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# AN INVESTIGATION OF THE PULMONARY SURFACTANT SYSTEM IN CHILDREN WITH SEVERE RESPIRATORY SYNCYTIAL VIRUS INFECTION

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Submitted for the degree of PhD to the University of Glasgow Faculty of Medicine

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# ABSTRACT

#### BACKGROUND

Severe infection with Respiratory Syncytial Virus (RSV) is an important cause of respiratory failure in infants and young children. Pulmonary surfactant is a surface-active complex of phospholipids and proteins which lines the alveolar surface of the lung. Clinical similarities of severe RSV infection to Respiratory Distress Syndrome of the newborn (RDS) and Adult Respiratory Distress Syndrome (ARDS) suggest that surfactant abnormalities may be involved in the pathogenesis of the disease.

#### HYPOTHESIS

The hypothesis tested in this study is that the pulmonary surfactant system is dysfunctional in severe RSV infection, due to deficiency, abnormal composition, damage or inhibition.

#### PATIENTS AND METHODS

#### Patients

Non-bronchoscopic bronchoalveolar lavage was performed on 18 children ventilated for severe RSV infection and 16 children ventilated for surgical procedures and post operative care. Sequential daily samples were collected from those patients ventilated for more than 1 day.

#### Methods

Bronchoalveolar lavage fluid was examined for surfactant function, surfactant content and composition, indices of surfactant damage and evidence of surfactant inhibition as follows:

#### Surfactant function

1. Surfactant activity in whole BAL sample - shake and click tests

2. Surface activity of an organic extract of BAL fluid - pulsating bubble surfactometer (PBS)

Surfactant content and composition

1. Surfactant proteins A,B and D - enzyme-linked immunosorbent assays (ELISA)

2. Total phospholipid - gas chromatography-mass spectrometry (GC-MS)

3. Phospholipid classes - high performance thin layer chromatography (HPTLC) and scanning densitometry

4. Phospholipid long chain fatty acids - GC-MS

#### Indices of surfactant damage

1. Malondialdehyde (a lipid peroxidation product) - high performance liquid chromatography

2. Nitrotyrosine (an index of damage by peroxynitrite) - ELISA

3. Lysophosphatidylcholine and glycolipids - HPTLC

Surfactant inhibitors and inhibitory capacity of BAL proteins

1. Total protein - Lowry method

2. Albumin (a surfactant inhibitor) - immunoturbimetry

3. C-reactive protein (a surfactant inhibitor) - immunoturbimetry

4. D-dimer fibrin degradation product (a surfactant inhibitor) - ELISA

5. Surface activity of bovine surfactant plus proteins from 40,000g supernatant of BAL fluid - PBS

#### RESULTS

The following results were found in ventilated patients with RSV infection compared to controls:

#### Surfactant function

- 1. Surfactant activity in the whole BAL sample, measured by shake and click tests was reduced.
- 2. Surfactometer studies of organic extracts of BAL fluid (adjusted to 1mM phospholipid) showed no evidence of functional impairment. This measurement assessed surfactant function in isolation from surfactant inhibition or deficiency.

#### Surfactant content and composition

- 1. Surfactant proteins A, B and D were reduced. The reduction in protein A was small but significant. Median surfactant protein A concentration was lower on the first day of ventilation than on the last in children with RSV infection. No pattern was evident for surfactant proteins B or D.
- 2. Surfactant protein B was reduced when expressed per µg phospholipid
- 3. Total phospholipid content of BAL was reduced.
- 4. The combined fraction of phosphatidylcholine and sphingomyelin (PC&S which could not be separately distinguished by densitometry) was reduced. Phosphatidylserine and phosphatidylglycerol (PG) fractions were increased. There was no difference between groups in the proportion of phosphatidylinositol detected. The changes in PC&S and in PG were small. Phosphatidylinositol and PG varied reciprocally throught the illness in patients with RSV. The proportion of phosphatidylethanolamaine (PE) present in BAL fluid could not be assessed, as PE co-migrated with a glycolipid fraction in samples from the RSV group.
- 5. The 14:0, 16:0 and 16:1 species of phospholipid fatty acids were reduced. The 18:0 and 18:1 species were increased. No difference was found in 18:2 and 20:4 species between groups. *Indices of surfactant damage*
- 1. Malondialdehyde was reduced and nitrotyrosine was increased.
- 2. Lysophosphatidylcholine and glycolipids were increased.
- Evidence for surfactant inhibition
- 1. Total BAL protein was elevated, as was the D-dimer fibrin degradation product.
- 2. There was no difference in BAL albumin between patients with RSV and controls.
- 3. C-reactive protein was not detected in any BAL samples
- 4. Pulsating bubble surfactometer studies showed that 1mg/ml of BAL supernatant proteins from

patients with RSV inhibited bovine surfactant more than 1mg/ml of BAL supernatant proteins

from controls.

#### CONCLUSIONS

It was concluded that in children with severe RSV infection, surfactant was dysfunctional. There was evidence that two mechanisms contributed to this:

1. Pulmonary surfactant proteins and phospholipids were deficient.

2. Surfactant surface activity was inhibited.

Surfactant phospholipid and fatty acid composition was abnormal, and surfactant damage was present. However, the surface active properties of an organic extract of BAL fluid were intact. This indicated that damage to surfactant and change in composition did not reduce surface activity. There was minimal damage to lipids by peroxidation.

In conclusion, the pulmonary surfactant system is abnormal in children with severe RSV infection. Surfactant abnormality may be an important factor leading to respiratory failure in these children.

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## **AUTHOR'S DECLARATION**

I declare that the work contained in this thesis is original, and is the work of one author. Dr Margaret Kerr.

Collection and analysis of bronchoalveolar lavage samples was carried out by the author, with the exception of the analyses listed below;

- Maiondialdehyde was assayed in Dr John Paterson's laboratory at the Crichton Royal Hospital, Dumfries.
- Nitrotyrosine was measured in the laboratory of Dr Mike Jacobs, Royal Free Hospital, London.
- 3. Fibrinogen and fibrin degradation products were determined by Dr Ann Rumley in the Department of Medicine, Glasgow Royal Infirmary.
- Albumin and C-reactive protein assays were performed by myself and Mr John Black at the Royal Hospital for Sick Children, Glasgow.

Signed

(Dr Margaret H.Kerr) Date ^

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### 1. INTRODUCTION

#### **1.1. RESPIRATORY SYNCYTIAL VIRUS INFECTION**

#### 1.1.1. EPIDEMIOLOGY AND DIAGNOSIS

#### Epidemiology

Respiratory Syncytial Virus (RSV) is one of the most important viral pathogens of infancy and early childhood. It causes annual epidemics of respiratory disease mainly in children under one year of age. Epidemics are of 3-5 months in duration and occur most commonly in winter and early spring (Kim et al. 1973). The disease is widespread in the community, and, during an epidemic, 44% of families with young children may be affected (Hall et al. 1976). If a child becomes infected with RSV, the risk of hospitalisation is between 1 and 2 percent (Sims et al. 1976). Usually, if hospital admission is required, the infection has spread from the upper respiratory tract to the small airways (bronchiolitis) or the lung parenchyma and alveoli (pneumonia).

Although only a small percentage of children who contract RSV infection become ill enough to require hospital care, during an epidemic, cases of RSV bronchiolitis and pneumonia account for a sizeable proportion of the workload in a children's hospital. In one study, nearly 85% of children admitted to hospital with respiratory disease in an 'epidemiologic' year (ic. from the beginning of one epidemic to the beginning of the next) had viral bronchiolitis or pneumonia (Mufson et al. 1973). When infants are hospitalised with these conditions, RSV is the pathogen involved in over 70% of cases

(Sims et al. 1976). While severe infection can occasionally be fatal, especially in infants with underlying disease such as congenital heart defects and immunosuppression, mortality rates for hospitalised children with the virus are now around 1% (Wang et al. 1995; Navas et al. 1992)

#### Diagnosis

Culture of RSV may be difficult, as the virus is labile. Diagnostic tests based on the detection of viral antigens in respiratory epithelial cells by immunofluorescence are now both very rapid and highly sensitive and specific. Such tests have replaced culture-based systems. Recent work has been directed towards more efficient methods of viral sampling (Mackie et al. 1991) and to the development of near-patient diagnostic tests. Molecular techniques based on polymerase chain reaction are also now available. The increased sensitivity of testing methods may reveal that an even greater proportion of bronchiolitis and pneumonia is due to RSV infection than has hitherto been documented.

#### 1.1.2. CLINICAL COURSE

The clinical course and severity of RSV infection varies greatly. Typically, the illness starts with rhinorrhoea and pharyngitis which persist for one to three days before the development of a cough. Narrowing of the small airways is a characteristic of the disease, and this gives rise to an audible wheeze. Rhinorrhoea and intermittent low grade pyrexia persist throughout the illness. The clinical condition may worsen, with increased coughing, wheezing and crepitations accompanied by intercostal recession and hyperexpansion (Ackerman and Salva, 1994).

At the most severe end of the spectrum, small airways become obstructed by plugs of mucus and cellular debris, giving rise to atelectasis and increased pulmonary shunting. Airway resistance increases, work of breathing is increased, and respiratory failure with hypoxaemia and metabolic acidosis may supervene. In such children, poor air entry, central cyanosis, tachypnoea, listlessness and, occasionally, periods of apnoea herald a need for urgent ventilatory support. The support required ranges from continuous positive airways pressure to full intermittent positive pressure ventilation or, in very severe cases, extracorporeal membrane oxygenation.

#### **1.1.3. PATHOLOGICAL CHANGES IN SEVERE RSV INFECTION**

At a microscopic level, the consequences of severe RSV infection involve damage to the respiratory epithelium, increased mucus secretion, a fibrinous exudate and invasion of the alveoli and bronchi by inflammatory cells.

Information on the histopathological changes which accompany severe RSV infection has been gleaned from animal studies and from post mortem studies of lung sections from patients who died of the disease.

#### **Human studies**

While RSV infection is common, pathological data are surprisingly sparse. The available data on the human lung come mainly from fixed post-mortem specimens. The series are small (Holzel et al. 1963; Wright et al. 1997; Gardner et al. 1970), often being case reports of one or two fatal cases.

Aherne and colleagues (1970) studied post mortem lung tissue from 9 patients in which RSV was recovered, and who were classed as having RSV bronchiolitis or pneumonia. In all these patients, there was necrosis of the bronchiolar epithelium and a peribronchiolar infiltrate of lymphocytes. Small airways were blocked by plugs of mucus, cell debris and fibrin, leading to subsegments of alveolar collapse. These patients were considered to have 'bronchiolitis'. In four patients, in addition to the changes of bronchiolitis, the process extended to the alveoli (pneumonia), and in these patients, pathological features included inflammatory infiltration of the air spaces and parenchyma, alveolar oedema and exudate and focal necrosis of areas of lung. The pathological picture in the group with pneumonia was heterogeneous, with different features prominent in each patient.

Gardner et al (1970) reported post mortem findings from two infants who died within two hours of onset of lower respiratory tract symptoms. Lung sections from these patients showed 'acute bronchiolitis' with epithelial necrosis, peribronchiolar infiltration of lymphocytes, increased mucus secretion and thick plugs of fibrin in the bronchioles. In these children, the alveoli were enlarged - presumably due to gas trapping behind partially obstructed small airways - but contained no cellular infiltrate. In a third child who died after 5 days of 'suspected pneumonia', an inflammatory exudate containing mononuclear cells was seen in the small bronchioles, alveoli and alveolar ducts, and cellular infiltration of interstitial tissue was evident.

Another case report from Halzel et al (1963) reported desquamation of cilated columnar epithelium, lymphocytic infiltration of bronchial walls, and small areas of

consolidation in a post mortem lung specimen from a 10 month old boy who had died from RSV bronchiolitis.

Recently, Wright and colleagues (1997) reported findings from immunostaining of post mortem lung tissue from two patients who had died of 'severe RSV pneumonitis'. In both cases, viral antigen staining was demonstrated in bronchial and bronchiolar epithelial cells. Alveolar pneumocytes staining positive for viral antigens were seen lining distorted alveolar spaces in one of the two cases with no distinction being made between type I and type II pneumocytes. In the second case, staining was confined to small airways. The number of antigen positive cells varied from airway to airway in both cases.

To date, only one study has reported on bronchoalvcolar lavage (BAL) findings. Lavage fluid from ventilated infants with severe RSV infection was examined, and found to contain around 80% neutrophils, 10% lymphocytes and 10% mononuclear phagocytes. Eosinophils accounted for less than 1% of cells seen (Everard et al. 1994). These findings seem to be at odds with the peribronchiolar infiltrate of lymphocytes described by previous authors (Aherne et al. 1970; Holzel et al. 1963; Gardner et al. 1970) who studied post mortem tissue sections. However, Everard and colleagues (1994) suggest that this discrepancy may be explained by rapid migration of neutrophils into the airway lumen in vivo.

#### Animal studies

As yet, no fully satisfactory animal model of human RSV infection has been developed. It is difficult to reproduce the clinical picture of severe RSV infection by inoculating

animals with human RSV, and responses to bovine RSV vary between species. There are, however, enough histopathological similarities between human RSV and animal models for the latter to be of some use in understanding human disease.

Infection with Respiratory Syncytial Virus is followed by the clinical symptoms of a lower respiratory tract illness only in large animals. For example, calves infected with the bovine strain of the virus develop necrosis of the bronchiolar epithelium and an extensive peribronchiolar infiltrate of inflammatory cells. In these animals, viral antigens can be demonstrated by immunofluorescence in the cells of alveolar walls and bronchiolar epithelium. However, infection of calves with a human isolate of RSV does not lead to development of the clinical signs of respiratory disease (Stott and Taylor, 1985). Lambs experimentally infected with bovine RSV have been found to develop focal areas of hung consolidation, necrosis of pulmonary epithelial cells and accumulation of cell debris (Cutlip and Lehmkuhl, 1979). In adult sheep exposed to bovine RSV, viral antigen has been found in bronchiolar epithelium, Type I pneumocytes, Type II pneumocytes, alveolar macrophages and mononuclear cells within the alveolar spaces (Meehan et al. 1994).

Experimental RSV pneumonia has been reproduced in monkeys after inoculation with a strain of RSV known to cause severe pulmonary infection in humans. These animals developed areas of consolidation with thickening of the alveolar walls which were infiltrated by polymorphonuclear leukocytes and mononuclear inflammatory cells. Multinucleated giant cells (syncytia) with eosinophilic cytoplasmic inclusions were present in the alveoli along with proteinaceous fluid and inflammatory cells. In these primates, viral antigen was detected by immunofluorescence in occasional tracheal

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epithelial cells, and, more extensively in alveolar cells, lung parenchyma and bronchiolar epithelium (Richardson et al. 1978).

By contrast, attempts to inoculate small animals eg. mice, rats, guinea pigs and ferrets with human RSV have not produced clinical signs of lower respiratory tract disease (Graham et al. 1988; Prince et al. 1978; Hegele et al. 1993; Stott and Taylor, 1985).

### **1.2. THE PULMONARY SURFACTANT SYSTEM**

#### **1.2.1. INTRODUCTION**

Pulmonary surfactant is a surface-active complex of proteins and phospholipids which lines the alveolar surface of the lung. Surfactant helps to maintain alveolar patency throughout the respiratory cycle, and thus allows adequate pulmonary gas exchange. Other functions attributed to pulmonary surfactant include protection of cell surfaces, assistance in keeping the alveoli free of fluid, participation in the defence against micro-organisms and maintenance of small airway patency.

#### 1.2.2. SURFACTANT PHOSHOLIPIDS

The surfactant lipid-protein complex consists of approximately 90% lipids and 5-10% surfactant-specific proteins (King et al. 1973). Phospholipids account for 80-90% of the surfactant lipids, with the remaining 10-20% comprising cholesterol, triacylglycerols and free fatty acids (King, 1984).

#### **Phospholipid classes**

Six different phospholipid classes have been isolated from pulmonary surfactant. In humans, phosphatidylcholine (PC) is the most abundant, at around 60 - 80% of total phospholipids, with phosphatidylglycerol (PG) the next most abundant at around 10 -20%. In adult humans, the relative proportions of phospholipids vary considerably between studies (reviewed by Ratjen et al (1996)). Recently, Ratjen and colleagues (1996), using high performance liquid chromatography (HPLC), characterised phospholipid classes in normal children between 3 and 15 years of age. In this age group, children had a higher proportion of PC (>80%) and a lower proportion of PG (<10%) than adults. There are at present no data on the phospholipid classes present in surfactant from normal children under 3 years old.

The other surfactant phospholipid classes, which are less abundant than PC and PG in humans, are phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (S). Each of these classes individually accounts for less than 10% of the total phospholipid complement of lung lavage fluid in humans (Ratjen et al. 1996).

In other animal species, PC is also the most abundant class. However, it can be seen from Table 1 that PG accounts for a relatively small proportion of surfactant phospholipid in some animals (chicken, monkey, cat and pig).

#### Total fatty acid composition of surfactant phospholipids

Several analyses have been performed on the total fatty acid composition (as opposed to individual molecular species) of PC and PG in humans and animals (Tables 2 and 3).

The results of these analyses show that 16:0 is the predominant fatty acid in human PC, at around 73%. Human neonates have less 16:0 (58.9%) and more 18:1 and 18:2. Palmitic acid is also the predominant fatty acid in other species of animals (accounting for between 64 and 87% of total PC lipid). The predominant fatty acids of PG can be seen from Table 3 to be 16:0 and 18:1.

#### **Phospholipid species - PC**

All phospholipids in surfactant are composed of a charged, hydrophilic head group, and two hydrophobic hydrocarbon tails. The hydrocarbon tails vary with age, between different samples, and between animal species.

Hunt and colleagues (1991), using HPLC, examined the molecular species of lavage PC from adults undergoing bronchoscopy, and found 67.6% to consist of the 16:0/16:0 (dipalmitoylphosphatidylcholine - DPPC) species, 12.7% to be 14:0/16:0, 7.9% 16:0/16:1, 6.1% 16:0/18:2 and 5.8% 16:0/18:1. Lavage PC was enriched in 14:0/16:0 and 16:0/16:0 compared to lung tissue PC and contained less 16:0/18:1 and 16:0/18:2 than tissue PC. There is evidence of variation with age in that in lavage PC from neonates, the same authors found 51.1% 16:0/16:0, 12.4% 14:0/16:0, 16:0% 16:0/16:1, 8.2% 16:0/18:2 and 12.7% 16:0/18:1.

The dipalmitoyl species is also a major component of animal surfactant PC. Work done on rabbit surfactant indicates that the dipalmitoyl (16:0/16:0) species also accounts for over 50% of PC in these animals. Three other species: palmitoyl-oleoyl (16:0/18:1), palmitoyl-linolcoyl (16:0/18:2) and palmitoyl-palmitoleoyl (16:0/16:1), together make up around 40% (Hayashi et al. 1990). While Khan et al (1995) found that 16:0/16:0 represented 39% of all PC's in a lipid extract of calf lung surfactant, disaturated species: 16:0/14:0 plus 16:0/16:0 together accounted for 54%.

The fatty acid composition of surfactant PC may have important implications for surface activity, and this is discussed in later sections.

#### **Phospholipid species - PG and PI**

Approximately 60% of PG in rabbits was found by Hayashi and colleagues (1990) to be composed of a roughly equal mixture of dipalmitoyl and palmitoyl-oleoyl species. The species pattern of PG and PI in rat surfactant has been shown to be similar, containing about 26% 16:0/18:1 species and 15% 16:0/16:0 species (Rustow et al. 1988).

# Table 1 Proportions of phospholipid classes in animal surfactant. All numbers given are percentages

	calf (a)	monkey (b)	cat (c)	dog (c) dog (g) dog (h)	rabbit (c) rabbit (f)	rat (c)	pig (d)	frog (c)	chicken (c)
PC	84	83.2	86.3	81.3 75 74 9	83.6 79.8	87.0	67.1	76.3	79.2
РG	5	1.4	2.1	11.1 10 12.3	8.0 3.7	8,3	2,0	9,1	0
PE	3	2.1	3.2	2,0 5 3.9	3.5 3.5	0.7	+ <b>PS</b> 7.1	6,4	5,2
PS				3 3.0	3.1 +P1			1.5	3.0
PI	4	10,3		2 2.4			3,3	4.5	8.9
S	2	1.3		3 3.8	1.7		2.7	0.8	2.9
LPC	<1	0.7			0.6		0.5		1

(a) (Hall et al. 1994) (b) (Egberts et al. 1987) (c) (Shelley et al. 1984) (d) (Body, 1971) (e) (Hallman and Gluck, 1976) (f) (Keough et al. 1985) (g) (Kumar et al. 1985) (h) (Clerex et al. 1989)

#### Table 2. Percentages of fatty acids in bronchoalveolar lavage PC

14:0	3.1	human (b) neonatal (d) human (e) human (h) 3.5	cat (b) turkey (g) 3.8	dog (b) dog (i) 2.6	rabbit (b) rabbit (f) rabbit (g) 2.3	rat (b)	pig (c)
		4.1 7.4 3.2	2.3	5	2.9 4.0		
14:1	1.8						
14:2	0.5	2.6 (d)			<u> </u>		
16:0	64.0	73.1 58.9 74.5 72.5	65.7 86.6	65.8 68	71.6 79.5 86.9	72.8	73.4
16:1	7.5	7.3 6.9 2.1 5.6	2.8	7.4 12	5.5 1.7	10.4	6.3
16:2	0.4		<u> </u>				
18:0	2.4	3.2 4.2 3.1 3.9	3.9 4.0	6.7 6	1.6 1.6 1.1	2.4	3.0
18;1	17.3	8.9 12,7 7.0 10.0	13.9 5.6	12.8 7	11.9 10.8 4.8	4.7	10.4
18:2	1.6	2.0 10.1 4.2 4.8	3.0 1.5	1.8	5.7 3.3 3.1	3.7	0.8
18:3	0.3						
18:4	0.4						
20:4	0.5						

(a) (Yu et al. 1983) (b) (Shelicy et al. 1984) (c) (Body, 1971) (d) (Motoyama et al. 1976) (c) (Post et al. 1982) (f) (Keough et al. 1985) (g) (Fujiwara et al. 1970)
(h) (Pison et al. 1989) (i) (Kumar et al. 1985)

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### Table 3. Percentages of fatty acids in bronchoalveolar lavage PG

cow (a)	human (b) human (c)	cat (b)	dog (b)	rabbit (b)	rat (b)
0.8	1.4 (b)				
0.6					
					· · · · · · · · · · · · · · · · · · ·
51.2	22.2 31.4	36.0	54.5	50,5	58.9
4.3	3.0 1.1	0.8	3.3	3.2	4.0
0,4					
4.7	16.7 13.9	6.3	12.6	5.3	5.8
33,0	51.3 40.4	31.0	23.4	33.5	19.2
2.9	4,0	11.2	1.6	5.0	8.7
0.5					
0,3					
1.0					
0.3					<u> </u>
	cow (a)         0.8         0.6         51.2         4.3         0.4         4.7         33.0         2.9         0.5         0.3         1.0         0.3	cow (a)       human (b) $0.8$ $1.4$ (b) $0.6$ $1.4$ (b) $0.4$ $2.2.2$ $31.4$ $4.3$ $4.7$ $16.7$ $13.9$ $33.0$ $51.3$ $40.4$ $2.9$ $4.0$ $6.0$ $0.5$ $0.3$ $1.0$ $0.3$ $0.3$	cow (a)       human (b)       cat (b) $0.8$ $1.4$ (b)	cow (a)human (b) human (c)cat (b)dog (b) $0.8$ $1.4$ (b) $$	cow (a)human (b) human (c)cat (b)dog (b)rabbit (b) $0.8$ $1.4$ (b) $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.6$ $0.4$ $0.4$ $0.4$ $0.4$ $0.4$ $0.4$ $1.0$ $0.3$ $0.3$ $0.3$ $0.3$ $0.3$

(a) (Yu ct al. 1983) (b) (Shelley et al. 1984) (c) (Post et al. 1982)

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# **1.2.3. THE ROLE OF SURFACTANT PHOSPHOLIPIDS IN THE ALVEOLUS**

#### Lamellar bodics

Surfactant is synthesised and packaged into lamellar bodies in type II cells which, together with type I cells, line the alveoli. Lamellar bodies are granular structures visible in the cytoplasm of type II cells in frozen sections or on electron microscopy. They are composed of surfactant phospholipid bilayers tightly packed around each other and woven into a complex array with a variety of proteins. Once formed, these granules are secreted from the Type II cells by exocytosis into an aqueous hypophase which is thought to line the alveolus (Figure 1).

#### Tubular myelin

As well as lamellar bodies, electron microscopic studies (Bastacky et al. 1995) suggest the presence, in the hypophase, of tubular myelin, a mesh-like matrix formed from surfactant lipids and proteins. It has been suggested that tubular myelin provides a pool of surfactant for adsorption to the air-liquid interface in the alveoli (Williams, 1992) (Figure 1). However, tubular myelin does not appear to be essential for surfactant function as this lattice is not present in all animal species (Wetzstein et al. 1980). Furthermore, recent work has shown that in mice who lack surfactant protein A (a protein needed for formation of tubular myelin), tubular myelin is not present yet lung compliance is normal, indicating satisfactory surfactant function (Korfhagen et al. 1993).



Figure 1. Surfactant secretion and morphology

#### Reduction of surface tension and the surfactant monolayer

In 1929, Von Neergaard noted that excised lungs, when filled with fluid, required a lower transpleural pressure for inflation than air filled lungs. From this observation he speculated that a substance was present in the lung lining which acted to reduce surface tension. Von Neergaard's paper, written in German, was all but forgotten until the 1950's, when studies of pulmonary mechanics and foam derived from lung-tissue pointed to a similar conclusion (Mead et al. 1957; Pattle, 1958; Clements, 1956; Macklin, 1954).

Surfactant phospholipids are thought to align in a monolayer at the air-liquid interface in the alveolus, orientated with their hydrophobic tails towards the air phase, and their hydrophilic heads in the liquid phase (Figure 1). In simple terms, these phospholipids pack together at the interface, reducing the area of contact between air and liquid phases, thus reducing surface tension, and preventing alveolar collapse. As the alveolar volume becomes less during expiration, the phospholipid molecules pack even closer together, reducing the contact between air and liquid even further, and thus reducing surface tension further still.

Several models of the arrangement of surfactant in the alveolus have been proposed.

#### i) Alveolar surfactant models - the bubble

If the alveolus is considered as a bubble connected by a tube to air, this is potentially a very unstable situation. If the air pressure outside the bubble is greater than the recoil pressure  $(2\gamma/r)$  from Laplace's Law for a sphere where  $\gamma$  is the surface tension and r is the radius of the bubble), the bubble will rapidly expand and eventually burst. If, on the

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other hand, the outside air pressure is less than the bubble recoil pressure, the bubble will empty itself, and eventually collapse. As alveoli are of different sizes, there is no single outside air pressure which could balance  $2\gamma/r$  for all alveoli, as the radius, and thus the recoil pressure varies greatly between alveoli. In this situation, alveolar 'bubbles' would tend to empty into each other and collapse or burst. Clearly since this is not what happens in the lung, some mechanism must be operating to prevent this from happening.

Clements and colleagues (1961) suggested that a monolayer of surfactant phospholipids lining the alveolus would reduce surface tension ( $\gamma$ ), and thus reduce bubble recoil. These phospholipids would keep on reducing surface tension (and thus recoil) as the bubble got smaller and the molecules packed more densely. This reduction in  $\gamma$  would eventually exceed the reduction in radius on the denominator of Laplace's equation, thus achieving an equilibrium recoil pressure which is the same for all alveoli. This theory, however, assumes that the alveoli behave as though they are separate and mechanically independent units. Objections to the 'bubble' model have been raised on the grounds that alveoli are mechanically interdependent via connective tissue forces, and via a continuous inerconnecting aqueous hypophase and surfactant monolayer (Hills, 1988).

#### ii) Alveolar surfactant models - water repellancy

Hills proposed a model of the alveolus in which the alveolar aqueous hypophase is seen as discontinuous. Surfactant is directly adsorbed to the alveolar epithelium, in a waterrepellent layer. Pools of water, with their convex surfaces orientated in to the alveolar cavity are found covered with a surfactant monolayer at the 'corners' of the alveoli.
Hills maintained that the continuous alveolar lining was found only in the pathological state when alveolar water increased due to, for example, increased capillary hydrostatic pressure in cardiac failure (Hills, 1988).

## iii) Alveolar surfactant models - the surfactant shell

In contrast, in a third model, Bangham proposed a pulmonary surfactant system where a mixed monolayer of DPPC and PG when compressed repeatedly generates an irreversible collapse phase of liposomes enriched with PG, leaving a monolayer progressively enriched in DPPC. This DPPC monolayer then provides a mechanically rigid 'shell' which stabilises the alveoli, and acts as a splint to stop small alveoli from collapsing into large ones. Bangham proposed that these patches of solid DPPC remain functional throughout life, slowly disappearing, and being replenished from lamellar bodies (Bangham et al. 1979; Bangham, 1987).

# Evidence from electron microscopy

The nature of the surfactant monolayer, and the existence of the aqueous hypophase have been difficult to demonstrate microscopically due to evaporation of water with use of a vacuum during EM visualisation, and disturbance of aqueous and phospholipid layers on fixation. However, Bastacky and colleagues (1995) have recently used rapid freezing of rat lung, followed by low temperature scanning electron microscopy, which preserves lung water in a frozen, hydrated state to visualise the alveolar lining layer. Using this technique, they demonstrated a thin, continuous aqueous hypophase covering the entire alveolar surface. Additionally, Schurch and co-workers (1995), using different fixation techniques, demonstrated a continuous surfactant film lining the alveoli which was thicker than a phospholipid bilayer. This indicates that the putative

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'surfactant monolayer' may actually be a surfactant 'multilayer'. The same authors performed in-vitro studies showing recruitment of extra surfactant from a 'surface associated reservoir' into the interface of an expanding bubble. This suggested that such an arrangement could operate in vivo as the reservoir of surplus surfactant in the multilayer which could be incorporated into the surface active film.

## Compression, spreading and squeeze out

The ideal features of an effective pulmonary surfactant are as follows:

1. The ability to achieve high dynamic surface pressure and low surface tension when compressed at the air liquid interface.

2. The ability to spread rapidly from the hypophase.

3. The ability to respread immediately after compression and monolayer collapse.

4. The ability to sustain a low surface tension for sufficient time to prevent alveolar collapse (Clements, 1977; Vodyanoy et al. 1990).

Pure DPPC at 37°C has been described as 'a solid, like candle wax' (Widdicombe, 1987). The phase transition temperature for DPPC is 42°C. Above this temperature, it assumes a more internally mobile liquid-crystalline form. The two rigid, saturated acyl tails of dipalmitoylphosphatidylcholine (DPPC) molecules make them ideally suited to packing together in an alveolar surfactant monolayer to achieve a high surface pressure (satisfying conditions 1 and 4 above). However, fluid unsaturated chains are much more suitable for rapid spreading and respreading, and rigid chains are detrimental to this ability.

Some indication of the influence of acyl chains on spreading and compression of surfactant monolayers comes from a study of lamellar body material isolated from rabbit lungs. Fetal lamellar body films were found to respread better than adult lamellar body material, and this was thought to be related to the finding of relatively high levels of unsaturated lipids in fetal rabbit lung (5 times more 16:1 at position sn-2 of PC in fetal compared to adult material). Also, adult lamellar body material achieved a higher surface pressure than fetal material, supporting the idea that saturated chains make for superior compressibility. Study of the enthalpy and entropy of collapse suggested that adult lamellar body films, with lower concentrations of unsaturated lipids, undergo on compression, a monolayer collapse with energetics similar to that of DPPC monolayers. In contrast, fetal lamellar body films tolerated film compression with a rapid, reversible loss of molecules from the surface, providing for better respreading during expansion (Vodyanoy et al. 1990).

Supplementation of DPPC in vitro with the more fluid lipids cholesterol or dioleoyl PC has also been shown to enhance respreading of DPPC after dynamic post-compression collapse (Notter et al. 1980).

Phase transition temperature influences compressibility and spreading of surfactant. It appears to be the phase transition temperatures of different PC species which mediates their spreading and compressibility at body temperature. Hawco and colleagues (1981) studied various mixtures of PC with different fatty acid tails. All could reach a minimum surface tension of 0mN/m, as long as the temperature of the mixture was below the gel to liquid crystalline transition temperature of the lipids under observation. This was the case regardless of whether or not the PC's were saturated.

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Those above their transition temperature were unable to reach a minimum surface tension below 15mN/m, even when highly compressed.

Clements (1977) discussed the dynamic changes which may occur in surfactant during the respiratory cycle. He noted that adsorption of lung surfactant to the interface in vitro proceeds towards an equilibrium state in which the surface tension is 24 - 28 mN/m, and that values of surface tension less than this are achieved by compressing the film and packing the molecules closer together. In this state, the film is metastable, as material escapes from the interface. Clements remarked that this escape happens much more slowly from films of pure DPPC at 37°C, and therefore, minimum surface tension returns very slowly to equilibrium with DPPC films. On the other hand, unsaturated lipids escape much faster, and it may be impossible to drive the surface tension below equilibrium. Temperature affects phospholipid escape due to differences in behaviour of lipids above and below their phase transition temperature. Clements suggested that in inflation, the film is a mixture of lipids, and in deflation, less stable lipids are 'squeezed out'. So perhaps in inspiration, the film may liquefy, and when we breathe out, the film may become almost pure solid DPPC.

# The role of PG

The second most abundant phospholipid in human surfactant, PG, also appears to play a role in the reduction of surface tension in the lung. This acidic phospholipid may act as an adjuvant in surfactant adsorption. Obladen and colleagues (1983) found that inclusion of PG in a suspension of DPPC liposomes increased the rate of PC adsorption to the air-liquid interface. King and MacBeth (1981) also found that 14%

DPPG in 85.7% DPPC showed enhanced adsorption compared to pure DPPC. Interestingly, pure DPPC mixed with PG has a melting point below body temperature.

However, it appears that surfactant function in vivo does not specifically require the presence of PG. The pulmonary surfactant of some animals contains another acidic phospholipid, PI in place of PG (Table 1). Also, Hallman and co-workers (1985) manipulated dietary precursors of PG and PI to favour synthesis of PI in experimental animals whose surfactant normally contained PG. They found that surfactant function in these animals was not adversely affected by replacement of PG with PI.

