Effect of pulmonary surfactant protein A and neutral lipid on accretion and organization of dipalmitoylphosphatidylcholine in surface films

Shou-Hwa Yu[1,*,§] and Fred Possmayer[*,†,§]

Department of Obstetrics and Gynaecology,* Department of Biochemistry,† and MRC Group in Fetal and Neonatal Health and Development,§ The University of Western Ontario, 339 Windermere Road, London, Ontario, Canada N6A 5A5

Abstract The effects of surfactant-associated protein A (SP-A) on lipid adsorption to the air–water interface and accumulation of dipalmitoylphosphatidylcholine (DPPC) in the surface region were investigated at 37°C. Dispersions used were bovine pulmonary lipid extract surfactant with or without neutral lipid (NL). Lipid adsorption was examined with the Wilhelmy plate technique and DPPC accumulation by monitoring surface radioactivity from [^{14}C]DPPC with a scintillation probe. SP-A enhanced the rate of lipid adsorption, while both SP-A and NL increased the extent of DPPC accumulation. At the specific radioactivity used [^{14}C]DPPC monolayers were undetectable: the surface radioactivity arose from surface-associated DPPC beneath the monolayer. At the highest concentration studied (0.3 mg lipid/ml), NL greatly enhanced DPPC accumulation and SP-A attenuated this effect. Langmuir-Blodgett (L-B) films were prepared from [^{14}C]DPPC-labeled dispersions (0.3 mg lipid/ml) at equilibrium surface tension. Autoradiographs of L-B films from lipid extract surfactant exhibited a diffuse misty appearance, while NL promoted formation of heterogeneous DPPC aggregates. Addition of SP-A to lipid extracts without NL generated DPPC aggregates; more uniform larger aggregates appeared in the presence of SP-A and NL. Radiation measurements confirmed that the L-B films were composed of more than monolayers. SP-A did not increase DPPC levels in films deposited from lipid extracts unless NL was present. These results indicate that neutral lipid cooperates with surfactant-associated protein A to organize dipalmitoylphosphatidylcholine in the surface films and enhance formation of a DPPC-rich reservoir below the air–water interface.—**Yu, S-H., and F. Possmayer.** Effect of pulmonary surfactant protein A and neutral lipid on accretion and organization of dipalmitoylphosphatidylcholine in surface films. *J. Lipid Res.* 1996. **37:** 1278–1288.

Supplementary key words surfactant-associated protein A • cholesterol • adsorption • surface tension • Langmuir-Blodgett film

Pulmonary surfactant is essential for normal lung function. Surfactant stabilizes the lung by reducing the surface tension of the alveolus during expiration (1–5). Dipalmitoylphosphatidylcholine (DPPC) is the principal material responsible for reducing the surface tension of the alveolus to very low values. At physiological temperatures, DPPC alone adsorbs and spreads very slowly to form a surface film. However, pulmonary surfactant does so readily. It is believed that the main function of non-DPPC components in pulmonary surfactant is to fluidize DPPC, thereby assisting DPPC adsorption, spreading and respreading (1–5). Nonetheless, their exact functional relationship in pulmonary surfactant is not clear.

Pulmonary natural surfactant consists of approximately 90% lipids and 10% surfactant-associated proteins, namely SP-A, SP-B, and SP-C (6). Phospholipid (PL) accounts for approximately 96% of the total lipids in bovine pulmonary surfactant (7). Of the total PL, 40% is DPPC, 37% unsaturated phosphatidylcholine (PC), 3% dimyristoylphosphatidylcholine (DMPC), 12% phosphatidylglycerol (PG), 2–3% each of phosphatidylethanolamine (PE) and sphingomyelin (7). The amount of neutral lipid in pulmonary surfactants varies from 4% (w/w) in bovine (7) to 7% in canine surfactant (5). Cholesterol is the major component of the neutral lipid (approximately 90% in bovine). This sterol could act as a fluidizer to enhance the adsorption and respreading of DPPC (8, 9). However, cholesterol destabilizes surface films under compression and impairs the surface tension lowering ability of surfactants in the absence of SP-A (10–14).

Approximately 80–90% of the total surfactant protein

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; L-B film, Langmuir-Blodgett film; NL, neutral lipid; PL, phospholipid; bLES, bovine lipid extract surfactant; SP-, surfactant-associated protein.

[1]To whom correspondence should be addressed.

is SP-A. Investigations into the biophysical functions of surfactant proteins have demonstrated that the low molecular weight hydrophobic proteins, SP-B and SP-C, significantly improve the surface activities of surfactants (15–17). SP-A enhances the ability of SP-B to promote PL adsorption and surface tension lowering in the presence of calcium (16–20).

Our previous studies showed that SP-A suppressed the appearance of radioactive cholesterol at the surface and stabilized surfactant films containing cholesterol at surface tensions compressed to below equilibrium (10). These results indicated that SP-A may be able to influence the composition of the lipids in the surface films. Moreover, studies conducted with the captive bubble tensiometer revealed that addition of SP-A to bovine lipid extract surfactant (without neutral lipid) decreased the extent of surface area reduction required to achieve near zero surface tension. This suggested that SP-A either promoted the selective transport of DPPC to the surface monolayer or generated a very effective mechanism for the removal of non-DPPC lipids during lateral compression (21). We have attempted to clarify these suggestions by labeling surfactant preparations with [^{14}C]DPPC and monitoring surface radioactivities. We have also investigated the effects of neutral lipid on the transport of DPPC to the surface region in the presence and absence of SP-A.

For clarification it should be noted that in this paper "adsorption" will be used to indicate monolayer formation and "surface films" will refer to the monolayer plus monolayer-associated surfactant material. Material detected by the surface radioactivity probe will be referred to as being in the surface region.

MATERIALS AND METHODS

Materials

Dipalmitoyl-1-[^{14}C]phosphatidylcholine and [^{14}C]glucose were purchased from New England Nuclear. DPPC was from Sigma. Concentrations of ^{14}C were verified with a scintillation counter (Beckman LS 6000 IC). Purity of radioactive and non-labeled DPPC was confirmed by thin-layer chromatography. Other chemicals and reagents (analytical grade) were from BDH (British Drug House) Inc., Toronto. Cholesterol determination kit was obtained from Boehringer Mannheim, Toronto.

