Dipalmitoylphosphatidylcholine and cholesterol in monolayers spread from adsorbed films of pulmonary surfactant

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Abstract **Pulmonary surfactant forms a surface film that consists of a monolayer and a monolayer-associated reservoir. The extent to which surfactant components including the main component, dipalmitoylphosphatidylcholine (DPPC), are adsorbed into the monolayer, and how surfactant protein SP-A affects their adsorptions, is not clear. Transport of cholesterol to the surface region from dispersions of bovine lipid extract surfactant [BLES(chol)] with or without SP-A at 37°C was studied by measuring surface radioactivities of [4-^{14}C]cholesterol-labeled BLES(chol), and the Wilhelmy plate technique was used to monitor adsorption of monolayers. Results showed that transport of cholesterol was lipid concentration dependent. SP-A accelerated lipid adsorption but suppressed the final level of cholesterol in the surface. Surfactant adsorbed from a dispersion with or without SP-A was transferred via a wet filter paper to a clean surface, where the surface radioactivity and surface tension were recorded simultaneously. It was observed that 1) surface radioactivity was constant over a range of dispersion concentrations; 2) cholesterol and DPPC were transferred simultaneously; and 3) SP-A limited transfer of cholesterol. These results indicate that non-DPPC components of pulmonary surfactant can be adsorbed into the monolayer. Studies in the transfer of [1-^{14}C]DPPC-labeled BLES(chol) to an equal or larger clean surface area revealed that SP-A did not increase selective adsorption of DPPC into the monolayer. Evaluation of transferred surfactant with a surface balance indicated that it equilibrated as a monolayer. Furthermore, examination of transferred surfactants from dispersions with and without prespread BLES(chol) monolayers revealed a functional contiguous association between adsorbed monolayers and reservoirs.**—Yu, S-H., and F. Possmayer. **Dipalmitoylphosphatidylcholine and cholesterol in monolayers spread from adsorbed films of pulmonary surfactant.** ***J. Lipid Res.*** **2001.** 42: **1421–1429.**

Supplementary key words air/water interface • cholesterol • DPPC • L-B film • SP-A

Pulmonary surfactant is a mixture of 90 wt% lipids and 10 wt% proteins (1) that forms a thin layer lining the alveolar epithelium (2). The main function of pulmonary surfactant is to reduce surface tension at the air/alveolar interface, thereby preventing collapse of the lung during expiration (3, 4). Dipalmitoylphosphatidylcholine (DPPC) is the only major component of pulmonary surfactant (~40 wt%) that is capable of reducing the surface tension at the air/alveolar interface to near zero during compression (5). As a result it is generally accepted that the ability of surfactant films to attain low surface tension involves DPPC enrichment during film compression. At physiological temperatures DPPC alone adsorbs slowly, while pulmonary surfactant adsorbs readily. This indicates that minor surfactant components are important for DPPC adsorption. It has been shown that the surface film formed from a dispersion of pulmonary surfactant is composed of a monolayer and a functional monolayer-associated surfactant reservoir (6, 7). Although the composition of the adsorbed monolayer has not been determined, results from surface tension-surface area isotherms of adsorbed surfactant films suggest that the surface monolayer may be enriched in DPPC by preferential adsorption of DPPC (3, 4, 8). The extent to which components other than DPPC are adsorbed into the monolayer is not clear.

Pulmonary surfactant proteins (SP) consist of two small hydrophobic proteins, SP-B and SP-C, that amount to about 2 wt% of total surfactant (9, 10), and two hydrophilic glycoproteins, SP-A and SP-D (11, 12). SP-A is the most abundant protein in the surfactant with a monomeric molecular mass of 28,000–36,000 Da (13). In its native state, SP-A exists as an octadecamer of ~650,000 Da (14). This intriguing protein has a high affinity for DPPC (15) and possesses diverse functions in the surfactant system (16, 17). With respect to the biophysical functions of surfactant, SP-A interacts with the head groups of DPPC and aggregates these molecules in spread monolayers of DPPC (18). SP-A facilitates surface tension lowering of DPPC spread monolayers (18) and increases the adsorption rate

Abbreviations: BLES, bovine lipid extract surfactant; DPPC, dipalmitoylphosphatidylcholine; L-B film, Langmuir-Blodgett film; SP, surfactant-associated protein.

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of pulmonary surfactants (6). SP-A also interacts with gel-like regions of spread monolayers of porcine surfactant lipid extract, resulting in reorganization of rigid-phase lipid (19). SP-A improves the surface activity of bovine lipid extract surfactant (BLES), but whether this is the result of selective adsorption of DPPC into the monolayer is not clear (20).

BLES(chol) is a bovine pulmonary surfactant extract depleted of SP-A. Although the cholesterol content in BLES(chol) is low (~4 wt%), this sterol is able to cooperate with exogenous SP-A to enhance the accumulation of DPPC-rich layers under the interface (6). It is known that cholesterol preferentially interacts with DPPC over fluid PC (21). Cholesterol packs in the intermolecular cavities of DPPC (22) and cannot be squeezed out readily from DPPC/cholesterol spread monolayers during compression (18). SP-A is able to impede the interaction of cholesterol with DPPC in DPPC/SP-A/cholesterol spread monolayers and decrease the surface area reduction required to reach near zero surface tension (18).

Previously, using [26-^{14}C]cholesterol-labeled BLES(chol), we have shown that SP-A decreased the detectable surface radioactivity of [^{14}C]cholesterol in the surface films formed from dispersions of BLES plus exogenous SP-A and cholesterol (23). Because a surface film is composed of a monolayer as well as the reservoir and the surface radioactivity detected arose mostly from the monolayer-associated reservoir (6), we were unable to determine whether cholesterol was adsorbed into the monolayer or the content of cholesterol in the monolayer was affected by SP-A. In the present study we attempt to isolate monolayers from the adsorbed films by the wet bridge technique, developed by Schindler (24) and Heyn, Egger, and Gaub (25) to transfer surfactants adsorbed from dispersions to clean surfaces. Our results show that the properties of the transferred surfactants are consistent with the formation of monolayers. We have used this approach to examine further the effect of SP-A on the adsorption of cholesterol and DPPC.

MATERIALS AND METHODS

Materials

[4-^{14}C]cholesterol and [1-^{14}C]DPPC were purchased from New England Nuclear (Boston, MA). All reagents (analytical grade) were obtained from BDH (Poole, UK). Concentrations of ^{14}C radioactivity were verified with a scintillation counter (LS 6000 JC; Beckman, Fullerton, CA). Distilled water purified through a Millipore (Danvers, MA) Milli-Q four-cartridge system was used in all experiments.

Purification of bovine SP-A

SP-A was purified by HPLC as described previously (6). Briefly, 10 mg of bovine pulmonary surfactant was suspended in 1 ml of 6 M urea-0.05% trifluoroacetic acid (TFA) and filtered. A 200 μl filtrate was applied into a 30 × 38 cm C_{18} reversed-phase column. SP-A was eluted with a linear gradient of 2-propanol in 0.05% TFA at a flow rate of 0.8 ml/min.

