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STUDIES ON LUNG SURFACTANT REPLACEMENT IN RESPIRATORY DISTRESS SYNDROME

RAPID FILM FORMATION FROM BINARY MIXED LIPOSOMES

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Binary mixed liposomes were prepared from dipalmitoylphosphatidylcholine (DPPC) and a minor compound, e.g., egg phosphatidylglycerol (PG) at a ratio of 9:1. Using different preparative techniques, large unilamellar vesicles (LUV), small unilamellar vesicles (SUV) or multilamellar vesicles (MLV) were obtained and were studied with an electron microscope for morphology, with a Wilhelmy balance for spreading and surface tension lowering potential, and in the surfactant-depleted isolated rat lung for their ability to restore expiratory lung capacity. Only the simultaneous investigation of phospholipids by negative staining and thin sectioning allows unequivocal classification of liposomes. The surface-active structures prepared with the technique of Bangham et al. (Bangham, A.D., Hill, M.W. and Miller, N.G.A. (1974) in Methods in Membrane Biology (Korn, E., ed.), Vol. 1, pp. 1–68, Plenum Press, New York) at room temperature are LUV. LUV containing DPPC:PG at a ratio of 9:1 rapidly spread to a film with high surface tension lowering potential. Within 5 min after injection into the subphase they rise to the surface and form a film at the air/liquid interface able to lower the surface tension to less than 1 mN/m at compression. SUV of the same chemical composition, however, are immediately surface-active only when spread directly onto the surface. MLV exhibit poor surface activity. LUV or pure DPPC, applied onto the surface, are weakly surface active within 5 min. DPPC vesicles injected into the subphase at 37°C do not adsorb to any film with surface tension lowering potential in this time. The minor compounds PE, PI, PS, PA, lysoPC enable DPPC to form surface-active films after application on saline at 37°C. Removal of surfactant decreases the expiratory lung capacity of the isolated rat lung from 49.7 to 12.4% at 4 cmH_2O. After substitution with natural surfactant, the expiratory lung capacity is twice that of the washed lung (25.9%), but the original distensibility of the native lung is not restituted. The effect of LUV containing DPPC:PG at a ratio of 9:1 is also remarkable (21.2%).

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PG, phosphatidylglycerol; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; lysoPC, lysophosphatidylcholine; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; MLV, multilamellar vesicles.

Introduction

The absence of phosphatidylglycerol (PG) in the pulmonary surfactant of infants with respiratory distress syndrome was demonstrated by Hallman et al. [1] and is indeed characteristic of this

disease [2–4]. This finding has stimulated surfactant research in two directions. First, the purely quantitative aspect of surfactant deficiency or even diminished surfactant phosphatidylcholine [PC] was left in favor of qualitative changes in surfactant function as a consequence of disturbed phospholipid interaction. Second, attempts to develop surfactant substitutes, which had failed in earlier studies with pure dipalmitoylphosphatidylcholine [5–7] were started again with preparations including phosphatidylglycerol [8,9]. As long as phosphatidylglycerol was absent from the tracheal fluid of preterm infants, we found a rise in phosphatidylinositol to be associated with recovery from respiratory distress syndrome, and concluded that the 'minor compound' plays a significant role in maintaining alveolar stability [4]. The addition of an unsaturated minor compound modifies the physical properties of the major compound dipalmitoylphosphatidylcholine (DPPC). In vitro studies have shown that pure DPPC having a gel-to-liquid-crystalline transition at 41°C, does not spread at the air/liquid interface at body temperature [10].

In the present study, we obtain additional information on this minor component, especially concerning the question as to whether enhanced surface spreading or film adsorption from the subphase is a function unique to phosphatidylglycerol. Since previous findings [11,12] suggested that synthetic phospholipids need a specific, highly organized structure for spreading without organic solvents, we chose different preparations in order to obtain various types of liposome as model precursors for monolayer formation.

Methods and Materials

Materials

All phospholipids were purchased from Sigma (München): DL-α-dipalmitoylphosphatidylcholine, grade I, crystalline, synthetic; L-α-dipalmitoylphosphatidyldimethylethanolamine, crystalline; L-α-phosphatidylethanolamine, type III, from egg yolk; L-α-phosphatidyl-DL-glycerol, ammonium salt, grade I, from egg yolk lecithin; L-α-phosphatidylinositol, ammonium salt, grade III, from soya bean; L-α-phosphatidyl-L-serine, from bovine brain; L-α-phosphatidic acid, sodium salt, grade I, from egg yolk; sphingomyelin, from bovine brain; L-α-palmitoyllysophosphatidylcholine, synthetic.

