Surface properties, morphology and protein composition of pulmonary surfactant subtypes

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Separation of surfactant subtypes is now commonly used as a parameter in assessing the amount of active compared with inactive material in various models of lung injury. The protein content, morphology and surface activity were determined of the heavy and light subtype isolated by differential centrifugation. Here we report the presence of surfactant proteins B and C in the heavy subtype but not in the light subtype. Adsorption studies revealed that separation of fast adsorbing bronchoalveolar lavage resulted in slowly adsorbing heavy and light subtypes. Surfactant, reconstituted from heavy and light fractions, did not show a high adsorption rate. It is concluded that the isolation procedures might result in a loss of fast adsorbing surfactant structures.

Surface area cycling was used as a model *in vitro* for the extracellular surfactant metabolism. The heavy subtype is converted into the light subtype during conversion. Conversion performed with resuspended heavy subtype revealed the generation of a disparate subtype. Furthermore it was found that the conversion was dependent on preparation and handling of the samples before cycling. Finally, adsorption studies at low surfactant concentrations revealed a delayed adsorption of lipid-extracted surfactants compared with natural surfactants. These observations emphasize the importance of the (surfactant-associated protein A-dependent) structural organization of surfactant lipids in the adsorption process.

INTRODUCTION

In the alveolar walls of the lung, type II cells produce and secrete pulmonary surfactant. This surfactant lines the alveolar walls and reduces the surface tension at the air/liquid interface of the alveoli on exhalation. Pulmonary surfactant is a mixture of approx. 90 % phospholipids and 10 % proteins. Four surfactant-associated proteins, designated SP-A, SP-B, SP-C and SP-D, have been identified [1,2]. SP-B and SP-C are hydrophobic proteins and as such they associate with the surfactant lipids. SP-A also associates with lipids and is, like SP-D, a collagenous carbohydrate-binding protein. Each protein has a specific role in surfactant function. SP-B and SP-C play a major role in decreasing surface tension by pulmonary surfactant. These proteins are known to facilitate the insertion of lipids into the monolayer at the air/liquid interface [3,4]. SP-A and SP-D might play an important role in lung defence [5]. Furthermore SP-A is important for the integrity of large surfactant aggregates [6] and prevents surfactant inactivation [7,8].

The extracellular surfactant structures have various sizes and densities depending on the stage of the extracellular surfactant metabolism. Pulmonary surfactant is secreted by type II cells as lamellar bodies. These organelles form tubular myelin, which in turn is thought to represent the reservoir for the lipid monolayer at the air/liquid interface. Subsequently, small vesicles are excluded from the monolayer and these remnants of the extracellular surfactant metabolism are recycled to the type II cells [9]. Pulmonary surfactant can be separated into subtypes with different morphologies and characteristics. Surfactant is usually subfractionated into heavy and light subtypes. The dense or heavy subtype contains tubular myelin, lamellar bodies and large vesicles. This subtype has the ability to decrease surface tension. It contains large amounts of SP-A [10] and SP-B [6]; it is the precursor of the light subtype. The light subtype is less surface-active, consists mainly of small vesicles [11,12] and contains small amounts of SP-A [6,10].

Separation of the subtypes can be achieved by sucrose density gradient centrifugation or by differential centrifugation [11,13,14]. Extracellular surfactant metabolism can be mimicked *in vitro* by surface area cycling, as described by Gross and Narine [15]. During cycling the heavy subtype is partly converted into the light subtype. Here we report studies of surfactant subtypes obtained by differential centrifugation before and after surface area cycling. The surface activities of the various surfactant fractions were correlated with morphology and surfactant protein content.

MATERIALS AND METHODS

Animals

Male Wistar rats (WU-strain, 7 weeks old, 180 ± 20 g, SPF obtained from Harlan Sprague Dawley Co., Zeist, The Netherlands) were allowed to acclimate for 1 week before the onset of the experiment. Rats were killed by abdominal bleeding under Nembutal (60 mg/kg of body weight, intraperitoneal) anaesthesia. Lungs were removed and weighed. Subsequently, bronchoalveolar lavage (BAL) was performed five times with a volume of 40 ml/kg of body weight with 0.15 M NaCl/1.5 mM CaCl_2 (pH 7). The BALs were centrifuged at 200 *g* for 10 min at 4 °C to remove cells and cellular debris. From the remaining BAL samples were taken for lipid and surfactant protein analysis, pulsating-bubble surfactometry, electron microscopy and surface balance experiments.