Preparation of BLES(chol)

BLES(chol) was produced by chloroform-methanol extraction of bovine pulmonary surfactant, using the method of Bligh and Dyer (26). BLES(chol) retains all lipid components of the surfactant and surfactant proteins, SP-B and SP-C, but not SP-A or SP-D (6).

Preparation of samples

A desired amount of BLES(chol) was mixed with [4-^{14}C]cholesterol or [1-^{14}C]DPPC in chloroform–methanol 1:1 (v/v). The solvent was evaporated under N_2 and the residue was hydrated with 37°C saline-1.5 mM $CaCl_2$ (1 mg/100 μl), with or without 5 wt% SP-A. The suspension was shaken with a wrist shaker at room temperature for 60 min and incubated at 37°C for 2–3 h. The specific radioactivity of [4-^{14}C]cholesterol-labeled BLES(chol) samples was 1.5 μCi/mg lipid and that of [1-^{14}C]DPPC-labeled BLES(chol) samples was 2.8 μCi/mg lipid.

Studies of lipid adsorption and transport of cholesterol to the surface region

Adsorption of lipid to the air/water interface was studied by the Wilhelmy plate technique (6). Transport of cholesterol from a [4-^{14}C]cholesterol-labeled BLES(chol) dispersion to the surface region was monitored by measuring the surface radioactivity of [4-^{14}C]cholesterol with a B1 Bicron scintillation probe having a 2.5-cm-diameter window (Labserco, Ontario, Canada). The probe was positioned 5 mm above the surface of a dispersion in a round Teflon dish (2.5 cm in diameter), containing 4 ml of saline-1.5 mM $CaCl_2$. Samples, preincubated at 37°C, were injected through an injection hole into the dish that was sitting in a water bath (37 ± 0.5°C) enclosed within a temperature-regulated box (37 ± 0.5°C).

Studies of transfer of surfactant to a clean surface

Two Teflon dishes, sitting in a 37°C water bath, with diameters of 2.5 cm (D1 with a surface area of 4.9 cm^2, holding 4 ml of saline-1.5 mM $CaCl_2$) and 5 cm (D2 with a surface area of 19.6 cm^2, 11 ml of saline-1.5 mM $CaCl_2$), were bridged with a 1.3 × 3.5 cm^2 strip of ashless filter paper that was suspended with a Teflon tape-wrapped wire. The B1 Bicron scintillation probe was positioned 5 mm above the subphase of D2 and a 5-mm-wide platinum plate was dipped into D2. After 30 min of equilibration, a 37°C preincubated sample was injected into D1 in all experiments for [4-^{14}C]cholesterol-labeled BLES(chol) and for some experiments with [1-^{14}C]DPPC-labeled BLES(chol). Both surface radioactivity and surface tension were recorded simultaneously with a dual recorder. All experiments were performed in a temperature-controlled box at 37 ± 0.5°C. In some experiments with [1-^{14}C]DPPC-labeled BLES(chol), two dishes with an equal surface area (D1 and D2 having a diameter of 4 cm and a surface area of 12.6 cm^2) were used.

Langmuir-Blodgett (L-B) films and autoradiography

L-B films were deposited from [4-^{14}C]cholesterol-labeled samples on 1 × 1 cm^2 microscope glass coverslips at 37°C as described previously (6). Surface films formed from dispersions of BLES(chol) in D1 or transferred surfactants in D2 were deposited after the surface radioactivity became constant. Surface tension was maintained at ~24 mN/m during deposition. Autoradiographs were obtained by exposing the L-B films to X-ray films for 7–10 days at 4°C.

Surface properties of the transferred surfactants

Surfactant was transferred from a 0.2 mg/ml dispersion of BLES(chol) in a dish with a surface area of 4.9 cm^2 to a clean surface (13 cm^2) on the rhomboid surface balance with a strip of filter paper (1.3 × 3.5 cm^2). The change in surface tension at the interface of the balance was monitored by the Wilhelmy plate technique. The filter paper was removed 40 min after the equilibrium surface tension (~24 mN/m) was established at the

interface of the surface balance. The surface tension increased slightly to 28–29 mN/m on removal of the filter paper. The equilibrium surface tension was re-established by compressing the balance about 2 mm. Expansion of the surface was performed at a speed of 5 mm/min (2 cm^2/min) for 1 min, with several repetitions at 10-min intervals. Surface tension at the interface of balance was recorded continuously. All experiments were performed at 37 ± 0.5°C.

Transfer of surfactant from dispersions of BLES(chol) injected under prespread monolayers of BLES(chol) to a clean surface

Two Teflon dishes with the same surface area of 12.6 cm^2 or different surface areas of 4.9 cm^2 (D1) and 19.6 cm^2 (D2) containing saline-1.5 mM $CaCl_2$ were bridged with a strip of filter paper (1.3 × 3.5 cm^2). The B1 scintillation probe was positioned 5 mm above D2 and the platinum plate was also dipped into D2. The filter paper was lifted up from D2 after 30 min of equilibration. A sample of 5 μl of 1 mg/ml unlabeled BLES(chol) in hexane–methanol 95:5 (v/v) was spread on D1 to form a monolayer with a surface tension of 24–25 mN/m. Ten minutes after spreading, a sample of 37°C preincubated [1-^{14}C]DPPC-labeled BLES(chol) was injected into D1 and the filter paper was subsequently returned to D2. Surface radioactivities and surface tensions were monitored simultaneously when the surface area of D2 was 19.6 cm^2, while separate recording experiments were performed when the surface area of D2 was 12.6 cm^2. The changes in surface tension in D1 were also recorded in separate experiments. All experiments were performed at 37 ± 0.5°C and the concentration of all samples in D1 was 0.15 mg/ml with a specific radioactivity of 2.8 μCi/mg lipid.

RESULTS AND DISCUSSION

Transport of cholesterol from BLES(chol) to the surface region

The radiation-emission range of ^{14}C in water is 2 × 10^6 to 3 × 10^6 Å, which is considerably longer than the thickness of a phospholipid monolayer (~20 Å). Consequently a large proportion of the surface radioactivity detected can be from ^{14}C-labeled BLES(chol) below the monolayers (6). Therefore, we refer to material detected by the scintillation probe as being in the surface region.

Transport of cholesterol from BLES(chol) to the surface region was studied by labeling BLES(chol) with [4-^{14}C]-cholesterol. Dashed lines 2–4 in **Fig. 1A** represent surface radioactivities from 0.1, 0.15, and 0.25 mg/ml BLES(chol). Transport of cholesterol was lipid concentration dependent. Surface radioactivities from dispersions of 0.15 and 0.25 mg/ml increased rapidly, followed by a slower phase of augmentation. The appearance of surface radioactivity was much slower at the lowest concentration (0.1 mg/ml). Dashed line 1 in Figure 1A showed that cholesterol alone did not move to the surface region, but was transported to the surface by moving along with other surfactant components.