There are interesting differences in other animal species. For example, PG and Pl are both absent in the rattlesnake (Daniels et al. 1995). Here, however, removal of surfactant has only a marginal effect on lung compliance. Perhaps in these animals, surfactant has a different role, for example acting as an anti-glue in folding and unfolding lung faveoli.

#### Temperature and reptilian studies

As stated earlier, normally, the temperature at which DPPC changes from an ordered gel phase to a more internally mobile liquid-crystalline phase is around 42°C. Some interesting light can be thrown on the function of surfactant by studies of surfactant in amphibians, who undergo dramatic alterations in body temperature as part of life.

In a seminal paper on this subject, Lau and Keough (1980) investigated the composition of surfactant taken from turtles kept at different temperatures for 1 month. In BAL surfactant from these animals, stearate and palmitate rose with

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increasing temperature, while myristate and palmitoleate fell, and the proportion of saturated fatty acids in PC from BAL was higher in samples from turtles at a higher temperature. This suggests that the turtles responded to increasing temperature by acquiring lipids in their surfactant with a higher transition temperature. Similarly, lizards which habitually maintain a high body temperature (37-40°C) possess relatively high levels of disaturated PC and cholesterol in their surfactant (Smits et al. 1994). In another species of lizard which may sustain a nocturnal temperature fall from 37 to 10°C, 48% of fatty acids in the total lipid fraction were found to be saturated, compared with 64% in the rat (a homeothermic animal). The saturated 16:0 species, which has a relatively high phase transition temperature, predominated in both rat and lizard, but the percentage of this fatty acid was greater in the rat (Daniels et al. 1989). These studies suggest that body temperature is an important factor in determining surfactant fatty acid composition.

As well as altering the degree of saturation of their surfactant phospholipids, amphibians respond to acute or chronic changes in body temperature by altering the amount of cholesterol in their surfactant. Cholesterol inhibits van der Waals forces between opposed acyl chains. Below the transition temperature, cholesterol molecules align to disrupt acyl-acyl interactions, allowing increased movement of the tails and enhancing fluidity. Toad surfactant has a relatively high cholesterol content and a low level of saturation of surfactant phospholipids, which helps surfactant in these animals to remain fluid at relatively low water temperatures (Daniels et al. 1994). This suggests that the proportions of different lipids and the level of saturation of phospholipids in the surfactant of any animal may be specifically designed to allow optimal function at that animal's body temperature. In humans, this is 37°C.

# 1.2.4 SURFACTANT LIPID FUNCTION IN THE SMALL AIRWAYS

Until recently, most considerations of surfactant physical activity have been confined to its role in the alveolus, where it lowers surface tension and protects against alveolar collapse. Evidence is increasing, however, that surfactant may also play an important part in maintaining the patency of small airways in the lung.

In 1991, Liu and colleagues reported that pulmonary surfactant could maintain the patency of a narrow glass capillary tube, preventing it from becoming blocked with fluid. The capillary tube can be thought of as analagous to the small airways in the lung. This analogy would suggest that an intact and functioning surfactant system may be necessary to maintain small airway patency. Enhorning et al. (1995), using excised rat lungs, subsequently provided evidence to suggest that surfactant did indeed act to maintain the patency of small airways in these lungs.

#### 1.2.5. IMMUNOLOGICAL FUNCTIONS OF SURFACTANT LIPIDS

In addition to their surface active properties, surfactant lipids may possess immunomodulatory properties. Evidence suggests that the immunological activity of surfactant lipids depends on what class or species of lipid is studied.

Phosphatidylcholine, PG and PI have been shown to inhibit T lymphocyte proliferation,

while PE, S and cholesterol appear to stimulate the growth of lymphocyte colonies in response to phytohaemagglutinin (PHA) (Wilsher et al. 1988). Wilsher and colleagues (1988) found that the suppression of proliferation by PC, PG and PI depended on the relative proportions of these phospholipids present. It is interesting to note that the immunosuppressive lipids PC and PG are prominent in pulmonary surfactant. It is tempting to speculate that this helps explain why humans do not mount an immune response to surfactant lipids.

Increasing concentrations of DPPC have been shown to suppress concanavalin A induced proliferation of rat splenocytes. (Spleen cells were used rather than immune cells from the lung which may already have been influenced by surfactant) (Kremlev et al. 1994).

Unsaturated phosphatidylcholines have been shown to inhibit superoxide generation by neutrophils in vitro stimulated by 1,2-dioctanoyl-sn-glycerol, while saturated PC's had no effect. Individual surfactant lipid components have also been demonstrated to have different effects on priming of alveolar macrophages for oxidative responses (Hayakawa et al. 1992).

The immunological functions of whole surfactant may prove complex to unravel. As described above, different lipids have different effects on cells of the immune system. In addition, there is evidence that both surfactant lipids and surfactant proteins participate in pulmonary defence mechanisms. The immunological role of surfactant proteins is discussed in the next section.

# **1.2.6. SURFACTANT COMPOSITION - PROTEINS**

As noted earlier, proteins account for 5-10% of the composition of pulmonary surfactant. Four proteins have been named as surfactant proteins (SP): SPA, SPB, SPC and SPD. However, it is interesting to note that although SPD is designated as a surfactant protein, 70% of SPD is recovered in the supernatant after high speed centrifugation of alveolar surfactant, while SPA, SPB and SPC are recovered in the surfactant lipid pellet . Also, although surfactant proteins A, B and C are present in lamellar bodies, SPD is not (Johanssen and Curstedt, 1997).

Surfactant protein A has immunological functions, and affects the surface-activity of surfactant lipids. Surfactant proteins B and C, two small hydrophobic proteins, have functions mainly associated with modulating surface activity, while the most recently discovered protein, surfactant protein D appears to play a role in local immune defences in the lung.

# Surfactant protein A

Human surfactant protein A (SPA) is a glycoprotein (White et al. 1985) with a similar tertiary structure (but not amino acid sequence) (Benson, 1993) to the immunologically active complement protein, C1q, and to a group of carbohydrate binding proteins, the C-type lectins. C-type lectins are extracellular proteins which possess disulfide bonds and bind to carbohydrate ligands in a calcium dependent manner (Van Golde, 1995; Benson, 1993).

The quaternary structure of SPA is shown in Figure 2. Polypeptide chains of SPA form into trimeric units, and six of these trimers associate in vivo to form the SPA oligomer

which has been likened in shape to a 'bunch of tulips'. As indicated in Figure 2 the protein has a collagenous domain and a carbohydrate recognition domain, and these sites appear to mediate immunological functions . Surfactant protein A has been identified not only in the lamellar bodies of Type II alveolar cells, where it is closely associated with surfactant lipids, but also in pulmonary alveolar macrophages and in non-cilated bronchiolar cells (Clara cells) (Walker et al. 1986). In fact, lamellar bodies have a significantly lower content of SPA relative to BALF indicating that secretion of SPA may occur separately from phospholipids (Doyle et al. 1994; Johanssen and Curstedt, 1997). Surfactant protein A has been shown, by immunoelectronmicroscopy, to reside in the corners of the tubular myelin lattice (Voorhout et al. 1991), and, in vitro, it is an essential factor in the formation of this matrix (Williams et al. 1991).

Surfactant protein A facilitates the formation of a lipid-rich, surface-active film at the air-liquid interface. In the presence of surfactant proteins B and C, SPA improves the surface active properties of a lipid surfactant extract (Chung et al. 1989), suggesting a co-operative association between proteins and lipids in the formation of a surfactant film. Kuroki and Akino (1991) demonstrated calcium-dependent binding of SPA to the hydrophobic region of PC, and it is possible that this binding affects the packing of PC molecules at the air-liquid interface (King et al. 1986).

However, a study of mice in whom the SPA gene was ablated ('SPA knockout mice'), showed that, in these mice, surfactant function was reduced at low phospholipid concentrations, but normal at higher concentrations. These mice also had normal pulmonary compliance (Korfhagen et al. 1993). This suggests that SPA may not be essential to surfactant function in health, but may be important when lung pathology

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compromises the secretion of phospholipids. One other interesting quality of SPA highlights a potential role for SPA in maintenance of surfactant function in disease. That is, the protection which SPA offers to surfactant phospholipids from inactivation by plasma proteins which flood into the alveolus in some respiratory diseases (Cockshutt et al. 1989).

The homology between SPA and C1q and the C-type lectins has stimulated research into the interaction between SPA and pulmonary defence mechanisms. Surfactant protein A has been shown to enhance concanavalin A-induced proliferation of rat splenocytes in vitro (Kremlev et al. 1994). Additionally, Malhotra and colleagues (1990) demonstrated that SPA binds to the C1q receptor on phagocytic cells, and binding of SPA to the C1q receptor on human monocytes has been shown to mediate phagocytosis of staphylococcus aureus by these cells (Geertsma et al. 1994). Phagocytosis of staphylococcus aureus by alveolar macrophages is also enhanced by SPA (Van Golde, 1995; van Iwaarden et al. 1990) and SPA has been shown to bind, in a calcium dependent manner to pneumocystis carinii (Zimmerman et al. 1992).

As well as the interactions with microorganisms described above, SPA has also been shown to interact with viruses, interactions which appear to be mediated via the protein's carbohydrate moiety (Van Golde, 1995; Van Iwaarden et al. 1991). Surfactant protein A acts as an opsonin in phagocytosis of herpes simplex type 1 virus by alveolar macrophages (Van Iwaarden et al. 1991) and has been shown to bind to the influenza A virus (Malhotra et al. 1994), and to cells infected by the influenza B virus (Van Golde, 1995).

Macrophage function is enhanced by SPA. There is evidence that SPA stimulates phagocytosis of foreign particles and micro-organisms by alveolar macrophages, causes secretion of reactive oxygen species from these cells (reviewed by Van Golde, 1995) and increases macrophage chemotaxis (Wright and Youmans, 1993). Alveolar macrophages have been shown to possess specific SPA receptors (Oosting and Wright, 1994; Pison et al. 1992).

There is evidence to suggest that SPA has a role in the regulation of phospholipid secretion and clearance (Hawgood, 1992). Type II pneumocytes have an SPA receptor on their surface (Kuroki et al. 1988) and, in vitro, SPA inhibits secretion (Rice et al.1987) and stimulates uptake (Wright et al. 1987) of lipids by type II alveolar cells . However, SPA knockout mice have no alterations in surfactant phospholipid pool sizes, suggesting that SPA is not solely responsible for regulation of surfactant secretion (Korfhagen et al. 1993).

In summary, then, there is evidence that surfactant protein A facilitates the formation of a phospholipid film at the air-liquid interface, assists in the formation of tubular myelin, modulates the secretion and clearance of surfactant, protects surfactant against inactivation by plasma proteins, acts as an opsonin and enhances the defensive functions of alveolar macrophages.

#### Surfactant proteins B and C

Surfactant proteins B and C (SPB and SPC) are low molecular weight (around 9 and 3.5 kD respectively), hydrophobic proteins which remain associated with surfactant phospholipids during organic extraction. Surfactant protein B mRNA has been found in type II pneumocytes and Clara cells, but not alveolar macrophages, type I cells or pulmonary interstitial cells. Surfactant protein C has been demonstrated in the lamellar bodies of type II pneumocytes (Benson, 1993).

Addition of SPB to a phospholipid mixture significantly improves the adsorption and surface-tension lowering properties of that mixture and this effect is further enhanced by the addition of calcium (Curstedt et al. 1987; Revak et al. 1988). Surfactant protein B is palmitoylated, and it is thought that SPB may associate with the polar head groups of phospholipids by electrostatic interactions with the positively charged amino acids, whereas the hydrophobic portions of SPB interact with the phospholipid acyl chains to modulate surface properties of the monolayer (Benson, 1993; Longo et al. 1993). In vitro experiments have also indicated that SPB, along with phospholipids, calcium and SPA is necessary for formation of tubular myelin (Suzuki et al. 1989; Williams et al. 1991).

Surfactant protein C also participates in the lowering of surface tension, enhancing the surface active properties and adsorption rate of a phospholipid mixture (Whitsett and Baatz, 1992). Like SPB, SPC is palmitoylated on cysteine residues, and SPC has been shown to produce rapid adsorption of a DPPC/PG suspension. Adsorption was enhanced by palmitoylated and by depalmitoylated SPC, although there was a trend for the depalmitoylated form not to enhance adsorption to as great an extent (Qanbar et al.

1996). Palmitoylated SPC cysteinc residues may intercalate with the phospholipid matrix of the surfactant film (Benson, 1993), increasing surfactant film stability, and lateral pressure within the hydrophobic region at the air-liquid interface (Horowitz et al. 1992). Qanbar and colleagues (1996) suggested that SPC may be involved in a process of selective adsorption whereby DPPC appears preferentially at the air-liquid interface. It has also been suggested that SPC may enhance surfactant respreading by preventing phospholipids from detaching from the surface layer upon area compression (Qanbar et al. 1996).

Surfactant proteins B and C have been shown to act in a co-operative manner to protect surfactant against inhibition by serum (Amirkhanian et al. 1993) and SPB has been found to protect a lipid surfactant extract from functional inhibition by fibrinogen (Seeger et al. 1992).

#### Surfactant protein D

Surfactant protein D is the most recently isolated lung-specific protein (Persson et al. 1988; Kuroki et al. 1991). This protein is made up of four subunits; each of which is a disulphide bonded trimer. Surfactant protein D is similar to SPA, in that it possesses a collagen-like region and a globular head which has C-type lectin activity (Figure 2).

Surfactant protein D, like SPA, has been implicated in the immunological defence of the lung. It has been shown to bind to alveolar macrophages in vitro (Miyamura et al. 1994a) and to enhance formation of reactive oxygen species in rat alveolar macrophages (Van Iwaarden et al. 1992). Alveolar macrophages have been shown to have specific SPD receptors (Miyamura et al. 1994a). Surfactant protein D also binds to *E.Coli*, probably by associating with bacterial endotoxin (Van Golde, 1995) and has been shown to inhibit the haemagglutination activity of influenza A virus, mediated by its carbohydrate binding domain (Hartshorn et al. 1994).

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Figure 2. Quaternary structure of SPA and SPD

# 1.2.7 SURFACTAN'T SECRETION AND CLEARANCE

#### Stimulation of surfactant secretion

Various physiological and pharmacological mechanisms enhance the secretion of surfactant (Rooney, 1985; Morton, 1989). These include stimulation of  $\beta$  adrenoreceptors on the surface of type II pneumocytes, cholinergic stimulation, lung distension and binding of SPA to the cell surface. Glucocorticoids also have a stimulatory effect on secretion, possibly by their permissive action on catecholamine receptors. Fatty acids, specifically the long chain polyunsaturated arachidonic and eicosopentanoic acids promote surfactant secretion possibly by effects on membrane fluidity. Linoleic, oleic and palmitic acids also stimulate secretion of surfactant, but to a lesser extent (Baybutt et al. 1994). Surfactant synthesis is enhanced by glucocorticoids, oestrogen, mechanical ventilation and vagal stimulation (Rooney, 1985; Morton, 1989).

#### Surfactant disposal and recycling

Spent surfactant lipids may be subject to a number of fates including recycling by type II cells. Studies on newborn rabbits have shown that 95% of surfactant PC is recycled by the alveolar epithelium (Robertson, 1985). Surfactant may also be taken up by pulmonary lymphatics, migrate upwards to larger airways, or be metabolised by phospholipases (Figure 3) in type II cells and in alveolar macrophages.

Surfactant protein A, SPB and SPC re-enter the Type II cell whose lamellar bodies are rich in phospholipids, SPB and SPC. Recycling of SPD has not been demonstrated. Clara cells do not appear to take part in recycling. There is some suggestion that surfactant proteins and phospholipids may be recycled separately (Rooney et al. 1994).

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Figure 3. Action of phospholipases

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# **1.3. SURFACTANT IN LUNG INFLAMMATION**

The pulmonary surfactant system can be adversely affected by disease. One of the most complicated disease processes which can result in surfactant dysfunction is acute inflammatory lung disease. The main histological characteristics of this are the presence in airways, alveoli and lung parenchyma of inflammatory cells and a proteinaceous alveolar exudate. Two diseases in which severe acute lung inflammation is seen frequently are pneumonia and Acute Respiratory Distress Syndrome (ARDS). The main pathological features of these two conditions are summarised in Table 4.

Table 4.	Clinical	features	of ARDS	and	pneumonia
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	PATHOLOGICAL FEATURES	CLINICAL FEATURES
ARDS	Interstitial and alveolar ocdema. Haemorrhage, cellular debris and proteinaceous fluid in the alveoli. Inflammatory cellular infiltrate around alveolar walls.	Triggered by eg. trauma, haemorrhagic shock, burns. Tachypnoea, hypoxaemia, low pulmonary compliance. Mortality around 50%.
PNEUMONIA	Inflammatory cells in the lung parenchyma and alveoli, Alveoli filled with exudate. May be necrosis of lung tissue and abscess formation.	Malaise, fever, cough, pleurític pain, tachypnoea, tachycardia, cyanosis, hypoxia.

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Pneumonia arises from pulmonary infection by micro-organisms. The infection may be restricted to one or more lobes, or may be generalised to the entire lung. Infection of the lung with bacteria or viruses results in an inflammatory reaction (Table 4), the purpose of which is to destroy the invading pathogen.

Acute respiratory distress syndrome can arise from a panoply of primary pulmonary and systemic insults including pneumonia, burns, sepsis, trauma and massive blood transfusion. The final common pathway is a state of lung inflammation with increased capillary permeability, invasion of inflammatory cells and ingress into the alveoli of oedema fluid containing plasma proteins.

Surfactant abnormalities have been shown to occur as the result of pneumonia and of ARDS. There is evidence for surfactant dysfunction in ARDS, and alterations in the absolute amount of surfactant components have been seen in both conditions. There is also potential for surfactant damage or inhibition in pneumonia and in ARDS, as a consequence of the inflammatory response.

There are several animal models for inflammatory lung disease including intravenous administration of fatty acids or n-nitroso-n-methylurethane and administration of intratracheal endotoxin. Lung irradiation has also been studied, as it produces an alveolar leak of oedema fluid which is also seen in ARDS. Irradiation studies produce a model in which the effects of this fluid on surfactant can be studied in the absence of the other inflammatory events. Surfactant has been studied extensively in animal models in an attempt to elucidate the pathology of ARDS in humans.

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Both human and animal studies of the pulmonary surfactant system in lung inflammation are reviewed below. The aspects of surfactant dysfunction, inhibition, deficiency and damage are considered.

# **1.3.1. SURFACTANT DYSFUNCTION**

# Respiratory distress syndrome of the newborn

In 1959, Avery and Mead noted that premature infants who died with a form of respiratory failure - Respiratory Distress Syndrome of the newborn (RDS) - were surfactant deficient.

Over a decade later, administration of exogenous surfactant was shown to benefit rabbits born prematurely with RDS (Enhorning and Robertson, 1972). Eventually, after many therapeutic trials following on from early replacement studies in human neonates (Morley et al. 1981; Fujiwara et al. 1980), surfactant replacement has become a well established therapy for newborn infants with RDS.

# Acute respiratory distress syndrome

Acute Respiratory Distress Syndrome (ARDS) was first described by Ashbaugh and colleagues in 1967 as a syndrome of hypoxaemia, poor lung compliance and tachypnoea occurring in adults, in which there were clinical and radiological similarities to infantile RDS. Because of these similarities, Petty and Ashbaugh (1971) suggested that the pattern of adult respiratory failure which they had observed be called the Adult Respiratory Distress Syndrome (ARDS). By this time, the role of surfactant deficiency in RDS of the newborn was well established, and implicit in the title 'adult RDS', was

the suggestion that surfactant function was abnormal in this newly-described condition. The title 'Acute Respiratory Distress Syndrome' (ARDS) has since been adopted, as it has become evident that the condition is not confined to adults.

#### Surfactant dysfunction in ARDS

In their original description of ARDS, Ashbaugh and associates (1967) described an increase in the minimum surface tension produced by surfactant from post mortem lung extracts of patients who had died from ARDS, suggesting surfactant dysfunction. Subsequent studies have confirmed these findings. Hallman and colleagues (Hallman et al. 1982; Spragg and Hallman, 1983) found that the minimum surface tension of BAL surfactant purified by density gradient centrifugation (to remove surfactant inhibitors) was far greater in patients with ARDS than in controls, suggesting an intrinsic surfactant abnormality independent of inhibitory substances. Gregory and co-workers (1991) studied a crude surfactant pellet obtained by high speed centrifugation of bronchoalveolar lavage fluid, and also found increased minimum surface tension in surfactant from patients with ARDS, compared to normal subjects. Gregory and colleagues also found raised minimum surface tension in surfactant from patients who were considered to be at risk for ARDS.

# Surfactant function in animal models of lung injury

Intravenous injection of a free fatty acid mixture containing predominantly oleic acid has been used to produce a clinical picture in experimental animals, similar to that seen in humans with ARDS. Hall et al (1990) obtained surfactant from BAL fluid of oleicacid treated rabbits, by evaporation of lavage fluid to dryness under nitrogen. This surfactant had a reduced ability to lower surface tension compared to that from the lungs of healthy controls. A later study by Hall and co-workers (1994), showed that a crude surfactant pellet from BAL of fatty acid injured animals also had lower surface activity than a similar pellet obtained from controls. As surfactant in these two studies was not subjected to lipid extraction or density gradient centrifugation which would remove inhibitory proteins, the importance of plasma protein inhibition on surfactant function cannot be accounted for.

However, another animal model suggests that surfactant function is similar in surfactant obtained by high speed centrifugation, and that purified by density gradient centrifugation. N-nitroso-n-methylurethane (NNMU) is another compound which causes lung injury similar to ARDS in animal models. Liau and colleagues (1987) obtained surfactant by two different methods from BAL in dogs injured with NNMU. Both a crude surfactant pellet from centrifugation at 27,000 x g and a surfactant fraction purified from BAL by density gradient centrifugation were tested for surface activity. The surface properties of both surfactant preparations were reduced to a similar degree in the injured animals. This indicates that either density gradient centrifugation did not remove surfactant inhibitors to any greater extent than simple centrifugation or that inhibition was not a significant cause of surfactant inhibition. Surfactant taken from the lungs after recovery functioned normally in vitro, suggesting that the surfactant dysfunction was temporary.

Intra-tracheal introduction of endotoxin in animals induces a condition similar to ARDS with hypoxia, reduced compliance, interstitial alveolar oedema and haemorrhages. In guinea pigs whose lungs had been injured with endotoxin, maximum and minimum surface tensions of a lipid extract of BAL fluid were increased compared あって アンステレンサー いたい しゅうびんしん

्राजे जिस्से जन्म राजस्थित की कार्यसम्पूर्ण अवकार्यने का किस्सार जन्म हो का कार्यसम्पूर्ण प्रकृति जन्म कि स्थाने का ज to control. Surfactant inhibition by non-sedimentable protein from a 105,000 x g supernatant of BAL fluid from the endotoxin group was also greater than control on a mg/mg basis (Tahvanainen and Hallman, 1987). This study established that the surface activity of hydrophobic components of BAL fluid was abnormal, and also that the inhibitory capacity of BAL proteins was increased. This would suggest an intrinsic surfactant abnormality. However, it is interesting to note that endotoxin was administered intratracheally, and it is possible that lipid soluble components of this bolus could have interfered with surface activity of a lipid extract of BAL.

## Inhibition or intrinsic abnormality?

In the human and animal studies of lung injury reviewed above, different methods of purification of surfactant were used before surface activity was studied. The evidence from these studies suggests that both inhibition by proteins and intrinsic surfactant abnormality are factors causing the surfactant dysfunction seen in lung injury. The role of inhibition is explored further in the next section.

# **1.3.2. INHIBITION OF SURFACTANT FUNCTION**

## Inhibition by proteins

Altered capillary permeability in acute lung inflammation and infection leads to leakage of proteins and other constituents of plasma into the alveolus. Granulocytes, which invade the lung in the inflammatory response, have been shown to cause lung oedema as a consequence of their releasing oxygen radicals (Shasby et al. 1982) and increased protein has been found in BAL from patients with ARDS (Veldhuizen et al. 1995). 一日日本人一下了了了了了。""这些是这些"这些人",这些人们不是一个人的

Kobayashi et al (1991) demonstrated in vitro and in vivo inhibition of surfactant by oedema fluid and it has been shown that various plasma proteins (Table 5) inhibit the surface activity of surfactant in vitro. Holm and colleagues (1988) suggested that inhibitory proteins may compete with surfactant phospholipids for adsorption at the air-liquid interface. Additionally, Schurch and co-workers (1995) have suggested that proteins may prevent additional surface active material being recruited from the surface-associated reservoir to the surface active film. Addition of surfactant protein A has been shown to reverse the inhibitory effects of some blood proteins on lipid extract surfactant (Cockshutt et al. 1990).

# Table 5. Substances which inactivate surfactant

Albumin (Keough et al. 1988; Seeger et al. 1985) C-reactive protein (Amirkhanian and Taeusch, 1993; Li et al. 1989) Elastin (Seeger et al. 1985) Erythrocyte membrane lipids (Holm and Notter, 1987) Fibrin degradation products (Seeger et al. 1993b; O'Brodovich et al. 1990b) Fibrin monomer (Seeger et al. 1985) Fibrinogen (Keough et al. 1988; Amirkhanian and Taeusch, 1993) Globulius (Keough et al. 1988; Seeger et al. 1985) Haemoglobin (Holm and Notter, 1987) Lysophosphatidylcholine (Amirkhanian and Taeusch, 1993) Meconium (Moses et al. 1991) Oleic acid (Hall et al. 1992) Phospholipases (Enhorning et al. 1992; Holm et al. 1991) Platelet activating factor (Amirkhanian and Taeusch, 1993) Polymerising fibrin (Seeger et al. 1993a) Pulmonary oedema fluid (Kobayashi et al. 1991)

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# Inhibition in radiation injury

Radiation pneumonitis is associated with leakage of proteins from the microvasculature into the alveoli, in the absence of other inflammatory events which occur in ARDS. It therefore provides some insight into the role of plasma proteins in surfactant dysfunction. Gross and colleagues (1991) studied surfactant in mice which were irradiated, and killed when pneumonitis had developed. Surfactant which was isolated by centrifugation of BAL after death showed abnormal surface activity. However, when extraneous material was removed from the pellet by equilibrium centrifugation, surface activity was the same in irradiated and sham-irradiated mice.

In 4 patients with mesothelioma (a malignant tumour of the pleura), who received irradiation of the hemithorax, a 105,000 x g crude surfactant pellet from BAL showed increased maximum and minimum surface tension after irradiation. There was an increase in soluble protein in the supernatant of BAL, and lyophilised supernatant from these patients increased the minimum surface tension of human amniotic fluid surfactant. This suggests that surfactant inhibition by protein does indeed contribute to the pathogenesis of radiation induced injury (Hallman et al. 1990).

# Inhibition by lysophosphatidylcholine

Another potential source of surfactant inhibition arises from lysophosphatidylcholine (LPC), a phosphatidylcholine breakdown product, and increased concentrations of this have been found in BAL fluid from patients and animals with lung injury.

Surfactometer studies of surfactant inhibition by LPC reveal a complex picture which has not as yet been fully elucidated. Some in vitro experiments have shown that

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addition of LPC or phospholipases (which break down PC to LPC) to surfactant inhibits surface activity (Holm et al. 1991; Enhorning et al. 1992). The inhibitory effect of LPC on surfactant is counteracted by addition of palmitic acid and may be due to the 'conical' molecules of LPC disrupting the packing of 'cylindrical' PC molecules at the air-liquid interface (Cockshutt et al. 1991). Lysophosphatidylcholine also has been shown to sensitise a lipid extract of surfactant to inhibition by fibrinogen in vitro (Cockshutt and Possmayer, 1991).

However, against this, Lema and Enhorning (1997) showed that substitution of LPC/palmitic acid (simulated hydrolysis products of DPPC) for DPPC led to increased surface activity of the mixture containing LPC when measured in pulsating bubble and capillary surfactometers (dynamic in vitro models of the alveolus and small airway respectively). In the presence of albumin, activity was inhibited in the capillary surfactometer but not the pulsating bubble surfactometer. The authors suggested that lipids other than DPPC may be hydrolysed by phospholipases in vivo and that this may disrupt a delicate balance in the distal parts of the lung.

There is evidence to suggest that LPC is increased in ARDS and in animal models of this condition, possibly due to increased phospholipase activity. Veldhuizen and colleagues (1995) found increased levels of LPC in cell-free BAL supernatant from patients with ARDS while Pison and colleagues (1987), studying sequential cell-free BAL fluid samples from patients with ARDS due to trauma found an increase in lysophposphatidylcholine on the day of trauma. However, surface activity was not tested in either of these studies, so the effects of this increase in LPC on surfactant function remain unknown.

Studies of BAL fluid from animal models of hung injury have revealed an increase in the LPC fraction (Liau et al. 1987; Tahvanainen and Hallman, 1987; Casals et al. 1989). In one of these studies, a lipid extract of BAL fluid also showed increased maximum and minimum surface tensions (Tahvanainen and Hallman, 1987). This would suggest that some abnormality in the hydrophobic fraction of surfactant was causing abnormal surface activity. In another, surface activity of surfactant fractions obtained by density gradient centrifugation and by high speed centrifugation was similarly impaired, indicating either that inhibitory proteins were eliminated to a similar degree by both preparation methods or that some intrinsic abnormality of surfactant was present independent of inhibition (Liau et al. 1987).

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It is impossible, from these in vivo studies to establish a causal link between LPC and surfactant inhibition in lung inflammation, and the in vitro evidence for surfactant inhibition by LPC is conflicting. However, evidence presented above does give grounds for speculation that LPC per se or in combination with plasma proteins may inhibit surfactant in inflammatory lung disease. The situation in vivo is so complex that LPC is only likely to be one part of a larger picture of surfactant dysfunction involving protective surfactant proteins, inhibitory plasma proteins, abnormalities of surfactant lipid composition and quantity and damage to surfactant.

# 1.3.3. SURFACTANT LARGE AND SMALL AGGREGATES --IMPLICATIONS FOR FUNCTION

Centrifugation can separate surfactant recovered from BAL into large aggregates (LA), which are easily sedimentable, and small aggregates (SA) which resist sedimentation during high speed centrifugation. Work on rabbit surfactant has shown

that the phospholipid concentrations and phospholipid compositions of large and small aggregate fractions are similar. However a lipid extract of large aggregates was shown to be effective in improving dynamic compliance in ventilated pre-term rabbits, whereas a lipid extract of small aggregates did not. Small aggregates of surfactant have also been shown to be less surface active in vitro than large aggregates (Yamada et al. 1990). Large aggregates from injured lungs are converted in vitro more rapidly to small aggregates than are large aggregates from normal lungs (Gunnison and Finkelstein, 1997). It is thought that small aggregates may represent an effect form of surfactant at the end of its 'life cycle' in the alveolus.

# Surfactant large and small aggregates in ARDS

Small aggregate/large aggregate ratio has been found to be significantly higher in samples from ARDS patients compared to control (Lewis et al. 1997; Veldhuizen et al. 1995). This may contribute to the surfactant dysfunction seen in ARDS.

# Surfactant large and small aggregates in animal models

In rabbits injured with IV fatty acid (Hall et al. 1994), the recovery of large aggregates of surfactant was reduced, and this was not mimicked by the addition of serum to BAL from healthy animals. Supplementation of BAL from injured animals with large aggregates improved surface activity. The authors speculated that conversion of large particles to small ones may be due to serine protease activity, and such enzymes are known to be elevated in injured lungs. Alternatively, they suggested that phagocytosis of large particles by leucocytes or decreased secretion of new lamellar bodies may have been responsible for the decrease in large aggregates.

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# **1.3.4. SURFACTANT PHOSPHOLIPID DEFICIENCY OR EXCESS**

Changes occur in the amount of phospholipids and proteins present in pulmonary surfactant in response to lung inflammation from ARDS or pneumonia.

# Acute respiratory distress syndrome

It is not clear whether the amount of surfactant phospholipids is reduced in ARDS. Several authors have found that the total phospholipid level in BAL fluid centrifuged to remove cell debris (Gunther et al. 1996; Hallman et al. 1982; Spragg and Hallman, 1983; Pison et al. 1990) was not different from control. Jacobson et al. (1993) examining tracheal aspirates from patients with ARDS under polarised light for the presence of characteristic surfactant particles, found no difference in occurrence of these particles from control. Offenstadt and colleagues (1981), on the other hand, found an increased concentration of phospholipids in cell free BAL supernatant from patients with ARDS compared to control. In contrast, Gregory and colleagues (1991) found decreased levels of phospholipid in the crude surfactant pellet of BAL fluid from patients with ARDS and patients at risk for ARDS, compared to normal controls.

#### Pneumocystis carinii pneumonia

Pneumocystis carinii pneumonia (PCP), which occurs in patients with HIV infection, has become of particular interest as it causes a generalised pneumonia (ie. not just affecting one lobe of the lung), and the changes evident on chest radiographs can be similar to those seen in RDS of the newborn and ARDS. This would suggest that surfactant deficiency may be a factor contributing to the pathogenesis of PCP.

Several researchers have studied phospholipid profiles in HIV-infected patients with PCP. A decreased lipid concentration in BAL fluid centrifuged to remove cells from patients with PCP was found by two groups (Escamilla et al. 1992; Hoffman et al. 1992), while Rose and colleagues (1994) found no reduction in total phospholipid in cell-free lavage samples from patients with AIDS-related lung disease (including PCP). However, it has not been unequivocally established that surfactant lipid concentrations are altered in this disease.

# Nosocomial pneumonia in neonates

Griese and colleagues (1996) found that phospholipid concentration was reduced in a crude surfactant pellet of tracheal aspirates from neonates with nosocomial pneumonia in neonates during deterioration and at the peak of infection.

# Animal studies

When lung injury was produced in guinea pigs with intra-tracheal endotoxin and ventilation with 100% oxygen, reduced surfactant phospholipids were found in a surfactant pellet obtained by high speed centrifugation (Tahvanainen and Hallman, 1987). In contrast, in fatty acid injured rabbits, normal amounts of phospholipid were found in cell free BAL compared to controls (Hall et al. 1994). This suggests that the method of injury may have important consequences for the change seen in phospholipid level.