Separation and analysis of surfactant subtypes

Heavy surfactant subtype was obtained by centrifugation of the BAL at 40 000 *g* for 15 min at 4 °C [13]. The supernatant contains the light subtype from which samples were taken for lipid analysis, pulsating-bubble surfactometry and electron micro-

Abbreviations used: AR, adsorption rate; BAL, bronchoalveolar lavage; SP, surfactant-associated protein; SUV, small unilamellar vesicles.

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scopy. The pelleted heavy subtype was resuspended in conversion buffer [10 mM Tris/0.15 M NaCl/1 mM CaCl_2 /1 mM MgCl_2 /0.1 mM EDTA (pH 7.4)] at 4 °C at a concentration of approximately 0.25 mg/ml phospholipid with a Dounce homogenizer. During resuspension, samples were kept on ice while the loosely fitting pestle was gently passed up and down (ten times) preventing the generation of air bubbles in the liquid. Samples were taken for lipid and surfactant protein analysis, pulsating-bubble surfactometry, electron microscopy and surface balance experiments.

Conversion *in vitro* by surface area cycling

Resuspended heavy subtype (2 ml) was placed in capped plastic tubes (Falcon 2058) and the tubes were attached to a disc that rotated at 40 rev./min for 3 h at 37 °C [15]. The surface area varied from 1.1 cm² to 9.0 cm² twice each cycle. After cycling, the heavy and light subtypes were separated. Again, samples were taken for lipid determination, pulsating-bubble surfactometry, electron microscopy and surface balance experiments.

Surfactant analysis

The phospholipid content of the samples was determined by lipid extraction by the method of Bligh and Dyer [16] and subsequent phosphorus analysis as described by Bartlett [17].

Quantification of SP-B and SP-C was performed by the method of Van Eijk et al. [18]. With this method proteins are separated according to their size by gel filtration on Sephadex LH-60 in an acidified dichloromethane/methanol/0.1 M HCl (30:65:5, by vol.) mixture. This method was also used to purify large quantities of biologically active SP-B and SP-C. The proteins were detected by the absorption of the amide backbone groups at 228 nm and quantified with quantitative amino acid analysis in comparison with standards.

Surface activity studies: preparation of natural surfactants

Natural surfactant fractions of the BAL, light and cycled light fractions were centrifuged for 3 h at 100 000 *g* and 4 °C and resuspended at a concentration of 1 mg/ml phospholipid in a buffer containing 10 mM Hepes, 150 mM NaCl and 3 mM CaCl_2 , pH 7.4, for pulsating-bubble surfactometry. Heavy and cycled heavy fractions were centrifuged at 100 000 *g* and 4 °C for 30 min and used at a concentration of 0.75 mg/ml phospholipid. Samples were resuspended with a Dounce homogenizer and incubated for 1 h at 37 °C. Natural surfactants used for the surface balance experiments were centrifuged for 3 h at 100 000 *g* at 4 °C and resuspended at a concentration of 1 mg/ml in a buffer consisting of 25 mM Hepes and 150 mM NaCl, pH 7.0, and stored at –20 °C.

Surface activity studies: preparation of small unilamellar vesicles (SUV)

The hydrophobic surfactant components were isolated by extraction by the method of Bligh and Dyer [16]. SUV were prepared from lipid-extracted surfactant. Lipids dissolved in chloroform were dried under a continuous stream of nitrogen at room temperature. The remaining film was hydrated at 60 °C in a buffer consisting of 25 mM Hepes and 150 mM NaCl, pH 7.0, for surface balance experiments. For pulsating-bubble experiments samples were hydrated in a buffer consisting of 10 mM Hepes, 150 mM NaCl and 3 mM CaCl_2 , pH 7.4. The samples were kept at 60 °C for 10 min and vortex-mixed for 1 min. Then the samples were sonicated four times with a Branson sonicator with a 0.5 inch (1.27 cm) flat-topped disrupter tip for 20 s (10 s

intervals) at 30 W and 55 °C. The resulting SUV were kept on ice and used within 8 h of preparation.