Preparation of lipid extract surfactants bLES and bLES(chol)

In order to distinguish between lipid extract surfactants with and without neutral lipid, we refer to lipid extract surfactant containing neutral lipid as bLES(chol) as cholesterol is the major component of the neutral lipid. bLES(chol) was produced by chloroform–methanol extraction of bovine pulmonary natural surfactant using the method of Bligh and Dyer (22) as described previously (7, 11). bLES(chol) retains all the components of natural surfactant except SP-A. Lipid extract surfactant without neutral lipid (bLES) was obtained (7, 11) by acetone precipitation of bLES(chol). Approximately 10 vol of cold acetone were added to a concentrated chloroform–methanol solution of bLES(chol). The suspension was left at -20°C overnight and the precipitated PL, SP-B, and SP-C were collected by centrifugation. bLES(chol) and bLES were dissolved in chloroform–methanol 9:1 and stored at -20°C. Acetone precipitation resulted in loss of neutral lipid and recovery of approximately 90% of the phospholipid. Compositions of phospholipid and protein were similar before and after acetone precipitation (7). Concentrations of phospholipid in bLES and bLES(chol) were determined by the method of Rouser, Fleischer, and Yamamoto (23). Qualitative analysis of neutral lipid was performed by thin-layer chromatography using a solvent system of chloroform–methanol–water 65:25:4. Cholesterol was determined by an enzymatic method following the manufacturer's instructions: the content found was similar to that obtained from gas–liquid chromatography (7).

Preparation of surfactant-associated protein A (SP-A)

SP-A was isolated from natural surfactant and purified by HPLC as previously described (16). Approximately 10 mg natural surfactant was suspended in 1 ml 6 M urea/0.05% trifluoroacetic acid, centrifuged, and filtered. The clear solution was collected and 200 µl of the solution was applied to a 30 × 3.8 cm C_{18} reverse-phase column (Waters Scientific). SP-A was eluted with a linear gradient of 2-propanol in 0.05% trifluoroacetic acid at a flow rate of 0.8 ml/min. Content of protein was determined through the procedure of Lowry et al. (24). Purity of SP-A was estimated by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis (16).

Preparation of samples

A desired quantity of lipid extract surfactant (mg of PL) in chloroform–methanol was dried under N_2 (in 10 × 70 mm glass tubes) and the residue was hydrated by adding 100 µl saline, 1.5 mM $CaCl_2$ with or without 10% SP-A (% wt of PL). [^{14}C]DPPC-labeled samples were prepared by mixing a desired amount of lipid extract surfactant with [^{14}C]DPPC in chloroform–methanol. The solvent was evaporated under N_2 and the residue was hydrated with 100 µl saline, 1.5 mM $CaCl_2$ with or without 10% SP-A. The mixtures were then shaken with a wrist-action shaker at room temperature for 15 min and incubated at 37°C for 2–4 h. The specific radioac-

tivity in all samples was 0.5 μCi/mg PL. The [^{14}C]glucose solution was prepared by mixing 1.1 μCi [^{14}C]glucose with 2.25 mg glucose in 100 μl H_2O resulting in a specific activity of 0.5 μCi/mg glucose.

Studies on lipid adsorption

The adsorption of lipids to the air–water interface was studied with the Wilhelmy plate technique as described previously (25). Briefly, a sand-blasted platinum plate was dipped into a round Teflon dish (2.5 cm dia. × 2 cm depth) containing 7.5 ml saline, 1.5 mM $CaCl_2$ in a temperature-regulated sand bath (37 ± 1°C) enclosed within a temperature-controlled box (37 ± 1°C). A sample of bLES(chol) or bLES (0.75, 1.50, or 2.25 mg PL) with or without 10% SP-A in 100 μl saline, 1.5 mM $CaCl_2$ was injected into the subphase through an injection hole after 2–4 h of incubation at 37°C. These samples gave final concentrations of 0.1, 0.2, and 0.3 mg PL/ml dispersions in the subphase. Surface tension was monitored with a TSAR 1 computer controlled transducer readout (TECH-SER, Inc., Torrance, CA). Samples were stirred slowly (≈30 rpm) with a magnetic stirrer. Distilled water purified through a Millipore Milli-Q four cartridge system was used. Each experiment was repeated three to four times (n ≥ 4).

Surface radioactivity measurements

Radiation emitted from ^{14}C, in dispersions or solutions, to the air was monitored using a Bicron B_1 scintillation probe (Bicron Corporation, Newbury, OH) coupled to a Berthold Multi-Logger (Labserco Ltd., Oakville, Ontario, Canada). The Bicron B_1 scintillation probe is a detector for alpha, beta, and gamma radiations. It is a 3.5-cm diameter cylinder with 2.5-cm diameter window (area ≈4.8 cm^2). This probe has a detection temperature range of -40°C to 50°C. Efficiency of the probe was determined at room temperature by applying various amounts of [^{14}C]DPPC in chloroform on microscope slide glass cover slips (Fisher Scientific) and measuring the radioactivity by setting the probe on the glass plate after the solvent had evaporated. This positioned the window of the probe about 1.5 mm above the radiation source. An efficiency of 14 ± 1% was observed. The efficiency fell to 4 ± 0.5% when the probe was positioned 8 mm above the plate.

The surface radioactivities of [^{14}C]DPPC-labeled bLES monolayers at equilibrium surface tension with various specific radioactivities of [^{14}C]DPPC were also measured with the B_1 scintillation probe at 37 ± 1°C. The monolayers were formed by spreading [^{14}C]DPPC-labeled bLES, in hexane–methanol 95:5, on a subphase of saline, 1.5 mM $CaCl_2$. In order to prevent condensation of moisture on the window, the probe was positioned 8 mm above the subphase. A linear relationship between specific radioactivities and surface radioactivities was observed with the line passing through the origin.

Studies on transport of dipalmitoylphosphatidylcholine (DPPC) to the surface region

The experiments on transport of DPPC from the surfactant mixtures to the surface region were performed as for the adsorption of lipids, except that instead of surface tension, the surface radioactivity was measured by detecting the radiation emitted from [^{14}C]DPPC to the air with the Bicron B_1 scintillation probe. The probe was positioned 8 mm above the subphase before sample injection. A sample of [^{14}C]DPPC-labeled bLES or bLES(chol), pre-incubated at 37°C for 3 h in the presence or absence of 10% SP-A, was injected into the subphase. The surface radioactivity was recorded until a constant value was obtained. Each experiment was repeated three to five times (n ≥ 4).

