Development of synthetic lung surfactants

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Abstract We have previously reported the development of a reconstituted lung surfactant consisting of an organic solvent extract of natural bovine lung surfactant supplemented with synthetic lipids. This "artificial" surfactant was used successfully to treat surfactant deficiency states both in animals and humans. We now report on the successful testing of a synthetic lung surfactant consisting of *1*) a lipid-bound protein isolated from natural lung surfactant and *2*) the lipids present in the "artificial" lung surfactant and now used in the same concentration but in a synthetic, commercially available form. The synthetic lung surfactant possessed the in vitro and in vivo surface properties characterizing the "artificial" lung surfactant. In order to identify the components of the synthetic lung surfactant that are responsible for the required surface properties, a series of 25 simple mixtures was prepared. Of these, three possessed surface properties very similar to those of the "artificial" lung surfactant and the synthetic lung surfactant, in vitro as well as in vivo. These three mixtures had four components in common. Besides dipalmitoyl phosphatidylcholine and the lipid-bound protein, they each had a saturated fatty acid, palmitic or stearic, and they each had an acidic phospholipid, phosphatidylglycerol or phosphatidylserine. — **Tanaka, Y., T. Takei, T. Aiba, K. Masuda, A. Kiuchi, and T. Fujiwara.** Development of synthetic lung surfactants. *J. Lipid Res.* 1986. **27:** 475–485.

Supplementary key words dipalmitoyl phosphatidylcholine • acidic phospholipid • saturated fatty acid • lipid-bound protein • surface tension–area diagram • surface spreading • surface adsorption • dynamic respreading • lung pressure–volume characteristics

Surfactant replacement therapy for the respiratory distress syndrome (RDS) of the newborn has been a goal since the first suggestion that this syndrome was due to surfactant deficiency (1, 2).

Early results with an aerosol preparation containing dipalmitoyl phosphatidylcholine (DPPC), the major component of lung surfactant, were not encouraging (3). In the past decade, in their studies on premature animals, Enhorning and Robertson (4) and Enhorning et al. (5) demonstrated that a crude natural surfactant, harvested by lung lavage, suspended in saline, and applied to the airways, protected against the respiratory complications resulting from a surfactant deficiency. These studies have been verified by others using premature sheep (6, 7) as well as rabbits (8).

In 1980, Fujiwara et al. (9), using a reconstituted bovine surfactant lipid (10), demonstrated a marked improvement in lung expansion and gaseous exchange in ten infants with severe RDS. Recently, confirmatory results have been reported in several well-controlled clinical trials (11–14).

Of particular importance is the observation that these successful clinical results were obtained with lipid extracts or reconstituted lipids, both containing very low protein concentrations (10, 12, 15, 16). Encouraged by these results, we have attempted to better define the functional characteristics of the individual lung surfactant components (17–23). By carefully and systematically altering the composition of the complex mixture that we knew was efficacious, we hoped to be able to eliminate some of the constituents without a loss of efficacy. Our ultimate goal was to develop a surfactant, consisting of synthetic components only, that could be used successfully for treating neonatal RDS.

The results to be presented indicate that surface properties, analogous to those of natural lung surfactant, can be obtained with a mixture of synthetic lipids to which is added a fraction of lipid-bound protein, isolated from lung surfactant lipids (LSL). The synthetic lipids used were similar to those present in the surfactant that had been found to be very effective when tested in animal (20–22, 24) and clinical trials (9, 25).

Abbreviations: LSL, lung surfactant lipids; ALS, artificial lung surfactant; SLS, synthetic lung surfactant; PC, phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; PG, phosphatidylglycerol; SM, sphingomyelin; DMPC, dimyristoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; POPC, palmitoyl oleoyl phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; γ_{min}, minimum surface tension; γ_{max}, maximum surface tension; P-V, lung pressure–volume; RDS, respiratory distress syndrome.

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MATERIALS AND METHODS

Materials

DPPC, egg phosphatidylglycerol (PG), cow brain sphingomyelin (SM), and cholesterol were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Dimyristoyl phosphatidylcholine (DMPC), distearoyl phosphatidylcholine (DSPC), dioleoyl phosphatidylcholine (DOPC), palmitoyl oleoyl phosphatidylcholine (POPC), cow brain phosphatidylserine (PS), pig liver phosphatidylinositol (PI), pig liver phosphatidylethanolamine (PE), and palmitoylglycerols were obtained from Serdary Research Labs. (London, Ontario, Canada). Fatty acids were obtained from Tokyo Kasei Co. Ltd. (Tokyo).

These materials were analyzed by thin-layer chromatography (one spot) and by gas-liquid chromatography (> 99.5%). All other chemicals used were reagent grade or better. Distilled water from all-glass stills was used in all measurements of in vitro and in vivo surface activities.

Preparation of artificial lung surfactant (ALS)

The LSL isolated from minced bovine lung tissues by the previously described procedure (17, 21) was dissolved in chloroform–methanol 2:1 (v/v). By adding DPPC palmitic acid, dissolved in the above solvent, the concentrations of disaturated phosphatidylcholine (PC) and fatty acids in LSL were adjusted to 47.5% and 8.5%, respectively. After evaporation of the solvent, the residues were suspended in distilled water containing 10% ethanol. The suspension was warmed to 45°C for 30 min and then lyophilized. The surfactant obtained was dispersed in saline by sonication for 5 min in an ice bath (Super Sonic Vibrator UR-150P; Tomy Co. Ltd., Tokyo) and was stored under nitrogen at −20°C. The concentration of DPPC was assumed to be that of disaturated PC.

Isolation of lipid-bound protein

LSL dissolved in chloroform–methanol 2:1 (v/v) was loaded on a column of Sephadex LH 20 packed in the same solvent. The column was eluted with that solvent and protein fractions were collected.

The detailed physicochemical and surface properties of the lipid-bound protein have been described elsewhere (22).