Pulsating-bubble surfactometry

Natural and extracted surfactant samples used for pulsating-bubble surfactometric measurements were prepared as described above. The surface tension (γ) of the surfactant samples was measured with a pulsating-bubble surfactometer (Electronetics Corporation, Amherst, NY, U.S.A.) as described by Enhörning [19]. The sample chamber was filled with 20 μl of surfactant and the formed bubble was left to equilibrate for 1 min at minimum bubble size. The bubble was then pulsed so that its radius (*R*) oscillated between a maximum of 0.55 mm and a minimum of 0.4 mm. The pressure (*P*) difference across the surface is calculated with the formula of Laplace, $\Delta P = 2\gamma/R$. Measurements were done at a rate of 20 pulsations per min for 5 min.

Adsorption experiments

Experiments were performed at 37 °C in a temperature-controlled box. Measurements were made by the Wilhelmy plate method with a Cahn 2000 electrobalance connected to a computer. A 5.5 ml Teflon trough contained a buffer subphase consisting of 25 mM Hepes and 150 mM NaCl, pH 7.0. The subphase was stirred continuously. Samples were injected under the surface through an injection hole into the subphase, followed directly by an injection of CaCl_2 (3 mM final concentration in trough). The surface pressure was monitored for 15 min. Phospholipid (10–50 nmol) was injected into the subphase. Lipid-extracted surfactant was injected as SUV. Natural surfactant was prepared by the protocol mentioned above.

Electron microscopy

Fractions were obtained as described above. The BAL and light fractions were pelleted by centrifugation at 100 000 *g* for 3 h at 4 °C. Heavy fractions were obtained by centrifugation for 15 min at 40 000 *g* and 4 °C. Pellets were fixed for 1 h at room temperature with 2.5% (w/v) glutaraldehyde in conversion buffer. Samples were then post-fixed for 1 h with 2% (w/v) osmium tetroxide, stained *en bloc* for 1 h in 2% (w/v) aqueous uranyl acetate, dehydrated in a graded series of acetone and embedded in Durcupan epoxy resin (Fluka). Ultrathin sections of 50 nm were cut on a Reichert UltracutS (Leica, Wien, Austria) and stained for 2 min with Reynolds lead citrate [20]. Sections were observed and photographed in a Philips CM10 electron microscope at 80 kV.

Statistical analysis

Unless indicated otherwise data were analysed with a one-way analysis of variance (ANOVA). The multiple comparison test by Bonferroni ($P < 0.05$) was used to test for differences between groups when the *F* statistic was significant ($P < 0.05$). If data violated the assumption of homogeneity of variances, even after log transformation, the data were analysed with the non-parametric Kruskal–Wallis test.

RESULTS

Surfactant subtypes and conversion *in vitro*

Isolation of the heavy and light subtypes from rat lavage resulted in $65 \pm 5\%$ heavy and $35 \pm 5\%$ light subtypes [21]. Subsequent

Figure 1 Effect of isolation and processing procedures on the morphology of surfactant subtypes

From BAL (A), heavy subtype (B) and light subtype (C) were isolated by differential centrifugation. Heavy subtype resuspended before (D) and after (E) surface area cycling revealed degradation of large aggregate structures. Subsequently the cycled heavy subtype (F) and cycled light subtype (G) were separated by differential centrifugation. Scale bar, 1 μ m.

surface area cycling of the heavy subtype resulted in the generation of the light subtype ($10 \pm 6\%$ of total phospholipids present after cycling; $n = 9$). The amount of light subtype generated increased significantly ($29 \pm 12\%$; $n = 12$; $P < 0.01$) if surface area cycling was performed with heavy subtype fractions that had been frozen in liquid nitrogen.

