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THE ROLE OF INFLAMMATION IN HYPEROXIA-INDUCED LUNG INJURY

by

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ABSTRACT

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The development of acute (HMD) and chronic (BPD) lung disease in premature human neonates is characterised, in part, by pulmonary inflammation. One of the pathophysiological determinants of lung injury includes exposure to high inspired concentrations of oxygen. Due to the practical and ethical problems associated with the study of these diseases a guinea pig (GP) model of prematurity has been developed. In this thesis the role of inflammation, following exposure to oxygen in the development of acute and chronic lung disease was investigated using the GP model of prematurity.

Delivery of preterm GP's (3 days premature) by Caesarian section in to air (21% oxygen), results in lung injury, evidenced by an alteration in the permeability of the lung which leads to an increase in lung wet weight and bronchoalveolar fluid (BALF) protein. BALF phospholipase A_2 activity was also raised and suggests that pro-inflammatory lipids may be present in the lung. Following an acute period of oxygen exposure, lung injury and mortality increased. The presence of activated neutrophils (PMN's) and leukotriene B_4 (LTB_4) in BALF indicated an on going inflammation which was investigated further.

Neutrophils have the potential to cause extensive tissue injury through the generation of superoxide. Although BALF cells from both preterm and term animals exposed to 95% oxygen generated significantly less superoxide than air exposed animals. As it proved difficult to isolate and study alveolar neutrophils, superoxide production from peritoneal PMN's of term and preterm pups was studied. No significant difference between term and preterm PMN superoxide generation was found. To further study the relationship between PMN's and lung injury, a specific antisera to GP PMN's was used to deplete these cells from the circulation. However, PMN depletion did not alter the degree of lung injury or survival. The role of PAF and LTB_4 in HMD in oxygen-induced lung injury was investigated using specific receptor antagonists. Treatment with the PAF antagonist, WEB2086, blunted inflammation and injury. However, no reduction in survival was observed. A similar observation was attained using the LTB_4 antagonist, U75302, although this was attributed to the possible antioxidant effects of the vehicle. These observations suggest that injury to the lung probably involves a number of different mediators.

The role of inflammation in prolonged (28 days) hyperoxic exposure was also studied. Qualitative microscopic analysis of premature GP's exposed to 28 days 85% oxygen demonstrated changes similar to those observed in human infants with BPD. Although inflammation was present through out the exposure period, the observed induction of antioxidants may have contributed to reduction in lung injury that occured between days 14 and 28. This thesis reports a description of the GP model of prematurity and the role of particular inflammatory mediators in the development of HMD and BPD.

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Abbreviations

6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
α1PI	Alpha-1-proteinase inhibitor
AA	Arachidonic acid
ANOVA	Analysis of variance
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BASO	Basophil
BCA	Bicinchoninic acid
BPD	Bronchopulmonary dysplasia
BPI	Bactericidal permeability increasing protein
BSA	Bovine serum albumin
C3b	Complement component 3b
C5a	Complement derived protein 5a
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CPAP	Continous positive airways pressure
CS	Control sera
Cu/Zn	Copper/Zinc
DAD	Diffuse alveolar damage
DAG	Diacylglycerol
DAUDA	11-(dansylamino)undecanoic acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
DOPG	Dioleoylphosphatidylglycerol
DPN	Dinitrophenylhydrazine
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Disaturated phosphatidylcholine
DTPA	Dietheylenetriaminepentacetic acid
EDTA	Ethylenediaminetetra acetic acid
EIC	Elastase inhibitory capacity
ELF	Epithelial lining fluid
EOS	Eosinophil
FABP	Fatty acid binding protein
FAD	Flavin adenine dinucleotide
FCA	Freunds complete adjuvant.
FICA	Freunds incomplete adjuvant
FMN	Flavin mononucleotide
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSH-Rd	Glutathione reductase
GS·	Thiyl radical
GSOO·	Peroxysulphenyl radical
GSSG	Oxidised glutathione
H^+	Hydrogen ion
H&E	Haemotoxylin and eosin
HBSS	Hanks buffered salt solution
HCl	Hydrogen chloride

GSH-Px	Glutathione peroxidase
GSH-Rd	Glutathione reductase
$GS^{\cdot}$	Thiyl radical
$GSOO^{\cdot}$	Peroxysulphenyl radical
GSSG	Oxidised glutathione
H^+	Hydrogen ion
H&E	Haemotoxylin and eosin
HBSS	Hanks buffered salt solution
HCl	Hydrogen chloride
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HMD	Hyaline membrane disease
H_2O	Water
H_2O_2	Hydrogen peroxide
IFNδ	Interferon gamma
IgG	Immunoglobulin G
IL-1	Interleukin-1
IP_3	Inositol-1,4,5-triphosphate
IPPV	Intermittent positive pressure ventilation
LPS	Lipopolysaccharide
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
Lymp	Lymphocyte
MAC	Membrane attack complex
MACS	Macrophages
MBP	Major basic protein
ND	Not determined
Mn	Manganese
MONO	Monocyte
MSB	Martius Scarlet Blue
NA	Neutrophil antisera
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$O^{2\cdot-}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
ODS	Octadecylsilicone
OZ	Opsonized zymosan
PAF	Platelet activating factor
PC	Phosphatidylcholine
PCA	Perchloric acid
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGD_2	Prostaglandin D_2

PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PI	Phosphatidyl inositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulphonylfluoride
PMN	Polymorphonuclear leukocyte
PPE	Porcine pancreatic elastase
$R^{\cdot}$	Free radical
RDS	Respiratory distress syndrome
RPM	Revolutions per minute
R.T.P	Room temperature and pressure
SAL	Saline
SLAPN	Succinyl-trialanyl-p-nitroanilide
SOD	Superoxide dismutase
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
TBHP	t-butyl hydrogen peroxide
Tc	Technetium
TCA	Trichloroacetic acid
TGF-b	Transforming growth factor b
TNF	Tumor necrosis factor
TRH	Thyrotropin releasing factor

CHAPTER 1

INTRODUCTION

1. Introduction.

During the last three decades, advances in neonatal intensive care have vastly improved the prognosis of infants born at an increasingly earlier stage in gestation. However, concurrent with these advances has been an increase in the incidence of neonatal acute lung disease (hyaline membrane disease: HMD). The management of this condition involves the use of mechanical ventilation and supplemental oxygen which are both believed to contribute to the development of a more persistent, chronic form of lung injury termed bronchopulmonary dysplasia (BPD). The exact pathological factors involved in the aetiology of BPD are unknown, although oxygen-induced pulmonary inflammation has been implicated as a primary candidate. However, to date, the exact mechanism by which this form of inflammation leads to the development of BPD is unclear. This is largely due to the ethical and practical problems associated with the study of these sick infants and also to the lack of suitable small animal models of prematurity in which to investigate possible pathogenic determinants of BPD. Consequently, the guinea pig model of prematurity was developed and in the present study the role of oxygen-induced inflammation in the development of acute and chronic injury to the immature lung was investigated.

1.1 Human lung development.

As infants are now delivered at increasingly earlier stages in gestation, a clearer understanding of the morphological and biochemical development of the lung is essential so that effective respiratory support can be offered following premature delivery. However, the problem in assessing normal lung development, is obtaining normal lung tissue. Although tissue obtained to date has come primarily from abortive fetus's or from infants that had breathed for varying periods of time before death, published studies now available offer a relatively clear picture of normal human lung development.

1.1.1 Structural development.

At about week four of gestation, the first indication of the future respiratory tree is a groove running lengthwise along the floor of the developing pharynx. This groove becomes a ridge and grows caudally to become a tube. The caudal section of this tube eventually becomes the trachea and the distal section develops two knob-like enlargements which grow to form the rest of the lung. As the trachea lengthens anterior to and parallel with the

developing oesophagus, mesenchymal cells surround the tube and gives rise to future connective tissue and cartilage. By week twelve, rudiments of the 16-20 'C'-shaped tracheal cartilages are present. Mucosal glands also develop at this point and the lining of the trachea becomes ciliated. This period of airway formation, termed the pseudoglandular stage is usually completed by about week 17 (Hislop and Reid, 1974). During this phase of development there is also dichotomous branching of the airway to reach some 15-26 generations (Bucher and Reid, 1961). The epithelium lining the developing lung is undifferentiated and capillaries show no specific relationship to the airways. At about the 19th or 20th week, primitive respiratory bronchioles develop (Inselman and Mellins, 1981) which are lined with undifferentiated cuboidal epithelial cells. By week 28, these bronchioles have subdivided and developed into transitional ducts and primitive saccules. A small number of alveoli can be identified at this stage as shallow depressions in the terminal airspaces. Type I and II pneumonocytes are differentiating (Campieche *et al.*, 1963), with surfactant detectable in type II cells (Meyrick and Reid, 1970). This period of development is also characterised by growth of capillaries towards the respiratory bronchioles and saccules to form the initial blood-air barrier. These changes close the canicular stage of development and from week 29 to term, alveolar development and proliferation occurs. Progressive development of the blood-air barrier and increased condensation of elastic tissue within the walls of airways and at the tips of developing alveolar septa also ensue. At birth, alveolar numbers may reach 100 million, a value approximately one third the number in the adult (Hislop *et al.*, 1986). Mature type II cells continue to secrete surfactant into the respiratory units, although adult surfactant composition is not reached until term (Rooney, 1985). Alveolarisation and growth of the lung continues during the early neonatal period until adult alveoli numbers are reached.

1.1.2 Biochemical development.

1.1.2.1 Pulmonary surfactant.

Pulmonary surfactant, secreted from epithelial type II cells, is a complex mixture of lipids, carbohydrates and proteins that coats the interior surfaces of the lung. Spreading over the alveoli, surfactant lowers the contractile force of the air-liquid interface and thereby reduces the tendency of alveoli to collapse during expiration (Clements, 1977). The majority of the lipid component of adult surfactant, isolated from bronchoalveolar lavage

fluid, is phospholipid (PL) (Figure 1.1). Phosphatidylcholine (PC) makes up 70-80% of the surfactant phospholipids and approximately 60% of PC contains two saturated fatty-acyl constituents. This disaturated PC (DSPC) is largely dipalmitoylphosphatidylcholine (DPPC) which is the major surface active component and responsible for the decrease in surface tension (Clements, 1977). The phosphocholine in DPPC is polar and hydrophilic, while the two palmitic acid residues are non-polar and hydrophobic. The choline part of the molecule associates with the liquid phase in the alveoli and the palmitic acids orientate towards the air. DPPC molecules become closely packed when the surface area is reduced, which confers stability to the surface film. The other two major PL classes include phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). Proteins comprise 5-10% of the total mass of surfactant of which three are non-serum derived. These proteins, SP-A, SP-B and SP-C are thought to have important structural and functional roles (Possmayer, 1988). The developmental changes in pulmonary surfactant are well documented in many animal species including man (Brumley *et al.*, 1967; Gluck *et al.*, 1972; Hunt *et al.*, 1991). The phospholipid composition of surfactant changes during late gestation, from a surfactant characterised by less PC, more phosphatidylinositol (PI) and no PG, to the surfactant characteristic of the adult lung which is enriched for DPPC and PG. Although the same changes in phospholipid composition occur with development in primates and rodents as they do in humans, human changes in pulmonary phospholipid composition occurs much earlier, with the major changes occurring over the last 30% of gestation (Clements and Tooley, 1977). The developmental changes in surfactant proteins are less clear. However, SP-A is not detected in amniotic fluid much before 30 weeks of gestation and rises during the third trimester in parallel with surfactant phospholipids (King *et al.*, 1975). As a

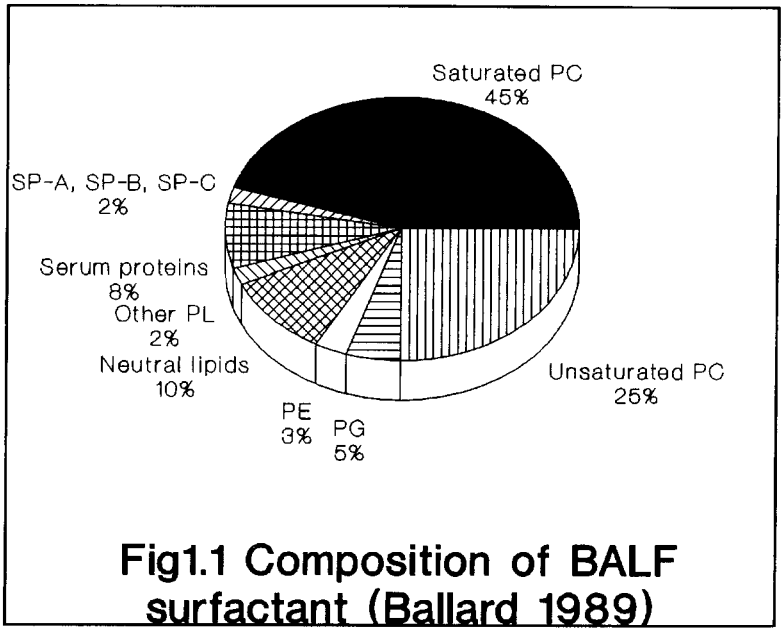


Fig1.1 Composition of BALF surfactant (Ballard 1989)

consequence of this late surge in surfactant production, infants born prematurely may have insufficient stores of this compound and therefore less able to maintain alveoli integrity. This would result in collapse of alveoli, a reduction in the gaseous exchange surface area, reduced oxygen uptake and the subsequent fall in blood PO_2 levels. This will result in a respiratory drive to increase oxygen uptake and the development of respiratory distress.

1.1.2.2 Pulmonary antioxidants.

At birth the fetus leaves a hypoxic uterine environment [PO_2 *in utero*.66-3,99 KPa (20-30mmHg)] to enter a relatively hyperoxic environment [13.3 Kpa(100mmHg)], which involves a 5-fold increase in oxygen concentration. This initial oxidative insult and the use of oxygen therapy for infants who develop respiratory distress as a consequence of prematurity is thought to pose a significant free radical mediated oxidative stress on the neonatal lung. Exposure of tissue to increased levels of oxygen results in an increase in the generation of reactive partially reduced species of oxygen (Freeman and Crapo, 1981; Turrens *et al.*,1982; Freeman *et al.*,1982). These include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$), all of which are capable of damaging tissue (Halliwell, 1991). To combat this potential oxidant threat, tissues have a variety of enzymatic and non-enzymatic antioxidants (Table 1.1) which are capable of preventing and minimising this damage. Included in the first line of defence are the non-enzymatic antioxidants. This group contains a vast array of compounds whose primary function may not one of antioxidant activity, such as albumin. It also contains compounds whose primary function is in the defence of free radical damage, such as vitamin E (Burton and Ingold, 1986). However, all are known to act as antioxidants to some degree and to protect tissue from free radical mediated injury.

Nonenzymatic antioxidants are found in all pulmonary cells and within the epithelial lining fluid (ELF) that coats the surface of alveoli (Pacht *et al.*,1986; Cantin *et al.*,1987; Pacht and Davis, 1988). Antioxidants within this fluid are the first to encounter oxidants, either taken in during respiration or following release of superoxide from resident inflammatory cells. The dominant small molecule in ELF is reduced glutathione (GSH) with levels in a normal adult lung being 150-250μM compared to $<5\mu$M in plasma (Cantin *et al.*,1987). Even higher levels, millimolar quantities, of this compound is found in pulmonary tissue (Jenkinson *et al.*,1988). Glutathione (L-τ-glutamyl-L-cysteinylglycine) is

Table 1.1 PULMONARY ANTIOXIDANTS

Catergory	Tissue site	Action
Enzymatic		
Catalase	Peroxisomes	The dismutation of H_2O_2.
Superoxide dismutase:-		
Cu/Zn	Cytosol and extracellular	The dismutation of $O_2^{\cdot -}$ to H_2O_2.
Mn	Mitochondrial	
Glutathione peroxidase	Cytosol and mitochondrial	The reduction of hydroperoxides
Glutathione reductase	Cytosol and mitochondrial	Reduction of low MW disulfides
Nonenzymatic		
Fat soluble.		
Vitamin E	Cell membranes and extracellular	Breaks lipid peroxidation chains.
ß-carotene	Membranes	Reacts with ROO.
Bilirubin	Blood and tissue	
Water soluble.	Intra and extracellular	
Vitamin C		Scavenges $O_2^{\cdot -}$ and .OH radicals. Regeneration of vitamin E.
Uric acid	Fluids	Scavenges $O_2^{\cdot -}$ and .OH.
Glutathione	Intra and extracellular	Substrate in glutathione redox cycle. Scavenges $O_2^{\cdot -}$

a tripeptide which performs a variety of physiological and metabolic functions which include the synthesis of proteins and DNA, transport, enzyme activity, metabolism and the detoxification of hydrogen peroxide, organic peroxides, free radicals and foreign compounds (Meister, 1982; Meister and Anderson, 1983). This detoxification aspect of GSH can operate in one of two ways. Firstly it can either act directly on free radicals (Travis, 1987) or as part of a redox cycling system involving glutathione peroxidase, glutathione reductase and the hexose monophosphate pathway (Fig 1.2). The most reactive group of GSH is the sulphydryl of the cysteinyl side chain. This group can serve as a donor of electrons and thereby act as a nucleophile, a reductant and a scavenger of free radicals. The reactions involving GSH as a reductant usually leads to the formation of glutathione disulphide (GSSG). Reactions with free radicals ($R^{\cdot}$), may give rise to sulphur-centred thiyl ($GS^{\cdot}$) radicals of GSH;

$$GSH + R^{\cdot} \text{ ----> } GS^{\cdot} + RH$$

These thiyl radicals ultimately give rise to GSSG and in the presence of oxygen, superoxide can form via the peroxysulphenyl radical ($GSOO^{\cdot}$). The superoxide anion can then be removed by the action of superoxide dismutase as described in section 1.1.2.2.1.

$$GS^{\cdot} + O_2 \text{ ------> } GSOO^{\cdot}$$
$$GSOO^{\cdot} + GSH \text{ ------> } GSSG + O_2^{\cdot -} + H^+$$

Other nonenzymatic antioxidants within ELF include vitamins C and E, lactoferin and plasma proteins including albumin, ascorbate, urate, caeruloplasmin and transferrin (Snyder *et al.*, 1983; Pacht *et al.*, 1986; Reynolds, 1987; Pacht and Davis, 1988; Davies and Pacht, 1991). Caeruloplasmin and transferrin do not interact directly with oxidants but are involved in the removal of free iron and copper which are remarkably good promoters of free radical reactions (Halliwell, 1987). The developmental profile of all these compounds in the lung during gestation has not been fully clarified and thus the susceptibility of the premature neonate to oxidative injury due to nonenzymatic antioxidants is unknown. As the majority of these molecules are blood born and enter the lung as a consequence of the semipermeable nature of the epithelial-endothelial barrier, several groups have assessed the

total antioxidant capacity of plasma (Sullivan, 1986; Sullivan and Newton, 1988; Karmazsin *et al.*, 1990; Miller *et al.*, 1993) at different periods during gestation and compared this with term and adult levels. In all these studies, plasma antioxidant capacity of preterm infants is lower than adults and the more premature the infant the lower the plasma's ability to withstand an oxidative insult. The second line of defence includes the enzymatic antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase (Autor *et al.*, 1976; Fryer *et al.*, 1986; Strange *et al.*, 1988; Strange *et al.*, 1990; McElroy *et al.*, 1990).

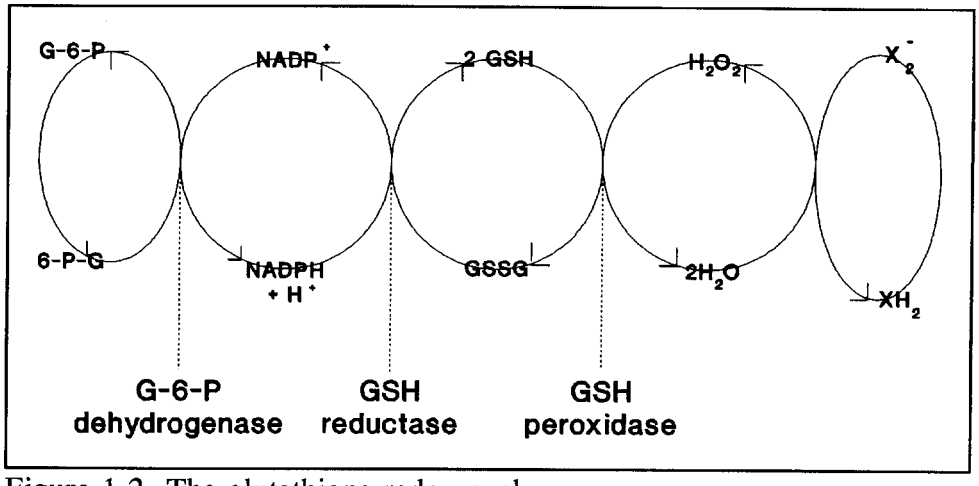


Figure 1.2. The glutathione redox cycle

As with nonenzymatic antioxidants these antioxidants are also found in all cells and ELF of the lung (Heffner and Repine, 1989) and include;

1.1.2.2.1 Superoxide dismutase (SOD).

Originally described as erythrocuprein, SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen as shown:

$$2O_2^{\cdot-} + 2H^+ \text{----------} > H_2O_2 + O_2$$

Four isoforms have already been identified (McCord and Fridovich, 1969) in aerobic organisms, two of which, Copper/Zinc (Cu/Zn) and Manganese (Mn) SOD are found in man (Marklund, 1980). Cu/Zn SOD is a 32Kd molecular weight enzyme consisting of two subunits each containing one copper and one zinc ion at the active site. Mn SOD is found

specifically in bacteria and the mitochondrial matrix of all animals (Keele *et al.*, 1970). This is a 40Kd molecular weight enzyme that consists of four subunits each with a Mn^{3+} ion at the active site. Unlike Cu/Zn SOD this enzyme is not inhibited by cyanide ions and being situated in the mitochondria is ideally located to remove superoxide anions generated by the respiratory chain. Both forms of SOD are found in numerous cell types within the lung and a unique extracellular Cu/Zn SOD has been located in ELF (Marklund, 1980).

1.1.2.2.2 Catalase (CAT).

Catalase, primarily located in peroxisomes (Tolbert, 1981) and identified in ELF (present as a result of cell death), removes hydrogen peroxide produced either metabolically or by the dismutation of superoxide:

$$2H_2O_2 \text{ ----------} > 2H_2O + O_2$$

Catalase is found in virtually all aerobic cells. It has a molecular weight of 240Kd and consists of four subunits each containing an Fe^{3+} protoporphyrin group (Deisseroth and Dounce, 1970).

1.1.2.2.3 Glutathione reductase (GSH-Rd).

Glutathione reductase plays a central role in the GSH redox system and is found in various tissue of animals and man (Meister, 1975). The primary function of this enzyme is to maintain glutathione in the reduced state by reduction of the disulphide (GSSG) at the expense of reducing equivalents of NADPH generated by the hexose monophosphate pathway (Fig 1.2).

1.1.2.2.4 Glutathione peroxidase (GSH-Px).

Glutathione peroxidase is a tetrameric selenoprotein composed of identical subunits (21Kd). Found in a multitude of organs such as liver, kidneys and lung (Flohe, 1982) it catalyses the reduction of a wide variety of hydroperoxides. It will protect biomembranes from attack by hydrogen peroxide or other hydroperoxides and in a concerted reaction with phospholipases and acylating enzymes, it may contribute to the repair of peroxidized phospholipids in biomembranes. The function of this enzyme depends on GSH generated

in the GSH cycle (Fig 1.2). Glutathione peroxidase may also be involved in the handling of the multitude of lipoxygenase products, all of which are hydroperoxides. The enzyme could therefore contribute to the metabolism of a variety of important mediators of inflammation such as the prostaglandins and leukotrienes.

1.1.2.2.5 Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH).

Although not specifically antioxidants, these enzymes are important by virtue of their catalysed reactions that supply NADPH for the regeneration of GSH (Jacob and Jandhl, 1966) in the GSH redox cycle. Cells deficient in G6PDH activity are sensitive to oxidative stress as demonstrated by the haemolysis that occurs in erythrocytes of patients with G6PDH deficiency (Cohen and Hocstein, 1961).

Due to the practical and ethical problems associated with obtaining healthy fetal lung tissue, few studies have described the pulmonary antioxidant status of human fetus's during development. The data accumulated so far contradicts many of the observations seen in animals. In animal studies, antioxidant enzyme activities increase progressively towards term (Tanswell and Freeman, 1984; Frank and Groseclose, 1984; Rickett and Kelly, 1990). However in humans only catalase activity has been observed to increase with gestation (Autor *et al.*, 1976; Fryer *et al.*, 1986; Strange *et al.*, 1988; McElroy *et al.*, 1990), the rest are expressed at a constant rate. The reason for this discrepancy between animal and human results is unclear, although the suitability of tissue used in human studies maybe partly to explain. The functional significance of the increase in pulmonary catalase activity in the absence of coordinated changes in the other enzymatic antioxidants is not presently understood. Catalase activity has been localised to peroxisomes in the developing lung. Although not well investigated, histological studies have shown that peroxisomes increase in number concomitant with an increase in cytosolic lamellar body stores of surfactant. In the study of McElroy *et al* 1992 , a strong correlation between tissue catalase and DPPC was observed during gestation and was hypothesised that peroxisomes may contribute to surfactant production or metabolism, catalase being present to mop up hydrogen peroxide generated.

1.1.3 Hormonal control of lung development

Numerous studies conducted over the past decade have established that fetal lung maturation is influenced by several endogenous hormones (Ballard, 1982). The mechanism of action for these compounds is largely unknown. However, to date there is extensive information regarding the role of glucocorticoid and thyroid hormones in pulmonary development. From the first observed association between glucocorticoids and lung development (Buckingham *et al.*, 1968) subsequent studies in several species including man have supported a role for steroids in lung growth and development (Liggins, 1976; Ballard *et al.*, 1977). During late gestation, both circulating fetal corticoids and thyroid hormones increase in concentration, an event that is temporally associated with surfactant release (Fisher *et al.*, 1977; Murphy *et al.*, 1980). Cortisol and cortisone from the adrenal cortex are produced as early as week 18 of gestation, with both adrenal size and steroid production increasing through out this period (Murphy, 1973). Cord blood cortisol levels start to increase around 35 weeks and continues until birth (Murphy *et al.*, 1980). Treatment of fetal lungs with glucocorticoids is associated with increased flattening of epithelial cells and thinning of tissue mesenchyme. Physiologically, steroids increase maximal lung volume, improve pulmonary compliance and increase surfactant release. This well documented effect of glucocorticoids on the surfactant system has been specifically located at the level of phospholipid synthesis. Although mechanisms are not yet established, some studies have demonstrated glucocorticoid-induced increases in the activity of both cholinephosphate cytidyltransferase and phosphatidic acid phosphatase (Rooney *et al.*, 1979), enzymes that normally increase in activity shortly after birth. It has also been proposed that corticosteroids act through specific receptors that stimulate *de novo* synthesis of specific proteins (Ballard and Ballard, 1972) and possibly through their actions in other tissues. A number of clinical observations support a relationship between glucocorticoids and the occurrence of respiratory distress. Respiratory distress is seen more frequently in infants delivered by caesarian section (Fedrick and Butler, 1972), labour being known to increase fetal cortisol levels (Murphy and Diez D'Aux, 1972). It is also more common in males than females with amniotic fluid lecithin/sphingomyelin (L/S) ratio and corticoid concentrations rising more slowly in males (Torday *et al.*, 1981).

A possible physiological role for thyroid hormones in fetal development is supported by a number of observations. Fetal serum T_3 levels increase during the final events of lung

maturation (Fisher *et al.*,1977) and T_3 receptors have been identified in fetal lung, apparently derived from type II cells (Lindenberg *et al.*,1978). Treatment of fetuses with thyroid hormone accelerates lung morphologic development (Wu *et al.*,1973). *In vitro* studies have also shown that thyroid hormones stimulate the rate of choline incorporation into PC (Smith and Torday, 1974) and intra-amniotic injection of T_4 stimulates the appearance of phosphatidylcholine in amniotic fluid (Mashiach *et al.*,1979), an effect thought to be mediated through binding to nuclear receptors and subsequent enzyme induction. In two clinical studies, low concentrations of T_3 and T_4 were associated with the occurrence of respiratory distress (Redding and Pereira, 1974; Cuestas *et al.*,1976). However, this was not confirmed in a later report (Hadeed *et al.*,1980). As a consequence of the roles of glucocorticoids and thyroid hormones in lung development during gestation, a recent clinical trial of the combined use of betamethasone and thyrotropin-releasing hormone (TRH) have demonstrated a significant reduction in the incidence of chronic lung disease in the preterm infants (Ballard *et al.*,1992).

Other hormones such as epidermal growth factor, Interferon-g, TGF-b and insulin may also have significant effects on lung development (Snyder and Mendelson, 1987; Whitsett *et al.*,1987; Ballard *et al.*,1988). A role for insulin in pulmonary development has been further supported by the observed low concentrations of SP-A in amniotic fluid samples from diabetic pregnancies (Katyal *et al.*,1984; McMahan *et al.*,1987; Snyder *et al.*,1988) and may account for the increased incidence of respiratory distress in infants of diabetic mothers.

1.1.4 Hormonal control of pulmonary antioxidant development.

As endogenous hormones have such a profound effect on pulmonary development, it is not surprising that development of the antioxidant enzyme system is also influenced by these compounds. As with many other aspects of pulmonary development, the majority of studies have concentrated on the role of glucocorticoids. Both *in vitro* and *in vivo* studies in rats have shown that treatment with dexamethasone increases the maturational rate of SOD, CAT and GSH-Px (Frank *et al.*,1985; Randhawa *et al.*,1986). Treatment of rats with metyrapone, an inhibitor of glucocorticoid synthesis, blunts this maturational process (Sosenko *et al.*,1986). The role of thyroid hormones is less clear. However, Tanswell *et al,* 1986 have shown that treatment of lung cell cultures with T_3 increased the activity of

Cu/Zn SOD without altering the activity of Mn SOD. Thus it would appear that maturation of the antioxidant system may be under tight hormonal control. As most of these studies have been performed in animals it is still unclear as to the role of these hormones in human fetal antioxidant development.

The preceding sections have shown that the development of the lung *in utero* is a highly regulated process and that infants born prematurely are likely to have lower concentrations of pulmonary surfactant, pulmonary enzymatic and circulating levels of antioxidants than term infants. Consequently, preterm neonates may not have functionally effective lungs and thus may be more susceptible to oxidative injury. The development of HMD is the initial outcome of these deficiencies. This form of acute lung injury may either be transient or develop into a more chronic form of injury, depending on the extent of immaturity of the organ. In the following sections the development of acute and chronic lung disease in the premature neonate will be described in more detail.

1.2 Prematurity and lung disease.

1.2.1 Hyaline membrane disease (HMD).

The development of respiratory distress in newborn infants describes the early onset of respiratory difficulty with tachypnea (>60 breaths/min), cyanosis, sternal retractions and grunting, hypoxemia and a chest radiograph showing a reticulogranular pattern (Joint Working Group of the British Association of Perinatal Medicine, 1992). The most common diagnosis of respiratory distress includes transitional respiratory distress, transient tachypnea of the newborn, hyaline membrane disease, aspiration syndromes, air-leak syndromes, persistent pulmonary hypertension and pulmonary hypoplasia (Robertson, 1985). HMD (also known as idiopathic respiratory distress syndrome or type I respiratory distress syndrome (RDS), is an acute form of lung injury which occurs almost exclusively in the preterm infant. The development of HMD occurs immediately after birth and the management of the condition is primarily directed at reversing the development of hypoxia. This is initially achieved by the use of supplemental oxygen, but in more severe cases mechanical ventilation is often required. Over the first 7 days of life infants who develop HMD will follow one of two clinical paths. The majority (75%) will recover, the rest will require more prolonged treatment. Those infants who do require prolonged treatment may

develop a more persistent, chronic form of injury termed bronchopulmonary dysplasia (BPD) (Robertson, 1985).

1.2.1.1 The pathology of HMD.

The primary pathophysiological abnormality associated with HMD is a deficiency in pulmonary surfactant (Lauweryns, 1970; Tomashefski *et al.*, 1985). The consequence of this is a failure of the infant to maintain alveolar integrity and for large areas of the lung to collapse. This results in a reduction in gaseous exchange and the subsequent development of hypoxia. Although a deficiency in pulmonary surfactant is the primary abnormality in the development of HMD, other secondary abnormalities may be present and participate in the initiation and progression of the disease (Fig 1.3) .

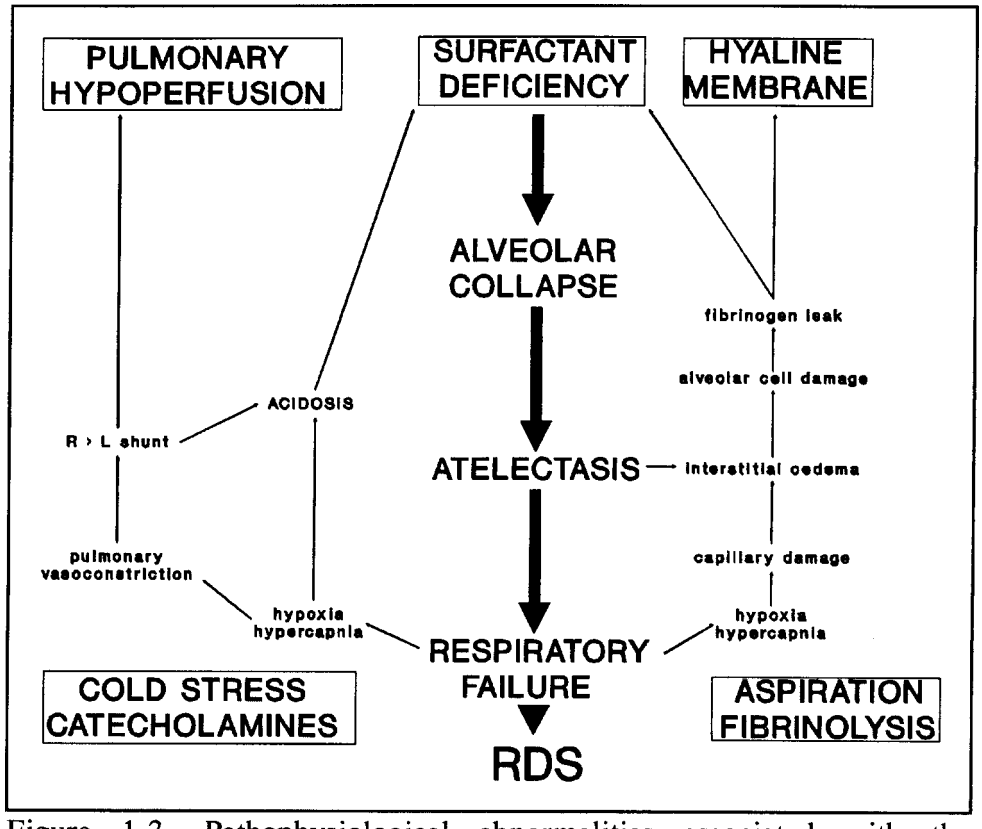


Figure 1.3. Pathophysiological abnormalities associated with the development of HMD (Adapted from Campbell *et al* 1976)

Clinical and physiological studies have shown that HMD starts at birth and runs most of its course over the first 3-5 days of life. Initially, death of the very premature infant

immediately follow birth (< 6hr) is primarily due to respiratory distress with little evidence of hyaline membrane formation. Infants who die at this early stage usually have anatomically immature lungs which are unable to support life, regardless of sufficient surfactant stores. The pathological findings of infants who have survived for 6hr or more include widespread atelectasis and the presence of hyaline membranes in the more central regions of the lung. These membranes consist of layers of eosinophilic staining material which tend to become thicker the longer the infant survives. They contain cellular debris (Esterly *et al.*, 1966; Lauweryns, 1970; Finlay-Jones *et al.*, 1974), plasma proteins (Gitlin and Craig, 1955; Van Breeman *et al.*, 1957), nucleic acids, lipids (Gregg and Bernstein, 1961) and iron (Finlay-Jones *et al.*, 1974). Macroscopically the lungs are heavy, do not float in water (Lauweryns, 1970) and cannot be inflated under normal transbronchial pressures. Ultrastructurally, cellular damage is seen as vacuolization of capillary endothelium and sloughing of epithelium into the airway lumen (Gandy *et al.*, 1970; D'Ablang *et al.*, 1975; Merritt *et al.*, 1981). Lungs with HMD are also highly permeable to solutes (Jefferies *et al.*, 1984) and as such are invariably characterised by pulmonary oedema (Desa, 1969) despite normal left atrial pressures (Rudolph *et al.*, 1961). Infants who survive this acute stage of lung injury show evidence of repair with increased numbers of type II cells lining the denuded parts of the alveoli (Boss and Craig, 1962).

The pathological features in fatal cases of HMD are similar in many ways to oxygen induced diffuse alveolar damage (DAD) of adult humans. Although there is some degree of pathological similarity between the two, there are many qualitative and quantitative differences too. Atelectasis is a predominant feature of HMD but a more variable secondary component of hyperoxic induced DAD. This is undoubtedly due to the fact that surfactant deficiency is a primary facet of HMD, whereas altered surfactant function is only an epiphenomenal feature of oxygen toxicity. Although described as thickened (Lauweryns, 1970), or congested and dilated with increased pinocytic vesicles (Gandy *et al.*, 1970), capillaries do not appear to be a prime site of pathology in HMD, whereas the capillary endothelium is a consistent target of oxygen toxicity. Although it is clear that HMD may not be initiated by hyperoxia, it has not been established that the pathological alterations of the lungs observed in fatal cases at autopsy are entirely unrelated to antecedent oxygen therapy.

1.2.1.2.Incidence and mortality from HMD.

As a consequence of improvements in intensive care techniques, mortality from RDS among preterm newborns has decreased in recent years. However, RDS is still a major cause of morbidity and mortality in newborns, with approximately 1% of all infants worldwide developing this disease (Farrell and Avery, 1975). The clinical risk factors associated with the development of RDS include gestational age (Usher *et al.*,1971), perinatal asphyxia (Jones Jr *et al.*,1975), sex of the infant (Lankenau, 1976), route of delivery (Usher *et al.*,1964) and previous delivery of a premature infant with RDS (Graven and Misenheimer, 1965).

1.2.2 Bronchopulmonary dysplasia (BPD).

Infants who do not recover from HMD within the first 7 days and who require prolonged intensive and supportive therapy are often destined to develop a more chronic form of lung injury termed BPD. First recognised by Northway and colleagues in 1967, this form of unresolved lung disease most likely represents a nonspecific reaction of the lung to a slowly resolving acute lung injury. As such, BPD may develop in individuals receiving high concentrations of oxygen by mechanical ventilation for diseases other than respiratory distress (Barnes *et al.*,1969; Churg *et al.*,1983). However, the evolution of BPD from HMD in premature neonates is by far the most common.

1.2.2.1 The pathology of BPD.

The pathological abnormalities associated with BPD are based on autopsy data and thus represent the more extreme end of the disease spectrum. Detailed descriptive pathological studies of BPD reveal a chronological progression from an initial exudative stage of DAD characteristic of HMD to a regenerative stage of repair. Macroscopically the lungs have a grossly abnormal appearance. They are firm, heavy with an irregular surface demonstrating alternating area's of emphysematous and atelectatic lesions (Northway *et al.*,1967; Taghizadeh and Reynolds, 1976; Stahlman, 1979; Sobonya *et al.*,1982). There is also widespread bronchial and bronchiolar mucosal hyperplasia and metaplasia that reduce the lumen in many of the small airways. Except for the hypertrophy of peribronchiolar smooth muscle that persists throughout the disease, the involvement of the small airways is more prominent during the early stages of the disease. In addition there is interstitial oedema and

an increase in fibrous tissue with focal thickening of the basal membrane separating capillaries from alveolar spaces. The lymphatics are frequently dilated and in many cases there are vascular changes resulting from pulmonary hypertension, such as medial muscle hypertrophy. Recent examination of tracheobronchial aspirates demonstrate large numbers of exfoliated bronchial epithelial cells (D'Ablang *et al.*, 1975; Merritt *et al.*, 1981; Rothberg *et al.*, 1986) with prominent chromocenters, suggestive of cell regeneration.

1.2.2.2 Incidence and mortality from BPD.

Most reports on the incidence and mortality of BPD are retrospective. Information from this type of study is limited due to the use of differing criteria for the diagnosis of BPD, the type of institution, the retrospective nature of the studies, different patient populations, changing survival rates with time and differences in clinical management of the disease. Using the original diagnosis of Northway and colleaques, 1967, the incidence of BPD varies between 2.4% and 68%. However, the criteria of Bancalari *et al*, 1979 is now generally accepted. These include the use of intermittent positive pressure ventilation (IPPV) for three or more days during the first week, clinical signs of respiratory disease, including tachypnea and sternal retractions, for more than 28 days, supplemental oxygen for more than 28 days and a chest X-ray with evidence of persistent radio-dense strands with areas of increased radiolucency. Using these criteria, between 2.5 and 4.2% of all infants requiring ventilatory support develop BPD (Bancalari *et al.*, 1979; Mayes *et al.*, 1983). Saigal and colleagues, 1984 prospectively evaluated the outcome of infants born live with birth weights less than 1000g from a geographically defined region. One hundred and seventeen of 225 infants survived of which 45% developed BPD. In another retrospective study of data from 1973 to 1978, the incidence of BPD in HMD survivors was less than 10% in infants weighing greater than 1500g and was 20 to 40% in infants weighing 1000 to 1500g (Tooley, 1979), indicating that birth weight and hence the extent of prematurity is an important risk factor in the development of BPD. Mortalities in the first year range from 30 to 60% in infants born less than 1250g (Mayes *et al.*, 1983; Lindroth and Mortensson, 1986). Following weaning from their respiratory support, mortality rates at discharge from hospital varies between 11 and 20% (Mayes *et al.*, 1983; Shankaran *et al.*, 1984; Sauve and Singhal, 1985).

1.2.2.3 Long term prognosis of infants with BPD.

Although there is usually a rapid resolution of the disease during the first two years of life (Lindroth and Mortensson, 1986), these infants remain at high risk from respiratory infections (Mayes *et al.*, 1983) such as respiratory syncytial virus (O'Brodovich and Mellins, 1985) which often necessitates readmission to hospital and ventilatory support. Other complications of BPD include increased airway resistance (Coates *et al.*, 1977), bronchial hyperactivity (Smyth *et al.*, 1981), cardiovascular disease, delayed growth and poor neurodevelopmental outcome (Mayes *et al.*, 1983; O'Brodovich and Mellins, 1985; Lindroth and Mortensson, 1986). Data concerning the longer term effects of BPD is limited, but most infants who survive to become adolescents and young adults develop some degree of pulmonary dysfunction consisting of airway obstruction, hyperactivity and hyperinflation (Northway *et al.*, 1990).

1.2.2.4 The aetiology of BPD.

There are a number of factors that may have a bearing on the development of BPD, including intubation, the degree of lung immaturity, the severity of HMD and sex of the infant (Edwards *et al.*, 1977; Goetzman, 1986; Avery *et al.*, 1987; Kraybill *et al.*, 1989). As such the aetiology of BPD can not be satisfactorily explained by any one factor and probably results from the interaction of several (Mayes *et al.*, 1983; Lindroth and Mortensson, 1986). However, the condition is almost exclusively found in infants who are receiving mechanical ventilation with high concentrations of supplemental oxygen. As such barotrauma and oxygen toxicity are believed to be principal contributing factors (Rhodes and Hall, 1975; Edwards *et al.*, 1977; Heimler *et al.*, 1988; Kraybill *et al.*, 1989). This has been recently confirmed in a retrospective multivariate regression analysis of 412 infants observed over a 10 year period, from 1980 to 1990 (Iqbal *et al.*, 1989). This study showed that over this period there was a significant reduction in the risk of BPD which was largely accounted for by a decrease in the duration of ventilation and supplemental oxygen.

1.2.2.4.1 Mechanical ventilation.

Although prolonged periods of positive pressure ventilation in adults does not induce detectable pulmonary damage (Splaingard *et al.*, 1983) this is not the case for the premature neonate (Fitzhardinge *et al.*, 1976; Lee *et al.*, 1984). The use of positive pressure and

positive end expiratory pressure to ventilate the poorly compliant lungs of the premature infant invariably causes damage. A published survey of 1625 infants in eight neonatal centres concluded that the comparatively low incidence of BPD in one of the centres was likely due to the reduction in the reliance of mechanical ventilation (Avery *et al.*, 1987). A year later, a retrospective case study of 99 infants found that intubation and mechanical ventilation were the most powerful predictors of the later development of the disease, which was independent of oxygen concentration and gestation (Heimler *et al.*, 1988). However, in a recent report of the Joint Working Group of the British Association for Perinatal Medicine (Joint Working Group of the British Association of Perinatal Medicine, 1992) no consensus on the ventilator management of RDS was arrived at and it was suggested that further controlled clinical trials be undertaken.

1.2.2.4.2 Supplemental oxygen.

The rationale for implicating oxygen in the development of BPD has largely been based on correlations between the duration and pressures of hyperoxia with the occurrence and predictability of the disease (Northway *et al.*, 1967; Frank *et al.*, 1978). In association with the fact that preterm infants may have insufficient pulmonary antioxidant protection and that hyperoxic exposure results in increased free radical flux within the lung, oxygen has been further implicated in the development of BPD.

In the original description of BPD (Northway *et al.*, 1967), it was concluded that oxygen exposure was the single most important determinant of the development of lung disease in the preterm neonate, an observation that has been confirmed in later studies (Nash *et al.*, 1967; Rhodes and Hall, 1975; Edwards *et al.*, 1977; Bonnet *et al.*, 1983). Biochemically, a role for oxygen in BPD has also accumulated over the years. The activity of plasma and lung fluid alpha-1-proteinase inhibitor (α1PI), the major inhibitor of neutrophil elastase, is significantly lower in preterm infants receiving oxygen therapy than in infants not given oxygen (Bruce *et al.*, 1981). Activity also directly correlates with the concentration and duration of oxygen administered (Bruce *et al.*, 1982) and also with the incidence of BPD (McCarthy *et al.*, 1984; Rosenfeld *et al.*, 1986). As neutrophil elastase activity is increased in the lungs of these infants (Gerdes *et al.*, 1988; Walti *et al.*, 1989), it has been suggested that exposure to high concentrations of oxygen results in to an imbalance in the protease-antiprotease profile of the lung leading to tissue damage (Gerdes *et al.*, 1988; Walti *et*

al., 1989). This has been further supported by an observed increase in urinary desmosine excretion, a byproduct of elastin degradation, in infants exposed to high concentrations of oxygen (Bruce *et al.*, 1985).

1.2.3 Treatment procedures for HMD and BPD.

At present there are three major strategies that are employed to prevent the development of BPD. The first is to prevent premature birth through improved obstetrical follow up and care. The second is to administer treatment to the fetus that accelerates fetal lung development such as glucocorticoids and thyroid hormones. The third is to optimise the postnatal treatment of infants who develop HMD through the availability of novel medications and techniques. For example, endotracheal administration of exogenous surfactant and antioxidants, artificial ventilation and extracorporeal membrane oxygenation. However, in those infants who develop BPD there is no reliable treatment once the disease is established, except for the maintenance of adequate oxygenation, temperature homeostasis and nutrition. The major emphasis on treatment therefore is in prevention.

1.2.3.1 Surfactant replacement.

The clinical potential of surfactant treatment for respiratory distress was first demonstrated by Fujiwara *et al.*, 1980 . A synthetic surfactant was prepared from an organic solvent extract of bovine lung (surfactant TA) from which 10 infants with severe disease were treated. Up to 1985 small randomized controlled trials using several different surfactant preparations have demonstrated significant decreases in pneumothorax and death associated with premature delivery (Hallman *et al.*, 1985; Enhorning *et al.*, 1985; Shapiro *et al.*, 1985). By 1992, 35 randomized controlled trials have been reported and the results reviewed by Jobe, 1993 . Two strategies were evaluated, one a prophylactic treatment in the delivery room to prevent the development of respiratory distress and lung injury and the other after a diagnosis of RDS was confirmed. The analysis also evaluated the effects of synthetic and natural surfactants. Overall the results demonstrated that there was a 30 to 40% reduction in mortality by 28 days of age with a significant reduction in air leaks (pneumothorax and pulmonary interstitial emphysema). However, the incidence of BPD was not lowered and it was suggested that this may have been due to different diagnostic criteria used to classify BPD.

1.3.3.2 Hormonal therapy.

The concept of accelerated fetal lung maturation first appeared in 1969, when Liggins noted that infusion of dexamethasone in pregnant ewes had a stimulatory effect on lung aeration of prematurely delivered lambs (Liggins, 1969). To date there are numerous controlled trials which provide clear evidence that corticoid administration to women at risk from early delivery reduces the risk of RDS by 50%, a reduction which is independent of gestational age and gender. The first reports describing the use of steroids in human infants with BPD was in 1974 (Philip, 1974). Marked pulmonary improvement had occurred in two infants given corticosteroids for reasons other than respiratory distress. Several uncontrolled trials followed (Pomerance and Puri, 1982; Donn *et al.*, 1983; Schick and Goetzman, 1983) which have reported short term improvements in pulmonary function. In the first controlled double blind cross-over study (Mammel *et al.*, 1983), treatment with dexamethasone at 0.5mg/Kg/day demonstrated a significant improvement in ventilator-determined respiratory rate, peak inspiratory pressure, fractional inspired oxygen concentration and alveolar-arterial oxygen gradients within 3 days of therapy. However, in this and subsequent trials there have been concerns about possible side effects including the risk of infection, systemic hypertension and adrenal suppression (Katz and Murphy, 1988).

1.2.3.3 Antioxidant therapy.

Another strategy which is under assessment is the use of antioxidants for treatment of HMD and BPD. Vitamin E is the most widely studied antioxidant and in the early work of Ehrenkranz *et al* in 1978 , vitamin E supplementation was shown to decrease the incidence of BPD in infants with RDS. However, later more detailed studies, did not confirm the initial promise of this vitamin as a protective agent (Ehrenkranz *et al.*, 1979; Abbasi *et al.*, 1980; Saldanha *et al.*, 1982). Another antioxidant investigated has been SOD. In a randomised double-blind trial of 45 infants the ability of SOD to prevent the development of BPD in premature neonates with RDS was assessed (Rosenfeld *et al.*, 1984). Patients treated with SOD required fewer days of continuous positive airways pressure ventilation (CPAP) and were less likely to develop chest radiographic and clinical (wheezing, pneumonia) abnormalities characteristic of BPD than control infants. Although the study was positive the results have yet to be confirmed and the potential obstacles of SOD treatment evaluated.

1.3 Oxygen toxicity.

Thus, infants who are born premature are more likely to develop acute (RDS/HMD) and chronic (BPD) lung disease than term neonates. These infants therefore require supplemental oxygen to maintain adequate blood PO_2 levels. Increased oxygen exposure leads to increased free radical flux and tissue damage. It is not surprising therefore, that this form of therapy is a known pathogenic determinant in the development of BPD. However, the exact mechanisms by which exposure to elevated concentrations of oxygen leads to tissue injury in these infants is not fully understood. As such a clear understanding of changes associated with oxygen-induced tissue damage needs to be assessed. In the next few sections this will be addressed.

Oxygen is toxic to aerobic organisms above normal atmospheric partial pressures and concentration (Bean, 1945). High concentrations and pressures of oxygen are used in a variety of clinical and nonclinical settings in addition to the treatment for RDS and BPD. Hyperbaric exposure is experienced in *sub aqua* diving, and normobaric hyperoxic exposure is used in the treatment of various disorders, such as the adult respiratory distress syndrome (ARDS). In all these situations it is the lung that is the first organ exposed to the high concentrations and pressure of oxygen. Exposure of normal healthy adults to high concentrations of oxygen results in symptomatic, physiological and anatomical changes in the lung. These changes include coughing, chest soreness, pulmonary oedema, increased physiological dead space, arteriovenous shunting with reductions in vital capacity and lung compliance and pulmonary inflammation (Comroe *et al.*,1945; Giammona *et al.*,1965; Caldwell *et al.*,1966; Nash *et al.*,1967; Burger and Mead, 1969). These changes remained unexplained until 1954, when it was hypothesised that the toxic effects of oxygen exposure was the result of an excessive production of oxygen centred free radicals (Gerschman *et al.*,1954). This theory was established primarily as the result of observed similarities between the toxic effects of oxygen and the toxic effects of ionising radiation. Ionising radiation acting on aqueous solutions, results in the generation of hydrogen and hydroxyl radicals, which can then react further with molecular oxygen to produce a variety of oxygen centred free radicals. This hypothesis was later strengthened by the superoxide theory of oxygen toxicity, which suggested that the formation of superoxide radicals *in vivo* largely accounted for the toxic effects of oxygen (Fridovich, 1975; Fridovich, 1978). In the years following these initial observations, the role of free radicals in oxygen-induced lung injury

has been firmly established (Heflin and Brigham, 1981). Recently the superoxide and hydroxyl radicals have become directly measurable in oxygen-induced cells by electron paramagnetic resonance spectroscopy (Zweier *et al.*, 1989).

1.3.1 Free radicals.

A free radical is defined as any molecular species containing one or more unpaired electrons (Halliwell and Gutteridge, 1984). These radicals can be formed by one of two mechanisms, either by absorption of radiation (1) or by the effect of redox reactions (2).

1) Homolytic bond fission: $AB \text{ ----------> } A^{\cdot} + B^{\cdot}$
2) Electron transfer: $A^{-} + B \text{ ---------> } A^{\cdot} + B^{\cdot -}$

1.3.1.1 Oxygen centred free radicals.

Molecular oxygen can undergo a stepwise reduction to yield a series of reactive oxygen radicals species (Fig 1.4). Addition of a single electron to oxygen generates the superoxide anion.

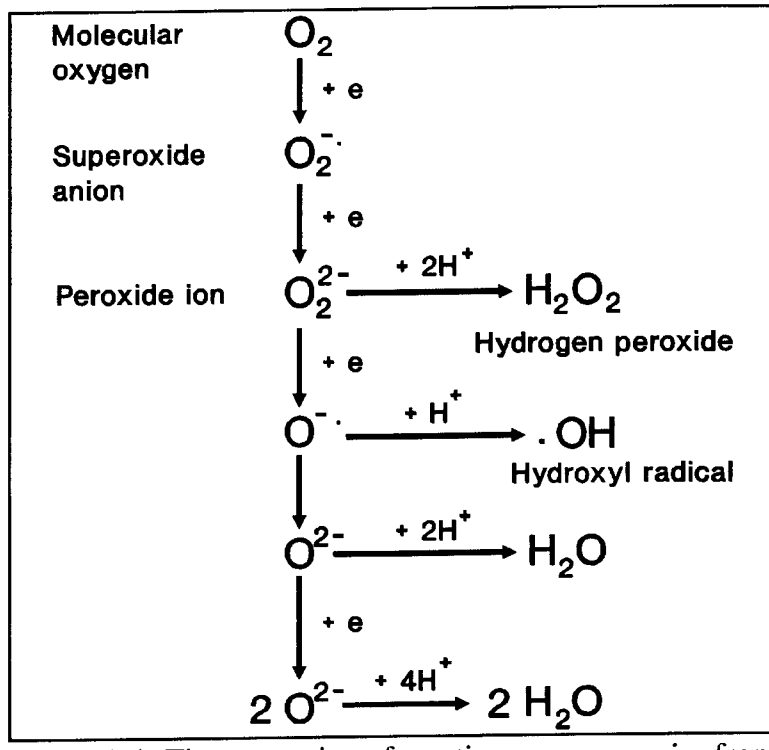


Figure 1.4. The generation of reactive oxygen species from molecular oxygen.

This radical can be further reduced to yield the peroxide anion which can then immediately protonate to form hydrogen peroxide (Slater, 1984; Halliwell and Gutteridge, 1984). Further reduction and protonation of the peroxide ion may generate the more potent reactive oxygen species, including the hydroxyl radical (Slater, 1984; Massaro, 1986).

1.3.1.2 Sources of oxygen centred free radicals.

Following the discovery in 1968 of SOD, an enzyme specific for a free radical substrate (McCord and Fridovich, 1969), it became clear that reactive oxygen species and free radicals were possibly formed in living organisms. Apart from the direct production of free radicals by tissue during exposure to hyperoxia, there are several biological molecules and systems that are known to generate oxygen centred free radicals. These include glyceraldehyde, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), adrenalin (Fridovich, 1981), the oxidation of iron in haemoglobin (Gutteridge, 1986) and also the activation of the respiratory burst oxidase in inflammatory cells (Morel *et al.*, 1991). Another important source of superoxide radicals is the respiratory chain. Electrons passing through the chain may 'leak' from protein carriers and pass directly onto molecular oxygen. This results in the reduction of oxygen and the formation of superoxide. Production of reactive oxygen has been detected in *in vitro* studies using whole rat lung and rat lung mitochondria preparations (Freeman and Crapo, 1981). Further investigations have shown that superoxide is generated at the NADH dehydrogenase and ubiquinone-cytochrome C level of the respiratory chain (Turrens *et al.*, 1982).

During exposure to high concentrations of oxygen, hydrogen peroxide is also generated in tissue (Lazarow and DeDuve, 1976; Turrens *et al.*, 1982). This occurs by the spontaneous or catalytic dismutation of superoxide according to the following reaction:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{----------} > H_2O_2 + O_2^{\cdot-}$$

The superoxide radical and hydrogen peroxide are generated in normal and oxygen exposed tissue and are toxic to cells at very high concentrations. However, current evidence suggests that these molecules pose little threat to the cell, at concentrations generated in tissue, by their direct action (Fridovich, 1986). Much of the damage induced by hyperoxia is thought to be dependant on the actions of the hydroxyl radical (Halliwell and Gutteridge,

1984). There is little direct evidence for the production of this radical in oxygen exposed tissue, although intracellular conditions favour its production. The hydroxyl radical can be generated via the Fenton reaction in which ferrous ions produce ·OH from hydrogen peroxide by a single electron reduction according to the reaction:

$$H_2O_2 + Fe^{2+} \text{----------} > Fe^{3+} + OH^- + OH\cdot$$

To date, considerable evidence exists implicating oxygen centred free radicals in a wide spectrum of diseases (Klebanoff and Clark, 1975; Fridovich, 1975; Hafeman and Lucas, 1979; Fridovich, 1978; Thorne *et al.*, 1980; Johnson *et al.*, 1981) and in which inflammation is a major component.

1.4 Inflammation.

Inflammation can be best described as 'the reaction of vascularized living tissue to injury'. This response is closely intertwined with the process of repair. Inflammation serves to destroy, dilute and contain injurious agents, but in turn sets in motion a sequence of events to heal and repair the damaged tissue. However, both these processes may be potentially harmful. Inflammation may be acute or chronic, depending on certain qualifications. Acute inflammation is relatively short lived (1 min-3 days), with the main characteristics being exudation of fluid and plasma proteins and the emigration of leucocytes, predominantly neutrophils into the injured tissue. Regardless of the nature of the injurious agent, acute inflammation is usually stereotypic. Chronic inflammation, on the other hand, is less uniform, of longer duration and is associated histologically with the presence of lymphocytes, macrophages and the proliferation of blood vessels and connective tissue.

1.4.1 Acute and chronic inflammation.

The local and clinical signs of acute inflammation include pain (dolor), fever (calor), redness (rubor), swelling (tumor) and loss of function. These signs of the inflammatory response occur as the result of; 1. changes in vascular flow and calibre; 2. changes in vascular permeability, and; 3. leucocyte exudation. These three reactions usually overlap and share common mechanisms. First there is a transient vasoconstriction of arterioles,

which depending on the severity of the injury, may last from a few seconds to several minutes. This is followed by arteriole vasodilatation and flow of blood to new microvascular beds in the area of injury. At this stage the increased blood flow through the injured tissue may result in a fleeting transudation of protein-poor fluid into the extravascular space. Increased permeability of the microvasculature with increased out-pouring of protein-rich fluid slows the circulation through the effected area. Histologically the area is typified by the presence of dilated small vessels packed with red blood cells, a condition termed stasis. Finally, as stasis develops peripheral leucocytes, the majority being polymorphonuclear leucocytes (neutrophils: PMN), begin to adhere and migrate through the vascular wall into the interstitial tissue. This event is controlled by a host of compounds secreted by affected tissue and by blood cells that arrive at the site of injury. These compounds orchestrate the response of the organism to the injury and are collectively called mediators of inflammation. Acute inflammation may have one of four outcomes, (1) resolution, (2) healing by scarring, (3) abscess formation or (4) chronic inflammation. The histological hallmarks of chronic inflammation are (1) infiltration of the tissue by mononuclear cells, principally macrophages, lymphocytes and plasma cells; (2) proliferation of fibroblasts and in many instances small blood vessels; (3) tissue destruction and (4) increased connective tissue (fibrosis). The infiltration of monocytes into the tissue is a particularly important component of chronic lung inflammation. Monocytes begin to emigrate relatively early in the acute phase of inflammation and within a 48-72 hours may become the predominant cell type. The macrophage is a central figure in chronic inflammation because of the great number of biologically active products it can produce (Nathan, 1987). Some of these products are directly toxic to tissue (oxygen metabolites, proteases), others cause the influx of other inflammatory cells and still others cause fibroblast proliferation and collagen deposition. All these products will contribute to progressive tissue damage and to the consequent functional impairment.

1.4.1.1 Cellular events during inflammation.

The accumulation of neutrophils is one of the most important features of an inflammatory reaction. Although these cells engulf and degrade bacteria, immune complexes and cellular debris they may also prolong the inflammatory reaction and increase tissue injury by the very same mechanisms that are used during these defensive procedures. The sequence of

accumulation of neutrophils can be divided into (1) margination and adhesion, (2) emigration towards a chemotactic stimulus, (3) phagocytosis and intracellular degradation and (4) extracelluar release of leucocyte products. As blood flow through the injured tissue is reduced, white blood cells begin to come into contact with the endothelium. These cells first tumble along the walls of endothelial cells and eventually come to rest, such that in the area of injury, most if not all of the endothelial cells are lined with these cells. Recent evidence suggests that specific adhesion molecules are expressed on the endothelial cell surface which complement similar molecules on the leucocytes (Bevilacqua, 1987; Dustin and Springer, 1988). The surface expression of these molecules are induced, enhanced or altered by a variety of inflammatory mediators, including leukotriene B_4 (LTB_4), platelet activating factor (PAF), interleukin-1 (IL-1), tumor necrosis factor (TNF), and complement derived protein 5a (C5a). Certain inflammatory mediators such as C5a stimulate a rapid rise in these proteins on the surface of leucocytes (Harlan, 1985) and IL-1 stimulates the production of adhesion proteins on endothelial cells (Bevilacqua, 1987). TNF a cytokine pivotal in the development of inflammation increases the adhesion molecules on both the leucocyte and endothelial cell (Dustin and Springer, 1988). As certain mediators increase expression others may serve to terminate the adhesive events (Wheeler, 1988). The importance of these receptors is shown in the existence of a genetic deficiency in leucocyte adhesion proteins which is characterised by recurrent bacterial infections and impaired leucocyte adhesion (Anderson and Springer, 1987).

Following adhesion, leucocytes move to the junctions between endothelial cells and insert large pseudopods (Marchesi, 1961). After crawling between these cells they eventually assume a position between the endothelial cell and the basement membrane. Depending on the strength and type of chemotactic signals in the vicinity, leucocytes may remain at this site for a short period or traverse the membrane and escape into the extravascular space. The cell type within the inflammatory lesions may vary with the age of the inflammatory event and the type of stimulus released. In most types of acute inflammation the neutrophil predominates but is replaced by the macrophage at a much later time point. Both exogenous and endogenous substances can act as attractants for inflammatory cells (Becker and Ward, 1980). The most significant chemotactic factors for neutrophils include, bacterial products, components of the complement system and products of the lipoxygenase pathway of arachidonic acid, particularly LTB_4 (Ford-Hutchinson *et al.*, 1980). Inflammatory cells

contain specific receptors for the major chemotactic factors and once occupancy of at least 20% of the receptors occur, a series of intracellular events develop that triggers the assembly of contractile elements responsible for cell movement. Essentially, occupancy of receptors activates phospholipase C which results in the hydrolysis of a membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes the release of calcium from intracellular stores, and it is this increase in cytoplasmic calcium that triggers the assembly of contractile elements responsible for cell movement (Stossel, 1985; Stossel, 1988). Increased intracellular calcium also activates phospholipase A_2 (PLA_2), which triggers the formation of arachidonic acid metabolites. In addition, DAG, through its activation of protein kinase C, is involved in various phases of leucocyte degranulation and secretion (Sha'afi and Molski, 1988). The phagocyte moves by extending pseudopods in the direction of the chemotactic agents and pulls the remainder of the cell towards it. The movement is the result of rapid association and dissociation of actin fibres, controlled by intracytoplasmic calcium gradients (White *et al.*, 1983).