Analytical grade methanol and chloroform were purchased from Merck (Darmstadt). Sterile 0.15 M sodium chloride was purchased from Fresenius (Bad Homburg) and was free of surface-active contaminations.

Preparation of liposomes

Large unilamellar vesicles (LUV) were prepared below the phospholipid phase transition temperature according to Bangham et al. [13] by two different techniques:

(a) The lipids (10 mg) were dissolved in chloroform (30 ml) and were added to a round 100 ml flask together with 20 glass spheres of 3 mm diameter. After evaporation of the chloroform at 30°C, the thin lipid film coating the glass wall was suspended by gentle agitation of the spheres in 200–500 μl of 0.15 M NaCl at room temperature, resulting in a milky dispersion.

(b) Liposomes were prepared according to Morley [9] as in (a). Then the dispersion was freeze-dried at −40°C in a Christ Beta lyophilizer for 12 h. Electron microscopy was performed for both preparations and showed unilamellar vesicles of 0.3–2 μm diameter.

Small unilamellar vesicles (SUV) were prepared by the following techniques:

(a) LUV were sonicated for 5 min using a microtip at 0°C and full power (Branson B 12 ultrasound cell disrupter). During sonication, the solution became almost clear.

(b) A second method employed to prepare SUV was detergent dialysis from micelles [14] using a flow-through dialyzer (Lipoprep® bilayer liposome preparation device, Diachema Ltd., 8135 Langnau, Switzerland) at 46°C for 24 h. Micelles were prepared with a phospholipid : sodium cholate ratio of 1 : 0.9 (w/w) by dialysis against 1 mM phosphate buffer (pH 7.3), adjusted to 0.16 ionic strength with NaCl. For both methods, electron microscopy reveals uniform vesicles whose diameter is predominantly 50–60 nm.

Multilamellar liposomes (MLV) were prepared above the phospholipid phase transition temperature. 10 mg phospholipid were mechanically suspended in 500 μl of 0.15 M NaCl at 45°C by vortexing for 2 min. A milky suspension resulted

which consisted of multilamellar vesicles with a diameter of 0.3–2 μm as shown by electron microscopy.

Electron microscopy

For negative staining, vesicles were adsorbed to carbon-coated grids and stained with 1% uranyl acetate (pH 4.5). For thin sectioning, samples were fixed simultaneously with 0.5% OsO_4, 1.25% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) for 1 h at 0°C. After fixation, vesicles were embedded in 1% agar, dehydrated with ethanol, and plastic-embedded in Epon. Sections were stained with lead citrate. All specimens were observed at 60 kV with a Philips EM 201 electron microscope.

Surface tension measurements

Dynamic surface tension measurements were performed using a modified Wilhelmy balance equipped with a large Teflon-coated, temperature-regulated Langmuir trough. All measurements were done under a hood and at a subphase temperature of 37°C. The film surface was 392 cm^2 and the subphase consisted of 200 ml 0.15 M NaCl. Compression of the film to 20% of initial area was achieved with a tightly fitting dam-type Teflon barrier advanced at constant speed, one compression/re-expansion cycle lasting 4 min. For spread films, samples containing 0.25 mg of phospholipid were applied onto the saline surface with a Hamilton syringe in 1 μl portions. For adsorbed films, samples containing 1 mg of phospholipid were injected into the 200 ml subphase 1 cm below the surface. Surface tension/area diagrams were recorded with an *XY*-recorder and evaluated in terms of the following parameters: Maximal surface tension (γ_{max}) after 5 min as indicator of spreading activity, minimal surface tension (γ_{min}) after compression to 20% of area as indicator of surface tension lowering potential, hysteresis area during first compression/re-expansion cycle as indicator of the phospholipid amount in the film.

