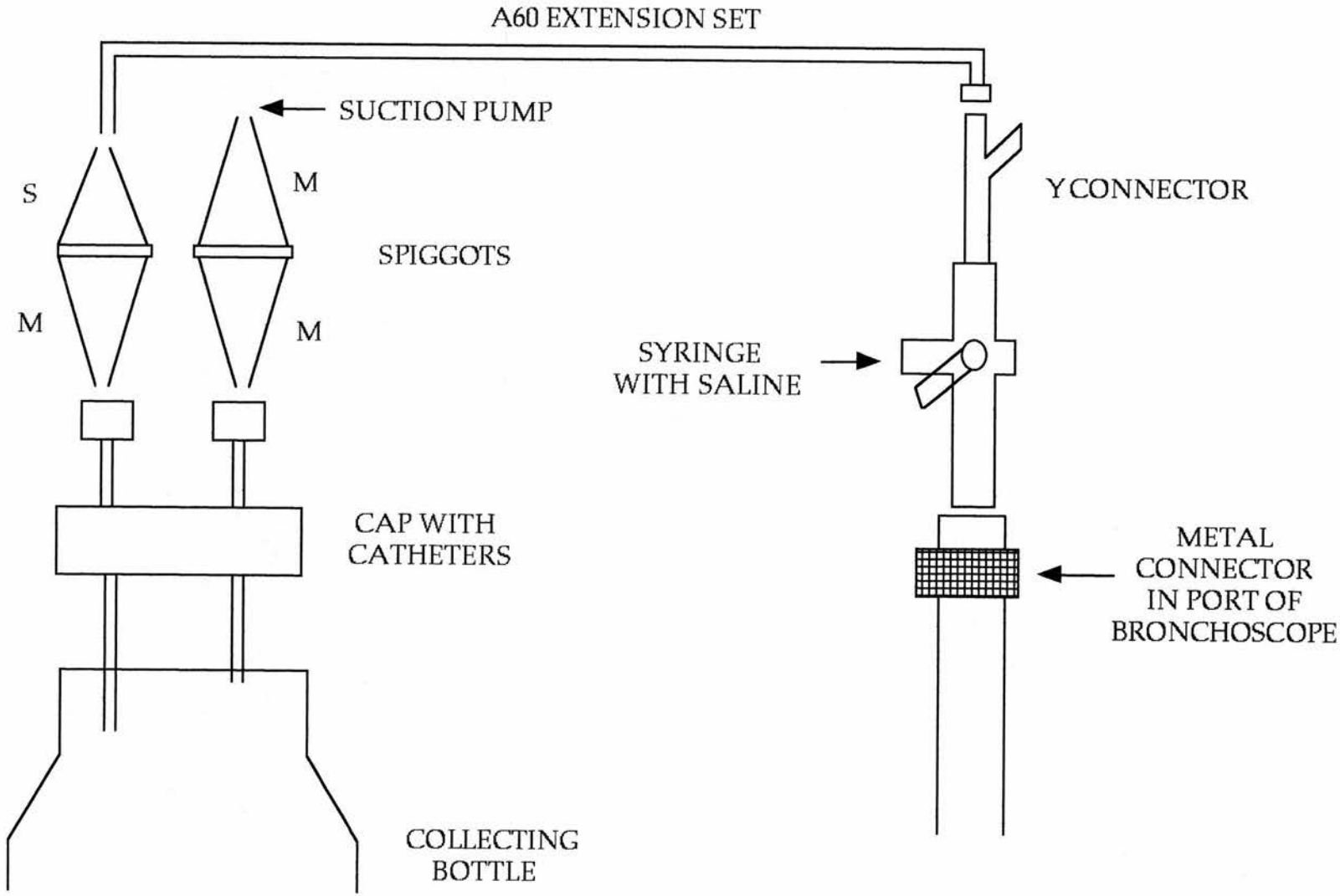
2.5.2. Bronchoalveolar lavage kit.

The BAL kit is shown in Figure 2.5. The spiggots, Y-connectors, bottles and caps were obtained from A. and J. Beveridge, Edinburgh, Scotland and re-used after sterilisation. The catheters (14 suction catheters, 50cm) were supplied by Mediplast, Taby, Sweden, the A60 extension set (1250mm wide bore) by Avon Medicals, Hythe, England and the 2 way tap (865.00) by Vygon, Cirencester, England.

2.5.3. Technique. (Figure 2.6).

The bronchoscope was wedged into a segment of the middle lobe or lingula (or in 1 case the left lower lobe). Two hundred and forty mls of warmed normal saline in 30ml aliquots was introduced and aspirated immediately. Subjects were given oxygen (2-3 litres per min) throughout the procedure.

Figure 2.5. Bronchoalveolar lavage kit (M-medium, S-small)


A60 EXTENSION SET
SUCTION PUMP
S
M
M
M
SPIGGOTS
Y CONNECTOR
SYRINGE
WITH SALINE
CAP WITH
CATHETERS
METAL
CONNECTOR
IN PORT OF
BRONCHOSCOPE
COLLECTING
BOTTLE

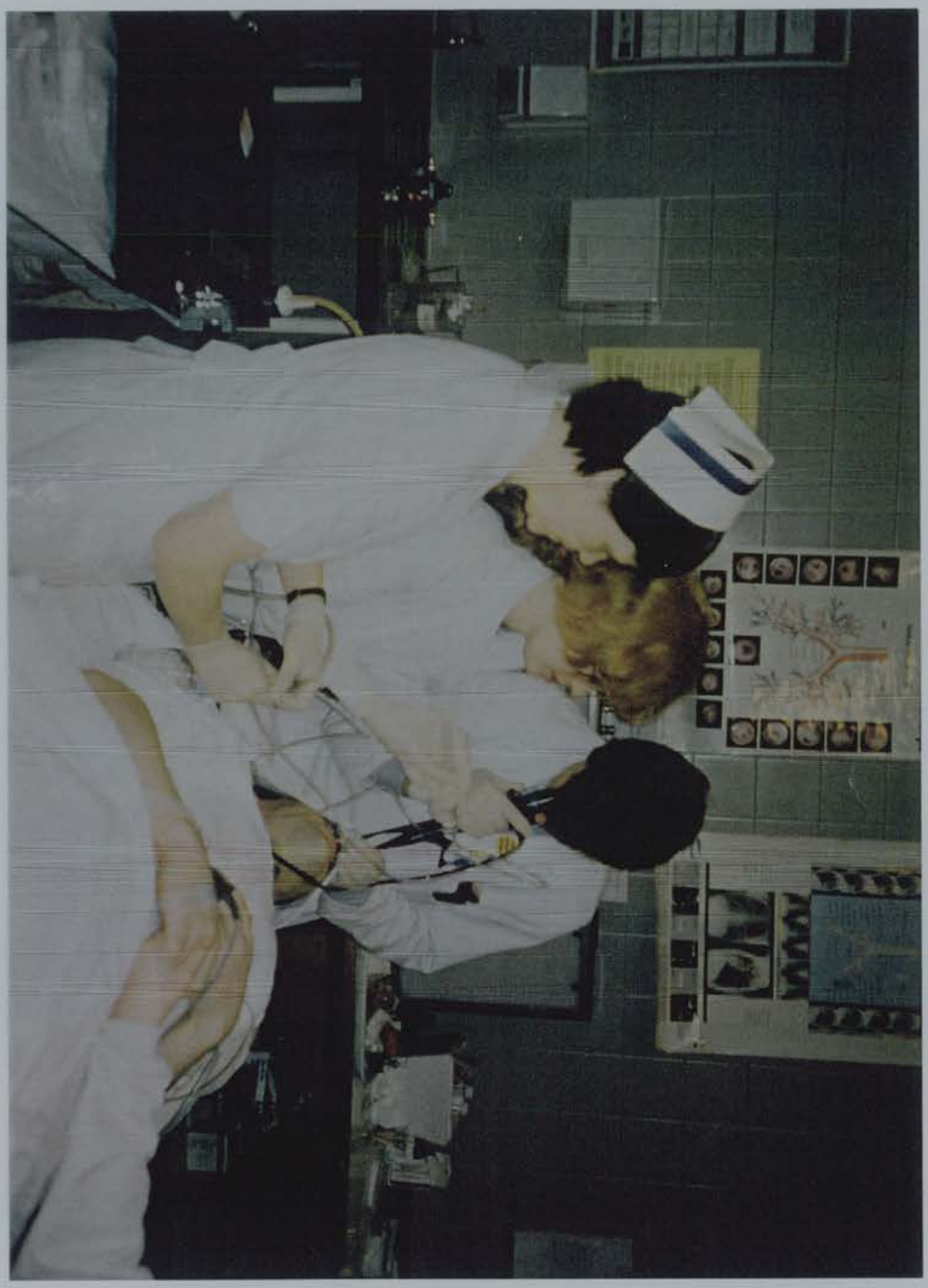


Figure 2.6. Bronchoalveolar lavage

2.5.4. Processing.

The bronchoalveolar lavage fluid (BALF) was immediately filtered through a sterile funnel (A. and J. Beveridge, Edinburgh, Scotland) through 4 sterile gauze swabs and then centrifuged at 250g for 10 minutes at 4°C to remove most of the cells. The supernatant was centrifuged again at 1000g for 10 minutes at 4°C to produce completely cell free fluid. The cell pellet was rinsed in phosphate buffered saline (PBS) and counted using a haemocytometer. Viability was ascertained by exclusion of Trypan blue. Cell differentials were performed on cytospin preparations (Shandon, Pittsburgh, PA, USA) stained with Diff Quik.

2.6. BIOCHEMICAL ASSAYS.

2.6.1. Albumin.

Serum albumin was assayed by a standard technique. Albumin in BALF was measured by an immunoturbidometric method (Boehringer Mannheim, Lewes, England). Concentrations of BALF constituents were calculated in ELF using the albumin method assuming its level in ELF to be 10% of the plasma albumin level (Ward *et al.*, 1993).

2.6.2. Superoxide anion.

Generation of $O_2^{\cdot -}$ by mixed BAL leucocytes (2.5×10^5 cells) was measured by SOD ($37.5 \mu g ml^{-1}$) inhibitable reduction of ferricytochrome c (Johnston *et al.*, 1975). After 120 minutes incubation at 37°C in 95% air and 5% CO_2 the samples were spun at 1400g for 5 minutes at 4°C and release of $O_2^{\cdot -}$, either spontaneously or when stimulated with phorbol myristate acetate (PMA) $1 \mu g ml^{-1}$ was measured. The difference in absorbance of the supernatant fluid, in the presence or absence of SOD, was determined using a Pye Unicam 8700 series UV/VIS spectrophotometer at 550nm. The amount of reduced cytochrome c was calculated based on an extinction coefficient of $21.0 mM^{-1} cm^{-1}$ for cytochrome c. Measurements without SOD were performed in triplicate and those with SOD single estimates.

2.6.3. Products of lipid peroxidation.

The level of plasma or BALF products of lipid peroxidation was measured as TBARS (Yagi, 1976). This is the most popular method for

measuring the extent of lipid peroxidation, although the fluorescent assay used is not as sensitive as HPLC (Petruska *et al.*, 1990), which was however not available to us. We have recently demonstrated increased lipid peroxidation in the plasma of healthy chronic smokers with the fluorescent assay (Rahman *et al.*, 1996). Blood was spun at 3000 rpm for 10 minutes at room temperature. EDTA at a final concentration of 1mM was added to BALF. Plasma 100μl or BALF 500μl in duplicate were mixed with 10% perchloric acid (v/v), pH 2.5. The resulting supernatant was reacted with 0.67% TBA (w/v) at 95°C for 15 minutes. After cooling the coloured complex was extracted with n-butanol and read at 532nm against a blank containing no plasma; 0.1nmole to 1nmole of 1, 1, 3, 3-tetramethoxypropane was used as an internal standard. The final result was expressed as μmoles of MDA formed per litre of plasma or BALF.

2.6.4. Trolox equivalent anti-oxidant capacity.

The anti-oxidant capacity of plasma and BALF was measured as TEAC (Figure 2.7) calculated by defining the concentration in mmol/l of Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbansaeure), a vitamin E analogue, having the equivalent anti-oxidant capacity to a 1mmol/l sample of the plasma or BALF under investigation (Miller *et al.*, 1993). The working solution of Trolox used was 2.5nmoles in 10μl PBS at pH 7.4. Metmyoglobin was prepared and purified after mixing stock myoglobin type III (400μM) in 5mmol/l PBS at pH 7.4 with an equal volume of freshly prepared potassium ferricyanide (740μM). The mixed solution was dialysed at 4°C in dialysis tubing against 200 times its volume (400mls) of PBS with change of buffer 3 times, twice after 2 hours and finally after 15 minutes and the absorbance (A) checked at 490, 560, 580 and 700nm on a Pye Unicam 8700 spectrophotometer.

Whitburn's equation was applied to calculate the final purified metmyoglobin (MetMb) concentration (Whitburn *et al.*, 1982):

$$(\text{Met Mb}) = 146\ A_{490} - 108\ A_{560} + 2.1\ A_{580}$$

The background was corrected by subtracting the reading at 700nm. The final concentration was diluted down to approximately 70μM.

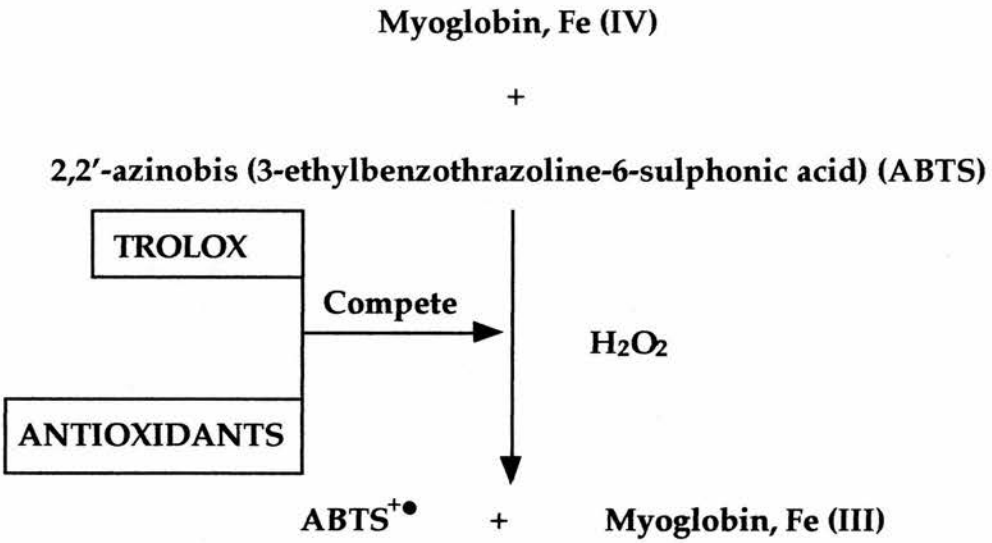


Figure 2.7 Measurement of Trolox equivalent anti-oxidant capacity

Ten mls of venous blood was withdrawn into a lithium-heparin tube, centrifuged and the plasma removed and stored at -20°C prior to analysis. BALF was processed as in 2.5.4. without further modification. The reaction mixture (1ml total) contained metmyoglobin 70μmol l^{-1} (36μl), ABTS 500μM (300μl) and an appropriate volume of PBS mixed with Trolox (0-50μl) or plasma (10μl) or BALF (250-500μl). The reaction was initiated by the addition of freshly prepared H_2O_2 450μmol l^{-1} (167μl). This gave final concentrations of 2.5μmol l^{-1} MetMb, 150μmol l^{-1} ABTS and 75μmol l^{-1} H_2O_2. The mixture was inverted and incubated at room temperature for 12 minutes. The production of the coloured radical $ABTS^{+\cdot}$ was measured on the spectrophotometer at 734nm. All measurements were performed in quadruplicate. The stability of the measurement was studied in plasma as shown in Table 2.1.

2.6.5. Glutathione.

Reduced and oxidised (GSSG) glutathione in BALF and mixed BAL leucocytes was estimated according to the method of Tietze (Tietze, 1969) (Figure 2.8) using a Unicam Pye 8700 scanning spectrophotometer at 412nm. A total of $2x10^6$ cells were used in each assay. After spinning at 100rpm at 4°C for 5 minutes the supernatant was discarded. Two mls of freshly prepared cold 0.6% sulphosalicylic acid (SSA) was added to the pellet, sonicated on ice and vortexed thoroughly. The sample was then centrifuged at 2500rpm at 4°C for 10 minutes and split equally for analysis of GSH and GSSG. For analysis in BALF a 2ml sample was also split equally and both cell supernatant and BAL treated as follows. The GSH sample was kept on ice until use. Twenty μl of 2-vinyl pyridine (2-VP) was added to the GSSG sample, vortexed and kept at room temperature for 1 hour. Sixty μl of triethanolamine solution diluted 1:2 with distilled water was added and the sample then kept on ice. Assay tubes were set upas follows, reagents being added in this order; 700μl buffer (600μl if adding 100μl sample), 100μl dithionitrobenzoic acid (DTNB) (freshly prepared in buffer and sonicated in a water bath for 5-10 minutes if necessary), 100μl GR (in buffer), 100μl β-NADPH (in buffer). If sample rather than blank was being measured 100μl was added at this stage. The assay tubes were then mixed and read at 412nm for 2 minutes and the increase in absorbance recorded. Standards for GSH and GSSG were prepared in buffer, 5 to 50μl volumes added to the assay tubes and again

Table 2.1. Stability of the measurement of Trolox equivalent anti-oxidant capacity (mmol l^{-1}). *sample haemolysed. RT-room temperature.

Subject First reading	0hr	Blood 1hr at RT spun	Plasma kept on ice 1hr	Blood 4hr at RT spun	Plasma left on ice 4hr	Plasma -20°C 24hr	Plasma -20°C 1wk	Plasma -20°C 2wk	Plasma -20°C 4wk
1	1.30	1.36	1.12	1.39	1.29	1.33	1.23	1.35	1.47
2	1.32	1.20	0.97	1.14	1.36	1.37	1.25	1.32	1.45
3	1.32	0.98	1.08	1.37	1.63	1.20	1.25	1.35	1.34
4	1.26	1.47	1.26	1.19	1.46	1.23	1.35	1.24	1.49

Subject Second reading	0hr	Blood 1hr at RT spun	Plasma kept on ice 1hr	Blood 4hr at RT spun	Plasma left on ice 4hr	Plasma -20°C 24hr	Plasma -20°C 1wk	Plasma -20°C 2wk	Plasma -20°C 4wk
1	1.14	1.32	1.30	1.17	1.30	1.28	1.00	1.40	1.59
2	1.23	1.29	1.38	1.28	1.47	1.37	1.11	1.40	1.24
3	1.20	1.25	1.19	1.02	1.17	1.25	1.11	1.24	1.33
4	1.17	1.24	1.29	0.99	1.28	1.17	1.21	1.46	1.29

Subject Third reading	0hr	Blood 1hr at RT spun	Plasma kept on ice 1hr	Blood 4hr at RT spun	Plasma left on ice 4hr	Plasma -20°C 24hr	Plasma -20°C 1wk	Plasma -20°C 2wk	Plasma -20°C 4wk
1	1.36	0.98	0.84	1.03	0.92	1.25	1.32	1.34	1.55
2	0.97	0.95	0.86	0.82	0.89	1.26	1.40	1.34	1.43
3	1.19	0.93	0.73	0.53*	0.81	1.21	1.38	1.25	1.27
4	0.99	0.96	0.75	1.04	0.97	-	1.29	1.17	1.39

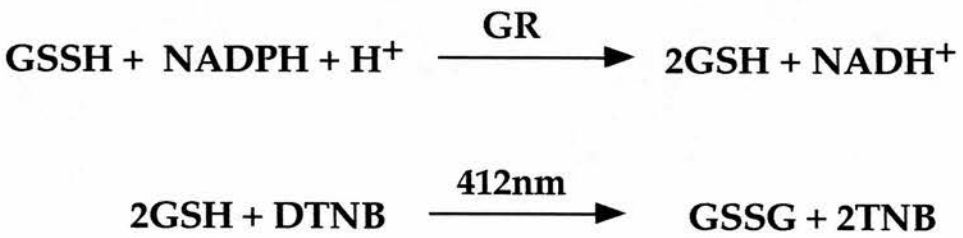


Figure 2.8 Measurement of glutathione

the buffer volume altered appropriately. Buffer was prepared by adding 16mls 0.1M potassium di-hydrogen phosphate (KH_2PO_4) to 84mls 0.1M di-potassium hydrogen phosphate (K_2HPO_4) both diluted in distilled water made up to 200ml. Finally 5mM EDTA-Na_2 was added to give 0.1M K-P 5mM EDTA buffer pH 7.5. All measurements were performed in duplicate.

2.7. OZONE EXPOSURE.

2.7.1. Ozone exposure *in vivo*.

Compressed air was purified and filtered through Purafil (Purafil Corporation, Sutton Coldfield, England) and activated carbon filters (Spirax Monnier IX1, Spirax Sarco, Cheltenham, England), which removed NO_X and hydrocarbons and then through a HEPA filter (Spirax Monnier IC3A). It was warmed and humidified and passed along Teflon tubing at 140 litres per minute to a perspex face mask and shield worn by the subject (Figure 2.9). All fittings in contact with ozone were made of either stainless steel or Teflon. Ozone was produced by passing medical grade oxygen through an electric arc ozonator Type BA. 023 (Wallace and Tiernan, Tonbridge, Kent, England) and was injected into the air stream at a sufficient distance from the subject to ensure good mixing of the ozone. A sample of the air ozone mixture was drawn from the mask and monitored continuously by an ozone analyser (Series 49-100/103, Thermoelectron Corporation, Warrington, England) . with reference to a primary ozone source (Institute of Terrestrial Ecology, Edinburgh University, Scotland).

2.7.2. Ozone exposure *in vitro*.

A549 cells were exposed to ozone in a specially constructed exposure chamber (Figure 2.10) in a negative pressure cabinet (Figure 2.11). Again all fittings in contact with ozone were made of either stainless steel or Teflon. Medical grade air was further filtered and purified as above and passed to both the ozone and control exposure chambers at 2 litres per minute. Ozone was produced by passing medical grade oxygen through a UV ozone analyser and generator (Series 49-100/103, Thermoelectron Corporation, Warrington) and injected into the air stream passing to the ozone exposure chamber. The ozone concentration in the chamber was

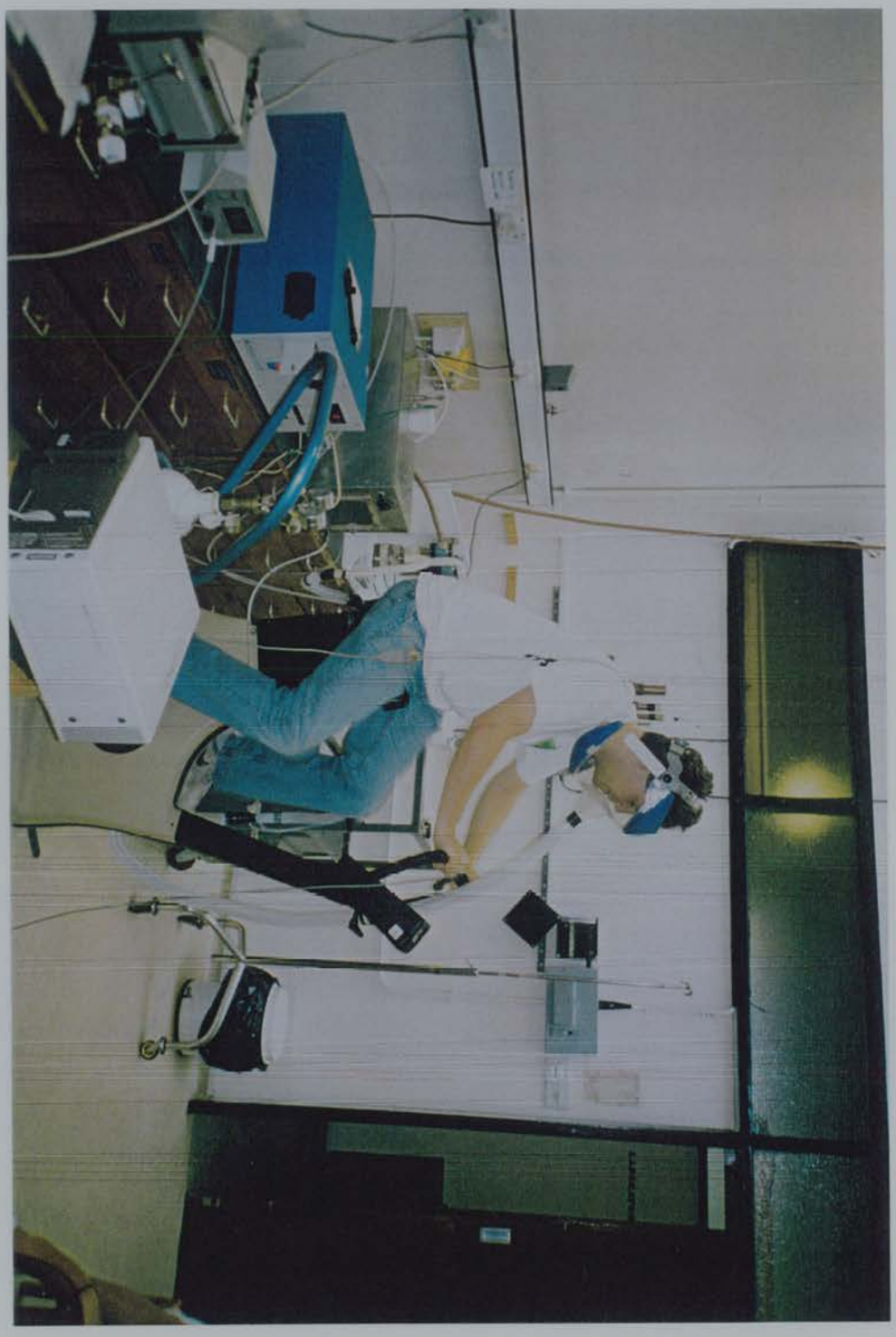


Figure 2.9. *In vivo* ozone exposure

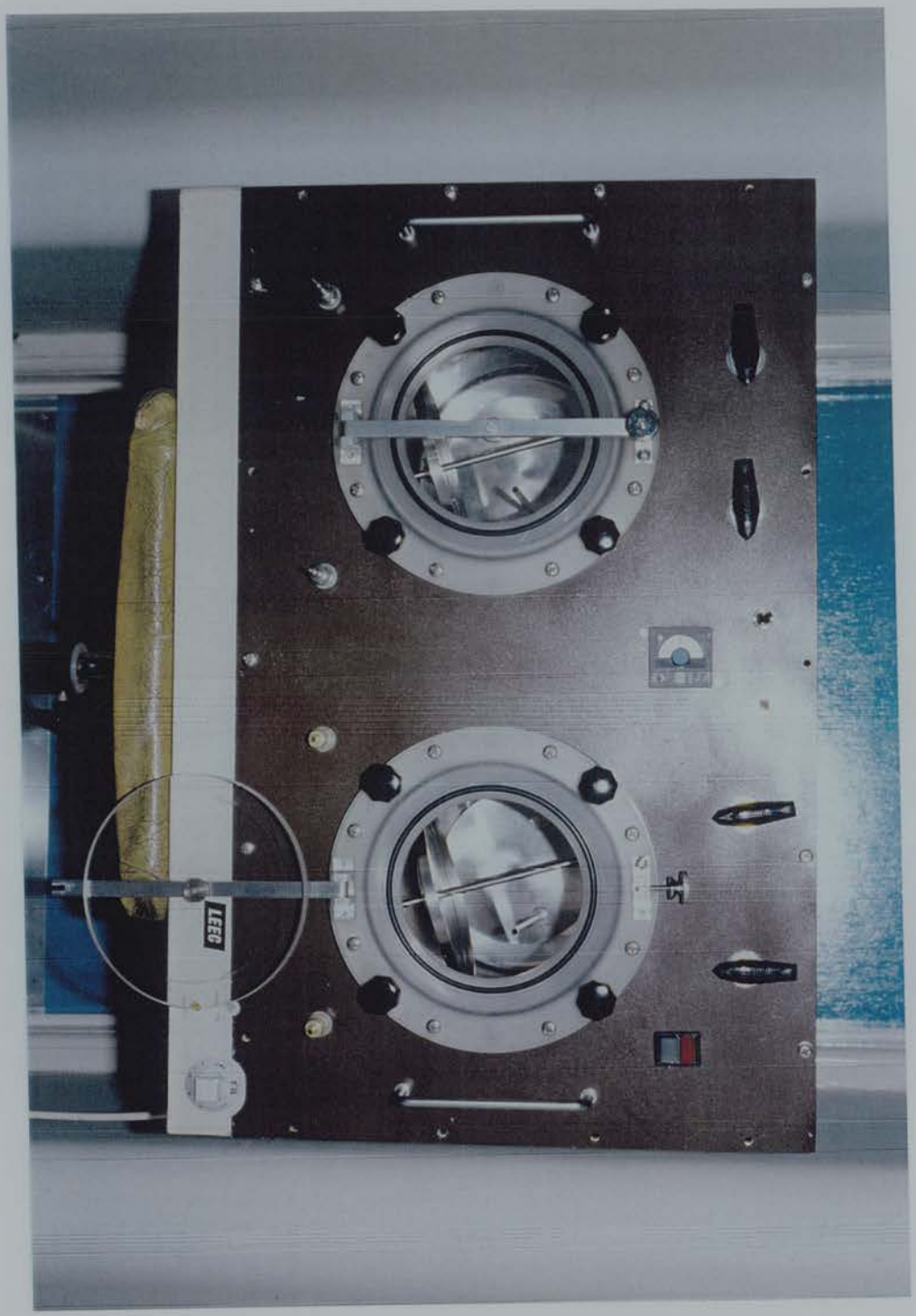


Figure 2.10. *In vitro* ozone exposure chamber

Figure 2.11. Negative pressure cabinets

allowed to equilibrate for 20 minutes according to the formula $t_{99}=K \times V/F$ where V is the volume of the chamber (8.2 litres), F is the total flow through the chamber (2 $lmin^{-1}$), t_{99} is the time required to attain 99% of the equilibrium concentration and K is a constant which for 99% is 4.605. It was continuously monitored by the above analyser. The chambers were humidified and the temperature maintained at 37°C.

2.8. *IN VITRO* STUDIES.

2.8.1. Cell culture.

The alveolar type II epithelial cell line, A549, was maintained in continuous culture in 75ml flasks at 37°C, 5% CO_2 in Eagle's minimal essential medium (MEM) which contained 10% foetal bovine serum (FBS), 72μgml^{-1} benzylpenicillin, 50μgml^{-1} streptomycin, L-glutamine 20mM and sodium bicarbonate ($NaHCO_3^-$). When required, confluent monolayers of A549 cells were washed twice with Dulbecco's PBS. Thereafter, trypsin-EDTA solution was added to detach the cells. The cells were then washed with MEM containing 10% FBS at 200g for 10 minutes to neutralize the trypsin and resuspended in MEM with 10% FBS.