Phagocytosis, degranulation and cellular activation of the respiratory burst constitutes the major benefits and disadvantages that are derived from the accumulation of phagocytes at an inflammatory loci (Silverstein, 1977; Becker, 1987). Phagocytosis involves three distinct but interrelated steps. The first is attachment to the leucocyte. Most bacteria or extraneous matter are not recognised until they are coated by naturally occurring serum factors called opsonins. The two major opsonins are IgG, a natural antibody already present against foreign matter, and C3b a factor generated by activation of complement by immune or non immune mechanisms (Unkeless and Wright, 1988). Opsonised particles then attach to specific receptor sites on the leucocyte and are then engulfed. Engulfment is the second step in phagocytosis. Extensions of the leucocyte membrane flow around the particular object to be engulfed and fuse to form a vacuole containing the opsonised particle. This membrane then fuses with intracellular lysosomal granules releasing their contents which eventually digest and destroy the particle. The final step is killing and degradation. There are two independent mechanisms by which this is achieved. The first is the oxygen-dependant bactericidal mechanism. The observation that phagocytic cells produce toxic metabolites of oxygen was first reported in 1961 (Iger *et al.*, 1961) using a system in which hydrogen peroxide was detected in the medium of activated neutrophils. With this information and

the fact that neutrophils exposed to opsonised microorganisms consumed oxygen from the surrounding buffer in a non-mitochondrial (cyanide insensitive) event (Baldridge and Gerard, 1933; Sbarra and Karnovsky, 1959), initiated the proposal that the oxygen consumed in phagocytosis was converted to an agent capable of killing the ingested microorganisms. The respiratory burst occurs in all so-called professional phagocytes, with the intensity of the activation depending on the cell type, animal species, and for macrophages, on the functional state of the cell (Sklar *et al.*, 1981; Nathan, 1982). During phagocytosis several intracellular events occur which include, a burst in oxygen consumption, glycogenolysis, increased glucose oxidation via the hexose-monophosphate shunt, and production of active oxygen metabolites (Silverstein, 1977; Babior, 1984). The generation of oxygen metabolites is due to the activation of an oxidase (NADPH oxidase) that utilises NADPH in the one electron reduction of molecular oxygen, shown below;

$$2O_2 + NADPH \text{ ---------- } 2O_2^{\cdot -} + NADP^+ + H^+$$

Superoxide released into the vacuole can then be further reduced to generate other oxygen metabolites in the phagosome and aid in the destruction of the opsonised particles.

The second mechanism is oxygen-independent. Lysosomal granules contain a variety of enzymes and proteins such as bactericidal permeability increasing protein (BPI) (Weiss, 1985), major basic protein (MBP) (Joung, 1986), lysozyme and a host of other proteases. Both these mechanisms serve to destroy the engulfed particle. However, membrane perturbations in leucocytes may result in the release of these products into the extracelluar space. These powerful products of activated inflammatory cells can cause tissue damage and thus amplify the effects of the initial stimulus. The actual release of lysosomal enzymes into the extracelluar space during phagocytosis may occur in at least three ways. Release of phagocytic contents may occur as the result of partially closed vacuoles. White cells exposed to a potentially ingestible complex may release lysosomal contents directly into the extracelluar space, and finally, the death of leucocytes will result in the rupture of these cells and the spilling of their lysosomal contents.

1.4.1.2 Chemical mediators of inflammation.

Oxygen metabolites that cause injury to tissue initiates the synthesis and release of a variety of chemical signals or mediators which are integral components of the inflammatory reaction. These chemical mediators can originate from plasma, cells or damaged tissue and can be divided into a number of groups which are shown in Table 1.2.

Table 1.2 **Mediators of inflammation**

CELLS	
PRE-FORMED	NEWLY SYNTHESISED
Histamine	Prostaglandins
Serotonin	Leukotrienes
Lysosomal enzymes	Platelet activating factor
	Cytokines
PLASMA	
Complement system	
Kinin system	
Clotting-fibrinolytic system	

1.4.1.2.1 Vasoactive amines.

The vasoactive amines, histamine and serotonin, are usually found in high concentrations during the early stages of inflammation. These high concentrations fall sharply within a short period of time and as such these mediators are believed to be responsible for the very early increases in vascular permeability and vasodilatation. Although found predominantly in tissue mast cells they are also associated with circulating inflammatory cells such as the platelet. Stored preformed in granules, they are immediately available on cellular activation.

1.4.1.2.2 Plasma based mediators.

The complement, kinin and clotting systems are three interrelated systems that generate highly vasoactive and chemotactic compounds important in inflammation.The complement system consists of at least 20 plasma proteins (Ross, 1986) which function by regulating a

series of biological reactions serving in the defence against bacterial agents (Figure 1.5). These reactions include increasing vascular permeability, chemotaxis, opsonization and lysis of target organisms. Consisting of activating and effector sequences, activation of the system occurs rapidly via the classical pathway. Alternatively, the system may be activated more slowly by the alternate pathway. Through the production of a variety of cleavage products, both pathways converge into the common production of a membrane attack complex (MAC) (Muller-Eberhard, 1986). The principal components of the complement system that have biologic activity in inflammation include C3a, C5a, C3b, C3bi and C5b-9. C3a and C5a both increase vascular permeability, but only C5a is chemotactic for inflammatory cells. C5a also increases the adhesion of leucocytes to endothelium by inducing leucocyte adhesion molecules. The permeability-increasing components of C3a and C5a are called anaphylatoxins and act mainly by liberating histamine from tissue mast cells and platelets.

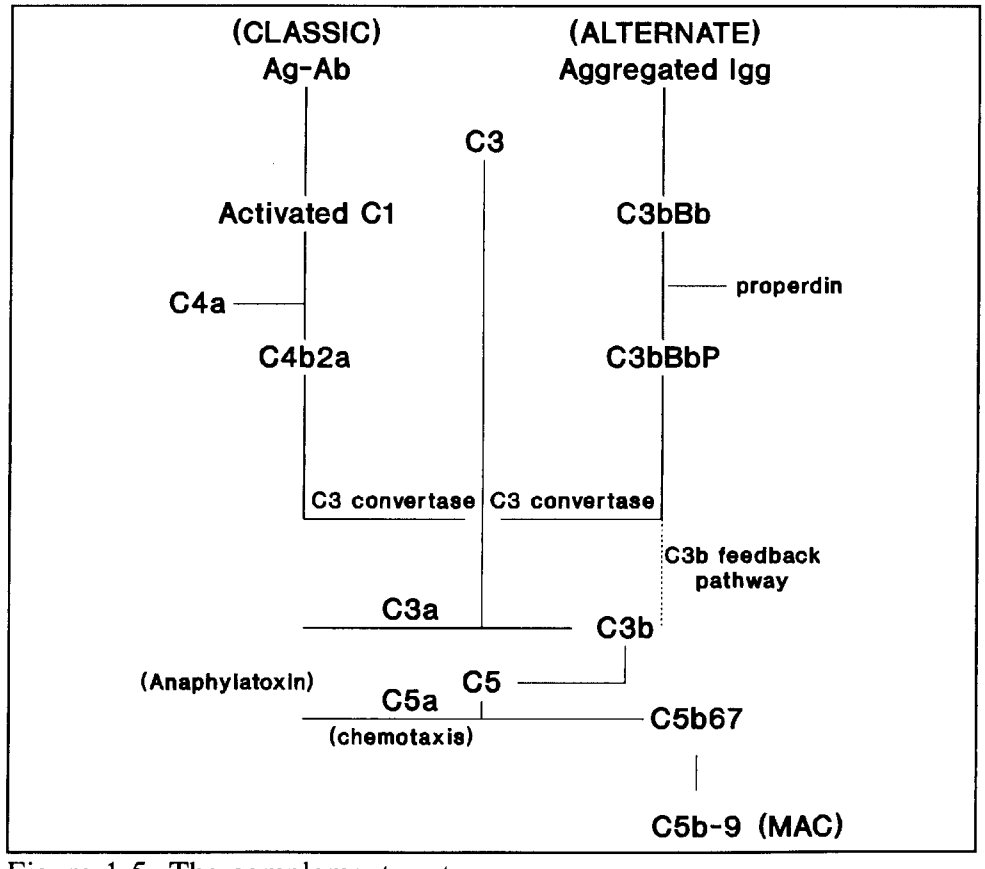


Figure 1.5. The complement system

C5a can also activate the lipoxygenase pathway of arachidonic acid metabolism in

inflammatory cells, thus leading to additional mediator release. C3b and C3bi are important opsonins and are recognised by specific receptors on inflammatory cells. Once the receptor is occupied the cell becomes activated and releases of lysosomal enzymes and activated oxygen species into the vacuole that develops as the cell phagocytosis the opsonin. C5b-9, the MAC is the final product of the complement system and is known to injure parenchymal cells (Muller-Eberhard, 1986). MAC can also stimulate arachidonic acid metabolism and generate activated species of oxygen from inflammatory cells.

The second system within this group is the kinin system (Figure 1.6) (Cotran *et al.*, 1991).

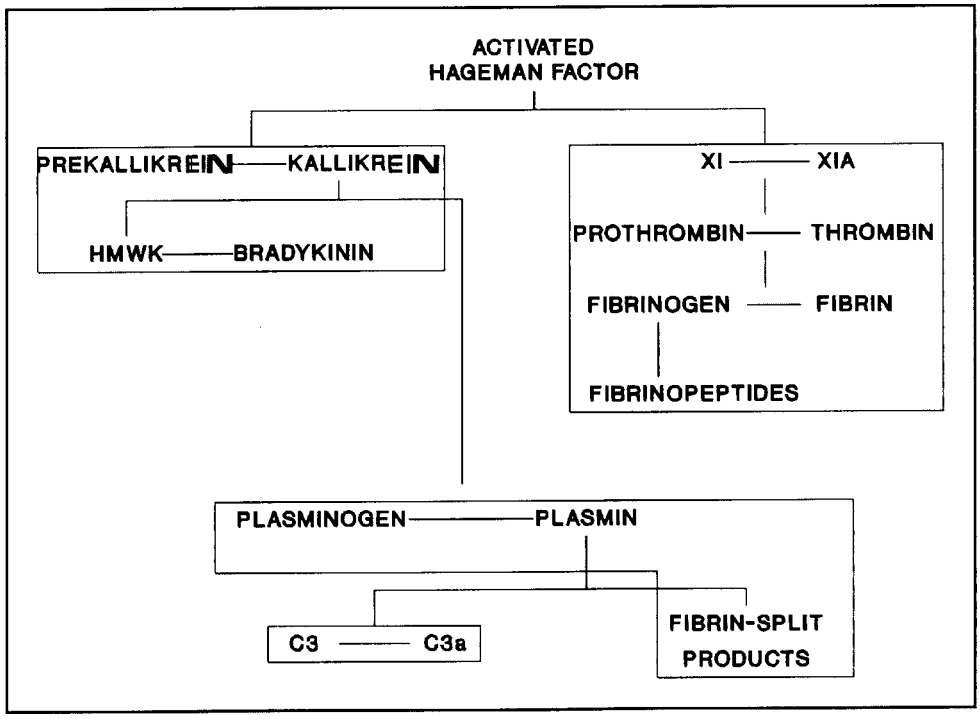


Figure 1.6. The kinin system.

This system is triggered by surface activation of the Hageman factor. The final result is the release of the vasoactive nonapeptide bradykinin, a potent agent that increases vascular permeability. Although not chemotactic, bradykinin causes smooth muscle contraction, dilatation of blood vessels and pain.

The clotting system is the last of the three systems discussed in this section (Figure 1.6) (Cotran *et al.*, 1991). This system consists of a series of plasma proteins that can be activated again by the Hageman factor, with the final step of the cascade resulting in the

production of fibrinopeptides which can increase vascular permeability and which are chemotactic for neutrophils.

1.4.1.2.3 Platelet activating factor.

From its initial description as a mediator of allergic reactions (Benveniste *et al.*, 1972; Henson, 1970), platelet activating factor (PAF) has come to be recognised as an extremely potent lipid mediator of inflammation. To date most of the studies of PAF have emphasized the release of PAF from cells into the medium following treatment of one kind or another. Recent studies have provided evidence that much of the PAF that is synthesised is retained intracellularly. However, the extreme potency of the lipid emphasises the need for only minute amounts to be released in order to produce significant biological effects (Henson, 1970). PAF is produced by, and acts upon, a wide variety of cell types including neutrophils, macrophages and endothelial cells (Braquet *et al.*, 1987; Sturk *et al.*, 1989). It stimulates many different target cells and in each case the cell responds in a manner for which it is preprogrammed for. In contrast to many effects of extracellular PAF, a number of reports have emphasised that the majority of this lipid that is synthesised is retained intracellularly (Lynch and Henson, 1985). In neutrophils the proportion of PAF retained following stimulation varies with the stimulus, but is never less than 70% of the total, and following phagocytosis is usually greater than 95% (Lynch and Henson, 1985). In endothelial cells it appears the retention of PAF is even greater (Prescott *et al.*, 1984).

PAF has also been implicated in several lung diseases, in particular, asthma (Morley *et al.*, 1987) and the adult respiratory distress syndrome (Barnes *et al.*, 1989) and as such there has been considerable interest in the use of antagonists in these conditions. PAF promotes platelet aggregation and the subsequent release of histamine and serotonin, vasodilation, bronchoconstriction and increased vascular permeability (Mojarad *et al.*, 1983). In addition, PAF also increases leucocyte adhesion to endothelial cells, stimulates the synthesis of lipoxygenase and cyclooxygenase products (Voekel *et al.*, 1982; Lefer *et al.*, 1984; Nieminen *et al.*, 1991), tumor necrosis factor (TNF) (Bonavida *et al.*, 1989) and is chemotactic for most inflammatory cells. The injection of PAF causes neutrophil accumulation and migration to extravascular spaces in guinea pigs (Bonnet *et al.*, 1983; Humphrey *et al.*, 1982). *In vitro* studies using cultured human umbilical cord endothelial cells release PAF following stimulation (Camussi *et al.*, 1983). The majority of the PAF synthesised

remains cell bound where it may react with, and activate circulating inflammatory cells (Henson and Johnston Jr, 1987). Thus PAF may thus elicit most of the cardinal signs of inflammation.

1.4.1.2.4 The cytokines.

The cytokines are a family of peptides that are released from a variety of nonlymphoid cells and act as mediators of many aspects of the response to trauma. The cytokine family is extensive and includes the interleukins (IL) and tumor necrosis factor (TNF). Cytokines may exert their effects on cells in three different ways. First there is the autocrine effect, where they act on the same cell from which they are secreted. The paracrine effect, where cells in the immediate vicinity are acted upon and the endocrine effect, where the cytokines act systematically, producing more widespread cellular responses. As they are released from a variety of cells and effect just as many, these polypeptides exert a host of actions on inflammation as shown in Figure 1.7. TNF is secreted preferentially by activated macrophages but also by natural killer cells and neutrophils (Dubravec *et al.*, 1990). It appears to play a central role in the initiation, development and augmentation of inflammation and acts on a diverse range of cell types having diverse actions including cytostasis, cytotoxicity and in contrast, cell activation

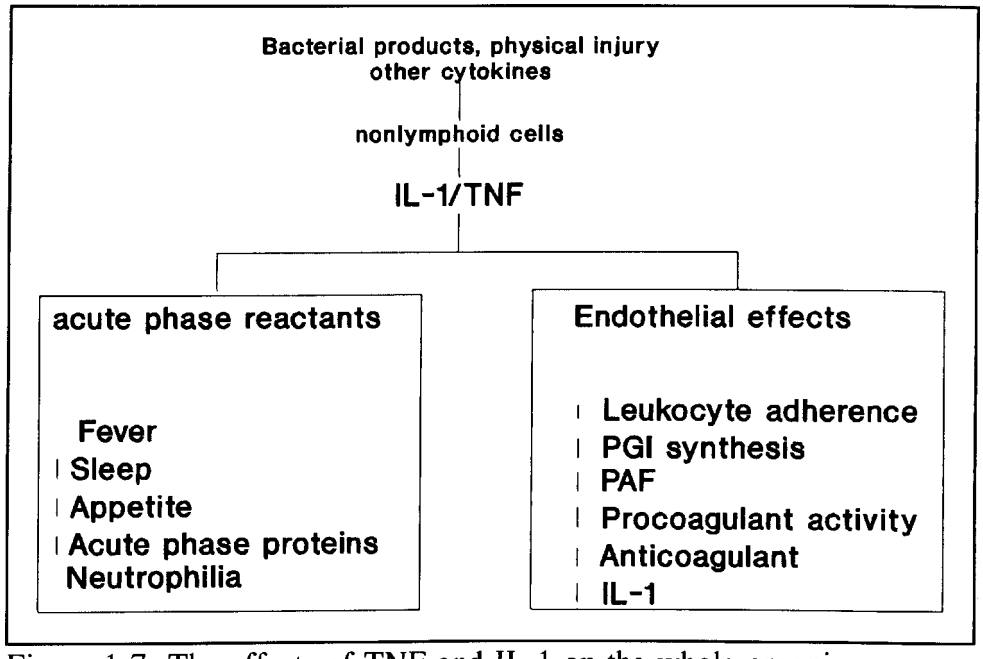


Figure 1.7. The effects of TNF and IL-1 on the whole organism.

and proliferation. It is clear that the net effect of TNF on a cell depends not only on the target cell itself but on the presence or absence of other cofactors.

Intratracheal instillation of TNF results in the induction of lung tissue antioxidants (Tsan *et al.*, 1990; Tsan *et al.*, 1992). It enhances the neutrophil dependent increase in vascular permeability, leukotriene biosynthesis in inflammatory cells and activation of neutrophils to secrete proteases is also observed. TNF activates neutrophils and increases their adherence and post adherence activities on vascular endothelial cells (Gamble *et al.*, 1985; Naworth and Stern, 1986; Klebanoff *et al.*, 1986; Shalaby *et al.*, 1987). Increased TNF-mediated adherence is possibly due in part to translocation of the iC3b (CR3) receptor adherence glycoprotein from intracellular stores to the membrane surface of neutrophils (Todd *et al.*, 1984).

TNF has also been reported to be a weak but direct stimulator of neutrophilic superoxide anion production (Shalaby *et al.*, 1987) and myeloperoxidase-H_2O_2-halide activity (Klebanoff *et al.*, 1986). TNF also primes neutrophils for a second stimulus such as FMLP or PMA. The result is a greater than additive increase in oxidative metabolism (Atkinson *et al.*, 1988). TNF may act directly or through the induction of other inflammatory mediators. TNF stimulates IL-1 production from endothelial cells (Libby *et al.*, 1986; Bachwhich *et al.*, 1986) and macrophages (Naworth and Stern, 1986). Evidence suggests that the effects of TNF may be mediated by prostaglandins (Kettlehut *et al.*, 1987). Both negative and positive feedback mechanisms for the control of TNF exist. Glucocorticoids inhibit TNF biosynthesis if administered to macrophages prior to stimulation with lipopolysaccharide (LPS) (Beutler *et al.*, 1986). Interferon gamma (IFN) increases LPS induced TNF production (Collart *et al.*, 1986). A phosphodiesterase inhibitor such as pentoxyfylline blocks TNF mRNA accumulation, while dexamethasone decreases mRNA translation (Han *et al.*, 1990) and may be one of the ways in which dexamethasone may exerts its beneficial effects.

Interleukin-1 (IL-1) is released by macrophages upon stimulation with endotoxin (Fuhlbrigge *et al.*, 1987). Depending on the concentration, IL-1 can act as a weak chemoattractant for neutrophils and also a weak stimulator of H_2O_2 production (Ozaki *et al.*, 1987). IL-1 suppresses human neutrophil adherence to Dacron fibres (Berger *et al.*, 1988) and induces antioxidant enzymes in a variety of cell types (Masuda *et al.*, 1988; DiSilvestro *et al.*, 1991). Cytokines which are induced together *in vivo*, have been used

together to look for synergism with respect to their role in inflammation. Simultaneous intratracheal instillation of both IL-1 and TNF results in pulmonary antioxidant induction (White *et al.*, 1987). Simultaneous intraperitoneal injection of recombinant IL-1 and TNF at suboptimal levels increased the percentage and number of peritoneal neutrophils in mice (Sayers *et al.*, 1988). This infiltration was greater than the effect of either cytokine alone. Intravenous administration of recombinant human TNF results in three separate responses. There is an initial fall in the number of circulating neutrophils (0.5 hours) which is then followed by two separate peaks of increased circulating neutrophils (1.5 and 6 hours). This response has been suggested to be due to a TNF-induced IL-1 production (Ulich *et al.*, 1987).

Interactions between lipids and cytokines may also occur. Lipoxygenase metabolites, such as LTB_4 enhance TNF production following stimulation with PAF (Dubois *et al.*, 1989) or LPS (Schade *et al.*, 1989). Evidence for this comes from the inhibition of TNF production using lipoxygenase inhibitors. Restoration of LTB_4 in this situation restores the production of TNF. In comparison to the stimulatory action of lipoxygenase products, cyclooxygenase metabolites appear to be inhibitory. PGE_2 inhibits TNF and IL-1 production at high concentrations (Renz *et al.*, 1988; Kunkel *et al.*, 1988). In both cases the inhibition may involve an increase in cellular cAMP (Knudsen *et al.*, 1986). PAF can also enhance TNF production in rat macrophages (Dubois *et al.*, 1989) and human monocytes (Bonavida and Braquet, 1988; Rola-Pleszczynski *et al.*, 1988; Poubelle *et al.*, 1991) and also IL-1 production in human monocytes in a concentration-dependant manner (Poubelle *et al.*, 1991). The activation pathway may involve phospholipase A_2 as lipoxygenase inhibitors can abrogate the effect of PAF (Dubois *et al.*, 1989).

1.4.1.2.5 Arachidonic acid metabolites.

Although arachidonic acid derivatives are involved in inflammation, this represents only one of a variety of biological and pathological functions of these metabolites. Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid that is derived either from the conversion of the essential fatty acid linoleic acid or obtained directly from the diet. It is normally esterified in the carbon 2 position of PC and PI. Through the action of cellular phospholipases activated by a variety of mechanisms, arachidonic acid is liberated and utilized to make eicosanoids. Biosynthesis of the individual metabolites of AA occurs by

one of two major pathways (Figure 1.8).

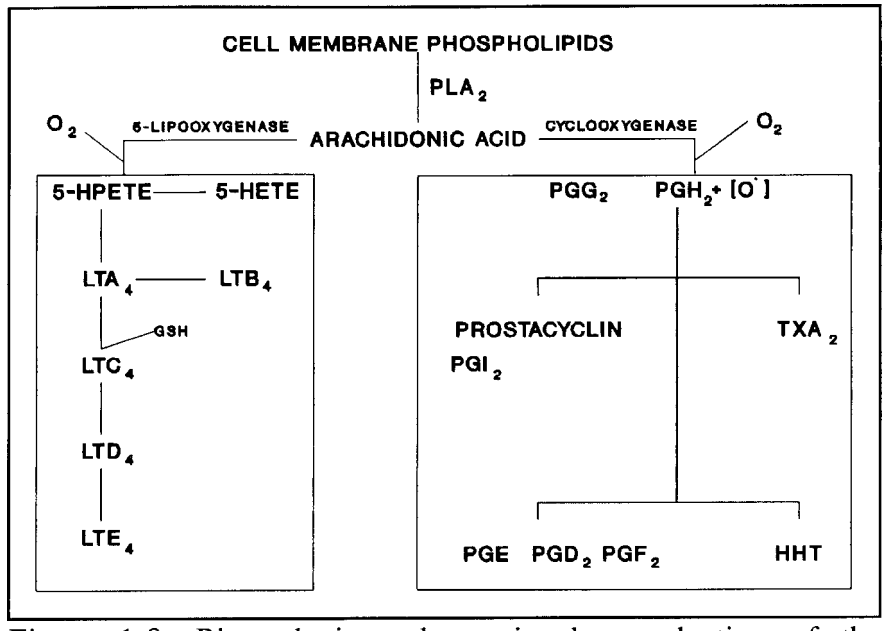


Figure 1.8. Biosynthetic pathway in the production of the eicosanoids.

In the first pathway AA is rapidly transformed by a fatty acid cyclooxygenase enzyme to prostaglandin endoperoxide PGG_2, which itself is converted enzymatically to PGH_2. PGH_2 is then converted into one of three possible products. These products include, thromboxane A_2 a potent platelet aggregator and blood vessel constrictor with a vary short half-life (seconds), prostacyclin (PGI_2), a potent inhibitor of platelet aggregation and vasodilator, found predominantly in vessel walls and the more stable prostaglandins. These include PGE_2, $PGF_2\alpha$ and PGD_2 which all have a variety of actions on vascular tone and permeability.

In the second pathway, AA is converted into a variety of hydroperoxy derivatives by a fatty acid lipoxygenase. The 5-hydroperoxyeicosatetraenoic acid (5-HPETE) derivative can undergo further conversion to an unstable 5,6-epoxy derivative, leukotriene A_4 (LTA_4), via the intermediate hydroxyeicosatetraenoic acid (HETE). LTA_4 can be enzymatically converted to leukotriene B_4 or by the addition of a glutathione residue to leukotriene C_4. LTC_4 can then be converted to leukotriene D_4 (LTD_4) and subsequently to leukotriene E_4 (LTE_4). These derivatives are designated leukotrienes on the basis of their conjugated triene chain and their initial isolation from leucocytes.

PGE_2 and PGI_2 cause dilation of the microcirculation, with effects observed at doses of 0.1 - 10ng. They also produce erythema as does PGD_2 which is not as potent (Solomon *et al.*,1968; Flower *et al.*,1976; Higgs *et al.*,1979). PGE_2 and PGI_2 predominate in most forms of acute inflammation, however, where mast cell degranulation is central to the pathology of the disease, PGD_2 may play a more important role, as this prostaglandin is the main cyclooxygenase product of mast cells. The role of lipoxygenase products in vascular tone is less clear. This may be due to species and tissue differences (Peck *et al.*,1981; Bisgaard *et al.*,1982). Prostaglandins are only weak induces of vascular permeability *in vivo*. Early studies demonstrated that PGE_2 at a dose in excess of 0.1ug is required to induce oedema in rat skin, and that this effect can be mediated either by the release of histamine (Crunkhorn and Willis, 1971) or by inflammatory cell dependent mechanisms (Williams and Morley, 1973; Johnston *et al.*,1976; Williams and Peck, 1977). Prostaglandins are also weak induces of leucocyte accumulation. Injections of PGE_2 or PGI_2 can enhance chemotactic factors, presumably by increasing local blood flow (Movat *et al.*,1984; Van zee *et al.*,1991).

Leukotrienes may induce vascular permeability. LTC_4 and LTD_4 are the most potent, (Dahlin *et al.*,1981). These leukotrienes are at least 1000 times more effect than histamine. However, these compounds do not cause leucocyte chemotaxis or adhesion. In contrast LTB_4 is not only one of the most potent neutrophil chemotactic factors known (Ford-hutchinson *et al.*,1980), It also increases neutrophil adhesion to endothelial cells (Gimbrone *et al.*,1984) and stimulates human monocytes to produce TNF proportionally to the level of the stimulus (Gagnon *et al.*,1989). Exogenously administered LTB_4 may induce prostacyclin synthesis and increase capillary permeability (Jackson *et al.*,1986). Prostaglandins of the E series and PGI_2 can suppress acute inflammatory reactions (Aspinall and Cammarata, 1969; Zurier and Quagliata, 1971). They can also decrease macrophage activation and in particular reduce the secretion of IL-1 (Knudsen *et al.*,1986). The effect of PGE_2 on the production of TNF especially from macrophages is more clear-cut. PGE_2 inhibits the production of mature TNF protein possibly by down regulating the level of mRNA(Kunkel *et al.*,1986). Eicosanoids can also stimulate the synthesis of PAF and adhesion proteins (Hemler *et al.*,1979; Hageman *et al.*,1986). Thus not only are the lipoxygenase and cyclooxygenase pathways active during inflammation, they also interact with other mediators of inflammation.

In conclusion, there is considerable evidence that during inflammation, the production of eicosanoids, PAF and cytokines may be interrelated. IL-1 and TNF induce PG synthesis in various cells (Mizel *et al.*, 1981; Albrightson *et al.*, 1985; Bachwhich *et al.*, 1986) and PG in turn modulate cytokine production. In contrast, leukotrienes (LT) can augment IL-1, IL-6 and TNF production and vice versa. TNF can also induce the synthesis of PAF in several cell types, including endothelial cells, neutrophils and macrophages (Bussolino *et al.*, 1986; Koobayashi *et al.*, 1991; Valone and Epstein, 1988), while PAF can augment the production of IL-1 and TNF. Such a positive feedback loop has potential amplification of the inflammatory responses. However, this may be counterbalanced by the negative feedback action of other cytokines (Koobayashi *et al.*, 1991; Schindler *et al.*, 1990).

1.5 Hyperoxic induced inflammation and lung injury.

As oxygen has been implicated in the pathological changes observed in infants who develop BPD, a clear understanding of the morphological and biochemical changes associated with oxygen exposure needs to be addressed.

Hyperoxia results in a dose dependant increase in mitochondrial superoxide (Turrens *et al.*, 1982) and hydrogen peroxide (Freeman and Crapo, 1981; Turrens *et al.*, 1982). Production of these metabolites can also be demonstrated in isolated nuclei and microsomes derived from endoplasmic reticulum (Turrens *et al.*, 1982; Freeman *et al.*, 1982; Yusa *et al.*, 1984). These highly reactive moieties have the capacity to cause extensive tissue damage either through direct oxidation of amino acids (Greenwald and May, 1980; Tsan *et al.*, 1990) or by initiating a self-propagating chain reaction termed lipid peroxidation (Halliwell and Gutteridge, 1984). They also have the capacity to react with DNA, resulting in strand breakage (Weitberg *et al.*, 1985; Birnboin and Kanabus, 1985) a factor that may be important in mutation and cancer (Clark *et al.*, 1985). Superoxide has also been shown to react with plasma (Petrone *et al.*, 1980) resulting in the generation of chemotactic factors similar to C5a (Shingu and Nobunaga, 1984). Vogt *et al*, 1987 has shown that hydrogen peroxide causes conformational changes in C5 enabling it to initiate the MAC complex and activate complement, changes that can be blunted by the administration of SOD (Turrens *et al.*, 1984; Tanswell and Freeman, 1987). Oxygen based free radicals are also capable of activating the lipoxygenase and cyclooxygenase pathways (Lands, 1979; Hemler *et al.*, 1979; Hemler and Lands, 1980), a series of reactions that lead to the release further

injurious compounds and an alteration in the endothelial-epithelial permeability of the lung. This is achieved through the activation of a membrane phospholipase (DelMaestro, 1980; Iwata *et al.*, 1986) and the liberation of free arachidonic acid (Freeman and Crapo, 1982; Chan *et al.*, 1982; Gurtner *et al.*, 1983) and to the production of leukotrienes, prostaglandins and thromboxanes. *In vitro* studies using systems that generate free radicals, results in the release of prostacyclin (Burghuber *et al.*, 1984) and thromboxane (Shasby *et al.*, 1982; Burghuber *et al.*, 1984; Farrukh *et al.*, 1985). Prior treatment of the cells with cyclooxygenase inhibitors prevents the release of these compounds (Shasby *et al.*, 1982; Farrukh *et al.*, 1985; Jacobson *et al.*, 1986). Cultured pulmonary artery endothelial cells pre-exposed to hyperoxia released LTB_4 when treated with a Ca^{2+} ionophore (Jackson *et al.*, 1986). This has been further supported by Taniguchi *et al.*, 1992 who demonstrated increased levels of LTB_4 in the BAL fluid of oxygen exposed rats. Furthermore, the administration of AA861, a 5-lipooxygenase inhibitor, significantly reduced the damaging effects of hyperoxia. Increased levels of 5-HETE have also been found in the BAL fluid of rats exposed to high levels of oxygen (Burghuber *et al.*, 1984). Through these events, thromboxane could increase pulmonary vasoconstriction leading to increased microvascular pressures and increased exudate formation (Tate *et al.*, 1984). Leukotrienes may act directly to further increase vascular permeability and also attract inflammatory cells (Perez *et al.*, 1980). Thus both cyclooxygenase and lipooxygenase products may be actively involved in tissue injury. Mediators other than the AA metabolites, such as the cytokines, have also been implicated in the aetiology of oxygen-induced lung injury. Recent studies in mice using an anti-TNF antibody have shown increased survival in hyperoxia (Jensen *et al.*, 1992). However, other studies have shown that pretreatment with TNF also affords protection against subsequent oxygen exposure (Tsan *et al.*, 1990; Warner *et al.*, 1991). This paradox in the role of TNF is yet unclear, though undoubtedly, timing and concentration of circulating TNF will have a major influence on the animals response to hyperoxia. Although inhibition studies with IL-1 and oxygen toxicity have not been done, IL-1, which has similar biological actions as TNF, also protects animals from the lethal effects of oxygen exposure (White *et al.*, 1987; Tsan *et al.*, 1991). The mechanism by which this is achieved may be via the induction of tissue antioxidants (Wong and Goeddel, 1991). The role of other cytokines in oxygen-induced lung injury is also unclear, however, IL-6 does appear to augment survival by increasing the effects of TNF in the rat (Tsan *et*

al., 1992). These changes result in the attraction of white blood cells to the area of injury. Once at the site of injury, inflammatory cells may then release proteases and generate further oxygen centred free radicals. The contribution of inflammatory cells to hyperoxic injury through the respiratory burst oxidase and subsequent generation of other toxic substances is controversial. Although in animal models of oxygen toxicity the influx of neutrophils and increased vascular leakage are coincident (Matalon and Egan, 1981; Matalon *et al.*, 1982), a role for this cell type in the development of lung injury is unclear. Neutrophil depletion studies have to date been inconclusive, with mixed results and differing conclusions being reached (Shasby *et al.*, 1982; Barry *et al.*, 1982; Raj *et al.*, 1985; Barry and Crapo, 1985).

Since the observations of J. Lorrain Smith, 1899 there have been numerous pathological studies confirming the gross and light microscopic features of hyperoxic-induced lung injury. Lesions attributable to pulmonary oxygen toxicity have fallen into two distinct categories. The first, DAD and the second, those lesions observed following prolonged oxygen therapy for HMD. In most animal species, DAD implies widespread injury to all elements of the alveolar-capillary wall and includes changes related to an acute destructive phase as well as proliferative and reparative phases. However, the extent and rapidity to which these lesions develop depends largely on a number of variables which include age, nutrition, endocrine status and previous exposure to oxidants (Deneke and Fanburg, 1980; Deneke and Fanburg, 1982).

In animal studies, the early or acute destructive phase of DAD is remarkably consistent between most species and usually develops within the first 48 hours. It is initially characterised by endothelial cell swelling with diffuse pulmonary congestion and oedema, sometimes associated with intra-alveolar haemorrhage, necrosis of alveolar cells and epithelial desquamation. Oedema is widespread, occurring at intraalveolar, interstitial, perivascular and peribronchiolar sites (Gould *et al.*, 1972; Massaro, 1986). Tracheal and bronchiolar damage also develops within the same time span. These changes consist of bronchiolar and airway epithelial swelling, decreasing proximally from the alveolar ducts. After 72 hours extensive capillary endothelial necrosis is observed. Morphometric studies indicate a 50% reduction in capillary numbers with focal epithelial cellular and organelle swelling. The thickness of the air-blood barrier may double by 72 hours. Upper airway epithelial necrosis and detachment with peribronchiolar swelling ensues in addition to

mitochondrial swelling, luminal blebbing and loss of cilia. Oxygen-induced hyaline membrane formation has also been described. They line all or part of the terminal airway and spaces, including terminal and respiratory bronchioles as well as alveolar ducts and sacs (Pratt *et al.*, 1979). Although containing necrotic components of airways, the fundamental component appears to be polymerized insoluble form of fibrin. Decreased fibrinolytic factors prior to the development of hyaline membranes may impede fibrinolysis and be responsible for the formation and maintenance of these membranes (Viscardi *et al.*, 1992).

The proliferative phase of DAD is an overlapping continua of the many initial alterations observed during the early phase of oxygen toxicity. This phase consists primarily of (1) replacement of alveolar epithelial surfaces by granular pneumonocytes (pneumonocytic metaplasia) and (2) fibrous scarring predominantly of the interstitium. Morphometric data of the transformation of type I to type II cells lining the alveoli reveal a two-fold increase by week one and a 22 fold increase by week two in the lungs of the oxygen exposed monkey (Kapanci *et al.*, 1969). This change in the cell type is potentially reversible, as type II cells may subsequently give rise to new type I cells following cessation of oxygen. The proliferation of fibroblasts and collagen production in the alveolar interstitium of the lung begins approximately one to two weeks after the start of the exposure. The quantity and consistency of the proliferative changes depend not only on the severity and duration of exposure but also on the animal species under study.

In man, within 18 hours exposure to 95% oxygen there is a 50% increase in lung lavage albumin concentration (Davis *et al.*, 1983). Recently Griffith *et al*, 1986 demonstrated that exposure to 30-50% oxygen for 48 hours was associated with a dose-dependant increase in bronchoalveolar lavage albumin. This increased alveolar-capillary membrane permeability in adults has also been studied in infants with HMD (Smedly *et al.*, 1986). Using inhaled technetium-labelled diethylenetriamine pentaacetate (^{99m}Tc-DTPA) there was increased clearance of the label during the first 72 hours. Hyaline membranes appearing as fine granular material have also been noted as membranous pneumonocytes and endothelial cells became progressively more severely altered and sloughed at 2-6 days. Proliferative changes, hyperplastic granular pneumonocytes replacing alveolar lining cells and interstitial fibrosis characterised the lungs after 6 days. Morphometric data obtained from these two studies reveal a progressive increase in thickness of the air-blood barrier and a striking reduction in the diffusion capacity by 12-13% of normal by 6-13 days.

A comprehensive clinicopathologic study of pulmonary oxygen toxicity in man was undertaken by Nash and colleques in 1967 . Seventy patients dying after prolonged ventilator therapy were compared with 70 patients who died without artificial ventilation. Hyaline membranes were observed in greater frequency lining the respiratory bronchioles, alveolar ducts and alveoli of the respiratory treated group compared to control. Furthermore, severe interstitial oedema and fibrosis was also found in greater abundance in this group. The respirator treated group was subdivided into those having received 21-90% and 90-100% oxygen and further subdivided into those who received oxygen for 0-10 days or greater than 10 days. The quantitative features of pathologic changes (lung weight, hyaline membranes, interstitial oedema and fibrosis and alveolar cell hyperplasia) were greater in the group of patients having received high concentrations of oxygen for longer than 10 days. The important hallmarks of DAD, interstitial oedema and fibrosis and alveolar hyperplasia were twice as prevalent in patients requiring greater than 10 days therapy of 90-100% oxygen. The incidence of hyaline membranes and that of interstitial oedema and fibrosis were 2-3 times greater in the 90-100% oxygen group than the 0-90% oxygen group. Thus there are a multitude of mechanisms that exist by which exposure to high concentrations of oxygen can directly or indirectly injure the lung. As such observations of the inflammatory changes that are observed in the lungs of infants who develop may indicate which of these mechanisms and free radicals are most important.

1.6 Pulmonary inflammation in the development of HMD and BPD.

The potential role of inflammation in the development of HMD and BPD is suggested by cytological, biochemical and histopathological studies confirming the presence of inflammatory cells and mediators within the lung. Bronchoalveolar lavage performed at < 24 hours of age on controls and infants who are destined to develop HMD and BPD, show low BAL cell counts consisting mainly of alveolar macrophages. Between 48 and 96 hours there is a dramatic increase in the cell counts of these infants, mainly due to the influx of neutrophils. At one week of age, a time when most neonates with RDS alone are recovering, the BAL cell counts return to control values with few neutrophils and a predominance of macrophages. Those infants who are destined to develop BPD have persistently elevated cell counts for up to 5 weeks which consist mainly of neutrophils. An increase in alveolar macrophage counts is seen early on in the development of BPD but

tends to fall of as BPD progresses (Merritt *et al.*, 1983; Nishida *et al.*, 1990). These observations have recently been supported (Arnon *et al.*, 1993) and implicates the neutrophil in lung injury, early on in the development of BPD. In this study the macrophage counts were also significantly lower in infants destined to develop BPD than in those who do not. However in contradiction to this, circulating neutrophil counts rise exponentially during the last 10 weeks of gestation (Davies *et al.*, 1992) and as the incidence of BPD increases with decreasing gestational age the role of these cells in the initiation and progression of lung injury to BPD is unclear. As both macrophages and neutrophils have the potential to causes tissue injury (Nathan, 1987; Weiss, 1989), the function of these cells in HMD and BPD have been investigated. Although there is no data on the functional state of the alveolar neutrophil, alveolar macrophages from infants with BPD are activated (Jacobson *et al.*, 1993). No change in the activation state of this cell from infants with RDS was observed. As described earlier, there are two mechanisms by which inflammatory cells can cause injury. The oxygen dependant mechanism is principally ascribed to the production of superoxide by the NADPH oxidase system. The release of superoxide and the generation of longer lived and more potent free radicals are known to cause tissue damage (Schraufstatter and Cochrane, 1991). The superoxide generating capacity of neutrophils from preterm infants has been assessed by a number of groups (Shigeoka *et al.*, 1981; Peden *et al.*, 1987; Kugo *et al.*, 1989). The results from these studies have been inconclusive, as some have found increased (Shigeoka *et al.*, 1981) and others a reduced (Kugo *et al.*, 1989) capacity to generate superoxide compared to inflammatory cells from term infants and adults. However, all these studies have been performed on blood and not on BALF neutrophils. These studies need to be undertaken as the functional capacity of blood neutrophils may not equate to those in the lung. Although the capacity of alveolar macrophages to generate superoxide has not been measured in preterm neonates, the subsequent generation of hydrogen peroxide from superoxide has been determined (Clement *et al.*, 1988). They found increased hydrogen peroxide production from these cells. If increased free radical production is occurring from macrophages and neutrophils, then the lung may be under additional oxidative stress, in addition to that from supplemental oxygen. This will lead to increased tissue damage if antioxidant defences cannot contain the oxidative insult.

Antioxidants in ELF are assumed to be the first line of protection for the lung.

Glutathione levels in ELF have been assessed from BALF studies of infants with RDS and BPD. Early on during the development of lung injury in these infants GSH levels in ELF are reduced compared to infants who do not develop BPD (Grigg *et al.*, 1993), an observation also seen in blood (Smith *et al.*, 1993). Increased quantities of the oxidised form of the antioxidant, GSSG has also been detected in the blood of these infants and is possibly indicative of increased oxidative attack. Other antioxidants that have been measured include the enzymatic tissue antioxidants (Strange *et al.*, 1988; McElroy *et al.*, 1992; Dobashi *et al.*, 1993). There are now several studies that have indicated that infants born prematurely may be deficient in these antioxidants and in particular catalase (McElroy *et al.*, 1992). However, a recent publication has also assessed the antioxidant status in infants with HMD and BPD (Dobashi *et al.*, 1993). In this study the immunological staining associated with Cu/Zn SOD was increased in infants with HMD and BPD. However, MnSOD staining was unaltered in HMD lungs and increased in lungs with BPD. These changes appear to correlate with those changes seen in animals studies following oxygen exposure (Sosenko and Frank, 1987; Petrone *et al.*, 1980).

The second mechanism that operates in these cells is oxygen independent and involves the release of intracellular proteins and proteases. Numerous studies published to date have shown that proteases such as elastase are released into the lungs of neonates with HMD and BPD (Merritt *et al.*, 1983; Walti *et al.*, 1989; Yoder *et al.*, 1991). Naturally occurring inhibitors of elastase, which include α-1-PI are known to be low in the circulation and in BALF. Studies have shown that this reduced protective layer to proteases is due primarily to a gestational phenomena (Lee *et al.*, 1978; Rosenfeld *et al.*, 1986) and as a consequence of oxidative inactivation in the lungs (Merritt *et al.*, 1983). Alpha-1-PI is easily inactivated by oxidation of the active site methionine group of the inhibitor (Swaim and Pizzo, 1988). This leads to a loss of inhibition and an imbalance in the protease-antiprotease profile. This may explain why increased levels of desmosine (a unique crosslinking amino acid of elastin) and hence lung injury are elevated in the urine of infants who develop BPD.

Thus at present, inflammatory cells within the lungs of these infants may be activated and releasing superoxide and proteases. As a consequence of reduced antioxidant and antiprotease protective mechanisms due primarily to the immaturity of the lung and oxidative inactivation of inhibitors, lung injury develops. A whole host of other mediators have been identified in the lungs of infants with HMD and BPD that have been associated

with the development of other lung diseases and which might be important in the development of BPD. Studies have shown considerable chemotactic activity of tracheal aspirates in infants at high risk of developing BPD (Groneck *et al.*, 1993). Several studies have shown that the major chemotactic factors, C5a and LTB_4 are all present in the lungs of these infants (Mirro *et al.*, 1990; Groneck *et al.*, 1993). The presence of C5a suggests activation of the complement system. Although the liver is the major site of synthesis of complement proteins, components of both pathways are produced in lung tissue. C2, C4 and factor B have been found to be produced by macrophages (Cole *et al.*, 1983) and type II epithelial cells (Strunk *et al.*, 1988). Additionally type II cells are capable of synthesising C3 and C5 (Rothmann *et al.*, 1989). Secretion of these factors may well contribute to the inflammatory process seen in these infants as neutrophil elastase can cleave locally produced C5 to C5a (Janoff, 1985). As well as the complement system, the kallikrein-kinin system is also activated. This system may play a fundamental role in the development of shock like conditions associated with RDS, as it results in the formation of the very potent vasodilator bradykinin, which increases vascular permeability (Saugstad *et al.*, 1982). In a recent study (Saugstad *et al.*, 1992), this system was shown to be activated in the first few days after delivery in infants destined to develop RDS. How this system is involved in the development of HMD and BPD is at present unclear and requires much more study. However, both the kinin and complement system are activated, which can only mean possible deleterious consequences for such infants.

Other important mediators of inflammation that have been measured in the preterm neonate are those derived from membrane lipids. The reason for this is that the observed physiological abnormalities associated with BPD are also associated with the actions of PAF and eicosanoids. In an extensive study carried out by Stenmark *et al* 1987, lipooxygenase and cyclooxygenase products and PAF were found in the BALF of infants with BPD. Both 5 and 12 hydroxyeicosatetraenoic (HETE) acid were also present in high concentrations. Though their biologic actions are not clear, reports suggest that they may act as cellular chemoattractants. Leukotriene B_4 was also found and as this lipid is one of the most potent chemoattractants, especially for neutrophils, this may explain the presence of elevated neutrophil numbers in the lungs of these infants. Although detected in lower concentrations LTD_4 could participate in bronchoconstriction, oedema and pulmonary vasoconstriction. The presence of thromboxanes and prostacyclin in increased quantities also suggests that

these lipids may be involved in the observed lung injury in these infants. Finally PAF was also found an observation later confirmed in a sequential study demonstrating the presence of PAF as early as day one after premature delivery (Koyama *et al.*, 1993). Finally, the role of cytokines in the development of HMD and BPD are now being actively investigated. In a recent study TNFα has been found in BALF of infants with RDS (Murch *et al.*, 1992). TNFα levels rose during the development of RDS which mirrored the changes in the macrophage population.

At present there are no specific inhibitors of the inflammatory mediators available for use in the preterm infant with BPD. However, cromolyn sodium, first characterised as a mast cell stabilising agent has been found to have a variety of other potential antiinflammatory effects. These include inhibition of neutrophil activation (Kay *et al.*, 1987), and antibody-mediated cytotoxicity (Rand *et al.*, 1988) and inhibition of neutrophil chemotactic response to LTB_4 and PAF (Bruijnzeel *et al.*, 1989). However, in a randomized blinded trial, cromollyn therapy was found to have no effect on the outcome of infants who developed BPD (Watterberg and Murphy, 1993). Another compound that has been used extensively, is the synthetic glucocorticoid, dexamethasone (Gerdes *et al.*, 1988; Bourchier, 1988; Cummings *et al.*, 1989). This compound has been shown to be effective in blunting the inflammatory response associated with BPD and improving the outcome of susceptible infants (Murch *et al.*, 1992; Groneck *et al.*, 1993). Dexamethasone acts by inhibiting the action of tissue phospholipases and consequently the myriad of lipid mediators that are potentially released. This action of dexamethasone indirectly suggests that tissue phospholipases may be involved in the inflammatory events observed in these infants.

Therefore in the setting of chronic lung injury in infants, there is a significant airway inflammatory response characterised by an increase in cell numbers, concentration of eicosanoids and PAF. The known pulmonary effects of these mediators raise the possibility that they could participate in the physiologic abnormalities associated with BPD.

1.7 Animal models of HMD and BPD

One of the principal problems associated with the pathogenesis of CLD is the lack of adequate material for detailed systematic analysis. This is effectively due to the ethical and practical problems associated with this group of patients. Thus to address some of the questions regarding the pathogenesis of BPD and the role of specific mediators of

inflammation during the development of BPD an appropriate animal model would be very beneficial. As a consequence, a workshop convened in 1979 under the auspices of the National Heart, Lung and Blood Institute in the U.S.A. produced a detailed list of requirements that should be met before an appropriate animal model of BPD be established. These included ;

1. Pathological changes must be similar to, if not indistinguishable from human BPD.
2. Viability of preterm animals following delivery.
3. Susceptible to hyaline membrane formation.
4. Sufficient size to permit appropriate physiological and biochemical analysis to be performed on the lung.
5. Availability of timed gestations.
6. Appropriate control data for comparison.
7. Resources available to maintain and study the animals.

In response to these guidelines a number of models have been developed. Two of the most important models include the monkey (Kessler *et al.*, 1982; Jackson *et al.*, 1987) and the baboon (Coalson *et al.*, 1982; Coalson *et al.*, 1988; Meredith *et al.*, 1989; Coalson *et al.*, 1992). Both these animals if delivered at about 80% of gestation require mechanical ventilation and supplemental oxygen to remain in a viable state, a situation analogous in part to the human. These animals once treated with oxygen and mechanical ventilation demonstrate radiographic and histological evidence of acute and chronic lung injury similar to preterm human infants with HMD. However, the monkey model has not been exploited to its full potential and the baboon lung is anatomically different to that of the human. However, more specifically, the baboon shows little evidence of inflammation so typical in BPD (Coalson *et al.*, 1992). Also the high cost of maintenance for these primates limits the usefulness of this model in the study of BPD. Consequently, a number of small animal models have appeared which include the preterm rat (Tanswell *et al.*, 1989; Chen *et al.*, 1994), goat (Egan *et al.*, 1980), rabbit (Lorenzo, 1985; Ikegami *et al.*, 1987; Frank and Sosenko, 1991), lamb (Jobe *et al.*, 1983; Jobe *et al.*, 1985) and guinea pig (Kelly *et al.*, 1991). The preterm rat, although satisfying a number of important criteria, is of little

use in the study of neonatal lung disease. The survival of 1 day premature rat in air is less than 10% after 36hr (Tanswell *et al.*,1989), and unlike the human infant alveolar development does not start until after normal term delivery. The preterm rabbit, goat and lamb, all of which have shown initial promise, have yet to be assessed beyond the first few days of oxygen exposure.

The preterm guinea pig offers considerable scope as an animal model for the study of BPD (Rickett and Kelly, 1990; Kelly *et al.*,1991; Hunt *et al.*,1991). Compared to other small-sized mammalian species the 68 day gestation period of the guinea pig is exceptionally long (hamster: 16 days, rat: 21 days, rabbit: 31 days). Hence, at birth the pups display advanced physiological and lung morphological maturity (Lechner and Banchero, 1982), conditions analogous to the human infant. Guinea pigs are born with open eyes, a full coat of fur, the potential to regulate their own body temperature and eat solid food. The survival rate of in this species is much higher following premature delivery than other small mammals (Lorenzo, 1985; Tanswell *et al.*,1989). Delivery of viable preterm guinea pigs can be achieved up to eight days before term (68 days). However, these animals die within 24 hours due to respiratory complications (Kelly *et al.*,1991). The survival rate increases with increasing gestational age such that by 65 days (3 days before term) greater than 90% of the guinea pigs delivered survive beyond 24 hours (Kelly *et al.*,1991). Pups delivered at 65 days of gestation develop respiratory distress which is characterised by sustained (>1 h) tachypnea. Analysis of surfactant at this time point shows that not only is the total lung phosphatidylcholine content significantly less than term animals but that the concentration of the major surface reducing component of surfactant, DPPC, is also less. The antioxidant status of the guinea pig lung has previously been assessed (Rickett and Kelly, 1990). Although there is a gradual increase in Mn-SOD, Cu/Zn-SOD, GSH-Px and CAT through gestation, pups delivered 3 days premature appear to only be deficient in CAT and Cu/Zn-SOD (Rickett and Kelly, 1990). Recent studies on the antioxidant status of human preterm infants (McElroy *et al.*,1992) also demonstrate that CAT may be deficient in these infants (McElroy *et al.*,1992). Thus, as with the human, the preterm guinea pig may be predisposed to oxidative lung injury. Possibly as a consequence of these deficiencies, morphometric analysis of preterm guinea pigs exposed to 21% oxygen show a reduction in percentage airspace with widespread atelectasis (Kelly *et al.*,1991). Further analysis of the preterm guinea pig lung show increased tissue inflammatory cells and

protein, indicative of lung injury (Kelly *et al.*, 1991). Thus the preterm guinea pig is shown to be a viable model in which to study the role of oxygen in the development of BPD. On exposure of this model to high concentrations of oxygen, the histological, morphological and biochemical changes appear to be similar to that of infants developing BPD (Kelly *et al.*, 1991). There is evidence of fibrin deposition, cellular debris and increased numbers of neutrophils in the alveoli. As a consequence of the numerous aspects of the preterm guinea pig as a model of prematurity, it is used in the present study to investigate the role of inflammation in oxygen-induced injury to the immature lung.

In summary, the maturation of the guinea pig leads to animals which demonstrate precocious physical, physiologic and morphologic maturity that is similar to the human newborn neonate. If delivered 3 days prematurely these animals are surfactant and antioxidant deficient and develop lung injury similar to that seen in human infants who develop HMD. As such, it makes this animal a good small-sized model in which to investigate the role of particular pathogenic determinants in the development of oxygen induced acute and chronic lung injury.

1.8 Research proposals.

The specific aims of the thesis are;

1. To establish and extend previous observations on development of lung injury in the air-exposed preterm guinea pig. This will entail a comparison of the inflammatory events following natural and Caesarian delivery of term and preterm guinea pigs respectively. Also the inflammatory changes associated with acute exposure to 95% oxygen for 3 days will be assessed.
2. Assess the role of the neutrophil in the development of oxygen-induced lung injury. This will be achieved firstly by assessing capacity of neutrophils from the lung and peritoneum of term and preterm guinea pigs to generate superoxide and secondly by depleting circulating neutrophils from the oxygen-exposed preterm pup and assessing the extent of lung injury.
3. Assess the role of lipid mediators of inflammation (PAF and LTB_4) associated with the development of acute and chronic lung injury in the preterm human neonate.
4. Characterise the inflammatory and histological changes associated with long term (28 days) oxygen exposure of the immature guinea pig lung.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials.

Unless stated otherwise in the text, all chemicals used were obtained from Sigma Chemical Co. (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK).

2.2 Animals.

Virgin female Dunkin-Hartley strain guinea pigs (500g) were obtained from the School of Biochemical and Physiological Sciences animal facility at the University of Southampton. The guinea pigs were housed in groups of three in a room controlled for temperature (22°C), humidity (45%) and light (12 hour light/dark cycle) before entering the separate breeding colony established by the Neonatal Respiratory Research Group (NRRG). Food and water were supplied *ad-libitum* which included pelleted dry feed (RGP; Labsure, Manea, UK) and hay. Guinea pigs were caged with a male at least three days before ovulation and daily vaginal examination of the female guinea pigs was undertaken to establish an ovulatory cycle. The date of conception was determined by inspection of daily vaginal smears. These were obtained by flushing the vagina with 1ml of tap water from a smooth tipped glass pipette. The liquid recovered was air dried on a glass slide, stained with Ehlich's haematoxylin for 10 minutes, washed in tap water, differentiated in acid alcohol (0.1M HCl, 75% ethanol) for five seconds, washed again with tap water and stained in 1% eosin for two minutes. The slide was then washed again and air dried overnight at room temperature. The slides were then examined under the microscope for the presence of leucocytes. The change from the predominance of non-nucleated cornified epithelial cells to leucocytes denoted the occurrence of ovulation within the last ten hours (Ecobichon, 1984). The closure of the vaginal opening by the appearance of a membrane signalled the correct time for removal from the male. The failure of the vagina to reopen for the next cycle was taken as a sign of successful mating. This occurred in approximately 70% of cases and those females not pregnant were reintroduced to the male before the start of the next cycle. Using this system, normal gestation of 68 $\pm$ 1 day could be reliably determined.

2.3 Caesarian section.

Preterm guinea pigs required for study were obtained by surgical Caesarian section under general anaesthesia. Pregnant females were anaesthetized with 2-4% halothane (ICI,

Macclesfield, UK) maintained with nitrous oxide (0.4 litres/min) and oxygen (1-2 litres/min). Using sterile procedure, the abdomen of the supine guinea pig was shaved prior to the application of chlorhexadine spray. An incision was made through the skin and peritoneum and the gravid uterus removed from the abdominal cavity. The pups were quickly delivered by hysterotomy, to prevent undue sedation which impaired the spontaneous onset of breathing. The umbilical cord was then double clamped, cut and the pups were resuscitated by gentle rubbing in a warm stream of air, weighed, numbered and placed with a surrogate mother. The anaesthetized female was then sacrificed by cutting the diaphragm and severing the major vessels supplying the heart.

2.4 Hyperoxic exposure.

All acute exposure regimes were performed in air tight purpose built perspex chambers (25 litres) which contained a wire grid 2cm above the base covered with a layer of sterile sawdust (figure 2.1). The grid was lined with sterile hay. Pelleted food was supplied in a dish placed inside the chamber and an externally fitted water bottle provided. The chambers were supplied with either 21% or 95% oxygen from cylinders of oxygen and air (BOC) attached to a pressure reducing and mixing regulator. A flow rate of 5 litres/min was used and the oxygen content in each chamber checked using an oxygen analyzer (Servomex, Crowbrough, U.K.). A temperature of 20 $\pm$ 2°C and a relative humidity of 65 $\pm$ 10% were maintained in each chamber (Fig 2.1). For exposure regimes lasting 28 days, animals were maintained in 25 litre perspex chambers for 14 days and then transferred into polyproperlyene boxes with wire mesh tops. These boxes were placed into another air tight perspex chamber (200 litre) and the oxygen concentration monitored as for the acute exposure regime. In all experimental procedures surrogate dams were used to provide milk and were rotated between 21% and 95% oxygen every day and every two days in animals exposed to 85% oxygen.

2.5 Bronchoalveolar lavage (BAL) and tissue collection.

Pups were anaesthetized by intraperitoneal injection of pentobarbitone (50mg/kg). Following the onset of deep anaesthesia the trachea was isolated and a 14g cannula inserted and secured. The lungs were then lavaged *in-situ* with 10ml (5x2ml) of sterile saline (37°C) of which 80-90% was normally recovered. The BAL fluid (BALF) was immediately placed

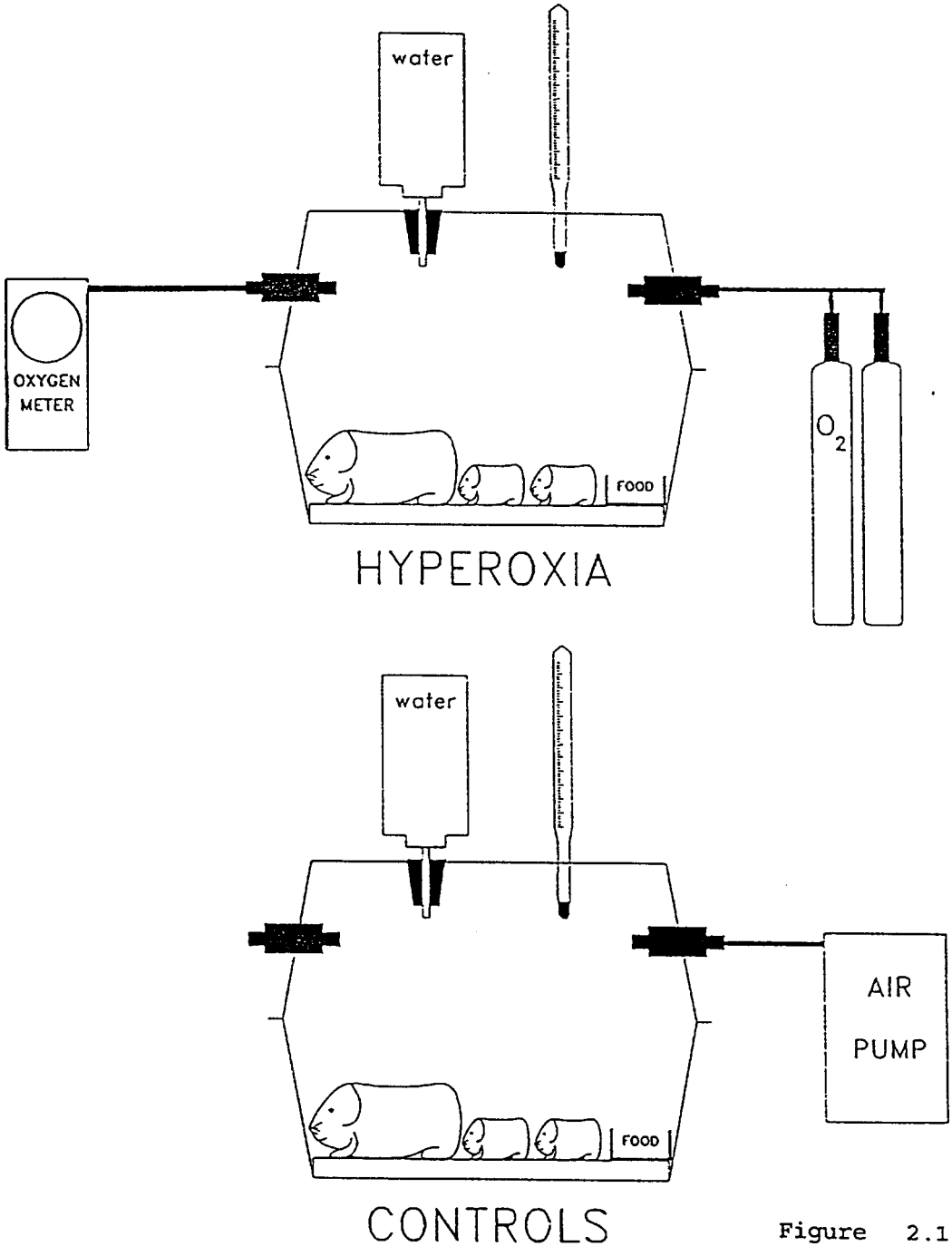


Figure 2.1

on ice and the cellular component pelleted by centrifugation at 1500rpm (200g) for 10 minutes at 4°C. The acellular fraction was then aliquoted out into 0.5ml fractions and frozen at -70°C for subsequent biochemical analysis. Following BAL, the thoracic cavity was opened and 1ml of blood was drawn from the heart and kept in a heperanised container on ice. The animal was then exsanguinated by severing the abdominal aorta and the pulmonary circulation flushed with 10ml of sterile saline (37°C) to remove the remaining blood from the lungs. The lungs were dissected out, trimmed of any extraneous material, washed, weighed and frozen at -70°C.

2.6 Neutrophil antibody preparation.

Guinea pig anti-neutrophil antibodies were raised by a modification of a previously described method (Gleich *et al.*, 1975). Briefly, guinea pig neutrophils were obtained by instillation of 50ml of oyster glycogen (0.1% w/v) in distilled water into the peritoneum of adult guinea pigs. Seventeen hours later, the guinea pig was placed under general anaesthesia with 2-4% halothane (ICI, Macclesfield, UK) and maintained with nitrous oxide (0.4 litres/min) and oxygen (1-2 litres/min). The peritoneal cavity was then flushed with 50ml of saline (37°C) and the neutrophils purified using a percoll density gradient. Neutrophils partitioning at a density of 1.099gm/ml were recovered and washed twice in Hanks buffered salt solution with glucose. Neutrophils were then emulsified in Freunds complete adjuvant, incomplete adjuvant or saline and injected intramuscularly and subcutaneously into two New Zealand white rabbits over a period of 8 weeks as shown in Table 2.1. Two weeks after the last injection, the rabbit was bled by heart puncture, and a serum fraction obtained by spinning the red blood cells down at 2500rpm for 15 minutes at 4°C. The serum was then adsorbed to remove any antibodies to inflammatory cells other than the neutrophil. This was achieved by mixing the serum with $5.0x10^7$ cells from a peritoneal wash of an adult guinea pig pretreated with an intraperitoneal injection of horse serum. Treatment with horse serum resulted in a mix of inflammatory cells which included 51% macrophages, 42% eosinophils, 5.7% lymphocytes and 0.04% mast cells. The mixture was incubated at 37°C for 30 minutes and then transferred to 4°C for a further 30 minutes. The suspension was then centrifuged at 2500rpm, 4°C for 15 minutes and the treated serum further adsorbed against red blood cells. To remove antibodies that cross-react with red blood cells, blood was collected from adult guinea pigs in a heparinized container. The blood was spun at 1000rpm for 15 minutes at 4°C and the platelet rich plasma and white blood cells removed. The remaining suspension was respun at 2400rpm for a further 15 minutes at 4°C and the rest of the plasma removed. Five millilitres of treated antisera was added to 0.5ml of packed red blood cells and incubated as for that performed with white blood cells. The suspension was then centrifuged at 1000rpm and the antisera removed, aliquoted and frozen at -20°C ready for use. Two methods were used to assess the specificity of the neutrophil antiserum Firstly an antibody-dependant complement mediated cytoxicity test (Gewerz *et al,* 1980) and a direct binding fluorescence assay using fluroscein isothiocyanate (FITC) labelled anti-rabbit IgG-Fc (Cammisule, 1981) on a fluorescence

activated cell sorter. Doubling dilutions of neutrophil antisera were made in a 96-well plate and to which 100μl of a cell suspension ($1x10^6$) was added, incubated at R.T.P. for 30 minutes and sedimented by centrifugation (200g, 10mins). The cells were then washed in 200μl of HEPES-HBS containing 0.3% BSA and again sedimented. One hundred microliters of a 1 in 5 dilution of fresh rabbit serum, as a source of complement, was then added and the plate incubated at 37°C for 1 hour in a 5% CO_2 chamber. Cytotoxicity was assessed by the addition of 10μl of a fluorescent viability stain (0.5mg ethidium bromide and 0.15mg of acridine orange per ml of PBS). Viability was then assessed under a fluorescence microscope. Greater than 90% of the added neutrophils were killed at a dilution of 1/800.

