Eur Respir J 1999; 14: 565–573
Printed in UK – all rights reserved

European Respiratory Journal
ISSN 0903-1936

Surfactant abnormalities in idiopathic pulmonary fibrosis, hypersensitivity pneumonitis and sarcoidosis

A. Günther, R. Schmidt, F. Nix, M. Yabut-Perez, C. Guth, S. Rosseau, C. Siebert, F. Grimminger, H. Morr, H.G. Velcovsky, W. Seeger

Surfactant abnormalities in idiopathic pulmonary fibrosis, hypersensitivity pneumonitis and sarcoidosis. A. Günther, R. Schmidt, F. Nix, M. Yabut-Perez, C. Guth, S. Rosseau, C. Siebert, F. Grimminger, H. Morr, H.G. Velcovsky, W. Seeger. ©ERS Journals Ltd 1999.

ABSTRACT: Bronchoalveolar lavage fluids (BALF) from patients with idiopathic pulmonary fibrosis (IPF; n=36), hypersensitivity pneumonitis (HP; n=32) and sarcoidosis (n=44) were investigated for their surfactant properties and compared to healthy control subjects (n=29).

The phospholipid (PL) and protein concentration, the PL:protein ratio, PL subclasses, and the surfactant apoproteins (SP)A and SP-B were quantified in BALF. Large surfactant aggregates (LSA) were measured by means of ultracentrifugation and assayed for surface activity using the pulsating bubble surfactometer.

As compared to controls, SP-A concentrations, LSA content and PL:protein ratios were significantly decreased in all groups, whereas PL and SP-B concentrations remained unchanged. Changes in the phospholipid profile, with reduced percentages of phosphatidylcholine (not significant) and phosphatidylglycerol and increased fractions of phosphatidylinositol and sphingomyelin ($p<0.05$), occurred more in IPF than in HP, and not in sarcoidosis. Surface activity was found to be severely impaired in IPF (minimum surface tension (γ_{min}) ~15–20 $mN \cdot m^{-1}$), but only modestly affected in HP and sarcoidosis (γ_{min} ~5 $mN \cdot m^{-1}$) compared to controls (γ_{min} ~0 $mN \cdot m^{-1}$). Reconstitution of pelleted surfactant material with soluble BALF proteins further increased γ_{min} values.

In conclusion, moderate changes in biochemical and physical surfactant properties are encountered in hypersensitivity pneumonitis and sarcoidosis, but pronounced disturbances occur in idiopathic pulmonary fibrosis.

Eur Respir J 1999; 14: 565–573.

Dept of Internal Medicine, Justus-Liebig-University, 35385 Giessen; Klinik für Lungen- und Bronchialheilkunde, 35753 Greifenstein; FRG.

Correspondence: A. Günther
Medizinische Klinik II
Klinikstrasse 36
35392 Giessen
Germany
Fax: 49 6419942359

Keywords: Bronchoalveolar lavage fluid
hypersensitivity pneumonitis
idiopathic pulmonary fibrosis
pulmonary surfactant
sarcoidosis

Received: November 14 1997
Accepted after revision April 17 1999

This study was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 547 "Kardiopulmonales Gefäbsystem").

The pulmonary surfactant system, which covers the alveolar lining layer, is mainly composed of lipids and specific apoproteins [1, 2]. A complex interaction of these constituents results in the reduction of surface tension at the air/liquid interface to near zero values at minimum alveolar radius, thereby preventing alveolar collapse and atelectasis. Biochemical and biophysical abnormalities of this delicate material are encountered in acute respiratory distress syndrome (ARDS) [3–6] and are thought to be involved in the pathogenetic sequelae of this syndrome [7, 8]. In summary, the following mechanisms may underlie surfactant disturbances in acute lung injury: 1) lack of surface active material due to impaired synthesis or increased "consumption", 2) changes in the (phosplio)lipid or apoprotein composition, 3) maldistribution of surfactant subtypes with a predominance of smaller, biophysically less active, surfactant aggregates, 4) surfactant inhibition by plasma-derived proteins, possibly with particular impact of fibrin, and 5) decomposition of surfactant components by proteolytic, phospholipolytic or oxidative attack.

If survived, the acute exudative phase of ARDS is regularly followed by the development of fibroproliferative changes in the lung (honeycombing), with a typical restrictive pattern in lung function. Chronic interstitial lung diseases such as idiopathic pulmonary fibrosis (IPF) share at least some inflammatory features with ARDS, the most prominent being neutrophil influx into the alveolar compartment [9, 10]. In view of such similarities in inflammatory mechanisms, it has been suggested that surfactant abnormalities may also play a major role in IPF, and this suggestion is supported by the finding of marked surfactant disturbances in an experimental model of pulmonary fibrosis using the antineoplastic agent bleomycin [11–13]. Previous investigations in IPF patients reported a reduction in total pelletable phospholipids and the surfactant apoprotein surfactant protein (SP)A as well as changes in the surfactant phospholipid composition, but controversy exists as to whether these changes are specific to IPF or may be present to a similar extent in other chronic interstitial lung diseases [14–21]. Moreover, measurement of biophysical surfactant function and determination of the concentrations of the functionally important hydrophobic apoproteins have, as yet, not been performed, and the distribution of the lavageable surfactant material between functionally active large (LSA) and more inactive small

surfactant aggregates (SSA) has not been taken into account. In the present study, biochemical and biophysical surfactant characteristics in IPF patients, who were untreated at the time of the initial lavage, were compared to those in control subjects and in two other types of chronic interstitial lung disease, namely hypersensitivity pneumonitis (HP) and sarcoidosis.

Materials and methods

Materials

The monoclonal antibody directed against SP-A (PE10) was a generous gift from T. Akino (Dept of Biochemistry, Sapporo Medical College, Sapporo, Japan) and the monoclonal antibody against SP-B (8E5B) was kindly provided by Y. Suzuki (Chest Disease Research Institute, Kyoto, Japan). Both antibodies have been successfully used for the determination of these two apoproteins in bronchoalveolar lavage (BAL) fluid BALF [6, 22]. Recombinant human SP-A was placed at our disposal by K.P. Schäfer (Byk Gulden, Constance, Germany). Human SP-B was isolated from BAL by means of extraction using an LH60 column, according to the method of WARR *et al.* [23], and was shown to possess a purity of >95%. Bovine serum albumin was purchased from Paesel and Lorei (Frankfort on the Main, Germany). Microtitre plates (Maxisorp® and Polysorp® with certification) were obtained from Nunc (Wiesbaden, Germany). The protein determination kit (bicinchoninic acid) was obtained from Pierce (*via* Bender & Hohbein, Munich, Germany). Biotinylated sheep antimouse antibody was obtained from Amersham Buchler (Brunswick, Germany). Avidin/biotin/horseradish peroxidase (AB complex) was from Dakopatts (Hamburg, Germany). 2,2′-Azino-di-[3-ethyl-benzthiazolin-sulphonate-(6)] (ABTS) was obtained from Boehringer (Mannheim, Germany). Tween 20 was purchased from Sigma (Munich, Germany) and Triton X-100 was from Serva (Heidelberg, Germany). Silica 60 plates and all other chemicals were from Merck (Darmstadt, Germany).