2.8.2. Epithelial permeability (Figure 2.12).

A549 cells were grown to confluence in 10mm Nunc tissue culture treated inserts in MEM plus 10% FBS. This was replaced for the exposure and permeability assay by MEM plus 2% bovine serum albumin (BSA). The luminal medium was removed during the exposure to allow ozone to interact with the cell monolayer as the inserts were not large enough to allow medium to rock back and forth over the monolayer. After exposure the luminal medium was replaced and a 5μl aliquot of ^{125}I-BSA was added to each insert for 30 minutes. Two standard 5μl reference aliquots of ^{125}I-BSA and the proportion having passed through the monolayer to the well were counted in a gamma well counter (LKB, Wallac, Finland) before and after precipitation with 20% trichloroacetic acid (TCA). Epithelial permeability was then expressed as a percentage of both the total and bound ^{125}I activity in the well with reference to the standard aliquots of ^{125}I-BSA. Studies were performed in triplicate.

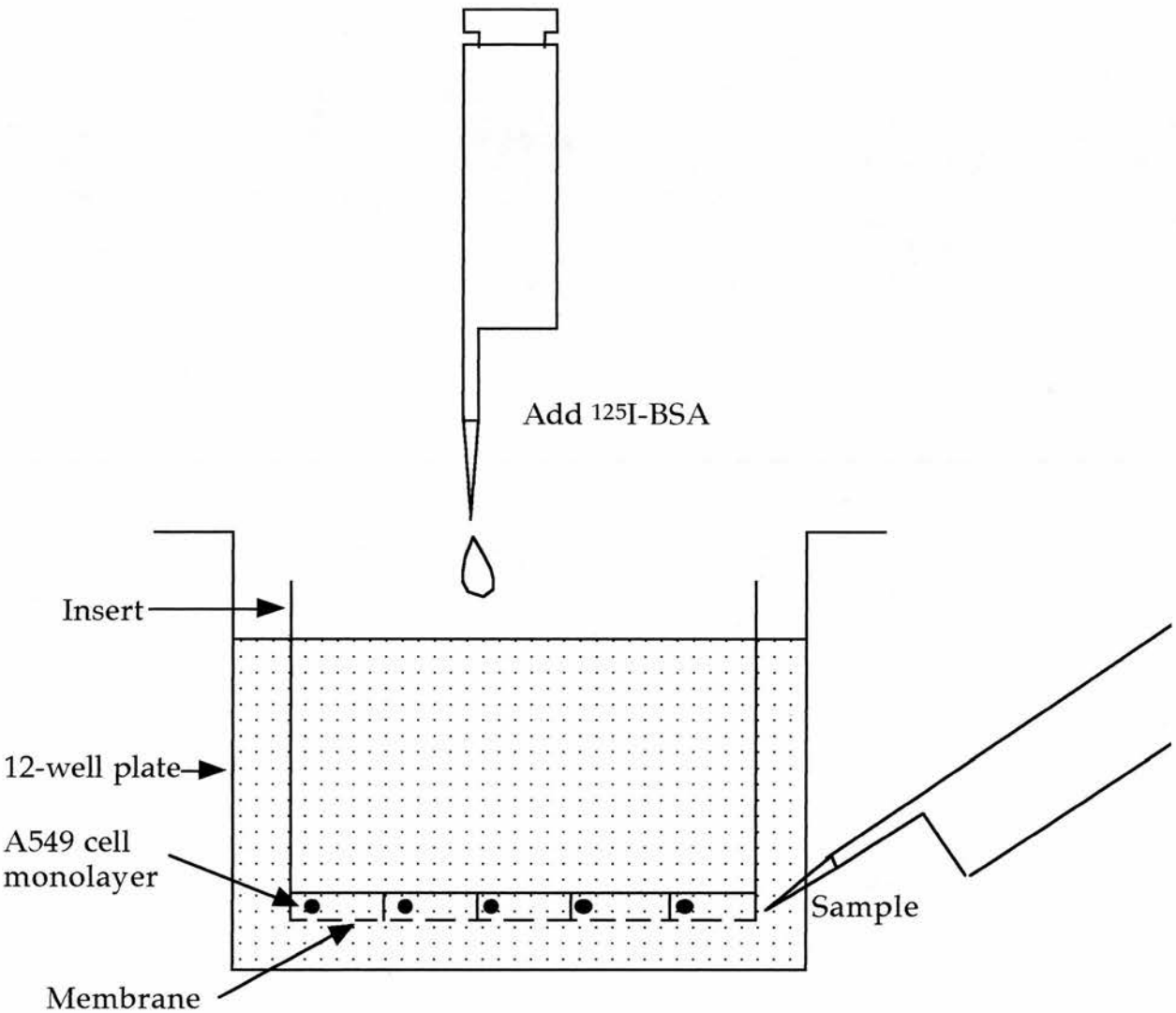


Figure 2.12. *In vitro* epithelial permeability assay

2.8.3. Measurement of glutathione.

A549 cells ($3x10^6$) were seeded onto 30mm plastic petri dishes at a concentration of $1x10^6 ml^{-1}$ and grown overnight in MEM plus 10% FBS. Ozone exposure was performed in 0.9mls serum free medium. This allowed 50% of the cells to be exposed to ozone for 30 seconds in each minute due to rotation of the tilted platform. Following exposure the medium was removed and the cells washed twice with PBS and the cells removed by incubation with trypsin-EDTA 10% for 2 minutes at 37°C. Cell numbers were counted using a haemocytometer and viability estimated by Trypan blue exclusion. Total and reduced glutathione were measured as above in the medium and the cells by the method of Tietze (Tietze, 1969). Studies were performed in triplicate.

2.8.4. Measurement of protein mixed disulphides.

Cells were prepared as for the measurement of GSH and the cell pellet retained for the measurement of protein mixed disulphides (PrSSG). To quantify GSH released from PrSSG, the cell pellet obtained after 0.6% SSA was homogenised and treated with sodium borohydride ($NaBH_4$) to release GSH from the PrSSGs then neutralized with phosphoric acid and spun at 15 000g for 15 minutes (Burchill *et al.*, 1978). GSH released from PrSSG was determined by the Tietze method involving DTNB-GSSG reductase as described above (Tietze, 1969) with slight modifications (Roberts and Francetic, 1989). The increase in the absorbance at 412nm was recorded for 2 minutes at 25°C on a Pye Unicam 8700 series UV/VIS spectrophotometer. The results were expressed as the amount of GSH, in nmoles, released from PrSSG per mg of protein.

2.8.5. Cytoskeletal assessment.

A549 cells ($4x10^6$) were seeded onto Nunc single chamber glass slides at a concentration of $1x10^6 ml^{-1}$ and grown overnight in MEM plus 10% FBS. Ozone exposure was performed in 0.9mls serum free medium. Following exposure the medium was removed and the cells washed twice with PBS and then fixed in 0.75ml $0.1mgml^{-1}$ lysophosphatidylcholine in 3.7% v/v formaldehyde for 15 minutes at 37°C. The cells were washed twice further in PBS and then incubated in PBS containing 20% normal rabbit serum for 10 minutes at room temperature. The serum was then drained and

the cells incubated with 0.15μM rhodamine-conjugated phalloidin containing 0.2% BSA and 0.1% sodium azide for 20 minutes at room temperature. Following 2 further washes with PBS the cells were mounted in 50% glycerol in PBS and sealed with clear nail varnish. The slides were divided along their length into 4 equal areas and 100 cells were assessed in each of the 2 end areas. Each cell was considered to have either normal or disrupted cytoskeleton on light microscopy (LM). The number of cells with normal cytoskeleton was then expressed as a percentage. Slides were viewed with an Olympus BH-2 RFCA fluorescent microscope (Olympus Optical Co. (U.K.) Ltd., London, U.K.) and photographed with an Olympus OM-4Ti camera using Kodak Ektachrome 64Ti colour film.

2.9. STATISTICS.

All data are presented as mean±SE.

CHAPTER 3. A COMPARISON OF 3 METHODS OF MEASURING ^{99m}Tc-DTPA CLEARANCE FROM LUNG TO BLOOD AS AN INDEX OF AIRSPACE EPITHELIAL PERMEABILITY AND THEIR REPEATABILITY.

3.1. INTRODUCTION.

One of the earliest demonstrations of the permeability of the respiratory epithelium was in 1857 when curare was seen to produce paralysis and death when introduced into the respiratory tract of a dog (Bernard, 1857). In recent years it has been extensively studied by measuring the lung clearance of inhaled ^{99m}Tc-DTPA. This technique was first used as a means of assessing regional ventilation as a replacement for the radioactive gas ^{133}xenon (Taplin and Chopra, 1978). The inhaled ^{99m}Tc-DTPA particles were noted to pass from the lungs to the blood at a rate of about 1% per minute. It was expected that diseases which thickened the alveolar-capillary membrane such as interstitial lung disease (ILD) would exhibit a reduced rate of clearance. However the converse was found to be true (Rinderknecht *et al.*, 1980). Increased rates of ^{99m}Tc-DTPA lung clearance occur in numerous conditions, including ARDS (Barrowcliffe and Jones, 1989), *Pneumocystis carinii* pneumonia (PCP) (Robinson *et al.*, 1991), after cytotoxic agents (O'Doherty *et al.*, 1986) and amiodarone (Terra Filho, 1989), ozone exposure (Kehrl *et al.*, 1987) and acute asthma (Lemarchand *et al.*, 1992). Cigarette smoking produces a rapidly reversible increase in ^{99m}Tc-DTPA clearance (Jones *et al.*, 1980, Minty *et al.*, 1981, Mason *et al.*, 1983, Kennedy *et al.*, 1984). Peripheral deposition is associated with faster clearance (Oberdorster *et al.*, 1986) as is positive end expiratory pressure (Nolop *et al.*, 1987) and raised lung volume (Nolop *et al.*, 1986).

Despite extensive use of this technique in studies of alveolar-capillary membrane integrity, a comparison of the different methods, as they are employed in practice, has not been performed. A wide range of normal t50 values for ^{99m}Tc-DTPA clearance has been quoted in the literature [44.4-117.5 minutes] (Table 3.1). This is likely to reflect the sensitivity of ^{99m}Tc-DTPA clearance to changes in lung volume (Rizk *et al.*, 1984, Nolop *et al.*, 1986, Nolop *et al.*, 1987). However the method of correcting for background activity may also contribute (Rinderknecht *et al.*, 1980, O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). Two groups have compared

uncorrected and corrected analyses on complete clearance curves (O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). However uncorrected analysis is normally restricted to the first 7-10 minutes of the clearance curve (Rinderknecht *et al.*, 1980, Mason *et al.*, 1983). I studied 8 non-smokers and 7 smokers, each on 2 occasions, with the aim of comparing 3 methods of analysis, including their repeatability, as they are employed in practice.

3.2. METHODS.

3.2.1. Subjects (Table 3.2).

The non-smokers (M:F 8:0) had normal ventilatory capacity which was significantly higher than the smokers (M:F 4:3), who had mild airflow limitation. The non-smokers were also significantly younger. There was no significant difference in subject characteristics between visits 1 and 2 in either the non-smokers or smokers. All were free of respiratory infection and had no significant medical problems.

3.2.2. Study design.

Subjects attended for an initial sham study, performed without isotope, in order to accustomise them to the test and eliminate as much as possible any variations in TV, inspiratory flow and functional residual capacity. They were then studied on 2 occasions (visits 1 and 2) over variable time intervals (1-30 weeks) with initial and return studies overlapping to help negate any possible influence of change in the test set up with time.

On each of the 3 occasions smokers were asked to refrain from smoking for 12 hours. Spirometry and COHb measurements were performed on all visits prior to study.

3.2.3. Stability of ^{99m}Tc-DTPA.

In 1 non-smoker and 4 smokers thin layer chromatography of the residual ^{99m}Tc-DTPA in the nebuliser and urine demonstrated 0% and <0.2% dissociation respectively into DTPA and free pertechnetate (Zimmer and Pavel, 1977).

Reference	Non-smokers	Smokers	Method
Jones *et al.*, 1980	59±5	20±2	C
Mason *et al.*, 1983	81±10	15±1	U
Kennedy *et al.*, 1984	110±22	42±5	C
O'Doherty *et al.*, 1985	83±6	30±3	U
	70±6	20±3	C
Langford *et al.*, 1986	66	33	U
	60	27	C
	58	26	C
Nolop *et al.*, 1986	55±7	22±4	C
Nolop *et al.*, 1987	52±6	28±5	C
Thunberg *et al.*, 1989	91±5	28 (range 22-46)	C

Table 3.1. Values for ^{99m}Tc-DTPA clearance (t50, minutes, mean±SE) from lung to blood in non-smokers and smokers, in previous studies, uncorrected (U) or corrected (C) for recirculating background activity.

	Non-smokers (n=8)		Smokers (n=7)		
	Visit 1	Visit 2	Visit 1	Visit 2	NS v S
Age (years)	32±2		46±4		$p<0.01$
FEV_1 (litres)	4.5±0.2	4.5±0.2	3.1±0.5	3.1±0.5	$p<0.001$
%Predicted	102.8±3.3	102.5±3.4	88.9±8.9	89.1±7.9	$p<0.05$
FVC (litres)	5.4±0.2	5.5±0.2	4.3±0.5	4.2±0.6	$p<0.01$
COHb (%)	0.9±0.1	1.1±0.1	2.5±0.5	2.8±0.4	$p<0.001$
RR (per min)	10±1	9±1	12±1	12±1	$p<0.01$

Table 3.2. Subject characteristics (mean±SE) in the non-smokers (NS) and smokers (S) studied. RR-Respiratory rate.

3.2.4. Ethical approval.

The study was approved by the local medical ethics committee and all subjects gave written informed consent.

3.2.5. Statistical analysis.

The subject characteristics in the non-smokers and smokers were compared between the 2 groups by the unpaired t-test and within the groups, between visits 1 and 2, by the paired t-test.

For comparison the 3 methods of analysis were combined, the data transformed to natural logarithms, and a hierarchical analysis of variance (ANOVA) performed. The mean values between visits 1 and 2 were compared for each technique by paired t-test. Correlation between the uncorrected t50 values and the correction factors for the 2 corrected methods of analysis was performed by a 2 variable parametric analysis.

Repeatability of the 3 methods of analysis was defined according to the single determination standard deviation and the 95% range of the repeated measurements (Chinn, 1991). For all 3 methods the difference between the values for ^{99m}Tc-DTPA clearance at visits 1 and 2 was related to the mean of these values. The data were therefore log transformed and the standard deviation of the differences between log values for visits 1 and 2 was calculated for each method and divided by $\sqrt{2}$ to give the single determination standard deviation. The 95% range, expressed as $\pm$ k, is derived from this as $\pm$ 2 x the single determination standard deviation. This may then be antilogged to x/÷ antilog(k) to indicate the limits around a single measurement, on a numerical scale, that must be regarded as possible values for the true measurement (Chinn, 1991). The values expressed in the tables are numerical. A level of <0.05 was considered statistically significant. Statistical advice was given and the hierarchical ANOVA performed by Dr. W Adams, Department of Medical Statistics, Edinburgh University, Edinburgh. All other calculations were performed on Statistica/Mac™, Release 1.7, Copyright StatSoft, Inc. 1991-1992.

3.3. RESULTS.

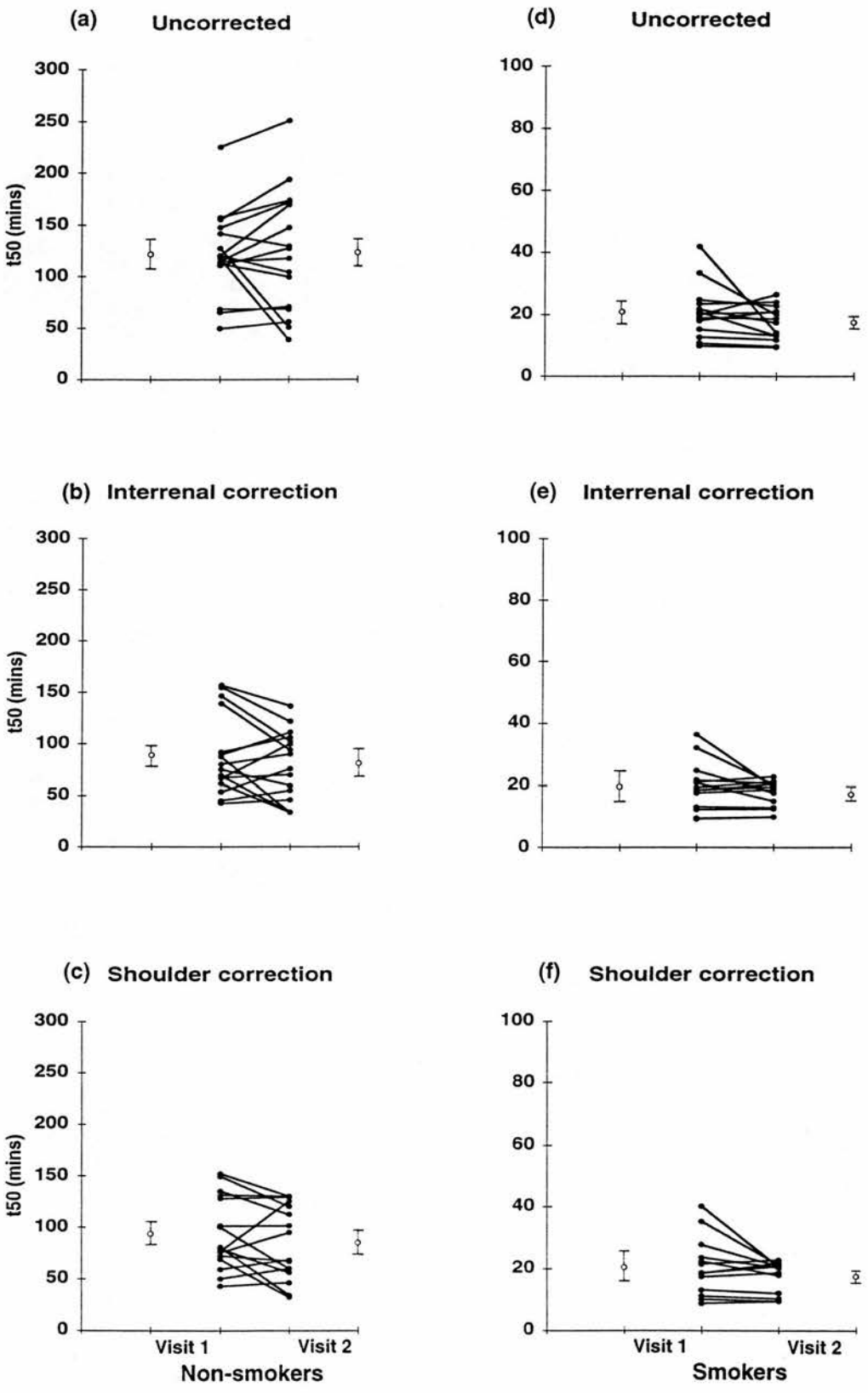
Individual t50 values are shown in Figure 3.1. Values for right and left lungs are shown. Absolute and percentage differences from visits 1 to 2 are given in Table 3.3. The uncorrected values in non-smokers were higher on visit 2 in 4 subjects, lower in 4 subjects and in 2 subjects showed no consistent change. There were no consistent differences between the 2 visits with the 2 background corrected methods in non-smokers nor with any of the 3 methods in smokers.

Mean t50, regression coefficients and their standard error values and correction factors (inter-renal and shoulder corrected methods) for the time-activity curves are shown in Table 3.4. All the clearance curves were monoexponential. The regression coefficients for the curves are given to show the tightness of fit to a monoexponential line. They are however rather insensitive to different qualities of fit around curves with similar slopes and are more sensitive to increases in slope. Thus the r values for the smokers are better than those for the non-smokers whose clearance curves have such a small slope. The standard error values for the curve fitting are thus also given in this study, the combination providing a better indication of the variation in the data from which the time-activity curves were calculated.

Mean t50 values in non-smokers were significantly higher than in smokers with each of the 3 methods of analysis ($p<0.001$). In non-smokers the uncorrected method gave higher mean t50 values than the inter-renal ($p<0.001$) and shoulder ($p<0.001$) methods of correction. The inter-renal method gave lower values than the shoulder corrected method ($p<0.05$). In smokers there was no difference in the values obtained between the 3 methods of analysis. There was no correlation between the uncorrected t50 values and the correction factors for the background corrected methods of analysis overall, or in non-smokers and smokers separately.

There were no significant differences between the mean values obtained at visits 1 and 2 in either non-smokers or smokers for any of the 3 methods of analysis. The repeatability of the 3 methods of analysis is given in Table 3.5. There was no significant difference between the

Figure 3.1. ^{99m}Tc-DTPA lung clearance (t50, mean$\pm$SE) values at visit 1 and visit 2 in 8 non-smokers (a-c) and 7 smokers (d-f) for the 3 analysis methods.


(a) Uncorrected
(d) Uncorrected
(b) Interrenal correction
(e) Interrenal correction
(c) Shoulder correction
(f) Shoulder correction
t50 (mins)
300
250
200
150
100
50
0
100
80
60
40
20
0
Visit 1
Visit 2
Non-smokers
Smokers

Non-smokers		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
	Difference	Right lung								Left lung							
Uncorrected	A (mins)	-79	49	-16	25	17	7.0	-0.1	32	-77	-13	-12	26	17	5.8	2.9	39
	%	-67	41	-13	17	15	14	-0.2	28	-60	-12	-8.6	11	11	8.9	2.5	25
Inter-renal	A	-36	-16	2.4	-32	14	3.1	33	-44	-28	-27	22	-20	10	9.6	22	-46
	%	-52	-21	3.5	-21	15	7.4	51	-30	-45	-31	40	-13	13	21	25	-33
Shoulder	A	-46	-24	-5.4	-29	1.1	3.2	19	-0.8	-37	-40	8.6	-23	-0.4	10	49	-22
	%	-58	-30	-7.4	-19	0.9	7.5	25	-0.6	-54	-40	15	-15	-0.4	21	64	-16

Smokers		1	2	3	4	5	6	7	1	2	3	4	5	6	7
	Difference	Right lung							Left lung						
Uncorrected	A (mins)	0.5	-1.1	-28	-7.6	-0.7	-0.04	3.0	-2.0	-0.4	-13	-4.3	-2.3	0.8	6.7
	%	2.1	-10	-66	-37	-5.4	-0.2	17	-8.0	-4.1	-40	-20	-15	3.7	34
Inter-renal	A	-1.1	0.5	-17	-5.8	0.04	2.1	1.2	1.8	0.4	-12	-7	-0.2	1.1	1.9
	%	-5.2	5.2	-46	-28	0.3	11	6.3	8.8	4.4	-37	-28	-1.2	6.4	10
Shoulder	A	-3.3	0.7	-20	-4.5	-1.0	1.8	1.4	1.4	-0.6	-14	-6.8	-1.1	1.3	3.3
	%	-14	7.7	-49	-20	-9.2	9.4	8.4	6.5	-5.7	-40	-25	-8.2	7.5	18.2

Table 3.3. Absolute (A) and percentage (%) difference in ^{99m}Tc-DTPA clearance from lung to blood between visits 1 and 2 in individual subjects.

Table 3.4. ^{99m}Tc-DTPA clearance from lung to blood in non-smokers and smokers showing time (minutes) to 50% clearance (t50), regression coefficients (r) and their standard errors (minutes, SEr) for slope and tightness of monoexponential fit to the time-activity curves and the correction factor (CF) values for the curves. Each value is given as mean$\pm$SE.

		Visit 1								Visit 2							
		Right lung				Left lung				Right lung				Left lung			
		t50	r	SEr	CF	t50	r	SEr	CF	t50	r	SEr	CF	t50	r	SEr	CF
Non-smokers																	
Uncorrected	Mean	106	0.81	22.7		137	0.76	34.6		110	0.81	24.2		136	0.77	37.7	
	SE	11.2	0.03	3.61		16.4	0.04	6.61		18.3	0.04	7.33		23.7	0.05	10.8	
Inter-renal	Mean	88.7	0.95	5.15	1.14	88.8	0.96	4.77	1.32	79.4	0.96	4.12	1.08	81.6	0.96	4.39	1.18
	SE	14.1	0.01	1.54	0.08	14.1	0.01	1.18	0.11	11.3	0.01	1.10	0.04	11.7	0.01	1.24	0.03
Shoulder	Mean	94.8	0.92	7.25	1.34	92.7	0.90	7.37	1.61	84.5	0.94	5.74	1.46	86.0	0.92	6.83	1.59
	SE	13.0	0.02	1.56	0.12	12.9	0.02	1.42	0.23	13.8	0.01	1.51	0.10	12.7	0.02	1.78	0.08
Smokers																	
Uncorrected	Mean	20.8	0.99	0.88		20.6	0.99	0.67		16.0	0.99	0.58		18.5	0.99	0.81	
	SE	3.9	0.00	0.30		2.8	0.00	0.11		2.0	0.00	0.11		2.2	0.00	0.19	
Inter-renal	Mean	19.5	0.99	0.48	1.22	19.5	0.99	0.41	1.33	16.7	0.99	0.32	1.05	17.6	0.99	0.38	1.14
	SE	3.3	0.00	0.16	0.07	2.8	0.00	0.12	0.06	1.7	0.00	0.06	0.08	1.8	0.00	0.05	0.04
Shoulder	Mean	20.3	0.98	0.60	1.78	20.4	0.98	0.62	1.73	16.7	0.98	0.55	1.70	18.0	0.98	0.65	1.74
	SE	3.9	0.00	0.16	0.11	3.3	0.00	0.17	0.12	1.8	0.01	0.12	0.14	2.0	0.01	0.14	0.07

	All			NS			Smokers		
	U	IR	S	U	IR	S	U	IR	S
s	1.30	1.23	1.24	1.33	1.27	1.29	1.27	1.18	1.19
95% range (x/÷)	1.69	1.51	1.54	1.77	1.61	1.66	1.62	1.40	1.41

Table 3.5. Single determination standard deviation (s) and 95% range for repeated measurements of ^{99m}Tc-DTPA clearance, from lung to blood, on 2 occasions, according to the three methods of analysis. The values have been transformed from natural logarithms. All-non-smokers (NS) plus smokers, U-uncorrected analysis, IR-inter-renal correction, S-shoulder correction.

methods in all subjects or in non-smokers and smokers analysed separately.

3.4. DISCUSSION.

I have compared 3 methods of analysing the clearance of ^{99m}Tc-DTPA from the lungs to blood, as they are employed in practice. Table 3.1 shows that the values obtained in previous studies have varied greatly. Although the mechanism of ^{99m}Tc-DTPA transport across the alveolar capillary membrane is not known the most important factor affecting ^{99m}Tc-DTPA clearance is thought to be its sensitivity to change in lung volume. It may be increased within 1 to 2 minutes of an increase in lung volume (Rizk *et al.*, 1984, Nolop *et al.*, 1986, Nolop *et al.*, 1987). This may be due to an increase in surface area available for clearance of ^{99m}Tc-DTPA, greater access to thinner alveolar capillary membrane, change in lung surface characteristics, increase in epithelial permeability, widening of intercellular junctions, recruitment of more permeable lung units, change in the surfactant layer or increased negative pulmonary interstitial pressure (Barrowcliffe *et al.*, 1987, Smith *et al.*, 1992). However the method of allowing for background activity may also contribute to the wide range of normal values (Rinderknecht *et al.*, 1980, O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). This study examines the effect of the method of analysis employed on the absolute values and repeatability of ^{99m}Tc-DTPA clearance, in particular the incorporation of a correction for recirculating background activity.

Lung clearance of ^{99m}Tc-DTPA is affected by many other factors. Bronchial absorption of ^{99m}Tc-DTPA is slower than alveolar absorption when mucociliary clearance is taken into account (Barrowcliffe *et al.*, 1987). This may be due in part to binding of ^{99m}Tc-DTPA to mucus (Barrowcliffe and Jones, 1987). Aerosols with MMAD less than 2μm will be predominantly deposited peripherally (Barrowcliffe and Jones, 1987). In some studies in humans clearance has been found to be significantly faster from apical compared to basal regions. This has been attributed to larger alveolar surface area and thus increased permeability in the apices (Rinderknecht *et al.*, 1980). An anterior-posterior difference has been found in the supine position with more rapid clearance posteriorly. This

is thought to be due to the increased perfusion in the dependent dorsal regions (Thunberg *et al.*, 1989). ^{99m}Tc-DTPA clearance is also affected by removal from blood (Rizk *et al.*, 1984). It is increased by exercise perhaps due to recruitment of capillaries (Meignan *et al.*, 1986).

One must ask whether a background correction is required. The initial studies in the U.K. incorporated a correction for recirculating background activity with the use of a second scintillation probe placed over the thigh, the first collecting activity over the lung (Jones *et al.*, 1980, Minty *et al.*, 1981). An intravenous injection was given to assess the contribution of soft tissue and blood activity. This form of correction assumes that activity collected over a non-thoracic area reflects the situation in the soft tissues and vessels of the thorax and that ^{99m}Tc-DTPA given intravenously in this way is distributed in the same fashion as that absorbed through the airspace epithelium. Time is required following the bolus for equilibration with the soft tissues. Background correction, by monitoring accumulation in the thigh, has been validated by Barrowcliffe and co-workers in pigs. The left lung was selectively intubated to receive ^{99m}Tc-DTPA and an intravenous bolus was given after 30 minutes. No difference was found in the corrected t50 from the left lung derived from background activity in either the right lung or thigh (Barrowcliffe *et al.*, 1988). Studies performed in North America have not used a correction since analysis has been confined to the first 7-10 minutes of the clearance curve (Rinderknecht *et al.*, 1980, Mason *et al.*, 1983). This makes the assumption that insufficient background activity accumulates over this short period to constitute a problem. In smokers however considerable background activity can be seen already accumulating over the first 7-10 minutes of analysis although this may only become significant where the t50 is less than 10 minutes. O'Doherty and co-workers used an intravenous correction employing a gamma camera technique and background regions over both shoulder areas. It appears from their data that about 30% of the total lung activity constitutes background at 30 minutes in smokers. They found that the correction factor increased with the permeability of the alveolar capillary membrane although I found no such relationship (O'Doherty *et al.*, 1985b). Langford and colleagues modified this technique with a background region between the kidneys (Langford *et al.*, 1986). They claimed to have found a technique that did

not require an intravenous bolus as the increase in activity in the background region was the same as that over the lungs giving a correction ratio of 1. This has however not been our experience with this method and I believe that an intravenous correction is required with it.

In our study there was no difference between the 3 methods of analysis in the t50 values obtained in smokers. Previous studies comparing uncorrected and corrected analyses have shown the corrected t50 values to be significantly less in both smokers and non-smokers. However these studies did not compare the methods as they are used in practice. Uncorrected analyses were performed on the complete clearance curves rather than the first 7-10 minutes only (O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). It is likely that uncorrected and corrected t50 values for such curves will differ thus demonstrating the validity of background correction. In my view it is desirable to use a background correction in studies where clearance may be rapid, particularly when it may be biexponential, to avoid the confounding effect of rapidly accumulating background activity. In smokers considerable background activity can be seen accumulating over the first 7-10 minutes, thus the claim that there is little recirculating background activity during uncorrected analysis of this period seems unlikely in curves having a t50 of less than 10 minutes, of which in this study there was only 1. Background correction allows a longer duration of data acquisition without including recirculating background activity (Barrowcliffe *et al.*, 1987). There is no way of knowing in advance how rapid ^{99m}Tc-DTPA clearance will be or if a curve is going to be biexponential, although studies in smokers have consistently yielded monoexponential clearance curves. Background correction enables the analysis of any degree of change in the ^{99m}Tc-DTPA clearance curve from normal non-smoker to the effects of a range of heterogeneous pathologies which produce biexponential curves, that is, the corrected curve can be used to study the evolution of ^{99m}Tc-DTPA clearance in an individual with a progressive then resolving lung injury.