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# **1.3.5. DEFICIENCY OR EXCESS OF SURFACTANT PROTEINS**

#### Acute respiratory distress syndrome

Gregory and colleagues (1991) also found decreased levels of SPA and SPB in the crude surfactant pellet from patients with ARDS, compared to normal subjects and decreased SPA in surfactant from patients who were considered to be at risk for ARDS. Gunther and colleagues (1996) demonstrated a reduction in SPA but not SPB in cell-free BAL supernatant from patients with ARDS.

#### AIDS-related pneumonia and bacterial pneumonia

Surfactant protein A levels have also been studied in pneumonia. Increased concentrations of SPA were found in cell-free lavage fluid from patients with AIDSrelated pneumonia due to Pneumocystis Carinii and also to other pathogens (Phelps and Rose, 1991), while Baughman and colleagues (1984) found reduced levels of SPA in cell-free bronchoalveolar lavage samples from patients with non-AIDS related bacterial pneumonia. A recent report details a reduction in SPA (but not SPB) in BAL filtered and centrifuged to remove cells from patients with ARDS and/or pneumonia (Gunther et al. 1996).

#### Surfactant protein C

It should be noted, at this point, that the extreme insolubility of SPC in aqueous media has hampered its detection in disease states, and therefore, no information is available on any changes in the levels of this protein which may occur in response to pulmonary inflammation.

# **1.3.6. CHANGE IN SURFACTANT COMPOSITION - PHOSPHOLIPID** CLASSES

## Infant respiratory distress syndrome

Early studies found reduced levels of PG in tracheal effluent from infants with RDS (Obladen, 1978; Bose et al. 1984). This stimulated research to elucidate changes in phospholipid composition of surfactant in ARDS.

## Adult respiratory distress syndrome

Pison and colleagues found that in cell-free BAL fluid from patients with ARDS due to multiple trauma PG was reduced and PI increased at 6 hours after trauma, and PC was reduced at 24 hours after trauma. In a subgroup of these patients who had severe respiratory failure, reductions in PC and PG and an increase in PE and sphingomyelin were found throughout the course of the disease (Pison et al. 1990; Pison et al. 1987). Two other studies found PC and PG to be reduced and sphingomyelin to be increased in cell-free BAL supernatant from patients with ARDS (Veldhuizen et al. 1995; Spragg and Hallman, 1983). Hallman et al (1982) also found PS to be increased. Additionally, Gregory and colleagues (1991) found decreased levels of PC and PG in a crude surfactant pellet from BAL in patients with ARDS.

The most consistent findings to emerge from these studies of ARDS appears to be a reduction in PC and PG, and an increase in sphingomyelin.

## Pneumonia

No changes were found in phospholipid composition of tracheal aspirates in nosocomial infection in ventilated preterm human neonates (Griese et al. 1996).

#### Animal models of lung injury

Several studies have investigated changes in phospholipid classes in animal models of lung injury, and the changes which occur seem to be specific to the model studied. However, in general as with studies of ARDS, a pattern of reduced PC and PG emerges.

In rabbits injured with IV oleic acid, surfactant purified from BAL by density gradient centrifugation showed reduced levels of PG and increased sphingomyelin (Casals et al. 1989). In endotoxin - injured guinea pigs, PG and PC were found to be reduced in a lipid extract of cell-free BAL fluid whereas PI and sphingomyelin were increased (Tahvanainen and Hallman, 1987). In NNNMU injured dogs PC and DSPC were found to be reduced in cell-free BAL. In these animals, reduction in respiratory compliance showed a significant linear correlation with quantities of PC in BAL (Ryan et al. 1981). In another study of dogs similarly injured, DPPC, PG, PC and PE were all reduced but increased towards late recovery. PI was increased compared to controls. In this study, surfactant composition was studied in cell-free lavage, crude surfactant pellet from high speed centrifugation and in this pellet after purification by density gradient centrifugation. The method by which surfactant was obtained did not appear to affect the composition of surfactant phospholipids measured (Liau et al. 1987). In contrast to studies of other animal models, Hall and colleagues (1994) found minimal change in phospholipid classes from cell free BAL fluid in fatty acid injured rabbits.

The evidence from animal models of lung injury therefore suggests that, although the pattern of phospholipid change probably depends on the method of injury employed, the most consistent pattern to emerge is a reduction in PC and PG.

## Animal models of infection

Calves inoculated with bovinc herpes virus or parainfluenza 3 virus showed reduced PC and increased PE in (Engen and Brown, 1991), while pigs inoculated with pasteurella multocida showed an increase in relative PI and a reduction in PG in BAL (Sachse, 1989).

# 1.3.7. CHANGE IN SURFACTANT COMPOSITION - FATTY ACID SPECIES Human disease

Two groups have studied fatty acid species in BAL fluid centrifuged to remove cells from patients with ARDS. Baughman and colleagues (1984) reported a reduction in phospholipid palmitic acid (16:0), and a greater proportion of stearic acid (18:0) and oleic acid (18:1) in ARDS and in pneumonia. Hallman and colleagues (1982; 1983) also found reduced 16:0, but in addition increased 16:1 increased 18:2 and increased 20:4 in PC from patients with ARDS. Both these authors found reduced saturated fatty acids and disaturated PC in ARDS.

It is interesting to note that lung tissue PC contains less 16:0/16:0 but is enriched in the 16:0/18:1 and 16:0/18:2 species compared to BAL PC (Hunt et al. 1991). Since 18:1 and 18:2 species are typically found in cell membranes, this raises the possibility that the changes in fatty acid content noted in studies of ARDS may be due to contamination of BAL with cell membrane fragments from pulmonary epithelial cells destroyed by the disease process.

# **Animal studies**

A study of bronchoalveolar lavage from dogs injured with NNNMU found increased PC 16:0 in the experimental group whereas 16:1 and 18:1 were less (Ryan et al. 1981). The reason for the discrepancy between findings in this model and human studies is not clear.

It appears that an alteration in fatty acids recovered from BAL occurs in acute inflammatory lung disease in humans. However, it is not possible to determine whether this is due to an alteration in surfactant phospholipids or to contamination of BAL with fragments of membranes from cells damaged in the disease process.

# **1.4. OXIDANT DAMAGE TO SURFACTANT**

# 1.4.1. REACTIVE OXYGEN SPECIES AND SCAVENGING IN THE ALVEOLUS

The lipid and protein components of surfactant are susceptible to damage from reactive oxygen molecules which may be released into the alveolus in lung inflammation. Pathways for the generation of these reactive molecules are shown in Figure 4

Oxidant injury in the lung may be limited by the presence in alveolar lining fluid of catalase, superoxide dismutase, reduced glutathione (McIlroy et al. 1992; Cantin et al. 1987; Matalon et al. 1990), vitamins C (Snyder et al. 1983) and E (Pacht et al. 1986), Caeruloplasmin and transferrin (Pacht and Davis, 1986) which act as 'scavengers' of reactive molecules.
## Peroxynitrite

One particular reactive oxygen molecule that has been attracting great interest recently is peroxynitrite, a reactive molecule formed by combination of superoxide and nitric oxide, both of which are released by activated alveolar macrophages (Ischiropoulos et al. 1992) (Figure 4). The role of peroxynitrite in lung injury is interesting as it is generated by a pathway which is separate from the Haber Weiss reaction. The antioxidants listed above, which are found in alveolar lining fluid act by keeping the participants in the Haber Weiss reaction at low levels. However, peroxynitrite is generated by a different mechanism (Figure 4), and may therefore be acting relatively unchecked in the alveolus to cause hung injury.





Figure 4. Pathways of free radical generation and molecular damage

Peroxynitrite can participate in several oxidative pathways which have the potential to induce lipid peroxidation (Radi et al. 1991), DNA damage and protein damage (Figure 4). Exposure of proteins to peroxynitrite can cause cysteine oxidation, tryptophan oxidation, protein fragmentation and tyrosine nitration. The product of this last reaction, nitrotyrosine, has been shown to be a specific marker for protein damage caused by peroxynitrite (Ischiropoulos and Al-Mehdi, 1995).

#### **1,4,2. OXIDANT DAMAGE AND SURFACTANT**

#### Peroxynitrite and surfactant

Haddad and colleagues (1993) studied the effects of peroxynitrite on surfactant in vitro and demonstrated that surfactant components could be damaged structurally and functionally by this molecule. The detailed findings of Haddad's study are summarised below.

Surfactant proteins were degraded by exposure to peroxynitrite in vitro as evidenced by SDS-PAGE of SPA, SPB and SPC. Additionally, an increased content of 3-nitro-L-tyrosine residues was demonstrated in SPA after exposure.

The surface tension lowering properties of surfactant were also shown to be susceptible to damage. Calf lung surfactant extract (CLSE - an organic extract containing surfactant phospholipids, SPB and SPC) exposed to peroxynitrite, lost surface activity and an increased concentration of lipid peroxidation products was found in CLSE after exposure, suggesting damage to surfactant lipids. Interestingly, if CLSE was supplemented with SPA before contact with peroxynitrite, the damaging effects on surface tension were not seen, implying that SPA may have a protective function.

To investigate the influence of the lipid composition of surfactant on oxidative damage, Haddad et al studied two surfactant preparations. When Exosurf (a synthetic surfactant containing only the DPPC lipid species) or a mixture of surfactant lipids (containing various phospholipid and fatty acid species) were incubated with peroxynitrite, only the surface activity of the lipid mixture decreased. This suggests that unsaturated lipids are the site of peroxidative injury.

This extensive in vitro study establishes that peroxynitrite has the potential to cause functionally significant damage to the lipid and protein components of surfactant.

### Other reactive species

There is evidence that reactive molecules other than peroxynitrite can cause damage to surfactant. Gilliard and colleagues (1994) exposed CLSE to ferrous chloride and hydrogen peroxide, and demonstrated a subsequent loss of surface active properties and an increase in indicators of lipid peroxidation. This indicates that activation of the Haber Weiss reaction can lead to surfactant damage (Figure 4). However, as discussed above, extensive scavenging mechanisms may offset damage by this route in vivo.

In summary, therefore, it seems that reactive species released during the inflammatory response in the lung have great potential to cause damage to pulmonary surfactant. The microenvironment of the alveolus has, however a defence system in place which

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may mitigate some of this damage. The picture is still sketchy and further work will be required before the effects of reactive molecules on surfactant are fully understood.

# 1.4.3. EVIDENCE FOR OXIDANT ACTIVITY IN THE LUNG IN ARDS Nitrotyrosine

Peroxynitrite-induced damage may well be significant in the clinical setting of inflammatory lung disease. Immunohistochemical studies (Haddad et al. 1994; Royall et al. 1995) have shown an increase in nitrotyrosine in postmortem sections of lungs from patients with ARDS and with pneumonia, indicating damage by peroxynitrite to surfactant proteins or to other proteins present in the lung.

## Lipid peroxidation

Roumen and colleagues (1994) measured serum lipofuscin - a pigment formed by combination of lipid peroxidation products with amino-groups on phospholipids and proteins. They found that in patients who had undergone major surgery or traumatic injury, those who subsequently developed ARDS had higher serum lipofuscin levels on the first day in intensive care than those who did not. They also found that serum lipofuscin level was inversely correlated with PaO2/FiO2 ratio (a measure of respiratory function) on the first day of intensive care stay. This indicated that poor pulmonary function was associated with a higher lipofuscin level, and by implication, with increased oxidant damage in the lung.

## Hydrogen peroxide

As can be seen from figure 4, Hydrogen peroxide is produced in the Haber Weiss reaction. Hydrogen peroxide has been found to be increased in the expiratory gas condensate from intensive care patients who developed ARDS compared to those who did not (Baldwin et al. 1986; Sznadjer et al. 1989). In Baldwin's study (1986), breath hydrogen peroxide was correlated positively with plasma lysozyme (an indicator of neutophil turnover) in both groups. However, interestingly, in a subgroup of 5 patients with pneumonia but not ARDS, breath peroxide was low, despite what the authors referred to as 'the probable accumulation of activated neutrophils in their lungs'. Sznadjer and colleagues (1989) studied intensive care patients with ARDS, patients with acute hypoxaemic respiratory failure (AHRF) with pulmonary infiltrates on chest X ray and patients with AHRF but without infiltrates. Infiltrates were due either to pulmonary contusion or to pneumonia. In contrast to Baldwin, Sznadjer found that expired hydrogen peroxide levels were similarly high in the ARDS and the 'AHRF with infiltrates' group, and significantly lower in the 'AHRF without infiltrates' group. The reason for this discrepancy is unclear, although Sznadjer and colleagues did study a larger number of patients than Baldwin, and used a different assay technique to measure hydrogen peroxide.

#### $\alpha$ -1-proteinase inhibitor

Alpha-1-proteinase inhibitor, a substance which helps to mitigate lung damage in the inflammatory response, was found inactivated in the BAL fluid from patients with ARDS. It was possible to reactivate this inactivated  $\alpha$ -1-PI by reduction, suggesting that it had been inactivated by oxidation (Cochrane et al. 1983).

## Plasma indicators of lipid peroxidation

Acute respiratory distress syndrome is ultimately a multi-systems disorder, with death often occurring due to failure of organs other than the lungs. Plasma lipid peroxides have been found to be increased in patients with ARDS (Cross et al. 1990). It is possible that, if lipid peroxides were studied in BAL from these patients, this increase would be reflected in BAL fluid, not necessarily as a result of lipid peroxidation occurring in the lungs, but as a result of leakage of plasma into the alveoli.

## 1.4.4. LUNG ANTIOXIDANT ACTIVITY IN ARDS

Two studies have demonstrated an increase in caeruloplasmin and transferrin in BAL fluid from patients with ARDS compared to control (Krsek-Staples et al. 1992; Lykens et al. 1992). Lykens and colleagues suggested that increased levels of these proteins were responsible for the greater antioxidant capacity of broncholaveolar lavage fluid which they found in ARDS.

A study of patients with ARDS who had increased lipid peroxidation products in their plasma also revealed a deficiency of plasma vitamin E in these patients (Cross et al 1990). This suggests that, in severe lung injury, the balance of antioxidants is altered in plasma and alveolar lining fluid.

## 1.4.5. THE EFFECTS OF HYPEROXIA ON SURFACTANT

The clinical and histological changes of ARDS are very similar to those seen in hyperoxic lung injury (Dedhia and Banks, 1994), possibly due to the common factor of oxidant damage to lung tissues. Hyperoxia has been shown to increase oxygen radical production by mitochondria in rat lungs (Freeman and Crapo, 1981) and there is evidence to suggest that hyperoxia-exposed lung epithelial cells are more susceptible to damage by products secreted from neutrophils in vitro (Suttorp and Simon, 1982). Also, hyperoxia can impair the bactericidal ability of pulmonary macrophages (Suttorp and Simon, 1981). It is difficult to disentangle the effects of hyperoxia and the effects of lung inflammation in vivo, as patients and animals with severe lung inflammation invariably need to be ventilated with high concentrations of oxygen for survival.

Several animal studies have been performed to investigate the effect of hyperoxia on pulmonary surfactant. In rats exposed to hyperoxia, total phospholipids (Balaan et al. 1995; Valimaki et al. 1975), total proteins (Balaan et al. 1995) and unsaturated fatty acids in PC and PG (Valimaki et al. 1975) were found to be incressed in lung washings. Balaan and colleagues (1995) exposed rats to 100% oxygen, and found that maximum and minimum surface tension of surfactant from these animals was increased. In contrast, rabbits (Holm et al. 1985) and mice (Gross and Smith, 1981) exposed to hyperoxia developed reduced alveolar phospholipid levels.

Rabbits exposed to 100% oxygen showed increased alveolar permeability (measured by detection of labelled cyanocobalamin) and reduced surface activity of cell free lavage concentrated to 1mM phospholipid. When these measurements were repeated in the recovery phase at 200 hours after exposure, lavage protein and surface activity had returned to the same as that in controls, while phospholipids were double compared to control (Holm et al. 1985). Increased alveolar permeability was offset by administration of calf lung surfactant extract to oxygen-exposed rabbits (Engstrom et al. 1989).

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Primates ventilated for 7 days with high inspired oxygen concentrations developed an inverse ratio of PC to PI in a 'high speed' pellet of surfactant purified by dialysis (King et al. 1989). However, exposure of rats to 95% oxygen resulted in no change in proportions of phospholipid classes in cell free lung washings after 60 hours (Valimaki et al. 1975).

Therefore, it seems that the picture found in hyperoxic lung damage is quite similar to that in ARDS, with an alveolar protein leak, alterations in surfactant phospholipid composition and quantity (with both increases and decreases found), and functional abnormality of surfactant.

## **1.5. SURFACTANT REPLACEMENT IN ARDS**

If surfactant is dysfunctional in acute lung injury, then surfactant replacement therapy may have an important role. However, the complexity of the impact which ARDS has on the pulmonary surfactant system, and the role of factors other than surfactant deficiency has been emphasised by initial attempts to use surfactant replacement therapy in ARDS. A multi-centre trial was conducted recently on the effects of aerosolised synthetic surfactant in sepsis-induced ARDS. No improvement was found in 30 day survival (Anzueto et al. 1996). However, more recently, administration of a bovine surfactant (containing SPB and SPC) was shown, in a pilot study, to reduce mortality substantially in ARDS (Gregory et al. 1997).

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In a paediatric population with ARDS, Exosurf treatment did not improve survival, but with Exosurf, dynamic compliance improved and there was a tendency for gas exchange to stabilize earlier (Perez-Benavides et al. 1995).

## 1.6. SUMMARY

From the review of studies above, it can be seen that the factors possibly leading to surfactant dysfunction in acute inflammatory lung disease are complex and far from understood. These may be summarised as:

- It is a matter of some controversy as to whether or not there is a deficiency of surfactant phospholipids in ARDS.
- There is evidence for intrinsic alterations in surfactant composition, specifically a reduction in PC and PG and an increase in sphingomyelin. Whether these affect surfactant function is not clear.
- 3. Fatty acid species seen in BAL fluid from patients with ARDS are different from control. However, this must be interpreted with caution in the light of possible contamination of BAL with membrane fragments.
- 4. It is likely that surfactant in the inflamed lung is inhibited by plasma proteins (and possibly by LPC which can exaccrbate this inhibition). Yet this has to be considered in the light of the balance between phospholipids and surfactant proteins which protect surfactant from inhibition.
- An imbalance between small and large aggregates of surfactant may lead to surfactant dysfunction in vivo.

 Surfactant may be injured by high levels of oxygen needed in therapy, or by oxidants gernerated in the inflammatory response. However, this damage may be offset by antioxidants in the alveolar lining.

## **1.7. SURFACTANT IN SEVERE RSV INFECTION**

#### **1.7.1. SURFACTANT DYSFUNCTION**

The radiological and clinical features of severe RSV infection would suggest that surfactant function may be reduced in this condition. The appearances seen on chest radiographs from ventilated infants with RSV can be similar to those seen in ARDS and in infantile RDS, where surfactant function is known to be poor. As in ARDS, ventilated children with RSV often require positive end expiratory pressure and high peak airway pressures to maintain oxygenation. These ventilatory requirements are indicative of a tendency to alveolar collapse and increased small airways resistance or decreased lung compliance - all of which could result from surfactant dysfunction.

Severe RSV infection, like ARDS and pneumonia, is a form of inflammatory lung disease, and there are important pathological similarities between these three conditions. All are characterised by an infiltrate of inflammatory cells, an alveolar exudate and the presence of cell debris in the alveoli. As in ARDS and pneumonia, there is the potential for surfactant dysfunction in severe RSV infection. Potential causes of this dysfunction are deficiency or abnormal composition of surfactant, damage to surfactant or inhibition of surface activity. These factors are summarised in Figure 5 and reviewed below.

## 1.7.2. POTENTIAL DEFICIENCY OR ABNORMAL COMPOSITION OF SURFACTANT

Alterations in the absolute and relative amounts of surfactant phospholipids and proteins have been found in BAL fluid from patients with ARDS and with pneumonia. It is possible that, given the parallels between RSV and these inflammatory lung diseases, a similar pattern of surfactant abnormality may be seen in severe RSV infection.

Molecular participants in the inflammatory response may affect the amount of surfactant present in the alveolus during RSV infection. Recent in vitro work by Haddad and colleagues (1996) suggests that nitric oxide and peroxynitrite (which are produced and secreted locally in the inflammatory response) may decrease synthesis of surfactant. Since RSV is associated with an inflammatory infiltrate, this is, then, potentially a mechanism for surfactant damage.

Other mechanisms, specific to infection with RSV, could also reduce the amount surfactant phospholipids and proteins present in the alveolus. For example, viral invasion of type II pneumocytes may perturb surfactant synthesis. Also involvement of surfactant proteins A and D in the local immune response to micro-organisms may lead to alterations in the alveolar concentration of these proteins in viral infections such as RSV.

In summary, it is possible to hypothesise that altered amounts of phospholipids and proteins as well as changes in phospholipid or fatty acid proportions may occur in RSV infection and could cause impairment of surfactant surface activity and immune

function. Changes in relative proportions of surfactant phospholipids or fatty acid species would have a theoretical potential to affect surface activity, as fatty acid or phospholipid composition of surfactant may be important in the packing of lipids at the air-liquid interface (section 1.2.3.).

## 1,7,3, POTENTIAL DAMAGE TO SURFACTANT

An infiltrate of inflammatory cells - probably mainly neutrophils - occurs in RSV infection. These cells release reactive molecules which may damage surfactant per se, or, in the case of nitric oxide and superoxide, unite to form peroxynitrite which has been proven to damage surfactant in vitro.

## 1.7.4. THE EFFECT OF HYPEROXIA

Ventilation with high concentrations of inspired oxygen has been shown to alter surfactant composition and to cause surfactant dysfunction in experimental animals (section 1.4.5.). If this also occurs in humans, the high concentrations of inspired oxygen needed to maintain tissue oxygenation in severe RSV infection could lead to similar changes.

## **1.7.5. POTENTIAL SURFACTANT INHIBITION**

Leakage of proteinaceous fluid from the pulmonary capillaries is common in inflammatory lung diseases, and there is evidence that such leakage is present in RSV infection. A fibrinous exudate fills a proportion of the alveoli in the lungs of patients

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who have died from severe RSV infection, and oedema fluid permeates the lung parenchyma and alveoli. Oedema fluid, containing plasma proteins, especially fibrinogen and fibrin degradation products is well known to cause inhibition of pulmonary surfactant in vitro. It is highly likely that this mechanism operates in the lungs of patients with RSV.

## 1.7.6. RSV AND SURFACTANT - CURRENT EVIDENCE

Until now, little investigation of the state of the pulmonary surfactant system in RSV infection has been carried out. Recent studies of tracheal aspirate samples from patients ventilated for severe viral bronchiolitis and pneumonitis have found reduced levels of SPA (Dargaville et al. 1996; LeVine et al. 1996). In addition to studying tracheal aspirates, Dargaville (1995) obtained BAL samples from some patients. However, when SPA was measured in these BAL samples there was no significant difference from the control group.

Dargaville's finding (1995) of a discrepancy between levels of SPA in BAL and tracheal aspirate is interesting. Bronchoalveolar lavage theoretically samples a lower part of the respiratory tract than aspiration of tracheal secretions. As RSV is a disease whose inflammatory manifestations are seen relatively distally in the bronchial tree, and as surfactant functions at a similar distal level in the lung, BAL would possibly give a better picture of the pulmonary surfactant system in RSV infection.

Surfactant protein B levels were not found to be different from controls in a study of ventilated children with viral pneumonitis (LeVine et al. 1996).

Dargaville and colleagues (1996) also found reduced levels of surfactant DPPC in tracheal aspirates, and reduced surface activity of the surfactant pellet recovered from patients with viral bronchiolitis. A recent preliminary study by Skelton and colleagues (1994) demonstrated inhibition of surfactant and a reduced PG fraction in tracheal aspirates from patients with viral bronchiolitis.

Therefore, it would appear that qualitative and quantitative abnormalities of surfactant phospholipid and a reduction in SPA, with reduced surfactant function are present in severe RSV infection.

## Preliminary evidence from surfactant replacement studies

If surfactant is dysfunctional, then replacement therapy might be helpful. Recently two groups have published case reports of clinical improvement following administration of exogenous natural surfactant to children with severe viral pneumonia (Vos et al. 1996; Putz et al. 1996).

## 1.7.7. SUMMARY

The pathological features which may lead to abnormalities of the pulmonary surfactant system in severe RSV infection are summarised in Figure 5. These provide theoretical grounds on which to base a hypothesis that surfactant is dysfunctional, due to deficiency, abnormal composition, damage or inhibition in respiratory failure due to RSV infection. Recent studies have shown evidence for a reduction in surfactant function and amount in this condition. However, the complex range of potential causes





# 2. HYPOTHESIS AND AIMS

## 2.1. HYPOTHEISIS

Pulmonary surfactant is dysfunctional in respiratory failure due to Respiratory Syncytial Virus infection, due to deficiency, abnormality in composition, damage or inhibition.

## 2.2. AIMS

The aims of this study were to determine whether pulmonary surfactant is dysfunctional, deficient, abnormal in composition, damaged or inhibited in severe RSV infection. The study to be described investigated pulmonary surfactant in a group of ventilated children with severe RSV infection versus controls. In the RSV group, the profile of surfactant change throughout the disease process, and the relationship of surfactant components in BAL fluid to alveolar arterial oxygen ratio (as a measure of disease severity) was also investigated.

The specific aims were:

1. To assess surfactant function by performing functional tests on whole BAL fluid and a lipid extract of BAL fluid at a known concentration of phospholipid.

 To ascertain whether surfactant is deficient or abnormal in composition in severe RSV infection by analysis of the phospholipid and protein components of pulmonary surfactant.

3. To search for evidence of damage to pulmonary surfactant in severe RSV infection by quantifying markers of free radical damage and surfactant breakdown

4. To assess surfactant inhibition and surfactant inhibitors in severe RSV infection by:

a) quantifying proteins in BAL fluid

b) evaluating the inhibitory potential of proteins in BAL fluid

# 3. PATIENTS AND CLINICAL DATA

## 3.1. PATIENTS

#### 3,1,1. STUDY GROUPS

The children studied were all intubated and artificially ventilated. Two groups were studied:

1. The study group was composed of children who were mechanically ventilated in the intensive care unit with a diagnosis of respiratory failure due to RSV infection. Diagnosis of RSV infection was made by immunofluorescence of nasopharyngeal secretions. Patients were either diagnosed as 'RSV positive' on admission to the intensive care unit, or diagnosis was made on clinical suspicion, and subsequently confirmed by immunofluorescence of nasopharyngeal secretions. All ventilated infants with RSV were patients in the medical intensive care unit at the Royal Hospital for Sick Children, Glasgow.

2. The control group were children undergoing mechanical ventilation for surgical procedures. These patients did not have any clinical or radiological evidence of primary lung parenchymal disease. They came from two sources. The first group (n=8) were being ventilated in the surgical intensive care unit at the Royal Hospital for Sick Children, Glasgow to allow adequate analgesia following surgical procedures. The second group (n = 8) were intubated for routine surgical procedures, and were studied

after induction of anaesthesia and before surgery (Table 6). The median age in the study group was 3.1 months (range 0.3 - 42.1), while that in the surgical group was 0.4 months (range 0.0 - 42.0). The age distribution in each group is shown in Figure 6.

Group	number	gender (m:f)	<b>median age</b> (months) (range)
ventilated to facilitate analgesia after surgery*	8	6;2	0,1 (0.0-0.5)
ventilated for routine procedures**	8	3:5	6.3 (0.0-42,0)
ventilated for severe RSV infection	18	11:7	3.1 (0.3-42.1)

Table 6. Children in the RSV and control groups.

\*Infants ventilated after operative procedures (eg. gastroschisis repair, repair of oesophageal atresia/tracheosophageal fistula, inguinal hernia repair) in the surgical intensive care unit at the Royal Hospital for Sick Children, Glasgow.

\*\*Children intubated for routine procedures (eg. dental extraction, CT scan, inguinal hernia repair) and studied after induction of anaesthesia and before surgery

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Figure 6. Age at sampling of patients with RSV infection and patients undergoing surgery. It can be seen that the distribution of ages is similar in the two groups.

## 3.1.2. ETHICAL APPROVAL AND INFORMED CONSENT

Approval for study of the patient groups detailed in section 3.1.1. was given by the Ethical Committee of the Royal Hospital for Sick Children, Glasgow. The procedure of sample collection and the purpose of the study was explained to parents or guardians before their child's participation, and witnessed written consent obtained. One copy of the consent and information form (Appendix 1) was given to parents or guardians, and one copy was retained alongside data collected in the study.

## 3.2. COLLECTION AND STORAGE OF CLINICAL DATA

## 3.2.1. RECORDING OF PATIENT DATA

Clinical data on patients studied were collected daily at the time of sampling using a standard form (Appendix 2). The parameters recorded included diagnosis, arterial alveolar oxygen ratio, drug therapy and details of any significant clinical incidents occurring in the preceeding 24 hours.

# 3.2.2. ARTERIAL ALVEOLAR OXYGEN RATIO AS A MEASURE OF DISEASE SEVERITY

The arterial alveolar oxygen ratio provides an accurate and relatively non-invasive measure of pulmonary function. It utilises measurements of arterial oxygen tension and a calculated measure of "ideal alveolar gas" (Appendix 3).

The form of the equation which is commonly used clinically for calculation of ideal alveolar oxygen content is :

$$P_{AO_2} = F_{iO_2}(P_B - P_{U_2O}) - P_{ACO_2}(F_{iO_2} + \frac{1 - R_{O_2}}{R})$$

(alveolar  $P_{CO_2}$  is assumed to be equal to arterial  $P_{CO_2}$  in practice)

The arterial alveolar oxygen tension ratio has been demonstrated to be a reasonably stable index of pulmonary function in the presence of different inspired oxygen concentrations (Gilbert and Keighley, 1974). It has been evaluated theoretically and

clinically in adult intensive care patients by Zetterstrom (1988), and was found to reflect shunt fraction (the proportion of blood which passes through the lungs but is not oxygenated) more accurately at different levels of inspired oxygen concentration than other methods in common use (eg. arterial oxygen tension, arterial oxygen saturation, and arterial alveolar oxygen difference).

Severe infection with RSV is associated with bronchial and bronchiolar plugging, and areas of atelectasis. These conditions, which resolve slowly with recovery would be expected to produce an increase in shunt fraction, and hence a decrease in arterial alveolar oxygen ratio. For this reason, arterial alveolar oxygen ratio was chosen as an index of disease severity for use in this study.

The equation above was used to calculate ideal alveolar partial pressure of oxygen. Inspired oxygen concentration ( $F_{102}$ ) was measured with a paramagnetic oxygen analyser. Barometric pressure ( $P_B$ ) was measured with a mercury barometer. Partial pressure of water vapour  $P_{AH 20}$  was assumed as 47mmHg. Alveolar carbon dioxide tension ( $P_{ACO2}$ ) was assumed to be equal to arterial PCO<sub>2</sub> (Riley and Cournand, 1949), and this was measured with a CO<sub>2</sub> electrode in a standard blood gas analyser. The respiratory quotient, R, was assumed to be 0.8. Arterial PO<sub>2</sub> was measured with an oxygen electrode in a blood gas analyser. In all intensive care patients, an arterial line was already in situ for blood sampling and invasive blood pressure recording and arterial blood samples were taken from this line.

The alveolar oxygen tension was calculated, and arterial  $PO_2$  was divided by this value to give the arterial/alveolar oxygen ratio.

# 4. MATERIALS

## 4.1. CHEMICALS

All chemicals used in this work were of analytical grade and were purchased from BDH Limited or Sigma Chemical Company.

## 4.2. BUFFERS AND OTHER REAGENTS

All water used in preparation of buffers and other reagents was deionised (Elgastat Spectrum de-ioniser) unless otherwise stated. The formulation of buffers used in this work is detailed in Appendix 4.

## 4.3. GLASSWARE AND PLASTICWARE

All glassware was washed with a lipid-free detergent, rinsed in acetic acid, and then rinsed twice with de-ionised water. 'Immulon 4' (Dynatech Ltd, Billinghurst, Kent) microtitre plates were used in this study .

## 4.4. PIPETTES

The same set of pipettes was used within each assay. Pipettes were calibrated at 6 monthly intervals.

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# 5. METHODS

## 5.1. COLLECTION AND STORAGE OF BAL SAMPLES

# 5.1.1. NON BRONCHOSCOPIC BRONCHOALVEOLAR LAVAGE Background

Bronchoalveolar lavage (BAL) is usually performed in adults and older children via a flexible fibreoptic bronchoscope inserted through a tracheal tube or laryngeal mask - or directly through the vocal cords. The flexible bronchoscope is advanced through the bronchial tree, and wedged in a known segmental bronchus, where saline is instilled through the bronchoscope. The instilled fluid, mixed with the contents of airway fluid distal to the wedged end of the instrument, is then aspirated through a suction channel. The smallest available flexible fibreoptic bronchoscope with a suction channel has an external diameter of 3.5mm (Wood, 1993). This bronchoscope can only be passed through a tracheal tube connector of 4.5mm or greater internal diameter. This makes bronchoscopic lavage impossible in small, ventilated infants where tracheal tube internal diameter is less than 4.5mm.

As an alternative, non-bronchoscopic BAL involves inserting a blindly directed suction catheter into the bronchial tree, wedging this catheter and then instilling and aspirating a volume of saline solution. The use of this technique in intubated infants with small artificial airways has been described in the past decade (Alpert et al. 1992), as a safe and effective method of obtaining alveolar lining material. More recently, a simplified technique has been described (Kombourlis and Kurland, 1993). Cytological examination of lavage samples by these workers revealed the presence of alveolar macrophages, indicating that alveolar lining material had been obtained by the nonbronchoscopic method.

Pulmonary surfactant should be largely confined to the most distal parts of the bronchial tree, where it is produced and recirculated. This makes it likely that this method of lavage will yield a more representative sample of alveolar fluid than, for example, tracheal aspiration (the method usually employed to study surfactant in small children). This is supported by some evidence that the phospholipid composition of fluid collected by fibreoptic bronchoalveolar lavage is different from the composition of that obtained from tracheal aspirates and washings (Widdicombe, 1987).