Methods

Study population. Spontaneously breathing patients (112, age 15–75 yrs) with interstitial lung diseases, who were still untreated for interstitial lung disease at the time of the initial lavage, were included in the study between January 1994 and December 1997. Information as to the duration of disease was obtained from an admission questionnaire and from a review of the patient's medical record. The patients were compared to 29 healthy never smoking subjects (16 male, 13 female, mean age 26 yrs) without a history of cardiac or pulmonary disease, and with normal chest radiographs and pulmonary function test results. These controls were included during the same recruitment period. The study protocol was approved by the local ethics committee and informed consent was obtained from each subject prior to entry into the study. Classification into IPF, HP and sarcoidosis was performed according to the following criteria.

Idiopathic pulmonary fibrosis (n=36). Major criteria (required): 1) unknown aetiology; 2) restrictive pattern in pulmonary function; 3) bilateral fine or coarse reticular or reticulonodular pattern on chest radiography or bilateral reticular pattern and patchy opacification of airspaces on computed tomography (CT)/high resolution CT (HRCT); and 4) increased neutrophil counts on BAL, which may be accompanied by an elevated percentage of lymphocytes or eosinophils. Minor criterion: histological findings in transbronchial lung biopsy or in transthoracic biopsy suggestive of usual interstitial pneumonia or desquamative interstitial pneumonia pattern.

Hypersensitivity pneumonitis (n=32). Major criteria: 1) history of periodically recurring or permanent complaints upon exposure to an inhalative antigen; 2) detection of precipitating antibodies; 3) patchy interstitial infiltrates in both lungs by chest radiography or CT/HRCT; and 4) elevated BAL lymphocyte counts with decreased CD4/CD8 ratio.

Sarcoidosis (n=44). Major criteria: 1) abnormal chest radiography results: bilateral hilar adenopathy without (I, n=10) or with (II, n=19) pulmonary infiltrates such as coarse reticulonodules or fluffy cotton wool confluent shadows, or infiltrates without hilar adenopathy (III, n= 15); 2) elevated BAL lymphocyte counts with increased CD4/CD8 ratio; and 3) Loefgren's syndrome (n=3). Minor criterion: epithelioid cell, noncaseating granuloma on lung biopsy.

There was no overlap between the groups of patients. Routine lung function testing was performed in all patients. All BALs were undertaken for diagnostic purposes on admission of the patients to the authors' pulmonary units. At the time of study, patients with HP and IPF reported that they had had complaints for ~15–40 months, together with progressive dyspnoea (table 1). Accordingly, lung function both at rest and during exercise was impaired. Patients with sarcoidosis reported a slightly shorter duration of complaints, had less dyspnoea and displayed more moderate alterations in lung function (table 1).

Table 1. – Demographic and physiological data on initial lavage

	Control	HP	IPF	Sarcoidosis
Patients n	29	32	36	44
Age yrs	26.0±3.7	51.4±2.8	57±2.5	44.8±2.1
Sex F/M	13/16	22/10	16/20	20/24
Smoking history n				
Neversmoker	29	24	17	25
Exsmoker	0	7	15	13
Current smoker	0	1	4	6
Dyspnoea*	0	1.3±0.2	1.7±0.2	1.0±0.2
Duration months	-	26.7±7.3	35.8±11.7	19.5±7.2
P_{a,O_2} % pred	104±6	87±5	85±4	90±2
FEV_1 % pred	104±6	70±3	68±4	79±3
FVC % pred	103±2	67±3	65±3	81±3
TLC % pred	105±5	85±4	78±4	90±3
Haemoglobin $g \cdot L^{-1}$	141±3	141±3	141±3	151±3

Data are presented as mean±SEM. *: scored by each patient on a 0 (no dyspnoea) to 4 (severe dyspnoea at rest scale). Lung function parameters and blood gas analysis percentage predicted values were based on the body mass index of the patients [24]. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; F: female; M: male; P_{a,O_2}: arterial oxygen tension; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity.

Bronchoalveolar lavage. BAL was performed in spontaneously breathing subjects during routine flexible bronchoscopy. The distal end of the bronchoscope was wedged into a segmental bronchus of the right middle lobe or the lingula. Ten 20-mL portions of saline were instilled, aspirated, cooled to 4°C and pooled. The overall fluid recovery ranged 55–70%. The lavage fluid was immediately centrifuged at $300 \times g$ (4°C, 10 min) to sediment cellular material, which was stained and counted according to standard techniques. Overall 40,000 cells were subjected to a cytospin and stained according to the Pappenheim method (2 min in May-Grünwald solution, followed by 10 min in Giemsa solution and final rinsing with water), and 100 cells were always counted. The supernatant was split into aliquots and subsequently frozen and stored at -80°C until further use.

Determination of phospholipid and protein content. Phospholipids (PLs) were extracted from a 2 mL BAL aliquot according to the method of BLIGH and DYER [25]. Upon evaporation of the organic phase under nitrogen, organic phosphorus was determined using a colorimetric phosphorus assay based on the method of ROUSER *et al.* [26]. All determinations were performed in duplicate and the results are expressed as PL concentration in the original BAL fluid. In addition, the PL concentration was corrected by means of the urea quotient (BAL/blood), according to RENNARD *et al.* [27]. Protein content was quantified using a commercially available kit based on the method of O.H. Lowry [28], and the PL:protein ratio calculated on the basis of PL and protein concentrations in the original BAL fluid.

Analysis of phospholipid classes. Separation of phospholipid classes was achieved by means of high-performance thin layer chromatography using silica 60 plates. In brief, 30 μg of extracted PLs and various amounts (0.42–62.5 μg) of a standard mixture containing phosphatidylcholine (PC). phosphatidylglycerol (PG), phosphatidylinositol (PI), sphingomyelin (SPH), phosphatidylethanolamine (PE), lyso-PC, cardiolipin (CL) and phosphatidylserine (PS) were applied with a Linomat IV applicator (Camag, Muttenz, Switzerland). Chloroform:methanol:acetic acid: distilled water (50:37.5:3.5:2) was used as mobile phase, and the phospholipids were stained with molybdenum blue reagent according to GUSTAVSSON [29], and quantified by means of densitometric scanning at 700 nm with a TLC Scanner II (Camag, Muttenz, Switzerland). Under these conditions, the densitometric quantification displayed high coincidence with colorimetric determination of phospholipid concentration and variance ranged 0.06–0.79%, depending on the PL analysed.