Control studies on the radiation emitted from ^{14}C injected below pre-formed monolayers

DPPC or bLES(chol), in hexane–methanol 95:5, was spread on 7.5 ml saline, 1.5 mM $CaCl_2$ in a round Teflon dish (2.5 cm dia. × 2 cm depth) to determine the amounts required to attain the equilibrium surface tension (≈25 mN/m). These amounts of DPPC or bLES(chol) were spread on a clean Teflon dish (2.5 cm × 2 cm) containing 7.5 ml saline, 1.5 mM $CaCl_2$ but without the platinum plate. The B_1 scintillation probe was then positioned 8 mm above the subphase. Twenty minutes after spreading, a sample of bLES(chol) (2.25 mg PL/100 μl saline, 1.5 mM $CaCl_2$ containing 1.1 μCi [^{14}C]DPPC) pre-incubated at 37°C for 3 h was injected into the subphase through the injection hole. The sample was stirred slowly (≈30 rpm) and the radiation emitted to the air was monitored for 2 h. All the experiments were performed at 37 ± 1°C and repeated at least twice (n ≥ 3). Similar experiments were also performed by injecting glucose solution (2.25 mg/100 μl H_2O containing 1.1 μCi [^{14}C]glucose) into the subphase (7.5 ml saline, 1.5 mg $CaCl_2$) with or without pre-spread bLES(chol) at 37 ± 1°C.

Langmuir-Blodgett (L-B) film deposition and autoradiography

The Langmuir-Blodgett film deposition technique is well described (26–28). Normally, an L-B film is made by depositing an insoluble monolayer on a solid substrate from a spread monolayer at a constant surface tension. A dipping procedure is commonly used. When a hydrophilic substrate (e.g., a glass slide) is lowered through an interface covered with a monolayer there is

no transfer, but as the slide is raised a monolayer is deposited with the hydrophilic headgroups on the substrate and the hydrophobic tails oriented toward the air (26–28). In our studies we applied this technique to a dispersion instead of a spread monolayer. Surface films formed from dispersions of [^{14}C]DPPC-labeled bLES or bLES(chol) with or without 10% SP-A were monitored until the surface radioactivity became constant. A precleaned microscope glass cover slip (Fisher Scientific) was further cleaned with chromic acid and thoroughly rinsed with water. The glass plate was lowered into the subphase and raised slowly through the air–water interface. The plate was raised at a speed of 1 mm/min and the area of deposition was 1 cm × 1.5 cm. The concentration of all dispersions in the subphase was 0.3 mg PL/ml saline, 1.5 mM $CaCl_2$. All film depositions were carried out at 37 ± 1°C. During the film deposition, the surface tension at the interface remained at equilibrium due to adsorption of surfactant from the subphase. L-B films were also made from [^{14}C]DPPC-labeled bLES(chol) dispersions injected under pre-formed monolayers and from ^{14}C-labeled glucose solutions with and without pre-spread bLES(chol) as in the control studies (with 0.3 mg/ml glucose in the subphases).

Autoradiographs of L-B films were made by exposing the plates to X-ray films for 48 h at 4°C. The radiation emitted from [^{14}C]DPPC in the L-B films was also measured with the B_1 scintillation probe at room temperature by setting the probe on the plate.

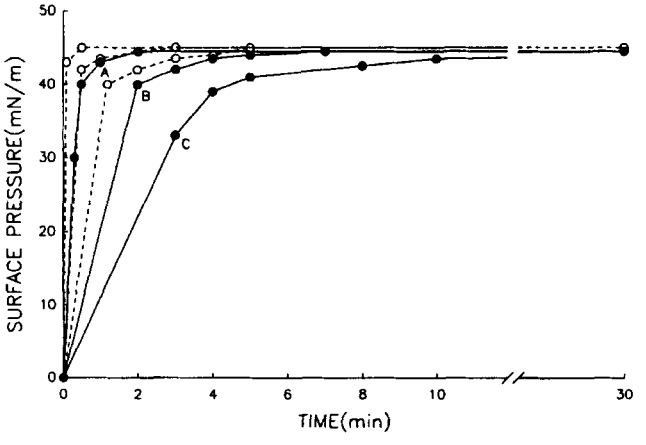

Fig. 1. Surface pressure–time adsorption isotherms of bLES(chol) in the presence and absence of SP-A. Lipid adsorption was studied with the Wilhelmy plate technique as described in the text. Three solid lines represent adsorption curves of lipids from dispersions of lipid extract surfactant with final concentrations of: A, 0.3; B, 0.2; and C, 0.1 mg PL/ml saline, 1.5 mM $CaCl_2$, respectively. Three dashed lines were the corresponding concentrations of lipid extract surfactant plus 10% SP-A. Curves are means for four experiments with SE < 3 mN/m.

Statistical methods

The final surface radioactivities achieved at three concentrations of bLES with and without SP-A (see Fig. 2) were compared using a two-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test for significant differences between group means. The final surface radioactivities for bLES(chol) depicted in Fig. 3 were analyzed in the same manner. Statistical differences between the effects of neutral lipid (cholesterol) and SP-A on the final radioactivities achieved with 0.3 mg PL/ml medium (see Figs. 2 and 3) were determined using a three-way ANOVA and Duncan's Multiple Range Test. A two-way ANOVA and Duncan's Multiple Range Test were also performed to establish differences in [^{14}C]DPPC deposited in the L-B films in Table 1, part I. A two-tailed *t*-test was used to establish differences with the control studies involving preformed monolayers (see Fig. 4 and Table 1, part II). Data are expressed as mean ± SE. Differences were considered significant when $P < 0.05$.

