THE RESOLUTION OF ACUTE LUNG INFLAMMATION IN INFANTS WITH SEVERE RESPIRATORY FAILURE TREATED WITH EXTRA-CORPOREAL MEMBRANE OXYGENATION, STUDIED BY NON-BRONCHOSCOPIC BRONCHOALVEOLAR LAVAGE

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V. Abbreviations

AM	alveolar macrophage
ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
CDH	congenital diaphragmatic hernia
CINC	cytokine-induced neutrophil chemoattractant
CLD	chronic lung disease
CPB	cardiopulmonary bypass
CRP	C reactive protein
DNA	deoxyribose nucleic acid
ECMO	extracorporeal membrane oxygenation
ELF	epithelial lining fluid
ELISA	enzyme linked immune sorbent assay
fMLP	N-formyl-metionyl-leucyl-phenylalanine
GCSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-monocyte colony stimulating factor
15-HETE	15-hydroxyeicosatetraenoic acid
HFOV	high frequency oscillatory ventilation
HMD	hyaline membrane disease
ICAM	intercellular adhesion molecule
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-3	interleukin-3

IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-13	interleukin 13
IL-RA	interleukin-1 receptor antagonist
IRDS	idiopathic respiratory distress syndrome
kDa	kilo dalton
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LRTI	lower respiratory tract infection
Mac-1	macrophage 1
MAS	meconium aspiration syndrome
MIP-1 / -2	macrophage inflammatory proteins 1 / 2
MCP	monocyte chemoattractant protein
M-CSF	monocyte colony stimulating factor
mRNA	messenger ribonucleic acid
NB-BAL	nonbronchoscopic bronchoalveolar lavage
NF-κB	Nuclear Factor-kappa B
PAF	platelet activating factor
PDGF	platelet derived growth factor
PEEP	positive end-expiratory pressure
PGE_2	prostaglandin E_2
PMN	polymorphic neutrophil
PPHN	persistent pulmonary hypertension of the newborn

PPV	positive pressure ventilation
PVM	pneumonia virus of mice
PVR	pulmonary vascular resistance
RANTES	receptor-activated normal T-cell expressed and secreted
ROS	reactive oxygen species
RSV	respiratory syncythial virus
SC IgA	secretory component of immunoglobulin A
sTNFR-1 / -2	soluble tumour necrosis factor receptor-1 / -2
TGF β	transforming growth factor beta
TNF-α / -β	tumour necrosis factor alpha / beta

Dedicated to

My parents Francis and Annebeth

and to

My wife Rita

and

Our children Lisa, Jocelyn and Hiram

Chapter 1. Introduction

1.1 Respiratory failure in near-term newborn infants and the role of inflammation.

1.1.1 Introduction

Neonatal respiratory disease occurs in 2-3% of all newborn babies (Field 1996) and is the commonest reason for admission of infants to the intensive care unit. In the mature infant common conditions leading to severe respiratory failure are meconium aspiration syndrome (MAS), persistent pulmonary hypertension of the newborn (PPHN), congenital diaphragmatic hernia (CDH), viral and bacterial pneumonia and hyaline membrane disease. (Hansen 1998b) The severity of respiratory failure ranges over a wide spectrum. Many infants will be able to maintain adequate gas exchange through spontaneous respiration and require additional inspired oxygen only. More severe cases require respiratory support with endotracheal intubation and positive pressure ventilation (PPV). Even though the initial etiology of severe respiratory failure may be varied, there is a degree of overlap in the subsequent pathophysiology characterized by acute inflammation (Bohn 1998, Hansen 1998a). This is because the lung is limited in the type of response it generates to any given type of injury. Typically polymorphic neutrophil (PMN, from here on referred to as neutrophil) mediated acute inflammation is seen in bacterial pneumonia (Askin 1998). Further evidence suggests that it may also be important in viral bronchiolitis and meconium aspiration syndrome (Davey 1993, Everard 1994). Secondly, treatment with positive pressure ventilation and additional inspired oxygen in high concentration causes additional lung injury and acute inflammation through barotrauma and oxygen toxicity (Bohn 1998, Hansen 1998a). Ventilated newborn infants with refractory disease develop a clinical picture of respiratory distress syndrome with bilateral infiltrates on chest radiogram and oxygenation impairment, similar to acute

respiratory distress syndrome (ARDS) observed in adults. Studies in human preterm neonates and adults support the model of a neutrophil-mediated acute lung injury underlying both idiopathic respiratory distress syndrome (IRDS) and ARDS (Anderson 1983, Donnelly 1992, Repine 1992, Fujishima 1995, Kotecha 1996a). According to the prevailing paradigm of acute lung injury, regardless of the nature of the initial insult, the process of acute lung injury in the lung can be divided into four phases. In neonatal IRDS (hyaline membrane disease) the triggering event is surfactant deficiency, either primarily due to prematurity or secondary to other causes such as infection or meconium aspiration syndrome, resulting in alveolar collapse and overdistention of the respiratory and terminal bronchioles (Askin 1998, Cleary 1998, Hansen 1998b, Greenough 1999). In the subsequent few hours following the initial insult there is alveolar epithelial cell necrosis with detachment of the epithelial layer from the basement membrane and disruption of the endothelial layer. This leads to plasma exudation into the interstitium and alveolar space and the formation of hyaline membranes. Then follows a second phase of exudative alveolitis with sequestration of neutrophils in the pulmonary capillaries and migration into the lung interstitium and alveolar space. According to this model, resident cells in the lung such as alveolar macrophages and epithelial cells release chemotactic agents in response to noxious stimuli, complement components or endotoxin. The chemotactic signal activates circulating neutrophils and causes them to marginate in the circulation. Through a complex mechanism of adhesion and transmigration through the capillary endothelium, activated neutrophils reach the lung interstitium. There they release a range of histiotoxic products in the form of reactive oxygen species (ROS) and proteolytic enzymes. Neutrophils appear to have a physiological role such as phagocytosis and killing of bacteria, but the acute inflammatory response may lead to a secondary injury, with disruption of the capillary endothelium and the alveolar epithelium. This results in further exudate entering the interstitium of the lung and the alveolar space with additional impairment of gas

exchange. Resolution of the exudative alveolitis is followed by a fibro-proliferative phase with proliferation of type II alveolar epithelial cells and migration and proliferation of myofibroblasts in lung tissue. This results in collagen deposition and interstitial remodeling and varying degrees of lung fibrosis. In premature neonates who recover from IRDS, acute inflammation resolves after several days. However, protracted and disordered acute inflammation and subsequent fibro-proliferation, result in chronic lung disease (CLD). Very similar observations have been made in older children and adults with ARDS in response to a wide range of insults (Durmowicz 1998, Redding 1998). However little is known about the resolution of this inflammatory process, in relation to the clinical outcome in mature human newborn infants with severe respiratory failure.

1.1.2 Mechanisms and cellular markers of the acute inflammatory response in the lung

Given here is an overview of the main cellular components of the lung at the alveolar level in normal conditions, as well as a more detailed discussion of cellular mechanisms of inflammation in acute lung injury. At the alveolar level, the lung can be divided into three anatomical compartments. First, the air-surface interface consists of the alveolar epithelium and the vascular endothelium provides a second interface between the blood and the lung. Each of these layers has a basement membrane, which is fused over a large portion of the gas exchange surface. Thirdly, the interstitium forms the separation between the epithelium and endothelium. The alveolar epithelium is composed of type 1 and 2 cells. Type 1 cells are about half in number compared to type 2 cells, but comprise 93-97 % of the alveolar surface due to their flattened shape (Evans 1989, O'Brodovich 1998). Type 2 cells comprise about 16% of the total cell number in the lung but cover only 3-7% of the surface area of the alveolar space. Epithelial cells have very tight cell-to-cell junctions including tight junctions, intermediate junctions, gap junctions and desmosomes. In this way the epithelial cells form an effective

mechanical barrier and also restrict the movement of macromolecules and certain ions (Rennard 1997, O'Brodovich 1998). Type 2 cells have several specific functions including synthesis, storage and secretion of surfactant, and regulation of volume and composition of epithelial lining fluid (ELF) by transepithelial solute transport. Following lung injury and destruction of the epithelial barrier, type 2 cell proliferation increases rapidly to replace the damaged type 1 cells (Voelker 1989, O'Brodovich 1998). Finally, epithelial cells play an important role in the acute inflammatory response (Rennard 1997). It has been found that epithelial cells are able to produce chemoattractants for different white cell populations. For instance stimuli such as bacterial endotoxins, viral infection, neutrophil elastase and cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF) and interferon-γ (IFN-γ), have been shown to induce production of eicosanoids (arachidonic acid metabilites) like 15-hydroxyeicosatetraenoic acid (15-HETE) and leukotriene B_4 (LTB_4) and the chemokine interleukin-8 (IL-8). These factors have a wide range of immune modulating properties including activation and attraction of neutrophils.

In between the epithelial and endothelial layers lies the interstitial space. This space contains the largest number of cells of all the compartments in the lung, around 36%. The majority of cells here are fibroblasts and smooth muscle cells, with some macrophages and lymphocytes. The fibroblasts produce extracellular matrix consisting mainly of three types of insoluble protein: collagen, elastin and glycosaminoglycans (Abscher 1989, O'Brodovich 1998). The main function of the extracellular matrix is to provide a structural framework and fibrous support for the lung. However the cellular and non-cellular components of the interstitium are also involved in cell growth and differentiation, water and solute movement in the lung and indirectly in surfactant synthesis.

The endothelium contributes to around 30% of the total cell mass in the lung (O'Brodovich 1998). At the alveolar level, the endothelial cells form a continuous unfenestrated layer, which has several active functions. The cells are separated by small gaps allowing for free movement of water and small ions, but restricting the movement of proteins. At the luminal side, epithelial cells produce coagulation factors such as von Willebrand factor and plasminogen activator, which are important for platelet function and maintenance of vascular patency (Henke 1997, O'Brodovich 1998). Endothelial cells are also capable of producing vasoactive substances in particular prostaglandins and nitric oxide, which are involved in control of vascular smooth muscle tone and pulmonary vascular resistance (Morin 1995). In addition, the endothelium plays a crucial role in the activation and recruitment of neutrophils into the lung in response to acute lung injury (Burnett 1997, Downey 1999). Under physiological circumstances, neutrophils are able to migrate through the lung without adverse effects. It is once neutrophils are activated, they are sequestered in the lung tissue and become involved in acute lung injury. This occurs through a stepwise process of tethering, triggering, strong adhesion and finally transendothelial migration. Initially under the influence of histamine and pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-8 and tumour necrosis factor α (TNF-α), endothelial cells express selectin surface receptors (E- and P-selectin). Neutrophils constitutively express surface receptors of the selectin family called E-selectin. Interaction of these receptors results in marginated neutrophils tethering to the endothelial surface in areas of inflammation. The pro-inflammatory cytokines also modulate the expression and activation of neutrophil transmembrane glycoproteins belonging to the intergrin family and endothelial cell intercellular adhesion molecules or ICAMs (triggering), which are involved in strong adhesion of neutrophils to the endothelium. In particular, the β_2 -integrin macrophage-1 (Mac-1 or CD 11a,b,c /CD 18) specifically binds to intercellular adhesion molecule 1 (ICAM-1 or CD 54) on the endothelial surface. Furthermore integrin adhesiveness is known to be increased by

bacterial products, such as N-formyl-metionyl-leucyl-phenylalanine (fMLP), whilst endothelial ICAM-1 expression is upregulated by bacterial lipopolysaccharide. The final step is for the neutrophils to migrate across the endothelium into the interstitium. Several studies have shown that the migration of neutrophils occurs along concentration gradients of chemotactic factors such as LTB_4 and IL-8, so that neutrophils accumulate near sites of active inflammation (Burnett 1997). Finally, the endothelial layer may itself become involved in acute lung injury (Henke 1997). In areas where the endothelium is denuded, the basement membrane is covered with fibrin. More extensive destruction of the capillary endothelium leads to complete thrombosis with impairment of the pulmonary microcirculation and increased pulmonary vascular resistance.

1.1.2.1 Macrophages in the lung

The inflammatory cell population in the lung can be divided into a resident population consisting of alveolar macrophages, dendritic cells, lymphocytes and mast cells. Various inflammatory processes, such as acute lung injury or asthma stimulate the recruitment of exogenous inflammatory cell populations, in particular neutrophils and eosinophils (Wilmott 1998). The two main cell populations involved in acute inflammation in association with acute lung injury, as described above, are macrophages and neutrophils.

Macrophages comprise the largest population of resident inflammatory cells in the lungs. They are distributed over the pleural, interstitial, intravascular and alveolar compartments, and are able to migrate between these compartments (Poulter 1997). The majority are located in the alveolar space and in the human adult it is estimated that the total number of macrophages in the lungs amounts to 23×10^9 or 50 - 100 macrophages per alveolus (Wilmott 1998). However in prenatal animals alveolar macrophage (AM) numbers have been found to be much lower,

with a rapid increase in the first 24 hours postnatally (Frank 1989). Animal studies into the origin and turnover of macrophages suggest that in health the macrophage population is relatively constant. Macrophages originate in the bone marrow as monocytes and are subsequently recruited from the bloodstream into the lung where they mature (Poulter 1997, Wilmott 1998). It is thought that a small proportion derive from local cell division. The turnover time is estimated at around 23 to 28 days and they are most likely removed from the lung mainly via mucociliary clearance and possibly also through migration to regional lymph nodes. During acute inflammation, the macrophage population rapidly expands through monocyte influx, with some contribution from proliferation of the resident macrophage population. A number of factors produced by epithelial cells, interstitial cells, monocytes and activated T-lymphocytes are involved in the regulation of maturation, differentiation and proliferation of macrophages. These include monocyte - colony stimulating factor (M-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) (Wilmott 1998). Like the skin, the lung is unique in that it is constantly exposed to the outside environment. Macrophages present in the lung form the first line of defense against particulate matter and microbes inhaled into the lungs (Poulter 1997, Wilmott 1998). Macrophages phagocytose inhaled particles, cellular debris and microorganisms and have surface receptors for immunoglobulins, complement and bacterial lectins, which aid in this process. They possess both anaerobic and aerobic bacteriocidal mechanisms especially lysosomal acid hydrolase and reactive oxygen intermediates for the intracellular killing of phagocytosed microorganisms. Macrophages are also capable of extracellular killing of microorganisms through the release of reactive oxygen species, proteases and acid hydrolases. In this way they are able to kill small numbers of gram positive bacteria. However for larger inocula and the killing of gram negative bacteria and viruses, neutrophil recruitment and specific immune activation through antigen presentation, are required. Possible side effects of uncontrolled

microbicidal activity are local tissue damage and promotion of acute inflammation. In addition, activated macrophages release a range of mediators including eicosanoids, complement factors, interleukin-1β, interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and the specific neutrophil chemoattractants interleukin-8 (IL-8) and macrophage inflammatory proteins (MIP) 1 and 2, which regulate acute inflammation and recruit neutrophils (Geba 1994, Poulter 1997). Other cytokines released, such as transforming growth factor β (TGF β) and fibronectin, promote fibroblast proliferation. Recently, it has been found that alveolar macrophages from healthy neonates and infants release less IL-1 and TNF on stimulation with bacterial lipopolysaccharide (LPS) *in vitro* compared to older children (Grigg 1999). Also, respiratory burst generation was found to be reduced in neonatal macrophages (Grigg 1999).

1.1.2.2 Neutrophils and acute lung inflammation

Neutrophils have a secondary role in the defense against invading organisms. It is only once local defense mechanisms have been breached that neutrophils are recruited in large numbers to ingest and destroy microorganisms (Burnett 1997). Neutrophils originate in the bone marrow as stem cells and develop over a period of 10 to 14 days through various precursor stages into their mature form. They are distributed between the bone marrow, blood and the tissues. In the human adult the marrow produces around 1.5×10^9 cells per kg blood per day, and has a large reserve pool of immature and mature neutrophils available for recruitment. Around half of the neutrophils in the blood circulate, while the other half marginates along the walls of small arteries forming a further reserve pool for rapid mobilisation (Stein-Streilein 1989, Burnett 1997). The circulation half-life is around 8 hours and neutrophils are able to survive in tissues for a further 24 to 48 hours. In the lung, the largest component of neutrophils is located in blood vessels and large numbers of neutrophils are sequestered in the vascular bed during their passage through the lung. In addition, under normal circumstances the

airspace also contains some neutrophils, constituting up to 2% of cells found during bronchoalveolar lavage. During infection and acute lung injury a multitude of substances are produced at the local site involved in activation and attraction of neutrophils (Stein-Streilein 1989, Burnett 1997). Early in this process, complement factors originating from exudated plasma and resident inflammatory cells are activated. Resident inflammatory cells at the local site such as macrophages and activated endothelial cells, are also involved in the production of arachidonic acid metabolites such as LTB_4 and cytokines like TNF-α and IL-8. Injured extracellular matrix releases components such as collagen and laminin. Furthermore, invading microorganisms themselves release factors which activate and attract neutrophils. These include formyl peptides and LPS, with the latter binding to neutrophils via the CD 14 receptor. The process of neutrophil adhesion to the vascular endothelium and transendothelial migration has been described in section 1.1.2. In an animal model of surfactant deficiency, Kawano showed that neutrophils play a major role in producing histological evidence of lung injury, which can be significantly reduced by neutrophil depletion (Kawano 1987). Accumulation of neutrophils is prominent in most patients with ARDS, but ARDS can also develop in neutropenic patients (Stein-Streilein 1989, Repine 1992). In the tissues, activated neutrophils use three main mechanism for intracellular killing of microorganisms. The first step is the recognition of both non-opsonised bacteria as well as bacteria and particles opsonised with complement and immunoglobulins, followed by phagocytosis. Subsequently intracellular killing occurs through respiratory burst generation and the delivery into the phagosome of cytotoxic proteins stored in the cytoplasm and neutrophil granules. These granules contain a range of proteins including defensins, cationic antimicrobial protein (CAP) 37 and 57, serine proteases, such as elastase, cathepsin G and protease 3, and metalloproteases including collagenase and gelatinase (Burnett 1997). The cytotoxic proteins are essential for host defense against invading bacteria and, at the tissue level, their activity is contained by a shield

of anti-proteases such as α_1-protease inhibitor, α2-macroblobulin and secretory leukoprotease inhibitor (Weiss 1989). Reactive oxygen species have the potential to inactivate these anti-proteases. If present in sufficient concentrations, proteases are able to break through the protective anti-protease shield and are able to cause additional tissue injury (Weiss 1989). Although elevated levels of proteases have been found in BAL fluid of adults with ARDS and neonates with CLD, presently, there is no firm evidence from *in vivo* studies in humans to show that reactive oxygen metabolites and proteases directly cause acute lung injury (Ogden 1984, Weiss 1989, Lee 2001). However, indirect evidence from experimental models of acute lung injury implicates, that specific inhibitors of both reactive oxygen metabolites from respiratory burst generation as well as neutrophil proteases, can ameliorate endotoxin induced lung injury, reperfusion lung injury and injury due to direct infusion of activated neutrophils (Stein-Streilein 1989, Lee 2001). It appears most likely that during acute lung injury there is imbalance of the inflammatory response, either due to an overwhelming initial insult or an inappropriately severe host response.

For acute lung injury to resolve successfully with restoration of normal lung tissue it is necessary that the neutrophils are removed in an orderly fashion. It has recently been shown that programmed cell death or apoptosis is an important mechanism by which this is achieved (Savill 1997, Haslett 1999). When neutrophils undergo apoptosis, they remain intact and the intracellular contents that promote inflammation and tissue damage are contained. Also, apoptosis reduces the ability of neutrophils to degranulate. Neutrophil apoptosis is promoted by exposure to TNF-α, ingestion of bacteria and nitric oxide donors. Factors which have been found to inhibit neutrophil apoptosis include LPS, GM-CSF and corticosteroids. Following apoptotic death, neutrophils are ingested and removed by tissue macrophages. This further contains neutrophil mediated tissue injury, by preventing disintegration of apoptotic

neutrophils. Furthermore, ingestion of apototic neutrophils by macrophages down regulates the pro-inflammatory activity of macrophages and induces the release of factors which promote resolution of inflammation, such as TGF-β and PGE_2. Neutrophil lifespan is extended in tissues (Jones 1996) and this, together with modulation of neutrophil apoptosis by cytokines and other inflammatory mediators, (Colotta 1992, Lee 1993) suggests active control of cell death during the course of inflammation *in vivo*. Studies in rats following LPS stimulation (Cox 1995) and in human infants with hyaline membrane disease (Grigg 1991), have confirmed that apoptosis of neutrophils and subsequent ingestion by macrophages occurs during resolution of acute lung inflammation. Apoptosis of neutrophils present within the lung is inappropriately suppressed in patients with acute respiratory distress syndrome (ARDS) with BAL fluid being anti-apoptotic to human neutrophils (Matute-Bello 1997). This anti-apoptotic effect was observed to be maximal in the early stages of ARDS (Matute-Bello 1997) and to decline at later time points.

1.1.3 Specific cytokines and the pulmonary inflammatory response

The second half of the 20th century has seen a rapid expansion in the scientific knowledge of a group of soluble bioactive factors known as cytokines, which have a complex multifunctional role in regulating cell-cell interactions (reviewed in Geba 1994). By agreement the term interleukin (IL) was chosen for a subgroup of these mediators whilst others continue to be referred to by their initial eponyms. Cytokines are glycosylated peptides and are produced transiently by an effector cell following an appropriate stimulus. Their effects are mediated by binding to specific receptors on target tissues and can be divided into four main groups: 1) intracrine effects: alteration of cell phenotype without being transported outside the cell; 2) autocrine effects: regulation of its own effector cell function by binding to receptors on the

cell membrane; 3) paracrine effects: by binding to cell membrane receptors of nearby cells; 4) endocrine effects: by entering the bloodstream and mediate effects on distant target tisssues. Cytokines are multifunctional molecules that regulate a wide spectrum of immune and non-immune biological events. The same cytokine can have different effects depending on its concentration, the state of activation or maturation of the target cell and the interaction with other cytokines in the local microcellular environment. Individual cytokines interact with each other in networks and the ultimate response is determined by the sum of signals received by the cell membrane and subsequent modulation of these signals by the receptor cell. Appropriate functioning of these regulatory processes is essential for normal growth, repair and development of an organism. Many human diseases are characterised by dysregulated or abnormal cytokine production, which can be seen as a malfunction of a normal response (Geba 1994). As already alluded to, certain groups of inflammatory mediators and cytokines play a pivotal role in the generation, maintenance and resolution of acute lung inflammation by mediating cell-cell interactions as described above. Discussed here are several specific cytokines, which have been shown to have an important role in the pulmonary acute inflammatory response and which can serve as markers of this process (Geba 1994).

1.1.3.1 Tumour necrosis factor-α (TNF-α)

Tumour necrosis factor is synthesised in two related forms, TNF-α and TNF-β, which share about 30% of amino acid homology and bind to the same receptor (Ulich 1993, Tracey 1997, Nelson 2000). The genes for these two cytokines are located in close proximity within the major histocompatibility complex on chromosome 6. TNF-α is produced mainly by monocytes and macrophages and is thought to be more potent than TNF-β, which is mainly produced by lymphocytes. TNF-α has been more widely studied, especially in relation to

acute inflammation in sepsis and ARDS and I shall therefore concentrate on TNF-α. Two forms of the TNF-α protein exist: a membrane bound form of 26 kDa molecular weight and a soluble form of 17 kDa. Regulation of TNF-α production occurs both at the transcriptional and posttranscriptional level. One of the most potent stimulants for TNF-α production is LPS, but other stimuli include fungal proteins, viral antigens, other cytokines such as MIP-1, TNF itself, IL-1, IL-2 and GM-CSF, and factors involved in the induction of cellular stress such as hypoxia, ROS, complement activation and temperature shock. In the lung TNF-α derives mainly from alveolar macrophages, which produce five to six times more TNF-α than peripheral blood monocytes. *In vitro* stimulation of AMs with LPS increases both TNF-α mRNA as well as TNF-α protein excretion and this response can be augmented with interferon-γ priming. *In vitro* studies have also shown that epithelial cells, endothelial cells and vascular smooth muscle cells are able to produce TNF-α in response to LPS (Ulich 1993, Nelson 2000). Several inhibitors of TNF expression have been identified. Glucocorticoids inhibit TNF-α production both *in vitro* in macrophages and *in vivo* in models of LPS induced pneumonitis (Ulich 1993). Other compounds capable of reducing TNF- α expression are cyclosporin, pentoxifylline and ethanol. Mediators which have been found to down-regulate TNF- α expression, include IL-4, IL-6, TGF-β and PGE_2. The release of TNF-α from leukocytes can be inhibited by serine proteases (Ulich 1993).

TNF receptors have been identified in virtually all mammalian cell types and two types of TNF receptors, which bind both TNF-α and β, have been distinguished (Manogue 1991). One receptor has a molecular weight of 55 kDa (type 1) and is expressed on epithelial and haemopoietic cell lines. The second receptor has a molecular weight of 75 kDa (type 2) and the extra-cellular domains of the two receptors are very similar. Both receptors appear in

truncated form in the circulation and the soluble forms exhibit anti-inflammatory properties (see 1.1.3.7).

In the lungs, TNF-α produced by AMs and other cells can be measured by bronchoalveolar lavage. Both intratracheal LPS challenge in a rat model and ARDS in humans leads to elevated levels of TNF-α in BAL fluid but not in plasma, suggesting local production at the site of lung injury (Ulich 1993, Nelson 2000). Release of TNF-α has effects on several cell populations (Manogue 1991, Ulich 1993, Nelson 2000). In neutrophils, important functions are upregulated such as antibody dependent cellular cytotoxicity, phagocytosis, acid production and relasease of ROS and lysosomal enzymes. Macrophages which are the main source of TNF- α in the lung, also undergo activation in response to TNF- α. Macrophages exhibit enhanced cytosolic activity, increase expression of lipid mediators like PGE_2 and production of cytokines such as IL-1, colony stimulating factors and further production of TNF- α. Endothelial cell activation by TNF- α causes vasodilatation and increased regional blood flow by induction of vasodilators like prostaglandins and nitric oxide. TNF- α is also a stimulus for the expression of adhesion molecules on the endothelial cell surface and for the expression of the cytokines IL-1 and IL-8, which are important in the activation and recruitment of inflammatory cells, in particular neutrophils (see 1.1.2 , 1.1.3.2 and 1.1.3.4). Studies in animal models have shown that intravenous injection of recombinant TNF causes an ARDS-like lung injury with increased lung permeability (Horvath 1988, Stephens 1988). In the study by Stephens (Stephens 1988), there was no significant increase in PMN count in BAL fluid in response to TNF injection, but a significant increase in PMN per alveolus was found on histology following TNF injection relative to control. However, direct injection of bacteria in the bloodstream caused significant neutrophilia in BAL fluid, showing that multiple factors in addition to TNF are likely to be involved in causing the clinical picture of

ARDS in sepsis. Imai studied the effects of surfactant washout with saline and conventional mechanical ventilation on lung injury in a rabbit model (Imai 1999). In control subjects, the intervention caused significant lung injury in terms of higher lung injury scores and significant increase in BAL fluid PMN counts and TNF concentration. In study subjects, pre-treatment with an anti-TNF antibody attenuated this lung injury with a significant reduction in PMN counts and TNF concentration in BAL fluid. In human adults and infants with normal lungs very little TNF is present in BAL fluid (Suter 1992, Bagchi 1994, Park 2001). In the early phase of ARDS in humans and IRDS in preterm infants, significant amounts of immunologically active TNF appear in BAL fluid (Murch 1992, Suter 1992, Park 2001). Persistence of TNF in BAL fluid in adults with ARDS is associated with poor outcome (Meduri 1995). Similarly, development of CLD in preterm infants with IRDS was associated with increased TNF concentrations in BAL fluid after the first week (Murch 1992, Bagchi 1994). Several investigators have studied the biological activity of TNF in BAL fluid in both adults with ARDS as well as in IRDS (Suter 1992, Bagchi 1994, Pugin 1996). These investigations found low biological activity of TNF present in BALF during the early phase ARDS or IRDS. However, Bagchi (Bagchi 1994) found rising immunological and biological activity of TNF in the third and fourth week of life in infants who developed CLD. On the one hand, the low biologic activity of TNF early in ARDS and IRDS suggests the presence of specific inhibitors (see 1.1.3.7). Alternatively, the biological activity of TNF is measured in the aqueous phase and it is possible that TNF has significant effects when bound to tissue receptors.

1.1.3.2 Interleukin -1 (IL-1)

Interleukin-1 appears in two forms, IL-1α and IL-1β, which are coded for by two separate genes on chromosome 2. Gene translation produces precursor molecules of around 270

aminoacids in size (molecular weight 31 kilo Dalton), which undergo post-translational modification to mature peptides of 155 aminoacids with a molecular weight of around 17.5 kDa. Human IL-1α and IL-1β share about 26% homology and bind with equal affinity to two distinct cell membrane receptors. In addition they share many biological activities (Aksamit 1993). In the healthy steady state IL-1 concentrations in the body are negligible. IL-1 is produced in many tissues including lung, liver, spleen, blood and kidney by a wide range of cells. However the main source appears to be stimulated monocytes and macrophages, which produce IL-1α and Il-1β in a ratio of 1 : 10 (Aksamit 1993). Stimuli which increase expression of IL-1 in monocytes and macrophages include IL-1 exposure, TNF-α, GM-CSF, endotoxin, adherence to plastic and glass, phagocytosis and viral gene products. Endothelial and vascular smooth muscle cells also produce IL-1 in response to IL-1, TNF-α and endotoxin. Furthermore, these stimuli can act synergistically with each other and with IL-2 and interferon-γ. IL-1 production can be increased by increased gene transcription rates or by increased IL-1 mRNA stability prolonging mRNA half-life. Agents able to suppress IL-1 production include prostaglandin E_2 and glucocorticoids. If corticosteroids are present before stimulation of the cells, they can inhibit gene transcription. Steroids also act by reducing IL-1 mRNA stability.

In humans two types of IL-1 receptor have been identified (Aksamit 1993, Tocci 1997). The type 1 receptor has a molecular mass of around 80 kD and consists of a 319 aminoacid extracellular domain, a 20 aminoacid transmembrane domain and a cytoplasmic domain of 213 aminoacids. Almost all nucleated cells express type 1 IL-1 receptor but the number of receptors per cell varies widely between cell types. Type 1 IL-1R binds both types of IL-1, but the affinity for IL-1 receptor antagonist (see 1.1.3.6) is far greater than the affinity for IL-1. Several factors can increase the expression of type 1 IL-1R including IFN-γ, IL-1, IL-2, IL-4,

PDGF (platelet derived growth factor) and PGE_2. Type 2 IL-1 receptor has a molecular mass of around 60 kD and is expressed on B cells, monocytes and macrophages. After binding of IL-1, the the type 2 receptor half-life is shortened and the number of receptors is downregulated. Also, binding to the type 2 IL-1R does not result in signal transduction. In this way, type 2 IL-1 R may serve as a regulator of IL-1 activity. Cell surface expression of type 2 IL-1R is increased by IL-1, IL-4, PGE_2, corticosteroids and haemopoietic growth factors.

The release of IL-1 has widespread systemic effects. Given here are those effects that are of importance to acute lung inflammation and sepsis. IL-1 is one of the early general activators of the host immune system in response to injury with activated monocytes and macrophages as the main source (Aksamit 1993). One of the most important roles is activation of both T and B cells. In response to IL-1, neutrophils become more metabolically active, increase thromboxane synthesis and degranulate. IL-1 also induces IL-8 synthesis resulting in neutrophil chemotaxis to the site of injury. In combination with PDGF, IL-1 stimulates fibroblast proliferation. IL-1 also induces the release of a wide range of cytokines and growth factors including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, G-CSF, GM-CSF, interferon-γ, PDGF and TNF. IL-1 is involved in the acute phase response in several ways (Aksamit 1993). Through increased prostaglandin synthesis in the hypothalamus, IL-1 is a potent stimulus for fever. In the liver synthesis of acute phase proteins is increased both through direct stimulation and through the generation of other cytokines such as IL-6. Furthermore, IL-1 is involved in some of the endocrine and the metabolic effects of the acute inflammatory response (Aksamit 1993). IL-1 indirectly triggers the release of corticosteroids. It reduces the activity of cytochrome P-450 and adipocyte lipoprotein lipase. IL-1 also induces anorexia and a negative protein balance. Many of these effects are potentiated by the presence of TNF. In terms of haematological effects, IL-1 is a growth factor for bone marrow and also triggers the release

colony stimulating factors and IL-6, as mentioned before. It also causes leukocytosis by release of neutrophils from the bone marrow. The vascular effects of IL-1 include hypotension and profound changes in endothelial cell function, which are important in the host defense to localized infection or injury (Aksamit 1993). These include increased procoagulant activity, release of PAF (platelet activating factor) and altered vascular permeability. IL-1 is also involved in the early phases of leukocyte chemotaxis and adhesion through its role in the induction of E-selectin and ICAM-1 expression as described in paragraph 1.1.2.

In animal models of acute lung injury, IL-1β can be detected in lung tissue as well as in macrophages and neutrophils early in the course of the disease (Xing 1994). Furthermore, intravenous administration of IL-1 produces a clinical picture similar to ARDS. In models of endotoxic shock mortality can be reduced with recombinant IL-1 receptor antagonist, an inhibitor of IL-1 (Aksamit 1993). In humans with ARDS, IL-1β is present in the early phase in BAL fluid and is able to upregulate ICAM-1 expression *in vitro*. Persistently high concentrations of IL-1β in BAL fluid of adult patients with ARDS are associated with poor outcome (Martin 1999).

1.1.3.3 Interleukin-6 (IL-6)

Interleukin-6 is produced by a wide variety of cells including monocytes, alveolar macrophages, fibroblasts, endothelial cells, mesangial cells, keratinocytes and B and T lymphocytes (Zitnik 1993, Geba 1994). The gene for IL-6 has been located on chromosome 7. The major transcription product is a 1.3 kilobase mRNA and its primary translation product consists of 212 aminoacids. On movement into the endoplasmic reticulum, a 28 amino acid

hydrophobic signal peptide is cleaved. The resulting 184 amino acid (molecular weight 21 kilo Dalton) undergoes further tissue specific post-translational modification in terms of glycosylation, formation of disulfide bridges and phosphorylation, resulting in a final molecular weight between 17 and 85 kD. The size of the IL-6 molecule is in part tissue specific. Production of IL-6 can be modulated directly through increased gene transcription, by cytokines such as IL-1, TNF, PDGF and TGF-β, as well as by endotoxin and RNA and DNA viruses. In addition, cytokine induced IL-6 production is in part mediated through stabilisation of IL-6 mRNA, resulting in prolonged mRNA half-life and increased IL-6 production. It has been observed that different cells produce IL-6 in response to different stimuli. Transcription of this gene can be inhibited by TGF-β, retinoic acid, glucocorticoids and agents that increase cyclic adenosine monophosphate (cAMP). A wide variety of cells express the IL-6 receptor on their cell membrane including epithelial, neural, fibroblast and haemopoietic cells. The receptor is 80 kD in size and forms a multimeric complex with a 101 kD glycoprotein (gp 130).

Studies *in vitro* and vivo models have shown a wide range of effects of IL-6, including B-cell differentation and immunoglobulin production, T-cell proliferation and stimulation of heamatopoiesis (Zitnik 1993, Geba 1994). IL-6 produced at the local site of inflammation has an endocrine effect on the liver and brain. In the liver IL-6 stimulates the production of acute phase proteins such as C-reactive protein (CRP), haptoglobin, α_1-antitrypsin, fibrinogen and α2-macroglobulin. Other hepatic effects include induction of uptake of aminoacids and zinc, and reduction in gluconeogenesis. In the central nervous system IL-6 stimulates the release of corticotrophin releasing factor (CRF) and adrenocorticotrophic hormone (ACTH). It also stimulates pituitary hormone production and direct production of corticosteroid by ardrenal

cortical cells. In humans increased IL-6 levels have been found in a range of chronic inflammatory disorders and malignancies (Zitnik 1993, Geba 1994).

The role of IL-6 in local lung infection and sepsis resulting in lung and systemic inflammation is complex and has both pro- and anti-inflammatory aspects (Zitnik 1993, Tilg 1997, Xing 1998). In the lungs, cells found to have IL-6 producing capabilities are fibroblasts, alveolar macrophages, endothelial cells and tracheal epithelal cells. *In vitro*, human lung fibroblasts produce IL-6 in response to IL-1, TNF and TGF-β in a synergistic fashion. However endotoxin has little effect on IL-6 production by lung fibroblasts. Similarly monocytes increase IL-6 synthesis in response to IL-1 but not endotoxin. Alveolar macrophages on the other hand produce large amounts of IL-6 in response to endotoxin, but do not increase IL-6 production in response to IL-1 or TNF. This suggests a primary role of AMs in the defense against invading organisms, with secondary involvement of fibroblasts by AMs and recruited blood monocytes, resulting in amplification of the local inflammatory response. The effects of IL-6 can be classed as both pro- and anti-inflammatory (Tilg 1997, Xing 1998). As described above IL-6 is involved in the induction of acute phase proteins such as C reactive protein (CRP). *In vitro* studies have shown that CRP is capable of inducing synthesis IL-1, TNF and IL-6 in human monocytes and alveolar macrophages. On the other hand, studies in animal models of sepsis have shown that IL-6 does not directly contribute to the systemic effects of the acute inflammatory response. Furthermore, IL-6 is involved in suppressing synthesis of TNF in tissue macrophages and upregulation of the expression of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and soluble TNF receptor (sTNFR, see paragraph 1.1.3.5) in circulating monocytes. Finally, the acute phase proteins have been found to have anti-inflammatory properties. For example, in *in vivo* models of endotoxin induced lung

injury, CRP ameliorates PMN influx and pulmonary endothelial injury. Also, bleomycin induced pulmonary fibrosis in hamsters is reduced by administration of α_1-antitrypsin. The current evidence suggests that, in the presence of endotoxin the acute phase proteins appear to stimulate synthesis of both IL-1 and IL-1RA in equal amounts. In the absence of endotoxin there is preferential induction of synthesis of IL-1RA, which is involved the down regulation the acute inflammatory response once the source of endotoxin has been removed (Tilg 1997).

1.1.3.4 Interleukin-8 (IL-8)

Interleukin-8 was discovered in the 1980's as one of the main factors directly involved in neutrophil activation and recruitment (Strieter 1993). It belongs to a group of related and structurally similar chemotactic cytokines called α-chemokines, for which the genes are located in close proximity on chromosome 4 (Oppenheim 1991, Martin 1999). Another term used for this group is C-X-C chemokines, based on the typical configuration of two amino terminal cysteines separated by a non-conserved amino acid (Kunkel 1997). The basic transcription product of the IL-8 gene consists of 99 amino acids. This polypeptide undergoes further proteolytic cleavage resulting in several different mature forms of IL-8. The molecular mass of the IL-8 polypeptide is 8.35 kD and the molecule is relatively resistant to proteolysis and denaturation. *In vitro* studies have demonstrated that most cells present in alveolar capillary membrane are capable of producing IL-8, but that this is dependent on the type of stimulus (Strieter 1993). Monocytes, alveolar macrophages and endothelial cells express IL-8 in response to both exogenous stimuli e.g. LPS as well as endogenous stimuli such as IL-1 and TNF. Interferon-γ released by lymphocytes acts synergistically with exogenous and endogenous factors in the upregulation of IL-8 expression. In addition, adherence of monoctyes and alveolar macrophages to plastic or physiological surfaces is also a strong stimulus for IL-8 expression. On the other hand, pulmonary smooth muscle cells, fibroblasts

and epithelial cells only produce IL-8 in response to IL-1 or TNF, suggesting a secondary role in the amplification of the acute inflammatory response. Several agents have been shown to be able to suppress IL-8 production in a cell specific manner (Strieter 1993). Pre-treatment with PGE_2 or dexamethasone was able to inhibit LPS induced IL-8 production in monoctyes, but only dexamethasone inhibited this response in alveolar macrophages. Fibroblast expression of IL-8 is also inhibited by dexamethasone. IL-4 also suppresses IL-8 synthesis in monocytes following LPS stimulation, but not in endothelial cells and fibroblasts. *In vitro* studies have shown that neutrophils possess approximately 20,000 specific IL-8 receptors on their surface. IL-8 binding appears to play an important role in the regulation IL-8 receptor expression (Strieter 1993). During neutrophil activation, IL-8 shares intracellular signal conduction pathways with other neutrophil activating agents such as fMLP, complement factor 5a, PAF and LTB_4, but IL-8 has been found to be 10 to 100 times more potent.

IL-8 has a wide range of effects in terms of neutrophil activation (Strieter 1993). It leads to expression of the CD11b/CD18 leukocyte integrin complex on the cell surface, which is important in the adhesion of neutrophils to the endothelial surface (see paragraph 1.1.2). Furthermore neutrophils undergo shape change, increase cytosolic calcium and release arachnidonic acid metabolites. Also, there is activation of intracellular mechanisms involved in killing of ingested micro-organisms (see paragraph 1.1.2) such as exocytosis of lysosomal enzymes and respiratory burst generation. *In vitro* studies have shown that IL-8 is chemotactic for neutrophils, basophils and T lymphocytes (Strieter 1993). In animal models systemic and local injection of IL-8 produces marked granulocytosis and neutrophil influx. In these models PMN infiltration is associated with venular wall damage and plasma leakage, suggesting a prime role for endogenous IL-8 in neutrophil recruitment and neutrophil mediated acute inflammation. In humans at risk of ARDS and with established ARDS, IL-8 has been detected

in BAL fluid and has been found to be the dominant chemoattractant compared to other chemokines in BAL fluid (Martin 1999). Persistence of ARDS in adults and development of CLD in premature neonates are both associated with persistently elevated levels of BAL IL-8 (Groneck 1994, Kotecha 1995, Meduri 1995, Martin 1999).

1.1.3.5 The role of anti-inflammatory cytokines in the resolution of the acute inflammatory response

Anti-inflammatory cytokines and specific cytokine inhibitors play a prominent role in the complex and intricate network that regulates the human immune response (Opal 2000). The purpose of these cytokines is to maintain homeostasis by limiting the potentially deleterious effects of excessive or sustained pro-inflammatory reactions. In the inflammatory milieu there is a dynamic balance between pro- and anti-inflammatory cytokines and the net effect on the inflammatory response is dependent on a range of factors. These include the partial agonist effects of some anti-inflammatory cytokines, timing of cytokine release, presence of local tissue factors, cytokine receptor density, and tissue responsiveness. Disturbances of control of the inflammatory response by genetic, environmental or microbial elements act by two main mechanisms. Anti-inflammatory cytokines either provide insufficient control over the pro-inflammatory signal resulting in excessive inflammation or there is overcompensation in response to pro-inflammatory activity, resulting in immune suppression and increased infection risk. Three anti-inflammatory cytokines and their role in acute lung disease will be considered, as they act as direct antagonists of the pro-inflammatory IL-1β and TNF-α: interleukin-1 receptor antagonist (IL-1RA), soluble tumour necrosis factor receptor-1 (sTNFR-1) and soluble tumour necrosis factor receptor-2 (sTNFR-2). I am aware of other anti-inflammatory cytokines such as interleukin 4 and interleukin 10, which are not direct

antagonists of pro-inflammatory cytokines, but have anti-inflammatory effects on different types of inflammatory cells (Holter 1997, Powrie 1997).

1.1.3.6 Interleukin-1 receptor antagonist (IL-1RA)

The gene for IL-1RA is located on chromosome 2 in close proximity to the genes for IL-1α and β (Aksamit 1993, Opal 2000). The mature protein contains 152 amino acids and has a molecular weight of 17 kD. IL-1RA shares around 26% homology with IL-1α and about 19% homology with IL-1β. IL-1RA is produced by monocytes and macrophages in response to a range of stimuli including LPS, IgG (immunoblobulin G), zymosan and the anti-inflammatory cytokines IL-4, IL-6, IL-10 and IL-13. Some of these stimuli also induce production of IL-1 and the balance of IL-1 and IL-RA is dependent on the type of stimulus and the differentiation state of the cells (Aksamit 1993, Opal 2000). In addition, DNA (deoxyribo nucleic acid) polymorphism of one of the regulatory regions of the gene may influence the synthetic rate of IL-1RA and result in an altered host response (Opal 2000). IL-1RA has similarly high affinity as IL-1α and β for the type 1 IL-1 receptor (80 kD) but has low affinity for the type 2 IL-1 receptor (68 kD). When IL-1RA binds to the IL-1 receptor, the accessory protein involved in intracellular signal transmission is not engaged and cellular activation by IL-1 is prevented. In a rabbit model of LPS and IL-1β induced lung injury, Ulich found that simultaneous instillation of IL-1RA, attenuated lung injury (Ulich 1991). In human adults, IL-1RA can be detected in BALF of healthy individuals and concentrations increase significantly at the onset of ARDS (Park 2001). A highly significant correlation was found between low concentrations of IL-1RA in BAL fluid and mortality in ARDS (Donnelly 1996). In survivors of ARDS the molar ratio of IL-1β over IL-1RA declines in the first 72 hours, suggesting a net anti-inflammatory signal which serves to dampen the inflammatory response (Park 2001). This study did not compare survivors and non-survivors of ARDS.

1.1.3.7 Soluble tumour necrosis factor receptor 1 (sTNFR-1) and 2 (sTNFR-2)

The extracellular domain of both the type 1 and type 2 TNF receptor (see 1.1.3.1) may be cleaved and appears in the circulation, urine and BAL fluid in low concentrations in healthy volunteers (Van Zee 1992, Opal 2000, Park 2001). A marked increase in the concentration of sTNFR1 and 2 has been detected in inflammatory conditions such as sepsis and ARDS and the pattern of increase mimics that of TNF-α (Girardin 1992, Spinas 1992, Suter 1992, Van Zee 1992, Ertel 1994, Park 2001). *In vitro* studies have shown that stimulated neutrophils shed TNF receptors from the cell membrane in response to stimulation with fMLP, complement and GM-CSF, resulting in a specific and marked reduction in TNF binding capacity (Porteu 1990). In high concentrations TNFRs inhibit TNF-α activity both *in vitro* and in *in vivo* models of endotoxaemia (Aderka 1992, Van Zee 1992). These observations suggest a protective effect of TNFRs both by desensitizing target cells against the effects of TNF-α and by inhibiting activity of circulating TNF-α. However, *in vitro*, at low concentrations TNFRs may stimulate the effects of TNF-α on B lymphocyte and fibroblast proliferation and also prolong TNF-α activity by preventing the natural decay of TNF-α (Aderka 1992). In human disease it has been observed that the molar ratio of TNF-α over TNFR may be important. For instance in meningococcal sepsis, patients with a higher molar TNF-α /TNFR ratio were more likely to die (Girardin 1992). In adults with ARDS, molar TNF-α /TNFR ratios were significantly lower compared to controls in the early phase of their disease, and the TNF-α /TNFR ratio gradually increased with time to reach normal values by the third week (Park 2001).

1.1.4 Common conditions in near-term newborn infants resulting in severe respiratory failure

In near-term newborn infants the commonest causes for severe respiratory failure are persistent pulmonary hypertension of the newborn, meconium aspiration syndrome, congenital diaphragmatic hernia, viral and bacterial pneumonia and hyaline membrane disease (Lyrene 1984, Bartlett 1995, Morin 1995, 1996, Hansen 1998, Lakshminrusimha 1999). By the time these infants fail conventional management and require ECMO, their primary lung disease will have been compounded by a degree of ventilation induced lung injury and oxygen toxicicty. In the next six sections follows a more detailed discussion of each of these clinical entities, with particular emphasis on the role of acute lung inflammation.

1.1.5 Persistent pulmonary hypertension of the newborn (PPHN)

During foetal life, the placenta serves as the main organ for gas exchange. The lungs are filled with foetal lung fluid and pulmonary blood flow is only about 10% of the total ventricular output. The low blood flow to the lungs is achieved through constriction of pulmonary arterial bed, resulting in high pulmonary vascular resistance and diversion of blood away from the pulmonary circulation via the ductus arteriosus and the foramen ovale into the systemic circulation (Lyrene 1984, Morin 1995, Lakshminrusimha 1999). At birth the lungs suddenly become the main organ for gas exchange and the foetal pulmonary circulation undergoes structural and functional adaptation: there is a sustained decrease in pulmonary vascular resistance with a five to ten fold increase in pulmonary blood flow. This is achieved in the first 24 hours of life through the dilatation and recruitment of non-muscularised arteries and in the first few weeks by a reduction in arterial muscularisation. Also, lung growth contributes to the increase in cross sectional arterial diameter and reduction in pulmonary vascular resistance (PVR) (Haworth 1981, Geggel 1984, Lakshminrusimha 1999). The initial trigger for this acute

change in pulmonary haemodynamics appears to be the onset of respiration (Lyrene 1984) and nitric oxide has been identified as one of the key mediators of involved in successful transition (Kinsella 1995, Morin 1995, Lakshminrusimha 1999). However, the overall control of pulmonary vascular tone before and after birth is complex and influenced by a range of additional factors which either increase or decrease pulmonary vascular resistance, and these stimuli can be divided into endogenous mediators and mechanical factors (see table 1.1).