## Method

A 35cm long 2mm external diameter catheter (in children under 18 months), or a 75cm long 2.7mm external diameter catheter (in children over 18 months) (Combicath, Panmedical Ltd, Paisley, Scotland) was used for this procedure (Figure 7). Patients were pre-oxygenated for 5 minutes prior to the procedure by increasing the inspired oxygen concentration delivered via the ventilator mixer to 100%. The ventilator tubing was then disconnected from the patient's tracheal tube connector, and the catheter advanced through the tracheal tube until it was felt to 'wedge'. The catheter was then withdrawn approximately 2mm. A 3ml (in children under 3kg), 5ml (in children 3-13 kg) or a 7ml (in children over 13kg) aliquot of sterile 0.9% saline solution warmed to body temperature was instilled, and then immediately aspirated. These volumes were felt to be more appropriate than weight adjusted volumes which would have led to

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lavage with excessive quantities of saline in older children. Saline was instilled from and aspirated into a 10ml syringe, and aseptic technique was observed at all times. When the 35cm catheter was used, each aliquot of saline was 'chased' by approximately 0.5ml of air to flush out the catheter dead space. With the 75cm catheter, an aliquot of approximately 1ml of air followed the saline. After lavage, the patient was briefly reconnected to the ventilator (with the mixer still set at 100%), and the lavage procedure subsequently repeated until a total of three aliquots had been collected. After the collection of these aliquots, tracheal suction was performed to collect any residual fluid or secretions. The duration of the entire collection procedure was approximately five minutes. The inspired oxygen concentration was then gradually weaned down to its original value over five minutes following the procedure. Sample collection in the children studied was performed at 24 hour intervals from intubation until extubation. Samples were always taken at least one hour after physiotherapy or tracheal suctioning by nursing staff.

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Figure 7. System used for sampling BAL fluid

## 5.1.2. PATIENT MONITORING

Throughout the pre-oxygenation period, during the lavage and suctioning, and subsequently, while the inspired oxygen concentration was being weaned back to its original value, the child's clinical condition, arterial oxygen saturation,

electrocardiogram, pulse rate, ventilator pressures and invasive blood pressure were monitored by the experimenter and intensive care nursing staff. Chest auscultation was performed on the patient before and after sampling. Auscultation allowed assessment of air entry to both lungs before and after the procedure. Any change in air entry could indicate either dislodgement of a mucus plug or inadvertent endobronchial intubation. Both of these are theoretical complications of BAL which can be remedied by further suction (to remove the mucus plug) or by repositioning of the tracheal tube. Neither of these complications occurred during this study.

#### 5.1.3. CENTRIFUGATION PROTOCOL AND STORAGE OF SAMPLES

Samples were centrifuged at 250 x g for 5 minutes to precipitate cellular debris. The supernatants were then decanted, aliquoted and, within 30 minutes of collection, stored at -70°C until analysis.



## 5.2. ASSESSMENT OF SURFACTANT FUNCTION

#### 5.2.1. THE SHAKE AND CLICK TESTS

### Background

The shake test (Clements et al. 1972) and the click test are quick, in vitro tests of surface activity performed on a whole sample of lavage fluid (Skelton and Jeffrey, 1994; Todd and John, 1983).

The shake test is performed by shaking BAL fluid with ethanol in a test tube and observing the formation of bubbles. The ethanol, at a concentration of 47.5% excludes proteins, bile salts or salts of free fatty acids which may otherwise cause a foam. At this fractional concentration of ethanol, only double chain phospholipids from BAL surfactant should compete effectively for the surface film (Clements et al. 1972).

In the click test, bubbles formed in the shake test are suspended under a cover slip in air free water (obtained by boiling deionised water) in the cavity of a glass slide, and examined under a microscope. If active surfactant is present, 'clicking' is seen as repeated shrinking and expanding of the bubbles. The 'clicking' is thought to be caused by repeated diffusion of air out of the bubbles into the air-free water. This diffusion makes the bubbles flatter against the cover slip. The surface film of active surfactant then suddenly reforms in the original spherical shape, making the bubble appear to contract, and the cycle begins again. If active surfactant is not present, this does not happen, and the bubbles gradually shrink and disappear without clicking as air diffuses into the water.

Skelton and Jeffrey (1994) found that the positive predictive value of both the shake test and the click test for diagnosis of Respiratory Distress Syndrome (where surfactant function is poor) in neonates was 100%. The negative predictive value of the click test was 93% and that of the shake test was 80%.

The click and shake tests are useful because they provide an integrated assessment of whole BAL fluid and provide an overall test of surfactant function. They give no indication of the reason for surfactant dysfunction. Any dysfunction found by this method could be due to deficiency of surfactant lipids or proteins, altered composition of the lipid fraction, damage to surfactant or inhibition of surface activity.

## Method

Two hundred microlitres of lavage fluid were mixed with an equal volume of 95% ethanol in a 12 x 75mm glass test tube and the mixture was vortex mixed for 15 seconds. In accordance with Skelton's description (1994), the shake test was considered 'positive' if a rim of bubbles surrounded more than ¾ of the tube circumference after vortex mixing.

The bubbles were then removed with a pipette and placed on a cover slip. The cover slip was inverted onto the cavity of a slide containing air-free water and the slide was observed under 10x magnification. In accordance with Skelton's description (1994), the click test was considered 'positive' if bubbles were seen 'clicking', as described above.

## 5.2.2. THE PULSATING BUBBLE SURFACTOMETER

## **Background and principle of operation**

The Wilhelmy balance was extensively used in early studies of the physical properties of pulmonary surfactant. In this balance, surface tension of a layer of surfactant on the surface of liquid in a trough is measured. Enhorning (1977) pointed to several disadvantages of this method - that is the comparatively large sample volume needed, the difficulty in cleaning the instrument, and the length of time needed to make the measurement of surface tension. Additionally, he noted that the surface area of the surfactant film in the Wilhelmy balance was flat, unlike the curved surface in the alveoli, and also that the frequency of compression and expansion was much slower than that of breathing.

The pulsating bubble technique was first conceived in an attempt to produce a realistic alveolar model (Adams and Enhorning, 1966). This surfactometer employs a 25µl sample chamber in which an air bubble is formed. The small sample chamber is filled with the fluid to be studied and then inverted (Figure 9). A bubble of air forms in the liquid at the end of the air inlet. The bottom of the sample chamber is then seated into the surfactometer to connect to a metal tube which passes through a container of water at 37°C. The sample chamber and attached metal tube are pushed down until the chamber is immersed in water. The end of the air inlet remains above the surface of the water, thus sustaining the bubble. The water container is fixed to the stage of a microscope and has a transparent side which allows viewing of the sample chamber while it is immersed. The bubble is aligned so that its edges abut onto vertical hairlincs on the eyepiece of the microscope, which are a known distance apart. The sinusoidal

pulsator unit - an arrangement of pistons - then moves a specified volume of liquid out of the sample chamber, and back in again, causing the bubble to alternate between a maximum and a minimum radius (r). A pressure transducer measures the pressure drop ( $\Delta P$ ) across the bubble (Figure 10).

According to the Young Laplace law,  $\Delta P = 2\gamma/r$  (for a sphere), where  $\gamma$  is the surface tension. As the radius (r) and the pressure drop ( $\Delta P$ ) are known, surface tension at maximum and minimum radius can be calculated (Enhorning, 1977). The bubble is not, in fact, spherical in solutions containing aliphatic substances. However, Hall and colleagues (1993) have shown that use of the spherical case of the Young Laplace equation results in a minimal absolute error in practice. The same authors demonstrated that viscosity effects were not a source of error at minimum and maximum bubble radii. This is because the error due to viscosity effects can be shown to be  $-2\kappa \cdot 1/r \cdot dr/dt$  where  $\kappa$  is a term describing viscosity and r is the radius of the bubble. At maximum and minimum radii, the term dr/dt, the derivative of the radius with respect to time becomes equal to zero, and so does the error.



Figure 9. Filling the surfactometer sample chamber



Figure 10. Pulsating bubble surfactometer - mechanism of action
#### Sample chamber preparation

Measurements of surface tension in the pulsating bubble surfactometer are very sensitive to artefacts due to sample chamber contamination. A cleaning procedure was therefore adopted before sample chamber use (personal communication B. Robertson, Stockholm 1995).

Each sample chamber was rinsed 'in situ' for 5 minutes while connected to the surfactometer, using distilled de-aerated water. The sample chambers were stored in 5% Extran MAO3 (Merck Ltd, Darmstadt, Germany), and then transferred to a fresh solution of 5% Extran MAO3, and vortex mixed manually for 5 minutes. After rinsing for five minutes in tap water, the chambers were sonicated for 1 hour in fresh 5% Extran MAO3. They were then rinsed in running tap water for 5 hours, and sonicated in distilled water for 30 minutes. Finally, the chambers were air dried.

#### Method - Surfactometer studies of a lipid extract of BAL fluid

In preparation for analysis in the surfactometer, BAL samples were centrifuged at 40,000g for one hour (Sorvall Superspeed centrifuge) to obtain a surfactant pellet. The 40,000g surfactant pellet was subjected to a chloroform:methanol extraction (as described in section 5.4.1.) in order to remove potential interfering proteins which may have been pelleted along with surfactant. The dried lipid was then immediately resuspended in suspension buffer to a concentration of 1mM (750  $\mu$ g) phospholipid, taking account of centrifugation losses.

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The organic extraction and adjustment of concentration allowed the effects of any damage to surfactant lipids or hydrophobic proteins to be assessed in the surfactometer independently of variations in phospholipid concentration or interfering proteins.

The sample was transferred to a clean surfactometer sample chamber via a disposable insulin syringe and the bubble cycled at a temperature of 37° C, and a rate of 20 cycles per minute. Recordings of maximum and minimum surface tension were made for 10 minutes.

## **5.3. QUANTIFICATION OF SURFACTANT PROTEINS**

The measurements made of surfactant components on each BAL sample are summarised in Figure 8.

#### 5.3.1. SURFACTANT PROTEIN A

#### Background

The enzyme linked immunosorbant assay (ELISA) used to quantify surfactant protein A is an antigen capture assay (McMahan et al. 1987). In this technique, microtitre plate wells are coated with an antibody to SPA. Samples or standards containing SPA are added and the SPA binds to the antibody on the plate. A second antibody to SPA is then added, followed by a horseradish peroxidase conjugate which binds to the second antibody. The conjugate is then reacted with a substrate to give a colorimetric end point. This assay was developed and validated in the Childrens Hospital Medical

Centre, Cincinnati, Ohio, and has been used in studies of surfactant from BAL fluid (LeVine et al. 1996; Gregory et al. 1991). Standards and antibodies used in this assay were a gift from Professor J. Whitsett (Children's Hospital Medical Centre, Cincinnati).

#### Method

A microtitre plate (96 wells) was coated with 100µl per well of a 1:200 dilution of goat anti-human SPA antibody in 0.1M sodium bicarbonate. The plate was allowed to stand overnight at 4°C

The plate was then washed three times with SPA/SPB wash buffer and dried thoroughly. Human albumin 2.5% and goat serum 2.5% were added to 82b buffer to make a blocking buffer. One hundred microlitres per well of this blocking buffer were added to the plate, and the plate was allowed to stand for 15 minutes at room temperature. The blocking buffer was removed, and the wells blotted dry.

Standard concentrations of SPA (100, 75, 50, 25, 10 and 5 ng/ml) were prepared in PBS-NP40. The samples of lavage fluid to be tested were diluted to 1:50, 1:200, 1:800 and 1:3200 with PBS NP-40. Samples, standards and a blank (PBS-NP40) were added in duplicate at 100µl per well, and the plate incubated for 2 hours at 37°C. The contents of each well were discarded and the plate washed three times with wash buffer and blotted dry.

One hundred microlitres of polyclonal rabbit anti human SPA diluted 1:400 in 82b with human albumin 2.5% and goat serum 2.5% were added to each well. The plate was incubated for one hour at 37°C. After incubation, the contents of each well were discarded, and the wells washed three times with wash buffer and blotted dry.

One hundred microlitres of goat anti-rabbit horseradish peroxidase conjugate diluted 1:1000 with SPA/SPB dilution buffer with human albumin 2.5% and goat serum 2.5% were added to each well. The plate was then incubated at 37°C for one hour. The contents of each well were discarded, and the plate washed three times with wash buffer and blotted dry.

Substrate solution was prepared by adding 5ml of 10x stock concentrate substrate buffer to 35ml water, and then adding this dilution to 5ml of water containing 50  $\mu$ l of 30% hydrogen peroxide, and 5ml of water containing 200mg of orthophenylenediamine.

One hundred microlitres of substrate solution was added to each well, and colour development was observed. Colour development was stopped with 100µl per well of 9M sulphuric acid and absorbance read at 490 nanometres on a Dynatech MR5000 plate reader. The absorbance was read versus the concentrations of standards, a standard curve constructed, and the concentrations of unknowns read from this curve.

#### 5.3.2. SURFACTANT PROTEIN B

#### Background

The ELISA for surfactant protein B, unlike that for surfactant protein A only utilises one surfactant protein specific antibody (Pryhuber et al. 1991). The assay was developed and validated in the Children's Hospital Medical Centre, Cincinnati, and has been used extensively in a clinical context for assessment of tracheal aspirate samples from neonates suspected of congenital SPB deficiency. Standards and antibodics used in this assay were a gift from Professor J. Whitsett (Children's Hospital Medical Centre, Cincinnati).

#### Method

A ninety six well microtitre plate was coated with 100µl per well of 0.1M sodium bicarbonate. The plate was then allowed to stand overnight at 4°C. The plate was washed with wash buffer three times and blotted dry.

One hundred microlitres of 82b with 5% human albumin were added to each well, and the plate was allowed to stand for 15 minutes at room temperature. The 82b/albumin was removed, and the plate blotted dry.

Surfactant protein B standards were made diluted in PBS-NP40. The concentrations of the standards were 100, 75, 50, 25, 10 and 5 ng per ml. Samples were diluted 1:2 and 1:8 with PBS-NP40. One hundred microlitres of standard, sample or blank (PBS-NP40) were added to each well in duplicate, and the plate was incubated for 2 hours at 37°C. The contents of each well were discarded and the plate washed three times with wash buffer and blotted dry.

Polyclonal rabbit anti-human SPB antibody diluted 1:1000 in 82b with 5% human albumin was added at 100µl per well. The plate was incubated for 1 hour at 37°C. The contents of each well were discarded and the plate washed three times with wash buffer and blotted dry.

A horseradish peroxidase conjugate of goat anti-rabbit IgG diluted 1:1000 with dilution buffer with 5% human albumin was added to each well at 100µl per well. The plate was incubated for 1 hour at 37°C. The contents of each well were discarded and the plate washed three times with wash buffer and blotted dry.

One hundred microlitres of substrate solution (prepared as described for SPA ELISA) were added to each well, and colour development observed. Development was stopped with 100  $\mu$ l per well of 9M sulphuric acid and absorbance read at 490 nanometres on a Dynatech MR5000 plate reader. The absorbance was read versus the concentrations of standards, a standard curve constructed, and the concentrations of unknowns read from this curve.

#### 5.3.3. SURFACTANT PROTEIN D

#### Background

The principle of the ELISA for surfactant protein D is similar to that for surfactant protein A. That is, it is an antigen capture assay. This assay was developed and validated (Miyamura et al. 1994b) in the MRC Immunochemistry Unit Oxford, and

standards and antibodies used were a gift from Professor K. Reid (MRC Immunochemistry Unit, Oxford).

#### Method

Rabbit anti-human SPD IgG was made up at  $5\mu$ g/ml in coating buffer. One hundred microlitres per well of this dilution were added to each well of a 96-well microtitre plate. The plate was left to stand overnight at 4°C.

The wells were washed three times with wash buffer, and then to each well was added 100  $\mu$ l of wash buffer with 3% bovine serum albumin (to block unoccupied binding sites). The plate was then left to stand at room temperature for 2 hours.

Meanwhile, standard solutions of SPD (2000, 1000, 500, 125, 62.5, 31.3 and 15.7ng per ml) were prepared diluted in wash buffer.

The blocker was discarded, and the plate washed three times with wash buffer and blotted dry. Standards, samples and blank were added in duplicate at 100µl per well. The plate was incubated at room temperature overnight.

At the end of this period, the plate was washed three times, and complete dryness ensured. Biotinylated anti-SPD IgG, diluted 1:1000 in wash buffer was then added at 100µl per well and the plate incubated for 3 hours at room temperature. The plate was washed three times with wash buffer, and dryness ensured. Extravidinalkaline phosphatase conjugate diluted 1:1000 with wash buffer was added at 100µl per well and incubated for 1 hour at room temperature.

The conjugate was discarded, and the plate washed three times and blotted dry. Fifty microlitres per well of developing buffer with Img/ml paranitrophenolphosphate was added. The plate was incubated in the dark for 30 minutes, and optical density read at 405 nanometres in a Dynatech MR 5000 plate reader. The absorbance was read versus the concentrations of standards, a standard curve constructed, and the concentrations of unknowns read from this curve.

## 5.4. PHOSPHOLIPID ANALYSIS

#### 5.4.1. LIPID EXTRACTION

#### Background

Organic extraction is a preliminary to analyses of lipids in biological tissues. Two methods of lipid extraction were used in this study: a chloroform methanol extraction and a propan-1-ol hexane extraction. The former method produces a 95-99% recovery of total lipids from tissue samples (Christie, 1982a). The latter was adopted in this study for relatively selective extraction of phospholipids, and has been shown to extract 86.8% of DPPC, 3.8% of tripalmitin and 16.4% of palmitic acid into the propan-2-ol layer (Ip et al. 1977).

1. A.

#### Choloroform-methanol extraction

After thawing, an aliquot of lavage fluid samples was placed in a test tube. An equal volume of methanol and a twice equal volume of chloroform were added, so that sample, methanol and chloroform were present in the ratio of 1:1:2 (v:v:v) (Bligh and Dyer, 1959).

The chloroform, methanol and water were first mixed vigorously by hand for 30 seconds, and then vortex mixed for the same time. After centrifugation at 1000 x g for 5 minutes, the upper aqueous layer was discarded, and the lower chloroform lipid-containing layer aspirated and transferred to a glass boiling tube, placed in a heating block, and evaporated to dryness under a stream of nitrogen at 45°C. The dried lipid was then taken up into 200µl of chloroform, and stored at -70°C until analysis.

#### Propan-2-ol - hexane extraction

A modification of the method of Ip and colleagues (1977) was used to extract phospholipids from BAL fluid. Five hundred microlitres of BAL fluid was placed in a test tube. To this was added 1ml of n-hexane, 1.75 ml of propan-2-ol and 500µl of 40mM sulphuric acid. The mixture was shaken vigorously for 30 seconds and then vortex mixed for 30 seconds. After centrifugation for 5 minutes at 1000g, the lower propan-2-ol layer was decanted and retained for analysis.

#### 5,4,2. SEPARATION OF PHOSPHOLIPIDS BY THIN LAYER CHROMATOGRAPHY

#### Background

Thin layer chromatography was used in this study to separate the phospholipid classes PC, PG, PE, PS, PI, S and LPC. In this technique, samples are applied close to the edge of a glass plate coated with a thin layer of silica gel. The plate is immersed in a tank containing a mixture of solvents, and the samples are fractionated according to the different adsorption and solubility characteristics of their component lipids. The plate can then be sprayed with an agent which renders the lipids visible. The lipids thus seen can then be either quantified on the plate by scanning densitometry or scraped off and quantified by other means.

#### Method

Silica gel thin layer chromatography plates (10 x 10 cm LHPK silica gel 60Å) were obtained from Whatman Inc, Clifton New Jersey. The plates were first washed to full length in n-hexane: diethyl ether 1:1 (v:v). The plates were then activated by heating at  $110^{\circ}$ C for 1 hour. The mobile phase was prepared by mixing methyl acetate, propan-2ol, chloroform, methanol and 0.25% potassium chloride in a ratio of 10:10:10:4:3.6 (v:v:v:v) respectively (Olsen and Henderson, 1989). The solvent mixture was allowed to equilibrate with air in a sealed, unlined tank for 10 minutes.

Surfactant lipids were extracted from lavage fluid as described in section 5.4.1. (chloroform and methanol extraction). The 200µl of chloroform used to re-dissolve the dried lipid was evaporated to dryness under a stream of nitrogen at 45°C. The product was then dissolved in 10µl of chloroform. The 10µl sample was applied to the preadsorbent layer of the plate. A standard solution of LPC, S, PC, PS, PI, PG and PE was also applied to each plate in a separate lane. The plates were then developed in the mobile phase to within 0.5cm of the top of the layer. The plates were allowed to dry in air, and then dried in an oven at 110°C for 30 minutes.

To allow visualisation of the phospholipid spots on the chromatography plate, the plate was sprayed until damp with 3% (w:v) cupric acetate in 8% (v:v) orthophosphoric acid. The plate was then charred in a pre-heated oven at 160°C for 20 minutes (Figure 11).

Plates were scanned at 340nm using a Shimadzu CS-9001 PC dual wavelength TLC scanner (Dyson Instruments, Houghton le Spring, UK) linked to a Shimadzu data recorder. Results were obtained by integration of peak areas and calculation of peak area percent for each phospholipid class (Olsen and Henderson, 1989).

In selected samples, glycolipids were visualised by spraying the plates with a 0.5% solution of α-naphthol in a 1:1 mixture of methanol:water (Siakotos and Rouser, 1965). After spraying, the plates were allowed to dry in air and then was heated at 120°C. Glycolipids appeared as purple spots, and other lipids as yellow / white spots (Figure 12). Glycolipid detection was qualitative only.



Figure 11. HPTLC of BAL phospholipids. Typical samples from the surgical control group (Lane 1) and the RSV group (Lane 2) are shown. A double glycolipid band is clearly shown at the top of Lane 2.



Figure 12. Visualisation of glycolipids on HPTLC. Typical runs for samples from children with RSV infection (Lane 1) and surgical patients (Lane 2) are shown. Glycolipids appear as purple spots; other lipids as yellow/white spots.

#### 5.4.3. FATTY ACID COMPONENTS OF PHOSPHOLIPIDS

#### Background

In this study, fatty acids of phospholipids were derivatised to fatty acid methyl esters. The resulting esters were separated by gas chromatography and identified by mass spectrometry.

#### Method

The propan-2-ol layer from a propan-2-ol hexane extract of BAL fluid was decanted into a 5ml capacity toughened glass 'Reactivial' with a teflon seal.

Five hundred microlitres of 0.5 molar methanolic sodium hydroxide were added, and the mixture was heated at 130°C for three minutes on an aluminium heating block. The mixture was allowed to cool, and then 750µl of 14% boron trifluoride in methanol (w:v) was added. This mixture was heated for 5 minutes, again at 130°C and allowed to cool.

Two millilitres of saturated sodium chloride solution were added, and the lipids extracted with an equal volume of petroleum ether. The petroleum ether layer was decanted and evaporated to dryness at 45°C under a stream of nitrogen. The resultant dried lipid was re-dissolved in 50 microlitres of n-hexane. The relative proportions of fatty acid methyl esters were assessed by gas chromatography-mass spectrometry (Hewlett Packard Series II 5890 gas chromatograph with SGE BP X 70 high resolution polar capillary column and 5972 series mass selective detector with automatic peak integration).

## 5.4.4. QUANTIFICATION OF TOTAL PHOSPHOLIPID Method

Lavage fluid phospholipids were extracted, derivatised and analysed as described in section 5.4.3. with the addition of 1µg of heptadecanoic acid (as an internal standard) immediately before the derivatisation stage. The ratio of total fatty acid methyl ester peak areas (derived from the sample) to heptadecanoic acid peak area was calculated. The conversion factor 1.371 (Christie et al. 1970) derived from the ratio of the molecular weight of heptadecanoic acid methyl ester to diheptadecanoyl phosphatidylcholine was then used to calculate an approximation to the amount of total phospholipid present in the sample in µg/ml.

## 5.5. ASSESSMENT OF SURFACTANT DAMAGE

The measurements made of surfactant damage on each BAL sample are summarised in Figure 8.

#### 5.5.1. MALONDIALDEHYDE

#### Background

The malondialdehyde (MDA) assays were kindly performed in Dr John Paterson's laboratory at the Crichton Royal Hospital, Dumfries. Malondialdehyde was detected in lavage fluid by formation of a thioarbituric acid adduct of MDA and detection of the adduct by high performance liquid chromatography (HPLC) (Young and Trimble, 1991).

#### Method

#### Formation of the thiobarbituric acid adduct

Ten microlitres of BAL fluid were mixed with 250µl of 0.44M phosphoric acid, 450µl of HPLC grade water, and 250µl of 4.61M thiobarbituric acid. This mixture was incubated in sealed glass tubes at 100°C for 60 minutes. It was then cooled in ice to 4°C for 15 minutes.

#### **HPLC**

Two hundred microlitres of this mixture were added to  $360\mu$ l of HPLC grade methanol and  $40\mu$ l of 1M sodium hydroxide. This neutralised the sample and precipitated proteins. The sample was centrifuged at  $1000 \ge 1000$  s for 7 minutes, and the supernatant pipetted into an injection vial.

#### Standards

Thiobarbituric acid adducts of MDA standards (prepared from 1,1,3,3tetramethoxypropane in distilled water) were treated in exactly the same way as samples.

Fifty microlitres of supernatant were injected into the HPLC column (phenosphere 5 ODS 3 column: 150 x 4.60mm). The thiobarbituric acid adduct of MDA was detected by spectrofluorimetry with an excitation wavelength of 529nm and an emission wavelength of 541nm. The column was flushed with 50% methanol between runs. The mobile phase contained 50% methanol 50% phosphate buffer, and a flow rate of 0.5ml/min was used. Malondialdehyde concentrations were determined by integration.
of peak area and comparison to a standard curve. Standards used were 1.2mM,
4.8μM, 2.4μM, 1.2μM, and 0.6μM.

#### 5.5.2. NITROTYROSINE

#### Background

Nitrotyrosine in lavage fluid was quantitated using a competitive ELISA kit supplied by TCS Biologicals Ltd. (Khan et al. 1995) in the laboratory of Dr Mike Jacobs, Royal Free Hospital, London.

#### Method

The plates for this assay are supplied already coated with a predetermined amount of nitrated protein. Samples and standards are then added to the plates. An antinitrotyrosine antibody is added, and any nitrotyrosine present in the samples and standards competes with that attached to the plates for binding to the antibody. The less nitrotyrosine present in the sample or standard, the more binds to the plate. This antibody binding to the immobilised antigen on the plate is then detected quantitatively to construct a standard curve.

Nitrated BSA standards (ten serial 1:2 dilutions starting from 100µg/ml), samples (diluted 1:1 v:v) in Dilution Buffer (PBS/Tween/ovalbumin) and a blank (Dilution Buffer) were added in triplicate to the plate. Rabbit anti-nitrotyrosine polyclonal antibody was then added at 100µl per well and the plate incubated for 2 hours at 37°C.

The plates were washed twice with Wash buffer (PBS-Tween provided in the kit) and blotted dry. Donkey anti-rabbit IgG HRP-conjugated antibody was added at 100µl per well, and the plate incubated for 1 hour at 37°C.

Substrate solution was prepared by dissolving a 1 mg tablet of tetramethylene benzidine in 1 ml of dimethysulphoxide, making up to 10ml with the Phosphate-citrate buffer supplied and adding 4µl of 30% hydrogen peroxide.

The plates were washed twice with wash buffer and blotted dry. Substrate solution was added at 100µl per well and the blue colour allowed to develop for 30 minutes at room temperature. Fifty microlitres of 2M sulphuric acid were added to each well to stop the reaction, and the absorbance read at 450nm in a microplate reader.

A standard curve was constructed, and values for the samples calculated from this curve.

#### 5.5.3. THIN LAYER CHROMATOGRAPHY

The HPTLC plates which had been run and developed as described in section 5.4.2. were inspected to determine qualitatively whether any phospholipids other than PC, S, PS, PI, PG and PE were present. Specifically, the presence or absence of lysophosphatidylcholine was sought.

## 5.6. ASSESSMENT OF SURFACTANT INHIBITION AND MEASUREMENT OF POTENTIAL INHIBITORS

The measurements made to assess whether surfactant inhibition was present or not are summarised in Figure 8.

#### 5.6.1. TOTAL PROTEIN

#### Background

The total protein content of lavage fluid was estimated by the Lowry method (1951). There are two steps to this method. The first is the reaction of protein and copper in alkali. The second is the reduction of the Folin (phosphomolybdic-phosphotungstic) reagent by the copper treated protein. The blue colour produced by this second step can be detected in a spectrophotometer at 750nm.

#### Method

Protein standards were prepared to give concentrations of 0.125, 0.25, 0.5, 1, 2 and 4 mg/ml.

Tubes containing 10µl water (blank), 10µl of each standard, and 10µl of sample were set up in duplicate. Lowry's reagent A (1ml) was added to each tube, mixed immediately and allowed to stand at room temperature for 10 minutes. Folin-Ciocalteau reagent (100µl) was then added, mixed immediately and allowed to stand at room temperature for 30 minutes. Optical density was then read at 750nm, and the protein concentrations derived from a standard curve.

#### 5.6.2. QUANTIFICATION OF ALBUMIN

#### Background

This assay was performed in the Biochemistry Department at the Royal Hospital for Sick Children with the help of Mr John Black. This assay, which utilises reagents from a Roche kit in an automated analyser is in routine clinical use for quantification of urinary albumin in Yorkhill Hospital.

#### Method

Bronchoalveolar lavage fluid was diluted in 154mM sodium chloride solution with 0.5% Tween 20. Albumin standards (192, 96, 48, 24, 12 and 6  $\mu$ g/ml) and blank were also made in the saline/Tween solution. Anti-serum to albumin (Roche 07 2996 5) was added to samples and standards and the precipitin formed was measured turbidimetrically at 340nm in a Cobas Fara II analyser. The concentration of albumin in samples was then read from a standard curve (Gerbaut, 1987).

#### 5.6.3. QUANTIFICATION OF C-REACTIVE PROTEIN

#### Background

This assay was performed in the Biochemistry Department at the Royal Hospital for Sick Children with the help of Mr John Black. The assay for C-reactive protein (CRP) is, like that for albumin, an immunoturbidimetric technique. This technique utilises a CRP assay kit (Roche) and is in routine clinical use for quantification of plasma CRP in Yorkhill Hospital.

#### Method

Bronchoalveolar lavage fluid was assessed undiluted. C-reactive protein standards (Roche 07 1604 9) (40, 20, 10, 5 and 2.5  $\mu$ g/ml) and blank were made up in 154mM sodium chloride solution. Antiserum to CRP (Roche 07 2184 0) and 'Accelerator 1 solution' (Roche 07 2186 7) was added to samples and standards and the precipitin formed was determined turbidimetrically at 340nm in a Cobas Fara II analyser. The concentration of CRP in samples was then read from a standard curve (Fink et al. 1989).

## 5.6.4. QUANTIFICATION OF FIBRIN DEGRADATION PRODUCTS Background

The assay for FDP was performed by Dr Ann Rumley in the Department of Medicine, Glasgow Royal Infirmary. An ELISA kit was used to detect the presence of cross linked fibrin degradation products containing D-dimer in BAL fluid (Dimertest Gold EIA kit, Agen Biomedical Ltd, Queensland, Australia) (Hart et al. 1994).

#### Method

Plate wells were supplied already coated with monoclonal anti D-dimer antibody DD-3B6 in PBS with 1g/l sodium azide as preservative. One hundred microlitres of PBS with Tween 20 (0.05%) were added to each well. To this was added 25µl of sample, standard (2,000 to 32 ng/ml in doubling dilutions made up in PBS/Tween) or blank (PBS/Tween). The contents of the wells were mixed gently and incubated on a plate mixer at room temperature for 15 minutes.

The wells were washed three times with PBS/Tween and then dried. Fifty microlitres of mouse monoclonal anti-FDP peroxidase conjugate were added to each well, mixed gently and incubated on a plate mixer for 15 minutes at room temperature. The wells were then washed three times as before.

The peroxidase substrate supplied (2,2'Azino-bis (3-Ethylbenzthiazoline Sulfonic Acid)) was activated by the addition of 0.3% v/v hydrogen peroxide. This was added at  $100\mu$ l/well to each well, and incubated on a plate mixer for 15 minutes at room temperature.

Fifty microlitres of stopping reagent were added to each well and mixed. Absorbance was read at 405nm, and unknowns read from a standard curve.

#### 5.6.5. SURFACTOMETER STUDIES OF SURFACTANT INHIBITORS IN BAL FLUID.

The concentration of protein in the BAL fluid supernatant after centrifugation at 40,000 x g for 1 hour was assessed by the Lowry method, as detailed above. An aliquot of this supernatant was lyophilised and then re-suspended to 1mg/ml protein in suspension buffer. Survanta, a commercially available animal surfactant (2.5mg/ml)

was added to this, and the mixture assessed in the pulsating bubble surfactometer for 10 minutes as described above. It should be noted that Survanta contains the small hydrophobic proteins, SPB and SPC. The inhibitory effect of supernatant proteins on lipid-only surfactant (eg. Exosurf) was not studied.

## 5.7. STATISTICS

Minitab version 10.5 was used to perform all statistical tests.

#### **Determination of normality**

Data distributions were inspected in each group, for each BAL constituent measured. Measures of kurtosis and skewness were calculated, and the Kolmogorov-Smirnov goodness-of-fit test performed (Conover, 1971). From this information, it was decided whether or not the population was normally distributed.

#### Continuous data

Continuous data in this study were not found to be normally distributed, by the procedure described above. Because of this, non-parametric methods, principally the Mann Whitney U Test, were used. Ninety five percent confidence intervals for the median were also calculated, and these were quoted in conjunction with the median and range.

#### **Categorised variables**

Results comprising categorised variables were analysed using the Chi-Square Test.

# Assessment of changes occurring throughout the disease progress in patients with RSV infection

In the 10 children with severe RSV infection who were ventilated for more than 3 days, results from each assay were plotted chronologically on the same diagram as arterial alveolar oxygen ratio. The plots were then inspected, and from this, any relationship between the BAL constituents and disease severity (as assessed by arterial alveolar (a/A) oxygen ratio) or duration of ventilation could be seen. Since initial inspection suggested no relationship between any measured variable and severity measured by a/A ratio, no formal statistical tests were performed.

Also, in children with RSV infection who had been ventilated for more than two days, the values of BAL constituents measured on the first and last days of ventilation were compared using the Mann Whitney U test.