Lung surfactant replacement

Pressure/volume diagrams of surfactant-depleted isolated rat lungs were recorded using a modification of the technique of Rüfer [15]. Adult male Sprague-Dawley rats (450–500 g) were killed with pentobarbital. After perfusion of the pulmonary circulation with 30 ml of Ringer's solution at 32°C, both lungs and heart were excised. Leakage was excluded. The first pressure/volume diagram was recorded in an incubator at 35°C during ventilation with a mechanically driven bulb syringe connected to a Hewlett-Packard 1280 pressure transducer and simultaneously to a 2.5 mm portex tube ligated to the trachea. The greater part of pulmonary surfactant was removed and stored at −18°C or reconcentrated by centrifugation according to Enhörning et al. [16] to 10 mg phospholipid/ml. A second pressure/volume diagram was recorded in the surfactant-depleted lung and 54 mg/kg of the liposomal test solution was instilled into the tracheal tube. The lung was ventilated with humidified air using a time-cycled ventilator at the following settings: ventilation rate 14/min; peak inspiratory pressure 25 cm H_2O; positive endexpiratory pressure 4 cm H_2O; flow 1 l/min. After 5 min ventilation at 37°C, a third pressure/volume diagram was recorded.

The expiration loop was evaluated according to Ikegami et al. [17] by comparing the lung volumes retained at 4 and 25 cm H_2O pressure. The experiment lasted for 90 min.

Results

Liposome morphology

With different preparative techniques and temperatures liposomes of highly different structure and size are obtained (Fig. 1). Large unilamellar vesicles are somewhat inhomogeneous in size distribution, whereas small unilamellar vesicles are of uniform size. There is no morphological difference with changing chemical composition. (Fig. 2) Liposomes containing 10% egg PG are similar in size to liposomes of DPPC only; the electric charge does not greatly alter morphologic features. Electron-microscopic analysis shows that the surface-active structures prepared with the technique of Bangham et al. from DPPC at room temperature are large unilamellar vesicles (Fig. 1a,1c). Only the simultaneous investigation of phospholipids by negative staining and thin sectioning allows unequivocal classification of liposomes. As shown in Fig. 1a, multilamellar structures can be simulated by large unilamellar vesicles due to superposition

