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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ADULT HUMAN BRONCHOALVEOLAR LAVAGE: ASSAY FOR PHOSPHOLIPID LUNG PROFILE

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SUMMARY

High-performance liquid chromatography has been used to separate pulmonary phospholipids from adult human bronchoalveolar lavage. A solvent system consisting of acetonitrile—water (80:20) as solvent A and pure acetonitrile as solvent B was used with a silica column (Bio-Sil HP 10) coupled to an Si-100 Polyol precolumn. A linear gradient from 87.5 to 25% of solvent B was found to separate all biologically relevant surfactant phospholipids in the following sequence and composition: phosphatidic acid (1.1%), phosphatidylglycerol (10.6%), phosphatidylinositol (9.9%), phosphatidylethanolamine (3.6%), phosphatidylserine (4.5%), phosphatidylcholine (60.8%), sphingomyelin (8.1%) and lysophosphatidylcholine (1.6%). These results were very similar to the phospholipid pattern obtained by two-dimensional thin-layer chromatography. It is concluded that high-performance liquid chromatography is a useful and rapid method for the separation of phospholipids in biological fluids containing pulmonary surfactant.

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INTRODUCTION

The phospholipid composition of biological fluids containing pulmonary surfactant drew attention in perinatal medicine when it became established that surface activity is due to defined phospholipid classes [1—3]. For the determination of foetal lung maturity, numerous biochemical and biophysical surfactant tests in amniotic fluid were developed, which have recently been reviewed by Tsao and Zachmann [4]. However, surfactant research has expanded beyond the borders of perinatal medicine. The phospholipid lining layer of the airways is affected in adult respiratory distress syndrome [5], chronic obstructive lung disease [6], sudden infant death syndrome [7] and alveolar proteinosis [8]. Inflammatory mediator reactions with cellular membranes contribute to phospholipid metabolism [9]. Substitution of natural [10, 11] or artificial [12, 13] surfactants may become a treatment of choice for several respiratory disorders. Bronchoalveolar lavage has been proposed for diagnostic and therapeutic purposes [14] and for obtaining human surface-active material for replacement therapy [15]. All these clinical situations require rapid and reproducible identification and quantitative analysis of all pulmonary phospholipids. To determine the phospholipid composition in biological sources, thin-layer chromatographic (TLC) methods are widely used [16—20]. Besides the major surfactant component, dipalmitoyl phosphatidylcholine (DPPC), interest has focused on two minor components, phosphatidylglycerol (PG) and phosphatidylinositol (PI) [21]. One-dimensional TLC, however, gives incomplete information on this phospholipid pattern, whereas two-dimensional TLC is very time consuming. For this purpose, alternative techniques such as high-performance liquid chromatography (HPLC) would be useful. Up to now, several HPLC methods for phospholipid separation have been described [22—32]. The purpose of our study was to establish an HPLC method for the separation of phospholipids in biological fluids containing pulmonary surfactant and to compare this method with an established TLC technique [18].

EXPERIMENTAL

Chemicals

The following phospholipids were purchased from Sigma (Munich, F.R.G.): phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), sphingomyelin (SPH), and the phosphatidylcholines (PC), dipalmitoyl phosphatidylcholine (DPPC), dilinoleoyl phosphatidylcholine (DLPC), diarachidoyl phosphatidylcholine (DAPC) and lysophosphatidylcholine (LPC). Standard solutions with these phospholipids were prepared at concentrations between 25 and 1000 μg/ml in chloroform—methanol (2:1). HPLC-grade solvents from Merck (Darmstadt, F.R.G.) were used throughout all experiments. Water was deionized and distilled twice in the presence of potassium permanganate.

Bronchoalveolar lavage

Normal bronchoalveolar lavage was obtained from adults of both sexes

undergoing surgical intervention. Patients were free of pulmonary disease. Written informed consent was obtained in each case before the procedure. Anaesthesia was carried out intravenously. A bronchofiberscope (Pentax, FB-15A) was directed through the endotracheal tube into a segmental bronchus. Five subsequent 20-ml volumes of 0.15 *M* sodium chloride (Fresenius, Bad Homburg, F.R.G.) were instilled and withdrawn under negative pressure. Recovery was 40—70% by volume. The lavage fluid was centrifuged (180 *g*, 10 min, 4°C) to sediment the cellular material. The supernatant fluids from ten patients were pooled after aliquots of 10 ml were separated for individual analysis. Samples were stored at —70°C.