Preparation of synthetic lung surfactant (SLS)

Chloroform–methanol 2:1 (v/v) solutions of phospholipids, fatty acids, acylglycerols, cholesterol, and the lipid-bound protein isolated from LSL were mixed in suitable proportions by agitation for 2 min. After evaporation of the solvent, a small amount of 10% ethanol was added, the mixture was kept at 45°C for 30 min, and was then lyophilized for removal of the organic solvent. The lyophilized material was then dispersed in saline by sonication and stored in the same manner as ALS.

The chemical compositions of ALS, SLS 1,[2] and various mixtures (indicated by the Arabic numerals) are given in **Table 1** and **Table 2**, respectively.

Chemical analysis

Individual phospholipids were separated by two-dimensional thin-layer chromatography (20 × 20 cm, precoated with Silica gel 60, E. Merck) in two solvent mixtures: chloroform–methanol–water 65:25:4 (v/v) and chloroform–methanol–7 N ammonia solution 230:90:15 (v/v); the lipid spots were quantified by measuring the phosphorus content after digestion with 70% perchloric acid (26). After separation with thin-layer chromatography (5 × 20 cm, precoated with Silica gel 60, E. Merck) in a solvent mixture containing petroleum ether–diethyl ether 3:1 (v/v), fatty acids, acylglycerols, and cholesterol were quantitated according to the procedure of Itaya and Ui (27), the acetylacetone method (28) and the *o*-phthalaldehyde method (29), respectively. Protein content was determined by the method of Lowry et al. (30) with the addition of 1% sodium dodecyl sulfate to the reagent to prevent the turbidity caused by lipids (31). Crystalline bovine serum albumin was used as a standard. Carbohydrate content was measured by the method of Dubois et al. (32). Disaturated PC content was measured according to the method of Shimojo, Abe, and Ohta (33).

Surface tension–area diagram

Surface activity was measured with a modified Wilhelmy surface tension balance (Kyowa Co. Ltd., Tokyo) as described previously (21). Unless otherwise specified, aqueous suspensions containing 81 μg of lung surfactant phospholipids were spread on a clean surface of normal saline (50 ml). Temperature was controlled at 37 ± 0.5°C with water circulating around the trough. After aging for 1 min, the surface film was compressed and expanded from a maximum surface area of 54 cm^2 to a minimum area of 21.6 cm^2 in 2.4 min. Cycling was continued until no further change was observed which, with few exceptions, required no more than 4–5 cycles.

Surface area at 10 dynes/cm of the fourth cycle was expressed as percent of the total area.

Dynamic respreading

Dynamic respreading after compression past collapse was measured by the method of Turcotte et al. (34) in some mixtures of special interest. The given dry surfactant mixtures were spread from hexane–ethanol 9:1 (v/v) on a saline subphase. The surface concentration used was 13 $Å^2$/molecule. Surface tension–area diagrams were recorded as before at 25°C subphase temperature.

[2]The 25 experimental mixtures studied begin with SLS 2. SLS 1 refers to the artificial lung surfactant.

TABLE 1. Chemical composition of artificial lung surfactant (ALS) and synthetic lung surfactant (SLS) 1

Chemical Composition	ALS[a] (Mean ± SD, n = 4)	SLS 1[a]
Phospholipids	84.7 ± 4.5	84.5
Disaturated phosphatidylcholine	47.5 ± 0.2	47.5[b]
Other phosphatidylcholine	16.6 ± 1.9	16.5[c]
Lysophosphatidylcholine	0.3 ± 0.1	0.5[d]
Phosphatidylglycerol	3.8 ± 0.5	4.0[e]
Phosphatidylserine	2.1 ± 1.1	2.0[f]
Phosphatidylinositol	1.4 ± 0.5	1.5[g]
Phosphatidylethanolamine	6.2 ± 0.8	6.0[h]
Sphingomyelin	6.8 ± 1.8	6.5[i]
Fatty acids	8.5 ± 0.2	8.5[j]
Acylglycerols	5.5 ± 1.9	5.5
Triacylglycerols	4.4 ± 1.9	4.0[k]
Diacylglycerols	0.7 ± 0.1	1.0[l]
Monoacylglycerols	0.4 ± 0.1	0.5[m]
Cholesterol	0.4 ± 0.1	0.5
Protein	0.8 ± 0.4	1.0[n]

[a]All values are % weight.
[b]Dipalmitoyl phosphatidylcholine was used.
[c]A mixture of dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine-dioleoyl phosphatidylcholine-palmitoyl oleoyl phosphatidylcholine (2.7:5.0:0.4:0.4, w/w).
[d]Monopalmitoyl phosphatidylcholine.
[e]Egg phosphatidylglycerol.
[f]Cow brain phosphatidylserine.
[g]Pig liver phosphatidylinositol.
[h]Pig liver phosphatidylethanolamine.
[i]Cow brain sphingomyelin.
[j]Palmitic acid.
[k]Tripalmitoylglycerol.
[l]Dipalmitoylglycerol.
[m]Monopalmitoylglycerol.
[n]A lipid-bound protein isolated from lung surfactant lipids.

Surface spreading rate

The surface spreading rate was measured in a round Teflon trough (2 cm deep and 5 cm in diameter) with a platinum plate supported by the strain gauge of a surface balance (Kyowa Co. Ltd.) dipping into a 30-ml saline solution. As soon as an aliquot containing 29.4 μg of surfactant phospholipids was layered onto the surface of the saline subphase, the rate at which the surface tension was lowered was measured for 2 min at 37°C.

Surface adsorption rate

While stirring at 60 rpm, 1.5 mg of surfactant phospholipids was injected deep into the 30-ml saline solution in the round Teflon trough. The change in surface tension with time was measured for 2 min at 37°C.

