A synthetic surfactant based on a poly-Leu SP-C analog and phospholipids: effects on tidal volumes and lung gas volumes in ventilated immature newborn rabbits

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Submitted 14 February 2003; accepted in final form 24 July 2003

Johansson, Jan, Margareta Some, Britt-Marie Linderholm, Andreas Almén, Tore Curstedt, and Bengt Robertson. A synthetic surfactant based on a poly-Leu SP-C analog and phospholipids: effects on tidal volumes and lung gas volumes in ventilated immature newborn rabbits. J Appl Physiol 95: 2055–2063, 2003. First published August 1, 2003; 10.1152/japplphysiol.00153.2003.—Available surfactants for treatment of respiratory distress syndrome in newborn infants are derived from animal lungs, which limits supply and poses a danger of propagating infectious material. Poly-Val—poly-Leu analogs of surfactant protein (SP)-C can be synthesized in large quantities and exhibit surface activity similar to SP-C. Here, activity of synthetic surfactants containing a poly-Leu SP-C analog (SP-C33) was evaluated in ventilated premature newborn rabbits. Treatment with 2.5 ml/kg body wt of 2% (wt/wt) SP-C33 in 1,2-dipalmitoyl-sn-3-glycero phosphoryl choline (DPPC)-1-palmitoyl-2-oleoyl-sn-3-glycero phosphoryl choline (POPC)-1-palmitoyl-2-oleoyl-sn-3-glycero phosphoryl choline (POPG), 68:0:31, 68:11:20, or 68:16:15 (wt/wt/wt) suspended at 80 mg/ml gave tidal volumes (VT) of 20–25 ml/kg body wt, with an insufflation pressure of 25 cmH2O and no positive end-expiratory pressure (PEEP), comparable to the VT for animals treated with the porcine surfactant Curosurf. Nontreated littermates had a VT of ~2 ml/kg body wt. The VT for SP-C33 in DPPC-egg phosphatidyglycerol-palmitic acid [68:22:9 (wt/wt/wt)], DPPC-PopG-palmitic acid [68:22:9 (wt/wt/wt)], and DPPC-POPC-POPG [6:2:2 (wt/wt/wt)] was 15–20 ml/kg body wt. Histological examination of lungs from animals treated with SP-C33-based surfactants showed incomplete, usually patchy air expansion of alveolar spaces associated with only mild airway epithelial damage. Lung gas volume after 30 min of mechanical ventilation were more than threefold larger in animals treated with Curosurf than in those receiving SP-C33 in DPPC-POPC-POPG, 68:11:20. This difference could be largely counterbalanced by ventilation with PEEP (3–4 cmH2O). An artificial surfactant based on SP-C33 improves VT in immature newborn animals ventilated with standardized peak pressure but requires PEEP to build up adequate lung gas volumes.

respiratory distress syndrome; surfactant protein C; membrane peptide; peptide synthesis; secondary structure; leucine

ATTEMPTS TO IDENTIFY ANALOGS of the two hydrophobic pulmonary surfactant proteins (SP) B and C to formulate a synthetic surfactant preparation for treatment of respiratory distress syndrome (RDS) are going on in several laboratories (6, 17, 26, 37). These efforts stem from the fact that SP-B and SP-C are the only proteins present in modified natural surfactant preparations currently used in clinical practice and appear to be essential for optimal function of such preparations. SP-B is a 17.4-kDa homodimer of two 79-residue polypeptide chains containing several amphipathic α-helices and probably interacts with the superficial parts of phospholipid membranes (19). SP-C is a 4.2-kDa lipopeptide and is composed of one 37-Å-long α-helix that can span a phospholipid bilayer (18) or interact in a tilted orientation with a phospholipid monolayer (9).