Surfactant proteins in surfactant subtypes

Heavy subtype contained SP-B (5 ± 2 ng/nmol of phospholipid) and SP-C (15 ± 6 ng/nmol of phospholipid). SP-B and SP-C could not be detected in the light subtype in 160 nmol of phospholipid. Although the detection limit for quantification is

Figure 2 Surface activity of the surfactant subtypes measured by pulsating bubble surfactometry

Samples were analysed at a concentration of 0.75 or 1.0 mg/ml phospholipid at 20 pulsations per min. Symbols: ○, surface tension at minimal bubble size; ●, surface tension at maximal bubble size. (a) Heavy, (b) light, (c) cycled heavy and (d) cycled light subtypes.

Figure 3 A representative pressure/time curve for the adsorption of surfactant

Surfactant was injected into the subphase and changes in surface pressure were recorded with a surface balance. AR is the tangent to the curve at its point of inflection; the lag time, τ , is the intercept of the baseline with the slope of the curve; π_{eq} is the equilibrium surface pressure.

1 μ g for SP-B and 4 μ g for SP-C [18], smaller amounts of protein could be detected. In heavy surfactant subtypes SP-B and SP-C peaks could be detected in as little as 90 nmol of phospholipid. These results indicate that levels in light subtype are much less than 2.8 ng/nmol of phospholipid and much less than 8.4 ng/nmol of phospholipid for SP-B and SP-C respectively. SP-A levels had previously been determined; BAL contained 28 ± 10 ng/nmol of phospholipid and heavy subtype contained 45 ± 0 ng/nmol of phospholipid. SP-A could not be detected in the light subtype [21].

Figure 4 Lag time and $1/AR$ are linearly correlated for both natural (+) and extracted (Δ) surfactant

Parameters were collected from pressure/time curves as represented in Figure 3. The correlation coefficient, χ^2 , is 0.93 ($n = 35$) for natural surfactant and 0.91 for extracted surfactant ($n = 26$). Phospholipid (20 nmol per assay) was injected into the subphase, resulting in a final concentration of 3.6 μ M phospholipid.

Morphology of surfactant subtypes

Several surfactant structures, i.e. tubular myelin, lamellar bodies and vesicles, were present in the BAL (Figure 1A). Subsequently, heavy and light subtypes were isolated by differential centrifugation. The pellet contains the heavy subtype and the supernatant the light subtype. The light subtype consisted of small vesicles, of which most were found to be unilamellar (Figure 1C). The heavy subtype comprised predominantly tubular myelin and lamellar bodies; vesicles were almost absent from this fraction (Figure 1B). As described in the Materials and methods section the heavy subtype pellet had to be resuspended for surface area cycling. Resuspension seriously affected the morphology of the heavy subtype. It resulted in dilatation and unfolding of lamellar bodies. Tubular myelin was unaffected (Figure 1D). After surface area cycling, the surfactant consisted of parallel membranes and small multilamellar structures (Figure 1E). Separation of the light subtype generated, at this point named the cycled light subtype, from the remaining heavy subtype was performed by differential centrifugation. The cycled light subtype and the remaining heavy subtype (cycled heavy) revealed little morphological difference. Both consisted of large parallel membrane structures (Figures 1F and 1G).

Pulsating-bubble surfactometry

The surface activity of the heavy surfactant subtype varied between 29 ± 4 and 8 ± 8 mN/m at maximum and minimum bubble sizes respectively (Figure 2a). The light surfactant subtype remained at high surface tensions from 53 ± 2 to 25 ± 4 mN/m (Figure 2b). The cycled heavy subtype was still capable of reaching low values; the cycled light subtype remained at high surface tension (Figures 2c and 2d).

Adsorption at the air/water interface

Adsorption experiments were performed for all surfactant subtypes, both extracted and natural, by injecting 20 nmol of phospholipid into the 5.5 ml subphase (final concentration 3.6 μ M). The curves of the surface pressure against time thus recorded were sigmoidal and showed a lag (Figure 3). Three parameters are indicated: the lag time, τ , the adsorption rate,

Figure 5 AR values of natural (●) and lipid extract (○) BAL are not significantly different and are proportional to their concentrations

AR values were collected as shown in Figure 3. The correlation coefficient $\chi^2 = 0.93$.