In the direct binding assay with FITC labelled anti-rabbit IgG-Fc, the specific fluorescent antibody detection limit for neutrophils was at a dilution of 1/10,000 with maximum fluorescence occurring at a dilution of 1/5,000. No detectable fluorescence was seen for other cell types even at a dilution of 1/50.

Table 2.1 Protocol for raising neutrophil antisera in New Zealand white rabbits

WEEKS	NEUTROPHILS INJECTED	NEUTROPHIL PURITY (%)	EMULSIFIER
0	$2.9x10^7$	98.4	FCA
2	$5.6x10^6$	97.3	FICA
3	$4.2x10^7$	98.2	FICA
7	$3.7x10^7$	98.9	SALINE
8	$3.7x10^7$	97.2	SALINE

FCA:- Freunds complete adjuvant. FICA:- Freunds incomplete adjuvant.

2.6.1 Peritoneal neutrophil collection.

Adult, term or preterm guinea pigs were anaesthetized with 2-4% halothane (ICI, Macclesfield, UK) maintained with nitrous oxide (0.4 litres/min) and oxygen (1-2 litres/min). Ten milliliters per 100g body weight of 0.1% Oyster glycogen (w/v) in 0.9% NaCl (37°C) was then instilled into the peritoneal cavity of the animals. The animals were left to recover and 16 hours later they were reanaesthetized and the peritoneal cavity washed with 0.9% NaCl (37°C) to recover the elicited neutrophils. This was achieved by instilling 10ml aliquots of saline into the peritoneal cavity and allowing the fluid to drain

into a collection vessel. This was repeated 5 times and the recovered fluid combined.

2.7 Inflammatory cell purification of guinea pig blood and BALF by discontinuous percoll density gradients.

Blood from term or preterm guinea pigs was obtained by cardiac puncture following pentobarbitone overdose. Blood drawn into an heperanized syringe was mixed with 6% dextran (1:1) and left to stand for 45 minutes at R.T.P. The clear upper layer, rich in inflammatory cells, was decanted off into microfuge tubes and spun at 1600rpm for 10 minutes (R.T.P). The supernatant was then discarded and the red blood cells in the pellet lysed with 1ml of 0.2% NaCl. The suspension was vortexed for 30 seconds after which 1ml of 1.6% NaCl was added and mixed. The suspension was respun at 1600rpm for 10 minutes and the supernatant again discarded. The pellet was resuspended in Hanks buffered salt solution with glucose (HBSG) and layered on top of the percoll gradient. BALF and peritoneal cells were removed by centrifugation at 1500rpm for 10 minutes (4°C), the supernatant discarded and the pellet resuspended in 1ml of HBSG before applying to the percoll gradient. Differing percoll densities were prepared by mixing differing volumes of HBSG and 90% percoll to obtain densities ranging from 1.1gm/ml to 1.06gm/ml. One millilitre of each density was placed on top of 1ml of a higher density until 10 layers were achieved. One ml of prepared cells was then placed on top and the mixture spun at 1700rpm for 15 minutes (R.T.P). Cells collecting at the interface between each density were harvested, placed in a microfuge tube and spun at 2600rpm for 20 minutes (R.T.P). The supernatant was discarded and the pellet washed once in HBSG. Washed cells were resuspended in 1ml of HBSG, a total and differential cell count performed and an appropriate volume used to measure superoxide production as described in section 2.10.4

2.8 Light and electron microscopy.

Pups were anaesthetized by intraperitoneal injection of pentobarbitone (50mg/kg). Following the onset of deep anaesthesia the trachea was isolated and a 14g cannula inserted and secured. The thoracic cavity was then opened and the lungs inflated with air to a constant pressure of 10cm H_2O. The lungs were then perfused via the right ventricle for 60 minutes with half-strength Karnovskys fixative. The heart and lung preparation was then dissected from the thorax *en bloc* and immersed in full-strength Karnovskys fixative (2.5%

glutaradehyde and 2.5% paraformaldehyde) for 24 hours. The left caudal lobe was then bisected from the hilum to the periphery and the facing sections processed for light and electron microscopy as described previously (Town, 1990).

2.8.1 Light microscopy.

For light microscopy, 4μm sections were cut and stained with haematoxylon and eosin (H&E) and Martius Scarlet Blue (MSB) by the Department of Histology, Southampton General Hospital. Colour photographs were taken for descriptive purposes.

2.8.2 Electron microscopy.

For electron microscopy, four tissue pieces were cut from the central portion of the caudal lobe, stored overnight in cacodylate/sucrose buffer (pH 7.4, 850 mosmol/l), post fixed in 2% (w/v) osmium tetroxide for 2h and then stained *en bloc* with 1.5% (w/v) uranyl acetate before being embedded in Spurr resin. Two of these blocks were then selected at random for thick (0.5μm) sections, which were stained with toluidine blue and an appropriate area was selected for thin sectioning (0-90nm). Thin sections were mounted on high-transmission copper grids and were stained with lead acetate. Each thin section was qualitatively assessed and photographed on an Hitachi 7000 electron microscope (Hitachi, Tokyo, Japan).

2.9 Total and differential leucocyte count.

2.9.1 BALF.

Exposure to elevated concentrations of oxygen leads to pulmonary and circulatory changes in the cellular population that are characteristic of an inflammatory response (Crapo *et al.*, 1980; Fox *et al.*, 1981; Chen *et al.*, 1994). Thus to assess whether the premature guinea pig develops inflammation following delivery and exposure to high concentrations of oxygen, BALF and blood total and differential cell counts were measured in the following way.

Total cell counts were performed on BALF before centrifugation using a Neubauer haemocytometer. The number of cells in 0.1mm^3 grid were counted and the total number of cells per ml of BALF calculated. A volume of BALF equating to 50,000 cells was used for differential cell counts. The cells were spun down onto slides by centrifugation in a

cytospin at 150g for 10 minutes. The slides were then air dried overnight and stained with May-Grunwald-Geisma. Differential counts were performed on 200-300 cells per slide. The results were initially expressed as a percentage then converted to absolute numbers for statistical analysis.

2.9.2 Blood.

Fifty microlitres of blood and 150μl of 1.5% acetic acid containing methylene blue (0.1%) were incubated at room temperature for 3 minutes to lyse red blood cells. A sample of this solution was then placed on a Neubauer haemocytometer and white blood cell number calculated as for BALF cells. Differential cell counts were performed on May-Grunwald-Geisma stained blood smears. One hundred cells per slide were counted and the results expressed as a percentage before being converted to absolute values, expressed per ml of blood.

2.10 Biochemical assays.

2.10.1 Blood and BALF elastase inhibitory capacity (EIC)

An imbalance between proteinases and their appropriate antiproteinases, especially elastase and α1-proteinase inhibitor (α1-PI) have been implicated in the development of acute and chronic lung disease in the preterm infant. The concentration of α1-PI can be measured by assessing the percentage inhibition of exogenously administered elastase in serially diluted plasma or BALF. This leads to the titration curve shown in figure 2.2, which is due to the partitioning of elastase between α2-macroglobulin and α1-PI. This standard method was employed to measure the concentration of functional α1-PI. Immunological levels were not measured because a proportion of the total concentration would be from oxidised and elastase complexed α1-PI, both functionally inactive. The results give an indication of the functional antiprotease screen lining the lung with respect to protection from neutrophil elastase. Other protease-inhibitor capacities can be measured by substituting elastase with other proteases.

The capacity of blood and BALF to inhibit the degradation of the synthetic substrate Succinyl-trialanyl paranitroanilide (SLAPN) by exogenously administered porcine pancreatic elastase (PPE) was determined as a measure of the EIC. One hundred microlitres of BALF or appropriately diluted plasma (1/100 - 1/500 in buffer: 0.2M Tris-HCl pH 8.0) was

serially diluted in a microtitre plate with Tris-HCl buffer (0.2M Tris-HCl pH 8.0). Fifty microlitres of active site titrated PPE (11.5ng/ml) was then added to each well and preincubated at 37°C for 15 minutes. Fifty microlitres of SLAPN (2mM in buffer) was then added and incubated at 30 minutes at 37°C. The absorbance at 410nm was then recorded and the remaining activity, expressed as a percentage of the total activity associated with 50μl of PPE. The percentage activity remaining was then plotted against the volume of BALF or plasma and the linear portion of the curve extrapolated to the amount of BALF or plasma required to completely inhibit the added PPE. This method of determination of EIC is applicable even in the presence of α_2-macroglobulin (Fig 2.2). Interassay variation for blood EIC was 8.7% and for BALF EIC 10.3%

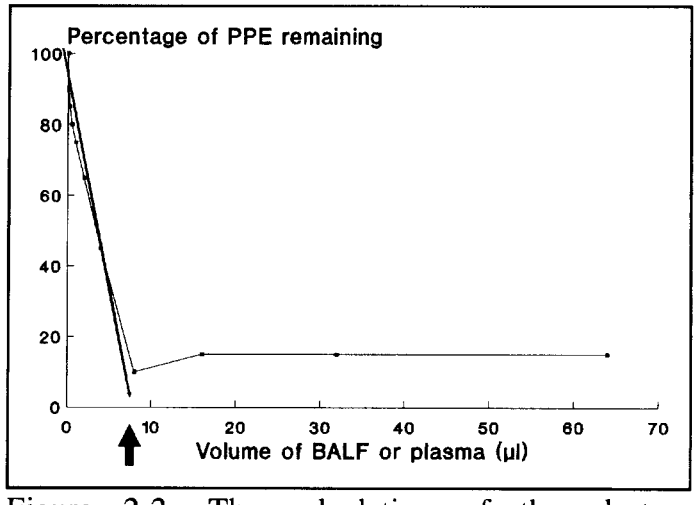


Figure 2.2. The calculation of the elastase inhibitory capacity of plasma or BALF.

2.10.2 BALF, lung tissue and plasma platelet activating factor (PAF).

Platelet activating factor is associated with the development of chronic lung disease in the oxygen treated preterm infant (Koyama *et al.*, 1993) and measured by an adapted method of Lysy *et al* 1992. Briefly, the method involves following the rate of rabbit platelet aggregation after addition of sample. The sensitivity of this method equates with well with other methods of PAF detection including HPLC and radioimmunoassay. However, this method is less time consuming than HPLC analysis and less expensive than radioimmunoassay.

Reagents: Wash buffer, pH 6.5

2.6mM Potassium chloride, 1.0mM Magnesium chloride $6H_2O$, 137.0mM Sodium chloride

12.0mM Sodium hydrogen carbonate, 5.5mM Glucose, 0.25% Bovine serum albumin, 0.2mM EGTA.

Aggregation buffer pH 7.4

2.6mM Potassium chloride, 1.0mM Magnesium chloride $6H_2O$, 137.0mM Sodium chloride. 12.0mM Sodium hydrogen carbonate, 5.5mM Glucose, 0.25% Bovine serum albumin, 1.3mM Calcium chloride $2H_2O$.

2.10.2.1 Sample preparation.

Frozen lung samples were homogenised in 10 volumes of 0.01M potassium phosphate containing 0.03M potassium chloride buffer for 30 seconds in an Ultraturax (Jane & Kunkel, Staufen, Sweden) homogeniser. The tissue was then mixed vigorously with 10 times the volume of chloroform/methanol (2:1) mixture. The chloroform layer was then removed with a siliconised pasteur pipette and dried under a stream of nitrogen. Plasma and BALF were extracted in the same manner, with 10 volumes of chloroform/methanol and dried. The dried lipid was then resuspended in aggregation buffer (500μl) of which 5μl to 25μl were assayed for PAF.

2.10.2.2 Rabbit platelet preparation.

Following decapitation, rabbit blood was collected in non-heparinised containers containing 0.1M EDTA (1ml per 10ml of blood) and gently mixed by rotation. The blood was then centrifuged at 1400rpm (340g) for 20 minutes at R.T.P, the platelet rich supernatant removed with a sterile siliconised pasteur pipette and placed again into a non-heparinised blood tube. Acetyl salicylic acid (ASA: 4mM [200μl/ml of supernatant]) was then added and gently mixed for 30 minutes at R.T.P. The supernatant was then respun at 2800rpm for 15 minutes at 21°C, and the supernatant from this spin discarded. The remaining pellet, containing platelets, was washed twice in 10ml of wash buffer and spun at 2800rpm for 15 minutes at 21°C. After the second wash, the pellet was resuspended in aggregation buffer and 20μl of this was made up to 20ml in isotone II solution (Coulter electronics Ltd, UK). This solution was further diluted (1/10) and the number of platelets counted using a coulter counter (Coulter electronics, Hialeah, Florida, USA) and checked against platelet chex (Streck Laboratories Inc, UK), a platelet standard. The platelets were

then appropriately diluted to give 6 x 10^8 platelets/ml of which 200μl was added to a siliconised aggregation tube and incubated for 5 minutes at 37°C. Ten microlitres of sample or standard (2nM - 10nM PAF in chloroform) was then added and the aggregation followed on an aggregometer (Payton Associates, Scarborough, Ontario, Canada), attached to a pen recorder for 2 minutes. Results were calculated against the standard curve and either expressed per gram tissue, per ml of BALF or per ml of plasma. Interassay variation of standards was 5.3%.

2.10.3 BALF leukotriene B_4 (LTB_4).

Two standard methods frequently used to measure LTB_4 concentrations include HPLC and radioimmunoassay. Although HPLC analysis is more sensitive it is technically more difficult and laborious. Preliminary studies with preterm guinea pigs had detected BALF LTB_4 using the radioimmunoassay and as such this technique was used for all samples. BALF LTB_4 concentrations were measured based on the method of Taniguchi *et al* 1986. Briefly, ethanol was added to BALF samples to a final concentration of 10%, which was then acidified to pH 3.0 with 0.1M HCl. The samples were then passed through an octadecylsilyl silica cartridge and successively washed with 10% aqueous ethanol (20ml), distilled water (20ml), petroleum ether (20ml) and finally methylformate (10ml). The last fraction was dried under nitrogen and the residue dissolved in 250μl of radioimmunoassay (RIA) buffer. Using this method recovery of ^3H LTB_4 from BALF was 60%. Measurement of LTB_4 was performed using an enzyme immunoassay (EIA) kit (Metachem Diagnostics Ltd, Northhampton, UK) based on the competition of variable amounts of LTB_4 in samples with a fixed amount of alkaline phosphatase labelled LTB_4 for a limited number of sites on the rabbit anti-LTB_4 antibody coated on wells of a microtitre plate. One hundred microliters of standard (0-5000pg/ml) or sample was added in duplicate to the precoated antibody wells and gently agitated for 15 seconds. A further 100μl of rabbit anti-LTB_4 antibody was then added, again gently mixed and incubated overnight at 4°C. After incubation 100μl of LTB_4 alkaline phosphatase conjugate was added to all wells and incubated at 4°C for 3 hours. All wells were then aspirated and washed 3 times with 400μl of wash buffer. Three hundred microlitres of para-nitrophenyl phosphate substrate was finally added, incubated at 37°C for 2 hours. Absorbance was read after addition of 50μl of stop reagent and sample concentrations read against standards. Interassay variation was 11.6%.

2.10.4 BALF inflammatory cell superoxide production.

Superoxide production was measured using the superoxide dismutase inhibitable reduction of cytochrome C as previously described (Petreccia *et al.*, 1987). Although sensitivity of the assay is less than that obtained with fluorescence probes, the fact that the colorimetric method could be scaled down on to microtiter plates was an influential factor in the choice of this assay. Microtiter plates enabled many more recordings to be made from the same sample and the lower sensitivity is compensated by many more readings. Also a greater through put of samples could be achieved making the assay less time consuming than other methods.

Reagents: Hanks buffered salt solution with glucose (HBSG). 120μM cytochrome C, 1.8mM calcium chloride, 1.0mM magnesium chloride in Hanks buffered salt solution $\pm$ 25μg/ml superoxide dismutase. 200ng/ml phorbol myristate acetate (PMA) in dimethyl sulfoxide. 5 X 10^6 inflammatory cells/ml Hanks buffered salt solution.

Procedure: To 90μl of prewarmed (37°C) cytochrome C solution in HBSG (with and without superoxide dismutase) in individual wells of a 96 well microelisa plate (EL340, Bio-Tek, Winooski, U.S.A.) 10μl of PMA (10ng/ml final concentration) was added. One hundred microlitres of inflammatory cells (5 X 10^5 cells) in HBSG, prewarmed to 37°C was then added to each well and the initial absorbance measured at 550nm. The plate was incubated at 37°C for 15 minutes and the final absorbance measured. The production of superoxide was then calculated from the differences in absorbance (final - initial), with and without superoxide dismutase and from the extinction coefficient of cytochrome C (E_{550}=2.1x$10^4 M^{-1}cm^{-1}$). To obtain the maximum rate of superoxide production and lag phase, absorbance was measured every 15 seconds for 30 minutes and tabulated as shown in Figure 2.3. The maximum rate of superoxide production was calculated from the slope and the lag phase from the addition of agonist to the point of maximum change in absorbance. The rate of superoxide production was expressed as nmoles superoxide/minute/10^5 cells and the lag phase in minutes (Fig 2.3).

2.10.5 BALF and lung tissue protein.

Protein was measured using the colorimetric bicinchoninic acid (BCA) method as

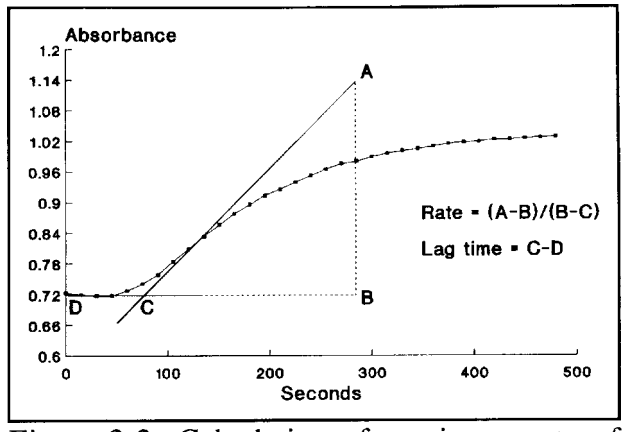


Figure 2.3. Calculation of maximum rate of superoxide production and lag phase following stimulation with PMA.

previously described (Smith *et al.*, 1985). Although as sensitive as the Lowry protein assay, the simplicity of the method and the ability to scale down to microtiter plates was a deciding factor on its use.

BCA is a chromogenic reagent that reacts with Cu^{+} ions following its reduction by protein and forms a coloured complex that absorbs at 562nm. The BCA solution was mixed in a 50:1 ratio with 4% Cu^{++} sulphate and added to 10μl of sample (BALF or tissue homogenate) or standard (0.2 - 1mg/ml) in a microelisa plate. The plate is incubated at 37°C for 30 minutes in a EL340 microelisa plate reader and the absorbance read at 550nm. The protein concentration was then determined by comparison with the range of known standards. Quality control samples were run in each assay. The inter-assay variation was 0.5%.

2.10.6 BALF neutrophil elastase activity.

An imbalance between proteinases and the appropriate antiproteinases, especially elastase and α1-PI, has been implicated in the development of acute and chronic lung disease in the preterm infant receiving supplemental oxygen. Thus in addition to the measurement of EIC, neutrophil elastase activity in BALF was measured. Elastase released from neutrophils can be found in one of three compartments, free within the extracellular medium, sterically but not proteolytically inhibited by α2-macroglobulin or proteolytically inhibited by covalent binding to α1-PI. Elastase activity was measured using two methods. The first method utilised a low molecular weight synthetic substrate methoxy-succinyl-ala-ala-pro-val-p-nitroanilide that measures free and α2-macroglobulin bound elastase and is based on the

procedure described by Castillo *et al.* 1979. This substrate is the most specific of the low molecular weight substrates for neutrophil elastase and on cleavage of the p-nitroanilide moiety by elastase can be monitored colorimetrically at 410nm. Similar substrates have been employed but using fluorometric chromogens and although slightly more sensitive than the above colorimetric assay, the method employing the p-nitroanilide substrate above is sensitive enough to pick up neutrophil elastase activity in BALF.

Fifty microlitres of BALF or standard (porcine pancreatic elastase:- 0.72-2.16ng/ml) and 100μl of buffer (0.2M Tris-HCl, pH 8.0) were preincubated in individual wells of a 96-well microtitre plate at 37°C for 30 minutes. Prewarmed substrate (50μl; 2mM in buffer) was then added to each well, covered in aluminium foil and incubated in a 37°C water beth for 24 hours. The absorbance at 410nm was measured and elastase concentration determined from the standard curve. Interassay variation as assessed by BALF QC was 3.5%.

The second method utilises tritiated elastin, the natural substrate for elastase, and is therefore a unique assay for neutrophil elastase. This substrate is to large to enter the α2-macroglobulin-elastase complex and therefore only measures free elastase activity. This method is based on the method of Banda *et al*, 1981. Briefly, dry elastin powder (2.5g: <400 mesh, type E60; Elastin products, St Louis, MO, U.S.A.) was moistened with ethanol and then suspended in 50 ml of distilled water. The suspension was then pH'd to 9.2 with 100mM NaOH before addition of NaB^3H_4 (25mCi) in 3mM NaOH. The mixture was mixed for 10 minutes at R.T.P. and then 250mg of cold $NaBH_4$ added. This suspension was further mixed for 2 hours in a well ventilated hood after which the pH was adjusted to 3.0 with glacial acetic acid. After a further 30 minutes of mixing, the labelled elastin was collected following centrifugation at 10000g for 30 minutes. The pellet was then repeatedly washed with distilled water until the counts in the wash was equivalent to background values. The elastase assay involved 200μg of labelled elastin in 200μg of 100mM Tris/HCl, pH 7.8 (containing 5mM $CaCl_2$, 0.02% NaN_3) to which 100μl of BALF or plasma was added. The mixture was then incubated for 1 hour at 37°C. the mixture was then centrifuged for 3 minutes in a microfuge and 100μl of supernatant was counted by liquid scintillation spectrometry. One unit was take as the solublisation of 1μg of elastin/hour/37°C.

2.10.7 BALF phospholipase A_2 activity.

The presence of LTB_4 and PAF in the BALF of preterm infants with chronic lung disease (Groneck *et al.*, 1993; Koyama *et al.*, 1993), implicates PLA_2 as a major contributor to the alteration in lung structure and function seen in these babies. Although not as sensitive as methods that use radiolabelled substrates, it is much less laborious. Preliminary studies had also shown that the method was sensitive enough to detect PLA_2 activity in guinea pig BALF samples and as such was used in the thesis.

The method is based on the displacement from rat liver, fatty acid binding protein (FABP) of the fluorescent probe 11-(dansylamino)undecanoic acid (DAUDA) by long chain fatty acids liberated from dioleoylphosphatidylglycerol (DOPG) by phospholipase A_2 as previously described (Wilton, 1990).

Reagents: 200mM Tris buffer containing 0.1M sodium chloride and 2.5mM calcium chloride at pH 8.0, DOPG (10mg/ml), DAUDA ($1x10^{-4}$M) in methanol, FABP (0.38mg/ml).

Procedure: A stock assay cocktail was prepared by adding 5ml of Tris buffer, 4.67ml of Analar water, 50μl of DOPG and 100μl of DAUDA. 967μl of this mixture was added to a 1ml plastic fluorometer cell kept at 37°C in a Perkin-Elmer LS3B fluorescence spectrophotometer. The solution was excited at 350nm and the fluorescence emission was measured at 500nm. The fluorescence was adjusted to zero and 33μl of FABP was added. Once the resultant increase in fluorescence was scaled to read 90% on the chart recorder, 10μl of BALF or plasma was added to the mixture and the initial rate of fall in fluorescence was measured. Calibration of the assay was achieved by adding known amounts of oleic acid in the absence of sample and measuring the rate of fall in fluorescence. Rates measured for samples are expressed as pmoles of oleic acid equivalents per minute.

2.10.8 BALF conjugated dienes.

Exposure to elevated oxygen concentrations leads to increased free radical production in lung tissue (Freeman *et al.*, 1982) and to the development of conjugated dienes in various lipids. This method has been employed for many years in the food industry and although more precise and sensitive methods are now available for the detection of oxidative damage

to lipids, the spectrophotometric analysis of conjugated dienes is simple, straight forward and enables large numbers of samples to be analysed quickly.

Briefly the method is based on the absorbance of a lipid extract at 233nm published by Reckagel *et al* 1984. BALF (800μl) was mixed with 3ml of a methanol:chloroform mixture (2:1, V/V). The solution was then extracted with an equal volume of a chloroform:water mix (1:1 v/v) and mixed well. The solution is then spun at 2000rpm for 10 minutes, the lower phase removed and dried under a stream of nitrogen. The dried material was then washed 3 times in a chloroform:methanol:water mixture (1:16:15.7, v/v/v) and again dried under nitrogen and stored at -70°C. For analysis, the sample was redissolved in HPLC grade cyclohexane and read against a cyclohexane blank at 233nm. The concentration of conjugated dienes were then calculated from the extinction coefficient $\Sigma=2.8 \times 10^3 M^{-1}cm^{-1}$. Interassay variation of repeated sample was 6.7%.

2.10.9 BALF dipalmytlphosphatidylcholine (DPPC).

The initial development of RDS in the preterm neonate is due to surfactant deficiency. Dipalmitoylphosphatidylcholine (DPPC) is the major surface reducing component of surfactant and is used as a marker of surfactant concentrations.

The assay is based on the published method of Takayama *et al* 1977 as adapted by M. Cabral, 1992. The method entails the hydrolysis of PC by phospholipase D (PLD) to liberate choline. Choline is subsequently oxidised by choline oxidase to betaine and hydrogen peroxide. In the presence of peroxidase, 4-aminoantipyrine and phenol, hydrogen peroxide yields a red quinone dye which can be read at 500nm. Previous methods used in the analysis of DPPC have included HPLC which apart from being time consuming and technically difficult does not enable large smaple numbers to be quickly processed. The method of Takayama *et al.,* 1977 is adapted for microtiter plates in the present thesis, is as sensitive as the original method (Cabral 1992) and enables large sample numbers to be analysed.

Reagents: 1mM DPPC in 10mM Tris/HCl buffer pH 8.0 containing 0.25% Triton X-100 50mM Tris/HCl buffer pH 8 (containing 2g triton X100/litre)

Colour reagent: PLD (45 units), choline oxidase (100units), 4-aminoantipyrine (12mg),

phenol (20mg). Calcium chloride (8mg), 100ml Tris/HCl buffer.

One hundred microlitres of BALF or DPPC standards (0 to 50nmoles/250μl) was added, in duplicate, to a microtitre plate to which 10μl of PLD was then added and incubated for 50 minutes at 42°C. The colour reagent was then added and the plate incubated for a further 50 minutes at 37°C. Absorbance at 492nm was then assessed using a Titretek Multiscan Plus Spectrometer. Results are expressed as nmoles of DPPC per ml of BALF. Intrassay variation of standards was 12.3%.

2.10.10 Lung tissue and plasma protein carbonyls.

This assay was based on the published method of Levine *et al* 1990. Other methods of analysis of oxidatively modified proteins are avaliable and more sensitive. However, this method does not require elaborate preparations and can be performed quickly with large numbers of samples. The assay is a well recognised standard method for analysing proteins with oxidative damage.

Reagents: 50mM phosphate buffer pH 7.4 (containing 5mM EDTA), 20% Trichloroacetic acid (TCA). 2M HCl (containing 0.1% dinitrophenylhydrazine [DNP]), 1:1 (v/v) ethanol: ethyl acetate and 6M guanidine-HCl.

Procedure: Lung tissue was homogenised in 50mM phosphate buffer to give a protein concentration of 1mg/ml. Plasma was diluted with phosphate buffer to give a concentration of 1mg/ml. One hundred microlitres of homogenate or plasma was then added to 500μl of 20% TCA and vortexed mixed for 1 minute. The solution was then centrifuged at 12000rpm for 10min, the supernatant removed and the pellet resuspended in 500μl of 2M HCl containing 0.1% DNP and incubated at room temperature for 1 hour. The mixture was then respun (12000rpm) and the pellet resuspended in 500μl of 20% TCA. The mixture was again spun at 12000rpm for 10 minutes and the pellet washed three times with 1ml of ethanol:ethyl acetate. After addition of wash solution, the pellet was left to stand for 10 minutes before centrifugation. Finally, 6M quanidine-HCl was added to the final pellet and the mixture incubated at 37°C. After 60 minutes the mix was spun (1200rpm) and the supernatant read at 370nm against samples that did not contain DNP. The protein content

of the final pellet was measured using the BCA method and the absorbance standardised to protein. The results were then calculated as nmoles carbonyls/mg protein.using E_{370} = 2.1 x $10^4 M^{-1}$ cm^{-1}. Interassay variation of repeated plasma sample was 8.8%.

2.10.11 BALF ß-glucuronidase activity.

ß-glucuronidase activity, is a standard, non-specific marker of inflammatory cell activation and as such was measured in BALF by the spectrophotometric determination of phenolphthalein liberated from phenolpthalein mono-ß-glucuronic acid. Duplicate 125μl samples of BALF were incubated at 56°C in the presence of 100μl of 0.03M phenolphthalein mono-ß-glucuronic acid, pH 4.5 and 75μl of 0.2M acetate buffer pH 4.5. One hundred and twenty five microlitres of 0.1M 2-amino-2-methyl-1-propanol buffer pH 11.0 with 0.2% sodium lauryl sulfate was added after 1hr and the optical density measured at 550nm. Calibration curves were constructed with phenolphthalein (0-8μg) and the results expressed as units of ß-glucuronidase activity. One unit represents the activity required to generate 1μg of phenolphthalein under the assay conditions.

2.10.12 Lung tissue and BALF reduced and oxidised glutathione

The assay for oxidised and reduced glutathione is an adaption of the method of Tietze *et al* (1969). Although both reduced and oxidised glutathione can be detected at much lower concentrations using HPLC, levels found within BALF and lung tissue are easily measured using this colourimetric assay. This assay also requires little preparation and following scaling down to microtiter plates, large number of samples and replicates can be assayed.

Reagents: 0.2N Perchloric acid (PCA), Vinylpridine, 125mM phosphate buffer pH 7.5 (containing 6mM EDTA), 0.3mM NADPH in phosphate buffer, 6mM DTNB, glutathione reductase (15ul stock/ml 3.6M $(NH_4)_2SO_4$), 20mM Glutathione.

Procedure: Freshly removed lung tissue (0.2g) was added to 3ml of 0.2N PCA, homogenised and centrifuged at 2000rpm (4°C) for 10 minutes. The supernatant was diluted 1:5 with distilled water and assayed directly for oxidised and reduced glutathione. Freshly obtained BALF was used undiluted for glutathione measurement. NADPH and DTNB were mixed together just prior to the assay in a ratio of 7:1 (v/v) and 160μl of this added to 40μl

of sample or standard (0.01nmol/ml - 1.0nmol/ml) in a microtitre plate. The plate was then incubated at 30°C for 5 minutes after which time 50μl of glutathione reductase was added and the rate of change in absorbance, measured over 1 minute at 405nm was assessed. To assess the amount of oxidised glutathione, 1ml of sample was incubated for 1 hour at R.T.P. with 10μl of vinylpyridine. Samples were then assayed as for reduced glutathione. Results were calculated from the standard curve and tissue levels expressed as μg/g tissue and BALF levels expressed as ng/ml BALF. Interassay variation was 6.3%.

2.11 Lung tissue preparation for antioxidant analysis.

The typical response of a tissue to oxidants is the increase in production of tissue antioxidants. To assess the initial antioxidant status and response of the immature lung to hyperoxic exposure lung tissue antioxidant analysis was undertaken. With all the enzymatic antioxidants, all methods employed are standard protocols that have been verified by the NRRG. If analysis of the antioxidant nature of particular cell types are required newer methods should be assessed.

Frozen lung samples were homogenised in 10 volumes of 0.01M potassium phosphate containing 0.03M potassium chloride buffer for 30 seconds in an Ultraturax (Jane & Kunkel, Staufen, Sweden) homogeniser. The homogenate was then sonicated on ice (MSE Soniprep, Leics, UK) at setting of 18μ by 6 ten second bursts. One hundred microlitres of the sonicated homogenate was then added to 900μl of 50mM phosphate buffer and stored at -20°C for DNA (2.11.1) and protein (2.10.5) analysis. The remainder was incubated on ice for 30 minutes with 1% by volume of absolute alcohol. The samples were then mixed with 10% by volume of 10% Triton-X100 and then centrifuged at 10,000g for 5 minutes. The supernatant was then split into four portions and stored at -20°C until assayed for antioxidants.

2.11.1 DNA.

DNA was measured using a previously described and well recognised method (Richards, 1974). Although not as sensitive as fluorometric methods the method of Richards was adequate for the quantity of tissue that was taken.

Reagents: Diphenylamine reagent: Diphenylamime (40g/l) and Paraldehyde (0.1ml/l) in

concentrated acetic acid, 4M perchloric acid and amyl acetate.

Procedure: DNA standards (0-500μg) or samples (0.5ml of homogenate) were incubated with an equal volume of 4M perchloric acid at 70°C for 20 minutes. After cooling to room temperature, 0.6ml of diphenylamine reagent was added, mixed and left to stand for 24 hours. The resulting blue colour was extracted into 1.5ml of amyl acetate by vortex mixing, and the upper blue phase removed and measured optically at 600nm. Interassay variation for quality control tissue homogenate was 7%.

2.11.2 Catalase (CAT) activity.

The CAT activity of the supernatants was assessed by following the catalytic reduction of hydrogen peroxide at 240nm as previously described (Aebi, 1984).

Reagents: 50mM phosphate buffer pH 7.0, 30mM hydrogen peroxide (340μl of a 30% hydrogen peroxide was added to 100ml of the phosphate buffer).

Procedure: Fifty microlitres of standards (1.25 - 15 units catalase/ml) or sample (diluted 1:10) were added to 950μl of phosphate buffer and mixed. Five hundred microlitres of 30mM hydrogen peroxide was then added to start the reaction and the decomposition of the hydrogen peroxide was followed continuously at 240nm using a spectrophotometer. Catalase activity was defined as K.U (Kilo units) and expressed per mg DNA. Interassay variation of tissue homogenate quality control using this technique was 6%.

2.11.3 Glutathione peroxidase (GSH-Px) activity.

Glutathione peroxidase activity was assessed using a perviously described (Beutler, 1979). Briefly, in the presence of glutathione, GSH-Px reduces tertiary butyl hydrogen peroxide to its corresponding alcohol. The oxidised glutathione that is generated is then reduced by glutathione reductase in the presence of NADPH. The oxidation of NADPH was then followed at 340nm, the rate being proportional to the activity of the GSH-Px present.

GSH-Px

$$2GSH + \text{t-butyl-OOH} \xrightarrow{\text{GSH-Px}} GSSG + H_2O + \text{t-butyl-OH}$$

GSH-Rd

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSH-Rd}} 2GSH + NADP^+$$

Reagents: 0.1M Tris-HCl pH 8.0 containing 5mM EDTA, t-butyl hydrogen peroxide (TBHP) (1/10000 dilution of stock was made in distilled water in a glass volumetric flask). Substrate solution: 0.2mg/ml NADPH, 0.614mg/ml glutathione and 1u/ml glutathione reductase all in the Tris-HCl buffer.

Procedure: To 1ml of substrate solution, 50μl of supernatant was added and mixed. The reaction was started with the addition of 100μl of TBHP and followed at 340nm for 2 minutes at 37°C on a LKB reaction rate analyzer. Glutathione peroxidase activity was calculated from the extinction coefficient of NADPH ($\epsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). Interassay variation using a quality control tissue homogenate was 3%.

2.11.4 Glutathione reductase (GSH-Rd) activity.

Glutathione reductase activity was measured according to the method of Langley *et al* 1993.

Reagent: 0.1M Sodium phosphate buffer pH 7.6 (containing 0.1mM NADPH, 0.5mM EDTA and 1.0mM GSSG)

Procedure: To 30μl of sample in a microtitre plate 200μl of reagent was added and the absorbance followed for 1 minute (30°C) at 340nm.

2.11.5 Superoxide dismutase (SOD) activity.

Both copper/zinc (Cu/Zn) and manganese (Mn) SOD activities were determined using the pyrogallol autooxidation method according to Marklund 1985 . Briefly, SOD activity is assessed by the inhibition of oxidation of pyrogallol at pH 8.2. Cu/Zn SOD and Mn SOD were distinguished by the inhibition of Mn SOD activity by cyanide.

Reagents: 0.1M Tris-HCl, pH 8.2, working buffer (50ml 0.1M Tris/HCl, pH 8.2, 49ml distilled water and 1.0ml of 0.1M diethylenetriaminepentacetic acid (DTPA)), 24mM pyrogallol in 0.01mM HCl and 33mM potassium cyanide.

The working buffer was vigorously aerated for 20 minutes at room temperature and adjusted to pH 8.2. Catalase (final concentration 1μM) is added to remove hydrogen peroxide and prevent production of superoxide anions in the solution.

Procedure: Total SOD activity was measured by adding 50μl of sample or standards (1 - 10 units/ml) to 1ml of working buffer. The reaction was started by the addition of 50ul of 24mM pyrogallol. The reaction was followed continuously in a reaction rate spectrophotometer at 420nm at 25°C. Cu/Zn-SOD activity was determined in the same way as for total SOD with the exception of the working buffer which contained cyanide at a final concentration of 2mM. Mn-SOD activity was calculated as the difference between the total SOD activity and the Cu/Zn-SOD activity. Interassay variation for tissue homogenate was 8%.

2.11.6 Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphoglucuronate dehydrogenase (6PGDH) activity.

Both G6PDH and 6PGDH activities are based on the standard method of Ninfali *et al* 1990 adapted for the plate reader. As with most of the other methods, adaption of this standard assay to the plate reader permitted large numbers of samples to be analysed very quickly. Briefly, the activity of the two enzymes were measured by following the increase in absorbance of NADPH following addition of glucose-6-phosphate (G6P) and 6-phosphoglucuronic acid (6PG) to the reaction mixture. The rate of 6PGDH on its own was measured in the same way, but following the addition of 6-phosphoglucuronic acid only to the reaction medium. The activity of G6PDH was then calculated from the difference in rate of absorbance between the two reactions.

Reagents: Homogenisation buffer.

5mM NaK phosphate buffer pH 8.0, 25mM ß-Mercaptoethanol, 3mM NaF, 0.2% Triton X-100, 1mM PMSF.

Assay buffer.

1M Tris/HCl pH 8.0 containing 5mM EDTA, 100mM $MgCl_2$, 2mM NADP, 6mM Glucose-6-phosphate and 6mM 6-phosphoglucuronic acid.

Frozen lung samples were homogenised in 10 volumes of 0.01M potassium phosphate containing 0.03M potassium chloride buffer for 30 seconds in an Ultraturax (Jane & Kunkel, Staufen, Sweden) homogeniser. To measure the rate of both enzymes (G6PDH and 6PGDH) and 6PGDH on its own, two solutions were made up. Solution A contained Tris, MgCl, NADP and ± G6P/6PG and solution B contained Tris, MgCl, NADP and ± 6PG. The concentrations of ingredients was such that when 190μl of each solution was made up to 200μl with 10μl of sample, the assay concentrations were met. The increase in absorbance was followed over 1 minute at 25°C and the rate of NADPH formation calculated. Blank solutions containing no substrate were also included. The rate of increase in absorbance for 6PG was subtracted from that of the G6P/6PG rate to give the activity of G6PDH. One unit of activity was defined as the amount of enzyme that catalysed the formation of 1μmole of NADPH per minute using the extinction coefficient of $6.3x10^3$ $mol^{-1}lcm^{-1}$ for NADPH. Interassay variation was 9.1%.

2.11.7 Lung tissue collagen

Lung tissue collagen was measured according to the method of Campa *et al* 1990 . Briefly, tissue collagen was measured by assessing the derivatised hydroxyproline content of an acid hydrolysate of lung tissue. Hydroxyproline is found in high concentrations in collagen and in low concentrations in other proteins. Thus the measurement of hydroxyproline relates almost exclusively to collagen. This method is an adaption of an older colorimetric assay in which hydroxyproline is measured following liberation by acid hydrolysis. Derivatisation of hydroxyproline and the detection by HPLC increases the sensitivity of the assay and allows much smaller samples to be analysed. Frozen lung samples were homogenised in 10 volumes of 0.01M potassium phosphate containing 0.03M potassium chloride buffer for 30 seconds in an Ultraturax (Jane & Kunkel, Staufen, Sweden) homogeniser. The proteins were then precipitated by adding ethanol to a final concentration of 67% (V/V) and leaving at 4°C overnight. The samples were then centrifuged at 4°C for 30 minutes at 30,000g and the pellet washed twice with 67%

ethanol. Samples were then dried under nitrogen with P_2O_5. Proteins were hydrolysed in 6M HCl at 110°C for 16hr and then decolorised with charcoal before filtration (0.65μm pore size filter, Millipore, Ltd, UK). The hydrolysates were then evaporated to dryness under vacuum at 45°C and the residue dissolved in 1ml of water. Aliquots (0.1ml) of the hydrolysates were buffered with 0.1ml of potassium tetraborate (0.4M, pH 9.5) to which 0.1ml of 12mM NBD-Cl in methanol was added. Derivatization proceeded at 37°C for 20 minutes in the dark. Addition of 0.05ml of 1.5M HCl and 0.15ml of 167mM sodium acetate, pH 6.4, in 26% acetonitrile (V/V) halted the reaction. An aliquot of the sample (100μl) was then chromatographed using an LKB single pump gradient system (Pharmacia/LKB, Milton Keynes, U.K.) with a reverse phase cartridge column (LiChroCART LiChrospher 250mm length x 4 mm diameter, 5μm particle size, 100RP-18, Merck/BDH, Poole, UK). The NBD derivatives were eluted with an acetonitrile gradient and detection was achieved by monitoring absorbance at 495nm. The signal was processed on a Trio chromatography computing integrator (Rivector, Sandy, Beds, UK) for quantitative analysis and the hydroxyproline content was determined by comparing the peak area of samples to that obtained from standard solutions derivatised and separated under identical conditions. Values in text are given as micrograms of collagen assuming a hydroxyproline content of 12.2%.

2.12 Statistical analysis.

Survival curves were compared using the Mantel-Haenszel test. Differences between groups were analyzed by 2-way analysis of variance (ANOVA). Where a significant effect was noted, differences between individual groups was measured by the two-tailed Mann-Whitney U test for non-parametric data. A p value less than 0.05 was considered significant.

CHAPTER 3

THE INFLAMMATORY RESPONSE OF THE IMMATURE GUINEA PIG LUNG FOLLOWING 3 DAYS EXPOSURE TO 95% OXYGEN

3.1 Introduction.

To date, BPD is the most commonest form of unresolved neonatal lung injury. However, the mechanisms that lead to the morphological, biochemical and structural alterations that are observed within the lungs of these infants is unclear. Although several studies have implicated oxygen-induced inflammation as a major factor (Edwards *et al.*, 1977; Stenmark and Voelkel, 1991), the mechanism by which inflammation injures the immature lung is not fully understood. The reason for this has been due primarily to the ethical and practical problems associated with the study of this group of patients and which has resulted in a lack of appropriate biochemical and histological data. As such, this has necessitated the development of animal models in which to study the role of oxygen-induced inflammation in the development of injury to the immature lung. Exposure to oxygen, at concentrations often required by neonates who develop respiratory distress, is capable of initiating pulmonary inflammation and injury in adult humans and various species of animals (Clark and Lambertsen, 1971; Gould *et al.*, 1972; Escobedo *et al.*, 1982; Delemos, 1991). However, the use of adult or newborn animals, while highlighting certain fundamental aspects of a tissues response to hyperoxia, do not mimic the unusual situation that is observed in the premature human neonate in which there is a combined biochemical and anatomical pulmonary immaturity at the onset of oxygen exposure.

The guinea pig model of prematurity, developed within the University of Southampton (Kelly *et al.*, 1991) satisfies a number of important criteria (Workshop in bronchopulmonary dysplasia, 1979) and includes the necessity for pulmonary immaturity. However, at present the inflammatory response of the immature lung following delivery into 21% oxygen and following an acute hyperoxic exposure (95% oxygen) is unknown. Thus in the present study a comparision between the inflammatory response of the lung from term and preterm pups in air and preterm pups exposed to 21% and 95% oxygen for 3 days will be assessed .

3.2 Experimental protocol.

Guinea pigs delivered naturally or by Caesarian section at 65 days of gestation were randomly allocated to receive either 21% or 95% oxygen for 72hr (Fig 3.1). Body weights were monitored every 24 hours and after 3 days, animals were sacrificed by pentobarbitone overdose. The trachea was then isolated, a cannulae inserted and a BAL performed using 10ml (5x2ml aliquots) of nonpyrogenic saline (37°C). The fluid recovered was placed

immediately on ice. The thoracic cavity was then opened and a blood sample taken by direct cardiac puncture into an heparinised syringe and also placed immediately on ice. Following this, the pulmonary circulation was flushed of blood with a further 10ml of saline (37°C) and the lungs removed, blotted dry, weighed and frozen in liquid nitrogen.

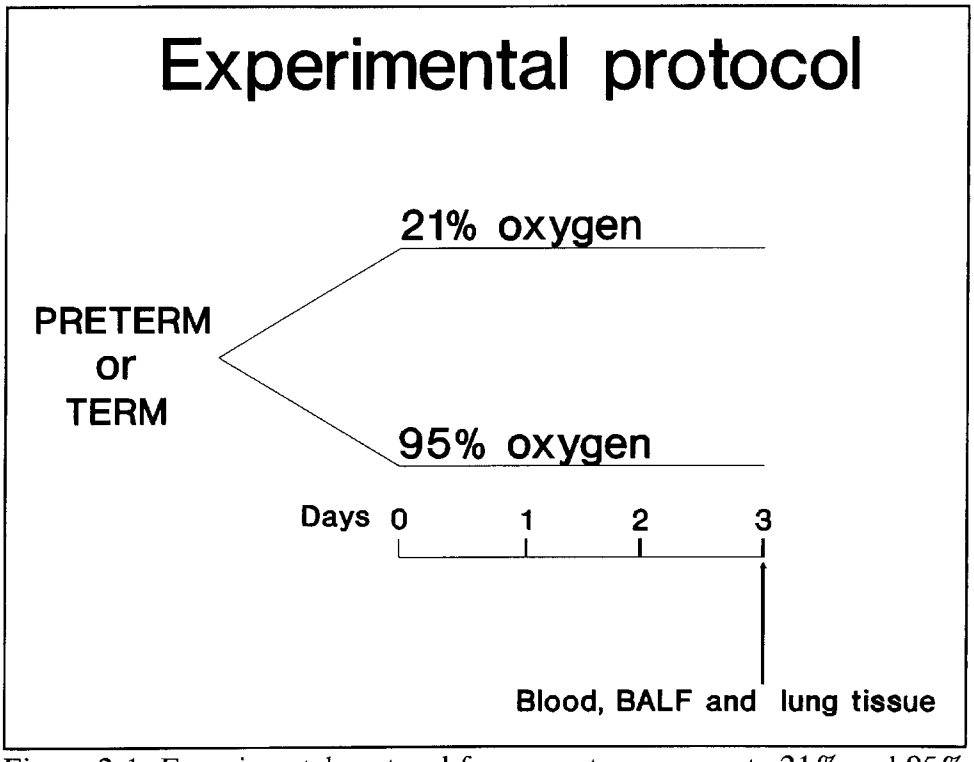


Figure 3.1. Experimental protocol for an acute exposure to 21% and 95% oxygen for 3 days.

An aliquot of the BALF was then taken for measurement of total and differential cell counts as described in Section 2.9.1. The BALF was then spun at 1500rpm for 10 minutes (4°C) to pellet the inflammatory cells and the supernatant aliquoted out into 500μl lots and stored at -70°C ready for analysis. In preterm animals, BALF was assessed for total protein (as a marker of lung injury), neutrophil elastase activity, EIC, LTB_4, PAF, ß-glucuronidase, phospholipase A_2, and conjugated dienes. Lung tissue was assessed for the antioxidant enzymes (CuZn SOD, Mn SOD, GSH-Px, CAT). BALF protein and PLA_2 activity were also assessed in term pups. Total and differential blood cell counts, enzymes of the pentose phosphate pathway (G6PDH and 6PGDH) and plasma and lung tissue protein carbonyls were measured in term and preterm animals. Blood samples obtained from guinea pigs of differing gestational ages (samples from Dr Ricketts) were assayed for EIC. Methods used for all measurements are described in Chapter 2.

3.3 Results

3.3.1 Survival.

Term pups did not exhibit any respiratory problems following birth and all survived until the end of the study. Animals delivered at 65 days of gestation developed transient respiratory distress which was characterised by tachyopnea, cyanosis and rib retraction and which resolved within 4-6 hours after delivery. In those animals that did not survive the 72 hour study period, respiratory distress continued until death, regardless of the exposure regime. As such, the mortality rate of guinea pigs delivered prematurely (Fig 3.2) and exposed to air was significantly greater than equivalently exposed term animals. Those animals exposed to 95% oxygen for longer than 24 hours also developed a subsequent respiratory distress. In these animals there was a significant increase in mortality in both the term and preterm groups when compared to equivalently aged matched air controls. However, no significant difference in survival was observed between oxygen-exposed term and preterm animals.

3.3.2 Body weight.

The body weights of term and preterm guinea pigs during exposure to 21% and 95% oxygen are shown in Table 3.1. Body weights of all animals exposed to 21% oxygen fell during the first 48 hours. As such, preterm animals were significantly lighter than birth weight at all time points studied. Those animals delivered naturally and exposed to 21% oxygen were significantly heavier than equivalent air-exposed preterm pups. No significant difference in body weight was observed between air and oxygen-exposed animals.

3.3.3 Lung wet weight

Preterm guinea pigs exposed to 21% oxygen had significantly heavier lungs than equivalently exposed term pups, whether expressed as total lung weight (Fig 3.3A) or when normalised to body weight (Fig 3.3B). Following exposure to 95% oxygen, lung weight was significantly heavier in both term and preterm pups compared to air controls. When the data was expressed per gramme body weight, only lungs from term animals remained significantly elevated. No significant difference in the recovery of BAL fluid was observed between term and preterm animals exposed to either 21% or 95% oxygen (Preterm: 21% O_2; 8.7±0.4, 95% O_2; 8.8±0.3: Term: 21% O_2; 8.8±0.4, 95% O_2; 8.8±0.2).

Figure 3.2. The effect of exposure to 21% and 95% oxygen for 3 days on the survival rate of term and preterm guinea pigs.

Guinea pigs delivered by Caesarian section at 65 days of gestation or naturally delivered term animals were exposed to 21% or 95% oxygen for 3 days and the percentage survival assessed every 24 hours.

---.--- Term 21% oxygen
---+--- Preterm 21% oxygen
---*--- Term 95% oxygen
---[]--- Preterm 95% oxygen

All term animals exposed to 21% oxygen survived. There was a significant reduction in survival of preterm pups in 21% oxygen ($p < 0.05$) compared to equivalently exposed term pups. A significant reduction in survival was also seen for term ($p < 0.05$) and preterm ($p < 0.01$) guinea pigs exposed to 95% oxygen when compared to their appropriate air controls. Data represents the survival of between 21 and 35 animals per group.

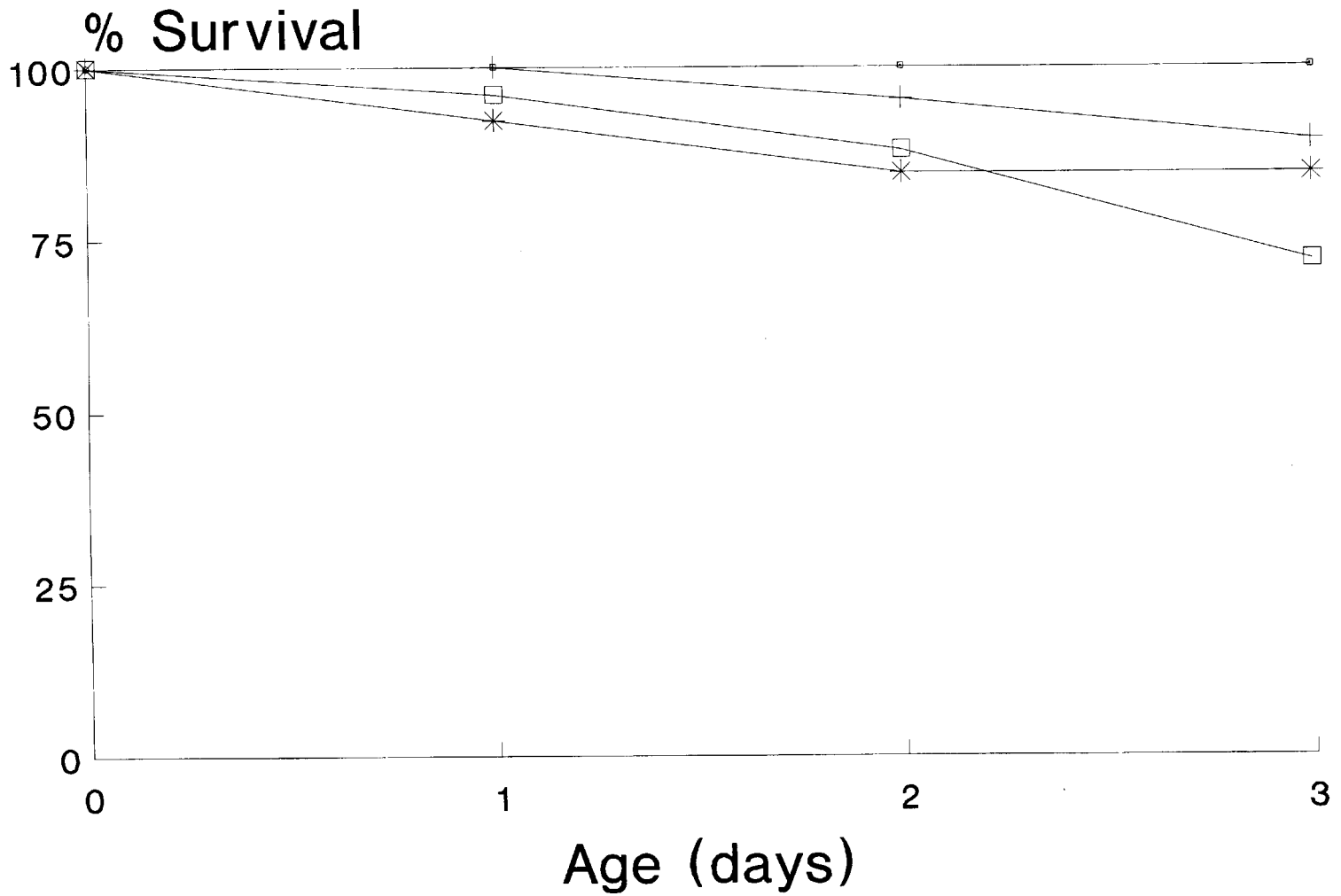
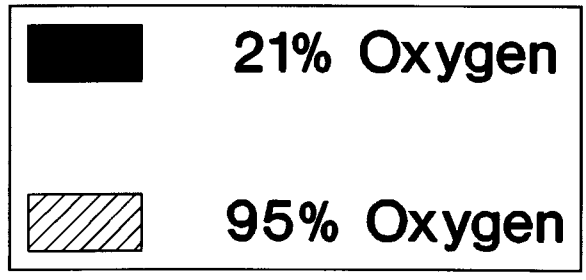

% Survival
100
75
50
25
0
0
1
2
3
Age (days)

Figure 3.3. The effect of exposure to 21% and 95% oxygen for 3 days on lung wet weights of term and preterm guinea pigs.

Guinea pigs delivered by Caesarian section 3 days before term and term animals delivered naturally were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed. The thoracic cavity was then opened and the pulmonary circulation flushed of blood with 10ml of sterile saline (37°C). The lungs were removed, blotted dry, weighed and frozen in liquid nitrogen.



Data represents the mean and standard deviation of between 11 and 15 animals per group. There was a significant effect of oxygen exposure and age on total lung wet weight (O_2: $F=28.8$, $p<0.001$; Age: $F=4.9$, $p<0.05$) and lung wet weight-body weight ratio (O_2: $F=19.5$, $p<0.001$; Age: $F=8.1$, $p<0.05$). * $p<0.05$, *** $p<0.0005$ compared to 21% oxygen; + $p<0.05$, +++ $p<0.0005$ compared to equivalently exposed term animals.

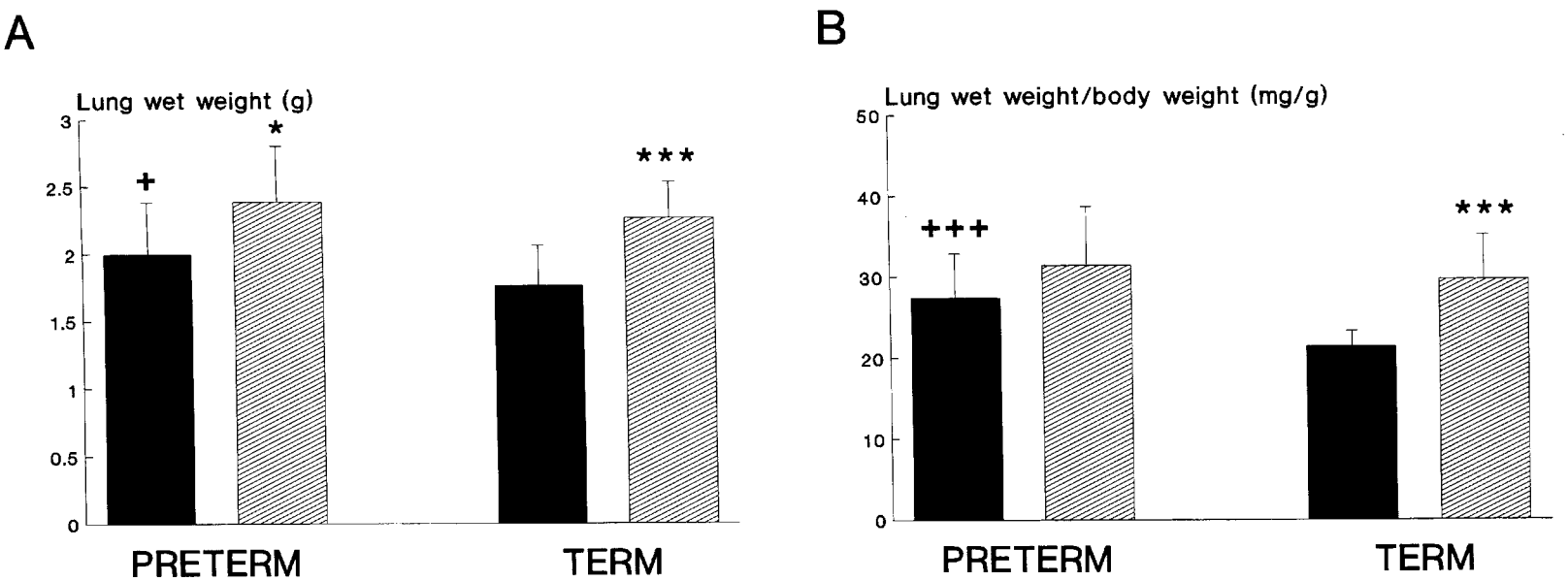

A
Lung wet weight (g)
3
2.5
2
1.5
1
0.5
0
+
*
***
PRETERM
TERM
B
Lung wet weight/body weight (mg/g)
50
40
30
20
10
0
+++
***
PRETERM
TERM

Table 3.1. The effect of exposure to 21% and 95% oxygen for 3 days on the body weights of term and preterm guinea pigs.

	% oxygen exposure			
Age	Preterm		Term	
hr	21	95	21	95
0	80 ± 11++**a**	83 ± 11**a**	101 ± 11**a**	85 ± 11+**a**
24	72 ± 10+++**b**	78 ± 11**a**	98 ± 11**a**	81 ± 10+**a**
48	71 ± 10+++**b**	77 ± 12**a**	97 ± 12**a**	78 ± 12++**a**
72	72 ± 11+**b**	77 ± 12**a**	100 ± 20**a**	80 ± 14**a**

Data represents the mean and standard deviation of between 15 and 25 term and preterm pups per group. There was a significant effect of oxygen exposure ($F=18.0$, $p<0.001$), age ($F=74.2$, $p<0.001$) and interaction between age and oxygen ($F=53.7$, $p<0.001$) on body weight. + $p<0.05$, ++ $p<0.005$, +++ $p<0.0005$ compared to term animals exposed to 21% oxygen. Data in each exposure and age group containing different symbols are significantly ($p<0.05$) different from each other.

No significant difference in lung weight was observed between oxygen-exposed term and preterm pups, regardless of data expression.

3.3.4 Lung protein and DNA.

The effect of exposure to 21% and 95% oxygen on lung tissue protein and DNA from term and preterm animals is shown in Table 3.2. There was significantly more protein in the lungs of air-exposed preterm animals than in similarly exposed term animals. However, on normalisation of the data to lung wet weight, this significance was lost. Following exposure to 95% oxygen there was a significant increase in the concentration of lung protein in term animals compared to similarly exposed preterm pups. Total lung DNA content of term lungs was also significantly greater than equivalently aged animals exposed to air. On normalisation to lung weight, this significance remained. A significant correlation between lung wet weight and total lung protein ($r=0.68$, $p<0.01$) was observed.

Table 3.2. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue protein and DNA of term and preterm guinea pigs.

% O_2	Age	Protein		DNA	
		mg	mg/g	mg	mg/g
21	Preterm	130 ± 5	66 ± 5	9.0 ± 4.5	3.5 ± 0.6
	Term	115 ± 11+	58 ± 14	8.5 ± 1.0	4.1 ± 0.6
95	Preterm	146 ± 21	58 ± 7	8.1 ± 1.5	3.3 ± 0.4
	Term	147 ± 25	67 ± 4+	6.6 ± 1.5*	2.9 ± 0.5**

Data represents the mean and standard deviation of between 6 and 8 animals per group. There was a significant effect of oxygen on tissue protein ($F=7.3$, $p<0.05$) and DNA ($F=7.7$, $p<0.01$) concentrations and total tissue protein content ($F=5.3$, $p<0.05$).
* $p<0.05$, ** $p<0.005$ compared to 21% oxygen. + $p<0.05$ compared to preterm.
Abbreviations: DNA:- deoxyribonucleicacid.

3.3.5 Blood inflammatory cell counts.

No significant difference in total cell counts was observed between air-exposed term and preterm animals (Table 3.3). Following exposure to 95% oxygen all cell types increased in number. This increase was significant for total, neutrophil and lymphocyte populations from term animals and neutrophil and monocyte populations from preterm animals when compared to air controls. Lymphocyte numbers from oxygen-exposed term pups was significantly greater than that from equivalently exposed preterm pups.

3.3.6 BALF inflammatory cell counts.

No effect of exposure to 21% or 95% oxygen was observed on the recovery of BALF from either preterm (21% oxygen:- 8.86 ± 0.29ml, 95% oxygen:- 8.79 ± 0.26ml) or term (21% oxygen 8.64 ± 0.24, 95% oxygen 8.67 ± 0.26) animals. Total BALF cell and macrophage numbers from air-exposed term animals was significantly higher than similarly exposed preterm pups (Table 3.4). Following exposure to oxygen, there was a significant increase in neutrophil numbers in both term and preterm animals compared to air controls. The number of macrophage from oxygen exposed term animals was significantly higher than equivalently exposed preterm pups.

Table 3.3. The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential blood cell count of term and preterm guinea pigs.

Cell type	% O_2	Cell number (10^4/ml)	
		Term	Preterm
Total	21	144 ± 37	163 ± 87
	95	267 ± 60**	215 ± 100
Neutrophils	21	38 ± 12	72 ± 65
	95	122 ± 39***	126 ± 72*
Monocytes	21	8 ± 5	5 ± 4
	95	13 ± 10	9 ± 9*
Lymphocytes	21	87 ± 21	81 ± 31
	95	130 ± 34*+	77 ± 30

Data represents the mean and standard deviation of between 8 and 15 animals per group. There was a significant effect of oxygen on total cell ($F=6.97$, $p<0.05$), monocyte ($F=5.04$, $p<0.05$) and neutrophil ($F=9.33$, $p<0.005$) cell numbers. There was a also a significant effect of age on lymphocyte numbers ($F=2.63$, $p<0.05$). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ compared to 21% oxygen; + $p<0.05$ compared to equivalent preterm pups.