Non-smokers have less background activity than smokers and thus differences between the uncorrected and corrected t50 values in this study are likely to be due to technical factors. In non-smokers the clearance curves have such a small slope that the peak activity following

inhalation was less sharply differentiated than in the smokers and was thus more difficult to identify. This may explain the higher values obtained by the uncorrected analysis of the first 7 minutes of the curve in non-smokers compared to the corrected values obtained from the first 20 minutes. The lower t50 values obtained from the inter-renal compared to the shoulder method of correction in non-smokers was probably due to the greater vascularity of the inter-renal background.

Previous studies of intra-individual coefficients of variation (CV's) are given in Table 3.6. Rizk and co-workers studied 5 anaesthetised, paralysed and ventilated dogs using an uncorrected method of ^{99m}Tc-DTPA lung clearance. They found a mean t50 of 24.6±5.3 (±SD) minutes which was not significantly different when repeated at least 72 hours later (26.7±7.9 minutes). There was substantial intra-individual variation but a CV is not quoted (Rizk *et al.*, 1984). O'Doherty and colleagues studied 7 non-smokers and 6 smokers on 2 occasions and found that the correction factors were not significantly different between visits (O'Doherty *et al.*, 1985b).

Smith and co-workers found that the least variability between and within subjects with an uncorrected method was given by monoexponential analysis after inhalation of ^{99m}Tc-DTPA by tidal breathing, followed by scanning for 30 minutes, compared to VC inhalation of isotope, shorter scanning times and biexponential analysis. They found that several VC manouevres prior to measuring ^{99m}Tc-DTPA clearance resulted in faster clearance rates for 10 to 20 minutes. This was associated with a more rapid curvilinear clearance suggesting a transient difference in pulmonary permeability, surface area or volume of ELF. They recommended that subjects rest for 20 minutes prior to inhaling the aerosol of ^{99m}Tc-DTPA to avoid alterations in clearance rates from deep breathing. In normal subjects, intersubject and probably also intrasubject variability is minimised with delivery of the aerosol supine rather than sitting (Smith *et al.*, 1992). This is probably because it likely reduces the difference in regional clearance rates between upper and lower regions (Rinderknecht *et al.*, 1980, Mason *et al.*, 1983). It has also been reported that keeping the number of ^{99m}Tc-DTPA molecules per number of aerosol particles constant improves intrasubject repeatability (Groth *et al.*, 1989). Smith

Reference	Subjects (n)	Method	Scan period (mins)	CV (%)	Studies
Oberdorster *et al.*, 1984	1	U	0-60	8-18	4
Nolop *et al.*, 1986	6	C	0-20	12	2
Groth *et al.*, 1989	8	U	0-15	7	2
Thunberg *et al.*, 1989	10	C	15-90	9.3-11.4	2
Smith *et al.*, 1992	10	U	0-30	±18	2

Table 3.6. Previous studies of the repeatability of ^{99m}Tc-DTPA clearance from lungs to blood within subjects. U-uncorrected, C-corrected, CV-coefficient of variation.

and colleagues recommended that submicronic particles with GSD less than 1.6μm should be employed to minimise variability (Smith *et al.*, 1992). Measurements of total lung projections improve repeatability (Thunberg *et al.*, 1989)

In this study there was no difference in the mean values for ^{99m}Tc-DTPA clearance between 2 visits in either non-smokers or smokers for any of the 3 methods of analysis. Ozone inhalation, 0.4ppm for 2 hours, in normal non-smokers, for example, has been shown to change the t50 for ^{99m}Tc-DTPA clearance from 117±16 (SE) to 40±10 minutes (Kehrl *et al.*, 1987). Repeatability in this study did not differ between the 3 methods of analysis nor between non-smokers and smokers. It would have seemed likely that smokers would have greater variability in epithelial permeability over time than non-smokers due to variations in their smoking habit but this was not seen.

In conclusion values for ^{99m}Tc-DTPA clearance from lung to blood obtained from 2 background corrected methods of analysis are the same as those from an uncorrected analysis in a group of smokers with t50 values greater than 10 minutes. There is no difference in the repeatability of the technique between the 3 methods of analysis in either smokers or non-smokers. I believe that background correction is necessary as it allows the analysis of any form of curve, the nature of which cannot be predicted prior to study. Background correction using an inter-renal region allows rapid analysis. Further attempts to standardise this and other aspects of the technique are likely to reduce the range of results in the literature.

CHAPTER 4. EPITHELIAL PERMEABILITY, INFLAMMATION AND OXIDANT STATUS IN THE AIRSPACES OF CHRONIC SMOKERS.

4.1. INTRODUCTION.

Cigarette smoke was first shown to disrupt the respiratory epithelium in guinea pigs in whom penetration of the tracer horseradish peroxidase (HRP) was observed at all levels after exposure to cigarette smoke (Simani *et al.*, 1974). This was reported to be maximal at 30 minutes occurring during the exudative phase of an inflammatory reaction and returning to normal by 12 hours in association with the repair phase (Hulbert *et al.*, 1981). Increased airspace epithelial permeability is found in smokers (Jones *et al.*, 1980) and is rapidly reversible after stopping smoking (Minty *et al.*, 1981, Mason *et al.*, 1983). It occurs within 3 days of starting smoking, is unrelated to nicotine (Minty *et al.*, 1984) and is abolished by filtration of the smoke to remove all the particulate material (Minty and Royston, 1985).

The total number of inflammatory cells and the number of neutrophils in the airspaces of smokers are increased (Hunninghake and Crystal, 1983, Martin *et al.*, 1985, Koyama *et al.*, 1991). They show increased oxidative metabolic responses (Hoidal *et al.*, 1981) and enhanced cytotoxic potential (Davis *et al.*, 1988). Cigarette smoke contains 10^{16} free radicals per puff (Janoff *et al.*, 1987). The airspaces of smokers are thus exposed to oxidants both inhaled from cigarette smoke and released from inflammatory leucocytes. An oxidant-antioxidant imbalance has been suggested but never proven in the lungs of smokers and a number of lung diseases including COPD (Rahman and MacNee, 1996).

Alterations in anti-oxidants in blood and in the lungs of cigarette smokers have varied depending on the anti-oxidant studied. Smokers have increased levels of the anti-oxidant GSH in their ELF (Cantin *et al.*, 1987). GSH and its redox enzymes are important intracellular anti-oxidants crucial in maintaining cell function and in protecting the epithelium from oxidant stress.

It has been suggested that the leak in the respiratory epithelium in smokers occurs at the corners where epithelial cells meet rather than

along their lateral surface (Walker *et al.*, 1982). These corners occur in greatest concentration in the distal portion of membranous bronchioles and in the portion of respiratory bronchioles covered by cuboidal epithelium (Hogg 1983). Since smoking causes inflammation of terminal and respiratory bronchioles (Niewoehner *et al.*, 1974) and the maximum leak occurs with the exudative phase of the inflammatory reaction it has been proposed that the leak is associated with the repeated acute injury caused by cigarette smoke (Hogg 1983). Increased epithelial permeability leaves the interstitium of the lung open to other insults for example viruses, bacteria, pollutants and allergens.

Previous human studies have not attempted to define the acute and the chronic effects of smoking. The aim of this study was to compare the chronic and acute effects of cigarette smoking on epithelial permeability, inflammation and oxidant status in the airspaces of chronic smokers.

4.2. METHODS.

4.2.1. Patients.

Fourteen regular cigarette smokers underwent ^{99m}Tc-DTPA lung scans after abstaining from cigarette smoking for 12 hours (chronic smoking) and 1 hour after smoking 2 cigarettes (G2, Imperial Tobacco) (Figure 4.1) to a standard protocol (acute smoking). The characteristics of these subjects are given in Table 4.1. There was no change in spirometry following acute cigarette smoking. The COHb was significantly higher prior to the acute smoking than after 12 hours abstinence from cigarettes and was further increased after smoking 2 cigarettes.

Each smoker also underwent bronchoscopy and BAL after either chronic smoking (n=8) or acute smoking (n=7). Seven control subjects who had never smoked were also studied with bronchoscopy and BAL. The characteristics of these patients are given in Table 4.2. The non-smokers were significantly younger than the acute group ($p<0.05$) in whom the FEV_1 ($p<0.05$) but not %predicted was significantly lower. COHb in the non-smokers was significantly lower than in either the acute ($p<0.001$) or chronic ($p<0.01$) smoking groups.

Figure 4.1. Acute smoking, exhaling into a safety hood as described in Section 4.2.1.

		Chronic	Acute Pre smoking	p value A pre v C	Acute Post smoking	p value A post v A pre
Age (years)	40.1±3.7					
	28-66 (range)					
FEV_1 (litres)		3.4±0.2	3.4±0.2	ns	3.4±0.2	ns
%predicted		90.6±4.7	91.4±4.9	ns	90.5±4.9	ns
FVC (litres)		4.3±0.2	4.4±0.2	ns	4.4±0.2	ns
Ratio (%)		77.5±3.0	76.3±2.1	ns	76.4±2.3	ns
Cigs daily	23.2±2.0					
	15-40					
Pack years	32.3±4.7					
	7-65					
COHb (%)		2.2±0.2	5.7±0.6	<0.001	8.1±0.6	<0.001
RR (per min)		11.4±0.9			11.8±1.1	ns v C

Table 4.1. Subject characteristics (mean±SE, n=14) in smokers who abstained for 12 hours (chronic smoking-C) and 1 hour following acute smoking of 2 cigarettes (acute smoking-A). RR respiratory rate.

		Chronic smokers	Acute smokers	Non-smokers	p value
n		8	7	7	
M:F		6:2	5:2	5:2	
Age (years)		40±4	44±4	32±1	<0.05 A v NS
		26-52 (range)	26-56	26-36	
FEV_1 (litres)		3.7±0.2	3.3±0.4	4.4±0.3	<0.05 A v NS
%predicted		94±5	90±8	107±6	ns
Cigs daily		20.6±2.2	23.6±3.4	n/a	ns
		15-30	15-40		
Pack years		24±4	33±7	n/a	ns
		7-33	7-65		
COHb (%)	Pre	3.0±0.2	4.8±0.5	1.2±0.1	<0.01 C v A
					<0.01 C v NS
					<0.001 A v NS
	Post		7.1±0.5		p<0.001 v pre

Table 4.2. Subject characteristics (mean±SE) in smokers (chronic smoking-C, acute smoking-A) and non-smokers (NS) prior to undergoing fibreoptic bronchoscopy and bronchoalveolar lavage.

None of the subjects had a history of respiratory infection within 6 weeks of the study.

4.2.2. Stability of ^{99m}Tc-DTPA.

In 3 chronic smokers thin layer chromatography of the residual ^{99m}Tc-DTPA in the nebuliser and urine demonstrated 0% and <1.7% dissociation respectively into DTPA and free pertechnetate (Zimmer and Pavel, 1977). In 1 acute smoker these figures were 0% and 2.4% respectively.

4.2.3 Ethics.

Ethical permission was obtained from the local medical ethics committee and all patients gave informed written consent.

4.2.4. Statistics.

In the ^{99m}Tc-DTPA lung clearance study all comparisons were made by paired t-test. In the BAL study all comparisons were made by one way ANOVA except where only 2 groups could be compared in which case either an unpaired or paired t-test was performed as appropriate. Superoxide anion production by mixed BAL leucocytes was analysed by two way ANOVA. Correlation was performed by a 2 variable parametric analysis. Statistical advice was given by Dr. TJ Peters, Senior Lecturer in Public Health Medicine, Department of Social Medicine, University of Bristol, Bristol. All calculations were performed on Statistica/Mac^{TM}, Release 1.7, Copyright StatSoft, Inc. 1991-1992. A level of <0.05 was considered statistically significant.

4.3. RESULTS.

4.3.1. ^{99m}Tc-DTPA lung clearance.

In the chronic smoking group t50 was 16.7±1.3 (mean±SE) minutes. This was further and significantly reduced after acute smoking to 14.8±1.0 minutes ($p<0.01$) (Figure 4.2). Individual values are shown in Figure 4.3. Mean values for ^{99m}Tc-DTPA clearance for right and left lungs are given in Table 4.3. Although there was a significant difference between the right and left lungs in both the chronic ($p<0.05$) and acute smoking groups ($p<0.05$) ^{99m}Tc-DTPA clearance was significantly greater following acute

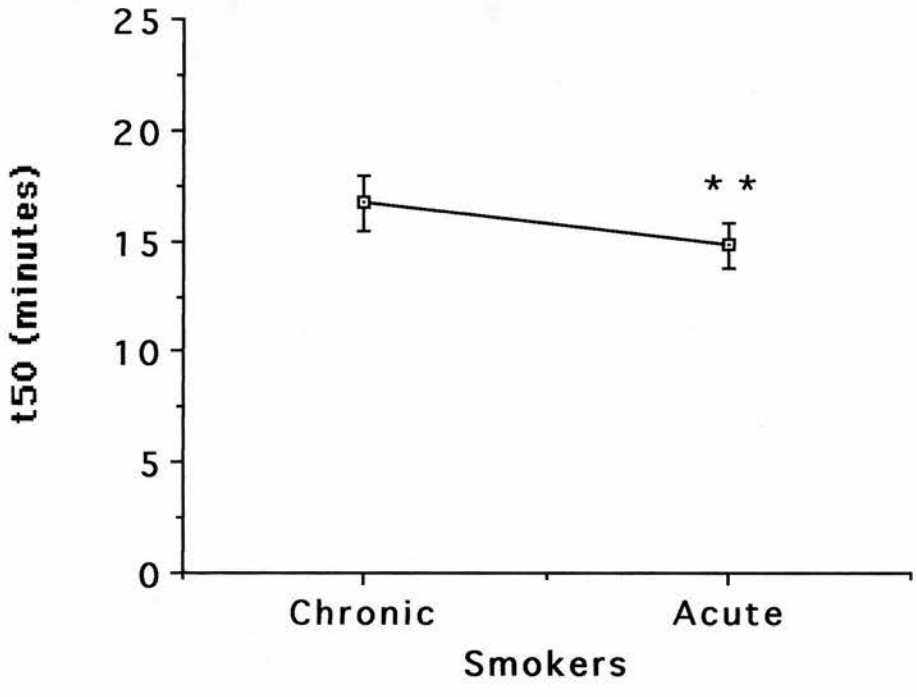


Figure 4.2. Time for 50% clearance of ^{99m}Tc-DTPA from lungs to blood (t50) in the chronic and acute smoking groups. Mean±SE, n=14, ** $p<0.01$.

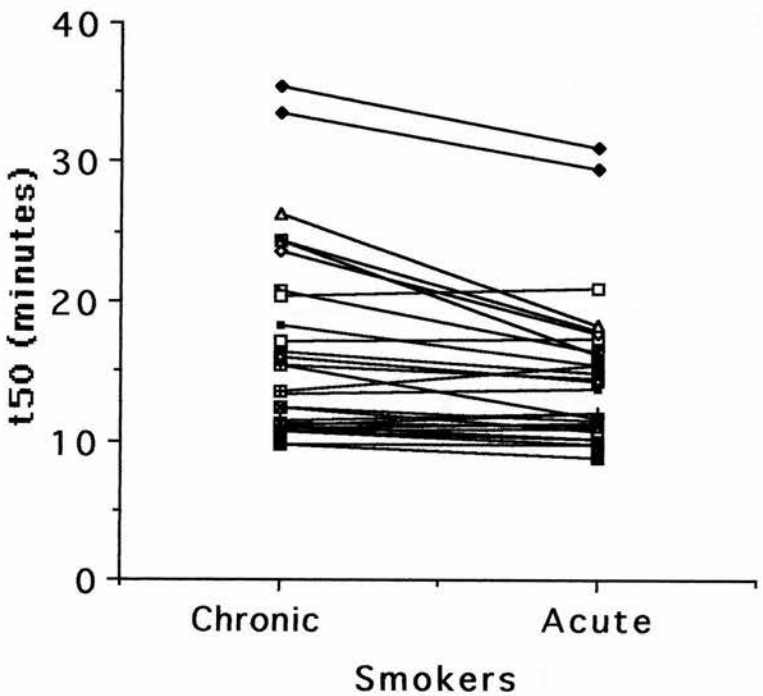


Figure 4.3. Individual values for ^{99m}Tc-DTPA lung clearance (t50) in chronic and acute smoking groups.

	Right lung			Left lung		
	t50	r	CF	t50	r	CF
Chronic smoking	15.7±1.8	0.99±0.00	1.37±0.14	17.6±2.0	0.99±0.00	1.64±0.10
Acute smoking	14.1±1.4	0.99±0.00	2.03±0.23	15.4±1.5	0.99±0.00	2.03±0.20

Table 4.3. Time to 50% clearance of ^{99m}Tc-DTPA from the lungs to the blood (t50), regression coefficients (r) for the time activity curves and correction factors for recirculating background activity (CF) in paired groups of smokers after abstaining for 12 hours (chronic smoking) and after smoking 2 cigarettes 1 hour before study (acute smoking). Values are given for the right and left lungs and are expressed as mean±SE. Although there was a significant difference between the right and left lungs in both the chronic ($p<0.05$) and acute smoking groups ($p<0.05$) ^{99m}Tc-DTPA clearance was significantly greater following acute smoking regardless of the lung studied (right lung $p<0.05$, left lung $p<0.05$). All regression coefficients were 0.98 or greater for the semi logarithmic time activity curves.

smoking regardless of the lung studied (right lung $p<0.05$, left lung $p<0.05$). All correlation coefficients were 0.98 or greater for the semi logarithmic time activity curves.

4.3.2. Bronchoscopy and bronchoalveolar lavage.

The characteristics of the BAL are given in Table 4.4. The total number of cells recovered was 6.5 fold greater in the acute smoking group than in the non-smokers and 11.4 fold greater in the chronic smoking group. There was no significant difference in the volume of BALF recovered in the 3 groups nor in the viability of the cells recovered which ranged from 72-94%.

There was a 3 fold increase in the percentage of neutrophils in BALF obtained from the acute smoking group compared to the chronic smoking group ($p<0.05$) and a 2.5 fold increase compared to the non-smokers (ns). There was a trend towards an increase in the percentage of eosinophils in the acute smoking group. Regarding absolute numbers significantly increased numbers of macrophages ($p<0.05$) and lymphocytes ($p<0.01$) were observed in BALF obtained from the chronic smoking group compared to the non-smokers. Neutrophil numbers were significantly increased in BALF obtained from the acute smoking group compared to the non-smokers ($p<0.05$).

There was a trend towards an increased albumin concentration in BALF (Figure 4.4) and ELF volume (Figure 4.5) in both smoking groups (Table 4.5). The values are in keeping with those given by Rennard using either albumin or urea to correct for the concentration of other substances inELF assuming the concentration of albumin in ELF to be one tenth that of plasma and the concentration of urea to be equal, as it is assumed to be freely diffusible (Rennard *et al.*, 1986).

Superoxide release (Table 4.6) from mixed leucocytes obtained from chronic ($p<0.01$) and acute ($p<0.001$) smokers was significantly increased compared to non-smokers (Figure 4.6). When unstimulated cells and cells stimulated with PMA $1\mu gml^{-1}$ were analysed separately only a trend towards an increase was seen in the 2 smoking groups compared to non-smokers. No significant increase was seen on stimulation with PMA in

	Chronic smokers	Acute smokers	Non smokers	p value
Return (mls)	151.2±9.0	157.9±11.8	170.0±14.5	ns
Total cells (x10^6)	95.7±29.0	54.8±9.0	8.4±1.6	<0.05 C v NS
% Viability	84.4±2.8	87.3±2.0	83.0±2.5	ns
Differential				
Macrophages (%)	96.3±0.4	94.4±0.9	95.9±1.0	ns
x10^6	92.2±28.1	52.0±9.0	8.12±1.58	<0.05 C v NS
Lymphocytes	2.97±0.38	3.21±0.58	3.18±0.79	ns
	2.79±0.79	1.56±0.26	0.22±0.04	<0.01 C v NS
Neutrophils	0.59±0.25	1.96±0.53	0.79±0.29	<0.05 C v A
	0.61±0.24	0.99±0.32	0.05±0.01	<0.05 A v NS
Eosinophils	0.12±0.08	0.46±0.18	0.11±0.05	ns
	0.08±0.06	0.25±0.11	0.006±0.004	ns

Table 4.4. Bronchoalveolar lavage characteristics and cell differential (% and absolute numbers x10^6) in smokers (chronic smoking-C, acute smoking-A) and non-smokers (NS).

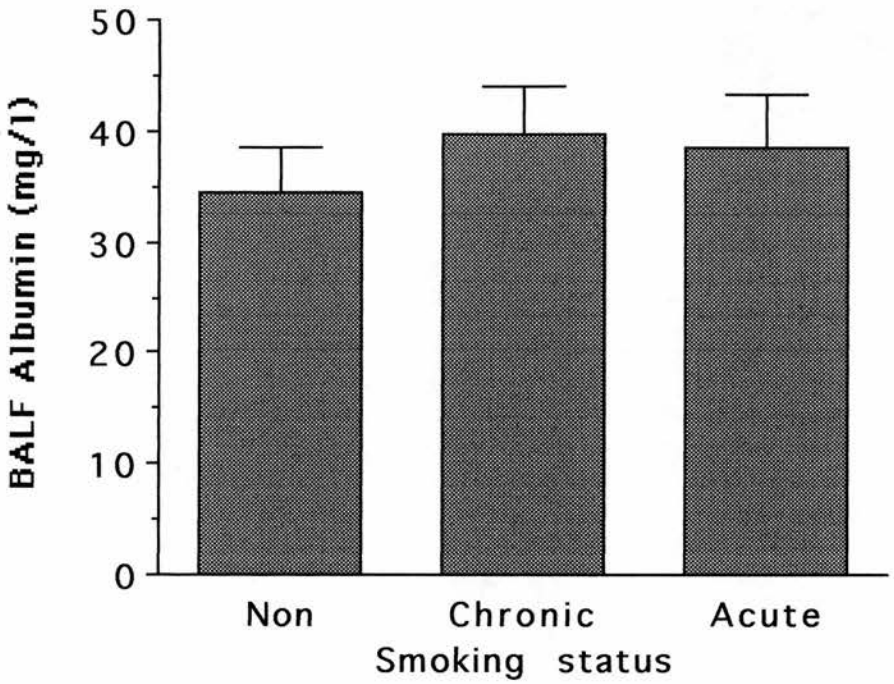


Figure 4.4. Bronchoalveolar lavage fluid (BALF) albumin in non-smokers and in the chronic and acute smoking groups. Mean±SE.

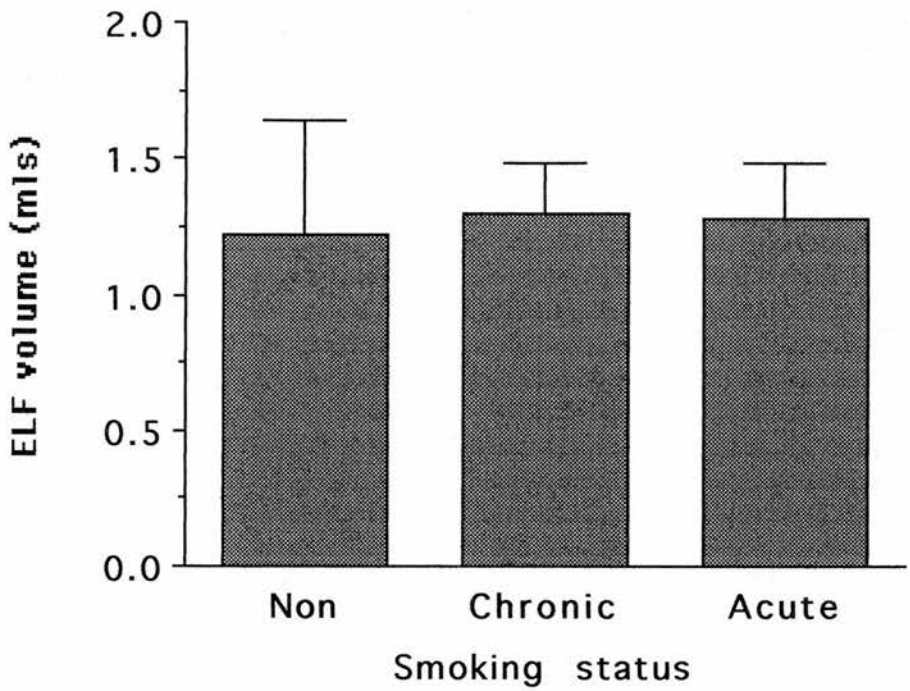


Figure 4.5. Epithelial lining fluid (ELF) volume in non-smokers and in the chronic and acute smoking groups. Mean±SE.

	Chronic	Acute	Non	p level
BALF albumin (μgml^{-1})	39.6±4.4	38.5±4.7	34.5±4.0	ns
Plasma albumin ($mgml^{-1}$)	46.0±0.8	46.1±0.6	47.7±1.1	ns
BALF volume (mls)	151.2±9.0	157.9±11.8	170.0±14.5	ns
ELF volume (mls)	1.30±0.18	1.28±0.16	1.22±0.16	ns

Table 4.5. Albumin levels in bronchoalveolar lavage fluid (BALF) and epithelial lining fluid (ELF) volume in the chronic and acute smoking groups and in non-smokers.

		Chronic smokers	Acute smokers	Non-smokers	p level
Superoxide anion nmol $2.5x10^5$ cells^{-1} 120 mins^{-1}	Unstimulated	17.6±3.1	18.4±2.8	4.6±1.0	ns
	PMA 1 μgml^{-1}	18.7±2.8	23.6±2.8	6.3±0.1	ns
Products of lipid peroxidation μmol l^{-1}	Plasma	2.99±0.75	3.06±0.42	1.51±0.21	<0.05 C v NS <0.05 A v NS
	BALF	0.11±0.05	0.30±0.13	0.05±0.05	ns
	ELF	11.28±5.55	38.8±17.1	6.56±6.56	ns

Table 4.6. Superoxide anion release from mixed bronchoalveolar lavage leucocytes and products of lipid peroxidation in plasma, bronchoalveolar lavage fluid (BALF) and epithelial lining fluid (ELF) in the chronic (C) and acute (A) smoking groups and in non-smokers (NS). PMA Phorbol myristate acetate. Overall $O_2^{\cdot -}$ release from both groups of smokers cells was greater than from non-smokers cells (C v NS $p<0.01$, A v NS $p<0.001$).

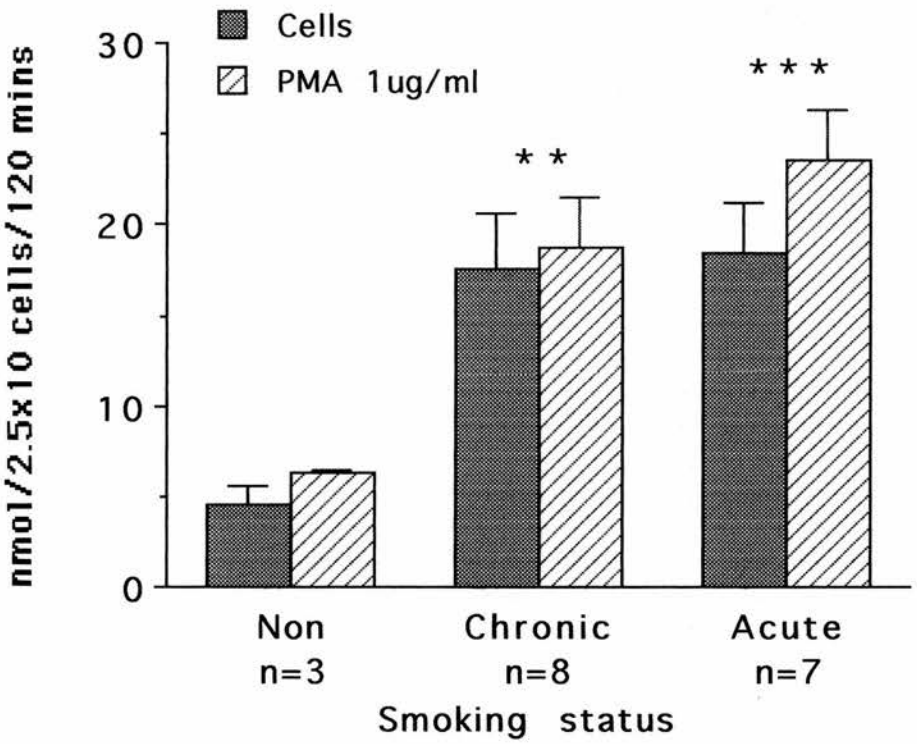


Figure 4.6. Superoxide production (mean±SE) from mixed bronchoalveolar lavage (BAL) leucocytes, unstimulated and stimulated with phorbol myristate acetate (PMA) 1μgml^{-1} in non-smokers (NS) and in the chronic (C) and acute (A) smoking groups. ** $p<0.01$ C v NS, *** $p<0.001$ A v NS.

any of the 3 groups although a trend towards this was seen in the acute smoking group.

Products of lipid peroxidation (Table 4.6) were significantly increased in plasma in both the chronic ($p<0.05$) and acute ($p<0.05$) smoking groups compared to the non-smokers although there was no difference between the 2 groups (Figure 4.7). In BALF (Figure 4.8) and in ELF (Figure 4.9) products of lipid peroxidation doubled in the chronic smokers and increased 6 fold in the acute smokers compared to non-smokers although these changes did not reach statistical significance.

TEAC (Table 4.7) was significantly reduced in plasma in both smoking groups ($p<0.001$) (Figure 4.10). There was a trend towards a further reduction in the acute smokers but this did not reach statistical significance. Conversely a significant increase in TEAC was seen in BALF (Figure 4.11) in both smoking groups ($p<0.05$) compared to the non-smokers although again there was no significant difference between the 2 smoking groups. The same pattern was seen in ELF (Figure 4.12) although the changes did not reach statistical significance.

GSH (Table 4.7) was increased 2 fold in mixed BAL leucocytes (Figure 4.13), BALF (Figure 4.14) and ELF (Figure 4.15) in chronic smokers compared to non-smokers. This increase was abolished following acute A weak correlation between t50 for ^{99m}Tc-DTPA clearance in the chronic smoking group (Table 4.8) and pack years was found ($r=0.59$, $p<0.05$) and a trend observed for age ($r=0.57$, $p=0.052$). There was no relationship between t50 and the number of cigarettes smoked daily or COHb. No correlation was found between t50 for the chronic and acute smokers together and any of the variables measured as shown in Table 4.9. Although the percentage of neutrophils in BALF in the acute smoking group was significantly higher than in the chronic smoking group there was no correlation with t50 in these 2 groups.