Table 1 AR values for natural and lipid-extracted surfactant subtypes at a concentration of 3.6 μ M phospholipid

	Adsorption rates (mN/m per s)	
	Natural surfactant	Extracted surfactant
BAL	0.09 ± 0.04	0.070 ± 0.002
Heavy subtype	0.02 ± 0.02	0.03 ± 0.01
Light subtype	0.03 ± 0.01	0.02 ± 0.01
Cycled heavy subtype	0.02 ± 0.01	0.03 ± 0.01
Cycled light subtype	0.03 ± 0.01	0.03 ± 0.01
Heavy + light subtypes	0.015 ± 0.010	—

AR, and the equilibrium surface pressure, π_{eq} . The lag time τ (in s) is the intercept of the baseline of the curve with the tangent to the curve at its point of inflection. AR is the tangent coefficient (in mN/m per s) and π_{eq} is the value of the surface pressure at elongated times. We focused our attention on AR and τ . An analysis of results thus collected showed a clear correlation between τ and AR. Figure 4 shows that τ is linearly correlated with $1/AR$. This observation indicates that one of the two kinetic parameters is sufficient to characterize the adsorption process. In what follows we discuss the curves in terms of the AR parameter only. The correlation depends on how the samples were prepared. The linear regression equation for natural surfactants is described by a coefficient of 2; that for extracted surfactant has a coefficient of 5.

We studied the changes in AR values as a result of increasing concentration of both natural and extracted BAL. A linear correlation was found between the phospholipid concentration and the AR for both natural and lipid-extracted BAL (Figure 5). The two curves did not differ significantly.

Experiments including natural surfactant subtype fractions revealed that BAL had the highest AR values, indicating that it adsorbs readily on the surface (Table 1). When BAL is separated into heavy and light, the AR decreases. Reconstituted BAL, containing 65 % heavy subtype and 35 % light subtype, gave a low AR (0.015 mN/m per s). Further treatment by cycling and subsequent separation into cycled heavy and cycled light subtypes had no effect on AR. Similar tendencies were observed when the surfactant subtypes were extracted (Table 1).

DISCUSSION

Surfactant subtypes and *in vitro* conversion

Surface area cycling is a model *in vitro* of extracellular surfactant metabolism. During cycling the heavy subtype is converted into the light subtype. The amount of light subtype thus generated can be influenced by a number of parameters: temperature, surface area change, time and pH [15,22]. One factor that has remained unattended by many investigators is the handling of the heavy subtype. Our results illustrate that pretreatment determines the yield of light subtypes generated by cycling. This might provide an explanation for the different yields mentioned by other investigators, although the possibility cannot be excluded that the animal species used might also influence the degree of conversion. For example, surface area cycling of heavy subtype frozen at -20°C resulted in a 50 % yield of light subtype [13]. In contrast, for heavy subtype isolated from rabbits, conversions of 38 % were found [23]. We provide evidence that handling of the heavy subtype before surface area cycling does influence the degree of conversion. We performed surface area cycling on freshly isolated large aggregates and found $10 \pm 6\%$ light subtype, whereas freezing in liquid nitrogen resulted in $29 \pm 12\%$ light subtype. This increased conversion may result from damage to surfactant aggregate structures as a consequence of freezing. Ice crystals formed during freezing might destroy the large aggregated structures, increasing the conversion into small structures. Also lamellar bodies are known to be very sensitive to freezing. The conclusion can be drawn that to secure a good reproduction of the situation *in vivo*, freezing the samples should be avoided.

Surfactant proteins in surfactant subtypes

Surfactant proteins B and C were measured by a new quantitative method for the determination of surfactant proteins B and C in lipid-extracted surfactant [18]. The heavy surfactant subtype is defined as the SP-A rich subtype [10,13]; the light surfactant subtype has been described as devoid of SP-A and SP-B [13]. Our results are in agreement with these observations, which were obtained by applying other non-quantitative methods. The heavy subtype contained three times as much SP-C as SP-B. The SP-B and SP-C contents in the light subtype were less than 2.8 ng/nmol of phospholipid and 8.4 ng/nmol of phospholipid respectively.

