Effects of various forms of surfactant protein C on tidal volume in ventilated immature newborn rabbits


Preparation of MNS, Normal SP-B, and Normal SP-C

Bronchoalveolar lavage (BAL) fluid from recently slaughtered pigs was centrifuged (150 g for 10 min) to remove cell debris, and the supernatant was further centrifuged (2,000 g for 1 h, 4°C). Modified natural surfactant (MNS) consisting of 98.0% phospholipids, 0.9% other lipids, and 1.1% hydrophobic SPs (0.37% SP-B and 0.72% SP-C) was obtained from the pellet by extraction with chloroform-methanol solution (2:1 vol/vol), by washing with 0.5% saline, and by acetone precipitation. More precise chemical compositions and isolation methods for MNS are presented elsewhere (20, 21).

MNS was dissolved again in chloroform-methanol (1:1 vol/vol) containing 0.1 N hydrochloric acid at 5%. From this solution, we could separate two types of protein fractions by using Sephadex LH-60 column chromatography (Pharmacia Biotechnology, Uppsala, Sweden) (7) with the aid of ultraviolet (280 nm) absorbance using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan) to obtain peak fractions. We then concentrated the fractions under reduced pressure and dissolved them in a chloroform-methanol solution (1:1 vol/vol) (29). The final weight ratio of protein in surfactant to phospholipids was 1:1. The physicochemical and biological properties for the different surfactants are presented elsewhere (20, 21).

PULMONARY SURFACTANT IS PRIMARILY composed of phospholipids and surfactant proteins (SPs) (17) and reduces surface tension of the air-liquid interface by forming a surface film (28, 31). Hydrophobic SPs (SP-B and SP-C) are important for facilitating surface adsorption of phospholipid molecules and for promoting tidal volume (17, 20). The regular form of SP-C is known to be monomeric, with a hydrophobic valine-rich α-helix at the carboxy terminal (15–17). The amino terminal part of regular SP-C is hydrophilic, and in most species the two cysteine residues are attached to the palmitoyl groups via thioester bonds with an overall stoichiometry ratio of close to 1:1 between the cysteine residues and the palmitoyl groups (6, 16). However, the relationship between molecular configuration and the physiological function are not yet completely understood.

Two types of abnormal SP-Cs are known to accumulate in the lungs of patients with pulmonary alveolar proteinosis (PAP). One type is monomeric but poor in palmitoyl groups, and the other type is dimeric and also deficient in palmitoyl groups (32, 34, 39). Information on the relationship between the configuration and function of SP-C may be obtained from analyses of these abnormal SP-Cs. Numerous investigations on surfactants consisting of synthetic lipids and SPs or the analogs have recently been conducted to develop artificial surfactant for therapeutic use (8, 27). The techniques used in these investigations can be applied to analysis of SP-C function. In the present study, several reconstituted surfactants (RSs), consisting of synthetic phospholipids, normal SP-B, and abnormal SP-Cs from PAP patients, were administered to surfactant-deficient immature newborn rabbits. Tidal volumes of the animals were then measured under pressure-controlled ventilation to evaluate the physiological function of abnormal SP-Cs.

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Hitachi, Tokyo, Japan). With further assessments described below, the protein first eluted from the column was used in the present study as normal SP-B (nSP-B), with the protein eluted second representing normal SP-C (nSP-C).

Each protein fraction was further assessed by use of tricine SDS-PAGE under the nonreducing condition and also under the reducing condition with 2-mercaptopethanol (30). Presence of SP-B was determined by Western blotting with biotinylated anti-SP-B antibody (8B5E) (33) and by amino terminal sequence analysis using a gas-phase protein sequencer (PSPQ-10, Shimazu, Kyoto, Japan) after blotting on a polyvinylidene difluoride membrane (Sequi-Blot, 0.2 μm, Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin et al. (38). The relative amount of protein on tricine SDS-PAGE was determined by use of a lane and spot analyzer (AE-6920, ATTO, Tokyo, Japan). The concentration of proteins was determined according the micro-Kjeldahl method (37).