The peptide analogs of SP-B or SP-C investigated so far include KL4, a 21-residue peptide that was designed based on sequence motifs found in SP-B and contains four repeats of the motif KLLLL (5). Airway instillation of KL4 surfactant reportedly improves lung function in animal models of surfactant dysfunction (5, 32) and meconium aspiration syndrome (7) as well as in babies with RDS (6). Several analogs based on the SP-C structure have been described. Synthetic SP-C analogs with the native poly-Val sequence are capable of forming effective surfactants (36). Surfactant based on recombinant SP-C (in which the native poly-Val sequence is present, the first residue corresponding to human SP-C is removed, Cys5 and Cys6 are replaced with Phe, and Met33 is replaced by Ile) improves lung function in premature newborn rabbits and lambs (8, 12) as well as in animal models of acute lung injury (16, 17). In our experience, synthetic analogs containing a poly-Val SP-C sequence exhibit low surface activity in vitro, mainly because these peptides do not fold into the helical conformation shown by native SP-C but have a strong tendency to form aggregates with the peptide in a β-sheet conformation (20). To overcome the difficulties with folding of poly-Val peptides, a poly-Val→poly-Leu substituted SP-C analog [SP-C(Leu)] was synthesized and found to be very similar to native

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SP-C in terms of secondary structure and in vitro surface properties (26). These observations are in line with the finding that SP-C, but not SP-C(Leu), converts into an aggregated β-sheet conformation and forms insoluble amyloid fibrils (11, 13, 21, 35). However, SP-C(Leu) mixed with 1,2-dipalmitoyl-sn-3-glycerophosphoryl choline (DPPC)-egg phosphatidylglycerol (PG)-palmitic acid (PA), 68:22:9 (wt/wt), has only a modest effect on lung function in premature newborn rabbits (26). This is explained by oligomerization of SP-C(Leu) into dimers, tetramers, hexamers, and probably also higher order oligomers, which made it difficult to suspend SP-C(Leu)/lipid mixtures at concentrations of >20 mg/ml, and hence the maximal dose given was lower than for modified natural surfactants (which can be suspended at concentrations up to 80 mg/ml) (26). The problem with peptide oligomerization was circumvented by synthesizing a modified peptide, SP-C(LKS), which contained three lysines incorporated in the SP-C(Leu) sequence in such a way that they cover the helix circumference and reduce peptide-peptide interactions (27). SP-C(LKS) does not form oligomers like SP-C(Leu), and combined with lipids it exhibits rapid adsorption and spreading (27). SP-C(LKS) surfactant can be suspended at 80 mg/ml, but also instillation of such preparations does not produce satisfactory improvement of lung function in premature newborn rabbits (J. Johansson, T. Curstedt, B. Robertson, unpublished observations). The reason for this is not well understood, but it is possible that introduction of positively charged Lys residues in the nonpolar middle and COOH-terminal regions interferes with the SP-C dipole moment (from the intrinsic helix dipole and from the positively charged NH2-terminal residues) and/or influences the peptide-membrane interactions. In the present study, we investigated the physiological activity of a novel SP-C analog, SP-C33 (Fig. 1), in different lipid mixtures. The design of SP-C33 attempts to improve the physiological activity previously seen with SP-C(Leu)/lipid mixtures (26) by taking advantage of the high solubility and lack of oligomerization of SP-C(LKS) (27).

MATERIALS AND METHODS

Lipids. DPPC, 1-palmitoyl-2-oleoyl-sn-3-glycero phosphor yl choline (POPC), 1-palmitoyl-2-oleoyl-sn-3-glycero phosphor glycerol (POPG), PG, and PA were purchased from Sigma Chemical. The lipids were used without further purification. The following five lipid mixtures and weight ratios were used for screening SP-C33 activity in different environments: DPPC-PG-PA, 68:22:9; DPPC-POPG-PA, 68:22:9; DPPC-POPC-POPG, 62:2:2; DPPC-POPC-PG, 68:21:10; DPPC-POPC-POPG, 68:16:15; DPPC-POPC-POPG, 68:11:20; and DPPC-PG-POPG, 68:31. The mixtures were selected based on 1) a high DPPC content, 2) replacing PA with POPG, and 3) varying the content of POPG between 10 and 31% weight at a constant ratio between saturated and unsaturated phospholipids.