Morphology of surfactant subtypes

Surfactant subtypes can be isolated by two different methods: by differential centrifugation [12,13] or by density-gradient centrifugation [11]. Workers in the field disagree over the use of these methods. Some prefer differential centrifugation for models of lung injury because inflammatory proteins can associate with the subtypes and alter their densities [14]. Furthermore a shift in density can also be induced by the reaction of the toxic agent with the surfactant. Our morphological results support the hypothesis that differential centrifugation is a useful tool for separating the heavy and light subtypes from freshly isolated lavage.

For further assessment of the surfactant the *in vitro* model for extracellular surfactant metabolism is often applied. To perform surface area cycling, the pellet of heavy surfactant subtype has to be resuspended. Our electron micrographs reveal that resuspension of the isolated heavy subtype affects its morphology. Large aggregated structures such as lamellar bodies unfold into membrane sheets. Surface area cycling gave rise to further degradation of the heavy subtype. Tubular myelin was converted

into parallel membrane sheets. Separation of the remaining heavy subtype from the generated light subtype gave two subtypes with few morphological differences. Our results show a disparate subtype; it has dilated lamellar bodies and unfolded tubular myelin and comprises a considerable number of concentric membranes.

Surface area cycling was first described and evaluated by Gross and Narine [15], using a continuous density gradient for the isolation of heavy and light subtype. Conversion from heavy subtypes to light subtypes took place by a quantized change in density and not by a continuous shift in density. The light subtype thus generated was morphologically identical with the light subtype freshly isolated from lung lavage. Unfortunately, the morphology of the heavy subtype remaining after conversion *in vitro* was not shown in that study [15]. Differential centrifugation, combined with conversion *in vitro*, was first evaluated by Veldhuizen et al. [13] as an alternative to the laborious separation of surfactant subtypes by density gradients. Their results do not reveal morphological changes as a result of resuspension of the heavy subtype. Results reported by Hall et al. [14] are in line with ours. They isolated the heavy subtype by differential centrifugation and resuspended it for subsequent analysis on a sucrose gradient. Their results showed that most of the material was present at a buoyant density from 1.02 to 1.06 g/ml, thus including the lighter subtypes. Most probably this shift in buoyant density originated from the degradation of large aggregate structures produced by resuspension.

These and our findings raise some questions as to whether differential centrifugation is to be recommended for the separation of subtypes if subsequent surface area cycling will be performed. The observed effects complicate the extrapolation to the situation *in vivo*. Therefore it is better to prevent resuspension of the pellet of the heavy subtype. A continuous sucrose gradient separation must be avoided in models of lung injury for the reasons explained above. Sucrose gradient centrifugation with a discontinuous gradient to isolate all material between 1.08 and 1.04 g/ml in one band might be a good alternative in models of lung injury.

Pulsating-bubble surfactometry

Lipid-extracted surfactant revealed a 'poor surfactant function' under pulsating-bubble surfactometry. Acetone precipitation fully restored the surface activity of extracted surfactant (results not shown). Recently, Wang et al. [24] have shown lipid-extracted surfactant to reach surface tensions of approx. 10 mN/m after 5 min (100 cycles) in a pulsating bubble. Lipid-extracted surfactant devoid of neutral lipids reached a tension of near zero after 5 min [24]. Surfactometry is frequently performed on lipid extracts obtained by acetone precipitation, which improves surface activity [13,25]. The composition of the surfactant lipids might be affected in toxic lung injury, so acetone precipitation should not be used in lung injury studies.

The light and heavy surfactant subtypes possess the surface activity expected on the basis of their definition. The cycled heavy subtype showed a good surfactant function; the cycled light subtype reached lower surface tensions, but could still be defined as not surface active. Our results on surface activity do not indicate abnormalities of the cycled heavy and cycled light subtypes, although the morphologies of these subtypes were seriously affected.

