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Age-dependency of surfactant phospholipids and surfactant protein A in bronchoalveolar lavage fluid of children without bronchopulmonary disease

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Age-dependency of surfactant phospholipids and surfactant protein A in bronchoalveolar lavage fluid of children without bronchopulmonary disease. F. Ratjen, B. Rehn, U. Costabel, J. Bruch. ©ERS Journals Ltd 1996.

ABSTRACT: Whilst alterations in surfactant components of bronchoalveolar lavage fluid (BALF) have been described in a variety of pulmonary diseases in adults, no information is available on the surfactant composition in children beyond the neonatal period. In order to obtain reference values for the paediatric age group, we have studied phospholipid profiles of BALF in children without pulmonary disease.

The study population consisted of 38 children aged 3–15 yrs (mean±SD 8±4 yrs) undergoing elective surgery for nonpulmonary illnesses. Surfactant composition was analysed by high pressure liquid chromatography using both an ultraviolet and a light-scattering detector.

Total phospholipid concentration was higher in children under 8 yrs of age and decreased with age. In contrast, the total protein concentration remained constant throughout the age range. No age-related changes in the relative composition of phospholipid fractions could be observed. Surfactant protein A concentrations were highly variable in all age groups.

This study provides the first data on surfactant components in bronchoalveolar lavage fluid of healthy children and will be the basis for future studies of children with pulmonary diseases.

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Pulmonary surfactant is a complex mixture of phospholipids and proteins that act to reduce the surface tension within the alveolus and are essential for the maintenance of normal pulmonary function. Deficiency of surfactant in neonates is known to cause the respiratory distress syndrome (RDS) [1], which can be treated successfully with replacement of exogenous surfactant material [2]. Recent studies utilizing bronchoalveolar lavage (BAL) have demonstrated that the total phospholipid concentrations as well as the ratio of phophatidylglycerol to phosphatidylinositol (PG/PI) are decreased in patients with the adult respiratory distress syndrome [3, 4] and in respiratory failure due to trauma [5, 6]. A reduction in the PG/PI ratio has also been observed in a variety of interstitial lung diseases [7–9]. Total phospholipid concentrations of bronchoalveolar lavage fluid (BALF) have been found to be diminished in patients with acquired immune deficiency syndrome (AIDS) both with and without *Pneumocystis carinii* pneumonia [10, 11]. BALF phospholipids are also reduced in cystic fibrosis patients due to phospholipases produced by Pseudomonas species that inhibit surfactant function [12, 13].

Few data exist concerning BALF phospholipids in children beyond the neonatal period. Abnormalities of surfactant or one of its constituents have been described in the sudden infant death syndrome [14, 15], in infants with bronchopulmonary dysplasia [16], and in children with recurrent cyanotic episodes [17]. Studies of the phospholipid composition in paediatric lung diseases have been difficult to interpret, since reference values for this age group are lacking. As part of a study to define BALF constituents in childhood, we have investigated the composition of surfactant in children without pulmonary disease.

Material and methods

The study population consisted of 38 children aged 3–15 yrs (mean±SD 8±4 yrs). All children were undergoing elective surgery for nonpulmonary illnesses (table 1). Children with an upper respiratory tract infection in the preceding 3 months, a lower respiratory tract infection or a history of hyperreactive airway disease were excluded from this study. The study was approved by the Ethics Committee of our institution. Written informed consent from both parents was obtained in all cases.

Table 1. – Diagnosis and operative procedure of children undergoing BAL

Diagnosis	Operative procedure	n
Hypertrophy of adenoids	Adenectomy	9
Hypertrophy of tonsils	Tonsillectomy	7
Hypospadias	Correction	7
Retentio testis	Orchidopexy	5
Vesicoureteral reflux	Correction	3
Conductive deafness	Tympanoplasty	2
Nasal fracture	Correction	1
Nasal septum deviation	Correction	1
Parotic cyst	Exstirpation	1
Urethra duplex	Correction	1
Epispadia	Correction	1