Pressure-volume (P-V) characteristics of neonatal lungs

For an evaluation of how the surfactant, instilled into the trachea of preterm rabbit neonates, might affect the pressure-volume relationship during lung expansion, we used a modification of a technique we have previously described (20, 22). At 27 and 30 days gestation the does used for these experiments were anesthetized with sodium pentobarbital and the fetuses were delivered by hysterotomy. Breathing was prevented and, after the animals had been weighed, the trachea was cannulated. A surfactant suspension containing 50 mg of phospholipids per kg body weight was instilled into the cannula which was then connected to a syringe and a water manometer. Pressure was increased to 30 cm H_2O for 2 min and then lowered gradually to 0 cm H_2O. For each neonate studied with surfactant, two littermates were matched as controls.

Statistical method

Student's *t* tests were used for evaluation of the difference in each variable between ALS and SLS preparations. All data are depicted as mean ± SD. Obtained data were also analyzed by regression analyses. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference.

TABLE 2. Chemical composition of synthetic lung surfactant (SLS) 2-26

SLS No.	Phospholipids[a] PC[b]	PG	PS	PI	PE	SM	FA[c]	Acylglycerols TP[d]	DP[e]	Chol[f]	Pro[g]
2	64.0	4.0	2.3	1.5	6.0	7.0	8.5	4.5	1.0	0.4	0.8
3	64.0	4.0	2.3	1.5	6.0	7.0	8.5	4.5	1.0	0.4	0.8
4	68.0	4.3	2.4	1.6	6.4	7.4	9.0				0.9
5	90.1						9.0				0.9
6	74.7	4.7	2.7	1.8	7.0	8.2					0.9
7	68.6	4.3	2.5	1.6	6.4	7.5	9.0				
8	68.0	22.1					9.0				0.9
9	68.0		22.1				9.0				0.9
10	68.0			22.1			9.0				0.9
11	68.0				11.1	11.1	9.0				0.9
12	68.0	22.1					9.0				0.9
13	68.0	22.1					9.0				0.9
14	68.0	22.1					9.0				0.9
15	74.7	24.4									0.9
16	68.0	22.1						9.0			0.9
17	68.0	22.1							9.0		0.9
18	68.0	22.1								9.0	0.9
19	68.6	22.3					9.0				
20	68.6	22.3					9.0				0.1
21	66.8	21.7					8.9				2.5
22	63.2	20.5					8.4				8.0
23	75.5	24.5									
24	91.0						9.0				
25	99.1										0.9
26							91.4				8.6

[a]All values are % weight.
[b]PC (phosphatidylcholine) used was dipalmitoyl phosphatidylcholine (DPPC) except for SLS 2 when it was a mixture of DPPC-dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine-dioleoyl phosphatidylcholine-palmitoyl oleoyl phosphatidylcholine (47.5:2.7:5.0:0.4:0.4, W/W).
[c]FA (fatty acid) was palmitic acid except for SLS 12 when it was palmitoleic acid, SLS 13 when it was stearic acid, and SLS 14 when it was oleic acid.
[d]TP, tripalmitoylglycerol.
[e]DP, dipalmitoylglycerol.
[f]Chol, cholesterol.
[g]Pro, a lipid-bound protein isolated from lung surfactant lipids.

TABLE 3. Surface properties of artificial lung surfactant (ALS) and synthetic lung surfactant (SLS)

Sample	γ_{min}	γ_{max}	Surface Area at 10 dynes/cm	Surface Adsorption 30 sec	60 sec	120 sec
	dynes/cm		%		*dynes/cm*	
ALS	5.2 ± 1.3	26.7 ± 1.8	76.8 ± 1.2	34.5 ± 3.5	31.1 ± 2.4	29.3 ± 2.3
SLS 1	5.8 ± 2.1	28.3 ± 1.7	75.3 ± 1.5	34.1 ± 2.6	31.4 ± 2.7	29.7 ± 2.3
2	7.3 ± 2.2	29.7 ± 2.0[a]	75.6 ± 2.1	32.1 ± 2.1	31.1 ± 2.3	30.3 ± 2.2
3	2.8 ± 1.5[a]	27.8 ± 2.2	79.5 ± 1.3[a]	33.0 ± 1.8	32.0 ± 1.8	31.1 ± 1.7
4	5.3 ± 1.2	26.6 ± 1.7	76.4 ± 1.7	31.8 ± 2.3	31.0 ± 2.2	30.4 ± 2.0
5	4.5 ± 2.1	48.0 ± 2.7[c]	52.1 ± 3.3[c]	58.8 ± 5.7[a]	53.4 ± 5.3[a]	47.3 ± 4.8[b]
6	8.8 ± 2.0[b]	38.2 ± 2.6[c]	43.9 ± 2.8[c]	38.2 ± 3.0	36.6 ± 2.9	35.2 ± 2.7[a]
7	3.1 ± 2.1	34.4 ± 2.2[c]	48.8 ± 3.1[c]	53.3 ± 3.4[a]	52.5 ± 3.1[c]	51.8 ± 3.0[c]
8	0.2 ± 0.2[c]	27.2 ± 1.6	76.7 ± 1.4	33.0 ± 2.7	31.9 ± 2.6	31.1 ± 2.3
9	0.3 ± 0.3[c]	23.2 ± 1.7[a]	32.2 ± 1.4	76.3 ± 1.4	31.0 ± 1.9	30.2 ± 1.8
10	7.0 ± 2.7	39.5 ± 2.7[c]	47.3 ± 3.0[c]	46.8 ± 4.0[b]	44.8 ± 3.4[b]	43.3 ± 3.2[b]
11	11.6 ± 2.3[c]	31.2 ± 2.6[a]		60.4 ± 5.5[a]	55.8 ± 4.4[b]	52.7 ± 3.8[c]
12	0.9 ± 0.6[c]	32.8 ± 2.8[b]	58.3 ± 2.6[c]	30.2 ± 2.8	29.2 ± 2.0	28.4 ± 1.5
13	0.6 ± 0.4[c]	30.8 ± 2.5[a]	75.2 ± 2.2	31.8 ± 1.0	31.1 ± 1.8	30.7 ± 1.8
14	0.6 ± 0.5[c]	31.2 ± 3.1[a]	69.3 ± 3.2[a]	30.7 ± 2.4	29.6 ± 1.9	29.0 ± 1.6
15	10.0 ± 1.9[b]	30.8 ± 1.7[b]	41.2 ± 2.5[c]	28.6 ± 2.7	28.2 ± 2.5	28.0 ± 2.3
16	0.2 ± 0.1[c]	23.3 ± 1.3[b]	71.1 ± 1.9[c]	32.9 ± 3.4	31.9 ± 3.3	31.2 ± 3.1
17	0.3 ± 0.2[c]	24.7 ± 1.5	69.2 ± 2.4[c]	41.8 ± 4.2	38.7 ± 3.8[a]	36.6 ± 3.7[a]
18	0.4 ± 0.2[c]	25.9 ± 1.0	59.7 ± 3.6[c]	45.1 ± 3.8[a]	44.4 ± 3.5[b]	43.8 ± 3.4[b]
19	2.3 ± 1.7[a]	34.7 ± 1.7[c]	51.2 ± 3.1[c]	55.2 ± 4.2[b]	51.9 ± 3.9[b]	49.8 ± 3.6[b]
20	1.0 ± 0.8[c]	26.5 ± 1.6	78.9 ± 1.5[a]	33.0 ± 3.1	31.8 ± 2.8	31.1 ± 2.6
21	2.1 ± 0.9[b]	27.4 ± 1.8	74.7 ± 1.8	32.2 ± 3.6	31.3 ± 3.1	30.7 ± 3.1
22	3.3 ± 1.6	26.2 ± 2.2	70.5 ± 2.8[b]	32.8 ± 3.3	31.8 ± 2.9	31.1 ± 2.8
23				70.4 ± 0.0[c]	70.4 ± 0.0[c]	70.4 ± 0.0[c]
24	22.0 ± 3.1[c]	55.9 ± 1.9[c]		70.4 ± 0.0[c]	70.4 ± 0.0[c]	70.4 ± 0.0[c]
25	2.3 ± 0.8[b]	20.0 ± 1.0[c]	69.8 ± 2.1[c]	29.4 ± 2.7	28.4 ± 2.5	27.7 ± 2.4
26				70.4 ± 0.0[c]	70.4 ± 0.0[c]	70.4 ± 0.0[c]