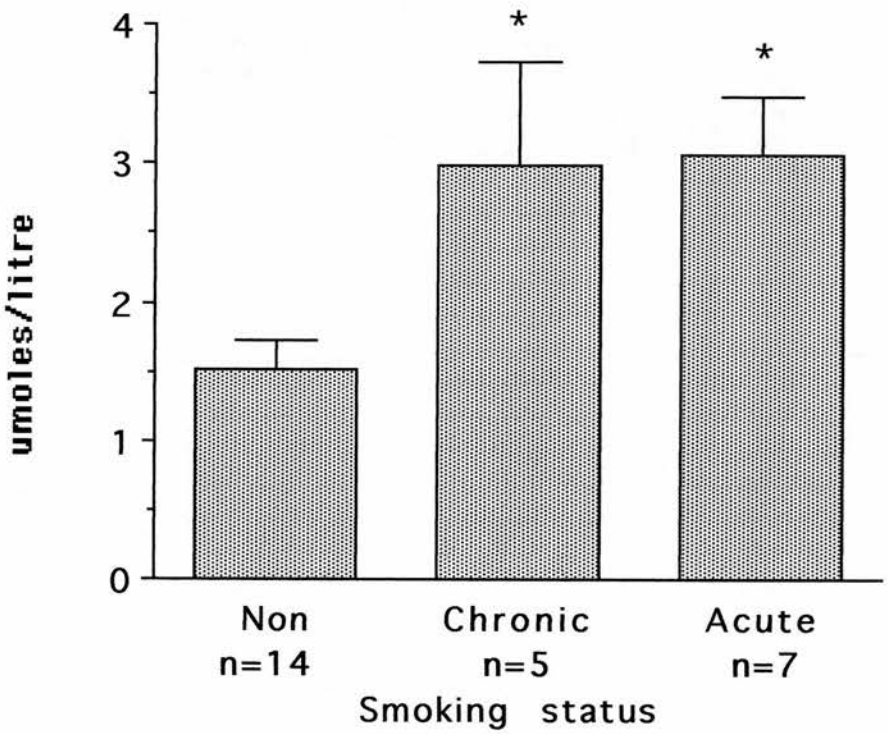


Figure 4.7. Plasma lipid peroxidation in non-smokers and in the chronic and acute smoking groups. Mean±SE, * $p<0.05$.

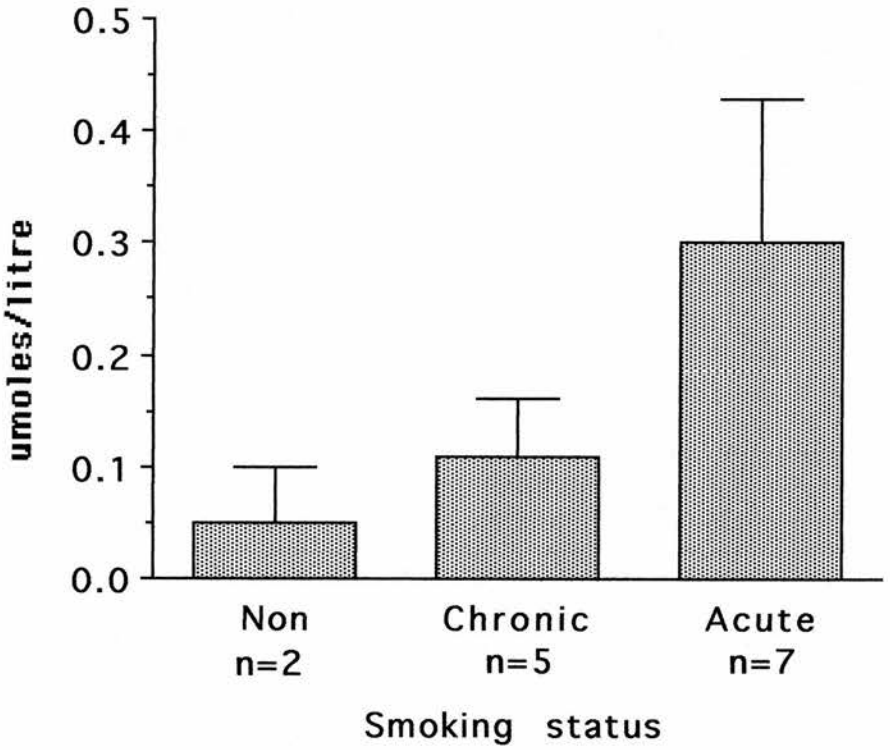


Figure 4.8. Bronchoalveolar lavage fluid lipid peroxidation in non-smokers and in the chronic and acute smoking groups. Mean±SE.

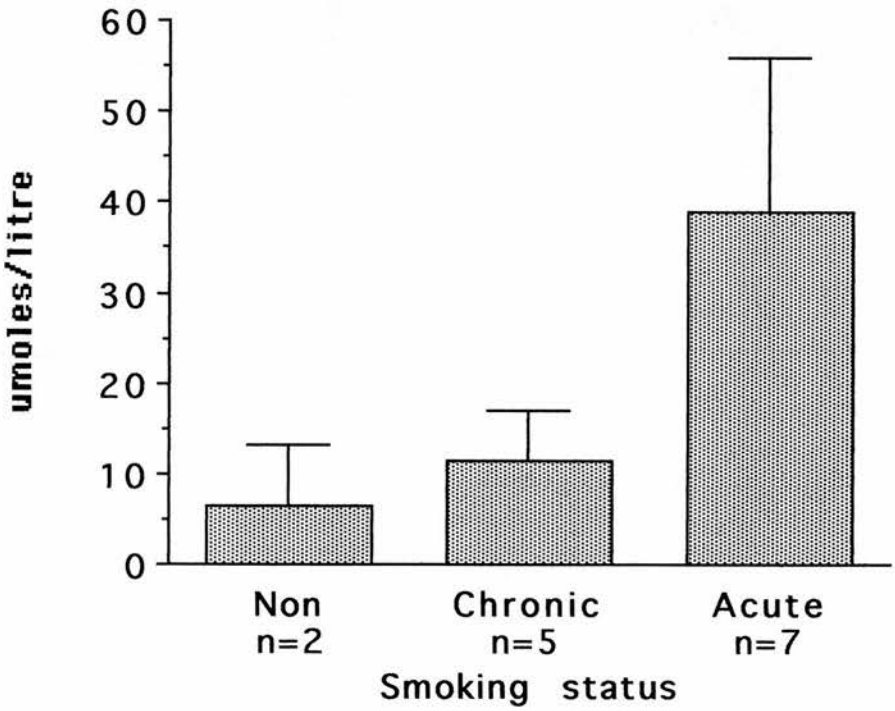


Figure 4.9. Epithelial lining fluid lipid peroxidation in non-smokers and in the chronic and acute smoking groups. Mean±SE.

			Chronic	Acute	Non	p level
TEAC	Plasma (mM)		0.66±0.07	0.41±0.03	1.31±0.10	<0.001 C v NS <0.001 A v NS
	BALF (μM)		23.2±5.6	25.3±5.9	8.2±1.6	<0.05 C v NS <0.05 A v NS
	ELF (μM)		3170±900	3740±1180	1160±230	ns
Glutathione	Cells	GSH	8.82±3.03	5.05±1.44	4.65±1.68	ns
	(nmol 10^6 cells^{-1})	GSSG	0.90±0.29	0.06±0.04	0.47±0.31	ns
	BALF (μM)	GSH	6.64±1.98	3.39±1.44	2.26±0.73	ns
		GSSG	0.51±0.23	0.019±0.019	0.32±0.21	ns
	ELF (μM)	GSH	762.7±257.1	402.6±137.9	353.7±125.3	ns
		GSSG	56.0±27.1	1.69±1.69	51.6±34.0	ns

Table 4.7. Trolox equivalent anti-oxidant capacity (TEAC) in plasma, bronchoalveolar lavage fluid (BALF) and epithelial lining fluid (ELF) and glutathione (reduced GSH, oxidised GSSG) in mixed bronchoalveolar lavage leucocytes, BALF and ELF in the chronic (C) and acute (A) smoking groups and in non-smokers (NS).

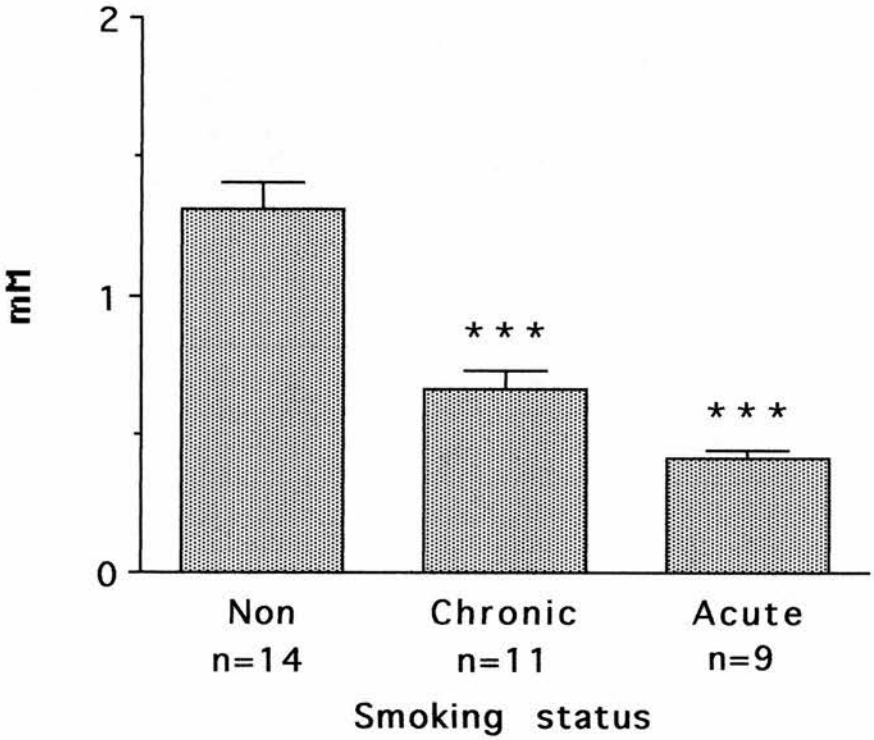


Figure 4.10. Trolox equivalent anti-oxidant capacity in plasma in non-smokers (NS) and in the chronic and acute smoking groups. Mean±SE, *** $p<0.001$ v NS.

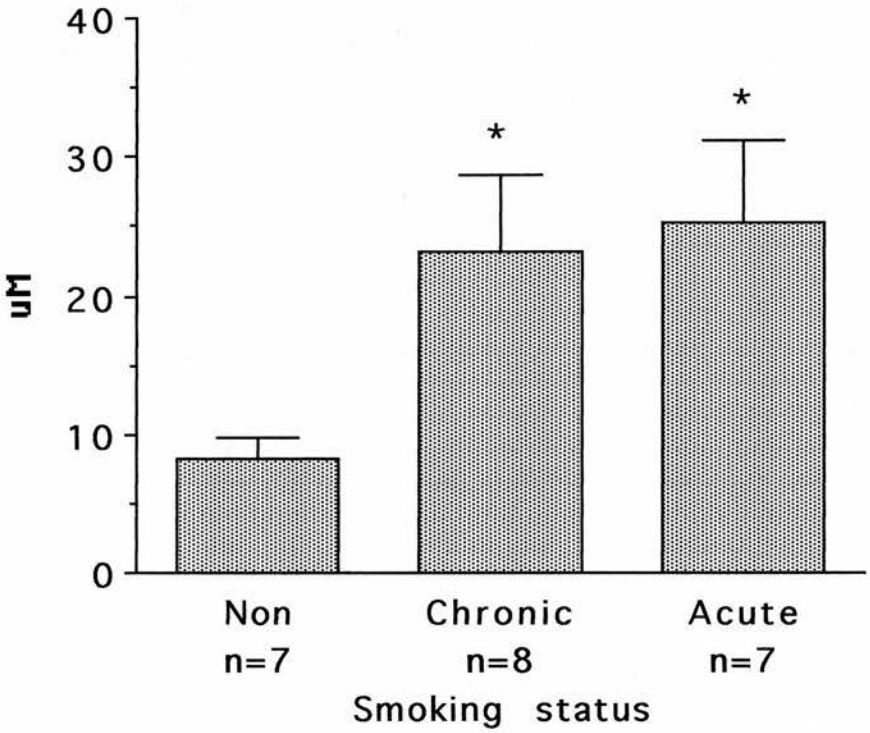


Figure 4.11. Trolox equivalent anti-oxidant capacity in bronchoalveolar lavage fluid in non-smokers and in the chronic and acute smoking groups. Mean±SE, * $p<0.05$.

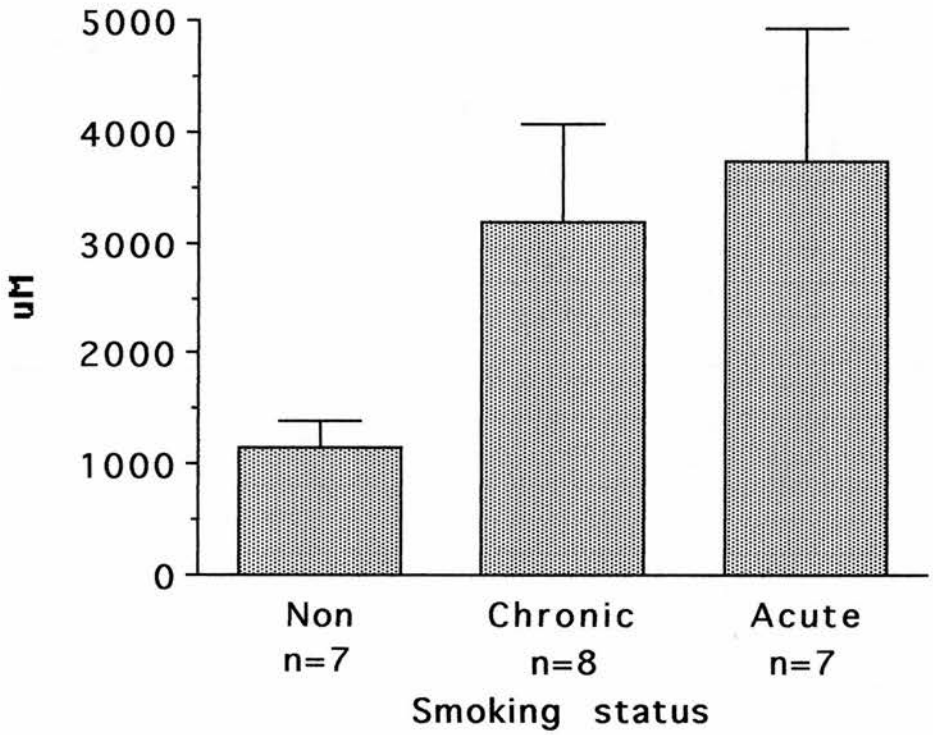


Figure 4.12. Trolox equivalent anti-oxidant capacity in epithelial lining fluid in non-smokers and in the chronic and acute smoking groups. Mean±SE.

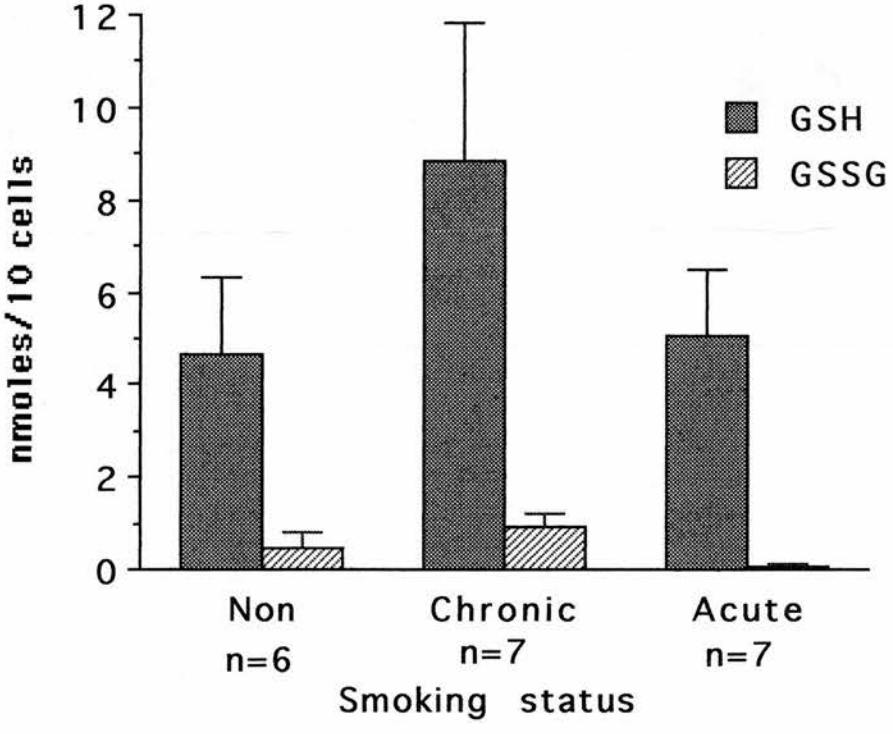


Figure 4.13. Reduced (GSH) and oxidised glutathione (GSSG) in mixed bronchoalveolar lavage leucocytes in non-smokers and in the chronic and acute smoking groups. Mean±SE.

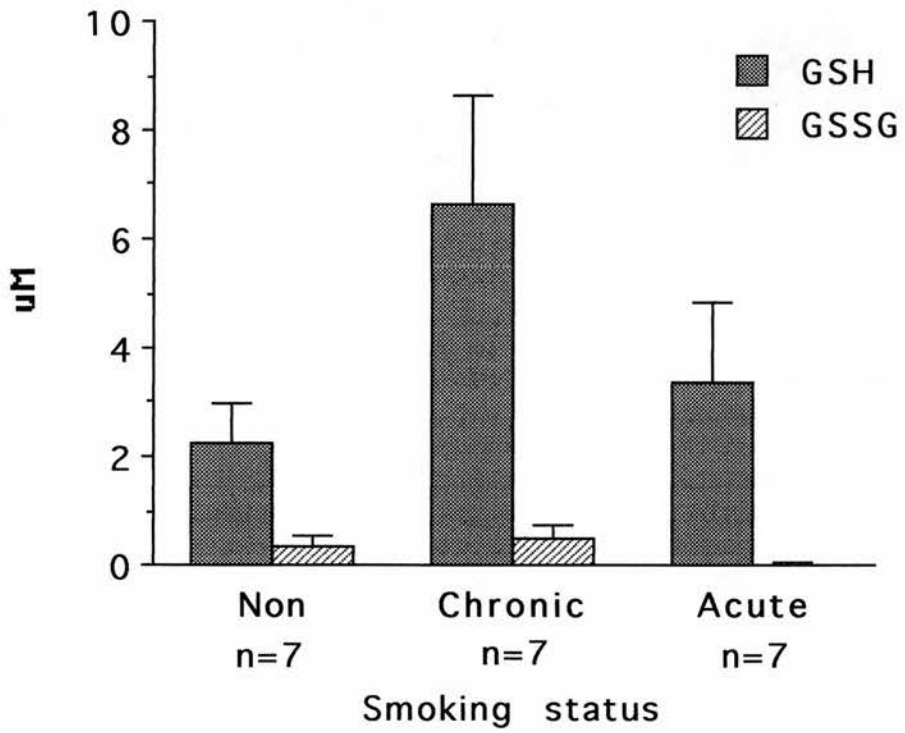


Figure 4.14. Reduced (GSH) and oxidised glutathione (GSSG) in bronchoalveolar lavage fluid in non-smokers and in the chronic and acute smoking groups. Mean±SE.

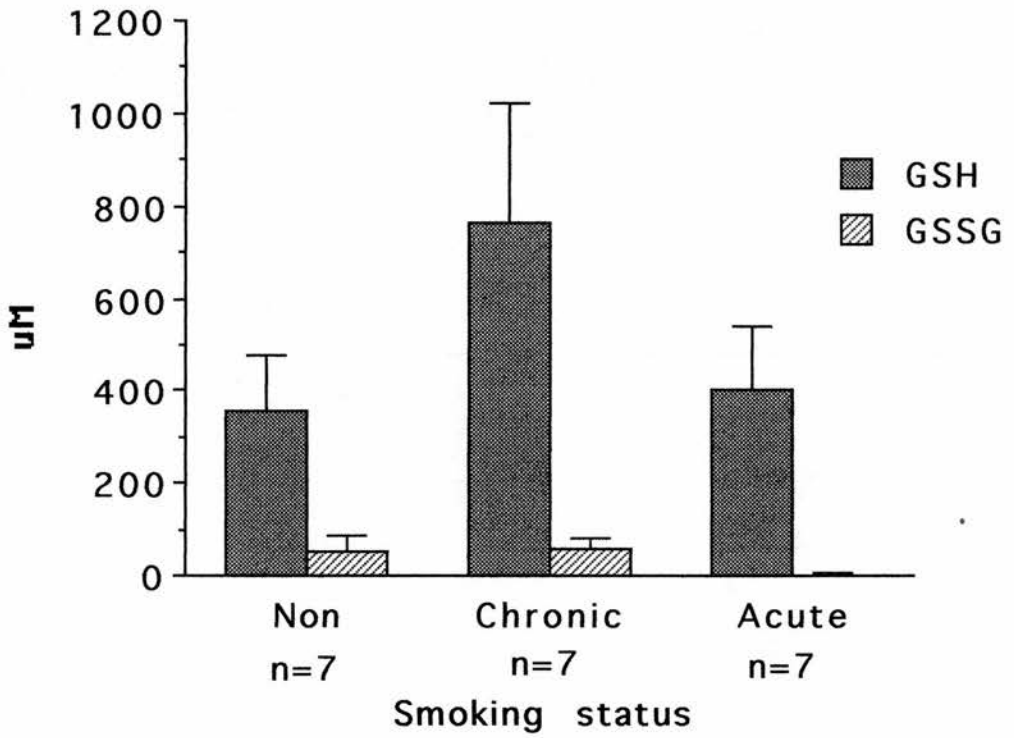


Figure 4.15. Reduced (GSH) and oxidised glutathione (GSSG) in epithelial lining fluid in non-smokers and in the chronic and acute smoking groups. Mean±SE.

Variable	r	p value	n
Age	0.57	<0.052	12
Cigs daily	0.36	<0.26	12
Pack years	0.59	<0.04	12
Pre COHb	-0.01	<0.96	12
Pre FEV_1	0.11	<0.74	12
Pre %predicted	0.23	<0.48	12
Pre FVC	0.19	<0.56	12
Pre ratio	-0.20	<0.54	12

Table 4.8. Correlations (r) between the time for 50% clearance of ^{99m}Tc-DTPA from lungs to blood (t50) in the chronic smoking group and baseline subject characteristics, carboxyhaemoglobin and spirometry.

Variable	r	p value	n	Variable			r	p value	n
Post COHb	-0.17	<0.44	24	Albumin	BALF		0.29	<0.30	15
Post FEV_1	0.12	<0.58	24		Plasma		0.18	<0.51	15
Post %predicted	0.20	<0.34	24	ELF volume			0.11	<0.69	15
Post FVC	0.18	<0.39	24	Superoxide anion	Unstimulated		0.03	<0.91	15
Post ratio	-0.17	<0.43	24		PMA 1 μgml^{-1}		0.15	<0.58	15
Return	-0.19	<0.49	15	Lipid peroxidation	Plasma		-0.13	<0.72	10
Total cells	-0.31	<0.27	15		BALF		0.49	<0.10	12
% Viability	0.14	<0.62	15		ELF		0.44	<0.15	12
Macrophages (%)	-0.32	<0.25	15	TEAC	Plasma		-0.15	<0.59	15
$x10^6$	-0.31	<0.26	15		BALF		-0.05	<0.87	15
Lymphocytes	0.13	<0.65	15		ELF		-0.12	<0.68	15
$x10^6$	-0.28	<0.32	15	Glutathione	Cells	GSH	-0.12	<0.68	14
Neutrophils	0.35	<0.21	15			GSSG	-0.17	<0.55	14
$x10^6$	0.03	<0.91	15		BALF	GSH	-0.13	<0.66	14
Eosinophils	0.12	<0.67	15			GSSG	-0.09	<0.76	14
$x10^6$	-0.05	<0.86	15		ELF	GSH	-0.21	<0.47	14
						GSSG	-0.14	<0.63	14

Table 4.9. Correlations between the time for 50% clearance of ^{99m}Tc-DTPA from lungs to blood (t50) in the chronic and acute smoking groups and carboxyhaemoglobin (COHb), spirometry, bronchoalveolar lavage characteristics and differential and oxidant and anti-oxidant measures. BALF-bronchoalveolar lavage fluid, ELF-epithelial lining fluid, TEAC-Trolox equivalent anti-oxidant capacity.

4.4. DISCUSSION.

The respiratory epithelium is a selectively permeable barrier separating the airways and airspaces from the submucosa and interstititium of the lungs and the vascular components of the respiratory tract. It lines the respiratory tract and acts as a barrier preventing the entry of potentially noxious agents such as bacteria, viruses, pollutants and allergens. Although cigarette smoke falls into this category, the lungs of cigarette smokers are rendered more permeable than those of non-smokers (Jones *et al.*, 1980). This is a rapidly reversible (Minty *et al.*, 1981, Mason *et al.*, 1983) and rapidly induced effect which is not produced by nicotine (Minty *et al.*, 1984) and relates neither to chronic airflow limitation (Huchon *et al.*, 1984), bronchial hyperreactivity (Kennedy *et al.*, 1984, O'Byrne *et al.*, 1984) or small airway function (Taylor *et al.*, 1988). Increased rates of ^{99m}Tc-DTPA lung clearance occur in numerous conditions, including ILD (Rinderknecht *et al.*, 1980), ARDS (Barrowcliffe and Jones, 1989), PCP (Robinson *et al.*, 1991) and acute asthma (Lemarchand *et al.*, 1992). In fact in fibrosing alveolitis the clearance of ^{99m}Tc-DTPA predicts the clinical course of the disease (Wells *et al.*, 1993) and in asbestos workers with and without asbestosis increased ^{99m}Tc-DTPA clearance is associated with worse functional and radiological outcome (Al Jarad *et al.*, 1993). Peripheral deposition (Oberdorster *et al.*, 1986) and deposition in the upper lobes (Mason *et al.*, 1983) is associated with faster clearance as is raised lung volume (Nolop *et al.*, 1986).

However the mechanism for increased ^{99m}Tc-DTPA clearance is not known. In guinea pigs exposed to cigarette smoke the tracer horseradish peroxidase has been seen to pass through the tight junctions linking epithelial cells at all levels and then to pass through the intercellular pathway (Simani *et al.*, 1974, Boucher *et al.*, 1980) with freeze fracture electron microscopy (EM) showing progressive disruption of tight junctions (Boucher *et al.*, 1980). In rabbits a significant increase in ^{99m}Tc-DTPA permeability following acute cigarette smoke exposure was associated with LM and EM changes of focal alveolar oedema and haemorrhage but no alveolar capillary membrane damage (Witten *et al.*, 1985). Burns and colleagues (Burns *et al.*, 1989) showed increased respiratory epithelial permeability to fluoroscein isothiocyanate dextran (FITC-D) after cigarette smoke exposure in guinea pigs and alveolar

epithelial damage. Epithelial tight junctions have a diameter of 6-10 A^{o} and are too small for the passage of FITC-D which has a 22 A^{o} radius. Transmission EM showed that the FITC-D diffused across damaged type I pneumocytes and entered alveolar capillaries through endothelial tight junctions which have a diameter of 40-58 A^{o}. Increased ^{99m}Tc-DTPA clearance occurred in rats, 15 minutes after exposure to cigarette smoke, lasting 8-9 minutes although no abnormality was found in the lung parenchyma on LM (Mordelet-Dambrine *et al.*, 1991). Cigarette smoke extract inhibited intercellular communication across gap junctions in primary hamster tracheal epithelial cells (Rutten *et al.*, 1988) and cigarette smoke condensate reduced gap junction numbers by at least 60% in primary chick embryo hepatocytes (van der Zandt *et al.*, 1990). In cultured rat hepatic epithelial cells and human fibroblasts the number of gap junctions and the rate of gap junction communication was inhibited by cigarette smoke condensate from burning but not heated cigarettes (McKarns and Doolittle, 1991). In monolayers of porcine pulmonary artery endothelium cigarette smoke extract caused a dose dependent increase in the flux of ^{125}I-BSA that was dependent on extracellular divalent cations. The effect was reversible, independent of the surface exposed (luminal or abluminal) and due to the vapour phase of the extract. It occurred without evidence of cell damage but subtle morphological changes were seen with focal retraction of individual cells and occasional areas where gaps had formed in the monolayer associated with focal areas of polymerised F-actin (Holden *et al.*, 1989).

It has been proposed that epithelial permeability is associated with the repeated acute injury caused by cigarette smoke (Hogg, 1983). I performed paired ^{99m}Tc-DTPA lung clearance scans in 14 life long smokers. On 1 occasion they were asked to refrain from smoking for 12 hours in order to eliminate the acute effects of cigarette smoke. Compliance was confirmed by COHb measurements. On another occasion they continued to smoke and had 2 cigarettes to a standard protocol over 10 minutes 1 hour prior to the lung scan. Airspace epithelial permeability was still increased in the first study, the chronic smoking study, and was increased further and significantly by the acute smoking. This was consistent for the right and left lungs analysed separately. The individual changes show this to be a very consistent effect. The change from 16.7±1.3 to 14.8±1.0 minutes

represents a change of 11% and indicates that the effect of cigarette smoke on epithelial permeability is indeed an acute one. This is consistent with previous reports indicating significant improvement in epithelial permeability as rapidly as 24 hours after the last cigarette (Mason *et al.*, 1983). The change in epithelial permeability seen in our study was not associated with any changes in respiratory function measured nor was there any evidence of dissolution of the ^{99m}Tc-DTPA compound into DTPA and free pertechnetate to explain the increase in clearance. It was not associated with any changes in BALF albumin concentration or ELF volume. There was no relationship between ^{99m}Tc-DTPA clearance and the number of cigarettes smoked daily, the duration of smoking or the COHb level either before or after smoking.

The total number of cells found in BALF obtained from cigarette smokers is elevated from 2.2-4 fold (Hunninghake and Crystal, 1983, Nagai *et al.*, 1988, McCrea *et al.*, 1994). The percentage of neutrophils in some studies has been no different in smokers compared to non-smokers (Nagai *et al.*, 1988, McCrea *et al.*, 1994) although in another it was 22 times greater (Hunninghake and Crystal, 1983). However the absolute number of neutrophils is consistently raised being 87, 1.6 and 2.6 fold elevated in these 3 studies (Hunninghake and Crystal, 1983, Nagai *et al.*, 1988, McCrea *et al.*, 1994). Long-term cigarette smoking also increases the number of neutrophils in the peripheral circulation and in lung parenchyma (Niewoehner *et al.*, 1988, McGowan and Hunninghake, 1989). Acute cigarette smoking has been shown to attract circulating neutrophils to the pulmonary vasculature (MacNee *et al.*, 1989), mediated by a change in cell deformability. Subsequent migration may be mediated through the release of chemotactic factors such as C5a, LTB4 (Sibille and Reynolds, 1990) and IL-8 (Kunkel *et al.*, 1991). Macrophages are increased in number at the site of development of centrilobular emphysema (Niewoehner *et al.*, 1974) and cigarette smoke can cause macrophages to release chemotactic factors for neutrophils (Hunninghake *et al.*, 1980). Total glutathione concentration is correlated with neutrophil numbers, levels of myeloperoxidase and elastase and chemotactic activity for neutrophils in BALF (Linden *et al.*, 1989).