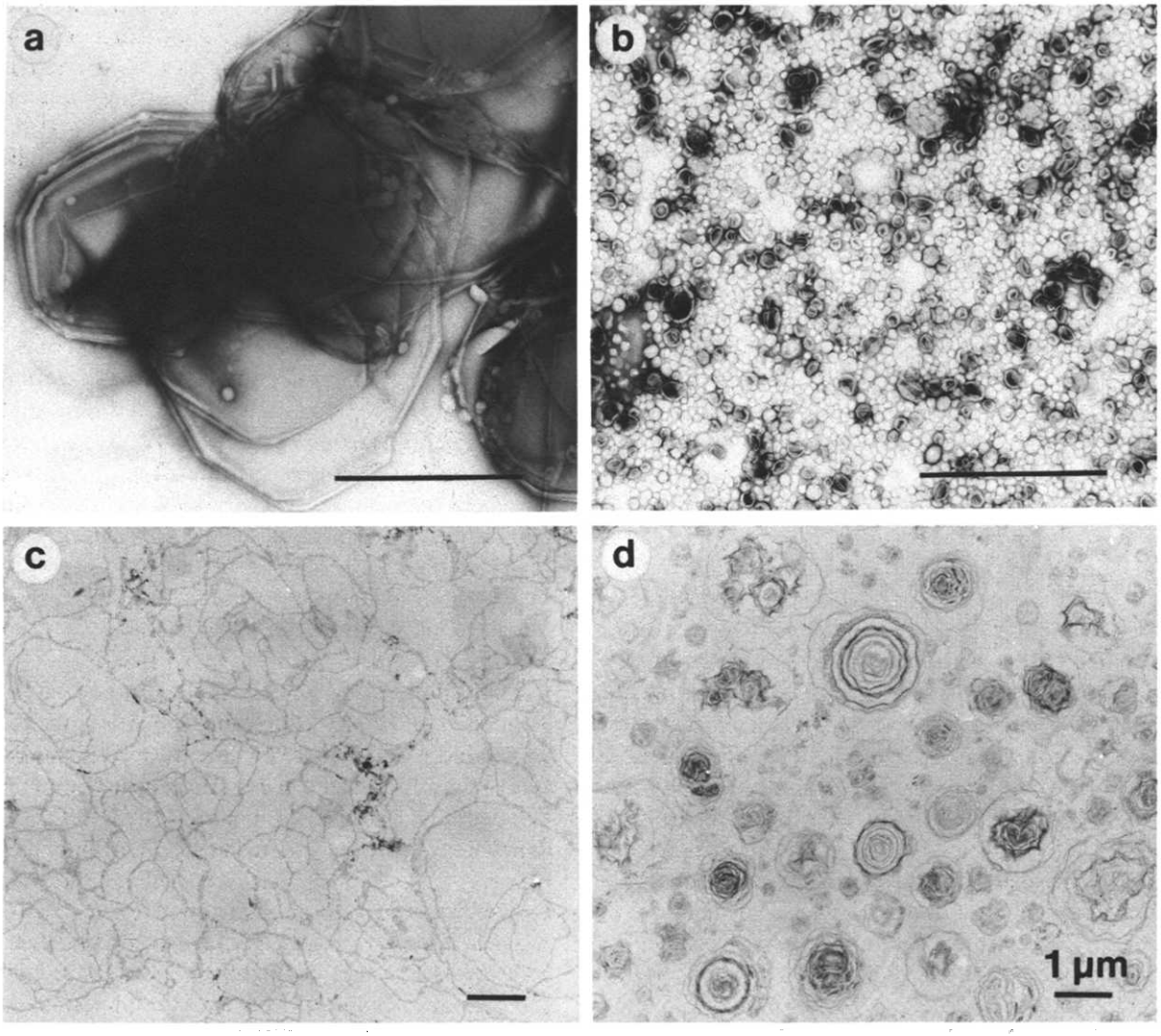

Fig. 1. Electronmicrographs of liposomes containing dipalmitoylphosphatidylcholine:egg phosphatidylglycerol at a ratio of 9:1 by weight. Bar corresponds to 1 µm. (a) Large unilamellar vesicles, negative staining with uranylacetate. A multilamellar appearence is simulated due to superposition of vesicles. (b) Small unilamellar vesicles, negative staining with uranyl acetate. (c) Large unilamellar vesicles, thin sectioning: note high variability in vesicle size. (d) Multilamellar vesicles, thin sectioning: note concentric lamellar structure resembling the type II cell lamellar bodies.

in the negative-stained specimen. Photomicrographs of sectioned liposomes (Fig. 1c) give the correct classification.

Surface activity of different types of liposome

As shown in Table I, large unilamellar vesicles containing DPPC:PG at a ratio of 9:1 rapidly spread to a film with high surface tension lowering potential. Within 5 min after injection into the subphase, they rise to the surface and form a film at the air/liquid interface able to lower the surface tension to less than 1 mN/m under compression in the Wilhelmy balance. Under these conditions, no difference in surface activity can be observed for spread and adsorbed films. Small unilamellar vesicles of the same chemical composition, however, are immediately surface-active only when spread directly on the surface. After injection into

