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Lung Surfactants. I.[1] Comparison of Surfactants Prepared from Lungs of Calf, Ox, Dog and Rabbit

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Lung surfactants were extracted either by lavage or by mincing method from lungs of calves, oxen, dogs and rabbits, and were purified in parallel. These lung surfactants were modified by adding some components in which they were apparently deficient; the contents of disaturated phosphatidylcholine, fatty acids and triacylglycerols were adjusted to 47, 7 and 7%, respectively. The native and modified lung surfactants were examined with respect to their surface properties *in vitro* and their lung pressure–volume characteristics *in vivo*.

No obvious differences were found in the chemical components of the lung surfactants from different mammalian species and of those prepared by the different extraction methods. Although there were no distinct differences in the phosphatidylcholine contents of the lung surfactants among the mammalian species, the content in the samples obtained by the lavage method was slightly higher than that in samples obtained by the mincing method.

Although all the native lung surfactants spread spontaneously and were rapidly adsorbed, they did not show good surface activities *in vitro* or good lung pressure–volume characteristics *in vivo*. In contrast, all the modified lung surfactants showed good surface properties *in vitro* and also gave normal lung pressure–volume characteristics to premature rabbit fetuses *in vivo* irrespective of the mammalian species from which the native surfactants had been derived or of the extraction method used.

Keywords——lung surfactant; modified lung surfactant; spreading; adsorption; surface activity; lung pressure–volume characteristic

Lung surfactants, present at the air–liquid interface of the alveoli, are essential for normal respiration; they lower the surface tension of the interface and prevent collapse of the lung.[2] Avery and Mead observed inadequate surface activity in the lung of neonates with respiratory distress syndrome (RDS).[3] A proposed new treatment for RDS is to instill a lung surfactant preparation into the lung *via* the trachea.[4] The lung surfactants have aroused the interest of many investigators, and numerous reports have been published.[5–7] However, many uncertainties and disagreements concerning the chemical composition and physiological properties of the surfactants still remain, though it is agreed that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is a major component.[8–10] No lung surfactant preparation with sufficient activity for the treatment of RDS has been obtained. The aims of this study were to resolve these disagreements and to obtain an effective medicine for the treatment of RDS.

We recently reported a procedure for the preparation of lung surfactant from minced bovine lung and determined its chemical composition. The lung surfactant was capable of spontaneous spreading and of lowering the surface tension to 10 dyn/cm and below at 30% compression of the surface area.[11] We also found that fatty acids and triacylglycerols, although they had previously been considered to inhibit surface activity,[12,13] were in fact indispensable components of lung surfactant in addition to DPPC and protein.[14] Lung surfactant has many components and, during extensive purification, it is practically impossible to recover the surfactant components alone from the surface of the alveoli. Thus, we tried to

modify lung surfactant by adding some components in which it was considered to be deficient. We succeeded in obtaining an artificially modified lung surfactant which very closely resembles the natural surfactant at 37 °C *in vitro* and gives good lung pressure–volume characteristics to mammals *in situ* and *in vivo*.[15,16] Fujiwara *et al.* established that the modified lung surfactant was an effective treatment for RDS.[17,18]

For the extraction of lung surfactant, a lung lavage method is more appropriate than a mincing method, because there are fewer contaminants from lung tissue and blood in the lavage method than in the mincing method. However, in some comparative studies of surfactants extracted by the lung lavage and the mincing methods, similar results have been obtained as regards chemical composition and surface properties.[10,19-21] In order to confirm the results obtained on the lung surfactant from minced bovine lung, we performed comparative experiments on lung surfactants extracted by the lavage and the mincing methods from the lungs of some other mammalian species, and also on modified lung surfactants prepared from these lung surfactants.

Materials and Methods

Materials——DPPC was purchased from Fluka (Buchs, Switzerland), and palmitic acid and tripalmitoylglycerol were purchased from Tokyo Kasei Co. (Tokyo). All other chemicals were of reagent grade.

Analysis of Phospholipid Phosphorus——Phospholipids content was determined by measuring phosphorus content after digestion with 70% perchloric acid[22] and was calculated on the assumption that phospholipids contained 4% phosphorus on average.