γ_{min} is minimum surface tension and γ_{max} is maximum surface tension. All values are mean ± SD; surface adsorption, n = 3; others, n = 5; [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; compared with ALS.

RESULTS

Surface properties of ALS and SLS 1

Both preparations exhibited rapid adsorption characteristics. Since spreading rates were very similar to adsorption rates, only the adsorption data are included in **Table 3.** When compressed, their films were able to yield a minimum surface tension (γ_{min}) of 5–6 dynes/cm. Surface tensions of less than 10 dynes/cm were achieved with only 20–25% surface compression. Upon expansion, the surface tensions rose to a maximum (γ_{max}) of 27–28 dynes/cm. The hysteresis loop was very reproducible from the second cycle and continued to be relatively stable up through the 10 cycles of dynamic compression–expansion. Surface tension–area diagrams obtained for the fourth cycle are shown in **Fig. 1.** These surface tension values of SLS 1 were not statistically different from those of ALS.

P-V characteristics of ALS and SLS 1

Quasi-static P-V characteristics of the lungs of prematurely delivered rabbit neonates (gestational age 27 days) after tracheal instillation of ALS and SLS 1 are shown in **Fig. 2.** For comparison, the data are given from littermate controls and from neonates at term (30 days), receiving no surfactant. The lung volumes at deflation to 5 cm H_2O were 4.2 ± 2.8 ml/kg body weight for the preterm neonates and 47.5 ± 6.2 ml/kg body weight for those delivered at term. These differences are statistically significant ($P < 0.001$). All premature neonates receiving surfactant had good P-V characteristics, not differing from those observed at term. The P-V characteristics of SLS 1 were not statistically different from those of ALS. When any material exhibited all of the in vitro surface properties characterizing ALS or SLS 1, we defined that material as an "acceptable" surfactant preparation.

Surface properties of SLS preparations (SLS 2–26)

Data for the surface properties of preparations 2–26 are given in Table 3, and the typical surface tension–area isotherms are shown in **Fig. 3** and **Fig. 4.**

SLS 2 and 3

SLS 2 was reconstituted to match the composition of SLS 1 with the exception that minor components, lyso-

phosphatidylcholine and monoacylglycerols, were eliminated. SLS 3 was identical with SLS 2 with the exception that the mixture of PC had been replaced with DPPC. Both SLS 2 and 3 displayed acceptable surface activity as defined above.

SLS 4–7

Some of the components were removed from SLS 3 to obtain SLS 4–7. As shown in Fig. 3, the surface tension–area diagram for SLS 4, which did not contain any neutral lipids, had a γ_{min} of about 5 dynes/cm, and a surface tension of 10 dynes/cm was reached with only 24% surface compression. These values were essentially the same as those for ALS. SLS 5, containing DPPC as the only phospholipid, showed an unacceptably low rate of surface adsorption and required high surface compression (48%) to yield a γ_{min} of 10 dynes/cm. The maximum surface tension was 48 dynes/cm (Fig. 4). In terms of the ability to generate a low minimum surface tension under dynamic compression, it is clear that SLS 5 is as effective a surfactant as ALS. However, SLS 5 displayed a poor dynamic respreading property and requried a significantly higher surface compression than ALS ($P < 0.001$) (**Table 4**). SLS 6 (no fatty acids) and SLS 7 (no lipid-bound protein) showed low rates of adsorption and required significantly higher surface compression. SLS 5–7 were thus eliminated.