3.3.7. BALF protein.

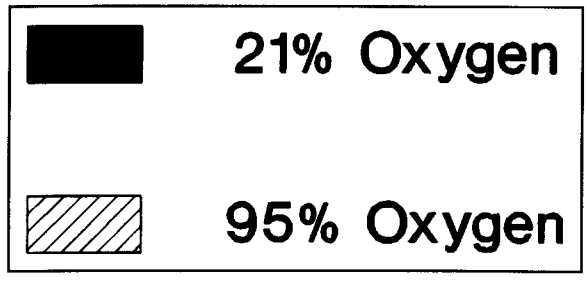
The protein concentration of BALF from term and preterm animals exposed to 21% and 95% oxygen is shown in Figure 3.4. The BALF of preterm pups exposed to 21% oxygen contains significantly more protein than similarly exposed term pups. Following hyperoxic exposure, this difference was lost. However BALF from both term and preterm animals had significantly more protein than their appropriate air controls. No correlation was observed between BALF protein and neutrophil numbers

3.3.8 Gestational profile of plasma EIC.

Figure 3.5 shows the change in plasma EIC during gestation and up to 10 days after birth. A significant correlation ($r=0.46$, $p<0.05$) between EIC and post conceptual age was observed.

Figure 3.4. The effect of exposure to 21% and 95% oxygen for 3 days on BALF protein from term and preterm guinea pigs.

Guinea pigs delivered by Caesarian section 3 days before term and term animals delivered naturally were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and BAL performed with 5 x 2ml aliqouts of sterile saline at 37°C. The BALF was then placed on ice before being spun at 1500rpm (4°C) for 10 minutes to remove the inflammatory cells. The acellular fraction of BALF was then aliqouted into 500μl lots and frozen at -70°C. Protein was measured using the method described in Section 2.10.5.



Data represents the mean and standard deviation of between 10 and 15 animals per group. A significant effect of oxygen exposure ($F= 18.6$, $p<0.001$) and age ($F=5.2$, $p<0.05$) was observed. *** $p<0.0005$ compared to 21% oxygen; +++ $p<0.0005$ compared to equivalently exposed term animals.

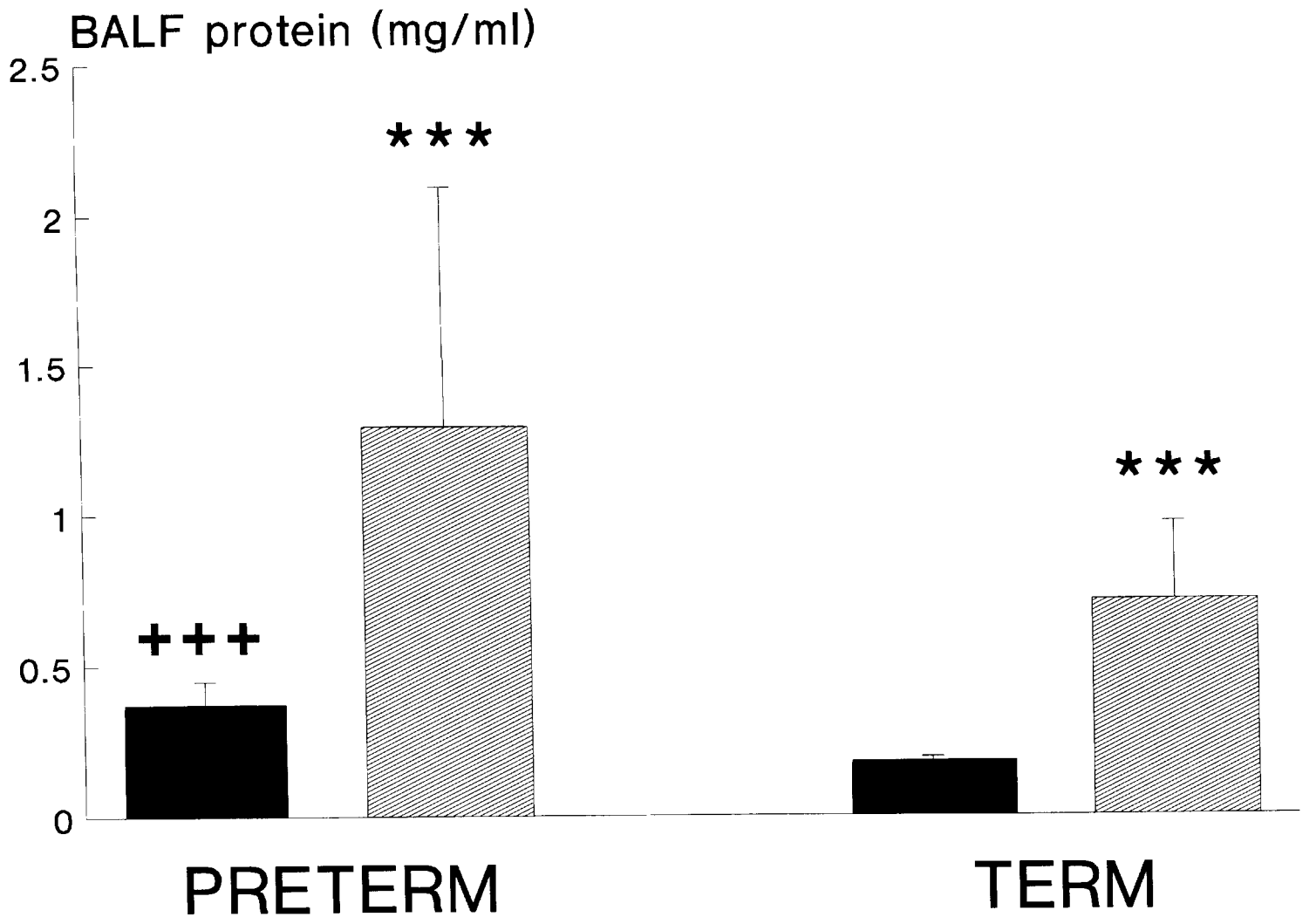

BALF protein (mg/ml)
2.5
2
1.5
1
0.5
0
***
***
+++
PRETERM
TERM

Figure 3.5. The effect of post conceptual age on the elastase inhibitory capacity (EIC) of plasma from neonatal guinea pigs

Timed pregnant females were sacrificed by cervical dislocation and the fetus's removed as described by Rickett, 1992. The fetus was killed by decapitation and the blood collected into heparinised tubes. Blood was then spun at 1500rpm for 10 minutes at 4°C and the plasma removed and stored in 100μl aliquots at -20°C. The EIC was then assessed as described in chapter 2.10.1. Linear regression analysis was then performed on the data and a significant correlation between EIC and gestational age observed ($r = 0.46$, $p < 0.05$).

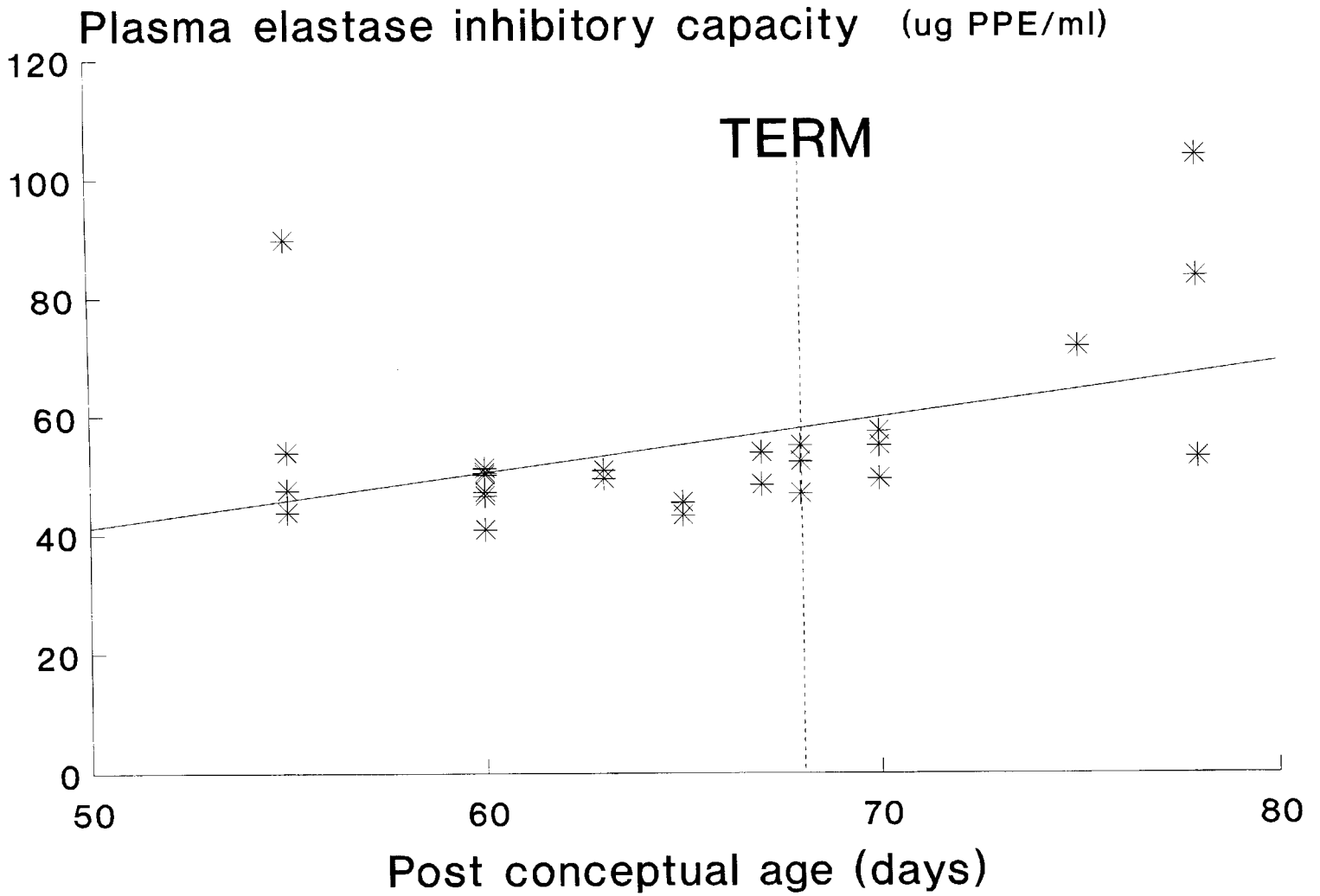

Plasma elastase inhibitory capacity (ug PPE/ml)
120
100
80
60
40
20
0
TERM
50
60
70
80
Post conceptual age (days)

Table 3.4. The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential BALF cell count of term and preterm guinea pigs.

Cell type	% O_2	Cell number (10^4/ml)	
		Term	Preterm
Total	21	110 ± 18+++	67 ± 32
	95	104 ± 18	82 ± 38
Neutrophils	21	0.6 ± 0.5	0.4 ± 0.7
	95	3.6 ± 1.4***	3.6 ± 2.8***
Eosinophils	21	64 ± 14	50 ± 24
	95	64 ± 12	56 ± 30
Macrophages	21	39 ± 12+++	16 ± 10
	95	34 ± 8+++	16 ± 10

Data represents the mean and standard deviation of between 8 and 15 animals per group. There was a significant effect of oxygen on total neutrophil ($F=24.45$, $p<0.001$) cell numbers and a significant effect of age on total ($F=11.23$, $p<0.005$) and macrophage ($F=42.53$, $p<0.001$) cell numbers. *** $p<0.0005$ compared to 21% oxygen; +++ $p<0.0005$ compared to equivalent preterm pups.

3.3.9 BALF mediator concentrations.

BALF beta-glucuronidase, LTB_4, neutrophil elastase and EIC (Table 3.5) were all significantly elevated above air controls following exposure to 95% oxygen. BALF phospholipase A_2 activity from air-exposed term pups was significantly lower than from BALF of equivalently exposed preterm pups. Following 95% oxygen, PLA_2 activity fell in preterm animals, but increased in term pups. PLA_2 activity correlated significantly with BALF protein ($r = -0.72$, $p<0.0005$) and was not detected in plasma of any animal. BALF PAF and neutrophil elastase (using the tritiated elastin assay) activity were not detected following exposure to either 21% or 95% oxygen. Neutrophil elastase activity, detected using a small synthetic substrate, was significantly elevated above air controls following oxygen exposure and significantly correlated with BALF protein ($r= 0.84$, $p<0.0001$) and not with BALF neutrophils.

3.3.10 Lung tissue antioxidant enzymes activities.

Term pups exposed to 21% oxygen had significantly higher lung tissue 6PGDH activity

than similarly exposed preterm animals. Following exposure to oxygen, Mn SOD and GSH-Px activities in preterm animals and G6PDH activity in term animals was significantly greater than air controls. As with air exposure, term pups exposed to oxygen had significantly higher 6PGDH activity than preterm animals (Table 3.6).

Table 3.5. The effect of exposure to 21% and 95% oxygen for 3 days on the BALF mediator concentrations from term and preterm guinea pigs

Mediator	AGE	21% oxygen	95% oxygen
Phospholipase A_2 (μmoles\min\ml)	Preterm	0.90 ± 0.19	0.58 ± 0.33*
	Term	0.24 ± 0.11+	0.52 ± 0.26
ß-glucuronidase (Units/ml)	Preterm	3.92 ± 5.92	53.40 ± 37.86**
PMN elastase (ng/ml)	Preterm	N.D	4.84 ± 5.17*
PMN elastase (ng ^{3}H elastin/ml)	Preterm	N.D	N.D
EIC (μg PPE/ml)	Preterm	0.50 ± 0.14	2.81 ± 2.34*
PAF (pmole/ml BALF)	Preterm	N.D	N.D
LTB_4 (pg/ml BALF)	Preterm	0.09 ± 0.14	0.55 ± 0.22**

Data represents the mean and standard deviation of between 6 and 15 animals per group. There was a significant effect of age ($F=10.12$, $p<0.005$) and a significant interaction between age and oxygen ($F=7.26$, $p<0.05$) on phospholipase A_2 activity. * $p < 0.05$, ** $p < 0.005$ compared to 21% oxygen; + $p<0.05$ compared to similarly exposed preterm animals. N.D:- Not detected. *Abbreviations*; PMN:- Polymorphonuclear neutrophil, EIC:- Elastase inhibitory capacity, PAF:- Platelet activating factor, LTB_4:- Leukotriene B_4.

3.3.11 Markers of oxygen-centred free radical damage.

No effect of oxygen exposure on BALF conjugated diene concentration (21% O_2: 6.2 ± 2.3μM; 95%O_2: 5.8 ± 1.4μM) was observed. Although there was no significant difference in tissue and blood protein carbonyl content between air and oxygen-exposed animals, lung tissue contained significantly more protein carbonyls than plasma, whether exposed to 21% or 95% oxygen (Fig 3.6).

Table 3.6 The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue antioxidant enzyme activities of term and preterm guinea pigs

Antioxidant	Age	21% oxygen	95% oxygen
Cu/Zn SOD (U/mg DNA)	Preterm	52.32 ± 18.42	41.80 ± 29.63
Mn SOD (U/mg DNA)	Preterm	2.83 ± 0.35	4.76 ± 2.37*
CAT (KU/mg DNA)	Preterm	11.01 ± 3.38	11.34 ± 4.99
GSH-Px (U/mg DNA)	Preterm	0.28 ± 0.10	0.47 ± 0.21*
6PGDH (U/mg DNA)	Preterm Term	0.13 ± 0.05 0.19 ± 0.03+	0.15 ± 0.03 0.19 ± 0.04+
G6PDH (U/mg DNA)	Preterm Term	0.14 ± 0.08 0.12 ± 0.05	0.20 ± 0.08 0.18 ± 0.08*

Data represents the mean and standard deviation of between 6 and 15 animals per group. There was a significant effect of oxygen exposure on G6PDH ($F=5.7$, $p<0.05$).
* $p < 0.05$, ** $p < 0.005$ compared to 21% oxygen, + $p<0.05$ compared to preterm.
Abbreviations; Cu/Zn SOD, Mn SOD:- Copper/Zinc and manganese superoxide dismutase, CAT:- Catalase, GSH-Px:- Glutathione peroxidase, 6PGDH:- 6phosphogluconic acid dehydrogenase, G6PDH:- Glucose-6-phosphate dehydrogenase, DNA:- Deoxyribonucleic acid.

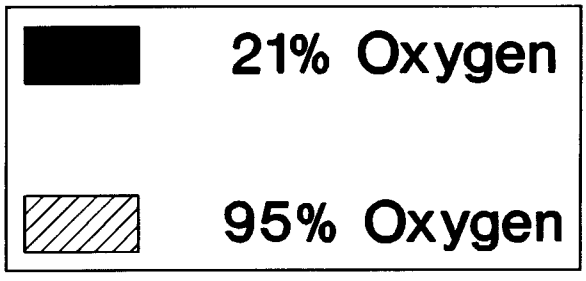
3.4. Discussion

The aim of this first study was to characterise the development of pulmonary inflammation and injury in the preterm guinea pig following delivery and exposure to an acute period of hyperoxia. The data presented confirms and extends those observations made previously within the model (Kelly *et al.*,1991; Hunt *et al.*,1991) and highlights particular areas in which more in depth analysis may be perused.

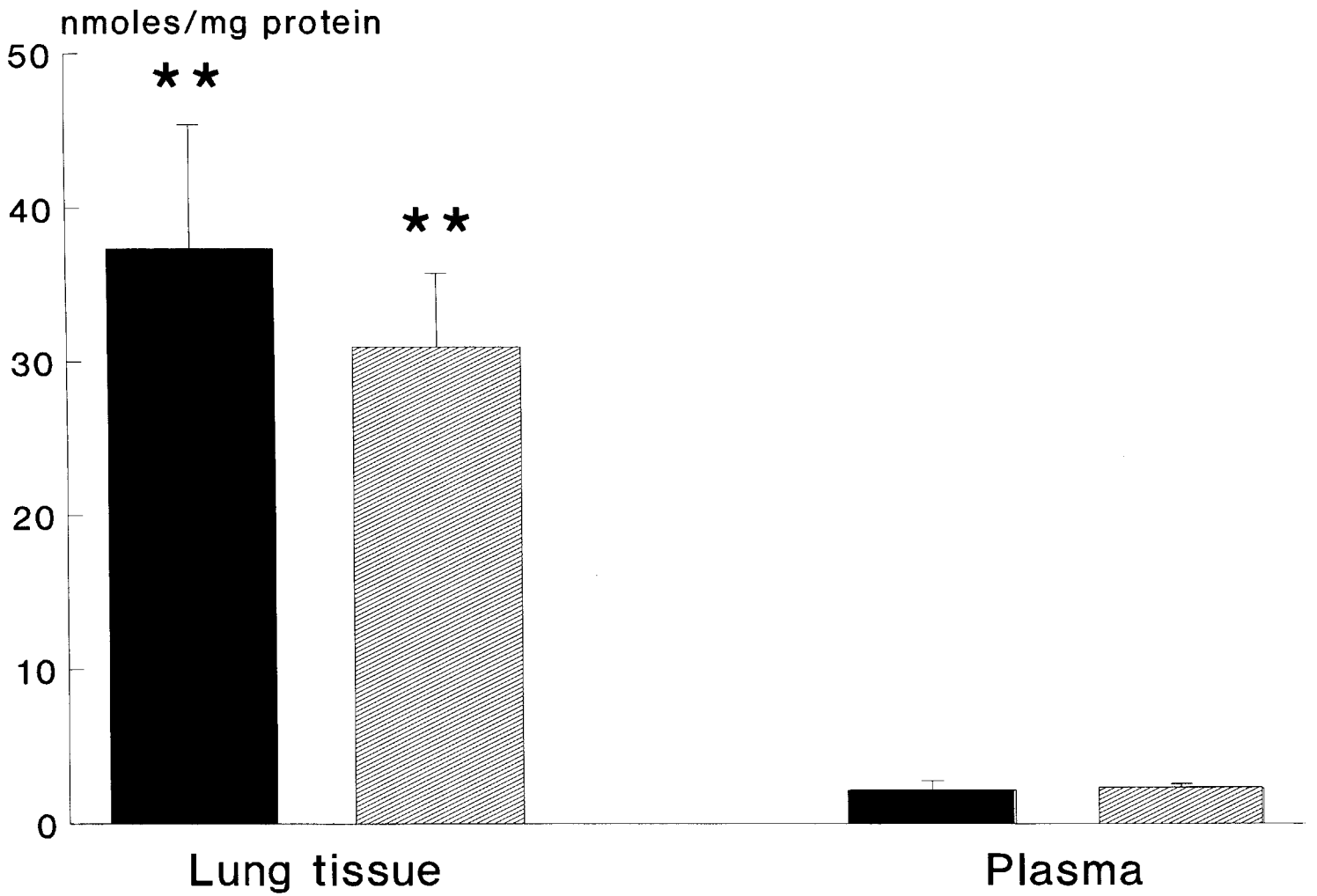
Studies performed within the Neonatal Respiratory Research Group (NRRG) at Southampton, have previously demonstrated that guinea pigs delivered at day 65 of gestation are pulmonary surfactant insufficient (Hunt *et al.*,1991). As surfactant insufficiency is the primary pathophysiological abnormality associated with the development of HMD, this may explain why on delivery preterm guinea pigs develop respiratory distress. However, BALF DPPC levels (the major surface tension reducing component of

Figure 3.6. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue and plasma protein carbonyl concentrations of preterm guinea pigs.

Guinea pigs delivered by Caesarian section 3 days before term were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed. The thoracic cavity was then opened, 1ml of blood withdrawn by cardiac puncture into an heperanised syringe and the pulmonary circulation flushed of blood with 10ml of sterile saline (37°C). The lungs were removed, blotted dry, weighed and frozen in liquid nitrogen. Blood was then spun at 1500rpm for 10 minutes at 4°C and the plasma removed and stored in 100μl aliquots at -20°C. Tissue and plasma carbonyl concentrations measured as described in section 2.10.10



Data represents the mean and standard deviation of 6 animals. There was a significant effect of tissue type ($F=21.2$, $p<0.001$). ** $p<0.005$ compared to plasma.


nmoles/mg protein
50
40
30
20
10
0
**
**
Lung tissue
Plasma

surfactant) from 3 day old air-exposed premature pups have been found to be approximately four times that of similarly exposed term animals (Cabral, 1992). The reason for this is unclear, although alveoli collapse and injury at birth, as a result of surfactant insufficiency may lead to type II cell damage and release of intracellular stores of this lipid (Cabral, 1992).

Respiratory distress in these animals was similar to that observed in the human preterm infant and is characterised by tachyopnea, cyanosis and rib retraction. Animals were also less active than their term counterparts and refused to feed from surrogate dams during the first few hours after delivery. Possibly as a result of this, an increase in mortality was observed following 72 hours exposure to air when compared to equivalently exposed term animals who did not experience the same respiratory problems following delivery. This survival rate is also similar to that found by Sosenko and Frank, 1987 and comparable to other animal models of prematurity delivered at the same point in gestation (95% of term) (Kessler *et al* 1982; Lorenzo, 1985). This increase in mortality, associated with premature delivery of human infants, guinea pigs and other animal models of prematurity, is associated with the degree of pulmonary immaturity (Usher *et al* 1971; Farrell and Avery, 1975; Morley, 1986; Kelly *et al.*,1991). In the preterm guinea pig, 60% of all animals delivered at day 63 of gestation die within the first 72 hour (Town, 1990) and delivery of animals much earlier than this results in nonviable pups or 100% mortality (Kelly *et al.*,1991).

Light and electron microscopic analysis of lung tissue from air-exposed preterm guinea pigs (Town, 1990) show evidence of widespread pulmonary injury including, atelectasis, increased interstitial thickness, deposition of fibrin and hyaline membrane formation within alveoli. Atelectasis (alveoli collapse) is a characteristic feature of human infants and primate models of prematurity who develop HMD (Finlay-Jones *et al.*,1974; Coalson *et al.*,1988). Lung collapse occurs as consequence of surfactant insufficiency and can be partially reversed by exogenous treatment with artificial surfactant (Shiki *et al.*,1987). In the premature guinea pig, alveolar collapse resulted in a reduction in the percentage airspace (Kelly *et al.*,1991). Increased interstitial thickness is principally a result of the accumulation of fluid (Town, 1990) and indicative of an alteration in the alveolar-capillary permeability. In the present study, this was seen as an increase in the wet weight and protein content of the lung and protein recovered following BAL. Hyaline membranes that form in these

animals and in human infants is composed of fibrin polymers and extensive cellular debris from both epithelial and degenerative inflammatory cells (Town, 1990). The results presented also agree with published studies (Jobe *et al.*, 1985; Koizumi *et al.*, 1985) in which ventilated and unventilated premature animals accumulate protein within alveoli and in preterm human neonates within 24 hours of delivery (Ogden *et al.*, 1984; Jeffries *et al.*, 1984). The presence of protein within immature guinea pig alveoli as well as within human alveoli at the onset of breathing, may aggravate problems that are already established as a consequence of surfactant insufficiency (Ikegami *et al.*, 1984; Ikegami *et al.*, 1986). Plasma proteins in the airways can lead to secondary surfactant dysfunction, as a number of these proteins have been shown to have an inhibiting effect on the function of pulmonary surfactant (Seeger *et al.*, 1985; Fuchimukai *et al.*, 1987). However, as unlike BALF DPPC levels, the functional properties of the recovered surfactant was similar to that of term pups (Cabral, 1992), the reason for which is unclear. However as the rate of alveoli protein accumulation decreases with increasing gestational age (Tabor *et al.*, 1992) and time after birth (Jobe *et al.*, 1985) the amount of protein found in the preterm guinea pig lung at 3 days may not be enough to effectively inhibit surfactant function. To establish whether function is altered, earlier time points would have to be examined.

Premature guinea pigs were also significantly smaller at birth and slower to achieve birth weight than equivalent term neonates. Smaller pups following premature delivery appears to be simply a result of early delivery. However, the inability to achieve birth weight at the same time as newborn term animals may be due to a number of factors. The development of acute lung injury in the premature pup will not only put an additional burden on the system to supply energy for increased respiration, but the immobility and refusal to feed will mean a reduction in essential energy intake. In addition, an immature digestive system, especially in the very immature human neonates, suggests that even if adequate food is taken in, the required nutrients may not be absorbed. Accretion of fat, glycogen, vitamins and minerals is known to occur late in gestation in the human neonate (Girard, 1985; Roux, 1985) and thus if this is also true for the guinea pig premature delivery may result in pups having low reserves of energy and micronutrients. This may culminate in death and explain the increase in mortality seen in these animals.

Pulmonary injury may also develop as a consequence of mechanisms other than the direct mechanical injury observed during alveoli collapse. At birth the fetus leaves a hypoxic

uterine environment (pO_2 2.66-3.99 kPa [20-30mmHg]) to enter a relatively hyperoxic environment (13.3 kPa [100mmHg]). This transition involves a five-fold increase in oxygen concentration and as such may pose a significant oxidative stress through increased production of oxygen centred free radicals (Freeman and Crapo, 1981; Turrens *et al.*, 1982). To combat this potential oxidative threat there are a variety of enzymatic and nonenzymatic antioxidants (Heffner and Repine, 1991). Several studies have demonstrated that pulmonary antioxidant development occurs over the last 15% of gestation in many animals species including the guinea pig (Sosenko and Frank, 1987; Frank and Sosenko, 1987; Rickett and Kelly, 1990). Those animals delivered prematurely would thus be more susceptible to oxidative injury. However, previous studies with the guinea pig has shown that *in utero* pulmonary enzymatic antioxidant activities are similar between animals at 65 (preterm) and 68 (term) days of gestation (Kelly *et al.*, 1993). However, following 3 days exposure to 21% oxygen, preterm pups have significantly lower antioxidant enzyme activities than equivalently exposed term animals. The reason for this is unclear, although delivery (preterm or term) results in a decrease in antioxidant activity (Kelly *et al.*, 1993). The possible increase in susceptibility to free radical damage is also highlighted in the present study by the lower activity of pulmonary 6PGDH. This enzyme in conjunction with G6PDH is involved in the production of reducing equivalents (NADPH) used in the detoxification of hydroperoxides by the glutathione redox system. The capacity to generate NADPH is therefore lower in preterm pups than in term animals and may possibly impair the function of the glutathione system. In fact lower tissue and BALF reduced glutathione concentrations have been observed in the preterm guinea pig, especially in those animals that have been fasted (Langley and Kelly, 1992), in the BALF of human preterm infants who develop BPD, (on the first day of life) (Grigg *et al.*, 1993) and also in patients who develop ARDS (Pacht *et al.*, 1991). Another possible reason why pulmonary glutathione concentration is reduced in the preterm neonate, may be due to the availability of cysteine (Deneke and Fanburg, 1989), as lower cord levels of cysteine may impair glutathione synthesis (Picone *et al.*, 1989). Circulating cysteine levels were not assessed in the preterm guinea pig, although impaired nutrition, as indicated earlier, may have some bearing on the availability of this amino acid. It is possible therefore to suggest that part of the observed damage to the lung on delivery of guinea pigs prematurely is due to increased oxygen centred free radical damage as a result of the inability of the immature lung to mount an

effective antioxidant response following birth.

Apart from the direct damaging effect these species may have on many cellular components, they are also capable of activating several enzyme systems involved in the initiation of inflammation, such as the phospholipases (PL) (Goldman *et al.*,1992). Phospholipase A_2 (PLA_2) is known to be associated with a variety of inflammatory diseases, and can generate a series of lipid mediators important in the initiation of inflammation and lung injury. PLA_2 may be derived from a variety of cell types including platelets (Vadas and Hay, 1980), neutrophils (Lanni and Becker, 1983) and macrophages (Vadas and Hay, 1980) and when given intratracheally is known to induce acute lung injury (Edelson *et al.*,1991). PLA_2 activity has also been found in bronchoalveolar lavage of patients with ARDS (Offenstadt *et al.*,1981) and although to date no evidence of activity has been reported in the BALF of infants with acute and chronic lung disease, PAF a product of PLA_2 activity has been identified in these infants (Koyama *et al.*,1993). In the present study, BALF PLA_2 activity was significantly greater in air-exposed preterm animals than in term pups. The absence of PLA_2 activity in the plasma may suggest either the release of locally produced PLA_2 by cells such as the macrophage or the presence of inhibitors within the plasma. Recently, Chilton and Murphy, 1986 have suggested a common precursor lipid in cell membranes that upon activation leads to the release of arachidonic acid and lysoplatelet activating factor. Metabolism of these two compounds results in the release of various prostaglandins, thromboxanes, leukotrienes and platelet activating factor, all of which have been identified in the BALF of infants with BPD (Stenmark *et al.*,1987). Leukotriene B_4, also a product of PLA_2 activity has been observed in the BALF of infants with BPD (Stenmark *et al.*,1987) and animal models of oxygen toxicity (Taniguchi *et al.*,1986). It is the most potent chemokinetic and chemotactic agent for PMN's of various species *in vivo* and *in vitro* (Ford-hutchinson *et al.*,1980; Palmer *et al.*,1980; Dahlin *et al.*,1981). LTB_4 is known to effectively stimulate PMN adhesion to endothelial cell surfaces (Gimbrone *et al.*,1984) with alveolar macrophages the largest producer of LTB_4 *in vitro* (Fels *et al.*,1982; Martin *et al.*,1984; Martin *et al.*,1987). Biosynthesis of LTB_4 by neutrophils is also well documented (Clancy *et al., 1983;* Palmer and Salmon, 1983; Henricks *et al.*,1986; Seeger *et al.*,1986) and thus there is a clear potential for LTB_4 to act as an endogenous amplifier of PMN-dependant responses in inflammation. The presence of detectable quantities of LTB_4 in the lungs of air-exposed preterm animals may therefore

LTB_4 in the lungs of air-exposed preterm animals may therefore explain the presence of increased numbers of tissue neutrophils (Kelly *et al.*, 1991).

Neutrophils have the potential to cause extensive tissue injury through the release of proteases, such as elastase, and activated species of oxygen (Fantone and Ward, 1985; Weiss, 1989). Previous studies with the model have demonstrated an imbalance in the protease-antiprotease profile of the lung, in favour of increased enzyme activity (Kelly *et al.*, 1991). To prevent tissue damage by elastase, the lung is protected by an antiprotease screen that consists primarily of alpha-1-proteinase inhibitor (α-1-PI). α-1-PI is the major neutrophil elastase inhibitor within the body and is small enough to move out of the circulation and into the lung epithelial lining fluid which covers the surfaces of alveoli. A reduction in the antiprotease screen (Merritt *et al.*, 1983; Walti *et al., 1989*), even in the presence of elevated protein is also seen in human preterm infants. This is thought to be due to the direct oxidative inhibition of α-1-PI (Johnson and Travis, 1978) by hyperoxia or from the release of oxygen centred free radicals of inflammatory cells (Padrines *et al.*, 1989). Circulating levels of α-1-PI have been directly related to gestational age and to the severity of acute lung injury (Singer *et al.*, 1976; Mathis *et al.*, 1973; Lee *et al.*, 1978). An imbalance in the elastase-α-1-PI profile within the lungs of premature infants has been suggested as a possible factor in the development of BPD (Merritt, 1982; Merritt *et al.*, 1983; Ogden *et al.*, 1984). In the present study, guinea pig α-1-PI activity was shown to be directly related to post conceptual age and as such delivery of animals before 68 days will result in a possible increased susceptibility to proteolytic attack thereby explaining the protease-antiprotease imbalance seen in these animals (Kelly *et al.*, 1991).

Sixty percent more cells were recovered from the lungs of term pups, due to the recovery of twice as many macrophages. As differences in the blood cell population were not observed this effect may represent local proliferation of macrophages. The pulmonary macrophage population is known to increase in number following birth (Evans *et al.*, 1987) by a combination of local proliferation and monocyte recruitment from blood. Why the macrophage numbers in the BALF of preterm animals is lower than that of term pups is unclear, although if these animals are susceptible to oxidative injury, alveolar proliferation can be blunted by exposure to increased oxygen concentration (Sherman *et al.*, 1988). This effect on macrophage numbers is also seen in infants with BPD (Arnon *et al.*, 1993) and the mechanism may be due to inhibition of lung protein and DNA synthesis (Northway Jr *et*

al., 1976; Frank and Groseclose, 1982). Though not detected in the presence study, PAF has been implicated in alterations in the alveoli-capillary permeability of the lung in air-exposed rabbits (Tabor *et al.*, 1992), premature baboons (Meredith *et al.*, 1989) and in infants who develop BPD (Koyama *et al.*, 1993). PAF is found in a number of inflammatory cells such as neutrophils (Betz and Henson, 1980) and macrophages (Arnoux *et al.*, 1980). It is extremely unstable with a half-life of less than 30 seconds, being rapidly degraded by highly specific acetylhydrolases found in plasma (Farr *et al.*, 1980) and in tissue microsomes (Blank *et al.*, 1981).

Therefore guinea pigs delivered 3 days before term develop respiratory distress characteristic of that seen in the human premature infant. In conjunction with a poor or inadequate nutritional intake during the first few days of life these animals develop hyaline membranes and other evidence of lung injury. However, mechanisms other than mechanical injury induced by surfactant insufficiency may operate in the development of lung injury. Lower levels of protective antioxidants or an inability to induce these proteins may lead to increased free radical production and the activation of PLA_2. This may in turn lead to the generation of a variety of proinflammatory compounds such as PAF and LTB_4 and the subsequent alteration in the permeability of the lung and attraction of inflammatory cells. As premature animals have a reduced antiprotease screen, the release of proteases following cellular activation will lead to an imbalance in the protease-antiprotease profile within the lung and the development of tissue injury. If these cells are also releasing activated species of oxygen, α-1-PI oxidation, PLA_2 activation and further tissue injury may ensue.

As a consequence of the drop in blood PO_2, infants become hypoxic and often require supplemental oxygen for survival, the concentration and duration of which depends on the extent of pulmonary immaturity. This will lead to further free radical production and an enhancement of the injury initiated by alveolar collapse and release of inflammatory mediators. In the present study, animals were exposed to 95% oxygen to assess the similarities between the response of the model and these changes observed in the premature human neonate. Seventy two hours exposure was taken as the end point of the study as by then HMD in human neonates has reached its zenith. The sequence of changes that occurs in the lungs of various species in response to pulmonary oxygen toxicity is very similar (Kapanci *et al.*, 1972; Crapo *et al.*, 1980; Crapo, 1986). Exposure to a lethal dose of oxygen is first associated with subtle changes in endothelial cell ultrastructure and a rapid

in the lung there is a rapid amplification of injury. Overt destruction of the endothelium begins shortly after PMN accumulation and as such they have been implicated in the observed tissue destruction.

Histologically, the lungs are severely affected. The main features are atelectasis, vascular congestion, pulmonary oedema, fibrin deposition and neutrophil infiltration (Town, 1990; Kelly *et al.*, 1991). Confirmation of these changes were observed in the present study as increased lung weight and total lung tissue and BALF protein. Although the extent of injury in both term and preterm animals was similar, it is unclear whether there were any differences in the time frame in which these injures developed. However, as a consequence, survival was significantly reduced. This in accordance with previous studies with the model (Rickett and Kelly, 1990; Kelly *et al.*, 1991) and with other models of oxygen toxicity (Lorenzo, 1985; Tanswell *et al.*, 1989; Frank and Sosenko, 1991). Sosenko *et al*, 1987 found that exposure of preterm guinea pigs (66 days gestation) to 95% oxygen resulted in an LT50 of 6.4 days, 4 days longer than adults. Although there was much more lung collapse in the oxygen-exposed guinea pigs than in air-exposed ones, BALF DPPC levels are significantly increased and no change in surfactant function was observed (Cabral, 1992).

In the present study lung tissue DNA was reduced in term animals as a consequence of oxygen exposure, a result not observed by Rickett, 1992 but similar to those obtained by Bucher *et al*, 1981 and Kimura *et al*, 1983 for preterm pups. However, no significant change in lung tissue DNA and protein are observed in infants with HMD (Wigglesworth *et al.*, 1987). The activity of two of the 4 enzymatic antioxidants measured increased over the 3 days in oxygen. However, although the activity of the enzymes involved in the pentose phosphate pathway increased in both term and preterm animals exposed to oxygen, this was only significant for G6PDH in term pups. This differential response to hyperoxia in term and preterm animals has also been seen in the enzymatic antioxidants following 85% oxygen exposure. Preterm animals are more able to initiate an antioxidant response to oxygen than term animals (Kelly *et al.*, 1993), a finding in accordance with previous studies of Frank *et al*, 1978 in the guinea pig and Chen *et al.*, 1994 in the preterm rat, but in contradiction to those findings in the preterm rabbit (Frank and Sosenko, 1991) and baboon (Jenkinson *et al.*, 1991). The reasons for these discrepancies in antioxidant enzyme inductions between the species is unclear, although the stage of lung development, the

inductions between the species is unclear, although the stage of lung development, the oxygen concentration the animals are exposed to and the length of time the animals are exposed to oxygen may be important in determining the nature and extent of the antioxidant response (Warshaw *et al.,* 1985; Frank and Sosenko, 1987; Sosenko and Frank, 1987; Kelly *et al.,*1993; Chen *et al.,*1994). Although antioxidant induction occurred, lung injury also developed. There are two possible reasons for this. Either the degree of antioxidant protection was not enough, or that other factors are involved in lung injury. The outcome of excess free radical production was not detected using the markers assessed in the present study. The concentration of BALF conjugated dienes and lung tissue protein carbonyls were no different to that of air-exposed animals.

The pulmonary inflammatory response seen after oxygen exposure was characterised by a substantial elevation in inflammatory cell numbers, particularly in the number of neutrophils recovered in the BALF. Increased numbers of neutrophils are also associated with the development of HMD and have been shown to be an independent predictor of the progression from acute to chronic lung disease (Merritt, 1982; Ogden *et al.,*1984). Elevated ß-glucuronidase activity, a marker of cellular activation, suggests that the inflammatory cells present within the lung are activated. This is also confirmed by the increase in elastase activity detected using the small synthetic substrate. However, this activity is associated with a large molecular weight fraction in BALF (Kelly *et al.,*1991) and no activity was detected using tritiated elastin as the substrate. A similar observation has been made by Wewers *et al,* 1988, who found that the elastase activity detected in the BALF of adults with ARDS was also associated with a large molecular weight protein. However, increased protease activity and hence an imbalance in the protease-antiprotease profile of the lungs of infants with HMD may predispose these neonates to BPD.

In the baboon model of HMD (Jackson *et al.,*1987) and in infants with HMD (Merritt *et al.,*1983; Ogden *et al.,* 1984) macrophages numbers are increased in the lungs later in the disease process. In the present study, the BALF macrophage population was unaltered. However, the blood monocyte population was significantly increased. As these cells are precursors of tissue macrophages, the time frame in which the present study was undertaken may not have been long enough to see the increase in BALF macrophages that is seen in the baboon study (Jackson *et al.,*1987). However, if pups are left to recover for 2 days in 21% oxygen the macrophage population is significantly increased above air controls (Town,

may reflect the release of elastin fragments, from the action of neutrophil elastase, which is known to be chemotactic for this cell (Hunninghake *et al.*, 1981).

In conclusion, prematurely delivered guinea pigs allowed to recovery in 21% oxygen develop respiratory distress and lung injury similar to that observed in the human premature neonate. Although mechanical injury as a consequence of alveolar collapse may be the primary consequence for the increase in alveolar-capillary permeability, evidence in the guinea pig model also implicates inflammation as another possible factor. Following exposure to 95% oxygen, lung injury increased. As the inflammatory response in these animals is markedly increased, it was speculated that inflammation in the premature human neonate receiving supplemental oxygen may participate in the development of HMD and BPD. In the following two chapters the role of two particular components of the resultant inflammation will be investigated, in particular the role of the neutrophil and the role of specific proinflammatory lipids liberated by the action of PLA_2 on the development of oxygen-induced acute lung injury.

CHAPTER 4

THE ROLE OF THE NEUTROPHIL IN OXYGEN-INDUCED LUNG INJURY IN THE PREMATURE GUINEA PIG

4.1 Introduction

The development of HMD and progression to BPD is characterised by an increase in the number of tissue and alveoli neutrophils (Merritt *et al.*,1983; Ogden *et al.*,1984; Walti *et al.*,1989; Yoder *et al.*,1991). These cells have the potential to cause extensive tissue damage through the release of activated species of oxygen and/or proteases (Wandall, 1988; Sha'afi and Molski, 1988; Thomas *et al.*,1988; Weiss, 1989; Stone, 1990), evidence for which has been observed in BALF obtained from preterm neonates (Bruce *et al.*,1981; Bruce *et al.*,1985). As oxygen has been implicated as a possible pathogenic factor in the development of BPD, the role of the neutrophil in the development of oxygen-induced acute lung injury is under question. However, hyperoxic-induced and many other forms of acute and chronic lung injury have been demonstrated in the absence of neutrophils (Schwartz *et al.*,1983; Winn *et al.*,1987; Hutson *et al.*,1990). As such the role of this cell as a primary mediator of cell injury *in vivo* remains unclear.

In the present study the preterm guinea pig model was used to investigate the role of the neutrophil in oxygen-induced acute injury to the immature lung. First by measuring the release of superoxide from neutrophils of preterm and term pups. A comparison of superoxide production from these two groups will show whether those animals that are delivered prematurely are more readily able to generate superoxide and thus impose an additional oxidative burden on an already oxidatively stressed lung. Second, whether selectively depleting these cells from the circulation of oxygen-exposed preterm animals will result in a reduction in the observed lung injury. This second experiment will also permit an assessment of the temporal relationship between lung neutrophils and lung antioxidant enzyme activities, observed previously in the model (Town *et al.*,1993), and whether neutrophil depletion leads to a change in the antioxidant profile of the immature lung.

4.2 Inflammatory cell superoxide production.

4.2.1 Materials and methods.

Newborn guinea pigs, or those delivered at 65 days of gestation (term = 68 days) were randomly allocated to receive either 21% or 95% oxygen for 72 hours (Fig 4.1). At the end of the 72 hour exposure period, pups were sacrificed by pentobarbitone overdose, blood taken and a BAL performed. To obtain as many cells as possible, 20mls (10 x 2ml aliquots)

of non pyrogenic saline (37°C) was used in the BAL procedure. Total and differential BALF cell counts were assessed and total superoxide production from 500,000 cells over 15 minutes following treatment with PMA (10ng/ml: final concentration) was measured as described in Section 2.10.4. Blood and BALF inflammatory cells from 3 oxygen-exposed preterm guinea pigs were combined and separated on a discontinuous percoll density gradient as described in Section 2.7.

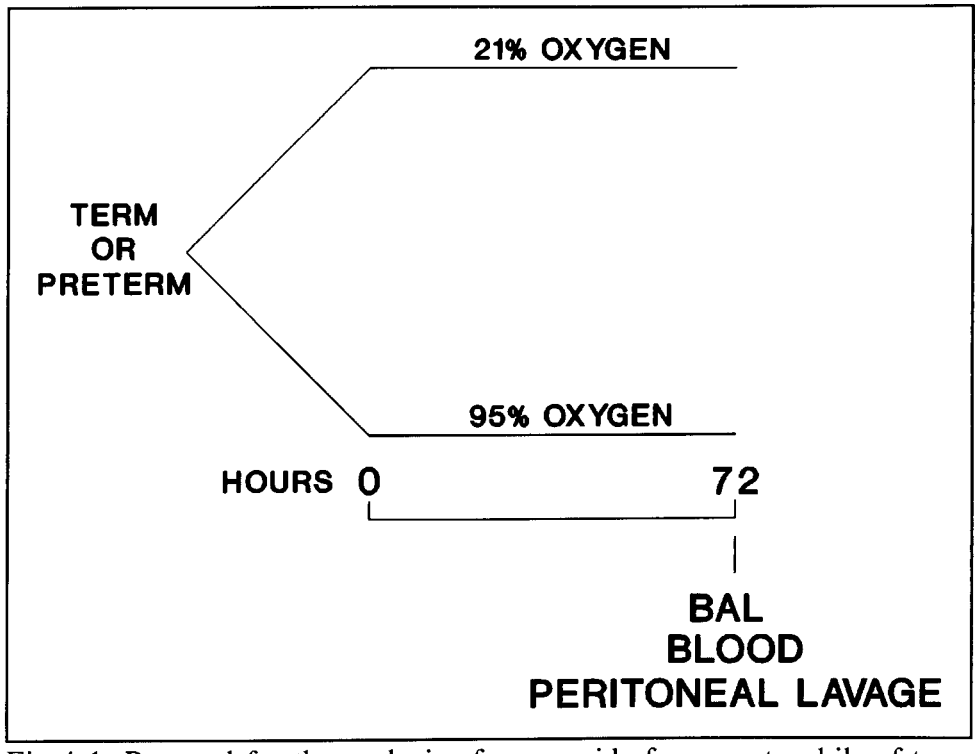


Fig 4.1. Protocol for the analysis of superoxide from neutrophils of term and preterm guinea pigs exposed to 21% and 95% oxygen for 3 days.

Total and differential blood and BALF inflammatory cell analysis was performed at each density. BALF inflammatory cells obtained at each density were washed in HBSS three times and 100μl of cell suspension in HBSG assessed for superoxide production. Neutrophils were also ellicted from the peritoneal cavity of air-exposed preterm guinea pigs as described in section 2.6.1 and treated with PMA at a concentration of 10 ng/ml per 500,000 cells for 30 minutes at 37°C. The change in density following this treatment was then assessed on a discontinuous density gradient as described in Section 2.7. Further neutrophils were elicited from the peritoneum of air-exposed term and preterm pups and

the maximum rate of superoxide production and lag phase measured following addition of several different concentrations of PMA (1 -100ng/ml). Total superoxide production from these cells (term and preterm) was measured over 15 minutes as described earlier.

4.2.2 Results

4.2.2.1 Survival

All animals exposed to 21% oxygen survived the 72 hours of the study (Fig 4.2). Although exposure to 95% oxygen resulted in a significant reduction in the survival of preterm pups compared to equivalently aged air controls, this was not observed for term pups.

4.2.2.2. Body weight

There was no effect of age on birth weight (Table 4.1). Although all animals lost weight over the period of the study, the rate of decline was greater during the first 24 hours than during the last 24 hours. However, as with birth weight, no difference in body weight was observed at any other time point during the study, either between term and preterm animals or between animals exposed to 21% or 95% oxygen.

Table 4.1. The effect of exposure to 21% and 95% oxygen for 3 days on the body weights of preterm and term guinea pigs.

% O_2	Treatment	Body weight (g)			
		0hr	24hr	48hr	72hr
21	Preterm	75 ± 14	69 ± 10	68 ± 11	70 ± 11
	Term	84 ± 12	75 ± 7	76 ± 12	78 ± 10
95	Preterm	81 ± 11	71 ± 14	71 ± 13	70 ± 13
	Term	83 ± 12	75 ± 9	76 ± 10	79 ± 8

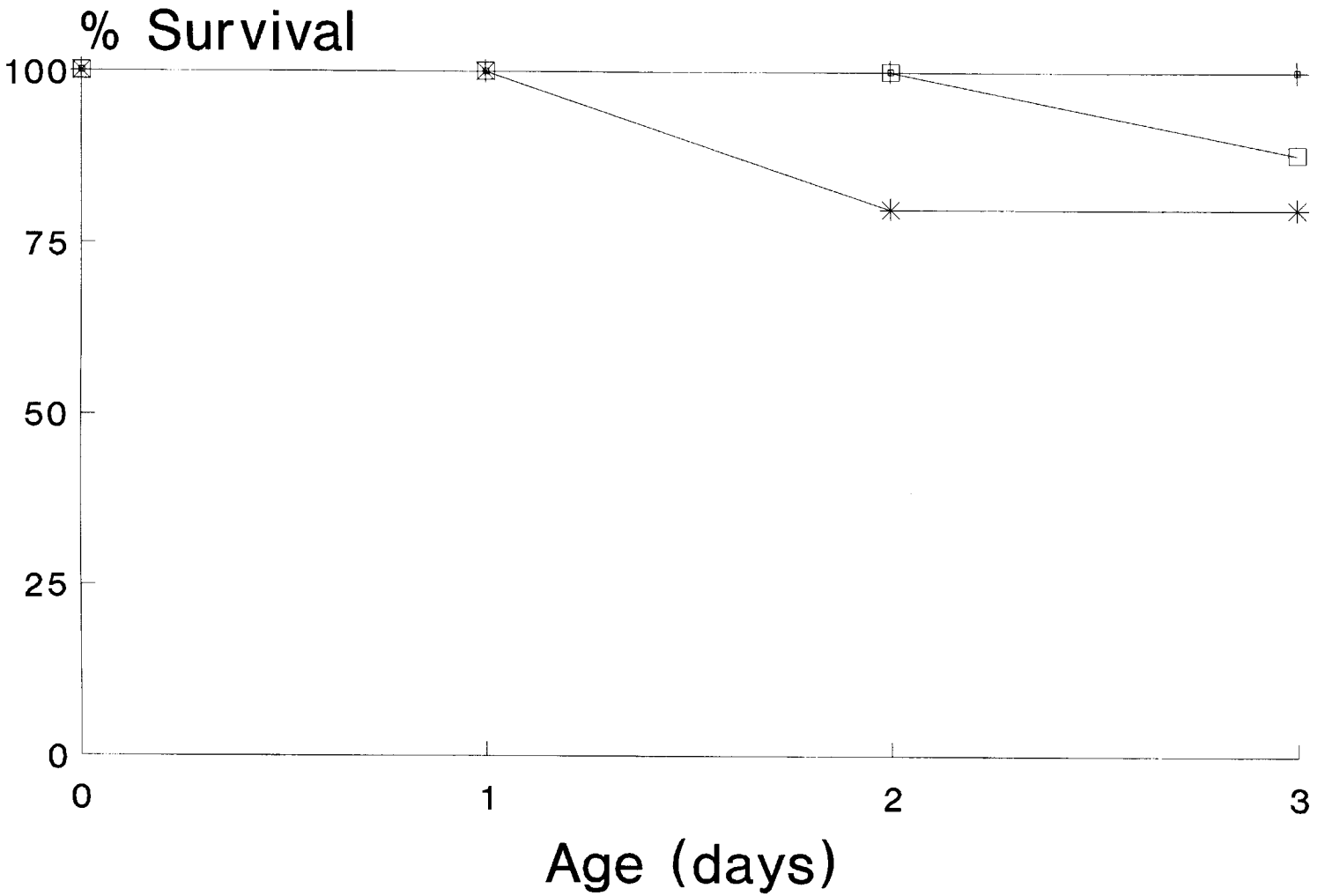
Data represents the mean and standard deviation of between 4 and 7 animals per group. There was a significant effect of time ($F=15.7$, $p<0.005$).

Figure 4.2. The effect of exposure to 21% and 95% oxygen for 3 days on the survival rate of term and preterm guinea pigs.

Guinea pigs delivered by Caesarian section at 65 days of gestation or naturally delivered term animals were exposed to 21% or 95% oxygen for 3 days and the percentage survival assessed every 24 hours.

---.--- Term 21% oxygen
---+--- Preterm 21% oxygen
---*--- Term 95% oxygen
--[]--- Preterm 95% oxygen

All animals exposed to 21% oxygen survived. There was a significant reduction in survival of preterm pups exposed to 95% oxygen when compared to equivalent animals exposed to air ($p < 0.05$). Data represents the survival of between 8 and 10 animals per group.


% Survival
100
75
50
25
0
0
1
2
3
Age (days)

4.2.2.3 BALF inflammatory cell counts.

Table 4.2 shows the effect of oxygen exposure on BALF inflammatory cell counts from term and preterm guinea pigs exposed to 21% and 95% oxygen.

Table 4.2. The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential cell count of BALF from term and preterm guinea pigs.

		BALF Inflammatory cell count (10^4/ml BAL)			
		TOTAL	PMN	MACS	EOS
Term	$21\%O_2$	48 ± 17	0 ± 0	14 ± 4	32 ± 10
	$95\%O_2$	60 ± 10	4 ± 3*	14 ± 2	42 ± 7
Preterm	$21\%O_2$	52 ± 15	1 ± 1	18 ± 7	33 ± 11
	$95\%O_2$	66 ± 11	4 ± 3*	17 ± 5	39 ± 5

Abbreviations. PMN:- Neutrophils, MACS:- Macrophages, EOS- Eosinophils. There was a significant effect of oxygen on BALF neutrophil numbers ($F=28.2$, $p<0.001$). Data represent the mean ± S.D of 4-7 animals per group. * $p<0.05$, compared to 21% oxygen.

The neutrophil count from oxygen-exposed term and preterm guinea pigs was significantly elevated above equivalently aged animals exposed to 21% oxygen. No significant difference was observed in total, macrophage or eosinophil cell counts between term and preterm or between 21% and 95% oxygen exposure.

4.2.2.4 BALF protein.

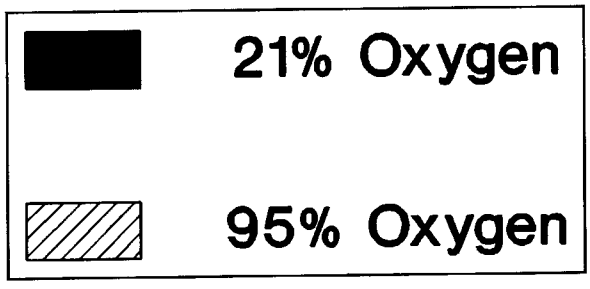
No significant difference in BALF protein was observed between preterm and term animals exposed to either 21% or 95% oxygen (Fig 4.3). There was a significant increase in BALF protein in both term and preterm animals relative to air controls following 72 hours exposure to 95% oxygen.

4.2.2.5 BALF inflammatory cell superoxide production.

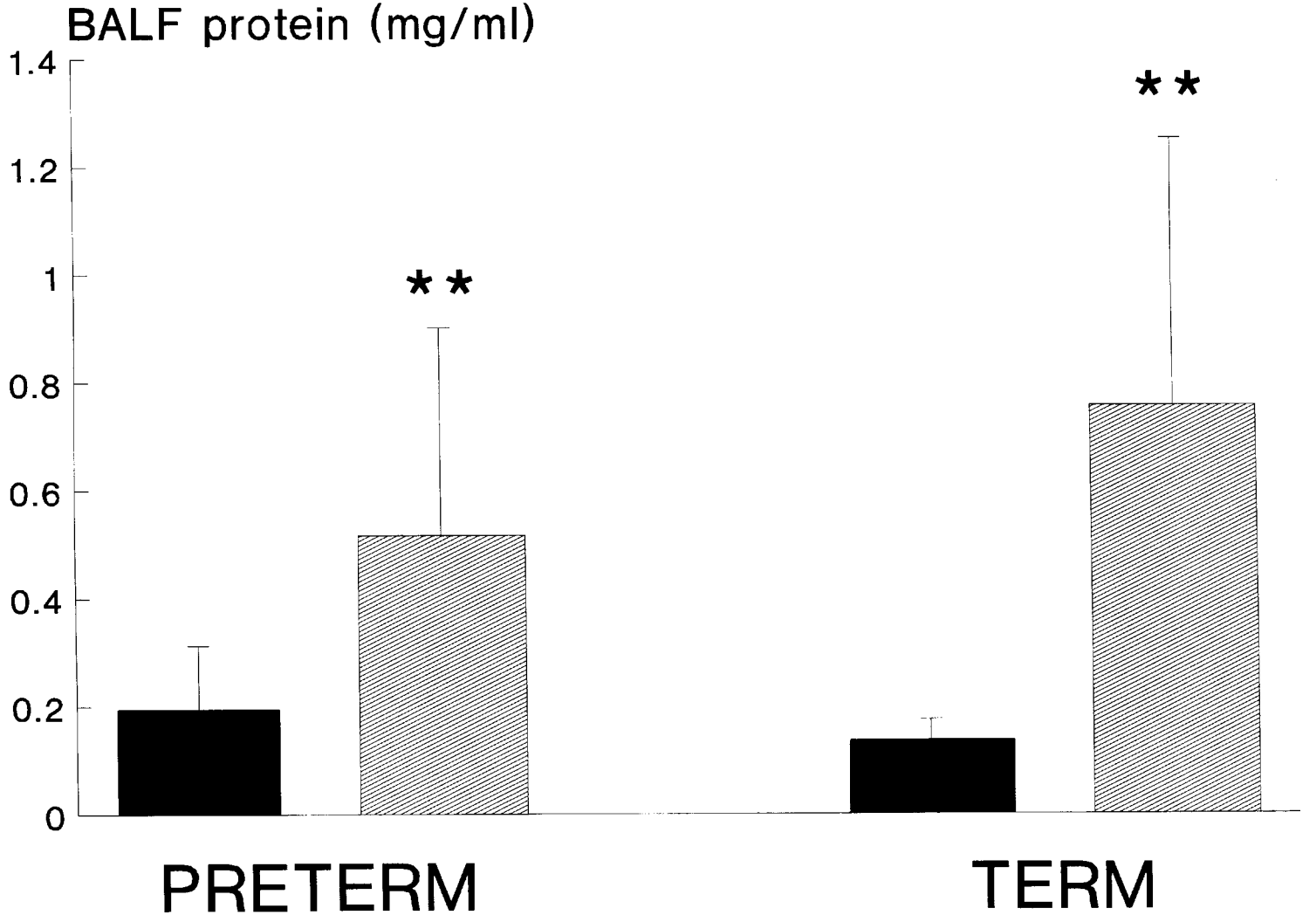
Although spontaneous superoxide production was observed from inflammatory cells isolated from the lungs of animals exposed to 21% and 95% oxygen (21% O_2; 5.0±6.9, 95% O_2: 1.9±2.2 nmol $O_2^{\cdot-}$/15min/5x10^5cells), no significant difference between exposure treatments was observed. However, animals exposed to 21% oxygen and treated with PMA

Figure 4.3. The effect of exposure to 21% and 95% oxygen for 3 days on BALF protein from term and preterm guinea pigs.

Guinea pigs delivered by Caesarian section 3 days before term and term animals delivered naturally were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed with 10 x 2ml aliqouts of sterile saline (37°C). The BALF was then placed on ice before being spun at 1500rpm (4°C) to remove inflammatory cells. The acellular fraction was then aliqouted into 500μl lots and frozen at -70°C. BALF protein was measured as described in Section 2.10.5



Data represents the mean and standard deviation of between 8 and 9 animals per group. A significant effect of oxygen exposure ($F=17.1$, $p<0.001$) was observed. ** $p<0.005$ compared to 21% oxygen.


BALF protein (mg/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
PRETERM
TERM

(10ng/ml), generated significantly less superoxide than to similarly exposed term animals (Fig 4.4). Oxygen exposure significantly blunted superoxide production in both term and preterm pups following treatment with PMA.

4.2.2.6. Purification of BALF and blood neutrophils by percoll density gradients.

Figure 4.5 illustrates the density profile of neutrophils in blood and BALF of oxygen-exposed preterm guinea pigs. At all densities assessed, eosinophils in BALF (Fig 4.5A and B) and lymphocytes in blood (Fig 4.5C and D) were the major contaminating cell types. Analysis of the density profile from the two compartments show that neutrophils from the lung tended to be denser than those of blood. Superoxide production assessed at each density from BALF cells of oxygen-exposed animals (Fig 4.6) correlated significantly with eosinophil numbers.

4.2.2.7. Density profile of PMA stimulated and unstimulated peritoneal neutrophil from air-exposed preterm and term guinea pigs.

Figure 4.7 shows the density profile before and after treatment with PMA (10ng/ml) of peritoneal neutrophils elicited from both term and preterm pups. Unstimulated peritoneal neutrophils from both groups of animals tend to be proportionally more denser than those obtained from the blood. Following stimulation, there was a shift towards proportionally more dense cells than that seen in the unstimulated population, towards a profile seen for neutrophils in BALF of oxygen-exposed animals.

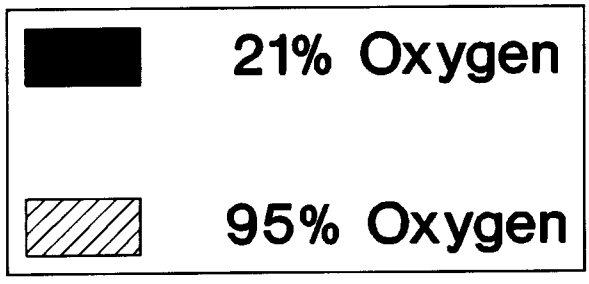
4.2.2.8 Superoxide production from peritoneal neutrophils of term and preterm guinea pigs.

Figure 4.8 shows the effect of different concentrations of PMA on the rate of superoxide production and lag phase. A significant effect of age was observed for both the rate of superoxide production and the lag phase. However, due to the small number of points at each PMA concentration, significant differences between each concentration of PMA was not observed.

Figure 4.9 shows the effect of PMA (10ng/ml) on the total superoxide production from peritoneal neutrophils of term and preterm animals. No significant difference was observed between neutrophils obtained from term or preterm pups.

Figure 4.4. The effect of PMA on the total superoxide production from term and preterm guinea pig BALF inflammatory cells following 3 days exposure to 21% and 95% oxygen.

Guinea pigs delivered by Caesarian section 3 days before term and term animals delivered naturally were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed with 10 x 2ml aliqouts of sterile saline (37°C). The BALF was then placed on ice before being spun at 1500rpm (4°C) for 10 minutes. The cell pellet was resuspended in one millilitre of HBSG, a total cell count performed and the suspension made up to 5 X 10^6 cells/ml HBSG. One hundred microlitres of this cell suspension was then used to measure superoxide production as described in Section 2.10.4



Data represents the mean and standard deviation of between 8 and 9 animals per group. A significant effect of oxygen exposure ($F=17.1$, $p<0.001$), age ($F=21.2$, $p<0.001$) and a significant interaction between the two ($F=4.7$, $p<0.05$) was observed. * $p<0.05$, ** $p<0.005$ compared to 21% oxygen, + $p<0.05$ compared to preterm.