During PPHN there is a failure of adaptation to extra-uterine life and this occurs through several possible pathophysiological mechanisms (Geggel 1984, Lakshminrusimha 1999). First there may be maladaptation whereby high vascular resistance is maintained in a structurally normal pulmonary vascular bed. This may occur during infection due to direct effects of endotoxins (Rojas 1984). Secondly, there may be abnormal muscularisation of the intra-acinar and pre-acinar arteries. This can occur *in utero* due to chronic hypoxia or develop in the postnatal period through maladaptation of the pulmonary vascular bed. Thirdly, there may be underdevelopment of the pulmonary vascular bed, with either a reduction in size or in number of pulmonary arteries. This is often seen in association with congenital diaphragmatic hernia (see 1.1.10). The overall clinical picture is determined by a complex constellation of interactions involving the pulmonary vascular bed, lung parenchyma and the heart. Depending on the balance between pulmonary and systemic vascular resistance a degree of extra-pulmonary shunting from the pulmonary to the systemic circulation occurs through the ductus arteriosus and the foramen ovale, resulting in systemic hypoxaemia and acidosis. PPHN is often associated with parenchymal lung disease such as meconium aspiration syndrome, pneumonia, RDS or congenital diaphragmatic hernia (Walsh-Sukys 2000). Parenchymal lung disease results in intrapulmonary shunting through loss of lung volume and reduced lung

Table 1.1. Factors that modulate pulmonary vascular resistance before and after birth (adapted from Kinsella 1995).

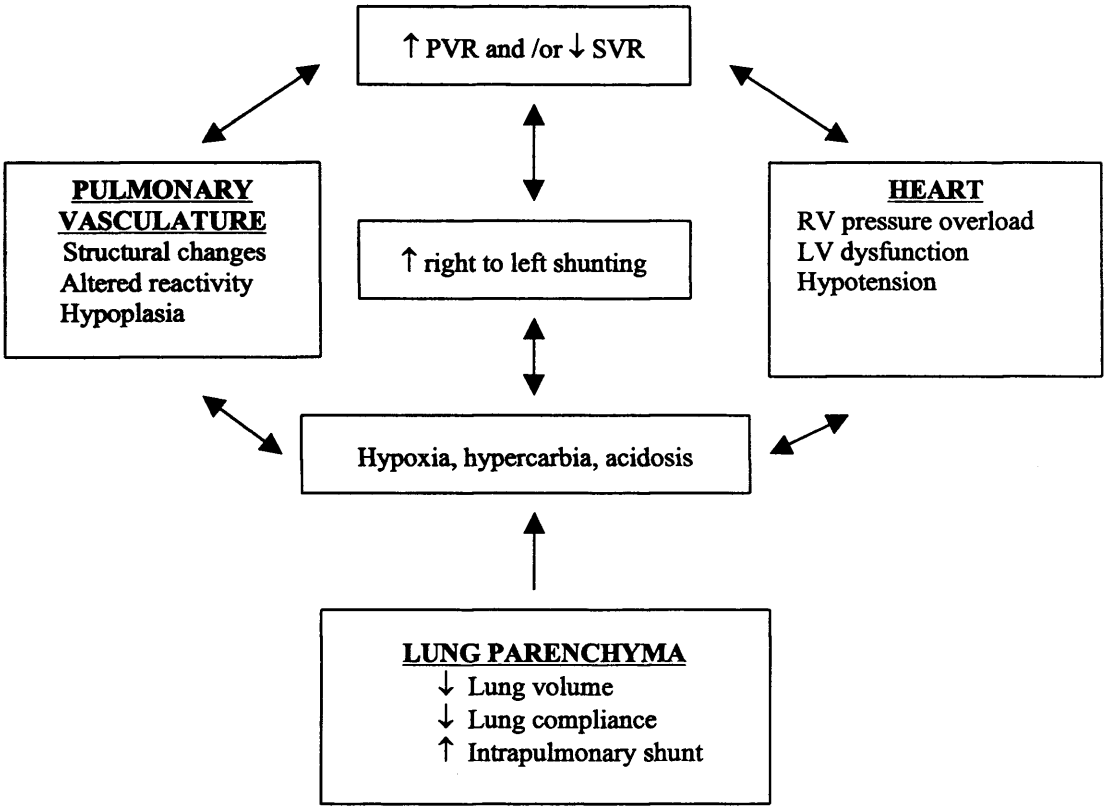
Reduce PVR	Increase PVR
Endogenous mediators or factors	**Endogenous mediators or factors**
Oxygen	Hypoxia
Nitric oxide	Acidosis
PGI_2, E_2, D_2	Endothelin-1
Adenosine, ATP, magnesium	Leokotrienes
Bradykinin	Thromboxanes
Atrial natriuretic factor	Platelet activating factor
Alkalosis	Calcium channel activation
Potassium channel activation	α-adrenergic stimulation
Histamine	$PGF_{2\alpha}$
Vagal nerve stimulation	**Mechanical factors**
Acetylcholine	Overinflation or underinflation
β-adrenergic stimulation	Excessive muscularisation, vascular remodeling
Mechanical factors	Altered mechanical properies of smooth muscle
Lung inflation	Pulmonary hypoplasia
Vascular cell structural changes	Alveolar capillary dysplasia
Interstitial fluid and pressure changes	Pulmonary thrromboemboli
Vascular shear stress	Main pulmonary artery distention
	Ventricular dysfunction, pulmonary venous hypertension

compliance and adds further to hypoxaemia, acidosis and hypercarbia, which in turn compound PPHN. In addition, PPHN can result in right ventricular pressure overload with subsequent left ventricular dysfunction, reduced tissue oxygenation and acidosis (see figure 1.1).

In the United Kingdom it is estimated that that PPHN occurs in 0.1 to 0.6 per 1000 live births (Greenough 1999). A recent large prospective study in 12 centres for neonatal intensive care

in North America, detected a prevalence of PPHN ranging between 0.43 and 6.82 per 1000 live births with an overall prevalence of 1.9 per 1000 (Walsh-Sukys 2000). Overall mortality

Figure1.1. Cardiopulmonary interactions in PPHN. (adapted from Kinsella 1995)



in this study was 22% (range between centres 4 to 33%) and newer treatment modalities such as high-frequency oscillatory ventilation, nitric oxide and ECMO did not reduce mortality. In the era prior to newer treatment modalities becoming available, mortality of PPHN ranged between 20 and 50% (Morin 1995). In the study by Walsh-Sukys *et al* (Walsh-Sukys 2000) the cause of PPHN was meconium aspiration syndrome in 41%, idiopathic in 17%, pneumonia in 14%, IRDS in 13%, either IRDS or infection (indistinguishable) in 14%, CDH in 10% and pulmonary hypoplasia in 4%. These data show that PPHN usually occurs in the context of other conditions such as MAS, RDS or infection which are associated with lung inflammation (see 1.1.10, 1.1.10, 1.18 and 1.1.10). Furthermore, severe cases require respiratory support with intubation and positive pressure ventilation which has the potential to create further lung injury through acute inflammation (see 1.1.10). Dobyns and workers studied newborn infants

with severe PPHN treated with ECMO and measured concentrations of eicosanoids (aranidonic acid metabolites) in BAL fluid (Dobyns 1994). This study showed that infants with PPHN requiring ECMO had higher concentrations of BAL eicosanoids than infants with mild PPHN requiring only conventional ventilation. In infants with good outcome on ECMO, concentrations of eicosanoids decreased whilst infants with poor outcome showed persistently elevated levels of BAL eicosanoids.

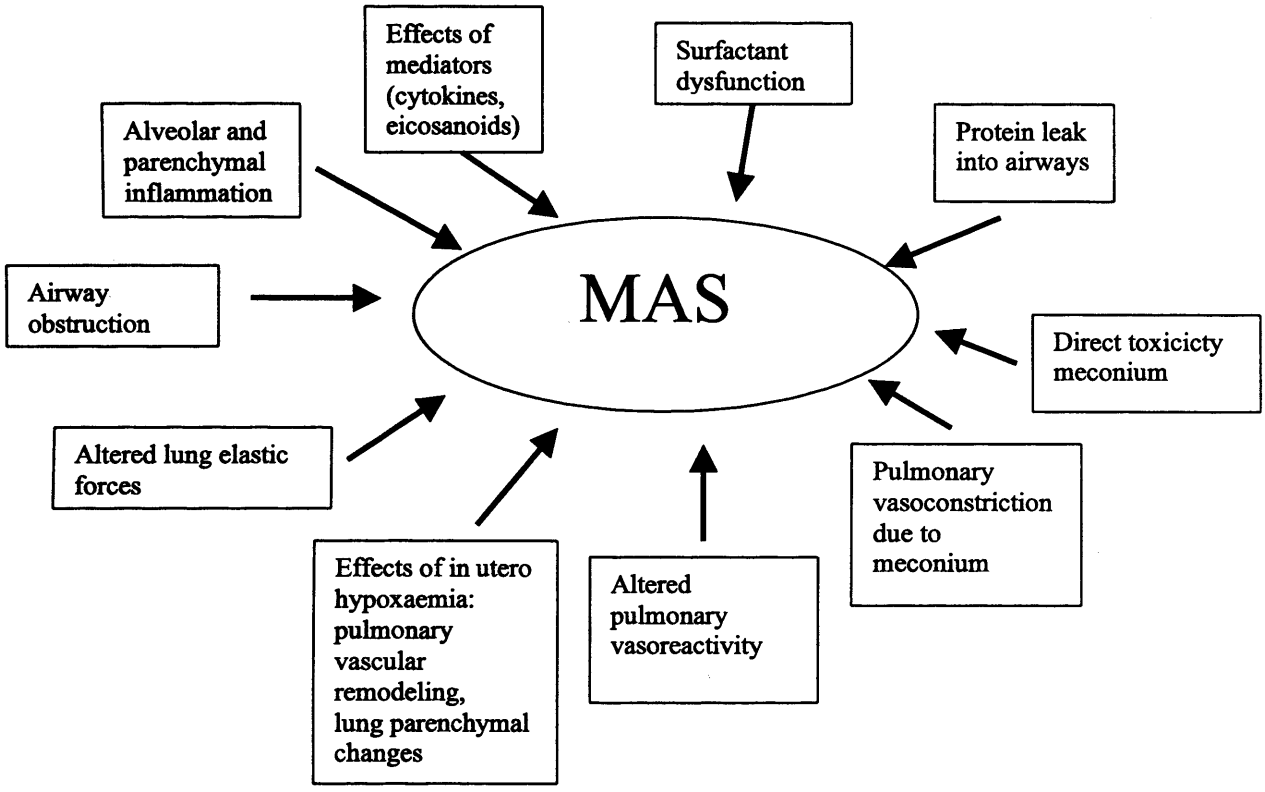
1.1.6 Meconium aspiration syndrome

Meconium aspiration syndrome (MAS) is defined as respiratory distress in association with the inhalation of meconium before, during or after birth, in the absence of any other direct cause of respiratory distress (Cleary 1998, Greenough 1999). Meconium stained amniotic fluid occurs in around 10 to 14% of deliviries. Only a small proportion will go on to develop MAS with an incidence in North America of 2 - 5 per 1000 deliveries and in Europe of 0.2 - 2 per 1000 deliviries. The mortality associated with MAS has a wide range between 0 and 40%, with a trend towards higher mortality rates in North America. In neonates with MAS respiratory failure is caused through two main mechanisms (see figure 1.2) (Cleary 1998, Greenough 1999). On the one hand inhalation of meconium below the vocal cords has several direct effects on the lungs including complete or partial airway obstruction, chemical pneumonitis, vasoconstriction, increased infection risk and surfactant inhibition. On the other hand, MAS often occurs in the context of acute or chronic intrauterine hypoxia, which is associated with pulmonary vascular remodeling resulting in PPHN (as discussed in section 1.1.5). Also, asphyxial lung injury is associated with surfactant inhibition. In clinical practice, severe MAS leads to a vicious cycle of hypoxaemia, acidosis, pulmonary hypertension and cardiac right to left shunting. Usually, it is not possible to distinguish which of the underlying mechanisms predominate in causing the clinical picture (see figure 1.2). Furthermore, it is at

present unclear whether severe MAS can occur from the inhalation of meconium alone in the absence of intrauterine asphyxia (Katz 1992, Thureen 1997).

Several investigators have studied the ability of meconium aspiration to produce acute lung injury and inflammation *in vitro* and in animal models. Using an *in vitro* model to study neutrophil chemotaxis by meconium, de Beaufort *et al* found significant concentrations of IL-8 present in human meconium and observed increased neutrophil migration when

Figure 1.2. Pathophysiological mechanisms involved in meconium aspiration syndrome.



incubated with human meconium (de Beaufort 1998). This effect on neutrophil migration could be inhibited by anti-IL-8 antibody in a dose-dependent manner. In animal models of MAS, it is standard practice to administer processed meconium solution via an endotracheal cannula in previously well animals. In unventilated rabbits, a progressive chemical pneumonitis develops with neutrophil influx, thickening of alveolar septae and hyaline

membrane formation starting at 6 hours, which progresses until 48 hours after instillation of meconium (Tyler 1978). Similar observations have been made in ventilated rats and piglets, with maximal acute inflammation occurring between 12 and 48 hours following meconium instillation independent of ventilation (Davey 1993, Cleary 1997, Soukka 1997b). The study by Davey showed that tracheal aspirates from rabbits with MAS, have significant *in vitro* neutrophil chemotactic activity. However, a 20% meconium solution had similar neutrophil chemotactic activity as pretreatment tracheal aspirates (Davey 1993). Several studies looking at interventions to improve outcome of MAS in animal models have studied effects on lung inflammation. In a porcine model, nitric oxide administration improved pulmonary hypertension and oxygenation, but not acute lung inflammation (Holopainen 1999). Similarly, Soukka found improvement in pulmonary hypertensive changes but not in pulmonary leukocyte sequestration, using pretreatment with methylprednisolone in a porcine model of MAS (Soukka 1997a). Wiswell and colleagues studied several modes of high frequency ventilation and surfactant administration in a piglet model (Wiswell 1994). This study showed no benefit from HFOV (high frequency oscillatory ventilation) or surfactant alone or in combination, on respiratory failure or lung histological changes. Ohama, using a rabbit model of MAS, compared surfactant administration with saline control during conventional ventilation (Ohama 1999). In this study, high dose surfactant did improve gas exchange and inflammatory changes (Ohama 1999). Two studies using different modes of high frequency ventilation in dogs and piglets, both showed improved lung injury scores compared to conventional ventilation (Keszler 1988, Wiswell 1992). Taken together these studies demonstrate that MAS in animal models is associated with acute lung inflammation, which may be exacerbated by ventilator induced lung injury. It is likely that antenatal events may also play a role. This is demonstrated by post-mortem studies in human newborn infants, who died following MAS, showing changes consistent with intra-uterine asphyxia in most, but also

hyaline membrane formation and pneumonic changes in the majority of cases (Seo 1990, Thureen 1997).

1.1.7 Congenital diaphragmatic hernia

In congenital diaphragmatic hernia (CDH) abdominal contents herniate posteriorly through the diaphragm via the foramen of Bochdalek, most commonly on the left side (Askin 1998, Adzick 2000). This is associated with a varying degree of ipsilateral and contralateral lung hypoplasia. Also, the pulmonary vascular bed may be hypoplastic with reduced numbers of pulmonary arteries and bronchial branches (Nobuhara 1996). Traditionally it is presumed that congenital diaphragmatic hernia is caused by failure of the pleuroperitoneal canal to close between the 7^{th} and 9^{th} week of foetal development. This results in herniation of abdominal contents into the thorax with compression and subsequent hypoplasia of the lung on the affected side (IJsselstijn 1998, Rosenberg 1998). Experiments in animal models suggest that lung hypoplasia may be the primary event and that underdevelopment of the diaphragm is either an associated or a secondary phenomenon (Iritani 1984, Jesudason 2000). In terms of pathophysiology several factors play a role (Nobuhara 1996, Askin 1998). First of all, the lung hypoplasia results in a smaller surface available for gas exchange. Also, pulmonary hypertension may be caused by a smaller cross-sectional area of the total vascular bed, as well as by increased muscularisation of the arteriolar walls and increased pulmonary vascular hyperreactivity (see 1.1.5). Furthermore, studies in animal models have suggested that there may be a degree of immaturity in CDH of enzyme systems involved in anti-oxidant, surfactant and endogenous nitric oxide production (IJsselstijn 1998). Finally, associated congenital abnormalities, in particular congenital heart disease, may contribute to the morbidity and mortality of CDH (Nobuhara 1996). Newborn babies with CDH, who develop severe respiratory failure at birth requiring intubation and positive pressure ventilation, are at risk of

ventilator associated lung injury (as discussed in 1.1.10). Wung reported that a ventilation strategy aimed at minimizing ventilation induced lung injury by allowing relative hypoxia and hypercapnia, resulted in a much improved mortality rate than expected (Wung 1985). The incidence of CDH is reported to lie between 1 per 2000 to 1 per 5000 live births (Adzick 2000, Beresford 2000). In antenally diagnosed cases of CDH, associated congenital abnormalities are found in 30 –50%. When the diagnosis is made antenatally, the mortality is around 58%, whilst for live births the mortality is around 48% (Beresford 2000). Neonates with severe respiratory failure at birth, require respiratory support with positive pressure ventilation. The aim is to stabilize the patient prior to surgical repair of the diaphragmatic hernia. In patients who are difficult to stabilise additional therapeutic interventions have been tried, including HFOV, inhaled nitric oxide and surfactant therapy (Hirschl 1996). The impact of the use of ECMO on outcome of CDH has been difficult to assess because there is variation between ECMO centers in patient selection, ECMO technique, timing of hernia repair and survival. Also very few controlled studies have been published (Lally 1996). Recent reports suggest that ECMO may improve survival in the highest risk group of patients with CDH (CDH Study Group 1999, Beresford 2000).

1.1.8 Viral and bacterial pneumonia

Viral lower respiratory tract infections are common in infants with up to 10% of infants developing bronchiolitis in the first year of life (Panitch 1993). Severe respiratory failure is uncommon in previously healthy infants, but a clinically more severe course may be seen in high-risk groups with congenital heart or lung defects, chronic lung disease, neuromuscular disorders, cystic fibrosis and immunosuppressed children (Penn 1993). Overall, the mortality is less than 1% but this may be higher in high-risk groups. The commonest cause of viral LRTI in infants is respiratory syncythial virus (RSV) but other common infectious agents are

parainfluenza virus type 1-3, influenza virus A and B, mycoplasma pneumoniae and adenovirus (Penn 1993). In terms of pathophysiology, several factors play a role including epithelial cell necrosis, sloughing of necrotic debris, loss of cilia, increased mucous secretion and submucosal oedema (Panitch 1993). These factors combine to produce the clinical picture of severe bronchiolitis with altered lung function due to expiratory flow limitation, reduced lung compliance and ventilation perfusion mismatch.

A significant body of evidence is emerging from both *in vitro* and *in vivo* studies, that viral infection of the respiratory tract causes an acute inflammatory response (Domachowske 2001). Studies using cultured respiratory epithelial cell lines have shown that RSV infection promotes cellular IL-8 production through activation of the transcription factor NF-κB (Nuclear Factor-kappa B) (Garofalo 1996). RSV infection also results in increased gene expression of surface molecules on respiratory epithelial cells such as ICAM-1 and CD-18, which are important for neutrophil recruitment and adhesion (see 1.1.2) (Domachowske 2001). In cultured alveolar macrophages, RSV infection resulted particularly in increased and sustained mRNA expression for TNF-α and increased TNF-α concentration in the supernatant (Becker 1991). In this study, there was also increased expression of IL-6 and IL-8 mRNA with increased concentrations in supernatant of these cytokines. Similarly, Influenza A infection of cultured airway epithelial cells also results in increased gene transcription of IL-6, IL-8 and RANTES (receptor-activated normal T-cell expressed and secreted) (Choi 1992, Matsukura 1996). The inflammatory response in the lung caused by mycoplasma infection has been studied by several authors in a murine model (Wubbel 1998, Hardy 2001, Martin 2001). Following infection, lung histological examination revealed an intense bronchopneumonia, with predominant neutrophil infiltration, which appeared most intense on day 3 to 4 (Wubbel 1998, Hardy 2001, Martin 2001). In the first 72 hours, infection with viable mycoplasma was also

associated with increased concentrations of TNF, IL-6 and functional IL-8 in BAL fluid (Hardy 2001). Finally, the potential of adenovirus infection to cause acute inflammation has been studied in cultured cells and animal models. Higginbotham and workers studied the response of isolated peripheral blood monocytes to infection with wild type 5 adenovirus (Higginbotham 2002). They found significant increase in IL-6 and IL-8 production and a small increase in TNF-α production. The ability of adenovirus infection to stimulate various cell types in the lung to produce pro-inflammatory cytokines may be dependent on cell type and and virus type.

Booth and Metcalfe (Leland Booth 1999) showed in cultured foetal lung fibroblasts and epithelial cells, that adenovirus type 7 but not type 5 induced expression of IL-8 mRNA and IL-8 production. Infection with both types resulted in prolonged IL-8 mRNA half-life. In a murine model Thorne and workers (Thorne 1999) showed that intratracheal instillation of adenovirus resulted in significant increase in BAL fluid concentrations of IL-6 and TNF-α at 3-6 hours and in neutrophil percentage at 12 –24 hours. Also using a murine model, Zsengeller *et al* (Zsengeller 2000) studied the role of alveolar macrophages in initiating an inflammatory response in the lung following infection with type 5 adenovirus. These authors showed that very rapidly following infection, adenovirus is distributed throughout the lung and quickly internalised by AM's. Internalisation of the virion is associated with production of the inflammatory cytokines TNF-α and IL-6 and the chemokines macrophage inflammatory protein-1α and –2 (MIP-1α and MIP-2). However, no such initiation of cytokine signalling occurred in airway epithelial cells or vascular endothelial cells.

Viral infection of the respiratory tract in humans is also associated with an acute inflammatory response. Everard and workers (Everard 1994) studied nasal lavage and BAL fluid differential cell counts in ventilated infants with RSV positive bronchiolitis. Both in the upper and lower

respiratory tract of these infants, neutrophils were the predominant cell type and neutrophil predominance did not appear to be related to duration of ventilation. A further study by the same group, (Abu-Harb 1999) showed increased IL-8 concentrations in nasal lavage samples obtained from unintubated infants admitted with RSV bronchiolitis. Experimental infection of human volunteers with influenza A virus, showed an early rise in IL-6 and IFN-α concentrations in nasal lavage fluids, which was associated with the onset of upper respiratory tract symptoms (Hayden 1998). In this study, nasal lavage fluid concentrations of TNF-α and IL-8 peaked later (day 3-6) and were associated with systemic and lower respiratory tract symptoms. The latter observation suggests a role for TNF-α and IL-8 in more severe lower respiratory tract infections with influenza A in humans. Mistchenko *et al* studied serum concentrations of IL-6, IL-8 and TNF-α in children with adenoviral disease (Mistchenko 1994). Serum concentrations of all three cytokines were related to outcome, with significantly higher serum concentrations in severe or fatal adenovirus infection.

The observed pro-inflammatory cytokine response and neutrophil predominance in respiratory lavage fluid following viral infection observed in animal models and humans, is at odds with post-mortem studies in children who died following severe viral lower respiratory tract infection. Aherne and workers studied the pathological changes in the lungs of 22 children who died following viral lower respiratory tract infection (Aherne 1970). In this study, the majority of subjects showed either acute bronchiolitis with epithelial cell necrosis and lymphocytic infiltration, or pneumonia with epithelial flattening, hyperplasia and necrosis and intralveolar thickening due to mononuclear infiltration. Only three patients showed predominant neutrophilic infiltration, of whom one had RSV infection and in two no infective agent was recovered. Becroft studied the histopathological changes in the lungs of five children following fatal infection of the lower respiratory tract with adenovirus (Becroft

1967). There was extensive bronchiolar destruction and necrosis. The bronchiolar lumen was filled with pleomorphic exudate, consisting of hyaline material, mucous, fibrin, neutrophils, macrophages and epithelial cells. Further down in the respiratory glands and alveoli, there was epithelial necrosis with predominantly monocytic inflilltration and relative paucity of neutrophilic infiltration.

The relative lack of neutrophilic infiltration in these post-mortem studies may have several possible explanations. These patients may have been at a more advanced stage in their disease compared to the subjects in animal and human studies referred to previously in this section. Also, sampling from different compartments (histology samples the interstitial space, whilst bronchoalveolar lavage samples the airways and alveolar space) could account for the observed differences. Another possible explanation is that an inability to mount an acute inflammatory response following viral infection of the lower respiratory tract, may be associated with worse outcome. Domachowske and workers studied this hypothesis in mice deficient of the pro-inflammatory chemokine MIP-1α, involved in neutrophil and eosinophil recruitment, or its receptor CCR-1 (Domachowske 2000). Following infection with pneumonia virus of mice (PVM), a paramyxovirus related to RSV, fully immune competent mice showed a marked increase in BAL total white cell numbers predominantly consisting of neutrophils, between day 3 and 6 after infection. Both MIP-1α and CCR-1 negative mice showed marked attenuation of this inflammatory response and higher levels of infectious virus were present in lung tissue compared to fully immune competent mice. CCR-1 deficient mice succumbed significantly earlier to PVM infection compared to immune competent mice. These data suggest that a MIP-1α /CCR-1-mediated acute inflammatory response is important in controlling viral replication and that this response prolongs survival in mice following PVM infection.

Early onset bacterial pneumonia has an estimated incidence of around 1.8 to 5 per 1000 livebirths and is usually caused by bacteria acquired before or during birth (Hansen 1998c, Dear 1999). The commonest causative organism is group B Streptococcus but other less common organisms include Haemophilus influenzae, Streptococcus pneumoniae, Listeria monocytogenes and Escherichia coli. The clinical picture can be very variable ranging from mild non-specific symptoms to severe respiratory failure mimicking RDS and septic shock.

In the airway, invading bacteria are recognised by resident cells such as macrophages, dentritic cells and epithelial cells, by virtue of distinct carbohydrates expressed on the bacterial surface. This recognition activates the innate immune system and results in an acute inflammatory response with influx of monocytes and neutrophils, which are essential for successful bacterial clearance (see 1.1.2) (Toews 2000). This process has been described in detail in studies into the sequential pathogenesis of pneumococcal pneumonia in mice, which showed a gradually spreading inflammatory response and tissue injury over the first few days following infection (Bergeron 1998, Fillion 2001). Initially there was an influx of PMNs into lung tissue and in BAL fluid, which were gradually replaced by monocytes. It was noted that PMN numbers decreased in blood and lung tissue over time, but remained elevated in BAL fluid until the death of the animals (see figure 1.3). In terms of cytokine response, TNF-α appeared early in BAL fluid, rising rapidly to high concentrations. IL-1 only appeared briefly in BAL fluid, but was present in significant concentrations in lung tissue, throughout the experiment. IL-6 appeared early in both BAL fluid and lung tissue and remained elevated in both (see figure 1.4). Two murine C-X-C chemokines (MIP-2 and KC), related to human IL-8, peaked within hours in both lung tissue and BAL fluid (Fillion 2001). In lung tissue levels remained elevated throughout whilst in BAL fluid concentrations had declined within the first

24 hours. The early peak in C-X-C chemokines correlated with neutrophil influx. A second wave of cellular influx by macrophages occurred at 48 to 72 hours, which was orchestrated in a synergystic manner by the C-C chemokines MCP-1 (monocyte chemoattractant protein 1), MIP-1α and RANTES. This study also showed that formyl peptides produced by Streptococcus pneumoniae make a significant contribution to recruitment of neutrophils and macrophages into the lung following infection. Several studies have addressed the role of inflammatory cells and pro-inflammatory cytokines in humans with bacterial pneumonia.

In adults with community acquired pneumonia with both gram positive and gram negative bacteria, BAL of the affected part of the lung showed significantly increased total white cell counts with neutrophil predominance and increased numbers of macrophages compared to non-involved areas and control subjects (Dehoux 1994, Boutten 1996). Similarly, BAL fluid concentrations of IL-1β, TNF-α, IL-6 and IL-8 were significantly increased in the affected parts of the lung compared to controls. Significant variability in the BALF cytokine concentration was noted, which may be explained by differences in duration of disease on presentation between study subjects. Alternatively, the method of BAL may be associated with significant variability. Measurement of cytokines in serum in these studies showed low concentrations of IL-1β, TNF-α and IL-8, but significantly increased concentrations of IL-6 compared to controls, suggesting that the infection remained limited to the lung. In children with cystic fibrosis, significantly increased concentrations of IL-1β were detected in BALF of patients with evidence of active infection, compared to non-infected cystic fibrosis patients (Wilmott 1990). There was also a positive correlation between BALF IL-1β concentrations and PMN numbers. Buck and workers found significantly increased concentrations of Il-1β and TNF-α in tracheal aspirates of ventilated newborns with pneumonia compared to non-

infected infants (Buck 1994). Furthermore, Papoff and workers observed that in ventilated premature newborns with proven

*Figure 1.3. Recruitment of inflammatory cells (means ± SEM) in BAL fluid as a function of time after infection of CD1 mice with 10^7 CFU of S. pneumoniae. *, $p < 0.05$ vs uninfected mice (0 h). (Adapted from Fillion 2001).*

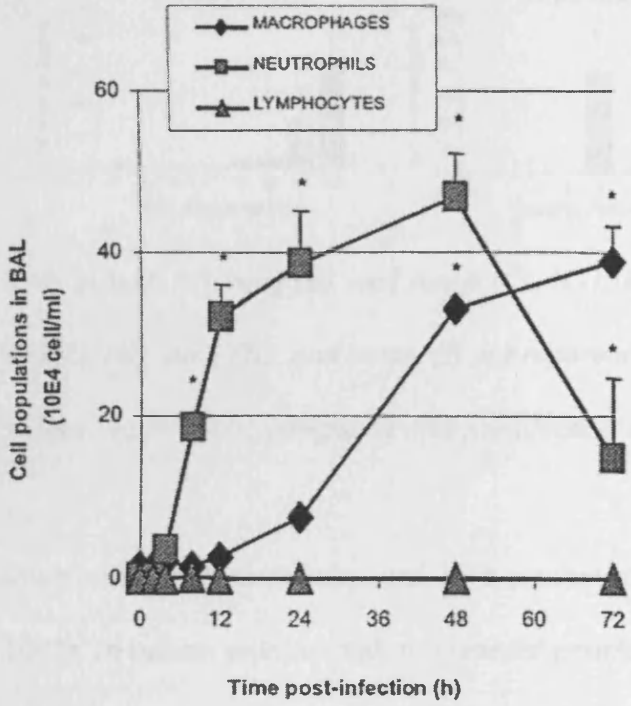
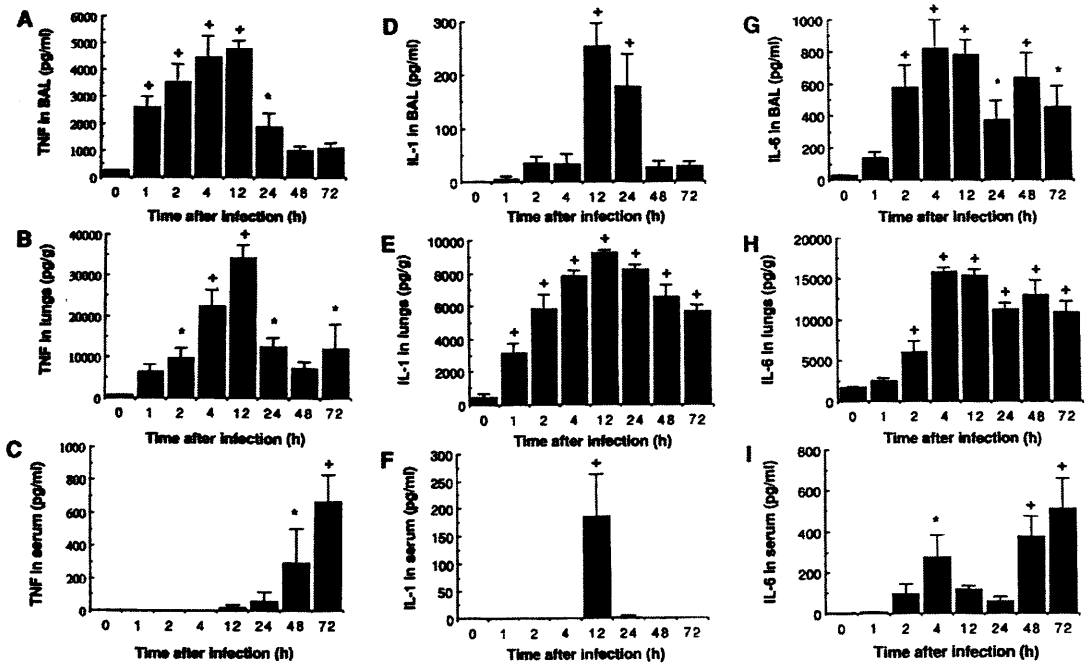


Figure 1.4. Mean (SEM) cytokine levels in cell-free BAL, lung homogenates previously cleared from blood, and serum of mice infected with 10^7 S. pneumoniae cells.



*TNF in BAL (A), lung (B), and serum (C), IL-1 in BAL (D), lung (E), and serum (F), and IL-6 in BAL (G), lung (H), and serum (I) are reported. *, $P < 0.05$ compared with preinfection values; +, $P < 0.01$ compared with preinfection values. (Adapted from Bergeron 1998)*

lower respiratory tract infections, IL-8 concentrations in BAL fluid were increased (Papoff 1995). In infants with normal or increased peripheral blood neutrophil counts, there was a positive correlation between IL-8 concentrations and neutrophil counts in BAL fluid. However no such correlation was observed in infants with low peripheral blood neutrophil counts. These findings suggest that adequate blood neutrophil numbers are required for recruitment into the lungs during lung injury.

Another model that has been used extensively to study the pulmonary response to infection both *in vitro* and *in vivo*, is exposure to endotoxin produced by gram negative bacteria such as

Escherichia coli. Dehoux and workers studied the *ex-vivo* cytokine production by peripheral blood monocytes and alveolar macrophages from patients with unilateral community acquired pneumonia and healthy control subjects (Dehoux 1994). Spontaneous IL-1β, TNF-α and IL-6 production by cultured AM's retrieved from the affected lobe in patients, and by peripheral blood monocytes from patients was significantly increased compared to controls. However, further stimulation with endotoxin of cultured AM's retrieved from the affected lobe, showed no increase in cytokine production, whilst AM's retrieved from unaffected parts of the lungs of patients and healthy controls did show a significant response following exposure to endotoxin. On the other hand, increased cytokine production in response to endotoxin was preserved in peripheral blood monocytes from both patients and healthy controls. These studies show that following initial increased cytokine expression by AM's in response to infection, there is either downregulation of this response or AM's have been maximally stimulated and cannot increase cytokine production further.

The group lead by Ulich, have extensively investigated the pulmonary inflammatory response to endotracheal instillation of endotoxin in rats (Tang 1995, Ulich 1995). These studies also show that instillation of endotoxin results in increased expression of IL-1 and TNF-α in the lung, which are involved in initiating the inflammatory response by inducing chemokines such as cytokine-induced neutrophil chemoattractant (CINC, an IL-8 like chemokine). These authors also demonstrated that the endothelial receptor ICAM-1 and the leukocyte integrin CD-11a are involved in neutrophil recruitment into the lung. Finally, in healthy adult volunteers, instillation of endotoxin into a pulmonary segment resulted in a two phase response (O'Grady 2001). In BAL fluid in the first 6 hours concentrations of TNF-α, IL-1β, IL-6, IL-8, L-selectin and IL-1RA peaked and this was associated with an influx of neutrophils. There was also an early increase in soluble TNFR-1 and -2 BAL concentrations,

which was sustained until 48 hours after instillation. Between 24 and 48 hours an influx was noted of monocytes, macrophages and lymphocytes and persistence of neutrophils until 48 hours. At that point neutrophil numbers declined significantly, suggesting removal of apoptotic neutrophils by macrophages.

1.1.9 Hyaline membrane disease

Idiopathic Respiratory Distress Syndrome (IRDS) caused by Hyaline Membrane Disease (HMD) is a condition predominantly affecting premature neonates and is rarely seen in near term neonates as a primary condition causing respiratory failure (Hansen 1998b, Greenough 1999). From 34 weeks of gestation the incidence falls from 15 % to less than 1% after 35 weeks of gestation (Lewis 1996). HMD was first described in the 1950s and was primarily attributed to developmental deficiency in the amount of surfactant lining the air-liquid surface of the lung. This deficiency may be worsened by associated lung injury caused by infection, oxygen or ventilation, which results in inhibition of surfactant by plasma proteins, which leak into the alveolar space. Surfactant consists of lipoproteins produced by type II pulmonary epithelial cells, and has surface tension reducing properties thereby preventing the alveoli from collapsing. Surfactant deficiency results in lower functional residual lung capacity and reduced lung compliance. This causes a marked increase in the work of breathing. In addition, there is marked intra-pulmonary shunting of blood and increased dead-space ventilation. These factors all contribute to the clinical picture of RDS in newborn babies. This syndrome is characterized by tachypnoea, accessory respiratory muscle use, expiratory grunting, cyanosis and respiratory acidosis. The chest radiograph shows a typical "ground-glass" appearance of diffuse, fine granular densities with an air-bronchogram. In severe HMD babies require support with mechanical ventilation, which may cause further lung injury (see 1.1.10).

Autopsy studies in infants have shown that the initial phase of HMD is characterized by an exudative phase with extensive hyaline membranes, proliferation of type 2 pneumocytes, septal wall thickening and early proliferation of fibroblasts (Anderson 1983). Bronchopulmonary dysplasia (BPD) or chronic lung disease (CLD) is heralded by a fibroproliferative phase with widespread interstitial fibrosis, emphysema and sometimes obliterative bronchiolitis. An acute inflammatory response associated with HMD has been observed in animal models as well as *in vivo* studies in humans. In ventilated, premature lambs with HMD, within hours an influx of neutrophils is seen in the lungs with increased neutrophil myeloperoxidase activity on histological examination of the lung (Kinsella 1997). Similarly, in premature humans with HMD a transient influx of neutrophils is seen into the lungs in the first week of life and this is followed by an influx of macrophages (Merritt 1983, Ogden 1984, Kotecha 1995, Jones 1996). Concurrently with the initial neutrophil influx, there is a rise in BAL fluid concentration of IL-8 and soluble ICAM-1 (Kotecha 1995, Jones 1996). This acute inflammatory response may be compounded by intra-uterine cytokine exposure due to prolonged rupture of membranes and chorioamnionitis (Grigg 1992b), and by post-natal infection. Worse outcome in terms of chronic lung disease is associated with a more profound and prolonged initial inflammatory response (Merritt 1983, Ogden 1984, Arnon 1993, Groneck 1994, Kotecha 1995).

1.1.10 Ventilator induced lung injury

Positive pressure ventilation (PPV) although life-saving, has the potential to cause further injury to the lungs through several mechanisms. It has been recognized for some time that PPV can cause disruption of the airspace wall and leakage of air into the interstitial space or the pleural space, classically described as barotrauma (reviewed in Haake 1987, Hansen

1998). The commonest presentation, and potentially most life threatening, of this is pneumothorax. More recently a different, more subtle form of injury has been described which is termed ventilator-induced lung injury (VILI) (reviewed in Dreyfuss 1998, Dos Santos 2000). The basic concepts of this type of injury are mainly based on observations in animal experiments, although application of some of these concepts in clinical practice has resulted in improved outcome. The principal mechanisms involved in VILI include regional overdistention of the lung, repeated opening and closing of atelectatic lung areas, and oxygen toxicity.

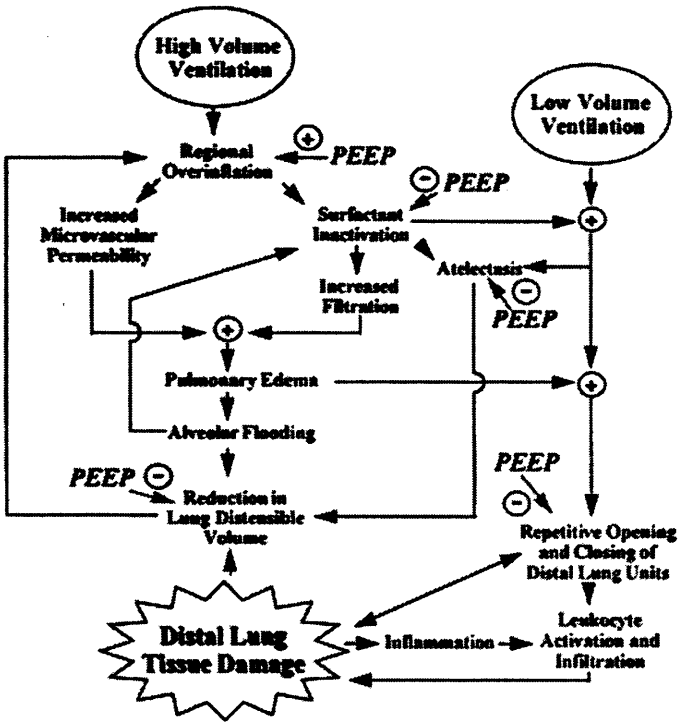
Lung overdistention has direct mechanical effects on the healthy lung causing rapid interstitial oedema formation, disruption of the alveolar epithelium and endothelium, and surfactant inactivation (Dreyfuss 1998). Lung collapse during PPV with repetitive opening and closing of lung units may also directly cause interstitial oedema and surfactant inactivation. The combination of these effects results in alveolar flooding with plasma exudate. This leads to regional lung volume loss and inhomogeneity of lung expansion, compounding the direct mechanical effects. Positive end-expiratory pressure (PEEP) may limit the damage caused by VILI (see figure 1.5).

The injured lung appears to be more sensitive to direct effects of positive pressure ventilation. Acute inflammation is probably a more delayed mechanism by which PPV causes lung injury and is usually only seen in experimental models with larger animals. It has recently been suggested that mechanical factors during PPV may induce an inflammatory response (Dos Santos 2000). Firstly, alveolar epithelial and endothelial cells which lose plasma membrane integrity, may directly or indirectly through calcium release activate key genes expressing mediators such as c-Fos and NF-κB. Similarly, shear forces and cell stretch may be involved

in inflammatory gene activation in endothelial cells and alveolar macrophages. Thirdly, exposure of circulating neutrophils to alveolar basement membrane components and, inflammatory cytokines and chemokines, will cause neutrophil activation and recruitment (see section 1.1.2.2).

The degree of inflammation caused appears to depend on the mode of ventilation. In perfused rat lungs, high volume ventilation causes increased mRNA expression of TNF-α and IL-6 and increased concentrations of these cytokines in BAL fluid and lung homogenate (Tremblay 2002). Similarly, in perfused mouse lung overventilation caused increased concentrations of TNF-α and IL-6 and chemokines in lung perfusate (Held 2001). A study into the effects of positive end-expiratory pressure on the degree of lung inflammation in a lamb model of HMD, showed that PEEP of 4 cm H_2O resulted in significantly less neutrophil infiltration and expression of IL-1β, IL-6 and IL-8 in the lungs, compared to PEEP of 0 or 7 cm H_2O (Naik 2001). In a surfactant washout model using rabbits, high frequency oscillation ventilation (HFOV) appeared to protect the lung from lung injury compared to conventional PPV. In animals receiving HFOV there was significantly less neutrophil activation and infiltration in the lung compared to animals that underwent conventional ventilation. Also, animals treated with conventional PPV showed evidence of increased lung TNF-α mRNA expression compared to animals treated with HFOV (Sugiura 1994, Takata 1997). In human adults with ARDS, ventilation with smaller tidal volumes and higher levels of PEEP is associated with a significant reduction in inflammatory cell numbers and cytokine concentrations in BAL fluid (Ranieri 1999). Several randomised controlled trials in adults with ARDS showed that reduced tidal volume ventilation result in

Figure 1.5. Flow diagram summarizing the contributors to mechanical ventilation-induced lung injury.



Positive end-expiratory pressure (PEEP) generally opposes injury or edema formation (minus sign) except when it contributes to overinflation (plus sign). (Adapted from Dreyfuss 1998)

significantly improved mortality (Amato 1998, ARDS Network 2000).

Similarly to VILI, oxygen toxicity causes lung injury, which is very similar in appearance to RDS due to other causes (Hansen 1998, Clark 1999). Exposure of the lung to high oxygen tension leads to the formation of reactive oxygen species through univalent reduction of oxygen. Oxygen radicals cause peroxidation of lipid membranes, resulting in the destruction of cell membranes and intracellular organelles. Also, reaction of these compounds with sulfhydryl enzymes can lead to inactivation and alterations in cellular metabolism. Finally, oxygen radicals can react with nucleic acid, resulting in damage to DNA. Several anti-oxidants are present in the lung, which can scavenge oxygen radicals, such as gluthatione and vitamins A and E. Oxygen toxicity is likely to occur when the scavenging capacity of the lung by anti-oxidants is exceeded.

Histological studies in experimental oxygen toxicity, show destruction of the alveolar epithelium and endothelium with interstitial oedema formation in the first week of exposure to high oxygen tension (Hansen 1984). At the same a time, an acute inflammatory response is noted with recruitment of large numbers of activated neutrophils into the lung, which is driven in part by chemokines like IL-8 (Hansen 1984, D'Angio 1995, Deng 2000).

1.2 Extra-corporeal membrane oxygenation in neonatal respiratory failure

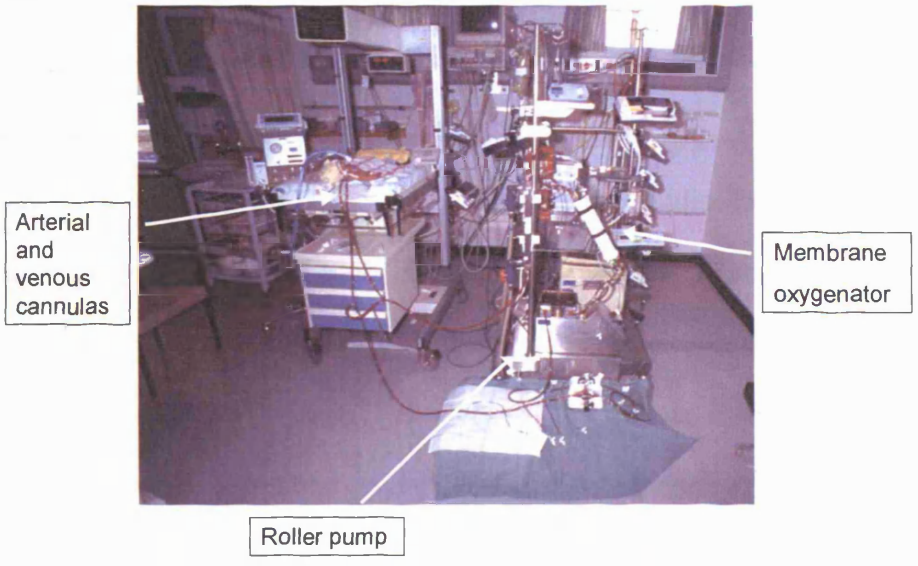
Over the past two decades, Extra-Corporeal Membrane Oxygenation (ECMO) has been developed as a modification of cardiopulmonary bypass (CPB), to support patients in the intensive care unit with severe cardio-respiratory failure (Kanto 1995). The main group of patients in which this technique has been developed, are near-term neonates (gestational age

over 34 weeks) with severe respiratory failure, who have failed to improve using conventional ventilation (Bartlett 1982). In the United Kingdom it is estimated that each year around 100 infants will fail "conventional support" and require support with extra-corporeal membrane oxygenation (Pearson 1992, UK Collaborative ECMO Trial Group 1996).

During ECMO gas exchange takes place outside the body, by draining de-oxygenated blood from the central venous system into the ECMO circuit (see figure 1.6). Blood then passes via a mechanical pump through a membrane oxygenator, where carbon dioxide removal and oxygen uptake take place. Oxygenated blood is then returned to the patient, either into the central venous system (venovenous ECMO) to provide respiratory support or into the arterial system (venoarterial ECMO) to provide cardiac support as well (Bartlett 1995). It is customary during ECMO to allow the native lung to "rest", in order to minimize further ventilator associated lung injury and to allow recovery of the original lung injury to occur. Once lung recovery has occurred and adequate gas exchange is maintained on low pressure ventilation, the patient is removed from extra-corporeal support and weaned from mechanical ventilation. As patients treated with ECMO are not directly dependent on their lungs for gas exchange they provide a safe model for the study of the resolution of lung injury.