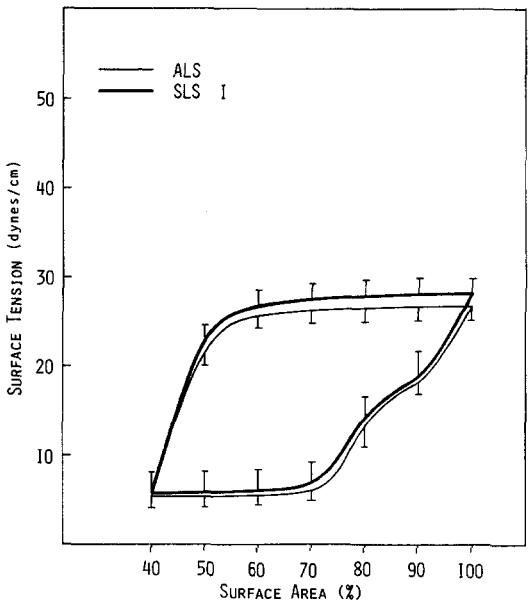

Fig. 1. Surface tension–area diagrams of artificial lung surfactant (ALS) and synthetic lung surfactant (SLS) 1. An aliquot of surfactant containing 1.5 μg of phospholipids/cm^2 surface area was layered onto the surface of saline solution. One min later, the surface of the solution was compressed and expanded between 54.0 and 21.6 cm^2 at a rate of 2.4 min/cycle at 37°C. Surface tension and surface area were continuously recorded. Means ± SD, n = 5.

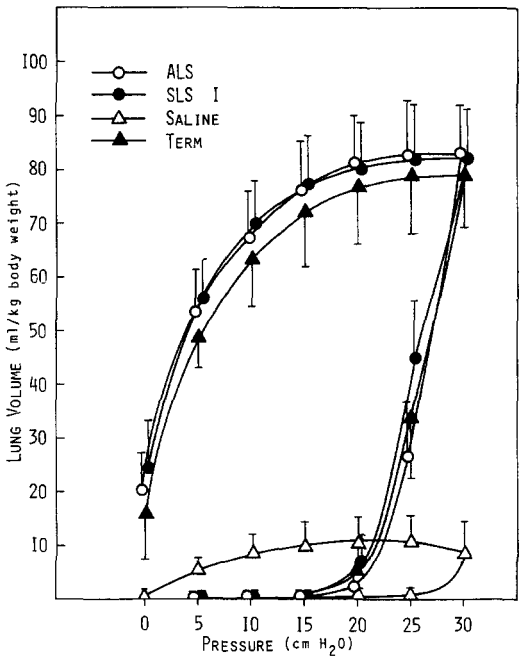

Fig. 2. Pressure–volume characteristics of premature rabbit neonates (gestational age 27 days) after tracheal instillation of artificial lung surfactant (ALS) and synthetic lung surfactant (SLS) 1 in comparison with those of neonates at term (30 days) receiving no surfactant. A sample of 50 mg of surfactant phospholipids/kg body weight was instilled. Air pressure was gradually increased to 30 cm H_2O and reduced to 0 cm H_2O at 37°C with a syringe pump. Mean ± SD, n = 4.

SLS 8–11

The purpose of designing these various preparations was to assess the effects of various acidic phospholipids on their surface properties (Figs. 3 and 4). Preparations 8 and 9 were found to be acceptable, but SLS 10, containing PI, had an unacceptably low rate of adsorption. SLS 11, containing PE and SM but no acidic phospholipids, was unable to attain $\gamma_{min} < 10$ dynes/cm even with full compression and had a very low rate of adsorption. As shown in Table 4, the respreading and surface compression properties of SLS 8 were identical to those of ALS. SLS 5, containing no acidic phospholipids, was able to lower the surface tension below 10 dynes/cm with greater surface compression (Fig. 4) and it also adsorbed slowly.

SLS 12–14

The effects of fatty acids on the surface properties of these preparations were investigated. They had excellent adsorption characteristics and were able to yield a γ_{min} of near zero with the application of various degrees of surface compression. SLS 13, containing stearic acid, had excellent surface properties similar to those of SLS 8, which contained palmitic acid.

SLS 15–18

These preparations consisting of a mixture (DPPC, PG, and the lipid-bound protein) with and without a

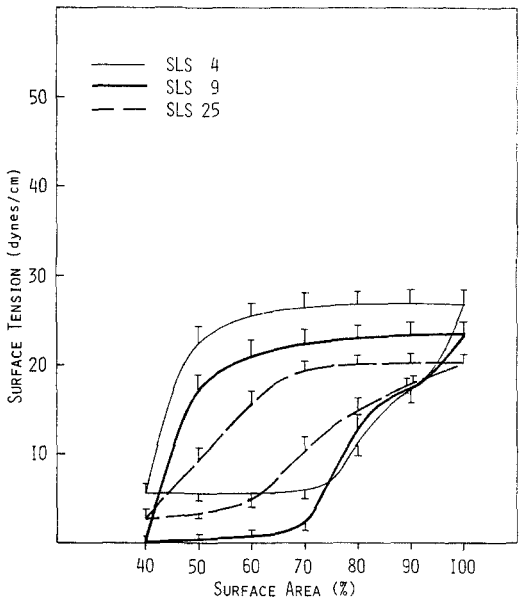

Fig. 3. Surface tension-area diagrams of synthetic lung surfactants (SLS) 4, 9, and 25. Details as in Fig. 1. Mean ± SD, n = 5.

neutral lipid (SLS 16-18 and 15, respectively) were examined for the effects of neutral lipids on the surface properties.

The aqueous dispersion of SLS 15 exhibited excellent adsorption, but did not yield a γ_{min} of less than 10 dynes/cm even with full compression at 37°C (Fig. 4). However, when this preparation was spread on the subphase from organic solution at 25°C, it exhibited the surface tension-area diagram with a minimum surface compression equivalent to that of ALS. This discrepancy is ascribed to the different spreading techniques (organic solvent vs. saline dispersions) and temperatures at which the measurements were made (25°C vs. 37°C).