The Wilhelmy plate technique was used to examine the surface-active properties of BLES(chol) in separate experiments. Solid lines 1 and 2 in Fig. 1A represent the adsorption isotherms for 0.1 and 0.25 mg/ml BLES(chol), respectively. These curves represent the rate of monolayer formation, which is lipid concentration dependent. The

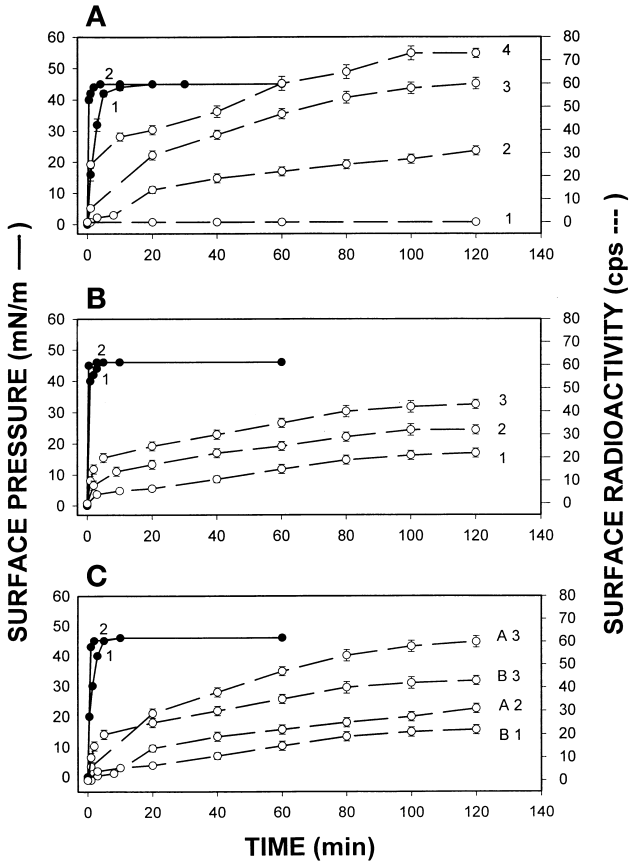

Fig. 1. A: Comparison of lipid adsorption and transport of cholesterol to the surface region from dispersions of BLES(chol) at 37°C. The two solid lines represent adsorption curves of lipid from BLES(chol) with final concentrations of *1*) 0.1 and *2*) 0.25 mg/ml saline-1.5 mM $CaCl_2$. Dashed line 1 shows a sample of 50 μg of [4-^{14}C]cholesterol-labeled cholesterol alone injected into 4 ml of saline-1.5 mM $CaCl_2$. Dashed lines 2–4 show transport curves of [4-^{14}C]cholesterol-labeled BLES(chol) at *2*) 0.1, *3*) 0.15, and *4*) 0.25 mg/ml saline-1.5 mM $CaCl_2$. Means ± SE for three or four experiments are plotted. B: Lipid adsorption and transport of cholesterol to the surface region from BLES(chol) in the presence of SP-A at 37°C. Experiments were performed as in (A). Solid lines 1 and 2 represent adsorption curves of 0.1 and 0.2 mg/ml BLES(chol) plus 5 wt% SP-A, respectively. The three dashed lines represent [4-^{14}C]cholesterol-labeled BLES(chol) with concentrations of *1*) 0.1, *2*) 0.12, and *3*) 0.15 mg/ml, all samples containing 5 wt% SP-A. Means ± SE of three or four experiments are plotted. C: Solid lines represent adsorption curves of 0.15 mg/ml BLES(chol) in the presence (2) and absence (1) of 5 wt% SP-A. Dashed lines show a comparison of surface radioactivities of [4-^{14}C]cholesterol-labeled BLES(chol) with [B1 and B3 from (B)] and without [A2 and A3 from (A)] SP-A at 0.1 and 0.15 mg/ml.

equilibrium surface pressure (46 mN/m) was established rapidly but was independent of lipid concentration. These results indicate that surfactant material continues to accumulate below the monolayers long after the monolayers were formed.

Effect of SP-A on the transport of cholesterol from BLES(chol) to the surface region

Similar experiments were performed as for BLES(chol) but with 5 wt% SP-A incorporated into BLES(chol).

Dashed lines in Fig. 1B represent surface radioactivities from 0.1, 0.12, and 0.15 mg/ml [4-^{14}C]cholesterol-labeled BLES(chol) plus SP-A. Initial transport of cholesterol was lipid concentration dependent and was accelerated by SP-A. The final levels of surface radioactivity were also lipid concentration dependent, but were lower in the presence of SP-A (Fig. 1C). Whether this is an indication of SP-A actually suppressing the transport of cholesterol to the surface region is not clear. This may be related to the ultrastructures of BLES(chol) in the subphase in the presence and absence of SP-A. Previously we have shown in electron micrographs (23) that SP-A affected the ultrastructures of BLES plus exogenous cholesterol.

Solid lines in Fig. 1B represent adsorption curves for BLES(chol) plus SP-A. SP-A accelerated the formation of monolayers. The equilibrium surface pressures are similar in the presence and absence of SP-A. Equilibrium surface pressure was attained much earlier than the final level of surface radioactivities, indicating a gradual accumulation of surfactant material under the monolayer.

Transfer of surfactant from BLES(chol) dispersions to clean surfaces

Surfactant was transferred from a dispersion of BLES(chol) (D1) to a clean surface (D2). The progressive increases in surface pressure and surface radioactivity of the transferred surfactant in D2 were recorded simultaneously. **Figure 2A**, **2B**, and **2C** show the results for surfactants transferred from 0.1, 0.15, and 0.25 mg/ml dispersions of [4-^{14}C]cholesterol-labeled BLES(chol). Solid lines represent the adsorption curves for monolayers. The Wilhelmy plate measures the surface tension at the air/water interface and monitors formation of the monolayer (3). The rate of surfactant transfer to D2 was lipid concentration dependent and was also directly proportional to the area of the paper bridge (27). Under our experimental conditions, transferred surfactants formed from 0.15 mg/ml (Fig. 2B) and 0.25 mg/ml (Fig. 2C) BLES(chol) dispersions attained the equilibrium surface pressure of 46 mN/m within 10 min, whereas the surface pressure of surfactants transferred from 0.1 mg/ml dispersions (Fig. 2A) rose to only 43 ± 1 mN/m (n = 3) even after 60 min of adsorption.

Dashed lines in Fig. 2A, 2B, and 2C represent the corresponding curves for surface radioactivities, which represent the movements of cholesterol from D1 to D2. The trends for surface radioactivity correlated closely with those for surface pressure, especially noticeable at low concentration (Fig. 2A). These results suggest that cholesterol is transferred simultaneously with DPPC and other surfactant lipids from D1 to D2.

Regardless of the dispersion concentration in D1, a constant level of surface radioactivity of 5.2 ± 0.3 cps (~300 cpm) was attained with all transferred surfactants in D2. Moreover, in each case the surface radioactivity became constant at the same time that the equilibrium surface pressure was established. The close similarities of the time courses for surface pressure and surface radioactivity in D2 over a range of dispersion concentrations in D1, and the consistency of the surface radioactivity in D2, led us to hypothesize that the transferred surfactants are present as monolayers. It is unlikely that there is any accumulation of excess surfactant material below the monolayer, otherwise a gradual increase of surface radioactivity in a lipid concentration-dependent manner should be observed (see Fig. 1). These results showed that the adsorbed surface films formed in D1 spread rapidly across the wet paper bridge to the clean surface (D2) until equilibrium surface pressure was achieved in both compartments. As the surface film equilibrates across the bridge, it forms a monolayer and cholesterol is present in that monolayer.