Institution of extra-corporeal support results in blood activation through several mechanisms (reviewed in Anderson 1983, Hornick 1996, Wan 1997). Contact activation occurs as blood comes into contact with the surface of the by-pass circuit and membrane oxygenator. Blood also undergoes shear stress due to the action of the mechanical pump and turbulent flow within the circuit and may be damaged by osmotic forces. These processes result in the activation of protein systems and cellular components of the blood, which resembles the

Figure1.6. Infant treated with veno-venous ECMO for severe viral pneumonia



inflammatory response seen during sepsis. Initially, the coagulation cascade is activated through clotting factor XIIa, which in turn results in activation of the kallikrein/bradykinin system. There is also direct activation of the complement system and direct platelet activation. Protein and platelet activation result in activation of circulating monoctyes, neutrophils and endothelial cells, with secondary release of arachnidonic acid metabolites, cytokines, proteolytic enzymes, nitric oxide and oxygen radicals. This process is in part responsible for the distant organ damage seen during cardiopulmonary by-pass. In particular neutrophil mediated acute lung injury has been observed in animal models of CPB (reviewed in Anderson 1983, Hornick 1996, Wan 1997). The inflammatory response during ECMO has been studied both *in vitro* and *in vivo* and may be milder than during CPB used for cardiac surgery. Yanagi and colleagues performed ECMO for 72 hours in a rabbit model. They observed a transient increase in plasma TNF-α and IL-1RA concentrations, but not IL-1β in the first 24 hours after the institution of ECMO (Yanagi 1996). A further group of rabbits that only underwent surgery for cannulation without initiation of ECMO, showed a similar peak in

plasma TNF-α and IL-1RA concentrations. Adrian and workers studied leukocyte activation and cytokine release in an isolated ECMO circuit (Adrian 1998). Significant increases in circulating concentrations of IL-1β, IL-8 and IL-1RA were noted, together with a fall in mean leukocyte and neutrophil numbers, over the first 24 hours. TNF-α and IL-6 concentrations showed statistically insignificant increases. *In vivo* studies in neonates treated with ECMO also show evidence of an acute inflammatory response in the blood associated with the initiation of ECMO. In the first 24 hours there is a decrease in circulating neutrophil and macrophage counts (Hocker 1991, DePuydt 1993, Plotz 1993). Also, during the same time frame neutrophil activation occurs associated with increased concentrations of IL-8 and TNF-α and complement activation (Hocker 1991, DePuydt 1993, Plotz 1993, Underwood 1995, Fortenberry 1996). Fortenberry showed a positive correlation between neutrophil activation and radiographic evidence of worsening lung injury following initiation of ECMO (Fortenberry 1996).

The selection criteria for ECMO in neonates with severe respiratory failure are summarised in table 1.2 (reviewed in Rosenberg 1995). ECMO is indicated for near term neonates with reversible, severe respiratory failure, which has failed to respond to conventional support. However, prolonged conventional ventilation beyond 10 to 14 days in infants with severe respiratory failure is associated with poor outcome, due to irreversible ventilator induced lung injury. The oxygenation index (OI) is commonly used as a measure of severity of respiratory failure. The OI is expressed as [mean airway pressure (in cm H_2O) x FiO_2 x 100] / PaO_2 (in mmHg). In the UK Collaborative ECMO Trial an OI of 40 was used as an indication for ECMO (UK ECMO collaborative trial group 1996). The commonest conditions leading to severe respiratory failure in near-term newborn infants are: meconium aspiration syndrome, PPHN, pneumonia/sepsis, HMD and congenital diaphragmatic hernia (see 1.1.5 – 1.1.10) (UK

ECMO collaborative trial group 1996, Roy 2000). With time the relative proportion of certain diagnostic categories has changed. From the late 1980's to the late 1990's the proportion of patients with CDH has increased and the proportion of cases with HMD has decreased (Roy 2000). At the same time there has been an increased use of newer treatment modalities such as HFOV, surfactant replacement and inhaled nitric oxide, which has resulted in reduced referral rates for ECMO, but no change in overall mortality (Hintz 2000). A gestational age of less than 34 weeks is associated with increased risk of intracranial bleeding due to the anticoagulation of blood during ECMO. Also, a birth weight below 2 kg makes the cannulation procedure technically very difficult and cannulas which would fit the major vessels of such small infants do not allow for sufficient flow to provide adequate support. Because of the use of systemic heparinisation during ECMO, significant coagulopathy or uncontrolled bleeding are also contra-indications for ECMO. In patients who fail to recover despite ECMO support, cerebrovascular events and haemorrhagic complications are commonly seen (Evans 1994).

Early non-randomised trials of ECMO in infants with severe respiratory failure showed a clear benefit of ECMO in terms of survival with good neurological outcome (Kanto 1995). These observations were confirmed by the UK Collaborative ECMO Trial, which showed a 27 % difference in mortality (32% mortality in the ECMO group versus 59% in the control group). The medium term neuro-developmental outcome is not significantly different between ECMO treated patients and controls. In addition ECMO patients appear less likely to develop chronic lung disease (UK ECMO collaborative trial group 1996, Vaucher 1996).

Table 1.2 Selection criteria for neonatal ECMO

Selection criteria for neonatal ECMO *(adapted from Rosenberg 1995)*
1. Reversible lung disease unresponsive to maximal conventional therapy
2. Mechanical ventilation ≤ 10 days
3. Gestational age ≥ 34 weeks, birth weight ≥ 2 kg
4. No significant coagulopathy or uncontrollable bleeding
5. No intracranial haemorrhage
6. No lethal congenital malformations
7. No major cardiac malformation

1.3 The study of lung inflammation *in vivo*

The options to study lung inflammation for research purposes in intubated newborn infants with respiratory failure are limited, compared to the methods available in animal models in the laboratory setting. For instance, to obtain lung tissue from human infants for research purposes by open lung biopsy carries significant risks and would be unethical.

Fibre-optic bronchoscopy with bronchoalveolar lavage has become a standard in both non-intubated and intubated adults for the study of the bronchoalveolar milieu. Although the newer neonatal bronchoscopes have a suction channel, it is often inadequate to permit satisfactory bronchoalveolar lavage of infants especially newborn ones. In routine clinical practice, intubated patients undergo regular endotracheal suction to aid pulmonary toilet. Recently, the technique of routine endotracheal suctioning has been modified to non-bronchoscopic bronchoalveolar lavage, to allow the study of cellular and non-cellular components at the air

exchange surface during health and disease (Ogden 1984, Alpert 1992, Grigg 1992a, Koumbourlis 1993, Riedler 1995). In the next section, the technique of NB-BAL and its role in the study of the development and resolution of acute lung inflammation in newborn infants, will be discussed in more detail.

1.4 Non-bronchoscopic bronchoalveolar lavage (NB-BAL)

In ventilated infants examination of epithelial lining fluid obtained by bronchoalveolar lavage can provide valuable information both for clinical and research purposes. Since first described by Ogden and colleagues (Ogden 1984) the technique of nonbronchoscopic bronchoalveolar lavage (NB-BAL), based on a modification of routine endotracheal suctioning performed in ventilated infants in intensive care, has been further developed (Alpert 1992, Grigg 1992a, Koumbourlis 1993, Riedler 1995). Recently, standards for acquisition and processing of BAL fluid in adults and older children have been formulated (Klech 1989). Non-bronchoscopic BAL in infants, however, has so far lacked any standardisation and there is significant variation in the lavage procedure in reported studies (Kotecha 1999, de Blic 2000).

Nonbronchoscopic BAL is indicated in ventilated infants in whom the diameter of the endotracheal tube (ETT) is too small to allow for the passage of a fibreoptic bronchoscope or in whom this would cause significant respiratory compromise. Because it is a blind method of sampling BAL fluid, it is only suitable in diffuse lung disorders and normals (Koumbourlis 1993, Riedler 1995). In clinical practice NB-BAL has been used for the identification of infectious agents in ventilated infants (Koumbourlis 1993, Riedler 1995, Grigg 1996) and in ventilated children with atelectasis (Koumbourlis 1993). As a research tool it has been applied in the study premature infants with respiratory distress syndrome defining the role of pulmonary inflammation and fibrosis (Merritt 1983, Bruce 1984, Ogden 1984, Gerdes 1988,

Walti 1989, Yoder 1991, Gerdes 1992, Grigg 1992, Heikinheimo 1992, Murch 1992, Ohlsson 1992, Watts 1992, Arnon 1993, Fujimura 1993, Groneck 1993, Speer 1993, Bagchi 1994, Sluis 1994, Groneck 1995, Kotecha 1995, Kotecha 1996b, Kotecha 1996c, Murch 1996, Watterberg 1996) and surfactant (Hallman 1986, Chida 1988, Hallman 1991, Gerdes 1992, Stevens 1992, Moya 1994, Kari 1995, Griese 1996, Schrod 1996). In this population several authors have studied the role of oxygen radicals (Gladstone 1994, Varsila 1995, Contreras 1996). Other groups have studied alveolar epithelial lining fluid constituents in children with congenital heart disease (Grigg 1996), inflammatory mediators and surfactant in neonates with persistent pulmonary hypertension on extracorporeal membrane oxygenation (Evans 1989, Lotze 1990, Dobyns 1994) and surfactant components in ventilated infants with pneumonia (LeVine 1996).

Review of the methodology of the above studies, highlights important differences in the methodology and site of sampling during NB-BAL. The aim of NB-BAL is to obtain alveolar epithelial lining fluid, which contains the cellular or noncellular constituents under investigation. Nonbronchoscopic bronchalveolar lavage is performed by blindly passing a suction catheter down the endotracheal tube followed by instillation of a set volume of normal saline 0.9 % in aliquots at room temperature or warmed to 37^0 C. Use of a three-way adaptor (Alpert 1992, Grigg 1992a, Koumbourlis 1993) connected to the endotracheal tube allows for minimal interruption of ventilation during BAL. Mechanical suction is then applied and the BAL fluid is collected in a suction trap. Some authors advocate blindly wedging the suction catheter with the child positioned so that the right lower lobe is sampled (de Blic 2000), whilst others use tracheal aspirates by suctioning just beyond the tip of the endotracheal tube. A further difference lies in the volume of lavage fluid instilled. Some groups use a volume based on body weight, whilst others instill a fixed volume of lavage fluid. Furthermore, different

types of catheters and varying levels of suction pressure are used, applied either with mechanical suction or manually using a syringe. Prior to processing, some authors opt to filter the BAL fluid to clear mucous whilst others fragment mucous by repeated aspiration. Also, many groups record the amount of fluid retrieved either as an absolute volume or a relative volume of the total volume instilled. Finally, in expressing the results of cell concentrations or non-cellular constituents, some authors choose to correct for dilution of the ELF by the lavage procedure by using an internal marker such as albumin, urea or secretory component of immunoglobulin A (SC IgA).

Bronchoalveolar lavage fluid consists of a mixture of diluent, usually normal saline and alveolar ELF. The non-cellular constituents are dissolved in this and in order to determine their concentration in the original ELF it is necessary to correct for dilution. To achieve this one can utilise either an external or an internal maker for dilution. Studies to develop markers for dilution have mainly been carried out in animal experiments and adults (Baughman 1983, Rennard 1986, Richter 1986, Marcy 1987, Ward 1992, Von Wichert 1993, Ratjen 1996) using fibreoptic bronchoscopy and examples of external markers include methylene blue, inulin and radio active tracers (^{99}Tc-DTPA, ^{51}Cr-EDTA). As internal markers the following have been utilised: potassium, calcium, total protein, albumin, urea and SC Ig A. In particular the latter four have been applied in NB-BAL. External markers are added to the diluent at a known concentration and remeasured once BALF is obtained. The concentration difference provides a factor for dilution of ELF. A general disadvantage with external markers is that the concentration difference before and after lavage is relatively small, creating a potential source for error. This may be a particular difficulty with NB-BAL in infants, where the volumes of diluent and BAL fluid are much smaller than in fibreoptic bronchoscopy in adults. There are

further specific difficulties with individual external markers making them unsuitable for use in intubated infants with lung disease, as outlined in the following two paragraphs.

Methylene blue has been advocated by Baughman and colleagues (Baughman 1983), who studied adults with interstitial lung disease. They also compared methylene blue dilution with inulin in dogs and found good correlation between both methods. The use of methylene blue dilution has been criticised for several reasons. First of all this method significantly overestimates the volume of ELF as compared to other dilution methods and morphological studies (Von Wichert 1993). This could be explained by binding of methylene blue to the epithelial surface as observed during fibreoptic bronchoscopy or loss through diffusion. Secondly, Richter and workers (Richter 1986) showed that methylene blue may chemically react with BALF components. They found that methylene blue sigificantly reduced the activity of alpha-1-protease inhibitor in BALF.

Baughman *et al* (Baughman 1983) comment on the difficulties of using inulin clinically as a marker. Dissolved in saline it is only stable for a limited period. Furthermore inulin is degraded if bacterial contamination occurs and therefore requires immediate processing. Animal experiments carried out by von Wichert *et al* (Von Wichert 1993) using inulin dilution led to bizarre results revealing negative volumes of ELF and casting doubt on its usefulness. In the same paper these authors show similar estimated ELF volumes by use of external radioactive markers compared to urea dilution and morphological methods. However in the clinical part of their study they observed some crossing of ^{99}Tc-DTPA into the blood stream, which is probably related to the degree of permeability of the alveolar capillary membrane.

They also comment that ^{99}Tc-DTPA is cleared from the lungs at an age dependent rate. Both these factors are difficult to quantify and result in increased estimated ELF volumes.

Internal markers of dilution have the general advantage of a relatively large difference in concentration between the BALF and the reference fluid, usually plasma, reducing the potential for error. However, conditions in the bronchoalveolar milieu in infants with severe respiratory failure may pose limits on the reliability of suggested internal markers. Infants with acute lung injury have increased permeability of the alveolar capillary membrane. The concentrations of albumin and total protein in ELF are dependent on alveolar capillary membrane permeability. This complicates comparison of results between normals and patients using albumin or protein as an internal marker (Klech 1989, Watts 1995, Dargaville 1999). Lysis of inflammatory cells in the in bronchoalveolar milieu may results in release of intracellular anions such as calcium and potassium, making these anions unsuitable as markers for dilution (Klech 1989). Furthermore, loss of respiratory epithelial cell integrity, increased levels of inflammatory cytokines and the presence of infection may alter the ELF concentration of SC IgA (Murch 1996, Dargaville 1999), a marker for dilution proposed by Watts and Bruce (Watts 1995).

Further limitations on the use of internal markers for correction for dilution are posed by the technique of BAL itself. Using sequential BAL in adults, several studies have shown that during fibreoptic BAL there is a net influx of urea into the ELF (Rennard 1986, Marcy 1987, Ward 1992). In contrast, theoretical models of sequential BAL predict a drop in urea concentration with each lavage, suggesting interference from the lavage procedure itself with urea concentration in ELF. In addition, Ward and colleagues (Ward 1992) have shown that

during BAL there is also a significant and rapid net influx of water into BALF, which results in dilution of internal markers. In order to reduce these effects, Rennard *et al* and Marcy and workers (Rennard 1986, Marcy 1987) have suggested alterations to the lavage procedure by minimising dwell times and avoiding sequential lavage. However single aliquot lavage may lead to incomplete retrieval of non-cellular components (Marcy 1987). Grigg *et al* showed that urea concentration remained constant during sequential lavage using NB-BAL (Grigg 1992a). Urea appears promising as an internal marker for dilution. Urea has been widely studied using fibreoptic bronchoscopy as a potential marker for dilution and has several advantages (Rennard 1986, Marcy 1987, Ward 1992, Von Wichert 1993, Ratjen 1996). Measurement of urea concentration is relatively easy. It has a low molecular weight and diffuses freely across membranes. Therefore in theory concentration in ELF and serum should be equal and remain unaffected by changes in alveolar capillary membrane permeability. In children with healthy lungs Ratjen and Bruch (Ratjen 1996), using fibreoptic bronchoscopy, have sampled a constant fraction of ELF with BAL adjusted to body weight and urea dilution. Dargaville and workers studied the validity of urea as a marker of dilution in intubated infants with healthy and diseased lungs, using NB-BAL (Dargaville 1999). They found that it performed well as a marker for dilution measuring surfactant components. Also, no significant additional influx of urea into BAL fluid was noted in injured lungs.

Watts and Bruce (Watts 1995) have studied the use of SC IgA, a protein secreted by respiratory epithelium, as a reference for dilution. These investigators found comparable concentrations SC IgA in ELF of healthy infants obtained by NB-BAL to those reported in adults. Also, they found no significant change in SC IgA in infants with altered permeability of the alveolar capillary membrane by diseases such as respiratory distress syndrome,

bronchopulmonary dysplasia and acute sepsis. In addition concentrations of SC IgA in ELF did not vary significantly with gestational or postnatal age during the first month of life. In contrast, as stated above, ELF concentration of SC IgA may be affected by loss of respiratory epithelial cell integrity, increased levels of inflammatory cytokines and the presence of infection (Murch 1996, Dargaville 1999).

The variation in methodology of NB-BAL makes comparison between studies difficult. Therefore, recent recommendations for the standardization of NB-BAL have been made (de Blic 2000). Also, it is currently unclear to which extent the sampling procedure affects the repeatability of measured cell counts and concentrations of non-cellular components in BAL fluid. NB-BAL has several further limitations in common with bronchoscopic BAL (reviewed in Reynolds 1987). On the one hand, bronchoalveolar lavage can provide information regarding the present and evolving status of disease processes affecting the lung. BAL is relatively easy to repeat and can therefore provide more dynamic information than for instance lung biopsy. Diseased tissue is sampled directly and the information obtained may be more representative than for example blood or sputum samples. However, BAL samples only the air exchange surface and changes here may not reflect those in the parenchyma or alveolar septa, complicating direct comparison with histologic lung sections. Another factor making this comparison more difficult is that BAL fluid may be contaminated with cells and proteins deriving from the conducting airways, potentially creating a false impression of spacial relationships of constituents within the alveolar space. Also, NB-BAL only provides information obtained from a small part of one lung, which may not be representative for both lungs. NB-BAL is further limited by the fact that no control can be exerted over the site from which sampling takes place, limiting its use to diffuse disease processes and healthy individuals. Nevertheless, as alluded to earlier in this section, NB-BAL has provided valuable

insights into the role of cellular and non-cellular constituents of ELF, in the evolution of lung inflammation in important conditions in newborn infants such as HMD and CLD.

Chapter 2. Hypothesis and research aims

2.1 Infants with severe respiratory failure requiring support with ECMO have uniformly inflamed lungs due to a combination of the underlying disease, ventilator induced lung injury and oxygen toxicity. Therefore, I hypothesised that two sequential, standardised non-bronchoscopic lavage procedures of the left and right lung of patients undergoing ECMO for severe respiratory failure will demonstrate good repeatability of NB-BAL for white cell counts and concentrations of non-cellular constituents.

In particular, I hypothesised that the following cell counts and pro-inflammatory cytokine concentrations show good repeatability between left sided and right sided NB-BAL samples from individual patients:

a. Total white cell count

b. Neutrophil total and differential cell count

c. Alveolar macrophage total and differential cell count

d. Lymphocyte total and differential cell count

d. Pro-inflammatory cytokines TNF-α, Il-1β, IL-6 and IL-8.

2.2 Similarly to premature infants with RDS, I hypothesized that infants with severe respiratory failure supported with ECMO who survive, show a transient inflammatory response in their lungs in terms of raised neutrophil counts and increased concentrations of pro- and anti-inflammatory cytokines in BAL fluid, which resolves with clinical improvement. Infants with severe respiratory failure who die, show a persistent inflammatory lung response which fails to resolve and lack of an anti-inflammatory response, as is seen in premature infants with chronic lung disease.

2.3 Neutrophil apoptosis and subsequent removal is an important mechanism for the successful resolution of acute inflammation. I therefore hypothesised that in infants with severe respiratory failure supported with ECMO who survive, neutrophil apoptotic activity is prominent in BAL fluid during the resolution of acute lung inflammation. In infants who die there is absence of neutrophil apoptotic activity associated with persistent acute lung inflammation. The cytokine TNF-α can induce neutrophil apopoptosis and I hypothesized that TNF-α concentration in BAL fluid is positively correlated with the pro-apoptotic activity.

Chapter 3. Repeatability of cellular constituents and cytokines concentration of lavage fluid obtained by non-bronchoscopic bronchoalveolar lavage of infants receiving extra-corporeal membrane oxygenation.

3.1 Introduction

Non-bronchoscopic lavage has become a valuable tool in the study of lung disease in intubated infants with conditions such as pneumonia, HMD and CLD (see section 1.4) (Alpert 1992, Henderson 1994, Kotecha 1995, Kotecha 1996b, Kotecha 1996c, Kotecha 1999, de Blic 2000). However, NB-BAL in infants has so far lacked standardisation and there is significant variation in the lavage procedure in reported studies (de Blic 2000). There are marked differences in choice of catheter size, site of sampling, volume of normal saline instilled and processing of samples after retrieval. The effect of these differences in sampling and processing on repeatability NB-BAL has been poorly studied (de Blic 2000).

In order to assess the repeatability of this method, lavages of multiple sites would be required – clearly not possible in sick infants requiring mechanical ventilation for severe respiratory failure as the underlying disease may be exacerbated. Extra-corporeal membrane oxygenation is used to support infants with severe, reversible respiratory failure who have failed conventional management (see section 1.2) (UK Collaborative ECMO Trial Group 1996). During ECMO, gas exchange occurs independently of the lungs. Infants receiving ECMO remain intubated and are ventilated mechanically with low airway pressures to prevent complete airway collapse and to permit recovery of the acute lung injury. Since gaseous exchange is independent of the lungs, ECMO provides a unique opportunity to perform NB-BAL of the right and left sides without compromising the infant. In addition, infants with

severe respiratory failure requiring support with ECMO have uniformly inflamed lungs due to a combination of the underlying disease, ventilator induced lung injury and oxygen toxicity.

In this part of my studies, I assessed repeatability of standardized NB-BAL by comparing cellular and non-cellular constituents in bronchoalveolar lavage fluid obtained by NB-BAL of each side in infants receiving ECMO for severe reversible respiratory failure. I hypothesized that two sequential, standardized lavage procedures of the left and right lung, will demonstrate good repeatability of NB-BAL for white cell counts and concentrations of non-cellular constituents.

3.2 Methods

3.2.1 Patient population

I recruited all infants less than 15 months of age with primary respiratory failure admitted to the Heartlink ECMO Centre, Glenfield Hospital, Leicester between October 1996 and January 1998. Those who required ECMO primarily for cardiac support after surgery for congenital heart disease were excluded. As is routine with ECMO, the oxygenation index (mean airway pressure x fraction inspired oxygen/arterial oxygen tension) on referral was used as a marker of severity of respiratory failure. The study was approved by the local ethics committee and informed consent was obtained from the parents to perform NB-BAL on the infants.

3.2.2 Nonbronchoscopic bronchoalveolar lavage procedure

I carried out all lavage procedures and the subsequent sample analysis was done in blinded fashion. The infants underwent standardised NB-BAL of the lower lobes of both lungs as soon as possible after cannulation for ECMO. Thereafter, bilateral NB-BAL was carried out once daily at the time of routine endotracheal suction until decannulation from ECMO. The lavage

procedure was deferred if clinically contraindicated due to cardio-respiratory instability, pulmonary haemorrhage or temporary interruption of extra-corporeal support. Standardised NB-BAL was performed according to the ERS Task Force guidelines and as previously described (Grigg 1992a, Kotecha 1995, de Blic 2000). Briefly, with the infant positioned supine and the head turned to the left a size 6 - 8 Fr suction catheter was gently passed through the end-porthole of the ventilatory circuit until it was wedged in the right lower lobe. An aliquot of 1 ml per kg bodyweight of normal saline at room temperature was instilled. This was retrieved after two – three ventilator breaths into a suction trap using a suction pressure of 5 to 15 kPa. Immediately thereafter, a second aliquot of 1 ml per kg of bodyweight of normal saline was instilled and sucked back. The two retrieved samples were pooled. I repeated the procedure with the head turned to the right to lavage the left lower lobe. The bronchoalveolar lavage sample from each side was processed separately. Macroscopically bloodstained samples were discarded. The most commonly encountered complication was transient bradycardia resulting from a vasovagal reaction to insertion of the suction catheter.

3.2.3 Processing of the returned BAL samples and cell counts

After the lavage procedure, I recorded the recovered BALF volume and the sample centrifuged within 10 minutes of collection, at 1800 rpm for 10 minutes at room temperature. The supernatant was removed and stored at –70°C until further analysis. The cell pellet was resuspended in a known volume of saline. I obtained the total white cell count (TWCC) with a haemocytometer and a differential cell count was obtained after staining a cell cytospin with Diff-Quick stain (Merck & Dade AG, Duedingen, Switzerland) and counting at least 300 cells per slide. I calculated the total cell counts for the various cell types using the TWCC and differential cell counts, and I expressed the results as cell number per ml of BALF.

3.2.4 Cytokine concentration measurement

Concentrations of the cytokines IL-6, IL-8 and TNF-α in the BALF supernatant were measured by an automated enzyme linked immune sorbent assay (ELISA) analyser (DPC Europe Ltd, Wales) as per the manufacturer's instructions. The analyzer utilizes a sandwich immunoassay with a solid phase of beads coated with monoclonal antibody for the cytokine under study. The beads are incubated with BAL fluid or plasma. Following a washing phase, a second incubation takes placed with polyclonal antibodies linked to an enzyme which catalyzes a chemiluminesence reaction with peroxide. The lowest sensitivity of the analyser was 1.7 pg/ml for TNF–α, 1 pg/ml IL-6 and 2 pg/ml for IL-8. In accordance with ERS Task Force guidelines (de Blic 2000), I did not attempt to correct for dilution and concentrations were expressed as picogram of cytokine per ml of BALF.

3.2.5 Statistical analysis

The absolute and differential cell counts and cytokine concentration for the left and right sides were firstly compared by linear regression, calculating the Pearson correlation coefficient (R-value), the p value and 95% confidence interval. Statistical significance was assumed at $p < 0.05$. Secondly, the Bland –Altman method (Bland 1986) was used. This consisted of plotting the difference between each pair of measurements against their mean value, then using the mean and standard deviation of the differences to calculate the 95% limits of agreement i.e. the range in which the difference may be expected to lie in the great majority (95%) of the samples. Ninety- five percent confidence intervals were calculated to indicate the precision of the limits of agreement.

Since the Bland Altman plots of absolute cell counts (Fig 3.2a, c and e) and of cytokine

concentrations demonstrated dependence of left-right variability on the overall magnitude (mean of left and right) – seen as a "funnelling" on the graphs – the corresponding limits of agreement were calculated using a logarithmic scale and presented as the ratio between left and right. (Logarithmic transformation was not performed for percentage of cells since funnelling was not observed, see Figures 3.2 b and d).

I hypothesised that the variability might be due to (a) the age of the child, (b) the underlying disease, (c) my experience of lavaging infants, (d) time spent on ECMO, (e) volume retrieved (volume retrieved from left side/volume retrieved from right side) or (f) survival. Linear regression, with random effects due to the multiplicity of measurements on each child, was used to examine the hypothesis separately for the various cellular and non-cellular constituents. Analysis was performed using a statistical package for microcomputers (SPSS Base 9.0, SPSS Inc., Chicago, Illinois, USA, STATA Statistical software 6.0, Stata Corp., College Station, Texas, USA).

3. Results

3.3.1 Patient Characteristics

The patient characteristics of the infants recruited are shown in Table 3.1 and the diagnostic groups in Table 3.2. As shown by the median age almost all infants were neonates with only 3 infants being greater than 30 days of age. One patient had an oxygenation index of 19.6 but because of multiple organ involvement he was placed on veno-arterial ECMO to support the circulation as well as the respiratory system. Median time on ECMO was 143 hours (range 71-406). Eleven patients received veno-venous ECMO and 9 were placed on veno-arterial ECMO. Fourteen survived and there were six deaths (Table 3.2).

Table 3.1 Patient Characteristics

		Range
Number of patients studied	20	
Number of NB-BAL	95	
Age at recruitment (days)	2	1 – 402
Weight (kg)	3.3	2.0 – 10
Male:Female	14:6	
Surfactant treatment	2	
Nitric Oxide	3	
Oxygenation index	46	19.6 – 97.1
VV:VA	11:9	
Time on ECMO (hrs)	143	71 – 406

Median value and range is given for age, weight, oxygenation index and time on ECMO.

VV:VA veno-venous : veno-arterial ECMO.

3.3.2 Comparison of BALF cell counts between the left and right sides

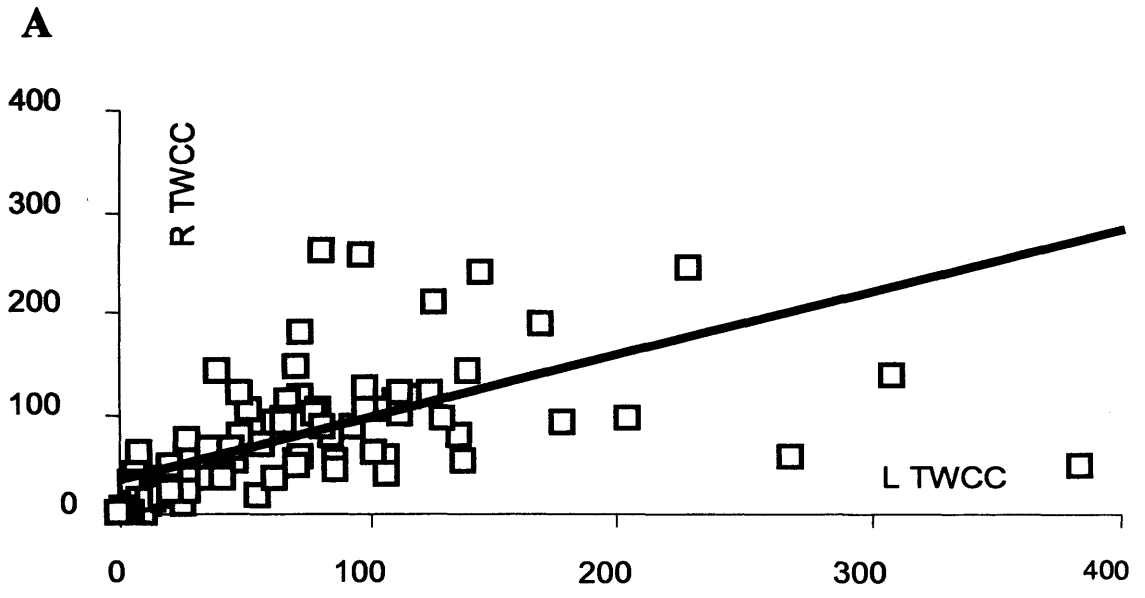
Ninety five paired NB-BAL samples were obtained. Total white cell counts (TWCC) were obtained from all samples but only 77 pairs were suitable for differential cell counts. The predominant cell types were neutrophils and alveolar macrophages. Few lymphocytes, eosinophils and epithelial cells were seen. Detailed data is therefore given in graphical form for neutrophils and alveolar macrophages only (Figures 3.1 and 3.2) and the results for all cell types are summarised in Table 3.3.

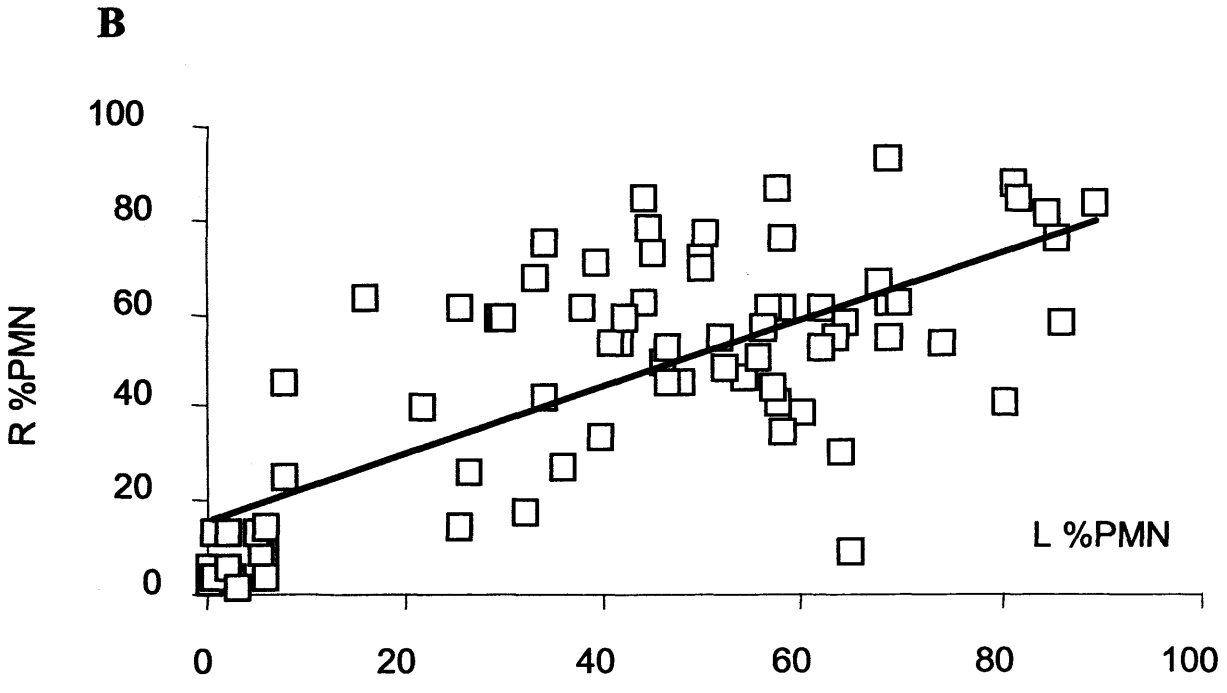
Table 3.2 Diagnostic groups in study (n=20) including survivors and non-survivors.

Disease Process	**Survivors** N=14	**Non-survivors** N=6
Meconium aspiration syndrome	6	0
Pneumonia	1	3
Persistent hypertension of the newborn	1	2
Sepsis	3	0
Congenital Diaphragmatic Hernia	2	1
Respiratory Distress Syndrome	1	0

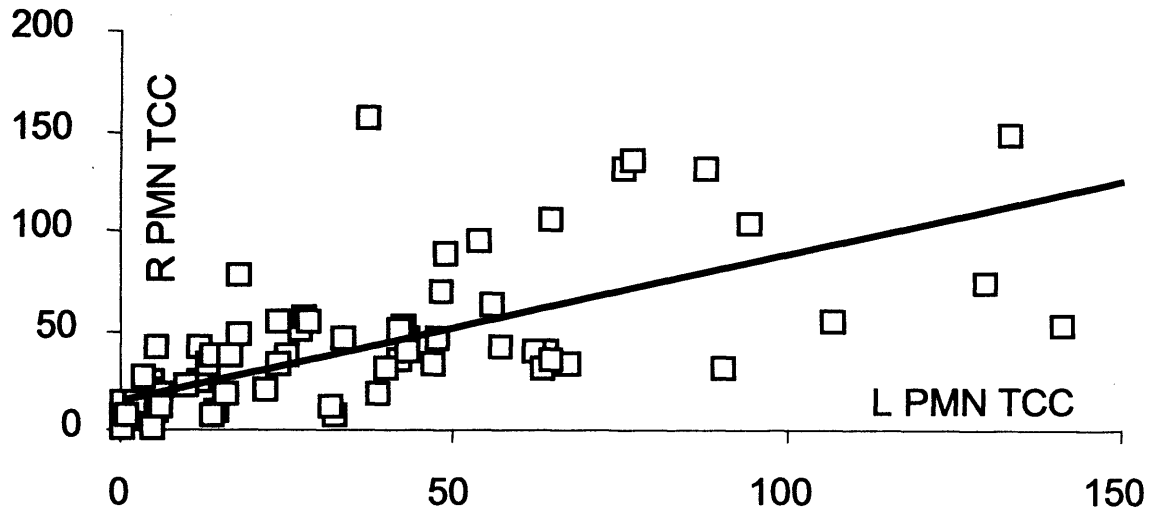
There was strong, positive correlation between the two sides for total white cell counts, percentage neutrophils, absolute number of neutrophils, percent macrophages and percent epithelial cells with R-values ranging from 0.70 to 0.84 (all $p < 0.001$, Fig 3.1, Table 3.3). For absolute cell counts of macrophages, lymphocytes and epithelial cells, and for percent lymphocytes the correlation between the two sides was weaker with R-values of between 0.44 and 0.56 (all $p < 0.001$, Fig 3.1, Table 3.3). Because correlation may not accurately reflect repeatability, I used the more appropriate Bland Altman analysis. I found that the mean difference between the right and left sides approached zero for all cell types (Table 3.3, Fig 3.2a-3.2e). In addition, the 95% confidence intervals always included zero. However, the limits of agreement for all cell types studied were wide ranging from –187.5 to +190.9x10^4/ml BALF for total white cell counts, -41.5 to +33.6% for percent neutrophils and -33.8 to +41.6% for percent alveolar macrophages (Table 3.3).

Figure 3.1 A – E. Correlation between the left and right total and differential cell counts of neutrophils and macrophages





C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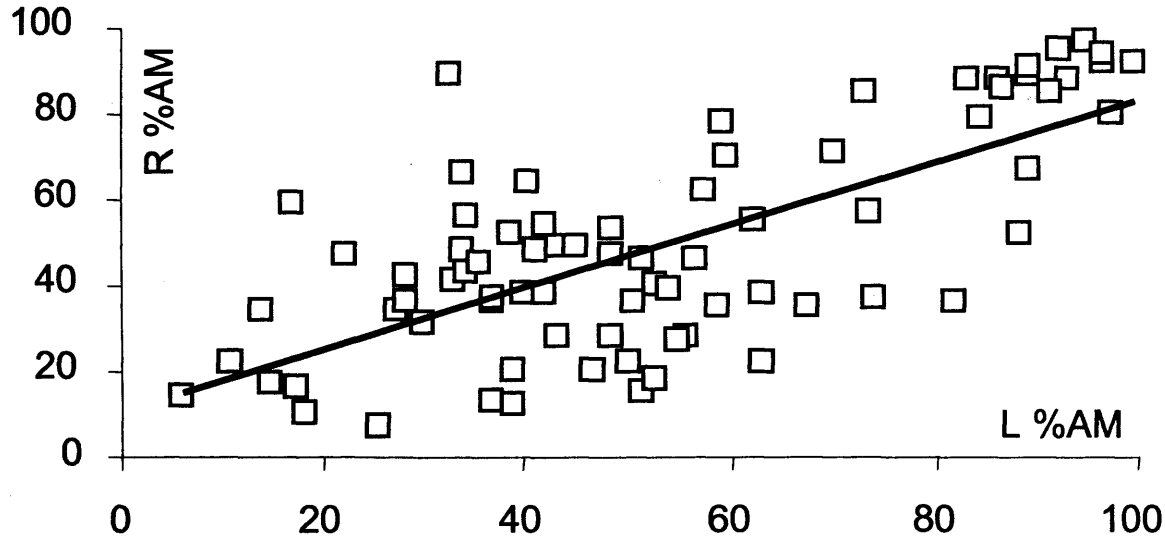
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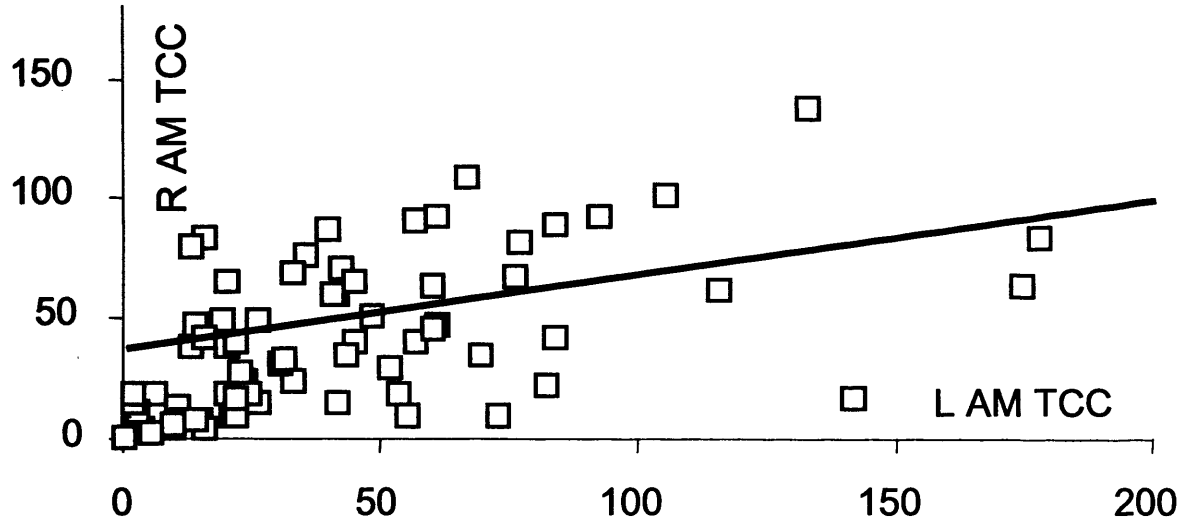


Correlation coefficients and linear regression lines are shown for results between the right and left sides for (a) total white cell counts (TWCC, R=0.79, p<0.001), (b) percent neutrophils (%PMN, R=0.72, p<0.001) and (c) neutrophil total cell counts (PMN TWCC, R=0.83, p<0.001), (d) percent alveolar macrophages (%AM, R=0.70, p<0.001) and (e) alveolar macrophages total cell count (AM TWCC, R=0.44, p<0.001). Cell counts are given in 10^4 cells per ml of BALF.

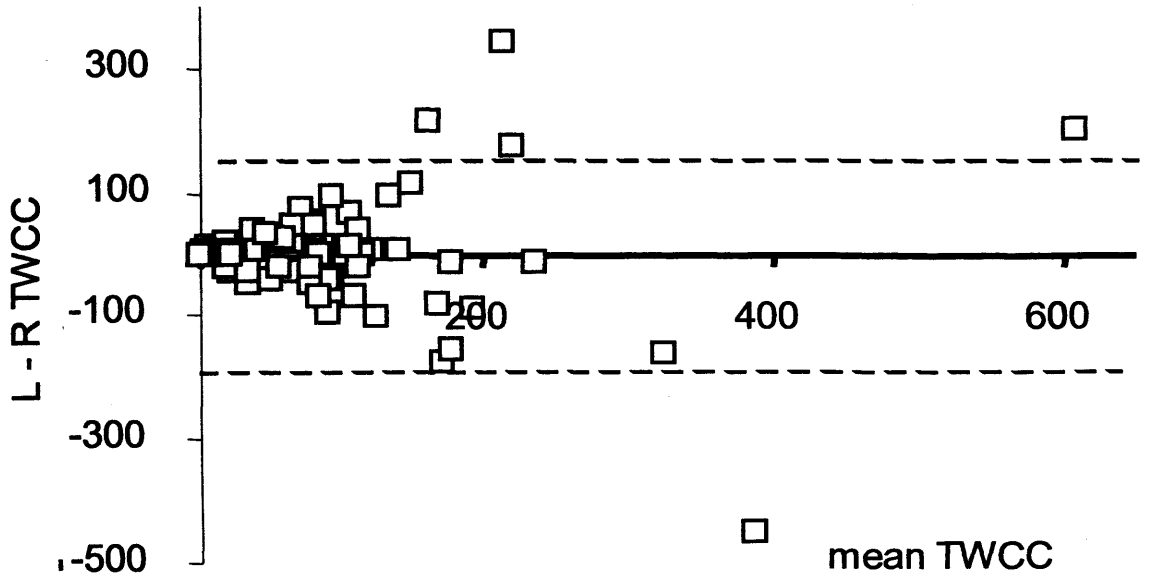
When the data was logarithmically transformed, where "funnelling" was seen, the corresponding limits of agreement (now shown as proportions due to transformation) were 0.15 (95% CI: 0.11 to 0.21) to 5.80 (95% CI: 4.19 to 8.05) for total cell counts, 0.13 (95% CI: 0.09 to 0.18) to 4.65 (95% CI: 3.24 to 6.67) for total neutrophils and 0.17 (95% CI: 0.12 to 0.24) to 6.62 (95% CI: 4.58 to 9.58) for total alveolar macrophages.

3.3.3 Comparison of cytokine concentrations and BALF volumes

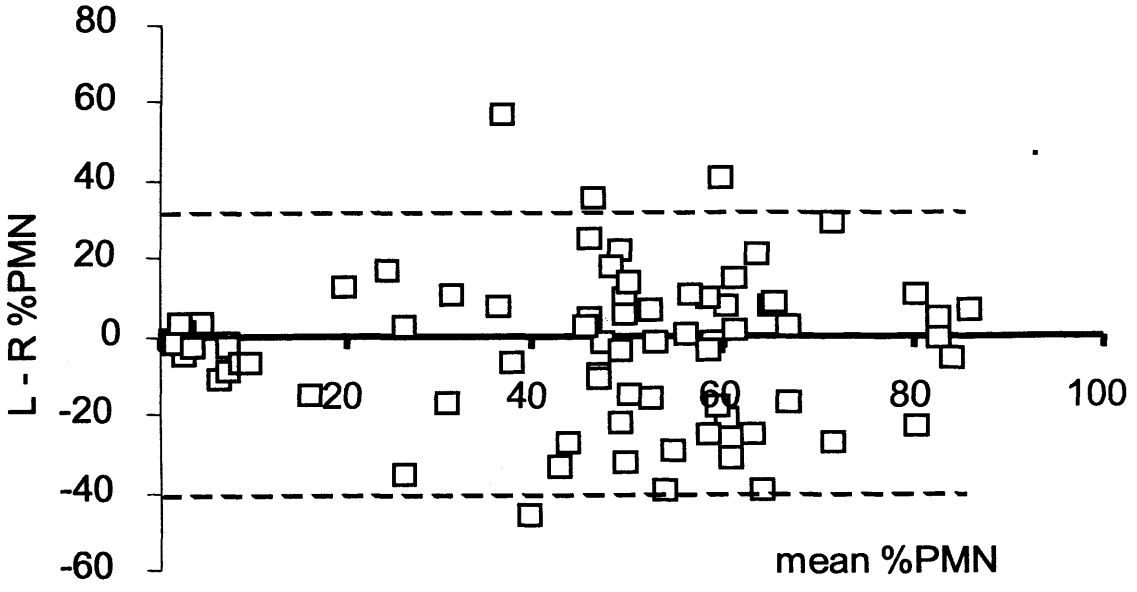
IL-8 concentration was estimated in 76 paired BAL samples, IL-6 in 74 pairs and TNF-α in 73 pairs due to the fact that for some of the BAL samples the volume retrieved was too small to measure all three cytokines. The mean concentrations were 4.96 pg/ml, 0.97 pg/ml and 0.18

Figure 3.2 A – E. Bland-Altman plots showing mean difference between the right and left sides

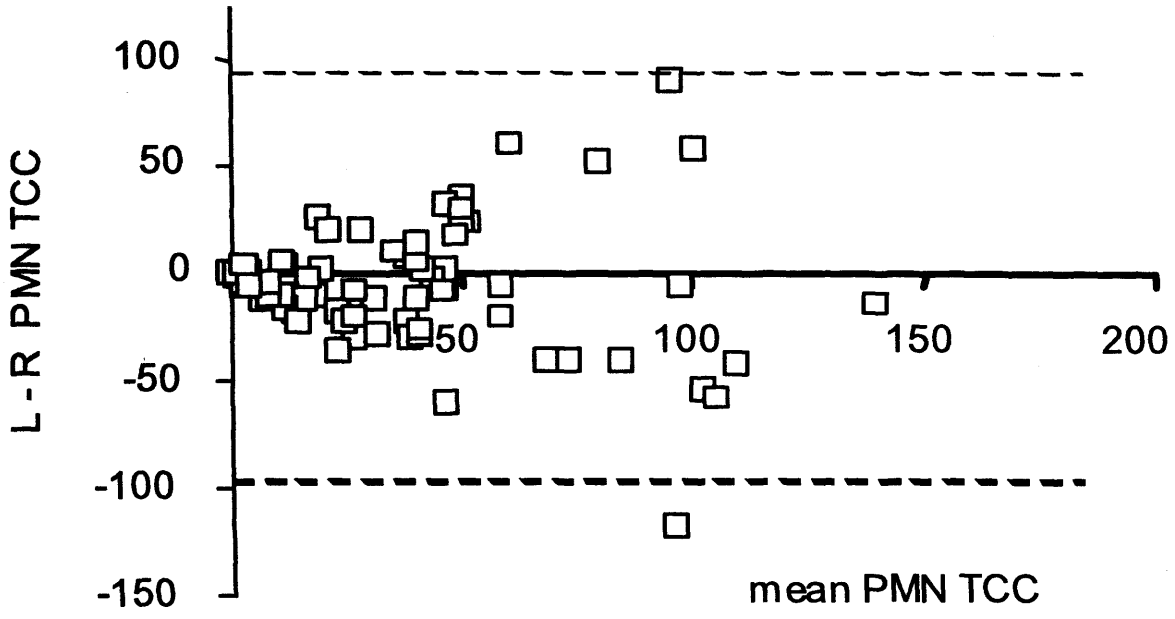
A



B



C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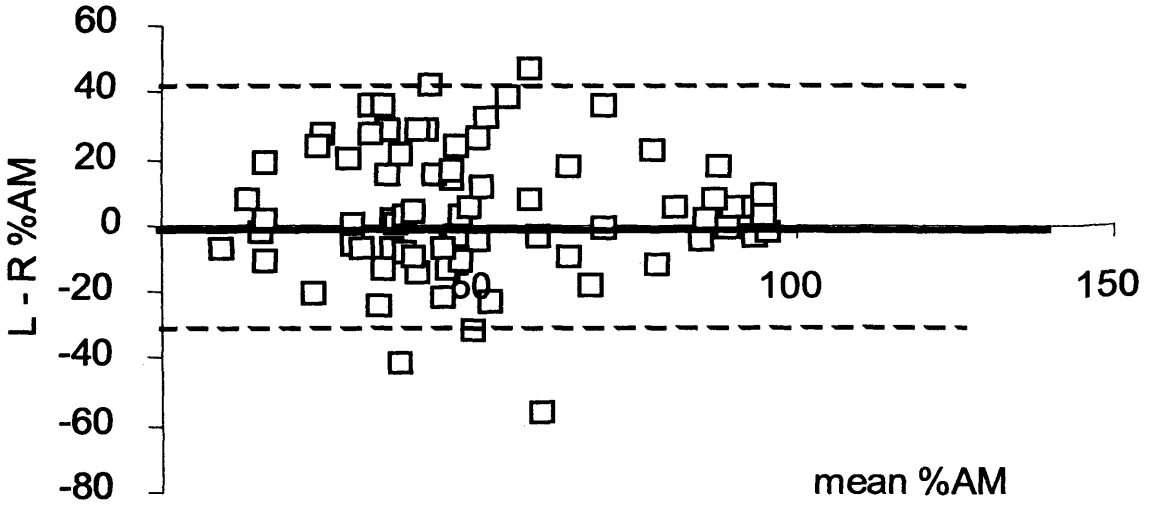
L - R PMN TCC
50
100
150
200
mean PMN TCC

D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0
-20
-40
-60
-80
L - R %AM
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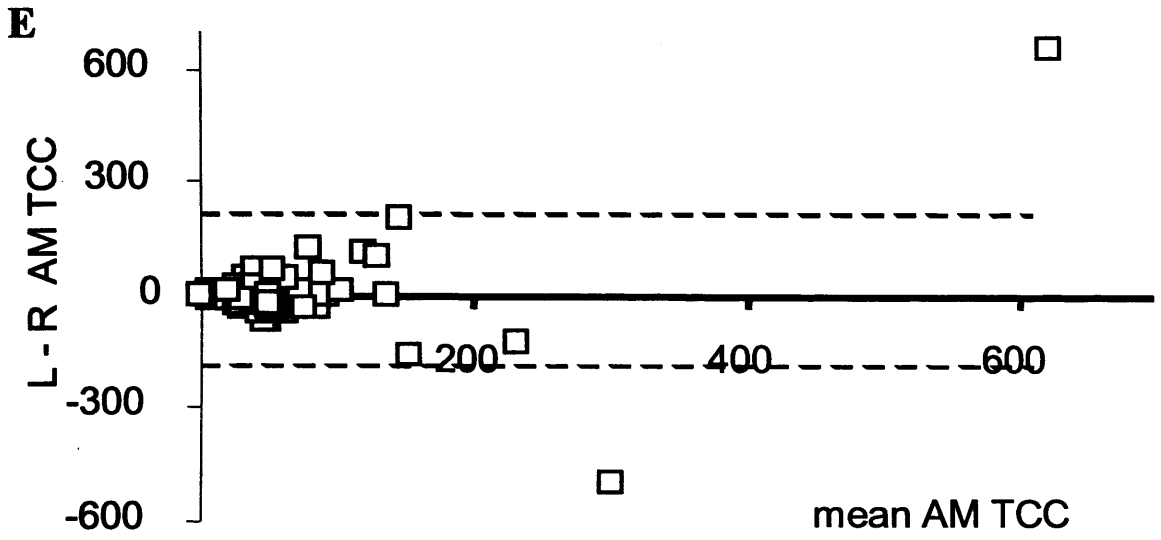
mean %AM



Bland-Altman plots showing mean difference between the right and left sides and limits of agreement for (a) total white cell counts (TWCC), (b) percent neutrophils (%PMN), (c) neutrophil white cell counts (PMN TCC), (d) percent alveolar macrophages (%AM), and (e) alveolar macrophages total cell counts (AM TCC). The thick black bar denotes the mean difference between the two sides and the dotted lines the limits of agreement. The cell counts are give in 10^4 cells per ml of BALF fluid. Please note the near-zero mean difference between the two sides and the wide limits of agreement for each parameter studied.

pg/ml for IL-8, IL-6 and TNF–α respectively for the left side and 4.92 pg/ml, 1.21 pg/ml and 0.14 pg/ml for the right side. Comparison between the left and right sides for IL-8 concentration demonstrated a moderate positive correlation ($R=0.66$, $p<0.001$, Table 3.4).