RESULTS

Effect of SP-A on the adsorption of lipids from bLES and bLES(chol)

Adsorption curves of lipids from bLES or bLES(chol) with or without 10% SP-A were determined by monitoring surface tension with a Wilhelmy plate at 37°C. **Figure 1** shows that lipid adsorption for 0.1, 0.2, and 0.3 mg PL/ml bLES(chol) was concentration-dependent in either the presence or absence of SP-A. Addition of SP-A to lipid extract surfactant improved the rate of adsorption, especially for the samples with the lowest lipid concentration. Lipid adsorption curves for bLES were indistinguishable from those for bLES(chol) in both the presence and absence of SP-A, indicating that neutral lipid in the lipid extract surfactant had little effect on the rate of monolayer formation at 37°C.

Effect of SP-A on the transport of DPPC from bLES to the surface region

Transport of DPPC from dispersions of bLES to the surface region was studied by labeling bLES with [^{14}C]DPPC and monitoring surface radioactivity with the B_1 probe placed 8 mm above the surface. The solid curves in **Fig. 2** (curves A, B, and C) represent surface radioactivities from 0.1, 0.2, and 0.3 mg PL/ml bLES without SP-A. The dashed curves (curves D, E, and F) are for the same concentrations of bLES plus 10% SP-A. The initial transport of DPPC was PL concentration-dependent in either the presence or absence of SP-A. Surface radioactivity (curves A, D, and E) increased rapidly initially followed by a slow phase of augmenta-

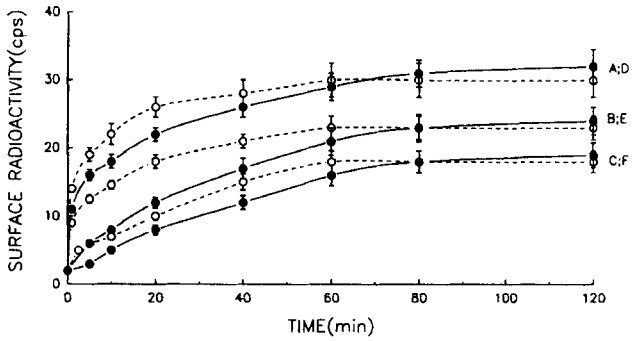

Fig. 2. Effect of SP-A on the transport of DPPC from bLES dispersions to the surface region. Transport of DPPC was examined by labeling the samples with [^{14}C]DPPC and measuring the radiation emitted from [^{14}C]DPPC to the air with a scintillation probe. Three solid lines represent transport curves of DPPC from bLES dispersions with final concentrations of: A, 0.3 mg; B, 0.2 mg; C, 0.1 mg PL/ml saline, 1.5 mM $CaCl_2$ with 0.5 μCi [^{14}C]DPPC/mg PL in all samples. Three dashed lines were DPPC transport curves from bLES plus 10% SP-A dispersions with final concentrations of: D, 0.3 mg; E, 0.2 mg; F, 0.1 mg PL/ml saline, 1.5 mM $CaCl_2$ with 0.5 μCi [^{14}C]DPPC/mg PL. The mean ± SE for four to five samples was plotted (n ≥ 4).

tion. SP-A accelerated the initial transport of DPPC but did not alter the final constant level of surface radioactivity (n = 4; P_{AD} ns, P_{BE} ns, P_{CF} ns). However, the radioactivities of [^{14}C]DPPC from the dispersions without SP-A took longer periods to reach the steady states. The final constant level of surface radioactivity was also PL concentration-dependent in either the presence or absence of SP-A.

Radiation emitted from ^{14}C has an emission range of 0.2–0.3 mm (3×10^6 Å) in water and about 20 cm in the air (29, 30), while monolayers are approximately 20 Å thick. Calculations based on an average molecular area of 50 Å^2 for DPPC in the monolayer at equilibrium surface tension, showed a 4.8 cm^2 monolayer of [^{14}C]DPPC would emit about 25 dps at the specific radioactivity (0.5 μCi/mg PL) used in the present studies. However, due to the low efficiency of the probe (14%), only about 3.5 cps of surface radioactivity would be detected when the probe was positioned very close to the surface. Consequently, when we spread a [^{14}C]DPPC-labeled bLES(chol) monolayer having a specific activity of 0.5 μCi/mg PL at equilibrium surface tension we were unable to measure any ^{14}C radiation with the probe positioned 8 mm above the surface at 37 ± 1°C. As the surface radioactivity of monolayers with a specific activity of 0.5 μCi/mg PL was too weak to be measured, the radioactivities being detected (Fig. 2) must be emitted from [^{14}C]DPPC underneath the monolayers. The gradual increases in surface radioactivities suggested accumulation of DPPC below the air–water interface. As the actual location of the [^{14}C]DPPC being detected is not clear, in this paper we will refer to the DPPC as being in the surface region.

Effect of SP-A on transport of DPPC from bLES(chol)

Figure 3 shows studies on the surface radioactivities detected after injection of [^{14}C]DPPC-labeled bLES(chol) with or without SP-A into the subphase. Increasing the concentration of bLES(chol) resulted in an increased accumulation of [^{14}C]DPPC in the surface region, in both the presence and absence of SP-A (n = 4; $P_{AB} < 0.01$, $P_{AC} < 0.01$, $P_{DE} < 0.05$, $P_{DF} < 0.01$). At 0.3 mg PL/ml, a high final surface radioactivity was observed with bLES(chol) but not with bLES(chol) plus SP-A ($P_{AD} < 0.01$). Comparison of Figs. 2 and 3 revealed that the initial transport of DPPC from bLES(chol) dispersions to the surface region (Fig. 3) was faster than from bLES (Fig. 2) at the corresponding concentrations, in either the presence or absence of SP-A. These results indicated that neutral lipid could accelerate the transport of DPPC from lipid extract surfactant to the surface region. At concentrations of 0.1 and 0.2 mg bLES(chol)/ml (Fig. 3, solid curves B and C) the final surface radioactivities were very similar to those for bLES (Fig. 2, solid curves B and C), but the level for 0.3 mg bLES(chol)/ml (Fig. 3, solid curve A) was much higher than that from 0.3 mg bLES/ml (Fig. 2, solid curve A, $P_{2A3A} < 0.01$). This suggested that at the highest concentration of bLES(chol) (0.3 mg PL/ml), the presence of neutral lipid resulted in the transport of more DPPC to the surface region. At the lower concentrations, SP-A enhanced the initial transport of DPPC (Fig. 3, dashed curves E and F) but the final values of surface radioactivity were very similar with or without SP-A (P_{BE} ns; P_{CF} ns). However, at the highest concentration of bLES(chol) (0.3 mg PL/ml), SP-A reduced the transport