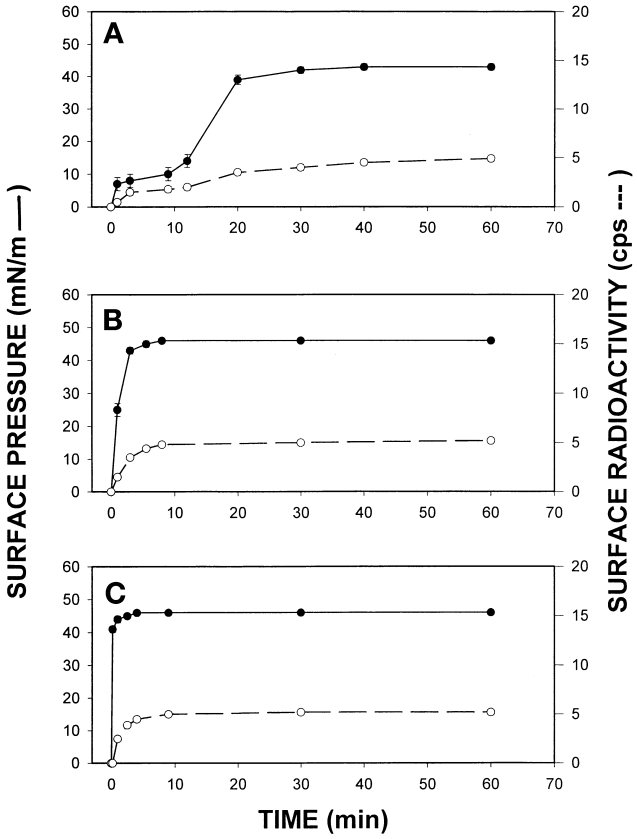

Fig. 2. Lipid monolayers formed from dispersions of BLES(chol) at 37°C. Transfer of surfactant from a [4-^{14}C]cholesterol-labeled BLES(chol) dispersion (D1 with a surface area of 4.9 cm^2) to a clean surface (D2 with a surface area of 19.6 cm^2) was carried out with a strip of filter paper (1.3 × 3.5 cm^2) as detailed in text. Changes in surface radioactivity and surface tension in D2 were recorded simultaneously. Solid lines represent adsorption curves of the transferred lipid and the dashed lines show the transfer of [4-^{14}C]cholesterol to the clean surface. Concentrations of dispersions are as follows: A, 0.1; B, 0.15; and C, 0.25 mg/ml saline-1.5 mM $CaCl_2$. Data represent means ± SE for n ⩾ 3.

Transfer of surfactants from dispersions of BLES(chol) in the presence of SP-A to clean surfaces

Figure 3A, **3B**, and **3C** reveal surface pressures (solid lines) and surface radioactivities (dashed lines) of transferred surfactants (D2) from dispersions (D1) of 0.07, 0.1, and 0.15 mg/ml [4-^{14}C]cholesterol-labeled BLES(chol) plus SP-A. Similar to Fig. 2, surface radioactivity and surface pressure corresponded closely. SP-A accelerated the

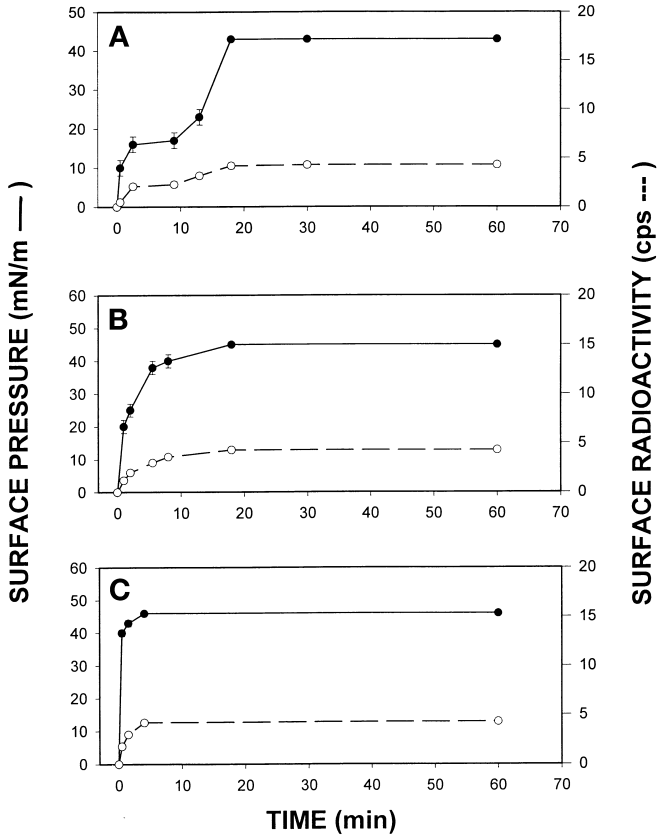

Fig. 3. Lipid monolayers formed from dispersions of BLES(chol) in the presence of SP-A. Experiments were carried out as in Fig. 2. Concentrations of dispersions are as follows: A, 0.07; B, 0.1; and C, 0.15 mg/ml [4-^{14}C]cholesterol-labeled BLES(chol) plus 5 wt% SP-A. The means ± SE for three or four experiments are plotted.

adsorption of surfactant in D1 and the initial appearance of surface radioactivity in D2. The same equilibrium surface pressure (46 mN/m) was observed in the presence and absence of SP-A. However, a consistently lower final surface radioactivity was observed (4.3 ± 0.2 cps or ~250 cpm) in the presence of SP-A with all concentrations of dispersions studied. These observations indicate that monolayers could be formed by transferring surfactants from dispersions of BLES(chol) plus SP-A to clean surfaces. Although the difference in the constant surface radioactivity due to SP-A was modest (~18%), it is highly significant ($P < 0.001$, by two-tailed t test).

SP-A accelerated adsorption of BLES(chol) in D1 and transfer of surfactant to D2 in a parallel fashion. However, the amount of cholesterol in surfactant transferred from dispersions of BLES(chol) plus SP-A appears to be lower than in surfactant derived from BLES(chol), suggesting that SP-A could restrain the adsorption of cholesterol. Previously we have shown that SP-A interacts with the hydrophilic head groups of DPPC in DPPC spread monolayers (18). Electron microscopic studies indicate that SP-A also interacts with the head groups of DPPC in bilayers (28, 29). Although cholesterol interacts with DPPC by packing into the hydrocarbon chains of DPPC molecules, hydrogen bonding between the β-OH of cholesterol and the hydrophilic C=O group of DPPC is essential for these interactions (30). We have presented evidence in earlier studies (18) that SP-A could impede the interaction of cholesterol with DPPC in monolayers formed by successive spreading of DPPC/SP-A/cholesterol. These observations suggest that SP-A competes with cholesterol for DPPC. Consequently, the presence of SP-A in dispersions of BLES(chol) could limit the association of cholesterol with DPPC.