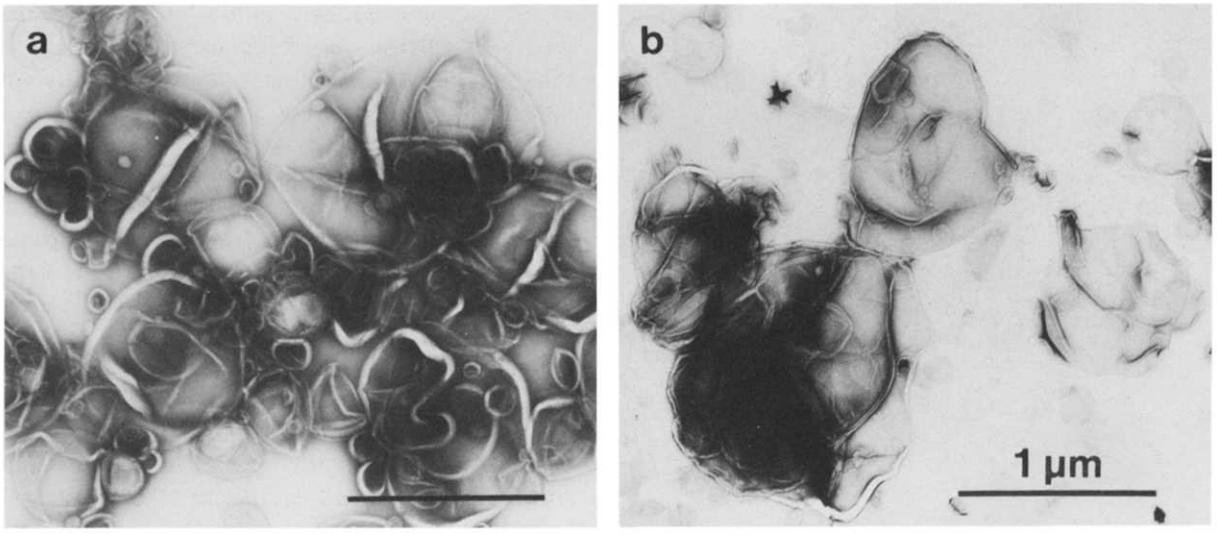

Fig. 2. Comparison of large unilamellar vesicles prepared from DPPC:egg PG at a ratio of 9:1(a) and from pure DPPC (b) after negative staining with uranyl acetate.

the subphase, they slowly adsorb to the surface, a surface active film is found after more than 10 min. Multilamellar vesicles exhibit poor surface activity despite their morphological similarity to pulmonary lamellar bodies. After injection into the saline subphase they do not rise to the surface but fall to the bottom of the Langmuir trough. Surface-active films are not formed during at least 2 h.

Comparison of large unilamellar vesicles of DPPC with and without egg PG

Addition of PG to DPPC greatly accelerates the formation of a surface-active film in the Wilhelmy balance (Fig. 3). Within 5 min at 37°C, DPPC/PG vesicles applied onto the surface form a film which reduces γ_{max} to about 50 mN/m, and γ_{min} to below 1 mN/m before and after compression to 20% of the initial area, respectively. Similarly, after injec-

TABLE I

SURFACE ACTIVITY OF DIFFERENT TYPES OF LIPOSOME WITH THE SAME LIPID COMPOSITION, DIPALMITOYLPHOSPHATIDYLCHOLINE:EGG PHOSPHATIDYLGLYCEROL AT A RATIO OF 9:1 BY WEIGHT

Liposome type (preparation)	Size (nm)	Spread films		Adsorbed films	
		γ_{max} (mN/m)	γ_{min} (mN/m)	γ_{max} (mN/m)	γ_{min} (mN/m)
Large unilamellar vesicles	300–2000				
Glass spheres at room temp.		53	<1	58	<1
Lyophilized		55	<1	56	<1
Small unilamellar vesicles	50–60				
Sonicated		65	<1	68	32
Lipoprep		65	18	70	52
Multilamellar vesicles	300–2000				
Suspension at 46°C		63	24	70	70

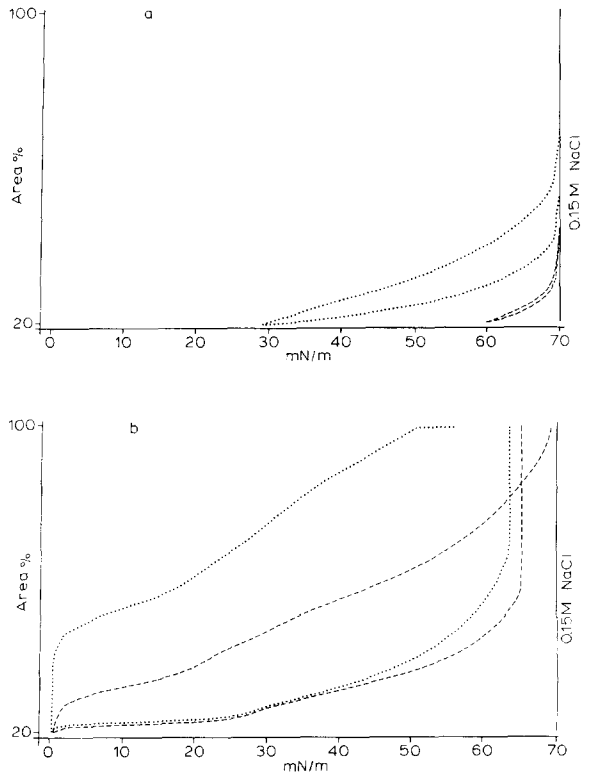

Fig. 3. Surface tension/area diagrams of films derived from 1 mg of large unilamellar vesicles on a subphase of 200 ml of 0.15 M NaCl. All measurements performed at 37°C and 5 min after application. ·····, spread film; — — — adsorbed film. (a) Dipalmitoylphosphatidylcholine: egg phosphatidylglycerol at a ratio of 9:1; (b) dipalmitoylphosphatidylcholine alone.

tion into the subphase, a surface-active film with essentially the same characteristics is formed (Fig. 3a). Pure DPPC vesicles, however, applied onto the surface, form a film which does not reduce γ_{max} within 5 min and which reduces γ_{min} to 28 mN/m only. DPPC vesicles injected into the subphase at 37°C do not adsorb to any film with surface tension lowering potential in this time (Fig. 3b). Even after 2 h adsorption time, no surface-active film is derived from DPPC vesicles at 37°C.