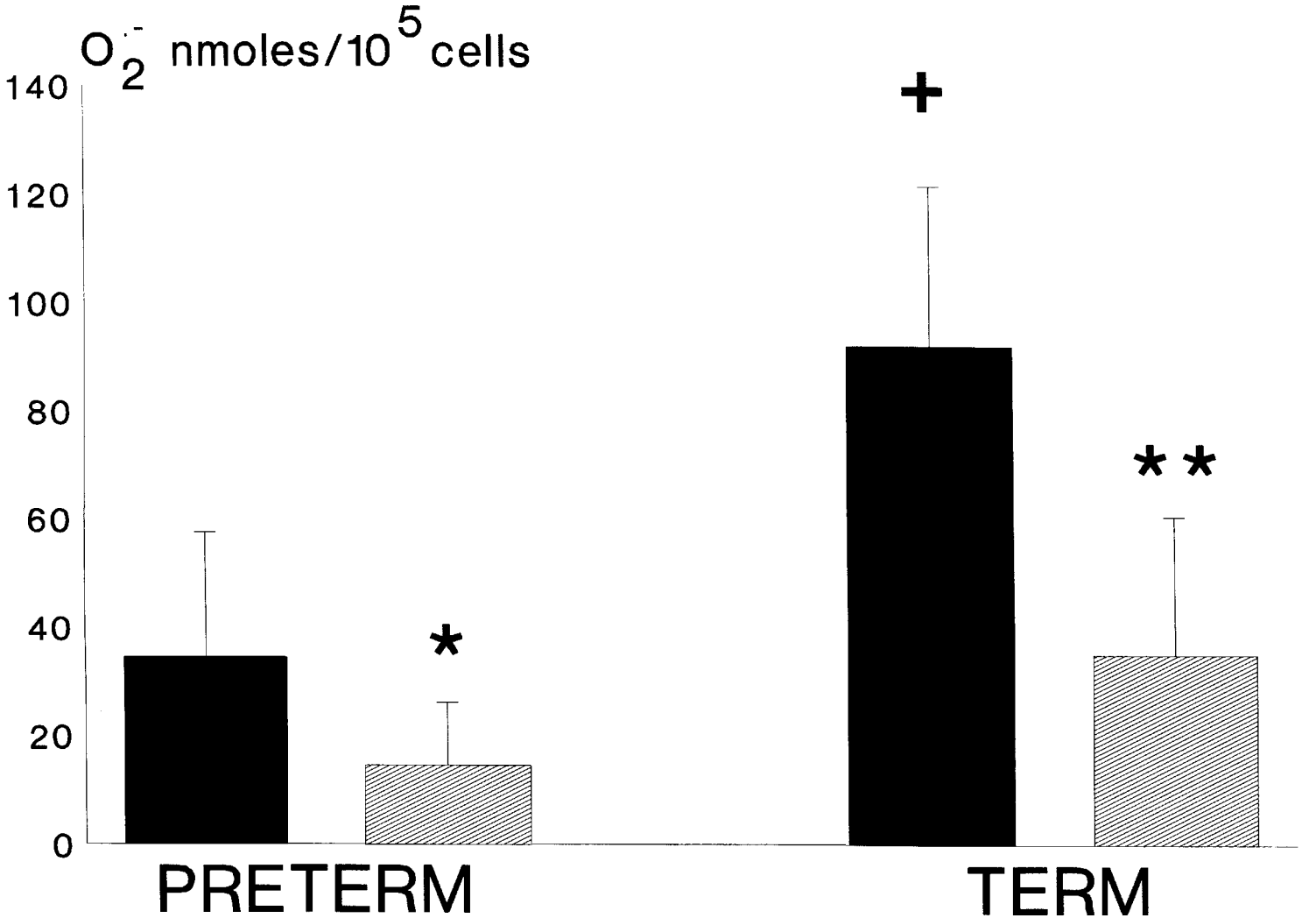
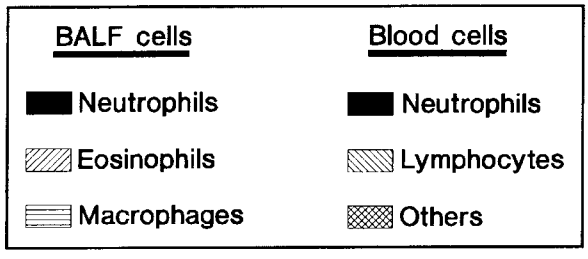
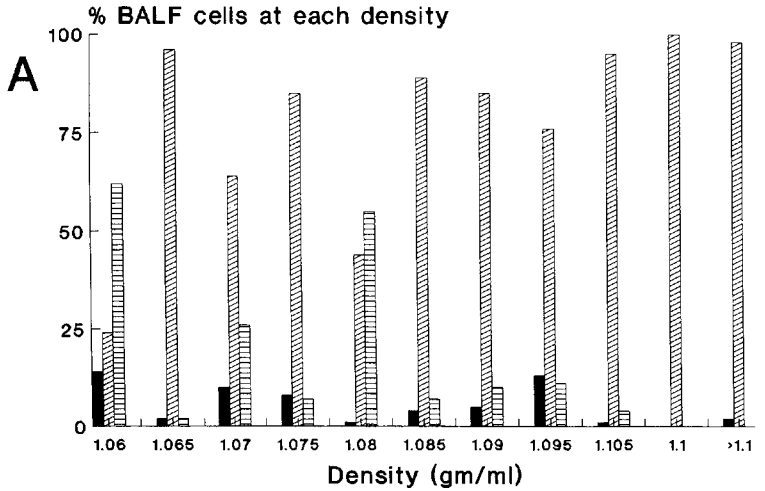

$O_2^{\cdot-}$ nmoles/10^5 cells
140
120
100
80
60
40
20
0
*
+
**
PRETERM
TERM

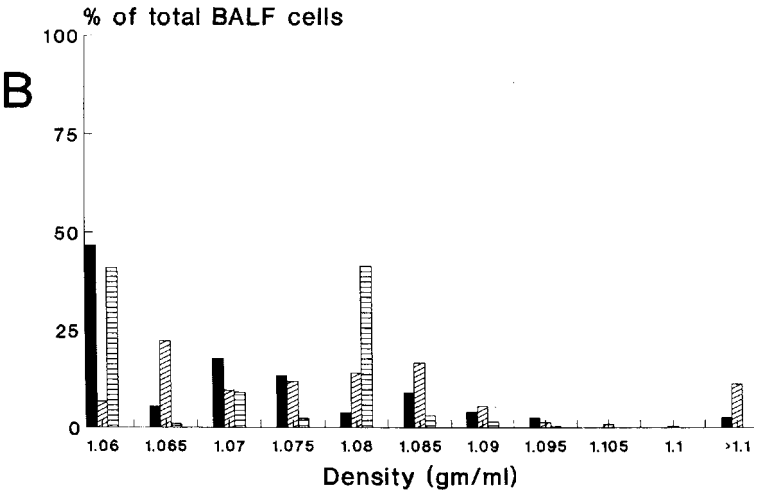
Figure 4.5. The density profile of BALF and blood inflammatory cells from preterm guinea pigs exposed to 95% oxygen for 3 days.

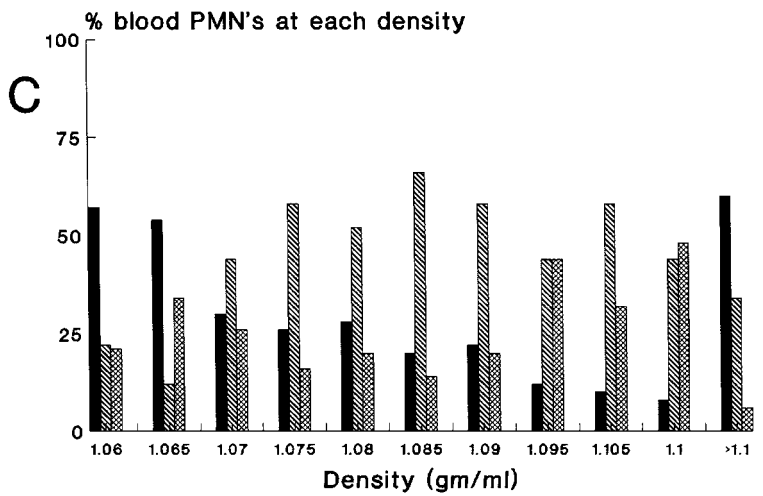
Guinea pigs delivered by Caesarian section 3 days before term were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed with 5 x 2ml aliqouts of sterile saline (37°C). The BALF was then placed on ice before being spun at 1500rpm (4°C) for 10 minutes. The cell pellet was then resuspended in one millilitre of HBSG and applied to a discontinuous percoll density gradient as described in Section 2.7. Following BAL, 1ml of blood was withdrawn by cardiac puncture and mixed with an equal volume of 6% dextran. The mixture was left to stand for 45 minutes after which the clear upper layer and the interface were removed and placed in a microfuge tube. Inflammatory cells were removed by centrifugation at 1500 rpm for 15 minutes (R.T.P) and washed twice in HBSG. The final pellet was resuspended in 1ml HBSG and applied to a discontinuous percoll density gradient. Total and differential cell counts were performed after separation at each density. Data is expressed firstly (A and C) as percentage cell type at each density and secondly (B and D) the percent cell type at each density as a total of that particular cell type recovered at all densities.



Data represents the mean of two percoll runs from a combined peritoneal wash of 3 preterm animals.


A
% BALF cells at each density
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)


B
% of total BALF cells
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)


C
% blood PMN's at each density
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)

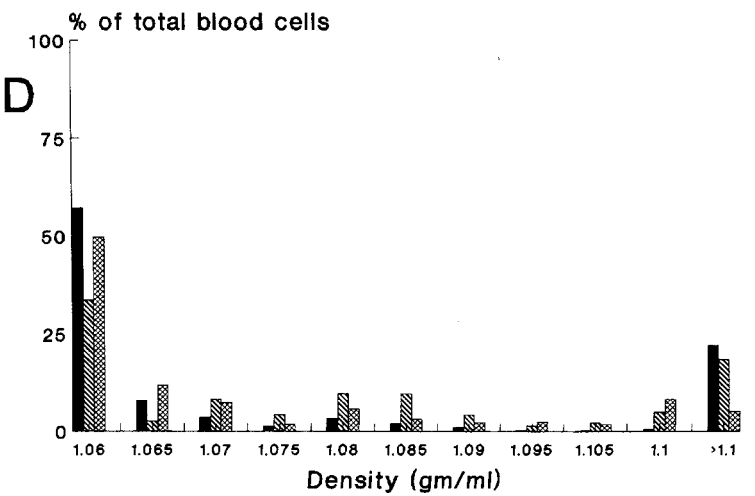

D
% of total blood cells
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)

Figure 4.6. Correlation between eosinophil numbers and total superoxide production from BALF inflammatory cells at each density.

Guinea pigs delivered by Caesarian section 3 days before term were randomly assigned to receive either 21% or 95% oxygen for 3 days. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed with 5 x 2ml aliqouts of sterile saline (37°C). The BALF was then placed on ice before being spun at 1500rpm (4°C) for 10 minutes. The cell pellet was then resuspended in one millilitre of HBSG and applied to a discontinuous percoll density gradient as described in section 2.7. Total and differential BALF cell counts were measured after separation at each density. The cells recovered were washed twice in HBSG and 100μl of cell suspension assayed for superoxide production. A significant correlation between eosinophil numbers and superoxide production was observed ($R = 0.85$, $p < 0.005$).

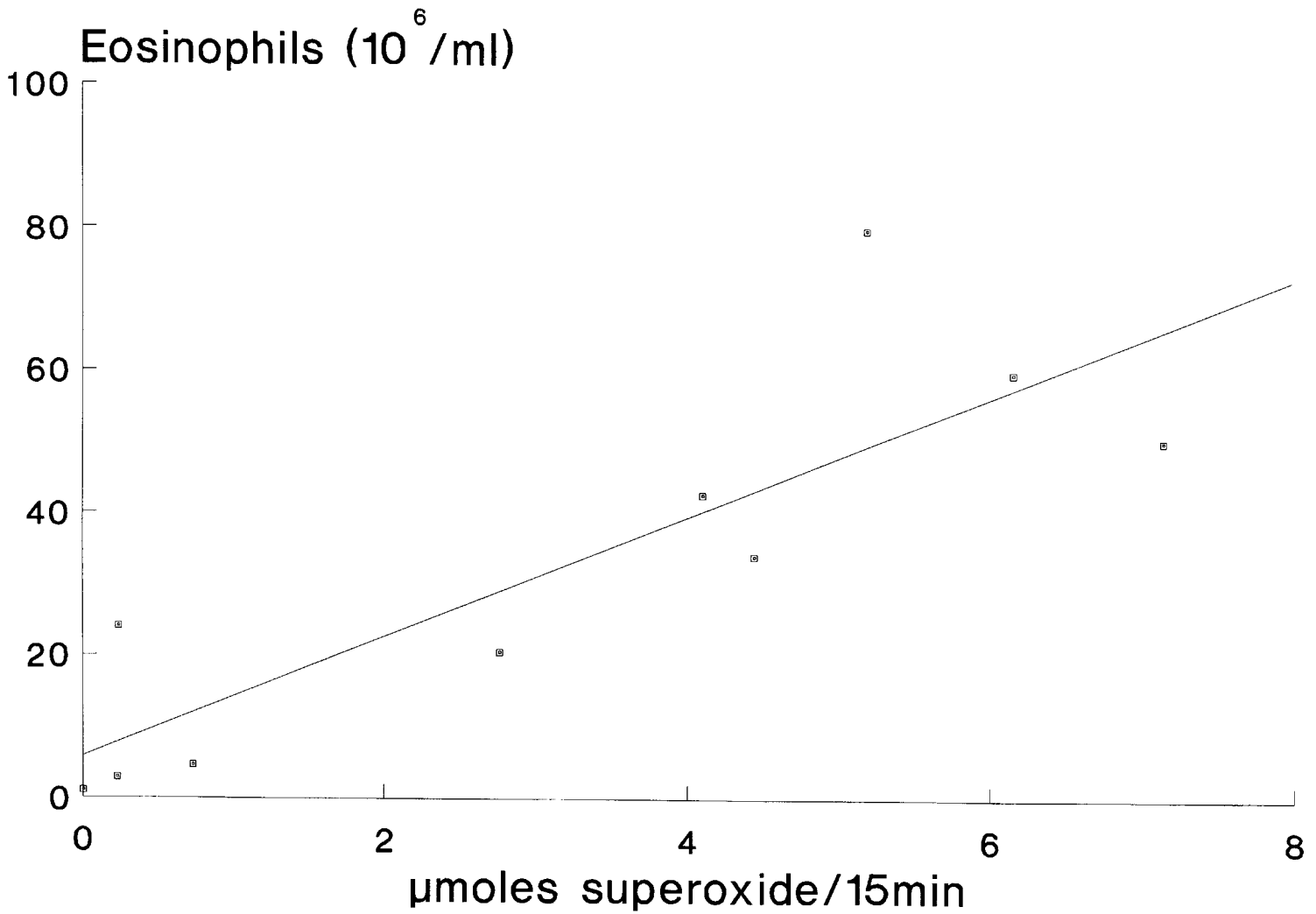
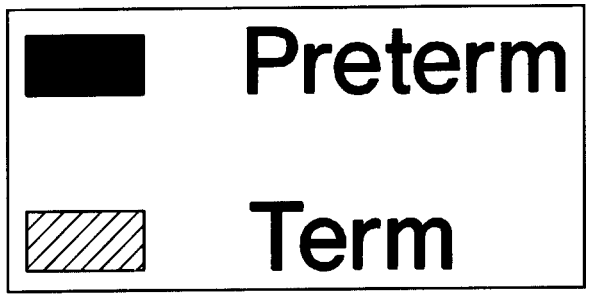
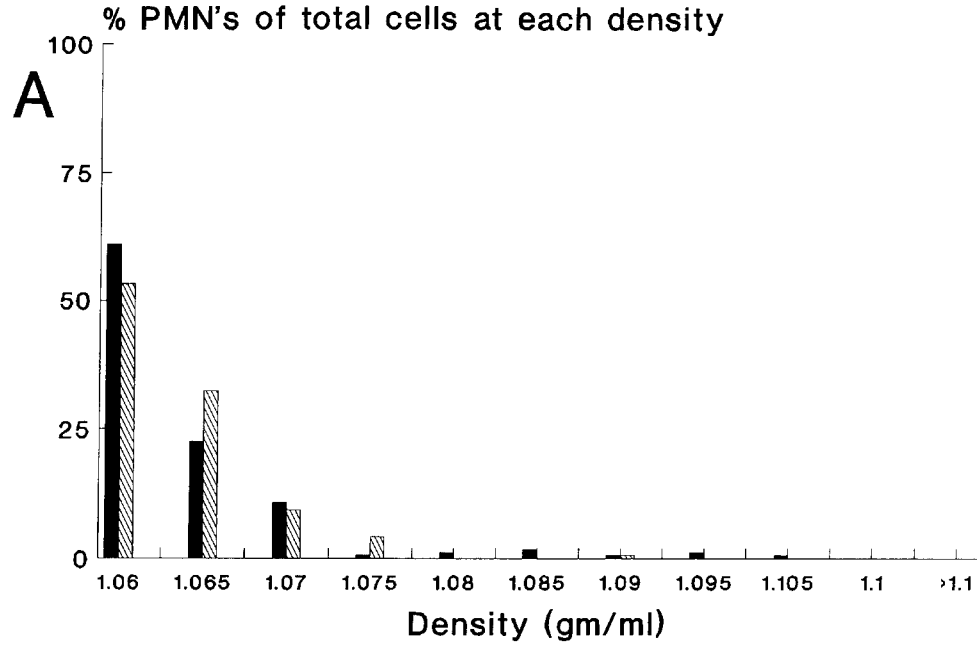

Eosinophils (10^6/ml)
100
80
60
40
20
0
0
2
4
6
8
µmoles superoxide/15min

Figure 4.7. The density profile of PMA treated peritoneal neutrophils from term and preterm guinea pigs exposed to 21% oxygen for 3 days.

Guinea pigs delivered by Caesarian section 3 days before term and term pups allowed to deliver naturally were exposed to 21% oxygen for 3 days. After 60 hours exposure, animals were given a 10ml intraperitoneal injection of 0.1% oyster glycogen in saline. Twelve hours later animals were sacrificed by cranial stunning and cervical dislocation and the peritoneal cavity washed with 50ml (5 x 10ml) of sterile saline (37°C). The cell suspension recovered after each wash was then combined and spun at 1500rpm for 10min (4°C). The cellular pellet was then resuspended in HBSG at a concentration of 5 x 10^6 cells/ml HBSG and separated into two groups, one exposed to PMA at a final concentration of 10ng/ml and the other to an equal volume of DMSO for 30min at 37°C. The suspension was then spun again at 1500rpm for 10min (4°C) and the cellular pellet redissolved in 1ml of HBSS and applied to a discontinuous percoll gradient. The total and differential cell count at each density was then assessed and the results expressed as percent neutrophils at each density as a fraction of the total neutrophil population, before (A) and after (B) PMA stimulation.



Data represents the mean of two percoll gradients (containing cells from 3 pups).


A
% PMN's of total cells at each density
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)

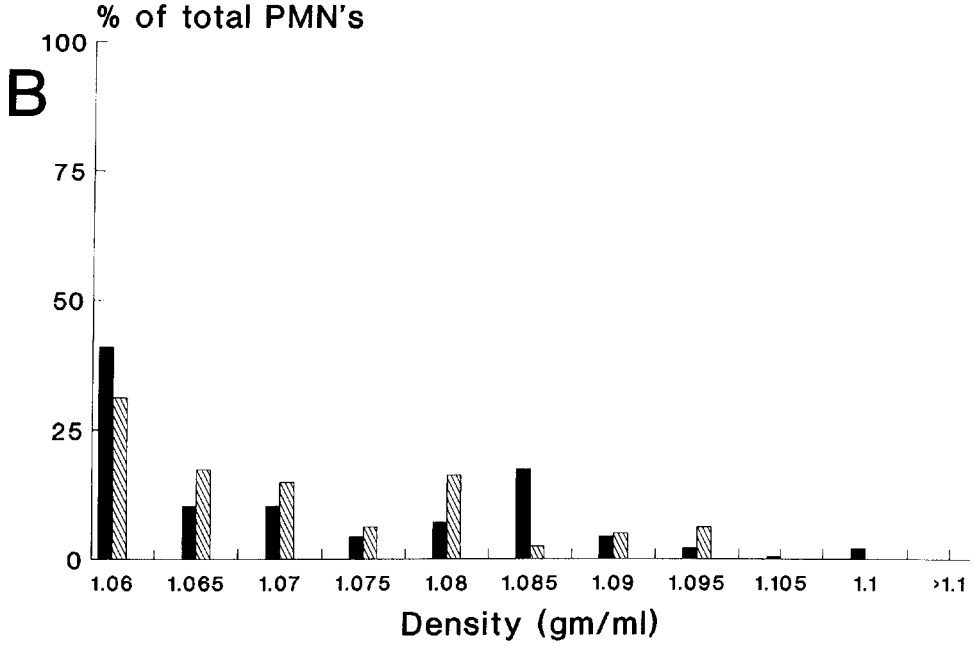
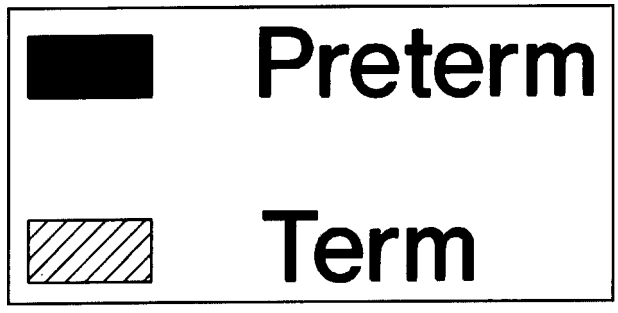
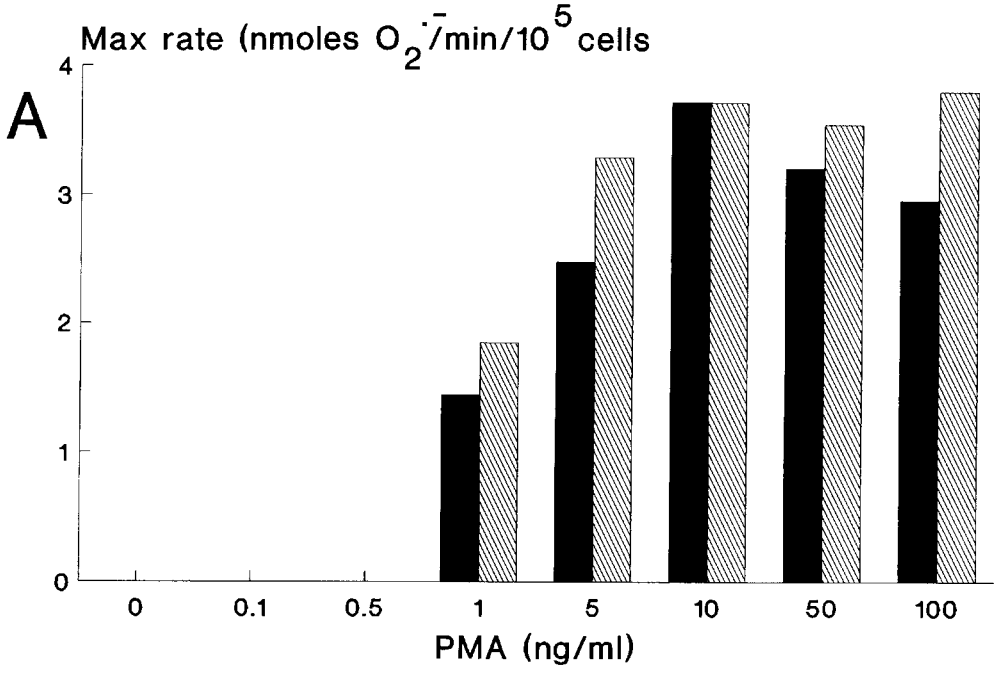

B
% of total PMN's
100
75
50
25
0
1.06
1.065
1.07
1.075
1.08
1.085
1.09
1.095
1.105
1.1
>1.1
Density (gm/ml)

Figure 4.8. The effect of treatment with PMA on the rate of superoxide production (A) and lag phase (B) of peritoneal neutrophils from term and preterm guinea pigs exposed to 21% oxygen for 3 days.

Guinea pigs delivered by Caesarian section 3 days before term and term pups allowed to deliver naturally were exposed to 21% oxygen for 3 days. After 60 hours exposure, animals were given a 10ml intraperitoneal injection of 0.1% oyster glycogen in saline. Twelve hours later animals were sacrificed by cranial stunning and cervical dislocation and the peritoneal cavity washed with 50ml (5 x 10ml) of sterile saline (37°C). The cell suspension recovered was then combined and spun at 1500rpm for 10min (4°C). The cellular pellet was then resuspended in HBSG at a concentration of 5 x 10^6 cells/ml and the superoxide production assessed at different PMA concentrations (0-100ng/ml) as described in section 2.10.4. The rate of superoxide production is shown in graph A and the lag phase in graph (B). Unstimulated superoxide production from term and preterm peritoneal neutrophils were, 0.35 ± 0.44 and 0.21 ± 0.57 nmole $O_2^{\cdot-}$/min/10^5 cells respectively (p=NS).



The data refers to the combination of cells from 3 term and preterm animals.


A
Max rate (nmoles $O_2^{\cdot -}$/min/10^5 cells
4
3
2
1
0
0
0.1
0.5
1
5
10
50
100
PMA (ng/ml)

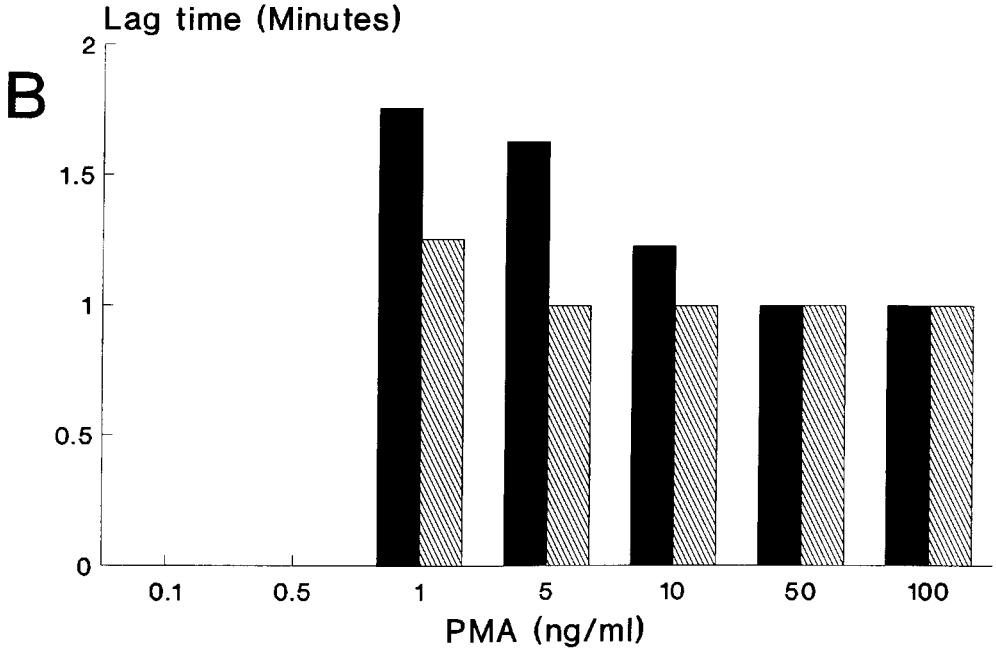
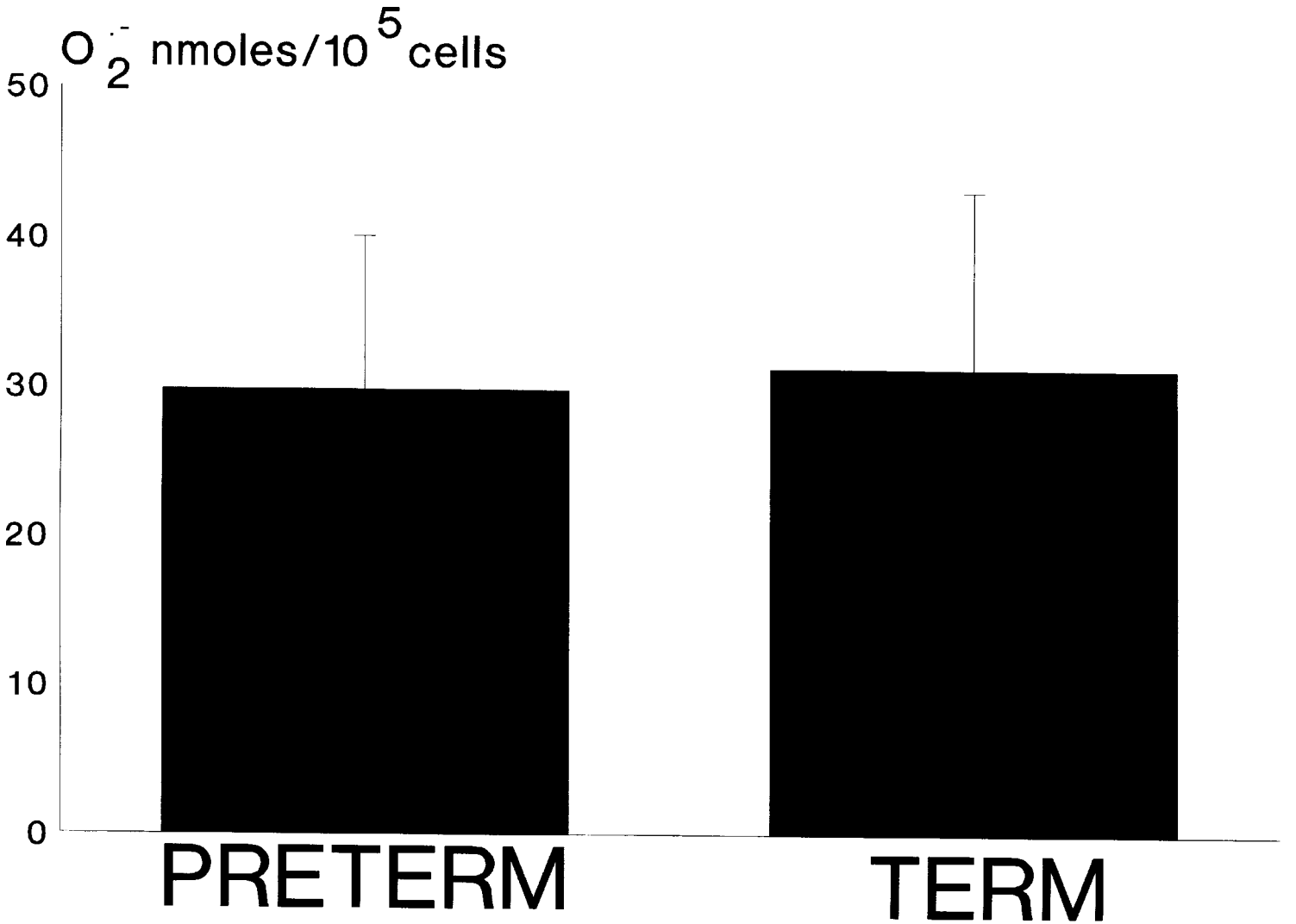

B
Lag time (Minutes)
2
1.5
1
0.5
0
0.1
0.5
1
5
10
50
100
PMA (ng/ml)

Figure 4.9. The effect of treatment with PMA on the total superoxide production of peritoneal neutrophils from term and preterm guinea pigs exposed to 21% oxygen for 3 days.

Guinea pigs delivered by Caesarian section 3 days before term and term pups allowed to deliver naturally were exposed to 21% oxygen for 3 days. After 60 hours exposure, animals were given a 10ml intraperitoneal injection of 0.1% oyster glycogen in saline. Twelve hours later animals were sacrificed by cranial stunning and cervical dislocation and the peritoneal cavity washed with 50ml (5 x 10ml) of sterile saline (37°C). The cell suspension recovered was then combined and spun at 1500rpm for 10min (4°C). The cellular pellet was then resuspended in HBSG at a concentration of 5 x 10^6 cells/ml and the superoxide production assessed following stimulation with PMA (10ng/ml) as described in section 2.10.4. No significant difference between term and preterm superoxide production was observed. Total superoxide production from unstimulated neutrophils from term and preterm animals were 3.22 ± 4.19 and 2.01 ± 3.05 nmoles $O_2^{\cdot-}/10^5$ cells respectively (p=NS). Data represents the mean and standard deviation of 5 animals.


$O_2^{\cdot-}$ nmoles/10^5 cells
50
40
30
20
10
0
PRETERM
TERM

4.2.3 Discussion

The development of HMD and the subsequent progression to BPD is characterised in part by the presence of inflammatory cells, in particular neutrophils, within the alveoli (Merritt *et al.*, 1983; Ogden *et al.*, 1984). Attention has focused on this cell type because of its capacity to generate superoxide, secrete proteases and release many other mediators of inflammation (Wandall, 1988; Sha'afi and Molski, 1988; Thomas *et al.*, 1988; Weiss, 1989; Stone, 1990), all potentially harmful to the host. Following release of superoxide, other more damaging oxygen-centred free radicals may be generated. These include, hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$) and via the action of myeloperoxidase, longer lasting oxidants may also be formed. These toxic oxygen species are known to damage tissue and as such, the role for inflammatory cell oxygen-centred free radicals in the development of neonatal acute lung injury is under investigation. Although the capacity of neutrophils to generate superoxide in term infants has been explored and compared to adults, there have been few studies on the functional capacity of neutrophils from preterm infants. Of those studies that have been done, only circulating and not alveolar neutrophils have been investigated (Gahr *et al.*, 1985; Peden *et al.*, 1987). As neutrophils are found within the alveolar lumen of the lungs of oxygen-exposed preterm guinea pigs, their role with respect to superoxide production and lung injury, was assessed.

No significant difference in the number of macrophages was observed between air-exposed term and preterm pups. The eosinophil population dominated at both ages, with this cell type constituting 60-70% of the total number of cells recovered following BAL. A further comparison demonstrated that BALF cells from preterm animals generated significantly less superoxide than equivalent cells from term animals following stimulation with PMA. Following percoll separation, a significant correlation was observed between eosinophils and total BALF cell superoxide production. The reduced capacity to generate superoxide therefore may be attributable to decreased eosinophil activity. This reduction in superoxide production by eosinophils is consistent with a number of published studies, that have focussed on different immune cells, in newborn (term and preterm) humans and other species (Sherman *et al.*, 1977; Sugimoto *et al.*, 1980; Strauss and Snyder, 1983; Kugo *et al.*, 1989; Okano *et al.*, 1991). Alveolar macrophages from 1-day old rabbits are able to ingest bacteria as effectively as macrophages from adult animals, but have a reduced capacity to produce superoxide on stimulation (Sherman and Lehrer, 1984). This reduction

in the capacity to generate superoxide has also been observed in blood neutrophils from human preterm and term infants (Peden *et al.*, 1987; Gahr *et al.*, 1985).

In the present study and those cited above, there are a number of reasons why these cells may produce less superoxide on stimulation. Prior studies on the maturation of lung alveolar macrophages demonstrate that key glycolytic, hexose monophosphate pathway (HMP) and lysosomal enzymes vary as a function of gestational and postnatal age (Nerurkar *et al.*, 1977). This gestational effect on key HMP enzymes has also been observed in the lungs of the preterm guinea pig (Chapter 3) and has been suggested to relate to bactericidal competence (Sugimoto *et al.*, 1980). As NADPH is involved in the direct reduction of molecular oxygen to superoxide (Weiss, 1989), a lower concentration of NADPH may translate to lower superoxide production. These enzymes also supply reducing equivalents to the GSH-redox system and as neutrophils from human neonates are known to be deficient in glutathione (Shigeoka *et al.*, 1981), this may predispose these cells to oxidative injury, leading to autooxidation, inactivation of the superoxide generating system and an observed reduction in superoxide release. Other possible reasons include the presence of fewer NADPH oxidases associated with the cells of preterm animals, although this has not been quantified. The inflammatory cells may also be down-regulated. This occurs following continued stimulation of inflammatory cells (Sha'afi and Molski, 1988) and further stimulation with PMA may not produce maximal superoxide production as seen in term pups.

Exposure of both preterm and term guinea pigs to hyperoxia resulted in an increase in the mortality rate and an influx of inflammatory cells to the lung, a response typical in this model. Although there was no difference in the inflammatory cell profile between term and preterm animals, there was a significant reduction in the ability of these cells, from both groups to generate superoxide. Previous studies on the effect of oxygen on cell function confirm the findings of the present study. Rister and Baehner, 1977 have shown that alveolar macrophages from oxygen-exposed guinea pigs produced less superoxide following stimulation with opsonised zymosan (OZ). Other cellular functions have also been showed to be altered following exposure to oxygen (Wolff *et al.*, 1978). More consistent evidence of damage and alteration to inflammatory cell function has been observed *in vitro*. A reduction in alveolar macrophage phagocytosis occurs following as little as 40% oxygen for 48h (Simon *et al.*, 1978; Raffin *et al.*, 1980). The chemotactic response elicited by fmlp is

48h (Simon *et al.*, 1978; Raffin *et al.*, 1980). The chemotactic response elicited by fmlp is also reduced in guinea pig alveolar macrophages following 2-3h hyperoxia (Bowles *et al.*, 1979). Nakamura and colleagues, 1988 found that exposure of rat alveolar macrophages to 95% oxygen for 72h reduced their capacity to generate superoxide. However, the majority of these studies have been performed on the alveolar macrophage and whilst these results are informative as to the effect of oxygen on cellular function it is unclear as to the effect on neutrophil function.

To assess the function of alveoli neutrophils, separation of these cells from the BALF of oxygen-exposed preterm pups was attempted using percoll density gradients. Although neutrophils were detected at all densities, the cells recovered were contaminated with eosinophils. On separation of blood cells using the same procedure, neutrophils again were found at most densities but this time contaminated by lymphocytes. As such it was impossible to purify neutrophils from either BALF or blood to much more than 50%. However, the distribution of neutrophils in blood and BALF were different. A higher proportion of BALF neutrophils were much denser than those cells observed in blood. As cell density increases following cellular activation, the density profile of BALF neutrophils indicates that these cells are potentially activated, an observation in agreement with other studies on the guinea pig (Kelly *et al.*, 1991). Because of the problem of separating out neutrophils from other cell types, it was decide to elicit neutrophils from the peritoneum. A purity of between 90 and 95% was obtained from both term and preterm animals. The density profile of these cells was between that found in the blood and that in the BALF of oxygen-exposed animals. Following activation with PMA the proportion of cells with a density greater than 1.07gm/ml increased to give a profile that was similar to that observed for BALF neutrophils, further evidence to suggest that these cells are activated and contributing to lung injury. Superoxide production was also assessed. Not only was the total amount of superoxide generated not significantly different between term and preterm animals, but also the lag phase and rate of superoxide production were similar. A trend for the superoxide production to be lower and the lag phase to be higher in neutrophils from premature animals at concentrations of PMA less than 10ng/ml was observed and as such, further studies are required to confirm this and see if other agonists can produce the same effect.

The results presented show no difference in neutrophil function between term and

such as the lung, as it is known that the production of oxygen radicals from inflammatory cells is dependant on the site of residence. Peritoneal macrophages release less superoxide than alveolar macrophages following stimulation (Drath and Karnovsky, 1975). PMN's in suspension respond differently to those attached to proteins such as lamin (Nathan, 1987), and thus what we observe *in vitro* may not necessarily occur *in vivo*. The maturational stage of the inflammatory cell is also important. Peripheral monocytes, precursors of tissue macrophages produce less hydrogen peroxide than mature macrophages (Nakagawara *et al.*, 1981). No account was made of the maturational stage of the neutrophils from preterm animals which needs to be addressed. Also, the state of activation of the inflammatory cell will also affect the cells response to stimulation (Fels *et al.*, 1987).

In summary, although there was a significant difference in BALF inflammatory cell superoxide production between term and preterm animals following stimulation with PMA, peritoneal neutrophils from these animals responsed similarly. Peritoneal and alveolar neutrophils were shown to be at different activational states and as such the extrapolation of results obtained from peritoneal cells as to the role of alveolar neutrophils in oxygen-induced lung injury may be incorrect. As there is such a difference between the two populations of neutrophils, the role of this cell type in oxygen-induced lung injury remains unanswered until improved techneques are avaliable to separate minor populations of neutrophils from BALF.

4.3 Neutrophil depletion

4.3.1 Materials and methods

Guinea pigs delivered at 65 days gestation were randomly allocated to receive either 21% or 95% oxygen for 72 hours and then returned to room air for a further 48 hours (Figure 4.10). Pups exposed to 95% oxygen were further randomly allocated to receive either saline (SAL), neutrophil antisera (NA) or control sera (CS) at a dose of 200μl/100gm body weight immediately after birth and then once every 24 hours. Each dose was given by intra-peritoneal (ip) injection. Pups exposed to 21% oxygen received only ip saline. Animals were taken at 72h and 120h after delivery and blood, BALF and lung tissue removed. Blood and BALF were immediately placed on ice and lung tissue frozen at -70°C. Total and differential blood and BALF cell counts, BALF protein, neutrophil elastase and ß-glucuronidase activities, and tissue catalase, glutathione peroxidase and superoxide

dismutase (Cu/Zn and Mn) activities, were measured according to the methods described in Sections 2.9 and 2.10.

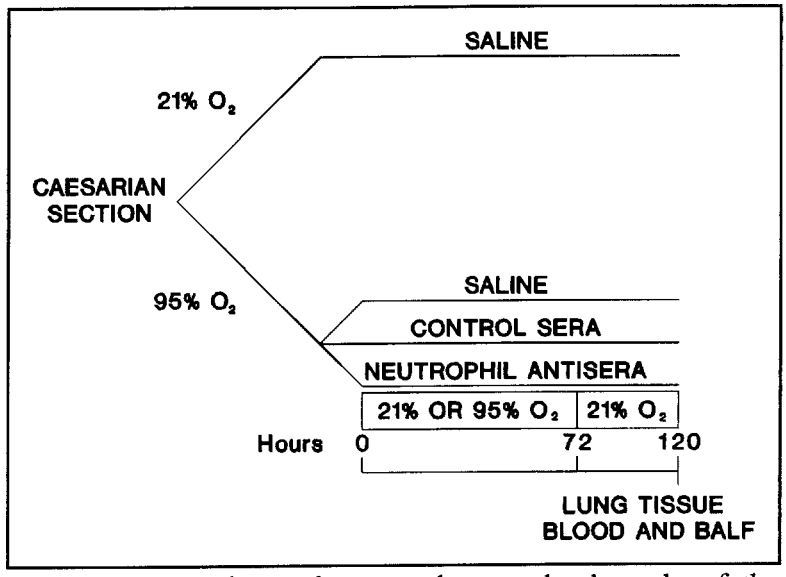


Fig 4.10. Experimental protocol to study the role of the neutrophil in oxygen-induced injury to the preterm guinea pig lung.

4.3.2 Results

4.3.2.1 Survival.

All animals exposed to 21% oxygen survived until the end of the study. Following exposure to 95% oxygen, there was a significant increase in mortality at 72hr and 120hr when compared to animals exposed to 21% oxygen (Table 4.3). No significant difference in survival was observed between NA, CS and SAL-treated animals exposed to 95% oxygen at either 72h or 120h days. There was also no significant difference in survival between 72h and 120h following any of the treatment regimes.

4.3.2.2 Body weights

Table 4.4 shows the effect of oxygen exposure and treatment with NA, CS and SAL on the change in body weight over 3 and 5 days following delivery. Pups in all groups lost between 10 and 20% of their initial body weight over the first 3 days. Although animals begun to gain weight after this initial period, pups were still lighter than birth weight. No effect of hyperoxia, NA or CS treatment on the percent change in body weight was

observed during the study.

Table 4.3. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on the survival rate of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

O_2 (%) 0 - 72hr	O_2 (%) 72 - 120hr	Treatment	Survival (%)
21	-	SAL	100
95	-	SAL	69.0 *
95	-	CS	65.2
95	-	NA	65.2
21	21	SAL	100
95	21	SAL	74.5 *
95	21	CS	72.7
95	21	NA	72.7

Abbreviations:- SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera. Data represents the percentage number of animals (6-10) surviving 72 hours exposure to 21% and 95% oxygen and following 2 days recovery in 21% oxygen. * $p<0.05$ compared to 21% oxygen.

4.3.2.3 Lung wet weight.

Although saline treated animals at 3 and 5 days that were exposed to 95% oxygen tended to have heavier lungs than equivalently treated air controls, this was not significant. No effect of, or difference between, NA and CS-treated animals was observed (Table 4.5). There were also no significant difference between group when the data was expressed per gram body weight.

4.3.2.4 Blood inflammatory cell count.

Total blood cell number was significantly elevated in saline treated pups exposed to 95% oxygen for 3 days and following 2 days recovery, when compared to equivalently treated animals exposed to air (Table 4.6). Although treatment with CS had no effect on the number of circulating inflammatory cells, treatment with NA did. NA treatment

significantly blunted the increase in the total cell count at 3 and 5 days, compared to equivalently exposed animals treated with CS or saline. Neutrophil numbers were also significantly elevated in oxygen-exposed saline and CS-treated animals at both time points compared to SAL animals exposed to air. Following treatment with NA, there were significantly fewer neutrophils at 3 and 5 days compared to all other treatments. The number of circulating neutrophils were also significantly lower at day 5 than equivalently treated animals at 3 days. Neither exposure to hyperoxia nor treatment with CS, NA or SAL had any effect on the number of circulating lymphocytes at day 3. However, following 2 days recovery from oxygen, the lymphocyte population was higher in SAL-treated animals than equivalently aged animals exposed to air. The lymphocyte count was also significantly higher in SAL-treated animals at day 5 than at day 3. Although monocyte numbers were elevated in saline and CS-treated pups exposed to oxygen at day 3 when compared to air controls, this did not reach statistical significance.

Table 4.4. The effect exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on the body weights of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

O_2 (%) 0 - 72hr	O_2 (%) 72 - 120hr	Treatment	Body weight (% of birth weight)
21	-	SAL	90 ± 11
95	-	SAL	91 ± 9
95	-	CS	83 ± 4
95	-	NA	82 ± 7
21	21	SAL	105 ± 17
95	21	SAL	96 ± 13
95	21	CS	92 ± 7
95	21	NA	90 ± 11

Abbreviations. SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera. Data represents the body weight of between 6 and 10 animals per group following 72 hours exposure to 21% and 95% oxygen and 2 days recovery in 21% oxygen.

Table 4.5. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on lung wet weights of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

O_2 (%) 0 - 72hr	O_2 (%) 72 - 120hr	Treatment	Lung wet weight (g)
21	-	SAL	2.08 ± 0.53
95	-	SAL	2.31 ± 0.35
95	-	CS	2.05 ± 0.11
95	-	NA	2.21 ± 0.47
21	21	SAL	1.80 ± 0.53
95	21	SAL	2.35 ± 0.51
95	21	CS	2.60 ± 0.87
95	21	NA	2.30 ± 0.30

Abbreviations. SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera. Data represents the mean and standard deviation of between 6-10 animals per group.

4.3.2.5 BALF inflammatory cell counts.

The BALF from oxygen-exposed SAL and CS-treated animals at 3 and 5 days contained significantly more leukocytes than equivalently treated air controls (Table 4.7). This trend was also observed following treatment with NA but was only significant on day 5. The total cell count from all groups at day 5 was greater than the corresponding group at day 3. No difference was observed between equivalently aged oxygen-exposed animals at either time point. Although no difference in the neutrophil count was seen between oxygen-exposed SAL and CS groups at either time point, both had significantly more cells than their appropriate air controls. Treatment with NA significantly blunted the influx of neutrophils into the lung when compared to oxygen-exposed SAL-treated animals at 3 and 5 days and CS-treated animals at day 5. This resulted in oxygen-exposed, NA-treated pups and SAL-treated air control animals having similar numbers of neutrophils recovered within BALF. There was no difference in the number of recovered macrophages between any group at day 3. However, following 2 days recovery from oxygen exposure, all treatment groups had significantly more macrophages than equivalent animals at day 3 and also those animals treated with SAL, CS and NA-treated animals had significantly more macrophages than

saline treated pups exposed to air. No significant difference in the eosinophil cell count was observed between any group at day 3. All oxygen-exposed animals at day 5 had significantly more eosinophils than air controls.

4.3.2.6 Lung tissue antioxidant enzyme activities.

No significant difference in pulmonary Cu/Zn SOD activity at either 3 or 5 days was observed between saline-treated oxygen and air-exposed animals (Table 4.8). Although there was no difference in Cu/Zn SOD activity between CS and NA-treated animals at 3 and 5 days, both treatment groups on both days had significantly lower antioxidant enzyme activity than equivalently aged saline-treated, oxygen-exposed animals. SAL-treated animals exposed to 3 days 95% oxygen and left to recover for 2 days had a significantly higher Cu/Zn SOD activity than equivalent animals at day 3. Pulmonary Mn SOD activity at day 5 in SAL and CS-treated animals was significantly higher than age matched SAL-treated pups exposed to air. At days 3 and 5, Mn SOD activity from animals treated with NA was significantly higher than equivalently exposed SAL-treated animals. Mn SOD activity at day 5 was also significantly higher than equivalent CS-treated animals. No difference in pulmonary catalase activity was observed between any groups at day 3. By day 5, SAL-treated, oxygen-exposed animals had significantly higher catalase activity than similarly treated animals exposed to air. Pups treated with either CS or NA had significantly less catalase activity than SAL-treated animals exposed to 21% or 95% oxygen and also to equivalently treated animals at day 3. Lung tissue GSH-Px activity from oxygen-exposed animals was significantly higher than equivalently treated air controls at both 3 and 5 days. Although there was no significant difference in GSH-Px activity between CS and NA-treated animals at either time point, they were both lower than equivalently exposed SAL-treated animals. This was significant for CS animals at both 3 and 5 days.

4.3.2.7 BALF protein.

All groups at day 3 and day 5 that were exposed to 95% oxygen had significantly more protein in their BALF than age matched saline treated animals exposed to 21% oxygen (Fig 4.11). The BALF protein concentration of all oxygen-exposed animals at day 5 was significantly lower than equivalently treated animals at day 3. No significant difference between any of the oxygen-exposed animals at either 3 or 5 days was observed.

Table 4.6. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on the total and differential blood cell count of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

			Inflammatory cell count (10^4 cells/ml blood)			
Days	% O_2	Treatment	Total	Neutrophil	Monocyte	Lymphocyte
3	21	SAL	140 ± 50	50 ± 30	10 ± 10	90 ± 50
	95	SAL	200 ± 40*	120 ± 30*	20 ± 10	70 ± 30
	95	CS	210 ± 130	110 ± 80	20 ± 10	80 ± 50
	95	NA	100 ± 30$&	10 ± 10$*&	10 ± 10	70 ± 30
5	21	SAL	110 ± 40	20 ± 10	10 ± 10	80 ± 30
	95	SAL	250 ± 90*	120 ± 70*	10 ± 10	120 ± 30*+
	95	CS	200 ± 70*	60 ± 30	10 ± 20	110 ± 30
	95	NA	110 ± 30$&	1 ± 1*+&$	10 ± 10	80 ± 10

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of treatment on blood neutrophils ($F=10.9$, $p<0.001$), age on lymphocyte numbers ($F=6.1$, $p<0.05$) and oxygen on monocyte numbers ($F=4.3$, $p<0.05$). $ $p<0.05$ compared to aged matched CS. * $p<0.05$ compared to aged matched SAL animals exposed to 21% oxygen. + $p<0.05$ compared to equivalently treated animals at 3 days. & $p<0.05$ compared to aged matched SAL animals exposed to 95% oxygen. *Abbreviations:* SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera.

Table 4.7. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on the total and differential BALF cell count of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

			Inflammatory cell count (10^4 cells/ml BALF)			
Days	% O_2	Treatment	Total	neutrophil	Eosinophil	Macrophage
3	21	SAL	49 ± 19	1 ± 1	32 ± 17	14 ± 10
	95	SAL	72 ± 37*	10 ± 11*	43 ± 22	22 ± 14
	95	CS	79 ± 42*	4 ± 2*	55 ± 40	15 ± 4
	95	NA	63 ± 23	1 ± 1$	44 ± 24	14 ± 3
5	21	SAL	66 ± 17+	1 ± 1	32 ± 10	33 ± 8+
	95	SAL	152 ± 69*+	21 ± 19*+	45 ± 19*	63 ± 23*+
	95	CS	139 ± 36*+	11 ± 9*	68 ± 36*	58 ± 36*+
	95	NA	105 ± 47*+	2 ± 3$	59 ± 33*	45 ± 19+

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of treatment on BALF eosinophil numbers ($F=6.3$, $p<0.05$), age on total ($F=23.3$, $p<0.001$) and macrophage ($F=82.1$, $p<0.001$) numbers and oxygen on total ($F=16.3$, $p<0.001$), macrophage ($F=8.6$, $p<0.005$), eosinophil ($F=10.6$, $p<0.005$) and neutrophil ($F=3.9$, $p<0.05$) cell numbers. **$** $p<0.05$ compared to aged matched CS. * $p<0.05$ compared to aged matched SAL animals exposed to 21% oxygen. + $p<0.05$ compared to equivalently treated animals at 3 days. **&** $p<0.05$ comapred to SAL animals exposed to 95% oxygen. *Abbreviations:* SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera.

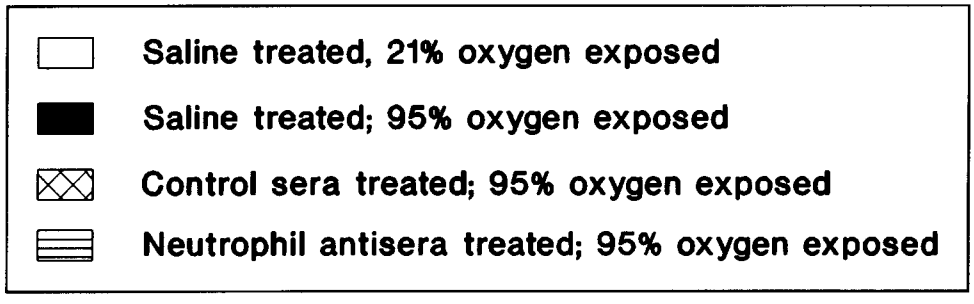
Table 4.8. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on lung tissue antioxidant enzyme activities of preterm guinea pigs treated with saline, control sera or neutrophil antisera.

			Antioxidant enzyme activity			
Days	% O_2	Treatment	Cu/Zn SOD (U/mg DNA)	Mn SOD (U/mg DNA)	CAT (U/mg DNA)	GSH-Px (U/mg DNA)
3	21	SAL	21.8±7.4	3.0±1.4	6.7±1.3	0.5±0.1
	95	SAL	20.4±4.9	3.5±0.8	7.2±1.0	1.0±0.4*
	95	CS	14.8±4.4&	5.0±3.2*	7.2±2.0	0.6±0.2&
	95	NA	12.1±5.1&	5.0±1.7*&	7.4±2.7	0.6±0.3
5	21	SAL	26.0±3.2	3.5±0.8	6.7±1.2	0.4±0.2
	95	SAL	28.8±7.6+	4.6±0.9*	8.9±2.4*+	0.7±0.4*+
	95	CS	12.7±2.2&	4.2±1.3*	4.5±0.6*+&	0.5±0.1&
	95	NA	14.5±2.9&	5.4±0.7*&$	4.7±0.8*+&	0.6±0.1

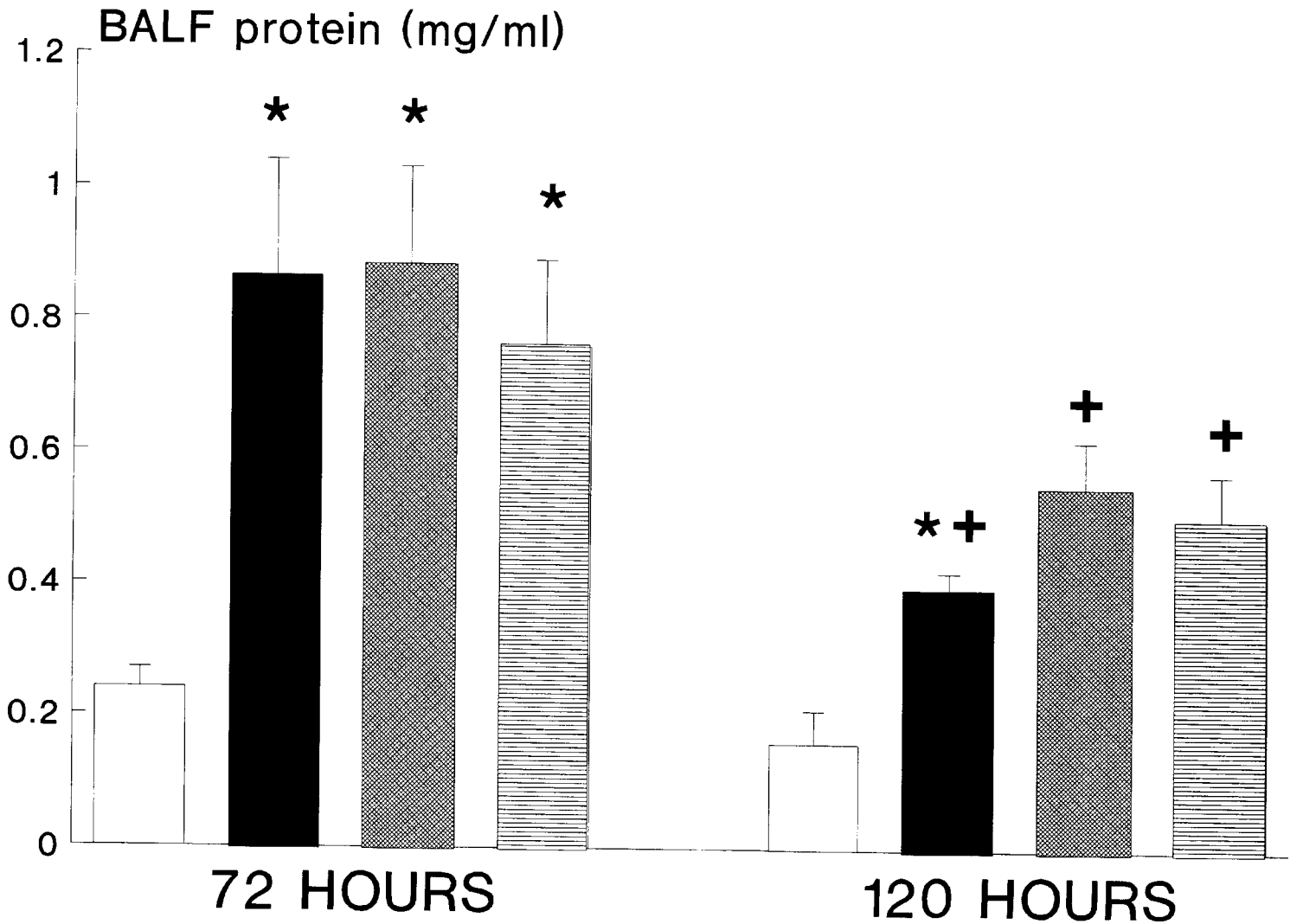
Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of treatment on Cu/Zn SOD ($F=10.6$, $p<0.005$), Mn SOD ($F=5.5$, $p<0.01$) and CAT ($F=4.4$, $p<0.05$). There was also a significant effect of age on GSH-Px ($F=5.8$, $p<0.05$) and oxygen exposure on Mn SOD ($F=6.9$, $p<0.05$). $ $p<0.05$ compared to aged matched CS. * $p<0.05$ compared to aged matched SAL animals exposed to 21% oxygen. + $p<0.05$ compared to equivalently treated animals at 3 days. & $p<0.05$ comapred to SAL animals exposed to 95% oxygen. *Abbreviations:* Cu/Zn SOD, Mn SOD:- Copper/Zinc superoxide dismutase, MN SOD:- Manganese superoxide dismutase, CAT:- Catalase, GSH-Px:- Glutathione peroxidase, DNA:- Deoxyribosenucleic acid, SAL:- Saline, CS:- Control sera, NA:- Neutrophil antisera, U:- Units.

Figure 4.11. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on BALF protein from preterm guinea pigs treated with saline, control sera or neutrophil antisera.

Guinea pigs were delivered at 65 days gestation and randomly allocated to receive either 95% or 21% oxygen for 72 hours. All pups were then returned to room air for a further 48 hours. Those animals exposed to 95% oxygen were further randomly allocated to receive either saline (SAL), neutrophil antisera (NA) or control sera (CS) at a dose of 200μl/100gm body weight immediately after birth and then once every 24 hours. Each dose was given by intra-peritoneal (ip) injection. Pups exposed to 21% oxygen received only IP saline. Animals were taken at 72h and 120h after delivery and blood, BALF and lung tissue removed.



There was a significant effect of treatment ($F=3.5$, $p<0.05$), age ($F=8.5$, $p<0.01$) and oxygen exposure ($F=17.6$, $p<0.001$) on BALF protein. * $p<0.05$ compared to aged matched saline-treated animals exposed to 21% oxygen. + $p<0.05$ compared to equivalently treated animals at 3 days. The data represents the mean and standard deviation of between 4 and 9 animals.


BALF protein (mg/ml)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*+
+
+
72 HOURS
120 HOURS

4.3.2.8 BALF neutrophil elastase activity.

Neutrophil elastase was not detected in BALF from air-exposed animals at either 3 or 5 days (Fig 4.12A). All groups at both time points that were exposed to oxygen had significantly higher neutrophil elastase activity than saline treated animals exposed to air. Although there was no significant difference in elastase activity between any of the oxygen-exposed treatment groups at 3 or 5 days, all groups at 5 days had significantly less elastase activity than equivalent animals at day 3.

4.3.2.9 BALF ß-glucuronidase activity.

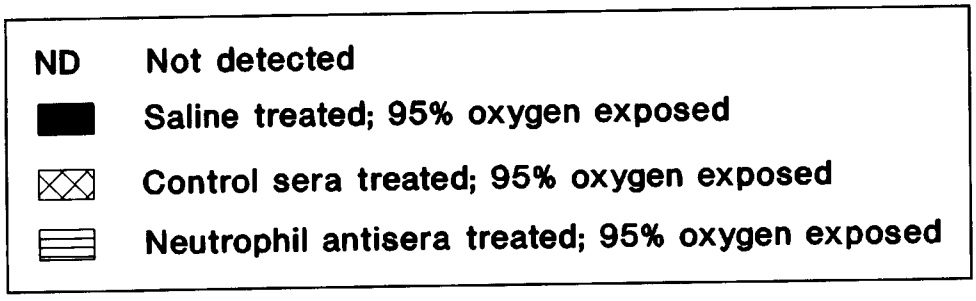
All treatment groups exposed to oxygen for 3 days and oxygen-exposed saline treated animals at 5 days had significantly more ß-glucuronidase activity than age matched air controls (Fig 4.12B). Animals exposed to oxygen and treated with either saline, CS or NA had significantly less BALF ß-glucuronidase activity than equivalently treated animals at day 3. No significant difference in activity was observed between CS and NA-treated animals at either time point.

4.4 Discussion.

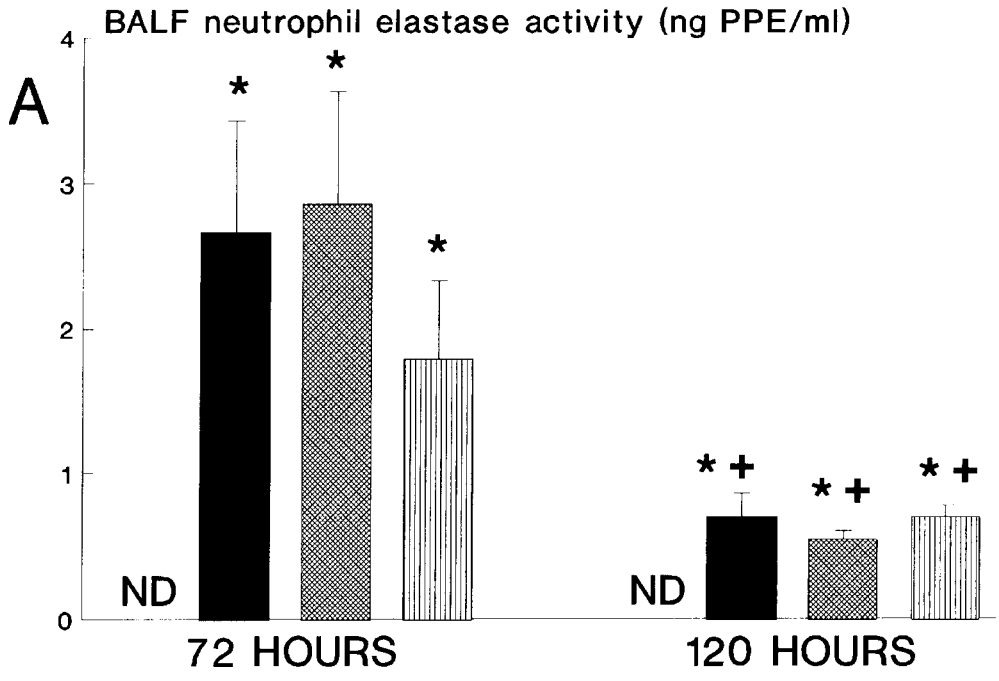
In adult animals, prolonged exposure of the lung to high concentrations of oxygen results in endothelial cell damage and oedema formation (Kistler *et al.*, 1967; Crapo *et al.*, 1980; Barry and Crapo, 1985; Frank, 1985). During this process, increased numbers of neutrophils accumulate within the interstitium and alveoli lumen, concomitant with an increase in chemotactic factors recoverable following BAL (Fox *et al.*, 1981; Merritt, 1982). These cells have the potential to cause extensive pulmonary endothelial, epithelial and connective tissue cell damage through the release of proteases (Hubbard *et al.*, 1991) and activated species of oxygen (Morel *et al.*, 1991). A correlation between the severity of lung injury and the number of neutrophils in BALF has been observed in oxygen-exposed rabbits (Shasby *et al.*, 1982) and in preterm guinea pigs (Kelly and Phillips, 1988) following 72 hours exposure to hyperoxia. As such a role for the neutrophil in the development of oxygen-induced acute lung injury has been proposed. However, several *in vitro* studies have shown that tissue injury may occur in the absence of inflammatory cells. Structural and metabolic abnormalities develop in lung cell cultures (Resnick *et al.*, 1974), and lung explants (Martin *et al.*, 1981) devoid of inflammatory cells after prolonged exposure to

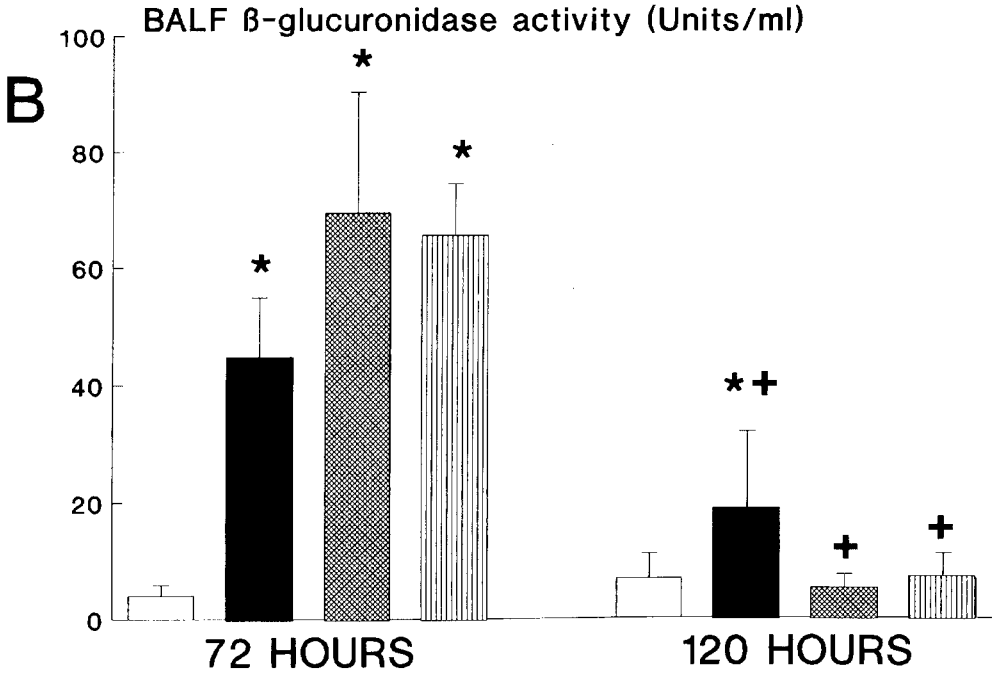
Figure 4.12. The effect of exposure to 21% and 95% oxygen for 3 days and following 2 days recovery in 21% oxygen on BALF neutrophil elastase (A) and ß-glucuronidase (B) activities from preterm guinea pigs treated with saline, control sera or neutrophil antisera.