Cholesterol exists as cholesterol-rich (DPPC-poor) and cholesterol-poor (DPPC-rich) domains in DPPC/cholesterol mixed monolayers formed by successive spreading of DPPC and cholesterol (18). Cholesterol also exists as cholesterol-rich and cholesterol-poor domains in biological membranes (31). Autoradiographs of L-B films from the present studies revealed cholesterol-rich domains in transferred surfactants from dispersions with (**Fig. 4A**) and without (Fig. 4B) SP-A. These autoradiographs showed an overall pattern similar to those deposited from dispersions of BLES(chol) in the presence (Fig. 4C) and absence (Fig. 4D) of SP-A. Autoradiographs of L-B films from dispersions were much darker because some surfactant materials under the monolayers were also deposited. The presence of SP-A in dispersions appeared to have no effect on the overall distribution of cholesterol-rich domains in L-B films deposited from transferred surfactants, although the direct measurement of surface radioactivity above the subphase revealed that about 18% less cholesterol was incorporated into surfactants transferred from dispersions with SP-A.

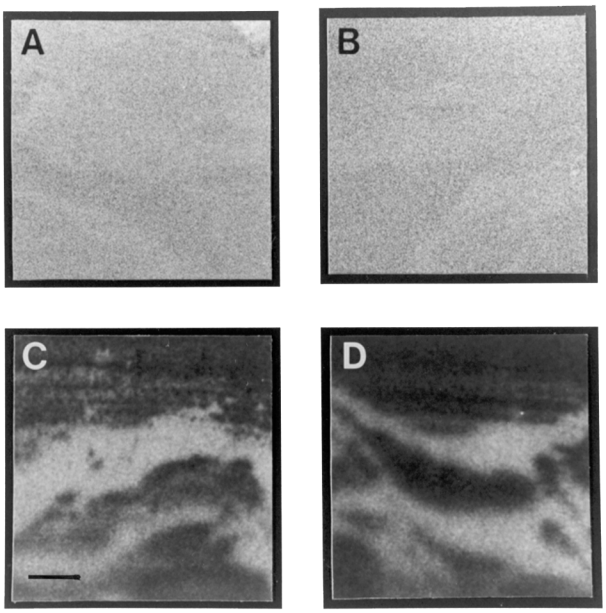

Fig. 4. Autoradiographs of L-B films. L-B films were deposited from transferred monolayers (D2) and dispersions (D1). Films A and B display transferred monolayers from dispersions of [4-^{14}C]-cholesterol-labeled BLES(chol) in the presence (A) and absence (B) of SP-A. Films C and D are deposited from dispersions of [4-^{14}C]-cholesterol-labeled BLES(chol)with and without SP-A. The bar indicates 2 mm for all graphs. The films presented are typical of films obtained from three or more experiments.

Effect of SP-A on transfer of DPPC from dispersions of BLES(chol) to clean surfaces

The presence of DPPC in transferred surfactant was examined using [1-^{14}C]DPPC-labeled BLES(chol) with and without SP-A. Surfactant was spread from a dispersion to a clean surface of equal area (**Fig. 5A** and **5B**) or to a larger clean surface area (D1 = one-fourth D2; Fig. 5C). Concentrations of dispersions were 0.1 mg/ml in Fig. 5A and 0.15 mg/ml in Fig. 5B and 5C. The results showed that the surface radioactivity reached a constant value of 4.2 ± 0.25 cps (~250 cpm) within 10 min for all samples studied. Similar to the cholesterol studies (see Fig. 2), concentrations of dispersions affected only the rate of transfer and not the final constant value of surface radioactivity in Fig. 5A and 5B. Surface pressures of the transferred surfactant correlated with surface radioactivity curves (results not shown). In addition, the time courses of the surface radioactivity curves for the transferred surfactants in D2 were different from those observed for transport of DPPC to the surface region from dispersions of [1-^{14}C]DPPC-labeled BLES(chol) (6). We have previously shown that it takes about 120 min for the surface radioactivity of [1-^{14}C]DPPC-labeled BLES(chol) dispersions to plateau with or without SP-A (6). In those studies the final constant levels of surface radioactivities were lipid concentration dependent. Furthermore, the previously reported values were considerably higher than the surface radioactivities observed for the transferred DPPC in the present studies, although the current studies used a higher specific radioactivity (2.8 vs. 0.5 μCi/mg lipid). The consistency of the surface radioactivities in transferred surfactants from various concentrations of dispersions with and without SP-A, and the close relationship between the surface pressure and surface radioactivity, indicate that transferred surfactants could be monolayers.

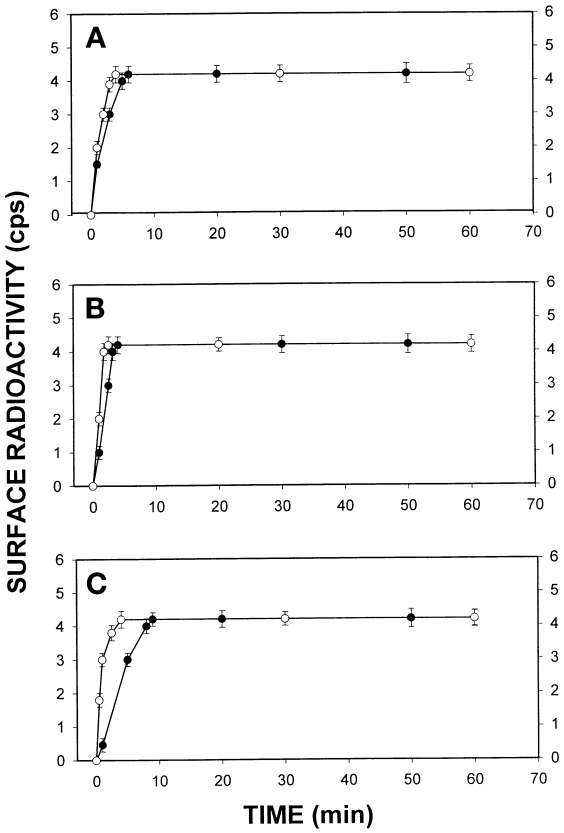

Fig. 5. Effect of SP-A on DPPC in monolayers transferred from dispersions of BLES(chol). Surfactants were transferred from dispersions of [1-^{14}C]DPPC-labeled BLES(chol) in the presence (open circles) and absence (solid circles) of 5 wt% SP-A with a specific radioactivity of 2.8 μCi/mg lipid at 37°C. A bridge of 1.3 × 3.5 cm^2 ashless filter paper was used in all experiments. In graphs A and B, surfactants were transferred from dispersions in a dish 4 cm in diameter (D1 with an surface area of 12.6 cm^2) to a clean surface having the same surface area (D2). The surface radioactivities in D2 were monitored with the B1 Bicron scintillation probe positioned 5 mm above the subphase. The concentration of dispersion in graph A is 0.1 mg/ml saline-1.5 mM $CaCl_2$ and in graph B it is 0.15 mg/ml saline-1.5 mM $CaCl_2$. Experiments in graph C were performed as in graphs A and B, except that surfactants were transferred from dispersions in a dish of 4.9-cm^2 surface area (D1) to a clean surface with an area of 19.6 cm^2 (D2). The concentration of the dispersion was 0.15 mg/ml saline-1.5 mM $CaCl_2$. Data represent means ± SE for n ≥ 3.