Lipid extraction

Lipids were extracted by the method of Folch et al. [33]. A 30-ml volume of chloroform—methanol (2:1) was added to 10 ml of lavage fluid, the mixture was stirred for 5 min and centrifuged (1100 *g*, 5 min, 4°C) to separate the methanol and chloroform layers. The chloroform layer was removed, dried under nitrogen and redissolved in 500 μl of chloroform—methanol (2:1) for HPLC analysis. Samples for TLC were redissolved in 30 μl of chloroform.

HPLC analysis

HPLC of phospholipids was carried out using the modified method of Nissen and Kreysel [30]. The HPLC equipment was from Kontron Analytic (Munich, F.R.G.) and consisted of two pumps (Model 410), a gradient former (Model 200), a spectrophotometer (Uvikon 720 LC) and a computing chromatography integrator (Hewlett-Packard, H-P 3390 A). The chromatographic analysis was performed at 30°C and at a flow-rate of 2 ml/min on a column (250 × 4 mm I.D.) prepacked with Bio-Sil HP 10, 10 μm (Bio-Rad, Munich, F.R.G.). A guard column (75 × 4.6 mm I.D.) was prepacked with Si-100 Polyol, 30 μm (Serva, Heidelberg, F.R.G.). The mobile phase consisted of acetonitrile—water (80:20), pH 6.0 (solvent A), and pure acetonitrile (solvent B). The solvents were degassed prior to use. A linear gradient from 87.5 to 25% of solvent B was formed between 5 and 15 min. The injected sample volume was 20 μl of lipid extract or phospholipid standard solution. The detector wavelength was set at 203 nm. Total procedure time was 50 min.

Two-dimensional TLC

Two-dimensional TLC was performed on 20 × 20 cm Pyrex plates which were coated with silica gel H (Merck) containing 5% ammonium sulphate, and on plain plates. The separation of phospholipids was carried out by the modified method of Gray [18] using chloroform—methanol—acetic acid—water (65:25:8:4) for the first dimension and tetrahydrofuran—formaldehyde—methanol—2 *M* ammonia (10:7:2:1) for the second dimension. Degasa tanks (230 × 220 × 115 mm; Heidelberg, F.R.G.) with silicone-sealed covers were used without filter-paper lining. The plate was developed with TLC solvents until the front had migrated 10 cm in both dimensions. Between runs, the plate was dried for 10 min at 70°C. Spots were visualized by charring on a hot plate at 280°C or by exposure to iodine vapor. On all plates, standards of PC and PG (100 μg each) were run in one dimension each. Phospholipid spots were

measured by reflectance densitometry using a Desaga Quick Scan densitometer connected to a Spectra Physics 4100 computing chromatography integrator. The latter was programmed in order to correct for zero calibration, baseline drift and incomplete separation of peaks [20]. Total procedure time was 3 h.

Phosphorus analysis

Phosphorus analysis was carried out according to Bartlett [34], and Kankare and Suovaniemi [35]. Chromatography was performed as described above. Phospholipid spots were scraped and charred with 0.5 ml of 5 *M* sulphuric acid at 180°C for 2 h. Two drops of 30% hydrogen peroxide were added and the sample was incubated at 180°C for another 2 h. After a 7-min incubation at 100°C with 3 ml of a reagent containing sodium hydrogen sulphite, sodium sulphate, amidole and ammonium molybdate, a photometric reaction developed. Absorption was measured at a wavelength of 820 nm.