## 6. METHODS DEVELOPMENT

## 6.1. PULMONARY FUNCTION TESTS

Initially, when this study was designed, it was intended to perform pulmonary function tests throughout the course of ventilation in patients with RSV infection, in order to monitor the disease process. The tests were to be carried out using a Sensormedics 2600 infant lung function cart which had been purchased by the Yorkhill Sick Childrens Fund before the project was concieved. The tests to be used were deflation flow volume, single breath compliance and resistance and functional residual capacity by nitrogen washout. Each of these investigations raised its own problems, as did the whole testing procedure, and these are summarised below.

#### 6.1.1. DEFLATION FLOW VOLUME

Forced deflation was first described in 1977 by Motoyama and colleagues as a means to produce flow limitation in the small airways of ventilated infants, and thus assess small airway function by inspection of flow volume loops. The technique involves applying a negative pressure of 40cmH<sub>2</sub>O to the infant's lungs, by means of a suction device and valve. A flow volume loop is generated as gas leaves the lungs via a pneumotachograph attached to the valve.

It was hoped to be able to study the function of the small airways in RSV infection in relation to surfactant measurements, as surfactant may play an important role in

maintaining the patency of these airways (Yager et al. 1989; Enhorning et al. 1995; Liu et al. 1991).

After preliminary evaluation in the intensive care unit, it was felt that the technique carried too much of a hazard to the patient, as there was a risk of potential dislodgement of a mucus plug into a major airway when the negative pressure was applied. It was therefore not used on grounds of patient safety.

#### 6.1.2. COMPLIANCE AND RESISTANCE

The Sensormedics 2600 infant lung function cart permits compliance and resistance of the respiratory system to be measured by the single breath occlusion method (LeSouef et al. 1984). In this technique, the infant's airway is occluded by a sliding valve at end inspiration, and mouth pressure is measured at this point. The valve then shifts, and the infant expires passively through a pneumotachograph, and a flow-volume curve is thus produced. In infants with healthy lungs, this curve is usually linear, allowing derivation of respiratory system compliance and resistance from the curve and the mouth pressure measurement as follows:

respiratory system compliance = tidal volume/mouth pressure at end inspiration respiratory system resistance = mouth pressure/flow at beginning of expiration The tidal volume and flow at the beginning of inspiration are derived from extrapolation of a linear flow volume loop to intersection with the flow and volume axes. Pulmonary surfactant deficiency would be likely to produce changes in respiratory system compliance and resistance. Therefore, it was intended to measure these parameters in conjunction with assessment of surfactant levels. Unfortunately, in ventilated infants in the intensive care unit with RSV infection, the relationship between flow and volume was found not to be linear. This was probably due to the presence of alveoli with widely differing time constants in the diseased lung. Additionally, the clinical condition of some infants with RSV infection who were preliminarily studied was too unstable for them to tolerate the procedure without significant desaturation. The technique was therefore abandoned.

## **6.1.3. FUNCTIONAL RESIDUAL CAPACITY BY NITROGEN WASHOUT** The 2600 pulmonary function cart allows measurement of functional residual capacity by nitrogen washout (Sivan et al. 1990). It was intended to measure functional residual capacity, as an indication of the volume of gas contained in open alveoli at end expiration. If this could be assessed in combination with surfactant measurements in infants with severe infection, it was hoped that alveolar patency could be compared with surfactant function.

The measurement of functional residual capacity was first validated with an air-filled syringe. Measurement of syringe voulme was frequently not reproducible with, for example, a coefficient of variation of 65.7% when measuring 40ml of air in a syringe. Dr Janet Stocks's team in Great Ormond Street, who were also validating the system had similar problems. The system was checked for leaks, breathing circuits were

replaced, and software and mechanical adjustments were made by the manufacturers. These adjustments improved the problem temporarily.

The technique of measuring functional residual capacity depended upon switching the infant into the 'washout circuit' at end expiration. This was easy enough when a syringe full of gas was being studied, however, it was obvious that end expiration would be difficult for the investigator to detect in an intensive care setting. New software which allowed detection of end expiration and automatic switching was installed in preparation for testing of the system in the intensive care unit. This produced widely varying values of functional residual capacity when a syringe was tested. At this point, further concerns about accidental extubation of the patient during testing were becoming obvious.

#### 6.1.4. RISK OF ACCIDENTAL EXTUBATION

One of the major concerns when using the 2600, was that accidental extubation of the infant might occur during testing. As the babies being tested were sick, and often deeply sedated and paralysed to allow mechanical ventilation, accidental removal of the tracheal tube through which they were being ventilated would be life threatening.

The factors predisposing to accidental extubation were as follows

1. Unexpected movement of the infant's head may occur at any time in an incompletely paralysed infant.

2. The valve and attached lung function testing system which were fitted on the end of the tracheal tube during measurements were heavy and cumbersome. 3. The tracheal tube was relatively slender, but not pliable enough to prevent dislodgement if subjected to excessive 'see-saw' movement.

4. The tube could not be kept reliably fixed with respect to the infant's head during testing.

5. Smooth disconnection of the valve assembly from the tube connector after testing was impossible, as the joint was of a 'friction fit' type, and the presence of water vapour and droplets in the joint exacerbated the problem of 'suction'. Often a great deal of force was required to disconnect the valve assembly from the tracheal tube.
6. The tester who held the valve had to remain motionless for a considerable period of time.

7. In FRC measurements, not only did the valve have to be held steady, but the infant also had to be ventilated manually, pressure kept upon the trachea to avoid air leaks, and test results assessed on screen simultaneously. This meant that it was difficult for one operator to give undivided attention to holding the valve.

In addition to these problems, there was no guarantee of a trained intubator nearby and available. Also recognition of extubation within the pharynx would be late if the tube was dislodged, as the usual cue of decreased ventilator pressure would not be present when testing was being performed.

Solutions to the problem of accidental extubation were sought. Improved fixation of the tube to the baby's face with a foam 'butress' around the tube under the infant's nose for added stability was tried. However, it was not possible to achieve a satisfactory level of stability with this arrangement. It was not possible to design a connection which removed the 'suction' when trying to disconnect the tube from the valve, while

still maintaining a gas-tight seal. A commercially-available 'friction-arm' was employed to hold the valve and tubing, however, this rendered the situation even more unsafe in the event of unexpected movements of the patient's head. A foam cushion was used to try to restrict the baby's head movements during testing, however this did not work. Introduction of a flexible link into the system, which would absorb movement was considered. However, this produced insurmountable difficulties in terms of maintaining a gas tight seal and in ease of disinfection.

Eventually, it was decided by the author that, as the problem of accidiental extubation could not be overcome, lung function testing could not be continued.

#### 6.1.5. MECHANICAL PROBLEMS

During the year in which the 2600 was being validated, repeated mechanical problems occurred. These are listed below.

1. A leak developed at the mouth pressure port of the occlusion valve used in compliance and resistance measurements. This necessitated return of the part to the manufacturer on two occasions.

2. The pneumotachograph became impossible to calibrate on two occasions due to a substantial positive or negative drift. This was found to be due to vibration of a circuit board and leaking pressure transducers. After several visits from engineers from England and Holland, and return of the machine to the manufacturers, these problems were eventually solved.

3. Functional residual capacity measurements were inaccurate. This was attributed to a residual vacuum drawing oil up a tube when the machine was switched off. When the

machine was switched on again, the oil was distributed throughout the mixing chamber, coating the nitrogen analyser, and rendering it inaccurate. A vacuum release was fitted, and this improved the readings obtained.

4. It became impossible to perform compliance and resistance measurements, as a persistent 'negative pressure' message appeared on the screen whenever a test was attempted. However, by this time, enough data had been collected to establish that this measurement was not physiologically appropriate in infants with severe RSV infection.
5. Intermittently, the footswitch which controlled valve operation did not work until the machine had been switched on for at least 15 minutes. This was very inconvenient, as the lung function cart was very cumbersome, and impeded the routine nursing care of patients in the intensive care unit due to its blocking of one side of the bedspace.
6. The computer would 'hang' when it was attempted to print the on-screen measurements. The manufacturers were not able to solve this problem.

7. Not infrequently, the computer would not 'boot up', and after switching on, would generate increasing powers of 2 which were displayed on the screen. The manufacturers could not explain this.

#### 6.1.6. SUMMARY

The measurement of pulmonary function was initially proposed in order to correlate the pathophysiological changes occurring in the lungs with changes in surfactant function. Unfortunately, the equipment available to perform pulmonary function tests was unsuitable for this purpose in terms of safety, appropriateness and reliability. Lung function testing was therefore abandoned.

## 6.2. THE CLICK AND SHAKE TESTS

A positive control (Survanta 2.5mg/ml) known to contain active surfactant, and a negative control (water) were assessed along with each batch of samples. On each occasion tested, Survanta reliably produced clicking and a positive shake test, whereas no clicking and a negative shake test were produced by water.

## 6.3. SURFACTANT PROTEIN A

This assay reliably produced standard curves with a r-squared value greater than 0.965. Intra assay coefficient of variation was 8.4% (5 measurements on equal aliquots of same BAL sample), and inter assay coefficient of variation was 11.5% (5 measurements on equal aliquots of same BAL sample).

## 6.4. SURFACTANT PROTEIN B

This assay reliably produced standard curves with an r-squared value greater than 0.965.

Intra assay coefficient of variation was 6.9% (5 measurements on equal aliquots of same BAL sample), and inter assay coefficient of variation was 7.3% (5 measurements on equal aliquots of same BAL sample).

## 6.5. SURFACTANT PROTEIN D

Initially, when the assay was performed in Oxford (by the author with the guidance of Dr J.Y. Wang), in accordance with the protocol being used at that time, an antibody

concentration of  $1\mu$ g/ml was used to coat assay plates. The changes in optical density obtained with this protocol were low, and development times were excessively long (over 3 hours). After discussion with Dr Wang, the coating antibody concentration was increased to  $5\mu$ g/ml. This produced greater changes in optical density, in a shorter development time.

In preparation for assay of the BAL samples in Glasgow, new chemicals were purchased and antibodies and standards were obtained from Oxford. When the assay was then attempted, it was impossible to produce a linear standard curve. Over a period of 5 months, chemicals were changed, new antibodies were obtained, and minor alterations were made to the assay protocol in consultation with Dr Wang. It was finally discovered that the standard sent had been mistakenly labelled as purified human SPD, when, in fact, it was recombinant SPD, which had been shown in Oxford to aggregate readily, and to be an unsatisfactory standard for generation of standard curves. In the week before he returned permanently to Taiwan, Dr Wang kindly purified some human SPD fom BAL taken from a patient with alveolar proteinosis, and this was used satisfactorily in generation of standard curves produced reliably had an r-squared value of greater that 0.965.

### 6.6. PROPAN-2-OL - HEXANE EXTRACTION

A modification of the method of Ip and colleagues (1977) was used to extract phospholipids from BAL fluid. This method has been shown to extract from a mixture containing an equal mixture of standards 86.8% of DPPC, 16.4% of palmitic acid and 3.8% of tripalmitin into the propan-2-ol layer (1p et al. 1977). A sample of BAL was A . 20 . . . .

extracted according to this method, and also with the chloroform methanol method, and the phospholipid fraction separated from other fractions in the extract by thin layer chromatography (using hexane:ether:acetic acid (60:40:1) as a solvent system -(Carroll, 1976)) and visualised under UV light with dichlorofluorescein. The sample extracted by this method appeared to contain contain no detectable amounts of lipids other than phospholipids. Although, according to Ip, the method does not produce a completely selective extraction of phospholipids, the amounts of triglycerides and free fatty acids present in BAL samples was probably small enough for them not to be present in amounts which could be visualised by this method.

## 6.7. SEPARATION AND QUANTIFICATION OF PHOSPHOLIPID CLASSES

Various methods have been used to separate and quantify relative proportions of surfactant phospholipid classes present in bronchoalveolar lavage fluid in human and animal studies of BAL fluid and amniotic fluid. Thin layer chromatography is a method widely used to separate phospholipid classes (Gunther et al. 1996; Escamilla et al. 1992; Gregory et al. 1991; Daniels et al. 1996; Griese et al. 1996; Guthmann et al. 1995). Once separated, the relative proportion of each phospholipid class can then be quantified by spectrophotometric methods or by scanning densitometry. Other methods which have been used to quantify phospholipid classes in studies of lung or amniotic fluid include high performance liquid chromatography (Ratjen et al. 1996; Pison et al. 1987) and enzymatic assay (Farquharson et al. 1988).

#### 6.7.1. THIN LAYER CHROMATOGRAPHY (TLC)

Initially, several TLC solvent systems were assessed using a mixture of phospholipid standards containing PC, PG, PS, PI, PE and sphingomyelin, and also using lipid extracts of tracheal aspirate and BAL fluid.

One dimensional and two dimensional TLC methods were used, and lipid extracts were spotted on 20 x 20 silica gel G, H and Whatman LK5 TLC plates. Separation of phospholipids by the following solvent systems in one dimension, or by combinations of these solvent systems in two dimensions was assessed. These included:

1. chloroform (60) methanol (20) ammonia (4) (Svennerholm and Vanier, 1973)

2. chloroform (65) methanol (25) formic acid (10) (Escamilla et al. 1992)

3. chloroform (65) methanol (25) water (4) (Rouser et al. 1967)

4. n-butanol (60) acctic acid (20) water (20) (Rouser et al. 1967)

5. chloroform (25) methanol (13) propan-1-ol (25) potassium chloride 0.25% (9) ethyl acetate (25) (Touchstone et al. 1979)

6. chloroform (30) methanol (9) propan-2-ol (25) potassium chloride 0.25% (6) triethylamine (18) (Touchstone et al. 1979)

7. methyl acetate (25) propan-2-ol (25) chloroform (25) methanol (10) potassium chloride 0.25% (9) (Olsen and Henderson, 1989)

8. chloroform (10) acetone (4) methanol (2) acetic acid (2) water (1) (Rouser et al. 1967)

#### 6.7.2. HIGH PERFORMANCE TLC (HPTLC)

Two solvent systems were assessed in one dimension using 10 X 10 HPTLC plates (Whatman).

1. chloroform (25) methanol (13) propan-1-ol (25) potassium chloride 0.25% (9) ethyl acetate (25) (Touchstone et al. 1979)

2. methyl acetate (10), propan-2-ol (10), chloroform (10), methanol (4) and 0.25% potassium chloride (3.6) (Olsen and Henderson, 1989)

The HPTLC methods proved to be superior to other TLC methods tested, in terms of clear and repeatable separation, and sensitivity of visualisation at low levels of phospholipid. Additionally, the time taken to complete a run was short, and up to 7 samples could be loaded per plate.

It was decided to use solvent system 2, as when this was scanned using a densitorneter, the peaks were more distinct than with system 1.

#### 6.7.3. VISUALISATION OF PHOSPHOLIPIDS ON CHROMATOGRAPHY PLATES

The following methods of phospholipid spot visualisation were assessed.

1. Sulphuric acid 50% in water charred at 180C (Christie, 1982a)

2. Sulphuric acid 50% in water + 0.6% potassium dichromate charred at 180°C (Smith and Seakins, 1976)

3. Rhodamine 6G (0.01% w/v) spray viewed under UV light (Christie, 1982a)

4. 0.1% (w/v) 2',7'- dichlorofluorescein in 95% methanol spray viewed under UV light (Christie, 1982a)
5. Molybdenum blue spray (Sigma Chemical Co) Diluted 1:1 with 4.2M sulphuric acid heated at 100C for 1 hour

6. Molybdenum blue spray (Sigma Chemical Co)

7. lodine vapour (Christie, 1982a)

8. Sulphuric acid 10% in ethanol (Smith and Scakins, 1976)

9. Bromothymol blue 0.1% in 10% aqueous ethanol with pH 6.8 (by adding cone.

Ammonia) (Smith and Seakins, 1976)

10. Molybdenum Blue Reagent (Dittmer and Lester, 1964)

11. Modified Molybdenum Blue Reagent (Ryu and MacCoss, 1979)

12. 3% cupric acetate and 8% orthophosphoric acid spray and charring at 180°C

(Olsen and Henderson, 1989)

The last method was adopted, as it gave the clearest and most sensitive visualisation of lipid spots.

#### 6.7.4. ENZYMATIC ASSAY

It was intended to use the enzymatic assay developed by Farquharson et al (1988) to quantify PC and PG in BAL fluid. The reactions involved in this assay are shown below.

phosphatidylglycerol phospholipase C glycerol phosphate + diglyceride phosphatidylcholine phospholipase C choline phosphate + diglyceride glycerol phosphate alkaline phosphatase glycerol + phosphate anion choline phosphate aikaline phosphatase choline + phosphate anion glycerol + ATPglycerokinaseglycerol phosphate + ADPcholine + ATPcholine kinasecholine phosphate + ADP

ADP + phosphoenolpyruvate pyruvate kinase pyruvate +ATP pyruvate + NADH + H+ lactate dehydrogenase lactate + NAD

A chloroform-methanol extract of BAL fluid was used for measurement in this assay. The change in optical density produced by degradation of NADH to NAD in the last reaction was measured at 340nm in a spectrophotometer. In this manner, PG was measured by first adding glycerokinase, and measuring the conversion of NADH to NAD produced by the pyruvate generated. Then PC was measured in the same sample by adding choline kinase and measuring the conversion of NADH to NAD resulting from the pyruvate generated in this second reaction.

The buffers, choline kinase and a reagent containing NADH, ATP and phosphoenolpyruvate used in this assay were provided in the 'Lecithin UV' kit produced by Boehringer Mannheim. Unfortunately, this kit ceased to be produced during the course of this study. When the NADH / ATP / phosphoenolpyruvate reagent was reconstituted from separate components in the proportions used in the kit, NADH was found to undergo spontaneous degradation, as measured by a steadily decreasing optical density at 340nm. It was not possible to ascertain from the manufacturers whether the combined reagent used in the kit was in any way different in composition from the reconstituted reagent. Further work in investigating this matter was precluded by illness and subsequent time constraints.

# 6.7.5. QUANTIFICATION OF SURFACTANT PHOSPHOLIPID CLASSES "Bartlett' phosphate assay

Initially, it was intended to quantify phosphorus in spots which had been scraped off TLC plates.

It was not possible to obtain COSHH clearance to use perchloric acid to digest phospholipids as recommended by Bottcher et al (1961). Therefore, the method of Bartlett (1959) was used, with the initial digestion step carried out by heating the sample at 160°C for 2 hours in 10N sulphuric acid, adding 30% hydrogen peroxide, and then heating for a further 2 hours. The optical density change produced by adding ammonium molybdate and Fiske and Subba Row reagent was measured as described by Bartlett in 50µg standards of PC, PG, PE, PS, PI and S. A standard curve constructed with inorganic phosphate standards (1 to 3µg) was linear with an r squared value of 0.98. Recovery of phospholipid was calculated from the ratio of molecular weight of each phospholipid standard to phosphorus. The phosphorus detected was read from the standard curve and phospholipid recovery calculated. This recovery varied from 85.5% for PG, through 77% for PC to 57.6% for PI.

Five separate determinations of phosphate by the above method were performed on 50µg of PC, PG, PE, PS, PI and S both as pure standards and after running on TLC and scraping from the plate. The '%TLC recovery' for each phospholipid was calculated as optical density change produced from TLC spot / optical density change produced from TLC spot / optical density change a mean of 90.6% for PC (sd 13.1) to 55.2% for PS (sd 16.3). The coefficients of

variation between runs for each lipid were also high (13.1 for PC to 56.6 for PE). It was decided that this system had several significant disadvantages:

1. Recoveries when measuring pure standards were fairly low

2. The recovery from TLC scrapings tended to be variable and low.

3. The procedure was time consuming (about 5 ½ hours per sample)

4. Sensitivity was likely to be a problem. If a 0.5ml sample of BAL fluid contained 10µg of phospholipid, and 1/10 of this was PG (as might be the case in a BAL extract), then it was calculated that this would produce a change in optical density of around only 0.010, without taking into account analytical losses.

### Other spectrophotometric methods of phospholipid quantification

The method of VanGent and Rosleur (1974) which utilises an ammonium molybdate reagent for quantification of phospholipids was assessed. When 100µg standards of phospholipids were subjected to the assay procedure, the change in optical density produced by PS, PI and PG were less than that produced by PC by at least a factor of 10.

The assay procedure of Stewart et al (1980), which uses ammonium hexacyanoferrate to produce a colour change was assessed. Again, this assay produced a great variation in changes in optical density, with PG, PE, PI and PS producing canges in OD of less than a fifth of that of PC, and S being about half that of PC.

These two methods were rejected due to the variable changes in optical density produced by each phospholipid.

## Charring and emulsification

Phospholipid classes may be charred on TLC plates, and the bands thus produced may then be suspended in an emulsifier-scintillator, and the amount of phospholipid quantified by scintillation counting (Christie, 1982a). This method is said to have an accuracy down to 10µg of lipid, which was thought not to be sensitive enough to detect the small amounts of individual lipid classes likely to be present in BAL fluid. Additionally, the equipment required for this method was not readily available.

## Charring and scanning densitometry

The method using HPTLC and charring followed by scanning densitometry as described in the methods section was assessed. Intra-plate repeatability was assessed by running 5 extracts of an equal volume of the same sample in separate lanes on one plate and inter-plate repeatability by running 5 equal extracts of the same sample on separate plates. The results are summarised in Table 7. These inter- and intra-run c.v.'s are fairly high, however, they are, in general lower than those obtained in the Bartlett phosphate assay.

	Inter-run coefficient of variation (%)	Intra-run coefficient of variation (%)	
Phospholipid species			
PC&S	9,5	4.5	
PS	19.9	11.6	
PI	18.3	12.0	
PG	6.6	13.7	

Table 7. Repeatability of HPTLC/densitometry measurements.

Five equal aliquots of the same sample were tested for inter-run repeatability. Five equal aliquots of the same sample were used for intra-run repeatability. Different samples were used for assessment of inter- and intra-run repeatability respectively.

Initially, an attempt was made to introduce a correction factor to compensate for the different charring characteristics of phospholipid classes. A commercial standard containing LPC, S, PC, PS, PI, PG and PE was run on plates along with samples. It became evident from inspection, however, that the charring characteristics of the phospholipids in the standard were both less uniform and different from those in the human samples. As Olsen and Henderson (1989) suggested, it was necessary to assume that all lipid classes had the same response factors, and the percentage composition was derived directly from the densitometer. It is unlikely that all

phospholipid classes in lavage fluid charred to the same degree, and this, undoubtedly introduces errors.

Mixtures of phospholipid standards from 4 to 25µg of total lipid were assessed by the HPTLC / charring / densitometry method, in an attempt to assess the effect of loading on the relative proportions given by this method. Variations in measured proportions were compatible with intra-run variation as assessed earlier, and in this range, there didi not appear to be relationship between relative proportion measured and loading. Therefore, the amount of sample used was adjusted if necessary to bring them within this range before spotting.

The use of HPTLC and scanning densitometry as a method to determine the relative proportions of phospholipid classes has significant limitations and the method should be regarded as semi-quantitative. Despite these considerable problems, the method of HPTLC, charring and densitometry was used in this study, as it was the best available at the time. Use of high performance liquid chromatography (HPLC) would have been preferable to the TLC method used, and HPLC methods have been used extensively to separate and quantify the proportions of surfactant phospholipids (Bernhard et al. 1994; Bruch et al. 1994; Pison et al. 1986; Dethloff et al. 1986; Bonnano et al. 1992). However, the facilities for this analysis were not available on site, and financial constraints prevented outside facilities from being used.

## 6.7.6. SPECIES OF FATTY ACIDS AND PHOSPHOLIPIDS

## High performance liquid chromatography (HPLC)

Individual PC species have been separated by HPLC and quantified fluorimetrically by Postle (1987) and this method has subsequently been used in clinical studies (Hunt et al. 1991; Ashton et al. 1994). However, neither the equipment nor the expertise necessary for this technique was available on site.

## **Disaturated species**

Phospholipid-containing samples can be treated with osmium tetroxide which oxidises unsaturated acyl groups. The unaffected disaturated molecules are then isolated chromatographically and quantified colorimetrically. Several authors have used this method to quantify the surface active DPPC species in BAL fluid (Clerex et al. 1989; Gibson and McMurchie, 1986; Neumann et al. 1990). However, Holm et al (1996) found recently that palmitoyl oleoyl PC (POPC) oxidised with osmium tetroxide gave a 51% recovery, where theoretically, there should have been none. This implies that the technique for quantifying disaturated species which uses osmium tetroxide gives falsely elevated values for DPPC due to the presence of POPC and perhaps other monounsaturated species. In confirmation of this, these authors performed gas chromatography on calf lung surfactant extract, and found the sn2 chain of PC to have 41% palmitic acid putting an upper limit of 41% on DPPC content. Osmium tetroxide assay of the same sample gave a value of 69% DSPC.

#### Silver nitrate chromatography

This method has been widely used in the past to separate fatty acid methyl esters on the basis of the number, configuration and position of double bonds (Christie, 1982b). This method was not in use locally, and was not investigated due to time constraints.

#### Gas-liquid chromatography mass spectrometry

Gas liquid chromatography has been widely used to separate fatty acid species in human and animal studies of BAL fluid (Shelley et al. 1979; King et al. 1986; Baughman et al. 1993; Gunnison and Finkelstein, 1997; Guthmann et al. 1995). This method was chosen for use in this study, as the equipment for HPLC was not available, and the osmium tetroxide method was considered to have limitations which precluded its use. Additionally, equipment for gas liquid chromatography was available on site. It was decided to use gas-chromatography mass spectrometry rather than GLC with flame ionisation detection, as an automated method for the former was in use. Also, due to problems with previous damage to septa and syringes, departmental policy confined use of the latter method to a limited number of staff members.

Samples were processed by the method of Ip (1977) which produces a relatively selective extraction of phospholipids into propan-2-ol. As described in section 6.6., this method only provides a relatively selective and incomplete extraction, and this will introduce an error. In retrospect, preparative TLC to isolate phospholipids prior to fatty acid analysis may have produced a more complete separation of phospholipids.

The high temperature used for derivatisation prompted concern that more fatty acid degradation might be taking place than in methods which used a lower temperature for

longer. In order to investigate this, one sample was split after extraction. One fraction was derivatised by the method of Ip; the other by refluxing with boron trifluoride methanol for 2 hours (Christie et al. 1970). Analysis of the variation between the samples showed it to be compatible with intra-run variation. It was therefore concluded that gross degradation of fatty acids was not taking place. Although the temperature used is high, the time of derivatisation is short, and this probably prevents excessive damage to fatty acids. Whether more subtle degradative effects were in fact occurring would require investigation of larger numbers of samples derivatised by the two methods.

### Repeatability and standardisation of fatty acid measurements

The inter and intra batch coefficients of variation (c.v.) for each fatty acid species percentage determined by this method were assessed by extracting, derivatising and running five aliquots of the same sample (a different sample was used for inter and intra run assessments respectively). The results are shown in Table 8.

Fatty acid species	14:0	16:0	16:1	18:0	18:1	18:2	20:4
inter- batch coefficient of variation (%)	*	2.6	8.6	5.6	3.6	9.5	*
intra- batch coefficient of variation (%)	24.8	1.7	3.1	4.3	2.9	3.5	2.6

Table 8. Repeatability of fatty acid measurements.

\* detectable amounts of the species not present in sample

Five equal aliquots of the same sample were tested for inter-run repeatability. Five equal aliquots of the same sample were used for intra-run repeatability. Different samples were used for assessment of inter- and intra-run repeatability respectively.

A standard containing an equal mass of each fatty acid methyl ester measured was run at the beginning and end of each batch of samples. The inclusion of these standards allowed a numerical correction to be made for non-uniform detector response between species.

## 6.7.7. PHOSPHOLIPID QUANTIFICATION

#### Background

As described above, problems were encountered in assessing an enzymatic assay, phosphate assay and other spectrophotometric methods used to quantify the surfactant phospholipids. In the method finally adopted, phospholipids were extracted into propan-2-ol and quantified by comparison with an internal standard in GC-MS. This method only gives an approximation of the total phospholipid content in BAL fluid for two reasons. Firstly, as described in section 6.6., the partition of phospholipids in the propan-2-ol layer is not complete. Secondly, the correction factor used is a general one derived from the molecular weights of the heptadecanoic acid species of phospholipid and fatty acid methyl ester (Christie et al. 1970). It is, therefore, not tailored to the contents of each individual BAL sample.

Despite these problems, the agreement between gravimetry and this method of quantification (shown in Table 9) was reasonable. However, it can be seen that the analytic recovery compared to gravimetry is in two cases over 100%. This is at variance with Ip's finding of an incomplete extraction of phospholipid into the propan-2-ol. This may be due to errors in gravimetry which could have been induced by vibration due to intermittent building work in the vicinity of laboratory. Although the balances were recalibrated regularly, it is possible that this may have caused inaccuracies in weighing phospholipids. Also, evaporation of the chloroform used to dilute the lipid standards may have resulted in a more concentrated standard being measured and giving a higher value. Alternatively, a lipid contaminant on the glassware or column could have led to more fatty acid methyl ester being measured than was added to the tubes. To minimise this, glassware was thoroughly cleaned, separate

glassware was used for BAL studies and blanks were run frequently. However, large amounts of lipid (from brain, liver, blood and food) were analysed routinely in the same laboratory and a small degree of contamination cannot be ruled out.

Heptadecanoic acid was used as an internal standard, as this was used in Christie's method, and was in use at the Scottish Agricultural College, Auchineruive where the method is currently being used in quantification of phospholipids. However, use of the diheptadecanoyl species of PC as an internal standard would have been more appropriate.

Table 9.	Phos	pholipid	quantifica	tion
		phonpho		

Phospholipid added (µg)	8.25	16.5	33.0
Phospholipid measured (µg)	8.0	18.4	33.8

A mixture of phospholipids (PC,PG,PS,PI,PE,S in the ratio 10:2:1:1:1:1) in chloroform was quantified by the method of section 5.4.4.

Illness, financial and time constraints precluded further assessment of other methods, and other locations in which phospholipids could be evaluated. Ideally, this method should have been assessed further before use, and, a more up to date and reliable method used. Further investigation may have found a laboratory where it was possible to carry out perchloric acid digestion of phospholipids, and allowed subsequent phosphate assay to be carried out. Also, another enzymatic assay is available (Furlong et al. 1992), and this may have proved more reliable than the method used.

#### Derivatisation

Ip and colleagues (1977), who described the extraction and derivatisation method used, reported large losses which they attributed to losses at the derivatisation stage. In order to investigate any losses which may have occurred due to this particular derivatisation method, a sample of lavage fluid was extracted and then the extract split into two equal aliquots. One aliquot was derivatised by the method of Ip, and the other, by refluxing for two hours with boron trifluoride methanol (Christie et al. 1970). The difference in recoveries of phospholipid between the aliquots was within 95% confidence limits for intra run variation. It is possible that the low recoveries obtained by Ip et al on adding DPPC to amniotic fluid are due to their use of linoleic acid (which may be present in amniotic fluid) as an internal standard. If endogenous linoleic acid was present in amniotic fluid, as well as that added as an internal standard, this would have increased the ratio of linoleic to palmitic acid measured, and hence led to an underestimation of the amount of DPPC present. Also, the chromatogram illustrated in Ip's paper shows a small broad peak for linoleic acid as opposed to that for palmitic acid (the most abundant species present). The large discrepancy between the peaks, and the lack of definition of the linoleic acid peak could have led to inaccuracies. A typical chromatogram obtained in this study using heptadecanoic acid as an internal

standard is shown in Figure 13. It can be seen that the internal standard peak is clearly defined.



Figure 13. A typical printout from gas chromatographyspectrometry of fatty acids.

## Reproducibility of phospholipid quantification

Intra-batch and inter-batch c.v. were assessed by analysing 5 aliquots of the same sample. Different samples were used for intra- and inter- batch evaluation respectively,

as sample volumes did not permit use of the same sample for both determinations. Intra and inter batch c.v. were 4.5% and 6.8% respectively.

## 6.8. MALONDIALDEHYDE

Various methods exist to measure MDA including spectrophotometry of MDA complexes, TLC, HPLC or GC. The most commonly used of these is formation of a thiobarbituric acid adduct of MDA or MDA-like substances, and subsequent quantification of this spectrophotometrically at 432nm. However, many substances other than MDA also form complexes with TBA which have a significant absorbance at around 532nm. These include other lipid peroxidation products, sugars, amino acids, biliverdin and urea. In order to eliminate the interference from these substances, prior separation of the MDA-TBA adduct may be performed by HPLC. This has two advantages in the measurement of BAL lipid peroxidation products. 1. Interfering substances are removed and 2. Sensitivity is increased. Initially, the spectrophotometric method without HPLC separation was assessed, and the treated BAL samples scanned through the 430-600nm range as recommended by Esterbauer (1991). No colour change was detected, and it was concluded that the levels in BAL were too low for detection by this method. For reasons of sensitivity and specificity, the HPLC method was adopted.

## 6.9. PULSATING BUBBLE SURFACTOMETER STUDIES

## Surface film 'creep'

A problem which has received attention in recent years is the expansion of surface film from the bubble up the capillary inlet tube of the sample chamber of the pulsating bubble surfactometer (PBS) (Putz et al. 1994b). Putz and colleagues calculated that this reduces the percentage compression of the film to 13% during pulsation. They studied surfactant preparations in an unmodified PBS, in a PBS in which the capillary tube had been kept dry during filling, and in a captive bubble surfactometer (CBS). In the CBS, a bubble is formed in an aqueous medium under an agarose ceiling (circumventing the need for a capillary inlet tube) (Putz et al. 1994a).

Putz et al found that in the unmodified PBS, adsorption times were longer, for example requiring 30 compressions for 1.25mg/ml Survanta to reach minimum surface tensions around 5mN/m (surfactant in the modified PBS and the CBS took only one or two compressions to reach this surface tension). They suggested that 13% compression did not provide enough reduction in surface area to squeeze out high compressibility components of the film early and allow rapid adsorption. However, with repeated cycling it was proposed that the monolayer became enriched in low-compressibility components (specifically DPPC), and that the nature of the coating on the capillary changed.

In the present study, to circumvent the problem of surface film 'creeping' up the capillary inlet tube in the surfactometer sample chamber, initial attempts were made to plug the outlet of the tube to keep it dry during filling. This procedure, however, was

difficult to implement repeatably in practice, and was not used. Surface tension measurements were taken at 10 minutes (200 cycles), and it was felt that this would give adequate time for adsorption. A commercial model of the CBS was not available at the time of this study.