As shown in Table 4, the cycle 6 to cycle 1 collapse ratio of the organic solvent spread films of SLS 15 was significantly lower than that of ALS, indicating an inferior respreading for SLS 15.

SLS 16 showed a rapid adsorption rate equivalent to that of ALS, but required a significantly higher surface compression than ALS in order to lower the surface tension below 10 dynes/cm ($P < 0.001$). Both SLS 17 and 18 required greater surface compression to lower the surface tensions below 10 dynes/cm and they also adsorbed slowly.

All kinds of neutral lipids improved the surface tension-lowering ability, but at the same time they adversely affected either adsorption or respreading facility (Table 3).

SLS 19-22

These preparations were examined for the effects of the lipid-bound protein on the surface properties. SLS 19-22 contained fixed quantities of DPPC, PG, and palmitic acid but varying concentrations of the lipid-bound protein. SLS 19, without this protein, showed a very slow adsorption and a very low collapse plateau ratio, and required greater compression to achieve a surface tension below 10 dynes/cm (Fig. 4 and Table 4).

Irrespective of the concentration, all SLS preparations containing the lipid-bound protein showed rapid adsorption and respreading, and were able to yield a γ_{min} of 0-5 dynes/cm (SLS 20-22). However, for the optimal surface activity, the concentration of the lipid-bound protein needed not to be greater than 0.9% by weight, as in SLS 8.

SLS 23-26

The components that are required for a complete return of surface activities similar to those of SLS 1 were examined with SLS 23-26. DPPC, PG, palmitic acid, and the lipid-bound protein, when tested individually, did not fulfill the criteria nor did a mixture of DPPC and PG (SLS 23). The addition of palmitic acid to the mixture of DPPC and PG (SLS 19) or to DPPC alone (SLS 24) did not improve the surface properties of SLS 19 or SLS 24.

The mixture of DPPC and the lipid-bound protein (SLS 25) showed rapid adsorption and was able to yield a γ_{min} of 2 dynes/cm. However, more than 30% surface area had to be compressed to lower the surface tension to less than 10 dynes/cm (Fig. 3) and the dynamic respreading property was significantly less than ALS (Table 4). The results obtained with SLS 23-26, as well as other preparations described above, suggest the importance of the lipid-bound protein.

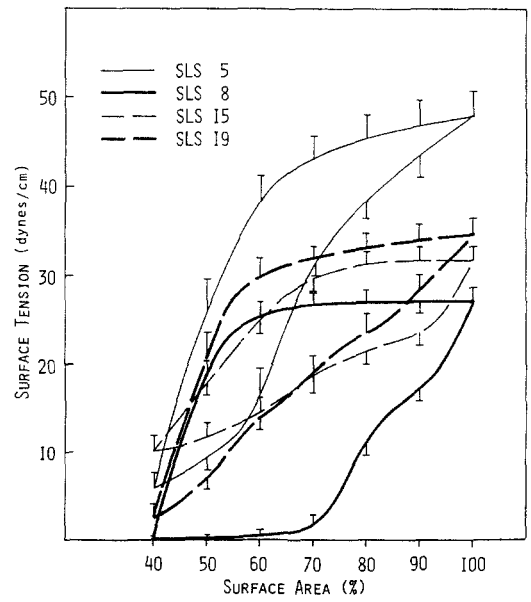

Fig. 4. Surface tension-area diagrams of synthetic lung surfactants (SLS) 5, 8, 15, and 19. Details as in Fig. 1. Mean ± SD, n = 5.

TABLE 4. Comparison of respreading and surface compression properties of artificial lung surfactant (ALS) and representative synthetic lung surfactant (SLS)

	ALS	SLS 5	SLS 8	SLS 15	SLS 19	SLS 25
Collapse plateau ratio						
Cycle 2:1	0.86 ± 0.03	0.91 ± 0.02 (NS)	0.85 ± 0.02 (NS)	0.89 ± 0.08 (NS)	0.46 ± 0.08**	0.53 ± 0.08**
Cycle 4:1	0.82 ± 0.06	0.51 ± 0.05**	0.73 ± 0.02 (NS)	0.66 ± 0.09 (NS)	0.16 ± 0.07***	0.30 ± 0.13**
Cycle 6:1	0.72 ± 0.02	0.42 ± 0.06**	0.65 ± 0.04 (NS)	0.43 ± 0.06**	0.12 ± 0.05***	0.21 ± 0.12**
% Surface area at 10 dynes/cm						
Cycle 2	73.2 ± 0.7	75.4 ± 3.1 (NS)	75.3 ± 2.8 (NS)	77.2 ± 5.2 (NS)	61.2 ± 2.3***	70.3 ± 3.8 (NS)
Cycle 4	71.1 ± 1.5	60.8 ± 6.7**	70.5 ± 3.7 (NS)	72.5 ± 5.8 (NS)	54.2 ± 7.4***	63.0 ± 5.5 (NS)
Cycle 6	69.2 ± 1.0	58.7 ± 1.4***	65.4 ± 1.3 (NS)	65.6 ± 2.4 (NS)	50.6 ± 2.5***	59.4 ± 5.4*

Surfactant films were spread from hexane–ethanol 9:1 (v/v) solution. Initial spreading to 13 Å^2/molecule, in a modified Wilhelmy surface balance at 25°C with saline subphase, compressed at 2.4 min/complete cycle 2.5:1. Values are expressed as mean ± SD, n = 3; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$, compared with ALS; % surface compression parameter well correlated with the collapse plateau ratio ($r = 0.879$, $P < 0.001$).

P-V characteristics of representative SLS

Fig. 5 and **Fig. 6** show the P-V data from lungs of premature (27 days) rabbit neonates after tracheal instillation of representative SLS preparations. SLS 4, 8, and 9 were acceptable surfactants according to surface balance results but SLS 5, 15, 19, and 25 were not.