Preparation of Abnormal SP-Cs

BAL fluid was obtained from therapeutic lung lavage of two PAP patients admitted to Kanazawa University Hospital. The chloroform-soluble fraction was obtained in the same manner as used for the preparation of MNS. This fraction was further separated into pulmonary alveolar proteinosis SP-B (SP-Bpap) and dimeric (dSP-Cpap) and monomeric forms of SP-C (mSP-Cpap) under the same methods used for isolation of nSP-C (32, 34). Purity and molecular weight were determined according to the methods described above. Amount of palmitoyl groups in nSP-C and abnormal SP-Cs were quantified using gas chromatography after hydrolysis with 0.1 mol/l of potassium hydroxide (6) and by methylation with boron fluoride-methanol (25).

Preparation of RSs

First, a synthetic phospholipid mixture (SPL) was prepared by mixing synthetic dipalmitoylphosphatidylcholine (Sigma Chemical, St. Louis, MO), synthetic dioleoylphosphatidylcholine (Sigma Chemical), and egg yolk phosphatidylglycerol (Sigma Chemical) at a weight ratio of 6:2:2 in chloroform-methanol (95:5) solution (36). The basic mixture was prepared by adding normal SP-B to SPL at a concentration of 0.7% (21). For RSs of the nSP-C series, nSP-C was added to the basic mixture at concentrations of 1, 2, or 3% (by weight). For the RSs of the mSP-Cpap series, mSP-Cpap was added to the basic mixture at concentrations of 1, 2, or 3%. For RSs of the dSP-Cpap series, dSP-Cpap was added to the basic mixture at concentrations of 1 or 2%. An RS containing dSP-Cpap at 3% could not be prepared because of limited supplies of the protein.

After evaporation of the organic solvent by blowing nitrogen, all test materials, i.e., MNS, SPL, basic mixture, and all types of RSs, were suspended in normal saline by repeatedly drawing them into and expelling them from a syringe, followed by incubation in an ultrasonic bath (Branson 3200, Yamato, Tokyo, Japan) for 3 min. The pH values of these suspensions of test material were corrected to 5.5, similar to that of MNS suspension, with 0.1 N sodium hydroxide (6) and by methylation with boron fluoride-methanol (25). Final concentration of the test material was adjusted at 50 mg/ml. Suspensions were stored at 4°C. We assessed surface activity of SPL, the basic mixture, RS containing nSP-C at 1%, SPL supplemented with nSP-C at 3% only (without SP-B), and MNS by using a pulsating-bubble apparatus (PBS; Electronetics, Buffalo, NY) (9). For this purpose, the suspension containing each test material at a concentration of 10 mg/ml was placed in a sample chamber kept at 37°C. An air bubble communicating with the ambient air was created in the suspension and then pulsated between radii of 0.40 and 0.55 mm at a speed of 40 cycles/min. After 5 min of pulsation, surface tension at maximum and minimum bubble sizes (γmax, γmin) were recorded.

Secondary Structure of Various SP-Cs

The circular dichroism (CD) spectra were obtained using a spectropolarimeter (J-710, JASCO, Tokyo, Japan) in a 0.2-mm cell at 25°C. Various SP-Cs (75 μg) were mixed with SPL (750 μg). These mixtures were dried and suspended in 1 ml of phosphate buffer (50 mmol/l, pH 6.0) (18). Two series of 10 scans from 240 to 200 nm were averaged, and protein-free SPL spectra were subtracted to yield the protein spectra. CD spectra were expressed as mean residue ellipticity calculated by protein concentration and estimation of the mean molecular weight of amino acid residues as 115. These spectra were analyzed for secondary structure with the aid of a neural network computer program (k2d, kindly distributed by M. A. Andrade through the World Wide Web; http://www.embl-heidelberg.de/~andrade/k2d.html) comprising a database of weights and a recall program for determining α-helix and β-sheet structure based on these weights (1, 12).