Table 3.3 Results of left and right sided mean total and differential cell counts, and correlation and mean difference between left and right sided mean total and differential cell counts

		Left	Right	R (P-value)	Mean Difference (left minus right) (95% CI)	LLA (95% CI)	ULA (95% CI)
TWCC		92.5	90.8	0.79 (p<0.001)	1.7 (-17.9 to 21.4)	-187.5 (-221.5 to -153.4)	190.9 (156.8 to 224.9)
PMN	%	42.8	46.7	0.72 (p<0.001)	-3.9 (-8.3 to 0.4)	-41.5 (-49.0 to -33.9)	33.6 (26.1 to 41.2)
	TWCC	49.0	50.4	0.83 (p<0.001)	-1.4 (-12.3 to 9.6)	-95.9 (-114.9 to -77.0)	93.2 (74.2 to 112.1)
AM	%	53.3	49.4	0.70 (p<0.001)	3.9 (-0.5 to 8.3)	-33.8 (-41.4 to -26.3)	41.6 (34.0 to 49.2)
	TWCC	60.4	56.4	0.44 (p<0.001)	4.0 (-19.9 to 27.9)	-202.7 (-244.2 to -161.3)	210.7 (169.3 to 252.2)
LYMPH	%	4.0	3.7	0.69 (p<0.001)	0.3 (-0.2 to 0.8)	-3.9 (-4.7 to -3.0)	4.6 (3.7 to 5.4)
	TWCC	3.3	3.8	0.56 (p<0.001)	-0.5 (-1.4 to 0.4)	-8.4 (-10.0 to -6.8)	7.4 (5.8 to 9.0)
EPI	%	9.7	10.5	0.84 (p<0.001)	-0.8 (-3.5 to 1.9)	-23.8 (-28.4 to -19.2)	22.2 (17.6 to 26.8)
	TWCC	7.4	10.3	0.54 (p<0.001)	-2.9 (-8.0 to 2.2)	-46.7 (-55.5 to -37.9)	40.9 (32.1 to 49.6)

Data for total white cell counts (TWCC), percent (%) and total cell counts (TWCC) shown for neutrophils (PMN), alveolar macrophages (AM), lymphocytes (LYMPH) and epithelial (EPI) cells. The mean for the right and left sides are given together with the Pearson's correlation coefficient and p-value of correlation. The mean difference including 95% confidence intervals (CI) are shown for each cell type together with the upper (ULA) and lower (LLA) limits of agreement obtained by the method described by Bland and Altman (25). Total cell counts were obtained from the total white cell count and differential count for each cell type

and is expressed as 10^4 cells per ml of BALF.

For TNF-α and IL-6 the correlation was weaker with R-values of 0.39 and 0.42 respectively (p=0.001, Table 3.4). The results for Bland Altman plots are shown in Figure 3.2. They showed that the difference between the two sides was close to zero but the limits of agreement were large for each cytokine studied. The limits of agreement were 3 –5 times greater or lower than the mean for each cytokine studied suggesting that the results from the two sides may vary up to several times.

Since marked "funnelling" (ie larger differences were noted as the magnitude of the measurements increased) was noted for all three cytokines studied, the analysis was repeated using logarithmically transformed data (Fig 3.3 and 3.4). For all three agents studied there was moderate correlation between the two sides for the logarithmically transformed data with an R value of 0.78 for IL-8, 0.75 for IL-6 and 0.64 for TNF–α (all $p<0.001$). Bland Altman analysis using logarithmically transformed data, thus shown as a ratio of left/right, were 0.82 (95% confidence interval 0.66 – 1.02) for IL-8, 0.74 (0.58 – 0.94) for IL-6 and 0.93 (0.80 – 1.07)] for TNF-α. Similarly, the limits of agreement were wide: 0.12 – 5.45 for IL-8, 0.09 – 5.87 for IL-6, and 0.27 – 3.16 for TNF–α, suggesting that the concentrations may vary by up to 10 times between the two sides.

Table 3.4 Comparison of concentration of cytokines in BALF obtained from the left and right sides.

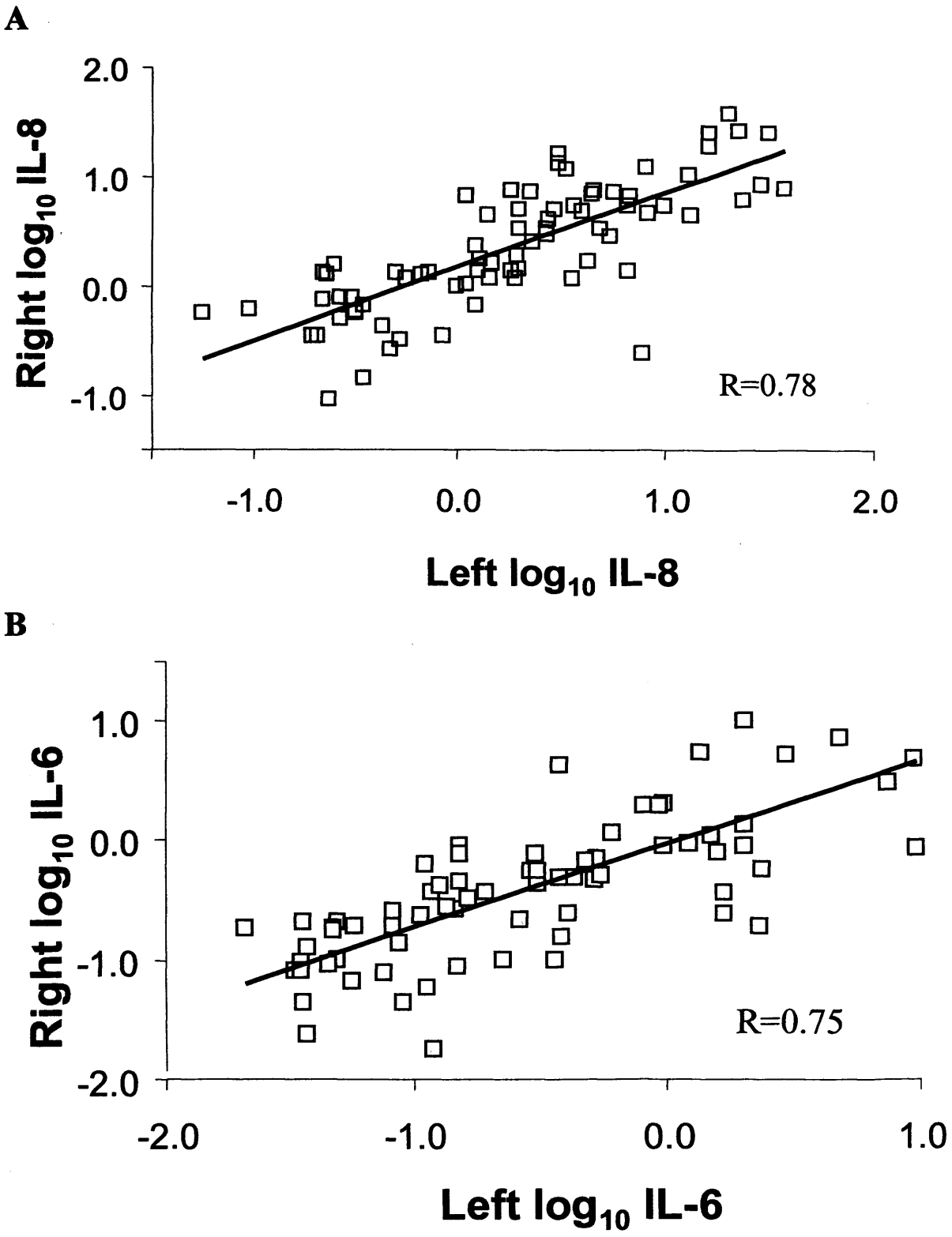
	Correlation		Bland – Altman analysis		
	R	**p-value**	**Difference (95% CI)**	**LLA (95%-CI)**	**ULA (95%-CI)**
IL-8	0.66	p<0.001	0.04 (-1.34 to 1.43)	-11.9 (-9.6 to -14.3)	12.0 (9.6 to 14.4)
Il-6	0.42	p<0.001	-0.25 (-0.77 to 0.27)	-4.7 (-3.8 to -5.6)	4.15 (3.3 to 5.1)
TNF-α	0.39	p<0.001	0.04 (-0.06 to 0.15)	-0.84 (-0.66 to-1.0)	0.92 (0.74 to 1.1)

The concentrations of cytokines were analysed by using Pearson's coefficient and by the Bland Altman plots of agreement. The Pearson's correlation coefficient (R) and p-value are given. The mean difference in cytokine concentration for the right and left sides together with the lower (LLA) and upper (ULA) limits of agreement are also given. 95% confidence intervals are shown in brackets.

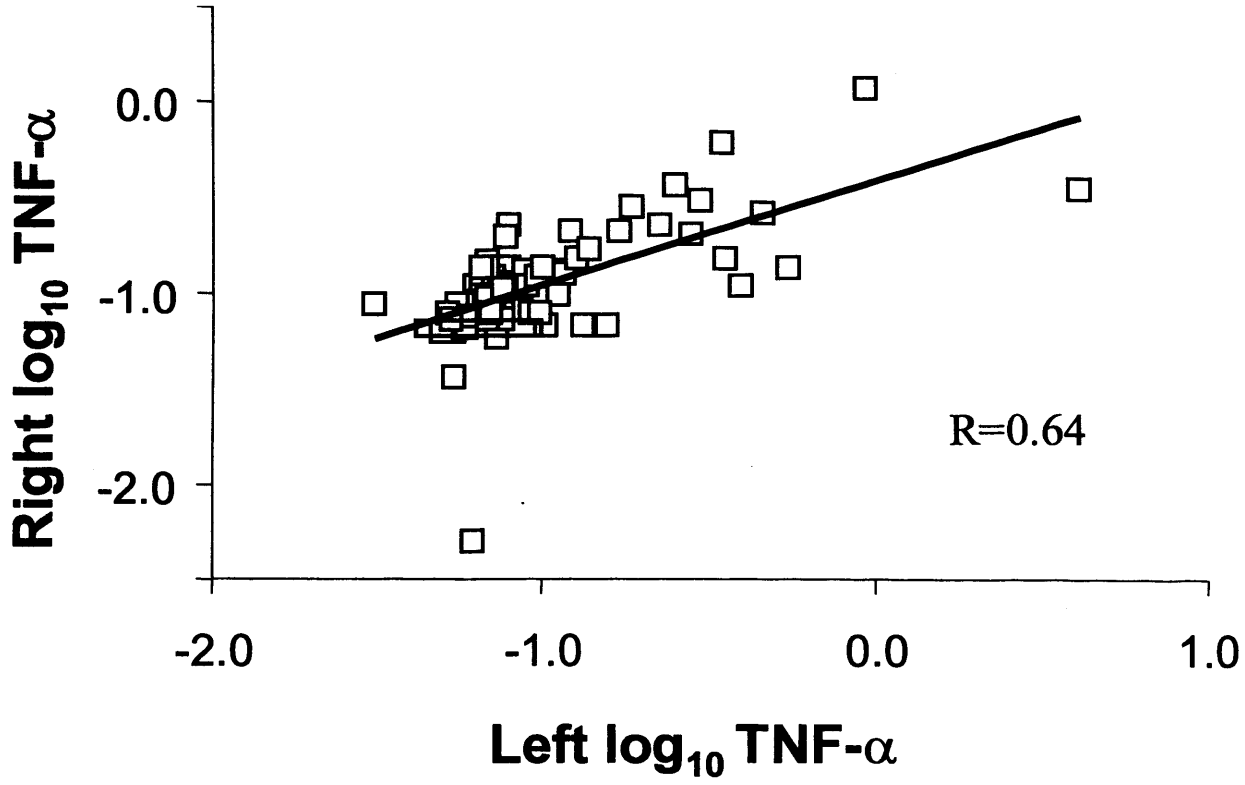
3.3.4 Potential sources of variability

To explain the large variability I noted for each cytokine studied, I examined a number of factors that may have accounted for the variability including (a) the age of the child, (b) underlying disease process, (c) time spent on ECMO, (d) my experience of performing NB-BAL, (e) survival or (f) volume retrieved affected the results. In the initial analysis, for cellular constituents, the only significant factor was age of the child on presentation ($p<0.05$ for total cell counts, absolute alveolar macrophages and neutrophil counts but not for percent neutrophils or macrophages).

Figure 3.3 A – C. Correlation between the values for IL-8, IL-6 and TNF-α concentrations for BALF samples from the right and left sides



C

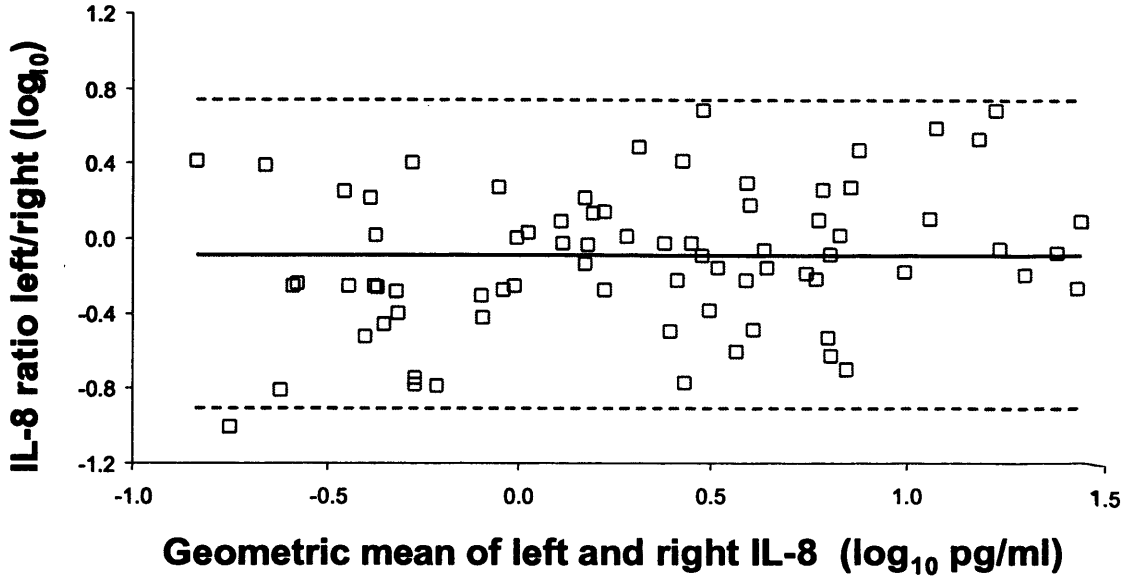


Correlation between the values for IL-8, IL-6 and TNF-α concentrations for BALF samples from the right and left sides. Pearson's correlation coefficient is given for each graph. P values for all graphs are <0.001.

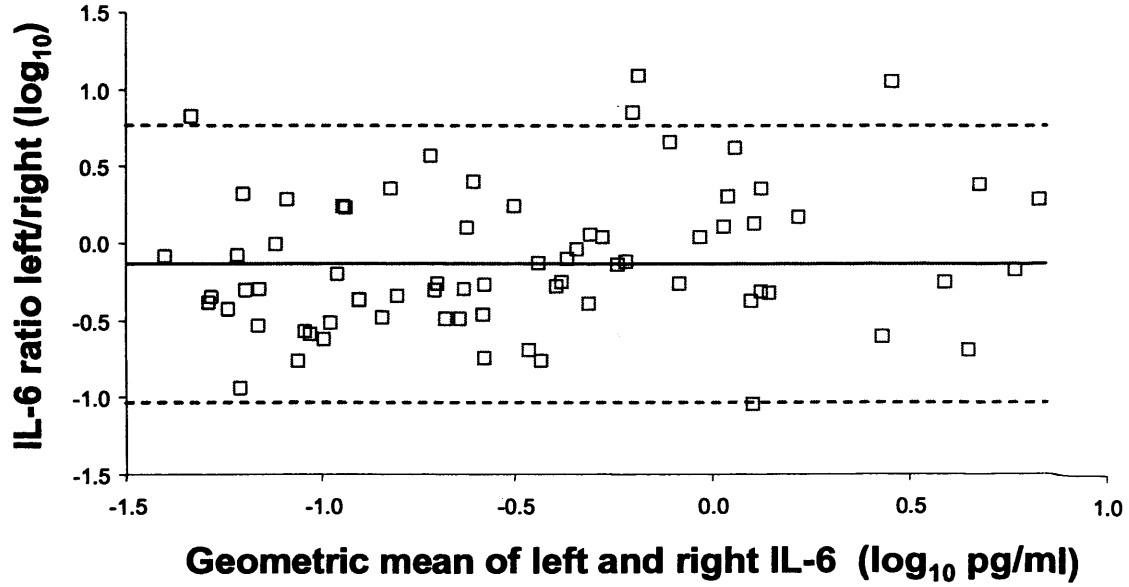
However, no significant contribution to variability for any cell type was noted from time on ECMO, underlying disease type, experience of the operator, volume of fluid retrieved or survival. The contribution of age to the variability was no longer noted when the analysis was restricted to infants aged under three months. When the random effects linear regression was applied to cytokine measurements, only the volume of fluid retrieved appeared to be significantly associated with the variability observed for IL-8 (p=0.003) and TNF–α (p=0.008) but not IL-6 (p=NS). Furthermore, the results showed a negative coefficient suggesting that

Figure 3.4 A – C. Bland Altman plots for IL-8, IL-6 and TNF–α

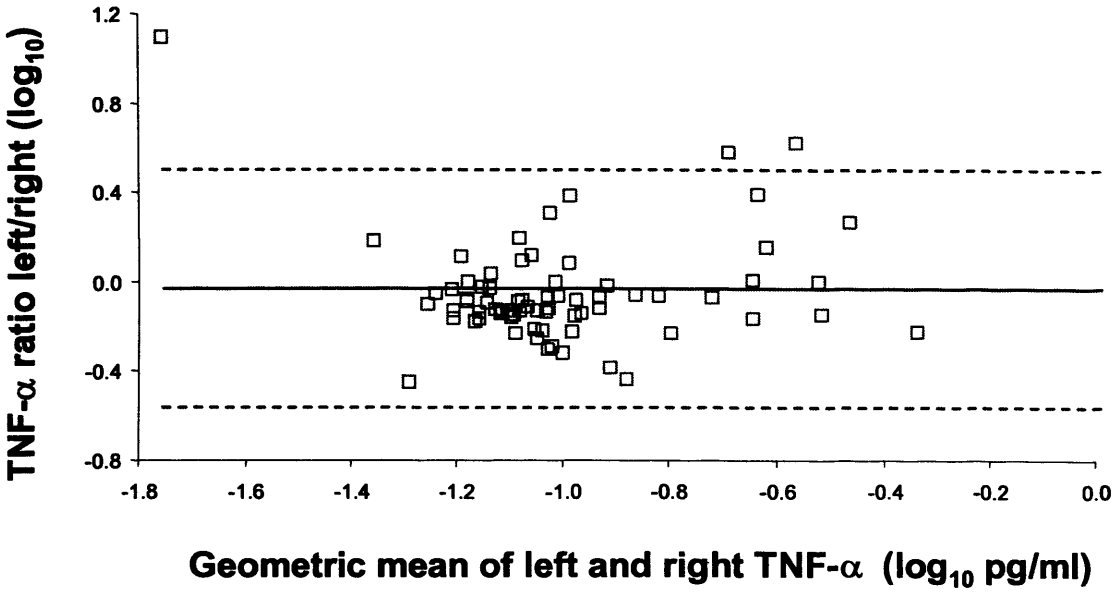
A



B



C



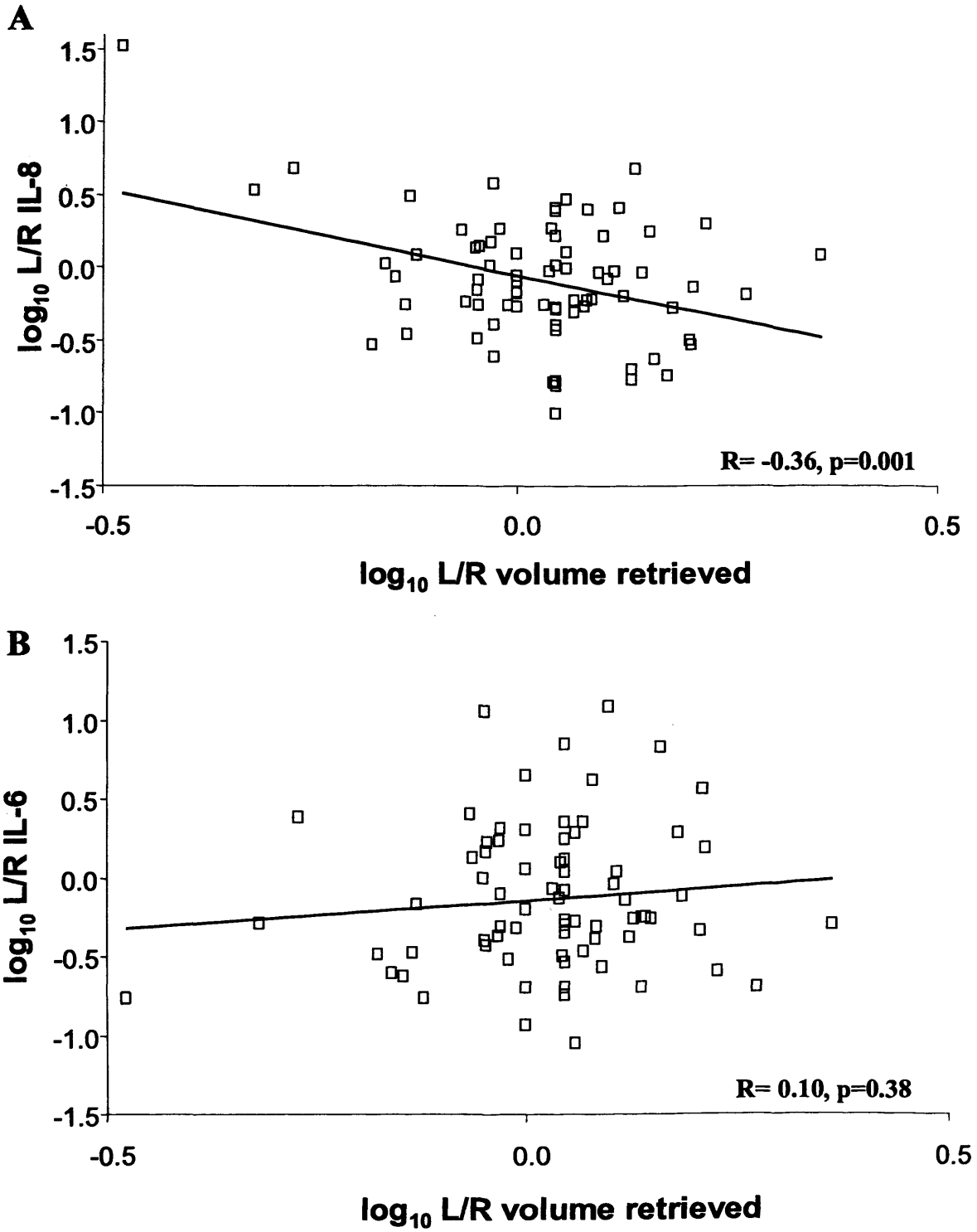
Bland Altman plots for the logarithmic transformed data is given for IL-8, IL-6 and TNF–α. Note that the mean difference between the two sides includes one for IL-8 and TNF–α but not IL-6. Also note the wide upper and lower limits of agreement for all three cytokines studied.

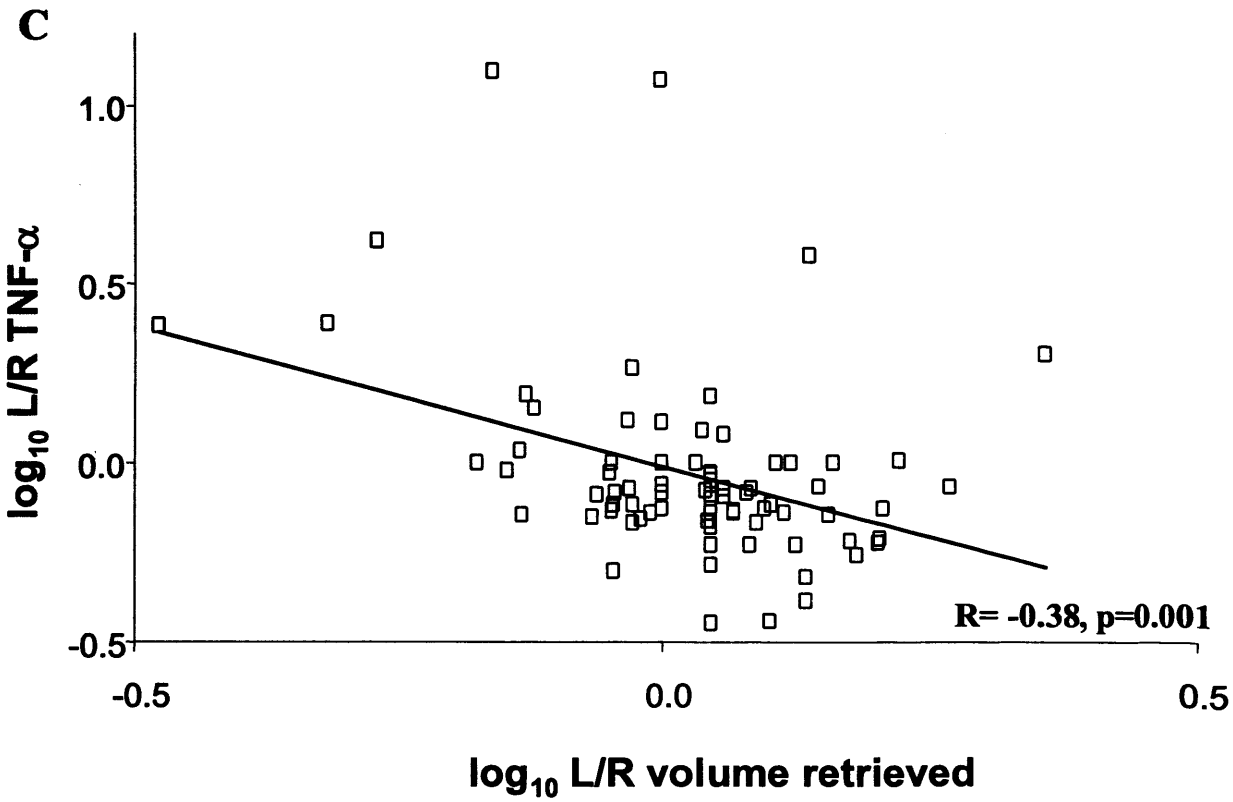
the larger the volume of fluid recovered the lower the concentration of IL-8 and TNF–α (Figure 3.5). I also re-analyzed the data using the difference between the volume retrieved from the two sides instead of the proportion of left to right of volume retrieved recovered and found the same results. Similar results were obtained for both untransformed and logarithmically transformed data.

3.4 Discussion

NB-BAL is a commonly used technique to study respiratory diseases of infants receiving mechanical ventilation. My data demonstrates a number of points: (1) great variability between the results for both cellular counts and cytokine concentrations obtained from the right and left sides, (2) volume of fluid retrieved but not age influenced the variability observed for cytokine concentrations and age but not volume retrieved affected cellular

Figure 3.5 A – C. Correlation of the logarithmic transformed data for the ratio left /right of volume retrieved and the logarithmic data for the ratio left/right for the cytokine concentrations





The logarithmic transformed data for the ratio left /right of volume retrieved is plotted against the logarithmic data for the ratio left/right for the cytokine concentrations to demonstrate if the concentration of cytokine was related to the volume of BALF recovered. Note that for IL-8 and TNF-α but not IL-6 the concentration of cytokine is inversely related to the BALF recovered.

counts, (3) the underlying disease process, time spent on ECMO, experience of the operator or survival did not affect the variability, and (4) the cytokine concentrations tended to be higher on the right side compared to the left.

I considered the Bland Altman method as the most appropriate way of analysing and presenting the agreement for the various measurements from the two sides (Bland 1986). I have however also presented the correlation coefficient and linear regression as these are often reported in the literature (Peterson 1988). For the absolute numbers of cell counts and for cytokine measurements, the variability between left and right seemed to directly dependent on

the overall magnitude of the measurement ("funnelling" noted on fig 2a, 2c, 2e). Therefore I repeated the Bland Altman analysis in these cases using logarithmically transformed data and accordingly presented the results in terms of the ratio between left and right values (rather than the absolute difference). The limits of agreement remained wide for both cellular and non-cellular constituents.

It will be noted that several measurements from each infant were used. Examination of the possible sources of variability was therefore carried out using random effects linear regression which accounts for the possible "clustering" of measurements made on the same child. To further ensure the validity of this approach I randomly selected one measurement from each child and found that limits of agreement for each cell type studied remained unchanged.

For each cell type studied, there was no significant difference between the mean results for the left and right sides. Correlation between left and right results was good for total counts of white cells and neutrophils, as well as for percentages of neutrophils, alveolar macrophages and epithelial cells. However correlation does not provide accurate information regarding the nature of the relationship between the results for the two sides. Furthermore, good correlation may be expected as the results relate to samples taken simultaneously from the same patient. In this situation, assessment of agreement using the method described by Bland and Altman results is in my opinion more appropriate. (Bland 1986) My findings show that there is considerable variability between the left and right for individual cellular measurements. This is reflected in the relatively wide 95% confidence intervals for mean differences between left and right results. Similarly, the limits of agreement between left and right cellular results were relatively large. The data suggest that the application of NB-BAL in studies based on individual results or on small groups of patients needs to be interpreted with caution.

For cytokine results, there was a trend towards the results being greater on the right than on the left as suggested by the ratios being less than one for all cytokines studied (Fig 3.4). Since I had included patients with congenital diaphragmatic hernia (all children had left sided hernias) one may have expected this observation. However, I was unable to attribute this to the underlying disease process. When factors that may have accounted for variability were examined, only age but not time on ECMO, my experience of performing BAL, underlying disease process, volume of fluid retrieved or survival were significantly associated with variability of cellular counts. The age effect was no longer significant when infants older than 3 months of age were removed from the analysis.

However, for cytokine measurements, the volume of fluid retrieved studied either as a proportion of left/right or left minus right yielded very interesting results (Fig 3.5). Fluid volume retrieved clearly appeared to be related to the variability observed for both IL-8 and TNF–α but not for IL-6. The negative coefficient suggested that the concentration of the cytokine decreased as proportion of volume recovered increased. This implies a dilutional effect of the saline instilled to obtain epithelial lining fluid. Current recommendations from the European Respiratory Society for both children and neonates state that BALF results should be expressed per ml of fluid especially as currently there are no satisfactory methods for estimating epithelial lining fluid (de Blic 2000). My data are in disagreement with this statement as the influence of the retrieved volume on results clearly needs to be taken into account. As I had followed the ERS guidelines, unfortunately I had made no attempts to estimate epithelial lining fluid volume with any of the methods presently available. I would have been able to demonstrate if this effect is corrected by representing the data per ml of ELF.

The methods for measuring the concentration of the cytokines may also influence the results. I used an automated cytokine analyser with extensive quality controls to ensure accuracy of the results. Using the controls and repeated measurements of cytokines in BALF wherever possible the results were almost always within 5% of each other. This variation is unlikely to account for the wide variability I have noted for the two sides. Due to the limited volume of fluid obtained from infants receiving ECMO it would be difficult to perform the measurements in triplicate or greater.

In conventionally ventilated infants a repeatability study would be unethical and dangerous. Patients undergoing extracorporeal support offer a unique opportunity for a repeatability study of NB-BAL, because they are not dependent on their lungs for gas exchange. The most commonly encountered complication was transient bradycardia. No major adverse events affecting patients occurred during the study. This is in agreement with other studies in conventionally ventilated infants (Grigg 1992a, Henderson 1994, Kotecha 1999, de Blic 2000).

Patients presenting for ECMO are unique. On the one hand they form a heterogeneous population in terms of underlying diagnosis. On the other hand, invariably these patients have been ventilated with high FiO2 and high mean airway pressures for a significant period of time, leading to radiologically uniform lung disease (see 1.1.10). In my study, there were apparent differences in white cell populations between the left and right lung for patients with congenital diaphragmatic hernia, meconium aspiration syndrome and persistent pulmonary hypertension of the newborn. Several studies in adults with sarcoidosis using fibreoptic bronchoscopy have shown good correlation for lymphocyte percentage between BAL samples from left and right lungs (Garcia 1986, Peterson 1988, Nugent 1989). A small group of

patients showed significant interlobar difference in lymphocyte percentage, which appeared to be related to focal infiltrates on chest x-rays in two studies (Peterson 1988, Nugent 1989). In other types of interstitial lung disease, a higher degree of interlobar variability was found, which was not related to chest x-ray appearance (Garcia 1986, Nugent 1989). In my study, I did not correlate the findings with chest radiological changes. Larger numbers of infants within each diagnostic group would be required to demonstrate any correlation between chest radiograph changes and cell counts.

Previous studies have investigated fluid and cell recovery but not repeatability with NB-BAL of ventilated human infants. Grigg *et al* demonstrated a significant difference in the percentage of macrophages between the first and second aliquot but not in volumes retrieved or in total cell counts (Grigg 1992a). Using fibreoptic bronchoscopy in human adults, two studies have addressed the effect of instilled fluid on volume and cellular recovery (Dohn 1985, Lam 1985). Both studies showed differences in differential cell counts for both lower and higher volume of BAL fluid instilled. These studies suggest that with small volume lavage, the returned cells originate mainly from the proximal airways while with larger volumes the distal airways including alveoli are sampled. This is supported by additional studies by Kelly *et al* investigating the distribution of fluid during the BAL procedure (Kelly 1987). While aspirating the first aliquot of radio-opaque BAL fluid under radiographic imaging, fluid movement towards the bronchoscope was seen only in the proximal airways. More distally located lavage fluid actually moved away from the bronchoscope. With further aliquots BAL fluid was retrieved from the entire lung segment.

In healthy human volunteers, Pingleton *et al* showed that fluid volume and total cell recovery were highest when the right middle lobe was lavaged (Pingleton 1983). There was no

difference between lobes in cell count per millilitre of BAL fluid. The pH and temperature of the instilled fluid did not affect cell or protein recovery. Similar observations were made by Laviolette and colleagues in normal dogs (Laviolette 1983). They found a significant difference in recovery of total number of cells between the left lower and right middle lobe, but not in percentage macrophages. In normal rats cell recovery was found to be dependent on the type of catheter and the use of bronchoscopic guidance (Reis 1994). To minimise the effects of these variables, I used a standardised method of NB-BAL and subsequent lavage fluid processing. Unlike BAL using fibreoptic bronchoscopy, NB-BAL is carried out blindly. Despite this it is likely that the suction catheter reached the intended site on most occasions. Placzek and Silverman studied catheter placement in NB-BAL (Placzek 1983). They showed that by turning the head to the left the suction catheter was passed into the right main bronchus in 20 out of 20 attempts. In a post-mortem study, Grigg *et al* (Grigg 1992a) confirmed that with the infant's head turned to the left the right lower lobe is lavaged. In my study, four lavage procedures in two patients were carried out at the time of routine chest X-ray examination, twice aiming at the right lower lobe and twice at the left lower lobe. On each occasion the catheter tip was seen to lie in the expected lower lobe bronchus. Since I was unable to attribute the variability to factors including the underlying disease process, my experience of performing BAL, fluid retrieval, days on ECMO, survival or age (in infants less than three months of age), it is likely that the variability is due to the technique itself. The placement of the catheter and distribution of the instilled fluid may contribute to the variability that I have reported.

In summary, my studies show that NB-BAL is a useful and reliable tool for the study of both cellular and non-cellular constituents in the bronchalveolar milieu in population studies. However, results obtained by this tool in studies involving individual patients should be

interpreted with caution. Also, the concentration of non-cellular constituents in BAL fluid appears dependent on the volume of fluid instilled during NB-BAL. Further studies are required to determine whether repeatability for non-cellular constituents can be improved, by using currently available methods to correct for dilution.

Chapter 4. Resolution of acute lung inflammation in infants with severe respiratory failure treated with extra-corporeal membrane oxygenation

4.1 Introduction

Near-term newborn infants with severe respiratory failure who require ECMO, show evidence of neutrophil-mediated acute lung inflammation (see sections 1.1.4 to 1.1.10, and 1.2) (Nakagawa 1997). Both pro- and anti-inflammatory cytokines have been shown to play an important role in the initiation, maintenance and resolution of acute lung inflammation in preterm infants (Groneck 1995, Kotecha 1995, Kotecha 1996a, Kotecha 1996c) and in adults with ARDS (Repine 1992, Martin 1999, Zimmerman 1999). Although absolute concentrations of the pro-inflammatory cytokines have often been reported in both adults and children with respiratory failure, it is likely that the ratio of the pro- and anti-inflammatory cytokines determines the outcome (Donnelly 1996, Martin 1997, Lentsch 1999). It is custom to express absolute cytokine concentrations in mass per volume measure of fluid (*eg* ng/ml or pg/ml). This method of expressing a cytokine concentration suffices when comparing the concentration of a particular cytokine between different groups of patients or at different time points. When net inflammatory activity is considered in terms of the ratio of pro- and anti-inflammatory cytokine, it should be realized that IL-1 and IL-RA, and TNF-α and TNFR molecules bind each other in a 1:1 ratio, but they each have different molecular weights (see sections 1.1.3.1, 1.1.3.2, 1.1.3.6 and 1.1.3.7). Therefore ratios of pro- and anti-inflammatory cytokine are expressed in terms of molar concentration rather than mass concentration.

Similarly to premature infants with RDS, I hypothesized that near-term newborn infants with severe respiratory failure supported with ECMO who survive, show a transient inflammatory response in their lungs in terms of raised neutrophil counts and increased concentrations of

pro- and anti-inflammatory cytokines in BAL fluid, which resolves with clinical improvement. Furthermore, I hypothesized that infants with severe respiratory failure who have a poor outcome and die, show a persistent inflammatory lung response which fails to resolve and lack of an anti-inflammatory response, as is seen in premature infants with chronic lung disease.

In order to test this hypothesis, I measured the individual cellular constituents and both pro- and anti-inflammatory cytokines in fluid obtained by NB-BAL in infants receiving ECMO for severe respiratory failure and compared the results in infants who survived with those who died, and with control infants undergoing cardiac procedures for congenital heart diseases.

4.2 Methods

4.2.1 Patient population

The study population has been described in section 3.2.1. Infants less than 12 months of age, with acyanotic congenital heart disease, but without respiratory disease or cardiac failure, undergoing routine cardiac catheterisation or elective cardiac surgery served as controls.

4.2.2 Sample collection

Standardised NB-BAL was performed according to the ERS Task Force guidelines (de Blic 2000) and as previously described (see section 3.2.2). The infants underwent daily NB-BAL until decannulation from ECMO. In survivors, NB-BAL was continued daily until removal of the endotracheal tube. Control patients underwent NB-BAL and blood sampling immediately after induction of anaesthesia but before any procedures including cardiopulmonary by-pass. Blood samples were drawn from the venous side of the ECMO circuit (central venous blood) in infants receiving ECMO and from indwelling arterial lines in survivors post-ECMO. After centrifugation within 10 minutes of collection, the plasma was stored at –70 °C until further

analysis.

4.2.3 Cell counts

Cell counts were performed and expressed as described in 3.2.3.

4.2.4 Measurement of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8, and anti-inflammatory cytokines IL-1RA, TNFR-1 and TNFR-2

Lavage fluid and plasma concentrations of the pro-inflammatory cytokines IL-8, IL-1β, IL-6 and TNF-α were measured by an automated ELISA analyser (DPC Europe Ltd, Wales) as described in section 3.2.4. Concentrations of the anti-inflammatory cytokines IL-1RA, soluble TNFR-1 and TNFR-2 in lavage fluid were measured by commercially available ELISAs (R&D Systems Europe, Abingdon, UK). These assays use an indirect sandwich immunoassay with 96 well plates coated with monoclonal antibody for the cytokine under study. The wells were incubated with BAL fluid or plasma followed by a washing phase. This was followed by a second incubation with an enzyme-linked polyclonal antibody. Following a second wash phase, a substrate solution was added and the colour development was to an intensity proportional to the amount of cytokine bound in the initial step. For each well the optical density was measured with a spectrophotometer set at a wavelength of 450 nm and standard solutions were used to calculate the final cytokine concentration. The lowest sensitivity of the assay was 14 pg/ml for IL-1RA, 1.5 pg/ml for TNFR-1 and 1.0 pg/ml for TNFR-2. The ratios of TNF-α over TNFR-1, TNF-α over TNFR-2 and IL-β over IL-1RA were calculated using the molecular weights of these cytokines (see 1.1.3.1, 1.1.3.2, 1.1.3.6 and 1.1.3.7). In accordance with ERS Task Force guidelines, (de Blic 2000) I did not correct for dilution during the NB-BAL procedure and concentrations are expressed as picogram of cytokine per ml of BAL fluid.

4.2.5 Statistical analysis

The data is presented as medians and interquartile ranges or ranges. The non-parametric method Wilcoxon-rank test was used to compare cytokine concentrations within groups and Mann-Whitney U-test was used to compare results between groups. Fisher's exact test was used to compare the two diagnostic groups. Statistical significance was assumed at $p<0.05$. Analysis was performed using a statistical package for microcomputers (SPSS Base 9.0, SPSS Inc., Chicago, Illinois, USA).

4.3 Results

4.3.1 Patient data

Between October 1996 and January 1998, twenty-two infants receiving ECMO and 35 control infants were enrolled into the study. Twenty of the 22 study patients were the same patients as described in section 3.3.1. In this part of my studies I enrolled two additional patients in the study group, because in these two additional patients only lavage samples from one lung were obtained for analysis, due to macroscopic blood staining of the contra-lateral lavage samples. Demographic data are summarized in Table 4.1. In the ECMO group, survivors were significantly younger compared to non-survivors (median age 1 versus 28 days, $p=0.04$). Overall the majority of patients were less than 30 days old ($n=18$), with 3 patients aged over 30 days amongst non-survivors and 1 in the survivors group. Median oxygenation index on admission was higher amongst survivors, but this difference was not statistically significant. Study patients who did not survive, were more likely to have received veno-arterial cannulation. One patient in this group was converted from veno-venous to veno-arterial cannulation to provide additional cardiac support. The group of non-survivors had a significantly longer median time on ECMO (237 hrs versus 133 hrs, $p<0.05$). The diagnostic groups amongst survivors and non-survivors are summarized in Table 4.2. All patients with

meconium aspiration syndrome survived. The group of non-survivors had a higher proportion of patients with pneumonia (n=4). This included 3 patients with viral pneumonia. The median age in the control group was 150 days (range 1- 390). None of these children had respiratory symptoms or cardiac failure.