I performed bronchoscopy and BAL in unpaired smokers and non-smokers. The smokers were asked to either abstain for 12 hours as above or to follow the acute smoking protocol. The total number of cells was significantly increased in the chronic smoking group and elevated 6.5 fold in the acute smoking group although this was not a statistically significant increase. These increases were largely due to increased numbers of macrophages although there were no changes in the percentage of macrophages in BALF. There was a significant increase in the percentage and number of neutrophils in BALF in the acute smoking group with no difference between the chronic smoking group and non-smokers. There was no correlation however between either the total number of cells or the number and percentage of neutrophils in BALF and ^{99m}Tc-DTPA clearance. It is however possible that acute cigarette smoking may induce a change in inflammatory leucocytes which makes them less likely to be sampled by BAL. The plasma membrane fluidity of rat alveolar macrophages is significantly reduced by exposure to cigarette smoke beginning as early as 2 weeks (Hannan *et al.*, 1989) and exposure of monolayers of bovine bronchial epithelial cells to cigarette smoke significantly increases adherence of both cocultured neutrophils and mononuclear cells (Robbins *et al.*, 1992).

Previous studies have either not controlled the smoking of the study subjects or have not looked at the acute effects of cigarette smoking. This study has done both and demonstrates that influx of neutrophils into the airspaces occurs within 12 hours of smoking. The effect is not the result of changes in respiratory function measured, BAL return, cell viability or differences in the smoking habit or duration of the 2 groups of smokers. Although I did not measure small airways function it is unlikely that the increased numbers of neutrophils found in the BALF of acute smokers was a sampling artefact resulting from acute small airways constriction as acute cigarette smoking also causes sequestration of neutrophils in the pulmonary vasculature in humans (MacNee *et al.*, 1989).

An imbalance between oxidants and anti-oxidants has been proposed in the pathogenesis of COPD (Taylor *et al.*, 1986) and there is increasing evidence of the importance of an oxidant anti-oxidant imbalance in the lungs in other diseases such as asthma, IPF, ARDS and cystic fibrosis

(Cross *et al*, 1994a). Cigarette smoke is the most important factor in the aetiology of COPD (United States Department of Health and Human Services, 1984) and contains 10^{16} free radicals per puff (Janoff *et al.*, 1987). In cigarette smokers increased numbers of inflammatory cells are found in the interstitium and BALF and macrophages accumulate around the junction between the terminal bronchiole and acinus (Niewoehner *et al.*, 1974). These cells are capable of releasing increased amounts of $O_2^{\cdot -}$ and thus contribute further to the increased oxidant burden found in the airspaces of cigarette smokers (Hoidal *et al.*, 1981). Cigarette smoke condensate has been shown to decrease epithelial cell adherence and increase detachment and lysis in a human type II alveolar epithelial cell line (A549) (Lannan *et al.*, 1994).

In keeping with previous studies I found $O_2^{\cdot -}$ production from inflammatory BAL leucocytes was significantly increased in smokers although I found no difference between chronic and acute smoking in this respect. Acute cigarette smoke exposure of neutrophils obtained from the peritoneal cavity of rats has been shown to damage the respiratory burst, this effect probably being initiated by the generation of $O_2^{\cdot -}$ with $OH\cdot$ generation a subsequent injurious factor (Tsuchiya, Suzuki *et al.*, 1992, Tsuchiya, Thompson *et al.*, 1992).

Increased levels of products of lipid peroxidation have been reported in the plasma and lung tissue of smokers (Petruzzelli *et al.*, 1990, Duthie *et al.*, 1991, Bridges *et al*, 1993, Morrow *et al.*, 1995, Rahman *et al.*, 1996). I was able to confirm this in plasma by the measurement of TBARS. In one study acute smoking of 3 cigarettes over 3 minutes had no effect on the levels of circulating products of lipid peroxidation but 2 weeks abstinence with compliance checked by measurement of urinary nicotine and cotinine resulted in a significant reduction (Morrow *et al.*, 1995). Similarly I found no difference between acute and chronic smokers in their levels of TBARS and no reduction after 12 hours abstinence. Frei and colleagues found that exposure of plasma to the gas phase of cigarette smoke induced lipid peroxidation once endogenous ascorbic acid had been oxidised completely. In addition cigarette smoke exposure caused oxidation of plasma PrSSG and albumin-bound bilirubin (Frei *et al.*, 1991). Cross and colleagues also found whole cigarette smoke induced

lipid peroxidation in plasma *in vitro* to be inhibited by ascorbic acid (Cross *et al.*, 1993). There are no previous reports of the effects of acute cigarette smoking on the levels of products of lipid peroxidation in BALF or ELF. The numbers I studied were too small to reach statistical significance but there appears to be a pattern suggesting that not only are TBARS increased in the BALF of cigarette smokers but that they are further increased by acute cigarette smoking. The same pattern was observed for ELF.

Anti-oxidant capacity is reduced in the plasma in chronic smokers (Taylor *et al.*, 1986). Measured in serum as the percentage reduction of auto-oxidation of ox-brain homogenate it is decreased in cigarette smokers (Galdston *et al.*, 1984). There is a strong relationship between a deficiency in the anti-oxidant capacity of plasma and a family history of lung disease (Taylor *et al.*, 1986). I have demonstrated that not only is the anti-oxidant capacity of plasma significantly reduced in chronic cigarette smokers but that the level following acute cigarette smoking is approximately two thirds of that in the chronic smoking group. Conversely in BALF the anti-oxidant capacity is significantly elevated in both the smoking groups with no difference between them. This may be due to the elevated airspace epithelial permeability found in cigarette smokers allowing influx of anti-oxidant molecules into the airspaces from the vascular space and the interstitium of the lung. One however might have expected a further increase following acute smoking if this were the case, in association with the further increase seen in epithelial permeability after acute cigarette smoking. In addition there was no correlation between ^{99m}Tc-DTPA lung clearance and BALF or ELF anti-oxidant capacity. On the other hand the increase in anti-oxidant capacity in the airspaces of chronic smokers may already have been maximal. Again the pattern was repeated in ELF but the changes did not reach statistical significance.

Cigarette smoke condensate increases epithelial permeability *in vitro* in epithelial cell monolayers and *in vivo* in rat lungs in association with changes in the homeostasis of the anti-oxidant GSH (Li *et al.*, 1994). A dose and time dependent reduction in intracellular GSH and formation of GSH conjugates occurs without elevation of GSSG (Rahman *et al*, 1995). Cigarette smokers have increased levels of GSH in ELF (800μm)

compared to non-smokers (400μm) (Cantin *et al.*, 1987). I have confirmed these levels not only in BALF and ELF but have also demonstrated the same pattern in mixed BAL leucocytes. These increases were abolished by acute cigarette smoking reinforcing the hypothesis that the repeated acute insult from cigarette smoking is important in disturbing the oxidant anti-oxidant balance in the lungs.

Other anti-oxidants are altered in smokers. In serum, ascorbic acid, vitamin E, β-carotene and selenium are decreased (Pelletier, 1970, Chow *et al.*, 1986, Bridges *et al.*, 1990, Duthie *et al.*, 1991, Van Antwerpen *et al.*, 1993). In leucocytes, vitamin E and ascorbic acid are decreased (Barton and Roath, 1976, Hemila *et al.*, 1984, Theron *et al.*, 1990). In erythrocytes GSH, SOD and catalase are enhanced (Toth *et al.*, 1986). In BALF, catalase activity (Greening *et al.*, 1985) and ascorbic acid are increased (Bui *et al.*, 1992) and vitamin E reduced (Pacht *et al.*, 1986). In alveolar macrophages ascorbic acid is increased (McGowan *et al.*, 1984). Increased activities of SOD and catalase have been demonstrated in the alveolar macrophages of young smokers (McCusker and Hoidal, 1990). However in elderly smokers SOD, glutathione-S-transferase (GST) and GP_X activities are reduced (Kondo *et al.*, 1994).

Thus I have demonstrated that the epithelial permeability found in cigarette smokers is an acute effect. Influx of neutrophils into the airspaces occurred following acute cigarette smoking. Alterations in the oxidant anti-oxidant balance in the plasma and in the airspaces were found, resulting in increased oxidant stress, particularly following acute smoking. No relationship was found between airspace epithelial permeability and any of the variables studied but further investigation, including non-smokers in both the ^{99m}Tc-DTPA lung clearance and the bronchoscopy and BAL studies, may yield further important results.

CHAPTER 5. EPITHELIAL PERMEABILITY, INFLAMMATION, OXIDANT STATUS AND THE CYTOSKELETON IN AIRSPACE EPITHELIUM FOLLOWING EXPOSURE TO OZONE *IN VIVO AND IN VITRO.*

5.1. INTRODUCTION.

Ozone is one of the constituents of photochemical pollution. Such pollution results in part from the increasing number of motor vehicles in use. NO_X react with VOC's and sunlight to produce ozone and a range of other oxidants. Concerns have been raised over this man-made ozone in the lower atmosphere and its possible adverse effects on health, in particular its possible role in the increased incidence of asthma and the association of higher levels with increased admissions due to asthma. Few measurements were made prior to 1970 but it would seem likely that prior to this levels were lower (Department of Health, 1991). Since then the evidence does not suggest any general upward trend (Lung and Asthma Information Agency, 1993/5).

Like cigarette smoke, ozone has been shown to produce increased respiratory epithelial permeability *in vivo* in humans (Kehrl *et al.*, 1987, Koren *et al.*, 1989, Devlin *et al.*, 1991), in animals (Bhalla and Crocker, 1986, Stutts and Bromberg, 1987, Bhalla *et al.*, 1990, Bhalla and Young, 1992, Kleeberger and Hudak, 1992, Nishikawa *et al.*, 1992) and *in vitro* (Cheek *et al.*, 1994, Yu *et al.*, 1994). Ozone also produces influx of neutrophils into the airspaces and evidence of inflammation in normal non-smokers (Koren *et al.*, 1989, Devlin *et al.*, 1991, Koren *et al.*, 1991, Schelegle *et al.*, 1991) and in asthmatics (Basha *et al.*, 1994). Neutrophil influx has also been demonstrated in animal studies (Bhalla and Young, 1992, Hyde *et al.*, 1992, Kleeberger and Hudak, 1992, Young and Bhalla, 1992).

Ozone has a standard redox potential of +2.07V and is therefore the second most powerful oxidant known behind fluorine. It is believed to cause damage to biological tissues either by direct reaction or through the formation of free radicals and reactive intermediates or by both mechanisms. Peroxidation of membrane lipids is thought to be a most important mechanism of ozone injury with oxidation of functional

groups and loss of activity of biomolecules including enzymes. As mentioned above alteration of membrane permeability occurs and there is induction of inflammation and initiation of secondary processes.

The GSH redox cycle is thought to play a crucial role in anti-oxidant defence against lipid peroxides and other damaging related oxygenated intermediates and can promote the repair of injured cells and tissues enabling the lung to withstand further injury (Mustafa, 1990). Intracellular GSH is depleted by ozone exposure *in vivo* in animal studies (Mountain, 1963, De Lucia *et al.*, 1975, Mustafa, 1990) and *in vitro* (Freeman and Mudd, 1981, Van der Zee *et al.*, 1987). Depletion of other anti-oxidants such as uric acid and ascorbic acid in BALF from humans, exposed *in vitro* to ozone, has also been demonstrated (Mudway *et al.*, 1994).

As with cigarette smoke the lungs may be rendered more susceptible to inhaled agents such as allergens, viruses and pollutants. This may in part explain the observation of increased susceptibility to allergen produced by ozone in asthmatic subjects (Molfino *et al.*, 1991). Regulation of the oxidant anti-oxidant balance, and in particular GSH may play an important role in maintaining the integrity of the respiratory epithelium. Bhalla and colleagues have suggested that this may involve the cytoskeleton (Bhalla *et al.*, 1990).

The aim of this study was to investigate epithelial permeability, inflammation and oxidant anti-oxidant balance in the airspaces of healthy non-smokers exposed to ozone and the effect of ozone on epithelial permeability, GSH levels and the cytoskeleton in A549 cell monolayers in culture. Ozone is a pure oxidant unlike cigarette smoke which contains 10^{16} free radicals in each puff, one of which is ozone itself (Janoff *et al.*, 1987). I therefore chose ozone as a model for the oxidant effects of cigarette smoke on the respiratory epithelium *in vivo* and *in vitro*,, in particular the acute effects in healthy non-smokers uncomplicated by the effects of chronic cigarette smoking.

5.2. METHODS.

5.2.1. Subjects.

Fifteen healthy non-smokers (M:F 12:3) between 21 and 50 years of age were recruited. Ten subjects were studied on 2 occasions. No subjects had any significant medical problems nor any history of respiratory infection within 6 weeks of the study.

5.2.2. Study design.

Five studies involved exposure to filtered air (FA). Of the remaining 20, 10 were to ozone 100ppb and 10 to 400ppb for 1 hour during a standard exercise protocol (alternate 15 minutes exercise and rest) on a bicycle ergometer (Figure 2.9) at an inspiratory minute ventilation (V_I) of 40 $l min^{-1}$ as assessed in a previous study breathing FA. COHb was measured prior to recruitment and on each visit prior to study to confirm the history of non-smoking. Spirometry was performed before and either immediately, 1 or 6 hours after exposure. Cardiac monitoring and oxygen saturation measurements were performed throughout each exposure. The clearance rate of ^{99m}Tc-DTPA from the lungs was measured to assess airspace epithelial permeability. Bronchoscopy and BAL were performed in each subject at least 2 weeks apart from the ^{99m}Tc-DTPA study. Ten studies were performed 1 hour after ozone exposure and 10, 6 hours after (5 at 100ppb and 5 at 400ppb).

5.2.3. Stability of ^{99m}Tc-DTPA.

Thin layer chromatography of the residual ^{99m}Tc-DTPA in the nebuliser and urine was performed in 2 subjects, 1 hour after FA and 1 hour after 400ppb ozone. There was 0% (nebuliser) and 0.4% (urine) dissociation into DTPA and free pertechnetate in the former and 0% and 1.1% dissociation respectively in the latter (Zimmer and Pavel, 1977).

5.2.4. *In vitro* studies.

Exposure of A549 cells to ozone 1000ppb for 1 hour *in vitro* and measurement of epithelial permeability, GSH, PrSSG and cytoskeletal assessment were performed as described in Chapter 2.7.2. and 2.8.

5.2.5. Ethics.

Ethical permission was obtained from the local medical ethics committee and all patients gave informed written consent.

5.2.6. Statistics.

5.2.6.i. *In vivo.*

The effects of 0, 100 and 400ppb ozone on spirometry prior to ^{99m}Tc-DTPA lung clearance scanning and prior to bronchoscopy and BAL and the effects on ^{99m}Tc-DTPA lung clearance, BAL characteristics, BALF and plasma albumin, ELF volume, $O_2^{\cdot -}$ release, BALF and ELF lipid peroxidation and TEAC, and GSH and GSSG in cells, BALF and ELF were compared by one way ANOVA with post hoc Scheffe test. These were performed separately for the 1 hour and 6 hour studies. In the analysis of the 6 hour studies the control data used as reference were the FA values from the 1 hour study. Data from the 1 and 6 hour studies were compared by independent t-test. ^{99m}Tc-DTPA lung clearance for the right and left lungs were compared by paired t-test. Pre and post values for lipid peroxidation and TEAC at 0, 1 and 6 hours post exposure were compared by paired t-test. GSH and GSSG were analysed separately. Correlation was performed by a 2 variable parametric analysis.

5.2.6.ii. *In vitro.*

Studies of monolayer permeability, thiols and the cytoskeleton were analysed by two way ANOVA with independent variables of air or ozone and experiment.

5.2.6.iii. Acknowledgement.

Statistical advice was given by Dr. TJ Peters, Senior Lecturer in Public Health Medicine, Department of Social Medicine, University of Bristol, Bristol. All calculations were performed on Statistica/Mac^{TM}, Release 1.7, Copyright StatSoft, Inc. 1991-1992.

5.3. RESULTS.

5.3.1. *In vivo.*

5.3.1.i. Subject characteristics and spirometry (^{99m}Tc-DTPA study).

There were no significant differences between baseline spirometry or age prior to ^{99m}Tc-DTPA lung clearance scanning or in RR during the inhalation of the ^{99m}Tc-DTPA between the 5 groups of subjects (Table 5.1). No significant effects of ozone on spirometry were seen immediately, 1 or 6 hours after exposure. The maximum fall in the mean FEV_1 of 0.4l was produced by 400ppb 1 hour after exposure (Figure 5.1). Subjects varied greatly in their sensitivity to ozone the maximum fall of 40% occurring in one subject unassociated with symptoms. Two subjects experienced a feeling of inspiratory limitation. Ozone sensitive and ozone resistant subjects have not been analysed separately because of the small numbers in each group. FVC followed the same pattern with similar magnitude as FEV_1. There was a trend for the RR to be higher in the ozone exposed groups although it did not reach statistical significance. This is as one would expect as it has been well demonstrated that ozone decreases the TV and thus increases the RR due to a limitation of inspiration perhaps due to discomfort. This may be due to stimulation of non-myelinated C-fibres possibly by PG's released during airway inflammation (Hazucha *et al.*, 1989).

5.3.1.ii. Spirometry (BAL study).

There was no significant difference in baseline FEV_1 prior to ozone exposure in the BAL studies and during the course of these studies FEV_1 followed the same pattern as above with a maximum fall of 0.4l with 400ppb but occurring immediately after exposure (Table 5.2). No measurements were made 1 hour after exposure as patients were being given premedication for the bronchoscopy at this point. As before none of the changes reached statistical significance.

5.3.1.iii. ^{99m}Tc-DTPA lung clearance.

^{99m}Tc-DTPA lung clearance did not change significantly after exposure to ozone at 100 or 400ppb either 1 or 6 hours after exposure regardless of whether the right and left lungs were analysed separately (Table 5.3) or together (Figure 5.2). The values are in keeping with those obtained for the healthy non-smokers in Chapter 3 (84.6±6.2 minutes). There was a

Table 5.1. Spirometry (mean±SE, n=5 except where indicated), respiratory rate (RR) and age (+range) in healthy non-smoking subjects before (Pre) and after (Post 0, 1 and 6 hours) exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. ^{99m}Tc-DTPA lung clearance scanning was then performed either 1 or 6 hours after ozone exposure.

Ozone	Spirometry	Age	Time of scan 1 hr post			Age	Time of scan 6 hrs post		
			Pre	Post 0 hrs	Post 1 hr		Pre	Post 0 hrs	Post 6 hrs
0ppb	FEV_1 (l)	35.4±4.7 24-50	4.0±0.3	4.0±0.3	3.9±0.3				
	%predicted		112.2±6.3	112.0±7.3	108.8±4.9				
	FVC (l)		4.9±0.4	5.0±0.4	4.8±0.4				
	Ratio (%)		82±2.5	81.2±2.3	82.4±2.7				
	RR (per min)		11.3±0.6						
100ppb	FEV_1	35.0±4.3 21-44	4.4±0.3	4.2±0.4, n=4	4.3±0.3	34.6±4.1 21-44	4.2±0.4	4.2±0.4	4.1±0.4
	%predicted		109.6±2.8	110.8±3.5	108.4±2.8		113.4±2.6	116.6±2.0	110.2±1.4
	FVC		5.2±0.5	4.9±0.5	5.0±0.5		4.9±0.5	4.9±0.6	4.7±0.5
	Ratio		85.8±4.6	87.5±5.9	87.4±4.2		85.8±2.5	87.2±3.1	87±2.0
	RR		14.6±1.9				15.0±1.8		
400ppb	FEV_1	33.2±2.2 28-41	4.4±0.2	4.1±0.4, n=4	4.0±0.3	37.2±3.7 30-50	4.1±0.2	3.9±0.2	3.8±0.2
	%predicted		104.6±3.3	99±9.3	94±8.2		107±6.5	103.4±8.5	100.0±7.5
	FVC		5.4±0.3	5.2±0.5	4.8±0.4		5.1±0.3	4.9±0.4	4.7±0.3
	Ratio		82.8±1.8	79.8±1.3	82±2.7		80.6±2.3	80.6±3.2	81.2±2.7
	RR		14.9±1.9				14.7±1.7		

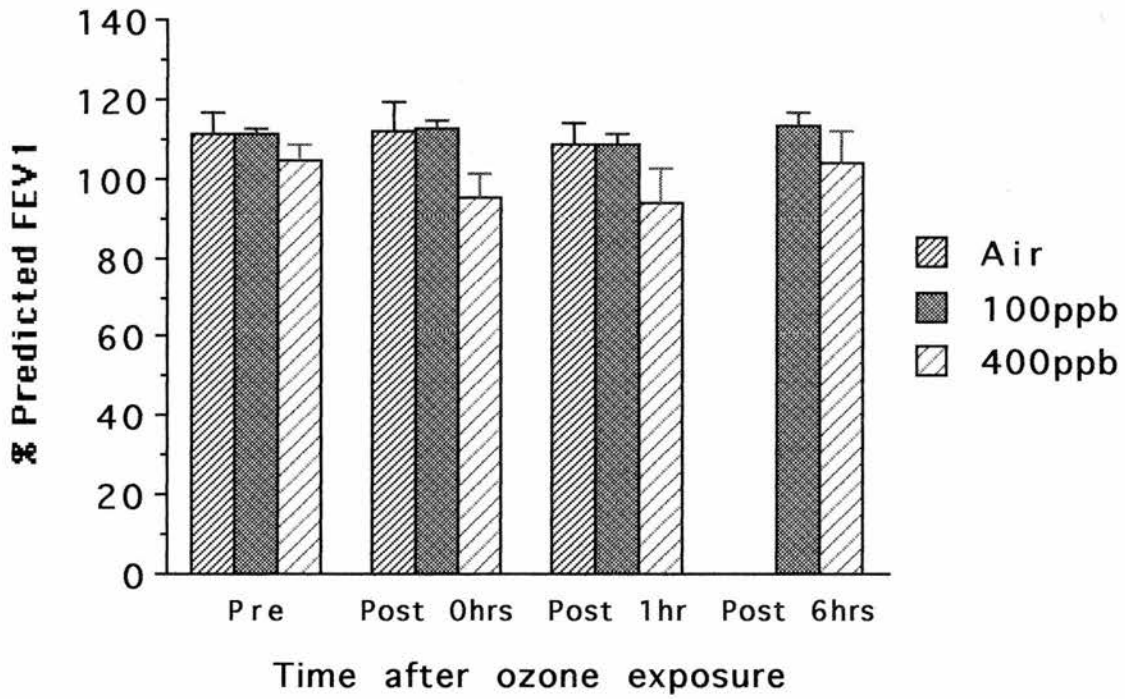


Figure 5.1. Spirometry (mean±SE, n=5) in healthy non-smoking subjects before (Pre) and after (Post 0, 1 and 6 hours) exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40 $lmin^{-1}$. The values are taken from both the ^{99m}Tc-DTPA and bronchoalveolar lavage studies.

Ozone	Spirometry	Time of BAL 1 hr post		Time of BAL 6 hrs post		
		Pre	Post 0 hrs	Pre	Post 0 hrs	Post 6 hrs
0ppb	FEV_1 (l)	4.0±0.3	4.1±0.3			
	%predicted	112.2±6.3	113.0±6.9			
100ppb	FEV_1	4.4±0.3	4.4±0.3	4.2±0.4	4.2±0.4	4.2±0.5
	%predicted	109.6±2.8	111.3±2.7	113.4±2.6	114.5±2.9	113.3±3.5
400ppb	FEV_1	4.4±0.2	4.0±0.4	4.1±0.2	3.8±0.3	3.9±0.3
	%predicted	104.6±3.3	95.7±8.9	107.0±6.5	100.1±10.5	103.5±8.4

Table 5.2. Spirometry (mean±SE, n=5) in healthy non-smoking subjects before (Pre) and after (Post 0 and 6 hours) exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. Bronchoscopy and bronchoalveolar lavage (BAL) were then performed either 1 or 6 hours after ozone exposure.

Time	Ozone	Right lung			Left lung		
		t50	r	CF	t50	r	CF
1 hour	0ppb	71.5±5.6	0.97±0.01	1.79±0.24	75.7±4.8	0.97±0.01	2.26±0.31
	100ppb	76.2±13.0	0.96±0.01	1.47±0.11	80.5±10.8	0.96±0.01	1.70±0.13
	400ppb	73.8±14.4	0.96±0.01	2.03±0.19	77.2±14.9	0.96±0.01	2.20±0.17
6 hours	0ppb						
	100ppb	68.4±6.8	0.97±0.01	1.66±0.05	75.6±10.0	0.97±0.01	1.80±0.08
	400ppb	61.1±8.6	0.97±0.01	1.53±0.17	61.6±8.6	0.97±0.01	1.93±0.11

Table 5.3. Time to 50% clearance of ^{99m}Tc-DTPA from the lungs (t50), regression coefficients (r) for the clearance curves and correction factors (CF) for recirculating background activity in healthy non-smoking subjects (n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. Values are given for the right and left lungs (mean±SE). There was no difference between the right and left lungs regardless of ozone level or time of ^{99m}Tc-DTPA scan.

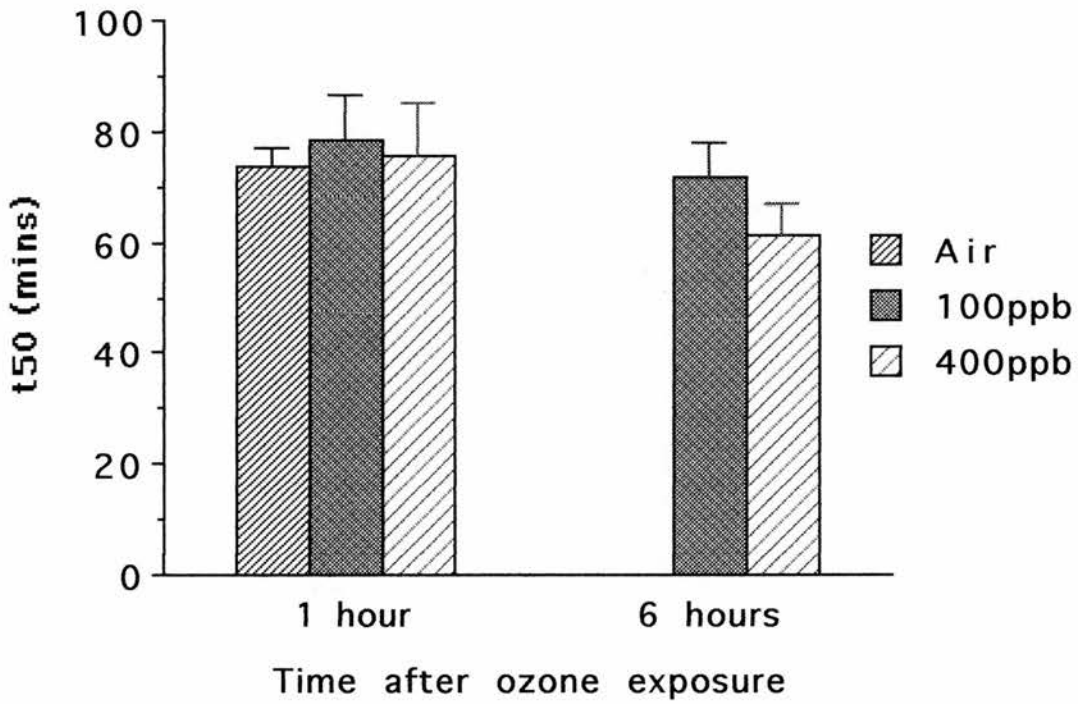


Figure 5.2. ^{99m}Tc-DTPA lung clearance (mean±SE, n=5) in healthy non-smoking subjects after (Post 1 and 6 hours) exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I of 40lmin^{-1}. There was no significant difference between 0, 100 and 400 ppb or between the 1 and 6 hour studies.

trend towards increased airspace epithelial permeability 6 hours after exposure of 400ppb. t50 tended to be higher for the left lung but this was marginal and not statistically significant. All correlation coefficients for the semi logarithmic time activity curves were greater than 0.92 and all curves were observed to be monoexponential.

5.3.1.iv. Bronchoscopy and bronchoalveolar lavage.

There were no significant differences between the groups in BALF recovery, total number of cells obtained or their viability (Table 5.4). The total number of cells obtained was not expected to increase following ozone (Koren *et al.*, 1989, Devlin *et al.*, 1991, Schelegle *et al.*, 1991). Inhalation of ozone 400ppb resulted in an increased percentage (FA, 0.8±0.2%, 400ppb, 2.4±0.7, $p<0.05$) and number (FA, 0.11±0.04 $x10^6$ cells, 400ppb, 0.31±0.09, $p<0.05$) of neutrophils in BAL 6 hours after exposure. There was a trend for lymphocytes to increase following 100 and 400ppb 1 hour after exposure but these changes were not statistically significant.

BALF albumin did not change significantly following ozone (Table 5.5) although 1 hour after inhalation of 400ppb the concentration rose from 29.4±3.6 to 40.7±3.2μgml^{-1} (Figure 5.3). After 6 hours it had fallen to 34.7±4.7μgml^{-1}. There were no changes in plasma albumin (Table 5.5). There was a statistically significant effect of ozone overall ($p<0.05$) on ELF volume (Table 5.5) but otherwise it followed the same pattern as BALF albumin increasing from 1.10±0.11 to 1.64±0.11mls ($p=0.057$) 1 hour after inhalation of 400ppb (Figure 5.4). Six hours after the level had fallen to 1.32±0.18mls.

$O_2^{\cdot -}$ production by mixed BAL leucocytes was reduced in general following inhalation of ozone at 1 or 6 hours after exposure (1 hour post, $p<0.05$, 6 hours post, $p<0.01$). This was statistically significant following 100ppb 1 hour after exposure (FA, 18.2±2.1nmol $2.5x10^5$ $cells^{-1}$ 120 $mins^{-1}$, 100ppb, 10.3±0.9, $p<0.05$) and 400ppb 6 hours after exposure (400ppb, 9.2±1.1, $p<0.01$) (Figure 5.5, Table 5.6). The value for the control group exposed to FA was 4 times that of the non-smoking controls (4.60±1.00) in Chapter 4. The major difference between the 2 groups of control non-smokers was the exercise performed by those in the ozone study.