RESULTS

The separation of phospholipids by HPLC with UV detection in the range 200—206 nm is handicapped by absorption of most of the solvent compounds regularly used in TLC. We therefore tested solvent systems containing hexane, isopropanol and water in several different compositions on silica columns for phospholipid separation, with regard to sensitivity and resolution. Phospholipid concentrations above 300 μg/20 μl were required for detection. It was

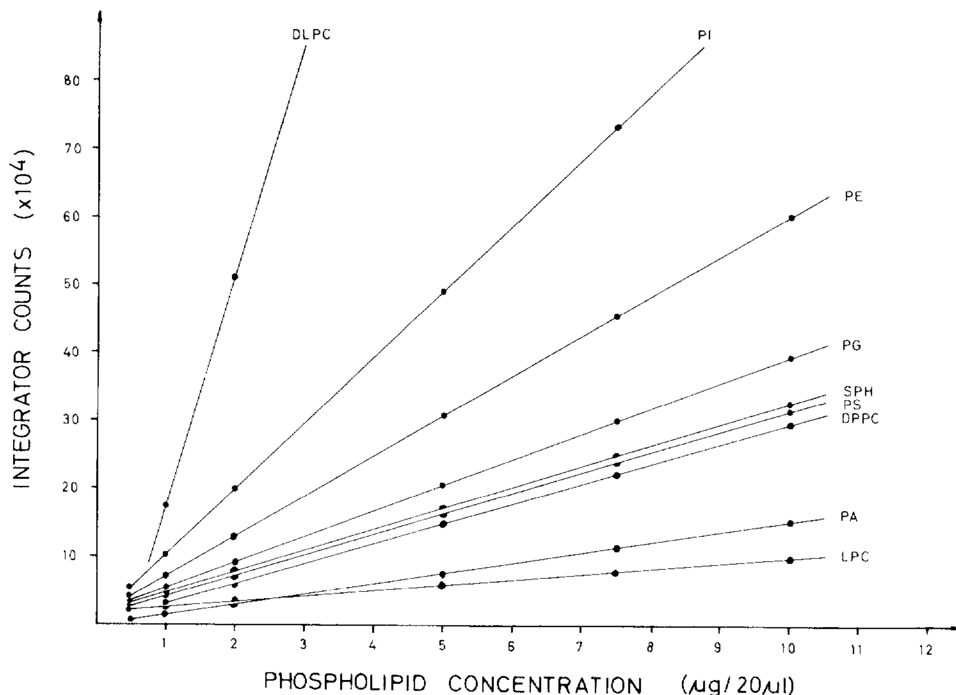

Fig. 1. Standard calibration graphs for different phospholipids obtained with the HPLC technique. Each point represents the mean value of three measurements. For DPPC and DLPC, linearity was observed up to at least 200 μg.

impossible to separate PC simultaneously from LPC and SPH, or PG from PI. In contrast, solvent systems containing acetonitrile as the main component allowed detection of phospholipid concentrations as low as 0.5 μg/20 μl. With the modified method of Nissen and Kreysel [30], all pulmonary phospholipids were separated in the following sequence: PA, PG, PI, PE, PS, PC, SPH, LPC. In distinct concentrations, PC (between 5 and 35 μg per 20 μl) and SPH (3.5 μg per 20 μl and above) appeared as split peaks in the chromatogram. The two species of PC co-chromatographed with synthetic DPPC and DLPC. DAPC co-migrated with PE in our system. However, concentrations of up to 250 μg/20 μl were needed to detect DAPC. Detection of PA was difficult owing to superposition of unidentified material in the solvent front.

Calibration curves for different phospholipids obtained from HPLC (Fig. 1) were linear in the range from 0.5 to 20 μg/20 μl. For DPPC and DLPC, linearity was found up to 200 μg/20 μl. The linear regression coefficient for each of the curves was 0.98 or higher.

To compare HPLC with TLC separation of phospholipids, pooled lavage material from adults without any respiratory disease was used. Typical chromatograms are shown in Figs. 2 and 3 for both methods.

With two-dimensional TLC, the following phospholipids ("lung profile")