Animal Experiment 1

Validation of MNS and immature newborn rabbit model. Twenty immature newborn rabbits were delivered by hysterectomy from three pregnant does at a gestational age at between 26 days 22 h and 27 days 5 h (term = 31 days). Animals were tracheotomized under anesthesia with intraperitoneal pentobarbital sodium (0.5 mg) and randomly assigned to MNS (n = 10) or nontreated (n = 10) groups. MNS group animals were administered 100 μl of MNS suspension (50 mg/ml) via tracheal cannula before taking their first breath, whereas control group animals were administered nothing. Animals were then transferred to a system of multiple-body plethysmographs kept at 37°C (20). After all animals were prepared, animals were relaxed with intraperitoneal suxamethonium bromide (0.02 mg) and subjected in parallel to pressure-controlled ventilation. The respirator unit (Servo 900B, Siemens-Elema, Solna, Sweden) delivered 100% oxygen at 40 breaths/min with a 50% inspiration time.

The peak inspiratory pressure (PIP) was first raised to 30 cmH2O for 1 min to facilitate distribution of administered materials, then lowered to 25 cmH2O for 15 min, and then to 20 cmH2O and 15 cmH2O for 5 min each. Finally, PIP was again increased to 25 cmH2O for 5 min. No end-expiratory pressure was applied to the ventilatory circuit. Individual tidal volumes were recorded at the end of each 5-min interval by using a pneumotachograph system described elsewhere (20). Electrocardiograms were recorded immediately after tidal volume measurements, and animals showing QRS complexes at a frequency of over 100 beats/min were considered survivors. After the animals were killed by an overdose of pentobarbital, the abdomen was opened to inspect the diaphragm and lungs. PAP patients were admitted to Kanazawa University Hospital. Animals were administered 100 μl of each
one of the RS suspensions (50 mg/ml) via the tracheal cannula and were ventilated in the same manner as in animal experiment 1. Tidal volumes at a PIP of 25 cmH2O were measured four times, and the last value (30 min after start of the ventilation) was used for comparisons. Animals with pneumothorax were excluded from statistical analyses.

mSP-Cpap series. Thirty-one immature newborn rabbits were delivered from five pregnant does and randomly assigned to groups receiving RSs (50 mg/ml) containing mSP-Cpap at concentrations of 0% (basic mixture, n = 7), 1% (n = 7), 2% (n = 10), or 3% (n = 7). The experiment was performed in a manner identical to the nSP-C series.

dSP-Spap series. Twenty-four immature newborn rabbits were delivered from four pregnant does and randomly assigned to groups receiving RSs (50 mg/ml) containing dSP-Spap series. The experiment was performed in a manner identical to the nSP-C series.

fitting the intergroup differences were examined by using analysis of variance followed by Scheffe’s test and by using analysis of variance followed by Tukey’s test with Bonferroni correction. Data for were examined by using Kruskal-Wallis test followed by Mann-Whitney’s test with Bonferroni correction. Levels of P < 0.05 were considered statistically significant.

Statistical Analysis

Data for tidal volume and surface tension were expressed as median and range (parentheses). Intergroup differences were examined by using Kruskal-Wallis test followed by Mann-Whitney’s test with Bonferroni correction. Data for body weight were given as means ± SD, and differences were assessed by using analysis of variance followed by Scheffe’s method. Differences in survival rate and incidence of pneumothorax were assessed by using Fisher’s exact test. Levels of P < 0.05 were considered statistically significant.