BAL: bronchoalveolar lavage

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed under general anaesthesia and tracheal intubation. All children received flunitrazepam (0.04–0.05 mg·kg^{-1} body weight (BW)) orally 1 h prior to the procedure. In children under 6 yrs of age, anaesthesia was initiated *via* inhalation of a nitrous oxide-oxygen mixture with 2.5 volume (vol) % halothane. Subsequently, a venous access was inserted and atropine (0.01 mg·kg^{-1} BW), vecoronium (0.05 mg·kg^{-1} BW) and alfentanil (0.05 mg·kg^{-1} BW) were given intravenously. The halothane concentration was then reduced to 1.5 vol %. In children older than 6 years a venous access was inserted first and methohexitone 1–1.5 mg·kg^{-1} BW was given followed by atropine, vecoronium and alfentanil as described above. After intubation, the oxygen concentration was raised to 100% with 1.5 vol % halothane during the bronchoscopy procedure. A flexible bronchoscope with an external diameter of 3.5 or 4.9 mm (Pentax, Hamburg, Germany) was wedged in the right middle lobe or one of its segments. BAL was performed with normal saline warmed to body temperature; three times 1 mL·kg^{-1} BW were used in all children. In subjects weighing less than 20 kg, BAL was performed in three portions. In children over 20 kg, 20 mL portions were instilled up to a total volume of 3×1 mL·kg^{-1} BW. The first sample was considered a primarily bronchial sample; subsequent samples were pooled for analysis. Studies on surfactant composition were performed on the pooled sample only.

Analysis of BALF

BALF was filtered through sterile gauze and centrifuged at 500×g for 10 min. The cell suspension was separated for the analysis of cellular components, which have been reported previously [18]. All studies of surfactant composition were performed on unfrozen specimens of the supernatant. The methodology of the surfactant analysis has been described in detail previously [19]. In summary, phospholipids were extracted from BAL fluids, according to FOLCH *et al.* [20], using a mixture of chloroform and methanol (2:1). The extract was concentrated under nitrogen at 40°C and resuspended in 200 or 300 µL chloroform-methanol. After filtering through a squirt filter (Millipore, Eschborn. Germany), 20 µL were injected into a high pressure liquid chromatography (HPLC) column. A Gynkotek HPLC-system was used for the analysis. Measurements were performed with an ultraviolet detector at 200 nm (UV-technique) and with a Sedex 45 light-scattering detector (Sedere, Vitry, France) as mass detector (MD-technique).

Results are presented for the MD-technique and compared to those obtained with the UV-technique.

Surfactant protein A analysis

A sandwiched enzyme-linked immunosorbant assay (ELISA) was developed using a highly purified recombinant human surfactant protein A (SP-A) antibody, which binds to the globular domain of SP-A. The modification of the methodology yielded a wider range of linear sensitivity which was necessary for the broad range of SP-A values, in particular at the lower side of the distribution. Standardization was performed with human SP-A produced by recombinant deoxyribonucleic acid (DNA) technology (antibodies and the standard solution were kindly provided by Prof. Dr. Schäfer, BYK Gulden, Konstanz, Germany). Immunoassay plates (96 wells) were coated overnight at 4°C with a monoclonal antibody directed against human SP-A.

All samples were diluted according to their protein content to a concentration of 5 µg·mL protein. The wells were filled with 100 µL diluted samples and standard solutions and incubated at 37°C for 4 h. Each plate comprised standard human SP-A in 5–1,000 µg·mL^{-1} dilution; the standard curve was linear in the given range. After appropriate washing the plates were incubated with a rabbit anti-human SP-A polyclonal antibody at 37°C for 2 h. The third antibody, conjugated with peroxidase, was a goat anti-rabbit immunoglobulin (IgG). The peroxidase determination was carried out by adding the substrate orthophenyldiamine (OPD) for 5–10 min, the reaction was subsequently interrupted with 1 M sulphuric acid. The peroxidized OPD was measured at 405 nm in a plate reader (Labsystems, Multiscan). All measurements were performed three times independently. The precision of this assay has been determined previously utilizing two independent triple assays in BALs of patients in which SP-A ranged 200–8,000 ng·mL^{-1}. The standard deviation over this range was below 10%.

Statistical analysis

All parameters were tested for normal distribution with the Kolmogorov-Smirnov test. The means, standard deviations (SD) and medians were calculated for all data. The agreement between the light-scattering MD and UV techniques was assessed with the mean difference technique proposed by BLAND and ALTMAN [21]. The phospholipid concentrations determined with the two techniques as well as age-related differences in surfactant phospholipids were compared with the Wilcoxon test.