Separation and Analyses of Phospholipids——Individual phospholipids were quantified by the above phosphorus analysis method after separation by two-dimensional thin layer chromatography (TLC) on a plate (20 × 20 cm, pre-coated with Silica gel 60, E. Merck) in two solvent systems: chloroform–methanol–water (65 : 25 : 4, v/v) and chloroform–methanol–7N ammonia (230 : 90 : 15, v/v). Areas corresponding to the separated lipid classes were visualized on the plate with iodine vapor, and scraped directly into hydrolysis tubes for analysis. Individual phospholipids were previously identified by co-chromatography with standards.

Separation and Analyses of Acylglycerols, Fatty Acids and Cholesterol——After separation by TLC on two plates (5 × 20 cm, pre-coated with Silica gel 60, E. Merck) in a solvent system of petroleum ether–diethyl ether (3 : 1, v/v), compounds were visualized on one plate with iodine vapor. The corresponding regions on the other plate were scraped off. Individual lipids had first been identified by comparison with standards. The lipids were each extracted with chloroform, and after evaporation of the chloroform, acylglycerols were measured by the acetylacetone method[23] as oleoylglycerol. Fatty acids were estimated according to the procedure of Itaya and Ui[24] as palmitic acid. Cholesterol was determined by the *o*-phthalaldehyde method.[25]

Analyses of Protein and Carbohydrate——Protein concentration was determined by the method of Lowry *et al.*[26] with 1% sodium dodecyl sulfate (SDS) to prevent the turbidity caused by lipids.[27] A standard protein, bovine serum albumin (Armour Pharm. Co.), was also prepared with SDS.

Carbohydrate was measured (as glucose) by the method of Dubois *et al.*[28]

Analysis of Disaturated Phosphatidylcholine——Disaturated phosphatidylcholine (DSPC) content was measured according to the method of Shimojo *et al.*[29]

Measurement of Surface Activity——Surface activity was measured in a Teflon trough (15 × 6 × 1.5 cm) on a modified Wilhelmy surface balance. In every case, the subphase solution used was 50 ml of 0.9% NaCl. The surface of the trough was compressed from 54.0 to 21.6 cm^2 in a compression–expansion cycle of 2.4 min. Before the cycle, an initial cycle was carried out to check whether the surface was clean. A sample of 81 μg of phospholipids was layered onto the surface and allowed to remain for 1 min before starting the cycle. The cycle was continued until no further change was observed. A surface tension–surface area diagram was established after 5—8 cycles. All operations were carried out at 37 ± 1 °C. No organic solvent was used to spread the sample on the surface.

The sample used was prepared as follows: lyophilized surfactant was suspended in 0.9% NaCl by gentle mixing and adjusted to a concentration of 20 mg phospholipids/ml. The mixture was sonicated and stored under N_2 gas at −15 °C until used.

Measurement of Spreading——Spreading was measured in a round Teflon dish (2.0 cm deep and 5.0 cm in diameter) on a surface balance at 37 ± 1 °C. A sample of 29.4 μg of phospholipids was layered onto a subphase which consisted of 30 ml of 0.9% NaCl, and the rate at which the surface tension was lowered was measured for 2 min.

Measurement of Adsorption——Adsorption was measured according to the method of King and Clements[10] with a slight modification. In the round Teflon dish, 1.5 mg of phospholipids was mixed with 30 ml of 0.9% NaCl at

37 ± 1 °C. Stirring was continued for 3 min, then the surface was aspirated to obtain a clean surface. Surface tension was measured for 4 min with continuous stirring at 60 rpm.

Measurement of Lung Pressure–Volume Characteristics——The measurement of lung pressure–volume characteristics in the premature rabbit fetus was carried out according to the method of Enhörning and Robertson[30] with several modifications. On the 27th day of gestation, a rabbit was anesthetized with sodium pentobarbital and a litter of fetuses was removed by caesarian section. Without allowing them to take their first breath, they were killed by exsanguination from the femoral artery and vein, and weighed. The trachea of a fetus was incised with scissors and a nylon tube was inserted. A sample of 1 mg of phospholipids (50 mg phospholipids/ml 0.9% NaCl) was instilled *via* the tube and the fetus was kept at 37 ± 1 °C. Air pressure in the tube was gradually raised to 30 cm H_2O with a syringe pump and lowered to 0 cm H_2O in a like manner. The volume of the lung was read at 5 cm H_2O intervals during the inflation and deflation. Three fetuses in each litter always served as controls.