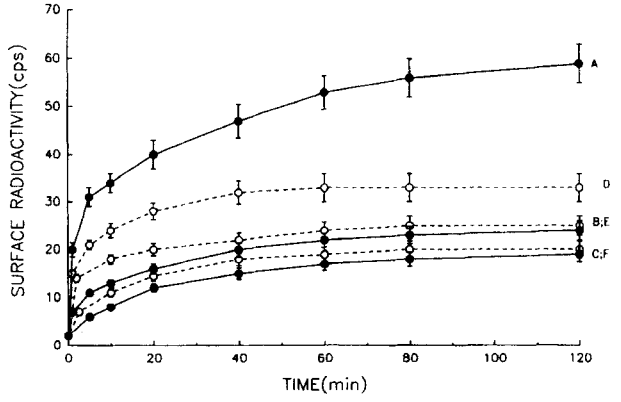

Fig. 3. Effect of SP-A on the transport of DPPC from bLES(chol). Experiments were performed as in Fig. 2. Solid lines were DPPC transport curves from bLES(chol): A, 0.3 mg/ml; B, 0.2 mg/ml; C, 0.1 mg/ml. Dashed lines were those from bLES(chol) plus 10% SP-A: D, 0.3 mg/ml; E, 0.2 mg/ml; F, 0.1 mg/ml. All samples were labeled with the same specific activity of [^{14}C]DPPC as in Fig. 2. The mean ± SE for four to five samples was plotted (n ≥ 4).

of DPPC (Fig. 3, dashed curve D) and the final surface radioactivity was close to that observed without neutral lipid (Fig. 2, dashed curve D). Statistical analysis of the final radioactivities for bLES(chol) and bLES(chol) plus SP-A (Fig. 3) by ANOVA revealed a significant interaction between these variables ($P < 0.001$). In addition, a significant interaction was also observed when the final radioactivities for bLES and bLES(chol) at 0.3 mg PL/ml were compared in the presence and absence of SP-A. These statistical data could relate to the apparent ability of SP-A to suppress the accumulation of [^{14}C]DPPC from 0.3 mg PL/ml bLES(chol).

Control studies on the radiation emitted from ^{14}C injected under pre-formed monolayers

In order to obtain some indication as to the nature and location of the accumulated [^{14}C]DPPC, control studies were conducted in which the air–water interface was pre-spread with cold DPPC or bLES(chol) to equilibrium surface tension before [^{14}C]DPPC-labeled bLES(chol) was injected into the subphase (final concentration was 0.3 mg PL/ml with 0.5 μCi [^{14}C]DPPC/mg PL). **Figure 4** shows that the initial transport of DPPC was very slow (Fig. 4, curves A and B). In contrast to the studies conducted with clean surfaces (Figs. 2 and 3), there was about a 1-min lag period initially with samples injected under either DPPC or bLES(chol) pre-spread monolayers. The surface radioactivity of [^{14}C]DPPC from surfaces pre-spread with DPPC (Fig. 4, curve A) progressed faster and attained a higher level than those pre-spread with bLES(chol) (Fig. 4, curve B, n = 3; $P_{AB} < 0.01$). These results suggested that molecules at the air–water interfaces can affect the transport of surfactant to the surface region. In addition, the accumulation of DPPC under pre-formed monolayers was lower than that observed without pre-formed monolayers (compare Fig. 4, curves A and B, with Fig. 3, curve A).

Figure 4, curves C and D reveal the radiation detected from a [^{14}C]glucose solution (0.3 mg/ml with 0.5 μCi [^{14}C]glucose/mg glucose) injected under a pre-formed monolayer of bLES(chol) (curve C) or a clean surface (curve D). In either case, the radioactivity became constant in less than 5 min, indicating that there was no gradual accumulation of glucose below the air–water interface. A small but significant (n = 3; $P_{CD} < 0.05$) increase in surface radioactivity was consistently detected from glucose solutions injected under pre-formed bLES(chol) monolayers. The basis for this phenomenon is not understood. This observation contrasts with the [^{14}C]DPPC-labeled bLES(chol) studies where a lower surface radioactivity was observed with a pre-formed monolayer.

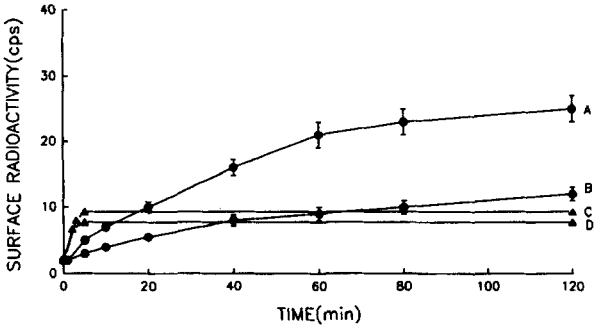

Fig. 4. Control studies on the radiation emitted from the subphase. In curves A and B, [^{14}C]DPPC-labeled bLES(chol) was injected into a subphase pre-spread with unlabeled DPPC (curve A) or bLES(chol) (curve B) at equilibrium surface tension. Concentration of bLES(chol) in the subphase was 0.3 mg PL/ml saline, 1.5 mM $CaCl_2$ with 0.5 μCi [^{14}C]DPPC/mg PL. [^{14}C]glucose solution was also injected into a subphase with (curve C) or without (curve D) pre-spread cold bLES(chol). Concentration of the glucose solution in the subphase was 0.3 mg glucose/ml saline, 1.5 mM $CaCl_2$ with 0.5 μCi [^{14}C]glucose/mg glucose. The mean ± SE for three to four samples was plotted (n ≥ 3).