SP-A affects the rate of lipid transfer but not the final surface concentration of DPPC in the transferred surfactant. This is consistent with results observed in the transport of DPPC to the surface region from [1-^{14}C]DPPC-labeled dispersions of BLES(chol), where SP-A also accelerated the initial rate of lipid transport to the surface region but did not affect the final constant values of surface radioactivities (6). Interestingly, a similar correlation was also observed for cholesterol studies in which the surface radioactivities from [4-^{14}C]cholesterol were suppressed in the presence of SP-A for both dispersions (Fig. 1) and transferred surfactants (Figs. 2 and 3).

We also observed a similar constant value of surface radioactivity for all transferred surfactants derived from dispersions with equal or one-fourth surface area (Fig. 5), and the constant value was independent of dispersion concentrations. These results show that transferred surfactants reached a constant concentration of surface radioactivity regardless of the surface areas in D2 or the concentrations of dispersions in D1. The ratio of surface area (D1:D2) affects the transfer as shown in Fig. 5; Fig. 5B and 5C derived from the same concentration of dispersions (0.15 mg/ml) with the same (D1 = D2 in Fig. 5B) and 1/4 (D1:D2 = 1:4 in Fig. 5C) surface areas, where the former attained constant surface radioactivity more rapidly than the latter. However, the rate of transfer was not linear with the transferred area, insofar as D1 = one-fourth D2 took about twice as long as D1 = D2 to reach the equilibrium surface pressure (not shown). The effect of SP-A on the rate of transfer was more profound for samples taking longer to reach equilibrium (compare Fig. 5B and 5C). From these observations it appears that the adsorption of surfactant from a dispersion (D1) and transfer of the surfactant from D1 to D2 is a continuous process, until the equilibrium surface pressure in D2 has been established.

Further characterization of the transferred surfactant

To investigate further the characteristics of the transferred surfactant, we transferred the surfactant from a dis-

persion of BLES(chol) to the clean surface of a rhomboid surface balance. Changes in surface tension at the interface of the surface balance were recorded. The filter paper was removed about 40 min after the equilibrium surface tension (~24 mN/m) in the surface balance was reached. The surface tension increased to 28–29 mN/m on removal of the filter paper, and the equilibrium surface tension was restored with a small compression of the balance. Experiments in surface area expansion began at equilibrium surface tension. **Figure 6** shows that surface tension at the interface of the balance increased markedly on expansion of the surface area and stayed constant during intervals where surface area was maintained constant. These results suggest that there was no excess surfactant material associated with the transferred surfactant to be adsorbed into the interface when the compression was halted. This is consistent with the transferred surfactant being a monolayer.

We have obtained the same surface concentration of radioactive DPPC in transferred surfactants from dispersions with equal or one-fourth surface area (Fig. 5). The question of how the transferred surfactant relates to the monolayer adsorbed from the dispersion and the monolayer-associated reservoir in D1 prompted us to pursue further investigation.

In **Fig. 7A** and **7B**, the increases in surface pressure and surface radioactivity of transferred monolayers were recorded in D2 after samples of [1-^{14}C]DPPC-labeled BLES(chol) were injected under prespread monolayers of unlabeled BLES(chol) in D1. Separate experiments depicted in Fig. 7C show the changes in surface pressure that occurred in D1 during such experiments. The initial rapid increases in surface pressure in Fig. 7A and 7B were due to the transfer of prespread monolayers. A corresponding effect was observed in Fig. 7C (D1), where there was a

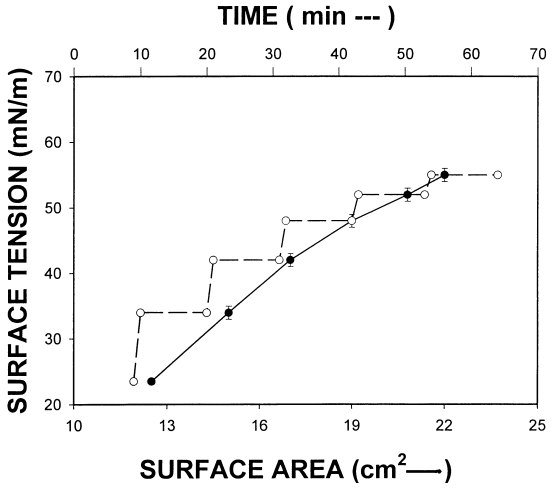

Fig. 6. Surface tension-surface area expansion isotherm of transferred monolayer at 37°C. Surfactant was transferred from a dispersion to the rhomboid surface balance until an equilibrium surface tension of 23–24 mN/m was reached. The surface balance was repeatedly expanded at 5 mm/min for 1 min with 10-min intervals. Surface tension at the interface of the balance was recorded through the experiments. Means ± SE for three experiments are plotted.

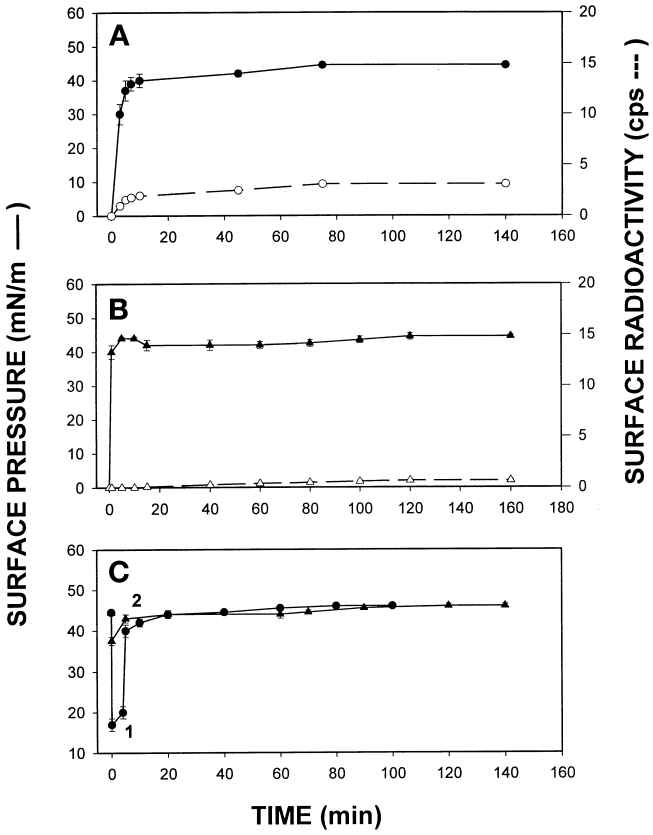

Fig. 7. Lipid monolayers transferred from dispersions with prespread monolayers of BLES(chol) at 37°C. Surfactant was transferred from prespread monolayer of unlabeled BLES(chol) with a dispersion of [1-^{14}C]DPPC-labeled BLES(chol) below (D1) through the filter paper (1.3 × 3.5 cm^2) to a clean surface (D2). In graphs A and B, surface tension (surface pressure) and surface radioactivity were recorded from D2. The surface areas of D1 and D2 in graph A were 4.9 and 19.6 cm^2, respectively, while those in graph B were equal at 12.6 cm^2. The concentration of all samples in D1 was 0.15 mg/ml BLES(chol) with a specific radioactivity of 2.8 μCi/mg lipid. Surface tensions in graph C were recorded from D1 in separate experiments with a BLES(chol) concentration of 0.15 mg/ml for both surface areas of D1 = 1/4 D2 in plot 1 and D1 = D2 in plot 2. All data recorded began with the injection of [1-^{14}C]DPPC-labeled BLES(chol) under a prespread monolayer of unlabeled BLES(chol) in D1 and connection of D1 to D2 with a filter paper, as described in text. Data represent means ± SE for n = 3.