Peptides. The peptide SP-C33 (Fig. 1) was synthesized and purified as described in detail previously for SP-C(Leu) (26) and SP-C(LKS) (27). O,O-dipalmitoylated SP-C33 (pSP-C33) was synthesized by treating SP-C33 dissolved in neat trifluoroacetic acid with 20 equivalents of palmitoyl-chloride for 10 min at room temperature (41). The covalent structures of both peptides were confirmed by electrospray mass spectrometry and NH2-terminal amino acid sequence analysis. As expected from the poly-Leu design (21), analysis of the secondary structures of SP-C33 and pSP-C33 in dodecylphosphocholine micelles by circular dichroism spectroscopy showed that they have a high α-helical content (~80%) and no detectable β-strand content. SP-C33 has one Lys residue introduced in the NH2-terminal part of the helix (at position 12) to prevent peptide oligomerization, with a minimal interference with the long stretch of residues with aliphatic side chains in SP-C (Fig. 1). Analysis of SP-C33 by SDS polyacrylamide gel electrophoresis showed a single band at the expected molecular mass, indicating absence of significant peptide oligomerization.

Preparation of synthetic surfactants. SP-C33 or pSP-C33 and lipids in weight proportions peptide/lipid = 0.02 were mixed in chloroform-methanol 98:2 (vol/vol), the solvents were evaporated, and the resulting peptide/lipid films were subsequently hydrated in 150 mM NaCl by repeated sonication at a lipid concentration of 80 mg/ml. Surfactant samples used for analyses in the pulsating bubble surfactometer were diluted in saline to working concentration (10 mg/ml). Surfactant suspensions at 80 mg/ml containing pSP-C33 in DPPC-PG-PA (68:22:9) sometimes were quite viscous and therefore difficult to administer to the experimental animals. Curosurf (Chiesi Farmaceutici, Parma, Italy) and Survanta (Abbott, Chicago, IL) were administered according to the manufacturer’s instructions. Curosurf was suspended at 80 mg/ml and given at a dose of 2.5 ml/kg body wt. Survanta was suspended at 25 mg/ml and given at a dose of 4 ml/kg body wt.

Fig. 1. Primary structures and helical wheel projections of human surfactant protein (SP)-C and SP-C33. In the helical wheels, positions 9–34 (which are in α-helical conformation (18)) are shown for SP-C (A) and the corresponding positions 7–32 are shown for SP-C33 (B). Residues with positively charged side chains are circled. The Cys residues at positions 5 and 6 in human SP-C are palmitoylated.
Pulsating bubble experiments. The dynamic surface properties of SP-C33 surfactants were evaluated with a pulsating bubble surfactometer at 37°C. The sensitivity to inhibition was tested by adding 40 mg/ml of albumin to the surfactant suspension. A bubble communicating with ambient air was created in a plastic test chamber containing ~20 μl of the sample fluid. The radius of the bubble was oscillated at a rate of 40 cycles/min between a maximum of 0.55 and a minimum of 0.40 mm, corresponding to a cyclic area compression of 50%. Values for dynamic surface tension at minimum (γmin) and maximum bubble size (γmax) were recorded during 5 min of pulsation.

In vivo experiments. The surfactant mixtures were tested in 173 preterm newborn rabbits, obtained by hysterotomy at a gestational age of 27 days (term, 31 days) (34). The animals were tracheotomized at birth, kept in plethysmograph boxes at a gestational age of 27 days (term, 31 days) (34). The animals of pulsation. (PEEP) was applied. Tidal volumes (VT) were recorded at otherwise ventilated with the same sequence of insufflation pressures as described above.

POPG (68:11:20) was chosen for these experiments because of the instability (see Results). We addressed this unexpected problem by conducting a seventh series of experiments comparing physiological effects of SP-C33 in DPPC-POPC-POPG (68:22:9) vs. Curosurf (series I), SP-C33 in DPPC-PG-PA (68:22:9) vs. Survanta (series II), SP-C33 in DPPC-POPC-POPG (68:22:9) vs. Curosurf (series IV), SP-C33 in DPPC-POPC-POPG (68:22:9) vs. Survanta (series V), and SP-C33 in DPPC-POPC-POPG (68:16:15) (series VI). Series I, II, and IV were designed to test the effects of SP-C33 or pSP-C33 in the lipid mixture DPPC-PG-PA (68:22:9), originally designed by Tanaka et al. (38), compared with the natural modification surfactants Curosurf and Survanta. Series III, V, and VI were designed to test the effects of SP-C33 in lipid mixtures with varying composition and contents of acidic lipids. During the course of this project, we gradually became aware of a discrepancy between VT and histological findings in animals receiving SP-C33-based surfactants, indicating alveolar instability (see Results). We addressed this unexpected problem by conducting a seventh series of experiments comparing physiological effects of SP-C33 in DPPC-POPC-POPG (68:11:20) and Curosurf in animals ventilated with or without PEEP (3–4 cmH2O). The phospholipid mixture DPPC-POPC-POPG (68:11:20) was chosen for these experiments because this mixture has optimal in vitro properties (see Fig. 3) and has a content of acidic phospholipids that is similar to that of natural surfactant. In these experiments, the animals were otherwise ventilated with the same sequence of insufflation pressures as described above.