Table 4.1 Demographic Data

	Survivors	**Non-survivors**	**Controls**
N	15	7	35
Age (d)	1 (1 – 120)	28 (1 – 402)*	150 (1-390)
Weight (Kg)	3.3 (2.5 – 4.1)	3.6 (2 – 10)	5.8 (2.2-9)
OI on admission	51 (19.6 – 97.1)	37 (9.4 – 76.5)	
Surfactant**	1	0	
Nitric Oxide	2	2	
VA:VV ECMO	4:11	6:2$^{\Psi}$	
Time on ECMO (h)	133 (71 – 304)	237 (94 – 406)*	

*Median (± Ranges) are given. * $p<0.05$ when survivors and non-survivors compared.*

*** surfactant use after admission. OI = oxygenation index, VA:VV veno-arterial:veno-venous cannulation. $^{\Psi}$ One infant was converted from VV to VA ECMO.*

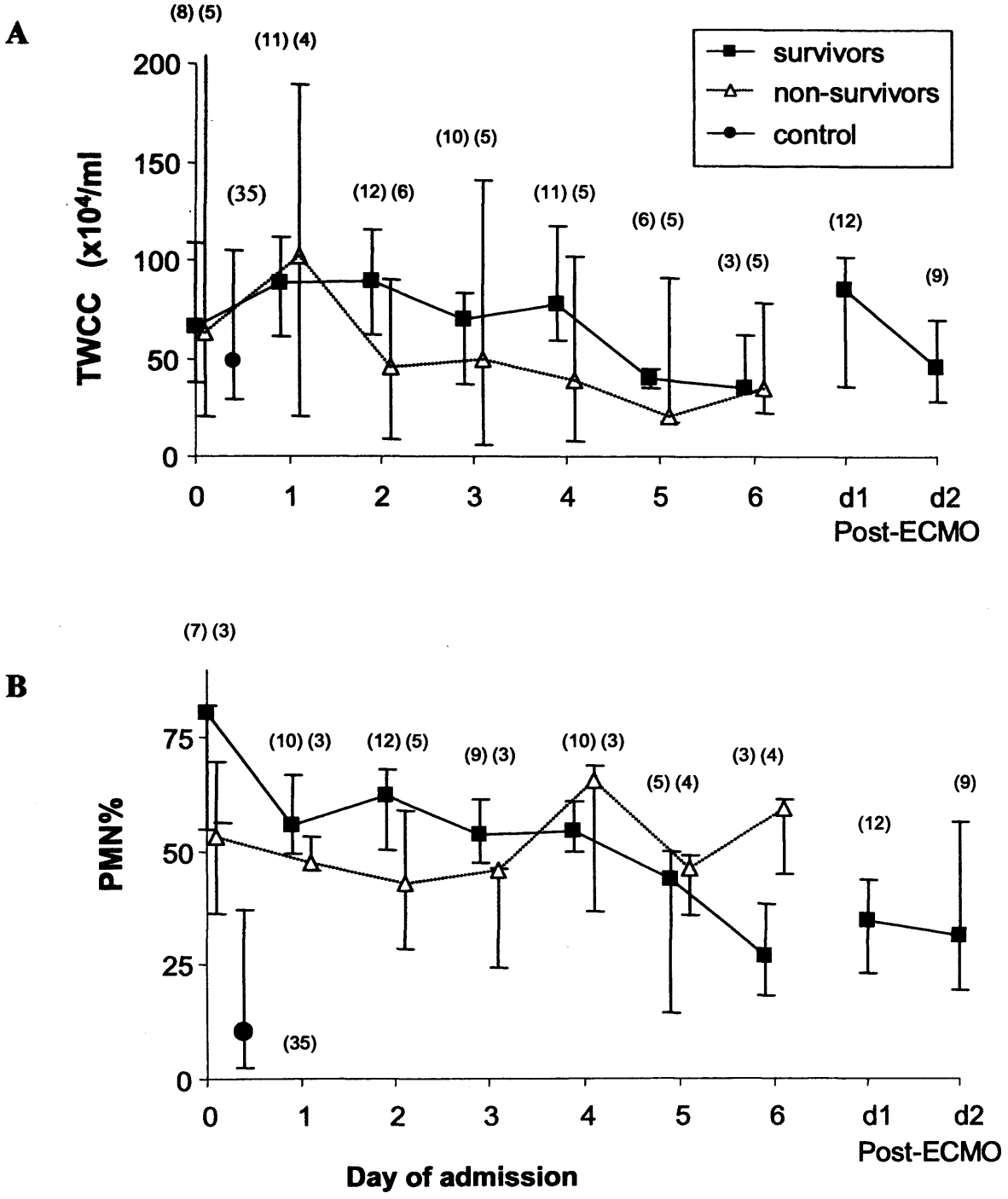
Table 4.2 - Diagnostic groups of infants receiving ECMO

Diagnosis	Survivors (N=15)	Non-survivors (N=7)
Meconium aspiration syndrome	7	0
Pneumonia	1	4
PPHN	1	2
Sepsis	3	0
Congenital Diaphragmatic Hernia	2	1
Respiratory Distress Syndrome	1	0

4.3.2 Cell populations in NB-BAL fluid

The cellular results from lung lavage fluid are shown in Figure 4.1. In the survivors, total pulmonary cell counts increased from 65.6 x 10^4/ml before cannulation to 88.5 x 10^4/ml after cannulation. Thereafter it decreased gradually to 34.7 x 10^4/ml by day 6 before increasing to 85.6 x 10^4/ml when placed on conventional ventilation after decannulation from ECMO. For the non-survivors the picture was very similar, in that the cell counts increased from 63.3 x 10^4/ml before cannulation to 102.0 x 10^4/ml after cannulation before decreasing to 34.8 x 10^4/ml on day 6. In the control infants the total cell count was 49x10^4. Only 10% of the cells were neutrophils in the control group but in the survivors this was 80.7% at presentation and this decreased to 34.5% by day 6 as the condition of the infants improved ($p = 0.05$). In contrast, these values were relatively static at approximately 50% in the non-survivors throughout the study period. Similarly, the percent alveolar macrophages in the survivors increased from 18% on presentation to 64.3% by day 6 ($p = 0.05$), but remained

Figure 4.1 A-E. Median total counts and cell percentages for white cells, neutrophils and macrophages in BAL fluid of study and control patients



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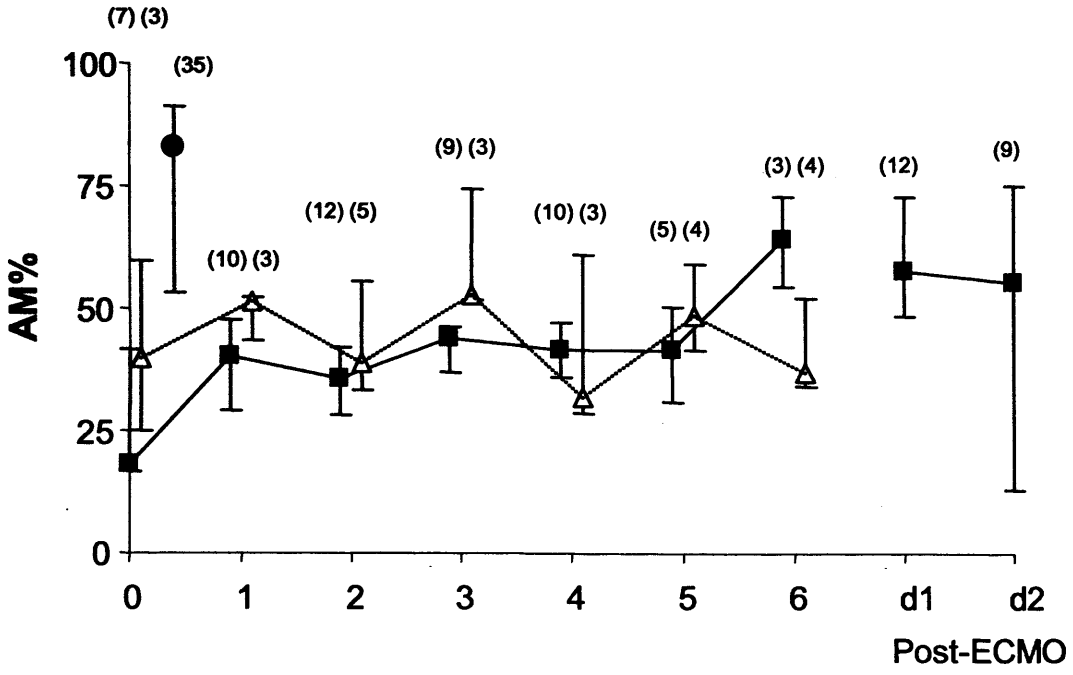
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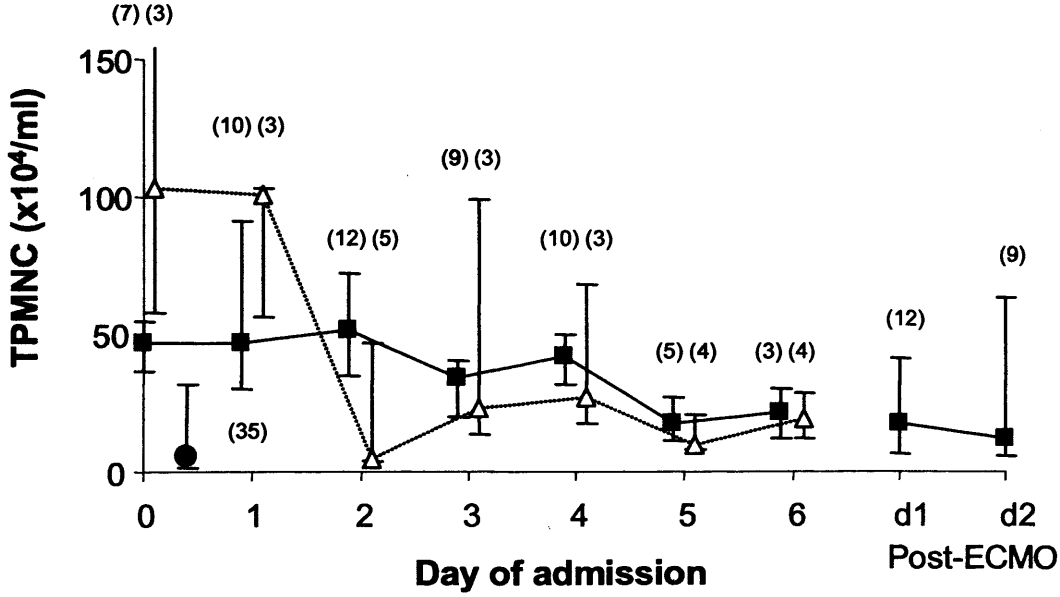
Post-ECMO

D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TPMNC (x10⁴/ml)
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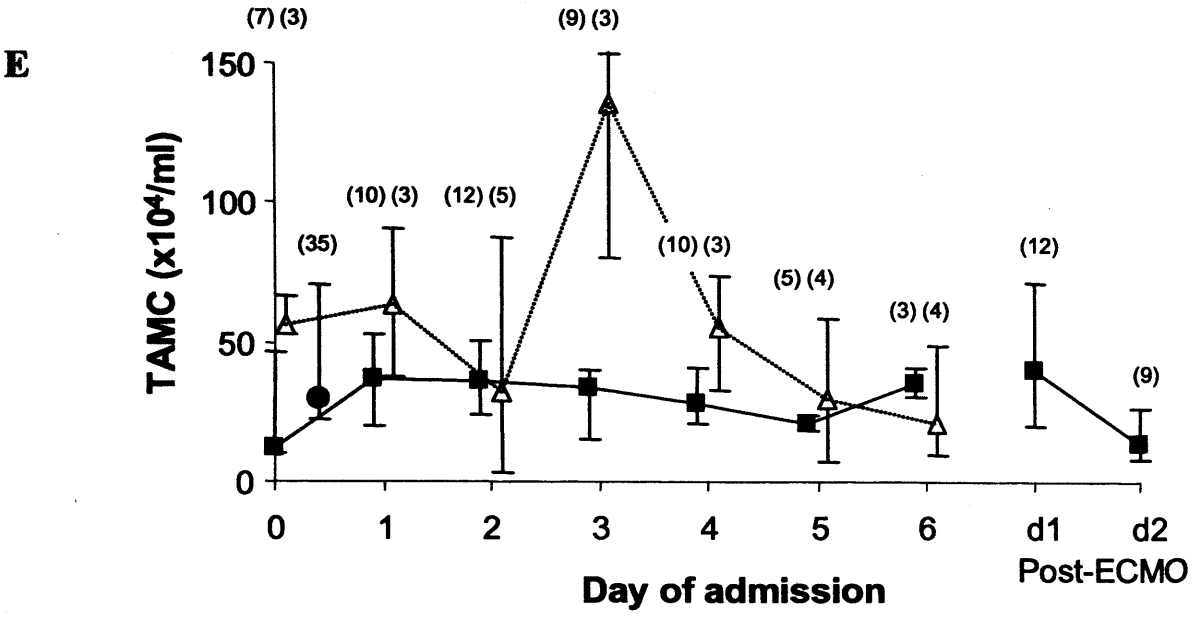
Day of admission



*Median and interquartile ranges are sh***Day of admission** *cell counts, (B) percent neutrophils, (C) percent alveolar macrophages, (D) absolute neutrophil counts, and (E) absolute macrophage counts in fluid obtained by non-bronchoscopic bronchoalveolar lavage. Numbers in brackets above bars denote number of samples obtained in each group per day.*

45 – 50% in the non-survivors of ECMO. In the control infants, 83% of the cells were alveolar macrophages. Absolute neutrophil counts were very high in the lungs of non-survivors at 103.2 x 10^4/ml on presentation and 100.6 x 10^4/ml after cannulation. This decreased rapidly remaining <27.3 x10^4/ml thereafter. When compared to control infants in whom absolute neutrophil counts were 5.5 x10^4/ml, the survivors had a count of between 45 – 50 x10^4/ml on days 0 – 2 before decreasing gradually to 17.7 x10^4/ml by day 5. Except for a large increase to 135.4 x10^4/ml on day 3 in the non-survivors the absolute alveolar macrophage counts were very similar in the three groups studied.

4.3.3 Pro-inflammatory cytokines in NB-BAL fluid and plasma

The results for the proinflammatory cytokines in NB-BAL fluid are shown in Figure 4.2. The pattern for each cytokine was very similar in the survivors of ECMO: the concentration of

pulmonary cytokine increased once the infant was placed on ECMO reaching a maximum on day 2 of ECMO before declining. A further increase was observed when the infants were placed on conventional positive pressure ventilation. By contrast, in the non-survivors the concentration of for each cytokine studied was relatively high at presentation but decreased rapidly thereafter to very low concentrations especially for IL-1β, IL-8 and IL-6.

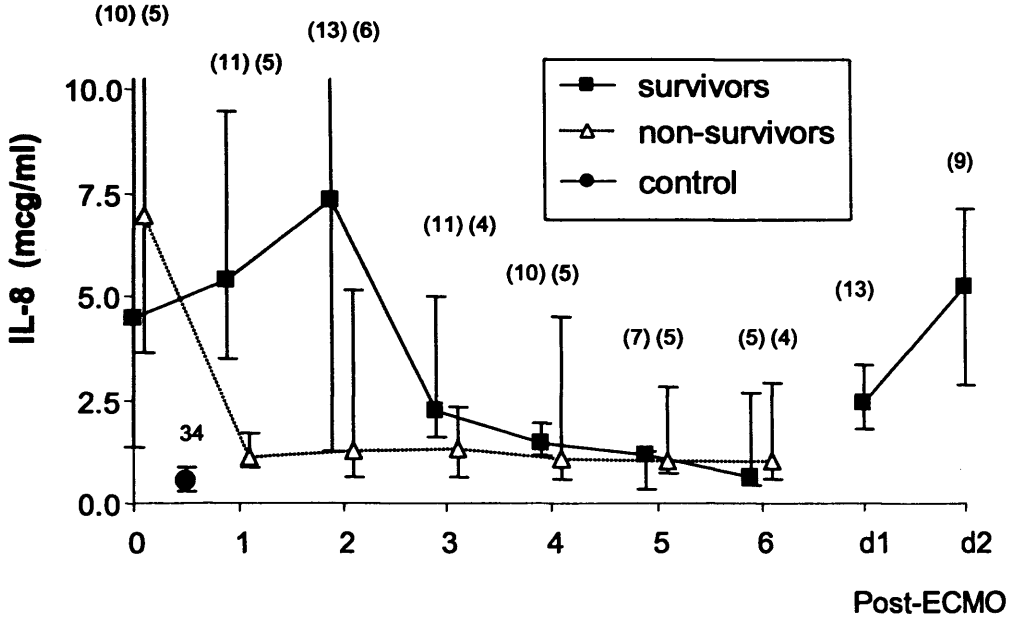
In plasma, the concentration of IL-8 and IL-6 was highest at presentation before declining in both the survivors and non-survivors (see Figure 4.3). The concentration of TNF-α increased in the non-survivors from 59.0 pg/ml to 144.0 pg/ml on day 2 before decreasing to 109.5 pg/ml on day 6. A similar picture was seen in the survivors in that the TNF-α increased from 80.4 pg/ml just before being placed on ECMO to 109.0 pg/ml on day 3 before decreasing to 78.9 pg/ml by day 5. In the control group, very low concentrations of IL-8 and IL-6 were found (IL-8 <5 pg/ml, IL-6 19.7 pg/ml) but the median concentration of TNF-α was 63.8 pg/ml.

4.3.4 Anti-inflammatory cytokines in NB-BAL fluid

The data for the anti-inflammatory cytokines in lung lavage fluid is shown in Figure 4.4. The pattern for all three agents studied was very similar to that observed for proinflammatory cytokines. In the non-survivors, the concentration of anti-inflammatory agents was initially high at presentation but decreased rapidly to low levels by day 3 – 4 of ECMO. In contrast, in the survivors, the initial concentration increased reaching a maximum after cannulation before declining more gradually over the following few days. Small increases were observed when the surviving infants were placed on positive pressure ventilation. Low concentration but high variability was seen in the control infants for all three anti-inflammatory agents studied.

Figure 4.2 A-D. Concentration of pro-inflammatory cytokines in BAL fluid of study patients and controls

A



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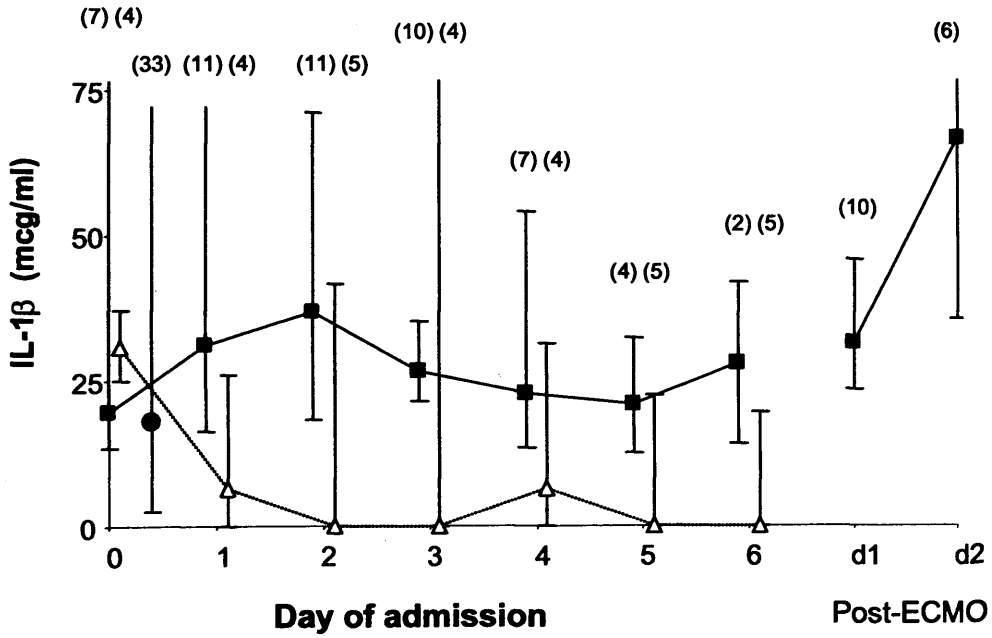

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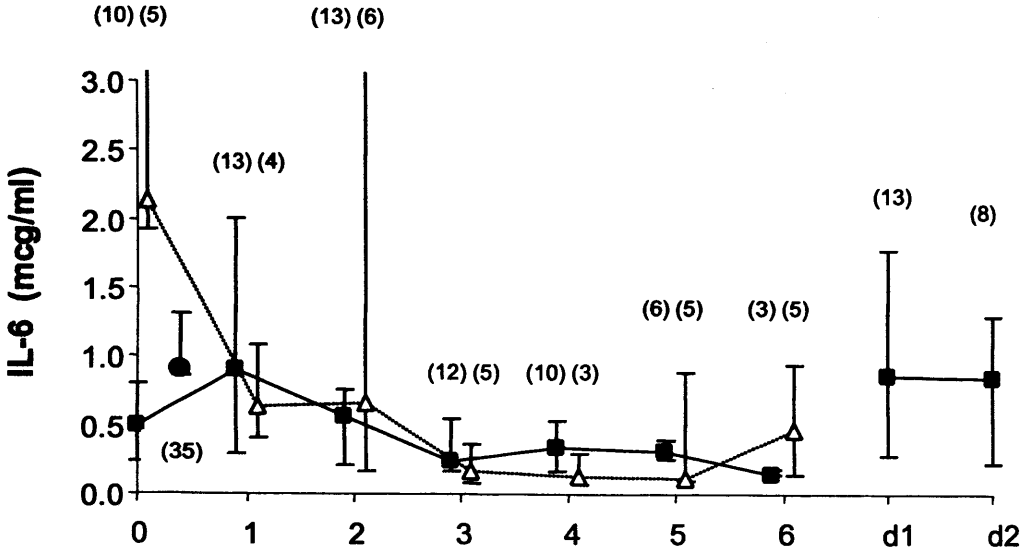

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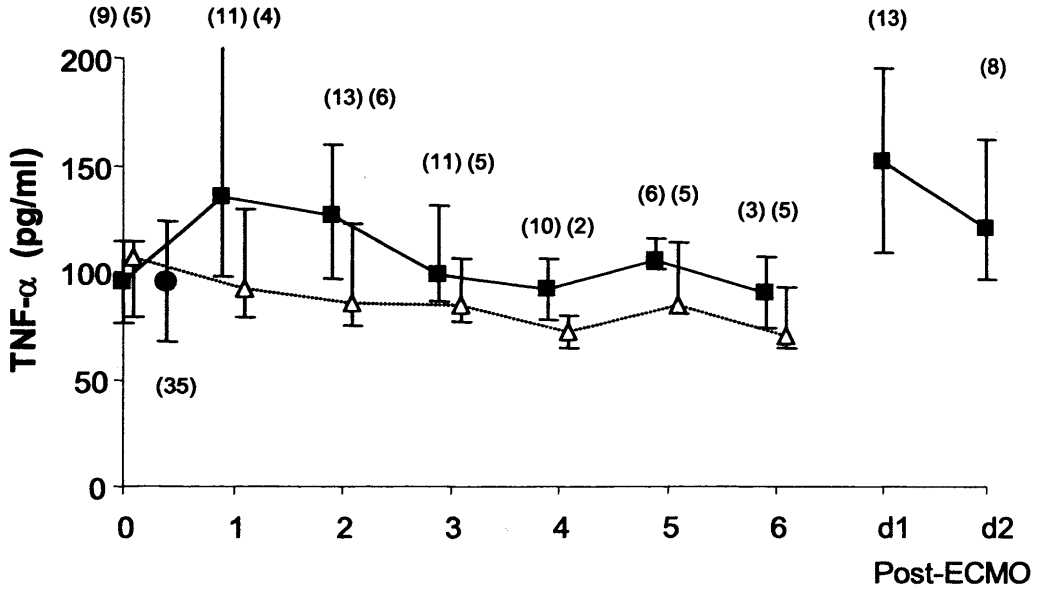


Day of admission

Median and interquartile ranges are shown for the proinflammatory cytokines (A) interleukin-8 (IL-8), (B) interleukin-1 beta (IL-1β), (C) interleukin-6 (IL-6) and (D) tumour necrosis factor-alpha (TNF-α) in fluid obtained by non-bronchoscopic bronchoalveolar lavage. Numbers in brackets above bars denote number of samples obtained in each group per day.

4.3.5 Molar Ratios of pro- and anti-inflammatory cytokines in BAL fluid

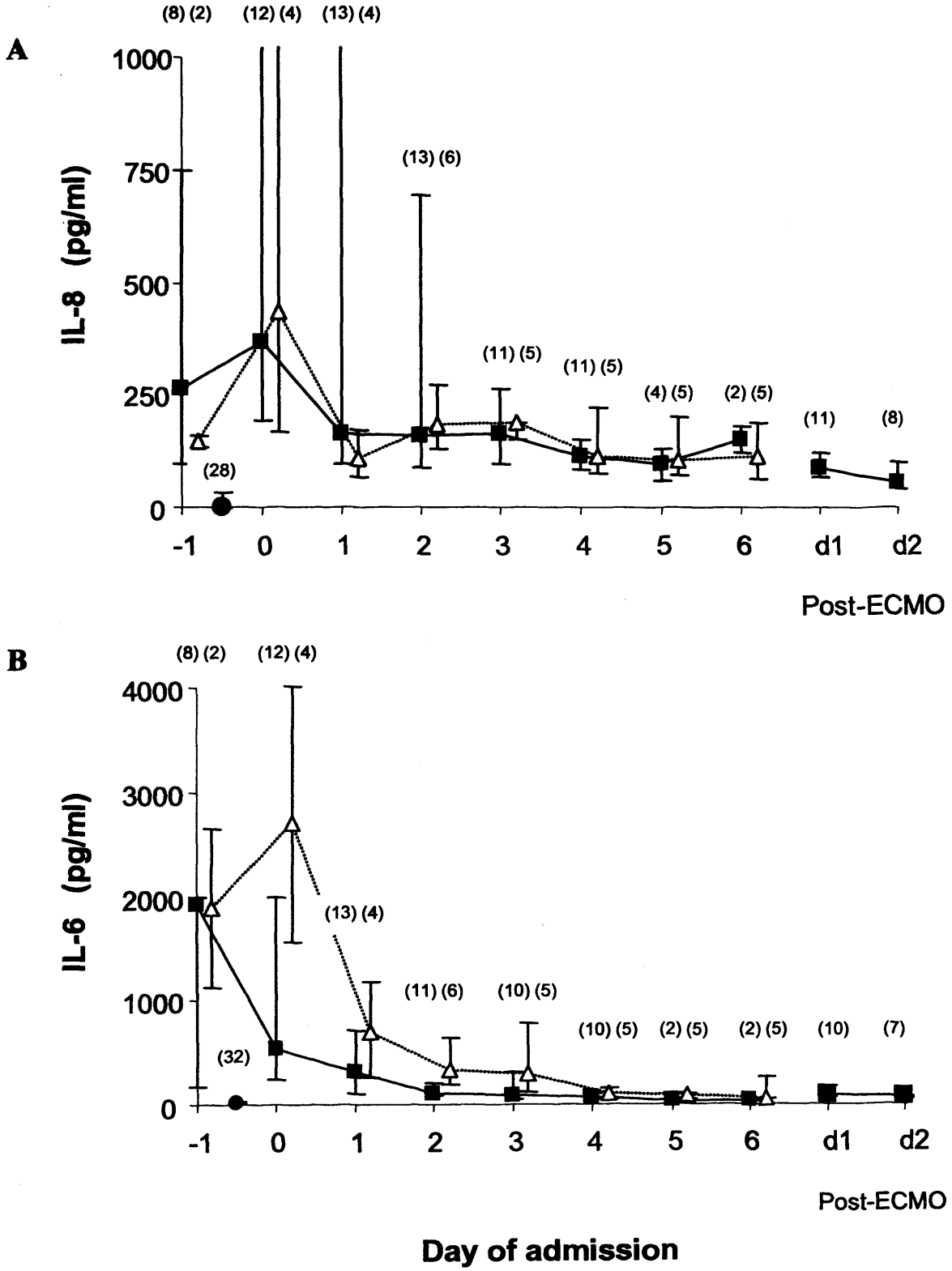
In this part of my studies, I expressed the data as molar ratios of pro- and anti- inflammatory cytokine, as it is likely that the balance between inflammatory and anti-inflammatory agents will determine outcome. The molar ratio for TNF-α/sTNF-R1 was relatively static at 5.0 in the survivors but increased from 2.4 at presentation to 11.6 on day 2 in the non-survivors (Fig 4.5A). The ratio in this group decreased to 3.1 by day 4 but a further increase was seen to 10.9 on day 6. Significant differences were noted between the two groups at day 2 ($p<0.05$). Similar changes were observed for the molar ratios TNF-α/sTNF-R2 in that the ratio remained relatively static at approximately 8.0 in the survivors but increased from 4.9 at presentation to a maximum of 29.6 on day 3 before declining to 4.5 on day 6 in the non-survivors of ECMO (Fig 4.5B). Significant differences were noted between the survivors and non-survivors at day 3 ($p<0.05$).

The ratio of IL-1β to sIL-1RA remained relatively static at 0.1 in the survivors throughout their course of ECMO (Fig 4.5C). In contrast, in the non-survivors, this ratio was 0.05 at presentation but decreased to undetectable levels between days 2 and 6 except for a marginal increase at day 4 to 0.03. The undetectable ratio of IL-1β to sIL-1RA in the non-survivors was due to undetectable IL-1β in the BAL fluid obtained from infants who did not survive. Significant differences were noted between the two groups at day 2 ($p<0.05$).

4.4 Discussion

In this study, I examined the role of pro- and anti-inflammatory cytokines in the resolution of acute lung injury in survivors and non-survivors receiving ECMO. When analyzing individual

Figure 4.3 A-C. Concentrations of pro-inflammatory cytokines IL-8, IL-6 and TNF-α in plasma of study patients and controls.



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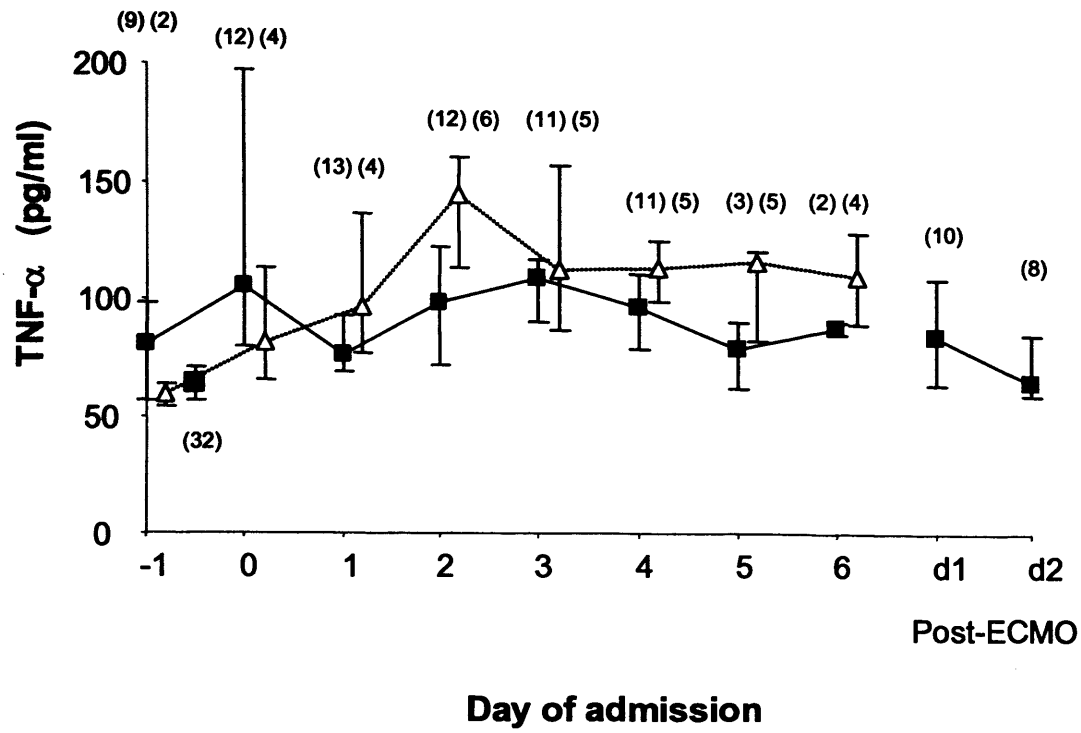
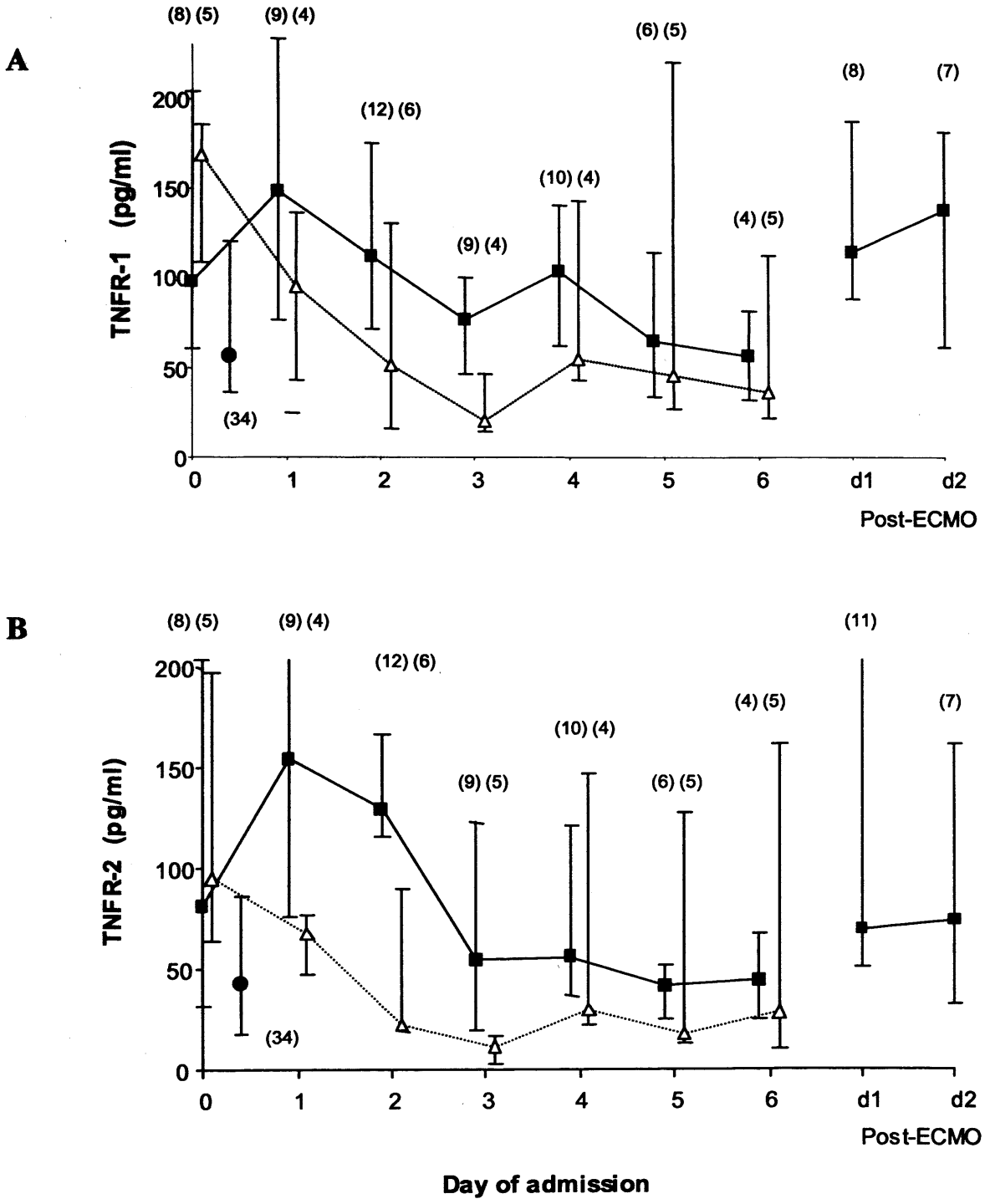


Median and interquartile ranges are shown for the proinflammatory cytokines (A) IL-8, (B) IL-6 and (C) TNF-α in plasma obtained from survivors and non-survivors of ECMO and control infants. Numbers in brackets above bars denote number of samples obtained in each group per day.

cell counts and BAL cytokine concentrations, essentially, two patterns emerge: in the survivors an appropriate inflammatory response was observed. The initially high proportion of neutrophils decreased with a parallel increase in percent alveolar macrophages in the lungs of infants who survived. Both pro- and anti-inflammatory pulmonary cytokines, increased during the first 24 – 48 hours after being placed on ECMO before declining as the condition of the infants improved. A relatively static molar ratio of TNF-α against sTNF-RI and RII and IL-1β to sIL-1RA was observed in the survivors. In the non-survivors, the absolute neutrophil numbers in lavage fluid were initially very high, as were the the concentrations of pro- and anti-inflammatory cytokines, but these all declined very rapidly to very low concentrations by 48 hours after being placed on ECMO.

Figure 4.4 A-C. Concentrations of anti-inflammatory cytokines IL-1RA, sTNFR-1 and sTNFR-2 in BAL fluid of study patients and controls



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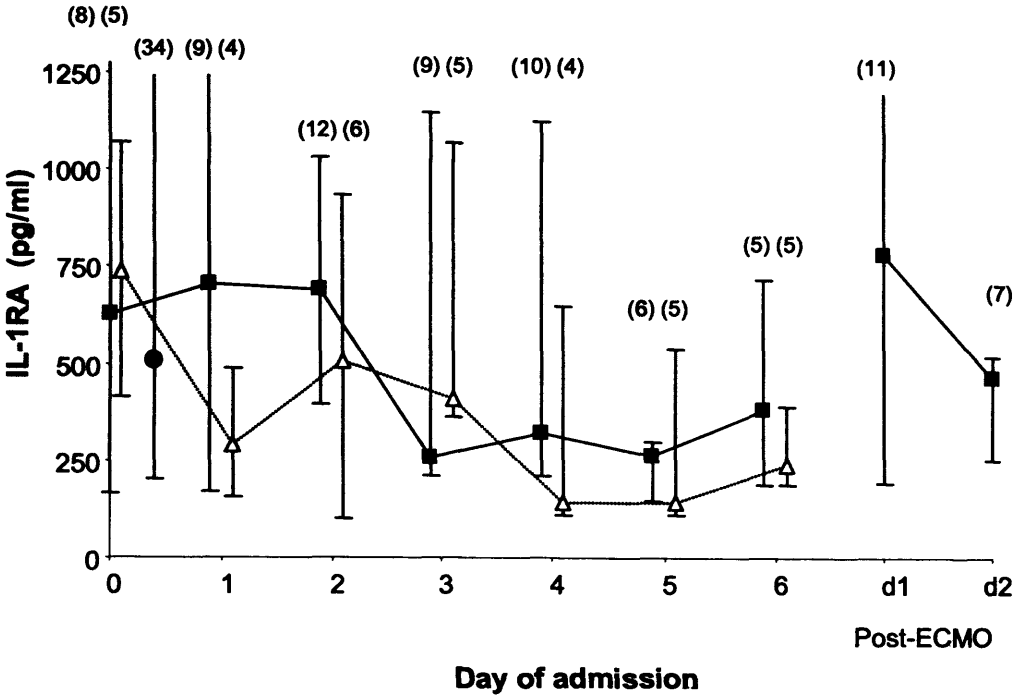


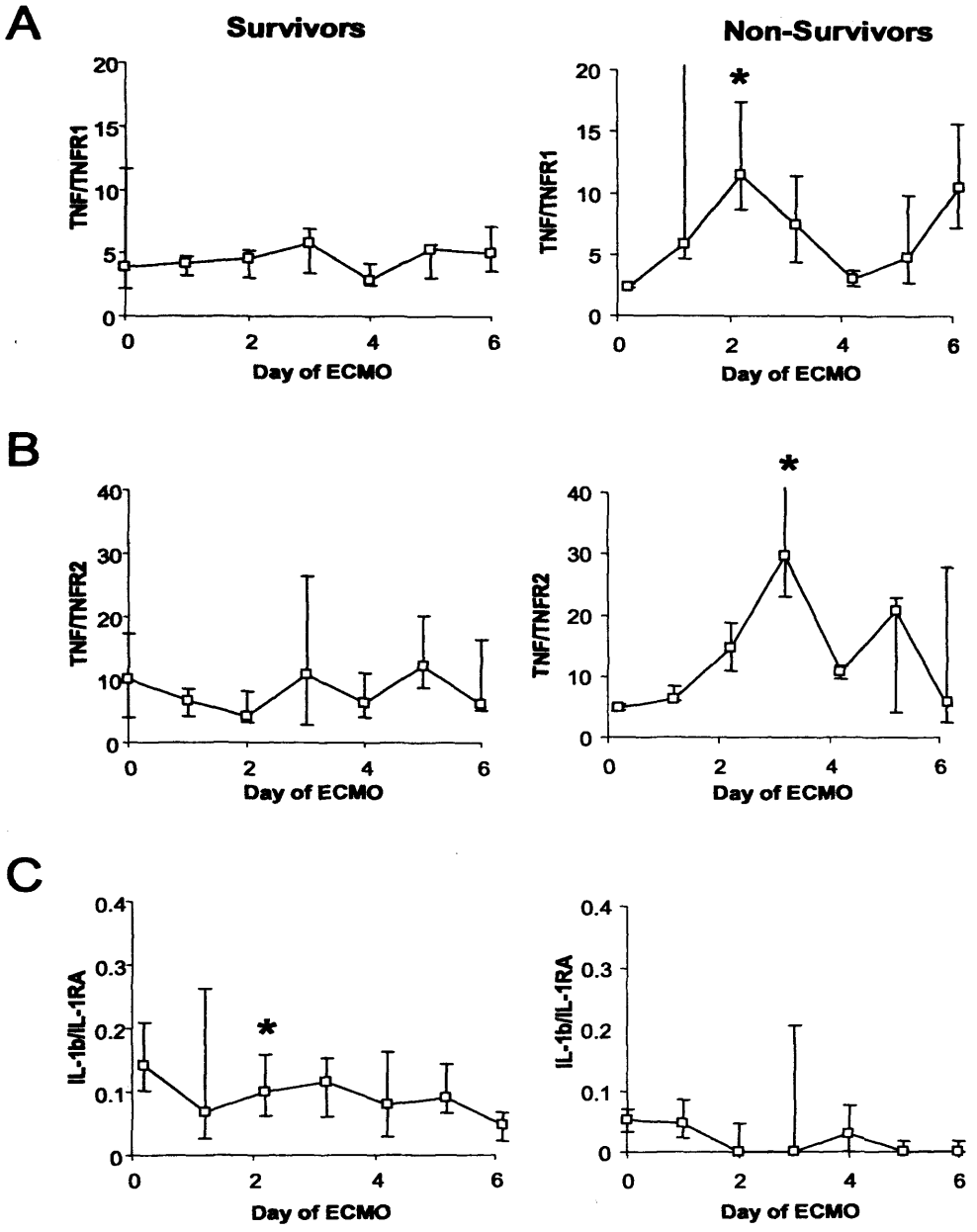
Median and interquartile ranges are shown for the anti-inflammatory cytokines (A) IL-1RA, (B) soluble TNF-receptor I and (C) soluble TNF-receptor II in fluid obtained by non-bronchoscopic bronchoalveolar lavage. Numbers in brackets above bars denote number of samples obtained in each group per day.

The ratio of TNF-α against its antagonists was increased at day 2-3, but the ratio of IL-1β to soluble IL-1RA was very low mainly due to undetectable IL-1β in BAL fluid. The findings suggest that, during the early recovery phase following acute lung injury, survivors mount an appropriate inflammatory response, during which there is a balance between pro- and anti-inflammatory cytokines. Patients who fail to recover appear unable to respond in this way. This observation is unlikely to be due to disease severity at presentation since the oxygenation index was actually greater in the group that survived when compared to the non-survivors. I did not find any underlying evidence of an immune or respiratory disease which may have explained the above findings. The underlying disease process may have contributed to the

patterns observed as more infants with an infective process did not survive.

Nakagawa *et al* also studied IL-8 concentrations and cell populations in BAL fluid of neonates with respiratory failure treated with ECMO (Nakagawa 1997). They found elevated levels of IL-8 and neutrophils early during ECMO treatment but both decreased with recovery. In our study, the survivors showed a similar trend. However, the non-survivors appeared to respond differently from other groups of patients with acute lung injury. Premature neonates, who go on to develop chronic lung disease have persistently elevated concentration of IL-6, IL-8 and ICAM-1 in lavage fluid samples infants (Groneck 1995, Kotecha 1995, Kotecha 1996a, Kotecha 1996c). Adults with ARDS who have persistent disease or poor outcome also show prolonged elevation of concentrations of pro-inflammatory cytokines and chemokines in BAL fluid (Meduri 1995, Goodman 1996). It is also possible that other mechanisms may be involved. Persistent pulmonary hypertension, alone or in combination with other mechanisms, contributes to respiratory failure in near-term newborn infants (Morin 1995). Dobyns and workers measured concentrations of prostaglandins and leukotrienes in BAL fluid of neonates with respiratory failure treated with ECMO, as markers of pulmonary hypertension (Dobyns 1994). They found a decline in levels over time in survivors, whilst patients with poor outcome had markedly elevated BAL fluid concentrations. Anti-inflammatory cytokines may be important in controlling acute inflammation in response to injury, and allow successful resolution to take place with survival of the organism (Martin 1997, Opal 2000). Donnelly and workers found a significant correlation between low concentrations of the anti-inflammatory cytokines IL-1RA and IL-10 in BAL fluid and mortality in adults with ARDS in the first 24 hours (Donnelly 1996). In adults with severe asthma, a concurrent increase in both pro- and anti-inflammatory cytokines was detected in BAL fluid compared to controls (Tillie-Leblond 1999). However, that study did not compare survivors and patients who died.

Figure 4.5 Molar ratios of pro-and anti-inflammatory cytokines in BAL fluid of study patients



*Median and interquartile ranges are shown for the ratios (A) TNF-α/sTNF-R1, (B) TNF-α/sTNF-R2, and (C) IL-1β/IL-1RA inBAL fluid. * = $p<0.05$ when the survivors and non-survivors of ECMO were compared.*

It has been observed in both infectious and non-infectious diseases that the molar ratio of TNF-α against its soluble receptors may be related to outcome. For instance in meningococcal

sepsis, patients with a higher plasma molar TNF-α /sTNF-R ratio had greater mortality (Girardin 1992). In adults who survived ARDS, the molar TNF-α /sTNF-R ratios in BALF were significantly lower compared to controls in the early phase of their disease but the ratio gradually increased with time to reach normal values by the third week (Park 2001). In adults, IL-1RA has been detected in BAL fluid obtained from healthy individuals and its concentration increases significantly at the onset of ARDS (Park 2001). A highly significant correlation has been reported between the low concentrations of IL-1RA in BAL fluid and mortality in ARDS (Donnelly 1996). In survivors with ARDS the molar ratio of IL-1β over IL-1RA declines in the first 72 hours, suggesting a net anti-inflammatory signal which serves to dampen the inflammatory response (Park 2001).

Inhaled nitric oxide (NO) is a selective pulmonary vasodilator and has been used in the treatment of severe hypoxaemic respiratory failure in newborn infants (Weinberger 1999). Inhaled nitric oxide has also been found to have anti-inflammatory properties. In animal models of IRDS (Kinsella 1997, Kinsella 1999) decreased lung neutrophil accumulation was noted with the use of inhaled NO. Following tracheal instillation of IL-1 in perfused rat lungs, inhaled nitric oxide reduced lung neutrophil accumulation (Guidot 1996). Similar observations were made in rodent models of sepsis (Friese 1996, Razavi 2002). No human studies have confirmed these observations. Kang *et al* used a rabbit model of intravenous LPS induced lung injury (Kang 2002). They showed both a reduction in neutrophil accumulation as well as a reduction in IL-1β and IL-8 production in alveolar macrophages. In my studies, four patients (2 in each group) received inhaled nitric oxide treatment prior to ECMO support. No details are available regarding the duration of inhaled NO treatment. Given that inhaled NO has the potential to cause a reduction in neutrophil accumulation in animal models, there may have been some suppression of neutrophil persistence in the non-survivors in my studies. On the

other hand, it is possible that the rapid decline in cytokine concentrations in the non-survivors was in part caused by inhaled NO.

It is now clear that ventilator induced lung injury causes a very similar inflammatory response compared to other types of lung injury (Dreyfuss 1998). Recent studies in both animal models as well as adults have shown that in addition to causing a pulmonary inflammatory response, positive pressure ventilation can also lead to systemic inflammation (von Bethmann 1998, Chiumello 1999, Ranieri 1999). Support with extracorporeal membrane oxygenation may be beneficial in refractory respiratory failure by providing "lung rest". In the present study, although the concentrations of proinflammatory cytokines were (non-significantly) greater in the plasma from non-survivors when compared to both survivors of ECMO and control infants they are unlikely to explain the patterns observed in the lungs of the non-survivors or to explain the poor outlook. The main disease process appears to be confined to the pulmonary environment especially in view of the concentration of IL-8 which in the lungs was an order of magnitude higher suggesting local production of IL-8 rather than primary systemic inflammation.

The role of pro-inflammatory cytokines in plasma of patients with severe lung injury, treated with ECMO is likely to be complex. Initiation of extracorporeal support both *in vivo* and *in vitro* has been shown to cause activation of the complement pathways and a transient inflammatory response due to contact between blood and the ECMO circuit (Moat 1993, Plotz 1993, Adrian 1998). Fortenberry and workers studied the effect of ECMO cannulation on neutrophil activation and cytokine expression *in vivo* (Fortenberry 1996). They observed significantly elevated plasma concentration pre-ECMO of IL-8 and IL-6, but not TNF-α. They also found a transient rise in IL-6 and IL-8 median concentrations in the first 24 hours after cannulation.

When studying individual cell counts and cytokine concentrations, I have refrained from applying extensive statistical analysis especially with the small numbers that I have been able to study. Instead, I have relied on observation of the patterns of change over time in infants who do or do not survive treatment with ECMO for severe respiratory failure. Such an approach has identified differing patterns of change of cellular and both pro- and anti – inflammatory cytokines between the two groups. I have also included a group of control infants without evidence of respiratory disease or cardiac failure. Nevertheless comparisons with this group of infants should also be interpreted with caution as these infants do have an underlying cardiac disease, which may alter the normal pulmonary environment e.g. by altering pulmonary blood flow.

In summary, in this part of my studies I studied patterns of change of white cells and pro-and anti-inflammatory cytokines in the lungs of infants with severe respiratory failure, who require support with ECMO. Survivors showed a trend towards an early transient inflammatory response in their lungs in terms of high initial BAL neutrophil percentage which declined with recovery. In this group, there was also a recurring trend towards a transient increase in BAL concentrations of IL-8, IL-1β and TNF-α. This response is matched by a transient increase in concentrations of the anti-inflammatory cytokines IL-1RA, sTNFR-1 and -2. Patients who subsequently died had relatively stable neutrophil percentages throughout. A pattern of rapid decline from very high concentrations in BAL was observed for both pro-and anti-inflammatory cytokines. Also, non-survivors showed higher ratios of TNF-α over its inhibitors sTNFR-1 and-2 in the first 48 hours. Taken together these observations suggest a failure in non-survivors to mount an appropriate, early inflammatory response which may be responsible for the poor outcome.