RESULTS

Characterization of Hydrophobic SPs

Tricine SDS-PAGE of the various SP-Cs is shown in Fig. 1A. Under the nonreducing condition, the molecular weight of nSP-C, dSP-Cpap, and mSP-Cpap were 4.9 kDa, 8.5 kDa, and 4.2 kDa, respectively. Under the reducing condition, the dSP-Cpap demonstrated a prominent staining band at 4.4 kDa and a faint one at 8.5 kDa, indicating the dimeric nature of dSP-Cpap. Tricine SDS-PAGE of nSP-B and SP-Bpap is shown in Fig. 1B. Under the nonreducing condition, nSP-B (lane 1) revealed a major band at 21–22 kDa and a faint one at 16 kDa. Under the reducing condition, the major band migrated to 11 kDa, but the faint band remained at 16 kDa. A similar band was also found in the MNS prepared after isolation by sucrose-gradient centrifugation according to Frosolono et al. (10) (data not shown). The amino terminal sequence of the major band at 21–22 kDa in the nonreducing condition represented Phe-Pro-Ile-Pro-Leu-Pro-Phe-X-Trp-Leu- (X was supposed to be “Cys”), which agreed with the result of the previous report (7). Densitometry indicated that the purity of the nSP-B was 84–85%. Two major bands were demonstrated for SP-Bpap, one at 21 kDa and the other at 30 kDa. Under the reducing condition, the major bands migrated to 10 kDa with a faint band at 16 kDa. Western blotting using anti-SP-B antibody is shown in Fig. 1C. The major bands of nSP-B (21 kDa) and SP-Bpap (21–30 kDa) demonstrated the binding to anti-SP-B antibody. Their faint bands at 16 kDa did not bind with antibody. A faint binding at 10 kDa was found in the blot of dSP-Cpap, indicating the contamination with SP-B monomer. On the basis of densitometry, the contamination was calculated to 1.5%.

Weight ratio of SP-Bpap, dSP-Cpap, and mSP-Cpap derived from BAL fluid of PAP patients was 27:23:50. Relative contents of palmitoyl groups in dSP-Cpap and mSP-Cpap were 42 and 38% of that contained in the identical weight of nSP-C, respectively.

Pulsating-Bubble Measurement

Findings of dynamic surface tensions are shown in Table 1. Both γmin and γmax of the basic mixture composed of SPL and nSP-B were significantly lower than those of SPL, indicating that nSP-B was functional. Similar findings were obtained on SPL supplemented with nSP-C only, of which γmin was, however, significantly higher than that of MNS. Neither nSP-B nor nSP-C alone could constantly lower γmin values of SPL to <5 mN/m. The RS consisting of SPL and both nSP-B and nSP-C demonstrated the same γmin and γmax values as MNS.

Secondary Structure of Various SP-Cs

CD spectra of nSP-C demonstrated minima at 208 and 222 nm (Fig. 2), a result characteristic of α-helix (11). The spectra of mSP-Cpap revealed smaller minima at these wavelengths than nSP-C. The minima of

![Image](image-url)
dSP-Cpap were somewhat smaller than those of mSP-Cpap (Fig. 2). The k2d program indicated that the α-helical content of the nSP-C was larger than those of mSP-Cpap or dSP-Cpap (Table 2).

Animal Experiment 1

Two of 10 animals died in the nontreated group, whereas all animals in the MNS group survived until the end of experiment. No pneumothorax was observed in the nontreated group, whereas 2 of the 10 animals in the MNS group were excluded because of pneumothorax. Body weight of all animals was 33.6 ± 3.4 g, without significant differences between the MNS and nontreated groups. As shown in Fig. 3, median tidal volume of the nontreated group was <2 ml/kg in four measurements at a PIP of 25 cmH2O, whereas the values of the MNS group was >24 ml/kg (P < 0.01 vs. nontreated group).

Animal Experiment 2

nSP-C series. Body weight of all animals was 33.6 ± 3.5 g, without significant differences among the four groups. All animals survived until the end of the experiment, and no cases of pneumothorax were observed. Changes in tidal volume are shown in Fig. 4.

Table 1. Dynamic surface tension of surfactants

<table>
<thead>
<tr>
<th></th>
<th>SPL</th>
<th>nSP-B</th>
<th>nSP-C</th>
<th>γmin (mN/m)</th>
<th>γmax (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic mixture</td>
<td>+</td>
<td>0.7%</td>
<td>–</td>
<td>23 (18–28)*</td>
<td>61 (48–66)*</td>
</tr>
<tr>
<td>RS containing nSP-C (1%)</td>
<td>+</td>
<td>0.7%</td>
<td>1.0%</td>
<td>4.6 (1.5–8.6)†</td>
<td>29 (26–40)†</td>
</tr>
<tr>
<td>SPL with nSP-C (3%)</td>
<td>+</td>
<td>–</td>
<td>3.0%</td>
<td>3.1 (0.9–4.4)†</td>
<td>31 (28–33)†</td>
</tr>
<tr>
<td>MNS</td>
<td>0.37%</td>
<td>0.72%</td>
<td>–</td>
<td>7.8 (4.2–13)##†</td>
<td>35 (28–44)†</td>
</tr>
</tbody>
</table>