At the end of the scheduled period of ventilation, animals were killed by intracerebral injection of lidocaine. Their abdomen was opened, and the position of the diaphragm was inspected for evidence of pneumothorax. The protocol for animal experiments complied with Swedish Animal Protection Law and was approved by the local Ethics Committee for Animal Experiments.

Determination of lung gas volumes. Lung gas volumes were assessed in series VII for reasons explained above. After the scheduled 30-min period of ventilation with 100% O2, with or without PEEP, the animals were ventilated for an additional 5 min with 100% N2 at a peak insufflation pressure of 25 cmH2O. The tracheal cannula was then clamped at end inspiration. The trachea was ligated, and the lungs were carefully excised and weighed. Lung volume was determined by water displacement technique as described by Scherle (30). Lung gas volume was calculated from the difference between lung volume (in ml) and lung weight (in g), based on the assumption that the density of lung tissue is the same as that of water. This leads to a slight underestimation of lung gas volume, which was corrected for in our calculations.

Histological examination of lungs. Lungs from the series of experimental animals treated with SP-C33 in DPPC-PG-PA (68:22:9) or Curosurf and from nontreated littermate controls (series I) were prepared for histological examination according to the conventional protocol. This modiﬁcation was introduced because histological examination of lungs from the first series of experiments revealed very little, if any, alveolar air expansion in animals treated with SP-C33-based surfactant, contrasting to comparatively large VT; we expected that differences in alveolar expansion between the groups would become more obvious if the lungs were ﬁxed at end inspiration. The excised lungs of animals in series VII were ﬁxed by immersion in 4% buffered formaldehyde after the lung gas volume had been measured.

The lungs were embedded in parafﬁn, and large transverse sections from the lower lobes, stained with hematoxylin and eosin, were examined by light microscopy with particular reference to the alveolar expansion pattern and evidence of epithelial necrosis in peripheral conducting airways. The relative number of aerated alveoli was estimated semiquantitatively according to a ﬁve-grade scale (0, 1–25, 26–50, 51–75, and >75%) (22). Airway epithelial necrosis was classiﬁed by using an arbitrary four-grade score (absent, mild, moderate, prominent). In animals from series VII, alveolar volume density (Vv) in the histological sections was determined by using computerized image analysis with total parenchyma as reference volume (2). The histological examination was blinded, i.e., performed without knowledge of the experimental conditions of individual animals.

Statistical evaluation. Values for dynamic surface tension and VT/kg body weight at different time points and insufflation pressures are given as means ± SD, and differences between groups were evaluated with ANOVA followed by Newman-Keuls test. The χ2 test (with Yates’s correction, when required) was used for analyzing differences in the incidence of pneumothorax and for the nonparametric morphological variables (grade of alveolar expansion and degree of airway epithelial damage). Correlation between lung gas...
volumes and alveolar \( V_v \) was assessed by linear regression analysis. The limit level for statistical significance was defined as \( P = 0.05 \).