Chapter 5. The role of neutrophil apoptosis in resolution of acute lung injury in infants with severe respiratory failure

5.1 Introduction

In my studies in chapter 4, I showed that infants with severe respiratory failure requiring ECMO who survive, have an increased proportion of neutrophils in BAL fluid, which declines with recovery. On the other hand, the proportion of neutrophils in BAL fluid from non-survivors remained static during the course of ECMO support. Previous studies have shown that apoptosis is important in the normal resolution phase of inflammation, because it leads to functional down-regulation (Whyte 1993) and to recognition and clearance of the apoptotic neutrophils by macrophages (see section 1.1.2.2) (Savill 1989). Therefore, I hypothesized (1) that apoptosis of airway neutrophils is a pre-requisite for resolution of acute lung injury in infants with severe respiratory failure requiring ECMO and (2) that poor outcome of severe respiratory failure might be attributable to inappropriate suppression of neutrophil apoptosis, resulting in persistent neutrophilia. I also examine the relationship between apoptotic activity of BAL fluid in study subjects and the BAL fluid concentration of TNF-α, a known promoter of apoptosis in fresh neutrophils (Murray 1997).

5.2 Methods

5.2.1 Patient population

The same population of infants was studied as described in 4.2.1. In summary, I recruited 20 infants with severe respiratory failure as a result of various conditions, including RDS, meconium aspiration syndrome and persistent pulmonary hypertension of the newborn. Of those recruited, 14 survived and 6 did not. A control group consisting of infants undergoing elective cardiac catherisation or cardiac surgery as described in 4.2.1 was also enrolled into

the study. Fully informed consent was obtained from the parents of each infant. The study was approved by the Leicestershire Health Authority Research Ethics Committee.

5.2.2 Bronchoalveolar Lavage

In the infants receiving ECMO, I performed BAL daily during ECMO treatment, as described in section 3.2.2. In survivors daily BAL continued after ECMO, until the patients were extubated.

5.2.3 Isolation of neutrophils

This part of my studies was carried out in collaboration with Professor Moira Whyte's group at the University of Sheffield. Human peripheral blood neutrophils were obtained from healthy adult volunteers. Ethical approval was obtained from the South Sheffield Research Ethics Committee and all subjects gave fully informed consent. Neutrophils were isolated from citrated venous blood by dextran sedimentation and centrifugation through a discontinuous plasma-Percoll (Amersham Pharmacia Biotech, Buckinghamshire) gradient as previously described (Haslett 1985). Purity was assessed by counting >500 cells on duplicate cytospin preparations, and was always >95%, with contaminating cells being almost exclusively eosinophils.

5.2.4 Assessment of neutrophil apoptosis in BALF

The apoptotic activity of the BALF was assessed as previously described (Matute-Bello 1997). Neutrophils were suspended at a concentration of 3.5 x 10^6/ml in Roswell Park Memorial Institute solution (RPMI), with 10% Fetal Calf Serum (FCS) and with penicillin and streptomycin (100U/L) (all from Life Technologies Inc., Glasgow, UK) (Renshaw 2000). The 'age' of neutrophils in culture was calculated designating this stage as time zero. 100 µl of the

neutrophil suspension was added to 100 μl of BALF from the infants, with normal saline used as a control. Cells were incubated in 96 well non-tissue culture treated Falcon "Flexiwell" plates (BD Pharmingen, Cowley, Oxford, UK) at 37°C in a 5% CO_2 atmosphere for 5 hours. A 5-hour time point was chosen because there is little (typically <5%) constitutive apoptosis in control (untreated) populations so that a pro-apoptotic effect of BALF upon neutrophils should be detected (Usher 2002). At 5 hours, apoptosis was quantified by assessment of nuclear morphology on Giemsa-stained cytocentrifuge preparations, counting >300 cells per slide on duplicate cytospins (Savill 1989, Renshaw 2000). This method has been shown to correlate closely with other measurements of neutrophil apoptosis, including Annexin V binding (Homburg 1995) and shedding of CD16 (Dransfield 1994). In addition, necrosis was assessed at all time points by exclusion of the vital dye, trypan blue, and was <2% unless otherwise indicated. In section 5.3.2, the apoptotic activity of BALF is expressed as the ratio of percentage of apoptotic neutrophils following incubation with the study sample over percentage of apoptotic neutrophils following incubation with saline control in the same experiment.

5.2.5 Measurement of TNF-α concentration in BALF

TNF-a concentration was measured by automated ELISA as described in 3.2.4.

5.2.6 Statistical analysis

The apoptotic activity of BALF is expressed as median ratio ± interquartile ranges, with each experiment using cells from separate donors and performed in duplicate. The apoptotic activity of BALF is given relative to that obtained for saline control. Thus values of >1.0 implied pro-apoptotic activity and <1.0 implied anti-apoptotic activity in BALF relative to saline. Data was analysed using the Wilcoxon Rank test for comparisons within groups and

the Mann-Whitney U test was used for comparisons between patient groups. TNF-α concentration in BALF and apoptotic activity were compared by linear regression, calculating the Pearson correlation coefficient (R-value), and the p value. Results were considered to be statistically significant where $p<0.05$.

5.3 Results

5.3.1 Patient data

The patient data are summarized in table 3.2. I obtained 73 BAL samples from 20 infants receiving ECMO [age 2 (1-402) days, weight 3.7 (2.5-10.0) kg]. Two fewer patients were enrolled into this part of my studies compared to my studies as described in chapter 4. This was because for these two patients insufficient volume of BAL fluid was available. Of the patients 14 survived and 6 did not. Of the survivors, 6 received ECMO for meconium aspiration syndrome, 3 for sepsis, 2 for congenital diaphragmatic hernia and one each for pneumonia, persistent pulmonary hypertension and respiratory distress syndrome. One infant with congenital diaphragmatic hernia, two with persistent pulmonary hypertension and three with pneumonia did not survive. The control group has been described in section 4.3.1 and table 4.1.

5.3.2 *In vitro* modulation of neutrophil apoptosis by BAL fluid

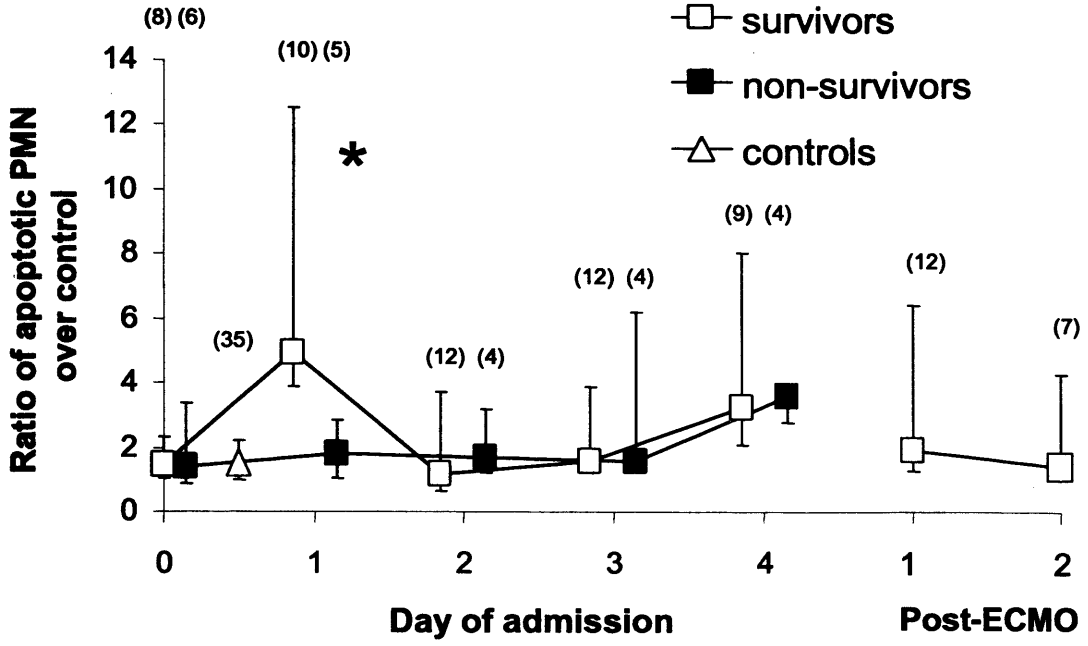
After cannulation for ECMO, there was little difference in apoptotic activity of BALF between survivors and infants who later died in pro-apoptotic activity of BAL fluid (see figure 5.1). At time point zero, in both groups activity was 1.4 x saline control. In the first 24 hours after institution of ECMO, there was a significant increase in pro-apoptotic activity to almost five times the activity in saline control in survivors ($p<0.05$), whilst the activity in non-survivors showed no significant change at any time point. After 24 hours the pro-apoptotic activity

returned to baseline levels and was not significantly different from non-survivors or control subjects. Control patients without lung disease also showed pro-apoptotic activity in BAL fluid compared to saline control (1.5 x saline control). No significant positive correlation was detected between activity of TNF-α in BALF and pro-apoptotic activity ($R = -0.05$, $p = 0.62$, see figure 5.2).

5.4 Discussion

The results of this part of my studies suggest that both resolution of inflammation and a favourable clinical outcome were associated with a transient increase in pro-apoptotic activity of bronchoalveolar lavage fluid. I have previously demonstrated a lack of resolution of pulmonary neutrophilia in the poor outcome group (see section 4.3.2). In this part of my studies this group lacked an early rise in apoptotic BALF activity on suspended neutrophils. TNF-α did not appear to be responsible for the increased pro-apoptotic activity in BAL fluid from survivors. Matute-Bello *et al* (Matute-Bello 1997) studied neutrophil apoptosis in the adult respiratory distress syndrome (ARDS) and demonstrated that neutrophil apoptosis was detected at low levels in BAL throughout the course of ARDS. Moreover, BAL fluid from ARDS patients suppressed constitutive neutrophil apoptosis, whereas BAL fluid from normal volunteers did not (Matute-Bello 1997). This inappropriate suppression of neutrophil apoptosis is analogous to the findings in the non-survivor group in my study and was abrogated by blocking antibodies to G-CSF and GM-CSF in the study by Matute-Bello (Matute-Bello 1997). In this study, the authors were unable to demonstrate a significant

Figure 5.1 In vitro modulation of neutrophil apoptosis by BAL fluid from study patients and controls compared to modulation by normal saline



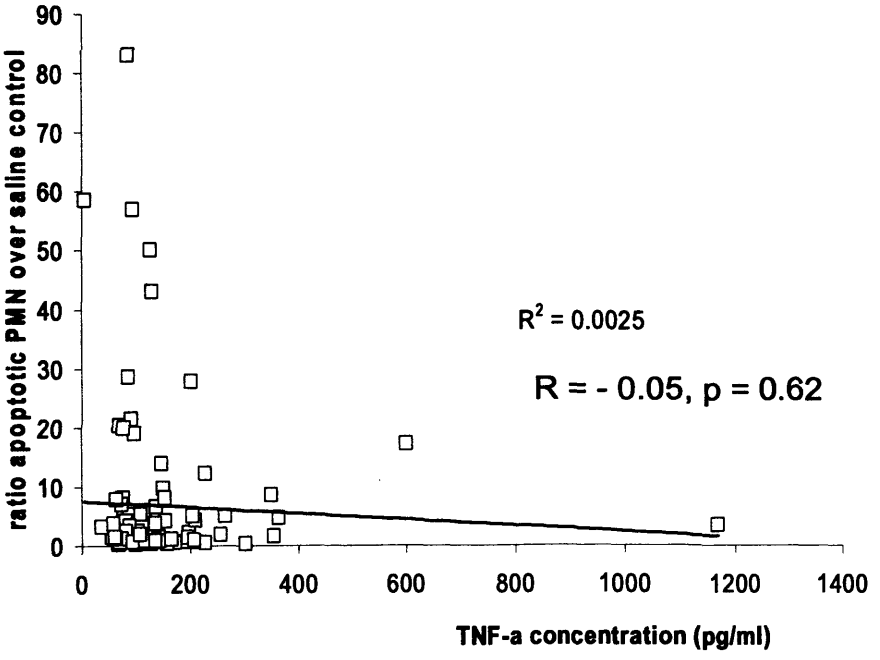
*Given are median ratio and interquartile ranges of percentage apoptotic neutrophils following incubation with BAL fluid over saline control, for survivors, non-survivors and control patients (see section 5.2.4 for details of methodology). * $p < 0.03$ compared to non-survivors. Numbers in brackets above bars denote number of samples obtained in each group per day.*

association between higher rates of neutrophil apoptosis and survival although mean apoptosis was higher in the survivors than in those who died (2.4% vs. 1.8%).

Allgaier *et al* have studied neutrophils purified from umbilical cord blood of neonates and have shown that they undergo apoptosis at a slower constitutive rate than adult neutrophils and also that they are more resistant to the effects of the ligation of the Fas death receptor (Allgaier 1998). It is possible, therefore, that age-related differences in apoptosis susceptibility had an

influence on the results observed. This is most likely attributable to the presence or absence of soluble modulators of apoptosis, and again gestational age and post-natal age, for example,

Figure 5.2 Correlation between TNF-α concentration in BAL fluid and ratio of percentage apoptotic neutrophils induced by incubation with BAL fluid over normal saline



could be associated with inability to elaborate a pro-apoptotic factor or factors in the lung.

I attempted to correlate TNF-α concentration with apoptotic activity in BALF. In general, the ability of cytokines and other inflammatory mediators to modulate neutrophil apoptosis is well recognised (Colotta 1992, Lee 1993) and many of these factors are present in BALF in both adults (Matute-Bello 1997) and neonates with RDS (Kotecha 1995, Kotecha 1996a, Kotecha 1996c). TNF-α concentration, which is known to be pro-apoptotic to human neutrophils at 5

hours (the time point used in my studies) (Murray 1997), was not significantly correlated with rates of apoptosis. The lack of positive association of TNF-α with neutrophil pro-apoptotic activity is not surprising, given the number of cytokines shown to be present in the inflamed lung. Identification of factors that correlate with outcome would, however, be of potential clinical use. Other potential factors that may modulate the neutrophil apoptotic process include FasL (Serrao 2001), and IL-2 (Lesur 2000) but we were unable to investigate these factors further due to the limited availability of BAL fluid.

To summarize, these studies have shown that persistent airway neutrophilia in non-survivors is associated with failure to produce a pro-apoptotic BALF. Conversely, resolution of lung inflammation in the survivors group is associated with a transient rise in pro-apoptotic activity in BALF early during recovery. These findings suggest that clinical outcome might be predictable from assessment of BALF early in the development of acute lung injury and also that strategies to induce neutrophil apoptosis might be associated with improved outcome.

Chapter 6. Conclusions

6.1 Repeatability of cellular constituents and cytokines concentration of lavage fluid obtained by non-bronchoscopic bronchoalveolar lavage of infants receiving extra-corporeal membrane oxygenation.

Infants with severe respiratory failure treated with ECMO, offer a unique opportunity to study the method of NB-BAL, as their respiratory function is maintained independently. By comparing mean cell counts and cytokine concentrations obtained from the left and right lung, I have shown that NB-BAL is a reliable tool for the study of lung disease in population studies of ventilated infants, particularly when cellular constituents are measured. On the other hand, the large variability associated with this method suggests that individual measurements should be interpreted with caution and that the clinical application of this method may be limited at present.

To my knowledge, the repeatability of NB-BAL has not been studied before in this way in human subjects but it has been studied in several animal models. Varner *et al* compared total and differential cell counts obtained segmental NB-BAL of one lung with whole lung lavage in rats (Varner 1999). In rats with healthy lungs, there was good agreement between the methods for mononuclear cells, neutrophils and oesinophils. In rats with lung inflammation (> 10% neutrophils), segmental BAL had significantly higher mononuclear percentage and lower neutrophil percentage than whole lung lavage. This may have been caused by a difference in the total number of mononuclear cells measured between the two methods of lavage. Alternatively, there may have been regional variation in the degree of lung inflammation. Also, the variability of the paired measurements was increased in rats with inflamed lungs

compared to rats with normal lungs. Three repeated segmental BALs over a four week period showed no effect on inflammation or airway resistance. Sweeney and workers studied cellular composition in BAL fluid obtained from the left and right lung in healthy horses (Sweeney 1992). They found no significant differences in total and differential cell populations apart from mast cells. McGorum *et al* compared cellular and non-cellular constituents in BAL fluid obtained from four different lobes in both healthy horses and horses with chronic obstructive pulmonary disease (McGorum 1993). They found no significant regional differences in total and differential cell counts, or BALF concentrations of albumin and urea in both healthy horses and horses with airway disease. These studies in animals support my findings in human infants, that NB-BAL is a valid tool for the study of cellular and non-cellular constituents in population studies.

Several factors appeared to be responsible in part for the observed variability between left and right sided results. For absolute counts of total white cells, neutrophils and macrophages a negative correlation was observed between difference between left and right results and age. This may well have been related to the fact that older children tended to have lower absolute cell counts compared to younger children. This observation is in agreement with studies in children without parenchymal lung disease undergoing bronchoscopic BAL (Midulla 1995).

For cytokine concentrations of IL-8 and TNF-α, the difference between right and left sided results and the volume of retrieved lavage fluid were inversely related . This finding suggests that correction for dilution may be necessary for non-cellular constituents of BAL fluid. However a "gold-standard" method to correct for dilution is currently lacking (see section 1.4). The approach I used of repeated NB-BAL in infants receiving ECMO, may be an

appropriate method to assess the validity of internal markers for dilution. In addition, my studies into the repeatability of cellular constituents and cytokines concentration of lavage fluid obtained by NB-BAL, need to be repeated using markers to estimate ELF volume such as urea and SC IgA, to determine if the observed variability decreases.

Other factors may have influenced the variability in cytokine concentrations I observed in my studies. First of all, the method of automated cytokine measurements uses relatively large volumes of BAL fluid. With only small volumes of BAL fluid available it was not possible to perform each measurement in triplicate. Secondly, although I did not observe an effect of underlying disease process on the variability, it cannot be excluded that there was such an effect due to the small numbers in the individual the subgroups (type II error). Finally, it is possible that in some subjects the disease process affected the lungs in a focal rather than a generalised manner.

In my studies comparing right and left sided cytokine concentrations in BAL fluid, I observed a trend towards higher right sided concentrations of IL-6, IL-8 and TNF-α. It is possible that this effect was caused by the fact that in normal bronchial anatomy the left main bronchus is longer than the right main bronchus. Therefore, on left sided catheter placement the tip may have been nearer to the central bronchi compared to right sided catheter placement. This may have resulted in a larger contribution of the bronchial compartment in the overall cytokine concentration in left sided samples.

Finally, during placement of the suction catheters at the beginning of the lavage procedures I was not able to confirm that the catheter reached the intended side on each occasion. In

practice this is difficult to do, because fibroptic bronchscopes small enough to perform adequate bronchoalveolar lavage in infants are currently not available. To check catheter position during each lavage procedure using radiography, would be associated with unacceptable radiation exposure. However, during my study, four lavage procedures in two patients were carried out at the time of routine chest X-ray examination. Twice the catheter was aimed at the right lower lobe and twice it was aimed at the left lower lobe. On each occasion the catheter was seen to lie in the intended lobe. Also, Placzek and Silverman studied catheter placement in NB-BAL (Placzek 1983). They showed that by turning the head to the left the suction catheter was passed into the right main bronchus in 20 out of 20 attempts. In a post-mortem study, Grigg *et al* (Grigg 1992a) confirmed that with the infant's head turned to the left the right lower lobe is lavaged. On the basis of these observations, I assumed that the catheter tip reached the intended side on most occasions.

6.2 Resolution of acute lung inflammation in infants with severe respiratory failure treated with extra-corporeal membrane oxygenation

My studies into the changes in BAL cell populations and pro- and anti-inflammatory cytokine concentrations in infants with severe respiratory failure showed two distinct patterns of change over time between survivors and non-survivors. Survival was associated with a transient, controlled inflammatory response. Both pro- and anti-inflammatory showed an initial transient rise with a significant decrease over time in the proportion of neutrophils in BAL fluid.

Failure to recover was associated with persistent neutrophilia and a rapid decline in the

cytokine response associated with an increased TNF-α / sTNFR ratio. These results suggest that infants with severe respiratory failure who go on to survive their illness, show a similar inflammatory response in the lungs compared to premature infants with IRDS and adults with ARDS who recover (see 1.1.2 and 1.1.3). The response in non-survivors is different from other types of patients with acute lung injury who have poor clinical outcome. Premature neonates, who go on to develop chronic lung disease have persistently elevated concentration of IL-6, IL-8 and ICAM-1 in lavage fluid samples infants (Groneck 1995, Kotecha 1995, Kotecha 1996a, Kotecha 1996c). Adults with ARDS who have persistent disease or poor outcome also show prolonged elevation of concentrations of pro-inflammatory cytokines and chemokines in BAL fluid (Meduri 1995, Goodman 1996).

The clinical implications of my observations are twofold. Firstly, BAL inflammatory markers such as neutrophil proportion and the TNF-α / sTNFR ratio may be developed into prognostic indicators for individual patients. This would require further study with larger, more homogenous patient populations. Secondly, further understanding of this apparent inability of non-survivors to mount an appropriate immune response, may lead to the possibility of therapeutic intervention. It could be speculated that the neutrophils of these patients are exhausted. Their ability to produce an appropriate response could be enhanced with immune modulating agents such as G-CSF and GM-CSF. However, the clinical use in individual patients of inflammatory markers in BAL may be limited by the variability I have observed in repeated measurements and further studies to reduce the variability of NB-BAL are required.

Although I observed differing trends between survivors and non-survivors in the change over time in cell populations and pro-and anti-inflammatory cytokines in BAL fluid, direct comparison of results between survivors and non-survivors on individual days showed very

few statistically significant differences. This may be explained by several factors. As I have shown in my studies as described in chapter 3, the method of NB-BAL is associated with significant variability. This may have been compounded by the fact that the number of non-survivors was relatively small. Also, the two groups were heterogeneous in terms of underlying diagnosis, with more infants suffering from infections amongst the non-survivors. Although survivors had a higher oxygenation index on presentation, these patients were significantly younger and a higher proportion of these infants may have had reversible PPHN compared to non-survivors, improving their chances of recovery. Finally, in the group of non-survivors more infants received VA – ECMO. On the one hand this may have been related to technical difficulties with establishing VV-ECMO. On the other hand, it is possible that these infants also had significant haemodynamic instability and multi-organ failure, increasing their mortality risk.

6.3 The role of neutrophil apoptosis in resolution of acute lung injury in infants with severe respiratory failure

In this part of my studies, an *in vitro* assay was used to study the ability of BAL fluid from study patients and controls to induce apoptosis in neutrophils from healthy volunteers. I found that BAL fluid from survivors showed a strong pro-apoptotic signal early during recovery compared to non-survivors, as part of a controlled transient inflammatory response. These results support the observations made in chapter 4, of a decreasing proportion of neutrophils in survivors over time following institution of ECMO support in response to the pro-apoptic signal. Conversely, non-survivors showed persistence of neutrophils as a proportion of total cells in BALF, which is likely to be related to the lack of pro-apoptoic signal in BALF.

Given the strength of the pro-apoptotic signal, further study is required to see if apoptotic activity can serve as a prognostic marker. Also, studies into the potential modulation of the apoptotic response as a therapeutic intervention are required. I did not assess the repeatability of the apoptotic activity measured by NB-BAL and it would be important to carry out such studies.

Morphology on high power light microscopy was used to assess apoptotic activity of BAL fluid. This method has been shown to correlate closely with other measurements of neutrophil apoptosis, including Annexin V binding (Homburg 1995) and shedding of CD16 (Dransfield 1994). However, we did not use a second method to confirm our findings of increased pro-apototic activity of BAL early during recovery in survivors, due to lack of sufficient quantities of BAL fluid. The cytospins made to estimate differential cell counts for my studies as described in chapter 3 and 4, were re-examined to count the percentage of apoptotic neutrophils and to compare results between survivors and non-survivors. The mean percentages of apoptotic neutrophils in BALF ranged between 1 and 3 %, and there was no significant difference between the two groups. On the one hand, this may in part have been due to the fact a significant number of cytospin samples was unsuitable for detailed morphological assessment with high power light microscopy. On the other hand, Matute-Bello and workers (Matute-Bello 1997) found very similar percentages of apoptotic neutrophils in BALF of patients with ARDS and observed no significant difference between survivors and patients who died.

In this part of my studies no significant positive correlation was found between pro-apoptotic activity and TNF-α concentration in BAL fluid, a known pro-apoptotic cytokine. However, the effect of TNF-α on neutrophil apoptosis may be dependent on neutrophil age and state of

activation. Murray *et al* studied the pro-apoptotic effect of TNF-α *in vitro* (Murray 1997). These authors found that there was an early pro-apoptotic effect on cultured human neutrophils incubated with TNF-α. In contrast, delayed apoptosis was observed when neutrophils were allowed to age or were activated by PAF, prior to incubation with TNF-α. Colotta and workers also found that TNF-α delayed apoptosis in cultured neutrophils in a dose dependent fashion (Colotta 1992).

In addition, the lack of positive correlation between BALF TNF-α concentration and apoptotic activity may be related to additional factors, as the overall apoptotic signal in BALF is likely to be determined by the balance between a range of pro-apoptotic factors and anti-apoptotic factors. In particular, the following factors have been found to promote neutrophil apoptosis: IL-10 and soluble Fas ligand, which is secreted by activated neutrophils (Cox 1996, Keel 1997, Serrao 2001). Factors that have been shown to delay neutrophil apoptosis include GCSF, GM-CSF, IL-1β, IL-2 and bacterial products such as LPS and exotoxins (Colotta 1992, Matute-Bello 1997, Lesur 2000, Matute-Bello 2000, Usher 2002). In order to asses the role of these factors in the resolution of acute lung inflammation in infants with severe respiratory failure, it would be useful to repeat my studies and correlate concentrations of these factors with apoptotic activity of BALF. Any significant positive or negative correlation could then be further explored in the *in vitro* assays with cultured neutrophils as I have described, using blocking antibodies against specific pro- or anti-apoptotic factors, to establish causality.

6.4 Limitations of studies

The population I studied was heterogeneous in terms of post-gestational age and underlying diagnosis. It is possible that this heterogeneity contributed to the clinical outcome, in addition to the underlying lung injury and inflammation.

I elected to discard visibly blood stained BAL samples because of concern of contamination by white blood cells and cytokines derived from the bloodstream.
Where possible I repeated sampling later the same day, but often repeat BAL samples were also blood stained. Presumeably, this was due to a combination of severe lung injury and also systemic heparinisation required to avoid clotting of blood in the ECMO circuit. This resulted in a reduced number of overall samples and may have contributed absence of statistically significant differences in inflammatory cell counts and cytokine concentrations between the study groups.

Although previous studies have shown that standardised blind placement of the suction catheter nearly always resulted in placement of the catheter in the expected lung segment, (Placzek 1983) I did not verify this systematically in my studies. It is possible that during the BAL procedures I performed, on occasions the suction catheter ended up in a different part of the lungs than intended. On the other hand, one may expect that in infants with this degree of severe lung injury there would be uniform inflammation throughout the lungs.

Blood sampling for cytokine measurement in patients on ECMO was done from the venous side of the ECMO circuit and consisted therefore of central venous blood. On the other hand, once patients were taken off ECMO but still ventilated arterial blood was used for cytokine measurement. The cytokine concentrations between venous and arterial blood samples could have differed, as capillary beds in the body and in the lung may differ in terms of production of inflammatory markers. I did not specifically study this.

The quantity of lavage fluid retrieved from infants is relatively small. This severely limits the

number of assays that can be performed and it was often not possible to repeat assays in triplicate. This may affect the reliability of some of the results and may have contributed to the observed variability. It also limits the ability to perform further confirmatory studies on samples to test hypotheses that initial results may have raised.

In my studies into the role of cytokine concentrations in BALF in the resolution of acute lung inflammation, I measured the immunological activity of the cytokines under study. Although specific trends were observed the differences were not statistically significant when directly compared between the two groups of survivors and non-survivors. It is possible that measuring the biological activity of these cytokines in *in vitro* assays could have been more informative. Pugin showed that in patients with ARDS, there may be significant differences in the immunologic and biologic activity of pro-inflammatory cytokines TNF-α and Il-1β in BALF (Pugin 1996). Also, given the variability I observed in both cellular and non-cellular constituents of BALF, the numbers of study patients may have been too small to detect significant differences between survivors and non-survivors, due to type II error. No previous studies using these methods in this population were available to me to estimate the required sample size, but my data can form the basis for future power calculations.

Finally, as I described in section 1.2, it is customary to reduce ventilator settings to "rest settings" following institution of ECMO. In the population I studied of infants with severe respiratory failure, a degree of lung atelectasis is likely to occur once rest settings are introduced as lung compliance is likely to be poor in the first few days following institution of ECMO. I did not study the effect of reduced lung volume on the results of my studies.

6.5 Future directions

In order to improve repeatability of measurement of cellular and non-cellular constituents of BAL fluid, I propose further clinical study in the following areas:

- Methods to correct for dilution particularly concentrating on internal markers for dilution such as urea and SC Ig A
- The use of balloon tipped catheters which may allow better sampling of the distal lung and avoid significant bronchial contamination
- To study the effect of age on the concentration of inflammatory markers in BALF in larger subgroups of differing ages
- To link concentrations of inflammatory markers of left and right sided BAL to measures of lung injury such as chest radiograph appearance, to determine whether differences in left and right sided results are related to differential lung injury
- To develop assays that can accurately measure concentrations of non-cellular constituents in BALF using small volumes, making repeated measurements possible

Understanding of the resolution of acute lung inflammation may be furthered by future study in the following areas:

- In my studies, infants with infection were more likely to die. One could hypothesize that an inability to mount an acute inflammatory response following viral infection of the lower respiratory tract, may be associated with worse outcome. Domachowske and workers studied this hypothesis in a model of mice deficient of the pro-inflammatory chemokine MIP-1α or its receptor CCR-1, infected with pneumonia virus of mice (Domachowske 2000). Fully immune competent mice showed a marked increase in BALF neutrophils, between day 3 and 6 after infection. Both MIP-1α and CCR-1 negative mice showed marked attenuation of this inflammatory response and CCR-1

deficient mice succumbed significantly earlier to PVM infection compared to immune competent mice. These data suggest that a MIP-1α /CCR-1-mediated acute inflammatory response is important in controlling viral replication and that this response prolongs survival in mice following PVM infection. Therefore further clinical studies with sufficient numbers may help identify whether there are similar differences in the pattern of change in inflammatory markers in BALF in the different diagnostic subgroups of infants with severe respiratory failure. Such differences may shed light on underlying mechanisms and may lead to therapeutic interventions to modulate acute lung inflammation.

- Further clinical studies may also reveal whether the ratio of TNF-α over its inhibitors sTNFR-1 and -2 in BALF and the pro-apoptotic activity of BALF, can serve as prognostic indicators
- Development of representative animal models of severe respiratory failure in infants with the use of ECMO support, can offer opportunities to further elucidate the role of individual inflammatory markers. These models can also be utilized to study therapeutic interventions aimed at modulating the inflammatory response in the lung

7. Publications and presentations stemming from this thesis

Publications

1. **Mildner RJ**, Killer HM, Taub NA, Field DJ, Kotecha S. Repeatability of cellular constituents and cytokines concentration of lavage fluid obtained by non-bronchoscopic broncho-alveolar lavage of infants receiving extra-corporeal oxygenation. Published in Thorax 2001; 56(12): 924-931 (see appendix 3).

2. **Mildner RJ**, Taub N, Vyas JR, Killer HM, Firmin RK, Field DJ, Kotecha S. Cytokine imbalance in infants receiving extra-corporeal membrane oxygenation for respiratory failure. Submitted for publication to the European Journal of Paediatrics.

3. Kotecha S, **Mildner RJ**, Usher L, Vyas JR, Currie AE, Lawson RA, Whyte MKB. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Published in Thorax 2003; 58(11): 961-7 (see appendix 4). Figure 4C in this publication differs in several respects with the corresponding figure 5.1 in my thesis. First of all, in the publication results are expressed as mean values with standard errors of the mean, whilst in my thesis results are expressed as medians and interquartile ranges. This was changed to maintain uniformity of expressing results in the paper with the other groups described with RDS and CLD. Also, there is no data point for survivors on day 4. This is because the mean value on day 4 in survivors was markedly outlying at 13.5% compared to a median value of 3.2 %. This marked discrepancy was caused by two survivors who had very high pro-apoptotic activity. Despite this, the mean activity was not statistically different compared to non-survivors. It was therefore decided to omit this data point altogether. Thirdly, the post ECMO data points in survivors were omitted from the graph in the paper as they did not add to the overall message.

Presentations

1. **Mildner RJ**, Killer HM, Taub NA, Field DJ, Kotecha S. Repeatability of cytokine concentrations of lavage fluid obtained by non-bronchoscopic broncho-alveolar lavage of infants receiving extra-corporeal oxygenation.
Poster presentation at American Thoracic Society Annual Congress, Toronto 2000.
Published in the American Journal of Respiratory and Critical Care Medicine 2000; 61(3):A 569.

2. **Mildner RJ**, Lawson RA, Usher L, Whyte M. The role of neutrophil apoptosis in the resolution of acute lung inflammation in infants receiving ECMO.
Oral presentation at European Respiratory Society Annual Congress, Madrid 1999.
Published in European Respiratory Journal 1999;16(suppl): 15s.

3. **Mildner RJ**, Taub NA, Killer HM, Firmin RK, Field DJ, Kotecha S. Resolution of acute lung inflammation in infants treated with ECMO.
Poster presentation at American Thoracic Society Annual Congress, San Diego 1999.
Published in American Journal of Respiratory and Critical Care Medicine 1999; 159(3): A153. Oral presentation at the Annual Spring Conference of the Royal College of Paediatrics and Child Health, York 1999. Published in Archives of Diseases in Children 1999; 80(Suppl 1): G2.

4. **Mildner RJ**, Killer HM, Taub NA, Field DJ, Kotecha S. Repeatability of non-bronchscopic bronchoalveolar lavage in infants receiving ECMO.
Oral presentation at European Respiratory Society Annual Congress, Geneva 1998.
Published in European Respiratory Journal 1998; 12(suppl 28): 7s.

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08 August 1995

Mr R K Firmin
Consultant Cardiothoracic Surgeon
Glenfield Hospital

Dear Mr Firmin

Cytokine response to extra corporeal membrane oxygenation in serum and bronchoalveolar lavage - our ref. no. 3903

Further to your application dated 24th July, you will be pleased to know that the Leicestershire Ethics Committee at its meeting held on the 4th August, 1995 approved your request to undertake the above-mentioned research subject to slight modifications to the information sheet to state "Patients requiring ECMO will have inflamed lungs. ECMO makes this worse in the first two days."

I would remind you, however, that your research project has been given approval only in relation to its acceptability from an ethical point of view. If, subsequently, departure from the methodology outlined in your protocol is contemplated, the Ethics Committee must be advised in order that the proposed changes may be approved. Also a report should be made to the Ethics Committee if any significant adverse reactions are noted during the course of the study. In addition, any NHS resource implications of your project must be discussed with the appropriate Trust Chief Executive. Similarly, it may be that the research project has implications for other disciplines and, if so, you are advised to discuss them with the appropriate departmental manager. Researchers should also be able to assure the Ethics Committee that satisfactory arrangements have been made for the labelling, safe storage and dispensation of drugs and pharmaceutical staff are always willing to provide advice on this.

Researchers' attention is also drawn to correspondence from the Regional Director of Public Health dated 28 January, 1991 relating to Clinical Trials which sets out revision of the procedures to be followed, and the Clinical Trials Indemnity Letter and Deed of Guarantee. Researchers should ensure that these indemnity arrangements have been complied with.

Researchers intending to study selective groups of patients in the community are reminded that their first approach should be to the individual patient's general practitioner to ascertain whether the particular patient was suitable for inclusion in the study. Equally, when the researcher contacts the patient it should be emphasised that the approach is made with the knowledge of the General Practitioner, with whom the patient may discuss this research, if the patient so wished.

Yours sincerely

M. Sursham
for Director of Public Health

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Appendix 2

INFORMATION SHEET FOR PARENTS

You are being asked to participate in a research project. The statement below explains what will happen if you agree to take part. It describes any risks or discomforts your baby may experience. Also it explains what we hope to learn as a result of you taking part.

You should not take part if you do not wish to do so.
If you decide not to take part, the treatment of your baby will not be affected by your decision.

Background and aim of study
Glenfield Hospital is a centre specialized in treatment of babies with severe breathing difficulties. This treatment is called ECMO (Extra Corporeal Membrane Oxygenation).

Children who need ECMO have inflamed lungs. Treatment with ECMO allows the lungs to rest and recover from this. We want to study the improvement of the lungs while on ECMO.

To know what our results mean, we need to compare the findings in children on ECMO with children who have healthy lungs. Children undergoing routine cardiac catheterisation are good candidates for this.

Every patient on a breathing machine undergoes regular cleaning of the tubes in the lungs to prevent them from blocking with phlegm. This is done by inserting a small volume of saline (salt water) into the breathing tubes in the lungs. This is immediately sucked back again using a thin plastic tube.

We wish to obtain lung fluid by a slight modification to this suctioning. With the baby lying on his/her back, we insert a volume of saline calculated on the baby's weight. A thinner tube will be used, which will reach further into the lung. The fluid obtained will then be examined in the laboratory.
Your baby will not have any additional procedure and the fluid will only be obtained at the time of regular suctioning.

If you feel able to allow us to use the lung fluid for our tests please let us know.
Your baby's treatment will not be affected in any way if you feel unable to participate in this study. Routine suctioning of the breathing tubes will continue as needed.

Please feel free to ask any questions you may have.

Dr Reinout Mildner
Registrar in Paediatrics

Dr Sailesh Kotecha
Senior Registrar in Paediatrics

Thorax 2001;56:924–931

Repeatability of cellular constituents and cytokine concentration in fluid obtained by non-bronchoscopic bronchoalveolar lavage of infants receiving extracorporeal oxygenation

R J Mildner, N A Taub, J R Vyas, H M Killer, R K Firmin, D J Field, S Kotecha

Abstract

***Background*—Since few studies have assessed the repeatability of non-bronchoscopic bronchoalveolar lavage (NB-BAL), we compared cellular counts and cytokine concentrations in fluid obtained by standardised NB-BAL from each side of 20 intubated infants receiving extracorporeal membrane oxygenation (ECMO).**

***Methods*—Total cell counts were obtained from 95 paired lavages and 77 pairs were suitable for differential counts and measurement of cytokine concentrations.**

***Results*—Moderate correlation was noted between the two sides for most cell types including total cell counts and percentages of neutrophils and macrophages (R=0.70–0.84) and for cytokine concentrations (IL-8 R=0.78, IL-6 R=0.75, TNF-α R=0.64, all $p \leq 0.001$). Using Bland-Altman analysis the mean difference between the two sides approached zero for cellular constituents (total cell counts mean difference 1.7, limits of agreement −187.5 to +190.9 × 10^4/ml; percentage neutrophils −3.9%, −41.5% to +33.6%; percentage macrophages 3.9%, −33.8% to +41.6%) but tended to be greater on the right for logarithmically transformed cytokine measurements (IL-8: left/right ratio 0.74, limits of agreement 0.12 to 5.45, IL-6: 0.93, 0.09 to 5.87, and TNF-α: 0.93, 0.27 to 3.16). Using linear regression with random effects to assess the variability, only the infant's age appeared to influence the cellular results but, for cytokines, only the volume retrieved affected the variability. The magnitude of the measurements, the underlying disease, the operator's experience, days on ECMO, or survival did not affect the variability.**

***Conclusion*—Measurements obtained by NB-BAL need to be interpreted with caution and strongly suggest that normalisation for the dilutional effects of saline is essential.**

(*Thorax* 2001;56:924–931)

Keywords: non-bronchoscopic bronchoalveolar lavage; bronchoalveolar lavage fluid; extracorporeal membrane oxygenation

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Although the newer neonatal bronchoscopes have a suction channel, it is often inadequate to permit satisfactory bronchoalveolar lavage (BAL) of infants, especially neonates. Non-bronchoscopic lavage (NB-BAL) has therefore been frequently used in intubated infants to study underlying disease processes.[1–4] NB-BAL is a simple modification of routine endotracheal suction of infants receiving mechanical ventilation. It has been used to study the bronchoalveolar milieu in intubated neonates and infants, particularly in the study of the neonatal respiratory distress syndrome and chronic lung disease of prematurity.[5–9]

NB-BAL in infants, however, has so far lacked any standardisation and there is significant variation in the lavage procedure in reported studies.[2] There are marked differences in choice of catheter size, site of sampling, volume of normal saline instilled, and processing of samples after retrieval. For instance, some investigators prefer "true" bronchoalveolar lavage with the suction catheter wedged in the right lower lobe,[5–7 10] while others have used tracheal aspirates.[11 12] Some investigators instil a fixed volume of normal saline[13 14] while others use a volume based on body weight.[5–7 10] The repeatability for each method and each individual variable has been poorly studied.[2]

NB-BAL has been used in infants receiving mechanical ventilation for underlying respiratory disease. Both cellular and non-cellular constituents (especially cytokines) have been studied in the returned lavage fluid. Indeed, with this technique it is now generally accepted that pulmonary inflammation is an important risk factor for the development of chronic lung disease of prematurity.[3 6 7] The recent report by the Task Force on Paediatric BAL organised by the European Respiratory Society suggested standardisation of NB-BAL of ventilated newborn infants.[2] An area of interest to both adults and children is the repeatability and reproducibility of the results obtained using BAL.

In order to assess the repeatability of this method lavages of multiple sites would be required, which is clearly not possible in sick infants requiring mechanical ventilation for severe respiratory failure as the underlying disease may be exacerbated. Extracorporeal membrane oxygenation (ECMO) is used to support infants with severe reversible respiratory failure who have failed on conventional management.[15] Common conditions leading to severe respiratory failure in this age group include meconium aspiration syndrome, persistent pulmonary hypertension of the newborn, neonatal respiratory distress syndrome (RDS), infection, and congenital diaphragmatic hernia. During ECMO gas exchange occurs independently of the lungs. Infants receiving ECMO remain intubated and are ventilated mechanically with low airway pressures to prevent complete airway collapse and to permit recovery of the acute lung injury. Since gaseous

Table 1 Diagnostic groups of survivors and non-survivors

Disease process	*Survivors (n=14)*	*Non-survivors (n=6)*
Meconium aspiration syndrome	6	0
Pneumonia	1	3
Persistent hypertension of the newborn	1	2
Sepsis	3	0
Congenital diaphragmatic hernia	2	1
Respiratory distress syndrome	1	0

exchange is independent of the lungs, ECMO provides a unique opportunity to perform NB-BAL of the right and left sides without compromising the infant. In this study we assessed the repeatability of NB-BAL by comparing cellular and non-cellular constituents in BAL fluid obtained by NB-BAL from each side in infants receiving ECMO for severe reversible respiratory failure.

Methods

PATIENTS

The parents of all infants less than 15 months of age with primary respiratory failure admitted to the Heartlink ECMO Centre, Glenfield Hospital, Leicester between October 1996 and January 1998 were invited to join the study. Those who required ECMO primarily for cardiac support after surgery for congenital heart disease were excluded. As is routine with ECMO, the oxygenation index (mean airway pressure × fraction inspired oxygen/arterial oxygen tension) on referral was used as a marker of severity of respiratory failure. The study was approved by the local ethics committee and informed consent was obtained from the parents to perform NB-BAL on the infants.

PROCEDURE

A single operator (RJM) carried out all lavage procedures and the subsequent sample analysis blindly. The infants underwent standardised NB-BAL of the lower lobes of both lungs as soon as possible after cannulation for ECMO. Thereafter, bilateral NB-BAL was carried out once daily at the time of routine endotracheal suction until decannulation from ECMO. The lavage procedure was deferred if clinically contraindicated due to cardiorespiratory instability, pulmonary haemorrhage, or temporary interruption of extracorporeal support. Standardised NB-BAL was performed according to the ERS Task Force guidelines and as previously described.[2 5-7] Briefly, with the infant positioned supine and the head turned to the left, a size 6–8 Fr straight suction catheter with two side holes was gently passed through the end porthole of the ventilatory circuit until it was wedged in the right lower lobe. An aliquot of 1 ml/kg bodyweight of normal saline at room temperature was instilled. This was retrieved after 2–3 ventilator breaths into a suction trap using a suction pressure of 5–15 kPa. Immediately thereafter a second aliquot of 1 ml/kg bodyweight of normal saline was instilled and sucked back. The two retrieved samples were pooled. The procedure was then repeated with the head turned to the right to lavage the left lower lobe. The BAL fluid samples from each side were processed separately. Macroscopically bloodstained samples were discarded. The most commonly encountered complication was transient bradycardia resulting from a vasovagal reaction to insertion of the suction catheter.

PROCESSING OF BAL FLUID SAMPLES

After the lavage procedure the volume of the recovered BAL fluid was recorded and the sample centrifuged within 10 minutes of collection at 1800 rpm for 10 minutes at room temperature. The supernatant was removed

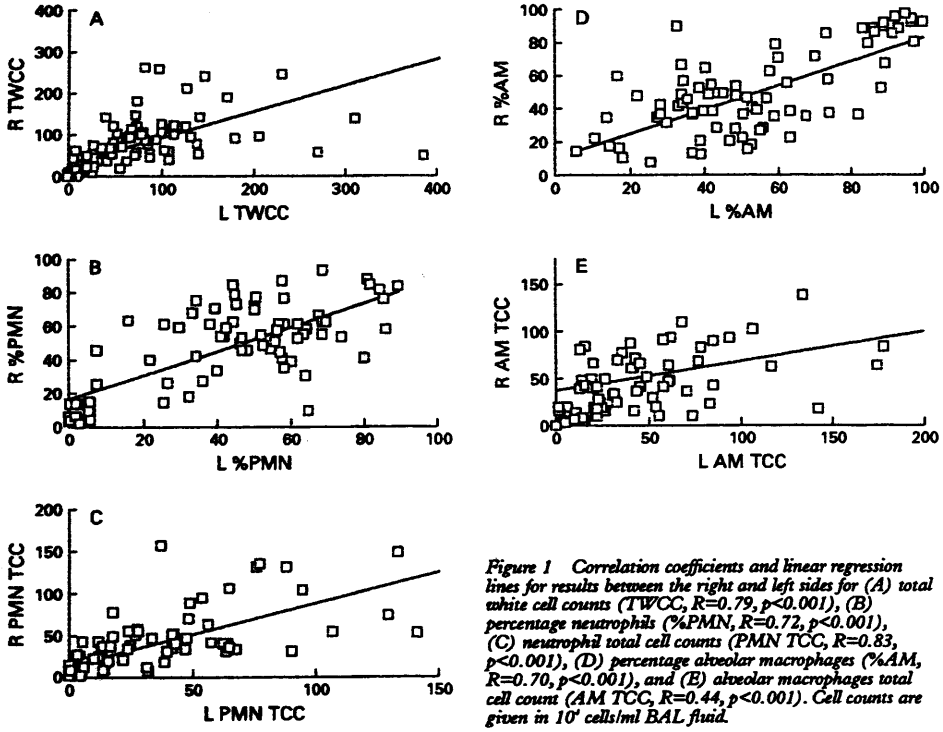

Figure 1 Correlation coefficients and linear regression lines for results between the right and left sides for (A) total white cell counts (TWCC, R=0.79, p<0.001), (B) percentage neutrophils (%PMN, R=0.72, p<0.001), (C) neutrophil total cell counts (PMN TCC, R=0.83, p<0.001), (D) percentage alveolar macrophages (%AM, R=0.70, p<0.001), and (E) alveolar macrophages total cell count (AM TCC, R=0.44, p<0.001). Cell counts are given in 10⁹ cells/ml BAL fluid.

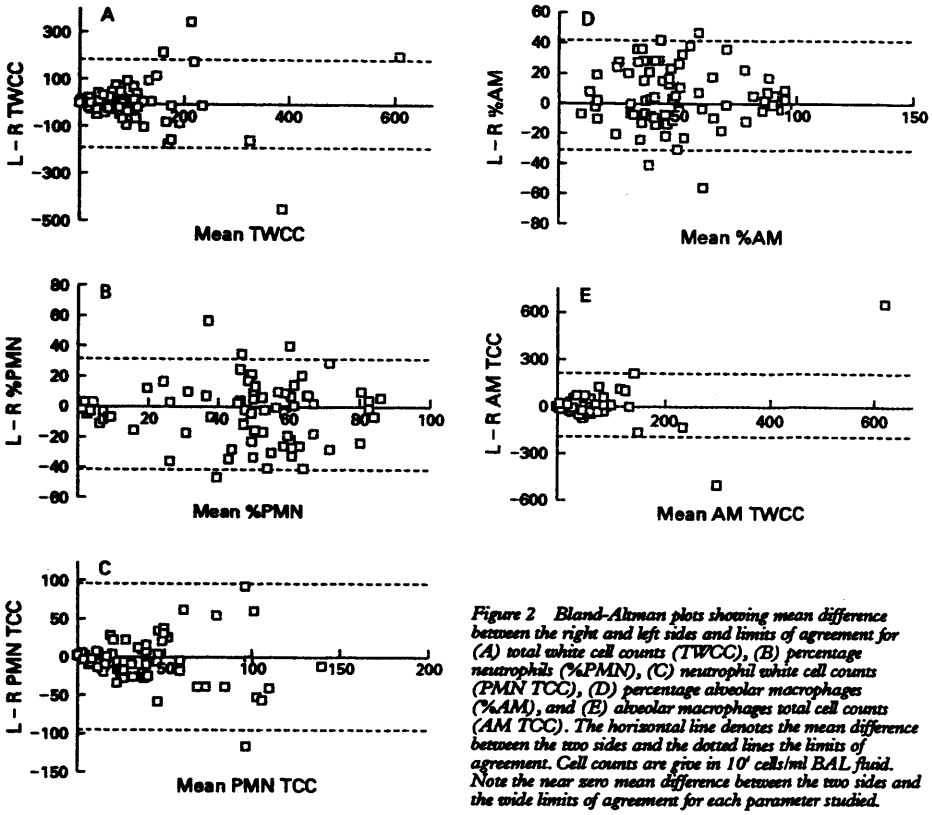

Figure 2 Bland-Altman plots showing mean difference between the right and left sides and limits of agreement for (A) total white cell counts (TWCC), (B) percentage neutrophils (%PMN), (C) neutrophil white cell counts (PMN TCC), (D) percentage alveolar macrophages (%AM), and (E) alveolar macrophages total cell counts (AM TCC). The horizontal line denotes the mean difference between the two sides and the dotted lines the limits of agreement. Cell counts are give in 10^7 cells/ml BAL fluid. Note the near zero mean difference between the two sides and the wide limits of agreement for each parameter studied.

and stored at −70°C until further analysis. The cell pellet was resuspended in a known volume of saline. The total cell count was obtained with a haemocytometer, and the differential cell count was obtained by staining a cell cytospin with Diff-Quick stain (Merck & Dade AG, Duedingen, Switzerland) and counting at least 300 cells per slide. The cell counts were performed by RJM after randomly relabelling the slides to ensure blinding. The total cell counts for the various cell types were obtained from the total cell count and differential counts and expressed as cell number/ml BAL fluid.