Quantification of surfactant protein B and surfactant protein A. The SP-B content of the BAL was determined using a solid phase adsorption-based enzyme-linked immunosorbent assay (ELISA), as recently described [22]. In brief, SP-B in samples or standard was bound to Polysorp microtitre plates and, by sequential washing steps with trifluoroethanol and diisopropylether/butanol, the influence of PL on the recovery of SP-B was eliminated. After saturation of free binding sites, SP-B was detected by means of a monoclonal antibody (8B5E) and the signal was amplified by use of a monoclonal biotinylated antimouse antibody and the AB complex technique with colouring of the plates by use of ABTS. Spiking of BAL fluid from seven patients with the SP-B standard yielded an excellent linear regression with a mean regression value of 0.998 and a slope of 1.085. SP-A was quantified employing a competitive ELISA protocol [6] based on monoclonal anti-SP-A (PE10). In brief, Maxisorp® microtitre plates were coated with recombinant human SP-A. Samples or standards were preincubated with PE10 in the presence of 2% Triton X-100 and were then applied to the precoated ELISA plates. Binding of free anti SP-A to the precoated SP-A was detected by application of a monoclonal biotinylated antimouse antibody and the AB complex as indicated above. Under these conditions, spiking of 23 different BAL aliquots resulted in a mean regression value of 0.963±0.010 with a slope of 1.052±0.056 (mean±SEM).

Concentration of lavage surfactant for biophysical measurements. BAL aliquots were centrifuged at $48{,}000 \times g$ (4°C, 1 h) to generate a crude surfactant pellet [5] and to separate the large surfactant aggregates (LSA) from the small surfactant aggregates (SSA) remaining in the supernatant. The PL concentration of the crude surfactant pellet was then determined, related to total BAL PL, and the percentage of material pelleted was assumed to represent LSA. By further addition of appropriate volumes of saline containing 3 mM Ca^{++}, the pellets were then adjusted to a final PL concentration of 2 $mg \cdot mL^{-1}$ (~2.7 mM), vigorously agitated on a vortex mixer for 1 min and taken for characterization of biophysical activity (see below).

Of the total BAL protein 2.7±0.43% was recovered in the $48{,}000 \times g$ pellet (mean±SEM, n=10); thus, the majority of proteins were removed from the LSA by this procedure. To address the influence of these soluble BAL proteins on surfactant function, the $48{,}000 \times g$ supernatant was applied to Macrosep centrifugal concentrators (cut-off 10 kDa, Filtron, Karlstein, Germany) and centrifuged at $2{,}500 \times g$ for 3 h at 4°C. This yielded a 16-fold (15.9± 2.1) concentration of proteins, and loss of proteins through the membrane amounted to 13.9±2.9%. Upon lyophilization and resuspension in 0.5 mL distilled water, the remaining PLs were separated by means of methanol/chloroform extraction according to BLIGH and DYER [25] (see above). Appropriate amounts of the upper protein-containing phase (methanol, aqueous) were lyophilized again and resuspended with the crude surfactant pellet in order to obtain corresponding PL:protein ratios as found in the original BAL fluid. For better resuspension of the lyophilized protein, the mixture was sonicated for 1 min (50 W, 25 kHz; Bandelin, Berlin, Germany).

Characterization of surface activity. The surface activity of the surfactant either alone or reconstituted with protein was assessed using a pulsating bubble surfactometer, as previously described [6, 30–32]. In brief, surfactant or surfactant protein mixtures were incubated for 30 min at 37°C after brief sonication. After filling the sample chamber with 30 μL of the sample, it was immediately transferred to the pulsating bubble surfactometer. Adsorption measurements were performed by machine-driven generation of the bubble at minimum bubble size and maintenance of it at this size for 12 s. The γ_{ads} value given in the present report refers to the surface tension

Table 2. – Phospholipid and protein content and differential cell count of bronchoalveolar lavage (BAL) fluid

	Control	HP	IPF	Sarcoidosis
Patients n	29	32	36	44
Phospholipid $\mu g \cdot mL^{-1}$	28.6±2.4	32.8±3.5	28.0±3.1	32.4±2.2
Phospholipid^{+} $mg \cdot mL^{-1}$	3.76±0.71	1.54±0.45**	1.65±0.38**	4.90±1.80
Protein $\mu g \cdot mL^{-1}$	76.8±9.4	262±30	248±27***	214±21***
Fluid recovery %	68.3±5.5	69.2±3.2	66.6±3.5	69.6±5.3
Total cell count $\times 10^6$	20.08±2.9	20.67±2.7	38.74±14.6	14.70±1.98
AM %	94.4±0.7	49.9±3.9	56.7±4.1	69.1±2.8
Lymphocytes %	3.5±0.7	32.1±4.2	14.7±2.7	24.8±2.5
Neutrophils %	2.7±0.5	17.5±4.0	26.5±4.3	5.4±1.1
Eosinophils %	0.4±0.2	2.5±0.6	4.5±1.2	0.8±0.2
CD4/CD8	ND	0.9±0.2	1.7±0.7	4.6±1.2

Data are presented as mean±SEM. $^{+}$: corrected for urea BAL/blood quotient. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; AM: alveolar macrophages; ND: not determined. **: p<0.01, ***: p<0.001 *versus* control.

obtained after 12 s of adsorption (= equilibrium surface tension value). Next, pulsation was started at a speed of 20 cycles·min^{-1} and continued for 5 min. Surface tension data were obtained at minimum and maximum bubble radius (γ_{min} and γ_{max}) after 5 min of oscillation.

Statistics. All data are given as mean±SEM or median values. Statistical evaluation of the differences between controls and HP, IPF or sarcoidosis groups was performed by applying an H-test (Kruskal-Wallis H-test) to assess principal significant diversity, followed by a comparison between the controls and each patient group using a nonparametric test (Mann-Whitney U-test/Wilcoxon rank sum W-test). Adjustment for multiple comparison according to Bonferroni was performed consistently by multiplying the significance level by the number of comparisons (three) undertaken. For evaluation of differences in surface activity upon reconstitution with BAL proteins, the t-test for matched pairs was used. The level of significance was set at p<0.05.

Results

As anticipated, relative lymphocyte counts were elevated in the BALF of patients with sarcoidosis and HP, whereas neutrophil influx was predominant in IPF (table 2). In all patient groups, BAL protein values were markedly and significantly increased as compared to control data, with the abnormalities in HP and IPF patients slightly exceeding those in sarcoidosis. In contrast, the PL concentration (uncorrected) in the original BALF did not significantly differ between controls and patients with interstitial lung diseases (table 2). Upon "correction" of the PL data according to the BAL/blood urea quotient, significantly decreased PL values were calculated for HP and IPF, but not for sarcoidosis (table 2). Due to the high alveolar protein load, the PL:protein ratio was significantly decreased in all patient groups, somewhat less prominently in sarcoidosis as compared to HP and IPF (fig. 1).