Adsorption of surfactant

The adsorption process might consist of at least two processes. Once the sample has been injected, material is transported

through the bulk liquid to accumulate at the air/liquid interface. The subsequent process includes spreading of the material along the surface, generating the surface pressure we observed. The two parameters, τ and AR, represent these processes. As during transport (through the bulk liquid) an increase in surface pressure is absent, τ is a measure of the rate of transport and other processes before spreading along the surface. A lag time was observed only when low surfactant concentrations were used [26,27]. AR clearly represents the process of spreading. The proportionality of τ to $1/AR$ indicates the coupling of these two processes.

Extracted surfactant has a lag time 2–3-fold longer than natural surfactant at a concentration of $3.6 \mu\text{M}$ phospholipid. Recalling that τ represents the rate of processes before spreading along the surface, we conclude that in natural surfactant subtypes these processes proceed faster than in extracted subtypes. The increased lag time might originate from the altered composition as a consequence of lipid extraction. Therefore we conclude that the hydrophilic components or specific structures present in the natural surfactant contribute predominantly to the processes before spreading. Natural surfactants include tubular myelin, which contains SP-A [28]. SP-A is known to enhance adsorption of lipids along the air/liquid interface in the presence of hydrophobic surfactant proteins [4,29,30]. SP-A added to lipids generates multilamellar structures. Both SP-A and SP-B are required for the formation of tubular myelin [31,32]. Schürch et al. [30] have shown that at low surfactant concentrations small amounts of SP-A can enhance lipid adsorption. These additive effects of SP-A might only be of relevance when surfactant concentrations are low. Lipid extract vesicles form a surface film after association with the interface [33]. SP-A might facilitate this process. At high concentrations the increased collision frequency of the vesicles is sufficient to form a surface film and to decrease τ . Both natural and lipid-extracted surfactants adsorb in a concentration-dependent manner. In view of the proportionality of $1/AR$ to τ , higher concentrations of surfactant give higher AR values and decrease the lag time. This corresponds to the mechanism explained above in which the lag time is influenced by the concentration of material in the bulk liquid. The broad scatter of data for the natural surfactants might result from the heterogeneous nature of the material.

Clearly a critical concentration of the material at the air/water interface must be reached to induce surface film formation. Once spreading has been initiated, insertion of lipids into the surface film takes place at equal rates when lipid-extracted and natural surfactant are compared. The initial AR values might therefore depend predominantly on the activity of the hydrophobic components of the surfactant.

Comparison of the AR values of the natural surfactant subtypes revealed some remarkable features: BAL has the highest AR of all the subtypes. Separation of BAL in heavy and light fractions results in two subtypes with a lower AR values. The low AR values of reconstituted BAL surfactant are not in line with synergism of these two subtypes in the BAL. Most probably the isolation procedures affect the absorption behaviour of the subtypes. The morphology of the heavy subtype was seriously affected by resuspension: lamellar bodies disintegrated into membrane sheets (Figure 1D), and this could affect the initial AR. In contrast, the activity of the heavy subtype measured by pulsating-bubble surfactometry was not equal to that of the light subtype. This suggests that the surface activity of the heavy subtype is higher than that of the light subtype if a surface film is present at the interface. Surface-film-associated SP-B and SP-C bind vesicles and insert lipids into the monolayer [3,33]. Owing to the presence of the hydrophobic proteins, more lipids are

associated with the surface film in the heavy subtype. In the adsorption studies by the Wilhelmy plate method the interface was empty and of constant size. The hydrophobic surfactant proteins are active only when present at the interface. Therefore the initial adsorption to an empty interface is not dependent on the activity of SP-B and SP-C. At later times the presence of SP-B and SP-C at the interface contributes to the AR. The cycled heavy and cycled light subtypes both express the same low AR values. This corresponds to the limited difference in the morphologies of these surfactant subtypes. The protein compositions of the light and cycled light subtypes differ: SP-A could be detected in the cycled light subtype but not in the light subtype [21]. This indicates once more that surface area cycling *in vitro* might not fully reflect the situation *in vivo*. Similar tendencies in the AR of lipid-extracted surfactant subtypes were found. These results emphasize again that extraction affects only the processes before spreading along the surface. These latter processes are dependent on the presence of SP-A, and thus aggregate forms, and the vesicle concentration.

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