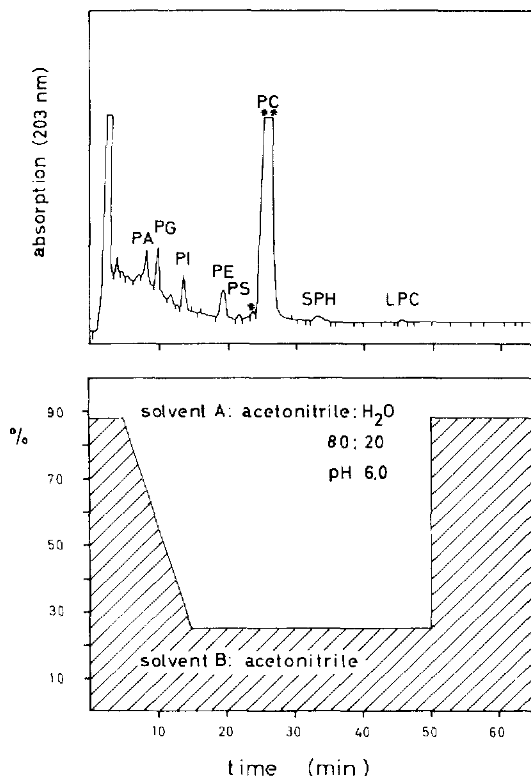

Fig. 2. A typical HPLC profile for adult human lung lavage is depicted at the top of the figure. At the bottom, the gradient for solvents A and B is shown. * = DLPC; ** = DPPC.

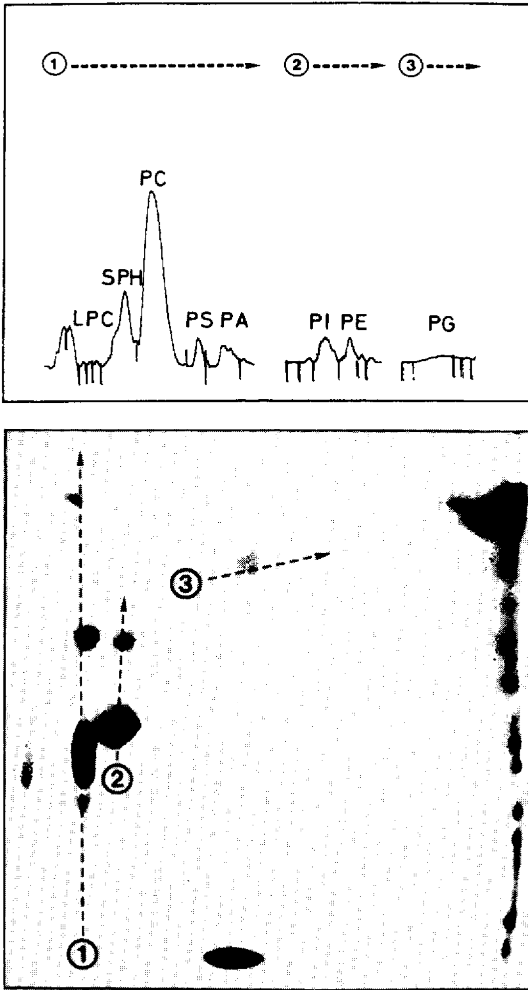

Fig. 3. A TLC plate after two-dimensional separation of lung lavage material is shown at the bottom; the computer printout of reflectance densitometry is shown at the top of the figure. Three subsequent scans (1—3) were performed to quantitate the phospholipids.

could be identified in lung lavage: PS, PA, PG, PI, PE, PC, SPH and LPC. Distribution of the compounds was analysed either by reflectance densitometry in comparison to standard phospholipid curves or by phosphorus determination. The latter method was performed by charring with 5% ammonium sulphate and by the methods of Bartlett [34], and Kankare and Suovaniemi [35]. PC was by far the major lavage phospholipid, followed by SPH and PG. Different phospholipid distributions were found using densitometry and phosphorus analysis.

With the HPLC technique, all pulmonary phospholipids were separated. PC was composed of DPPC and DLPC, with more than 90% of DPPC by counts. Comparison of different phospholipids by counts was limited by the different slopes of the calibration graphs. In this respect, values for PI were calculated to be higher by counts than by comparison with the standard calibration graph

TABLE I

COMPARISON BETWEEN THE TLC AND HPLC SEPARATION TECHNIQUES FOR PHOSPHOLIPIDS (PL) IN ADULT HUMAN LUNG LAVAGE

Mean values of three independent measurements are shown, respectively. Phosphorus (P) was analysed from silica gel H charred with 5% ammonium sulphate or from plain plates visualized with iodine and digested with sulphuric acid. Calibration of densitometry with standard phospholipids yielded a linear correlation to phosphorus content in the range of 10—100 μg. The μg values in the HPLC experiments were calculated from the calibration curves as shown in Fig. 1. PC values were calculated by the addition of DPPC and DLPC values with more than 90% of DPPC by counts. Percentages of phospholipids were estimated by counts ($\%PL_1$) or by μg values ($\%PL_2$). Last three rows: phospholipid ratios commonly used for clinical evaluation.