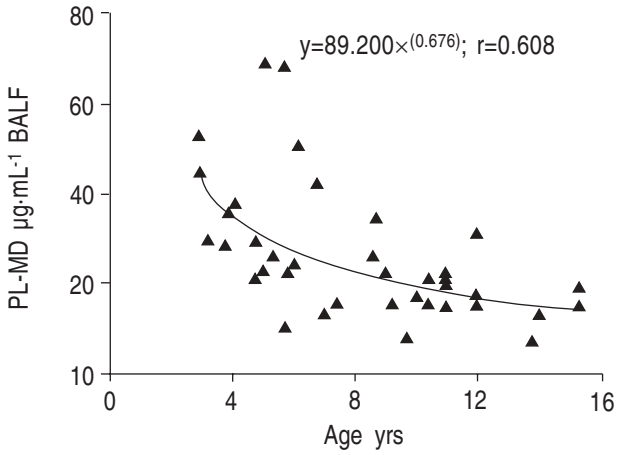

Fig. 1. – Total phospholipid concentrations, as determined by light-scattering mass detector (PL-MD), of bronchoalveolar lavage fluid (BALF) as a function of age. Each triangle represents one individual. The solid line is a power function of best fit.

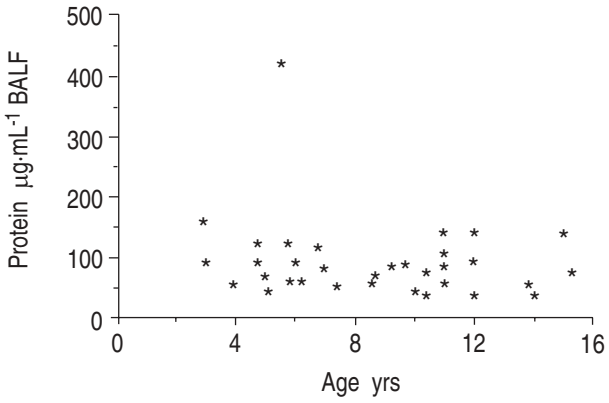

Fig. 2. – Protein concentration of bronchoalveolar lavage fluid (BALF) as a function of age. Each asterisk represents one individual.

Results

No side-effects related to the bronchoalveolar lavage were observed except for a low grade fever in three subjects that subsided spontaneously. The mean±SD recovery was 56±18% for the total BAL and 64±19% for the pooled sample. The relationship between age and total phospholipid (PL) concentration of BALF measured by the light-scattering MD-technique is shown in figure 1. The phospholipid concentration was higher in younger children and decreased with age. Most of these changes had occurred by the age of 8 yrs. If subjects were clustered for age, children under 8 yrs of age had approximately twice the amount of PL in their BALF (table 2). The relative distribution of the phospholipid components phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanol (PE) and sphingomyelin (Sph) were independent of age (table 3). Compared to adult reference values, a lower percentage of PG was found in the BALF of children (table 3). In contrast to the changes seen in phospholipids, the total protein concentration was constant throughout the age range (fig. 2).

The agreement between the light-scattering MD- and UV-technique for the total phospholipid concentrations in BALF is displayed in figure 3. Total phospholipid concentrations determined by the UV technique were significantly higher ($p<0.001$, Wilcoxon test). This difference was independent of the absolute phospholipid concentrations (fig. 3).

Surfactant protein A concentrations were highly variable throughout the age range studied (fig. 4). The SP-A concentrations were similar to those observed in adults (table 4). However, the range of values was more spread, in particular towards the higher values. A weak but significant correlation was observed between SP-A and the total phospholipid concentration of BALF determined by the light-scattering MD-technique ($r=0.46$; $p<0.01$).

Table 2. – Comparison of total phospholipid (PL) content in different age groups

Age	Total PL µg·mL^{-1}	n
3–8 yrs	35.2±16.9	18
8–15 yrs	18.6±6.7	20
Adults*	8.1±3.1	9

Values are presented as mean±SD. *: own unpublished results.