Guinea pigs were delivered at 65 days gestation and randomly allocated to receive either 95% or 21% oxygen for 72 hours. All pups were then returned to room air for a further 48 hours. Those animals exposed to 95% oxygen were further randomly allocated to receive either saline (SAL), neutrophil antisera (NA) or control sera (CS) at a dose of 200μl/100gm body weight immediately after birth and then once every 24 hours. Each dose was given by intra-peritoneal (IP) injection. Pups exposed to 21% oxygen received only IP saline. Animals were taken at 72h and 120h after delivery and blood, BALF and lung tissue removed .



There was a significant effect of age and oxygen exposure on BALF neutrophil elastase activity (Age: $F=16.2$, $p<0.001$; O_2: $F=13.7$, $p<0.001$) and ß-glucuronidase activity (Age: $F=17.5$, $p<0.001$); O_2: $F=9.7$, $p<0.005$). * $p<0.05$ compared to aged matched saline-treated animals exposed to 21% oxygen. + $p<0.05$ compared to equivalently treated animals at 3 days. The data represents the mean and standard deviation of between 4 and 9 animals.


A
BALF neutrophil elastase activity (ng PPE/ml)
4
3
2
1
0
ND
ND
72 HOURS
120 HOURS


B
BALF ß-glucuronidase activity (Units/ml)
100
80
60
40
20
0
72 HOURS
120 HOURS

hyperoxia. Further to this, acute lung injury may develop in individuals who have lower than normal levels of circulating neutrophils and who are classified neutropenic (Laufe *et al.*, 1986). As such the role of this cell type in hyperoxic-induced acute lung injury remains unclear. In the preterm human neonate the role of the neutrophil in the development of HMD and BPD is even more obscure. In these patients there is the additive complication of lower concentrations of tissue antioxidants (McElroy *et al.*, 1990; Dobashi *et al.*, 1993), lower circulating levels of protease inhibitors (Rosenfeld *et al.*, 1986; Kotas *et al.*, 1972; El-Bardeesy and Johnson, 1972) and the unknown functional capacity of neutrophils from an immature immune system. If inflammatory cell function from these neonates is similar to that of the adult or term neonate, then as a consequence of the immaturity of the protective mechanisms, the lung will be more susceptible to injury. As such the role of the neutrophil in injury to the immature lung associated with oxygen exposure is unclear and was investigated using the guinea pig model of prematurity. This was attempted by depleting these cells from the circulation using a specific neutrophil antibody raised against guinea pig neutrophils and assessing the extent of lung injury following exposure to oxygen.

The data presented showed that exposure to 95% oxygen for 3 days resulted in lung injury which was typified by an increase in BALF protein and the initiation of an inflammatory response. Inflammatory cells within the lungs of these animals were activated (increased elastase and ß-glucuronidase activities) and possibly contributing to the development of lung injury. During the recovery phase, all indices of lung injury were reduced, even in the presence of increased numbers of neutrophils and macrophages. Markers of cellular activation were much lower at this time point than at day 3 and suggests that a these cells although increased in number were not activated, not releasing proteases and possibly not causing injury. Following treatment with NA there was a significant reduction in circulating and alveolar neutrophils during exposure to and recovery from oxygen. However, this was not associated with a reduction in lung injury or an increase in survival.

In previous studies, the role of the neutrophil in the development of tissue injury and oedema formation during hyperoxia has been explored using several neutrophil depletion techniques. However, these studies have used many chemical agents to deplete circulating neutrophils including, nitrogen mustard (Shasby *et al.*, 1982; Raj *et al.*, 1985; Laughlin *et al.*, 1986; Kubo *et al.*, 1992), hydroxyurea (Kubo *et al.*, 1992) and cyclophosphamide (Lurie

et al., 1988). Although these agents deplete circulating neutrophils they have other non-specific side effects. Nitrogen mustard has been shown not to protect rabbits and sheep from hyperoxic injury. This compound exerts its cytotoxic effect by alkylating DNA which results in the interference of mitosis in proliferating tissue (Calabresi and Parks, 1980). As oxygen toxicity proceeds, in part, by damage to DNA (Clement *et al.*, 1985; Birnboin and Kanabus, 1985), the cytotoxic action of this compound may adversely effect the outcome of oxygen exposure studies. Hydroxyurea is a compound that exerts its effect by inhibiting the enzyme ribonucleoside diphosphate reductase. As with nitrogen mustard, hydroxyurea may also have an adverse effect on the response of the lung to oxygen exposure via its effects on DNA. However, hydroxyurea can also act directly as an antioxidant (Taylor *et al.*, 1983) and any reduction in lung injury may occur as a result of an increase in the antioxidant status of the lung. This compound may also cause severe lymphopenia (Kubo *et al.*, 1992) and as the role of this cell type in oxygen toxicity is unknown, the usefulness of this compound in hyperoxic exposure studies is questionable. Similarly, cyclophosphamide is toxic to the lung (Lurie *et al.*, 1988). It has been reported that cyclophosphamide and oxygen act synergistically to cause tissue injury (Morse *et al.*, 1985) and high concentrations of this compound may induce pulmonary changes in the absence of oxygen (Patel and Block, 1985). As such, a specific neutrophil antisera was used in the present investigation to try to overcome some of the problems associated with the use of these chemical agents. The administration of neutrophil antisera resulted in the blunting of the inflammatory cell influx associated with hyperoxia. There was an reduction in the number of circulating neutrophils to 20% of those present in air controls and 95% lower than CS-treated animals. This resulted in a decrease in the number of BALF neutrophils to that found in saline-treated air controls. Continuation of treatment during the recovery phase further reduced the circulating pool of neutrophils. Two days after the exposure period, blood neutrophil numbers were less than 1% of that of saline-treated animals and 10% of that number found in the blood after 3 days. However, even with this further reduction in circulating neutrophils, the alveolar neutrophil population remained unchanged at the level observed in air controls. At both time points, no reduction in lung injury or increase in survival was observed.

There are a number of possible reasons for these observations. Firstly, the neutrophil may not be a major contributor to in lung injury during a period of acute hyperoxic

exposure. Alternatively, the number of neutrophils that are normally present within the lungs of air-exposed animals are capable of causing the observed injury, but only when activated as they appear to be in oxygen-exposed animals. Similar BALF neutrophil elastase activity at 3 and 5 days in all oxygen-treated animals, despite the differences in the number of recoverable neutrophils, would support such an hypothesis. However, published data on neutrophil elastase activity in the BALF of animals and humans with acute lung injury suggests that this may also not be the case (Wewers *et al.*, 1988; Collins *et al.*, 1990). Injury to the lung results in an alteration in the alveoli-capillary permeability and the movement of plasma proteins into the lumen. Elastase released into this section of lung will partition between protease inhibitors including, α2MG, a large general purpose serine protease inhibitor (Sottrup-Jensen, 1989) not normally found in healthy lungs and other elastase inhibitors such as α-1-PI, the most abundant elastase inhibitor in healthy and injured lungs (Hubbard and Crystal, 1991). Alpha-1-PI completely inhibits the activity of elastase by binding tightly to the active site of the enzyme, whereas α2MG inhibits elastase by steric hinderance. Elastase cleaves a specific amino acid sequence within the active site of α2MG which then undergoes a conformational change. This 'locks' the enzyme within the interior of the protein. Although the enzyme is now unable to cleave large proteins such as elastin and collagen, small molecular weight peptides and other small substrates can enter the inhibitor-elastase complex and be cleaved. Results obtained in Chapter 3 showed that neutrophil elastase was not detected in any animal using the tritiated elastin substrate regardless of the concentration of oxygen they were exposed to, but was detectable with the aid of small synthetic substrates. In the present study, although there was a reduction in neutrophil numbers following treatment with NA, there was no change in the elastase activity detected using the synthetic substrate when compared to undepleted animals. It is hypothesised that regardless of the number of neutrophils present, elastase released will saturate the pool of α2MG present in the alveolar lumen. If this pool is similar in depleted and undepleted animals then the detectable elastase activity using the synthetic substrate is going to be the same. The rest of the elastase will be taken up by α1PI which cannot be measured using substrates for the enzyme. To investigate the total amount of elastase released one would have to measure this pool of elastase as well as the elastase associated with α2MG.

Other ways in which the role of the neutrophil has been investigated in hyperoxic

induced-lung injury has been through inhibition studies. Taniguchi *et al* 1986 demonstrated the presence of increased amounts of the chemoattractant LTB_4 in the BALF of oxygen-exposed rats. Following administration of the lipoxygenase inhibitor, AA861, BALF LTB_4 concentration fell concomitant with a reduction in the number of alveolar neutrophils and an increase in the survival rate. Also treatment of oxygen-exposed animals with dexamethasone blunts the associated inflammatory cell influx (Town *et al.*, 1993). Dexamethasone is a synthetic glucocorticoid which inhibits the activity of PLA_2 and the generation of arachidonic acid metabolites such as LTB_4. This has been confirmed in a recent study in preterm human neonates at risk of developing BPD. A reduction in the chemotactic activity of BALF to neutrophils was observed concomitant with a reduction in LTB_4 concentrations (Groneck *et al.*, 1993). However, survival data from this study was not presented. Inactivation of PLA_2 will result in the reduction in a whole host of leukotrienes and prostaglandins. As many of these lipids have been found in the lungs of infants with HMD and BPD (Stenmark *et al.*, 1987; Stenmark and Voelkel, 1991) and as they also have a variety of potentially deleterious effects on tissue, it is unclear whether the reduction in neutrophil numbers or the reduction in the number of pro-inflammatory lipids brings about the changes seen in the human preterm neonate and in animal models of oxygen toxicity.

To combat the deleterious effects of high concentrations of inspired oxygen, the lung has several antioxidant defence mechanisms (Fridovich, 1986; Heffner and Repine, 1989; Heffner and Repine, 1991). Antioxidant enzyme activity is known to increase following exposure to oxygen in all animal studies performed to date. The importance of this defence system has been shown following treatment that increases the antioxidant activity of the lung prior to oxygen exposure (Frank *et al.*, 1980; Frank, 1982; Frank *et al.*, 1989; White *et al.*, 1987; Frank, 1991). These methods are capable of prolonging the survival of these animals in oxygen. During inflammation, immune cells and host tissue can secrete a variety of cytokines, extracellular signalling proteins that have a role in homeostatic processes within the lung (Kelley, 1990). Two of these cytokines, interleukin-1 (IL1-ß and IL-1α) and tumor necrosis factor (TNFα) have various biological effects including the induction of antioxidant enzymes (White *et al.*, 1989). As the neutrophil is a known secretor of cytokines, the second aspect of the present study was to assess whether neutrophil depletion blunted the rise in antioxidant enzyme activity associated with hyperoxia. However, the effect of neutrophil depletion on antioxidant enzyme induction was complicated by the

response of the lung to control sera. Both control sera and neutrophil antisera decreased Cu/Zn SOD and catalase activity compared to untreated animals exposed to oxygen at day 5 and Cu/Zn SOD at day 3. Mn SOD activity was also altered by both treatments, however, at day 5 there was significantly greater Mn SOD activity in the lungs of antisera-treated animals than those treated with control sera. Further studies are therefore required to assess the factors present in rabbit serum that alters the antioxidant activity of guinea pig lung in response to hyperoxia. If the neutrophil participates in the induction of lung antioxidants through the release of IL1 and TNFα, one would expect a decrease in Mn SOD activity and not an increase, as the alveolar and hence presumably the interstitial neutrophil population was significantly reduced at both 3 and 5 days. Apart from these two cytokines, the neutrophil also releases a variety of other cytokines (Kelley, 1990), all possibly having a direct effect on the lung. Consequently, depleting the neutrophil may set in motion a series of changes that may mask the specific effects of TNFα and IL1 on the pulmonary antioxidant enzymes.

4.5 Summary and conclusion.

The aim of this Chapter was to address the role of the neutrophil during and following recovery from, acute oxidative-induced injury to the immature lung. The first section illustrated the difficulty of obtaining a pure population of alveolar neutrophils to assess the role of neutrophil derived superoxide in the development of oxygen-induced lung injury. As such, a comparison of the superoxide production from peritoneal neutrophils of term and preterm animals was assessed. No significant difference in superoxide release was observed, although differences in the density profile of the cells were different to those recovered from the lung and blood. As an increase in cell density occurs during activation, it was suggested that data obtained from these neutrophils may not reflect the role of the neutrophil in the lung. It was also discussed that age, location and state of activation all have a significant bearing on the way in which the cell will respond and as such, it may be difficult to extrapolate these results back to the lung.

The second part of the Chapter attempted to address the more fundamental question as to the extent to which neutrophils, by what ever mechanism, participates in lung injury and antioxidant enzyme activation. Although, great lengths were went to in order to specificaly deplete the circulating and alveolar neutrophils without unduely altering the lung in other

ways, the results illustrated that although a decrease in alveolar and circulating neutrophils was achieved, no reduction in tissue injury or survival was observed. Further, it was also shown that rabbit serum was a complicating factor in the assessment of the relationship between the neutrophil and lung tissue antioxidant enzyme induction.

In conclusion the work performed in these studies suggest that the neutrophil may not play an important role in oxygen-induced acute lung injury. In the meantime it has highlighted many unanswered questions that need to be examined before a conclusive assessment of the role of the neutrophil in injury to the immature lung is to be arrived at.

CHAPTER 5

THE ROLE OF LIPID MEDIATORS OF INFLAMMATION IN THE DEVELOPMENT OF OXYGEN-INDUCED LUNG INJURY IN THE PRETERM GUINEA PIG

5.1 Introduction.

The pathologic abnormalities associated with the development of HMD and BPD include pulmonary oedema, inflammation and hypertension (Stenmark and Voelkel, 1991). These features correlate with the known actions of the pro-inflammatory lipids, platelet activating factor (PAF) and leukotriene B_4 (LTB_4) (Heffner *et al.*, 1983; Mojarad *et al.*, 1983; Hamasaki *et al.*, 1984). These lipids have been detected in the BALF of preterm human and animal neonates who develop HMD and BPD (Stenmark *et al.*, 1987; Meredith *et al.*, 1989; Tabor *et al.*, 1992; Groneck *et al.*, 1993; Koyama *et al.*, 1993). They have also been detected in the BALF of various animal models of oxygen toxicity (Taniguchi *et al.*, 1986; Meredith *et al.*, 1989; Tabor *et al.*, 1992) and as such a role for these lipids in the development of oxygen-induced injury to the immature lung and the development of BPD has been speculated.

To assess whether PAF and LTB_4 are associated with the pathophysiological abnormalities observed following oxygen exposure, various inhibition studies have been undertaken. In the oxygen-exposed rat, treatment with the lipooxygenase inhibitor AA861 blunted the accumulation of BALF LTB_4 and neutrophils, which resulted in a significant increase in the survival rate for these animals (Taniguchi *et al.*, 1986). In the preterm rabbit, lung oedema associated with premature delivery was reduced following treatment with various PAF antagonists (Tabor *et al.*, 1992). As such, the associated development of lung injury following premature delivery in the human neonate and the subsequent development of BPD was investigated using the preterm guinea pig and specific receptor antagonists for PAF and LTB_4.

5.2 Materials and methods.

Guinea pig pups were delivered by Caesarian section 3 days before term and randomly assigned to receive either 21% or 95% oxygen for 3 days (Fig 5.1). A random subgroup of pups from each exposure group were allocated treatment with either WEB2086 (5mg/Kg body weight/i.p 12hr) the PAF receptor antagonist or an equivalent volume of saline, U75302 (15mg/Kg body weight/ i.p 12hr), the LTB_4 receptor antagonist or an equivalent volume of vehicle (5% ethanol; 1% tween 80). These doses have been previously shown to be effective in blunting the inflammatory response associated with allergen challenge in adult guinea pigs and therefore employed in the preterm guinea pig (Richards *et al.*, 1989;

Heuer 1988). After 3 days, all animals were sacrificed by pentobarbitone overdose, the trachea isolated and a cannulae inserted. A BAL was then conducted using 10ml (5x2ml aliquots) of non-pyrogenic saline (37°C), the recovered fluid being placed immediately on ice. Following BAL, the thoracic cavity was opened and a blood sample taken by direct cardiac puncture. The lungs were then flushed of blood with 10ml of saline (37°C), removed, blotted dry, weighed and frozen in liquid nitrogen. Total and differential blood and BALF cell counts, BALF protein, PLA_2 and neutrophil elastase activity and lung tissue antioxidant enzyme activities were measured as described in Sections 2.9 and 2.10.

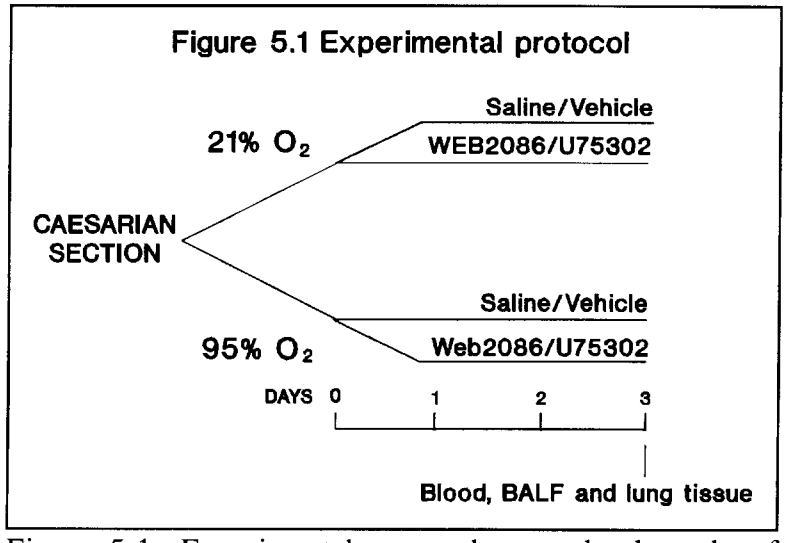


Figure 5.1. Experimental protocol to study the role of LTB_4 and PAF in the development of oxygen-induced injury to the immature lung of the preterm guinea pig.

5.3 Platelet activating factor.

5.3.1 Results.

5.3.1.1 Survival.

All animals exposed to 21% oxygen for 3 days (Fig 5.2) survived. Exposure to 95% oxygen resulted in a significant reduction in survival, regardless of treatment when compared to air controls. No significant difference in survival was observed between saline and WEB2086-treated animals exposed to 95% oxygen.

5.3.1.2 Body weights.

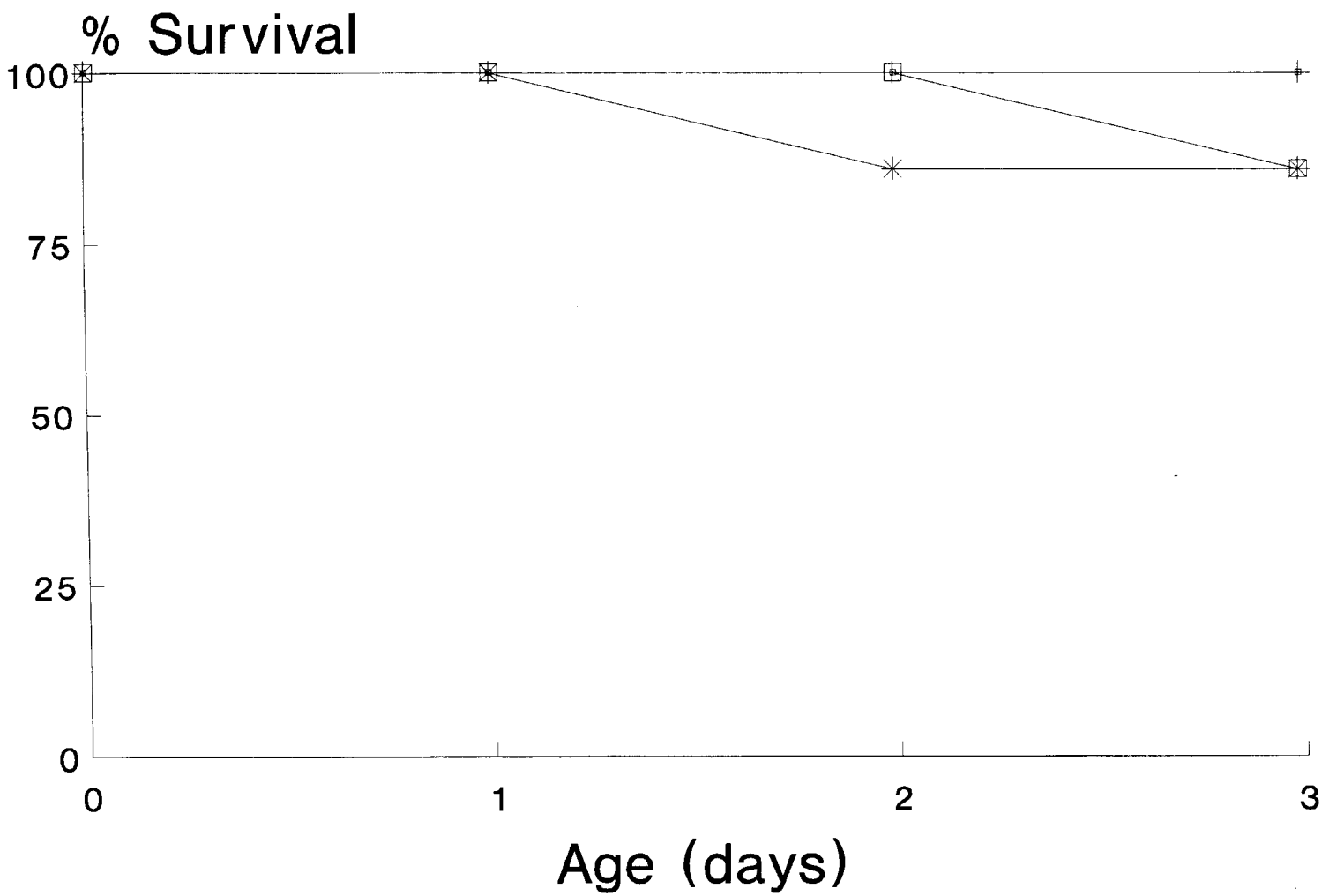
No effect of treatment or oxygen exposure was observed over the 3 days of the study (Table 5.1).

Figure 5.2. The effect of exposure to 21% and 95% oxygen for 3 days on the survival rate of preterm guinea pigs treated with either saline or WEB2086.

Guinea pigs were delivered by Caesarian section at 65 days of gestation and randomly assigned to receive either 21% or 95% oxygen. Pups were further randomly allocated treatment with either WEB2086 (5mg/Kg body weight/i.p 12hr) or an equivalent volume of saline for 3 days. The percentage survival was assessed every 24 hours.

---.--- 21% oxygen: Saline
---+--- 21% oxygen: WEB2086
---*--- 95% oxygen: Saline
---[]--- 95% oxygen: WEB2086

Data represents the survival of between 7 and 9 animals per group. There was a significant ($p < 0.05$) reduction in survival in oxygen-exposed animals, regardless of treatment, when compared to air controls.


% Survival
100
75
50
25
0
0
1
2
3
Age (days)

5.3.1.3 Lung and liver wet weights.

No significant difference in lung wet weight was observed between saline and WEB2086-treated animals exposed to air. Oxygen-exposed animals treated with saline or WEB2086 were consistently heavier than equivalently treated air controls (Fig 5.3A). This was significant in saline-treated animals only, however when the data was expressed per gram body weight this significance was lost and WEB2086-treated animals became significantly heavier than air controls (Fig 5.3B). No effect of drug treatment or oxygen exposure was observed on liver wet weight (Fig 5.3C and D).

Table 5.1. The effect of exposure to 21% and 95% oxygen for 3 days on the body weights of preterm guinea pigs treated with either saline or WEB2086.

% O_2	Treatment	Body weight (g)			
		0hr	24hr	48hr	72hr
21	SALINE	81 ± 15	71 ± 12	72 ± 13	73 ± 12
	WEB2086	78 ± 11	74 ± 10	77 ± 11	80 ± 12
95	SALINE	83 ± 14	78 ± 13	78 ± 13	79 ± 13
	WEB2086	83 ± 5	76 ± 5	73 ± 6	73 ± 8

Data represents the mean and standard deviation of between 6 and 9 animals per group.

5.3.1.4 Lung tissue DNA.

No effect of either oxygen exposure or treatment with WEB2086 on the total or concentration of lung DNA was observed (Table 5.2).

5.3.1.5 Blood inflammatory cell count.

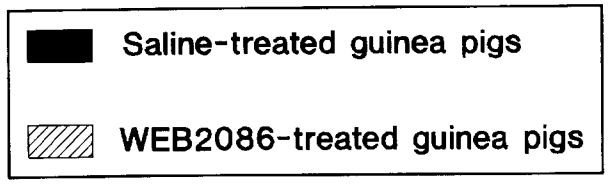
Although circulating total and neutrophil cell numbers tended to be higher in oxygen-exposed animals, no effect of drug treatment or oxygen exposure was observed (Table 5.3).

5.3.1.6 BALF inflammatory cell count.

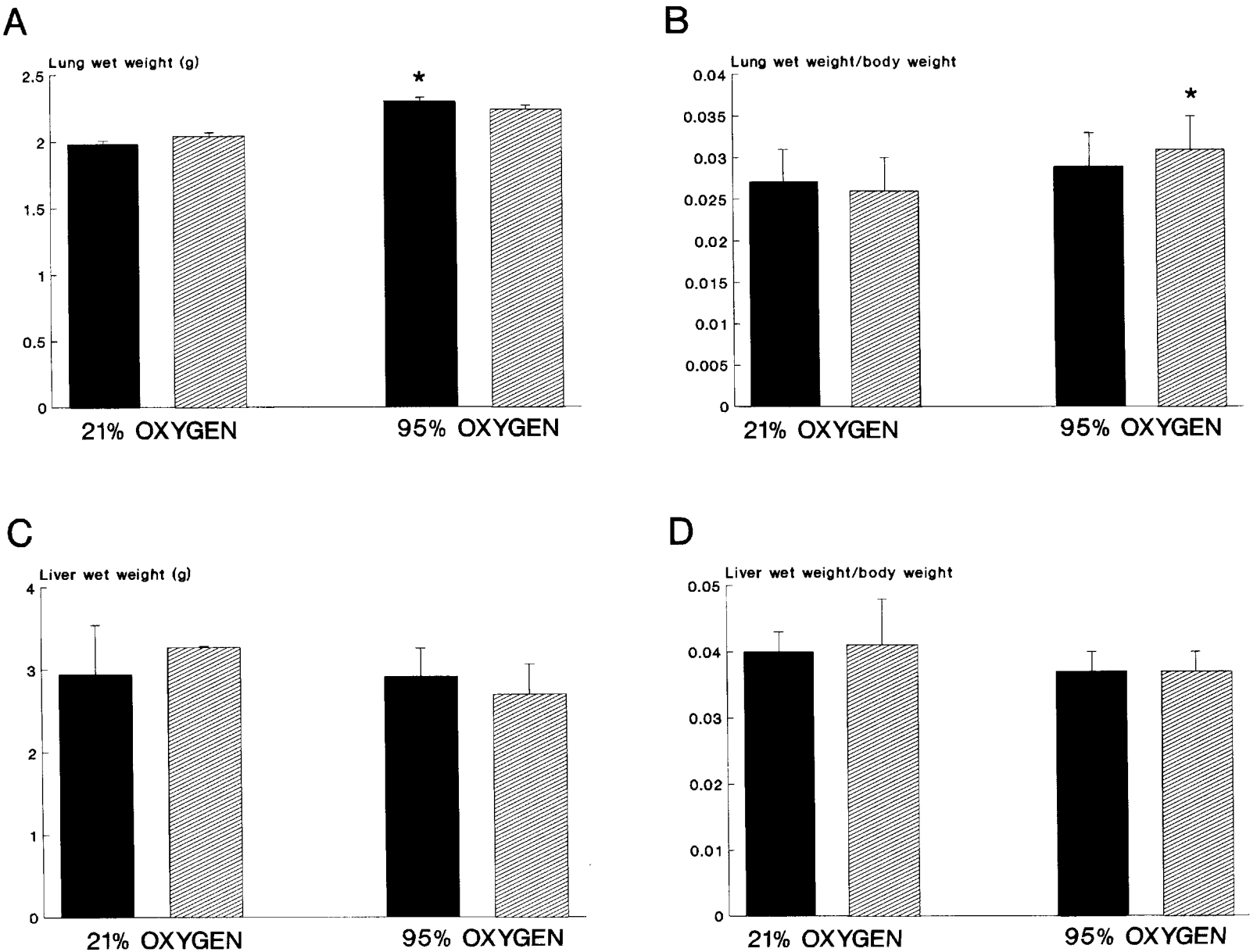
No significant difference in the total or individual cell count was observed between saline and WEB2086-treated animals exposed to air. The number of neutrophils recovered in the BALF of oxygen-exposed saline-treated pups was significantly greater than that recovered

Figure 5.3. The effect of exposure to 21% and 95% oxygen for 3 days on the lung and liver wet weights of preterm guinea pigs treated with either saline or WEB2086.

Guinea pigs were delivered by Caesarian section 3 days before term and randomly assigned to receive either 21% or 95% oxygen for 3 days. They were then further randomly assigned treatment either with WEB2086 (5mg/Kg body weight/i.p 12hr) or an equivalent volume of saline. After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed. The thoracic cavity was then opened, 1ml of blood withdrawn by cardiac puncture and the pulmonary circulation flushed of blood with 10ml of sterile saline (37°C). The lungs and livers were removed, blotted dry, weighed and frozen in liquid nitrogen.



There was a significant effect of oxygen exposure on total lung wet weight ($F=5.53$, $p<0.05$), lung wet weight/body weight ratio ($F=6.33$, $p<0.05$) and liver/body weight ratio ($F=4.45$, $p<0.05$). * $p<0.05$ compared to equivalently treated animals exposed to 21% oxygen.


A
Lung wet weight (g)
2.5
2
1.5
1
0.5
0
*
21% OXYGEN
95% OXYGEN
B
Lung wet weight/body weight
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
21% OXYGEN
95% OXYGEN
C
Liver wet weight (g)
4
3
2
1
0
21% OXYGEN
95% OXYGEN
D
Liver wet weight/body weight
0.05
0.04
0.03
0.02
0.01
0
21% OXYGEN
95% OXYGEN

from equivalently treated air controls (Table 5.4). Treatment with WEB2086 effectively blunted this rise in neutrophil numbers, which resulted in the recovery of equivalent numbers of neutrophils to that of saline-treated air control pups.

Table 5.2. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue DNA of preterm guinea pigs treated with either saline or WEB2086.

% O_2	Treatment	DNA	
		mg	mg/g
21	Saline	8.7 ± 3.3	4.3 ± 1.7
	WEB2086	9.1 ± 2.5	4.3 ± 0.6
95	Saline	7.4 ± 1.8	3.3 ± 0.9
	WEB2086	9.6 ± 1.7	4.2 ± 0.6

Data represents the mean and standard deviation of between 5 and 7 animals per group.
Abbreviations: DNA:- deoxyribosenucleicacid.

Table 5.3 The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential blood cell count of preterm guinea pigs treated with either saline or WEB2086.

% O_2	Treatment	Blood inflammatory cell count (10^4 cells/ml)					
		TOTAL	PMN	MONO	LYMP	BAS	EOS
21	Saline	163±99	77±65	4±3	81±35	1±1	0±0
	WEB2086	155±75	64±46	4±3	86±38	2±1	1±1
95	Saline	206±101	122±73	7±4	75±31	1±1	1±2
	WEB2086	204±128	93±61	6±4	103±62	2±1	1±2

Data represents the mean and standard deviation of between 7 and 9 animals per group.
Abbreviations: PMN: polymorphonuclear leucocyte, MONO: monocytes, LYMP: lymphocytes, BAS: Basophils, EOS: Eosinophils.

Although there was no rise in the macrophage population of saline-treated animals following exposure to hyperoxia, treatment with WEB2086 resulted in the recovery of significantly more macrophages than WEB2086-treated animals exposed to air. No effect

of drug treatment or oxygen exposure was observed on BALF eosinophil numbers.

5.3.1.7 BALF protein.

No significant difference was observed between saline and WEB2086-treated animals exposed to 21% oxygen. Following hyperoxic exposure, BALF protein concentration of saline-treated animals was significantly greater than saline-treated animals exposed to air (Table 5.5). Treatment with WEB2086 blunted this increase in BALF protein, such that no significant difference was observed between oxygen and air-exposed animals treated with WEB2086.

Table 5.4. The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential BALF cell count of preterm guinea pigs treated with either saline or WEB2086.

% O_2	Treatment	BALF inflammatory cell count (10^4 cell/ml)			
		TOTAL	PMN	MACS	EOS
21	Saline	82 ± 27	0.2 ± 0.5	20.9 ± 9.4	60.0 ± 20.7
	WEB2086	80 ± 43	0.2 ± 0.5	18.3 ± 10.3	61.0 ± 34.2
95	Saline	90 ± 41	2.0 ± 1.5*	20.5 ± 10.4	68.0 ± 32.3
	WEB2086	100 ± 26	0.5 ± 0.6	28.5 ± 12.8*	70.0 ± 16.0

Data represents the mean and standard deviation of between 7 and 9 animals per group. *Abbreviations*: PMN: polymorphonuclear leucocytes, MACS: macrophages, EOS: eosinophils. There was a significant effect of oxygen exposure ($F=10.7$, $p<0.005$), WEB2086 ($F=5.85$, $p<0.05$) and a significant interaction between the two ($F=5.58$, $p<0.05$) on BALF neutrophil numbers. * $p<0.05$; compared to 21% oxygen.

5.3.1.8 BALF neutrophil elastase activity.

No significant difference in elastase activity was detected between saline and WEB2086-treated animals exposed to air (Table 5.5). Exposure to hyperoxia resulted in a significant increase in elastase activity in saline-treated pups when compared to equivalent air controls. This increase in activity was blunted following treatment with WEB2086, although saline and WEB2086-treated animals were not significantly

different.

5.3.1.9 BALF phospholipase A_2 activity.

Animals exposed to 95% oxygen and treated with saline had significantly lower PLA_2 activity than animals exposed to air (Table 5.5). No significant difference in activity between treatments at either concentration of oxygen was observed. A significant negative correlation ($r = -0.6$, $p<0.005$) between PLA_2 activity and BALF protein was observed.

Table 5.5. The effect of exposure to 21% and 95% oxygen for 3 days on BALF neutrophil elastase and phospholipase A_2 activities and protein concentration of preterm guinea pigs treated with either saline or WEB2086.

% O_2	Treatment			
		Elastase ng/ml	Protein mg/ml	PLA_2 μm/min/ml
21	SALINE	N.D	0.39 ± 0.05	0.94 ± 0.15
	WEB2086	1.7 ± 2.9	0.47 ± 0.29	0.84 ± 0.14
95	SALINE	7.6 ± 6.9*	1.57 ± 1.07**	0.60 ± 0.38*
	WEB2086	2.9 ± 5.9	0.88 ± 0.41	0.84 ± 0.57

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of oxygen on protein ($F=12.31$, $p<0.005$) and elastase ($F=7.12$, $p<0.05$). * $p<0.05$, ** $p<0.005$; compared to 21% oxygen.

5.3.1.10 Pulmonary antioxidant enzyme activities.

Although antioxidant enzyme activities of oxygen-exposed, saline-treated animals tended to be higher than equivalent air controls (Table 5.6) this was significant only for Mn SOD. Treatment with WEB2086 blunted the rise in Mn SOD activity associated with hyperoxia. No significant difference in enzyme activities were observed between saline and WEB2086-treated animals at either oxygen concentration.

Table 5.6. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue antioxidant enzyme activities of preterm guinea pigs treated with either saline or WEB2086.

$\%O_2$	Treatment	Lung tissue antioxidant enzyme activities			
		CuZn SOD U/mg DNA	Mn SOD U/mg DNA	CAT KU/mg DNA	GSH-Px U/mg DNA
21	SALINE	52.2 ± 18.4	2.8 ± 0.8	11.0 ± 3.4	0.6 ± 0.2
	WEB2086	49.4 ± 9.4	2.4 ± 0.7	9.8 ± 3.4	0.5 ± 0.2
95	SALINE	65.0 ± 19.5	4.4 ± 1.9*	14.8 ± 4.0	0.8 ± 0.4
	WEB2086	45.29 ± 9.1	2.6 ± 0.4	11.4 ± 2.3	0.5 ± 0.2

Data represents the mean and standard deviation of between 6 and 9 animals per group. *Abbreviations*: Cu/Zn SOD: copper zinc superoxide dismutase, Mn SOD:- Manganese superoxide dismutase, CAT: catalase, GSH-Px:- Glutathione peroxidase.* $p<0.05$ compared to 21% oxygen.

5.3.2 Discussion.

One of the features of the immature lung, both clinically and experimentally is an abnormality in permeability (Gerdes *et al.*,1988; Yoder *et al.*,1991; Kelly *et al.*,1991; Tabor *et al.*,1992). This leads to the movement of plasma proteins into the alveolar lumen and the development of pulmonary oedema. Oedema is known to decrease with time after birth, increase with decreasing gestational age and is reduced following treatment with hormonal agents that induce lung maturation (Jobe *et al.*,1985; Tabor *et al.*,1990). The formation of oedema is thought to occur primarily as a consequence of mechanical damage following alveolar collapse due to surfactant insufficiency. However, as increased vascular permeability is also seen in other forms of acute lung injury (Rinaldo and Rogers, 1982), it has been speculated that they may share similar mechanisms, independent of mechanical damage. Platelet activating factor is a pro-inflammatory lipid that is released by a variety of different cell types and is one of the most important chemical mediators of inflammation (Evans *et al.*,1987). It has several biological actions which include polymorphonuclear leukocyte accumulation (Arnoux *et al.*,1988) and pulmonary oedema formation (Evans *et al.*,1987), both of which are observed in human premature neonates and in animals exposed to high concentrations of oxygen (Koyama *et al.*,1993; Meredith *et al.*,1989). As such, a

potential role for this lipid in the pathophysiology of chronic lung disease has been suggested. To examine the role of PAF in the development of acute inflammation and injury to the immature lung, a PAF receptor antagonist, WEB2086, was used in the preterm guinea pig following exposure to oxygen for up to 72 hours.

PAF is a recognised mediator of inflammation (Henson *et al.*, 1992) and in the lung it is synthesized either by a remodelling or *de novo* pathway. Released into the extracellular environment (Lee and Snyder, 1989) it may act upon a wide variety of different cell types (Lynch *et al.*, 1979; Zimmerman *et al.*, 1985) at concentrations as low as 1ng/Kg body weight. Intratracheal instillation of PAF into rabbits and baboons induces an inflammatory response which consists of desquamation of epithelial cells, polymorphonuclear leukocyte accumulation (Camussi *et al.*, 1983; Mojarad *et al.*, 1983; Evans *et al.*, 1987) and a dose-dependant increase in vascular permeability (Evans *et al.*, 1987). PAF can also initiate and prime inflammatory cells to release toxic oxygen species (Gay *et al.*, 1986; Collins *et al.*, 1990), proteases and metabolites of arachidonic acid, such as LTB_4 (Lin *et al.*, 1982; Voekel *et al.*, 1982; Tomashefski *et al.*, 1985). All these effects are blocked by the PAF receptor antagonists, BN 56203 (Evans *et al.*, 1987) and WEB2086 (Chung and Barnes, 1989). Accompanying these effects, there is also a fall in the circulating white blood cell count (predominately neutrophils). This is maximal after 5 min and returns to base line within 15 min (Chung and Barnes, 1989). The reason for this is unclear, although vasoconstriction of the pulmonary circulation and migration of cells into extravascular spaces maybe an explanation (Mojarad *et al.*, 1983).

In the present study PAF was not detected in the blood or BALF of air-exposed animals. Liver wet weights and survival following exposure to 21% oxygen, were unaffected by treatment with WEB2086 and suggests that the drug was well tolerated at the dose given. However, in contradiction to that observed in the premature rabbit (Tabor *et al.*, 1992), WEB2086 had no effect on the permeability of the air-exposed lung. In the preterm rabbit, PAF antagonists succesfully blunted the increase in oedema associated with premature delivery, suggesting that PAF may be involved in the initiation of lung injury, in this model, soon after delivery. PAF analysis was also undertaken 30 minutes after delivery in ventilated animals, a full 3 days before analysis was performed in the present study. However, as WEB2086 was administered every 12h, starting within an hour of delivery, if PAF was released into the lungs of the air-exposed premature guinea pig then the

antagonist could have blunted the increase in lung wet weight and BALF protein associated with these animals. Therefore the observed alteration in pulmonary permeability in these premature guinea pigs (chapter 3) may develop as a consequence of a mechanism that is independant of PAF release. Further to this, PAF is known to stimulate the release of TNF and IL-1. These cytokines can activate PLA_2 and thus if PAF is released within the lungs, treatment with WEB2086 would reduce cytokine production and consequently the activation of PLA_2. A decrease in the activity of BALF PLA_2 was not seen in the present study.

Following oxygen exposure, pulmonary inflammation, antioxidant enzyme induction and lung injury increased. These features have been observed previously within the model (Kelly *et al.*, 1991) and in premature human neonates who develop BPD (Merritt *et al.*, 1983; Ogden *et al.*, 1984; Dobashi *et al.*, 1993). Treatment with WEB2086 significantly blunted these responses, and although lung injury was reduced, survival was not. The reduction in inflammation and injury therefore suggests that PAF maybe released in the oxygen-exposed premature guinea pig. As in air-exposed animals, PAF was not detected in the BALF or blood following oxygen exposure, although it has been detected in the lungs of preterm human and baboon neonates who develop BPD (Meredith *et al.*, 1989; Koyama *et al.*, 1993). There are a number of possible reason for the absence of detectable quantities of PAF in both air and oxygen-treated animals, one of which is the instability of the lipid following release into the extracellular environment. Plasma contains an acetylhydrolase that inactivates PAF (Blank *et al.*, 1983), the activity of which varies with age. In the rabbit there is a five-fold increase in activity between days 21 and 30 of gestation and a further two-fold increase after birth (Maki *et al.*, 1988). Also intravenous administration of [^{3}H] PAF into adult rabbits has a half-life of approximately 30 seconds and as acetylhydrolase activity is much lower in adult than in newborn animals this further suggests that PAF may be rapidly inactivated. Alternatively, analysis of BALF after 3 days may not be the optimum time point in which to find PAF. Analysis at time points earlier than 72 hours are therefore required to fully assess the release of this lipid into the lungs of the preterm guinea pig.

Infusion of PAF into isolated rat lungs perfused with cell-free medium liberates LTC_4 and LTD_4 (Voelkel *et al.*, 1982) and when infused into the pulmonary artery of unanesthetized sheep results in the development of oedema. Similary, thromboxane A_2 and B_2 have been associated with PAF-induced acute lung injury (Hamasaki *et al.*, 1984). The blunting of the

inflammatory reaction observed in the preterm guinea pig treated with WEB2086 may thus be mediated via a reduction in the PAF inducible generation of leukotrienes (Voelkel *et al.*, 1982) and/or cyclooxygenase metabolites (Lefer *et al.*, 1984). The influx of neutrophils in the present study may occur indirectly as the consequence of PAF induced LTB_4 production and although LTB_4 was not assessed in the present study, it has been shown to be increased following oxygen exposure (chapter 3). Reduced neutrophil elastase activity in BALF, probably directly relates to the reduction in neutrophil numbers, although the direct inhibition of the effect of this drug on cellular activation may also occur as *in vitro* activation by PAF of neutrophils can lead to enzyme secretion and superoxide production (Wandall, 1988; Sha'afi and Molski, 1988; Thomas *et al.*, 1988; Weiss, 1989; Stone, 1990). A reduction in the release of these constituents may also have aided in the observed reduction in pulmonary oedema associated with exposure to oxygen.

PAF can also regulate the immune response to a given stimuli, either indirectly or by the production of cytokines which are potent modulators of immune function. Enhanced production of IL-1 and TNF from human monocytes and rat alveolar macrophages when cultured in the presence of graded concentrations of PAF (Poubelle *et al.*, 1991; Dubois *et al.*, 1989) have been reported. This may be part of an amplification loop since cytokines can also stimulate the synthesis of PAF (Valone and Epstein, 1988). This activation pathway may also involve the lipooxygenase enzyme since leukotrienes, generated by PAF, can also participate in immunoregulatory processes and modulate cytokine production (Rola-Pleszczynski, 1985). Leukotriene B_4 can enhance the production of IL-1 and TNF in rat alveolar macrophages and human monocytes (Rola-Pleszczynski and Lemaire, 1985; Rola-Pleszczynski *et al.*, 1986; Dubois *et al.*, 1989) and the generation of LTB_4 by PAF can be inhibited by the lipooxygenase inhibitors, NDGA and AA861 (Rola-Pleszczynski *et al.*, 1988; Dubois *et al.*, 1989). Both cytokines and eicosanoids have been identified in the BALF of infants with BPD (Groneck *et al.*, 1993; Murch *et al.*, 1992; Stenmark and Voelkel, 1991) and can be reduced following treatment with dexamethasone. As this steroid is known to inhibit the activity of PLA_2, the role of the eicosanoids in the modulation of immune function in these infants is further highlighted. TNF and IL-1 are known to increase the activity of the pulmonary antioxidant enzymes (Warner *et al.*, 1991; Masuda *et al.*, 1988) of which MnSOD is elevated in infants with BPD (Dobashi *et al.*, 1993). In the present study treatment with the antagonist blunted the increase in MnSOD activity

associated with oxygen exposure. Whether this was an effect related to an alteration in cytokine or eicosanoid release is not known as at 72 hours TNF levels were undetectable using either an immunassay or a bioassay (personnel communications). However, as LTB_4 is present within the BALF of these animals and that LTB_4 can induce cytokine production, TNF and IL-1 may be generated. As with PAF, the stability of the cytokines may preclude identification or the release of TNF may occur optimally at time points other than 72 hours. Thus to fully assess whether cytokines are involved in antioxidant induction in this model, measurements at earlier time points are required.

In summary, oxygen-induced lung injury, antioxidant enzyme induction and inflammation may develop partly through the induction and release of PAF. The timing of PAF release is not known and requires further study. PAF is known to induce the production and release of the cytokines and LTB_4. Leukotriene B_4 is an important mediator in many forms of acute and chronic lung inflammation and as such, the next section addresses the role of this lipid in the development of acute lung injury and inflammation in the oxygen-exposed preterm guinea pig.

5.4 Leukotriene B_4

The development of BPD in the human preterm neonate is characterised in part by the accumulation of neutrophils within the lung (Merritt *et al.*, 1983; Ogden *et al.*, 1984). These cells can release proteases and oxygen-centred free radicals that have been implicated in the development of lung injury (Gerdes *et al.*, 1988; Yoder *et al.*, 1991; Bruce *et al.*, 1985) and the presence of these cells in the lung has been attributed to the release of chemotactic factors. These factors include C5a and LTB_4, both of which have been identified in the lungs of infants who develop BPD (Stenmark *et al.*, 1987; Groneck *et al.*, 1993; Long *et al.*, 1984). In animals, the degree of oxygen toxicity has also been associated with the accumulation of inflammatory cells and correlated with an increase in the concentration of BALF LTB_4 (Taniguchi *et al.*, 1986). However, the role of LTB_4 in the development of injury to the immature lung has not been assessed. In the present study the role of LTB_4 in the development of hyperoxic-induced inflammation and injury to the immature lung of the preterm guinea pig was investigated using a novel structural analogue of LTB_4, U75302. This compound competitively inhibits the binding of $[^3H]LTB_4$ to its receptors on human neutrophils (Lin *et al.*, 1988) and is a selective antagonist of the myotropic activity of LTB_4

on isolated guinea pig lung parenchymal strips (Lawson *et al.*, 1989).

5.4.1 Materials and methods

The protocol followed is described in section 5.2.

5.4.2 Results.

5.4.2.1 Survival.

All animals treated with vehicle and exposed to 21% oxygen survived (Fig 5.4). However, there was a significant increase in mortality in those animals who were treated with U75302. Although the mortality rate of animals treated with U75302 significantly increased following exposure to 95% oxygen when compared to air controls, no difference in survival was observed between the two oxygen-exposed groups.

5.4.2.2 Body weights.

Animals treated with vehicle and exposed to 21% oxygen and animals treated with U75302 and exposed to 95% oxygen were significantly lighter than birth weight at days 1, 2 and 3. Vehicle-treated, oxygen-exposed pups also weighed less than their birth weight on day 3 of the study. Body weights were unaffected by oxygen exposure (Table 5.7).

5.4.2.3 Lung and liver wet weights.

The lung wet weight of vehicle-treated animals exposed to 95% oxygen was significantly greater than equivalently treated air controls (Fig 5.5A). When the data was expressed per gram body weight, this significance was lost and lungs of U75302-treated animals were significantly heavier than controls (Fig 5.5B). There was no effect of oxygen exposure on liver wet weight, however, animals treated with U75302 (total or per gram body weight), regardless of oxygen exposure, were significantly heavier than equivalently exposed vehicle-treated animals (Fig 5.5 C and D).

Figure 5.4. The effect of exposure to 21% and 95% oxygen for 3 days on the survival rate of preterm guinea pigs treated with either vehicle or U75302.

Guinea pigs were delivered by Caesarian section at 65 days of gestation and randomly exposed to 21% or 95% oxygen and treated with either U75302 (15mg/Kg body weight/ i.p. 12hr) or an equivalent volume of vehicle (5% ethanol, 1% tween 80). The percentage survival was assessed every 24 hours.

---.--- 21% oxygen: Vehicle
---+--- 21% oxygen: U75302
---*--- 95% oxygen: Vehicle
---[]-- 95% oxygen: U75302

Data represents the survival of between 7 and 9 animals per group. There was a significant ($p < 0.05$) increase in the mortality rate of air-exposed, U75302-treated animals when compared to equivalently exposed vehicle-treated pups. Exposure to hyperoxia resulted in a significant increase in mortality ($p < 0.05$) in vehicle-treated animals compared to air controls.

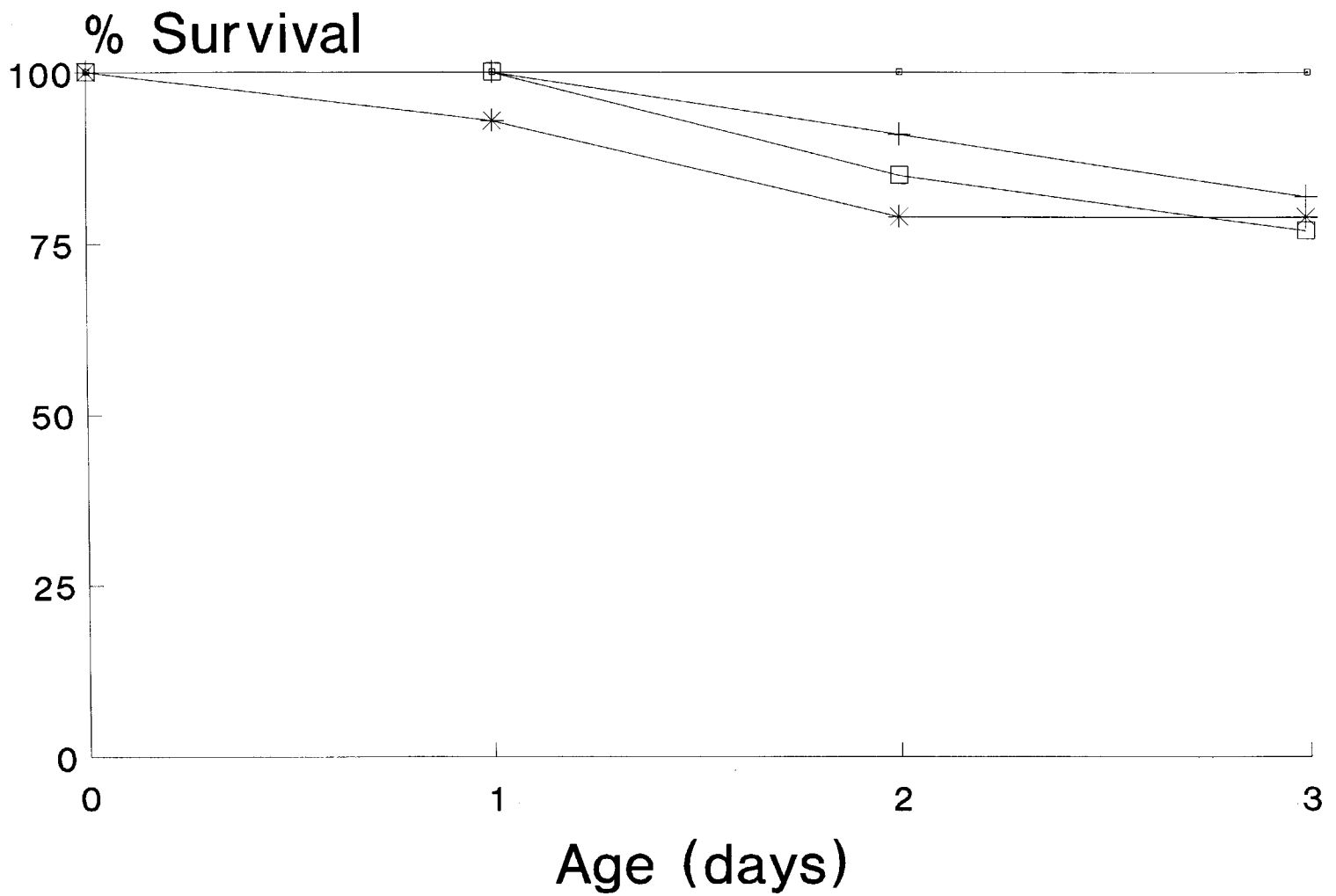
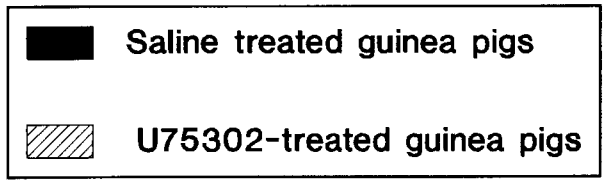

% Survival
100
75
50
25
0
0
1
2
3
Age (days)

Figure 5.5. The effect of exposure to 21% and 95% oxygen for 3 days on the lung and liver wet weights of preterm guinea pigs treated with either vehicle or U75302.

Guinea pigs delivered by Caesarian section 3 days before term and randomly assigned to receive either 21% or 95% oxygen for 3 days. Animals in each exposure groups were further randomly assigned treatment with U75302 (15mg/Kg body weight/ I.P 12hr) or an equivalent volume of vehicle (5% ethanol, 1% tween 80). After 3 days the animals were sacrificed by pentobarbitone overdose and a BAL performed. The thoracic cavity was then opened and the pulmonary circulation flushed of blood with 10ml of sterile saline (37°C). The lungs and livers were removed, blotted dry, weighed and frozen in liquid nitrogen.



There was a significant effect of drug on total liver wet weight ($F=9.75$, $p<0.005$) and liver/body weight ratio ($F=24.11$, $p<0.001$). * $p<0.05$ compared to equivalently treated animals exposed to 21% oxygen. + $p<0.05$, ++ $p<0.005$ compared to equivalently exposed animals treated with vehicle.

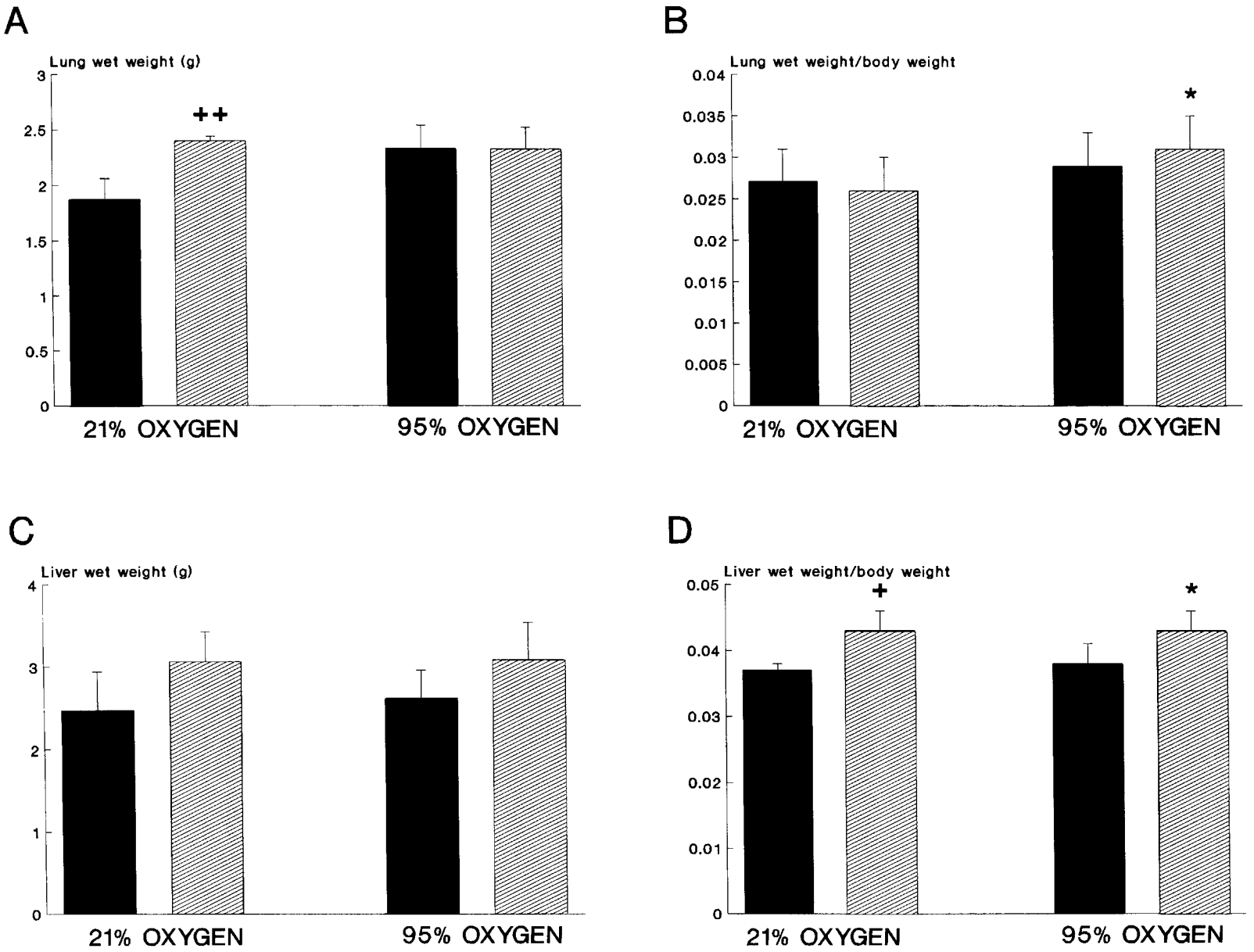

A
Lung wet weight (g)
3
2.5
2
1.5
1
0.5
0
++
21% OXYGEN
95% OXYGEN
B
Lung wet weight/body weight
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
21% OXYGEN
95% OXYGEN
C
Liver wet weight (g)
4
3
2
1
0
21% OXYGEN
95% OXYGEN
D
Liver wet weight/body weight
0.05
0.04
0.03
0.02
0.01
0
+
*
21% OXYGEN
95% OXYGEN

Table 5.7. The effect of exposure to 21% and 95% oxygen for 3 days on the body weights of preterm guinea pigs treated with either vehicle or U75302.

% O_2	Treatment	Body weight (g)			
		0hr	24hr	48hr	72hr
21	VEHICLE	78±9	71±9*	68±10**	67±12*
	U75302	81±3	76±5	72±6	71±8
95	VEHICLE	85± 11	78± 11	73±11	69±13*
	U75302	80±5	74±6*	72±8*	72±11*

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant interaction between oxygen exposure and drug treatment on body weight ($F=7.9$, $p<0.001$). * $p<0.05$, ** $p<0.005$; compared to birth weight.

5.4.2.4 Lung tissue protein and DNA.

No significant difference in total or concentration of tissue protein or DNA was observed between vehicle and U75302-treated animals at either 21% or 95% oxygen exposure (table 5.8). Following exposure to 95% oxygen total tissue protein increased in both treatment groups compared to equivalently treated animals in air. However, this was only significant following treatment with U75302. Although both vehicle and U75302-treated guinea pigs had lower lung tissue DNA concentrations than air exposed pups, this did not reach statistical significance.

Table 5.8. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue protein and DNA of preterm guinea pigs treated with either vehicle or U75302.

% O_2	Treatment	Protein		DNA	
		mg	mg/g	mg	mg/g
21	Vehicle	98.4±36.0	52.5±17.6	6.9±2.1	3.7±1.0
	U75302	105.4±8.7	46.5±9.3	6.8±2.1	3.0±1.2
95	Vehicle	126.0±11.2	50.1±5.3	5.6±1.5	2.5±1.4
	U75302	128.2±9.1+	52.5±3.2	6.1±1.5	2.7±0.6

Data represents the mean and standard deviation of between 5 and 7 animals per group. There was a significant effect of oxygen on tissue protein ($F=4.4$, $p<0.05$). + $p<0.05$ compared to 21% oxygen. *Abbreviations:* DNA:- deoxyribosenucleicacid.

5.4.2.5 Blood inflammatory cell counts.

There was no effect of oxygen exposure or treatment with U75302 on the total blood cell count (Table 5.9).

Table 5.10. The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential BALF cell count of preterm guinea pigs treated with either vehicle or U75302.

% O_2	Treatment	BALF inflammatory cell number (10^4 cell/ml)			
		TOTAL	PMN	MACS	EOS
21	Vehicle	65±25	1.0±0.8	20.1±8.2	44.5±18.0
	U75302	120±46+	1.9±2.1	29.6±13.3	88.5±32.9+
95	Vehicle	79±41	5.5±4.7*	17.4±15.5	55.6±26.3
	U75302	109±45	2.8±1.1	18.3±11.2	87.9±39.6

Data represents the mean and standard deviation of between 7 and 9 animals per group. *Abbreviations*: PMN: polymorphonuclear leucocytes, MACS: macrophages, EOS: eosinophils. There was a significant effect of oxygen exposure on neutrophil ($F=6.28$, $p<0.05$). There was also a significant interaction between oxygen and U75302 on eosinophil numbers ($F=27.19$, $p<0.005$). * $p<0.05$; 95% O_2 Vs 21% O_2, + $p<0.05$, U75302 compared to vehicle.