Preparation of Lung Surfactant

Animals——The lungs of calves and oxen were obtained directly from a slaughterhouse. Healthy mongrel dogs of either sex, weighing 8.6—13.8 kg, and male Japan white rabbits weighing 2.5—2.9 kg were also used. The animals were killed by exsanguination after injection of sodium pentobarbital. The lung was excised together with the trachea and weighed.

Extraction of Lung Surfactant——(1) Lung Lavage: The trachea was cannulated with a plastic cannula, then the lung was degassed with an aspirator and washed 5 times with 1.5—5 ml of 0.9% NaCl/g lung tissue. The washed solutions were mixed.

(2) Extraction from Minced Lung: The lungs were cut into small pieces with scissors, then ground with a meat grinder, and suspended in 5 volumes of 0.9% NaCl (v/w). The suspension was stirred for 30 min and filtered with a filter cloth. The residue was stirred with 1 volume of 0.9% NaCl (v/w) for 10 min and filtered again. The filtrates were then combined.

Separation of Lung Surfactant——Lung surfactants were separated from extracted solutions by the method described previously with several modifications.[11] All subsequent operations were carried out under 6 °C unless otherwise specified. The extracted solution was centrifuged at $9000 \times g$ for 30 min. The precipitate was suspended again in 10 volumes of 0.9% NaCl (v/w) and the suspension was centrifuged at $850 \times g$ for 10 min. The supernatant obtained was centrifuged at $9000 \times g$ for 60 min after being adjusted to a density of 1.20 g/ml by adding solid NaCl, and a floating white pellicle was obtained. The pellicle was suspended in distilled water and dialyzed. The dialysate was lyophilized and the lyophilized material was suspended in 100 volumes (v/w) of ethyl acetate at room temperature. After the removal of the soluble material by filtration, the material was dissolved in 100 volumes (v/w) of chloroform–methanol (2 : 1, v/w). After the removal of the insoluble material by filtration, 0.2 volume of water (v/v) was added to the filtrate with vigorous mixing and the mixture was stored for 24 h. The resulting lower solvent layer was separated and the solvent was removed with a rotary evaporator. The remaining material was suspended in distilled water and lyophilized to remove the solvent wholly. The surfactant obtained was stored under N_2 gas at -15 °C.

Modification of Lung Surfactant——The lung surfactant was dissolved in a small quantity of chloroform and desired volumes of chloroform solutions containing various kinds of lipid were added to an aliquot of the dissolved surfactant. The surfactant was adjusted to 47% DSPC, and to 7% fatty acids and 7% triacylglycerols. DPPC as DSPC, palmitic acid as fatty acid and tripalmitoylglycerol as triacylglycerol were used. After the chloroform had been evaporated off, a small amount of distilled water was added to the surfactant. The surfactant was sonicated and lyophilized. The lyophilized material was suspended in 0.9% NaCl. After sonication, the surfactant was stored under N_2 gas at -15 °C until used.

It is very difficult to measure the DPPC content in a lung surfactant, so DSPC content was used as an index of DPPC content in this experiment.

Results

Chemical Composition of Lung Surfactant

The chemical compositions of lung surfactants are shown in Table I. The greater part of the components was lipids, among which the main component was phospholipids (87—90%). Triacylglycerols, diacylglycerols, fatty acids and cholesterol were also found. Small amounts of protein and carbohydrate were detected. No obvious differences in chemical compositions of the lung surfactants were found among the species of the mammals employed or between preparations obtained by the two methods of extraction.