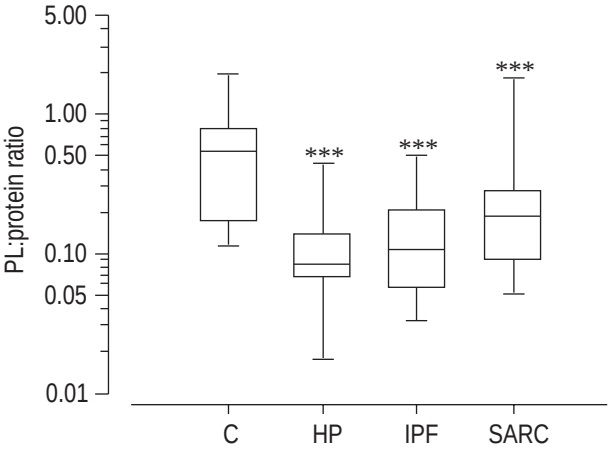

Fig. 1. – Phospholipid (PL):protein ratio in bronchoalveolar lavage fluid (BALF) based on uncorrected concentrations. Box plots show medians, the upper and lower hinges and extremes for each group. C: control (n=29); HP: hypersensitivity pneumonitis (n=28); IPF: idiopathic pulmonary fibrosis (n=29); Sarc: sarcoidosis (n=40). ***: p< 0.001 *versus* controls.

As compared to controls, analysis of the PL profile revealed substantial changes in IPF, moderate changes in HP and no alteration in sarcoidosis (table 3). The changes in IPF comprised a fall in the relative content of PC and PG, with concomitant rises in the proportions of PI, PE and SPH. Lyso-PC content was not increased. CL was detectable in trace amounts in controls and did not change among the patient groups (data not shown).

In all patient groups, SP-A levels in BALF were significantly decreased as compared to controls (table 4), with the most prominent reduction being found in IPF. In contrast, SP-B values remained unchanged or even appeared slightly increased (sarcoidosis, table 4). When related to total BALF protein content, the levels of both

Table 3. – Phospholipid (PL) profile of bronchoalveolar lavage fluid

	Control	HP	IPF	Sarcoidosis
Patients n	22	27	26	35
PC %	83.2±0.8	82.3±1.0	80.2±1.7	82.1±0.9
PG %	9.4±0.6	7.6±0.6*	4.5±0.5***	8.4±0.6
PI %	2.9±0.2	4.3±0.4	5.7±0.6***	3.4±0.6
PE %	1.7±0.1	2.0±0.3	2.9±0.6*	2.1±0.1
PS %	1.1±0.2	0.8±0.2	2.8±0.6*	1.1±0.2
SPH %	0.7±0.1	1.7±0.2***	2.8±0.9***	1.7±0.3**
Lyso-PC %	0.1±0.1	0.4±0.3	0.4±0.2	0.1±0.1

Data are presented as mean±SEM as a percentage of total PLs. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; PC: phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PS: phosphatidylserine; SPH: sphingomyelin. *: p<0.05, **: p< 0.01, ***: p<0.001 *versus* controls.

Table 4. – Apoprotein content of bronchoalveolar lavage fluid+

	Control	HP	IPF	Sarcoidosis
Patients n	25	30	34	41
SP-A				
ng·mL^{-1}	1529±136	1309±178*	1121±252***	1295±138*
% PL	5.0±0.4	5.2±0.8	4.7±0.8*	4.3±0.5*
SP-B				
ng·mL^{-1}	712±67	877±75	716±43	916±59*
% PL	2.6±0.2	3.2±0.3	3.7±0.5	3.2±0.2

Data are presented as mean±SEM. +: determined by enzyme-linked immunosorbent assay (see *Methods*).HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; PC: phosphatidylcholine; SP: surfactant protein; PL: phospholipid. *: p< 0.05, ***: p<0.001 *versus* controls.

apoproteins SP-A and SP-B were significantly reduced in all patient groups (not shown). In relation to total BALF PLs, the amounts of apoprotein were unchanged except for a significant reduction in SP-A:PL values in IPF and sarcoidosis. There was a significant correlation between both SP-B or SP-A concentrations in BALF and total BALF PL levels in all patient groups (not shown).

Centrifugation of the BALF for isolation of LSA displayed a reduction in the amount of PL material pelleted in all patient groups, both for relative (fig. 2) and absolute quantities (not depicted). However, due to the broad scatter of data, significance was only achieved for the relative amount of LSA in sarcoidosis and IPF.

The adsorption ability of the pelleted surfactant material, as assessed in the absence of soluble proteins, was reduced in IPF more than in HP and sarcoidosis (fig. 3a). The capacity to reach near zero γ_{min} was largely lost or reduced in all patient groups, with median values of ~19 mN·m^{-1} in IPF and ~5 mN·m^{-1} in HP and sarcoidosis, as compared to near 0 mN·m^{-1} in controls measured with the present technique. γ_{max} were moderately elevated in IPF patients only (not shown). Reconstitution of surfactant pellets with individual soluble BALF proteins, in the same ratio as observed in the original BALF, could be performed in a limited number of patients, in whom sufficient material was available. As indicated in figure 4, such reconstitution resulted in a further and significant loss of surface activity in all groups of patients, but not in controls. The differences in mean and median values in the absence of protein in figure 3, as compared to figure 4, are explained by the fact that the shortage of material was particularly evident upon use of BALF with low percentages of LSA, *i.e.* pelleted material. In addition, the γ_{min} in the absence and presence of proteins were significantly correlated (fig. 5), as were the gmin values in the presence of proteins and the PL:protein ratio (fig. 5).

To test the relationship between surfactant changes and lung function abnormalities (restriction), γ_{min} values were correlated with forced vital capacity (FVC) and total lung capacity (TLC) data. No significant correlation was noted for HP and sarcoidosis. In IPF, however, a significant correlation between FVC and γ_{min} (r=0.75, n=23, p=0.00004) and between TLC and γ_{min} (fig. 6) was observed: the capacity to reduce γ_{min} to near zero values was increasingly lost, the more pronounced the reduction in lung volume.

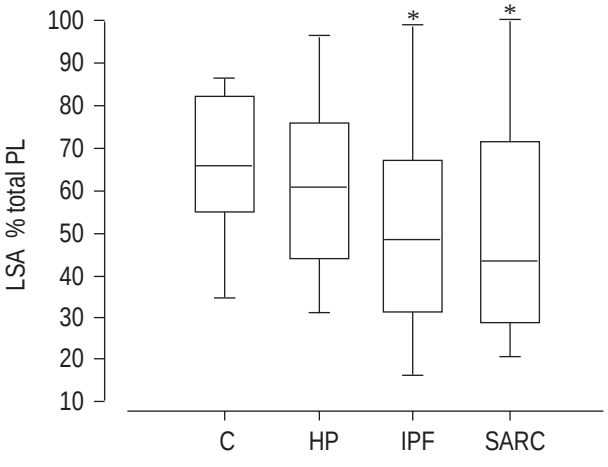

Fig. 2. – Concentration of large surfactant aggregates (LSA) in bronchoalveolar lavage fluid (BALF) relative to total phospholipids (PL) (48,000 × *g* pellet). Box plots show medians, the upper and lower hinges and outliers for each group. C: control (n=17); HP: hypersensitivity pneumonitis (n=23); IPF: idiopathic pulmonary fibrosis (n=24); Sarc: sarcoidosis (n=33). *: p<0.05 *versus* controls.