For comparison, the P-V loops of littermate controls and of term neonates are also shown. The loops obtained with the acceptable surfactants, SLS 4, 8, and 9 did not differ from those of term neonates. The loops with SLS 5, 15, 19, and 25 were better than those of control littermates, but they were clearly inferior to those obtained with SLS 4, 8, and 9.

DISCUSSION

The in vitro interfacial properties thought to be critical for good in vivo function are low minimum surface tension during dynamic compression, rapid adsorption of the surfactant from the subphase to the air–liquid interface, and good respreading after film compression past collapse (See Ref. 35 for review).

A consistent characteristic of the surface tension–area of the films spread from aqueous dispersion[3] of surfactant mixtures (Fig. 1) is the reproducible hysteresis similar to that observed in cycling experiments with alveolar lavage fluid. This reproducible hysteresis reflects the efficient reentry of the molecules that are ejected from the surface during compression. This specific property, dynamic respreading after film compression past collapse, has recently been extensively characterized by Notter et al. (15, 16) in a variety of well-defined films and shown to be a critical variable for physiological function in vivo (36). We evaluated the adsorption property in a series of separate experiments and the respreading property in another set of experiments with solvent spread films of representative mixtures. Since the latter was found to correlate well with the surface compression[4] necessary to reach 10 dynes/cm ($r = 0.879$, $P < 0.001$, Table 4), we used this surface compression parameter as an indirect measure of the respreading property.

The data presented show that a surfactant, having all of the above in vitro interfacial properties can be prepared by mixing *1*) DPPC, *2*) an acidic phospholipid (PG or PS), *3*) a saturated fatty acid (palmitic acid or stearic acid), and *4*) the lipid-bound protein, isolated from the lipids of natural lung surfactant.

This synthetic lung surfactant, SLS, was found to be able to convert the pulmonary mechanics of surfactant-deficient premature rabbit neonates to a mature level, while other mixtures, lacking any of these four components, were less effective.

Since ALS had the proper surface and physiological activity with less batch-to-batch variability as previously reported (17–23) and confirmed here, we formulated a surfactant, SLS 1, containing synthetic lipids of the same kind and in the same concentration as in ALS. In addition, we included the lipid-bound protein isolated from natural lung surfactant.

[3]Because we spread surfactant mixtures from a saline dispersion containing 81 μg of phospholipid onto the surface (54 cm^2), the surface concentration could not be well defined, nor could the subsurface or bulk phase concentration. However, the surface concentration of surfactants appears to correspond to the "surface excess" initial condition which is far beyond that required for monolayer coverage.

[4]Surface area required to reach minimal surface tension value in a given surfactant rather than that required to reach 10 dynes/cm may be used here as an indirect measure of the respreading property.

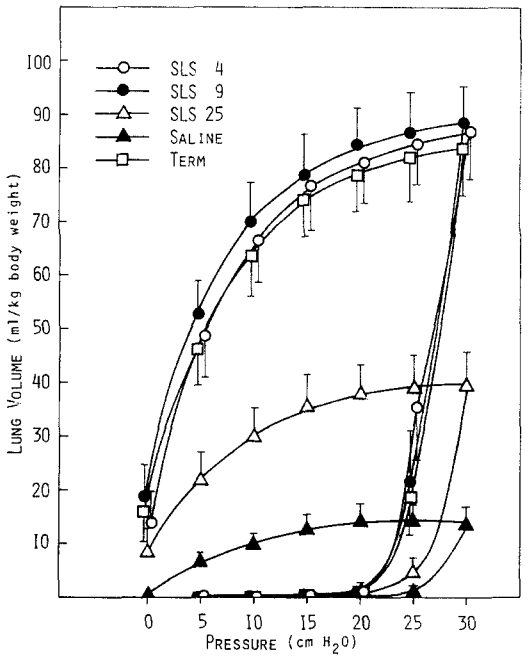

Fig. 5. Pressure-volume characteristics of premature rabbit neonates treated with synthetic lung surfactants (SLS) 4, 9, and 25 in comparison with those of term neonates (30 day gestation). Details as in Fig. 2. Mean ± SD, n = 4.

In order to identify the specific components of SLS 1 that are necessary for producing the requisite surface properties, a series of 25 mixtures was prepared, all mimicking SLS 1, but each with a distinct alteration of the composition.

Of these preparations, SLS 2, 3, 4, 8, 9, and 13 were functionally similar to SLS 1 with respect to respreading, adsorption, and the ability to lower surface tension during dynamic compression.

With the preparations SLS 4, 8–11, we examined the functional role of certain acidic phospholipids added to the mixture of DPPC, palmitic acid, and the lipid-bound protein. PG and PS were equally effective in improving the surface properties of SLS 5 containing DPPC, palmitic acid, and the lipid-bound protein. SLS 11, containing no acidic phospholipids, was minimally surface active. Phosphatidic acid also improved the surface properties of the mixture as did PG and PS (data not shown), but PI was not so effective.

Several workers (37, 38) reported no functional differences between surfactant containing PG or PI in animals, suggesting that the two phospholipids were interchangeable. Since, in this study, we failed to examine the effect of the mixture containing PI on P-V mechanics nor did we examine surface activity of lung-derived PI, we are uncertain whether the use of liver-PI accounts for our inferior results. Our results suggest that acidic phospholipids are necessary for the production of surface properties similar to those of natural surfactant. We presume that these acidic phospholipids, interposed among DPPC, enhance the lung surfactant to form stable multilamellar monomolecular films by providing a substantial electric charge. They may also readily associate with hydrophobic proteins through ionic linkage.

The egg PG and cow brain PS used in this study contained a total of 40–50% unsaturated fatty acids mainly oleic and linoleic acid as judged by gas–liquid chromatography. These unsaturated fatty acid residues probably enhance the fluidity of the DPPC films and also the mixture of lipids and the lipid-bound protein (39).