Phospholipid Composition of Lung Surfactant

The compositions of the phospholipids are given in Table II. The most abundant

TABLE I. Chemical Composition of Lung Surfactants

Chemical composition	Calf		Ox		Dog		Rabbit	
Extraction method	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)
Phospholipids	90.2±0.5	90.4±0.2	90.0±0.7	88.9±1.4	89.7±0.8	89.6±0.9	89.5±1.0	87.3±1.5
Triacylglycerols	1.2±0.5	0.8±0.3	1.4±0.4	1.4±0.8	1.6±0.6	2.7±0.6	1.7±0.3	1.8±0.3
Diacylglycerols	0.9±0.4	0.5±0.2	1.0±0.4	0.7±0.5	1.0±0.4	1.2±0.5	0.5±0.3	0.9±0.5
Fatty acids	2.9±0.8	3.3±0.7	3.1±1.4	4.6±2.0	2.8±0.8	4.7±1.0	4.7±1.5	4.4±1.1
Cholesterol	0.8±0.6	0.7±0.7	0.8±0.2	0.7±0.3	0.5±0.3	0.2±0.2	0.7±0.3	0.8±0.3
Protein	3.0±0.5	2.7±0.7	2.1±0.7	3.0±0.5	2.9±0.6	1.3±0.4	2.8±0.5	3.3±0.2
Carbohydrate	1.0±0.5	1.0±0.4	1.3±0.6	0.5±0.2	1.4±0.6	0.2±0.1	0.3±0.3	1.3±0.5

Mean±S.D., $n=4$.

TABLE II. Phospholipid Composition of Lung Surfactants

Phospholipid	Calf		Ox		Dog		Rabbit	
Extraction method	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)	Lavage (%)	Mince (%)
Phosphatidylcholine	73.5±3.4	71.9±3.3	72.7±4.1	64.1±1.2	73.3±2.9	68.5±3.4	75.3±3.0	66.3±3.1
Lysophosphatidylcholine	0.5±0.4	1.0±0.3	0.4±0.2	1.3±0.6	0.5±0.4	1.6±0.6	1.8±0.8	2.0±0.8
Phosphatidylethanolamine	6.3±0.8	6.6±0.8	5.9±1.2	8.9±3.2	5.9±1.2	4.6±1.1	4.7±1.1	5.8±1.5
Phosphatidylserine	5.2±1.0	5.1±1.0	3.6±0.8	4.3±0.9	5.1±1.1	5.4±1.0	4.0±0.9	4.9±1.4
Phosphatidylinositol	5.2±1.3	6.2±1.0	4.1±1.5	4.5±1.2	3.9±0.8	5.1±1.2	5.0±1.5	5.8±0.8
Phosphatidylglycerol	3.9±1.4	3.8±1.8	4.1±1.3	3.5±1.3	6.4±1.8	8.4±2.4	3.6±0.7	4.5±0.8
Sphingomyelin	4.3±2.9	2.5±1.2	7.5±2.1	10.3±1.8	3.6±0.4	4.3±0.7	3.6±0.4	6.2±2.0
Unknown phospholipids	1.1±0.2	2.9±0.8	1.8±0.9	3.1±0.9	1.3±0.7	2.1±0.8	2.1±1.1	4.5±1.4
DSPC/Surfactant	32.7±2.7	27.8±3.8	31.8±3.3	21.4±2.3	29.5±2.0	22.7±3.4	31.0±2.7	23.3±3.1

Mean±S.D., $n=4$.

component was phosphatidylcholine, 64—75%, and phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and lysophosphatidylcholine were contained at levels from 0.4—10.3%.

Although no distinct differences in the phosphatidylcholine content of the lung surfactants were found among the species of mammals studied, the content of the preparations obtained by the lavage method were higher than those in the mincing method. There were no clear differences in the contents of other phospholipids. The DSPC contents of lung surfactants obtained by the mincing method were 5—10% lower than those of surfactants obtained by the lavage method.