Although there was no effect of hyperoxia on neutrophil numbers, treatment with U75302 reduced the cell count by greater than 50%. Basophil and eosinophil numbers were significantly increased following treatment with U75302, regardless of oxygen exposure

5.4.2.6 BALF inflammatory cell count.

No effect of hyperoxia on the total cell count of vehicle-treated animals recovered by BAL was observed. However, following treatment with U75302 total cell number increased and was significant for pups exposed to 21% oxygen (Table 5.10). Exposure to 95% oxygen resulted in a significant increase in BALF neutrophils, which was blunted following treatment with U75302. BALF macrophage numbers were unaltered following treatment with U75302 or exposure to oxygen. The increase observed in total cell counts in U75302-treated animals was due to an increase in BALF eosinophils. Eosinophil numbers in air-

Table 5.9 The effect of exposure to 21% and 95% oxygen for 3 days on the total and differential blood cell count of preterm guinea pigs treated with vehicle or U75302.

		Blood inflammatory cells (10^4 cells/ml)					
% O_2	Treatment	TOTAL	PMN	MONO	LYMP	BASO	EOS
21	vehicle	179 ± 94	87 ± 78	3 ± 2	88 ± 29	1 ± 2	1 ± 2
	U75302	182 ± 73	48 ± 21	5 ± 4	99 ± 49	6 ± 2+	24±16++
95	Vehicle	235 ± 125	140 ± 104	4 ± 4	90 ± 33	1 ± 2	0 ± 0
	U75302	177 ± 37	51 ± 30	3 ± 3	107 ± 41	13 ± 1+	45 ± 6++

Data represents the mean and S.D of between 7 and 9 animals per group. *Abbreviations:* PMN: polymorphonuclear leucocyte, MONO: monocytes, LYMP: lymphocytes. There was a significant effect of U75302 on circulating neutrophil ($F=9.75$, $p<0.05$), basophil ($F=18.52$, $p<0.001$) and eosinophil ($F=25.37$, $p<0.001$) cell numbers. + $p<0.05$, ++ $p<0.005$; compared to vehicle.

exposed U75302-treated animals was significantly higher than equivalently exposed vehicle-treated animals.

5.4.2.7 BALF protein.

Exposure to 95% oxygen resulted in a significant increase in BALF protein when compared to equivalently treated air controls (Table 5.11). Treatment of oxygen-exposed animals with U75302 blunted this increase in BALF protein, such that the protein concentration was equivalent to animals exposed to air.

5.4.2.8 BALF neutrophil elastase activity.

Exposure to hyperoxia resulted in a significant increase in elastase activity in vehicle-treated animals when compared to air controls. Treatment of pups with U75302 reduced this activity by 40% but which was still significantly greater than U75302-treated air controls (Table 5.11)

Table 5.11. The effect of exposure to 21% and 95% oxygen for 3 days on BALF neutrophil elastase and phospholipase A_2 activities and protein concentration of preterm guinea pigs treated with either vehicle or U75302.

% O_2	Treatment			
		Elastase ng/ml	Protein mg/ml	PLA_2 μm/min/ml
21	VEHICLE	0±0	0.29±0.04	1.39±0.06
	U75302	0±0	0.31±0.07	1.03±0.52
95	VEHICLE	5.7±2.5*	0.43±0.08*	0.81±0.11
	U75302	3.4±1.6*	0.39±0.11	0.85±0.29

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of oxygen on protein ($F=8.63$, $p<0.05$), elastase ($F=6.52$, $p<0.05$) and PLA_2 ($F=7.17$, $p<0.05$). * $p<0.05$ compared to equivalently treated air controls. *Abbreviations*:- PLA_2:- Phospholipase A_2, N.D:- Not detected.

5.4.2.9 BALF Phospholipase A_2 activity.

Phospholipase A_2 activity of BALF from oxygen-exposed animals, regardless of

treatment, were consistently lower than equivalently treated pups exposed to air (Table 5.11). This was significant for vehicle-treated animals when the data was expressed per gram protein (data not shown).

5.4.2.10 Lung tissue antioxidant enzyme activities.

There was no effect of drug or oxygen exposure on any antioxidant enzyme measured (Table 5.12).

Table 5.12. The effect of exposure to 21% and 95% oxygen for 3 days on lung tissue antioxidant enzyme activities of preterm guinea pigs treated with either vehicle or U75302.

% O_2	Treatment	Lung tissue antioxidant enzyme activities			
		CuZn SOD U/mg DNA	Mn SOD U/mg DNA	CAT KU/mg DNA	GSH-Px U/mg DNA
21	VEHICLE	33.4 ± 12.7	2.4 ± 0.7	5.7 ± 3.5	0.3 0.2
	U75302	54.2 ± 36.2	2.6 ± 1.6	6.2 ± 2.9	0.5 ± 0.3
95	VEHICLE	67.8 ± 40.4	2.9 ± 1.4	13.1 7.6	0.8 ± 0.5
	U75302	56.7 ± 36.4	2.1 ± 1.0	8.3 ± 1.2	0.4 ± 0.7

Data represents the mean and standard deviation of between 6 and 9 animals per group. *Abbreviations*: Cu/Zn SOD: copper zinc superoxide dismutase, Mn SOD:- Manganese superoxide dismutase, CAT: catalase, GSH-Px:- Glutathione peroxidase.* $p<0.05$ compared to 21% oxygen.

5.4.3 Discussion.

Leukotriene B_4 is a product of arachidonic acid metabolism, and its major effect is directed towards phagocytes. Studies performed *in vitro* and *in vivo* demonstrate that LTB_4 promotes chemotaxis of phagocytes (Palmblad *et al.*, 1981; Lewis and Austen, 1984; Martin *et al.*, 1984; Martin *et al.*, 1987) and more specifically, promotes the chemotaxis of neutrophils in man (Martin *et al.*, 1989), rabbits (Staub *et al.*, 1985) and guinea pigs (Silbaugh *et al.*, 1987). It also causes neutrophil degranulation (Naccache *et al.*, 1979) and as the biosynthesis of LTB_4 by neutrophils is well documented (Palmer and Salmon, 1983; Henricks *et al.*, 1986; Seeger *et al.*, 1986) it may therefore act as an endogenous amplifier

of PMN-dependant responses in inflammation. However, instillation of LTB_4 into the lungs of humans (Martin *et al.*, 1989) and sheep (Staub *et al.*, 1985) does not alter the permeability of the lung. The absence of increased protein in lymph fluid in humans (Martin *et al.*, 1989) suggests that either or both the endothelial and epithelial cells are intact regardless of the movement of neutrophils across the interstitium into the alveolar lumen. Tanaguchi *et al.*, 1986 found increased BALF LTB_4 in the lungs of oxygen-exposed rats which correlated with BALF neutrophil numbers. The addition of the 5-lipooxygenase inhibitor, AA861, not only decreased the number of BALF neutrophils, but also increased the survival of these animals in oxygen. However, this relationship has been queried by others. Smith *et al* 1986 and 1990, studied oxidative lung damage in mice, found LTB_4 levels unchanged in BALF and as such the role of this lipid in oxidative-induced lung damage is unclear. In chapter 3 BALF LTB_4 was found in both air and oxygen-exposed animals. Whether it was released from resident macrophages or other cell types within the lung is unclear. However, the presence of this chemotactic agent may explain the increased number of tissue granulocytes previously observed in the model (Kelly *et al.*, 1991).

In the present study the use of U75302, at a concentration of 15mg/Kg body weight, was seen to be toxic to the premature animal. In all animals exposed to air, the drug caused a significant loss of body weight through out the study, which was over and above that normally seen after delivery. There was also an increase in liver wet weight and a significant reduction in survival. Further to this there was a dramatic alteration in the blood and lung inflammatory cell profile. Blood, basophils and eosinophil numbers were markedly elevated and neutrophil numbers lowered, an alteration which was reflected in the inflammatory cell population recovered following BAL. The total number of circulating monocytes and lymphocytes were similar to that found for untreated animals in chapter 3, and suggests that U75302 either alters the production of phagocytes or their release into blood. Therefore U75302 may be acting directly at progenitor blood cells or as an inflammatory stimuli for eosinophils and basophils. Further studies are thus required to fully assess the mechanism of this compound on the inflammatory cell profile of the premature guinea pig. Interestingly, although blood eosinophil numbers increased 20 to 50 fold in U75302-treated animals, BALF eosinophils only doubled. This would suggest a passive transfer of these cells into the guinea pig lung even following oxygen exposure when chemotactic factors are released. BALF neutrophil numbers increase 6 fold in the

vehicle-treated oxygen exposed animal and therefore the chemotactic factors generated in this model appear to specifically attract these cell types and not eosinophils.

To date, only one study has utilised U75302 to examine the role of LTB_4 in pulmonary inflammation (Richards *et al.*, 1989). In their study bronchopulmonary eosinophilia associated with antigen challenge was significantly reduced following treatment with the drug but the increase in neutrophils that was also associated with antigen challenge was unaffected. The reason for the difference between the results presented and that published is unclear. The drug was administered directly to the lung at a concentration of 30mg/Kg body weight but in the present study, U75302 was administered by i.p. injection. Also adult guinea pigs were used in their study and these are possibly more tolerant to the effects of the drug than premature guinea pigs.

Oxygen-exposure resulted in an increase in the mortality rate of both treatment groups (U75302 and vehicle-treated). As in air-treated animals, body weight fell throughout the study and the inflammatory cell profile of the blood was dramatically altered by U75302 treatment. The increase in circulating neutrophils associated with oxygen exposure was not apparent in the drug-treated animals, which may explain the decrease in BALF neutrophils observed in these animals. BALF protein was also decreaseded following drug treatment. However, vehicle-treated animals also have reduced BALF protein when compared to previous studies reported in this thesis. The reason for this is unclear, although Tween 80, which is used as a constituent of the vehicle, may act as an antioxidant and prevent free radical mediated tissue injury. Further studies with tween 80, at different concentrations will have to be undertaken to assess whether this vehicle is acting directly as an antioxidant.

Leukotriene B_4 also has an immunoregulatory function and is capable of altering lymphocyte responsiveness to immunological stimuli (Ford-hutchinson, 1985). When human monocytes are exposed to graded concentrations of LTB_4, their cell-free supernatants contain increased amounts of TNF (Gagnon *et al.*, 1989) and IL-6 (Poubelle *et al.*, 1991). The presence of LTB_4 and other metabolites of arachidonic acid have also been identified within BALF of preterm infants who develop BPD (Stenmark *et al.*, 1987; Groneck *et al.*, 1993). TNF has also been identified in the BALF of these infants and in both cases, treatment with dexamethasone significantly reduced BALF LTB_4 and TNF concentrations and the influx of neutrophils (Groneck *et al.*, 1993). As TNF and other cytokines are known

al., 1988), alterations in the concentration of BALF LTB_4 may thus have a bearing on the susceptibility of the neonate to oxidative injury. In previous chapters hyperoxic exposure leads almost invariably to an increase in Mn SOD activity. This is not seen following treatment with either vehicle or U75302. If tween 80 is acting as an antioxidant, reducing the effect of the free radicals, then an increase in tissue antioxidants is not required and any effect of U75302 on antioxidant enzyme induction is going to be overshadowed by the antioxidant effect of the vehicle.

In summary, treatment of either air or oxygen-exposed animals with U75302 was toxic to the premature guinea pig. Also as tween 80 was suspected of having antioxidant activity, the changes associated with U75302 and oxygen exposure were overshadowed by the effect of this constituent of the vehicle. If further studies in the premature guinea pig are to be carried out with this compound, a better vehicle would be required and a dose response curve established to ascertain whether the benefits of the compound, if any, outweigh its toxicity.

5.4 General discussion.

Reactive oxygen species are known to damage cells directly (Warren *et al.*, 1991; Freeman and Crapo, 1982), stimulate lipid peroxidation (Freeman and Crapo, 1982; Kellogg and Fridovich, 1977), activate phospholipase (Goldman *et al.*, 1992), liberate platelet activating factor, arachidonic acid and lead to the generation of various prostanoids, monohydroxy fatty acids and leukotrienes (Chan *et al.*, 1982; Freeman and Crapo, 1982). A whole host of these lipids have been identified in various models of oxidant-induced lung injury (Holtzman *et al.*, 1989; Lee *et al.*, 1990) and in humans with BPD (Westcott *et al.*, 1985; Stenmark *et al.*, 1987; Westcott *et al.*, 1986). However, whether one or all of these lipids involved in the development of neonatal lung disease is unknown.

Upon activation of a membrane phospholipase, the lipid 1-o-alkyl-2 arachidonyl-s, n-glycero-3-phosphocholine is acted upon to release lysoplatelet activating factor and arachidonic acid (Chilton and Murphy, 1986). Via a acetyltranserase enzyme, acetyl CoA is then added to give rise to the active lipid, platelet activating factor. As mentioned earlier, PAF increases microvascular permeability and induces pulmonary oedema (Ford-hutchinson *et al.*, 1980; Mojarad *et al.*, 1983). As PAF has been recently found in the BALF of infants with BPD (Koyama *et al.*, 1993), and as it is able to stimulate the release of both

lipooxygenase and cyclooxygenase metabolites (Voekel *et al.*, 1982; Lefer *et al.*, 1984) it may play a central role in the development of neonatal lung disease.

The prostanoids in BALF of infants with BPD include prostacyclin (PGI_2) and thromboxane (TXA_2) (Stenmark *et al.*, 1987), of which much greater quantities of TXA_2 are associated with the lungs of these infants than is prostacyclin. Prostacyclin is a potent pulmonary vasodilator (Dusting *et al.*, 1982), an inhibitor of platelet aggregation and is released in many forms of lung injury. It may modulate the effects of TXA_2, which causes pulmonary vasoconstriction, bronchoconstriction and platelet aggregation (Bowers *et al.*, 1979; Kadowitz *et al.*, 1982). Prostacyclin may also modulate the effect of leukotrienes, by increasing intracellular cyclic AMP, which inhibits the release of LTB_4 and counteracts the effects of LTC_4 and LTD_4 (Kuehl *et al.*, 1984). This protective effect can be seen in oxygen-exposed newborn rabbits. In these animals greater quantities of prostacyclin are found than is found in similarly exposed adult animals and may explain the increased susceptibility of adult animals to oxygen (Holtzman *et al.*, 1989). Treatment of BPD with indomethacin, a drug thought to be an antiinflammatory agent, acts via blockade of the cyclooxygenase pathway and may possibly lead to a decrease in the production of prostacyclin . Although it reduces thromboxane synthesis in rabbits, in infants with BPD, it increases BALF elastase and fibronectin levels, both detrimental to the infant (Gerdes *et al.*, 1988). Whether this effect in humans is a direct result of the reduction in the concentration of prostacyclin or to the shunting of arachidonic acid down the lipooxygenase pathway is unknown. In the oxidant-induced lung injury in the rat, cyclooxygenase blockade with indomethacin increases the concentration of 5-HETE, implying activation of the 5-lipooxygenase pathway.

Leukotrienes arise from a metabolic cascade involving 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene A_4. Leukotrienes C_4, D_4 and E_4, commonly referred to as the sulfidopeptide-containing leukotrienes are potent bronchoconstrictors (Hanna *et al.*, 1981) and are known to increase capillary permeability in both systemic and pulmonary capillary beds (Lewis and Austen, 1984; Dahlen *et al.*, 1981; Albert and Henderson, 1982). All these leukotrienes and HETE are found in BALF of infants with BPD (Stenmark *et al.*, 1987), the role of which, in the development of chronic lung disease is unclear. In the oxygen-exposed adult rat, administration of the 5-lipooxygenase inhibitor, AA861, not only decreased the concentration of LTB_4 but also the increase in mortality and BALF PMN numbers

(Taniguchi *et al.*, 1986) associated with oxygen exposure. In the glucose-oxidase treated rat, U60-257 another 5-lipooxygenase inhibitor, decreased lung oedema and the associated increase in 5-HETE (Burghuber *et al.*, 1984).

Glucocorticoids, steroids that reduce the adverse effects of BPD (Mammel *et al.*, 1987; Bourchier, 1988; Gerdes *et al.*, 1988; Cummings *et al.*, 1989), inhibit phospholipase activity and thus reduce the production of the prostanoids, leukotrienes and PAF. Experimental evidence shows that dexamethasone blocks the release of prostaglandins by histamine in the guinea pig lung, inhibit the release of arachidonic acid from inflammatory cells (Fahey *et al.*, 1981) and inhibit the generation of PAF from PMN's (Parente and Flower, 1985). In addition dexamethasone has shown to protect against the toxic effects of PAF, which are known to cause to release of eicosanoids (Myers *et al.*, 1983). Local treatment of the hamster cheek pouch with two glucocorticoids, dexamethasone and budesonide, at clinically relevant concentrations ($2x10^{-7}m$) resulted in the inhibition of bradykinin-induced increase in microvascular permeability (Svensjo and Roempke, 1985). Vascular leakage induced by histamine, LTC_4 and PAF was inhibited with methylprednisolone (Bjork *et al.*, 1985). However, one must also be aware that treatment with glucocorticoids may not always be beneficial. In the endotoxin treated rat, high dose administration of steroids does not prevent the increase in prostaglandin release and actually increased circulating levels of thromboxane B_2 and 6-ketoprostaglandin (Hofman and Ehrhart, 1983). Also in a study by Ogletree *et al,* although methylprednisolone treatment inhibited both prostacyclin and 12-HETE in lung lymph following endotoxin challenge, thromboxane levels remained unaltered (Ogletree *et al.*, 1986).

Thus in many forms of lung injury including BPD, many proinflammatory lipids are released that may have an adverse affect on the function of the organ. Also as there exists a potential network of lipids that can all influence the production and release of each other and as such there is the potential that blockade of any one product may not interrupt the cycle of events and reduce cellular injury. Even with the use of glucocorticoids, in some models of lung injury, these steroids are of benefit, but in others they are not. Thus although at present it is known that there are many lipid mediators released in to the lungs of infants developing BPD the precise interplay between them is far from clear and more work is required to unravel this complex web of interactions that lead to lung disease.

In the present study the results indicate that for PAF, although not detected in either blood or BALF, may be involved in the development of inflammation and injury in the oxygen-exposed premature guinea pig. Whether this is through the direct inhibition of some of the known actions of PAF on cellular activation and permeability or the reduction in the production of secondary pro-inflammatory compounds is not known. The role of LTB_4 in oxygen-induced inflammation and injury was less clear. The LTB_4 antagonist, U75302 was toxic to the premature guinea pig. Although the drug was successful in blunting the movement of neutrophils into the lung, this appeared to be due to an alteration in the inflammatory cell profile of the blood and not a direct inhibition of chemotaxis. Another confounding observation in this study was the fact that the vehicle, tween 80, may have been acting as an antioxidant. In conclusion PAF, at least, may be important in the development of oxygen-induced injury to the immature lung.

CHAPTER 6

THE EFFECTS OF EXPOSURE TO 85% OXYGEN FOR 28 DAYS ON THE IMMATURE LUNG OF THE PRETERM GUINEA PIG

6.1 Introduction.

The pathogenesis of BPD is complex and almost certainly multifactorial. Diagnosis of BPD includes the requirement for oxygen dependency for longer than 28 days. As such, the morphological and biochemical alterations within the lungs of these infants may develop partly as a consequence of prolonged exposure to high concentrations of oxygen. As a consequence of this, a number of groups have assessed the biochemical and morphological changes within the lungs of a variety of different animals species over weeks instead of days (Hayatdavoudi *et al.*, 1981; Pappas *et al.*, 1983; Holm *et al.*, 1987; Fracica *et al.*, 1988; Ohtsu *et al.*, 1989; Fracica *et al.*, 1991). However, as with many studies on the acute response to hyperoxia, many of these models have proved inappropriate, lacking the biochemical and anatomical immaturity at the onset of oxygen exposure, that is observed in human preterm neonates. Of those studies that have used preterm animals (Coalson *et al.*, 1982; Kessler *et al.*, 1982; Coalson *et al.*, 1992) the associated expense and commitment on time has resulted in slow progress in the elaboration of mechanisms involved in the development of BPD. In the present study, a detailed biochemical and histological description of the effects of prolonged exposure to hyperoxia (28 days) on the response of the immature lung of the premature guinea pig is described.

6.2 Materials and methods.

To mirror the high levels of oxygen often given to preterm infants who develop BPD, preliminary studies had shown that exposure to 95% oxygen for longer than 4 days resulted in unacceptably high mortality rates. An oxygen concentration 10% lower (85%) was adequately tolerated by the premature animals and allowed long term oxygen exposure studies to be undertaken. Guinea pigs were delivered by Caesarian three days before term and randomly assigned to receive either 21% or 85% oxygen for up to 28 days (Figure 6.1). All animals were sacrificed by pentobarbitone overdose, the trachea isolated and a cannulae inserted. A BAL was then conducted using 10ml (5x2ml aliquots) of non-pyrogenic saline (37°C), the recovered fluid being placed immediately on ice. Following BAL, the thoracic cavity was opened and a blood sample taken by direct cardiac puncture. The lungs were then flushed of blood with 10ml of saline (37°C), removed, blotted dry, weighed and frozen in liquid nitrogen. Total and differential blood and BALF cell counts, BALF protein, GSH, GSSG and PC concentrations, lung tissue CAT, GSH-Px, Mn SOD,

Cu/Zn SOD, GSH, GSSG and collagen were all measured as described in Sections 2.9 and 2.10. At day 28, between 4 and 5 animals were taken, sacrificed and their lungs inflated and fixed *in situ* for light and electron qualitative microscopic examination as described in Section 2.8.

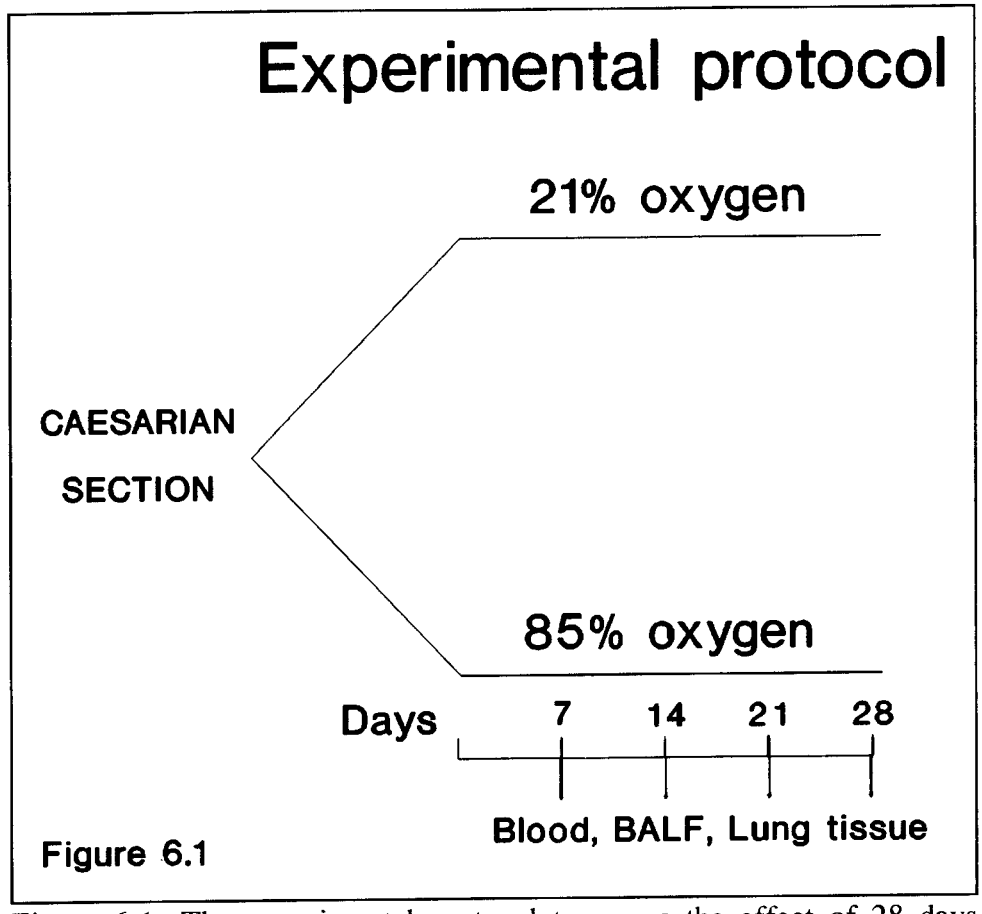


Figure 6.1. The experimental protocol to assess the effect of 28 days exposure to 85% oxygen on the immature lung of the preterm guinea pig.

Results. p386X**6.3**

6.3.1 Survival.

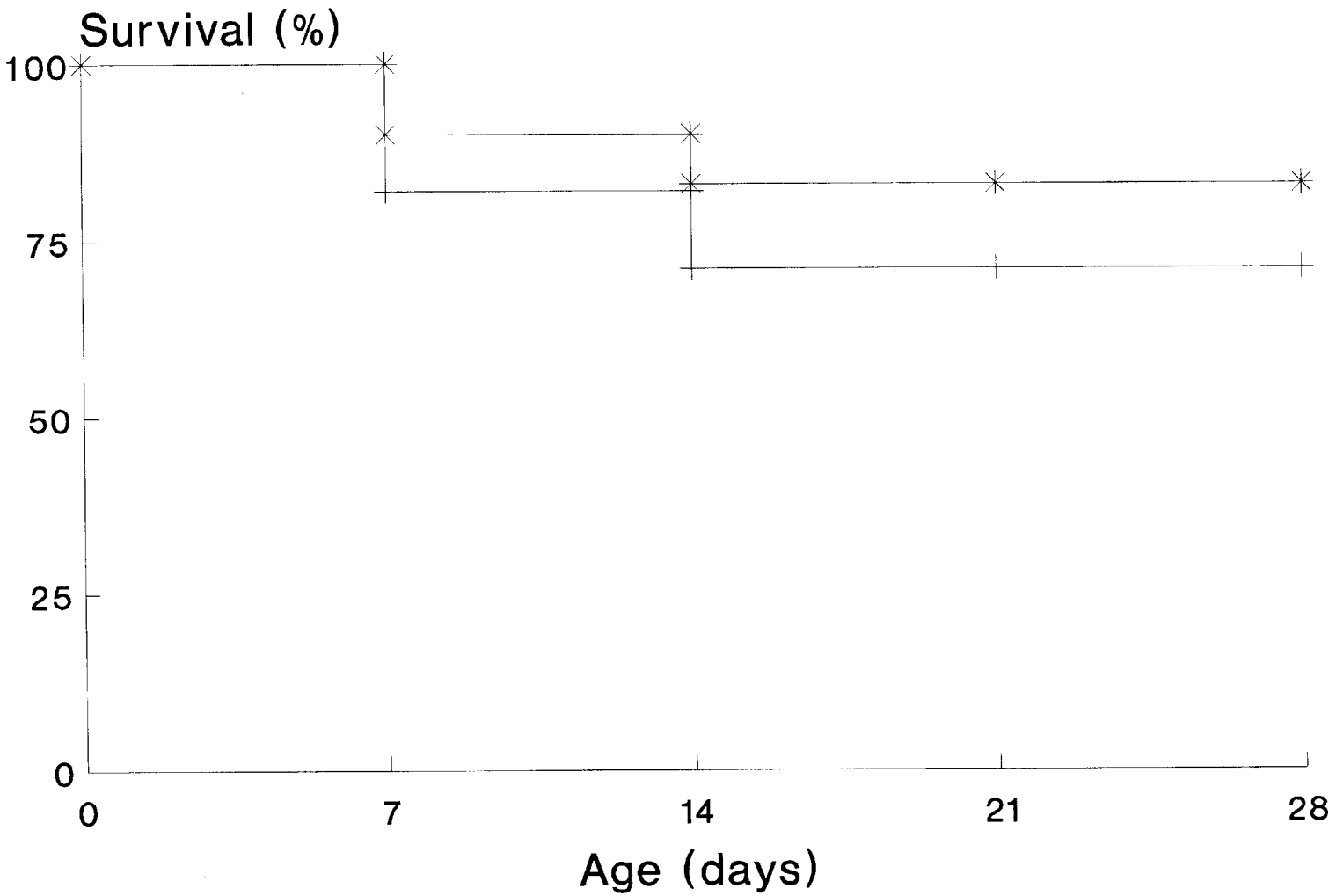
All preterm animals delivered by Caesarian section developed respiratory distress shortly after delivery. Oxygen-exposed animals also experienced a second period of respiratory difficulty which occurred between 7 and 14 days into the study. Animals that survived this period or respiratory difficulty survived until the end of the study. Survival of pups exposed to 85% oxygen was significantly reduced when compared to air controls (Fig 6.2).

Figure 6.2. The effect of exposure to 21% and 85% oxygen for up to 28 days on the survival rates of preterm guinea pigs.

Guinea pigs were delivered by Caesarian section 3 days before term and randomly assigned to receive either 21% or 85% oxygen for up to 28 days. Cages were checked daily for mortalities from which percentage survival was calculated. There was a significant effect of oxygen exposure ($p < 0.001$) on the survival of the premature guinea pig when compared to air controls.

----*---- 21% oxygen

----+---- 85% oxygen


Survival (%)
100
75
50
25
0
0
7
14
21
28
Age (days)

6.3.2 Body weights.

Following an initial drop in body weight shortly after birth, animals in both treatment groups gained weight over the 28 days. However, those pups that were exposed to oxygen were not only significantly lighter than equivalent air controls from day 7 onwards, they also grew at a significantly reduced rate (Fig 6.3).

6.3.3 Lung and heart wet weights.

Lung and heart wet weights increased with age, regardless of oxygen exposure (Table 6.1). Heart weights of air-exposed animals increased by 44% and lung wet weight by 14% from day 7 to 28 and as such, the lung/heart ratio fell during the study. When both lung and heart weights were normalised to body weight, the lung/body ratio fell as a consequence of a much faster increase in body weight to that of the lung. No change in the ratio of heart/body was observed over the 28 days of the study.

Following hyperoxic exposure, lung wet weights were consistently heavier and heart weights lighter than equivalently aged air controls. As such, when lung and heart wet weights were expressed as a function of body weight, the lung/body ratio fell. The heart/body ratio remain unchanged over the 28 days of the study and was similar to that observed in air controls. The lung/heart ratio increased up to day 14 and then fell towards the end of the study.

The volume of saline recovered following BAL fell with age and exposure to oxygen. Regression analysis of recovered BALF and lung wet weight demonstrated a significant negative correlation between the two ($p<0.0005$, $r=-0.5353$).

6.3.4 Lung tissue protein, and DNA.

Total lung tissue protein and DNA increased with age and following exposure to oxygen (Table 6.2). Lung tissue protein was significantly higher at all time points following oxygen exposure when compared to air controls. When the data were expressed per gram lung wet weight, there was only a significant effect of age and oxygen exposure on lung DNA, which reflected the dramatic changes observed in lung wet weight. There was also a significant correlation between total lung protein and lung wet weight ($p<0.001$; $r=+0.887$).

Figure 6.3. The effect of exposure to 21% and 85% oxygen for up to 28 days on the body weights of preterm guinea pigs.

Guinea pigs were delivered by Caesarian section 3 days before term. They were randomly assigned to receive either 21% or 85% oxygen for up to 28 days. Animals were weighed regularly over the first 10 days or until birth weight was acheived and then every so often. Data represents the mean and standard deviation of between 40 (day 7) and 8 (day 28) animals per exposure group. There was a significant effect of time ($F=27.7$, $p<0.001$), oxygen exposure ($F=268.9$, $p<0.001$) and a significant interaction between the two ($F=4.0$, $p<0.001$). * $p<0.05$, compared to 21% oxygen.

----*---- 21% oxygen

----+---- 85% oxygen

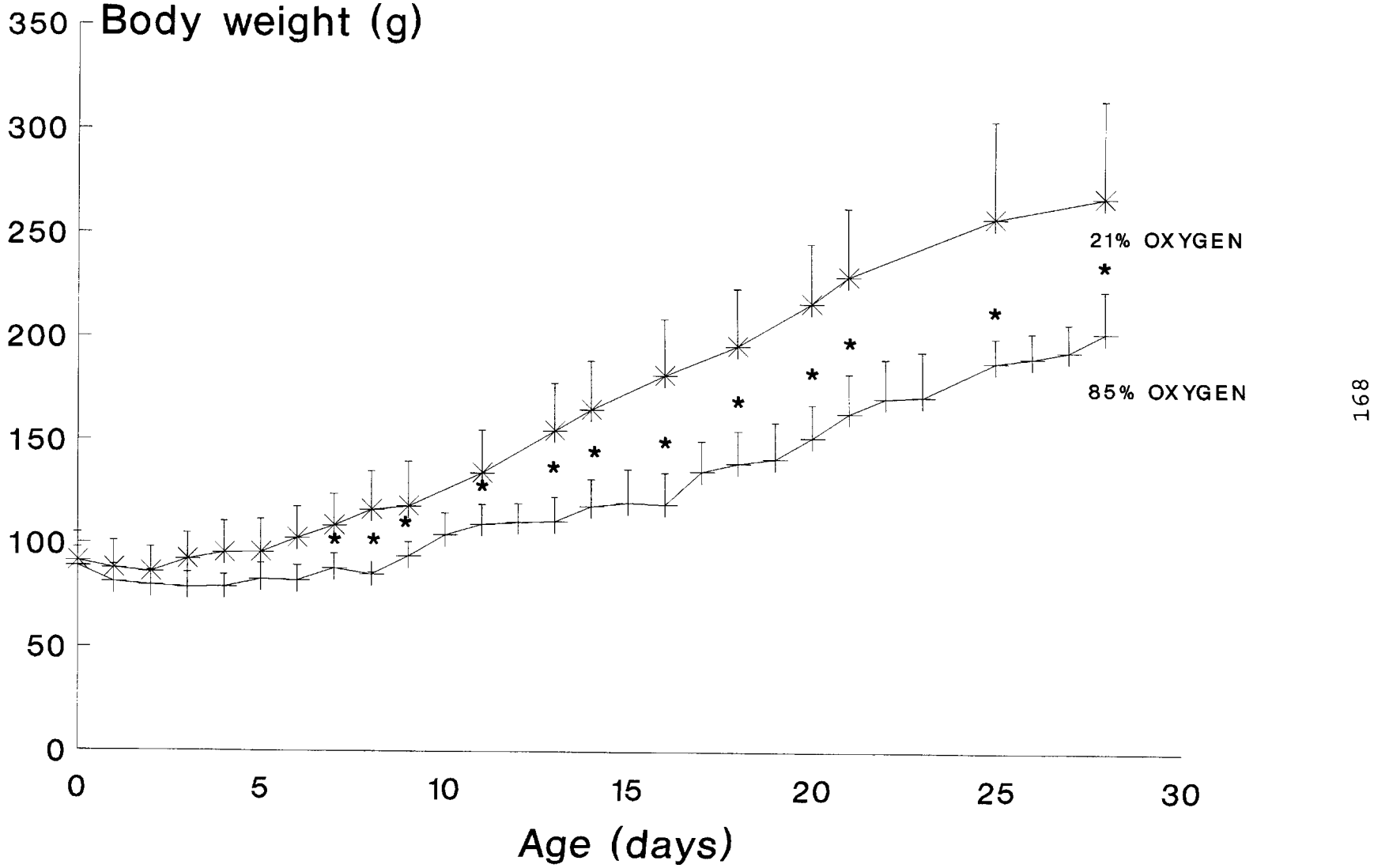

Body weight (g)
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
Age (days)
21% OXYGEN
85% OXYGEN

Table 6.1. The effect of exposure to 21% and 85% oxygen for up to 28 days on lung and heart wet weights of preterm guinea pigs.

% Oxygen	AGE (days)	LWW (g)	HW (g)	LWW/HW (g/g)	LW/BW (mg/g)	HW/BW (mg/g)
21	7	1.8±0.3**a**	0.36±0.02**a**	4.90±0.70**a**	17.7±3.7**a**	3.6±0.6
	14	1.8±0.3**a**	0.50±0.12**ab**	3.85±0.73**b**	13.6±1.2**a**	3.8±0.4
	21	2.1±0.4**a**	0.67±0.36**c**	3.19±0.32**c**	11.4±0.6**a**	3.6±0.2
	28	2.6±0.3**b**	0.95±0.12**d**	2.78±0.17**d**	10.0±0.6**a**	3.6±0.1
85	7	2.7±0.5****a**	0.35±0.03**a**	7.76±1.28****a**	34.1±8.1****a**	4.4±0.4*
	14	4.0±1.0*****b**	0.42±0.04**b**	9.84±3.15*****b**	40.7±15.3*****a**	4.1±0.4
	21	4.3±0.5*****b**	0.64±0.09**c**	6.79±0.74*****a**	28.2±5.4*****ba**	4.2±0.9
	28	4.6±0.9*****b**	0.73±0.11***c**	6.32±1.09*****a**	25.5±5.6*****b**	4.0±0.3*

Data represents the mean and standard deviation of between 5 and 8 animals per group. *Abbreviations*; LWW:- Lung wet weight, HW:- heart weight, LW/HW:- Lung to heart weight ratio, LW/BW:- lung to body weight ratio, HW/BW:- heart to body weight ratio. There was a significant effect of age on lung weight ($F=12.6$, $p<0.001$), heart weight ($F=56.3$, $p<0.001$), lung/heart ratio ($F=8.6$, $p<0.001$) and lung/body ratio ($F=6.0$, $p<0.005$). There was also a significant effect of oxygen exposure on lung weight ($F=131.7$, $p<0.001$), heart weight ($F=7.6$, $p<0.01$), lung/heart ratio ($F=116.0$, $p<0.001$), lung/body ratio ($F=97.8$, $p<0.001$) and heart/body ratio ($F=17.5$, $p<0.001$). A significant interaction between age and oxygen exposure was observed for lung weight ($F=3.7$, $p<0.05$), heart weight ($F=2.9$, $p<0.05$) and lung/heart ratio ($F=3.4$, $p<0.05$). * $p<0.05$, ** $p<0.005$, *** $p<0.001$ compared to equivalently aged air controls. Different letters within each exposure group are significantly different from each other.

6.3.5. Lung tissue collagen.

Total lung collagen increased with age (Fig 6.4A). Following exposure to oxygen there was significantly greater amounts of collagen than equivalently aged air controls at days 14, 21 and 28. On expression of the data per gram lung, tissue collagen concentration also increased with age (fig 6.4B).

6.3.6 Blood inflammatory cell counts.

No change in the total number of circulating leukocytes from air-exposed control animals were observed with age (Table 6.3).

Table 6.2. The effect of exposure to 21% and 85% oxygen for up to 28 days on lung tissue protein and DNA of preterm guinea pigs.

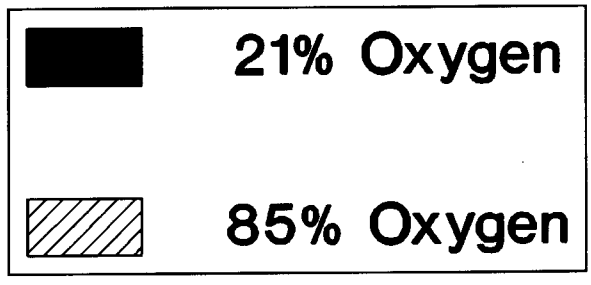
% O_2	AGE	Protein		DNA	
	days	(mg)	(mg/g)	(mg)	(mg/g)
21	7	96±9**a**	56±9**a**	7.2±1.1**a**	4.2±0.9**a**
	14	101±32**ab**	56±14**a**	5.1±1.4**b**	2.9±0.9**b**
	21	131±27**b**	62±3**a**	7.6±2.4**ac**	3.6±0.8**ab**
	28	181±39**c**	71±7**b**	10.4±3.6**ac**	3.9±1.2**ab**
85	7	168±57***a**	61±13**a**	6.8±1.7**ab**	2.5±0.4****a**
	14	205±32*****a**	52±7**a**	7.0±2.1**a**	1.8±0.6***b**
	21	263±49*****c**	61±6**a**	10.7±3.2**b**	2.5±0.5****ab**
	28	261±64***c**	57±13***a**	8.1±2.1**ab**	1.8±0.5***bc**

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of age and oxygen exposure on lung tissue protein (Age: $F=11.1$, $p<0.001$; O_2: 63.3, $p<0.001$) and DNA (Age: $F=5.6$, $p<0.001$; O_2: $F=3.3$, $p<0.05$) When the data was expressed per gramme lung wet weight there was a significant effect of age and oxygen exposure on lung tissue DNA only (Age: $F=3.7$, $p<05$; O_2: $F=48.6$, $p<0.001$). * $p<0.05$, ** $p<0.005$, *** $p<0.001$; compared to 21% oxygen. Data within each exposure group with different letters are significantly different to each other ($p<0.05$). *Abbreviations;* DNA:- Deoxyribosenucleic acid.

This observation held when individual populations of circulating neutrophils, monocytes and lymphocytes were assessed. In animals exposed to 85% oxygen, the total leukocyte and

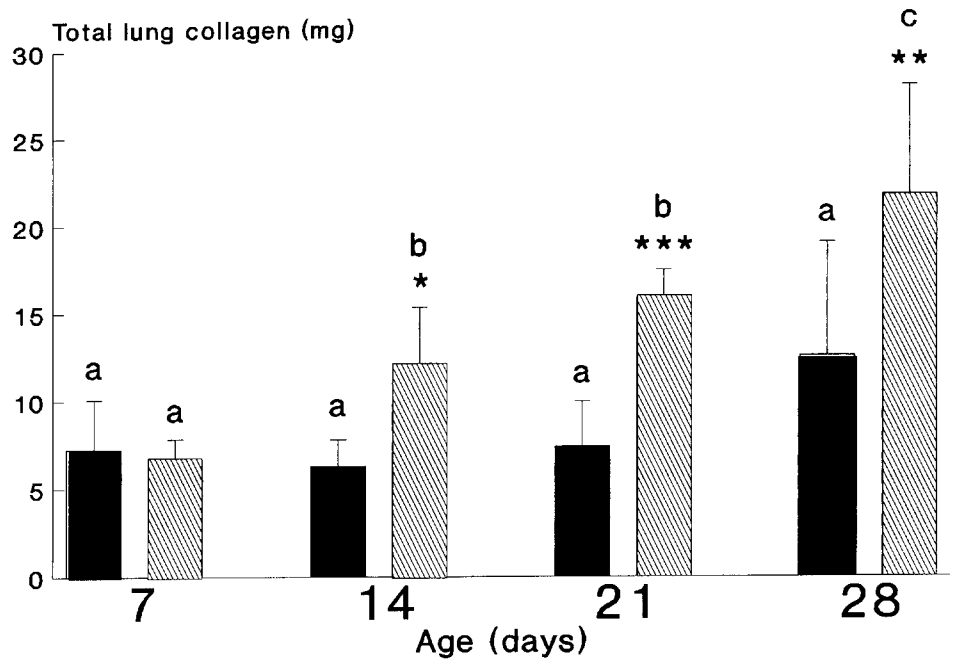
Figure 6.4. The effect of exposure to 21% and 85% oxygen for up to 28 days on the lung tissue collagen concentration of preterm guinea pigs.

Guinea pigs were delivered by Caesarian section 3 days before term and randomly assigned to receive either 21% or 85% oxygen for up to 28 days. After 7, 14, 21 and 28 days exposure,a random group of animals were sacrificed by pentobarbitone overdose and a BAL performed. The thoracic cavity was then opened and the pulmonary circulation flushed of blood with 10ml of sterile saline (37°C). The lungs were removed, blotted dry, weighed and frozen in liquid nitrogen.

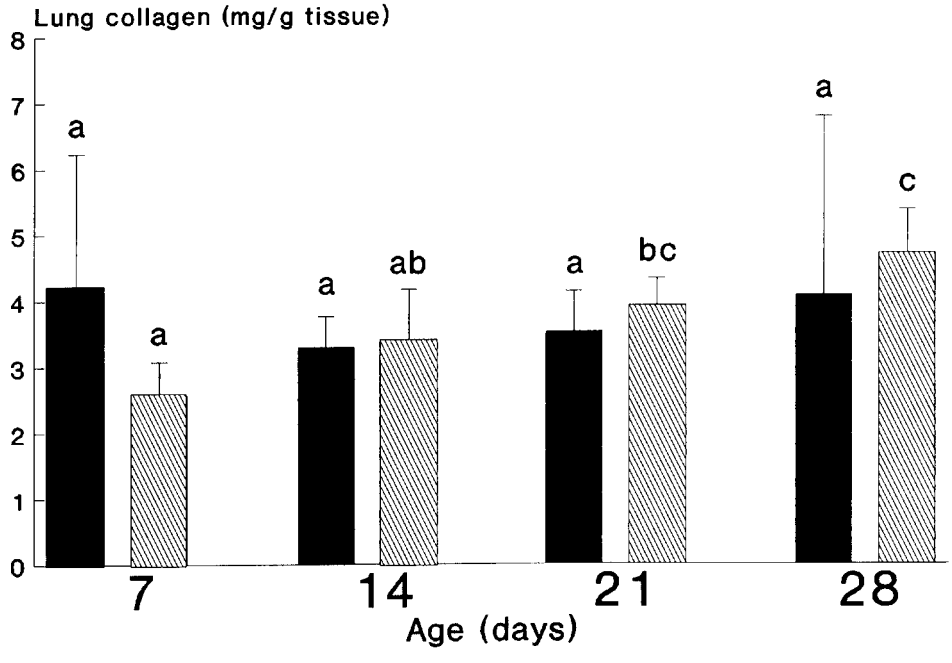


There was a significant effect of age on total lung collagen (fig 6.4A; $F=2.9$, $p<0.05$) and collagen concentration (fig 6.4B; $F=13.4$, $p<0.001$). There was also a significant effect of oxygen exposure ($F=21.1$, $p<0.001$) and a significant interaction ($F=3.2$, $p<0.05$) on total lung tissue collagen. Data within each exposure group with different letters are significantly ($p<0.05$) different to each other. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ compared to equivalently aged animals exposed to 21% oxygen.

A


Total lung collagen (mg)
30
25
20
15
10
5
0
a
a
a
b
*
a
b
***
a
c
**
7
14
21
28
Age (days)

B


Lung collagen (mg/g tissue)
8
7
6
5
4
3
2
1
0
a
a
a
ab
a
bc
a
c
7
14
21
28
Age (days)

individual cell populations were significantly increased. Total and neutrophil cell numbers were increased from day 7 and remained significantly elevated throughout the 28 days of the study when compared to air controls. Monocyte numbers were significantly elevated on day 7 falling to control levels by day 14.

6.3.7 BALF inflammatory cell counts.

Total BALF cell numbers from air-exposed animals decreased progressively throughout out the study, primarily as a result of the reduction in the eosinophil population (Table 6.4).

Table 6.3. The effect of exposure to 21% and 85% oxygen for up to 28 days on the total and differential blood cell count of preterm guinea pigs.

% O_2	AGE	Blood inflammatory cell count (10^4 cells/ml)			
	days	TOTAL	PMN	LYMP	MONO
21	7	166±50a	49±19a	107±36a	0.7±1.0a
	14	155±38a	46±9a	107±27a	2.5±3.1a
	21	176±58a	63±38a	103±32a	1.3±1.6a
	28	187±54a	56±25a	120±36a	1.2±1.4a
85	7	479±145a*	273±84***a	165±34a	13.9±8*a
	14	580±73a*	338±117***a	224±45a	1.6±1.7b
	21	483±176a*	352±156***a	123±53a	1.1±2.0b
	28	538±150a*	343±103***a	204±58a	3.3±2.9b

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of oxygen on total cell ($F=85.1$, $p<0.001$), neutrophil ($F=143.0$, $p<0.001$), lymphocyte ($F=38.0$, $p<0.001$) and monocyte ($F=12.1$, $p<0.001$) numbers. * $p<0.05$, *** $p<0.0005$; compared to 21% oxygen. *Abbreviations;* PMN:- Polymorphonuclear leukocyte, Lymp:- Lymphocyte, Mono; Monocyte. Different letters refer to significant ($p<0.05$) differences within each exposure group.

No change in the neutrophil or macrophage populations were observed with increasing age. Following oxygen exposure, the total BALF cell count increased with age, becoming significantly higher than control animals by day 14. Neutrophil numbers also increased in oxygen-exposed animals, reaching their peak within 14 days and remaining significantly

elevated above air controls until the end of the study. Macrophage numbers did not increase until day 14, where after they remained significantly elevated above air controls until day 28. Interestingly, following exposure to oxygen, eosinophil numbers were significantly lower relative to air-treated controls up to day 14 but by day 21 the population had increased and by the end of the study were significantly higher than air controls.

Table 6.4. The effect of exposure to 21% and 85% oxygen for up to 28 days on the total and differential BALF cell count of preterm guinea pigs.

% O_2	AGE	BALF inflammatory cell count (10^4 cells/ml)			
	days	TOTAL	PMN	MAC	EOS
21	7	78±13**a**	0±0**a**	35±7**a**	43±15**a**
	14	56±15**ab**	1±2**a**	33±7**a**	22±9**bc**
	21	54±20**b**	1±1**a**	36±14**a**	17±7**bc**
	28	47±11**b**	0±0**a**	34±11**a**	12±2**bd**
85	7	77±20**a**	13±6***a**	45±10**a**	15±9**a**
	14	167±42**b***	45±34***b**	106±28**b**	5±3**b**
	21	182±84**b****	41±30***b**	99±36**b**	28±19**ac**
	28	199±71**b*****	29±20***ab**	124±34***b**	31±15***c**

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of oxygen on total cell ($F=32.7$, $p<0.001$), neutrophil ($F=23.8$, $p<0.001$), and macrophage ($F=73.5$, $p<0.001$) BALF cell numbers. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; compared to 21% oxygen. *Abbreviations;* PMN:- Polymorphonuclear leukocyte, MAC:- Macrophage, EOS:- Eosinophil. Different letters refer to significant ($p<0.05$) differences within each exposure group.

6.3.8 BALF protein.

BALF protein concentrations of air-exposed animals increased with age (Table 6.5). Following oxygen exposure the protein concentration was significantly greater than air controls by day 7. Thereafter a progressive decline towards control values occurred, such that by day 14 no significant difference between air and oxygen-treated animals was observed.

6.3.9 BALF DPPC.

No change in BALF phosphatidylcholine (PC) concentrations were observed with age in air or oxygen-exposed animals (Table 6.5). Exposure to 85% oxygen resulted in a significant elevation in PC within 7 days which remained significantly greater than air controls until the end of the study.

6.3.10 BALF glutathione.

As with BALF PC, no change in reduced or oxidised glutathione was observed with age (Table 6.5). However, following oxygen exposure both reduced and oxidised glutathione were significantly elevated above air controls at day 7, which fell to control values by day 14.

Table 6.5. The effect of exposure to 21% and 85% oxygen for up to 28 days on BALF protein, phosphatidylcholine, reduced and oxidised glutathione of preterm guinea pigs.

% O_2	AGE				
	days	Protein (μg/ml)	PC nmol/ml	GSH nmol/ml	GSSG nmol/ml
21	7	43±19**ab**	109±26**a**	0.8±1.6**a**	0.1±0.1**a**
	14	58±33**ab**	123±41**ab**	0.3±0.3**a**	N.D **a**
	21	71±37**ac**	154±9**b**	0.7±1.1**a**	0.4±**a**0.1
	28	109±33**c**	139±10**ab**	0.5±0.4**a**	0±0**a**
85	7	988±844***a**	208±37****a**	4.9±4.0***a**	3.1±2.9***a**
	14	190±52**b**	212±38***a**	0.6±0.6**b**	1.3±2.2**ab**
	21	155±104**bc**	221±49****a**	1.6±2.3**ab**	0±0**b**
	28	118±28**c**	213±33****a**	2.1±1.8**ab**	0±0**b**

Data represents the mean and standard deviation of between 6 and 9 animals per group. There was a significant effect of age and oxygen exposure on BALF protein (Age: $F=6.3$, $p<0.001$; O_2 13.4, $p<0.001$), GSH (Age: $F=3.5$, $p<0.001$; O_2: $F=8.3$, $p<0.001$) and GSSG (Age: $F=3.0$, $p<0.001$; O_2: $F=6.5$, $p<0.001$). There was also a significant interaction between age and oxygen exposure for BALF protein ($F=7.5$, $p<0.001$) and GSSG ($F=3.1$, $p<0.5$). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; compared to 21% oxygen. *Abbreviations;* PC:- Phosphatidylcholine, GSH:- Reduced glutathione, GSSG:- Oxidised glutathione, N.D Not detected.

6.3.11 Lung tissue antioxidant enzyme activities.

Catalase activity decreased and reduced glutathione concentration increased in air-exposed control animals during the 28 days of the study (Table 6.6). Following exposure to oxygen, all antioxidant concentrations and activities were significantly elevated above control animals at some point during the 28 days. Lung tissue reduced glutathione was significantly elevated at day 7 and 14 following exposure to oxygen but returned to control values 7 days later (day 21). Oxidised glutathione followed a similar pattern to reduced glutathione, but was only significantly elevated at 7 days, returning to baseline levels by day 14.

6.3.12 Light and electron microscopic assessment of lungs exposed to 85% oxygen for 28 days.

In the present study, after 28 days exposure to 21% oxygen, the lungs of the preterm guinea pig were normal with no evidence of injury (fig 6.5A and B). Following exposure to 85% oxygen, there were a number of structural alterations to the pulmonary architecture that developed over the 28 days of the study. The most prominent of these alterations was an increase in interstitial thickness. A comparison of Figures 6.5A and C demonstrate an observable difference in thickness of the interstitium between the two treatment groups. This is seen much more clearly at higher power magnification (Fig 6.5B and D). Air-exposed animals have a thin septa that separates alveoli, whereas in those animals exposed to oxygen they are much thicker and contain areas in which no cells or structure can be seen. Large protein deposits are also observed beneath the surface of the alveoli (Fig 6.5D), the nature of which is unclear. A much more in depth view of these lungs are shown in figure 6.6. Figure 6.6A is an electron micrograph of a segment of lung from an air-exposed animal and highlights the fine structure of the septa between a capillary and the alveoli lumen. In contrast to this, the septa from oxygen-exposed animals (Fig 6.6C and D) are much thicker. The voids observed under the light microscope are also seen in the electron micrographs (Fig 6.6B and D). A whole host of cell types can be identified in these animals, including neutrophils within capillaries (Fig 6.6C), fibroblasts (Fig 6.6B) and other unknown cells (Fig 6.6B) within the interstitium. Strands of collagen deposits are also seen within the interstitium of oxygen-exposed animals and may explain the presence of fibroblasts and the protein deposits seen in light micrographs below the alveolar surface (Fig 6.5D and 6.7 A and B). Further examination of these strands show the typical banding

Table 6.6. The effect of exposure to 21% and 85% oxygen for up to 28 days on lung tissue antioxidant enzyme activities.

Antioxidant	Age (Days)	Percent oxygen exposure 21	85
CuZn SOD (U/mg DNA)	7	19.1 ± 4.1**a**	34.2 ± 11.4***a**
	14	30.8 ± 13.0**b**	48.3 ± 23.6**ab**
	21	24.2 ± 11.6**ab**	46.9 ± 11.3****ab**
	28	21.5 ± 8.4**ab**	52.0 ± 13.8****ab**
Mn SOD (U/mg DNA)	7	3.0 ± 1.4**a**	5.7 ± 2.5***a**
	14	3.0 ± 1.1**a**	10.0 ± 3.3****b**
	21	3.3 ± 1.1**a**	5.4 ± 1.4***a**
	28	3.9 ± 2.0**a**	7.7 ± 4.3**ab**
CAT (KU/mg DNA)	7	2.6 ± 0.6**a**	6.1 ± 2.1***ab**
	14	3.2 ± 1.7**ab**	7.9 ± 1.4*****a**
	21	2.0 ± 0.6**ab**	4.6 ± 1.5****b**
	28	1.8 ± 0.6**b**	4.8 ± 1.5***b**
GSH-Rd (U/mg DNA)	7	218.8 ± 33.5**a**	285.7 ± 53.1***a**
	14	294.6 ± 103.5**a**	418.8 ± 159.6**ab**
	21	279.1 ± 62.5**a**	370.2 ± 105.6**ab**
	28	239.6 ± 98.9**a**	480.9 ± 183.2***b**
GSH-Px (U/mg DNA)	7	0.38 ± 0.10**a**	0.8 ± 0.2****a**
	14	0.53 ± 0.28**a**	0.9 ± 0.3**a**
	21	0.52 ± 0.16**a**	0.7 ± 0.2**a**
	28	0.54 ± 0.31**a**	0.9 ± 0.4**a**
GSH (μmol/g T)	7	1.0 ± 0.3**a**	1.4 ± 0.2***a**
	14	1.2 ± 0.2**a**	1.7 ± 0.1*****b**
	21	1.3 ± 0.2**a**	1.2 ± 0.3**a**
	28	1.6 ± 0.1**a**	1.4 ± 0.6**ab**
GSSG (nmol/g T)	7	40.1 ± 56.2**a**	392.7 ± 333.3***a**
	14	18.9 ± 26.4**a**	11.4 ± 18.6**b**
	21	19.7 ± 30.2**a**	15.2 ± 2.2**b**
	28	25.2 ± 15.4**a**	4.4 ± 6.5**b**

Data represents the mean and standard deviation of between 5 and 8 animals per group. There was a significant effect of age on Mn SOD ($F=2.9$, $p<0.05$), CAT ($F=8.0$, $p<0.001$) and GSSG (6.5, $p<0.001$). There was a also a significant effect of oxygen on CuZn SOD ($F=29.6$, $p<0.001$), Mn SOD ($F=32.8$, $p<0.001$), CAT ($F=81.3$, $P<0.001$), GSH-Rd ($F=17.7$, $p<0.001$), GSH-Px ($F=19.0$, $p<0.001$) and GSSG ($F=3.9$, $p<0.05$). A significant interaction between age and exposure was observed for GSH ($F=5.5$, $p<0.005$) and GSSG ($F=5.5$, $p<0.005$). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; compared to 21% oxygen. Data within each exposure group with different letters are significantly different to each other ($p<0.05$). *Abbreviations;* CuZn SOD:- Copper/zinc superoxide dismutase, Mn SOD:- manganese superoxide dismutase, CAT:- catalase, GSH-Px:- glutathione peroxidase, GSH:- reduced glutathione, GSSG:- oxidised glutathione, T:- lung tissue.

of these strands show the typical banding pattern of collagen (Fig 6.7B and C) and in many areas larger contingents of fibroblasts may be observed (Fig 6.7D), many in close proximity to the collagen. Type II cell hyperplasia and alveoli inflammation are typical of many models of chronic oxygen toxicity and BPD. In the present study many alveoli contained neutrophils (Fig 6.8C) and although type II cell hyperplasia was not uniformly observed in all alveoli of oxygen exposed animals, it was apparent in some (Fig 6.8B). Biochemical analysis of BALF also demonstrated increased amounts of PC following oxygen exposure which in Fig 6.8A, B, 6.7B and C is confirmed histologically.

Structural alterations to the large conducting airways are also seen following oxygen exposure (Fig 6.9 and 10). Normal airway architecture is seen in figure 6.9A and B, where bronchial epithelial cells are intact. At higher magnification, these cells are functional and intact with tight junctions between each other (Fig 6.10A). Functional cilia are also observed in these animals (Fig 6.10B). Following oxygen exposure major alterations of the airway epithelia develop. Cells become metaplastic and hyperplastic (Fig 6.9C and D) with an increase in size that sometimes almost completely occludes the airway lumen (Fig 6.9C). At higher magnification these changes are seen as changes in the basic structure and type of the cell, with normal tight junctions disappearing and gaps developing between and beneath cells (Fig 6.10C). Further damage may develop as a consequence of hyperoxia. In some conducting airways, cell necrosis develops with bronchial cells sloughing off from the basement membrane and becoming free in the lumen (Fig 6.11A and B). Mucosal plug formation may then develop (Fig 6.12B) and further hinder effective gaseous exchange.

Finally, other changes distinct from those observed in the alveoli and conducting airways may occur. Capillaries are also seriously affected. Endothelial cells of air-exposed animals are usually flat with very little ruffling of the membrane (Fig 13D). Following oxygen exposure not only do these cells become activated with many ruffles (Fig 13B), but blebbing may also occur (Fig 13A). Endothelial cell hypertrophy is also seen in these animals (Fig 13C). Although there are many alterations that occur following oxygen exposure, widespread endothelial cell damage was not evident.

Figure 6.5. Histological section of lung from preterm guinea pigs exposed to 21% and 85% oxygen for 28 days.

Preterm guinea pigs were removed after day 28 exposure to 21% and 85% oxygen. Animals were sacrificed by pentobarbitone overdose, the trachea canulated and inflated to $10cm^3$ of water. The thoracic and peritoneal cavities were opened, the descending aorta cut and half strength Karnofskys fixative perfused through the pulmonary circulation via the ventricle for one hour. The trachea was then tied and the lung/heart preparation removed *enbloc* and immersed and kept in full strength Karnofskys for 24hr. The left upper lobe of the lung was removed, cut in half and one half taken for light microscopy, the other taken for electron microscopy.

Histological sections from animals exposed to 21% (fig 6.5A and B) and 85% (fig 6.5C and D) were stained with MSB. Sections (fig 6.5A and B) show thin septa between alveoli (arrowed:fig 6.5B) in air exposed animals. Increased interstitial thickening as a consequence of fluid accumulation (*: fig 6.5D) in oxygen treated animals in conjunction with large areas of protein deposition (arrowed:fig 6.5D) just below the alveoli surface. (Bars represent 500μm for figures A and B and 50μm for figures C and D).

A
B
C
D
*

Figure 6.6. Electron micrographs of lung from preterm guinea pigs exposed to 21% and 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Histological sections are from animals exposed to 21% (fig 6.6A) and 85% (fig 6.6B, C and D). Sections show thin septa between alveoli (arrowed:fig 6.6A) in air-exposed animals. Increased interstitial thickening as a consequence of fluid and cellular accumulation (starred: fig 6.6B and D) are seen in oxygen-treated animals. A neutrophil can be seen in a capillary (fig 6.6C) and numerous other unidentified cells within the interstitium (fig 6.6B). Collagen deposits (starred:fig 6.6D) just below the surface of the alveoli may explain proteinaceous material in light microscopic studies. Bars represent 10μm for all figures.

A
B
C
D

Figure 6.7. Electron micrographs of lung from preterm guinea pigs exposed to 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Sections show typical banding pattern of collagen and collagen deposits (starred: fig 6.7A and B) just below the surface of the alveoli. Numerous fibroblasts (arrowed:fig 6.7D) can also be seen in close conjunction with the collagen in the interstitium. Bars represent 1μm for figures A, B and C and 4μm for figure D.

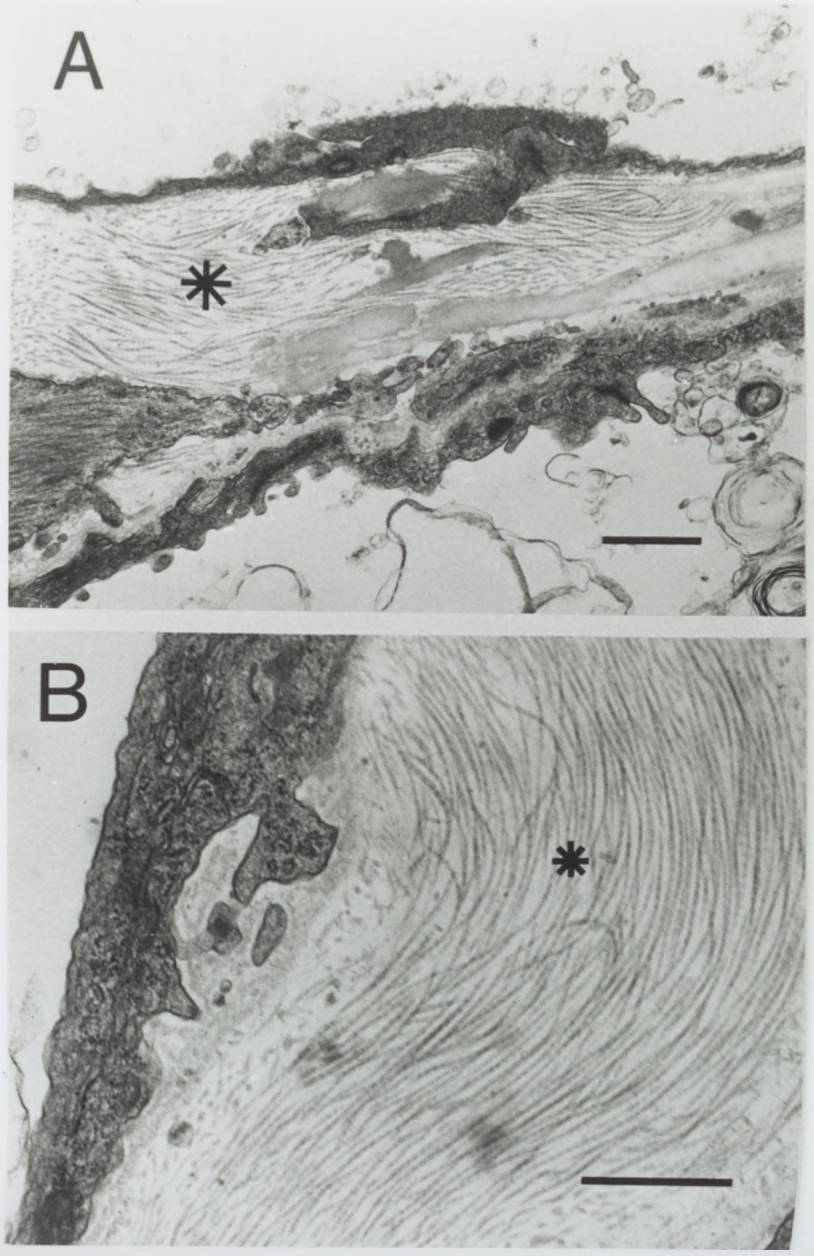

A
B
C
D

Figure 6.8. Electron micrographs of lung from preterm guinea pigs exposed to 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Sections show numerous inflammatory cells (arrowed:fig 6.8C) and over production of surfactant (fig 6.8A) in oxygen-treated animals. Type II pneumonocytes can been seen lining the alveoli in numbers not seen in air controls (arrowed:fig 6.8B). Bars represent 3μm for figure A, 2.5μm for figure B and 1.5μm for figure C.