The isolates of natural lung surfactant (40) or lamellar bodies (41) contain varying amounts of neutral lipids (up to 15% of the total lipid). Cholesterol and free fatty acids are the predominant neutral lipids in the lamellar bodies (41) isolated from lung tissues. The role of these neutral lipids in lung surfactant activity is still unknown.

The addition of palmitic acid to the mixture containing DPPC, PG, and the lipid-bound protein resulted in a complete return of nearly normal surface activities. Stearic acid had the same effect as palmitic acid.

Free fatty acids are present in varying amounts (1–10%) in the isolates of natural lung surfactant (40, 42) or lamellar bodies (41, 43). It is, however, not certain whether these lipids are components of natural surfactant or trace contaminants. Pfleger and Thomas (44) reported relative-

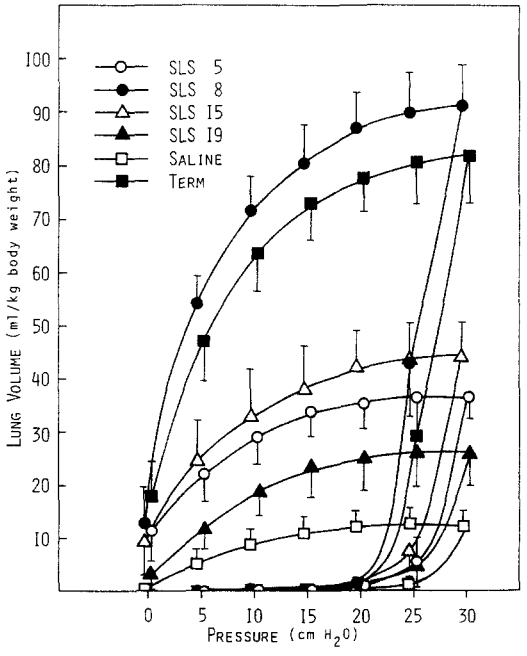

Fig. 6. Pressure-volume characteristics of premature rabbit neonates (27 days) treated with synthetic lung surfactants (SLS) 5, 8, 15, and 19. Details as in Fig. 2. Mean ± SD, n = 4.

ly high free fatty acid concentrations in the clear supernatant and washed lining cells of dog lung lavage as compared to surfactant fraction or lung tissues. These workers claimed that the supernatant lipids containing 8% PG and 6% free fatty acids are also surfactant. However, these workers did not report the surface activity data of these subfractions of lung lavage fluid.

Recent reports (12, 36) show that effective surface and physiological activity can be found in the lipids extracted from lung lavage without high free fatty acid concentrations.

Since the concentration of free fatty acids was chosen purely empirically for the experiments here, further study is clearly needed to determine whether the concentration of these fatty acids could be reduced, without a loss of efficacy, to the 2–3% that was found in the natural surfactant or lamellar bodies.

Although DPPC and PG can easily form bilayer films in aqueous solutions, their hydrophobic properties are not sufficient to form stable films. It seems likely that the stability of monomolecular films depends on the balance between hydrophobic and hydrophilic properties of the reconstituted lipids as in the case of binary films (45). Palmitic acid and stearic acid must be expected to interact with DPPC in mixed films by virtue of their fatty acid chain similarities. The chains are straight and tightly packed and there are specific chain-chain interactions. They might simply enhance the hydrophobic properties of the surfactant phospholipid films by shifting easily among the film components and adapting to the dynamic molecular action in compression–expansion cycles. Although the unsaturated fatty acids also enhance surface activity, the enhancement was not so striking as that in the case of palmitic acid (SLS 12 and 14). This result would be expected inasmuch as the chains of unsaturated fatty acids are bent and more loosely packed. Thus, specific chain-chain interaction may be lost due to partial local rotations along the chain.

Triacylglycerols, diacylglycerols, and cholesterol were inferior to those saturated fatty acids in enhancing the surface activities (Table 3). Although these neutral lipids improve the surface properties of the mixture containing DPPC, PG, and the lipid-bound protein only slightly, their intricate and flattened molecular shapes may impede the free movement of surfactant molecules in the films during dynamic compression–expansion cycles.

The surface properties of SLS 8 and 9, containing all the four components, i.e., DPPC, PG or PS, palmitic acid or stearic acid, and the lipid-bound protein, were identical to those of ALS. The latter contains other lipids that are not present in SLS 8 and 9 but are in the isolates of natural surfactant. They might be contaminants from other sources, e.g., blood or cell membranes. Although they had no disturbing effect on the surface activity, there might be a limit to what can be considered a permissible contamination.

Some of our lipid combinations resulted in a loss of surface activity. They may help to elucidate the mechanism behind the development of neonatal and, in particular, adult RDS. For instance, one of the four components we have found to be essential may be missing or there may be a detrimental excess of a single component, such as lysoPC. This will require further investigation with final testing in an animal model.

A single constituent of surfactant, when tested individually, was not able to exhibit any properties of the total complex. The physical states of hydrophobic/hydrophilic ratio, fluidity, molecular packing, and electric charge appear to be important for lung surfactant activities. Although pure synthetic lipids had profound effects on the physical state of DPPC, a small amount of lipid-bound protein exerted the strongest effect.

For instance, the addition of lipid-bound protein to DPPC strikingly enhanced the adsorption and surface tension-lowering facility (SLS 25). However, this mixture appeared to have poor respreading property and did not produce the satisfactory physiologic results in the animal model (Fig. 5) in contrast to those obtained with SLS 8. Thus, the lipid-bound protein alone is not sufficient to aid the DPPC molecule in producing all the necessary surface activity.

In summary, the three mixtures 8, 9, and 13 were active and had four components in common. Beside DPPC and the lipid-bound protein, they each had a saturated fatty acid, palmitic or stearic, and they each had an acidic phospholipid, PG or PS. The results emphasize that the surface properties of a surfactant consisting of pure synthetic lipids are enhanced by the inclusion of a small amount of the lipid-bound protein. Determinations of the properties of this lipid–protein complex are in progress in our laboratory.

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