CYTOKINE MEASUREMENTS

Concentrations of the cytokines interleukin (IL)-8, IL-6 and tumour necrosis factor TNF(α) in the BAL fluid supernatant were measured by an automated ELISA analyser (DPC Europe Ltd, UK) according to the manufacturer's instructions by an independent researcher (JRV). The lowest sensitivity of the analyser was 2 pg/ml for IL-8, 1 pg/ml for IL-6, and 1.7 pg/ml for TNFα. In accordance with ERS Task Force guidelines[2] we did not attempt to correct for dilution and concentrations were expressed as pg cytokine/ml BAL fluid.

STATISTICAL ANALYSIS

The absolute and differential cell counts and cytokine concentrations for the left and right sides were firstly compared by linear regression, calculating the Pearson correlation coefficient (R value), p value, and 95% confidence interval. Statistical significance was assumed at $p<0.05$. Secondly, the Bland-Altman method[16] was used. This consisted of plotting the difference between each pair of measurements against their mean value, then using the mean and standard deviation of the differences to calculate the 95% limits of agreement—that is, the range in which the difference may be expected to lie in 95% of the

Table 2 Total white cell counts (TWCC), percentage and total cell counts (TCC) for neutrophils (PMN), alveolar macrophages (AM), lymphocytes, and epithelial cells

		Left (mean)	Right (mean)	R (p value)	Mean difference (L–R) (95% CI)	LLA (95% CI)	ULA (95% CI)
TWCC		92.5	90.8	0.79 (<0.001)	1.7 (−17.9 to 21.4)	−187.5 (−221.5 to −153.4)	190.9 (156.8 to 224.9)
PMN	%	42.8	46.7	0.72 (<0.001)	−3.9 (−8.3 to 0.4)	−41.5 (−49.0 to −33.9)	33.6 (26.1 to 41.2)
	TCC	49.0	50.4	0.83 (<0.001)	−1.4 (−12.3 to 9.6)	−95.9 (−114.9 to −77.0)	93.2 (74.2 to 112.1)
AM	%	53.3	49.4	0.70 (<0.001)	3.9 (−0.5 to 8.3)	−33.8 (−41.4 to −26.3)	41.6 (34.0 to 49.2)
	TCC	60.4	56.4	0.44 (<0.001)	4.0 (−19.9 to 27.9)	−202.7 (−244.2 to −161.3)	210.7 (169.3 to 252.2)
Lymphocytes	%	4.0	3.7	0.69 (<0.001)	0.3 (−0.2 to 0.8)	−3.9 (−4.7 to −3.0)	4.6 (3.7 to 5.4)
	TCC	3.3	3.8	0.56 (<0.001)	−0.5 (−1.4 to 0.4)	−8.4 (−10.0 to −6.8)	7.4 (5.8 to 9.0)
Epithelial	%	9.7	10.5	0.84 (<0.001)	−0.8 (−3.5 to 1.9)	−23.8 (−28.4 to −19.2)	22.2 (17.6 to 26.8)
	TCC	7.4	10.3	0.54 (<0.001)	−2.9 (−8.0 to 2.2)	−46.7 (−55.5 to −37.9)	40.9 (32.1 to 49.6)

Means for the right and left sides are given together with the Pearson's correlation coefficient and p value of correlation. The mean difference including 95% confidence intervals (CI) are shown for each cell type together with the upper (ULA) and lower (LLA) limits of agreement obtained by the method described by Bland and Altman.[25] Total cell counts were obtained from the total white cell count and differential count for each cell type and are expressed as 10^7 cells/ml BAL fluid.

Table 3 Comparison of concentration of cytokines in BAL fluid obtained from left and right sides

	Correlation		Bland-Altman analysis		
	R	*p* value	Difference (95% CI)	LLA (95% CI)	ULA (95% CI)
IL-8	0.66	p<0.001	0.04 (−1.34 to 1.43)	−11.9 (−9.6 to −14.3)	12.0 (9.6 to 14.4)
IL-6	0.42	p<0.001	−0.25 (−0.77 to 0.27)	−4.7 (−3.8 to −5.6)	4.15 (3.3 to 5.1)
TNFα	0.39	p<0.001	0.04 (−0.06 to 0.15)	−0.84 (−0.66 to −1.0)	0.92 (0.74 to 1.1)

Concentrations of cytokines were analysed using Pearson's coefficient (*R*) and by Bland-Altman plots of agreement. The mean difference in cytokine concentration between the right and left sides together with the lower (LLA) and upper (ULA) limits of agreement with 95% confidence intervals are given.

samples; 95% confidence intervals were calculated to indicate the precision of the limits of agreement.

Since the Bland-Altman plots of the absolute cell counts and cytokine concentrations demonstrated dependence of left-right variability on the overall magnitude (mean of left and right)—seen as "funnelling" on the graphs—the corresponding limits of agreement were calculated using a logarithmic scale and presented as the ratio between left and right. (Logarithmic transformation was not performed for percentage of cells since funnelling was not observed.)

It was hypothesised that the variability might be due to (a) the age of the child, (b) the underlying disease, (c) the experience of the operator (RJM), (d) time spent on ECMO, (e) volume of BAL fluid retrieved (volume retrieved from left side/volume retrieved from right side), or (f) survival. Linear regression with random effects due to the multiplicity of measurements on each child was used to examine the hypothesis separately for the various cellular and non-cellular constituents. Analysis was performed using a statistical package for microcomputers (SPSS Base 9.0, SPSS Inc, Chicago, IL, USA; STATA Statistical software 6.0, Stata Corp, College Station, TX, USA).

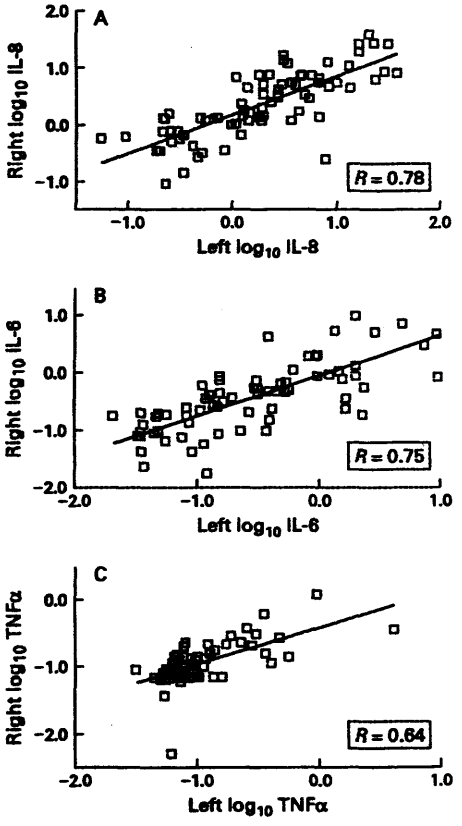

Figure 3 Correlation between the values for (A) IL-8, (B) IL-6, and (C) TNFα concentrations for BAL fluid samples from the right and left sides. Pearson's correlation coefficient is given for each graph. P values for all graphs are <0.001.

Results

PATIENT CHARACTERISTICS

Twenty infants (14 male) with a median age of 2 days (range 1–402) and weight of 3.3 kg (2.0–10.0 kg) were recruited. Almost all infants were neonates with only three infants being older than 30 days of age. The median oxygenation index was 46 (range 19.6–97.1). One patient had an oxygenation index of 19.6 but, because of multiple organ involvement, he was placed on veno-arterial ECMO to support the circulation as well as the respiratory system. The diagnostic groups are shown in table 1. Median time on ECMO was 143 hours (range 71–406). Eleven patients received veno-venous ECMO and nine were placed on veno-arterial ECMO. Fourteen survived and there were six deaths (table 1).

COMPARISON OF BAL FLUID CELL COUNTS ON LEFT AND RIGHT SIDES

Ninety five paired NB-BAL samples were obtained. Total white cell counts were obtained from all samples but only 77 pairs were suitable for differential cell counts. The predominant cell types were neutrophils and alveolar macrophages. Few lymphocytes, eosinophils, and epithelial cells were seen. Detailed data are therefore given in graphical form for neutrophils and alveolar macrophages only (figs 1 and 2) and the results for all cell types are summarised in table 2.

There was a strong positive correlation between the two sides for total white cell counts, percentage neutrophils, absolute number of neutrophils, percentage macrophages, and percentage epithelial cells with *R* values ranging from 0.70 to 0.84 (all p<0.001, fig 1, table 2). For absolute cell counts of macrophages, lymphocytes, and epithelial cells and for percentage lymphocytes the correlation between the two sides was weaker with *R* values of 0.44–0.56 (all p<0.001, fig 1, table 2).

When the results were compared by using the more appropriate Bland-Altman analysis the mean difference between the right and left sides approached zero for all cell types (table 2, fig 2A–E). In addition, the 95% confidence intervals always included zero. However, the limits of agreement for all cell types studied were wide ranging from −187.5 to +190.9 × 10^4/ml BAL fluid for total white cell counts, −41.5% to +33.6% for percentage neutrophils, and −33.8% to +41.6% for percentage alveolar macrophages (table 2).

When the data were logarithmically transformed—that is, where "funnelling" was seen—the corresponding limits of agreement (now shown as proportions due to transformation) were 0.15 (95% CI 0.11 to 0.21) to 5.80 (95% CI 4.19 to 8.05) for total cell counts,

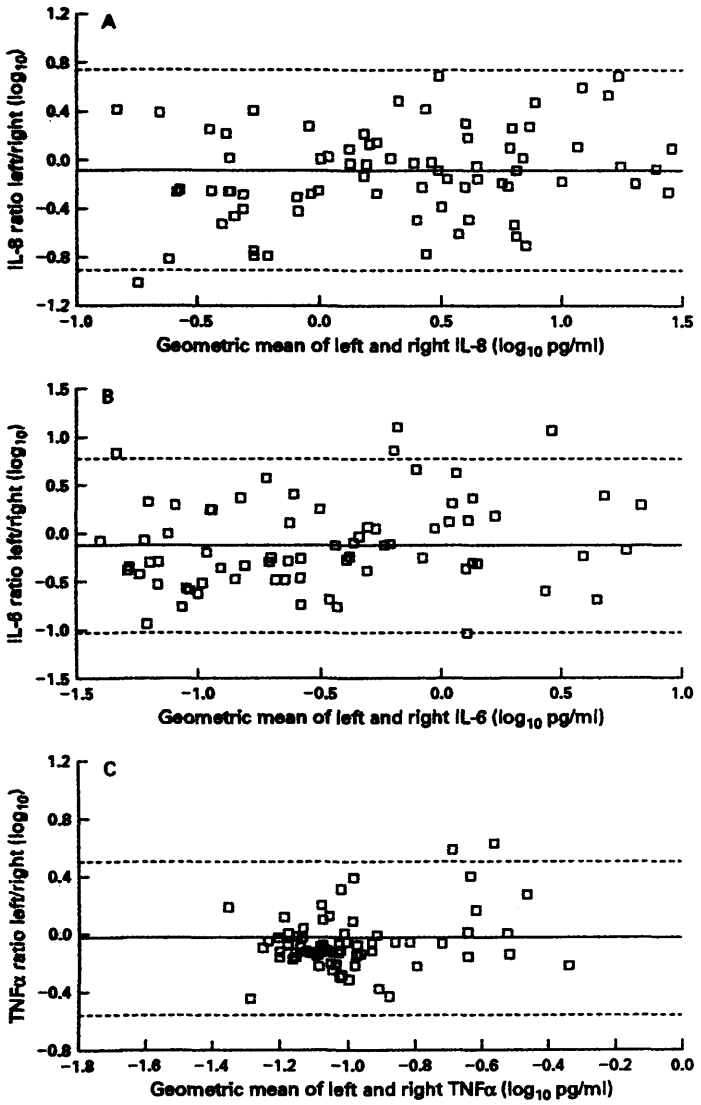

Figure 4 Bland-Altman plots for the logarithmic transformed data for (A) IL-8, (B) IL-6, and (C) TNFα. Note that the mean difference between the two sides includes one for IL-8 and TNFα but not IL-6. Also note the wide upper and lower limits of agreement for all three cytokines studied.

0.13 (95% CI 0.09 to 0.18) to 4.65 (95% CI 3.24 to 6.67) for total neutrophils, and 0.17 (95% CI 0.12 to 0.24) to 6.62 (95% CI 4.58 to 9.58) for total alveolar macrophages.

COMPARISON OF CYTOKINE CONCENTRATIONS

The concentration of IL-8 was estimated in 76 paired BAL samples, IL-6 in 74 pairs, and TNFα in 73 pairs due to the limited volume obtained for some samples. The mean concentrations were 4.96 pg/ml, 0.97 pg/ml and 0.18 pg/ml for IL-8, IL-6, and TNFα, respectively for the left side and 4.92 pg/ml, 1.21 pg/ml, and 0.14 pg/ml for the right side. Comparison between the left and right sides for IL-8 concentration showed a moderate positive correlation (R=0.66, p<0.001, table 3). For TNFα and IL-6 the correlation was weaker with R values of 0.39 and 0.42, respectively (p=0.001, table 3). The results of the Bland-Altman plots showed that the difference between the two sides was close to zero but the limits of agreement were large for each cytokine studied (table 3). The limits of agreement were 3–5 times greater or lower than the mean for each cytokine studied, suggesting that the results from the two sides may vary up to several times.

Since marked "funnelling"—that is, larger differences were noted as the magnitude of the measurements increased—was noted for all three cytokines studied, the analysis was repeated using logarithmically transformed data (figs 3 and 4, table 4). For all three agents studied there was moderate correlation between the two sides for the logarithmically transformed data with an R value of 0.78 for IL-8, 0.75 for IL-6, and 0.64 for TNFα (all p<0.001). Bland-Altman analysis using logarithmically transformed data, thus shown as a ratio of left/right, were 0.82 (95% CI 0.66 to 1.02) for IL-8, 0.74 (0.58 to 0.94) for IL-6, and 0.93 (0.80 to 1.07) for TNFα. Similarly, the limits of agreement were wide: 0.12–5.45 for IL-8, 0.09–5.87 for IL-6, and 0.27–3.16 for TNFα, suggesting that the concentrations may vary by up to 10 times between the two sides.

Table 4 Comparison of concentrations of cytokines in BAL fluid obtained from the left and right sides using logarithmic transformed data

	Correlation		Bland-Altman analysis		
	R	p value	Difference (95% CI)	LLA (95% CI)	ULA (95% CI)
IL-8	0.78	<0.001	0.82 (0.66 to 1.02)	0.12 (0.08 to 0.18)	5.45 (3.72 to 8.00)
IL-6	0.75	<0.001	0.74 (0.58 to 0.94)	0.09 (0.06 to 0.14)	5.87 (3.84 to 8.98)
TNFα	0.64	<0.001	0.93 (0.80 to 1.07)	0.27 (0.21 to 0.35)	3.16 (2.45 to 4.07)

Concentrations of cytokines were logarithmically transformed and analysed using Pearson's coefficient (R) and by the Bland Altman plots of agreement. The mean difference in cytokine concentration between the right and left sides together with the lower (LLA) and upper (ULA) limits of agreement with 95% confidence intervals are given.

POTENTIAL SOURCES OF VARIABILITY

To explain the large variability noted for each parameter studied we examined a number of factors that may have accounted for the variability including (a) the age of the child, (b) underlying disease process, (c) time spent on ECMO, (d) experience of the operator, (e) survival, and (f) volume retrieved. In the initial analysis the only significant factor for cellular constituents was age of the child on presentation (p<0.05 for total cell counts, absolute alveolar macrophages, and neutrophil counts but not for percentages of neutrophils or macrophages). However, no significant contribution to variability for any cell type was noted from time on ECMO, underlying disease type, experience of the operator, volume of fluid retrieved, or survival. The contribution of age to the variability was no longer noted when we restricted the analysis to infants aged under 3 months.

When random effects linear regression was applied to cytokine measurements, only the volume of fluid retrieved appeared to be significantly associated with the variability observed for IL-8 (p=0.003) and TNFα (p=0.008) but not IL-6 (p=NS). Furthermore, the results showed a negative coefficient suggesting that, the larger the volume of fluid recovered, the lower the concentration of IL-8 and TNFα (fig 5). We also reanalysed the data using the difference between the volume

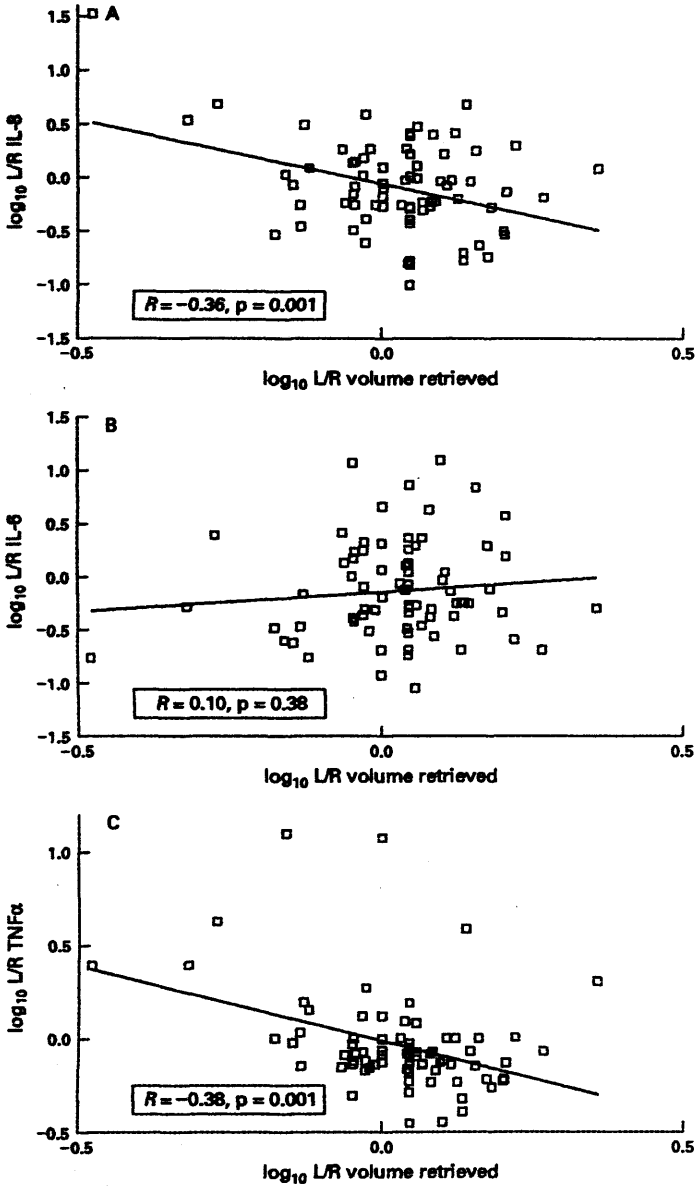

Figure 5 Logarithmic transformed data for the ratio of left/right volume retrieved plotted against the logarithmic data for the ratio of left/right cytokine concentrations to determine whether the concentration of cytokine was related to the volume of BAL fluid recovered. The results show that for IL-8 and TNFα but not for IL-6 the concentration of cytokine is inversely related to the volume of BAL fluid recovered.

retrieved from the two sides instead of the proportion of left to right of volume retrieved and found the same results. Similar results were obtained for both untransformed and logarithmically transformed data.

Discussion

NB-BAL is a commonly used technique to study respiratory diseases of infants receiving mechanical ventilation. Our results demonstrate a number of points: (1) great variability was noted for both cellular counts and cytokine concentrations when the left and right sides were compared; (2) the volume of fluid retrieved, but not age, influenced the variability observed for cytokine concentrations but, in contrast, age but not volume retrieved affected variability in cell counts; (3) neither the underlying disease process, time spent on ECMO, experience of the operator, nor survival affected the variability; (4) the cytokine concentrations tended to be higher on the right side than on the left.

We consider the Bland-Altman method to be the most appropriate way of analysing and presenting the agreement for the various measurements from the two sides.[16] We have, however, also presented the correlation coefficients and linear regression as these are often reported in the literature. For the absolute cell counts and for cytokine measurements the variability between left and right sides seemed to be directly dependent on the overall magnitude of the measurement ("funnelling" noted in fig 2A, C and E). We therefore repeated the Bland-Altman analysis in these cases using logarithmically transformed data and presented the results in terms of the ratio between left and right values (rather than the absolute difference). The limits of agreement remained wide for both cellular and non-cellular constituents.

It will be noted that several measurements from each infant were used. Examination of the possible sources of variability was therefore carried out using random effects linear regression which accounts for the possible "clustering" of measurements made on the same child. To further ensure the validity of this approach, we randomly selected one measurement from each child and observed similar limits of agreement for each cell or cytokine studied (data not shown).

For each cell type studied there was no significant difference between the mean results for the left and right sides. Correlation between left and right results was good for total counts of white cells and neutrophils, as well as for percentages of neutrophils, alveolar macrophages, and epithelial cells. However, correlation does not provide accurate information regarding the nature of the relationship between the results for the two sides. Furthermore, good correlation may be expected as the results relate to samples taken simultaneously from the same patient. In this situation, assessment of agreement using the method described by Bland and Altman results is more appropriate.[16] Our findings show that there is considerable variability between the left and right sides for individual cell measurements. This is reflected in the relatively wide 95% confidence intervals for mean differences between left and right results. Similarly, the limits of agreement between left and right cell results were relatively large. These data suggest that the application of NB-BAL in studies based on individual results or on small groups of patients needs to be interpreted with caution.

For cytokines there was a trend towards the results being greater on the right than on the left, as suggested by the ratios being less than one for all cytokines studied (fig 4). Since we had included patients with congenital diaphragmatic hernia (all children had left sided hernias), this observation may have been expected. However, we were unable to attribute this to the underlying disease process. Most of

the infants requiring ECMO had received several days of mechanical ventilation with high pressures and >90% inspired oxygen. The acute lung injury therefore resulted not only from the underlying disease process but also from the injury induced by conventional treatment. Since the right main bronchus is more vertical than the left, it is tempting to speculate that greater barotrauma or volutrauma was transmitted to that side by mechanical ventilation, resulting in greater lung injury on the right side as implied by our results.

When factors that may have accounted for variability were examined, only age but not time on ECMO, experience of the operator, underlying disease process, volume of fluid retrieved, or survival were significantly associated with variability in cell counts. The effect of age was no longer significant when infants older than 3 months of age were removed from the analysis.

However, for cytokine measurements the volume of fluid retrieved studied either as a proportion of left/right sides or the difference between the left and right sides yielded very interesting results (fig 5). The volume of fluid retrieved clearly appeared to be related to the variability observed for both IL-8 and TNFα but not for IL-6. The negative coefficient suggested that the concentration of the cytokine decreased as the proportion of the volume recovered increased. This implies a dilutional effect of the saline instilled to obtain epithelial lining fluid. Current recommendations from the European Respiratory Society for both children and neonates (for which one of the authors (SK) was a member) state that BAL fluid results should be expressed per ml of fluid, especially as currently there are no satisfactory methods for estimating epithelial lining fluid.[2] Our data are in disagreement with this statement as the influence of the retrieved volume on the results clearly needs to be taken into account. As we had followed the ERS guidelines, we had unfortunately made no attempt to estimate the epithelial lining fluid with any of the methods presently available.

The methods for measuring the concentration of cytokines may also influence the results. In the current study we used an automated cytokine analyser with extensive quality controls to ensure accuracy of the results. Using the controls and repeated measurements of cytokines in BAL fluid wherever possible, the results were almost always within 5% of each other. This variation is unlikely to account for the wide variability we have noted for the two sides. Because of the limited volume of fluid obtained from infants receiving ECMO, it would be difficult to perform the measurements in triplicate or more.

In conventionally ventilated infants a repeatability study would be unethical and dangerous. Patients undergoing extracorporeal support offer a unique opportunity for a repeatability study of NB-BAL because they are not dependent on their lungs for gas exchange. The most commonly encountered complication was transient bradycardia. No major adverse events affecting patients occurred during the study. This is in agreement with other studies in conventionally ventilated infants.[1 2 4 10]

Patients presenting for ECMO are unique. On the one hand they form a heterogeneous population in terms of underlying diagnosis while, on the other, they invariably have been ventilated with high F_{IO_2} and high mean airway pressures for a significant period of time, leading to radiologically uniform lung disease. We were particularly interested to determine whether the underlying disease process contributed to the variability in both cellular and cytokine constituents observed from the two sides, but were unable to contribute the variability to the underlying disease process despite the infants receiving ECMO for various diseases including congenital diaphragmatic hernia in which differences between the two sides may be expected. There were apparent differences in white cell populations between the left and right lungs in patients with congenital diaphragmatic hernia, meconium aspiration syndrome, and persistent pulmonary hypertension of the newborn. Several studies in adults with sarcoidosis using fibreoptic bronchoscopy have shown good correlation for the percentage of lymphocytes between BAL fluid samples from left and right lungs.[17–19] A small group of patients showed a significant interlobar difference in lymphocyte percentage which appeared to be related to focal infiltrates on chest radiographs in two studies.[18 19] In other types of interstitial lung disease a higher degree of interlobar variability was found which was not related to the appearance of the chest radiograph.[17 19] Infants who are placed on ECMO quickly develop similar radiological changes on both sides with an initial "white out" thought to be due to a transudative process followed by gradual resolution with time as pulmonary inflammation improves. Thus, one would expect similar findings for both cells and cytokines from both sides.

Previous studies have investigated fluid and cell recovery but not repeatability with NB-BAL of ventilated human infants. Grigg *et al* found a significant difference in the percentage of macrophages between the first and second aliquot but not in volumes retrieved or in total cell counts.[10] Using fibreoptic bronchoscopy in human adults, two studies have addressed the effect of instilled fluid on volume and cell recovery.[20 21] Both showed differences in differential cell counts for the lower and higher volume of BAL fluid instilled. These studies suggest that, with small volume lavage, the returned cells originate mainly from the proximal airways while, with larger volumes, the distal airways including alveoli are sampled. This is supported by additional studies by Kelly *et al* who investigated the distribution of fluid during the BAL procedure.[22] While aspirating the first aliquot of radio-opaque BAL fluid under radiographic imaging, fluid movement towards the bronchoscope was seen only in the proximal airways. Lavage fluid more distally actually moved away from the bronchoscope.

With further aliquots BAL fluid was retrieved from the entire lung segment.

In healthy human volunteers Pingleton *et al* showed that fluid volume and total cell recovery were highest when the right middle lobe was lavaged.[23] There was no difference between lobes in cell count/ml BAL fluid. The pH and temperature of the instilled fluid did not affect cell or protein recovery. Similar observations were made by Laviolette and colleagues in normal dogs.[24] They found a significant difference in the recovery of total number of cells between the left lower and right middle lobe, but not in percentage macrophages. In normal rats cell recovery was found to be dependent on the type of catheter and the use of bronchoscopic guidance.[25] To minimise the effects of these variables we used a standardised method of NB-BAL and subsequent lavage fluid processing. Unlike BAL using fibreoptic bronchoscopy, NB-BAL is carried out blindly. Despite this, it is likely that the suction catheter reached the intended site on most occasions. Placzek and Silverman studied catheter placement in NB-BAL[26] and showed that, by turning the head to the left, the suction catheter was passed into the right main bronchus in over 95% of cases. In a necroscopic study Grigg *et al*[10] confirmed that, with the infant's head turned to the left, the right lower lobe is lavaged. In the present study four lavage procedures in two patients were carried out at the time of routine chest radiography and, on each occasion, the catheter was directed as anticipated to the contralateral lobe. A catheter was inserted into both the right and left sides simultaneously with the head turned towards the opposite side and on the chest radiograph the two catheters were seen to have entered the expected contralateral lobes (data not shown). We can therefore make a reasonable assumption that the catheter entered the expected lobe at the time of NB-BAL. Since we were unable to attribute the variability to the underlying disease process, experience of the operator, fluid retrieval, days on ECMO, survival, or age (in infants less than 3 months of age), it is likely that the variability is due to the technique itself. The placement of the catheter and distribution of the instilled fluid may contribute to the variability reported.

In conclusion, our results have shown a wide variation in both cell counts and cytokine concentrations between the left and rights sides for standardised NB-BAL of infants receiving ECMO. The wide variation in cell counts may be related to the age of the infant but, interestingly, the concentrations of cytokines appeared to be influenced by the volume of fluid returned by the procedure, suggesting that normalisation for dilutional effects of saline is essential for cytokines but not for cells. The small differences between the two sides, especially in cell counts, suggests that the technique may be useful for population studies but individual results must be interpreted with caution.

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NEONATAL

The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants

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Background: The persistent airway neutrophilia observed in chronic lung disease of prematurity (CLD) may reflect inappropriate suppression of neutrophil apoptosis.
Methods: 134 bronchoalveolar lavage (BAL) samples were obtained from 32 infants requiring mechanical ventilation for respiratory distress syndrome (RDS): 13 infants (median gestation 26 weeks, range 23 to 28) subsequently developed CLD (CLD group), and 19 infants (gestation 31 weeks, range 25 to 39) recovered fully (RDS group). A further 73 BAL samples were obtained from 20 infants (median age 2 days, range 1 to 402) receiving extracorporeal membrane oxygenation (ECMO) for severe respiratory failure.
Results: Neutrophil apoptosis was increased in the RDS group (mean (SEM) neutrophil apoptosis on day 7 BAL: RDS 17.0 (8.6)% *v* CLD 0.7 (0.2)% ($p<0.05$)). BAL fluid obtained from RDS but not CLD patients was proapoptotic to neutrophils (apoptosis ratio BAL fluid/saline control: day 1, RDS 9.8 (5.5) *v* CLD 1.2 (0.1) ($p<0.05$); day 2, RDS 4.32 (2.8) *v* CLD 0.5 (0.4) ($p<0.05$)). There were similar findings in the ECMO group: survivors had proapoptotic BAL fluid compared with non-survivors (apoptosis ratio day 1, survivors 7.9 (2.1) *v* non-survivors 2.1 (0.7) ($p<0.05$)).
Conclusions: Inappropriate suppression of neutrophil apoptosis may be associated with a poor outcome in newborn infants with respiratory failure.

The neutrophil granulocyte is a major participant in the acute inflammatory response in tissues, being recruited from the circulation when local defences are overwhelmed. The constitutively short life span of the neutrophil is less than 24 hours in the circulation, and these cells are exquisitely sensitive to apoptosis (programmed cell death).[1 2] Apoptosis is important in the normal resolution phase of inflammation, as it leads to functional downregulation[3] and to recognition and clearance of the apoptotic neutrophils by macrophages.[1] Neutrophil life span is extended in tissues[4] and this, together with modulation of neutrophil apoptosis by cytokines and other inflammatory mediators,[5 6] suggests active control of cell death during the course of inflammation in vivo. Apoptosis of neutrophils present within the lung is inappropriately suppressed in patients with acute respiratory distress syndrome (ARDS), and the bronchoalveolar lavage (BAL) fluid of such patients is antiapoptotic to human neutrophils.[7] This antiapoptotic effect was observed to be maximal in the early stages of ARDS[7] and to decline at later time points. The suppression of apoptosis is predominantly mediated through elaboration of antiapoptotic cytokines.[7 8]

We have previously found that neutrophils that have been "aged" in vitro but have not undergone apoptosis show equivalent proinflammatory functions to freshly isolated neutrophils, emphasising the potential of these cells to cause tissue damage.[3] We have also reported that airway neutrophilia, shown on BAL studies, is associated with the development of chronic lung disease of prematurity (CLD) in preterm infants who require mechanical ventilation for neonatal respiratory distress syndrome (RDS).[9] We hypothesised that the persistent airway neutrophilia observed in CLD is caused by inappropriate suppression of neutrophil apoptosis. We therefore investigated neutrophil apoptosis in infants who developed CLD following neonatal RDS (CLD group) compared with those who recovered from RDS (RDS group). Specifically, we investigated the proportion of apoptotic neutrophils in bronchoalveolar lavage fluid at time points up to 14 days in infants in the two groups receiving mechanical ventilation; and whether BAL fluid from the CLD and RDS groups differs in its effects on constitutive neutrophil apoptosis in vivo.

METHODS

Patient groups

Infants with and without respiratory failure were recruited into the study and divided into the following groups:

- *Chronic lung disease of the newborn (CLD group)*: these infants initially required mechanical ventilation for RDS. At 28 days of age they remained oxygen dependent and had persistent chest *x* ray abnormalities.
- *Respiratory distress syndrome (RDS group)*: these infants were also ventilated for RDS but by 28 days were nursed in air and had a normal chest *x* ray.
- *Extracorporeal membrane oxygenation (ECMO group)*: these were infants with severe respiratory failure as a result of various conditions, including RDS, meconium aspiration syndrome, and persistent pulmonary hypertension of the newborn. Of those recruited, 14 survived and six did not.
- *Controls*: these infants received mechanical ventilation perioperatively for surgical conditions for non-pulmonary reasons, and were exposed to less than 28% inspired oxygen.

Infants eligible for the CLD, RDS, and control groups but with proven or strongly suspected sepsis were excluded from the study. The diagnosis of sepsis was based on one or more

Abbreviations: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; CLD, chronic lung disease of prematurity; ECMO, extracorporeal membrane oxygenation; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; RDS, respiratory distress syndrome; TNFα, tumour necrosis factor α; TRAIL, tumour necrosis factor related apoptosis inducing ligand; TUNEL, TdT mediated dUTP-biotin 3'-OH nick-end labelling

of the following: maternal fever or infection, prolonged rupture of membranes (>48 hours), positive blood cultures in the infant, positive endotracheal cultures, and raised or relative increase in blood white cell count or C reactive protein in the face of clinical suspicion of sepsis. Six infants were excluded from the study because organisms were identified from secretions obtained through the endotracheal tube or because of central line sepsis.

Fully informed, written consent was obtained from the parents of each infant. The study was approved by the Leicestershire Health Authority research ethics committee.

Bronchoalveolar lavage

BAL was done twice weekly in the ventilated preterm infants until 21 days of age or until extubation. In the infants receiving ECMO, BAL was done daily for the first four days of treatment. BAL was undertaken as previously described.[9–11] Briefly, with the infant lying supine and the head turned to the left, a 5 F catheter was gently inserted into the endotracheal tube until resistance was felt. Two aliquots of 1 ml per kg of saline (0.9%) were instilled through the catheter and immediately sucked back. The BAL samples were processed within 20 minutes of the lavage procedure. From the pooled aspirate, aliquots were taken for total and differential cell count. The remainder of the aspirate was centrifuged at 500 $\times g$ for 10 minutes at room temperature and the supernatant stored at −70°C for later analysis. All subsequent analyses were done by investigators who were blinded to the clinical diagnosis in each case.

Characterisation of BAL cells

Haemocytometer counting was used to obtain total cell counts per ml of BAL fluid. Cytocentrifuge preparations were made from a further aliquot of BAL fluid. At least 300 cells were counted on each cytospin to provide a differential cell count. In addition, apoptotic neutrophils, assessed morphologically by the typically condensed nuclei, were counted and expressed as a percentage of the total neutrophil count. As all the counts were done before classification of the infants to the retrospective diagnoses of CLD or RDS, the counts were blind to the outcome.

Isolation of neutrophils

Human peripheral blood neutrophils were obtained from healthy adult volunteers. Ethical approval was obtained from the South Sheffield research ethics committee and all subjects gave fully informed consent. Neutrophils were isolated from citrated venous blood by dextran sedimentation and centrifugation through a discontinuous plasma-Percoll gradient (Amersham Pharmacia Biotech, Buckinghamshire, UK) as previously described.[12] Purity was assessed by counting >500 cells on duplicate cytospin preparations and was always >95%, with contaminating cells being almost exclusively eosinophils.

Assessment of neutrophil apoptosis in BAL

The apoptotic activity of the BAL fluid was assessed as previously described.[7] Neutrophils were suspended at a concentration of 3.5×10^6/ml in Roswell Park Memorial Institute medium (RPMI), with 10% fetal calf serum and with penicillin and streptomycin (100 U/l) (all from Life Technologies Inc, Glasgow, UK).[13] The "age" of neutrophils in culture was calculated, designating this stage as time 0. A 100 µl aliquot of the neutrophil suspension was added to 100 µl of BAL fluid from the newborn infants, with normal saline used as control. Cells were incubated in 96-well Falcon "Flexiwell" plates (not tissue culture treated; BD Pharmingen, Cowley, Oxford, UK) at 37°C in a 5% CO_2 atmosphere for five hours. A five hour time point was chosen because there is little (typically less than 5%) constitutive apoptosis in control (untreated) populations, so a proapoptotic effect of BAL fluid upon neutrophils should be detected.[14]

At five hours, apoptosis was quantified by assessment of nuclear morphology on Giemsa-stained cytocentrifuge preparations, counting >300 cells per slide on duplicate cytospins.[1 13] This method has been shown to correlate closely with other measurements of neutrophil apoptosis, including Annexin V binding[15] and shedding of CD16.[16] In addition, necrosis was assessed at all time points by exclusion of the vital dye trypan blue, and was less than 2% unless otherwise indicated. In the results section, the apoptotic activity of BAL fluid is expressed as a ratio of the value in the saline control in the same experiment.

Cytokine assays

The concentrations of tumour necrosis factor α (TNFα), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin (IL)-10 were measured in BAL fluid using commercially available enzyme linked immunosorbent assays (ELISA) according to the manufacturer's instructions (R&D Systems, Oxfordshire, UK).

Statistical methods

Gestation and birth weight are expressed as the median values and range. Only one sample was obtained from any individual infant at any one time point, and the mean of results from two cytospins is given. Total and differential cell counts and the apoptotic activity of BAL fluid are expressed as mean (SEM) of the number (n) of independent experiments, with each experiment using cells from separate donors and carried out in duplicate. Data from the controls are only given for BAL fluid neutrophil apoptotic activity (see fig 4A) as too few cells were present on the cytocentrifuge preparations from these infants for reliable differential cell counts to be obtained.

The apoptotic activity of BAL fluid is given relative to that obtained for the saline control. Thus values of >1.0 imply proapoptotic activity and <1.0 imply antiapoptotic activity in BAL fluid relative to saline.

The Mann-Whitney U test was used for comparisons between patient groups, as the data were non-parametrically distributed. Cell count data were compared by analysis of variance (ANOVA) with the Bonferroni post-test, using the Prism 3.0 program (GraphPad Software Inc, San Diego, California, USA). Maximum values for each infant for BAL fluid neutrophil activity during the first week of age were also compared between the RDS and CLD groups. Correlations between gestation, birth weight, and cytokines with BAL neutrophil apoptotic activity were determined using the Pearson correlation coefficient (R value). Results are considered statistically significant at a probability (p) value of <0.05.

RESULTS

Patient characteristics

We obtained 134 BAL samples from 32 infants in the neonatal unit. The characteristics of these infants are shown in table 1. BAL samples were obtained from the CLD group at time points up to 21 days. For the RDS group, data are shown only up to 14 days for some of the analyses, as thereafter there were too few samples available. No clinical differences were noted between infants who were or were not treated with antenatal corticosteroids. There were no significant differences between the two groups for inspired oxygen or ventilation variables during the first day of life. However, when the gestational age and birth weight were compared there were differences between the two groups (birth weight,

Table 1 Patient characteristics

	CLD	RDS
Number	13	19
Gestation (weeks)	26 (23 to 28)	31 (25 to 39)†
Birth weight (g)	750 (630 to 1070)	1400 (720 to 4160)†
Antenatal dexamethasone	12/13 (92%)	10/19 (53%)
Surfactant*	12/13 (92%)	16/19 (84%)
PIP (mean first 24 hours)	17 (14 to 36)	16 (11 to 22)
MAP (mean first 24 hours)	8 (4 to 18)	7 (4 to 13)
Fio_2 (mean first 24 hours)	0.37 (0.24 to 1.00)	0.32 (0.21 to 0.76)

*Curosurf®, Chiesi Pharmaceuticals, Parma, Italy.
†Significant differences were noted between the two groups for birth weight and gestation (both $p<0.001$).
CLD, chronic lung disease; Fio_2, inspired fractional oxygen; MAP, mean airway pressure; PIP, peak inspiratory pressure; RDS, respiratory distress syndrome.

$p<0.001$; gestation, $p<0.001$). We also studied nine control infants (median birth weight 2240 g, range 900 to 3510; median gestation 36 weeks, range 26–40). These infants received mechanical ventilation but less than 28% inspired oxygen.

We obtained a further 73 BAL samples from 20 infants receiving ECMO (median age 2 days, range 1 to 402; median weight 3.7 kg, range 2.5 to 10.0). Of these, 14 survived and six did not. BAL samples were obtained daily for the first four days of ECMO treatment. Of the survivors, six received ECMO for meconium aspiration syndrome, three for sepsis, two for congenital diaphragmatic hernia, and one each for pneumonia, persistent pulmonary hypertension, and respiratory distress syndrome. One infant with congenital diaphragmatic hernia, two with persistent pulmonary hypertension, and three with pneumonia did not survive.

Total cell, absolute neutrophil, and alveolar macrophage cell counts

The mean (SEM) total cell counts in BAL fluid from the RDS group increased from 258 (74)$\times10^4$/ml on day 1 of age to 368 (107)$\times10^4$/ml by day 4, before decreasing rapidly to $<60.0\times10^4$/ml on days 10 and 14 (fig 1). In the CLD infants, however, the BAL fluid total cell counts were 124×10^4/ml at day 1, increased gradually to 382 (180)$\times10^4$/ml by day 10, before declining to 193 (105)$\times10^4$/ml by 17 days.

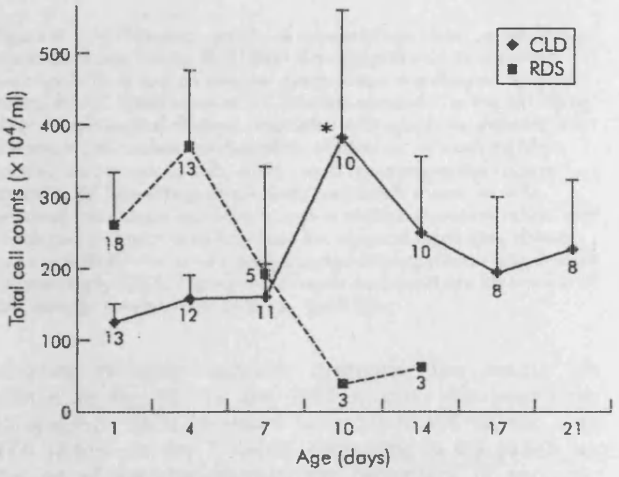

Figure 1 Total cell counts in bronchoalveolar lavage (BAL) fluid obtained from ventilated preterm infants who progressed to develop chronic lung disease of prematurity (CLD) and who recovered from the neonatal respiratory distress syndrome (RDS). The numbers beside each point are the number of BAL samples available for analysis. *$p<0.05$.

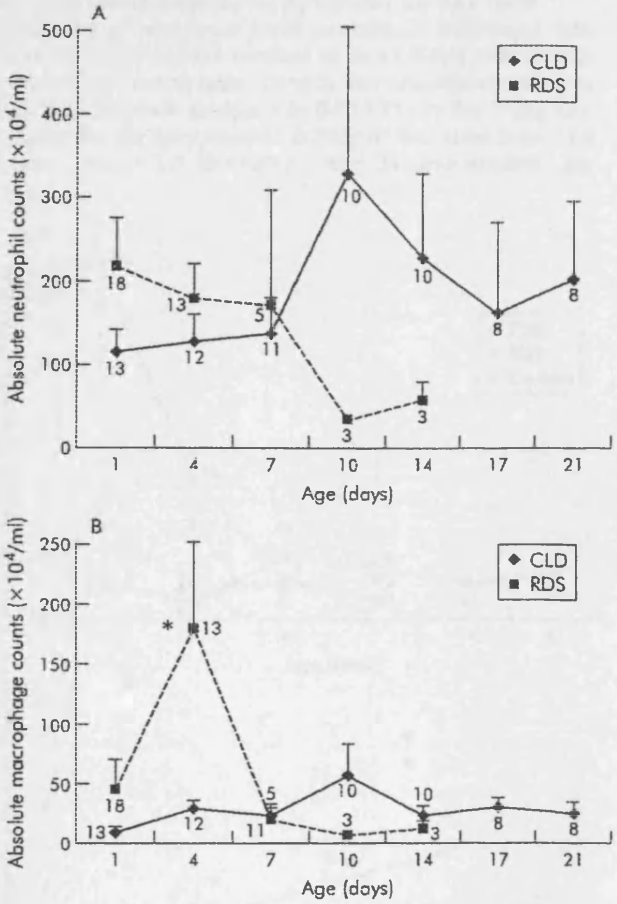

Figure 2 (A) Absolute neutrophil count and (B) alveolar macrophage count in bronchoalveolar lavage (BAL) fluid obtained from ventilated preterm infants who progressed to develop chronic lung disease of prematurity (CLD) and those who recovered from the neonatal respiratory distress syndrome (RDS). The numbers beside each point are the number of BAL samples available for analysis. *$p<0.05$.

There was a significant difference in BAL cellularity between the two groups on day 10: RDS, 39 (3.0)$\times10^4$/ml *v* CLD, 382 (180)$\times10^4$/ml ($p<0.05$). Absolute BAL neutrophil counts in the RDS group decreased from 215 (57)$\times10^4$/ml on day 1 to 176 (00)$\times10^4$/ml on day 4 before decreasing to $<60.0\times10^4$/ml on days 10 and 14 (fig 2A). In the CLD infants, the neutrophil counts increased from 116 (24)$\times10^4$/ml on day 1 to 326 (180)$\times10^4$/ml on day 10, before declining to 162 (106)$\times10^4$/ml by day 17. The difference in cellularity between the two groups was thus largely attributable to an increase in neutrophil numbers in the CLD group at day 10 (fig 2A), as we have shown in previous studies.[9]

BAL alveolar macrophage counts, in contrast, increased in the RDS group from 43 (27)$\times10^4$/ml on day 1 to 177 (74)$\times10^4$/ml on day 4, before decreasing to $<15.0\times10^4$/ml thereafter between 7 and 14 days of age (fig 2B). In the CLD group, the alveolar macrophage count was $<30.0\times10^4$/ml on all days studied, except for a slight increase on day 10, to 56 (26)$\times10^4$/ml. Counts were significantly higher in the RDS group than the CLD group at day 4 (RDS, 177 (74)$\times10^4$/ml; CLD, 29.0 (6.5)$\times10^4$/ml; $p<0.05$) but thereafter no differences were found between the two groups.