Autoradiographs of L-B films

Autoradiographs were obtained of L-B films deposited from dispersions of 0.3 mg PL/ml saline, 1.5 mM $CaCl_2$. **Figure 5A** shows a representative film deposited from a dispersion of bLES. [^{14}C]DPPC in the film had a faint homogeneous misty appearance with very little evidence of aggregates. When SP-A was mixed with bLES (Fig. 5B), a number of large DPPC aggregates appeared, although the film still had some small scattered particles. Figure 5C reveals an L-B film from dispersions of bLES(chol): DPPC appeared as large heterogeneous aggregates with a considerable amount of background mist. This indicated that the presence of neutral lipid can promote the aggregation of some of the DPPC (compare Fig. 5A and 5C). L-B films obtained from the dispersion of bLES(chol) plus SP-A (Fig. 5D) illustrated a more complete aggregation of DPPC. Although the aggregates were not as large as some of those from the dispersion of bLES(chol) (Fig. 5C), the background mist had virtually disappeared. These latter aggregates (Fig. 5D) were larger and more homogeneous than those from the dispersion of bLES plus SP-A (Fig. 5B). Figures 5E and 5F were films obtained from the control studies, where the air–water interface of the subphase was spread with either bLES(chol) (Fig. 5E) or DPPC (Fig. 5F) to equilibrium prior to the injection of [^{14}C]DPPC-labeled bLES(chol). Some of the radioactive lipid in the subphase was deposited on the plates: more lipid was deposited from bLES(chol) injected under pre-spread DPPC than from bLES(chol) injected under pre-spread bLES(chol). It is not surprising to observe small amounts of [^{14}C]DPPC deposited on the plates, as the interface was disturbed during the film-deposition

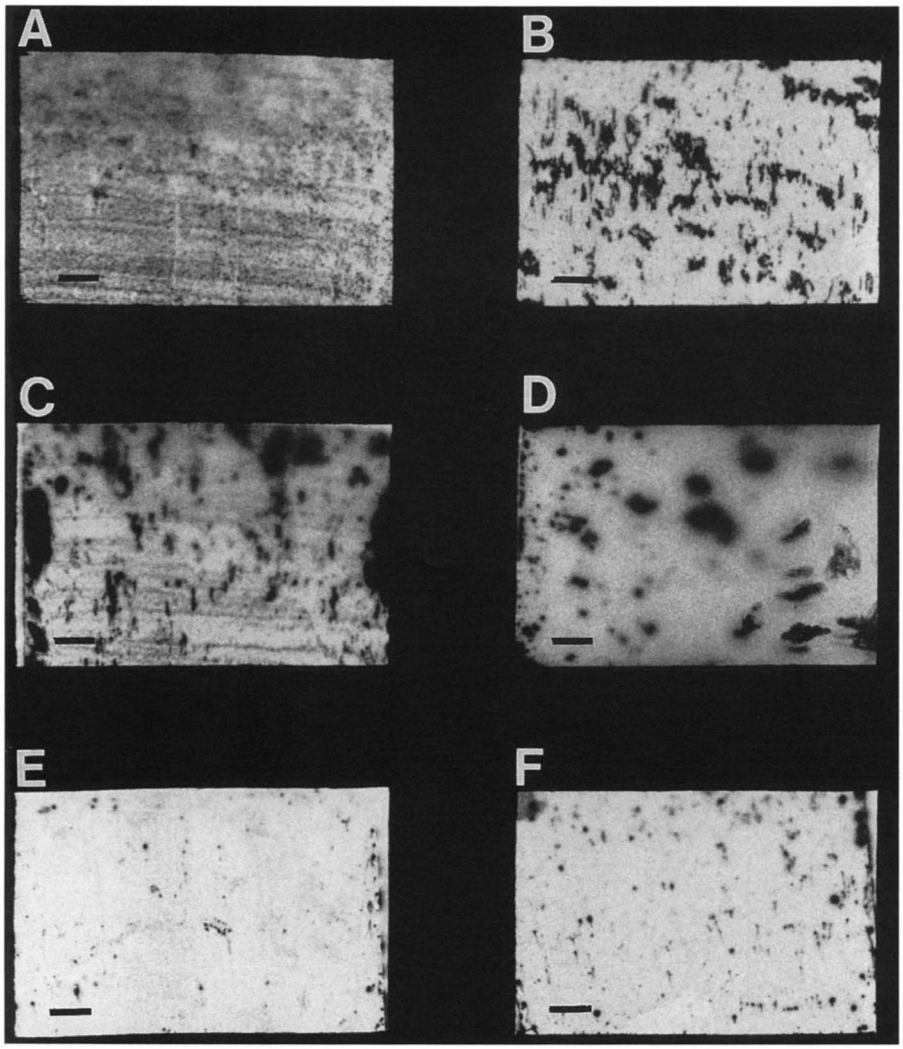

Fig. 5. Autoradiographs of Langmuir-Blodgett films. L-B films were deposited from [^{14}C]DPPC-labeled dispersions of: A, bLES; B, bLES plus 10% SP-A; C, bLES(chol); D, bLES(chol) plus 10% SP-A; E, bLES(chol) injected under unlabeled bLES(chol) pre-spread monolayers; F, bLES(chol) injected under unlabeled DPPC pre-spread monolayers. Concentration of all dispersions was 0.3 mg PL/ml saline, 1.5 mM $CaCl_2$ with 0.5 μCi [^{14}C]DPPC/mg PL. Bars indicate 1.5 mm in all graphs. The films presented are typical of films obtained from three or more experiments.

process. Some surfactant lipids in the subphase would adsorb instantaneously into the air–water interface during the deposition process in order to maintain the equilibrium surface tension, and this surfactant could have subphase material associated with it. Another possibility is that small amounts of [^{14}C]DPPC in the subphase may be associated with the pre-formed monolayer and deposited on the glass plate with it. Nonetheless, in comparison with the films deposited from the same dispersion without pre-spread monolayers (Fig. 5C), the control films contained much less radioactive lipid. These results indicated that bLES(chol) in the subphases, but not adhered to the monolayers, would not be deposited on the glass plates during the formation of L-B films. This suggests that the autoradiographs of L-B films obtained from surfactant dispersions (Fig. 5 A, B, C, and D) represent the organization of DPPC in the surface films and it is possible that these L-B films consist of more than monolayers. Autoradiographs of L-B films from [^{14}C]glucose solutions showed that [^{14}C]glucose was not deposited on the glass plate either in the presence or absence of pre-formed bLES(chol). These results indicated that glucose did not adhere to pre-formed bLES(chol) even though a significant increment in surface radioactivity was observed (Fig. 4, curve C versus curve D).