Discussion

Excellent adsorption and surface tension-reducing properties, with γ_{min} ~0 mN·m^{-1}, were noted in the control subjects, reflecting biophysical surfactant characteristics assumed to exist *in vivo* [33, 34]. These surface properties were largely lost in nearly all patients with IPF, and were reduced to a minor extent in HP and sarcoidosis. Differences in the time from onset of disease to initial lavage, smoking histories, age range, sex distribution and the degree of restriction noted upon pulmonary function testing did not explain the prominence of functional abnormalities in the IPF patients. The following biochemical and metabolic changes might underlie the severe disturbances in surfactant biophysics in IPF.

Reduction in the alveolar phospholipid pool

No difference in total BALF PL concentrations and BALF recoveries was found between control subjects and the three patient groups. However, these findings do not necessarily suggest constancy of the total alveolar PL pool, since differences in lavaged surface area cannot be excluded. When employing the urea quotient to relate the recovered PL quantities to the volume of alveolar lining layer washed out by the BAL procedure, a marked reduction in PL concentrations was noted, in particular in IPF and HP. Unfortunately, this method is itself prone to serious pitfalls [35, 36]. Hence, the PL concentration in the original BALF was relied upon. At first glance, the finding of unchanged PL concentrations appears to differ from previous investigations, reporting a decrease in total PL quantities recovered by means of lavage in IPF patients [16, 17]. However, in those preceding studies, measurements were performed only in a 72,000 × *g* pellet, and the results may thus reflect changes in total lavage PLs and in its proportion of LSA, which is here shown to be significantly reduced (see below).

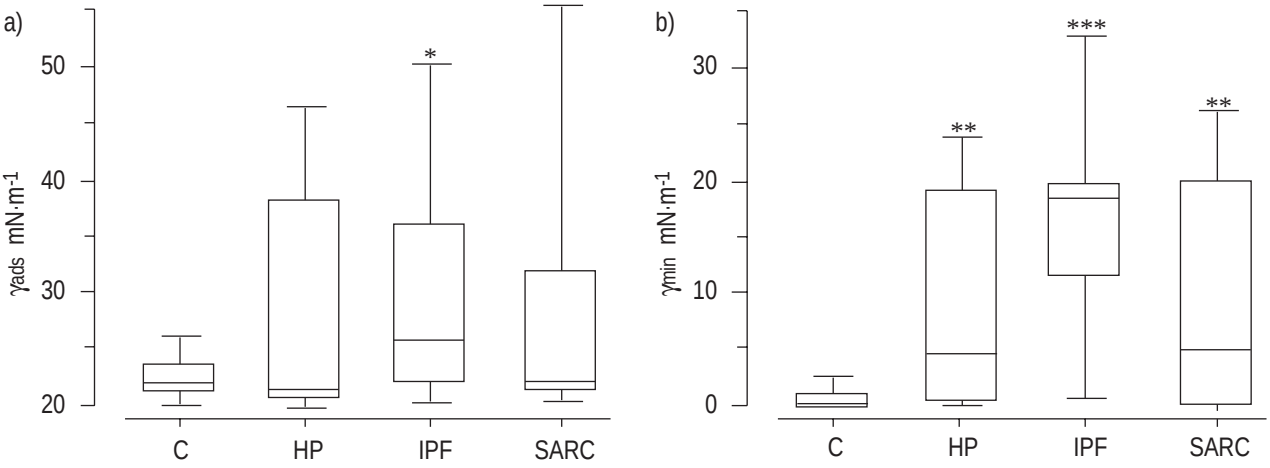

Fig. 3. – Biophysical surfactant function. All measurements were performed at a phospholipid concentration of 2 mg·mL^{-1} in a pulsating bubble surfactometer: a) surface tension after 12 s adsorption (γ_{ads}); and b) minimum surface tension (γ_{min}) after 5 min of film oscillation. Box plots show medians, the upper and lower hinges and extremes for each group. C: control (n=17); HP: hypersensitivity pneumonitis (n=22); IPF: idiopathic pulmonary fibrosis (n=23); Sarc: sarcoidosis (n=38). *: p<0.05, **: p<0.001, ***: p<0.001 *versus* controls.

Altered surfactant subtype distribution

The fraction of LSA was separated from SSAs by means of 48,000 × *g* centrifugation [30, 37], and was found to be markedly reduced in all patient groups with interstitial lung disease. In view of the established lower biophysical activity of SSA [30, 37, 38], such reduction in the percentage of LSA may be assumed to contribute to the impairment of surface activity *in vivo* but may not underlie the impaired surface activity of LSA *ex vivo*, since all bubble experiments were performed at 2 mg PL·mL^{-1}. It has to be borne in mind, however, that the metabolic steps underlying the conversion from LSA to SSA have hitherto not been fully elucidated, and a reduction in the proportion of LSA might be accompanied by additional changes in the (still) pelletable material not reflected in the centrifugation step. This view is supported by the recent observation of a significant correlation between the reduction in LSA concentration and the loss of biophysical activity of this subfraction in ARDS patients [6] and also in cycled lavage fluids from healthy rabbits [39]. Further studies are required to elucidate this aspect.

Changes in the phospholipid profile

A uniform pattern of changes in the PL profile was noted more in IPF than in HP (reduction in PC and PG and increase in SPH, PI and PE concentration), but was absent in sarcoidosis. The noted alterations in PL composition accord well with previous investigations in IPF [14–17] and HP [18]. The absence of alterations in PL profile in sarcoidosis favourably corresponds to the data of HONDA *et al.* [14] and LOW *et al.* [20]. Compositional changes in the PLs that comprise the surface-active material recovered at lavage may suggest functional alterations of type II cells, and in particular the decrease in the relative content of PG is a constant finding in virtually all models of severe acute lung injury and corresponds to an "immaturity" pattern of type II cell metabolic activity during gestation [2]. Alternatively, changes in the PL profile may