Binary mixtures with different minor compounds

With the exception of sphingomyelin and dipalmitoylphosphatidyldimethylethanolamine, all minor compounds tested enable DPPC formation of surface-active films after application on saline at 37°C (Table II). The ability to adsorb to the air/liquid interface after application into the subphase is different for binary mixtures of different phospholipid composition.

Liposomes in the surfactant-depleted rat lung

Removal of surfactant greatly decreases the lung volume retained during expiration. As illustrated in Fig. 4 and shown in Table III the total lung capacity at 4 cmH_2O falls from 49.7 to 12.4% after alveolar lavage. Table III compares pressure volume diagrams of isolated and surfactant-depleted rat lungs substituted with natural surfactant or with large unilamellar liposomes prepared from DPPC and egg PG at a ratio of 9:1. After substitution with natural surfactant, the expiratory lung volume is twice that of the washed lung (25.9% total lung capacity), but the original distensibility of the native lung is not restituted. The effect of the liposomal substitute is also remarkable (21.2% total lung capacity), even though somewhat inferior to that of natural surfactant.

Discussion

The data presented here confirm that liposomes of appropriate structure and phospholipid composition rapidly spread to surface films exerting

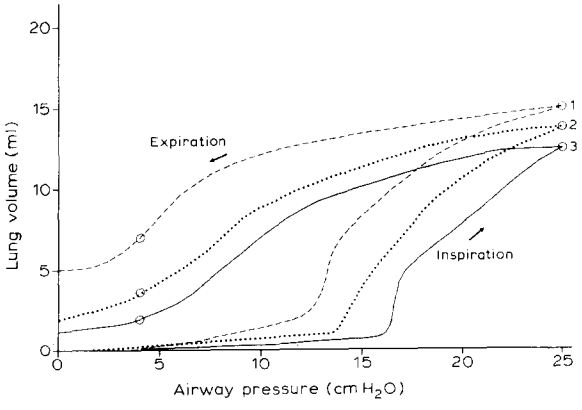

Fig. 4. Pressure/volume diagrams of an isolated rat lung ventilated with air at 34°C. First measurement (broken line) native lung. Second measurement (solid line) after surfactant depletion by ten subsequent washings with Ringer's solution. Third measurement (dotted line) after substitution of 54 mg/kg DPPC: egg PG at a ratio of 9:1 in large unilamellar vesicles. Circles define total lung capacity at 25 cmH_2O and expiratory lung capacity at 4 cmH_2O airway pressure.

TABLE II

SURFACE ACTIVITY OF LARGE UNILAMELLAR VESICLES OF DIFFERENT LIPID COMPOSITION

Samples contain the major compound DPPC and one varying minor compound at a ratio of 9:1 by weight. SM, sphingomyelin.

Minor compound	Spread films			Adsorbed films		
	γ_{max} (mN/m)	γ_{min} (mN/m)	Hysteresis area (cm^2)	γ_{max} (mN/m)	γ_{min} (mN/m)	Hysteresis area (cm^2)
None	70	27	29	70	58	1
	69	23	41	69	66	0
PDME	70	25	34	70	59	0.6
	70	40	4	70	70	0
PE	53	<1	150	68	12	60
	57	1	172	61	1	124
PI	62	1	195	68	20	67
	65	<1	175	69	17	58
PS	53	1	137	69	25	22
	58	<1	201	57	2	80
PG	43	<1	278	69	<1	90
	55	<1	277	69	<1	127
PA	53	<1	154	69	<1	72
	51	<1	159	70	<1	55
SM	69	12	76	69	55	1
	69	48	9	69	66	0
LysoPC	66	6	84	70	43	11
	56	2	208	69	57	1