	Return mls	Total cells x10^6	Viability %	Macrophages % x10^6	Lymphocytes % x10^6	Neutrophils % x10^6	Eosinophils % x10^6
1 hour post							
0ppb	176.0±11.7	13.6±1.5	91.0±2.5	97.0±0.4	2.0±0.3	0.8±0.2	0.2±0.1
				13.2±1.5	0.27±0.05	0.11±0.04	0.02±0.01
100ppb	168.0±14.6	9.9±1.4	90.5±1.5	94.1±1.1	4.7±1.5	0.6±0.2	0.6±0.2
				9.27±1.15	0.53±0.23	0.06±0.03	0.05±0.01
400ppb	192.0±3.7	15.6±3.3	93.4±1.2	92.4±2.9	6.3±2.9	0.8±0.5	0.5±0.2
				14.6±3.4	0.88±0.38	0.11±0.07	0.08±0.03
6 hours post							
100ppb	180±10.5	13.6±2.0	93.4±0.8	95.7±1.0	3.0±0.8	0.6±0.1	0.8±0.4
				13.0±1.9	0.38±0.08	0.08±0.03	0.12±0.06
400ppb	194.0±4	12.8±1.7	95.0±0.7	94.2±1.5	3.0±0.8	2.4±0.7*	0.5±0.4
				12.1±1.7	0.33±0.06	0.31±0.09!	0.06±0.05

* $p<0.05$ versus 1 hr Neutrophils Air
6 hrs Neutrophils 100ppb
! $p<0.05$ versus 6 hrs Neutrophils 100ppb

Table 5.4. Bronchoalveolar lavage characteristics and cell differential (% and absolute numbers x10^6) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

	BALF albumin $\mu g ml^{-1}$	Plasma albumin $mg ml^{-1}$	BALF volume mls	ELF volume mls
1 hour post				*
0ppb	29.4±3.6	46.2±0.9	176.0±11.7	1.10±0.11
100ppb	28.9±6.9	48.6±0.5	168.0±14.6	0.98±0.25
400ppb	40.7±3.2	47.4±0.8	192.0±3.7	1.64±0.11
6 hours post				
100ppb	23.1±5.0	48.2±1.4	180±10.5	0.85±0.17
400ppb	34.7±4.7	50.8±1.5	194.0±4	1.32±0.18

Table 5.5. Albumin levels in bronchoalveolar lavage fluid (BALF) and plasma and volume of BALF and epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I $40 l min^{-1}$. Overall there was a significant effect of ozone on ELF volume, *$p<0.05$, at 1 hour post exposure and a trend towards a difference between the levels of ozone studied (post hoc Scheffe test, 100 v 400ppb $p=0.057$).

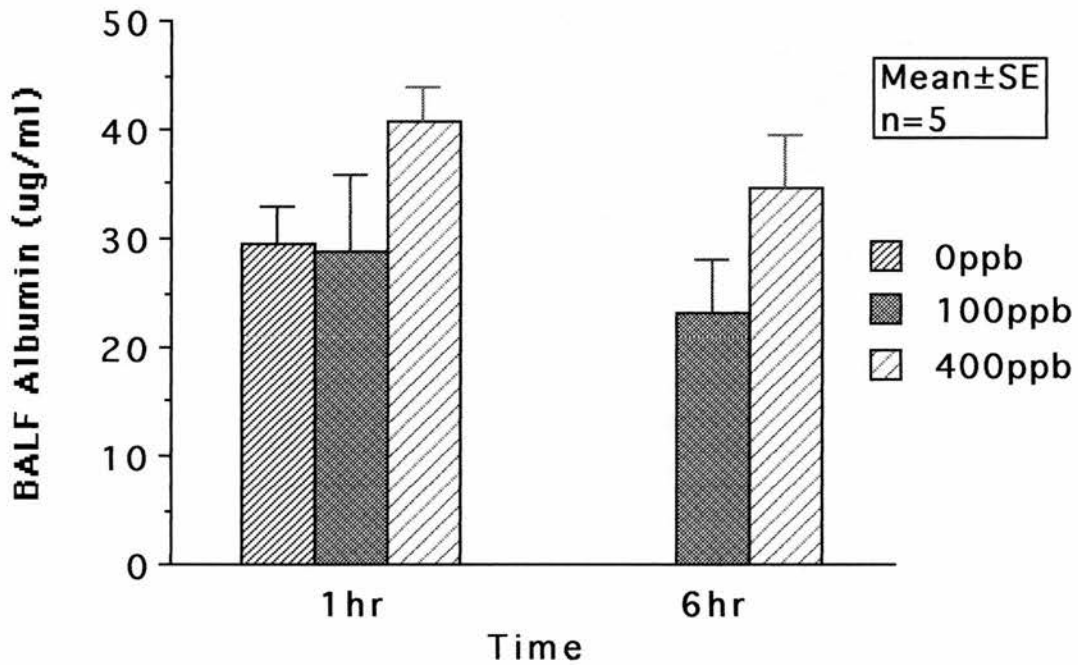


Figure 5.3. Bronchoalveolar lavage fluid (BALF) albumin in healthy non-smoking subjects after (Post 1 and 6 hours) exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

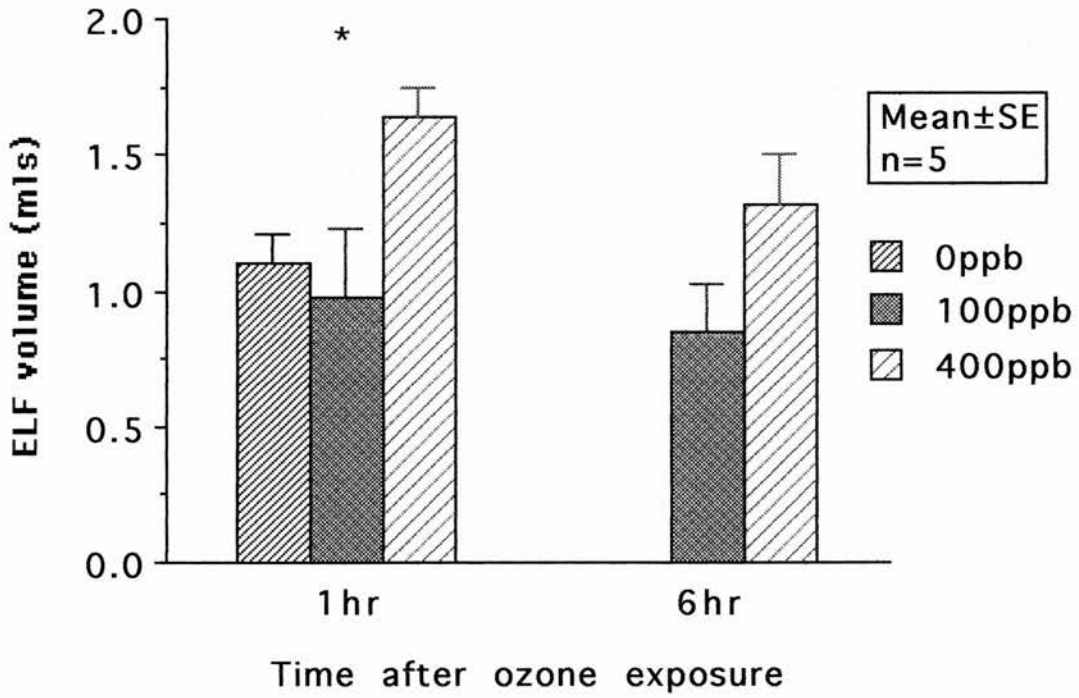


Figure 5.4. Epithelial lining fluid (ELF) volume in healthy non-smoking subjects 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. Overall there was a significant effect of ozone on ELF volume, *$p<0.05$, at 1 hour post exposure and a trend towards a difference between the levels of ozone studied (post hoc Scheffe test, 100 v 400ppb $p=0.057$).

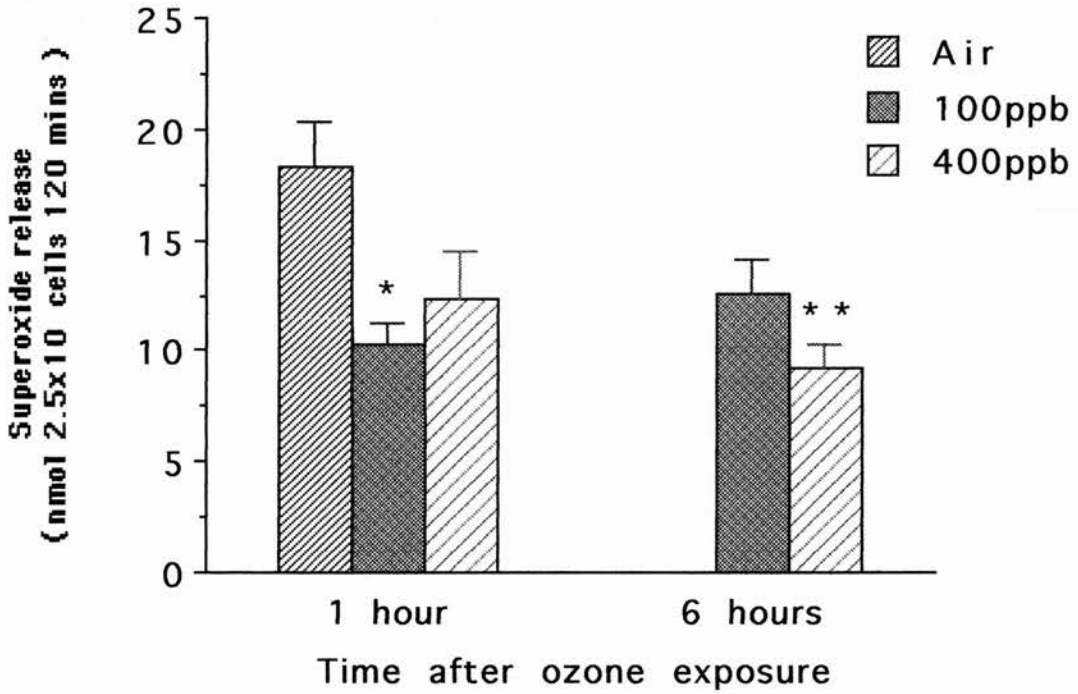


Figure 5.5. Superoxide anion release from mixed bronchoalveolar lavage leucocytes in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400 ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *p<0.05, **p<0.01 versus 0ppb 1hour.

Ozone	1 hour post	6 hours post
0ppb	18.2±2.1	
100ppb	10.3±0.9*	12.6±1.5
400ppb	12.3±2.2	9.2±1.1**

Table 5.6. Superoxide anion release from mixed bronchoalveolar lavage leucocytes in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400 ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *$p<0.05$, **$p<0.01$ versus 0ppb 1hour.

Increased $O_2^{\cdot -}$ production following exercise has been described previously (Boveris and Chance, 1973).

There were no significant differences in plasma products of lipid peroxidation between pre and post exposure levels (Figure 5.6, Table 5.7). Unlike $O_2^{\cdot -}$ production the control levels (1.47±0.06μmoll^{-1}) were very similar to those in the non-smoking control group (1.51±0.21) in Chapter 4. There were no significant differences between the groups for lipid peroxidation in BALF (Figure 5.7, Table 5.8). The control levels (0.30±0.01μmol^{-1}) were considerably higher than those in the non-smokers (0.05±0.05) in Chapter 4. In ELF there was a significant reduction both 1 and 6 hours following 400ppb (1 hour post, 34.4±1.4μmol^{-1}, $p<0.05$, 6 hours post, 44.4±4.2, $p<0.05$) compared to the control group (49.9±5.2) (Figure 5.8, Table 5.8). The control values were 7.5 times higher than the non-smoking values (6.56±6.56) in Chapter 4. The control values are in fact higher than those in the acute smoking group (38.8±17.1).

Plasma anti-oxidant capacity showed no clinically relevant changes following ozone exposure compared to the pre exposure values although the slight increase from 1.20±0.18 to 1.24±0.17mmol l^{-1} 1 hour following 400ppb was statistically significant (Figure 5.9, Table 5.9). There was no general trend to support the validity of this change. The control values pre exposure (0.98±0.13mmol l^{-1}) are somewhat less than those in the control group (1.31±0.10) in Chapter 4. Anti-oxidant capacity was not altered significantly in BALF (Figure 5.10, Table 5.10) or ELF (Figure 5.11, Table 5.10) by inhalation of ozone at 100 or 400ppb either 1 or 6 hours after exposure. Control levels (BALF, 7.9±1.1, ELF, 1356±288μmol l^{-1}) were similar to those in Chapter 4 (BALF, 8.2±1.6, ELF, 1160±230).

GSH in mixed BAL leucocytes (Figure 5.12, Table 5.11), BALF (Figure 5.13, Table 5.11) or ELF (Figure 5.14, Table 5.11) did not change following inhalation of ozone at 100 or 400ppb either 1 or 6 hours after exposure although there was a trend in cells, BALF and ELF for GSH to decrease 6 hours after 400ppb ozone The control levels (Cells, 1.54±0.57nmol 10^6 cells^{-1}, BALF, 1.04±0.11μM, ELF, 177.0±36.1μM) were lower than those in Chapter 4 (Cells, 4.65±1.68, BALF, 2.26±0.73, ELF, 353.7±125.3). There were no significant changes in GSSG in cells, BALF or ELF following ozone.

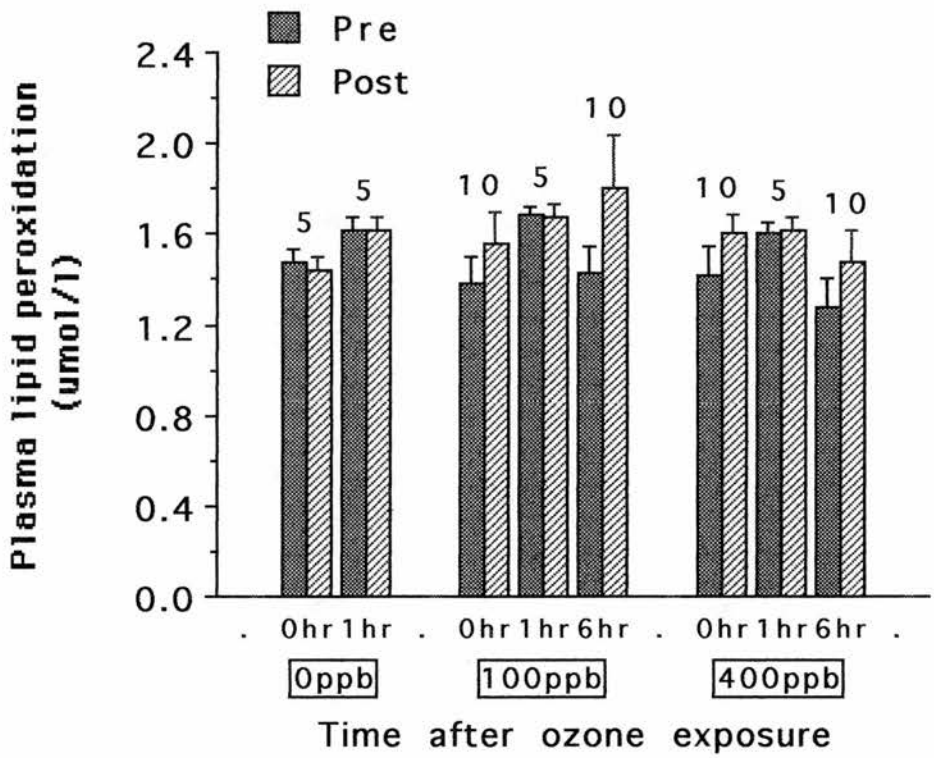


Figure 5.6. Products of lipid peroxidation in plasma in healthy non-smoking subjects (mean±SE, n=5, n=10) before and 0, 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

Plasma lipid peroxidation μmol l^{-1}	Time after ozone exposure					
	0 hours		1 hour		6 hours	
	Pre	Post	Pre	Post	Pre	Post
0ppb	1.47±0.06	1.44±0.06	1.61±0.06	1.62±0.06		
100ppb	1.38±0.12^	1.56±0.14^	1.69±0.03	1.68±0.05	1.43±0.12^	1.80±0.24^
400ppb	1.42±0.13^	1.60±0.08^	1.60±0.05	1.62±0.05	1.28±0.12^	1.47±0.14^

Table 5.7. Products of lipid peroxidation in plasma in healthy non-smoking subjects (mean±SE, n=5, n=10 where indicated^) before and 0, 1 and 6 hours after exposure to ozone 0, 100 or 400 ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

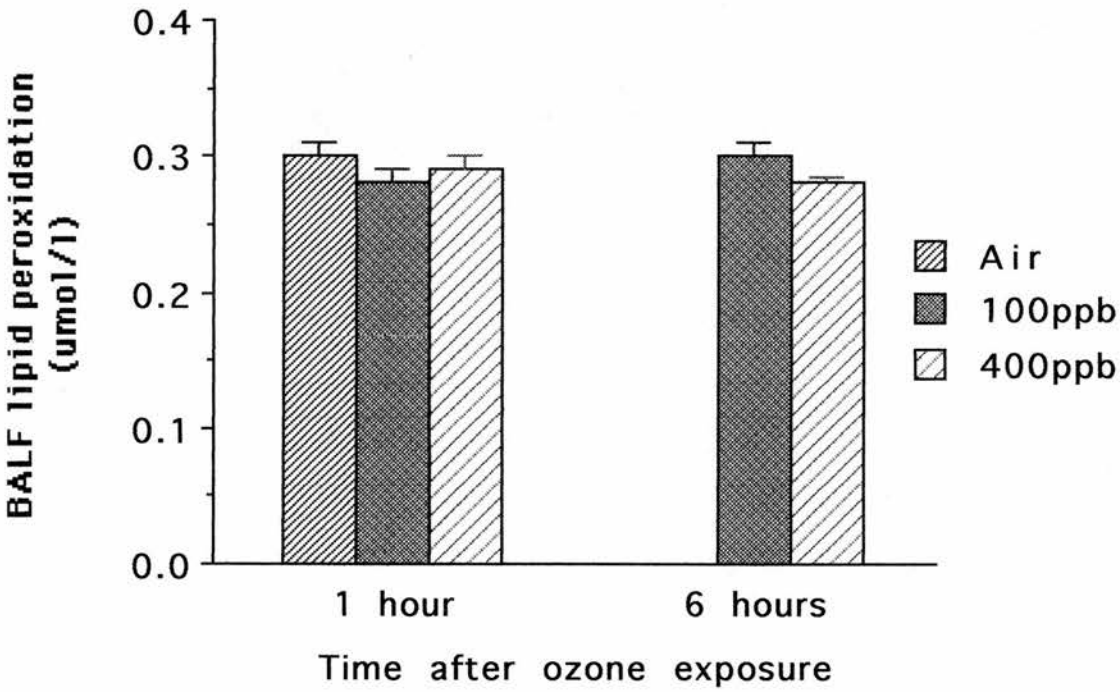


Figure 5.7. Products of lipid peroxidation in bronchoalveolar lavage fluid (BALF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

Ozone		Time after ozone exposure	
		1 hour	6 hours
0ppb	BALF μmol l^{-1}	0.30±0.01	
	ELF	49.9±5.2	
100ppb	BALF	0.28±0.01	0.30±0.01
	ELF	57.8±7.8	75.3±12.3
400ppb	BALF	0.29±0.01	0.28±0.005
	ELF	34.4±1.4*	44.4±4.2*

Table 5.8. Products of lipid peroxidation in bronchoalveolar lavage fluid (BALF) and epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *$p<0.05$ v 100ppb.

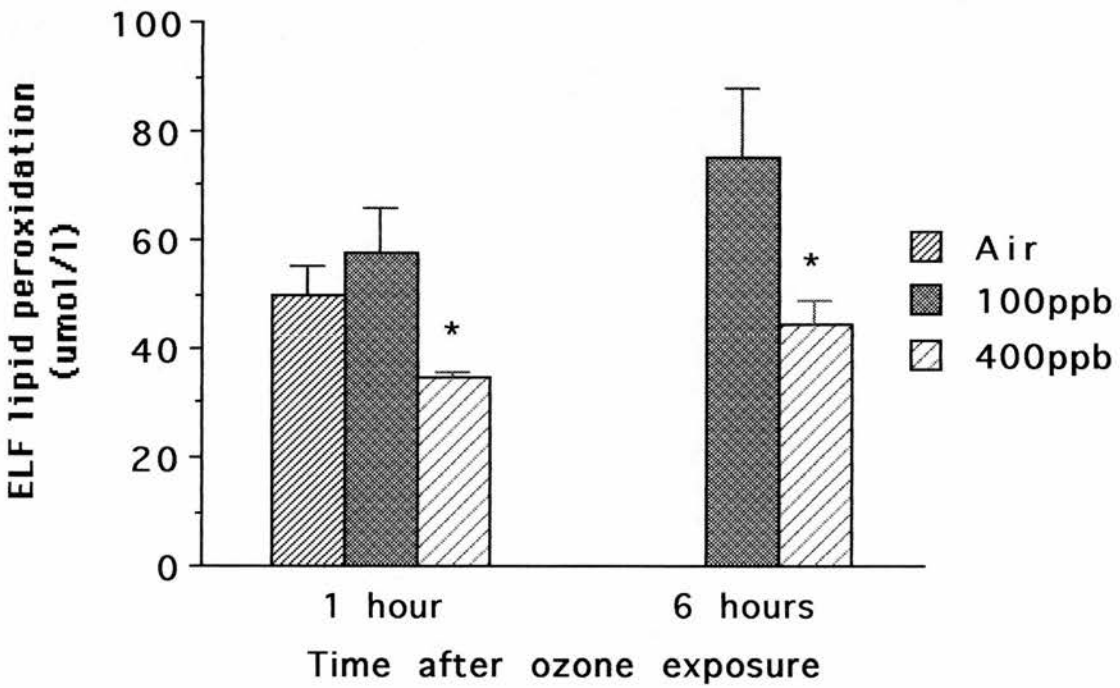


Figure 5.8. Products of lipid peroxidation in epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *p<0.05 v 100ppb.

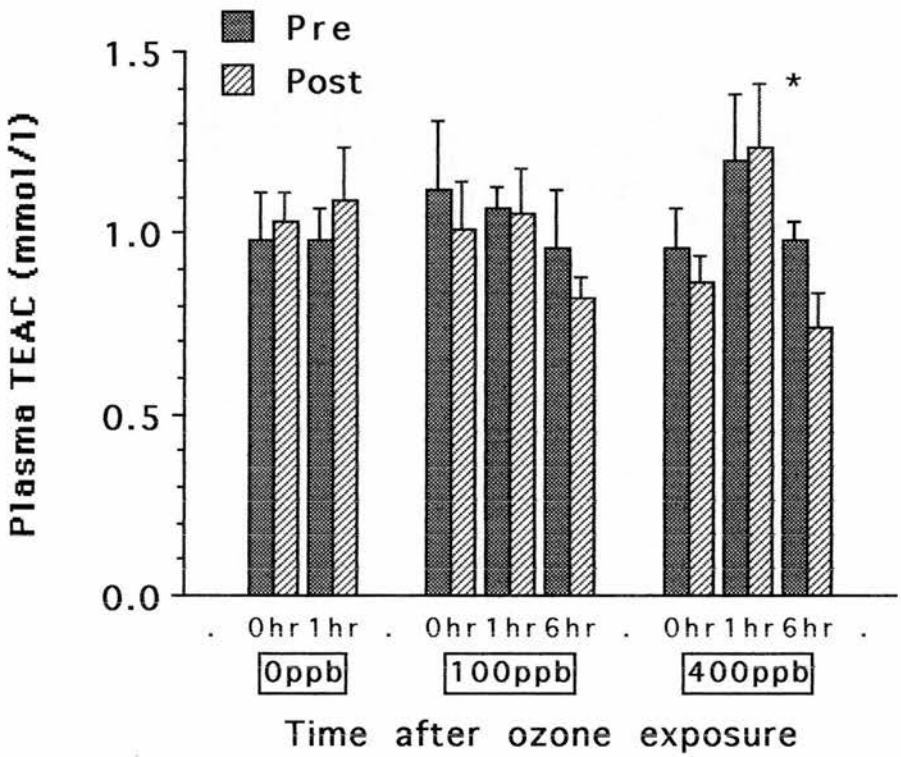


Figure 5.9. Trolox equivalent anti-oxidant capacity (TEAC) in plasma in healthy non-smoking subjects (mean±SE, n=5) before and 0, 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *$p<0.05$ pre v post, 400ppb 1 hour.

Plasma TEAC mmol l^{-1}	Time after ozone exposure					
	0 hours		1 hour		6 hours	
	Pre	Post	Pre	Post	Pre	Post
0ppb	0.98±0.13	1.03±0.08	0.98±0.09	1.09±0.15		
100ppb	1.12±0.19	1.01±0.13	1.07±0.06	1.05±0.13	0.80±0.08	1.03±0.20
400ppb	0.96±0.11	0.86±0.07	1.20±0.18	1.24±0.17*	0.79±0.04	0.88±0.04

Table 5.9. Trolox equivalent anti-oxidant capacity (TEAC) in plasma in healthy non-smoking subjects (mean±SE, n=5) before and 0, 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. *$p<0.05$ pre v post, 400ppb 1 hour.

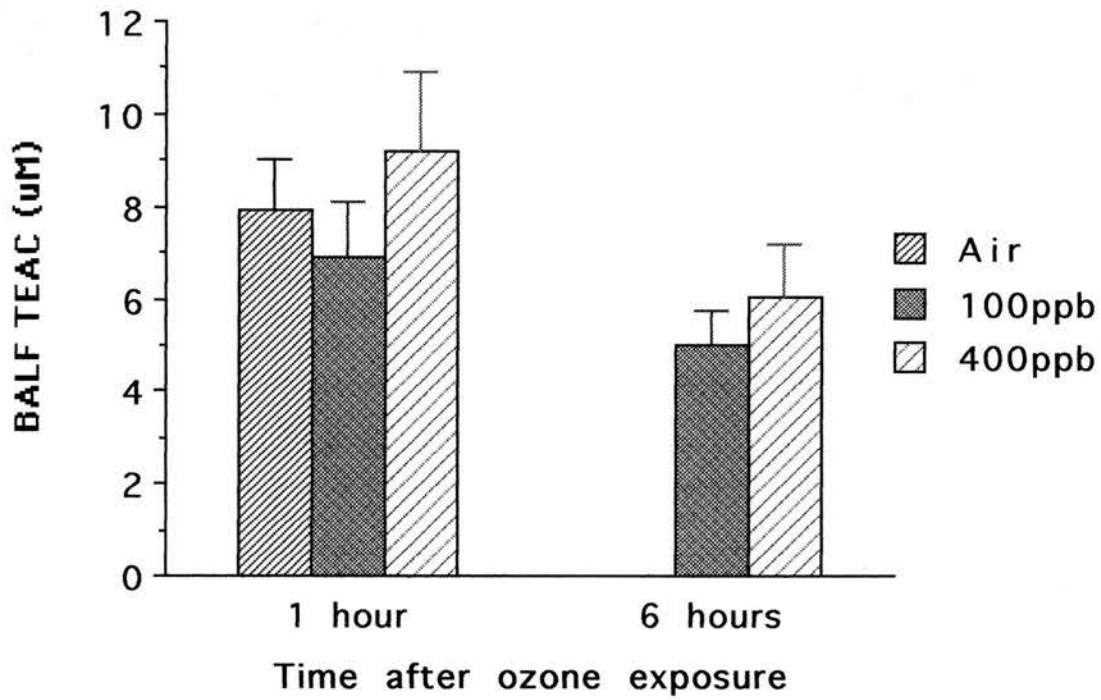


Figure 5.10. Trolox equivalent anti-oxidant capacity (TEAC) in bronchoalveolar lavage fluid (BALF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

Ozone		Time after ozone exposure	
		1 hour	6 hours
0ppb	BALF μmol l^{-1}	7.90±1.10	
	ELF	1356±288	
100ppb	BALF	6.90±1.17	5.00±0.75
	ELF	1546±470	1349±458
400ppb	BALF	9.20±1.70	6.05±1.10
	ELF	1049±161	931±165

Table 5.10. Trolox equivalent anti-oxidant capacity in bronchoalveolar lavage fluid (BALF) and epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

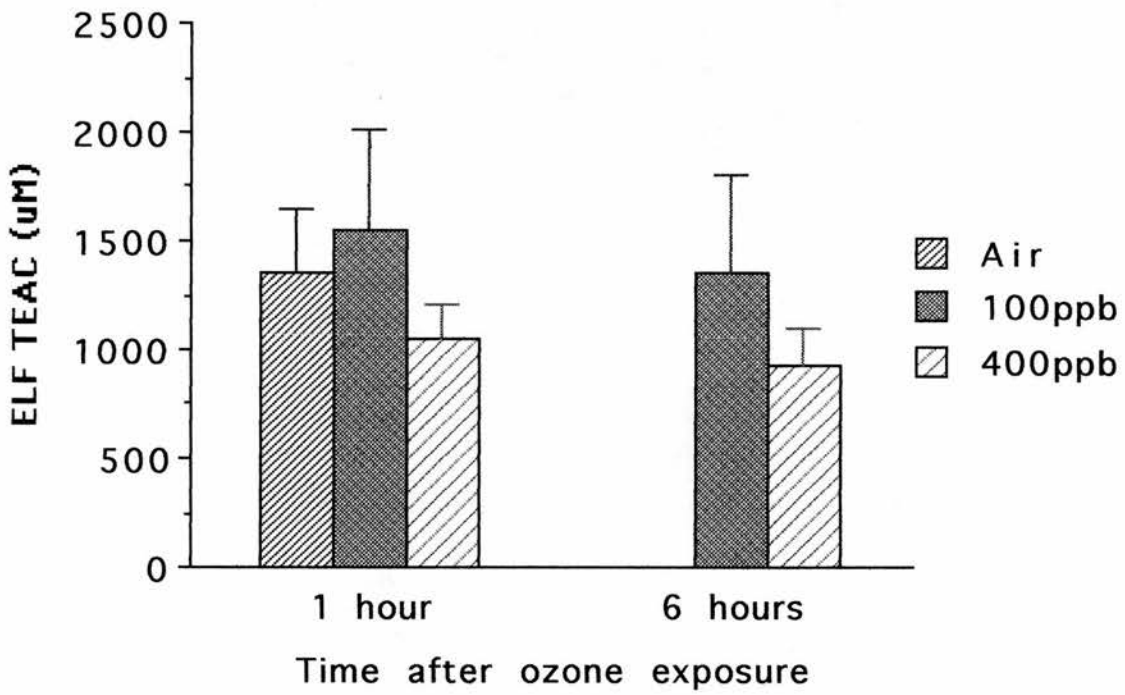


Figure 5.11. Trolox equivalent anti-oxidant capacity (TEAC) in epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