Values are median (range) of 8 measurements. SPL, synthetic phospholipid mixture; nSP-B and nSP-C, normal SP-B and normal SP-C, respectively; γmin and γmax, minimum and maximum surface tension after 5 min of pulsation, respectively; RS, reconstituted surfactant; MNS, modified natural surfactant. Phospholipid concentration was 10 mg/ml. *P < 0.05 vs. MNS. †P < 0.05 vs. SPL.

Tidal volumes with the basic mixture were larger than those seen in the nontreated group of animal experiment 1 at all PIPs (P < 0.01). At a PIP of 25 cmH2O, tidal volumes of animals receiving the RSs containing nSP-C at 2 or 3% were significantly larger than those receiving basic mixture (nSP-C at 0%). At PIPs of 15 and 20 cmH2O, tidal volumes of animals administered RS containing SP-C at 3% were significantly larger than those receiving basic mixture.

mSP-Cpap series. Body weight of all animals was 30.4 ± 4.0 g, without significant differences among the four groups. All animals survived until the end of the experiment, but one animal receiving the RS containing mSP-Cpap at 2% was excluded because of pneumothorax. Changes in tidal volumes are shown in Fig. 5. At a PIP of 25 cmH2O, median tidal volumes of all groups were only ~10 ml/kg.

dSP-Cpap series. Body weight of all animals was 30.2 ± 4.7 g, without significant differences among the three groups. All animals survived until the end of the experiment, and no cases of pneumothorax were observed. Changes in tidal volumes are shown in Fig. 6. At a PIP of 25 cmH2O, median tidal volume of the animals receiving the RS containing 2% dSP-Cpap was nearly twice that of those receiving basic mixture (P < 0.05).

DISCUSSION

In animal experiment 1, tidal volumes of the MNS group were significantly larger than those of the nontreated group. Although difference in tidal volumes might have been exaggerated by relationships between alveolar opening pressures and PIPs, the same pressure-controlled ventilation used in the present study has been used previously to evaluate the physiological activity of surfactant preparations (7, 20, 21). Sucrose-gradient centrifugation, which has been used to isolate

Table 2. Secondary structure of various SP-Cs assessed by use of circular dichroism

<table>
<thead>
<tr>
<th></th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Random Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>nSP-C</td>
<td>0.59</td>
<td>0.08</td>
<td>0.34</td>
</tr>
<tr>
<td>mSP-Cpap</td>
<td>0.41</td>
<td>0.11</td>
<td>0.47</td>
</tr>
<tr>
<td>dSP-Cpap</td>
<td>0.38</td>
<td>0.07</td>
<td>0.55</td>
</tr>
</tbody>
</table>

nSP-C, normal SP-C derived from porcine lungs; mSP-Cpap and dSP-Cpap, monomeric and dimeric forms of SP-C derived from lungs of patients with pulmonary alveolar proteinosis, respectively.
“complete” surfactant (10, 20, 21), was omitted from the present study. Lipid composition of MNS, therefore, may differ from that of complete pulmonary surfactant. Another difference between complete surfactant and MNS is the absence of SP-A and SP-D in MNS given that extraction with organic solvents results in removal of hydrophilic proteins (17, 20). However, we have found that this MNS promotes tidal volumes in surfactant-deficient immature newborn rabbits to a similar extent as complete surfactant (21). From these perspectives, we believe that the physiological activity of surfactant preparations can be assessed using the protocols of the present study and that MNS is a reasonable source for hydrophobic SPs.