TABLE III

LUNG VOLUME (ml) AT TWO DIFFERENT AIRWAY PRESSURES (cmH_2O) OF ISOLATED RAT LUNGS BEFORE AND AFTER REMOVAL OF THE NATURAL SURFACTANT, AND AFTER ADMINISTRATION OF EITHER NATURAL OR SYNTHETIC SURFACTANT

Type of substitution	Animal No.	Before removal			After removal			After substitution		
		25 cmH_2O	4 cmH_2O	Change (%)	25 cmH_2O	4 cmH_2O	Change (%)	25 cmH_2O	4 cmH_2O	Change (%)
None [a]	65	15.5	6.3	41.0	12.1	2.1	17.0	8.8	1.2	14.0
	66	13.1	5.5	42.0	9.3	0.8	9.0	10.0	1.2	12.0
	67	16.3	8.5	52.0	13.5	2.3	17.0	12.9	2.5	19.0
	68	15.4	7.3	47.0	14.8	1.5	10.0	15.0	2.0	13.0
Natural surfactant	46	18.0	8.5	47.2	16.0	1.3	8.1	16.0	6.0	37.5
	61	13.3	7.3	54.9	14.0	1.6	11.4	12.6	3.5	27.8
	62	17.5	9.1	52.0	16.0	1.3	8.1	15.3	3.0	19.6
	63	15.6	8.4	53.8	14.1	1.1	7.8	14.4	2.7	18.8
Synthetic surfactant [b]	53	14.4	8.2	56.9	12.8	1.5	11.7	12.3	2.2	17.9
	56	15.0	7.1	47.3	12.5	2.1	16.8	13.7	3.6	26.2
	57	15.8	9.3	58.9	15.9	2.8	17.6	15.1	3.6	23.8
	58	16.3	7.9	48.5	14.9	2.3	15.4	14.1	2.5	17.7
	60	13.8	6.1	44.2	15.9	1.7	10.7	15.3	3.1	20.3

[a] No substitution, but same type and duration of ventilation was used in controls.

[b] Large unilamellar vesicles containing DPPC:egg PG at a ratio of 9:1.

high surface pressures under compression. In addition, large unilamellar vesicles are able to adsorb rapidly to the surface of a saline subphase, another feature required for action as a surfactant [18]. Surface monolayers are formed by adsorption of vesicles to the air/liquid interface and lipid exchange from a vesicle layer in the subphase. The speed of this film adsorption increases with increasing vesicle size and with increasing concentration of vesicle in the subphase [19]. The lipid exchange occurs between the outer layer of the vesicles and the monolayer at the air/liquid interface. According to Schindler [19,20], vesicles below 100 nm diameter lead to a low monolayer surface pressure, equivalent to poor film adsorption. Multilamellar liposomes spread at the surface less readily than unilamellar vesicles and seem to retain a part of their internal content [21].

A major drawback in working with liposomes is that, even if the same relation of the synthetic phospholipids is maintained, minor variations in technique or temperature of preparation may greatly change the type or size (and thus, surface activity) of the structures obtained. Fascinated by the morphological similarity of MLV and lamellar bodies stored in the type II cell of the lung, we initially wanted to prepare MLV [22] and employed the method of Bangham et al. [13]. However, application of this method to DPPC at room temperature, i.e., below the phospholipid phase transition temperature, yielded LUV. As the gross structure of synthetic phospholipids highly influences the kinetics of film formation, we strongly suggest that preparations studied for their usefulness as surfactant substitute should be adequately characterized in morphology and reproducibility be electron microscopy through thin sectioning, as negative staining might give confusing aspects.

Binary mixed films from synthetic phospholipids display some essential features of pulmonary surfactant, but they are not surfactant. The functions of the alveolar lining layer are very complex. Some examples are: prevention of end-expiratory collapse of smaller alveoli by lowering the surface tension at the air/liquid interface, prevention of inspiratory overdistension of larger alveoli, prevention of lung edema by counteracting the oncotic pressure of the interstitium. Studies with the Wilhelmy balance focus on the surface tension lowering aspect only, yet even this simple analysis is complex enough. The surface tension lowering potential is influenced by the sample (amount, phospholipid composition, charge, particle size and structure) and by the conditions of measurement (trough size, temperature, subphase composition, calcium concentration, pH, cycling time, spread vs. adsorbed film). Surfactant protein [23] and inhibitors in the alveolar space [24] possibily have functions to be considered in the development of surfactant substitutes. Wilhelmy balance characteristics of a synthetic lipid preparation can be interpreted as a model only, giving useful information about various aspects of surfactant function.