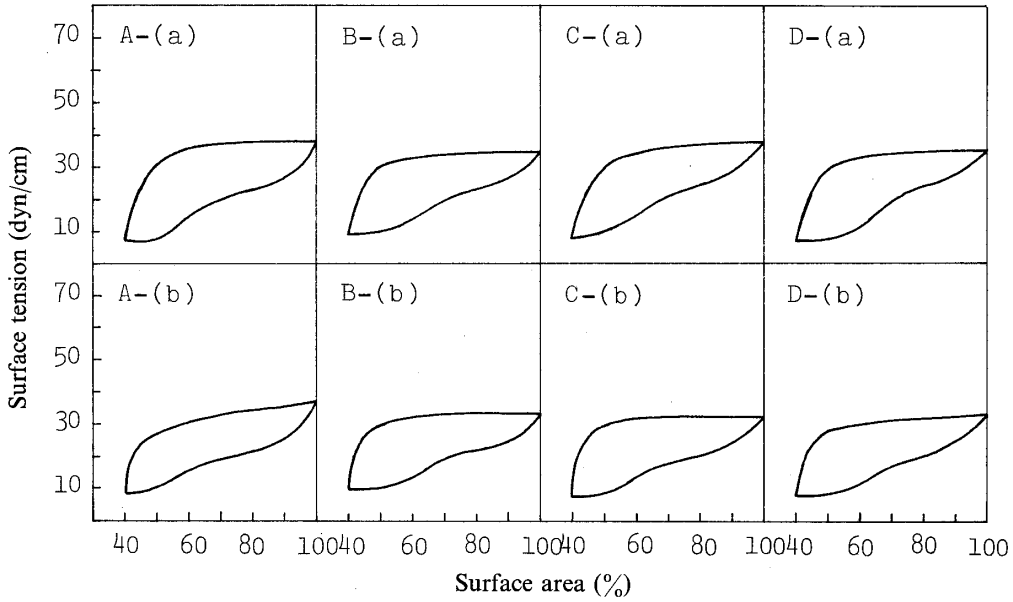

Fig. 1. Surface Activities of Native Lung Surfactants

A, calf; B, ox; C, dog; D, rabbit; (a), lavage method; (b), mincing method.

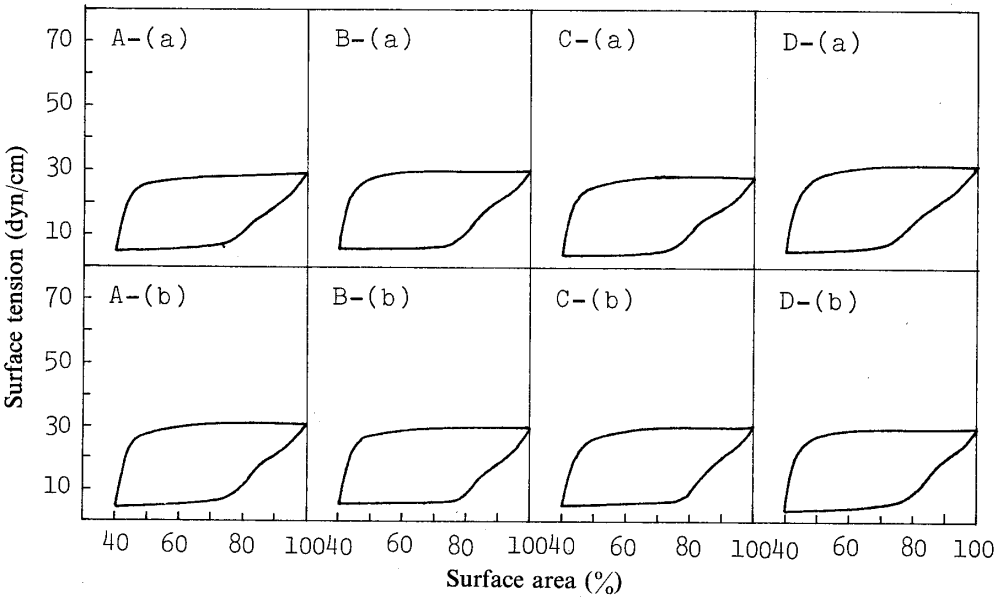

Fig. 2. Surface Activities of Modified Lung Surfactants

A, calf; B, ox; C, dog; D, rabbit; (a), lavage method; (b), mincing method.