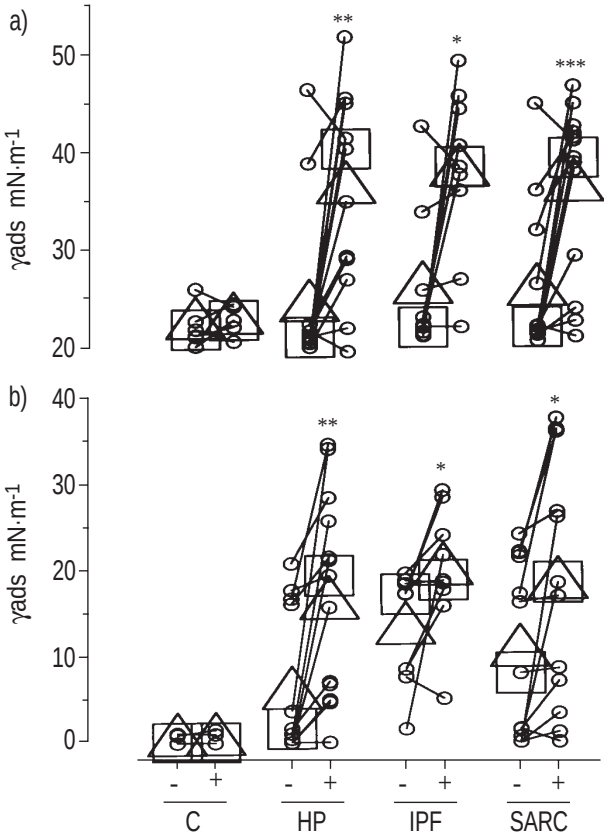

Fig. 4. – Inhibition of surface properties by soluble bronchoalveolar lavage fluid (BALF) proteins. Pelleted surfactant was recombined with BALF proteins at the same ratio as observed in the original BALF and measured at a phospholipid concentration 2 mg·mL^{-1} in a pulsating bubble surfactometer: a) surface tension after 12 s adsorption (γ_{ads}); and b) minimum surface tension (γ_{min}) after 5 min of film oscillation in the absence (-) or presence (+) of the proteins. Individual BALF (○), mean (△) and median values (□) are given for each group. C: control (n=5); HP: hypersensitivity pneumonitis (n=13); IPF: idiopathic pulmonary fibrosis (n=9); Sarc: sarcoidosis (n=13). *: p<0.05, **: p<0.01, ***: p<0.001 *versus* absence of protein.

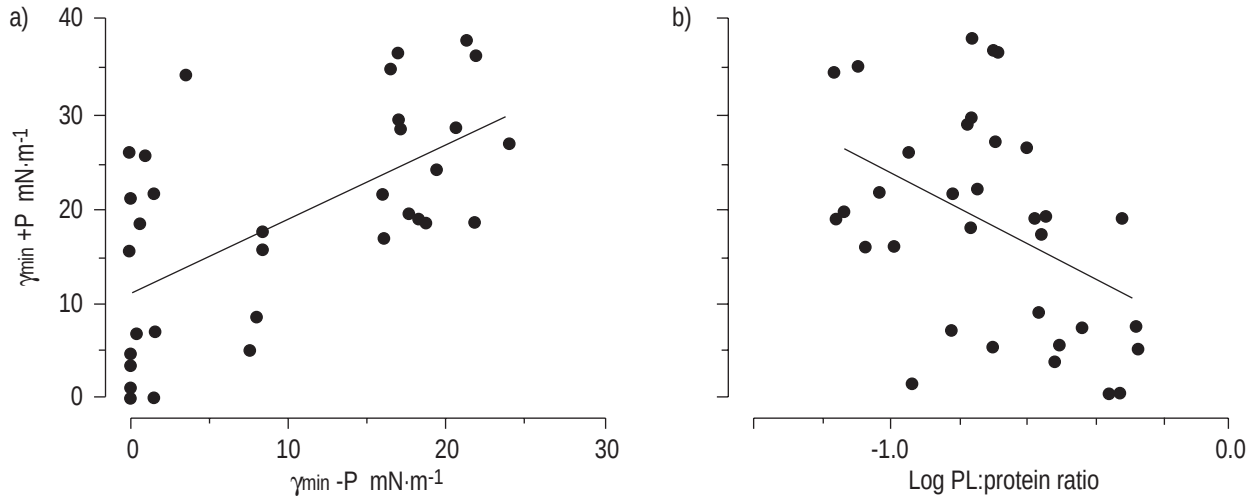

Fig. 5. – Correlation of: a) minimum surface tension (γmin) in the absence (-P) and presence (+P) of soluble BALF proteins n=35, r=0.63, p<0.01; and b) γ_{min} in the presence of protein and phospholipid (PL): protein ratio n=35, r=0.46, p<0.01.

result from contamination with cellular debris or leaked plasma lipids. However, as reflected by the BALF protein values, plasma leakage was evident in sarcoidosis without any compositional changes of PL, which questions the significance of this pathway of contamination.

Changes in the apoprotein content

When considering the SP-A and SP-B data in the BALF and the concentrations of these apoproteins after normalization to the PL content, a clear decrease in SP-A but not SP-B concentration was encountered in all patient groups, with the most prominent changes occurring in IPF. The finding of decreased absolute and relative SP-A levels in IPF favourably corresponds to recent studies of McCormack *et al.* [17, 21], although their changes in SP-A were much more prominent (~three-fold) than the present ones: as mentioned above, these measurements were performed in a 72,000 × *g* pellet, thus reflecting the amount of SP-A in the LSA fraction, whereas the apoproteins were quantified in the original BALF, independently of their distribution between surfactant subtypes, in the present study. The observation of decreased SP-A levels in sarcoidosis and HP differs from previous investigations into these entities, suggesting unchanged or even increased SP-A levels [18, 40]. Further studies should aim to characterize the distribution of surfactant apoproteins between the different subtypes under clinical conditions, since variations in the apoprotein content of the different surfactant subtypes have been described under experimental settings, *e.g.* upon *in vitro* conversion of BALF [30, 37].

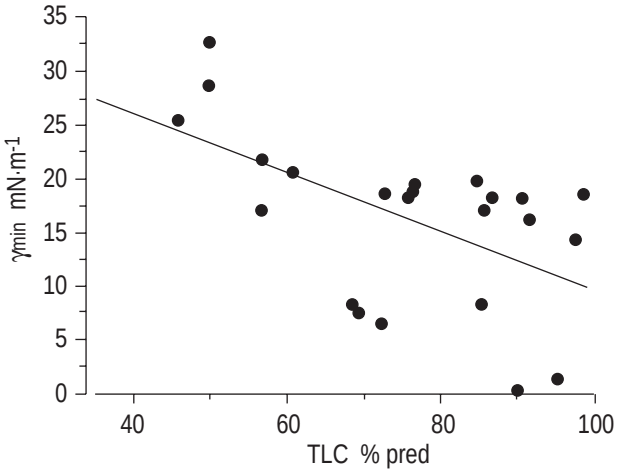

Fig. 6. – Correlation of minimum surface tension (γ_{min}) and total lung capacity (TLC) in idiopathic pulmonary fibrosis subjects (n=23, r=0.55, p=0.006).