Phospholipid	Silica gel H, charred with 5% ammonium sulphate				Silica gel H, visualized with iodine		HPLC-separated phospholipids in adult human lung lavage			
	Densitometry		Phosphorus determination		Phosphorus determination		UV absorption		Comparison with PL standards	
	Counts	%PL	nmol P	%P	nmol P	%P	Counts	$\%PL_1$	μg PL	$\%PL_2$
PA	114173	3.94	4.10	2.03	7.15	2.32	7279	0.39	0.50	1.05
PG	126350	4.36	5.75	2.85	18.75	6.09	208487	11.17	5.05	10.56
PI	192390	6.64	4.45	2.20	11.90	3.87	464087	24.87	4.75	9.94
PS	98306	3.39	5.10	2.53	11.90	3.87	77038	4.13	2.15	4.50
PE	131326	4.53	4.45	2.20	9.40	3.05	101220	5.42	1.70	3.56
PC	1686680	58.19	149.50	74.03	204.00	66.31	847710	45.42	30.05	60.77
SPH	534051	18.43	24.50	12.13	36.40	11.83	136370	7.31	3.85	8.05
LPC	15129	0.52	4.08	2.02	8.15	2.65	24064	1.29	0.75	1.57
PC/SPH ratio	3.16		6.10		5.60		6.22		7.80	
PI/PS ratio	1.96		0.87		1.00		6.02		2.21	
$\frac{PI+PG}{SPH}$	0.60		0.42		0.84		4.93		2.54	

for PI. In contrast, PG was determined to be lower by counts than by the calibration graph. PC was the main component of phospholipids in human lung lavage, with DPPC as the major species followed by PG, PI and SPH.

Compared with the TLC results, good agreement was seen for the phosphorus data, whereas values obtained from reflectance densitometry were sometimes only one-half those found by HPLC experiments. The results obtained with both methods are depicted in Table I.

To investigate interindividual variability of the phospholipid pattern, lipid extracts from ten patients were separated by the HPLC technique. One sample was lost during preparation. The results for the remaining samples are shown in Table II. Calculation was carried out by comparison with standard calibration graphs for the different phospholipids. Column performance was checked by running DPPC and SPH standards between every two samples.

DISCUSSION

The purpose of this study was to establish a fast HPLC method for the separation of phospholipids in biological fluids containing pulmonary surfactant. A comparison with an established TLC method was carried out.

Mobile phase composition turned out to be the limiting factor for sensitivity using UV detection in HPLC. In this respect, an acetonitrile—water solvent

TABLE II

PERCENTAGES OF PHOSPHOLIPID COMPOSITION IN ADULT HUMAN LUNG LAVAGE

Separation of phospholipid classes was carried out by the HPLC technique. Individual results of nine patients are shown. Values were calculated by comparison with standard calibration curves for the different phospholipids. Mean values and standard deviations (± S.D.) for the different phospholipids are shown in the last column. Last three rows: phospholipid ratios commonly used for clinical evaluation.

Phospholipid	Composition (%)									
	1	2	3	4	5	6	7	8	9	Mean ± S.D.
PA	1.05	1.43	N.D.*	N.D.	1.39	1.49	N.D.	1.19	1.15	0.85 ± 0.66
PG	11.55	8.27	7.50	9.94	13.39	9.97	11.35	8.31	9.92	10.02 ± 1.86
PI	6.42	7.06	9.74	7.34	9.14	9.05	9.82	7.61	8.72	8.32 ± 1.24
PS	3.85	4.68	7.95	6.12	4.62	5.17	N.D.	5.16	3.44	4.55 ± 2.16
PE	2.89	3.03	6.75	11.47	3.02	5.17	3.99	4.11	2.94	4.82 ± 2.81
PC	63.33	68.91	63.57	59.33	59.20	61.30	67.48	61.21	60.61	62.77 ± 3.44
SPH	9.31	6.61	4.50	5.81	7.73	7.85	7.36	8.04	9.09	7.37 ± 1.53
LPC	1.58	N.D.	N.D.	N.D.	1.51	N.D.	N.D.	4.37	4.13	1.29 ± 1.80
PC/SPH ratio	6.80	10.42	14.13	10.21	7.66	7.81	9.17	7.61	6.67	8.94 ± 2.38
PI/PS ratio	1.67	1.50	1.23	1.20	1.98	1.75		1.47	2.53	1.48 ± 0.69
$\frac{PI+PG}{SPH}$	1.93	2.32	3.83	2.97	2.91	2.42	2.87	1.98	2.05	2.59 ± 0.62