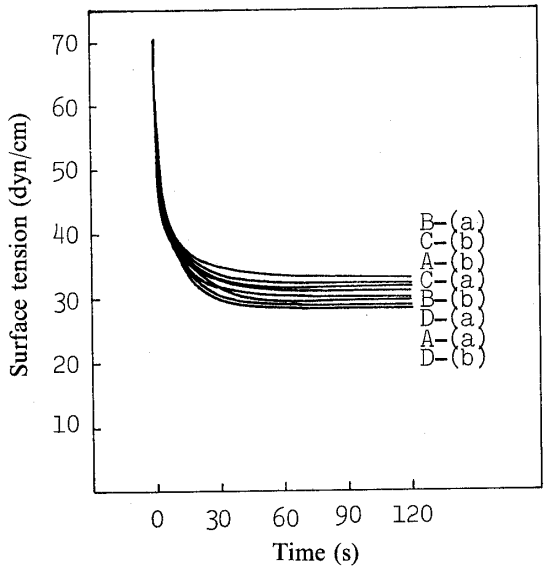

Fig. 3. Spreading Rates of Modified Lung Surfactants

A, calf; B, ox; C, dog; D, rabbit; (a), lavage method; (b), mincing method.

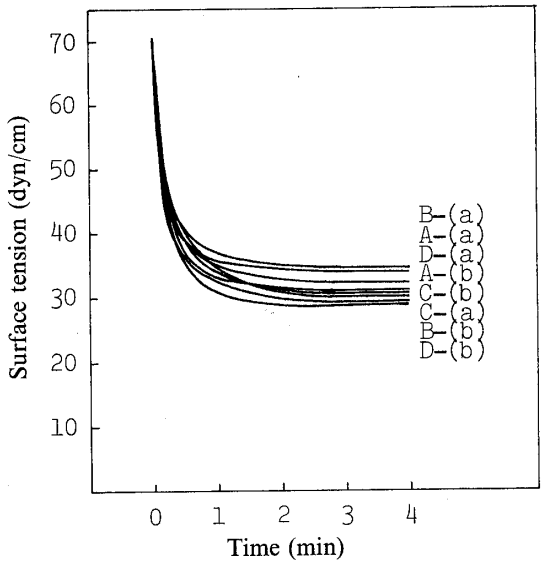

Fig. 4. Adsorption Rates of Modified Lung Surfactants

A, calf; B, ox; C, dog; D, rabbit; (a), lavage method; (b), mincing method.

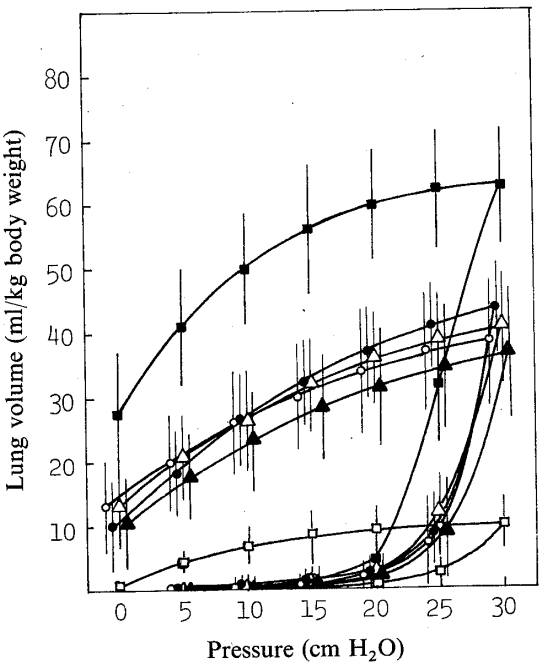

Fig. 5. Lung Pressure–Volume Characteristics of Premature Fetuses with or without Tracheal Instillation of Native Lung Surfactants

○, with native lung surfactant (calf, lavage); ●, (calf, mince); △, (ox, lavage); ▲, (ox, mince).
□, without native lung surfactant.
■, mature fetuses (gestation = 30 d).
Mean ± S.D., $n = 4$.