Inhibition of surfactant function by plasma proteins

A manifold increase in the alveolar protein load was noted in all patients with interstitial lung diseases, with the highest values in IPF patients; the PL:protein ratios were accordingly reduced. Such increased protein levels might result from both plasma protein leakage and local (over) production, and the exact protein profile has not yet been discovered for the currently investigated diseases. Inhibition of surfactant function by leaked plasma proteins has been established as an important mechanism of surfactant disturbance in experimental models of acute lung injury, and among the plasma proteins fibrin(ogen) and haemoglobin may be of major importance [31, 41–44]. In addition, evidence has been presented that such inhibition of surfactant by protein leakage occurs also under clinical conditions of ARDS [6]. The present technique of surface tension measurement first separated the pelletable surfactant material from the bulk of soluble proteins in the BALF, and >90% of proteins were removed by this step. However, it is conceivable that multimeric and/or high-molecular-weight proteins, closely associated with the surfactant compounds, copelleted during this centrifugation step and substantially contributed to the loss of surface activity of the LSA, mostly in IPF patients. One "pelletable" candidate previously shown to possess marked surfactant inhibitory capacity [32] is fibrin oligo- or polymer, which may be easily generated within the alveolar compartment upon fibrinogen leakage due to the predominance of procoagulant activities in the alveolar

compartment in interstitial lung disease and in particular in IPF patients [45]. In addition, inhibitory capacities of the soluble BALF proteins were directly shown in nearly all of the patients in the present study, in whom the relative content of LSA was high enough to allow a reconstitution of this subfraction with the individual lavage proteins according to the PL:protein ratio in the original BALF.

Further surfactant inhibitory mechanisms

Experimental studies established phospholipolytic cleavage of essential surfactant compounds as a pathogenetic mechanism resulting in loss of surface activity [46]. However, no increase in the small percentage of lyso-PC was noted in the present study. In addition, alterations of the apoproteins by proteases [47] or reactive oxygen species [48, 49], which might have been missed by the immunological detection of SP-A and SP-B currently undertaken, might well be operative. This suggestion is of particular interest for IPF patients, since neutrophil elastase was shown to possess the capability of degrading surfactant apoproteins [47], and high alveolar neutrophil counts are a characteristic feature of IPF.

Conclusions

In conclusion, a marked loss of surface tension-lowering capacity was noted in idiopathic pulmonary fibrosis, and such a change in biophysical surfactant function was clearly less prominent in hypersensitivity pneumonitis and sarcoidosis. Minimal surface tension values of >15 $mN \cdot m^{-1}$, as observed in the majority of idiopathic pulmonary fibrosis patients, must be assumed to result in severe loss of alveolar stability, at least if this holds true *in vivo* [33, 34]. Interestingly, the loss of biophysical surfactant function was significantly correlated with the degree of lung restriction in this patient group. Various mechanisms may be operative in effecting the impairment of surface properties, particularly observed in the idiopathic pulmonary fibrosis patients, including surfactant inhibition by leaked plasma proteins, changes in the phospholipid profile suggestive of altered type II cell metabolism, reduced apoprotein content or functional activity (presently not addressed), and catabolic steps resulting in a reduced percentage of functionally active large surfactant aggregates. A current hypothesis for the loss of gas exchange units in fibrosis is so-called "collapse induration" which means irreversible fusion of (denuded) alveolar basement membranes [50], with abnormalities in surfactant function promoting this process by favouring alveolar collapse and apposition of alveolar walls. The presently observed surfactant abnormalities, in particular in the idiopathic pulmonary fibrosis patients, may support this concept.

Acknowledgements. Portions of this paper were supplied by R. Schmidt in partial fulfillment for the degree of doctor of chemistry and by F. Nix in partial fulfillment for a medical thesis.

References

1. Hawgood S. Surfactant: composition, structure, and metabolism. *In:* Crystal RG, West JB, *et al*, eds. *The Lung*. New York, Raven Press, 1991; pp. 247–261.
2. Van Golde LMG, Batenburg JJ, Robertson B. The pulmonary surfactant system: biochemical aspects and functional significance. *Physiol Rev* 1988; 68: 374–455.
3. Hallman M, Spragg R, Harrell JH, Moser KM, Gluck L. Evidence of lung surfactant abnormality in respiratory failure. Study of bronchoalveolar lavage phospholipids, surface activity, phospholipase activity, and plasma myoinositol. *J Clin Invest* 1982; 70: 673–683.
4. Pison U, Seeger W, Buchhorn R, *et al*. Surfactant abnormalities in patients with respiratory failure after multiple trauma. *Am Rev Respir Dis* 1989; 140: 1033–1039.
5. Gregory TJ, Longmore WJ, Moxley MA, *et al*. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. *J Clin Invest* 1991; 88: 1976–1981.
6. Günther A, Siebert C, Schmidt R, *et al*. Surfactant alterations in severe pneumonia, ARDS and cardiogenic lung edema. *Am J Respir Crit Care Med* 1996; 151: 176–184.
7. Lewis JF, Jobe AH. Surfactant and the adult respiratory distress syndrome. *Am Rev Respir Dis* 1993; 147: 218–233.
8. Seeger W, Günther A, Walmrath HD, Grimminger F, Lasch HG. Alveolar surfactant and adult respiratory distress syndrome. Pathogenctic role and therapeutic prospects. *Clin Investig* 1993; 71: 177–190.
9. Crouch E. Pathobiology of pulmonary fibrosis. *Am J Physiol* 1990; 259: L159–L184.
10. Kovacs EJ, DiPietro LA. Fibrogenic cytokines and connective tissue production. *FASEB J* 1994; 8: 854–861.
11. Thrall RS, Swendsen CL, Shannon TH, *et al*. Correlation of changes in pulmonary surfactant phospholipids with compliance in bleomycin-induced pulmonary fibrosis in the rat. *Am Rev Respir Dis* 1987; 136: 113–118.
12. Horiuchi T, Mason RJ, Kuroki Y, Cherniack RM. Surface and tissue forces, surfactant protein A, and the pulmonary phospholipid components of pulmonary surfactant in bleomycin-induced pulmonary fibrosis in the rat. *Am Rev Respir Dis* 1990; 141: 1006–1013.
13. Osanai K, Takahashi K, Sato S, *et al*. Changes of lung surfactant and pressure-volume curves in bleomycin-induced pulmonary fibrosis. *J Appl Physiol* 1991; 70: 1300–1308.
14. Honda Y, Tsunematsu K, Suzuki A, Akino T. Changes in phospholipids, in bronchoalveolar lavage fluid of patients with interstitial lung diseases. *Lung* 1988; 166: 293–301.
15. Hughes DA, Haslam PL. Changes in phosphatidylglycerol in bronchoalveolar lavage fluids from patients with cryptogenic fibrosing alveolitis. *Chest* 1989; 95: 82–89.
16. Robinson PC, Watters LC, King TE, Mason RJ. Idiopathic pulmonary fibrosis. Abnormalities in bronchoalveolar lavage fluid phospholipids. *Am Rev Respir Dis* 1988; 137: 585–591.
17. McCormack FX, King TE, Voelker DR, Robinson PC, Mason RJ. Idiopathic pulmonary fibrosis. Abnormalities in bronchoalveolar lavage content of surfactant apoprotein A. *Am Rev Respir Dis* 1991; 144: 160–166.
18. van der Graaf EA, Jansen HM, Lutter R, *et al*. Surfactant protein A in bronchoalveolar lavage fluid. *J Lab Clin Med* 1992; 120: 252–263.
19. Lesur O, Mancini NM, Janot C, *et al*. Loss of lymphocyte modulatory role by surfactant lipid extracts from acute