*N.D. = Not detectable.

system generated the best transparency. Solvent systems such as those described by Hax and Geurts van Kessel [26], Nasner and Kraus [29], Hanson et al. [25] or Blank and Snyder [22], contain hexane, isopropanol and water, and in one case [25] also methanol, in various compositions. They yield less sensitivity and resolution of surface-active phospholipids. Our results are in accordance with those of Nissen and Kreysel [30], who studied phospholipids in human spermatozoa and from whom the greater part of the method was adopted.

All phospholipid classes known as "lung profile" [36] from TLC could be separated with this HPLC method. In addition, three different PC species were separated. DPPC proved to be the phospholipid with the highest concentration. This is well known from other methods [16, 37] and for other species [3, 38]. DLPC was of less importance. DAPC co-migrated with PE but was negligible, as was suggested for dog [39], rat [40], guinea pig [41] and bovine lung lavage [42]. Although DPPC could be separated from DLPC with a good resolution, the fatty acid content in PC species has to be determined with methods such as gas chromatography or rechromatography on HPLC. This will be a field of further investigation.

A main problem known from TLC is the absolute quantitation of phospholipids in biological fluids. So, it could be suggested from the different slopes of the phospholipid calibration graphs that calculation has to be based on internal

standardization or on the apparent molecular extinction coefficients [27]. Quantitation by independent methods, such as phosphorus analysis, is also possible after effluents from specific peaks are collected [32], but the time spent on this method is comparatively high. Jungalwala et al. [27] have shown that phospholipids containing unsaturated fatty acids give much greater UV absorption at 203 nm than phospholipids with saturated fatty acids. For the complex absorption behaviour of phospholipids, it has to be pointed out that quantitation is accurate for standardized compounds only. In biological fluids with a great variation of fatty acid composition, direct quantification of lipid classes might therefore be problematic, as suggested by Geurts van Kessel et al. [28].

It must be emphasized that data obtained by TLC and densitometry do not reflect the molecular distribution of phospholipids, as shown by the low PC content, the high SPH content and the different ratios obtained with densitometry and phosphorus determination. Differences in the phospholipid profile obtained with phosphorus determination from TLC and HPLC are within experimental error of both methods. In this respect, the phosphorus content of different phospholipid classes is not always the same. For example, the proportion of LPC is calculated too high whereas PI values are determined too low by phospholipid phosphorus.

Differences seen with both HPLC and TLC separation techniques suggested that each method has to be standardized for each laboratory employing these techniques for diagnostic purposes. Two-dimensional TLC of phospholipids, especially the simultaneous quantification of PC and PG, can evaluate the risk for neonatal respiratory distress syndrome with high specificity [43, 44]. This assay, however, cannot be processed automatically, is time consuming and is subject to the usual limitations of TLC: baseline drift due to inconstant background staining, incomplete spot separation and the relatively large sample size required for analysis. The latter is relevant if reliable detection of the minor compounds PG and PI is required, as in neonatal respiratory distress syndrome: minor phospholipids may become undetectable in small samples. Some of the difficulties known from two-dimensional TLC of phospholipids can be overcome by HPLC. Both sample size and total procedure time are markedly reduced.

In conclusion, the described HPLC method is useful for phospholipid separation of biological fluids containing pulmonary surfactant. This method permits reproducible identification and quantitative analysis of all relevant surface-active phospholipid classes in biological sources. Such a rapid quantitative method will be useful to determine foetal lung maturity, to study adult human lung disease (e.g. adult respiratory distress syndrome, alveolar proteinosis, chronic obstructive lung disease), to check exogenous material for surfactant substitution and to investigate inflammatory mediator reactions with cellular membranes.

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