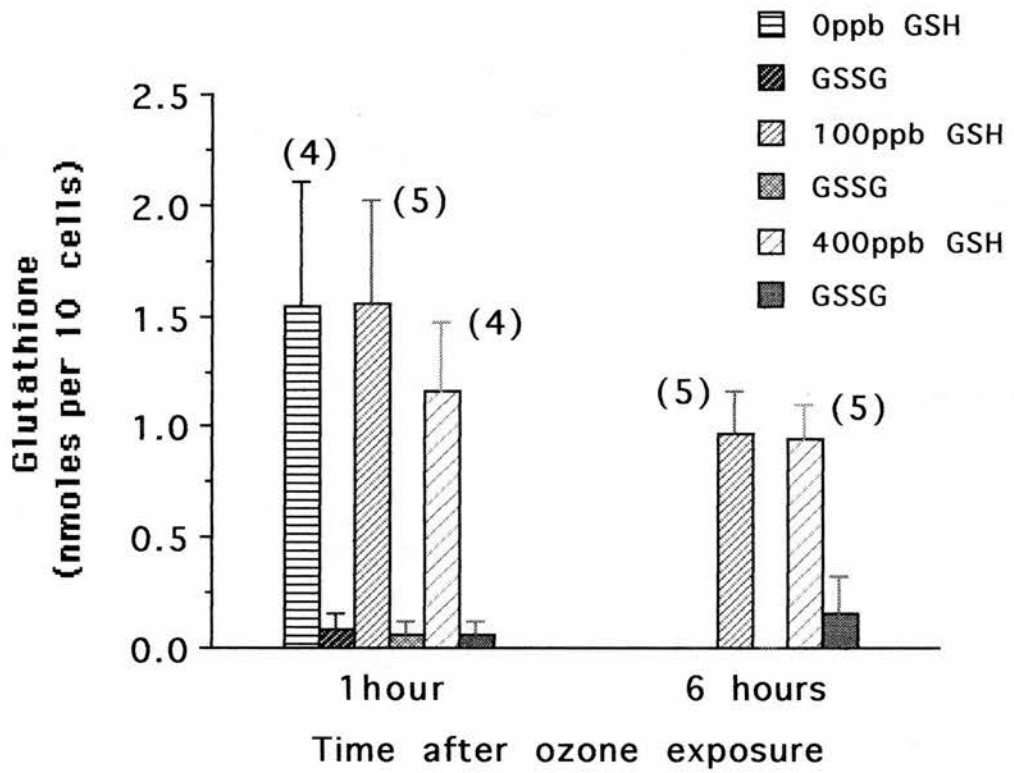


Figure 5.12. Glutathione (reduced GSH, oxidised GSSG) in mixed bronchoalveolar lavage leucocytes in healthy non-smoking subjects (mean±SE, n=5, n=4 where indicated) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

		Time after ozone exposure			
		1 hour		6 hours	
		GSH	GSSG	GSH	GSSG
Cells	0ppb	1.54±0.57^	0.08±0.08^		
(nmol 10^6 cells^{-1})	100ppb	1.56±0.46	0.06±0.06	0.97±0.19	0±0
	400ppb	1.16±0.31^	0.06±0.06^	0.94±0.16	0.16±0.16
BALF (μM)	0ppb	1.04±0.11	0.15±0.08		
	100ppb	1.22±0.21	0±0	1.04±0.14	0.06±0.04
	400ppb	1.33±0.32	0.10±0.08	0.81±0.13	0±0
ELF (μM)	0ppb	177.0±36.1	30.2±17.6		
	100ppb	258.8±94.2	0±0	208.8±50.0	18.7±13.4
	400ppb	156.8±34.6	10.5±8.2	96.7±15.5	0±0

Table 5.11. Glutathione (reduced GSH, oxidized GSSG) in mixed bronchoalveolar lavage leucocytes, bronchoalveolar lavage fluid (BALF), and epithelial lining fluid (ELF) in healthy non-smoking subjects (mean±SE, n=5, n=4 where indicated^) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

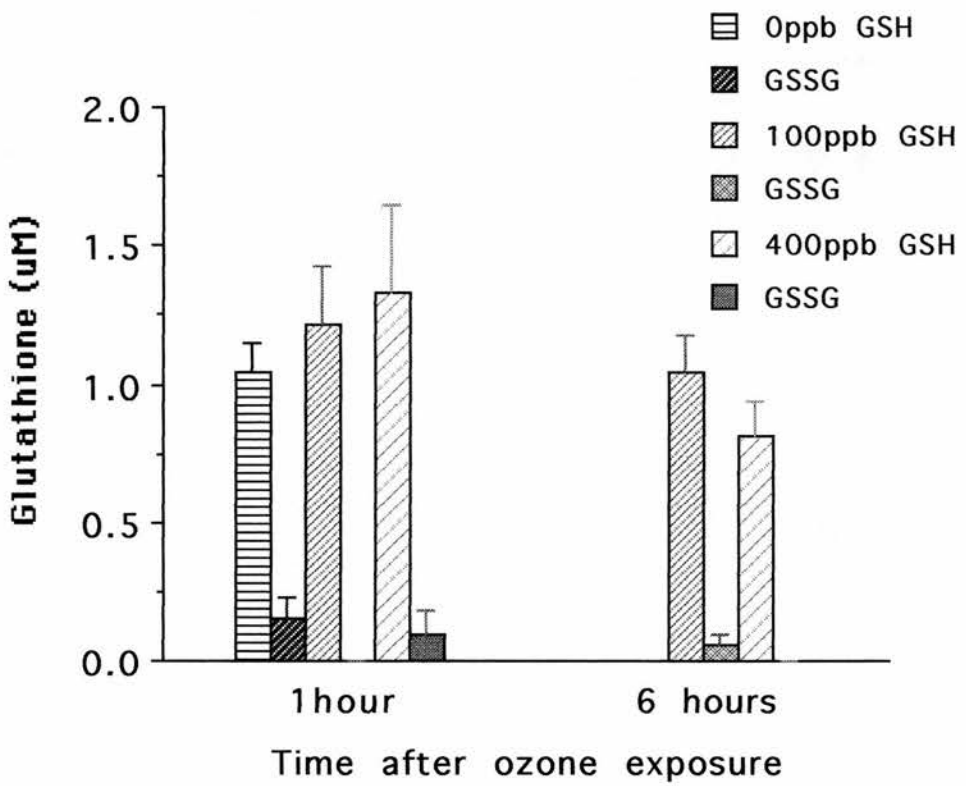


Figure 5.13. Glutathione (reduced GSH, oxidised GSSG) in bronchoalveolar lavage fluid in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

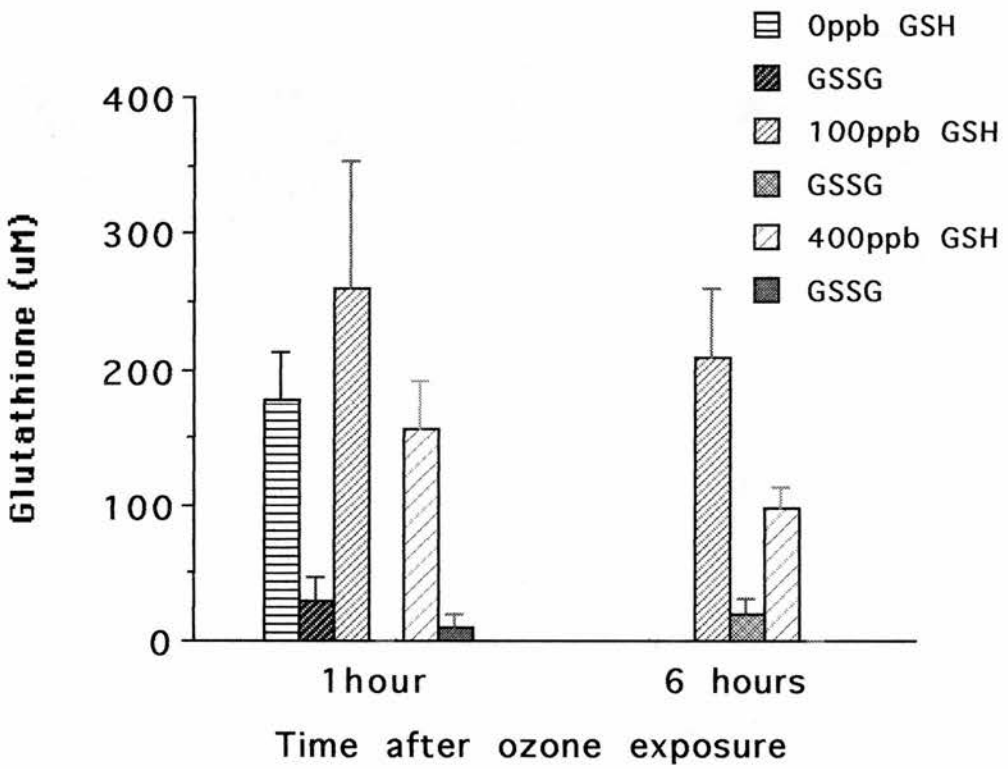


Figure 5.14. Glutathione (reduced GSH, oxidised GSSG) in epithelial lining fluid in healthy non-smoking subjects (mean±SE, n=5) 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}.

The levels were close to the limit of detection of the assay.

There were no correlations between ^{99m}Tc-DTPA clearance and any of the variables measured in particular the percentage or number of neutrophils, BALF albumin, ELF volume, $O_2^{\cdot -}$ production or products of lipid peroxidation in ELF (Table 5.12).

5.3.2. *In vitro.*

Epithelial permeability, intracellular and extracellular thiols and cytoskeletal study results in A549 cells *in vitro* are given in Table 5.13. Exposure of monolayers of A549 cells to ozone 1000ppb for 1 hour produced increased epithelial monolayer permeability regardless of whether ^{125}I bound to BSA and free (FA, 0.83±0.10%, Ozone, 1.07±0.11, $p<0.05$) or only bound ^{125}I (FA, 0.14±0.02, Ozone, 0.22±0.04, $p<0.05$) was measured (Figure 5.15). This was due to focal cell loss as demonstrated in Figure 5.16. These changes were abolished by the presence of medium covering the monolayer during exposure. The level of GSH measured in the cell medium increased significantly (FA, 0.12±0.03, Ozone, 0.28±0.04, $p<0.001$) indicating export of intracellular GSH (Figure 5.17). Although measured as total glutathione it is likely that the form exported was GSSG (Deneke and Fanburg, 1989), this form being toxic to cells (Deneke and Fanburg, 1989). There was no change in the intracellular concentration of GSSG, although as above the measurement was at the limit of detection of the assay, but there was a marked and statistically significant increase in the intracellular concentration of PrSSG (FA, 0.28±0.02nmoles 10^6 cells^{-1}, Ozone, 0.66±0.06, $p<0.001$) (Figure 5.17). There was no difference in cell viability between the cells exposed to FA (78.3±3.8%) and ozone (79.5±4.1). These changes were associated with a significant reduction in the percentage of cells containing normal cytoskeleton (FA, 60.6±3.2%, Ozone, 28.3±4.0, $p<0.001$) (Figure 5.18). Normal and disrupted A549 cell cytoskeleton are shown in Figure 5.19.

Table 5.12. No significant correlations were found between t50 and age, spirometry, bronchoalveolar lavage characteristics and differential or oxidant and anti-oxidant measures. Studies were performed in healthy non-smoking subjects before and 0, 1 and 6 hours after exposure to ozone 0, 100 or 400ppb for 1 hour during intermittent exercise at V_I 40lmin^{-1}. For spirometry, post exposure, the 1 and 6 hour data were combined (Post 1, 6 hrs) and for plasma lipid peroxidation and Trolox equivalent anti-oxidant capacity (TEAC) post exposure the 0 and 6 hour data were combined (Post 0, 6 hrs). BALF-bronchoalveolar lavage fluid, ELF-epithelial lining fluid, GSH-reduced glutathione, GSSG-oxidized glutathione.

Variable	r	p value	n	Variable			r	p value	n
Age	-0.02	<0.92	25	Neutrophils			-0.09	<0.68	25
Pre FEV_1	-0.05	<0.83	25	$x10^6$			-0.09	<0.68	25
Pre %predicted	-0.08	<0.70	25	Eosinophils			-0.03	<0.88	25
Pre FVC	-0.12	<0.56	25	$x10^6$			-0.06	<0.76	25
Pre ratio	0.24	<0.25	25	Albumin	BAL		-0.22	<0.29	25
Post 0 hrs FEV_1	-0.04	<0.87	23		Plasma		0.001	<0.99	25
Post 0 hrs %pred	-0.09	<0.68	23	ELF volume			-0.14	<0.52	25
Post 0 hrs FVC	-0.05	<0.83	23	Superoxide anion	Unstimulated		-0.10	<0.65	25
Post 0 hrs ratio	0.06	<0.77	23	Lipid peroxidation	Plasma	Pre hrs	-0.27	<0.19	25
Post 1, 6 hrs FEV_1	0.02	<0.93	25			Post 0, 6 hrs	-0.25	<0.24	25
Post 1, 6 hrs %pred	0.02	<0.94	25		BALF		-0.05	<0.80	25
Post 1, 6 hrs FVC	-0.07	<0.75	25		ELF		0.20	<0.33	25
Post 1, 6 hrs ratio	0.27	<0.19	25	TEAC	Plasma	Pre hrs	0.04	<0.84	25
Return	0.18	<0.39	25			Post 0, 6 hrs	0.15	<0.48	25
Total cells	-0.15	<0.46	25		BALF		-0.16	<0.44	25
% Viability	-0.03	<0.88	25		ELF		0.09	<0.66	25
Macrophages (%)	0.19	<0.37	25	Glutathione	Cells	GSH	0.23	<0.29	23
$x10^6$	-0.14	<0.52	25			GSSG	0.25	<0.26	23
Lymphocytes	-0.16	<0.43	25		BAL	GSH	0.24	<0.25	25
$x10^6$	-0.15	<0.46	25			GSSG	0.06	<0.79	25
					ELF	GSH	0.27	<0.19	25
						GSSG	-0.006	<0.98	25

	Permeability		Thiols				Cytoskeleton
	%	%	nmoles per 10^6 cells		nmoles per 10^6 cells		%normal
	Total iodine	Bound iodine	Cell GSH	Cell GSSG	Medium GSH	Cell PrSSG	
Air	0.83±0.10	0.14±0.02	5.94±0.87	0.14±0.05	0.12±0.03	0.28±0.02	60.6±3.2
Ozone	1.07±0.11*	0.22±0.04*	5.13±0.90	0.08±0.04	0.28±0.04***	0.66±0.06***	28.3±4.0***

Table 5.13. Epithelial permeability (n=7), intracellular and extracellular thiols (n=6) and cytoskeleton (n=4) in A549 cells *in vitro* following air or ozone 1000ppb for 1 hour. *p<0.05, ***p<0.001, Air v Ozone. GSH-reduced glutathione, GSSG-oxidized glutathione, PrSSG-protein thiols.

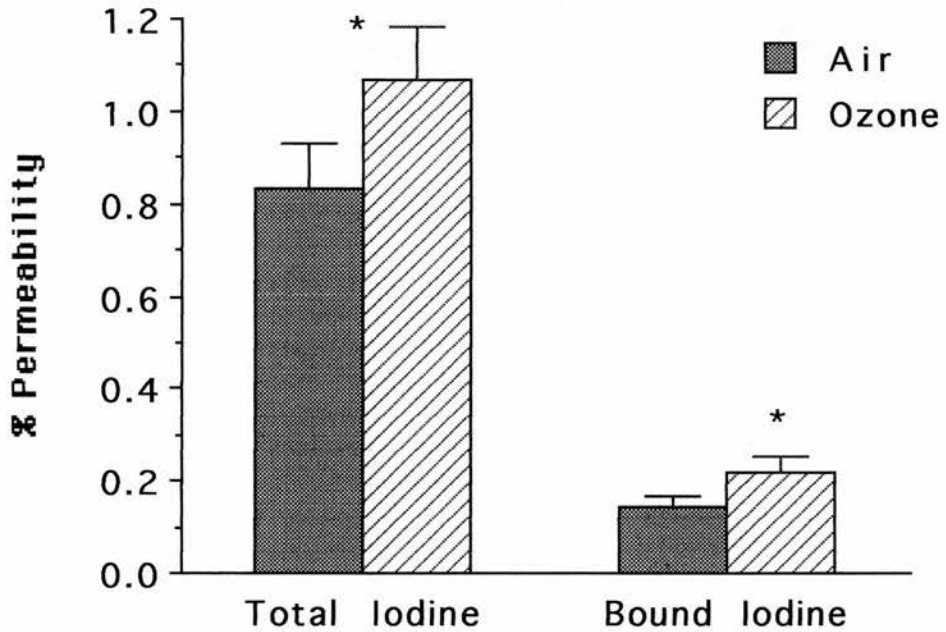


Figure 5.15. Epithelial permeability, assessed by the passage of ^{125}I-bovine serum albumin, in monolayers of A549 cells *in vitro* (mean±SE, n=7) following air or ozone 1000ppb for 1 hour. Epithelial permeability is expressed as a percentage of both the total and bound ^{125}I activity in the well with reference to standard aliquots of ^{125}I-BSA. *$p<0.05$, Air v Ozone.

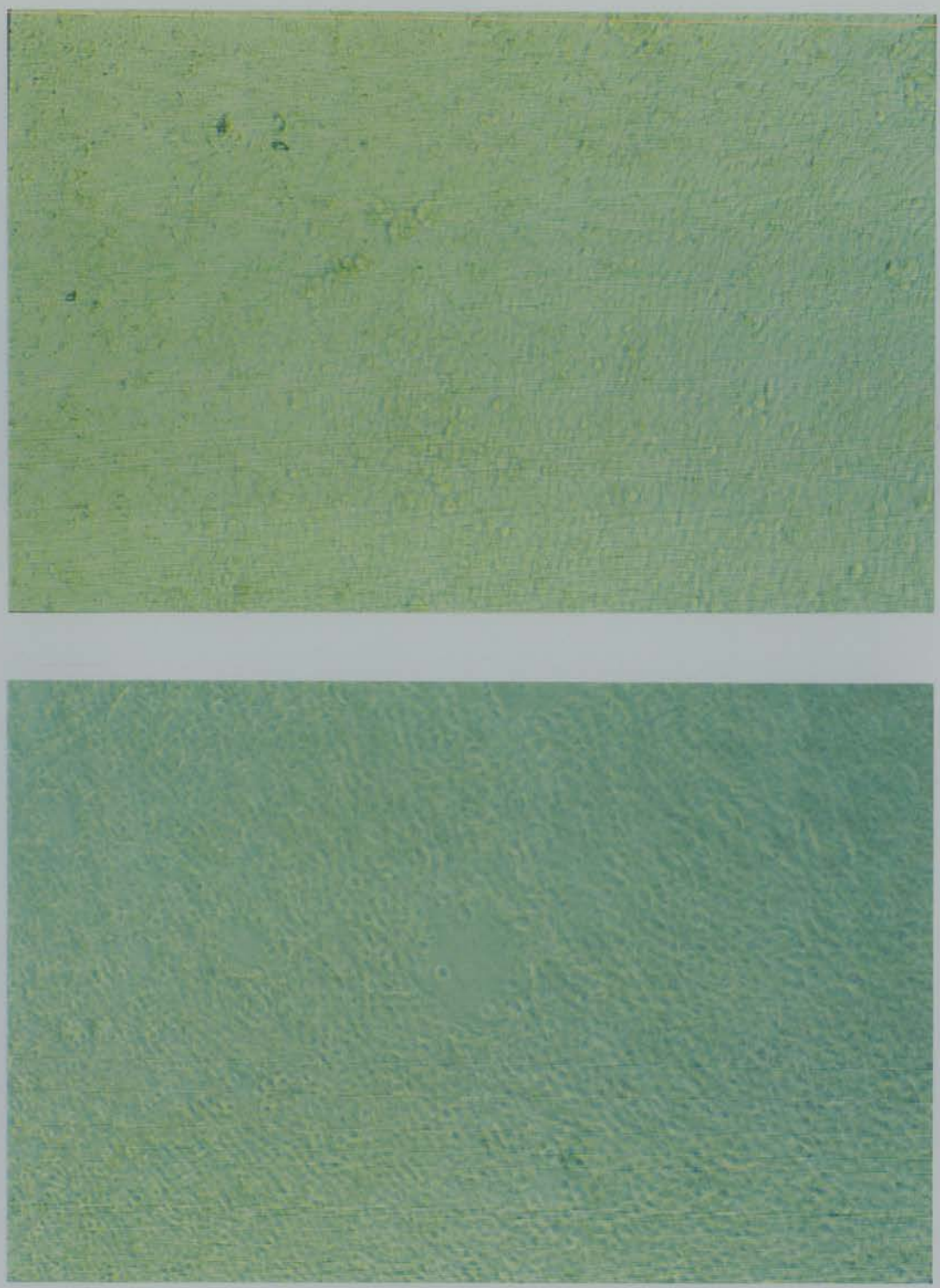


Figure 5.16. A549 cell monolayers *in vitro* following exposure to air (top plate) or ozone 1000ppb (bottom plate) for 1 hour demonstrating focal cell loss following ozone exposure.

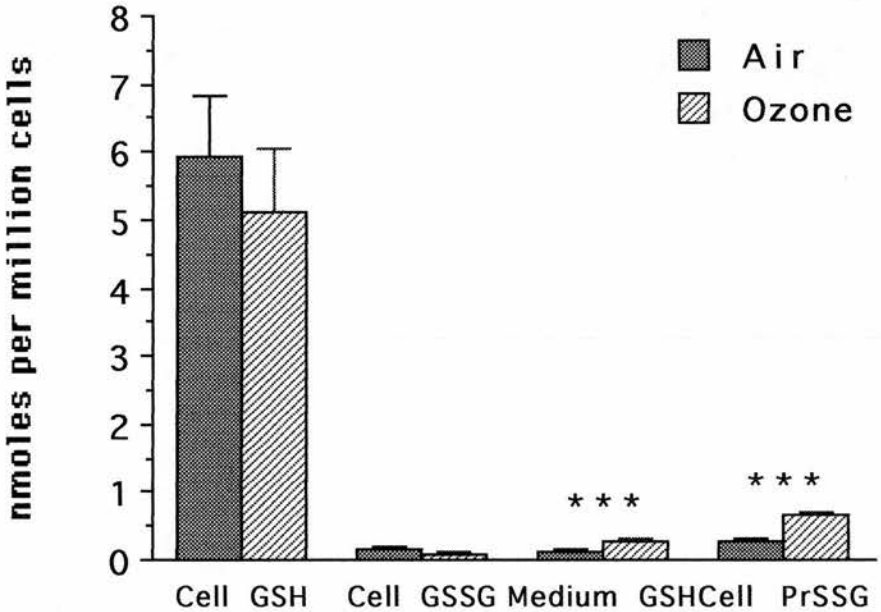


Figure 5.17. Intracellular and extracellular thiols in A549 cells *in vitro* (mean±SE, n=6) following air or ozone 1000ppb for 1 hour. GSH-reduced glutathione, GSSG-oxidized glutathione, PrSSG-protein mixed disulphides. ***p<0.001, Air v Ozone.

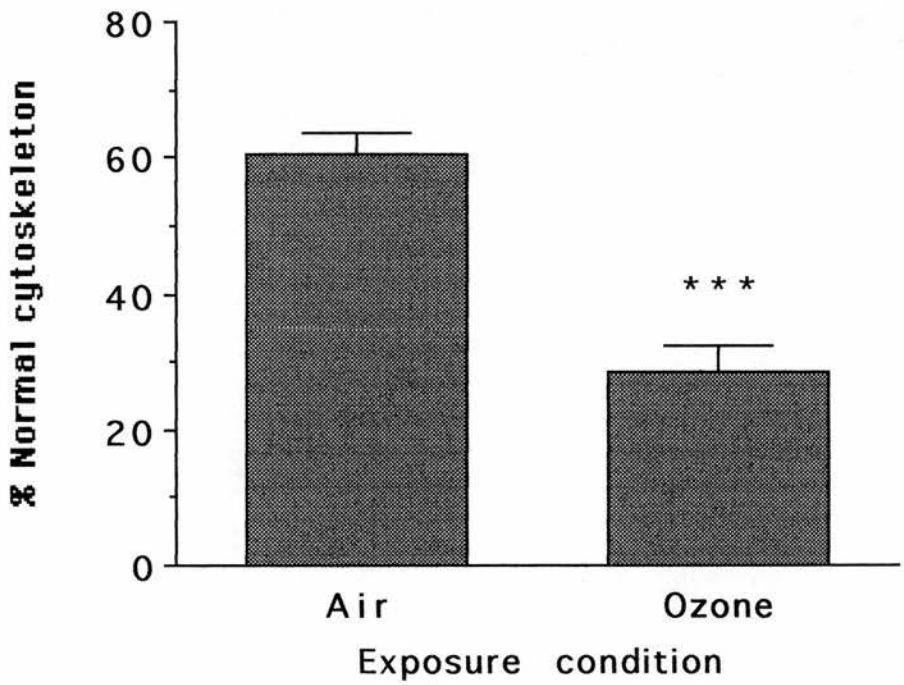


Figure 5.18. Cytoskeletal assessment in A549 cells *in vitro* (mean±SE, n=4) following air or ozone 1000ppb for 1 hour. The number of cells with normal cytoskeleton is expressed as a percentage. ***p<0.001, Air v Ozone.

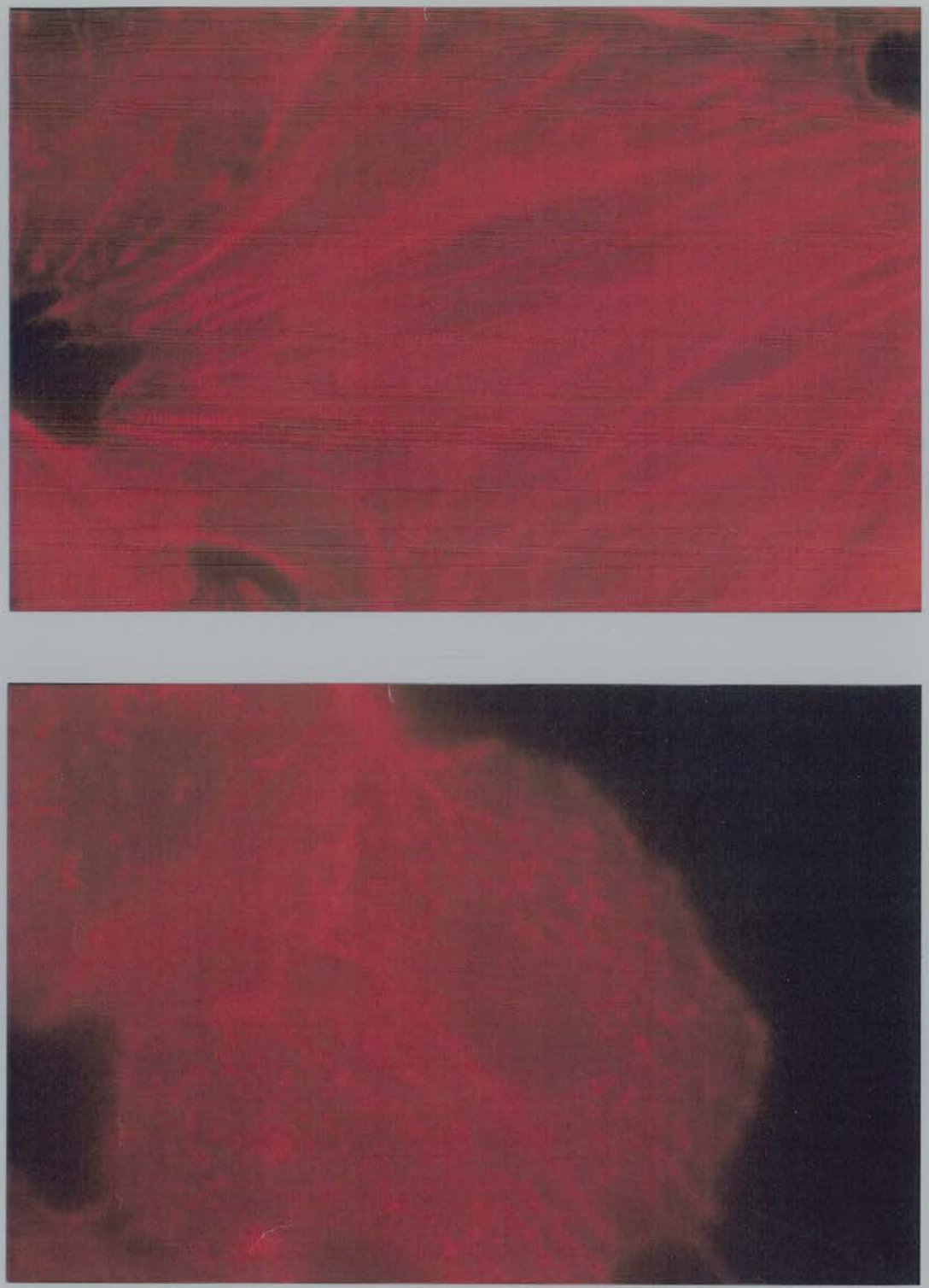


Figure 5.19. A549 cells *in vitro* following air (top plate) or ozone 1000ppb (bottom plate) for 1 hour. Ozone exposure produced significant disruption of the cell cytoskeleton.

5.4. DISCUSSION.

Like cigarette smoke, ozone has been shown to produce increased respiratory epithelial permeability. In healthy non-smoking males exposed to 400ppb ozone for 2 hours during intermittent high intensity exercise [expiratory minute ventilation (V_E) 66.8lmin^{-1}] 7 out of 8 showed increased ^{99m}Tc-DTPA lung clearance (Kehrl *et al.*, 1987). In rats exposed to 0.8ppm ozone for 2 hours ^{99m}Tc-DTPA transfer across the tracheal epithelium increased 2 fold and that of ^{125}I-BSA also increased but to a lesser degree. In ozone exposed rats the tracer HRP and ^{125}I-BSA both localised in the intercellular spaces between epithelial cells, throughout their length, with most at the basement membrane and in endocytic vesicles in all parts of the epithelial cells. HRP endocytic vesicles were seen to fuse with the cell membrane through a short stalk and their number and area increased significantly in both non-ciliated cells without secretory granules and ciliated cells. However neither HRP nor ^{125}I-BSA were seen in tight junctions. In air exposed animals HRP was only seen on the luminal cell surface and in endocytic vesicles (Bhalla and Crocker, 1986).

Oxidative stress induced by menadione in normal and cancer cells has been shown to produce cell membrane blebbing with a reduction in the amount of actin and PrSSG. Formation of high molecular weight aggregates occurred due to oxidative cross linking of actin which contains SH groups. All these changes were prevented by dithiothreitol (Bellomo *et al.*, 1990). The cytoskeleton therefore may also be a potential target for ozone. Bhalla and colleagues produced permeability to ^{99m}Tc-DTPA and ^{125}I-BSA in a main stem bronchus in rats exposed to ozone 0.8ppm for 2 hours and after *in vivo* treatment with cytochalasin D, which causes actin rich microfilament destabilisation. However there was no increase in permeability with vinblastine alone, which causes microtubule depolymerisation, although it did produce a slightly greater increase in permeability in combination with ozone than did ozone alone. When cytochalasin and vinblastine treatment were combined there was a synergistic effect on bronchoalveolar permeability suggesting that both microfilamentous and microtubular cytoskeletal elements do in fact participate in maintaining the integrity of the bronchoalveolar epithelium (Bhalla *et al.*, 1990).

In guinea pigs exposed to ozone 1ppm for 3 hours there was a 45% increase in potential difference and a 41% increase in short circuit current across tracheal epithelium. In both exposed and control animals short circuit current was equivalent to net ^{22}Na absorption but in the exposed group was disproportionately more sensitive to the specific Na^+ channel blocker amiloride (Ozone 44% v Control 13%) (Stutts and Bromberg 1987). This increase in active ion transport was present for up to 3 days after a single exposure (Bromberg *et al.*, 1991). It was concluded that the early effect of ozone is to increase the density or open time of Na^+ channels or to increase the Na^+ pump activity leading to a gradient for Na^+ entry. Increased permeability of the trachea to water soluble molecules also occurred after ozone exposure when measured *in vivo* and HRP was visualised in intercellular spaces (Bromberg *et al.*, 1991). This was no longer demonstrable after the 4th daily exposure and did not occur when permeability was measured *in vitro* after *in vivo* ozone exposure.