TABLE 1. Radiation emitted from [^{14}C]DPPC deposited on the glass plates

Part I Dispersions[a] without Pre-formed Monolayers[b] Dispersions	cps ± SE	n	Significance[c]
A. bLES	46 ± 2	4	P_{AB} n.s.
B. bLES plus SP-A	43 ± 4	4	$P_{BD} < 0.05$
C. bLES(chol)	68 ± 3	4	$P_{AC} < 0.01$
D. bLES(chol) plus SP-A	54 ± 2	4	$P_{CD} < 0.01$

Part II Dispersions[a] Injected under Pre-formed Monolayers[b] Dispersions	Pre-formed Monolayers	cps ± SE	n	Significance[d]
E. bLES(chol)	bLES(chol)	7 ± 1	3	$P_{EF} < 0.01$
F. bLES(chol)	DPPC	16 ± 1	3	

Radioactivities were measured directly from L-B films with a B_1 scintillation probe at room temperature.
[a]Concentration of all dispersions was 0.3 mg PL/ml with 0.5 μCi [^{14}C]DPPC/mg PL.
[b]Pre-formed monolayers were at equilibrium surface tension and unlabeled.
[c]Differences were analyzed using a two-factor (2 × 2) ANOVA and Duncan's Multiple Range Test.
[d]Differences were analyzed using a two-tailed *t* test.

Detection of [^{14}C]DPPC in L-B films

We have also measured the radiation emitted from [^{14}C]DPPC in L-B films deposited on the glass plates using the B_1 scintillation probe. The background count was 2 cps (counts per second). **Table 1** (A and B) reveals that L-B films deposited from bLES with or without SP-A had similar amounts of [^{14}C]DPPC. This indicates that SP-A did not promote the accumulation of more DPPC into the L-B films. In contrast to SP-A, the neutral lipid in bLES(chol) enhanced the incorporation of DPPC into the films (Table 1, C). Addition of SP-A to bLES(chol) resulted in a decreased amount of DPPC in the L-B films (Table 1, D). However, 25% more DPPC was associated with L-B films from dispersions of bLES(chol) plus SP-A (Table 1, D) than from dispersions of bLES plus SP-A (Table 1, B; $P_{BD} < 0.05$). Considerably less radioactivity was detected when unlabeled bLES(chol) or DPPC was spread prior to the injection of [^{14}C]DPPC-labeled bLES(chol) (Table 1, Part II). The radiation counts for the L-B films listed in Table 1 are much higher than those calculated for true monolayers (calculation as in the transport of DPPC section on an area of 1.5 cm^2), indicating that the L-B films consist of more than monolayers.

Two-factor ANOVA revealed a significant interaction between the effects of neutral lipid and SP-A on the deposition of [^{14}C]DPPC in L-B films. This indicates that at the concentration used (0.3 mg PL/ml), neutral lipid influences the effect of SP-A and/or vice versa. These results are consistent with those revealed in Figs. 2 and 3 (curves A and D) where SP-A suppressed the accumulation of [^{14}C]DPPC with 0.3 mg PL/ml bLES(chol).

DISCUSSION

The present studies focused on the effects of SP-A and neutral lipid on the transport of DPPC from dispersions to the surface region and on the organization of the transported DPPC in the surface films. Lipid extract surfactant adsorbs readily to the air–water interface and attains equilibrium surface tension within a few minutes (Fig. 1). Increasing the surfactant concentration and adding SP-A accelerated adsorption to form a monolayer but did not affect the final surface pressure. Similar adsorption rates were observed with dispersions of bLES and bLES(chol) at 37°C, as assessed by the Wilhelmy plate technique. In our earlier studies (16), using the pulsating bubble surfactometer, we have shown that the adsorption rates of lipids from bLES and bLES(chol) were similar at 37°C, although bLES(chol) adsorbed more rapidly at 24°C. These investigations indicated that the neutral lipid in pulmonary surfactant has little effect on the rate of formation of the surface active monolayer at 37°C.

When transport of [^{14}C]DPPC in lipid extract surfactant to the surface region was monitored with a Bicron B_1 scintillation probe, the surface radioactivity increased rapidly followed by a gradual increase lasting up to 2 h. In contrast to surface pressure, final surface radioactivity was surfactant concentration-dependent, and the initial transports of DPPC from the dispersions of bLES(chol) to the surface region (Fig. 3) was faster than those from bLES (Fig. 2) at the corresponding concentrations. With 0.1 and 0.2 mg PL/ml surfactant, similar final levels of surface radioactivity were observed for bLES and bLES(chol). SP-A accelerated [^{14}C]DPPC accumulation in the surface region. However, with 0.3 mg PL/ml bLES(chol) there was an unanticipated large increase in surface radioactivity over that observed with bLES. This increased accumulation of [^{14}C]DPPC in the surface region with 0.3 mg PL/ml bLES(chol) was attenuated by addition of SP-A. These results demonstrated that neutral lipid present in bLES(chol) influences the accumulation of surfactant in the surface region despite the apparent lack of an effect on equilib-

rium surface tension, and SP-A is capable of regulating the accretion of surfactant near the interface.

The present studies demonstrated that there was surfactant material associated with the surface monolayer. It has become evident that pulmonary surfactant exists in the alveolar lining as more than a single monolayer. Electron microscopy of rat and rabbit lungs fixed through vascular perfusion showed that the extracellular lining layer is composed of polymorphous films, trilayers and multilayers, and it is continuous (31–33). Freeze-fracture studies on rat lung revealed evidence of broad surface ridges and grooves apparently associated with adsorbing tubular myelin figures and surface-associated surfactant vesicles (34). The presence of a surface-associated surfactant reservoir formed during adsorption of bLES has been suggested through subphase depletion studies conducted with the captive bubble surfactometer (32). However, the nature of the lipid–protein structures associated with the surface is not known. Whether a relationship exists between the structural and functional surface-associated surfactant pools reported previously (31–34) and the [^{14}C]DPPC accretions described in the present studies must be established through further investigation.