Assessment of neutrophil apoptosis in cells obtained by bronchoalveolar lavage

The percentage of neutrophils that showed light microscopic features of apoptosis (fig 3A) was determined by a blinded

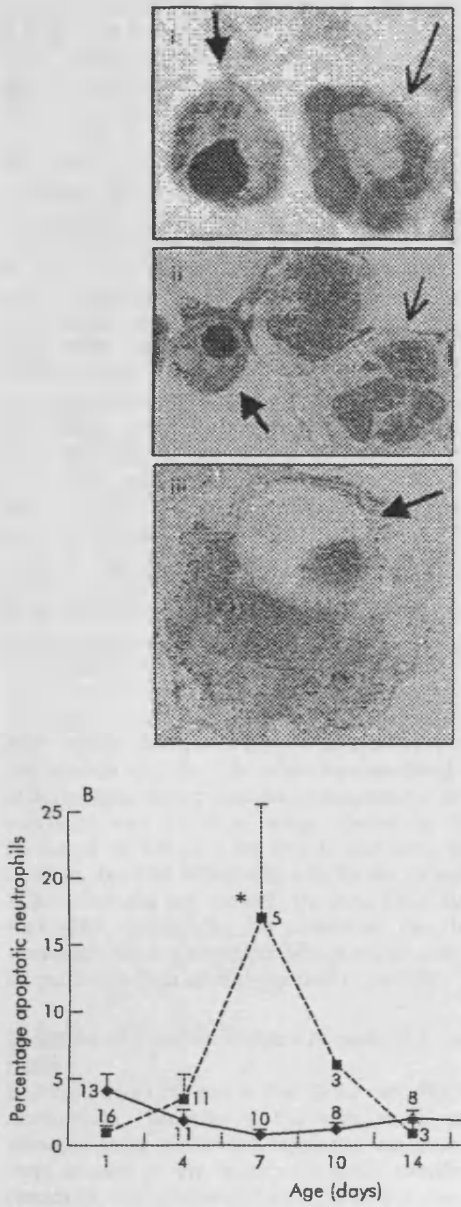

Figure 3 (A) Photomicrographs of cytocentrifuge slides prepared from bronchoalveolar lavage (BAL) fluid showing presence of apoptotic neutrophils (i, ii) and an alveolar macrophage engulfing an apoptotic neutrophil (iii). Solid arrowheads illustrate apoptotic neutrophils; these show morphological changes associated with apoptosis, including cell shrinkage and nuclear condensation. Unfilled arrowheads highlight healthy, non-apoptotic cells, which retain hypersegmented nuclear structure. (B) Percentage of apoptotic neutrophils shown for cells obtained from bronchoalveolar lavage in ventilated preterm infants who developed and recovered fully from the neonatal respiratory distress syndrome (RDS) *v* those who progressed to develop chronic lung disease of prematurity (CLD). The numbers beside each point are the number of BAL samples available for analysis. *p<0.05.

observer counting duplicate cytospins. The results are shown in fig 3B. In the RDS infants, the percentage of apoptotic PMN increased from 1.0 (0.4)% on day 1 to 17.0 (8.6)% on day 7, before decreasing to 0.8 (0.8)% on day 14 of age. In contrast, the percentage of apoptotic neutrophils in BAL from infants developing CLD was 4.1 (1.2)% on day 1 but <1.8% thereafter until 21 days of age. At day 7 there was a significantly greater proportion of apoptotic PMN in the RDS group than in the CLD group (p<0.05).

Modulation of neutrophil apoptosis by BAL fluid

Incubation of peripheral blood neutrophils with day 1 BAL fluid from RDS infants resulted in an 11.0-fold induction in apoptosis relative to saline control. The proapoptotic effect of BAL fluid lessened gradually to 0.6 (0.25) by day 7 (fig 4A). In contrast, the proapoptotic activity of BAL fluid from CLD infants was <2.0 throughout the 21 days studied. For

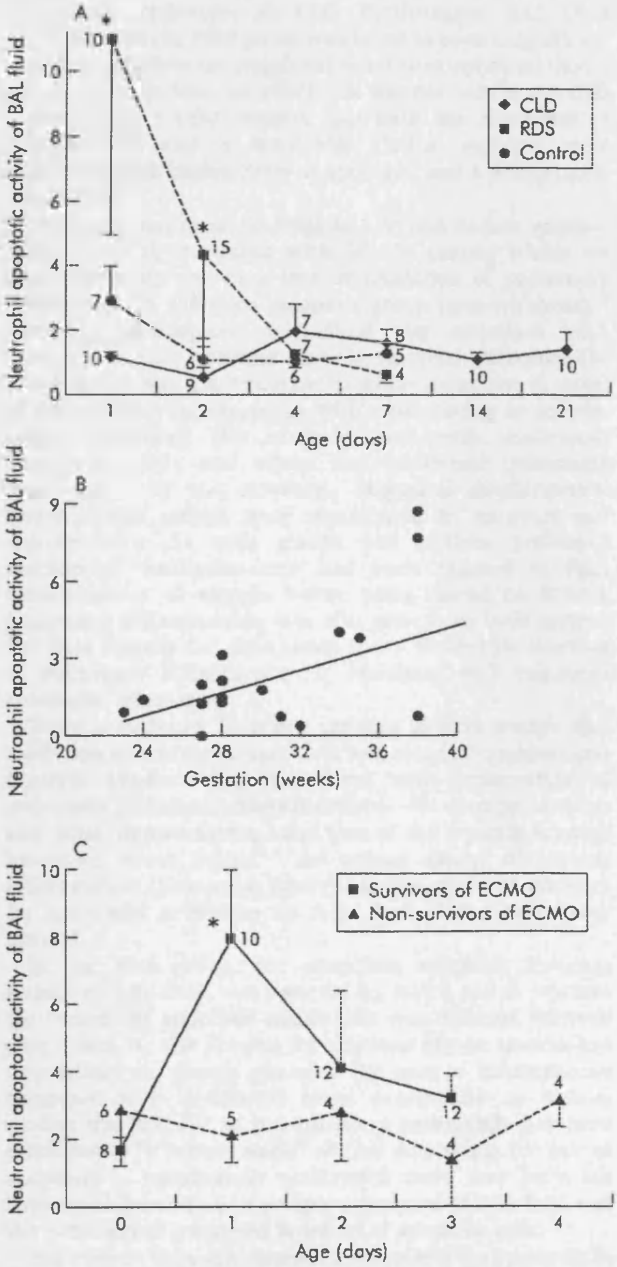

Figure 4 (A) Neutrophil apoptotic activity of bronchoalveolar lavage (BAL) fluid obtained from ventilated preterm infants who developed and recovered fully from the neonatal respiratory distress syndrome (RDS) and those who progressed to develop chronic lung disease of prematurity (CLD). (B) Moderate correlation between BAL fluid neutrophil apoptotic activity and gestational age ($R=0.50$, $p<0.05$). (C) Apoptotic activity of BAL fluid from infants who received extracorporeal membrane oxygenation for severe respiratory failure. The apoptotic activity of BAL fluid is expressed as a ratio of value for the saline control in the same experiment. The mean (SEM) percentage apoptosis for the saline control was 1.4 (0.2)%, n=10. The numbers beside each point are the number of BAL samples available for analysis. ECMO, extracorporeal membrane oxygenation. *p<0.05.

comparison, BAL fluid obtained from control infants showed a mean 2.9-fold induction of apoptosis on day 1, which fell to<1.3-fold thereafter until 7 days of age. The proapoptotic effect of RDS fluid on neutrophils was greater than that of CLD or control fluid on both day 1 and day 2 ($p<0.05$).

We also analysed the data by comparing the maximum BAL fluid neutrophil activity during the first week of life in CLD and RDS infants, and noted a significant difference between the two groups: CLD, 1.3 (interquartile range (IQR), 0.8 to 2.0) *v* RDS 1.9 (1.5 to 3.3) ($p<0.05$), mean difference 0.78, 95% confidence interval 0.11 to 1.83. The maximum value was achieved in the RDS infants at a median age of 2.0 days (IQR 1 to 2) and in the CLD infants at a later age of 4.0 days (IQR 2 to 7) (median difference 2.0, 95% CI 0.001 to 3.000, $p<0.05$). To determine if the BAL proapoptotic activity was related to either the gestation age or the birth weight of the infant, we compared gestational age and birth weight with the proapoptotic activity of the BAL fluid at day 1. A correlation was found between the BAL fluid neutrophil activity at day 1 and gestational age (fig 4B) ($R = 0.5$, $p<0.05$) but not birth weight ($R<0.10$, $p>0.05$).

To further address the possibility that proapoptotic BAL fluid was associated with a better clinical outcome, we studied a cohort of infants who were treated with ECMO. We had previously shown a significant improvement in BAL neutrophilia in this cohort who survived the course of ECMO when compared with infants who did not.[17] We hypothesised that those ECMO infants who survived might—as with the RDS cohort—have BAL fluid with a proapoptotic effect upon neutrophils and that this might be associated with resolution of acute lung injury. The BAL proapoptotic activity of ECMO survivors was 1.7 (0.4) when placed on ECMO (day 0), increased to 7.9 (2.1) on day 1, and then decreased to 3.1 (0.8) on day 3 of ECMO (fig 4C). In the infants who received ECMO but did not survive, the BAL fluid apoptotic activity was <3.1 throughout the treatment. On day 1 of ECMO treatment there was greater proapoptotic activity in BAL fluid of survivors than of non-survivors ($p<0.05$).

Relation of soluble factors to apoptotic activity of BAL fluid

Soluble factors present in BAL fluid can affect the survival of neutrophils.[7] Because of the very small sample volumes obtained from the infants (typically less than 0.5 ml/kg), we were limited to the study of a small number of candidate cytokines. We measured GM-CSF, as this has been shown to prolong neutrophil survival both in vitro[5] and in vivo[7 8]; TNFα, which has a proapoptotic effect upon neutrophils at the time point studied[18]; and IL-10, because high levels of this anti-inflammatory cytokine are proapoptotic[19 20] and are associated with a poorer outcome in patients with pneumonia.[21] Both GM-CSF[22] and TNFα[23] have been shown to be increased in infants who develop CLD compared with those who recover from RDS, and IL-10 may be deficient in infants who develop CLD.[24]

We measured BAL fluid levels of each of these cytokines by ELISA and correlated them with BAL fluid apoptotic activity in all the groups studied. All three cytokines were detected, either in all samples (TNFα) or in the majority of samples. No relations were identified, however, between BAL levels of TNFα, GM-CSF, or IL-10 on day 1 and the numbers of apoptotic neutrophils present in BAL fluid at day 1, nor with BAL neutrophil apoptotic activity at day 1(all $R^2<0.25$, $p>0.10$; data not shown). Comparisons were only made at day 1 as too few samples were available thereafter.

DISCUSSION

In these studies we showed that the development of chronic lung disease following neonatal respiratory distress syndrome is associated with persistent neutrophilia in BAL fluid, compared with preterm infants in whom RDS resolves, and we confirmed previous studies showing that this difference becomes apparent at day 10 of age.[9 25] We hypothesised that the observed difference in neutrophil numbers could reflect differences in the regulation of neutrophil apoptosis between the two groups. We showed that the CLD group had reduced neutrophil apoptosis at day 7 compared with the RDS group, suggesting that increased survival may be a mechanism of neutrophil persistence in CLD. Furthermore, BAL fluid obtained from the RDS group was found to have a significant proapoptotic effect on peripheral blood neutrophils on days 1 and 2 of ventilation—an effect that was not seen in the CLD group. These results suggest that both the resolution of inflammation and a favourable clinical outcome were associated with higher rates of apoptosis and a proapoptotic lavage fluid.

We confirmed these findings in a second patient group—those receiving treatment with ECMO—among whom we have previously shown a lack of resolution of pulmonary neutrophilia in the poor prognosis group (non-survivors).[17] Again, a proapoptotic BAL fluid was associated with improved clinical outcome (that is, survival). Although the ECMO group was a heterogeneous group of patients in terms of their underlying diagnosis, with some having an inflammatory pathology (for example, pneumonia, meconium aspiration, RDS) and others not (persistent pulmonary hypertension of the newborn, congenital diaphragmatic hernia), both groups were represented in survivors and non-survivors. As both groups had received prolonged mechanical ventilation and had been exposed to high concentrations of oxygen before being placed on ECMO, pulmonary inflammation was also present in both groups. The data support the observation that a favourable outcome of pulmonary inflammation is associated with enhanced neutrophil apoptosis.

There is evidence from our previous in vitro studies that inhibition of neutrophil apoptosis by a range of cytokines and bacterial products[3 5] is associated with preservation of neutrophil proinflammatory functions—for example, respiratory burst, degranulation—and thus of the capacity to cause bystander tissue injury.[3 5] An animal model of chronic inflammation (bleomycin injury) has also produced evidence for neutrophil activation up to 21 days after a pulmonary insult.[26]

In the RDS group, the maximum apoptosis inducing activity of BAL fluid was detected on days 1 and 2, whereas the "wave" of apoptotic neutrophils was detected between days 4 and 10. The reasons for this time lag are unclear but may reflect the greatly extended life span of inflammatory compared with peripheral blood neutrophils. In animal models the half life of inflammatory neutrophils has been estimated to be several days,[26] so that even where the rate of apoptosis is significantly accelerated there may be a lag between elaboration of a proapoptotic factor in BAL fluid and the detection of increased numbers of apoptotic cells.

The current data also support a major role for apoptosis in the clearance of neutrophils from the lung in neonatal pulmonary inflammation. For instance, the total neutrophil counts in the RDS group decreased from a mean of 176×10^4/ml at day 7 to 40×10^4/ml on day 10, concomitant with the identification of up to 17% apoptotic neutrophils in the BAL fluid.

Matute-Bello *et al* studied neutrophil apoptosis in ARDS and showed that neutrophil apoptosis was detected at low levels in BAL throughout the course of ARDS.[7] Moreover, BAL fluid from ARDS patients suppressed constitutive neutrophil apoptosis, whereas fluid from normal volunteers did not.[7] This inappropriate suppression of neutrophil apoptosis is

analogous to the findings in our CLD group and was abrogated by blocking antibodies to G-CSF (granulocyte colony stimulating factor) and GM-CSF.[7] In that study the investigators were unable to show an association between higher rates of neutrophil apoptosis and survival, although mean apoptosis was higher in the survivors than in those who died (2.4% *v* 1.8%).

Increased numbers of macrophages were noted in the RDS group at day 4 (fig 2A), preceding the increase in apoptotic neutrophils that was evident on day 7 (fig 2B). It is conceivable that the alveolar macrophages play a role in induction of neutrophil apoptosis that is likely to be in addition to the proapoptotic effect of BAL on days 1 and 2. Potential mechanisms for such induction by alveolar macrophages might include cell surface expression of death ligands—for example, Fas[15] or TRAIL (tumour necrosis factor related apoptosis inducing ligand)[27]—which are proapoptotic for neutrophils and are known to be expressed on macrophage populations.[28 29] In addition, these macrophages may play a role in clearance of apoptotic neutrophils. We attempted to quantify ingested apoptotic bodies and were able to detect examples (fig 3A), but at insufficient levels for a valid comparison of the RDS and CLD groups. A probable explanation is that light microscopic evaluation is substantially less sensitive than other methods—for example, TUNEL staining (which we found to be superior in unrelated studies on murine models of pulmonary inflammation) or electron microscopy.[7] Alternatively, clearance of macrophages that have ingested apoptotic cells may occur rapidly through lymphatics.[30] In the present studies, only two cytospins were available for each data point. In future studies, we hope to obtain additional cytospin preparations for immunohistochemistry (for macrophage death receptor expression) and TUNEL staining (for ingested apoptotic cells). Unfortunately, total cell numbers are too small for flow cytometry to be feasible for the majority of time points.

We considered whether the observed differences in BAL fluid modulation of apoptosis might be attributable to other factors to which the babies were exposed. Corticosteroids are known to inhibit neutrophil apoptosis, but at higher concentrations than are likely to be present in BAL fluid after antenatal dexamethasone treatment.[31] None of the infants in the study received postnatal corticosteroids. Hypoxia has also been shown to prolong neutrophil survival, but at levels of hypoxia of <1% oxygen.[32] The degree of respiratory failure was similar between the two groups at the onset of ventilation, and tissue oxygenation was maintained in most cases, so this is unlikely to be relevant to our studies. The volume of fluid returned after the BAL procedure was similar between the CLD and RDS groups. In accordance to the recent recommendations of the European Respiratory Society's task force on bronchoalveolar lavage studies in children, we did not normalise for dilution of BAL fluid by using urea, secretory component of IgA, or albumin.[33] Although one potential factor that may modulate neutrophil apoptosis is surfactant, the use of exogenous surfactant was similar between the two groups (CLD 92%, RDS 84% (NS)). Furthermore, previous studies with peripheral blood neutrophils have not suggested a major proapoptotic effect of surfactant.[34]

One clear difference between the CLD and RDS groups was that the CLD group was significantly more premature (median gestation 26 weeks compared with 31 weeks). It is possible that, with increasing prematurity, neutrophils are more resistant to apoptosis. We noted a relation between the apoptosis inducing effect of the first available BAL fluid sample from each infant and gestation but not birth weight. It needs to be clarified further whether the data observed reflect immature neutrophil apoptotic mechanisms in preterm infants, who are thus less likely to clear their neutrophils effectively. Allgaier *et al* have studied neutrophils purified from umbilical cord blood of neonates and have shown that they undergo apoptosis at a slower constitutive rate than adult neutrophils, and also that they are more resistant to the effects of the ligation of the Fas death receptor.[35] It is possible, therefore, that age related differences in apoptosis susceptibility may influence the results observed. Nonetheless, we have shown clear differences in the effects of BAL fluid of CLD and RDS patients upon apoptosis of healthy adult neutrophils. This is most probably attributable to the presence or absence of soluble modulators of apoptosis. Prematurity could also be associated with inability to elaborate a proapoptotic factor or factors in the lung.

Within the limitations of the sample volumes obtained we attempted to identify soluble factors that might influence rates of apoptosis. In general, the ability of cytokines and other inflammatory mediators to modulate neutrophil apoptosis is well recognised,[5 6] and many of these factors are present in BAL fluid in both adults[7 36] and neonates with RDS.[9–11 37] Raised levels of GM-CSF have been associated with ARDS[7 35] and CLD,[22] and blocking antibodies have been shown to abrogate the antiapoptotic effect of BAL fluid from ARDS.[7] Other studies, however, have not found this association,[36] but have instead shown increases in IL-8 and G-CSF (the latter also found by Matute-Bello[7]). A cohort study suggested that levels of G-CSF or GM-CSF in BAL fluid were not, however, predictive of development of ARDS.[36] We could not identify a correlation between GM-CSF levels and percentage neutrophil apoptosis, although a difference in GM-CSF levels between infants with RDS and CLD has previously been reported.[22] The same was true for TNFα, which is known to be proapoptotic to human neutrophils at five hours, the time point used in our studies.[18] Similarly, levels of IL-10—which are increased in ARDS[39 40] and are known to inhibit cytokine mediated delay of neutrophil apoptosis[19]—were not associated with rates of apoptosis or with different patient groups. A recent report by Oei and colleagues did not find a relation between IL-10 in tracheal fluid and neutrophil apoptosis in preterm infants at risk of developing CLD.[41] The lack of association of any single factor with the overall percentage of neutrophil apoptosis is not surprising, given the number of cytokines shown to be present in the inflamed lung. Identification of factors that correlate with outcome would, however, be of potential clinical use. Other potential factors that may modulate the neutrophil apoptotic process include Fas ligand[42] and IL-2,[43] but we were unable to investigate these factors further because of the limited availability of BAL fluid from ventilated preterm infants.

Conclusions

These studies have shown that persistent airway neutrophilia in chronic lung disease is associated with inappropriately low rates of neutrophil apoptosis and failure to generate a proapoptotic BAL fluid. Conversely, resolution of lung inflammation in the RDS group is associated with higher rates of apoptosis and proapoptotic BAL fluid. These findings suggest that clinical outcome might be predictable from assessment of BAL fluid early in the development of ARDS and also that strategies to induce neutrophil apoptosis could be associated with improved outcome.

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APPENDIX 5

TOTAL AND DIFFERENTIAL CELL COUNTS IN BAL FLUID IN PATIENTS AND CONTROLS

TWCC		DAY							
survivors	0	1	2	3	4	5	6	POST1	POST 2
PATIENT 10	72.25	46	89.5					109.5	
PATIENT 11		88.5	62.25					99.5	
PATIENT 12		176.8	89.75					100	28.75
PATIENT 13	103.9	106.5	107.3	81.8	78			220	206.7
PATIENT 14			119.4		69.2			161.1	52
PATIENT17	167.4	107.5	60		127				
PATIENT 18	49.85	236.4	125.3	215.1	150	46.05	90		
PATIENT 19		116.5	64.5	83.9	94.2	40.9	34.7	18.6	27.5
PATIENT 20		30.2		40	108	49.2		95.3	55.3
PATIENT 21				111.05	161.7	33.55	31	16.3	
PATIENT 22	122.3	75.63	140	36.25	63.2			37.6	114
PATIENT 24			115.7	64.2	55.3	38.55		72	
PATIENT 29	58.85	80.1	25.15	12.85	15.9			31.6	7.7
PATIENT 30	1.8		113.7	75.7				75.9	40
PATIENT 32	3.3	0.895	3.95	3.22	0.655	0.72			
non-survivors									
PATIENT 8	610.8	222.3	93.25	141.25	102	91	110.3		
PATIENT 9	63.25		81						
PATIENT 23		25.4	8.5	49.5	38.45	20.15	22.75		
PATIENT 25	3	5.6	1.75	0.5	2	17.4	34.8		
PATIENT 26			10.9	5.64	7.5	3.5	2.1		
PATIENT 27	193.3	178.6	1046	386.4	168.9	102.1	78.85		
PATIENT 31	20.45								

TPMNC	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST 2
PATIENT 10	60.56	26.99	44.26					40.87635	
PATIENT 11		42.12	4.092					2.31835	
PATIENT 12		96.77	20.36					20.33	19.645
PATIENT 13	37.93	51.8	68.68	49.97	47.4574			106.326	175.7
PATIENT 14			71.64		40.2342			67.662	6.4116
PATIENT17	136.7	74.53	43.8						63.5
PATIENT 18	35.42	140.3	74.36	133.13	96.425	37.98	39.14		
PATIENT 19		98.19	39.57	39.818	50.3917	17.73		9.369	5.8888
PATIENT 20		24.56		33.732	83.16	26.81		75.287	12.166
PATIENT 21				16.212	10.6151	0.665	3.143	4.09456	
PATIENT 22	46.59	41.07	99.86	19.448	31.3374			14.664	97.664
PATIENT 24			58.57	21.348	31.994	11.18		17.28	
PATIENT 29	48.65	26.66	16.71	8.1533	4.04997			6.32	0.231
PATIENT 30			87.92	40.628	43.5135			18.932	
PATIENT 32	2.787							2.233	3.6396
non-survivors									
PATIENT 8	529.4	100.6	4.086	4.0049	7.50668	6.688	3.491		
PATIENT 9	12.19		46.71						
PATIENT 23		12.46	5.075	22.624	27.2594	11.4	15.01		
PATIENT 25			0.595			7.83	23.15		
PATIENT 26									
PATIENT 27	103.3	106.3	407.6	176.47	109.6	47.28	45.91		

TAMC	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST 2
PATIENT 10	11.08	17.17	37.46					57.30135	
PATIENT 11		42.24	54.1					83.58	
PATIENT 12		73.98	63.52					75	7.5699
PATIENT 13	62.13	52.75	34.09	30.303	28.2004			327.8	26.189
PATIENT 14			43.82		28.0728			90.216	42.988
PATIENT17	30.13	28.67	15.2					60.1091	
PATIENT 18	12.38	86.65	49.45	79.587	48.58	6.889	46		
PATIENT 19		16.52	23.45	38.751	41.2677	20.9		22.32251	11.097
PATIENT 20		5.333		4.8	20.1528	18.8		16.51549	40.551
PATIENT 21				89.111	145.869	30.24	25.21	9.88758	
PATIENT 22	71.83	31.81	31.26	15.05	29.516			19.67608	13.304
PATIENT 24			53.42	40.421	21.1228	24.03		52.0776	
PATIENT 29	9.807	52.3	7.614	4.2691	11.3445			24.016	7.084
PATIENT 30			24.64	33.308				28.59153	19.868
PATIENT 32	0.404							7.3	8.0796
non-survivors									
PATIENT 8	56.26	118.7	87.67	135.36	92.4433	79.73	104		
PATIENT 9	50.31		31.86						
PATIENT 23		12.2	3.257	25.581	10.149	3.973	7.024		
PATIENT 25			0.419			8.46	10.65		
PATIENT 26									
PATIENT 27	76.37	63.36	624.3	204.79	55.3647	51.04	30.95		

PMN%	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST 2
PATIENT 10		49.67	24.67					20.33	34.165
PATIENT 11	83	58.67	50					37.33	
PATIENT 12		47.67	7.315					2.33	
PATIENT 13	36.49	49.34	65.17	60.33	54.33			48.33	85
PATIENT 14			60		60.835			42	12.33
PATIENT 17	81.67	69.33	73		50				
PATIENT 18	71.89	59.33	63.67	61.5	61.67	80.69	49.5		
PATIENT 19		84.3	60.87	47.33	61.33	43.83	27	31.66	28.33
PATIENT 20		81.33		84.33	77	50.16		79	22
PATIENT 21				16	9	2	9.665	25.12	
PATIENT 22	38.17	52.83	71.33	48.835	50.17			39	85.67
PATIENT 24			50.5	31.5	55	14.5		24	
PATIENT 29	82.84	39.67	67.17	63.5	26.5			20	3
PATIENT 30			77.33	53.67				57.33	47.33
PATIENT 32	80.69								
non-survivors									
PATIENT 8	86.17	47.34	4.665	2.835	7.335	6.665	3.17		
PATIENT 9	19.84		64.33						
PATIENT 23		44.17	59	46.5	71.835	56.5	59.65		
PATIENT 25			28.35			45.67	67.17		
PATIENT 26									
PATIENT 27	53.17	58.84	43	45.67	65.835	46.67	59		

AM%	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST 2
PATIENT 10	16	37.33	41					52.33	
PATIENT 11		47.67	87.02					84	
PATIENT 12		47.17	68.5					75	13.165
PATIENT 13	59.85	48.83	30.67	37.835	42.83			38.33	12.67

PATIENT 14			36.7		38			56	82.67
PATIENT17	18	26.67	25.33		47.33				
PATIENT 18	24.24	36.67	34.35	37	35	16.35	45		
PATIENT 19		14.17	36.88	46.33	35.33	50.67	64.33	59.66	61.33
PATIENT 20		17.66		12	18.66	41.83		17.33	73.33
PATIENT 21				78.315	88	90.17	81.67	60.66	
PATIENT 22	58.67	43.33	22.33	45.33	46			52.33	11.67
PATIENT 24			46.34	64.835	40.835	31.17		72.33	
PATIENT 29	16.5	59	29.5	33.17	70.33			76	92
PATIENT 30			21.67	44				37.67	49.67
PATIENT 32	16.59								
non-survivors									
PATIENT 8	9.835	51.34	93.67	95.835	90.665	88.67	94.34		
PATIENT 9	79		33.33						
PATIENT 23		53.17	38.67	50.665	25.5	24.89	35.82		
PATIENT 25			19.97			47.17	30.34		
PATIENT 26									
PATIENT 27	40	36.17	55.34	53	31.835	49.67	38.5		

controls	TWBC	N%	N x 10*4	AM%	AM x 10*4
CONTROL 6	842.5	79.7	671.47	20.3	171
CONTROL 7	78	1	0.78	93.3	72.77
CONTROL 10	163	19.67	32.062	69.7	113.6
COMTROL 11	309.4	10	30.94	82.7	255.9
CONTROL 19	170	67	113.9	32	54.4
CONTROL 20	40	35.3	14.12	58	23.2
CONTROL 24	33	7.67	2.5311	83	27.39
CONTROL 27	8.5	10.67	0.907	86.33	7.338
CONTROL 28	49.4	58	28.652	38	18.77
CONTROL 34	21.7	53.67	11.646	44.33	9.62
CONTROL 1	106.5	1.33	1.4165	76.66	81.64
CONTROL 8	169	22.67	38.312	77.3	130.6
CONTROL 13	28.2	5	1.41	86.7	24.45
CONTROL 14	92.2	10.3	9.4966	82	75.6
CONTROL 15	27.8	4.67	1.2983	88	24.46
CONTROL 17	47.4	23.2	10.997	61.8	29.29
CONTROL 21	17.2	2.66	0.4575	93.66	16.11
CONTROL 23	32.1	8.7	2.7927	89.3	28.67
CONTROL 25	32	2.67	0.8544	92	29.44
CONTROL 30	20.4	2.33	0.4753	94.67	19.31
CONTROL 35	49	12.67	6.2083	85.67	41.98
CONTROL 2	54.5	2	1.09	88	47.96
CONTROL 3	188.5	68	128.18	22.7	42.79
CONTROL 4	90	66.7	60.03	24.67	22.2
CONTROL 5	900	82.7	744.3	14.3	128.7
CONTROL 9	102.5	2	2.05	94.67	97.04
CONTROL 16	103	38.7	39.861	48.7	50.16
CONTROL 18	40	9	3.6	90.67	36.27
CONTROL 22	84	13.66	11.474	80.66	67.75
CONTROL 26	185.1	76.33	141.29	20.33	37.63
CONTROL 29	16.1	2.33	0.3751	96.67	15.56
CONTROL 31	32.1	7.33	2.3529	91.33	29.32
CONTROL 32	7.5	2	0.15	95.67	7.175
CONTROL 33	10.8	2	0.216	92	9.936
CONTROL 36	30	18.33	5.499	80	24

CYTOKINE CONCENTRATIONS IN BAL FLUID IN PATIENTS AND CONTROLS

IL-8 (mcg/ml)		DAY							
survivors	0	1	2	3	4	5	6	1 POST	2POST
PT 10	4.483	3.84	15.843					6.487	
PT 11	1.816	3.375	1.273					2.436	
PT 12	0	10.26	1.186					2.117	5.884
PT 13	37.5	18.687	4.596	3.266	1.361			4.08	8.048
PT 14			7.33		3.27			0.685	5.238
PT 17	10.8	29.525	7.325	1.674				3.347	
PT 18	26.745		25.428	8.431		1.183	4.705		
PT 19		5.37	1.277	1.566	1.439	4.425	0.613	0.176	0.544
PT 20		0.369		2.24	1.955	0.338		2.536	4.278
PT 21				1.528	0.3485	1.271	0.265	0.812	
PT 22	5.059	5.365	8.019	2.512	1.182			2.018	7.128
PT 24			5.353	6.689	4.897			1.796	2.888
PT 29	1.364	7.13	12.249	1.758	1.69	0	0	2.5085	0.964
PT 30	0.338	0	19.172	6.74	0	0	0	11.85	9.953
PT 32	0.421	0.672	0.421	0.773	0.572	0.0905			
non-survivors									
PT 8	3.665	1.088	0.489	1.33	0.586	0.733	0.568		
PT 9	11.634		7.795						
PT 23		1.145	0.66	0.613	1.595	1.007	1.035		
PT 25	0.605	0.141	0.615	0.167	0.585	7.46	7.202		
PT 26		1.907	2.331		0.214	0.323			
PT 27	6.936	3.372	6.201	22.787	13.216	2.811	2.925		
PT 31	37.5								

IL-6 (mcg/ml)	DAY								
survivors	0	1	2	3	4	5	6	1 POST	2POST
PT 10	0.373	0.625	0.624					1.171	
PT 11	0.241	0.368	0.25					0.87	
PT 12		1.114	0.0997					0.745	1
PT 13	10	10	3.072	0.763	0.559			1.776	2.472
PT 14	0	0	1.69	0	0.702			1.059	0.15
PT 17	0.805	2.635	0.771	0.146				2.196	
PT 18	4.88	7.2	0.567	0.783	0.157	0.427	0.24		
PT 19		0.905	0.164	0.24	0.269	0.895	0.137	0.2	0.143
PT 20		0.146		0.2005	0.615	0.328		1.777	1.068
PT 21				0.209	0.0975	0.304	0.139	0.118	
PT 22	0.0894	0.233	0.28	0.279	0.406			0.244	0.691
PT 24			0.212	0.482	0.451	0.236		0.748	
PT 29	0.508	1.375	0.765	0.0813	0.0435			0.2815	0.256
PT 30	0.496	0	0.952	0.629				2.49	1.991
PT 32	0.0881	0.185	0.077	0.095	0.183	0.0243			
non-survivors									
PT 8	4.57	0.492	0.0672	0.176	0.128	0.0788	0.14		
PT 9	0.56		0.855						
PT 23		2.04	0.059	0.0839	0.0175	0.0999	0.464		
PT 25	2.142	0.187	4.3	0.0879	0.4575	5.51	5.227		
PT 26			0.484	0.363		0.109	0.0855		
PT 27	1.925	0.776	10	9.63		0.894	0.95		
PT 31	10								

TNF-a (pg/ml	DAY								
survivors	0	1	2	3	4	5	6	1 POST	2POST
PT 10	95.2	116.5	193					197	
PT 11	60	98	121					115	
PT 12		209	66.5					140	264
PT 13	1168	351	364	154	76			201	228
PT 14			141		128			196	104
PT 17	114	239	160	23.8				236	
PT 18	301.5	601.6	203	149	66.9	102	125		
PT 19		147.5	84.9	76.8	93.3	135.5	57.3	68.3	54.9
PT 20		86.2		99.5	124	103		98.7	94.1
PT 21				97.2	91	119	90.9	92.4	
PT 22	110	97.4	97.2	129	99			152	138
PT 24			127	134	109	108		171.2	
PT 29	76.3	135	118	105	84.4			110	98
PT 30	68.8		141	96.9				156	141
PT 32	81.8	73.9	76.3	63	36.1	72.5			
non-survivors									
PT 8	79.1	103	83.3	106.5	57.3	66.7	70.5		
PT 9	114		135						
PT 23		81.5	72.5	76.9	87	81.4	5		
PT 25	64	73.1	61.5	61.3		85	93.6		
PT 26			87.4	84.8		114	65.2		
PT 27	107	210	256	355		226	164.5		
PT 31	206.5								

IL-1B (pg/ml)	DAY								
survivors	0	1	2	3	4	5	6	1 POST	2POST
PT 10	19.7	19.2	60.2					69.5	
PT 11		10							
PT 12		81.3						27.5	94.9
PT 13	528	85.2	39.3	22.9				34.6	119
PT 14			64		25.4			22.1	34.5
PT 17	35	179	34.6	35.5				46.5	
PT 18	169	447.2	104	115	53.8	24.9	56		
PT 19		31.2	14.9	21.3	17.9	55.4		12.8	
PT 20		13.5		30.6	20	16.6		66.6	
PT 21				13	0		0		
PT 22	16.9	97.3	185	21.5	71.8				203
PT 24			19.4	34.3	54.7			28.3	0
PT 29	10.3	28.8	33.2					10.2	
PT 30			92.9	72.2				44.2	38.5
PT 32	0	0	0	0		0			
non-survivors									
PT 8	35.2	13	0	0	12.6	0	0		
PT 9	21.3		41.6						
PT 23			0	0	0	0	0		
PT 25		0	0		0	26.6	119		
PT 26		0		0		0	0		
PT 27	26.4	65	99.8	473	88	22.3	19.5		
PT 31	43.6								

CONTROLS

CONTROL	IL-8 (mcg	CONTRO	IL-6 (mcg/	TNF-a (p	CONTRO	IL-1B (pg/ml)
2	0.3495	2	0.116	104	2	0
3	27.22	3	10	638	3	1000
4	2.9	4	0.102	124	4	132
5	7.582	5	1.11	340	5	212
6	6.593	6	6	52.3	6	487
7	0.162	7	0.0546	96.7	7	11.2
8	5.357	8	0.539	184	9	0
9	0.353	9	0.0298	130	10	211
10	13.698	10	1.011	189	11	22.9
11	2.196	11	1.016	63.4	13	0
13	0.0498	13	0.0175	62.1	14	12.2
14	0.31	14	0.103	120	16	79.6
16	3.39	16	0.494	166.5	17	0
17	0.197	17	0.041	100.5	18	39.2
18	0.68	18	0.0865	101	19	108
19	7.205	19	0.961	83.7	20	16.1
20	0.416	20	0.0913	66.7	21	14.4
21	0.2565	21	0.041	102.5	22	47.3
22	2.348	22	0.135	97	23	14.2
23	0.593	23	0.0868	95.5	24	0
24	0.149	24	0.0482	71.4	25	0
25	0.671	25	0.05	54.3	26	400
26	6.157	26	0.332	765	27	12.6
28	5.334	27	0.0453	68.8	28	255
29	0.129	28	1.583	181	29	0
30	0.106	29	0.0243	57.2	30	0
31	0.307	30	0.0245	56.3	31	10.1
32	0.241	31	0.0663	55.8	33	19.9
33	0.357	32	0.0428	67.2	35	31.7
34	0.451	33	0.0609	86.9	36	75.6
35	0.287	34	0.121	75.4		
36	1.391	35	0.109	72.2		
		36	0.0566	76.1		

ANTI-INFLAMMATORY CYTOKINE BALF CONCENTRATION FROM PATIENTS AND CONTROLS

IL-1RA (pg/ml)	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST2
PT 10	207.73	156.82	341.364					895	
PT 11	1312.3	1295.9	1481.36					1577.9	
PT 12		1719.5	1001					1615.9	500.5
PT 13	2208.6	169.55	529.545	299.55	125				
PT 14			977.92		1105.61		720.45		
PT 18	1051.4	2438.6	855.909		198.636	405.909	920.45		
PT 19		705	347.92	1147.2	1130.23	265	186.82	84.091	105.9
PT 20				214.09	245.909	258.636		527.92	530.2
PT 21				131.36	271.364	113.182	70.455	48.636	
PT 22	165	250.45	522.273	252.27	1282.54			82.535	195.9
PT 24			414.843	258.64	375.909	312.273		674.09	
PT 29	163.3	1584.1	1547.92	1861.8	2216.38			2034.8	649.5
PT 30	1644.1		1135.61	1449.5				1040.2	429.5
PT 32	94.843	80.228	150.228	117.92	124.074	42.5354	381		
nonsurvivors									
PT 8	737.73	402.27	190.455	364.09	137.727	112.273	87.727		
PT 9	416		822.535						
PT 23		179.99	74.0909	1072.3	149.545	98.6364	191.36		
PT 25	312.27	82.273	58.6364	26.818	35	142.535	241		
PT 26			973.182	411.36		537.727	391.36		
PT 27	1069.5	744.09	2323.18	1638.6	2154.07	1890.23	1622.5		
PT 31	2083.3								

TNFR-1(pg/ml)	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST2
PT 10	95.98	122.41	139.041					221.69	
PT 11	99.041	76.388	76.3878					189.52	
PT 12		208.48	82.8542					114.55	71.29
PT 13	458.84	251.29	174.959	86.388	86.3878			98.633	257
PT 14			97.2292		98.2708				
PT 18	439.45	413.12	198.224		31.4898	57.8163	80.469		
PT 19		148.22	55.1458	106.81	145.771	145.163	87	46.592	46.59
PT 20				107	143.735	128.633		187.02	149.3
PT 21				45.571	54.3469	72.5102	32.102	53.939	
PT 22	77.204	58.837	58.4286	45.98	131.813			105.56	52.1
PT 24			127.021	71.898	108.837	25.3673		78.429	
PT 29	125.35	233.48	197.229	99.938	202.229			315.56	137.9
PT 30	10.146		175.563	77.021				147.44	214.3
PT 32	2.4375	13.896	8.0625	13.271	0	0	30.979		
nonsurvivors									
PT 8	108.43	56.592	23.3265	46.5	60.4694	45.3673	20.878		
PT 9	185.25		157.646						
PT 23		142.71	13.1224	13.939	49.0408	26.7959	36.592		
PT 25	14.755	0.8776	79.449	0	25.1633	220.354	297.85		
PT 26			0	20.061		8.63265	22.102		
PT 27	168.43	134.55	147.816	148.63	392.021	263.688	112.65		
PT 31	324.31								

TNFR-2 (pg/ml)	DAY								
survivors	0	1	2	3	4	5	6	POST1	POST2
PT 10	56.653	76.453	113.72					265.05	
PT 11	106.65	104.79	121.72					259.2	
PT 12		337.07	116.968					55.187	30.25

PT 13	345.99	206.39	160.253	82.653	20.2533			48.253	220.8
PT 14			149.096		139.096				
PT 18	293.32	314.25	229.587		57.32	49.72	90.52		
PT 19		154.65	129.84	123.35	158.032	105.853	59.853	30.92	12.52
PT 20				13.853	49.5867	52.92		180.27	74.41
PT 21				31.373	33.0533	34.12	15.053	0.2533	
PT 22	38.12	33.587	27.5867	19.853	69.6277			67.926	35.7
PT 24			130.798	54.653	55.1867	21.9701		70.179	
PT 29	179.31	331.01	258.989	172.5	342.819			500	104
PT 30	12.606		186.011	152.71				196.76	255.6
PT 32	2.9255	18.245	5.26596	10.585	0	1.2234	28.777		
nonsurvivors									
PT 8	63.587	72.866	18.7867	17.253	29.9867	12.92	0.52		
PT 9	197.98		170.053						
PT 23		63.12	3.58667	2.7867	29.8533	17.32	28.653		
PT 25	4.806	0	20.3867	0	0.38667	128.777	165.9		
PT 26			23.9867	11.672		4.52	10.387		
PT 27	95.453	89.453	112.387	152.52	500	240.798	162.07		
PT 31	200.05								

CONTROLS	IL-1RA (pg/ml)		TNFR-1(pg/ml)		TNFR-2 (pg/ml)
2	544.84	2	107.646	2	25.3723
3	2230.5	3	171.898	3	132.387
4	373.3	4	57.4375	4	84.2021
5	1555.6	5	159.938	5	0
6	2251	6	505.771	6	358.457
7	456.38	7	114.521	7	46.4362
8	1814.1	8	97.6458	8	51.3298
9	529.46	9	44.9375	9	0
10	2164.1	10	269.104	10	217.606
11	188.69	11	217.438	11	99.3085
13	138.69	13	46.8125	13	27.9255
14	1277.9	14	24.7292	14	0
16	505.61	16	120.771	16	145.479
17	302.54	17	0	17	0
18	1587.9	18	13.8958	18	12.6064
19	621.77	19	158.271	19	135.798
20	201	20	67.0208	20	35.7979
21	124.84	21	44.3125	21	25.1596
22	1117.9	22	56.1875	22	43.5638
23	270.23	23	66.8125	23	63.6702
24	350.23	24	40.3542	24	43.2447
25	1034.8	25	15.1458	25	30.6915
26	871.77	26	140.146	26	188.989
27	145.61	27	24.7292	27	17.7128
28	1249.5	28	140.146	28	97.7128
29	87.92	29	13.8958	29	15.7979
30	158.69	30	46.1875	30	86.2234
31	1734.1	31	38.6875	31	15.0532
32	157.15	32	14.1042	32	16.4362
33	499.46	33	29.5208	33	17.8191
34	187.92	34	37.6458	34	80.6915
35	808.69	35	58.4792	35	54.734
36	314.84	36	36.1875	36	35.0532

CYTOKINE CONCENTRATIONS IN PLASMA OF PATIENTS AND CONTROLS

IL-8 (pg/ml)	DAY								
survivors	-1	0	1	2	3	5	6	POST 1	POST 2
PT 10	343	402	160	160					
PT 11	105	153	87.1	87.8				92.4	
PT 12	186		133	86				63.3	40.3
PT 13		7500	10593	948	172				57.7
PT 14		972	62	706				87.3	98.8
PT 15		203	366		288			125	99.9
PT 17	1775	1943	2011	454	237			114	
PT 18		5570	16991	6700	1031	131	92.5		
PT 19	405		484	58	66.4			50.2	35.8
PT 20			164		96.6	59.6		42.5	43
PT 21	54.8		45.5		61.3	133	208		
PT 22		100	117	92.6	95.8			179	
PT 24		300		157	139			76.4	
PT 29		334	1657	471	165			69.6	47.3
PT 30	19041	1544		695	711			242	133
PT 32	73.2	90.9	45.5	38.2		48.7			
non-survivors									
PT 8			69.1	94.9	97.7	72.6	56.7		
PT 9	123	2416		124					
PT 23			235	287	188	200	278		
PT 25		204	152	139	152	103			
PT 26	170			474	1178	201	160		
PT 27		61	61.4	228	189	65.2	62.3		
PT 31		665							

IL-6 (pg/ml)	DAY								
survivors	-1	0	1	2	3	5	6	POST 1	POST 2
PT 10	2000	431	141	139					
PT 11	182	130	70	109				180	
PT 13		2000	2000	2000	2371			61.6	
PT 14		2000	704	287				58.1	70.3
PT 15		634	1802		76.8			46.1	73.3
PT 17	1852	319	414	71.7	102			156	
PT 18	2000	9815	2764	892	308	48.3	33.9		
PT 19	2414		338	138	300			45.9	64.4
PT 20			249		343			154	93.2
PT 21	164		19.4		74.5		38.3		
PT 22		108	105	54.2	54.8			54.5	
PT 24		1485		90.8	55.7				65.7
PT 29		271	311	97.6	31			32.3	30.5
PT 30	2000	2000						203	146
PT 32	167	127	19.4	19.3		22.5			
non-survivors									
PT 8			313	169	57.8	46.7	45		
PT 9	361	5804		58.5					
PT 23			1081	719	888	107	824		
PT 25		3415	1500	1112	779	367			
PT 26	3421			372	293	84.2	61.1		
PT 27		208	99.4	285	114	44.6	53.5		
PT 31		2000							

TNF-a (pg/ml)

survivors	-1	0	1	2	3	5	6	POST 1	POST 2
PT 10	80.4	88.4	89.2	120					
PT 11	56.6	146	76.2	102				143	
PT 12	98.6		113	129				117	62.4
PT 13		321		98.8	89				59.5
PT 14		80.3	100	188				113	66.2
PT 15		452	107		127			89.5	89.2
PT 17	104	95.4	92.9	132	121			95.6	
PT 18	99.8	199	69.8	75.7	142	101	82		
PT 19	56.5		54.5		100				96.7
PT 20			68.7		113	78.9		60.1	57.2
PT 21	49.3		44.1		109		93.1		
PT 22		65.1	71	97.6	73			73.7	
PT 24		60.9		50.6	61.1			59	
PT 29		197	87.2	60.9	112			60.5	56.6
PT 30	97.5	76.6		94.1	91.2			79	83.2
PT 32	79.1	115	44.1	40.2		45.1			
non-survivors									
PT 8			78.8	135	87.1	82	76.8		
PT 9	49.6	179		163					
PT 23			199	187	157	116	125		
PT 25		91.5	115	62.1	73.4	134			
PT 26	68.5			153	167	120	137		
PT 27		71.7	72.1	106	112	71.4	94.1		
PT 31		48.5							

CONTROLS (concentrations in pg/ml)

control	IL-8	contol	IL-6	control		control	TNF-a	control	
2	0	2	14.2	33	17.5	2	57.7	33	56.8
3	101	3	177	34	53.1	3	29.2	34	58.3
4	0	4	24.2	35	19.2	4	76.6	35	87.1
5	53.8	5	20.8	36	19.9	5	77.5	36	58.2
6	289	6	90.5			6	85.1		
8	0	8	23.3			8	74.9		
9	0	9	16.7			9	65.4		
11	66.6	11	416			11	73.6		
12	30.3	12	231			12	53.3		
13	0	13	15			13	67.5		
14	0	14	20.5			14	67.4		
16	43.4	16	61.4			16	66.5		
21	0	17	14.1			17	47.8		
22	0	21	16.8			21	55.6		
23	0	22	16.7			22	60.1		
25	0	23	35.3			23	76.1		
26	0	24	19.3			24	65.6		
27	0	25	14.6			25	66.6		
29	0	26	13.7			26	51.4		
30	0	27	40.8			27	69.7		
32	0	29	16.9			29	52.3		
33	0	30	12.9			30	59.4		
34	35.2	31	19.5			31	50.1		
35	0	32	29.8			32	62.1		

APOPTOTIC ACTIVITY IN BAL FLUID FROM SURVIVORS, NON-SURVIVORS AND CONTROLS

DAY	0	1	2	3	4	5	6	POST1	POST2
PT 10	2.24	4.71	0.64	1.54		2.14		2.14	
PT 11	1.58	1.32	0.36					5.91	
PT 12		4.27	0.6					1.63	5.16
PT 13	3.53	8.61	4.71	4.12	8.08			27.76	12.22
PT 14			1.54	2	43.08			1.3	1.32
PT 17									
PT 18	0.4	17.29	5	9.68	3.38	4.52	50.05		
PT 19		13.84	28.61	5.27	56.95	6.59	20.35	20.35	1.42
PT 20		5.21		1.05	0.87	0.87		1.74	3.47
PT 21				3.31	2.11	2.94	21.44	1.14	
PT 22	0.84	18.97	0.79	1.27	1.12			8.12	
PT 24			0.43	1.22		5.24		0.5	
PT 29	1.1	3.74	1.42	1.38	2.31			3.15	0.33
PT 30	2.5		0.86	1.01				0.37	0.87
PT 32	1.28	1.28	3.42	1.76	3.24	3.42			
nonsurvivors									
PT 8*	1.4	2.4		1.01	3.82	0.32	0.65		
PT 9*	0.87	1.79	0.79						
PT 23*	1.39	4.12	6.95	20.03	3.44	2.38	58.5		
PT 25*	7.8	1.18	1.39	1.55	4.41	83.13	0.71		
PT 26*									
PT 27*	1.91	0.68	1.94	1.59	0.81	0.56	1.13		
PT 31*	0.88								

CONTROLS

Code	ACTVITY		
CON 10	1.509481	CON 32	2.044808
Con 13R	3.235294	CON 33	1.218817
CON 14L	2.352941	CON 34 R	1.053667
CON 16 L	0.545731	CON 35	1.793147
CON 17R	1.839547	CON 36 L	5.232581
CON 18 L	5.926646	CON 4	5
CON 19 L	0.995257	CON 5R	1.025992
CON 20	7.755545	CON 6 R	0.306845
CON 21L	0.387197	CON 7L	5
CON 22 L	3.971117	CON 8R	0
Con 23R	0.985085		
CON 24 R	0.3219		
CON 25 L	1.474967		
Con 26R	1.857556		
CON 27	1.600644		
CON 28R	1.365412		
CON 29 L	0.845396		
CON 2R	1.816305		
CON 3	0.500999		
CON 30R	1.551223		
CON 31 L	1.239405		

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