sudden decrease in surface pressure on the connection of D1 to D2, especially for D1 < D2 (Fig. 7C, plot 1). After an initial rapid increase in surface pressure to ~40 mN/m (Fig. 7A and 7B), a much slower transfer of surfactant was observed. It required approximately 80 min for D1 = one-fourth D2 (Fig. 7A) and 120 min for D1 = D2 (Fig. 7B) to reach a surface pressure of 44 mN/m, where surface pressure remained constant for long periods, whereas it took only 10 min or less for D2 to reach an equilibrium surface pressure of 46 mN/m in the absence of prespread monolayers (Fig. 5). Although some clean surface became available quickly in D1 on connection of D1 and D2, adsorption from dispersions (D1) was slow; it reached equilibrium surface pressure of 46 mN/m at about 80 min for D1 = one-fourth D2 (Fig. 7C, plot 1) and 110 min for D1 = D2 (Fig. 7C, plot 2).

Surface radioactivities in Fig. 7A and 7B reveal the transfer of surfactants from the adsorbed monolayers (not prespread monolayers) in D1. Transfers were slow initially, especially for D1 = D2 (Fig. 7B), because of less availability of surface area in D2. Transfers of [1-^{14}C]DPPC represented only about 15% in D1 = D2 (Fig. 7B) and 70% in D1 = one-fourth D2 (Fig. 7A) of those from dispersions without prespread monolayers (Fig. 5B and 5C), indicating that the initial transfer of material was from the unlabeled prespread monolayers.

The time courses and the amounts of radioactive DPPC transferred show that the radioactive surfactant injected below could not associate well with the prespread monolayers in D1, which contrasts with the rapid adsorption and transfer of surfactant observed in the absence of prespread monolayers (Figs. 2, 3, and 5). This is consistent with our previous studies (6) showing that L-B films deposited from [^{14}C]DPPC-labeled BLES(chol) dispersions contain considerably more radioactive material (68 ± 3 cps/cm^2) than those deposited from the same concentration of dispersions with prespread unlabeled BLES(chol) monolayers (7 ± 1 cps/cm^2). The autoradiographs of L-B films from dispersions depicted in Fig. 4C and 4D contain much more radioactive material than transferred surfactants shown in Fig. 4A and 4B. These observations suggest that adsorbed films possess more surfactant material than can be accounted for by a monolayer. Present studies indicate that the excess material associated with the monolayer in adsorbed films can be incorporated rapidly into the monolayer when unoccupied surface space becomes available, such as when D1 and D2 are connected. On the contrary, surfactant dispersions injected under prespread BLES(chol) monolayers interacted slowly with the prespread monolayer upon surface area expansion. The association of injected dispersion with the prespread monolayer is slow despite the presence of SP-B and SP-C in the surface, which can facilitate incorporation of surfactant lipid into the surface (32, 33).

Weibel and Gil (34) have reported the presence of a lamellar layer of phospholipids with three to six repeated distances of 38–51 Å on the alveolar epithelium of rat lungs. Lamellar structures were also observed in alveolar surface film of guinea pigs (7), in pulmonary lipid extract surfactant films (without SP-A) formed in a captive bubble (7), and at the interfaces of calf lung surfactant with or without SP-A (35). Our results can be interpreted as indicating that while the surface monolayers were being transferred to the clean surface, surfactant material from the reservoir was constantly adsorbed into the interface of D1. This would result in the continued "unraveling" of the lamellar layers comprising the reservoir, indicating that adsorbed monolayers are functionally contiguous with underlying lamellar layers. The results are consistent with earlier washout experiments, which demonstrated that after surfactant in the bulk phase was depleted, the monolayer-associated material can be incorporated into the surfactant monolayers during surface expansion (36). Taken together, the present studies and the evidence for lamellar structures at interfaces lead us to conclude that the lipid composition of transferred monolayers is similar to that of adsorbed films.

It is thought that the ability of pulmonary surfactant, which contains 40–50% DPPC, to achieve low surface tension during lateral compression is related to DPPC enrichment of the surface monolayer. A number of studies with reconstituted and lipid extract surfactants have shown that surface activity during initial film compression can be markedly improved by increasing the bulk concentration of the surfactant or by adding SP-A. Such studies have led to the suggestion that high bulk surfactant concentrations or the presence of SP-A during film formation leads to improved formation of a surface-associated reservoir, which promotes selective adsorption of DPPC (3, 8, 37). Although present studies provide strong support for the existence of a monolayer-associated reservoir, they do not provide evidence of selective adsorption of DPPC into the monolayer, indicating that some mechanism other than selective DPPC adsorption is involved in reducing surface tension to low values.

In summary, we have used a wet paper bridge to transfer surfactant from adsorbed films of BLES(chol) to clean surfaces. Experimental evidence suggests that the transferred surfactant forms a monolayer on the clean surface. Varying the concentration of [^{14}C]cholesterol-labeled BLES(chol) affected the transfer rate but not the final surface radioactivity of the transferred surfactant at equilibrium surface pressure, indicating that cholesterol can be adsorbed into the monolayer. SP-A accelerated the rate of surfactant transfer but decreased the final surface radioactivity of [^{14}C]cholesterol at equilibrium surface pressure by 18%. This observation confirms previous studies that indicated that SP-A impedes adsorption of cholesterol in natural surfactant into surface films. Transfer of surfactant from [^{14}C]DPPC-labeled BLES(chol) dispersions with and without SP-A to equal or larger clean surfaces led to identical surface radioactivities at equilibrium over a range of surfactant concentrations. There was no evidence of DPPC enrichment in the adsorbed monolayer with increasing surfactant concentrations or in the presence of SP-A. Prespread monolayers disrupted surfactant transfer from dispersions to clean surfaces. We conclude that the surface monolayer of surfactant film is contiguous with its reservoir.

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REFERENCES

1. Yu, S. H., N. Smith, P. G. R. Harding, and F. Possmayer. 1983. Bovine pulmonary surfactant: chemical composition and physical properties. *Lipids.* **18:** 522–529.
2. Bastacky, J., C. Y. C. Lee, J. Georke, H. Koushafar, D. Yager, L. Kenaga, T. P. Speed, Y. Chen, and J. A. Clements. 1995. Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. *J. Appl. Physiol.* **79:** 1615–1628.