The pressure/volume diagram of isolated washed lungs is also not directly comparable to respiratory mechanics in a breathing lung with surfactant deficiency. We have performed these measurements to see if our Wilhelmy data on large unilamellar vesicles can be applied to respiration physiology at all. Both results point in the same direction: LUV from DPPC/PG at a ratio of 9 : 1 adsorb from the subphase and rapidly spread to a film from the trachea which reaches the alveolar wall and which is able to lower the surface tension at compression. This study confirms that DPPC is a more effective surfactant substitute when used in combination with egg PG or another minor compound. In our previous study, a different method of preparation gave surface-active liposomes also with disaturated PG [11]. Ikegami et al. [25] found different synthetic phospholipid combinations to be less effective than natural surfactant in the surfactant-depleted isolated rat lung model. They employed sonication during the preparation of the phospholipids in saline, but not for the natural surfactant.

The function of the surfactant minor compound seems to be enhanced surface spreading. Respreading after overcompression of the film beyond film collapse is likewise enhanced in films containing PG as compared to pure DPPC [26].

Fig. 5 presents a schematic illustration of the steps we believe to be important during monolayer formation. For film spreading at 37°C, a minor phospholipid lowering the phase transition temperature is advantageous. Fast transformation from the vesicle layer to a monolayer is required for adsorption and replacement from a surfactant store

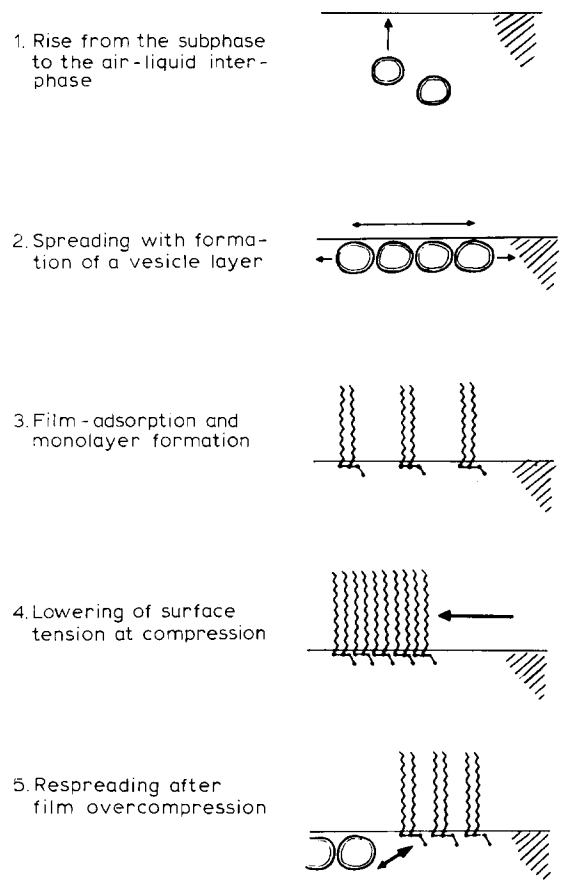

Fig. 5. Schematic illustration of the essential steps in the formation of a surfactant monolayer. The film is adsorbed from a vesicle layer in the subphase. During compression, the film consolidates and counteracts the surface tension at the air/liquid interface. After overcompression of the film, respreading from the subphase is required to reconstitute the monolayer.

in the subphase. For synthetic lipids, it can be achieved with large unilamellar vesicles. The lowering of surface tension at compression is the function of the fatty acid. Saturated PC monolayers withstand higher surface pressure due to their condensed film packing.

The data presented here do not suggest that enhancement of spreading is unique to PG. A negative charge is apparently not essential for this function. Minor compounds with a certain degree of unsaturated fatty acids, amounting up to less than 20% of all surfactant phospholipids [27], probably do not play any role in lowering the surface tension. We wish to speculate, however, that they determine the kinetics of film formation by enhancing adsorption of lipid exchange. If this hypothesis is substantiated, both the understanding of natural surfactant function and the properties required for a surfactant substitute may become clearer. Preparation of spreadable liposomes is one promising way towards the treatment of neonatal or adult respiratory distress syndrome.

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