The control studies (Fig. 4) revealed only a slow phase of [^{14}C]DPPC accumulation when [^{14}C]DPPC-labeled bLES(chol) was injected under pre-formed monolayers (curves A and B). However, a much more rapid initial transport, similar to the kinetics of monolayer formation (Fig. 1, curve A), was observed for the dispersion injected under a clean surface (Fig. 3, curve A). These results suggested that part of the [^{14}C]DPPC in the surface region may arise from a process associated with surfactant adsorption and spreading. Figure 4 also showed that the radioactivities detected from the dispersions injected under DPPC pre-spread monolayers (Fig. 4, curve A) were higher than those under bLES(chol) pre-spread monolayers (Fig. 4, curve B). This indicated that the chemical nature of the monolayer influences the association of bLES(chol) with the surface monolayer. The nature of such association must still be determined. Nonetheless, earlier studies (25) demonstrated that there was no DPPC exchange between DPPC pre-spread monolayers and liposomes in the subphase.

Cholesterol is known to interact with DPPC by packing into the cavities between DPPC fatty acid chains (13). Previous studies showed that cholesterol could be transported to the surface region when mixed with pulmonary surfactant (most likely carried along with DPPC), but not when injected by itself (10). The effect of concentration on the transport of DPPC from bLES(chol) dispersions to the surface region was surprising, where the transport capacity and the final surface radioactivity were considerably higher with 0.3 mg PL/ml than with 0.2 mg PL/ml (Fig. 3). This concentration effect was not observed with bLES that lacks neutral lipids (Fig. 2). It has been suggested that cholesterol exerts its influence through hydration of the β-OH group and ordering the water near the membrane surface (35, 36). However, the mechanism in which the concentration of bLES(chol) affected the transport of DPPC to the surface is not known.

Surprisingly, although addition of SP-A enhanced monolayer formation with both bLES and bLES(chol) (Fig. 1), this hydrophilic surfactant protein depressed the initial transport and the ultimate level of [^{14}C]DPPC detected at the surface with 0.3 mg PL/ml bLES(chol). The presence of SP-A also reduced the amount of DPPC associated with the L-B films (compare Table 1 C with D). In both experiments, SP-A did not decrease incorporation of DPPC into the surface region with bLES. SP-A binds to PL and aggregates PL vesicles in the presence of calcium (37–39). The binding affinity for DPPC is the highest among all PL (40). Electron micrographs of lipid–SP-A mixtures showed that SP-A particles were closely associated with the lipid bilayer surfaces (41). It was evident that SP-A affects lipid structure (10, 41). The ultrastructure of bLES plus exogenous cholesterol and SP-A, as examined by electron microscopy, is quite different from that of bLES plus cholesterol alone; the former has a multilayer-vesicular organization while the latter exhibits an irregular disordered structure lacking vesicles (10). These earlier studies (10) demonstrated that radioactive cholesterol from dispersions of [^{14}C]cholesterol-labeled bLES plus 5% cholesterol accumulated slowly at the surface, whereas an equilibrium surface tension was established rapidly. SP-A depressed the transport of [^{14}C]cholesterol and the ultimate surface radioactivity. These observations were interpreted as indicating that SP-A somehow interfered with the adsorption of cholesterol into the surface monolayer, but the present observations would suggest that SP-A modifies the manner in which cholesterol (as well as DPPC) becomes associated with the surface.

Studies (10) using the pulsating bubble surfactometer revealed that additional pulsations were required to produce near zero surface tension when exogenous cholesterol was added to bLES. Furthermore, compressed surface active monolayers with surface tensions below equilibrium returned to equilibrium surface tension more rapidly when cholesterol was present in the sample. The characteristics of cholesterol-supplemented bLES were similar to those observed with bLES(chol) (10, 11). Several studies (10–14) have indicated that cholesterol can penetrate deeply into the cavities between DPPC molecules and cannot be squeezed out readily from the monolayer through repeated compressions. We have observed that the surface destabilizing

effects of cholesterol could be diminished by SP-A (10). In addition, the surface tension lowering ability of natural surfactant (contains SP-A and cholesterol) is greater than that of bLES(chol) which lacks SP-A (11). Taken together, these results suggested that SP-A limited incorporation of cholesterol into the surface active monolayers and into the surfactant layers accumulating beneath the monolayers. It appeared that SP-A may be able to impede the penetration of cholesterol into the cavities of DPPC molecules. Nonetheless, it is clear from the present studies that cholesterol can influence the organization of DPPC in the surface films even in the presence of SP-A (compare Fig. 5B and 5D). However, the mechanism in which SP-A regulates the accumulation of DPPC in the surface region is not known. Further investigations are required to understand the interactions among DPPC, SP-A, and cholesterol, as well as other surfactant components.

Although SP-A had a profound effect on the organization of DPPC (compare Fig. 5A and 5B) in the absence of neutral lipid similar amounts of DPPC were deposited in the L-B films (Table 1, A and B). It was concluded from these results that SP-A did not promote the incorporation of additional DPPC into surface films formed from dispersions of bLES plus SP-A, but enhanced aggregation of DPPC. Neutral lipid generated the formation of heterogeneous aggregates (Fig. 5C) and increased the proportion of DPPC in the L-B films (Table 1, C). As elaborated above, surface tension studies indicated that some cholesterol is incorporated into the monolayer with DPPC resulting in monolayer destabilization (10). However, in the presence of both SP-A and neutral lipid, DPPC primarily formed relatively large aggregates (Fig. 5D), and more DPPC appeared in the L-B films (Table 1, D) than from the dispersion of bLES plus SP-A (Table 1, B). These observations indicate that neutral lipid and SP-A not only promote accumulation of DPPC in the surface films but also enhance the formation of large aggregates containing DPPC. It appears possible that these aggregates could play an important role in the rapid adsorption of DPPC during expansion and the squeeze-out of non-DPPC components during compression. As revealed in our earlier studies using the pulsating bubble surfactometer (11), natural surfactant films adsorbed to equilibrium are able to achieve near zero surface tension with one or two pulsations whereas multiple pulsations are required for bLES or bLES(chol). The physical nature of surfactant accretions below monolayers is not clear. Nonetheless, it is reasonable to suggest that this surface-associated surfactant could serve as a PL reservoir.

In summary, the present studies focused on the roles of SP-A and neutral lipid on the surfactant structure in the surface films. It was found that neutral lipid and SP-A affected the accumulation of DPPC in the surfactant layers under the interface. Moreover, both SP-A and neutral lipid produced striking effects on DPPC organization in the surface films.

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