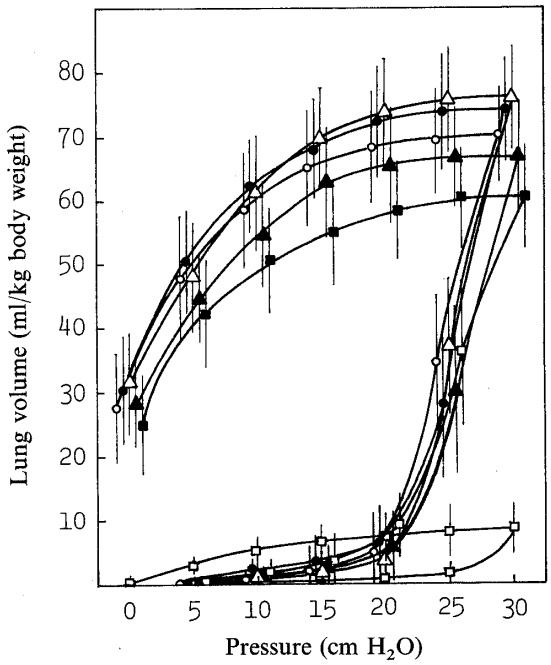

Fig. 6. Lung Pressure–Volume Characteristics of Premature Fetuses with or without Tracheal Instillation of Modified Lung Surfactants

○, with modified lung surfactant (calf, lavage); ●, (calf, mince); △, (ox, lavage); ▲, (ox, mince).
□, without modified lung surfactant.
■, mature fetuses (gestation = 30 d).
Mean ± S.D., $n = 4$.

Surface Properties of Lung Surfactant

The surface activities of the lung surfactants are shown in Fig. 1. The lowering velocity of the surface tension in compression was slow with all the lung surfactants and most of them

barely reached minimum surface tensions of less than 10 dyn/cm at full compression.

All the surfactants spread rapidly and surface tension reached equilibrium at 28—35 dyn/cm within 1 min. All the surfactants were quickly adsorbed and surface tension reached equilibrium at 29—34 dyn/cm within 1 min.

Surface Properties of Modified Lung Surfactant

The surface activities of modified lung surfactants are shown in Fig. 2. Irrespective of the mammalian species or the extraction method, all the modified surfactants showed high surface activities: a minimum surface tension of less than 10 dyn/cm at a surface area compressed to about 70% of its original area, and a large area of surface tension–surface area hysteresis loop.

As shown in Figs. 3 and 4, all the modified surfactants spread spontaneously and rapidly, and were quickly adsorbed. Both the spreading and adsorption rate were slightly improved by the modification of the native lung surfactants.

Lung Pressure–Volume Characteristics

Figure 5 shows the lung pressure–volume characteristics of prematurely derived rabbit fetuses (gestation = 27 d) after the tracheal instillation of the native lung surfactants from calves and oxen in comparison with those of fetuses without the instillation and with those of mature rabbit fetuses (30 d). All the native surfactants improved the characteristics of the premature rabbit fetus independently of the extraction method, but the improvements were only partial and the characteristics were inferior to those of the mature fetus.

As Fig. 6 shows, all the premature fetuses acquired good lung pressure–volume characteristics as a result of the instillation of the modified lung surfactants. All of them gave the premature fetuses respiratory functions equivalent to those of mature fetuses, independently of the extraction method.

Discussion

King and Clements reported that the DPPC content in a lung surfactant obtained by extensive purification was 62% of phosphatidylcholine.[10] DSPC contents of our native lung surfactants were 45—47 and 38—41% of phosphatidylcholine in the preparations obtained by the lavage and the mincing methods, respectively. These native surfactants did not show sufficient surface activities, but modified lung surfactants in which the DSPC content was adjusted to 47% showed good surface activities not only *in vitro* but also *in vivo*. This content corresponded to about 72% of the phosphatidylcholine in the lung surfactant. This result suggested that the content of DPPC in the surfactant of King and Clements is also rather less than that of natural lung surfactant in the alveoli, and it seems to be very difficult to obtain a lung surfactant preparation possessing a high DSPC content without adding DPPC to the preparation.

The second most abundant phospholipid in the surfactant from dog lung is phosphatidylglycerol and it is present at a level of about 9—11%.[31-33] Our results in dogs were in reasonable accord with this result (phosphatidylglycerol accounted for 6—8% of the phospholipids of dog lung surfactant), but it was present only at rather low levels in lung surfactants from other mammals.