Table 3. – Phospholipid components of total lipid content in healthy subjects

[Ref.]	Age	PC %	PG %	PI %	PE %	Sph %	PS %
Present study	<8 yrs	86.5±3.3	7.3±2.4	3.4±1.5	1.8±0.5	1.0±0.5	ND
Present study	>8 yrs	84.8±4.7	8.1±3.3	3.7±1.5	2.0±0.7	1.4±0.5	ND
Bruch*	Adults	67.3±17	16.2±9.8	7.3±6.4	3.6±1.2	5.6±2.1	ND
GREGORY [3]	Adults	76.3±2.1	11.6±1.2	3.9±0.8	3.3±0.5	1.5±0.1	0.2±0.1
HALLMAN [22]	Adults	73.0±2.3	12.4±0.6	2.7±0.2	2.6±0.3	3.7±0.5	1.0±1.0
HONDA [8]	Adults	76.7±1.9	10.8±1.6	2.6±0.2	3.5±1.4	2.0±0.4	1.1±0.4
LOW [23]	Adults	83.9±6.0	12.4±3.9	1.0±1.3	0.4±0.5	ND	6.0±0.7
PISON [5]	Adults	62.8±1.1	10.0±0.7	8.3±0.4	4.8±0.9	7.9±0.5	2.3±0.9
ROBINSON [7]	Adults	72.9±2.7	13.7±1.6	4.7±0.7	4.7±0.5	1.5±0.2	2.5±0.2

values are presented as mean±SD. *: unpublished reference values. PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanol; Sph: sphyngomyelin; ND: not done.

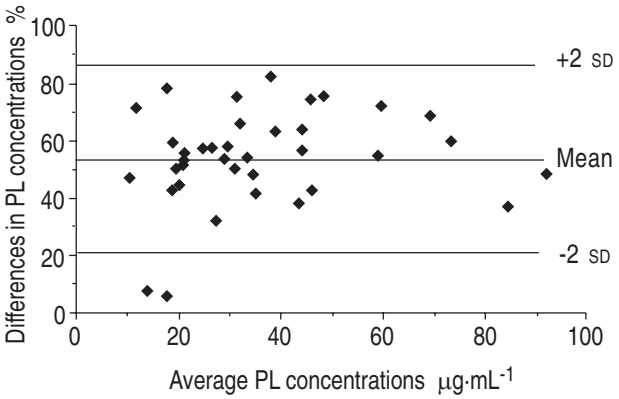

Fig. 3. – Comparison of the total phospholipid (PL) concentrations determined by the ultraviolet (UV) and light-scattering (MD) technique. The average concentrations for the two techniques are displayed on the abscissa; the differences (PL-UV – PL-MD) in percentage of the mean concentration are shown on the ordinate. Each triangle represents one individual. The solid lines represent the average mean±SD for the total population. PL concentrations were systematically higher with the UV-technique.

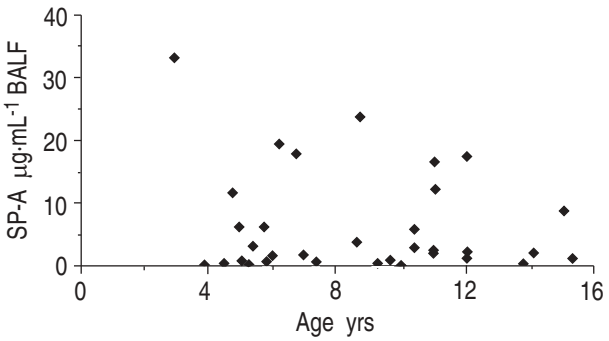

Fig. 4. – Surfactant protein A (SP-A) concentration of bronchoalveolar lavage fluid (BALF) as a function of age. Each triangle represents one individual.

Discussion

This study provides the first reference values for surfactant components obtained by BAL in children. We could demonstrate an age-dependency of surfactant components in our population of children without pulmonary disease. If subjects are clustered for age (table 2) children aged 3–8 yrs have approximately twice the amount of surfactant as compared to older children. Interestingly, the protein concentrations in BALF showed no age-dependency. Studies in rabbits and monkeys have demonstrated that BALF of new-born animals contains 5–10 times more surfactant material than BALF of adult animals [24, 25]. A recent study in rats suggested that the higher total phospholipid content in neonates decreases to adult levels during the first 50 days of life [26]. Our results indicate that a similar decrease in the total phospholipid content of BALF occurs in children between 3 and 8 yrs of age. No data for human new-borns are available at the present time, but it is likely that the lipid concentrations are even higher in the neonate. Further studies are warranted to elucidate the changes that are expected to occur in surfactant content between the neo-natal period and 3 yrs of age.