Adsorption data were not recorded in this study due to software and computer hardware difficulties. Perhaps, in the light of Putz et al.'s work such data would have been difficult to interpret, as the spreading characteristics of each sample in the capillary tube were not known.

## Centrifugation

Several studies have investigated the influence of centrifugation speed on the fraction of surfactant phospholipids which sediment. Oulton and colleagues (1979) recentrifuged the supernatant obtained by spinning amniotic fluid at 33,000 x g for one hour. When the supernatant of this fraction was spun again, at 40,000 x g for 1 hour, negligible amounts of phospholipid sedimented. Guthmann et al (1995) found that a mean 81.8% of phospholipids in BAL from rats sedimented when spun at 60,000 x g for 60 minutes. Ruiz-Budria and colleagues (1992) spun amniotic fluid at 65,000xg for 60 min and found 69.4% of PC in the pellet.

The results found from centrifugation of BAL fluid from RSV and surgical patients in this study are in broad agreement with other work on the proportion of phospholipid which sediments on centrifugation. In selected samples, the 40,000g supernatant was extracted, derivatised and run with an internal standard (as described in section 6.7.7.) to ascertain the amount of phospholipid which was not pelleted. From these samples,

the mean phospholipid recovery in the pellet after centrifugation was calculated to be 79.6% (standard deviation 5.14).

## **Density gradient centrifugation**

Several authors have utilised density gradient centrifugation to separate plasma proteins in BAL from the surfactant fraction, and thus to assess the surface activity of surfactant in the absence of interfering proteins.

In BAL fluid from dogs with NNMU lung injury, surfactant purified by density gradient centrifugation had a similar phospholipid composition to a 27,000 x g pellet which has not been density gradient centrifuged. The latter fraction contained 20-25% protein by weight, whereas the former contained 10-15% protein by weight. Perhaps surprisingly, the fraction separated by density gradient centrifugation had similar surface properties to the 27,000g pellet in injured dogs (Liau et al. 1987). In another study, centrifugation of rabbit BAL fluid at different speeds (100 x g for 20 min, 60,000 x g for 60 min and 100,000 x g for 16h) revealed that all subfractions contained serum proteins which were probably albumin and subunits of 1gG. All the subfractions contained secretory IgA. The 100,000 x g pellet contained most secretory IgA per microgram of protein (Wright et al. 1984).

Molecular species analysis of PC composition of lavage surfactant purified by sucrose density gradient centrifugation and that of cell-free lavage supernatant have been found by Hunt and colleagues (1991) to be very similar. Shelley et al (1977), using density gradient centrifugation found the fatty acid composition of lung surfactant washings to differ only slightly from that purified by density gradient centrifugation. The centrifugation speeds used in previous studies for density gradient centrifugation were above those available to the author, and there appears still to be protein in surfactant fractions after this purification process has been performed. For these reasons, it was decided to spin the BAL sample at  $40,000 \times g$  (the highest speed available on site), and then to perform an organic extraction to remove interfering plasma proteins. This has the disadvantage of removing surfactant protein A. However, it was felt that, as this protein has a limited role in surface activity of surfactant this was an acceptable loss.

#### **Dilution of surfactant**

Whitsett and colleagues (1986) studied calf lung surfactant extract in a pulsating bubble surfactometer, and found that concentrations down to 0.5mg/ml reached a surface tension of less than 1 dynes/cm (mN/m) in a pulsating bubble surfactometer. Concentrations of 2, 0.75 and 0.5 mg/ml phospholipid reached this minimum surface tension in 5, 7 and 12 minutes respectively.

Kobayashi and colleagues (1990) studied a lyophilised and resuspended acetone precipitable fraction of a lipid extract of a pellet from 2,000 x g centrifugation of porcine BAL. Study of this preparation in a pulsating bubble surfactometer revealed a minimum surface tension of less than 1.8mN/m at 5 minutes only at concentrations of over 3mg/ml. Unlike Whitsett and colleagues, this group did not study the surface tension of their surfactant preparation in the pulsating bubble surfactometer after 5 minutes. It may be that if a longer time for adsorption had been left, lower surface tensions could have been reached. Also, Kobayashi's preparation is quite different from

the CLSE preparation used by Whitsett and colleagues, as the latter is prepared with a higher centrifugation speed, and the acetone precipitation step is omitted. It is therefore possible that the two preparations would have different surface properties.

Dargaville and colleagues (1996), in their recent study of surfactant in viral bronchiolitis used a concentration of 1mMol phospholipid (750µg/ml DSPC) for assessment in a pulsating bubble surfactometer.

It was decided to adopt a concentration of 750µg/ml phospholipid for use in this study, as it was felt that this would allow comparison between this study and that of Dargaville, and use of a low concentration of phospholipid would allow the maximum possible number of samples (some of which contained little phospholipid) to be assessed. Also, Whitsett's work (1986) suggested that this concentration would be adequate for adsorption in the PBS if pulsated for a sufficient time. However, it must be acknowledged that the low surfactant concentration used may not in fact have been enough to allow adequate adsorption in all samples.

## 6.10. TOTAL PROTEIN

Lipids have been shown to interfere with the Lowry protein assay (Kessler and Fanestil, 1986; Eichberg and Mokrasch, 1969). Kessler et al. (1986) demonstrated that the bicinchoninic acid-based protein assay was more sensitive to phospholipid inhibition than was the Lowry assay, and a procedure for eliminating interference with the latter has been developed (Rodriguez-Vico et al. 1989). Many published studies of BAL fluid have used Lowry's original method, and this was the method used in the

## 7. RESULTS - SURFACTANT FUNCTION

## 7.1. INTRODUCTION

As described in the introduction to this thesis, there is evidence of reduced surfactant function in inflammatory lung disease. Studies of BAL fluid from patients with ARDS have found increased minimum surface tension in both crude surfactant pellets and in surfactant purified by density gradient centrifugation to remove inhibitory plasma proteins. Animal models of lung injury provide further evidence of surfactant dysfunction in pulmonary inflammation. In severe RSV infection, there is an inflammatory infiltrate, an alveolar capillary leak, and, in some cases, viral invasion of type II cells. Additionally, the lung histology of advanced infection is similar to that seen in ARDS. This would suggest that in RSV infection, surfactant may be dysfunctional due to several mechanisms. Surfactant may be damaged by products released from cells in the inflammatory infiltrate, deficient or abnormal in composition as a result of disturbed synthesis or inhibited by plasma proteins. Surfactant function was therefore investigated by performing click and shake tests on whole BAL fluid, and pulsating bubble studies on a lipid extract of BAL fluid.

The shake and click tests were performed in order to give an overall estimate of the functional status of pulmonary surfactant in cell-free supernatant of BAL fluid. These tests give no indication of the reason for surfactant dysfunction: they merely provide a 'positive' result with good surfactant function and a 'negative' result with poor surfactant function. No adjustment was made for the concentration of surfactant

present in BAL fluid, and no attempt was made to remove surfactant inhibitory proteins from the fluid. Previous studies (Skelton et al. 1996; Skelton and Jeffrey, 1994) have investigated the results of click and shake tests performed on tracheal aspirates. The results described below illustrate that these procedures can also be used in assessment of BAL fluid.

In order to assess surfactant function in isolation from the effects of inhibitory proteins or alterations in phospholipid concentration, a lipid extract of BAL fluid adjusted to a concentration of 1mMol (750µg/ml) phospholipid was studied in the pulsating bubble surfactometer. This allowed the effects of surfactant damage and change in phospholipid composition on surface activity to be studied.

## 7.2. RESULTS

## 7.2.1. THE SHAKE AND CLICK TESTS

Shake and click tests were performed on 66 samples of BAL fluid from 18 patients with RSV infection, and 28 BAL samples from 13 surgical patients. (It was not possible to assess surfactant function in all samples due to limited sample size.)

#### The shake test

The shake test for surface activity was positive in 90% of samples in the surgical group compared to 8% of samples in the RSV group ( $\chi^2 - 64.69$ ; d.f. = 1; p < 0.000).

## The click test

The click test for surface activity was positive in 96% of samples in the surgical group, compared to 11% of samples in the RSV group ( $\chi^2 = 62.72$ ; d.f. = 1; p < 0.000).

No relationship was seen between disease severity (as measured by arterial alveolar oxygen ratio) and surface activity as assessed by the shake and click tests.

The proportion of positive click and shake tests on the first and last days of ventilation in samples from children with RSV infection are shown in Figure 14. The proportion of patients showing a positive click test on the final day of ventilation was greater than on the first day. However, this difference was not statistically significant ( $\chi^2 = 3.022$ ; d.f. = 1; p = 0.082) 20 a 2



Figure 14. Click and shake tests on the first and last days of ventilation in children with RSV infection who were ventilated for more than one day. It was not possible to assess all 16 children ventilated for more than 1 day due to insufficient sample size.

## 7.2.2. SURFACTOMETER STUDIES OF LIPID EXTRACTS OF BAL FLUID

Evaluation of the surface activity of an organic extract of BAL fluid adjusted to 1mM phospholipid in the pulsating bubble surfactometer showed no significant difference in minimum or maximum surface tension attained in a 10 minute period between RSV and control groups (Table 10).

	No. of patients (samples)	min. surface tension mN/m median (range) (95% Cl for median)	max. surface tension mN/m median (range) (95% CI for median)
Children	15	21.4	47.3
with RSV infection	(34†)	(19.1-27.5) (20.6 - 21.7)	(35.2-67.8) (44.0 - 50.0)
Surgical	8	20.6	54,1
patients	(19†)	(17.2-24.0) (19.0 - 21.8)	(37.8-68.3) (46.1 - 56.3)
p value*		0.1295	0.0704

## Table 10. Surfactometer studies on lipid extract from BAL fluid.

\* Mann Whitney U test

Surfactant from BAL fluid was subjected to an organic extraction to remove interfering proteins, and adjusted to 1mM to compensate for variations in sample dilution. When tested in the surfactometer, surfactant adjusted in this way showed no difference in surface activity between RSV and control groups.

<sup>†</sup> It was not possible to assess all 71 samples in the RSV group and all 31 samples in the control group due to limited sample size and to insufficient amount of phospholipid present in some samples.

## 7.3. DISCUSSION

In this study, the click and shake tests of surfactant function, performed on whole BAL samples were found to be negative in the majority of patients with RSV infection, and positive in the majority of controls. These simple tests of whole surfactant function provide evidence that surfactant is not fully functional in severe RSV infection.

When potential inhibiting proteins were removed from the lipid fraction of BAL fluid by organic extraction, and phospholipid concentration was adjusted to the same level in all samples, no significant difference was found in surface activity of lavaged surfactant between RSV and surgical groups. Also, no significant difference in surface activity of the lipid extract was found between first and final days of ventilation. This implies that the surfactant which is present in RSV infection is functionally normal in the absence of inhibitors irrespective of disease progress. However, the method used to study the lipid extract has some limitations which are discussed below, and these make it difficult to conclude whether or not the hydrophobic fraction of surfactant is functionally normal in RSV infection.

The principal problem is that the values for maximum and minimum surface tension are high in both RSV and control groups in this study. The minimum surface tensions reported here are very similar to those found by Dargaville and colleagues (1996) in their study of ventilated infants with severe viral bronchiolitis and surgical controls. They studied crude 25,000 g surfactant pellets from tracheal aspirates resuspended at 1mM (750µg/ml DSPC) in the pulsating bubble surfactometer. The maximum and minimum surface tensions reported in the current study are also very similar to those reported by Raymondos and colleagues (1996). In Raymondos's study, surfactant from tracheobronchial aspirates of ventilated infants and children was resuspended at 3mg/ml after density gradient centrifugation at  $116,000 \times g$  and assessed in the pulsating bubble surfactometer. Using this method, mean minimum surface tension was found to be 17.4 mN/m and maximum surface tension was 53.9 mN/m.

However, the findings of Gregory and colleagues (1991) who studied crude 48,000 g surfactant pellets resuspended to 1.5mM phospholipid from adults with and without ARDS were somewhat different from the results described in the present study. Gregory's group found that, in the control group, the mean minimum surface tension was 7.44 dynes/cm (mN/m), and that the mean maximum surface tension was 35.76 dynes/cm. These values are considerably lower than the control values in this study.

The reasons for resuspension of surfactant lipid extract to 1mM phospholipid are explained in section 6.9. However, in view of the relatively high maximum and minimum surface tensions obtained, this concentration may not have been great enough to allow adsorption to the air-liquid interface in the pulsating bubble surfactometer. This might explain why the results differ from those of Gregory. On the other hand, the concentration of surfactant which Gregory used was still quite low, and may have even contained inhibitory proteins which could have sedimented with the surfactant pellet. Also, Raymondos and colleagues used a higher concentration of surfactant, and obtained higher surface tensions. Interestingly, the surfactant used in

Gregory's measurements was derived from adults, whereas the surfactant measured in the present study and those of Raymondos and of Dargaville was derived from children. The reasons for these variations are not clear, and it is difficult to compare results unless a standardised method is used.

It is interesting to note that the three studies on BAL from children produced fairly consistent results. The possible limitations of the surfactant concentration used in the present study should be acknowledged. However, the similarity of the surface tensions in this study with those found in other studies of children prompts speculation that BAL surfactant may be functionally normal in RSV infection compared to control in the absence of inhibitors.

Acute respiratory distress syndrome tends to increase the proportion of small aggregates present in BAL fluid (Lewis et al. 1997). If the small/large aggregate ratio was also increased due to lung injury in RSV, this would have led to less surfactant sedimenting at 40,000 x g. In this case, an assumption of 79.6% recovery would have led to an over-dilution of the pellet before assessment in the surfactometer, and, if anything, an apparently reduced surface activity of the lipid pellet in the RSV group. No attempt was made to study surfactant small/large aggregate ratio in BAL fluid from the two groups in this study. If different aggregate ratios were present in different BAL samples, this would have altered the proportion of phospholipid which pelleted at 40,000 x g. The assumption of a 79.6% recovery (derived from measuring recovery in a small number of samples) would then have introduced an error in the final concentration of surfactant assessed in the surfactometer. If this was the case, then a more accurate dilution volume for the surfactant pellet would have been obtained by

measuring the percentage of phospholipid pelleted in each individual sample. Unfortunately, total phospholipid amounts in the BAL sample and/or volumes of supernatant were, in many cases, too small to allow this measurement.

While acknowledging the above limitations, the data provide evidence of surfactant dysfunction in RSV infection, and perhaps suggest that abnormality of the hydrophobic fraction of surfactant may not be the origin of this dysfunction.

# 8. RESULTS - SURFACTANT COMPOSITION

## 8.1. INTRODUCTION

## 8.1.1. SURFACTANT PROTEINS A, B AND D

Variable alterations in surfactant proteins A and B have been shown in two major inflammatory lung diseases - ARDS and pneumonia. Reduced levels of surfactant protein A have been found in patients with ARDS (Gunther et al. 1996; Gregory et al. 1991), while SPB in ARDS was found to be reduced in one study (Gregory et al. 1991) and normal in another (Gunther et al. 1996). Surfactant protein A has also been found to be reduced in bacterial pneumonia which was not associated with HIV infection (Gunther et al. 1996; Baughman et al. 1984), and increased in AIDS-related pneumonia (Phelps and Rose, 1991). Two groups (LeVine et al. 1996; Dargaville et al. 1996) have found reduced surfactant protein A in tracheal aspirates from ventilated children with viral lower respiratory tract infection. LeVine's group also studied SPB, and found it not to be reduced in children with viral pneumonitis. No studies have hitherto investigated the levels of SPD in pneumonia, RSV infection or ARDS.

As described, in severe RSV infection there is a pulmonary inflammatory infiltrate. Surfactant proteins A and D play a role in the immune defense mounted by the lung against microorganisms, and consumption of these proteins during opsonisation could lead to reduced quantities being present in BAL fluid. Severe infection is also

associated with viral invasion of pulmonary cells including, in some cases, type II pneumocytes. Theoretically, this could lead to disruption of surfactant protein synthesis.

In the light of these pathological features, and the similarities with ARDS and pneumonia, SPA, SPB and SPD were measured by ELISA of BAL fluid.

## 8.1.2. SURFACTANT PHOSPHOLIPIDS

It is not clear how ARDS and pneumonia affect the concentration of phospholipids in BAL fluid. Several studies have found surfactant phospholipid levels in BAL fluid not to be different from control in ARDS (Jacobson et al. 1993; Gunther et al. 1996; Pison et al. 1990; Spragg and Hallman, 1983; Hallman et al. 1982), however, there are also studies which demonstrate increased (Offenstadt et al. 1981) and decreased (Gregory et al. 1991) phospholipid levels in this condition.

In the AIDS-related pneumocystis pneumonia, too, the picture is not clear, with two studies finding a reduced lipid concentration in BAL (Escamilla et al. 1992; Hoffman et al. 1992), and one study finding no reduction in total phospholipid in AIDS-related lung disease (including pneumocystis pneumonia) (Rose et al. 1994).

A recent study of tracheal aspirates from neonates with nosocomial pneumonia found a reduction in phospholipid concentration (Griese et al. 1996). Also, Dargaville and colleagues (1996) found reduced DSPC in tracheal aspirates from ventilated infants with severe viral bronchiolitis. As discussed above, viral invasion of type II

pneumocytes can occur in severe RSV infection, and this may affect the synthesis of phospholipids. In order to investigate these possibilities, phospholipids in BAL fluid were quantified.

## 8.2. RESULTS

## 8.2.1. SURFACTANT PROTEINS A, B AND D

Concentrations of SPA, SPB and SPD measured in lavage fluid were significantly lower in the group with severe RSV infection than in the group without RSV infection. This difference was present irrespective of whether surfactant protein levels were expressed per ml lavage fluid or per mg total protein (Tables 11 and 12, Figure 15).

Table 11. Surfactant proteins in BAL fluid.

	No. of patients (samples)	SPA μg/ml median (range) (95% CI for median)	SPB ng/ml median (range) (95% CI for median)	SPD ng/ml median (range) (95% CI for median)
Children with RSV infection	<b>18</b> (71)	5.6 (0.6-151.9) <sub>(3.8-7.6)</sub>	12.0 (0.0-60.8) (7.3 - 13.6)	130.3 (0.0-1486.0) (71.0-270.0)
Surgical patients	<b>16</b> (31)	9,0 (0,5-139.6) (6,1 - 22,5)	118.1 (0-778.2) (63.8 - 171.7)	600.4 (0.0-1869.0) (367.0 - 679.0)
p value*		0.0368	< 0.0000	< 0.0000

\* Mann Whitney U test

Surfactant proteins A B and D were found to be significantly reduced in RSV infection compared to control.

## Table 12. Surfactant proteins in BAL fluid expressed per mg total protein.

	no. of patients (samples)	SPA μg/mg protein median (range) (95% CI for median)	SPB ng/mg protein median (range) (95% CI for median)	SPD ng/mg protein median (range) (95% CI for median)	protein mg/ml median (range) (95% Cl for median)
Children with RSV infection	<b>18</b> (71)	10.4 (0.6-173.1) <i>(6.9-16.3)</i>	14.0 (0.0-190.0) <i>(7.8-24.1)</i>	242,8 (0.0-5680,0) <i>(98.0-450,0)</i>	0.49 (0.13-2.46) (0.44 - 0.70)
Surgical patients	<b>16</b> (31)	33.3 (0.5-654.4) <i>(10.1-80.8)</i>	342.0 (0.0-1441.1) (136.0-476.0)	1607.3 (0.0-88575.0) (916.0-3140.0)	0.36 (0.07-1.65) (0.21 - 0.59)
p value*		0.0011	<0,0000	<0.0000	0.0079

\* Mann Whitney U test

As a consequence of the high levels of total protein seen in the RSV group, expression of surfactant protein values per mg total protein magnified the differences between the two groups.



Figure 15. Surfactant proteins A, B and D in BAL fluid (95% CI for median shown)

## Sequential changes in patients with RSV infection

Surfactant protein levels were higher on the last day of ventilation than on the first in 12 of the 16 patients with RSV who were ventilated for more than one day. The median SPA level was  $2.3\mu$ g/ml on the first day compared with  $6.0\mu$ g/ml on the last day (p = 0.0135 Mann Whitney) (Figure 16). No significant difference was found between first and last days of ventilation in SPB and SPD levels (Figures 17 and 18). There was no clear relationship between sequential surfactant protein levels and disease severity (as measured by arterial alveolar oxygen ratio). Surfactant protein levels were highly variable throughout the course of ventilation (Figure 19).


Figure 16. Surfactant protein A concentration on first and last days of ventilation in children with RSV infection ventilated for more than 1 day (n=16). The median value of SPA on the final day of ventilation ( $6.0\mu g/ml$ ) was significantly higher than that on the first day ( $2.3\mu g/ml$ )(p = 0.0135 Mann Whitney).

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Figure 17. Surfactant protein B concentration on first and last days of ventilation in children with RSV infection ventilated for more than 1 day (n = 16). There was no significant difference between median initial and final levels of SPB.



Figure 18. Surfactant protein D concentration on first and last days of ventilation in children with RSV infection ventilated for more than 1 day (n = 16. In six of these children, SPD level was 0ng/ml on first and last days). There was no significant difference between median initial and final levels of SPD.



Figure 19. Surfactant protein levels in three children with RSV infection who were ventilated for more than 5 days. Levels of SPA, SPB and SPD are highly variable.

# 8.2.2. TOTAL PHOSPHOLIPID

### Total phospholipid in patients with and without RSV infection

The median total phospholipid level in the RSV group was significantly lower than that in the surgical group (Figure 20).



### Figure 20. Phospholipid levels in BAL fluid (95% CI for median shown)

The median level for children with RSV infection was 28.6 (range 0.9-177.5) $\mu$ g/ml. In surgical patients, the median was 82.6 (range 2.1 - 386.1)  $\mu$ g/ml p<0.000. It was not possible to assess all 71 samples from the RSV group due to insufficient sample size.

# Sequential changes in patients with RSV infection

In patients with RSV infection who were ventilated for more than 3 days, no consistent pattern of change in phospholipid level was found throughout the period of ventilation and no correlation was found with the arterial alveolar oxygen ratio. In patients who were ventilated for more than one day, no significant difference was found between median phospholipid level on the first day of ventilation and on the last day (Figure 21).



Figure 21. Phospholipid concentration on first and last days of ventilation in children with RSV infection ventilated for more than 1 day (n = 13). It was not possible to plot values for all 16 children ventilated for more than one day due to insufficient sample size. There was no significant difference between median initial and final phospholipid concentrations.

# 8.2.3. THE RELATIONSHIP BETWEEN PHOSPHOLIPIDS AND PROTEINS

When surfactant proteins were expressed per  $\mu g$  of phospholipid, there was no significant difference in SPA or SPD levels between the groups, but the level of SPB was significantly lower in the RSV group than in the surgical group (Table 13).

Lincar regression analysis was performed in RSV and in control groups to ascertain any correlation between surfactant protein and phospholipid levels. In the control group, both SPA and SPB were positively correlated with BAL phospholipid (rsquared values: SPA 0.344 p=0.001, SPB 0.489 p<0.000), and these significant correlations were not present in the RSV group. In neither the RSV nor the control group was SPD correlated with phospholipid level.

	No. of patients (samples)	SPA μg/μg phospholipid median (range) (95% C1 for median)	SPB ng/µg phospholipid median (range) (95% C1 for median)	SPD ng/μg phospholipid median (range) (95% Ci for median)
Children with RSV infection	<b>18</b> (68)	0.23 (0.02-9.11) (0.12-0.33)	0.27 (0.00-3,87) (0.11-0.38)	5.51 (0,00-200.32) (1.92-10.01)
Surgical patients	<b>16</b> (31)	0,15 (0.01-1,36) (0.10-0.24)	1.29 (0.00-6.47) (0.83-1.70)	5.60 (0.00-337.43) (3.59-15.16)
p value*		0.1323	< 0.0000	0.2551

Table 13. Surfactant proteins in BAL fluid expressed per  $\mu g$  phospholipid.

# 8.3. DISCUSSION

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# 8.3.1. SURFACTANT PROTEINS A, B AND D

The median level of SPA in the control group in this study (9.0 $\mu$ g/ml) was similar to the levels found by Ratjen et al (1996) in their study of BAL fluid from children without bronchopulmonary disease aged 3-15 years (mean 6.9 ± s.d. 9.2  $\mu$ g/ml).

The finding that the SPA concentration is reduced in BAL fluid from children with RSV infection is similar to the reports of Dargaville and co-workers (1996) and LeVine and colleagues (1996) who studied tracheal aspirates from ventilated children with viral pneumonitis. Interestingly, Dargaville et al (1995) in earlier preliminary data reported no significant difference in SPA in viral bronchiolitis compared to control when BAL samples rather than tracheal aspirates were studied. Indeed, consideration of confidence intervals suggests that the reduction in SPA in severe RSV infection is relatively modest.

In agreement with Dargaville and LeVine, it was found that SPA tended to increase between the first and last days of ventilation (Figure 16). However, in the present study, where sequential daily samples were taken, no consistent pattern of day to day change throughout the disease process could be discerned.

Surfactant protein B was found to be reduced in ventilated children with RSV infection. A provious study of tracheal aspirates from children with viral pneumonitis (LeVine et al. 1996) did not demonstrate this reduction. Two possible explanations for the difference spring to mind - sampling method and age distribution. In the present

study, sequential daily samples of BAL fluid were taken, whereas LeVine's group collected tracheal aspirates on selected days. Also in the present study, the ages of patients in the RSV and control groups are similar (Figure 6). In contrast, in LeVine's study, the mean age of the control group was 1.89 years ( $\pm$  2.37 SD), while the mean age in the group with viral pneumonitis was 0.37 years ( $\pm$  0.42 SD). The effect of age on SPB is not known, but surfactant phospholipid concentration in BAL fluid is known to decrease with increasing age (Ratjen et al. 1996). If SPB also decreases in the same manner, this could have masked any difference between the groups in LeVine's study.

# Limitations of surfactant protein measurements, and standardisation with other BAL components

The possibility of damage (by the disease process) to the tertiary structure of any of the surfactant proteins and consequent altered immunoreactivity should be acknowledged as a factor which may affect the results of ELISAs. Equally, the disease process could damage the functional properties of surfactant proteins, leaving immunogenic epitopes intact.

There has been concern about quantification of extracellular lung fluid proteins in BAL fluid, and debate about the most appropriate method of standardising alveolar protein levels to take account of possible variations in lavage dilution (Grigg and Venge, 1996). This concern could indeed be extended beyond the quantification of proteins to the quantification of any lavageable material.

It was found in this study that the total protein content of lavage fluid from patients with RSV infection was higher than that in the control group, reflecting the pulmonary

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inflammation occurring with infection. In these circumstances, and, in fact, in any inflammatory lung disease with a 'protein leak' into the alveoli, expression of BAL constituent concentrations per mg of total protein potentially introduces an error.

Dargaville and colleagues (1996) used urea as a marker in their studies of viral pneumonitis, and expressed surfactant constituents per ml of 'cpithelial lining fluid' as well as reporting values per ml of lavage fluid. However, the urea method is also known to be problematic due to the tendency for urea to enter the alveolus very rapidly on lavage (van de Graaf et al. 1991; Ward et al. 1992).

Due to the problems involved in using either urea or protein to standardise values, the concentrations of surfactant constituents were expressed per ml of lavage fluid in this study. Surfactant proteins were also expressed per µg of phospholipid. A standard collection technique was used by one investigator (MK), and lavage dwell time was very short in all lavages performed. There was no significant difference in percentage return of BAL fluid between controls and RSV patients (Table 14), indicating that lavage dynamics were similar in both groups. It should be noted that the reduction in surfactant proteins found in this study was present regardless of whether the values were expressed in mg/ml or mg/mg protein. However, when expressed with reference to phospholipid, only SPB was reduced in RSV relative to control, suggesting a global reduction in secretion of surfactant lipids and proteins, with a more profound reduction in SPB compared to other components.

Table 14. Percentage return on BAL.

	no. of patients (samples)	% return on BAL median (range) (95% (?! for median)
Children with RSV infection	<b>18</b> (71)	44.7 (22.0 - 60.7) (41.3 - 46.7)
Surgical patients	<b>16</b> (31)	46.7 (30.0 - 70.0) (41.3 - 56.7)
p value*		0.1342

\* Mann Whitney U test

There was no significant difference in percentage return on bronchoalveolar lavage between patients with RSV and with surgical conditions, indicating that lavage dynamics were similar in the two groups.

### 8.3.2. PHOSPHOLIPIDS

### Phospholipids in BAL fluid from patients with RSV infection

In this study, a reduction in surfactant phospholipid was found in patients with RSV

infection compared to controls. This supports the findings of Dargaville (1996).

It is interesting to note that in this study, no significant difference was found in median levels of phospholipid between the initial and final days of ventilation. This finding differs from that of Dargaville and colleagues (1996) who found that 'epithelial lining fluid DPPC' increased between the first and last days of ventilation. However, it is difficult to compare these results, as Dargaville's group studied DPPC in tracheal aspirates, and expressed the result corrected for the amount of urea in the sample. In this study, total phospholipid was measured in BAL fluid, and the results expressed in  $\mu$ g/ml. Any or all of these three factors - sampling method, phospholipid species measured, or method of standardisation might contribute to the differences between studies.

# Limitations of the method to quantify phospholipids, and comparison with published data

The similarity of the phospholipid amounts obtained in this study to those measured by Dargaville (1996) suggests that the method used in the present study gives a reasonable estimate of phospholipid quantity.

It is interesting to note that the total phospholipid values obtained in the control group in the present study (median 82.6  $\mu$ g/ml) are higher than those obtained by Ratjen et al (1996) in normal children over 3 years old. The mean value of phospholipid obtained by Ratjen et al was 35.2  $\mu$ g/ml, and they found that the quantity of phospholipid present rises with decreasing age. If surfactant phospholipid continues to rise with decreasing age in children under 3 years old, it would be likely that the phospholipid values found in the control group in the present study (a younger age group) would be higher than those in Ratjen's study.

While acknowledging the limitations of the method used to obtain an approximation of phospholipid levels in this study, it should be noted that the results are in broad agreement with other similar studies.

### 8.3.3. PHOSPHOLIPIDS AND PROTEINS

Interestingly, SPA and SPB were positively correlated with phospholipid levels in the control groups, but SPD showed no correlation with phospholipids. Surfactant proteins A, B and phospholipids are found in lamellar bodies, while SPD is not, and the correlation of SPA and SPB with phospholipids suggests that the secretion of these lamellar body products is linked. In the RSV group, this correlation disappears, possibly suggesting that synthesis or secretion of SPA, SPB and phospholipids is disrupted by the disease.

# 9. RESULTS - SURFACTANT COMPOSITION

# 9.1. INTRODUCTION

# 9.1.1. PHOSPHOLIPID CLASSES

Several studies have investigated the proportions of phospholipid classes present in BAL fluid from people with ARDS. From these studies has emerged a picture of reduced PC and PG, and increased sphingomyclin. Also, in studies of animal models of lung injury, reduction in PC and PG are the main changes seen. In tracheal aspirates from neonates with nosocomial pneumonia, no changes were found in phospholipid composition (Griese et al. 1996). Skelton and colleagues (1976) found reduced PG in tracheal aspirates from infants with viral bronchiolitis.

In the light of the findings described above, and in order to investigate the proportions of phospholipid classes present in BAL from children with severe RSV infection, a lipid extract of BAL fluid was separated by HPTLC, and the lipid classes quantified by charring and scanning densitometry.

### 9.1.2. FATTY ACID SPECIES

Studies of the fatty acid profile of BAL fluid in ARDS have found a reduction in phospholipid palmitic acid and increases in oleic (Baughman et al. 1984) and linoleic acids (Hallman et al. 1982; Spragg and Hallman, 1983). An animal study of dogs with acute lung injury, however, found increased palmitic acid and decreased oleic acid. The findings in human studies are consistent with contamination of BAL surfactant phospholipids by fragments of cell membrane which could have been damaged by the disease process. In this study, BAL samples were centrifuged at 250 x g in an attempt to remove cell debris before GC-MS analysis to ascertain relative proportions of phospholipid fatty acids. However, it is likely, given the nature of the pathological changes seen in severe RSV infection that some contamination from membrane fragments of necrotic cells was present, and this was supported by the results of fatty acid analysis.

# 9.2. RESULTS

### 9.2.1. PHOSPHOLIPID CLASSES

Examination of HPTLC plates revealed that the large phosphatidylcholine band and the small sphingomyclin band were not easily distinguished separately by scanning densitometry, and PC and S were therefore reported as a combined peak. Also, in the RSV group, PE co-migrated with a double glycolipid band (identified as described in section 5.4.2. (Siakotos and Rouser, 1965)), so that proportion of PE could not be reliably reported (Figures 11 and 12).

Phospholipid proportions were reported as percentages of the total (excluding PE).

# **Proportions of phospholipid classes**

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The combined PC & S fraction was significantly lower in the RSV group than in the control group, while PG accounted for a significantly higher proportion in the RSV group compared to control. However, the differences were small and the 95% confidence intervals for the medians overlapped. The proportion of PS was significantly increased in the RSV group. There was no significant difference between groups in the values for PI. (Table 15).

# Sequential changes in patients with RSV infection

The PG fraction and the PI fraction tended to follow a pattern of reciprocal change throughout the period of ventilation, with one fraction increasing as the other decreased (Figure 22). There was no consistent association of phospholipid proportions with disease severity as measured by arterial alveolar oxygen ratio. No predictable pattern of change was observed between the first and last days of ventilation. The pattern of change seen in the S&PC fraction and PS is shown in Figure 23.

# Table 15. Phospholipid classes in BAL fluid.

	<b>No.of</b> patients (samples)	S&PC % median (range) (95% CI for median)	PS % median (range) (95% CI for median)	PI % median (range) (95% C1 for medicn)	PG % median (rangc) (95% CI for median)
Children with RSV infection	<b>18</b> (69†)	58.2 (40.4-72.9) (56.3 - 60.4)	5.9 (2.2-11.4) (5.3-6.7)	11.3 (1.3-23.7) (10.3-14.0)	21.3 (8.3-49.3) (19.9 - 23.3)
Surgical patients	1 <b>4</b> (29†)	62.3 (51.2-80.2) (57.2 - 66.3)	4.0 (0.5-9.3) (2.8-4.7)	12.3 (2.8-25.8) (9.9-18.2)	18.7 (12.2-32.9) (16.4 - 20.8)
p value*		0.0317	0.0001	0.4404	0.0240

\* Mann Whitney U test

The proportion of PS in the RSV group was increased. Although statistically significant, differences in proportions of the S & PC fraction and the PG fraction between patients with RSV and controls were small as indicated by overlapping confidence intervals.