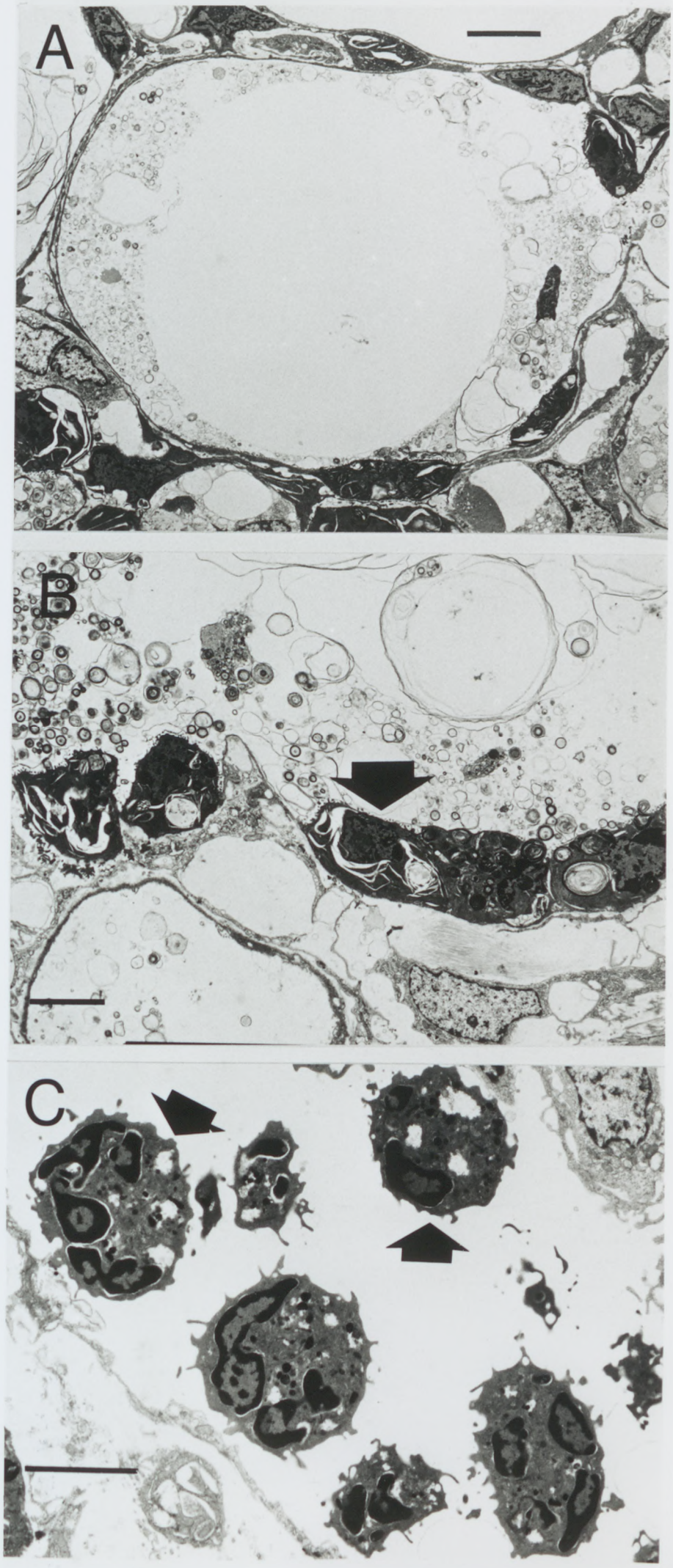

A
B
C

Figure 6.9 Histological section of lung from preterm guinea pigs exposed to 21% and 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Histological sections from animals exposed to 21% (fig 6.9A and B) and 85% (fig 6.9C and D) were stained with MSB. Oxygen exposure results in hyperplastic development of epithelial cells (fig 6.C and D) which almost completely excludes the airway lumen. Bars represent 100μm for figures A, C and D and 500μm for figure B.

A
B
C
D

Figure 6.10. Electron micrographs of lung from preterm guinea pigs exposed to 21% and 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Electron micrographs of epithelial cells from air-exposed animals demonstrate intact junctions (starred:fig 6.10A) with cilia (arrowed:fig 6.10B). Following oxygen exposure the typical structure of the airway is lost and edematous lesions develop beneath epithelial cells (starred:fig 6.10C). Bars represent 2μm for figure A, 0.5μm for figure B and 2.5μm for figure C.

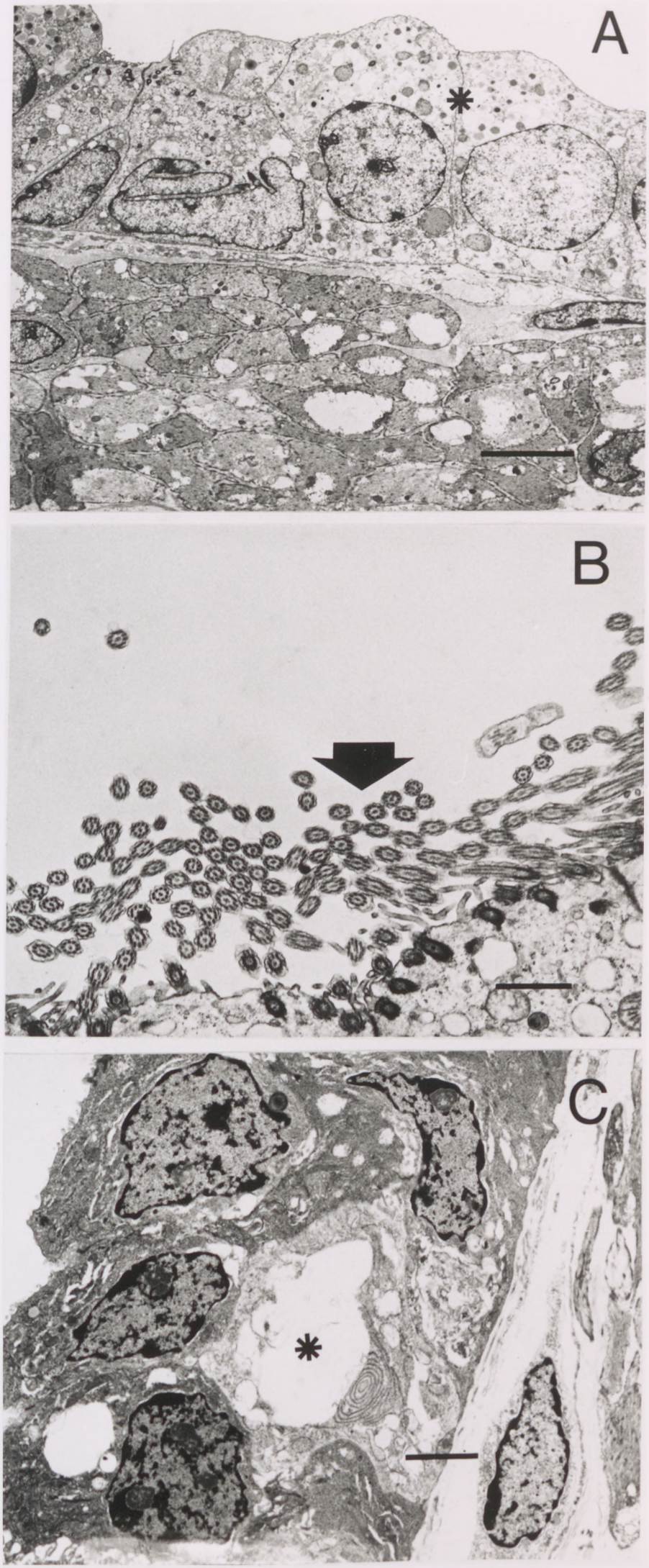

A
B
C

Figure 6.11. Histological section of lung from preterm guinea pigs exposed to 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Sections were stained either with hemotoxylin and eosin (fig 6.11A and B) or MSB (fig 6.11C and D). Oxygen-exposed guinea pig lungs show hyperplastic cellular development (arrowed:fig 6.11C) and necrosis of epithelial cells (arrowed:fig 6.11B). Bars represent 500μm for figures A and C, 100μm for figure D and 50μm for figure B.

A
B
C
D

Figure 6.12. Histological section of lung from preterm guinea pigs exposed to 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Sections were stained either with hemotoxylin and eosin (fig 6.12A) or MSB (fig 6.11B). Histological sections of oxygen-exposed guinea pig lungs show hyperplastic development (arrowed:fig 6.12A) of airway epithelial cells. Necrotic cells can also be seen forming mucous plugs within the the airways (arrowed:fig 6.12B) . Bars represent 500μm for figure A and 100μ for figure B.

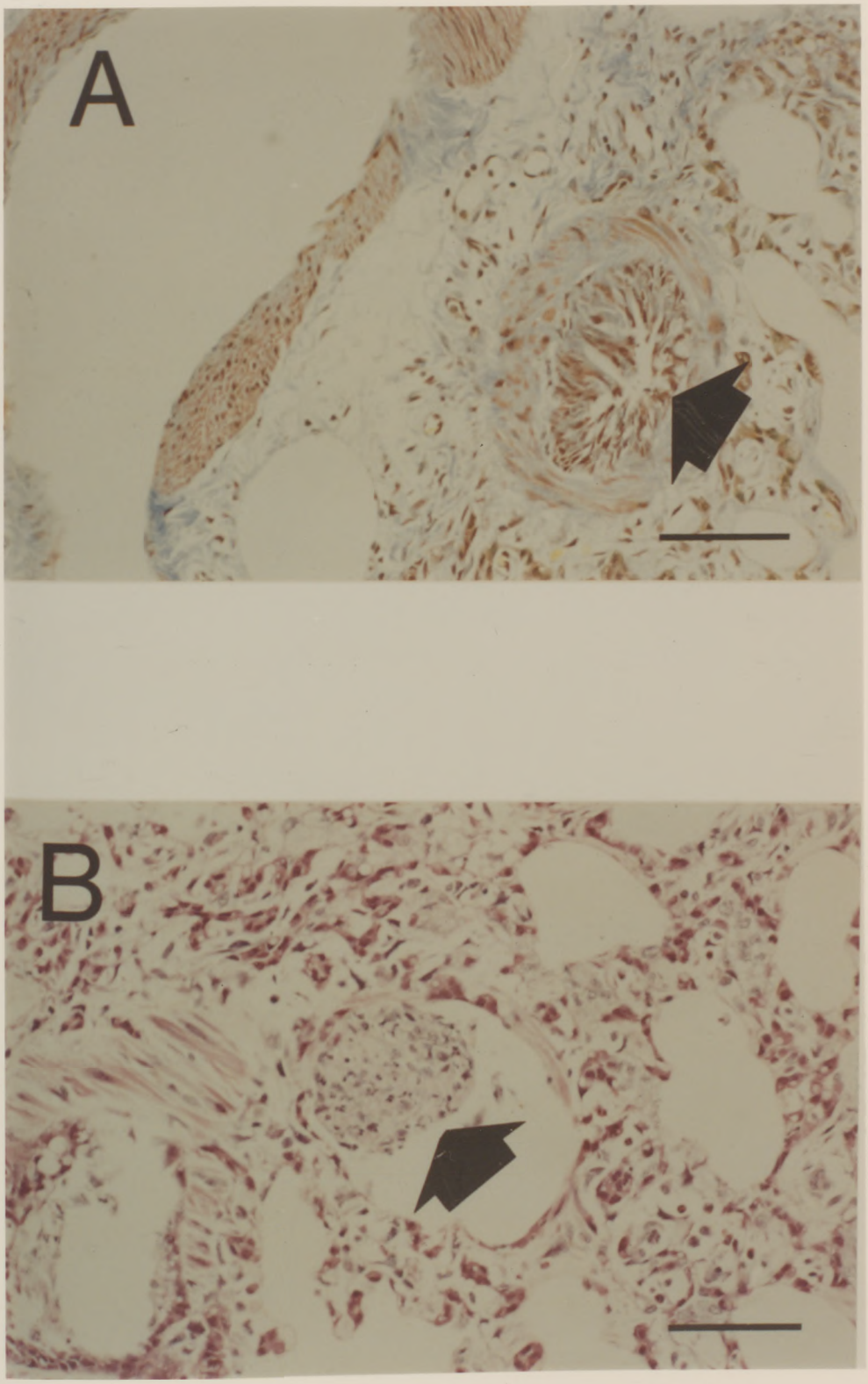

A
B

Figure 6.13. Electron micrographs of lung from preterm guinea pigs exposed to 21% and 85% oxygen for 28 days.

Lungs were prepared for histological analysis as described in Fig 6.5.

Flat unruffled endothelial cells can be seen for air-exposed animals (arrowed: fig 6.13D). Following oxygen exposure, endothelial cells quickly become damaged. Blebbing occurs (arrowed: fig 6.13A) with hypertrophic changes (arrowed: fig 6.13C) and membranes become ruffled (arrowed: fig 6.13B). Bars represent 1μm for all figures.

A
B
C
D

6.4 Discussion.

The diagnosis of BPD includes the following characteristics (O'Brodovich and Mellins, 1985): (1) a respiratory disorder that begins with acute lung injury during the first 2 weeks of life, (2) >28 days of postnatal life, (3) significant clinical (tachypnea), radiologic (hyperinflation) and blood gas tension abnormalities. The reason for attainment of 1 month of age before diagnosis of BPD is due to the fact that poor correlations between radiographic and pathological classifications are seen during the first 3 weeks of life (Edwards, 1977). The pathological and biochemical picture of BPD is not unique as many similar findings have been observed in animals exposed to high concentrations of oxygen. This has therefore lead to the hypothesis that exposure to an elevated concentration of oxygen for a period of not less than 28 days is the major etiological agent in the development of this disease (Banerjee *et al.*,1972; Bonikos *et al.*,1976; Edwards *et al.*,1977). However, many of these animal are adult or newborn and the lungs are therefore mature and able to maintain adequate blood PO_2 without respiratory support, a situation that is not seen in those infants who develop BPD. Also as the biochemical and histological response to oxygen varies with age it makes extrapolation of experimental observations to the human preterm neonate complicated. Therefore, several models of prematurity have been developed to include lung immaturity at the onset of oxygen exposure. Although the most suitable model currently available is the primate (Jackson *et al.*,1987; Meredith *et al.*,1989; Coalson *et al.*,1982), it requires extensive technical, financial and respiratory support and as such a small animal model of prematurity has been developed by the NRRG (Kelly *et al.*,1991). In the present study the response of the immature lung to prolonged exposure (28 days) to hyperoxia was assessed. Specifically, the inflammatory response of the lung during exposure and the eventual, after 28 days, structural changes were characterised.

All animals, regardless of the concentration of oxygen received, developed transient respiratory distress shortly after delivery, a condition that has been attributed to surfactant insufficiency (Hunt *et al.*,1991). Although no deaths occurred in air-exposed animals during the first 3 days, there was a 20% reduction in survival following the full 28 days of the study. These deaths occurred between days 3 and 14, a reason for which remains unclear and requires further study. A second episode of respiratory difficulty was encountered between days 7 and 14, but only in those animals exposed to 85% oxygen. These animals

were inactive, refused to feed and were in respiratory distress. Those that survived this period of respiratory distress tended to survive until the end of the study. However, the mortality rate was significantly higher (30%) than that of air-exposed animals. Although these results concur with a number of survival studies performed on other small animal models of oxygen toxicity such as the rat and mouse (Bonikos *et al.*,1976; Pappas *et al.*,1983) and prematurity (Tanswell *et al.*,1989; Lorenzo, 1985), several other models of prematurity highlight the differences that exist between premature neonates of different species, a phenomena that is also observed between the adult animals. For example, in the preterm rat, pups delivered 24 hours prematurely have very low survival rates in air (6% at 24 hours). In preterm rabbits although survival in air is significantly better, 20% of the premature animals die within the first 24 hours and 40% within 7 days (Lorenzo, 1985). However, even with exact mortality figures it is difficult to equate the results obtained in the present study with those obtained from human infants who develop BPD. In these infants diagnosis of BPD may vary slightly from institution to institution and hence the incidence and mortality from this condition will also vary. However, from available data it is quite clear that the mortality rate is higher in infants who develop RDS and even higher in those that go on to develop BPD (Fisch, 1985). In the premature guinea pig the mortality rate is higher in oxygen-exposed than in air-exposed animals, who also have a significantly higher mortality rate than equivalently exposed term pups.

As with previous acute studies, animals left to recover following Caesarian delivery lost weight over the first 48 hours. Thereafter air-exposed pups put on weight gradually and by the second week were growing by approximately 10 grams per day. Animals that were exposed to oxygen did not achieve birth weight until 10 days after the start of the experiment, some 7 days later than air controls. This alteration in growth has been observed in many other models of oxygen toxicity (Fracica *et al.*,1988; Hayatdavoudi *et al.*,1981) and in human neonates who develop BPD (Fisch, 1985), but as yet has not been confirmed in any other animal model of prematurity. As adaption to oxygen occurred around 10 to 14 days following delivery, animals began to grow, but this was at a much slower rate than that observed for air controls. The reason for this delay and slower rate of growth is unclear, although in human neonates weighing less than 1Kg at birth, low reserves of energy [less than 2% body fat, compared to 16% at full term (Sosenko and Frank, 1991)] and an increased energy expenditure of around 25% (Yeh *et al.*,1989) may occur in the

preterm guinea pig and thereby reducing that energy usually directed for growth.

The increase in body weight of air-exposed animals also reflected an increase in tissue groth. When normalised to body weight, lung wet weight fell with age and heart weight remained constant through out the study. Following oxygen exposure, lung and heart wet weights also increased with age, with lungs being significantly heavier and the hearts consistently lighter than equivalent tissue in animals exposed to air. However, smaller hearts in the oxygen-exposed group could be attributed to the fact that these animals were also consistently smaller than air controls. When the data was expressed per gram body weight, heart weights were then consistently heavier than air controls. These observations are inconsistent with those obtained in the newborn rat exposed to 80% oxygen for up to 12 days (Bucher and Roberts, 1981). In these animals a reduction in both body and lung wet weight leads to a lung to body weight ratio being lower for these animals than for air controls, a result similar to that obtained by Frank *et al* 1978 in the adult rat. Although the reason for this is unclear and may relate to the type of model used, age of the animal and the concentration of inspired oxygen, right ventricular hypertrophy has been observed in the human preterm neonate with BPD (Taghizadeh and Reynolds, 1976). Therefore the observed increase in heart weight, relative to body weight, in the preterm guinea pig may more closely represent what is actually occurring in the human infant with BPD.

The increase in lung wet weight with age and oxygen exposure correlated with a similar increase in lung tissue protein, again consistent with other long term oxygen exposure studies (Hayatdavoudi *et al.*, 1981; Bonikos *et al.*, 1976) in neonatal and adult animals. The accumulation of non-protein fluid from the circulation probably accounts for the majority of the 1 to 2 gram increase in weight, as total lung protein only increased by approximately 100mg over the 28 days exposure period. Increased tissue protein could be due to the presence of plasma proteins, cellular hypertrophy, collagen deposition and/or an increase in the number of inflammatory cells. Although histological assessment of the lung after 28 days oxygen exposure demonstrated an increase in interstitial cellularity this was not confirmed quantitatively or following analysis of total tissue DNA, an observation again in contradiction to a number of published studies (Bucher and Roberts, 1981; Yam *et al.*, 1978; Ohtsu *et al.*, 1989). In the response of the neonatal mouse lung to 18 days 80% oxygen, the DNA content of the lung fell by almost 30% when compared to air-controls (Ohtsu *et al.*, 1989). Similarly, in a study by Frank *et al* 1978 on adult rats the DNA

content of the lung also fell following 7 days exposure to 96-98% oxygen. The reasons for these discrepancies is unclear, although most of the reported effects on lung tissue DNA are from oxygen-exposed term neonates and not from premature animals. However, although total tissue DNA concentration is yet to be reported in another animal model of prematurity beyond 7 days exposure to oxygen, the observations in the premature guinea pig may support the published conclusion that this animal is more able to with stand an oxidative insult than either neonatal or adult animals (Sosenko and Frank, 1987).

Exposure to a high concentration of oxygen has been shown to cause alterations throughout the respiratory tract in humans and in many other animal species. In 1986 Crapo classified the stages of sublethal oxygen exposure into initiation, inflammatory, destructive, proliferative and fibrotic phases, the sequence of which show species variation in the duration and relative severity of each portion of the process. In all species yet studied, the first four phases occur quite rapidly and often overlap within the first 7-14 days. The initiation phase starts at the beginning of oxygen exposure and continues inversely with the concentration of inspired oxygen. A drop from 100% to 85% oxygen prolongs this first phase in the adult rat, such that the beginning of the inflammatory phase occurs on or around day 3, by which time all rats exposed to 100% oxygen have died (Barry and Crapo, 1985; Crapo *et al.*, 1980). This phase may be extended further, as in rabbits where exposure to 60% oxygen results in no detectable morphological or biochemical alterations after 7 days (Holm *et al.*, 1987). However, in these adult animals normal lung structure and function is present at the onset of oxygen exposure. In the preterm guinea pig as in the human neonate, lung structure is already compromised as a consequence of surfactant insufficiency (Hunt *et al.*, 1991) and in both cases this leads to structural damage following alveolar collapse (Kelly *et al.*, 1991). However, previous studies with the premature guinea pig have found increased BALF surfactant at 72 hours (Cabral 1993) in oxygen-exposed animals, which is also confirmed in the present chapter at all other time points. This is in accordance with results obtained by Holm *et al* 1987 on rabbits exposed to 60% oxygen for up to 21 days. However, why and how this increase in surfactant occurs remains speculative, although an increase in the number of type II cells associated with adaption to oxygen and to oxidative-induced type II cell damage may lead to an increased release of this material. As plasma proteins are also known to inactivate surfactant, the increase in this material may be in response to a reduction in the functional state of surfactant already lining

the surface of the alveoli. By increasing the amount of surfactant the animals are able to maintain alveolar integrity even in the presence of large quantities of functionally inactive surfactant.

In addition, premature neonates have a reduced ability to combat an increase in the generation of oxygen centred free radicals (Miller *et al.*, 1993) and following delivery from a relatively hypoxic uterine environment to a relatively hyperoxic external atmosphere this will lead to increased oxygen-centred free radical production in the lung (Freeman and Crapo, 1981; Turrens *et al.*, 1982). The reason for this reduced ability to combat an increase in tissue oxidants may be due to lower levels of protective antioxidants. In the preterm guinea pig lower levels of lung tissue GSH are observed at 72 hours (Langley and Kelly, 1994). In the human preterm neonate, plasma GSSG is higher than that found in term infants (Smith *et al.*, 1993) and may indicate increased oxidative damage as a consequence of significantly lower concentrations of BALF GSH which is observed in infants who develop BPD when compared to those neonates who do not succumb to the disease (Grigg *et al.*, 1993). In addition, there are a variety of enzymatic antioxidants that also aid in the prevention of oxidant damage. As these enzymes increase in activity during gestation (Frank and Sosenko, 1987), following premature delivery the immature lung may not contain the full antioxidant profile that is required for effective protection, this may be especially true for catalase (McElroy *et al.*, 1992).

As the name implies, the inflammatory phase is entered into as soon as changes in the inflammatory cell population within the lung develops. In the air-exposed preterm guinea pig, lung tissue PMN's were already increased in number 72 hours after delivery (Kelly *et al.*, 1991). This would indicate that inflammation develops even in the absence of exposure to elevated concentrations of oxygen. However, this response was transient, localised and quickly resolved as increased neutrophil numbers were not observed either in BALF or blood of these animals. The total number of alveolar macrophages also remained constant throughout the study. However, they were increasingly the predominant cell type, ranging from 45% at day 7 to 75% at day 28. This apparent increase in the macrophage population is not due to an increased number of cells but rather to a progressive fall in eosinophil numbers. This cell type constituted $>60\%$ of the total BALF cellular population at day 7, but only 25% at day 28. This developmental profile has also been observed in term guinea pigs (personnel communications) and represents the normal maturational process.

Following exposure to 85% oxygen, a significant alveolar inflammatory episode develops within 3 days (Barry *et al.*, 1982, Kelly *et al.*, 1993). Although no data is available with respect to the number of circulating inflammatory cells at this time point, at day 7 there was a 5-fold increase in the number of neutrophils and a 20-fold increase in the number of monocytes. Neutrophil numbers then remained elevated through out the study, but the number of monocytes fell to air control levels within 14 days after initiation of oxygen. Within the alveoli, neutrophil numbers were increased by day 7 and peaked at day 14. Alveolar macrophages on the other hand did not increase in number until day 14 and as with neutrophils the total number of cells remained elevated until the end of the study. The reason for this time delay between the peak in the number of inflammatory cells in the blood and the number in the alveoli is unclear, but may relate to the movement of these cells from the circulation into the alveoli. This movement of cells suggests the release of chemotactic factors, but what type and how many are released is unknown. In the oxygen-exposed rat (Fox *et al.*, 1981) and newborn guinea pig (Merritt, 1982) the influx of neutrophils into the lung was associated with a 10-20 fold increase in the chemotactic activity of BALF. Although the chemotactic factors involved in the present study were not examined, LTB_4 has been implicated in the rat (Taniguchi *et al.*, 1986). Leukotriene B_4 as well as C5a has also been implicated in the movement of inflammatory cells from the circulation into the lungs of the premature human neonate with BPD (Groneck *et al.*, 1993; Groneck *et al.*, 1993). In the present study the chemotactic factors involved are unknown and further studies using specific inhibitors, as performed in the acute model (chapter 5) may help in establishing their role in the development of injury to the oxygen-exposed immature lung of the premature guinea pig.

Similarly the inflammatory phase has been identified in various other models of oxygen toxicity. In the newborn guinea pig, exposure to $>90\%$ oxygen results in a significant increase in both BALF macrophages and neutrophils within 72 hours (Merritt, 1982). However, unlike the preterm guinea pig the order in which these cells enter the lung is reversed. Macrophages are first to enter, and increase in number between 24 and 48 hours after initiation of oxygen exposure. Neutrophil numbers also increase but this occurs after the influx of macrophages, sometime between 48 and 72 hours. Why there is this reversal in inflammatory cell movement in the newborn compared to the premature guinea pig lung is unclear and requires further study. In adult rats exposed to 85% oxygen this second

phase also occurs around day 3 (Hayatdavoudi *et al.*, 1981) and in the adult baboon (Fracica *et al.*, 1988; de los Santos *et al.*, 1987) inflammatory cell changes develop within 60 hours of oxygen exposure. In these primates, intravascular neutrophil numbers are increased and following a further 36 hours these cells are seen in the BALF. In the premature monkey vascular, interstitial and alveolar neutrophil numbers are increased during the development of acute HMD, some 2 to 3 days after delivery, and although there are further increases in the interstitial compartment during recovery this is not significant (Jackson *et al.*, 1987). In agreement with the present chapter, macrophage numbers were also increased and occurred much later than the increase in neutrophil numbers. However, BPD in these animals did not develop and thus the role of prolonged oxygen exposure on the inflammatory cell profile was not assessed. In another primate model of prematurity, premature baboons were maintained on high concentrations of oxygen and allowed to develop BPD (Coalson *et al.*, 1982; Coalson *et al.*, 1988; Coalson *et al.*, 1992). In this model tracheal aspirate samples were unable to identify any inflammatory changes associated with BPD, although it was discussed that such changes have been observed, and that were similar to those seen in the human premature infant with BPD. Overall, the influx of inflammatory cells into the oxygen-exposed lung is quite consistent, regardless of the age or species of the animal under investigation. Neutrophils are generally the first cell type to enter the lung and are then followed by the macrophages. However, the extent of alveolar inflammation and timing of this influx is dependant on the concentration and length of exposure to oxygen.

In the human premature infant neutrophils are rarely seen during the early phase of HMD and only increase in number as BPD develops (Ogden *et al.*, 1984; Merritt *et al.*, 1983). Cell numbers peak after about 3 weeks and from then on begin to fall towards baseline. However, even after 4 weeks there is still a substantial cellular pulmonary inflammation. Macrophages also constitute a large proportion of the cells recovered from these infants. The change in the number of these cells is similar to that for neutrophils but tend to increase in number only after that observed for the neutrophil. However, as for the neutrophil a role for the macrophage in the development of lung injury has been speculated. These cells have the potential to cause extensive tissue injury through the release of a multitude of proinflammatory compounds which not only injure tissue directly but also attract and activate other inflammatory cells such as the neutrophil (Nathan, 1987). In

human neonates with BPD, macrophages are known to generate more hydrogen peroxide than similar cells from control infants (Clement *et al.*,1988). However, alveolar macrophages are known to be functionally impaired following exposure to oxygen (Nakamura *et al.*,1988) and as stated earlier for the neutrophil, it is unclear whether the functional status of these cells are different to those of term infants. As such, their exact role in the development of BPD is not known and requires further study.

Sublethal exposure to oxygen associated with a sufficient inflammatory response results in the onset of the destructive phase. In most animal models increased numbers of neutrophils occur concomitantly with lung injury. This is usually manifested by an increase in lung wet weight due to the breakdown of the semipermeable nature of the lung. This alteration in permeability results in the flood of plasma proteins into the alveolar lumen, protein that can be sampled following BAL. In air-exposed premature guinea pigs, increased lung wet weight and BALF protein were observed at 72 hours (Chapter 3) when compared to equivalently aged term pups. In addition to the elevated numbers of tissue neutrophils (Kelly *et al.*,1991), it was speculated that these inflammatory cells may contribute to the observed injury. In the present chapter, BALF protein rose through out the period of study in air-exposed animals but not to the extent that it represented lung injury. Previous studies have shown that the concentration of BALF protein from air-exposed animals falls significantly between days 3 and 7 (Town 1990). Thus the increase observed in the present study likely reflects an increase in the number of alveoli being sampled. These results therefore highlight the problem of using BALF protein as a marker of altered pulmonary permeability and suggests that other methods should be investigated. These methods include the use of intravenously administered labelled albumin or trypan blue, both of which will be elevated in BALF if lung injury is present. Following oxygen exposure, a close correlation between neutrophils and lung injury has been previously reported (Kelly and Phillips, 1988) in the preterm guinea pig, with injury occurring some time between 24 and 48 hours following initiation of exposure to 95% oxygen. In animals exposed to 85% oxygen for 72 hours, no increase in BALF protein is seen (Ballard *et al.*,1992), although as stated earlier, there is a systemic and alveolar inflammation. Exposure at this concentration for longer, results in the development of lung injury that manifests at some point between days 3 and 7 as shown in this chapter. BALF protein peaks at day 7 and falls to control levels by day 14. However, lung wet weight remained elevated

through out the study. On comparison with preterm infants developing BPD, BALF albumin, although significantly greater than controls for at least the first 3 weeks, falls to control levels at the same point at which chronic lung disease is assessed (Ogden *et al.*, 1984). Therefore in the premature human and guinea pig, adaption to oxygen occurs that prevents the continuation of plasma protein movement into the alveoli. How and why this adaption develops is speculated later on in the discussion. In most animal species, this phase is also identified by extensive changes in the structure of most compartments of the lung. In the present thesis, light and electron microscopic examination of the lungs of the oxygen-exposed premature guinea pig was investigated to compare and contrast the alterations with those seen in the human infant with BPD.

First recognized by Northway and colleagues in 1967, BPD is a form of unresolved lung injury which progresses from the "stiff lung" disease of HMD to severe chronic obstructive pulmonary disease over the first few weeks of life. Chest radiographs show evidence of over inflation and alternating strands of opacification, interpreted as fibrotic strands. Pathologically, reports published in the literature represent the extreme end of the BPD spectrum and often contain non quantitative analysis and subjective descriptions of the observed abnormalities. However, these detailed descriptive studies have revealed a chronological progression from an initial exudative stage of diffuse alveolar damage to a regenerative stage with repair. At this later stage there is severe damage to the airway epithelial cells which are often necrotic and obliterating the lumen. Cystic dilation of distal airways also occurs and results in overexpanded regions of the lung that are interspersed with dense fibrotic areas. Although interstitial fibrosis is an essential feature of BPD, very little lung collagen measurements have been made in these infants. Distended lymphatics and oedematous interstitial spaces are also evident (Taghizadeh and Reynolds, 1976) and as with most pathological studies of BPD, no quantitative analysis of the pulmonary vasculature has been performed although arterial muscular hypertrophy is usually apparent (Desa, 1969).

There have also been numerous pathological reports on the effect of long term oxygen (60% to 100%) exposure on the lungs of newborn and adult animals (Hayatdavoudi *et al.*, 1981; Coalson *et al.*, 1988; Pappas *et al.*, 1983; Holm *et al.*, 1987). Ultrastructural and morphometric studies of adult rats exposed to 85% oxygen for up to 14 days show changes in type II but not in type I or endothelial cells during the first 5 days of exposure. By day

14, hypertrophy and proliferation of type II cells, proliferation of interstitial cells, interstitial inflammation and a decrease in the number of capillary endothelial cells have been described. This loss of capillary endothelial cells (Coalson *et al.*, 1992) which although not quantitated in the guinea pig may account for the observed increase in lung wet weight and BALF protein. Three types of minor structural alterations were observed in the endothelial cells of these animals. Although not widespread the most frequently observed alteration was cellular hypertrophy. Endothelial cell membranes were also often ruffled and in the larger blood vessels some cells contained vacuoles, changes that are frequently observed in neonates who have died from BPD (Bonikos *et al.*, 1976). Studies conducted in newborn mice (Pappas *et al.*, 1983) following exposure to 80% oxygen for up to 6 weeks results in significant pulmonary injury. After 4 weeks oxygen there is extensive bronchiolar submucosal inflammation, beneath degenerating mucosal cells. There is a patchy reduction of ciliated cells, some necrosis of airway epithelial cells, but not extensive enough to expose the underlying basement membranes. Alveolar walls also appear thickened, mainly attributable to an increase in fibroblastic cells, collagen and fluid. An important model that has been pursued with respect to the pathology of BPD is the premature baboon (Coalson *et al.*, 1988; Escobedo *et al.*, 1982; Delemos *et al.*, 1987). Exposure of the premature baboon for up to 11 days results in histopathological alterations that resemble BPD. There is often focal haemorrhage, interstitial oedema and increased connective tissue deposition has also been identified in the saccular walls. Medial hypertrophy, a feature often seen in neonates with BPD, in arteries accompanying airways was not seen. Ultrastructurally, endothelial vacuolisation and necrosis were evident. Increased mononuclear and fibroblasts are seen in the interstitium and the alveolar epithelium showed sites of type II cell hyperplasia.

Many of these alterations seen in the human and primate premature neonate with BPD have also been identified in the present chapter. In all three species, large deposits of collagen and increased numbers of fibroblasts are seen in the interstitial compartment. A major finding in the present study is the susceptibility of the airway epithelial cells to hyperoxia. Many of the airways in the guinea pig have regions of epithelial cell necrosis, cells sloughing of the basement membrane and forming plugs in the airways. In other airways there is extensive epithelial cell hyperplasia and hypertrophy, all characteristic features of the human infant with BPD. However, in both human and baboon infants, there are extensive areas of atelectasis and hyperexpansion, areas which are few and far between

in the premature guinea pig. This may possibly be due to the relatively mature state of the guinea pig lung at day 65 of gestation and to see this in the model, animals must be delivered earlier and kept alive. However, this may entail mechanical ventilation as the survival of these younger animals without effective respiratory support is very low.

A proliferative phase begins near to the time of the destructive phase. This proliferative response includes all major compartments in the alveolar septum and is probably responsible for the blunting of the destructive phase and the survival of the animal. If this is so, then in the present study, the proliferative stage starts at around day 14 as all animals that had reached this time point, regardless of the concentration of oxygen survived. In rats exposed to 85% oxygen there is a four-fold increase in the number of interstitial cells, which are predominately fibroblasts and monocytes. In the present study a similar increase in the number of monocytes must of occurred between days 7 and 14 as circulating monocyte numbers peaked at day 7 and alveolar macrophages peaked at day 14. The number of fibroblasts were also increased in number although no quantitative data was obtained. In rats an epithelial response also develops. There is an increase in the number and size of the type II cells, a response that is seen in other models of oxygen toxicity. Although in the present study, a change in the number and type of epithelial cells was not measured changes in the concentration of BALF protein without a concomitant change in lung wet weight indicates adaption to oxygen in these cells because if endothelial cell adaption had occurred then both lung wet weight and BALF protein would not be elevated.

Although these changes in the cellular profile of the lung may aid in the blunting of the inflammatory and destructive phases of oxygen toxicity, other aspects of the biochemical nature of the lung may also participate in these changes. one of these aspects includes the response of the antioxidant system of the lung. Hyperoxic exposure of lung tissue is known to result in increases in a variety of antioxidants. Tissue and BALF GSH and enzymes of the glutathione system in rats exposed to 80% oxygen for 5 days is significantly greater than air controls (Jenkinson *et al.*, 1988) and in the premature guinea pig there is also a marked increase in the antioxidant activity of the lung tissue, especially from the glutathione system following 3 days exposure to 85% oxygen (Kelly *et al.*, 1993). In the present study, both tissue and BALF glutathione (oxidised and reduced) concentrations are significantly elevated. BALF and lung tissue glutathione (reduced and oxidised) fell back to control levels by day 14 and remained at this level until the end of the study. similar changes were

observed in lung tissue enzymatic antioxidants, the reason for which is unclear, as the same concentration of oxygen is being inspired at day 28 as it is at day 7. Therefore these results suggest that the sustained increase in lung tissue antioxidants is not an essential prerequisite for survival in oxygen after 14 days. With the antioxidant activities of the lung returning to control values the tissue seems more than able to withstand the oxidative insult. Whether this is due to some other form of adaption that reduces the formation of free radicals in hyperoxia is unclear.

The final phase in sublethal exposure to oxygen is the fibrotic phase. In the present study increased collagen deposition was observed histologically throughout the lung, but appeared most prominent around the smaller airways. Biochemically, although the total lung collagen content of the oxygen-exposed animal was almost double that of the air controls, the tissue concentrations were similar. This discrepancy between the histological and biochemical data may be artifactual as the increase in tissue weight that is observed in the oxygen-exposed animals appears to be primarily due to increased water content of the lung and not lung tissue. Also the localised or regional deposition of collagen around the airways may only represent a minor part of the total collagen in the lung and any increase may be to small to be picked up biochemically. However, this patchy nature of the fibrotic response to hyperoxia is also seen in the preterm infant with BPD (Stocker 1986). In this report infants with long standing healed BPD had little or no fibrosis in areas of the lung that appeared normal or were overexpanded. However, in areas that were exposed to the full measure of respiratory support, such as the airways, were fibrotic. As such the data in the present study mimics the patchy nature of fibrotic lesions that develop in the premature human neonate. What is unclear at present is whether the lesions observed in the oxygen exposed premature guinea pig lead to impaired gaseous exchange and to the alteration of respiratory function that is also seen in these infants.

In summary, the data demonstrated that prolonged exposure to 85% oxygen resulted in the development of a persistent alveolar inflammation and structural changes to the lung at 28 days that resembled the lungs of infants with BPD. As such the data presented in this chapter demonstrates that the oxygen-exposed preterm guinea pig is a suitable model of prematurity in which to investigate the role of hyperoxia other factors thought to be involved in the development of BPD.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

The aetiology of BPD is multifactorial in origin but includes lung immaturity and the concentration of inspired oxygen. However, due to the practical and ethical problems associated with the study of these neonates the exact role of any one particular component in the development of this disease is unclear. In their original report published in 1967, Northway *et al* postulated that the pulmonary lesions observed in BPD were due to the effects of high concentrations of oxygen on lungs that were healing from RDS. This was later reinforced by other reports (Edwards *et al.*, 1977; Banerjee *et al.*, 1972; Rhodes and Hall, 1975), but as many of these infants also received mechanical ventilation it has made precise interpretation of the role of oxygen in neonatal lung injury difficult. Thus a number of animal models have been developed to assess the role of oxygen in the development of BPD. These models have shown that exposure to oxygen can produce severe functional and morphological changes in the lung that are also seen in infants with BPD (Winter and Smith, 1972). However, the problem that is encountered in many of these models is the fact that the lungs of these animals are mature and able to maintain adequate blood PO_2 levels without respiratory support. Also as adult and neonatal animals of various species exposed to high levels of oxygen respond differently according to length of exposure and concentration of inspired oxygen (Frank *et al.*, 1978) it has made extrapolation of experimental observations to the human preterm neonate complicated. Therefore several animal models of prematurity have been developed. However, only a few have made significant contributions to the understanding of neonatal lung disease and even fewer have meet the criteria laid down by the Workshop on BPD convened under the auspices of the National Heart, Lung and Blood institute in 1979. Those that have, such as the preterm monkey and baboon (Kessler *et al.*, 1982; Jackson *et al.*, 1987; Delemos *et al.*, 1987; Escobedo *et al.*, 1982), require extensive respiratory, financial and technical support. As a consequence, a small animal model of prematurity has been developed by the NRRG in the guinea pig (Kelly *et al.*, 1991) which offers a greater degree of flexibility in the type, nature and extent of experiments that can be undertaken.

A characteristic feature of the oxygen-exposed lung in infants who develop BPD and in most animal species, is the development of an inflammatory reaction (Barry *et al.*, 1982; Fox *et al.*, 1981; Ogden *et al.*, 1984; Merritt *et al.*, 1983), the components of which are known to be harmful. As such, a role for oxygen-induced inflammation in the development of injury to the immature lung has been speculated. However, an in depth assessment of

the consequences of specific inflammatory components on the lung of the premature human infant has proved problematic.

Therefore the specific aims of the thesis were two-fold. Firstly to characterise the development of oxygen-induced acute and chronic injury and inflammation in the immature lung and secondly to assess the role of specific components of the inflammatory reaction associated with acute oxygen-induced lung injury.

Following Caesarian section at 65 days of gestation (term = 68 days) all animals developed transient respiratory distress which was characterised by tachyopnea, grunting and rib retraction. This time point was chosen for a number of reasons. Firstly animals delivered at much earlier time points had unacceptably low survival rates (Kelly *et al.*, 1991). Secondly, the lungs of these animals were immature, an essential prerequisite for the study of HMD and BPD. On delivery, premature guinea pigs were significantly smaller and were also less able to maintain their initial body weight over the first 48 hours in air (preterm:- 11% drop in body weight, Term:- 4% drop in body weight) when compared to equivalently treated term animals. A number of reasons for this were put forward. Firstly, it was unclear whether acceptance of the premature pup by the surrogate dam was effective immediately. If not, feeding may have been delayed and not enough food ingested for adequate growth. Secondly, as these animals developed respiratory distress, the increase in energy expenditure in breathing necessary to maintain an adequate blood PO_2 level may have redirected that energy normally used for growth. Therefore, whether through respiratory distress and/or inadequate food intake, growth was adversely affected. However, within 3 to 5 days of delivery all premature pups had achieved birth weight and began to grow at a steady 10gm/day. These results however must be taken in context with the fact that the lower fall in percentage body weight observed in term animals was only seen in Chapter 3. In Chapter 4 the fall was 9.5% over the first 48 hours and equates to that observed in preterm animals. Also, these animals had a mean body weight that was similar to preterm animals at 65 days of gestation (mean and SD of preterm animals used in all Chapters: 81.5 ± 3.0) and possibly indicates that these pups were less mature than those animals used in Chapter 3. However, the greater number of term animals used in Chapter 3 more likely reflects the true weight at term. Apart from this discrepancy, the mean body

weight for preterm pups was very similar between studies (Body weight [CV%] for all preterm pups at birth is 3.7%).

The mortality rate for these animals over the first 3 days was significantly higher than that for term pups and is in accordance with many other animals delivered prematurely at the same time point in gestation (Kessler 1982, Lorenzo 1985, Jackson 1987). As in the human neonate, surfactant insufficiency was implicated as the primary pathophysiological abnormality associated initially with the development of respiratory distress and also to the increase in the mortality rates of these animals (Hunt *et al.*,1991; Kelly *et al.*,1991). However, surfactant replacement studies have not been performed on these pups and it is as yet unclear whether these observations are solely due to surfactant insufficiency.

On closer examination of these lungs, previous studies have demonstrated evidence of injury with hyaline membranes lining the alveoli, atelectasis of the parenchyma and areas of pulmonary oedema and fibrin deposits (Town 1990). All these features observed in the air-exposed premature guinea pig are also characteristic features of the human infant with HMD (Merritt *et al.*,1983; Gandy *et al.*,1970; Finlay-Jones *et al.*,1974). In addition, inflammation was present which was localised to the interstitial compartment of the lung (Kelly *et al.*,1991). It is unknown at present at which time point after delivery this inflammatory episode began or for how long it continues, as the number of neutrophils in the blood and BALF of these animals remained unaltered throughout the 28 days in which the animals were assessed. Also no quantitative data was obtained for tissue neutrophils at points beyond 3 days. However, these cells have the potential to cause extensive tissue injury through the release of proteases and activated species of oxygen (Strauss and Snyder, 1983; Smedly *et al.*,1986; Stone, 1990; Matteo and Smith, 1988) and as such have been implicated as major participants in the development of neonatal lung disease. In human infants, the possible effect of neutrophil proteases and free radical release on the lung is compounded by the fact that they often have lower than normal levels of circulating protease inhibitors (Andrew *et al.*,1983) and pulmonary antioxidants (Grigg *et al.*,1992; Grigg *et al.*,1993).

An imbalance in the protease-antiprotease profile of BALF from air-exposed animals has been shown previously (Kelly *et al.*,1991) and this imbalance was further highlighted by results shown in chapter 3, in which plasma α1-PI activity was shown to increase with increasing gestational age. As such, those animals delivered prematurely have lower

concentrations of circulating inhibitors than is associated with animals at term. Although, neutrophil elastase activity was not detected in the BALF of these animals, this does not indicate that elastase is not being released. The assay used in the thesis only measures the activity of free or α2-MG bound elastase and not immunological levels. Therefore elastase released in to the alveolar lumen may have been effectively inhibited by α1-PI. This inhibitor covalently binds to elastase and completely inhibits the enzymatic activity towards the substrate. Therefore even with no detectable elastase in BALF, elastase may be present and have contributed to the observed injury to the immature lung. To fully assess this assumption, immunological levels must be measured.

As neutrophils have the capacity to release oxygen centred free radicals that can injure lung tissue directly, there is a wide variety of antioxidants available to protect the lung from these harmful species (Heffner and Repine, 1991). Previous studies have shown that the enzymatic antioxidant profile of the guinea pig lung increases with increasing gestational age (Rickett and Kelly, 1990). As such, animals delivered early may have a reduced ability to protect themselves from an oxidative insult, such as that from activated inflammatory cells. In support of this, lower 6PGDH activity, an enzyme of the pentose phosphate pathway, was observed in these animals. This enzyme is part of an important biochemical pathway that generates reducing equivalents necessary for a wide variety of functions including the antioxidant activity of the glutathione cycle. However it is known that the neonatal animal of various species are relatively tolerant to hyperoxic stress. The basis of which is believed to be their ability to induce antioxidant defences (Yam *et al.*, 1978; Deneke and Fanburg, 1980) and consequently the basis of decreased lung injury and survival. In the guinea pig it has been shown that this response is dependant on the maturity of the lung and the concentration of oxygen the animal is exposed to (Kelly *et al.*, 1993). As such the preterm guinea pig is more able to induce antioxidant enzymes than term neonates after 72 hours exposure. However, this again does not explain why premature animals have a greater degree of lung injury than that seen in term pups, unless the degree in which the antioxidants are induced fails to protect against the increase in free radical production following exposure to 21% oxygen. Alternatively the observed injury may be independant of any free radical based mechanism. Also if there is a failure of the immature lung to effectively counteract the increase in oxygen centred free radical production, this also may lead to the inactivation of several important molecules that may further aid in lung

injury and organ disfunction. As discussed earlier, circulating concentrations of α1-PI may be low in these infants. This inhibitor can be inactivated by oxidation of the active site methionine residue (Swaim and Pizzo, 1988). If increased free radical production is present than this inhibitor could be inactivated and the protease-antiprotease balance upset in favour of tissue damage.

Inflammatory cells within the interstitium of these animals may also contribute to the development of lung injury through the release of superoxide. However, results on BALF inflammatory cell superoxide production showed and agreed with previously published data on blood neutrophils (Strauss and Snyder, 1983; Okano *et al.*, 1991), that these cells were less capable of generating superoxide than equivalently treated term animals. Thus although the premature guinea pig may have lower levels of protective antioxidants, the inflammatory cells present within the lung may release less superoxide once activated than term pups. This also raises the question as to whether the neutrophil from these animals are also capable of releasing the same amount of proteases as full term animal. Thus, it is difficult to establish whether the lungs of the premature guinea pig and human are relatively less able to withstand oxidative and proteolytic damage from invading inflammatory cells than neonatal or adult animals. What is clear, is that delivery into 21% oxygen is a significant oxidative insult to both term and preterm infants. As this will result in the increase in tissue oxidants, the preterm lung is initially more prone to oxidative injury. If this is the case then as a consequence, more inflammatory cells will invade this organ and the intensity of the resulting inflammatory event will be greater. This may in fact be what we are seeing in these animals as term guinea pigs do not experience a pulmonary inflammatory event following delivery (Town 1990).

The alveolar macrophage is another important inflammatory cell in BPD and a modulator of inflammation (Nathan, 1987). In the mature human lung of the newborn term infant the increase in macrophage numbers occurs as a consequence of local proliferation and monocyte recruitment (Evans *et al.*, 1987). However, in infants who develop BPD this increase is blunted (Grigg *et al.*, 1993). This is also seen in the air-exposed preterm guinea pig, the reason for which is unclear, although it has been postulated that this failure to increase in numbers is due to the absence of surfactant (Arnon *et al.*, 1993) or as a consequence of the cells susceptibility to oxidative damage (Sherman *et al.*, 1988). However, within 5 days of delivery the macrophage population doubles and remains at this

level for at least 28 days. This increase in numbers at day 5 is mirrored by a similar increase in the number of blood monocytes. As the monocyte is the precursor of the tissue macrophage this correlation is not surprising. However, within 2 days the number of circulating monocytes fall but the number of alveolar macrophages remained at the same level.

Not surprisingly it was also found that the lungs of these animals were heavier and had significantly more BALF and lung tissue protein than that of equivalently treated term pups. BALF protein and lung tissue wet weight have been used in the present thesis as markers of lung injury. These markers are all elevated at day 3 and fall as the animal adapts and grows in air. By day 28, although the concentration of BALF protein was greater than that of animals at 7 days of age, this may have been due to the sampling of larger areas of a much larger lung. However, these changes do highlight the problem that may be encountered if BALF protein is continued to be used as a marker of lung injury. Lung wet weight on the other hand can be expressed as a function of age using body weight and thus takes into account this effect of growth. However, both markers do indicate and agree with previously published observations (Kelly *et al.*, 1991) that lung injury does develop during the first few days in the air-exposed preterm guinea pig.

The increase in inflammatory cells within the lung associated with air-exposed premature guinea pigs, suggests the presence of chemotactic factor(s), such as LTB_4. Although LTB_4 was detected in the BALF of these animals it is unclear why very few neutrophils were found within the alveolar lumen. It is also unclear which cell type(s) are releasing these factor(s). As a consequence of the presence of this lipid in the BALF, it was suggested that other components of inflammation may also be present. This was further strengthened by the fact that BALF PLA_2 activity was greater in air-exposed animals than in equivalently exposed term pups. PLA_2 is central to the generation of prostanoids, leukotrienes and can be activated by reactive oxygen species (Goldman *et al.*, 1992) that may be increased in concentration in the immature lung. As a consequence of the presence of PLA_2 in the lungs, many other mediators may have also been released into the neonatal lung. Thus, this may indicate mechanisms other than direct mechanical damage due to alveolar collapse may operate to cause lung injury soon after birth. The sequence of events that lead to PLA_2 activation and eventual lung injury is shown diagrammatically in figure 7.1. Delivery of neonates into the world results in the exposure of the lung to a 5-fold increase in oxygen

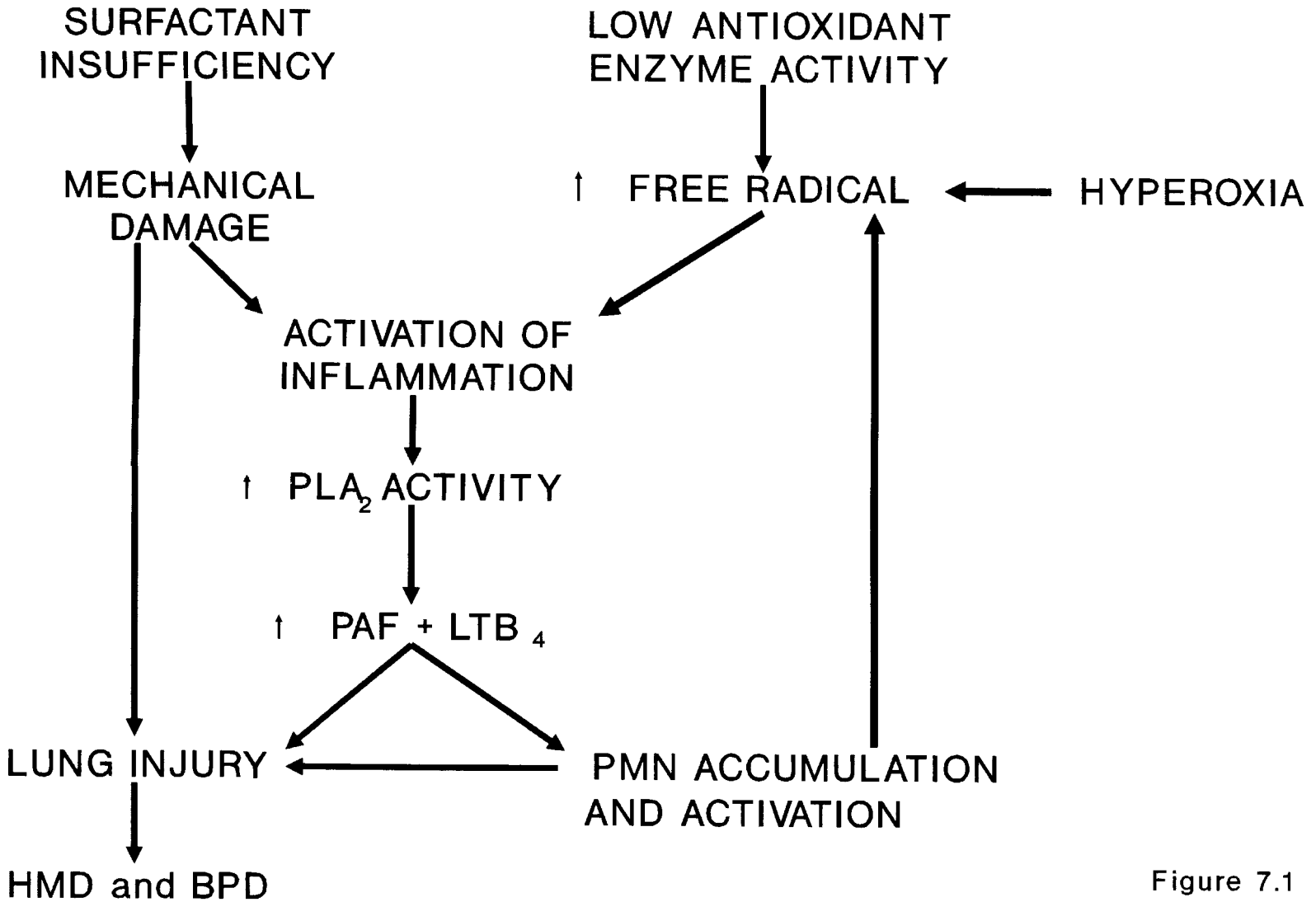

SURFACTANT INSUFFICIENCY
LOW ANTIOXIDANT ENZYME ACTIVITY
MECHANICAL DAMAGE
↑ FREE RADICAL
HYPEROXIA
ACTIVATION OF INFLAMMATION
↑ PLA2 ACTIVITY
↑ PAF + LTB4
LUNG INJURY
PMN ACCUMULATION AND ACTIVATION
HMD and BPD

Figure 7.1

concentration and consequently to an increase in the production of oxygen centred free radicals. In healthy newborn lungs this increase is prevented from damaging tissue by antioxidant protective mechanisms.

Therefore following premature delivery the preterm guinea pig maintained in 21% oxygen develops lung injury that is characteristic of HMD. The development of injury in these animals was suggested to occur by one or all the mechanisms shown in figure 7.1. Direct lung injury may occur as a result of insufficient surfactant. This leads to alveolar collapse and hence mechanical damage to the alveolar walls. Plasma proteins flood into the lung as a consequence and further inactivate surfactant. Hence a cycle of damage and inactivation may occur. Mechanical damage may also activate an inflammatory reaction with the release of pro-inflammatory lipids that not only have a direct effect on the permeability of the lung but also attract and activate inflammatory cells. These cells may then release more pro-inflammatory lipids and other compounds that injure tissue. Further to this as infants are antioxidant deficient, increased free radical production following birth will injure tissue directly, activate PLA_2, initiate inflammation and inactivate protease inhibitors. In all, lung injury in the premature neonate may be brought about by a combined effect of a number of immature physiological systems that are unable to cope effectively with the changes associated with birth.

Premature human neonates who develop respiratory distress soon after delivery often require extensive respiratory support, including the use of supplemental oxygen at levels as high as 100% for many weeks. As such the premature guinea pig was given 95% oxygen for up to 3 days and the development of an acute inflammatory response assessed. Following exposure to oxygen inflammation observed in air-exposed animals was greatly enhanced. Lung wet weight, BALF protein, circulating and alveolar neutrophils, LTB_4 and neutrophil elastase activity were all significantly increased, observations consistent with that found in human premature neonates with acute and chronic lung disease.

As the number of neutrophils increase concomitantly with an increase in lung injury (Kelly and Phillips, 1988; Fox *et al.*, 1981), a role for this cell type in disease has been speculated. To investigate this the role of neutrophil superoxide production from these cells was measured. Unfortunately, it was found difficult to extract the neutrophil from other cell types in BALF. However as with previously cited studies (Krieger *et al.*, 1985; Kelly *et*

al., 1991) it was shown that following exposure to hyperoxia that the superoxide production from BALF cells was significantly reduced. Reasons for this reduction in the capacity of inflammatory cells to generate superoxide was suggested and included the possibility of increased susceptibility to auto-oxidative damage due to lower cellular antioxidants and/or to the possibility of over stimulated and consequently down regulated inflammatory cells. These cells would thus require a much greater stimulus to generate the same quantity of superoxide as that of inflammatory cells from term animals. Thus the role of these cells in oxygen-induced lung injury with respect to free radical production is thrown into question. However, to clarify, superoxide production was assessed at 72 hours post delivery and what is at present unclear, is whether these cells had already produced superoxide at a much earlier time point. This is supported by the observation of Kugo *et al.*, 1989 who showed that although superoxide production is significantly decreased in inflammatory cells from adult rats exposed to 95% oxygen, elevated levels of superoxide are released at a much earlier time points. Therefore it would be necessary to repeat this first experiment, but assess superoxide production at regular intervals up to 72 hours. Neutrophils isolated from the peritoneum of term and preterm guinea pigs generated similar amounts of superoxide on stimulation with PMA. However, it was stated that although valid, these results may not highlight superoxide production from lung neutrophils as the response of inflammatory cells to stimuli is dependant on tissue site and initial activational state. Thus further studies are required to isolate the neutrophil from BALF.

The second experiment further attempted to assess the role of the neutrophil in hyperoxic-induced lung injury using a specific neutrophil antibody to deplete these cells from the circulation and hence the lung. Although circulating neutrophils were reduced to 7% of undepleted animals after 3 days, BALF neutrophils were only reduced by 60%. BALF protein concentration, lung wet weight and neutrophil elastase activity remained unchanged. It was suggested that either the remaining neutrophils in the lung were still able to cause the same extent of lung injury as observed in undepleted animals or that this cell type is not involved in hyperoxia-induced acute lung injury. It was also pointed out, that in the hyperoxia exposed preterm guinea pig, the neutrophil population constitutes approximately 5%-10% of the total cell population whereas in the preterm human neonate with RDS or BPD, the neutrophil constitutes greater than 90% of all cells recovered by BAL. Thus, although the neutrophil may be an important cell in lung injury in the human

neonate, the small number of neutrophils in the preterm guinea pig model may not have a significant role in hyperoxia-induced lung injury. The reason for the small number of neutrophils entering the lung during hyperoxic exposure is unclear, although the extent of pulmonary immaturity may have a bearing. The more immature the lung the greater the risk of lung disease and the greater the degree of inflammation. Thus to equate more closely with the human preterm neonate, premature guinea pigs must be delivered at earlier time points in gestation. However, this would complicate the assessment of the role of oxygen in lung injury, as previous observations in the guinea pig have shown that delivery at much earlier time points leads to the requiremnet for ventilation and the use of ventilation itself is an additional factor in BPD.

Another inherent problem with the preterm guinea pig model, again due to the inflammatory cell profile of the lung, is the fact that this animal is essentially eosinophilic. At birth, whether premature or not, the lung is populated with up to 90% eosinophils. In the human neonate, the major cell type present is the macrophage. When RDS or BPD is diagnosed, eosinophils are essentially absent from the lungs of the human neonate but constitute approximately 60% at day 3 and 15% at day 28 in the oxygen-exposedpreterm guinea pig. Like the neutrophil, the eosinophil has the potential to cause extensive tissue damage via mechanisms that are present within the neutrophil. In Chapter 4 it was shown that the majority of the superoxide generated by inflammatory cells recovered by BAL was associated with the eosinophil. Therefore regardless of the neutrophil influx, the tissue damage following exposure to hyperoxia may develop as a consequence of activated eosinophils. As such, the role of this cell type in the development of oxygen-induced lung injury will have to be explored further.

A further problem associated with the guinea pig in addition to other animal models is the variable nature of the response to a particular challenge. This is seen in the present thesis when BALF total and neutrophil cell numbers are compared between Chapters. Overal the percent CV (a measure of variation) for the total number of cells, whether exposed to 21% or 95% oxygen, is 18% and for neutrophils is 80%. The reason for the very high variation seen in the neutrophil count is due to the small number of these cells in BALF, especially in air exposed animals, and that the influx of these cells following exposure to oxygen is in itself highly variable. This variability is dependant on many other factors, such as nutrtion, quality of the surrogate mother, number of pups in each litter and

having animals of the correct gestational age.

In these two experiments, the role of inflammatory cells were assessed for their contribution to lung injury. Although at 72 hours the data suggests that inflammatory cells may not play a major role in lung injury, this is not known for sublethal exposure to oxygen at time points up to day 14 as shown in chapter 6. Further experiments are therefore required.

As in air-exposed animals lipid mediators were also implicated in the movement of inflammatory cells in to the lung of the oxygen-exposed animal and to the subsequent development of injury. Although LTB_4 concentration were significantly elevated in these animals, PAF was undetectable. However, following treatment with the PAF antagonist the inflammatory response associated with hyperoxia was blunted. This reduction with WEB2086 was also seen for BALF protein and neutrophil elastase activity. It was suggested that this reduction may be due to the direct inhibition of the PAF associated alteration of permeability and chemotaxis and the inhibition of the production of LTB_4 and hence the reduction in the neutrophil influx. Unfortunately, LTB_4 was not assessed in the BALF of the PAF antagonist treated animals. In the study to examine the role of LTB_4 directly using a novel receptor antagonist, the compound was found to be toxic to the premature guinea pig at he dose used. It was also suggested that the reduction in lung injury found in the vehicle treated animals may have been due to a possible antioxidant property of tween 80.

To further investigate the role of oxygen in the development of chronic lung disease the final experiment was undertaken at 85% oxygen for 28 days. This level of oxygen was used as the survival rate following exposure to 95% oxygen for longer than 3 days was unacceptible. Exposure was continued for 28 days continously as diagnosis of BPD is usually only taken after one month of age. Histologically, the lung structure was severly altered, with structural changes present that were identical to those observed in the human neonate with BPD. Also in accordance with the human infant, the oxygen-exposed premature guinea pig had a persistent pulmonary inflammation and evidence of widespread pulmonary fibrosis. It was concluded that the overal changes observed in this exposure regime were identical to that in the human infant. To establish the role of particular components of the the inflammatory reaction seen in these animals requires many of the same interventive procedures described in the acute studies of this thesis.

Thus in conclusion exposure of the preterm guinea pig to elevated concentrations of oxygen results in the development of inflammation and injury. The particular components of this inflammation may have a role in the development of injury especially following acute oxygen exposure but still requires further study. In animals exposed to oxygen for periods of up to 28 days, the biochemical and morphological alteration in the lungs are consistent with that seen in the preterm human neonate with BPD. As such it makes the preterm guinea pig one of the most appropriate small animal models of prematurity in which to investigate particular components of oxygen-induced inflammation in the development of injury to the immature lung.

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