An RS consisting of the same phospholipids as used in the present study and porcine SP-B (0.7%) and porcine SP-C (1.4%) could improve blood-gas findings in rats with acute lung injury in our previous experiment (36). Although the reconstituted materials without SP-B exhibit some surface activity (21, 26), SP-B is believed to represent an indispensable component to optimize the surfactant activity (21, 27). In the present study, the pulsating-bubble measurements revealed that surface tension of SPL was significantly decreased by adding nSP-B or nSP-C. However, surface tension did not decrease to the same level as MNS by either of the two SPs alone. Apparent molecular size, amino terminal sequence, and findings under Western blotting

Fig. 3. Tidal volumes (TV) of immature newborn rabbits at various peak inspiratory pressures (PIP) in animal experiment 1. v, Not given any material (nontreated group, n = 10); v, treated with modified natural surfactant (MNS group, n = 8). Values are medians (ranges). *P < 0.05 vs. nontreated group. Some ranges are hidden by symbols.

Fig. 4. TV in the nSP-C series of animal experiment 2 under PIP of 15 cmH2O (triangles), 20 cmH2O (circles), and 25 cmH2O (squares). Immature newborn rabbits were administered reconstituted surfactants (RSs) consisting of basic mixture (mixture of synthetic phospholipids and normal SP-B) and 0–3% nSP-C. RS without nSP-C (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per RS. Some ranges are hidden by symbols. *P < 0.05 vs. basic mixture.

Fig. 5. TV in the mSP-Cpap series of animal experiment 2 under PIP of 15 cmH2O (triangles), 20 cmH2O (circles), and 25 cmH2O (squares). Immature newborn rabbits were administered RSs consisting of basic mixture and 0–3% mSP-Cpap. RS without mSP-Cpap (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per RS. Some ranges are hidden by symbols.

Fig. 6. TV in the dSP-Cpap series of animal experiment 2 under PIP of 15 cmH2O (triangles), 20 cmH2O (circles), and 25 cmH2O (squares). Immature newborn rabbits were administered RSs consisting of basic mixture and 0–2% dSP-Cpap. RS without dSP-Cpap (0%) corresponds to the basic mixture (open symbols). Values represent medians (ranges); n = 7–10 animals per each RS. Some ranges are hidden by symbols. *P < 0.05 vs. basic mixture.
ting of nSP-B in the present study were consistent with those previously reported for regular porcine SP-B (7, 17, 33). In the present experiment, the basic mixture, i.e., mixture of SPL and nSP-B (0.7%), increased the tidal volume of immature newborn rabbits from <2 ml/kg to ~7.7 ml/kg. This only modest improvement may be because the SP-B concentration was lower than that used in a previous study (2.0%) (7). The concentration of SP-B used in our experiment may have been suboptimal, so that a larger tidal volume could be obtained by using a higher concentration. It was reported that addition of SP-B at a concentration of 2.0% reduced the γmin of an artificial surfactant, including phospholipids and an analog of SP-C, to nearly the same value as that of another surfactant preparation isolated from porcine lungs, but decreasing the SP-B concentration from 2.0 to 0.5% did not change the γmin significantly (27). Our results using a basic mixture containing SP-B at a concentration of 0.7% would, therefore, be valid as a yardstick for discriminating the physiological effects of various SP-Cs.

A γmin value similar to that of MNS was obtained when nSP-C was added at 1.0% to the basic mixture. Moreover, tidal volumes of the immature rabbits increased with the concentration of nSP-C. Regular porcine SP-C comprises 35 amino acid residues, and the residues 9–34 form an α-helix structure (19). Our findings, that nSP-C had a molecular size of 4.9 kDa and an α-helix content of 59%, do not contradict previously reported results for regular SP-C (4, 17). Given these considerations, tidal volumes of immature rabbits receiving the RSs containing nSP-C can be used as standards to evaluate the function of various SP-Cs.