† It was not possible to assess all 71 samples in the RSV group and all 31 samples in the control group due to insufficient sample size.









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#### 9.2.2. PHOSPHOLIPID FATTY ACID PROFILE

#### Fatty acid species in RSV and control groups

The median weight percent of the 14:0, 16:0 and 16:1 species of fatty acids was significantly reduced in the RSV group compared to the surgical group. Conversely, the median weight percent of the 18:0 and 18:1 species was significantly increased in the RSV group compared to control. There was no significant difference between groups in the proportions of 18:2 and 20:4 (Figure 24).

### Sequential changes in children with RSV infection.

The pattern of variation of 16:0 and 16:1 (Figure 25) and of 18:0, 18:1 and 18:2 (Figure 26) is shown in 10 children with RSV infection ventilated for more than three days. There was no consistent relationship with disease severity as measured by arterial alveolar oxygen ratio.

The median percentage of the 18:2 species was greater on the first day of ventilation (median 8.0%; range 2.4 - 18.3%) than on the last (median 5.6%; range 0.0 - 9.7%) (p = 0.0159 Mann Whitney). No other species showed a significant change between the first and last days of ventilation.



#### Figure 24. Fatty acid species in BAL fluid (95% CI for the medians shown)

The median (and range) percentages of fatly acid species wore as follows:  $14:0\ 0.0(0.0-6.7)\ RSV$ 2.0 (0.0-6.4) surgical p=0.0003. 16:0 42.8 (18.7-60.1) RSV; 52.6 (41.1-65.1) surgical p<0.0000. 16:1 8.0 (0.0-13.2) RSV; 13.8 (6.2-17.0) surgical p<0.0000. 18:0 13.7 (5.9-46.0) RSV; 6.0 (0.0-15.8) surgical p<0.0000. 18:1 23.1 (9.9-35.9) RSV; 14.1 (10.9-30.5) surgical p<0.0000. 18:2 and 20:4 - no significant difference.



Figure 25. Sequential changes in 16:0 and 16:1 species throughout the course of ventilation in the 10 children with RSV infection who were ventilated for more than 3 days



% 18:0 vs days of ventilation
% 18:1 vs days of ventilation
% 18:2 vs days of ventilation

Figure 26. Sequential changes in 18:0, 18:1 and 18:2 species throughout the course of ventilation in the 10 children with RSV infection who were ventilated for more than 3 days.

# 9.3. DISCUSSION

#### 9,3.1, PHOSPHOLIPID CLASSES

### Surfactant phospholipid classes in RSV and surgical patients

A statistically significant reduction was found in the combined S & PC class (p = 0.0317) and an increase in PG (p = 0.0240) in the RSV group. However, examination of confidence intervals reveals an overlap in both cases. This, taken with the relatively high p values would suggest caution in interpreting these results as biologically significant. An increased proportion of PS was found in children with RSV infection compared to control. The mechanism for and consequences of this finding are uncertain.

Unfortunately, when all the BAL samples were run, although PC and S could often be distinguished by the naked eye (Figure 11), it was not possible to make an adequate distinction for reporting when the scanning densitometer was used. The reporting of S and PC as a combined peak undoubtedly introduces an incomplete interpretation of the facts. It was also impossible to report the proportion of PE, due to its co-migration with glycolipid fractions in samples from patients with RSV infection (Figures 11 and 12). Additionally, as described before, charring characteristics of different phospholipid classes may vary. This makes it difficult to compare the results obtained from the control group in this study with published normal values. Also no normal data are available for children under 3 years of age, the age group of patients who were controls in this study.

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The findings in the present study contrast with some preliminary data in viral bronchiolitis from Skelton and colleagues (1996). Skelton reports a qualitative deficiency of PG early in the course of ventilation, which recovered by extubation. She studied 18 infants with bronchiolitis, and found that 'surfactant activity [by the click test] and PG were present in only 6/18 at intubation, but reappeared in all 18 by extubation'. In the present study, this pattern was not found and PG was seen to be present in all patients at all times. When the patients who were ventilated for more than 1 day were studied, PG rose from the first to the last day in 50% of patients and fell from the first to the last day in 50% of patients (Figure 27). It is not clear what method was used by Skelton et al for qualitative analysis of PG, and methodological differences along with sampling differences (tracheal aspirates vs BAL) may account for the divergence of these findings. Skelton's data are only available in abstract form at present, so it is not possible to comment in more detail.

Values of PS, PG and PI in the control group in this study are all high compared to other published normal data (as reviewed by Ratjen et al (1996)). As described earlier, the method of HPTLC and scanning densitometry used in the present study has significant limitations in terms of accuracy, and as several phospholipid classes were not clearly distinguished by densitometry, it is difficult to compare proportions found in this study with other published data.



Figure 27. Phosphatidylglycerol on the first and last days of ventilation in children with RSV infection (n = 14). Levels of PG could not be plotted for all 16 children due to insufficient sample volume. In 50% of children, there was a decrease from first to last days, and in 50%, an increase.

In order to investigate further whether the high PG levels found in this study were artifactual due to methodological problems, or whether, indeed they represented a true indication of PG in BAL, PG proportion was compared to fatty acid species proportion. As can be seen from Tables 2 and 3, the PG fraction of surfactant contains a higher proportion of 18:0 and 18:1 species and a relatively lower proportion of 16:0 species than PC. Regression analysis of results from samples in the control group showed significant positive correlations between both 18:0 and 18:1 species and PG fraction, and a significant negative correlation between 16:0 and PG. In the RSV

group, similar correlations were found with the exception of 18:1 which was not significantly correlated to PG. This gives somewhat greater validity to the proportions of PG found in this study. However, due to the limitations of the method, and differences from published values, their potential inaccuracy should be acknowledged.

The microsomal and mitochondrial fractions of adult human lung tissue have less PC, less PG, more PE and more S than the surfactant fraction (Post et al. 1982). Due to comigration of S and PE with other fractions on HPTLC in this study, it is difficult to assess the effect of any possible contamination of BAL fluid with membrane fragments from the organelles of damaged cells on the profile of phospholipid classes.

The finding that PI and PG increased and decreased in a reciprocal manner is consistent with the literature reviewed in section 1.2.3. The concentrations of these phospholipids are linked in a reciprocal manner both during lung development and in disease states (Akino, 1992). Although global variations in PG/PI ratio have been demonstrated before in other diseases, it is interesting to note the reciprocal day to day variation between these two phospholipids in some ventilated patients with RSV throughout the disease process (Figure 22).

# **9.3.2. PROPORTIONS OF PHOSPHOLIPID FATTY ACIDS IN BAL FLUID** The pattern found in this study was that of a reduction in myristic (14:0), palmitic (16:0), and palmitoleic (16:1) acids, and an increase in stearic (18:0) and oleic (18:1)

acids in patients with RSV infection compared to controls. Linoleic (18:2) and arachidonic (20:4) acids were not significantly different between the groups.

This pattern of shift is reminiscent of that found by Baughman and colleagues (1984) in adult intensive care patients with bacterial pneumonia. They found a reduction in the 16:0 species and an increase in the 18:0, 18:1 and 18:2 species in BAL fluid of patients with pneumonia compared to controls. A similar pattern was also seen in patients with ARDS, and, in this group, the 16:0 species was reduced, and the 18:0 and 18:1 species were increased compared to control. Hallman et al (1982) found reduced 16:0 and increased 18:0 and 18:2 in BAL from patients with ARDS.

High levels of inspired oxygen - comparable to those sometimes used in ventilated patients with RSV- can cause decreases in DPPC levels in experimental animals (King et al. 1989). Therefore, it is possible that the high inspired oxygen fraction needed in treatment of RSV infection could have been a contributory factor to the decrease in the palmitic acid fraction seen in the RSV group.

Phosphatidylcholine from microsomal membrane fragments and lung tissue contains more 18:0, 18:1 and 18:2 and less 16:0 species than surfactant (Rooney et al. 1975; Hunt et al. 1991; Schlame et al. 1988). Although centrifugation at 250 x g was performed before sample analysis to pellet cells and cell debris, it is likely that small intracellular membrane fragments contaminated analysed BAL fluid in this study. Membrane contamination could account for the pattern of fatty acid species seen in BAL from patients with RSV. According to the histological picture in severe infection, there is likely to be more breakdown of cells in the RSV group, and this was

the group with higher levels of 18:0 and 18:1 species, and lower levels of 16:0. It is interesting to note that in this study the other fatty acid species which is present predominantly in membrane fragments, 18:2, showed a reduction on the last day of ventilation compared to the first, and this may have been due to a reduction in cell necrosis with disease resolution.

Therefore, the changes in BAL fatty acid proportions seen in children with severe RSV infection are highly suggestive of the presence of membrane fragments in lavage fluid. The presence of similar changes in ARDS and pneumonia supports this, as pulmonary cellular necrosis can also occur in these conditions. However, the influence on surfactant synthesis of hyperoxia, or of other unknown effects of viral infection in type II pneumocytes cannot be ruled out.

# 10. RESULTS - INDICES OF SURFACTANT DAMAGE

# **10.1. INTRODUCTION**

Peroxynitrite is a reactive molecule formed by combination of nitric oxide and superoxide, which are released during the inflammatory response. There are no known specific scavenging mechanisms in the alveolar lining fluid which would offset peroxynitrite damage. Therefore this molecule may cause cellular and molecular damage during inflammatory lung disease (Haddad et al. 1993; Royall et al. 1995).

Nitrotyrosine is a specific marker for damage to proteins by peroxynitrite (Ischiropoulos and Al-Mehdi, 1995). Evidence of peroxynitrite damage is seen from the presence of increased nitrotyrosine in lung sections from patients who have died of ARDS (Royall et al. 1995; Haddad et al. 1994). Peroxynitrite has been shown, in vitro, to damage pulmonary surfactant phospholipids and proteins (Haddad et al. 1993). As this reactive molecule may be released during the pulmonary inflammatory response in severe RSV infection, in this study, BAL nitrotyrosine was measured, as a marker of damage to alveolar proteins.

As a non-specific marker of oxidant damage to lipids, BAL malondialdehyde (MDA) was also studied. Malondialdehyde is thought to originate in vivo mainly from oxidised linolenic, arachidonic and docosahexanoic acids and from certain prostaglandins via the action of platelet thromboxane synthetase. Linoleic acid is regarded as a weak precursor of MDA (Esterbauer et al. 1991). Malondialdehyde has been detected after in vitro exposure of calf lung surfactant extract to peroxynitrite (Haddad et al. 1993). Other indicators of lung lipid peroxidation have been measured in ARDS (Baldwin et al. 1986; Sznadjer et al. 1989; Cochrane et al. 1983), and MDA has been measured in BAL fluid in rats (Petruska et al. 1990). However, to the author's knowledge, no measurements of MDA have hitherto been made in human BAL fluid.

Glycolipids and lysophosphatidylcholine have been found on chromatographic analysis of BAL fluid in ARDS (Hallman et al. 1982; Rauvala and Hallman, 1984; Gregory et al. 1991). Glycolipids are found on cell surfaces and can act as receptors for viruses and bacteria and mediate interactions between cells. They are also known to bind SPA and Momoeda and colleagues (1996) identified them as receptors for SPA in the murine lung. Lysophosphatidylcholine is a breakdown product of PC resulting from phospholipase action. It is likely that the presence of these lipids in BAL fluid in inflammatory lung disease indicates damage to cellular and surfactant lipids. Lysophosphospatidylcholine and glycolipids were sought on HPTLC analysis of BAL fluid in this study.

# 10.2. RESULTS

### **10.2.1. MALONDIALDEHYDE**

Contrary to expectation, significantly lower amounts of malondialdehyde were found in lavage fluid from patients in the RSV group than in the control group (Figure 28).

Further, no correlation was evident with severity of illness or with duration of

ventilation.

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**Figure 28. Malondialdehyde levels in BAL fluid (95% CI for median shown)** Values for MDA from patients in the control group who had not had surgery are shown as yellow dots. The median level in patients with RSV was 0.00µmol/l (range 0.00-0.65). In surgical patients, the median level was also 0.00µmol/l (range 0.00-3.38) p=0.0005 Mann Whitney.

# **10.2.2. NITROTYROSINE**

Levels of nitrotyrosine per mg of total protein in BAL fluid were significantly higher in the RSV group (Figure 29). No clear relationship could be determined between nitrotyrosine level and disease progress. In particular, there was no significant difference between median values of nitrotyrosine on the initial and final days of ventilation (Figure 30).



# Figure 29. Nitrotyrosine levels in BAL fluid (95% CI for median shown)

Values for nitrotyrosine from patients who had not had surgery are shown as yellowdots. The median value in patients with RSV was 107.9ng/mg protein (range 0.0-11500.0). The median value in surgical patients was 0.0ng/mg protein (range 0.0-338.9). p<0.0000 Mann Whitney.



Figure 30. Nitrotyrosine levels on first and last days of ventilation in children with RSV infection ventilated for more than 1 day (n = 14). No significant difference was found between the median values on initial and final days (p = 0.1207 Mann Whitney).

# 10.2.3. EVIDENCE FOR SURFACTANT AND CELLULAR DAMAGE ON HPTLC

High performance thin layer chromatography was performed on 69 BAL fluid samples from 18 RSV patients, and 29 BAL fluid samples from 14 surgical patients (It was not possible to perform HPTLC on all samples due to insufficient sample volume).

# Lysophosphatidylcholine

A lysophosphatidylcholine band on HPTLC plates (Figure 9) was detected in 87% of samples from patients with RSV infection, and in 10% of controls ( $\chi^2 = 52.198$ ; d.f. =
1; p< 0.000). No clear relationship was found between duration of ventilation and the presence of LPC.

#### Glycolipids

Glycolipids were detected in 88% of samples from RSV patients, and in none of the control samples (Figures 9 and 10) ( $\chi^2 = 67.905$ ; d.f. = 1; p< 0.000 Chi Square). No clear relationship was found between duration of ventilation and the presence of glycolipids.

### **10.3. DISCUSSION**

#### **10.3.1. NITROTYROSINE**

Nitrotyrosine levels were found to be increased in lavage fluid from children with RSV infection. Low levels of nitrotyrosine were found in some control patients. There was no apparent distinction between nitrotyrosine levels in patients in the control group who had had surgery and those who had not (Figure 29).

Although the level of nitrotyrosine can be seen to fall sharply between the first and last days of ventilation in some patients (Figure 30), this was not a consistent finding, and there was no significant difference in the median levels on first and final days.

Because it is likely that nitrotyrosine concentrations are linked to the amount of protein present in the alveolus, nitrotyrosine levels were expressed per mg of protein in this study.

Neutrophils and macrophages which invade the lung in RSV infection produce nitric oxide and superoxide (Rosen et al. 1995), the majority of which is probably converted to peroxynitrite (Ischiropoulos et al. 1992). Peroxynitrite is known to cause nitration of tyrosine residues in SPA and in other proteins (Haddad et al. 1993). The finding that nitrotyrosine is elevated in RSV infection, therefore, is consistent with the inflammatory infiltrate seen in this condition and concords well with the presence of increased nitrotyrosine in immunocytochemical studies of lung sections from patients with other inflammatory hung diseases (Haddad et al. 1994; Royall et al. 1995).

#### **10.3.2. MALONDIALDEHYDE**

The finding that malondialdehyde is present in significantly lesser amounts in the lavage fluid of patients with RSV infection than in surgical patients appears at first contradictory, in the light of the raised amounts of nitrotyrosine (the other indicator of free radical damage) found in the RSV group. The presence of raised nitrotyrosine levels and reduced MDA levels in RSV would suggest that peroxynitrite is present in this condition, but, for some reason is not causing significant lipid peroxidation.

Potentially, there may be three reasons for the decreased appearance of MDA in lavage fluid from children with RSV compared to controls: less substrate, increased scavenging and an increase in MDA in the control group due to the effects of surgery.

As stated earlier, the main substrates for MDA formation are long chain unsaturated fatty acids. It is not possible to tell whether the proportions of fatty acids found on analysis of BAL fluid in this study have been altered due to lipid peroxidation. The proportionate amounts of arachidonic and linolcic acids did not differ significantly between the two groups, and docosahexanoic acid was not found in detectable amounts in any samples. Linolenic acid was found in small amounts in 4 members of the control group, and three of these had undetectable levels of MDA. It should not be overlooked that, although the percentages of arachidonic and linolenic acids did not differ between groups, the absolute amount of phospholipid was less in the RSV group. This could theoretically have led to less lipid substrate being available for lipid peroxidation. However, when amounts of MDA were expressed per  $\mu$ g phospholipid, MDA was still significantly lower in the RSV group (p = 0.0057 Mann Whitney). Also, free fatty acids and triglycerides were not quantified in the two groups, and these may have provided potential substrates. Additionally, the amounts of prostaglandins present in the two groups may differ.

Alveolar lining fluid is known to contain catalase, superoxide dismutase and reduced glutathione, all of which act to mop up the three participants in the Haber-Weiss reaction ( $O_2$ ,  $H_2 O_2$  and Fe<sup>+</sup>) (Figure 4) while leaving peroxynitrite untouched. As discussed in the introduction, the free radical scavenging capacity of lavage fluid from patients with ARDS is greater than that of controls. Perhaps the situation is similar in RSV infection which, like ARDS, is an inflammatory lung condition. It may be that while nitrotyrosine formation by peroxynitrite is increased in the patients with RSV, superoxide and hydrogen peroxide (the other reactive molecules capable of lipid peroxidation) are more efficiently scavenged in these patients, leading to less formation of MDA.

Plasma proteins are also known to act as free radical scavengers, and these are present in greater amounts in the lavage fluid of children with RSV infection. It is possible that proteins absorb the majority of damage by peroxynitrite (as evidenced by increased nitrotyrosine) and effectively shield lipids from harm. In vitro studies of the free radical scavenging properties of BAL fluid from patients with and without RSV will cast more light on this area.

Surgery inevitably results in tissue damage, and, as such is likely to cause a release of free radicals and prostaglandins. Both these factors could increase the levels of MDA in the circulation, and this might be transferred from the pulmonary capillaries to the fluid collected in bronchoalveolar lavage. The levels of MDA in eight samples from patients in the control group who had not had surgery are identified in Figure 28. It can be seen that values for 7 out of these 8 samples fall within the 95% confidence interval for the median in the control group. This would suggest that surgery did not have a significant impact on MDA levels.

#### **10.3.3. LYSOPHOSPHATIDYLCHOLINE AND GLYCOLIPIDS**

#### Lysophosphatidylcholine

In this study, qualitative analysis of HPTLC plates showed a spot migrating between the origin and the sphingomyelin band. By comparison with the relative migration of a commercial standard, and from Olsen and Henderson's (1989) original description of the method, this spot was assumed to be lysophosphitidylcholine (LPC). This lysophospholipid was found to be present in BAL from significantly more patients with RSV infection than from surgical controls. The finding of increased LPC in BAL from ventilated children with severe RSV infection is in accordance with other human and animal studies of inflammatory lung disease. Gregory and colleagues (1991) found increased LPC in BAL from patients with ARDS, and Casals and co-workers (1989) found that LPC was increased in rabbits with lung injury.

The increased levels of LPC seen in lung inflammation may be due to action of phospholipases on PC. In one study of adults with ARDS and pneumonia, phospholipase A2 activity was found to be lower in ARDS than control, however, when expressed on the basis of total phospholipid, normal controls had lower activities of this enzyme than patients with pneumonia or ARDS. Additionally, LPC levels were increased compared to controls, but this increase did not reach statistical significance (Hallman et al. 1982; Spragg and Hallman, 1983). In a study of oleic-acid injured rabbits, Casals group demonstrated significantly higher LPC and Phospholipase A activity in BAL and whole lung in injured animals (Casals et al. 1989).

#### Glycolipids

An unexpected finding in this study was the presence, on qualitative analysis of HPTLC, of two bands corresponding to glycolipids in the majority of BAL samples from patients with RSV infection, but in none from controls (Figure 12).

Glycolipids have also been found in BAL from patients with ARDS (Hallman et al. 1982; Rauvala and Hallman, 1984). Examination of BAL fluid in one study revealed on

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TLC two doublet bands identified as paragloboside and lactosylceramide (Rauvala and Hallman, 1984).

Neutrophils, erythrocytes and normal lung tissue contain glycolipids, including paragloboside (Yoda et al. 1982; Narasimhan and Murray, 1979; Macher and Klock, 1980; Vance and Sweeley, 1967).

It may be that the glycolipids found in lavage fluid from patients with RSV are simply an index of cellular damage. As discussed earlier, cellular damage is likely to be a feature of severe RSV infection (section 9.3.2.). It is also possible, however, that they are engaged in some more complex interaction with the virus or with surfactant proteins as SPA is thought to compete with microorganisms for glycolipid receptors on cell surfaces (Kuroki et al. 1992). More detailed studies will be needed to elucidate the exact nature of these glycolipids and to define the contribution made by glycolipids to the overall pathological picture in RSV infection. South States and States

## 11. RESULTS - INDICES OF SURFACTANT INHIBITION AND POTENTIAL INHIBITORS

### **11.1. INTRODUCTION**

Pulmonary inflammation leads to an alteration in capillary permeability, and leakage of oedema fluid and plasma proteins into the alveolus. One study of BAL fluid in ARDS study showed a correlation between the percentage of neutrophils in BAL and BAL protein (Weiland et al. 1986). Plasma proteins and ocdema fluid have been shown, in vitro, to inhibit the surface activity of pulmonary surfactant. It was considered likely from the histological picture of fibrinous alveolar exudates in severe RSV infection that surfactant inhibition would be present in this condition. Therefore, the inhibitory potential of lyophilised proteins from BAL was assessed by mixing these proteins at lmg/ml with a bovine lipid surfactant preparation, and studying the mixture in the pulsating bubble surfactometer.

Total protein and the surfactant inhibitors albumin, fibrinogen, fibrin degradation products and c-reactive protein were quantified in BAL fluid. These substances were the 'main suspects' for inhibition of surfactant in RSV infection. However, other substances such as globulins and haemoglobin were not quantified, and these may also have played a part in surfactant inhibition.

## 11.2. RESULTS

#### **11.2.1. TOTAL PROTEIN**

Total protein levels were significantly higher in lavage fluid from patients with RSV than in surgical patients (Figure 31, Table 12). Total protein levels showed no consistent relationship either to disease severity as measured by arterial alveolar oxygen ratio or to duration of ventilation.

#### **11.2.2. C-REACTIVE PROTEIN**

C-reactive protein was not detected in any of the BAL samples assayed.



Figure 31. Total protein and fibrin degradation products in BAL fluid (95% CI for median shown). Values for FDP's from patients in the control group who had not had surgery are shown as yellow dots. In patients with RSV, the median FDP level was 47ng/ml (range 1-1252) In surgical patients, median FDP level was 11ng/ml (range 1-108) Values for total protein are shown in table 12.

#### 11.2.3. ALBUMIN

Levels of albumin were not significantly different between RSV and control groups (Table 16).

Table 16. Albumin levels in BAL fluid.

	No. of patients (samples)	albumin µg/ml median (range) (95% CI for median)
Children with RSV infection	<b>18</b> (63†)	69 (7-420) (55 - 117)
Surgical patients	<b>11</b> (24*)	86 (17-388) (35 - 114)
p value*		0.6349

\* Mann Whitney U test

There was no significant difference in the level of albumin detected between RSV and control groups.

<sup>†</sup> It was not possible to assess all 71 samples in the RSV group and all 31 samples in the control group due to insufficient sample size.

#### **11.2.4. FIBRIN DEGRADATION PRODUCTS**

Fibrin degradation products (FDP) were significantly increased in lavage fluid from

RSV patients compared to control (Figure 31). Levels of FDP showed no consistent

relationship either to disease severity as measured by arterial alveolar oxygen ratio or to duration of ventilation (Figure 32).



# Figure 32. FDP concentration on first and last days of ventilation in children with RSV infection ventilated for more than 1 day. Although there was a dramatic fall in FDP concentration between the first and last days of ventilation in some patients, there was overall no significant difference between median FDP levels on the initial and final days of ventilation.

## 11.2.5. SURFACTOMETER STUDIES OF SURFACTANT INHIBITORS IN BAL FLUID

When proteins from the 40,000g supernatant of BAL fluid were resuspended to a concentration of 1mg/ml in suspension buffer containing 2.5mg/ml Survanta and tested in the surfactometer, minimum surface tensions achieved within a 10 minute period were significantly higher in the RSV group than in the control group (Table 17), indicating greater inhibition of surfactant function. There was no significant difference in the median level of inhibition (as assessed by minimum surface tension) between the first and last days of ventilation.

	No. of patients (samples)	min. surface tension mN/m median (range) (95% CI for median)	max, surface tension mN/m median (range) (95% CI for median)
Children with RSV infection	<b>16</b> (53†)	21.0 (1.1-35.5) (19.3 - 23.3)	46.2 (35.2-59.4) (45.2 - 47.3)
Surgical patients	10 (20†)	13.9 (3.4-27.9) (8.4 - 18.2)	47.6 (33.6-55.8) (44.7-48.8)
p valuc*		0.0086	0.6920
Survanta alone	5	3.8 (3.0-3.8)	52.0 (51.5-57.3)

 Table 17. Surface tension values achieved with 1mg/ml supernatant protein and

 2.5mg/ml Survanta.

\* Mann Whitney U test

Inhibition of Survanta, an animal surfactant was greater when 1mg/ml supernatant protein was added from BAL fluid of patients with RSV infection than when the protein was derived from BAL fluid of controls.

† It was not possible to assess all 71 samples in the RSV group and all 31 samples in the control group due to insufficient sample size or to insufficient amounts of protein in some samples.

## **11.3. DISCUSSION**

#### **11.3.1. TOTAL PROTEIN**

The total protein content of BAL fluid from children with RSV infection was found to be increased compared to control. This is likely to be due to leakage of protein rich fluid from pulmonary capillaries in the inflammatory response and is in keeping with the histological evidence of an alveolar exudate in RSV infection. To give a better picture of the molecules involved in this exudate, potential surfactant inhibitors were quantified in BAL fluid.

#### 11.3.2. FIBRINOGEN AND C-REACTIVE PROTEIN (CRP)

The concentration of CRP was assessed by immunoturbimetry, and fibrinogen by the thrombin time method (which uses the fact that the thrombin clotting time is inversely proportional to the amount of fibrinogen present) (Clauss, 1957; Alving and Bell,

1976). CRP was assessed in all patients and fibrinogen in selected samples from RSV and control patients. Neither CRP nor fibrinogen was detected in any samples. The lower limit of detection in the CRP assay (2.5 µg/ml) was within the range of the low amounts of CRP found in control patients in Li's study (1989) of BAL from ARDS patients, and would certainly have allowed detection of the levels elevated to the degree found in Li's ARDS group.

These results suggest that CRP and fibrinogen, known inhibitors of surfactant function, are unlikely to play a significant role in the inhibition of surfactant in RSV infection.

#### 11.3.3. ALBUMIN

Albumin levels, assessed by immunoturbimetry, were not significantly different between RSV and control groups. The levels of albumin found in the BAL in this study were similar to those found clscwhere in healthy adult human volunteers (Low et al. 1978). This suggests that albumin, an inhibitor of surfactant at relatively high levels of protein, does not have a specific role in the inhibition of surfactant in patients with RSV.

#### 11.3.4. FIBRIN DEGRADATION PRODUCTS (FDP)

The D-dimer FDP was found to be elevated in samples of BAL from children with RSV infection - a finding consistent with the histological picture of an alveolar fibrinous exudate. The D fibrin degradation product has been shown to be an extremely

potent inhibitor of surfactant in vitro - more potent than either fibrinogen or the E fibrin degradation product (O'Brodovich et al. 1990; Seeger et al. 1993).

As surgery increases the action of the clotting and fibrinolytic systems, patients in the control group who had not had surgery were identified on the graph of FDPs shown in Figure 31. From this, it appears that surgery does not have a substantial effect on FDP levels in BAL fluid and whether preoperative or post operative samples are considered, the conclusion that FDP's are raised in RSV infection remains.

It can be seen from Figure 32 that the level of FDP detected in BAL fluid fell dramatically between the first and last days of ventilation in some patients. The implication of this finding for surface activity is uncertain, as this fall in FDP was not reliably associated with the appearance of a positive click test.

#### 11.3.5. SURFACTOMETER STUDIES OF SURFACTANT INHIBITORS IN BAL FLUID

When the inhibitory capacity of BAL proteins was assessed, it was found that proteins from patients with RSV infection inhibited a bovine surfactant replacement preparation (Survanta) more strongly than those from control patients. This is consistent with the finding that a strongly inhibitory FDP was found in increased amounts in BAL from patients with RSV infection. It is also possible that other proteins which were not studied played a part in the inhibition of surfactant in these patients. Whatever the cause, this study provides clear evidence that surfactant inhibitors are present and are contributing to the observed surfactant dysfunction in RSV infection.

## **12. DISCUSSION**

## **12.1. SURFACTANT FUNCTION - SURFACE ACTIVITY**

## 12.1.1. SURFACTANT DYSFUNCTION AND DEFICIENCY OF SURFACTANT PHOSPHOLIPID AND SPB

In agreement with other authors (Dargaville et al. 1996; Skelton et al. 1996), surfactant function was found, in this study, to be reduced in whole BAL fluid. The factors contributing to the surfactant dysfunction in severe RSV infection emerged as complex.

In contrast to most studies of ARDS (Jacobson et al. 1993; Offenstadt et al. 1981; Gunther et al. 1996; Hallman et al. 1982; Spragg and Hallman, 1983; Pison et al. 1990), and in keeping with Dargaville's (1996) finding of reduced DSPC in viral bronchiolitis, the amount of surfactant phospholipid in BAL fluid was found in this study to be decreased in severe RSV infection.

There are histological clues to the underlying mechanism of the decrease in phospholipids. The first comes from the finding, in an immunohistochemical study, of RSV in type II pneumocytes, the cells which synthesise and secrete surfactant phospholipid (Wright et al. 1997). It is possible that the virus, once in the cells, reduces their surfactant output. Secondly, areas of lung may become necrotic in viral pneumonia (Aherne et al. 1970), and this would reduce surfactant production simply by wholesale cellular destruction. These particular features are in marked contrast to

the situation in ARDS, where there is hyperplasia of type II pneumocytes in the early stage of the disease, probably leading to normal or even increased surfactant phospholipid production.

In this study it was found that SPA and SPB but not SPD were positively correlated with phospholipid levels in the control groups. However, in the RSV group, this correlation disappears. This would support the hypothesis that the disease has a disruptive effect on the synthesis of surfactant components in type II pneumocytes.

As well as the effects of viral invasion on type II pneumocytes, indirect effects of RSV infection in the shape of the release of peroxynitrite and nitric oxide in the inflammatory response could affect metabolism and surfactant processing in these cells. Evidence for increased peroxynitrite production in RSV infection is seen in increased nitrotyrosine in BAL fluid and peroxynitrite has been shown to inhibit oxygen consumption and sodium transport in alveolar type II cells in vitro (Hu et al. 1994). Also nitric oxide, which is released from alveolar macrophages in the inflammatory response, has been shown to reduce synthesis of PC in vitro by 60% (Haddad et al. 1996). There is indirect evidence, from the presence of nitrotyrosine, that nitric oxide production is increased in severe RSV infection, and this mechanism may also act to reduce alveolar phospholipids.

The high oxygen levels used in treatment of severe RSV infection (Fig 33) could also be partly responsible for the reduction in surface active phospholipids in the infected infants in this study. Similar levels of oxygen have been shown to cause decreases in DPPC and surface activity in experimental animals (King et al. 1989). No significant

correlation was found in this study between inspired oxygen level and phospholipid level at time of sampling. However, the effect of increased fractional oxygen concentration on phospholipid level is a delayed one (King et al. 1989). It is not possible to disentangle the effect of increased oxygen in RSV infection from the other factors which may be responsible for a reduced phospholipid level, as oxygen is an integral part of the therapy of respiratory failure.



% inspired oxygen

Figure 33. Inspired oxygen concentrations in patients ventilated for RSV infection. Inspired oxygen concentration is shown on the day that each sample was taken in the group of patients with RSV infection. Surfactant protein B was also found to be reduced in severe RSV infection. This contrasts with Levine's (1996) finding of normal levels of SPB (per ml of BAL and per mg of total protein) in viral pneumonitis. The potential reasons for this divergence of findings are discussed in section 8.3.1. It is interesting to note that in severe RSV infection, the positive correlation between SPB and phospholipids is lost. This supports the idea that surfactant synthesis is in some way disrupted. Surfactant protein B is found in lamellar bodies of type II pneumocytes, and is thought to be secreted along with surfactant phospholipids. It is as though there is an 'uncoupling' of SPB and phospholipid levels in RSV, with both falling, but not to the same degree. The amount of SPB produced in the lung is increased, in animal models, by high inspired oxygen (Nogee et al. 1991). It may be that the high oxygen concentrations used in severe RSV infection offset some decrease in SPB in these patients. However, it is not possible to go beyond speculation in this matter.

Both surfactant phospholipids and SPB are important for surfactant function, and it is likely that some of the surfactant dysfunction observed in this study may be due to deficiency of these components.

#### **12.1.2. COMPOSITION AND FUNCTION OF THE SURFACTANT LIPID** FRACTION OF BAL

In agreement with Skelton et al (1996), and with studies of ARDS (Pison et al. 1990; Pison et al. 1987; Veldhuizen et al. 1995; Hallman et al. 1990; Gregory et al. 1991), the fraction of PC was found to be reduced in this study. However, a small increase was found in the PG fraction in patients with severe RSV infection. This is at variance with Skelton's (1996) finding of absent PG in infants with viral bronchiolitis. Also,

studies of ARDS (Pison et al. 1990; Pison et al. 1987; Veldhuizen et al. 1995; Hallman et al. 1990; Gregory et al. 1991) and infant RDS (Obladen, 1978; Bose et al. 1984), conditions which have pathological similarities to RSV, have shown a reduction in PG. The small degree of the increase in PG, and the inaccuracy of the HPTLC method used in this study, along with the fact that this finding is at variance with other studies makes it unlikely that an increase in PG is a significant contributor to the pathology of RSV. However, it should be said, in defense of this result, that the proportion of fatty acids which are found predominantly in PG increased with increasing levels of this lipid. Also, a PG spot was clearly visible on each HPTLC run in this study, and this does not support Skelton's finding that PG is at times absent in viral bronchiolitis. Further work with a more reliable method of quantification would be necessary to elucidate this matter. The uncertainty of this conclusion means that it is not possible to determine whether or not the phospholipid composition of surfactant adversely affects surface activity in severe RSV infection. Certainly, the surface activity of a lipid extract of surfactant in this study did not appear to be adversely affected by the presence of RSV infection. However, concerns about the surfactant extract tested being too dilute to adsorb fully to the air liquid interface must prompt caution in interpreting this as proof of a normally active lipid fraction.