The age dependent shift in lung surfactant observed in our study is not a methodological side-effect due to variable recovery or washing out during the BAL procedure. It may potentially be influenced by the mode of anaesthesia, which was initiated with nitrous oxide and halothane in children younger than 6 yrs, whereas in older children anaesthesia was induced intravenously. There is no evidence to suggest that any of the medications used for the induction of anaesthesia has a rapid effect on the surfactant pool in the lung, although halothane is known to reduce surfactant production [27]. However, this effect becomes relevant only after several hours and would not have affected our measurements as BAL was performed immediately after induction of anaesthesia. Moreover, the same regimen (nitrous oxide plus halothane) was used for the maintenance of anaesthesia in both groups. If at all, the longer halothane exposure in younger children would have resulted in lower phospholipid concentrations in this age group; the converse of what we have observed. To account for any potential influence of the mode in which anaesthesia was induced, we have analysed total phospholipid concentrations by stepwise linear regression analysis using 6 yrs as a break-point. Using this methodology, a significant negative correlation between age and PL concentrations is still observed for children older than 6 yrs ($r = -0.5$; $p=0.01$). These considerations make it unlikely that the differences in phospholipid concentrations observed between younger and older children are caused by the different methods of induction of anaesthesia.

Table 4. – Surfactant protein A (SP-A) concentrations in BALF of children as compared to healthy nonsmoking adults

	SP-A µg·mL^{-1}	n
Children	6.9±9.2 (0.07–33.7)	35
Adults*	3.6±4.1 (0.16–16)	17

Values are presented as mean±SD, and range in parenthesis. *: Bruch *et al*., unpublished reference values. BALF: bronchoalveolar lavage.

The biological significance of the higher surfactant concentrations in younger children without pulmonary disease is unknown. Lung growth consists of a rapid increase in the number of alveoli in the first years of life [28]. Whilst most of this growth by replication has been completed by the age of 2 yrs, marked changes in alveolar size occur throughout childhood [29]. This is reflected by a decrease in the number of alveoli per millilitre lung tissue from 790 at 3 yrs of age to 390 at 14 yrs of age [29]. As surface tension depends upon alveolar diameter, more surfactant will be needed to maintain a constant surface tension if the individual unit is smaller. It is, therefore, likely that the decrease in surfactant concentration observed in this study is related to this increase in alveolar size during childhood, since less surface active material is necessary to reduce the surface tension in a bigger alveolus.

Regarding disease states, it is well-known that deficiency of surfactant in the neonate causes the respiratory distress syndrome (RDS), which is amenable to replacement therapy with exogenous surfactant [2]. With the

exception of the new-born infant with RDS, little information is available about the lipid components on the alveolar surface in children. Studies in infants with bronchopulmonary dysplasia (BPD) have shown that phosphatidylcholine (PC), the most prevalent component of surfactant, is reduced in BALF of these infants [16]. Tracheal aspirates have been used for the analysis of surfactant in ventilated neonates [30]. Whilst tracheal aspirates are unlikely to render reliable information about the surfactant content of the epithelial lining fluid, phosphatidylcholine was found to be lower in neonates who subsequently developed RDS. However, PC in these tracheal aspirates was not found to be a good predictor for the development of chronic lung disease [30]. The total phospholipid content of BALF is reduced in children dying of sudden infant death syndrome [14], but it is unclear whether this is a primary phenomenon or secondary to phospholipases produced by infectious organisms [15]. Chronic bacterial infection is also the cause of reduced surfactant concentrations in patients with cystic fibrosis, who are chronically colonized with *Pseudomonas aeruginosa* which is known to produce significant amounts of phospholipase [12, 13].

Abnormalities of surfactant function have been described in two children with recurrent cyanotic episodes but the overall frequency of these changes is unknown [17]. As surfactant plays an important role in the initial host defence process [31], it is likely that changes in its composition occur relatively early in pulmonary disorders. Studies of the surfactant composition, therefore, bear the potential to increase our understanding of the pathogenesis of paediatric lung diseases.

Surfactant protein A was found to be highly variable in this group of children without pulmonary disease. This is in concordance with studies in adults where a wide range of concentrations has been observed in healthy subjects, although significant difference have been observed between healthy individuals and patients with pulmonary disease [32–34]. We have observed a weak correlation between SP-A and the total phospholipid content for the group as a whole; however, the r-value of 0.46 would imply that only 20% of the variability is explained by the variation in phospholipid concentrations. Whether SP-A will be a useful parameter to differentiate between healthy subjects and children with pulmonary disease remains to be determined.

In summary, we have studied the phospholipid composition of bronchoalveolar lavage fluid in 38 children without lung disease aged 3–15 yrs. The total lipid content was higher in younger children and decreased with age. These data provide a first insight into the phospholipid composition of the epithelial lining fluid in children. Further studies will address whether specific profiles of surfactant components can be observed in paediatric lung diseases.

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