3. Possmayer, F. 1997. Physicochemical aspects of pulmonary surfactant. *In* Fetal and Neonatal Physiology. Chapter 15. R. A. Polin and W. H. Fox, editors. W. B. Saunders, Philadelphia, PA. 1259–1275.
4. Veldhuizen, R., K. Nag, S. Orgeig, and F. Possmayer. 1998. The role of lipids in pulmonary surfactant. *Biochim. Biophys. Acta.* **1408:** 90–108.
5. Notter, R. H. 2000. Lung surfactants: basic science and clinical applications. *In* Lung Biology in Health and Disease. Vol. 149. C. Lenfant, editor. Mercel Dekker, New York.
6. Yu, S. H., and F. Possmayer. 1996. Effect of pulmonary surfactant protein A and neutral lipid on accretion and organization of dipalmitoylphosphatidylcholine in surface films. *J. Lipid Res.* **37:** 1278–1288.
7. Schürch, S., F. H. Y. Green, and H. Bachofen. 1998. Formation and structure of surface films: captive bubble surfactometry. *Biochim. Biophys. Acta.* **1408:** 180–202.
8. Goerke, J. 1998. Pulmonary surfactant: functions and molecular composition. *Biochim. Biophys. Acta.* **1408:** 79–89.
9. Hawgood, S., M. Derrick, and F. Poulain. 1998. Structure and properties of surfactant protein B. *Biochim. Biophys. Acta.* **1408:** 150–160.
10. Johansson, J. 1998. Structure and properties of surfactant protein C. *Biochim. Biophys. Acta.* **1408:** 161–172.
11. McCormack, F. 1997. The structure and function of surfactant protein-A. *Chest.* **111:** 114S–119S.
12. Crouch, E. C. 1998. Structure, biologic properties, and expression of surfactant protein D (SP-D). *Biochim. Biophys. Acta.* **1408:** 278–289.
13. Hawgood, S., and K. Shiffer. 1991. Structures and properties of surfactant-associated proteins. *Annu. Rev. Biochem.* **65:** 441–473.
14. Voss, T., H. Eistetter, and K. P. Schäffer. 1988. Macromolecular organization of natural and recombinant lung surfactant protein SP 28–36. *J. Mol. Biol.* **201:** 219–227.
15. Kuroki, Y., and T. Akino. 1991. Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine. *J. Biol. Chem.* **266:** 3068–3073.
16. Tino, M. J., and J. R. Wright. 1998. Interactions of surfactant protein A with epithelial cells and phagocytes. *Biochim. Biophys. Acta.* **1408:** 241–263.
17. Haagsman, H. P. 1998. Interactions of surfactant protein A with pathogens. *Biochim. Biophys. Acta.* **1408:** 264–277.
18. Yu, S. H., and F. Possmayer. 1998. Interaction of pulmonary surfactant protein A with dipalmitoylphosphatidylcholine and cholesterol at the air/water interface. *J. Lipid Res.* **39:** 555–568.
19. Worthman, L. D., K. Nag, N. Rich, M. L. F. Ruano, C. Casals, J. Pérez-Gil, and K. M. W. Keough. 2000. Pulmonary surfactant protein A interacts with gel-like regions in monolayers of pulmonary surfactant lipid extract. *Biophys. J.* **79:** 2657–2666.
20. Schürch, S., F. Possmayer, S. Cheng, and A. M. Cockshutt. 1992. Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant. *Am. J. Physiol.* **263:** L210–L218.
21. Guyer, W., and K. Bloch. 1983. Phosphatidylcholine and cholesterol interactions in model membranes. *Chem. Phys. Lipids.* **33:** 313–322.
22. Shah, D. O., and J. H. Schulman. 1967. Influence of calcium, cholesterol and unsaturation on lecithin monolayers. *J. Lipid Res.* **8:** 215–225.
23. Yu, S. H., and F. Possmayer. 1994. Effect of pulmonary surfactant protein A (SP-A) and calcium on the adsorption of cholesterol and film stability. *Biochim. Biophys. Acta.* **1211:** 350–358.
24. Schindler, H. 1979. Exchange and interaction between lipid layers at the surface of a liposome solution. *Biochim. Biophys. Acta.* **555:** 316–336.
25. Heyn, S., P. M. Egger, and H. E. Gaub. 1990. Lipid and lipid-protein monolayers spread from a vesicle suspension: a microfluorescence film balance study. *J. Phys. Chem.* **94:** 5073–5078.
26. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
27. Lawrie, G. A., G. T. Barnes, and I. R. Gentle. 1999. Self-assembled films of dimyristoylphosphatidylcholine. *Colloids Surfaces.* **155:** 69–84.
28. Palaniyar, N., R. A. Ridsdale, C. E. Holterman, K. Inchley, F. Possmayer, and G. Harauz. 1998. Structural changes of surfactant protein A induced by cations reorient the protein on lipid bilayers. *J. Struct. Biol.* **122:** 297–310.
29. Palaniyar, N., F. X. McCormack, F. Possmayer, and G. Harauz. 2000. Three dimensional structure of rat surfactant protein A trimers in association with phospholipid monolayers. *Biochemistry.* **39:** 6310–6316.
30. Blockhoff, H. 1974. Model of interaction of polar lipids, cholesterol, and proteins in biological membranes. *Lipids.* **9:** 645–650.
31. Liscum, L., and K. W. Underwood. 1995. Intracellular cholesterol transport and compartmentation. *J. Biol. Chem.* **270:** 15443–15446.
32. Oosterlaken-Dijksterhuis, M. A., H. P. Haagsman, L. M. G. van Golde, and R. A. Demel. 1991. Characterization of lipid insertion into monomolecular layers mediated by lung surfactant protein SP-B and SP-C. *Biochemistry.* **30:** 10965–10971.
33. Oosterlaken-Dijksterhuis, M. A., H. P. Haagsman, L. M. G. Golde, and R. A. Demel. 1991. Interaction of lipid vesicles with monomolecular layers containing lung surfactant protein SP-B or SP-C. *Biochemistry.* **30:** 8276–8281.
34. Weibel, E. R., and J. Gil. 1968. Electron microscopic demonstration of an extracelluar duplex lining layer of alveoli. *Respir. Physiol.* **4:** 42–57.
35. Sen, A., S-W. Hui, M. Mosgrober-Anthoony, B. A. Holm, and E. A. Egan. 1988. Localization of lipid exchange sites between bulk lung surfactants and surface monolayer: freeze fracture study. *J. Colloid Interface Sci.* **126:** 355–360.
36. Qanbar, R., S. Cheng, F. Possmayer, and S. Schürch. 1996. The role of the palmitoylation of surfactant-associated protein C in surfactant film formation and stability. *Am. J. Physiol.* **271:** L572–L580.
37. Veldhuizen, E. J., and H. P. Haagsman. 2000. Role of pulmonary surfactant components in surface film formation and dynamics. *Biochim. Biophys. Acta.* **1467:** 255–270.