The presence of glycolipids in the lipid fraction of surfactant from children with RSV infection is consistent with the finding of glycolipids in two studies of ARDS (Hallman et al. 1982; Rauvala and Hallman, 1984). In one of these studies, the glycolipids found were shown to reduce surface activity of BAL surfactant purified by density gradient centrifugation when tested in the pulsating bubble surfactometer (Rauvala and Hallman, 1984). Glycolipids may be influencing surface activity of surfactant in RSV

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infection. However, from the PBS studies performed in this study, it is not possible to draw any firm conclusions.

The presence of LPC in a large number of BAL samples from children with severe RSV infection corresponds with the finding of this lipid in ARDS. Lysophosphatidylcholine on its own appears to inhibit the surface activity of a lipid mixture, but when LPC combined with palmitic acid is added to a lipid mixture, this effect is reversed (Cockshutt et al. 1991), and surface activity may even be improved (Lema and Enhorning, 1997). As free palmitic acid was not measured in this study, it is not possible to say whether the presence of LPC in the sample could have been detrimental to surface activity.

Surfactant protein B participates in the lowering of surface tension of a phospholipid mixture. It is also co-extracted with surfactant lipids in a chloroform-methanol extraction. In order to investigate whether the reduction in SPB seen in whole BAL fluid affected surface tension of the lipid extract of BAL in this study, regression analysis was performed. No significant correlation was found between SPB concentration in a sample (expressed per ml of BAL fluid and per µg of phospholipid) and minimum or maximum surface tensions achieved by a lipid extract of that sample.

In conclusion, several changes in the composition of the hydrophobic fraction of BAL fluid were found in this study. There were minor alterations in phospholipid class and phospholipid fatty acid proportions, lysophosphatidylcholine and glycolipids were increased and SPB was reduced. Any of these changes could, in theory, adversely affect the surface activity of a lipid extract of surfactant. This study provides no

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10. 20 Miles

evidence of a detrimental effect of any of these changes on surfactant function. However, the concentration of BAL lipid extract used in surfactometer studies was at the low end of the range used in other studies, and therefore this conclusion should be interpreted with caution.

#### **12.1.3. INHIBITION OF SURFACTANT BY PROTEINS**

The concentration of total protein found in BAL fluid was increased in children with severe RSV infection. A similar picture occurs in ARDS, and lavage protein has been shown to correlate positively with the percentage of neutrophils in BAL fluid (Weiland et al. 1986). Neutrophils release free radicals in the inflammatory response, and are present in large numbers in BAL fluid from ventilated infants with RSV infection (Everard et al. 1994). It is likely that the integrity of the alveolar and pulmonary capillary endothelium is breached by free radical damage, and that proteins teak from the plasma into the alveolus in both ARDS and RSV.

The increase in fibrin degradation products found in this study is consistent with the fibrinous exudate seen in histological sections of the lung in severe RSV infection. Fibrinogen was not detected in the small numbers of exploratory samples from infants with RSV studied, therefore it was decided not to pursue this line of investigation further. It is possible, though unlikely that substantial amounts of fibrinogen were present in some but not others of the samples from children with RSV. It is more likely that fibrinogen in the inflammatory exudate was converted into the insoluble fibrin which was then deposited in the aveolus, and subsequently degraded by proteolytic enzymes. Jacobson and colleagues (1993) found fibrin/fibrinogen in BAL fluid from

patients with ARDS, and there can be little doubt that the fibrin/fibrinogen/FDP system is active in lung inflammation.

The finding of FDP in BAL fluid has implications for surfactactant inhibition, as fibrin degradation products are known to be potent inhibitors of surfactant function, however, the effect of FDP is difficult to separate from other proteins which might be causing surfactant inhibition. The minimum and maximum surface tensions of Survanta produced when Img/ml of BAL protein was mixed with the lipid did not correlate with levels of FDP/mg protein. Also albumin, another known inhibitor of surfactant activity was not elevated in the RSV group, and CRP, which has been shown to inhibit surfactant in ARDS (Li et al. 1989) was not detectable in any of the samples in this study. Therefore, it seems that some other proteinaceous surfactant inhibitor is at work in severe RSV infection.

Immunoglobulins, haemoglobin, elastin and fibrin monomer were not measured in this study, and all these substances are known surfactant inhibitors. Additionally, viral proteins were not quantified, and, although no work has been done in this area, it is possible that they may have surfactant inhibitory properties. Quantification of these other potential inhibitors in BAL in RSV infection would provide a clearer picture.

The BAL proteins tested for inhibitory capacity in this study were derived from a 40,000 x g supernatant of lavage fluid. This centrifugation procedure would have removed most SPA and SPB. However, some SPD may have been present in the supernatant. Both surfactant proteins A and B protect surfactant from inhibition by plasma proteins but it is not known whether SPD has a similar role. Theoretically, if

SPD did act to protect surfactant phospholipids from inhibition, and there was less of this protein in the supernatant from patients with RSV infection, the increased inhibitory capacity of total protein found in these samples could have been partly due to this reduced protection.

The reduction in SPB found in this study was still present when concentations of these surfactant proteins were expressed per mg of phospholipid. However SPA/ $\mu$ g phospholipid was not reduced in the RSV group compared to control. This suggests that in vivo, there would be less protection from SPB available to surfactant phospholipids against protein inhibition in severe RSV infection. However, as the full spectrum of potentially inhibitory proteins has not been studied in this condition, it is not possible to say whether the reduction in SPB exacerbates the problem or not. It is known that SPB offsets the inhibition of lipid mixtures by fibrinogen. However, it is not known whether this is also the case with FDP's. Surfactometer studies of FDP's and other plasma proteins, lipid mixtures and surfactant proteins would shed more light on this issue.

Finally, LPC was found in increased numbers of infants with severe RSV infection. The presence of lysophosphatidylcholine produced by phospholipase digestion of phospholipids in lipid extract surfactant has been shown to sensitize this extract to inhibition by fibrinogen (Cockshutt and Possmayer, 1991). Perhaps LPC also increases the sensitivity of surfactant phospholipids in RSV infection to inhibition by FDPs and plasma proteins which enter the alveolus during the inflammatory response.

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## **12.2. SURFACTANT AND THE ALVEOLAR IMMUNE** RESPONSE

Surfactant proteins A and D were found to be significantly reduced in patients with RSV infection. Surfactant proteins A and D are known to participate in local inflammatory mechanisms in the lung, and it is possible that these proteins opsonise RSV, and are subsequently phagocytosed in the inflammatory response to the virus. If this happens the concentration of SPA and SPD in the alveolus would be reduced.

A reduction in SPA or SPD during pulmonary infection could have several important consequences for the local immunological defenses in the lung. Both have been shown to bind to microorganisms and alveolar macrophages, enhancing the cytotoxic properties of these phagocytic cells (Hartshorn et al. 1994; Malhotra et al. 1994; Van Iwaarden et al. 1991; Anonymous1995; van Iwaarden et al. 1990; Van Golde, 1995). Additionally, SPA enhances phagocytosis by alveolar macrophages. This prompts us to ask : does RSV infection increase phagocytosis of surfactant proteins and microorganisms, which, in turn reduces the stimulation of alveolar macrophages to phagocytose and kill the infective agent, leading to a downward spiral of worsening infection? This is not likely to be the full picture, as severe RSV infection is not usually fatal, and spontaneous recovery occurs if the infant is supported through the most difficult days of the disease.

In vitro studies of the binding of surfactant proteins to the virus, and the effect of the virus on phagocytosis of these proteins would be useful as a preliminary attempt to elucidate the fate of SPA and SPD in RSV infection. As well as phagocytosis, it is possible that viral invasion of type II pneumocytes or damage to these cells by

peroxynitrite could reduce SPA synthesis in this disease. In animal models, an elevated inspired oxygen concentration increases the amount of SPA produced in the hung (Nogee et al. 1991). Patients in the RSV group were subjected to over 50% inspired oxygen throughout most of the period of ventilation (Figure 33) and it may be that the reductions seen in SPA would have been more dramatic had this not been the case.

### **12.3. MARKERS OF FREE RADICAL ACTIVITY**

The finding that nitrotyrosine is elevated in RSV infection is consistent with previous immunohistochemical studies of ARDS and pneumonia (Haddad et al. 1994; Royall et al. 1995). The presence of nitrotyrosine in all these conditions suggests increased peroxynitrite activity, as a result of invasion by neutrophils and macrophages.

The reactivity of peroxynitrite is affected by pH. At low pH, formation of the reactive trans peroxynitrite anion is favoured. In lung disease, the alveolar environment may be more acidic due to retention of CO2 behind partially occluded airways. The level of CO2 present in the alveolus is indicated by arterial CO2 (Riley and Cournand, 1949). The median arterial CO2 level was higher in the RSV group (48.1 - range 14.6-144.0 mmHg) than in the post-operative patients in the control group (30.6 - range 18.3-57.1 mmHg) (p<0.0001 Mann Whitney), leading to a lower median alveolar pH in the RSV group, which may have favoured peroxynitrite damage. However, when linear regression analysis was performed, no correlation was found between arterial CO2 and nitrotyrosine level.

Haddad and colleagues (1993) showed nitration of tyrosine residues in SPA to occur as a result of in vitro exposure to peroxynitrite. The findings of this study provide no evidence of damage specifically to SPA, but this might be inferred from the increased nitrotyrosine per mg of total protein. The functional consequences of this structural alteration have not been fully characterised. However, SPA exposed to peroxynitrite has been shown to have a reduced ability to cause lipid aggregation (Haddad et al. 1993), and tyrosine nitration is known to adversely affect the function of IgG (McCall and Easterbrook-Smith, 1989) and cytochrome P-450 (Janing et al. 1987). This prompts speculation that the functional properties of SPA may be compromised in RSV infection. Perhaps the stimulatory effects on alveolar macrophages are lost, reducing the cytotoxic activity and phagocytic ability of these cells during infection. Surfactant protein A might then no longer offer protection to surfactant phospholipids from the inhibitory effects of plasma proteins which flood into the alveolus in the inflammatory response. Surfactometer studies and in vitro studies of macrophage activity in the presence of peroxynitrite-exposed SPA would provide useful information in clarifying the functional effects of tyrosine nitration on SPA.

Three potential reasons for the reduced amount of MDA seen in RSV infection have been discussed earlier : reduced substrate, increased scavenging and the effects of surgery in the control group. The results of this study provide no evidence for a reduction in substrate as a causal factor, although this cannot be ruled out, as some substrates for MDA formation eg. prostaglandins and free fatty acids were not studied. Also it is impossible to tell whether the concentrations of fatty acids measured in RSV and control groups were affected by lipid peroxidation. The effects of surgery on MDA level do not appear to be significant, although the number of patients in the control

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group who had and had not been operated upon were relatively small. A larger study might show some effect of surgical tissue damage on BAL MDA, although it is difficult to imagine under what circumstances such a study would be performed. Probably the most likely explanation for the reduced concentration of MDA seen in BAL from patients with RSV is increased scavenging of oxidants by plasma proteins or surfactant proteins. Support is lent to the importance of this last mechanism by the finding of increased nitrotyrosine and increased total protein in BAL fluid from patients with RSV infection.

A fourth potential reason for the reduction in MDA in ptients with RSV infection is also suggested by the presence of nitrotyrosine in this group. It is interesting to note that the presence of nitric oxide, depending on its concentration, can either stimulate or inhibit lipid peroxidation in phosphatidylcholine liposomes in vitro (Rubbo et al. 1994). Increased nitric oxide production in children with RSV infection is inferred by the presence of increased nitrotyrosine in BAL. Nitric oxide may actually act in a defensive manner in patients with RSV, offering a potential explanation for the reduced amounts of MDA. However, it is not possible to go beyond speculation in this matter, as nitric oxide levels could not be directly measured.

## **12.4. SURFACTANT AND THE CLINICAL PICTURE**

## 12.4.1. ARTERIAL ALVEOLAR OXYGEN RATIO AND SURFACTANT LEVELS

The arterial arteriolar (a-A) oxygen ratio was used as an index of clinical condition in this study, and as such provided a relatively non invasive measurement of shunt 가려가 가려가 있는 것이 가지 않는 것이 있는 것이 가지 않는 것이 있다. 것이 있는 것이 가지 않는 것이 있는 것이 있는 것이 있는 것이 있는 것이 있는 것이 있는 것이 같은 것이 같은 것이 같은 것 옷에서 가지

fraction. It would have been preferable to have measured pulmonary function tests in addition to a-A ratio and to have combined these measurements to produce an index of disease progress which encompassed lung mechanics and gas exchange.

In this study no correlation was found between a-A ratio and parameters measured in BAL fluid. From a review of the literature it appears that alterations in the pulmonary surfactant system are not reflected by indices of oxygenation alone. In a study of adults recieving artificial surfactant after cardiopulmonary bypass, Macnaughton and colleagues (1994) found that A-a gradient, FRC and respiratory system compliance were not affected by administration of exogenous surfactant, however  $T_{LCO}$  was improved. Pison and colleagues studied sutfactant phospholipid classes in patients with ARDS secondary to multiple trauma, and found that the proportion of PC was inversely correlated with the respiratory failure score. This score was found by the following formula: 0.108 + (chest radiographic score x 0.1512) + (oxygenation index x)0.6208) + (mean PAP x 0.00725) - (respiratory system compliance x 0.00416), a complex score composed of radiographic, oxygenation, ventilatory and lung mechanical measurements. Survival or severity of trauma did not correlate with any surfactant data (Pison et al. 1989; Pison et al. 1990). In another study, reduction of PG showed little relation to the time of sampling during ARDS (Spragg and Hallman, 1983).

Also, in patients with idiopathic pulmonary fibrosis, phospholipid content in BAL was correlated with improvement in %FVC but arterial alveolar oxygen difference was not correlated with phospholipid content of BAL or with PG/PI ratio (Robinson et al. 1988). In study of neonates with RDS the percentage of tracheal wash palmitate rose in concert with a clinical grading score which reflected the amount of ventilatory support required (Balint et al. 1978). In another study, infants given dexamethasonc, logPC concentration in epithelial lining fluid was correlated with the degree of respiratory failure as measured by mean airway pressure x FiO2. However, among placebo treated infants, the association was not significant (Kari et al. 1995).

These studies suggest that measurement of arterial alveolar oxygen ratio alone, or, indeed pulmonary function tests alone provides a very inaccurate indication of the totality of clinical progress with relevance to changes in the pulmonary surfactant system.

#### **12.4.2. SURFACTANT AND DISEASE PROGRESS IN RSV INFECTION**

It can be seen from the results in this study that, surfactant function did not recover in all patients with RSV by the time ventilation was discontinued (Figure 14). Nor, with the exception of surfactant protein A, did surfactant components change in a consistent pattern between the first and last days of ventilation. Equally, the pattern seen in indices of surfactant damage or inhibition was highly variable from day to day. These findings prompt the question - if respiratory failure in RSV infection is due to surfactant dysfunction, why did that dysfunction and the factors contributing to it not resolve by the last day of ventilation?

There are several possible answers, all of which underline the complex nature of the disease. When ventilation is discontinued in RSV infection, the effects of the virus are still present, and the child often requires respiratory support eg. continuous positive

airways pressure or oxygen via nasal cannulae after extubation, so recovery is by no means immediate or complete at extubation. Some indication of this is given by the fact that 13 out of the 18 patients with RSV infection were still being ventilated with 50% or more oxygen on the day when the last sample was taken before coming off ventilation. It may be that children with resolving infection still have a surfactant dysfunction, however, this cannot be easily studied as BAL fluid is only available in the ventilated group.

Several types of RSV are known to cause respiratory failure, and the variable pattern of change seen in surfactant components in this study may be due to interactions with RSV which are specific to viral type. It is impossible to go beyond speculation in this matter, as, at present, viral typing is not performed routinely in children hospitalised with RSV.

Surfactant protein A levels were shown to increase between the first and last days of ventilation in 12 out of 16 children with RSV who were ventilated for more than one day. As discussed, SPA reduces inhibition of surfactant by proteins, and it may be that the presence of this protein is important for disease resolution.

It is likely that factors other than surfactant function, such as regeneration of the alveolar epithelium and clearance of mucus, contribute to the improvement in gas exchange which eventually allows the ventilated child with RSV to be extubated. This emphasises the conclusion that although surfactant abnormalities may contribute to the respiratory failure seen in severe RSV infection, they should be viewed in the context of the disease as a whole.

## **12.5. IMPLICATIONS FOR CLINICAL PRACTICE**

#### 12.5.1. SURFACTANT THERAPY IN VENTILATED CHILDREN WITH RSV INFECTION

This study provides evidence that surfactant function is abnormal, that surfactant phospholipids and proteins are deficient and that surfactant inhibition is likely to be a part of the pathological picture in severe RSV infection. It backs up previous evidence from Dargaville's (1996) and Skelton's (1996) studies of surfactant deficiency and dysfuntion in viral bronchiolitis. Also, two recent case reports point to an improvement in clinical condition following intratracheal instillation of surfactant in ventilated children with severe RSV infection (Vos et al. 1996). As discussed earlier, there is no satisfactory animal model for severe RSV infection, therefore surfactant replacement studies in animals with RSV would not be useful. In light of the evidence provided by the present study and other studies, it must be considered whether the next step is to replace surfactant in ventilated patients with RSV infection.

As described in the introduction to this thesis, surfactant replacement in ARDS, a condition with many similarities to severe RSV infection, has met with limited success. When considering the viability of surfactant replacement in RSV, several factors should be taken into account. These are the method of delivery, the type of surfactant used, cost effectiveness, and other potential therapies for RSV infection in the intensive care unit.

Exogenous surfactant can be delivered to the lungs either by nebulisation or by intratracheal instillation. Nebulised surfactant is preferentially distributed to normally

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ventilated parts of the lung (Macnaughton and Evans, 1994), and therefore may not reach the most severely affected alveolar units which are collapsed or lie behind narrowed airways in RSV infection. This is also true to a lesser extent of surfactant delivered by intratracheal instillation. However, Enhorning's work on surfactant function in the small airways (1995; 1991) suggests that surfactant might, in fact, improve airway narrowing in RSV, and lead to not only better small airway function and consequently improved gas exchange, but also better delivery of exogenous surfactant to the alveoli.

It is interesting to note that in a study of rabbits injured with NNMU (Lewis et al. 1991) supplementation with nebulised surfactant produced a modest physiological improvement, whereas intratracheal surfactant was not beneficial. In this study, only  $3.6 \pm 0.5\%$  of the amount of nebulised surfactant was recovered from the lung. In contrast, a trial of nebulised Exosurf produced no significant clinical improvement in patients with ARDS (Anzueto et al. 1996). However, another pilot study of intratracheally delivered natural surfactant produced a reduction in mortality from ARDS (Gregory et al. 1997). It is not possible to separate the influence of mode of delivery from the type of surfactant used in these two studies of ARDS. Natural surfactant has certain advantages over Exosurf which are considered below.

Natural surfactants (eg, Curosurf, Infasurf, Survanta) are manufactured by retrieval of surfactant from the lungs of slaughtered animals (pigs or cows) by bronchoalveolar lavage. An organic extraction is then performed on this fluid to obtain the clinical preparation. This results in this type of exogenous surfactant conatining all surfactant lipids and SPB and SPC. Exosurf, on the other hand, contains only phosphatidylcholine Ś

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solubilised in alcohol. As plasma proteins are present in the alveolus in ARDS, and SPB and SPC have been shown to protect surfactant phospholipids from inhibition by these proteins, it would seem that a natural surfactant replacement would be a more logical choice in this condition. The same arguement applies in RSV infection, where inhibition of surfactant by BAL proteins has been demonstrated in vitro. Additionally, SPB deficiency was found in this study. If surfactant were to be replaced therapeutically in RSV, it would be sensible to choose a surfactant known to contain hydrophobic surfactant proteins.

Also, calf lung surfactant extract (a natural surfactant) has been shown to reduce the increased alveolar permeability resulting from hyperoxia (Engstrom et al. 1989), and the increased protein in BAL fluid from patients with RSV would suggest that alveolar permeability is increased in this condition, and that exogenous natural surfactant might be helpful in stopping the leak.

In an ideal world, a surfactant containing phospholipid and proteins A, B and C would be the perfect replacement mixture to use in RSV infection or in ARDS. Surfactant protein A would be an especially desirable component, as not only does it offer some protection against free radical damage to lipid components of surfactant, but also, it offsets the inhibitory effects of fibrinogen (Cockshutt et al. 1989). Natural surfactant replacements containing surfactant protein A are, unfortunately, not commercially available due to purification difficulties. Synthetic analogues of SPB have been produced by genetic engineering in E.coli, however, SPA has more post translational modifications and is not suited to production in this way (Benson, 1993). 如此,我们也能是一个,我们就是一个是我们的是不是一个,我们就是我们的,我们就是我们的,我们就是不是我们的,我们就是我们的,你们就是你们的,你们就能能能能。""你们

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Exogenous surfactant therapy is expensive, but so is a day's stay in the intensive care unit. If administration of surfactant could be shown to shorten the intensive care time or offer benefits to the patient's long term wellbeing, it would be accepted as part of the inpatient treatment of severe RSV infection. However, if giving surfactant produced only transient clinical improvement, then it could not be considered an economically viable treatment.

It is possible that other advances in care of patients with RSV infection might supersede the need for surfactant, even it it does prove to have success in the clinical situation. Improved hygiene has reduced nosocomial spread of the virus (Madge et al. 1992) and immunoglobulin therapy has been shown to prevent infection in high risk infants (Everard 1995).

In summary, the evidence from this study supports the theory that surfactant is dysfunctional and deficient in severe RSV infection. Also case reports have shown a clinical improvement after surfactant therapy in this condition. However, there are several caveats which must be taken into account when designing a pilot study of the efficacy of surfactant replacement in clinical practice.

#### 12.5.2. SURFACTANT THERAPY IN SPONTANEOUSLY BREATHING CHILDREN WITH RSV INFECTION

Surfactant abnormalities may be present in children with resolving RSV infection who are no longer ventilated or in children with milder disease who have breathing difficulties but are not ventilated. In this situation, the pathological features of the disease are likely to be confined to the small airways, where surfactant supplementation could theoretically improve luminal patency (Liu et al. 1991; Enhorning et al. 1995; Yager et al. 1989). In these patients, the problem of a putative surfactant phospholipid deficiency could be addressed by therapy with surfactant in a powder form. To date, the only artificial surfactant available in this form is ALEC, and this has the advantage of relatively low cost and easy administration to patients who are not intubated and ventilated.

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## **13. CONCLUSIONS**

The main conclusions of this study are summarised in Figure 34.

### **13.1. SURFACE ACTIVITY OF SURFACTANT**

Surfactant is dysfunctional in whole BAL fluid from patients with severe RSV infection. The factors involved in producing this dysfunction are complex.

Surfactant function in a lipid extract of BAL from children with severe RSV infection was not different from control, however, the low concentration of phospholipids used in this study may have masked differences between the groups.

### **13.2. DEFICIENCY AND ABNORMAL COMPOSITION OF SURFACTANT**

In ventilated children with severe RSV infection, there are deficiencies of surfactant proteins A, B and D, and of surfactant phospholipids. It is likely that the surface tension-lowering and immunological functions of surfactant, and the protection offered against surfactant inhibition by plasma proteins are compromised in RSV infection by these deficiencies. Surfactant fatty acid composition is altered in RSV infection, in a pattern similar to that seen in ARDS. This is likely to be due to contamination of BAL fluid by membrane fragments.

### **13.3. DAMAGE TO SURFACTANT COMPONENTS**

There is evidence of alveolar protein damage by the peroxynitrite free radical in severe RSV infection. However, lipid peroxidation by free radicals is minimal. It is possible that the presence of proteins or other free radical scavengers in the alveolus offset damage to surfactant lipids by reactive molecules released in the inflammatory response.

Evidence for damage to phosphatidylcholine and pulmonary cells in severe RSV infection is present in the form of increased lysophosphatidylcholine and glycolipids in BAL fluid.

### **13.4.INHIBITION OF SURFACTANT**

Increased amounts of protein and fibrin degradation products are present in BAL fluid from ventilated children with severe RSV infection. Plasma proteins and FDPs are potent inhibitors of surfactant, and the conclusion that surfactant inhibition is present in severe RSV infection is supported by the finding that BAL proteins from children with RSV have a strong inhibitory effect on bovine surfactant.



### Figure 34. Surfactant abnormalities in severe RSV infection

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,如此是我们的是不是有些人的。""我们就是是这个人的,你们就是这些人的,我们就是这些人,就是我们的,我们就是我们的。"

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# **15. APPENDICES**

### **APPENDIX 1. CONSENT AND INFORMATION FORMS**

### INFORMED CONSENT FOR PARTICIPATION IN A STUDY OF THE EFFECTS OF VIRAL PNEUMONIA ON SURFACTANT

# An investigation of the pulmonary surfactant system in infants with viral pneumonia

We are studying how viruses affect the hungs of small children. To do this we want to look at samples of a natural chemical, surfactant, that stabilises the lungs and prevents the small air spaces from collapsing when someone breathes out.

To study surfactant in the lungs, we need to pass a fine suction tube into the small airways (via the breathing tube). The tiny samples which we get in this way will be taken to the laboratory and measured. The amount we remove is so small that it does not affect the working of the lungs. Usually, we will combine the collecting of samples with the suction that the nurses need to do to keep your child's airway free of secretions.

This technique has been tried and tested, and your child will be closely monitored all the time. If our study shows that sufractant is lacking in the lungs of children with pneumonia, this would pave the way for a trial of giving artificial surfactants such as are used in very small babies. This could help other children in the future recover more quickly from their pneumonia.

### Confidentiality

The medical history, the results of treatment, laboratory results and X rays may be published for scientific purposes, but neither your child's nor your identity will be disclosed, and confidentiality will be maintained at all times. You are free to withdraw from this project at any time without any penalty, and treatment will otherwise be the same as that given to other children with pneumonia.

I,\_\_\_\_\_(Parent/Guardian) agree that my child may participate in the study as detailed above. I understand that I may withdraw consent at any time.

Signed	(Parent)
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\_\_\_\_(Investigator)

\_\_\_\_\_(Witness)

(Date)

### INFORMED CONSENT FOR PARTICIPATION IN A STUDY OF SURFACTANT IN NORMAL INTUBATED CHILDREN

# An investigation of the pulmonary surfactant system in normal intubated children

We want to look at samples of the natural chemical, surfactant, that stabilises the lungs and prevents the small air spaces from collapsing when someone breathes out. Very little is known about this chemical in children with healthy lungs.

In operations such as the one your child is having, the anaesthetist always puts a breathing tube into the mouth. This tube goes down into the windpipe and helps your child breathe more easily while they are anaesthetised. The fact that this tube is already there makes it easy for us to take small samples of natural chemicals from the lungs.

To study surfactant in the lungs, we need to pass a fine suction tube into the small airways (via the breathing tube). The tiny samples which we get in this way will be taken to the laboratory and measured. The amount we remove is so small that it does not affect the working of the lungs. Usually, we will combine the collecting of samples with the suction that the nurses need to do to keep your child's airway free of secretions.

This technique has been tried and tested, and your child will be closely monitored all the time. Looking at surfactant in children with healthy lungs will help give us clues as to how surfactant works. It will also help us to understand what happens when it goes wrong, eg. in the lungs of children with lung disease.

### Confidentiality

The medical history, the results of treatment, laboratory results and X rays may be published for scientific purposes, but neither your child's nor your identity will be disclosed, and confidentiality will be maintained at all times. You are free to withdraw from this project at any time without any penalty.

I,\_\_\_\_\_(Parent/Guardian) agree that my child may participate in the study as detailed above. I understand that I may withdraw consent at any time.

Signed \_\_\_\_\_ (Parent)

\_\_\_\_\_(Investigator)

\_\_\_\_\_(Witness)

\_\_\_\_ (Date)

# APPENDIX 2. CLINICAL DATA FORM

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### APPENDIX 3. CALCULATION OF IDEAL ALVEOLAR GAS

The concept of "ideal alveolar" gas was first introduced in 1949 by Riley and Cournand . In a patient with lung disease, samples of expired gas give a very poor estimate of the composition of alveolar gas, due to the asynchronous emptying of alveoli with different time constants and different oxygen concentrations. Calculation of ideal alveolar gas circumvents this problem. The alveolar air equation allows estimation of an alveolar oxygen tension for the lung as a whole. The alveolar air equation can be derived with respiratory quotient (R) as a starting point.

Where

V = volume of gas per unit time

P = partial pressure of gas

F = fractional concentration of gas

R = respiratory quotient

 $P_{s}$  = barometric pressure

 $P_{H_{2}O}$  = partial pressure of water vapour, assumed to be 47mmHg

A = alveolar

i = inspired

e = expired

 $O_2 =$ oxygen  $CO_2$  carbon dioxide  $N_2 =$  nitrogen

$$R = \frac{VCO2}{VO2} \tag{1}$$

 $VCO2 = V_{eA} \times F_{ACO2}$  $VO2 = (V_{iA} \times F_{iO2}) - (V_{eA} \times F_{AO2})$ 

therefore, equation 1 becomes:  $R = \frac{V_{eA} \times F_{ACO2}}{(V_{iA} \times F_{iO2}) - (V_{eA} \times F_{AO2})}$ 

$$=\frac{F_{ACO2}}{\left(\frac{V_{LA}}{F_{EA}}\times F_{iO2}\right)-F_{AO2}}$$
(2)

As the amount of nitrogen in inspired and expired gas is the same,

$$\frac{N_{2\sigma}}{N_{2i}} = 1 \qquad \text{therefore, assuming that inspired } CO_2 = 0, \qquad \frac{V_{\epsilon A}(1 - F_{AO2} - F_{ACO2})}{V_{ia}(1 - F_{iO2})} = 1$$

$$\Rightarrow \frac{V_{iA}}{V_{cA}} = \frac{1 - F_{AO2} - F_{ACO2}}{1 - F_{iO2}}$$

Equation 2 may now be rewritten as:  $R = \frac{F_{ACO_2}}{F_{iO_2}(\frac{1-E_{AO_2}-E_{AO_2}}{1-E_{iO_2}}) - F_{AO_2}}$ 

This allows  $E_{402}$  to be determined,

$$F_{AO_2} = F_{IO_2} - \frac{F_{ACO_2}}{R} + F_{ACO_2} \times F_{IO_2} \times \frac{1-R}{R}$$

This equation can be simplified further to

$$F_{AO_2} = F_{iO_2} - F_{ACO_2}(F_{iO_2} + \frac{1 - R_{O_2}}{R})$$

1)

### **APPENDIX 4. BUFFERS**

Where buffers are prepared as concentrated solutions, the proportions given below represent the final composition after dilution with deionised water. Liquid additives are given as volume/volume percentages. Preservatives are given as weight volume percentages.

#### 82b

sodium chloride 150mM Tris pH 7.4 10mM bovine serum albumin 5mg/ml (Prepared as a 5 X solution, stored at -20°C and diluted before use)

#### PBS (phosphate buffered saline)

sodium chloride 137mM potassium chloride 2.7mM phosphate (as disodium hydrogen phosphate and potassium dihydrogen phosphate) 10mM pH 7.4

(prepared by dissolution of PBS tablets - Sigma Chemical Co)

#### PBS/NP-40

non ionic detergent NP-40 0.5% (v:v) in PBS

#### Phosphate buffer for MDA assay

sodium hydrogen phosphate 25mM pH 6.5

#### **SPA/SPB** dilution buffer

0.05% Tween 20 in PBS pH 7.4

#### SPA/SPB substrate buffer

sodium hydrogen phosphate 65mM citric acid 17mM merthiolate 0.001% pH 6.3 (Prepared as a 10 X solution and diluted before use)

### SPA/SPB wash buffer

Tween 20 0.05% Tris 10mM

merthiolate 0.001%

### pH 8

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(prepared as a 50 X solution and diluted before use)

### SPD coating buffer

sodium carbonate 35mM sodium bicarbonate 15mM sodium azide 0.05% pH 9.6

### SPD developing buffer

Tris 100mM

sodium chloride 100mM magnesium chloride hexahydrate 5mM adjusted to pH 9.6 with 1M hydrochloric acid

### SPD wash buffer

Tris 20mM sodium chloride 130mM Tween 20 0.05% calcium chloride 1mM sodium azide 0.05% adjusted to pH 7.4 with 1M hydrochloric acid

### Suspension buffer

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sodium chloride 150mM calcium chloride 1.5mM

## **APPENDIX 5. ABBREVIATIONS**

ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BSA	bovine serum albumin
CBS	captive bubble surfactometer
CLSE	calf lung surfactant extract
DPPC	dipalmitoylphosphatidylcholine
DSPC	disaturated phosphatidylcholine
ELISA	enzyme linked immunosorbent assay
GC	gas chromatography
GLC	gas liquid chromatography
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRP	horseradish peroxidase
IV	intravenous
LA	large aggregates
LPC	lysophosphatidylcholine
MDA	malondialdehydc
NNNMU	n-nitroso n-methylurethane
Р	partial pressure
PBS	pulsating bubble surfactometer. Phosphate buffered saline
PC	phosphatidylcholine
РСР	pneumocystis carinii pneumonia
PE	phosphatidylethanolamine

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PG	phosphatidylglycerol
PI	phosphatidylinositol
POPC	palmitoyloleoylphosphatidylcholine
PS	phosphatidylserine
RDS	respiratory distress syndrome
RSV	respiratory syncytial virus
S	sphgingomyelin
SA	small aggregates
SPA	surfactant protein A
SPB	surfactant protein B
SPC	surfactant protein C
SPD	surfactant protein D
TLC	thin layer chromatography

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