In the mSP-Cpap series, however, tidal volume barely improved with the increment in concentration. This result strongly suggests that mSP-Cpap lacks the function. Our previous experiment demonstrated that the amino terminal sequence of mSP-Cpap and dSP-Cpap coincided with that of human SP-C (34). The α-helix part allows the anchorage of SP-C in the phospholipid bilayer and monolayer (3) and is believed to be essential for surface activity (16). CD spectra indicated that the α-helix content in mSP-Cpap was 41%, about two-thirds of nSP-C. The possibility therefore exists that inefficiency of mSP-Cpap results from shortened α-helix structure. However, Qanbar et al. (29) found with the captive-bubble technique that RS containing palmitoylated SP-C required less compression to reach low surface tension than those with chemically de-palmitoylated SP-C. Gustafsson et al. (13) also have shown that RS composed of palmitoylated SP-C analog forms a stable surface film at the air-water interface but that composed of nonpalmitoylated analog does not. In the present experiment, palmitoyl content in mSP-Cpap was only 38% of that in nSP-C. As the cause of functional inefficiency of mSP-Cpap, therefore, we must also consider a deficiency in palmitoyl groups.

Interestingly, the RSs containing dSP-Cpap increased the tidal volume of immature newborn rabbits, whereas RSs with mSP-Cpap did not. It seems unlikely that contamination by SP-Bpap in the dSP-Cpap fraction improved the tidal volume, because the concentration of SP-Bpap corresponded to only 1.5% of 2% SPL, i.e., 0.03% of SPL. Baatz et al. (2) reported that dimeric SP-C from bovine lung predominantly consisted of β-sheets. In contrast, Creuwels et al. (5) reported that the dimeric SP-C was helical. Both studies demonstrated that dimeric SP-C improved surface activity of synthetic phospholipid mixtures. In the present experiment, the α-helical content of mSP-Cpap was similar to that of dSP-Cpap, although both values were lower than that of nSP-C. The palmitoyl content of mSP-Cpap was also similar to that of dSP-Cpap. The functional efficiencies, however, were clearly different in the present study. Data from the present study indicate that surfactant function is influenced not only by the α-helical content and degree of palmitoylation of SP-C but also by the presence of dimeric SP-C.

Surfactant forms surface film at the air-liquid interface, which is thought to be consisting of a phospholipid monolayer at the most superficial part and bilayers in the underlying part (22, 28, 31). Some researchers have speculated that palmitoylation of SP-C may link the monolayer to a neighboring bilayer and link two bilayers together by anchoring palmitoyl groups and the α-helix to different layers, because the palmitoylated SP-C analog demonstrated improved adsorption of surface-associated phospholipid layers to the air-liquid interface during surface expansion (13). Wang et al. (40) reported that regular SP-C facilitated the adsorption of both synthetic phospholipids and phospholipids isolated from natural surfactant better than depalmitoylated SP-C. The surface adsorption rate of surfactant molecules is an important factor in physiological activity (20, 31). Nonionic polymers such as dextran and polyethylene glycol are known to improve the surface adsorption rate of surfactant preparations, and presumably these polymers pull neighboring phospholipid layers together by removing water molecules (23, 24). Taking all these possibilities into consideration, we believe that each of two α-helix structures in the dSP-Cpap molecule anchored to different lipid layers and linked them together, improving surface adsorption and physiological function despite the reduced content of palmitoyl groups.

The helical structure of regular SP-C is reportedly able to unfold and form aggregates with β-sheets, leading to formation of amyloid fibrils (14, 35). Such fibrils have been found in BAL fluid from a PAP patient (14). In the present experiment, neither polymeric forms nor aggregates of SP-C with higher molecular weights could be found on the tricine SDS-PAGE, probably because only the chloroform-soluble fraction was used for isolation of abnormal SP-Cs. The palmitoyl content of mSP-Cpap was found to be 38% of that of nSP-C. The mSP-Cpap fraction could thus consist of either a largely monopalmitoylated species or a mixture of nonpalmitoylated and dipalmitoylated species. The respective concentrations of these three species were not determined. Nevertheless, results of the animal experiment indicated the importance of the palmitoyl content of nSP-C. We used SPL for the lipid component of
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