The role of phosphatidylglycerol in the surface activity of lung surfactant has been discussed.[33-35] However, its function has not yet been clearly defined. By loading an electric charge with 5—10% acidic phospholipids, cohesion of liposome particles is prevented and stable films are formed.[36] Phosphatidylglycerol, an acidic phospholipid, may thus contribute to the formation of stable surfactant films.

Although lung surfactants contain phosphatidylinositol and phosphatidylserine besides phosphatidylglycerol as acidic phospholipids, the reported contents of these acidic phospho-

lipids vary somewhat.[5] The contents of phosphatidylglycerol were 2—5% of the phospholipids in our modified lung surfactants and the total contents of acidic phospholipids were 8—13%. These total contents must provide an electric charge to maintain the surfactant films in stable condition.

Recently, the surface tension at the surface of the alveoli was directly measured in wettability experiments and it was found that the minimum surface tension on the surface was 9 dyn/cm or less and the maximum surface tension was about 30 dyn/cm.[37] The minimum and maximum surface tensions of the modified surfactants *in vitro* were 2—9 and 26—33 dyn/cm, respectively. These values are similar to those found in the wettability experiments. All the modified lung surfactants showed good surface activities irrespective of the mammalian species or the extraction methods.

Although there is still much confusion, some attempts have been made to evaluate lung surfactant preparations from biochemical and physicochemical viewpoints.[2,5,7] The following surface properties *in vitro* are regarded as relevant physiological criteria: 1) a minimum surface tension of at most 10 dyn/cm,[2,7,10] 2) spontaneous spreading on a subphase,[38,39] 3) adsorption at an air–liquid interface,[10] 4) the presence of surface tension–surface area hysteresis,[2] 5) a compressibility of 0.09 cm/dyn or less at 10 or 15 dyn/cm at 37 °C,[2,10] 6) an abundance of DPPC,[2,10] and 7) stability of low surface tension films with respect to the rate of change of the relative surface area.[2] A surfactant judged satisfactory by these criteria must finally be evaluated in terms of the pressure–volume characteristics of the excised mammalian lung *in situ*[40] or of the lung of the prematurely delivered mammalian fetus *in vivo*.[30]

Our modified lung surfactant is the first to satisfied all these criteria. Morley *et al.* reported a dry surfactant that satisfied two of the criteria, the required minimum surface tension and spontaneous spreading, in dry conditions, but the surfactant did not satisfy these criteria in wet physiological conditions.[39,41]

The modified lung surfactant described here showed the following properties. 1) The surfactant showed a minimum surface tension of 2—9 dyn/cm at about 70% surface area, *i.e.* 30% compression of the surface area. 2) The surfactant spread spontaneously and rapidly, and surface tension reached equilibrium at 28—34 dyn/cm within 1 min. 3) The surfactant was adsorbed quickly, and surface tension reached equilibrium at 28—34 dyn/cm within 1 min. 4) In the measurement of surface activity, surface tension fell immediately in compression and rose in expansion; furthermore, continuous cycling gave a large hysteresis loop, and the diagram was stable for more than 10 cycle. 5) the compressibility at 15 dyn/cm at 37 °C was about 0.01—0.02 cm/dyn and was far lower than 0.09 cm/dyn. 6) The DSPC content of the surfactant was 47% and it was the most abundant component in the surfactant. 7) The surface tension attained a minimum at about 70% surface area, was constant to the end of compression, and the low tension films were stable. Thus the modified lung surfactant satisfied all the above criteria irrespective of the mammalian species or the extraction method, and it also gave normal respiratory functions to the premature rabbit fetus *in vivo*.

Unfortunately, we could not measure the lung pressure–volume characteristics with the native and the modified lung surfactants from dogs and rabbits due to the lack of a sufficient quantity of the surfactants. However, the surface properties *in vitro* do suggest that the modified lung surfactants from the mammals would also give normal lung pressure–volume characteristics to the premature rabbit fetus, like the modified surfactants from calves and oxen.

Although the lung surfactant was modified with DPPC, palmitic acid and tripalmitoylglycerol, these are naturally occurring components in lung surfactant. The modified lung surfactant showed good surface activities *in vitro* and *in vivo*. Thus it is reasonable to assume that the composition, and physicochemical and physiological properties of the modified lung surfactant are very similar to those of natural lung surfactant in the alveoli.

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