hypersensitivity pneumonitis: comparison with sarcoidosis and idiopathic pulmonary fibrosis. *Eur Respir J* 1994; 7: 1944–1949.
20. Low RB, Davis GS, Bell DY, Giancola MS, Vacek PM. Lipids in bronchoalveolar lavage fluid from patients with sarcoidosis. *Thorax* 1987; 42: 926–932.
21. McCormack FX, King TE, Bucher BL, Nielsen L, Mason R. Surfactant Protein A predicts survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1995; 152: 751–759.
22. Krämer HJ, Schmidt R, Günther A, Becker G, Suzuki Y, Seeger W. ELISA technique for quantification of surfactant protein (SP-B) in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1995; 152: 1540–1544.
23. Warr RG, Hawgood S, Buchley DI, *et al*. Low molecular weight human pulmonary surfactant (sps): isolation, characterization and cDNA and amino sequences. *Proc Natl Acad Sci USA* 1987; 84: 7915–7919.
24. Quanjer PhH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault J-C. Lung volumes and forced ventilatory flows. Report working party. Standardization of lung function tests. European community for steel and coal. Official statement of the European Respiratory Society. *Eur Respir J* 1993; 6 (Suppl. 16): 5–40.
25. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem* 1959; 37: 911–917.
26. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 1969; 5: 494–496.
27. Rennard SL, Basset G, Lecossier D, *et al*. Estimation of epithelial lining fluid recovered by lavage using urea as a marker for dilution. *J Appl Physiol* 1986; 60: 532–538.
28. Smith PK, Krohn RI, Hermannson GT, *et al*. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985; 150: 76–85.
29. Gustavsson L. Densitometric quantification of individual phospholipids. *J Chromat* 1986; 395: 255–266.
30. Veldhuizen R, Inchley K, Hearn SA, Lewis JF, Possmayer F. Degradation of surfactant-associated protein B (SP-B) during *in vitro* conversion of large to small surfactant aggregates. *Biochem J* 1993; 295: 141–147.
31. Seeger W, Günther A, Thede C. Differential sensitivity to fibrinogen inhibition of SP-C-vs. SP-B-based surfactants. *Am J Physiol* 1992; 261: L286–L291.
32. Seeger W, Elssner A, Günther A, Krämer HJ, Kalinowski HO. Lung surfactant phospholipids associate with polymerizing fibrin: loss of surface activity. *Am J Respir Cell Mol Biol* 1993; 9: 213–220.
33. Bachofen H, Schürch S, Urbinelli M, Weibel ER. Relations among alveolar surface tension, surface area, volume, and recoil pressure. *J Appl Physiol* 1987; 62: 1878–1887.
34. Goerke J, Clements JA. Alveolar surface tension and lung surfactant. *In*: Macklem PT, Medds J, eds. Handbook of Physiology. Section 3. The Respiratory System. Vol. 2. Control of Breathing. Bethesda, MD, USA. American Physiological Society, 1986; pp. 247–261.
35. von Wichert P, Joseph K, Müller B, Franck WM. Bronchoalveolar lavage. Quantification of intraalveolar fluid. *Am Rev Respir Dis* 1993; 147: 148–152.
36. Effros RM, Murphy C, Ozker K, Hacker A. Kinetics of urea exchange in air-filled and fluid-filled rat lungs. *Am J Physiol* 1992; 263: L619–L626.
37. Veldhuizen RAW, Hearn SA, Lewis JF, Possmayer F. Surface-area cycling of different surfactant preparations: SP-A and SP-B are essential for large-aggregate integrity. *Biochem J* 1994; 300: 519–524.
38. Ueda T, Ikegami M, Jobe A. Surfactant subtypes *in vitro* conversion, *in vivo* function, and effects of serum proteins. *Am J Respir Crit Care Med* 1994; 149: 1254–1259.
39. Günther A, Schmidt R, Feustel A, *et al*. Surfactant subtype conversion is related to loss of surfactant apoprotein B and surface activity in large surfactant aggregates. *Am J Respir Crit Care Med* 1999; 159: 244–251.
40. Hamm H, Luhrs J, Guzman-y-Rotacche J, Costabel U, Fabel H, Bartsch W. Elevated surfactant protein A in bronchoalveolar lavage fluids from sarcoidosis and hypersensitivity pneumonitis patients. *Chest* 1994; 106: 1766–1770.
41. Holm BA, Enhorning G, Notter RH. A biophysical mechanism by which plasma proteins inhibit lung surfactant activity. *Chem Phys Lipids* 1988; 49: 49–55.
42. Venkitaraman AR, Baatz JE, Whitsett JA, Hall SB, Notter RH. Biophysical inhibition of synthetic phospholipid-lung surfactant apoprotein admixtures by plasma proteins. *Chem Phys Lipids* 1991; 57: 49–57.
43. Seeger W, Grube C, Günther A, Schmidt R. Surfactant inhibition by plasma proteins: differential sensitivity of various surfactant preparations. *Eur Respir J* 1993; 6: 971–977.
44. Seeger W, Grube C, Günther A. Proteolytic cleavage of fibrinogen: amplification of its surfactant inhibitory capacity. *Am J Respir Cell Mol Biol* 1993; 9: 239–247.
45. Chapman HA, Allen CL, Stone OL. Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. *Am Rev Respir Dis* 1986; 133: 437–443.
46. Holm BA, Keicher L, Sokolowski J, Enhorning G. Inhibition of pulmonary surfactant function by phospholipases. *J Appl Physiol* 1991; 71: 317–321.
47. Pison U, Tam EK, Caughey GH, Hawgood S. Proteolytic inactivation of dog lung surfactant-associated proteins by neutrophile clastase. *Biochim Biophys Acta* 1989; 992: 251–257.
48. Seeger W, Lepper H, Wolf HRD, Neuhof H. Alteration of surfactant function after exposure to oxidative stress and to oxygenated and native arachidonic acid *in vitro*. *Biochim Biophys Acta* 1985; 835: 58–67.
49. Gilliard N, Heldt GP, Loredo J, *et al*. Exposure of the hydrophobic components of porcine lung surfactant to oxidative stress alters surface tension properties. *J Clin Invest* 1994; 93: 2608–2615.
50. Burkhardt A. Alveolitis and collapse in the pathogenesis of pulmonary fibrosis. *Am Rev Respir Dis* 1989; 140: 513.