In guinea pigs, ozone 1ppm for 90 minutes produced an immediate increase in tracheal vascular permeability, as measured by the passage of intravenous Evan's Blue dye, in association with an immediate increase in airway responsiveness to methacholine. The magnitude of the changes, but not the duration, was increased by combination with 10 puffs of cigarette smoke. When exposed for only 30 minutes to ozone or 5 puffs of cigarette smoke singly, there were no effects although the combination did produce an immediate increase in permeability and airway responsiveness. Ten puffs of cigarette smoke singly did not increase tracheal vascular permeability but did increase airway responsiveness (Nishikawa *et al.*, 1992).

In a study of rat primary culture type II alveolar epithelial cells a dose dependent increase in monolayer permeability, as measured by a decrease in resistance, occurred following ozone exposure (Cheek *et al.*, 1994). In confluent canine bronchial epithelial cells 0.2-0.8ppm ozone produced a dose dependent increase in transcellular electrical resistance and mannitol flux. The increase in epithelial permeability was partly prevented by vitamins E and A and phalloidin which produces actin polymerisation. The effect of 0.2 and 0.5ppm were decreasing by 18 hours

post exposure but the effect of 0.8ppm was still increasing at this time point (Yu *et al.*, 1994).

The levels of ozone chosen for our study were felt to equate with the levels one might find in the U.K. (100ppb) and in the U.S. (400ppb), particularly in the Los Angeles basin. The lower level has been shown to produce inflammatory effects in the lungs (Devlin *et al.*, 1991) and the higher to produce airspace epithelial permeability (Kehrl *et al.*, 1987). Most studies to date have used ozone concentrations which are not relevant to the U.K. and are in fact often higher than those encountered in the U.S.. This is particularly the case when one considers the intense level of exercise used in many studies in association with exposure to ozone lasting several hours. It is probable that the concentration and duration of ozone exposure used in our study were too low or too short to result in increased epithelial permeability. However it is possible to speculate that with larger numbers in the study there would have been a statistically significant effect on epithelial permeability 6 hours after exposure to 400ppb ozone.

There was a significant effect of ozone overall on ELF volume suggesting an increase in epithelial permeability. There was however no significant effect of ozone on BALF albumin another measure of epithelial permeability. Both ELF volume and BALF albumin showed a trend towards an increase 1 and 6 hours after exposure.

Koren and colleagues studied human volunteers exposed to 0.4ppm ozone for 2 hours during intermittent exercise at V_E 35lmin^{-1} (m^2 body surface area)$^{-1}$ and performed BAL on 2 occasions, each subject acting as their own control. There was no change in the total number of cells but the percentage of neutrophils increased from 2.4±0.8 to 10.8±1.3 (mean±SE). This was associated with increases in protein, albumin and IgG. Neutral protease also increased with elevated HNE, in BALF and in neutrophils themselves. An increase was seen in plasminogen activator. Inflammatory indicators such as fibronectin, PGE_2 and C_{3a} also increased (Koren *et al.*, 1989). In a further study BAL was performed 1 hour after exposure to the same dose and duration of ozone at V_E 60lmin^{-1}. The percentage of neutrophils increased from 1.3±0.2 to 9.9±1.6 (mean±SE)

associated with increases in PGE_2 and IL-6 (Koren *et al.*, 1991). Schelegle and colleagues exposed normal humans to 0.3ppm ozone on 3 separate days for 1 hour at V_E 60lmin^{-1}. A significant neutrophilia occurred with onset at 1 hour and peak at 6 hours (Schelegle *et al.*, 1991). Devlin and colleagues exposed non-smokers to 80 or 100ppb for 6.6 hours with intermittent exercise at V_E 40lmin^{-1} and performed BAL 18 hours after exposure. Both doses produced a neutrophilia and elevated levels of PGE_2, lactate dehydrogenase, IL-6 and decreased phagocytosis of yeast by macrophages via complement-receptor. The lower dose was also associated with elevated α_1-Pi and the higher dose with elevated protein and fibronectin (Devlin *et al.*, 1991). Basha and colleagues studied non-smoking asthmatics and healthy volunteers exposed to ozone 0.2ppm and FA for 6 hours with 30 minutes alternating exercise at 5lmin^{-1} (lVC^{-1}) and 30 minutes rest. BAL was performed 18 hours after exposure. There was a significant increase in the percentage and number of neutrophils per ml of BALF in the asthmatics following ozone exposure (12.2±1.0%, $p<0.05$, 45 116±16 260ml^{-1}, $p<0.05$) compared to the asthmatics exposed to FA (1.6±0.6, 2 652±1 135) and to the control subjects exposed to ozone (6.8±0.7, 14 402±1 416) and FA (1.0±0.3, 1 494±624). This was associated with significantly greater increases in IL-8 and IL-6 (Basha *et al.*, 1994).

In my own studies I found, as many other groups have (Koren *et al.*, 1989, Devlin *et al.*, 1991, Schelegle *et al.*, 1991), that the total number of cells recovered in BAL did not change. The percentage and number of neutrophils increased 6 hours after the inhalation of 400ppb ozone. As in the smoking study in Chapter 4 this was not the result of sampling as the maximal reduction in airway calibre occurred immediately after exposure in the BAL studies. Our data are thus in keeping with that of Schelegle and colleagues where neutrophil influx into BAL peaked at 6 hours in pooled BAL (Schelegle *et al*, 1991). In our study no increase in the neutrophil content of BAL occurred 1 hour after exposure. Schelegle did however find a significant increase at 1 hour in the first or proximal aliquot of BAL. Had I not pooled the aliquots of BAL then it is possible that I might also have seen neutrophil influx beginning at 1 hour.

Neutrophils may contribute to epithelial permeability by the release of oxidants and arachidonic acid metabolites. In Rhesus monkeys exposed to 0.96ppm ozone for 8 hours there was a strong relationship between epithelial necrosis and emigration and retention of neutrophils at all airway levels (Hyde *et al.*, 1992). In rat trachea following 3 hours exposure to 0.8ppm ozone permeability to ^{99m}Tc-DTPA increased immediately, peaked at 8 hours, and returned to the control level at 24 hours. The total number of neutrophils in the interstitium increased at 12 hours and appeared to pass from the vasculature to the interstitium at 8 hours (Young and Bhalla, 1992). In a further study BAL protein, reflecting tissue injury and increased permeability, increased immediately after exposure, peaked at 8 hours, declined after 12 hours and returned to normal after 16 hours, at which point repair was felt to begin. Albumin, reflecting permeability only, increased over 0 to 4 hours after exposure, peaked 8 to 16 hours after and declined after 20 to 24 hours although it was still raised at this time. The persistent increase in permeability was thought to be due to the inability of new cells to form tight junctions, this being a late event occurring only in mature cells. Immediately after ozone exposure cell viability fell to 85% compared to 92% in control cells. It recovered rapidly and by 4 hours had risen to above 90% although it remained below that of the control cells. This fall, presumably due to cell destruction, suggests a rapid and direct toxic effect of ozone. The neutrophil count in alveolar septae increased 3 fold at 4 hours, peaked at 8 hours and returned to normal after 16 hours. It lagged behind the protein changes and coincided with the changes in albumin suggesting that the neutrophil influx was a consequence of the increased permeability. Morphological evidence of cell injury was seen with thickening of alveolar septae and increased parenchymal cellularity plus free cell accumulation. These changes began to occur at 12 hours with a sharp increase at 16 hours which continued through 20 and 24 hours. The time course of the morphological changes was later than that of the functional impairment and therefore not a direct measure of it. Variation was seen between animals in the time at which the morphological changes began with 2 out of 6 animals showing changes at 8 hours and 4 at 12 hours. Injury from ozone thus appeared to stop functionally at 16 hours and permeability to continue until cells matured sufficiently to form tight junctions (Bhalla and Young, 1992).

It would seem likely therefore that ozone produces an initial direct injury to cell membranes and possibly to macrophages, with increased permeability, resulting in increased release of chemotactic stimuli for neutrophils and thus an increase in their numbers. This secondary inflammatory response will result in further injury and permeability.

However following a study of rats inhaling 0.2 to 0.8ppm ozone, for 7 hours daily and for up to 4 days, Donaldson and colleagues concluded that epithelial injury by ozone at the concentrations used was likely to be a direct effect of the ozone and not an indirect effect of inflammatory leucocytes. They found an increase in neutrophils in BAL but no stimulation of any measures of the ability of leucocytes to cause injury to epithelial cells or extracellular matrix (Donaldson *et al*, 1993).

Ozone induced permeability in rats, exposed to 0.8ppm for 2 hours, was inhibited by pretreatment with cyclophosphamide, FPL 55712 (an LTD4 antagonist) and indomethacin although LTB4 had no effect on permeability. This may perhaps be explained by the short duration of the effect of LTB4 despite being chemotactic for neutrophils and causing neutrophil aggregation and degranulation (Bhalla *et al.*, 1992). However in mice exposed to 2ppm ozone for 3 hours, indomethacin, colchicine (a chemotaxis inhibitor) and cyclophosphamide, in a dose sufficient to produce granulocytopaenia, all resulted in a significant decrease in neutrophil influx at 6 and 24 hours but did not attenuate the increase in total protein in BALF induced by the ozone at either of these time points. The cyclophosphamide also abolished any neutrophil response in peripheral blood to ozone exposure (Kleeberger and Hudak, 1992). Although this may have been the result of species differences the first study employed 3 measures of epithelial permeability, ^{99m}Tc-DTPA clearance and both albumin and protein flux into BALF. Total protein, as mentioned above, is an indicator of both tissue injury and epithelial permeability and is thus less specific for the latter than the other 2 methods and this may explain the different results in the 2 studies. Moreover it is well known that permeability of the alveolar capillary membrane can occur in ARDS even in neutropenic patients (Sibille and Reynolds, 1990).

Neutrophils from ozone exposed rats have been shown to alter epithelial resistance *in vitro* but only when stimulated with PMA or fMLP (Bhalla *et al.*, 1993). Neutrophils have a synergistic effect on ozone (1ppm, 3 hours) induced airway epithelial injury when perfused in the isolated perfused rat lung (Joad *et al.*, 1993). The motility of neutrophils isolated from peripheral blood in rats exposed to 0.8ppm ozone for 2 hours and incubated with ARL-14 or primary culture type II cells is increased with spontaneous redistribution of actin filaments and greater adherence of the neutrophils to the epithelial cell lines. Actin caps formed at sites of contact and EM showed prominent surface modifications at sites of contact (Bhalla *et al.*, 1993).

Ozone produces solubilisation of elastin and increases its susceptibility to proteolysis by HNE, an effect which can be prevented by ascorbate, EDTA and uric acid (Winters *et al.*, 1994). Inactivation by ozone of α_1-Pi *in vitro* both in solution and in plasma and of bronchial leucocyte proteinase inhibitor (BLPI) in solution has been demonstrated. No inactivation of α_1-Pi occurred however in blood plasma in non-smoking volunteers exposed to 0.5ppm ozone for 4 hours on 2 consecutive days (Johnson, 1980, Johnson, 1987). However, if significant local inhibition of anti-proteases in the microenvironment of the lung were to occur following ozone exposure then susceptibility to protease mediated damage would increase.

Ozone has a standard redox potential of +2.07V and is therefore the second most powerful oxidant known next to fluorine. It is believed to cause damage to biological tissues either by direct reaction or through the formation of free radicals and reactive intermediates or by both mechanisms. Oxidant stress causes a loss of control of calcium distribution within the cell (Nicotera and Orrenius, 1987). Lipid peroxidation chain reactions are initiated with oxidation of functional groups and loss of activity of biomolecules including enzymes. Peroxidation of membrane lipids is thought to be a most important mechanism of ozone injury. Ozone, $O_2^{\cdot -}$ and $OH\cdot$ radicals are all capable of reacting with polyunsaturated lipids (LH) producing lipid free radicals. In the presence of molecular oxygen very reactive and toxic peroxy radicals are generated. They react further with LH producing

hydroperoxide and further lipid free radicals. Aldehydes may be the most important damaging compounds generated (Department of Health, 1991). Lipid peroxidation has been observed in red blood cells exposed to ozone (Goldstein and Balchum, 1967). *In vivo* evidence of lipid peroxidation has been found in lung tissue from ozone exposed animals including conjugated dienes (Goldstein *et al.*, 1969) and TBARS (mostly MDA) (Chow and Tappel, 1972, Sagai *et al.*, 1987). There are no such studies of lipid peroxidation in BAL. In aqueous solution ozone decomposes to form H_2O_2, and $O_2^{\cdot -}$, $OH^{\cdot}$ and hydroperoxyl (HO_2) radicals and reacts similarly with molecular oxygen (Mustafa, 1990). The types of free radicals produced *in vivo* are not certain but those that are produced are non-selective and fast reacting.

Ozone inhalation in healthy non-smoking volunteers is an oxidant model for acute cigarette smoking. Cigarette smoke contains a huge number of oxidant species whereas ozone exposure involves the inhalation of a single oxidant species. Studies of ozone may allow a clearer determination of oxidant effects on the airspaces. Although there have been a number of studies in humans on the effect of ozone inhalation on cellularity and other indicators of inflammation in the airspaces there is little if any data on the effect of ozone inhalation on the oxidant status of the airspaces in humans.

I found that although the production of $O_2^{\cdot -}$ was increased in the control group, as a result of exercise, ozone inhalation produced a reduction in $O_2^{\cdot -}$ production, 1 hour after 100ppb and 6 hours after 400ppb. However on reviewing the data it appears there is a general trend that both doses produced essentially the same effect, at both time points, although all the changes did not reach statistical significance. This effect of ozone is supported by data from previous studies (Amoruso *et al.*, 1981, Ryer-Powder *et al.*, 1986, Donaldson *et al.*, 1993) and may be related to the loss of activity of a cytochrome b_{558} (Ryer-Powder *et al.*, 1986).

There is limited data on the effect of ozone inhalation on lipid peroxidation in humans either in plasma, BALF or ELF. Dillard and colleagues and Lovlin and colleagues found that products of lipid peroxidation, exhaled pentane and plasma MDA respectively, were

increased by exercise (Dillard *et al.*, 1978, Lovlin *et al.*, 1987). I found no significant changes in plasma or BALF. In ELF and in BALF the control levels were again very much higher than those from the smoking study. In ELF there was a significant reduction in TBARS both 1 and 6 hours after 400ppb ozone The reverse trend seen in BALF albumin and ELF volume 1 and 6 hours after 400ppb raises the possibility that the decrease in products of lipid peroxidation seen in ELF could therefore be related to the anti-oxidant capacity of albumin or may be dilutional. It is interesting to speculate that epithelial permeability may thus have a protective role against oxidant stress.

Ozone causes oxidation of SH groups, amines, alcohol groups and aldehydes and other functional groups in proteins, enzymes, nucleic acids, membranes and other small and large biomolecules. This may alter the redox status of cells and their metabolic functions (Mustafa, 1990). Ozone oxidises SH, for example GSH and those found in proteins. The GSH redox cycle is thought to play a crucial role in anti-oxidant defence against lipid peroxides and other damaging related oxygenated intermediates. Alveolar macrophages have high levels of intracellular GSH approximately 5mM (Horton *et al.*, 1987). Various enzymes for example GR, GP_X, catalase, SOD and key enzymes of the pentose-shunt pathway (glucose-6-phosphate and 6-phosphogluconate dehydrogenases) play an important role in the anti-oxidant defence of the lungs and can promote the repair of injured cells and tissues enabling the lung to withstand further injury (Mustafa, 1990).

In murine L929 fibroblasts, exposed to ozone, GSH depletion occurred with an initial increase in GSSG. There was no increase in GSH or GSSG in the buffer covering the cells which were exposed for 10 seconds every 30 seconds (Van der Zee *et al.*, 1987). GSH in rat lung after exposure to ozone 4ppm for 6 hours fell by 40% but was recoverable with $NaBH_4$. There was no change in GSSG but PrSSG formation occurred peaking at 24 hours post exposure and reducing by 48 hours (De Lucia *et al.*, 1975). In rats inhaling 0.8ppm ozone for 6 hours daily BAL was performed immediately after exposure on day 1, 18 hours after this exposure and after the exposures on days 3 and 7. GSH was elevated in cells after day 3 and in BALF after 7 days with no change in GSSG in cells or fluid. GP_X

increased in cells after day 3 and GR was elevated immediately after the exposure on day 1 and after day 7 (Boehme *et al.*, 1992). In rats exposed to ozone long term GST, GP_X and SOD all increased in a dose dependent fashion although not all changed at all levels of the airways (Plopper *et al.*, 1994).

Other studies have shown that acute ozone exposure results in depletion and oxidation of GSH (Mountain, 1963, Freeman and Mudd, 1981). SH groups may be found at the active site of proteins, enzymes, coenzymes and other biomolecules and thus oxidation can alter the function of these molecules. Amino acids are another site of attack in proteins particularly cysteine, which itself contains an SH group, methionine, tryptophan and tyrosine (Mustafa, 1990). As mentioned above two examples of enzymes inhibited by ozone are α_1-Pi and BLPI (Johnson, 1980, Johnson, 1987).

I studied the total functional anti-oxidant capacity of plasma, BALF and ELF following ozone exposure. No clinically relevant or statistically significant changes occurred although the general trend in plasma was towards a reduction. In BALF and ELF there was also no evidence of any increase, as was seen in the smoking study with both acute and chronic smoking.

Similarly no significant changes occurred in GSH or GSSG levels in mixed BAL leucocytes, BALF or ELF. The data suggest a trend towards a decrease 6 hours after 400ppb ozone. It is certainly clear that there was no increase. The control values, not only for GSH, but also for TEAC, were generally lower than in the smoking study. Viguie and colleagues found that exercise was associated with a decrease in GSH and an increase in GSSG in blood with no change in total glutathione. Levels returned to baseline after 15 minutes of recovery (Viguie *et al.*, 1993).

In vitro I found that exposure of A549 type II epithelial cell monolayers to 1000ppb ozone for 1 hour produced focal cell loss. There was not surprisingly a marked increase in permeability to ^{125}I-BSA. I had hoped to find a dose of ozone which would have produced epithelial permeability without cell loss. Whilst some dissociation of the ^{125}I from the BSA occurred with time in storage this did not alter the result as

demonstrated by studies of both total ^{125}I and BSA bound ^{125}I. I found evidence of oxidant stress in the cells. Although there was only a trend towards a decrease in GSH intracellularly there was a significant increase in PrSSG and in the medium bathing the cells there was a significant increase in the level of GSH measured. As noted above the latter is likely to represent the export of intracellular GSSG which is toxic to cells. In cases where GSSG may accumulate it is either transported from the cells or reacts with protein sulphydryls via a mixed disulphide reaction (Deneke and Fanburg, 1989). Van der Zee and colleagues did find a significant depletion of GSH in murine L929 fibroblasts exposed to ozone with an initial increase in GSSG intracellularly although no GSH or GSSG was detectable in the extracellular buffer (Van der Zee *et al.*, 1987). They could not however rule out leakage as GSH and GSSG might both be readily oxidised in the medium, particularly as they used PBS.

The percentage of cells in our study showing a normal cytoskeleton also fell markedly. Disruption of the actin filaments occurred following ozone exposure resulting in aggregation of the actin. Actin molecules contain SH groups and our results are consistent with the study by Bellomo and colleagues where oxidant stress produced high molecular weight aggregates of oxidatively cross linked actin (Bellomo *et al.*, 1990). Epithelial permeability induced by ozone *in vitro* can be prevented not only by anti-oxidants but also by phalloidin which produces polymerisation of actin (Yu *et al.*, 1994). Bhalla and colleagues have shown that both microfilamentous and microtubular cytoskeletal elements participate in maintaining the integrity of the bronchoalveolar epithelium (Bhalla *et al.*, 1990) (Bhalla *et al.*, 1990).

Thus in summary I have shown an effect of the inhalation of ozone 400ppb for 1 hour on epithelial permeability during moderate intermittent exercise. ELF volume increased significantly overall although there were only trends towards an increase in ^{99m}Tc-DTPA lung clearance and BALF albumin. There was a significant increase in the percentage and number of neutrophils in BALF 6 hours after 400ppb ozone. Despite this the levels of $O_2^{\cdot -}$ production from mixed BAL leucocytes and products of lipid peroxidation in ELF decreased. This could not be explained by any increase in either the total functional anti-oxidant

capacity or the levels of GSH in either BALF, ELF or plasma. There was therefore no evidence of increased oxidant stress accompanying the above change in epithelial permeability and neutrophil influx into the airspaces. This contrasts with the *in vitro* studies in which a higher concentration of ozone was used. Focal cell loss and evidence of oxidant stress were seen with disruption of the cell cytoskeleton.

CHAPTER 6. SUMMARY.

In this thesis I have investigated the effects of cigarette smoke and ozone on the respiratory epithelium in particular focussing on their oxidant effects. Oxidants may be inhaled, as with cigarette smoke or ozone, or released into the airspaces by inflammatory leucocytes. There is evidence of an alteration in the oxidant anti-oxidant balance in many respiratory conditions including COPD (Taylor *et al.*, 1986, Cross *et al.*, 1994a, Rahman *et al.*, 1996). The oxidant anti-oxidant balance is critically important to the maintenance of normal cell function. The respiratory epithelium acts as a barrier against inhaled material for example cigarette smoke, pollutants such as ozone, bacteria, viruses and allergens and it maintains the composition and volume of the RTLF's. Cigarette smoke is the main aetiological agent in COPD and contains 10^{16} free radicals per puff (Janoff *et al.*, 1986). Ozone is the second most powerful oxidant known. Both cigarette smoke and ozone have been shown to produce airspace epithelial permeability (Jones *et al.*, 1980, Kehrl *et al.*, 1987). The mechanism of these effects is not known although it has been proposed that it is the repeated acute injury from cigarette smoking that results in increased epithelial permeability (Hogg, 1983). The most likely site for the increase in epithelial permeability produced by cigarette smoke is in the distal terminal and respiratory bronchioles (Niewoehner *et al.*, 1974, Walker *et al.*, 1982, Hogg, 1983). The effect of cigarette smoking on epithelial permeability occurs within 3 days of starting smoking (Minty *et al.*, 1984) and is rapidly reversible on stopping (Minty *et al.*, 1981, Mason *et al.*, 1983). Studies with cigarette smoke have shown passage of tracer molecules through progressively disrupted tight junctions and through both intercellular and cellular pathways (Simani *et al.*, 1974, Boucher *et al.*, 1980, Burns *et al.*, 1989). Studies with ozone have also shown passage through intercellular and cellular pathways but this has not been demonstrated through tight junctions (Bromberg *et al.*, 1981, Bhalla and Crocker, 1986).

The permeability of the respiratory epithelium can be studied by following the passage of ^{99m}Tc-DTPA from the lungs to the blood. My initial studies compared 3 methods of ^{99m}Tc-DTPA clearance and assessed their repeatability. Initial studies performed in the U.K.

employed a correction for recirculating background activity (Jones *et al.*, 1980, Minty *et al.*, 1981). An intravenous injection of ^{99m}Tc-DTPA was given and the assumption made that this would equilibrate in a background region in the same way as in the lungs. Background ROI's have been used over the inter-renal region (Langford *et al.*, 1986) and over both shoulders (O'Doherty *et al.*, 1985b). In North America studies have used only the first 7-10 minutes of the clearance curve (Rinderknecht *et al.*, 1980, Mason *et al.*, 1983) making the assumption that during this period there is no recirculating background activity. A wide range of normal values for t50, using uncorrected and corrected methods, has been quoted in the literature [44.4-117.5 minutes] (Rinderknecht *et al.*, 1980, O'Doherty *et al.*, 1985b, Langford *et al.*, 1986). Although comparisons between the uncorrected and corrected forms of the technique have been performed (O'Doherty *et al.*, 1985b, Langford *et al.*, 1986) these have compared analysis on the complete clearance curves. Not surprisingly it was found that the uncorrected technique gave significantly higher values. However this comparison is not one that reflects the practice of the methods of analysis as given above. We therefore compared uncorrected analysis on the first 7 minutes of the clearance curve with 2 methods of corrected analysis on the complete clearance curve pre intravenous injection. No difference was found in the values obtained in smokers although surprisingly the uncorrected analysis gave higher values in non-smokers. There was no correlation between the uncorrected t_{50} values and the correction factors for the background corrected methods of analysis. There were no differences between the values obtained at 2 separate visits with any of the 3 methods nor in their repeatability. Having completed these studies I feel that a background correction is necessary however, as it allows the analysis of any form of curve, the nature of which cannot be predicted in advance. The inter-renal background ROI is simple and rapid to apply and has been shown to equate most closely, in terms of background correction, to the lungs (Langford *et al.*, 1986) and this method was therefore used for further studies.

Previous human studies have not attempted to define the acute and the chronic effects of smoking on epithelial permeability, inflammation and oxidant status in the airspaces of chronic smokers. Fourteen chronic

cigarette smokers underwent ^{99m}Tc-DTPA lung scans after refraining from smoking for 12 hours (chronic smoking group) or after continuing to smoke until 1 hour before study (acute smoking group). ^{99m}Tc-DTPA clearance was significantly increased in the chronic smoking group and increased further and significantly after acute smoking. Each smoker also underwent bronchoscopy and BAL after either chronic or acute smoking. Seven healthy non-smokers were also studied. There was a trend towards increased albumin concentration in BALF and increased ELF volume in both smoking groups. Ozone exposure was also performed in 15 healthy non-smokers as an acute oxidant model. Subjects inhaled FA or ozone 100 or 400ppb for 1 hour, during intermittent exercise at V_I 40lmin^{-1}. There was a significant overall increase in ELF volume. Inhalation of 400ppb resulted in a trend towards an increase in BALF albumin and ELF volume 1 hour after exposure and an increase in ^{99m}Tc-DTPA clearance 6 hours after exposure. These data indicate that whilst increased epithelial permeability is readily found in chronic cigarette smokers, it is an acute phenomenon, supporting the proposal that it is the repeated acute insult from cigarette smoke that is responsible (Hogg, 1983). I believe that small numbers in the ozone studies prevented the above results reaching statistical significance but overall they would appear to support the hypothesis that increased epithelial permeability resulting from inhalation of an oxidant is an acute phenomenon.

The absolute number of neutrophils in the airspaces has been consistently raised in studies of smokers (Hunninghake and Crystal, 1983, Nagai *et al.*, 1988, McCrea *et al.*, 1994). Various chemoattractants have been proposed including C5a, LTB4 (Sibille and Reynolds, 1990) and IL-8 (Kunkel *et al.*, 1991). There is sequestration of circulating neutrophils in the pulmonary vasculature in cigarette smokers which is increased by acute cigarette smoking (MacNee *et al.*, 1989), mediated by a change in cell deformability (Selby *et al.*, 1991, Drost *et al.*, 1993). Ozone inhalation has been shown to cause neutrophil influx into the airspaces, beginning at 1 hour and peaking at 6 hours (Schelegle *et al.*, 1991). In my studies the total number of cells recovered in BAL was 11.4 fold greater in the chronic smoking group and 6.5 fold greater in the acute smoking group compared to the control non-smokers. The percentage and number of neutrophils were significantly increased in the acute smoking group. Following ozone

inhalation, although there was no increase in the total number of cells obtained, the percentage and number of neutrophils increased 6 hours after exposure to 400ppb. The increased number of neutrophils found in the airspaces of cigarette smokers is therefore also an acute phenomenon. The ozone studies support the hypothesis that oxidant inhalation results in an acute inflammatory response in the lungs.

$O_2^{\cdot -}$ release from mixed BAL leucocytes and products of lipid peroxidation in plasma were significantly increased in both the chronic and acute smoking groups compared to non-smokers. In BALF and ELF products of lipid peroxidation doubled in the chronic smoking group and increased 6 fold in the acute smoking group. TEAC was significantly reduced in plasma in both groups with a trend towards a further reduction in the acute smoking group. Conversely a significant and similar increase in TEAC was seen in BALF (with the same trend in ELF) in both smoking groups. GSH was increased 2 fold in mixed BAL leucocytes, BALF and ELF in the chronic smoking group compared to control non-smokers. This increase was abolished following acute smoking. Ozone inhalation however, resulted in a significant reduction in $O_2^{\cdot -}$ release from mixed BAL leucocytes and products of lipid peroxidation in ELF, both 1 and 6 hours following 400ppb. The major difference between the smoking and ozone studies was the exercise performed by those in the latter which resulted in greatly increased control values for $O_2^{\cdot -}$ release and products of lipid peroxidation in the group exposed to FA (Boveris and Chance, 1973, Dillard *et al.*, 1978, Lovlin *et al.*, 1987). Products of lipid peroxidation in plasma and in BALF were not altered by ozone inhalation, nor were TEAC in plasma, BALF or ELF or GSH in mixed BAL leucocytes, BALF or ELF, although there was a trend towards a decrease in GSH 6 hours following 400ppb. These studies indicate that in smokers there is an increased oxidant burden which is greatest following acute smoking, whilst conversely ozone inhalation resulted in a reduction in some variables associated with oxidant stress.

In vitro studies of A549 cells exposed to ozone 1000ppb for 1 hour produced epithelial monolayer permeability, due to focal cell loss, with an increase in the concentration of protein thiols in the cells and export of GSSG, and disruption of the cytoskeleton.

In conclusion I present evidence that the increase in epithelial permeability and neutrophil influx in the airspaces, produced by oxidant inhalation are acute phenomena. No relationship was found between them however, therefore further studies of the time course of oxidant induced changes in indices of epithelial permeability and neutrophil influx are required, to establish whether they are linked. In cigarette smokers they were associated with an alteration in the balance between oxidants and anti-oxidants, in the direction of increased oxidant stress, particularly following acute cigarette smoking. The reduction in anti-oxidant capacity found in the plasma of cigarette smokers associated with the increased level of anti-oxidant capacity in the airspaces suggests that anti-oxidant molecules may be transferred from the plasma to ELF following cigarette smoking perhaps as a result of increased epithelial permeability. GSH is thought to be particularly important in protecting the lungs from cigarette smoke (Rahman and MacNee, 1996). Although it is elevated in the airspaces of chronic smokers this increase is overwhelmed and abolished by acute cigarette smoking. The relative loss of a major component of the overall anti-oxidant capacity in the airspaces may be important factor in the acute effects of cigarette smoke on the lungs. Animal and *in vitro* studies lend support to the hypothesis that a reduction in GSH, resulting from cigarette smoke inhalation, may result in epithelial permeability (Li *et al.*, 1994). I have demonstrated that both are acute phenomena although further work is required to establish a link between them in smokers. In contrast *in vivo* ozone exposure resulted in a decrease in some measures of oxidant stress. Although the overall picture was complicated by the effect of exercise, the fact that anti-oxidant levels were maintained suggests that either the dose of ozone used was too low to affect them or that in healthy subjects they are not overwhelmed by the inhalation of a powerful oxidant. Consequently there was a less marked effect on epithelial permeability, despite the potentially increased oxidant burden associated with the influx of neutrophils into the airspaces. Studies combining ozone inhalation and another acute inflammatory state, for example asthma, would demonstrate whether the anti-oxidant defences in the lungs remain well maintained in other such circumstances. It is clear from the *in vitro* studies that ozone has the potential to produce increased oxidant stress in

the respiratory epithelium and airspaces, with profoundly damaging effects. In this era of increasing concern over the effects of environmental pollution this indicates the need for futher research in this area.

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