**RESULTS**

*In vitro surface activity of SP-C33 in different lipid mixtures.* The dynamic surface properties of 2% (wt/wt) SP-C33 or pSP-C33 in different lipid mixtures were evaluated with a pulsating bubble surfactometer (Fig. 2A). This showed \( \gamma_{\min} < 2 \text{ mN/m} \) after 5 min of pulsation for all mixtures except for pSP-C33 in DPPC-POPC-POPG (6:2:2) and \( \gamma_{\max} < 40 \text{ mN/m} \) for all mixtures except for SP-C33 in DPPC-PG-PA (68:22:9), and for both mixtures containing pSP-C33. Addition of 40 mg/ml of albumin to the SP-C33/lipid or pSP-C33/lipid mixtures increased \( \gamma_{\min} \) and \( \gamma_{\max} \) of all preparations (Fig. 2B). In particular, SP-C33 in DPPC-PG-PA (68:22:9) and mixtures containing pSP-C33 were strongly inactivated by albumin. To compare mixtures with a constant ratio of DPPC to unsaturated phospholipids but varying the contents of POPG, SP-C33 in DPPC-POPC-POPG (68:11:20), DPPC-POPC-POPG (68:2:3:15), and DPPC-POPC-POPG (68:21:10) were analyzed. This showed that the \( \gamma_{\min} \) values increased as the content of POPG was decreased.

Twenty percent was the minimum POPG content for which \( \gamma_{\min} \) values of < 5 mN/m were obtained (Fig. 3).

*In vivo activity of SP-C33 in different lipid mixtures.* One animal treated with SP-C33 in DPPC-PG-PA (68:22:9) in series II and one control animal in series VI showed evidence of pneumothorax at the end of the experiment. These two animals were not included in the final statistics. \( V_T \) recorded at different time points in various groups of experimental animals are summarized in Figs. 4–7. Compared with controls, all groups of surfactant-treated animals had a prominent increase in \( V_T \) throughout the experiment, except when the insufflation pressure was lowered to 15 cmH\(_2\)O. Initially, the effects of 80 mg/ml of SP-C33 in DPPC-PG-PA (68:22:9) were compared with those of the modified natural surfactant preparations of Curosurf and Survanta in their recommended clinical doses. This showed similar \( V_T \) for all three preparations at all time-points (Fig. 4), establishing that SP-C33 surfactant has physiological effects.

Next, we aimed at optimizing the effect of SP-C33-based surfactant by varying the lipid mixtures and investigating the effect of \( O,O \)-dipalmitoylated SP-C33. Figure 5A shows the effect of SP-C33 in DPPC-PG-PA (68:22:9) compared with that of SP-C33 or di-
palmitoylated SP-C33 in DPPC-POPC-POPG (6:2:2). The lipid mixture DPPC-POPC-POPG (6:2:2) was chosen to decrease the contents of DPPC and acidic lipids compared with DPPC-PG-PA (68:22:9), thus making the lipid mixture more similar to that of natural surfactant. SP-C33 in DPPC-PG-PA (68:22:9) gave significantly higher VT than the other mixtures. pSP-C33 in DPPC-POPC-POPG (6:2:2) gave low VT throughout the experiment, which did not differ from the untreated controls (n = 8). Levels of statistical significance are indicated for final recordings only: **P < 0.01 vs. control animals.

palmitoylated SP-C33 in DPPC-PG-PA (68:22:9) and modified natural surfactants in vivo. A: tidal volumes (VT) during 30 min of ventilation in preterm newborn rabbits treated with SP-C33 in DPPC-PG-PA (68:22:9) (80 mg/ml, 2.5 ml/kg, n = 10) or Curosurf (80 mg/ml, 2.5 ml/kg, n = 9), and for nontreated controls (n = 10). B: VT during 30 min of ventilation in preterm newborn rabbits treated with SP-C33 in DPPC-PG-PA (68:22:9) (80 mg/ml, 2.5 ml/kg, n = 9) or Survanta (25 mg/ml, 4 ml/kg, n = 8), and for nontreated controls (n = 8). Levels of statistical significance are indicated for final recordings only: **P < 0.01 vs. control animals.

Fig. 4. Effects of SP-C33 in DPPC-PG-PA (68:22:9) and modified natural surfactants in vivo. A: tidal volumes (VT) during 30 min of ventilation in preterm newborn rabbits treated with SP-C33 in DPPC-PG-PA (68:22:9) (80 mg/ml, 2.5 ml/kg, n = 10) or Curosurf (80 mg/ml, 2.5 ml/kg, n = 9) and for nontreated controls (n = 10). B: VT during 30 min of ventilation in preterm newborn rabbits treated with SP-C33 in DPPC-PG-PA (68:22:9) (80 mg/ml, 2.5 ml/kg, n = 9) or Survanta (25 mg/ml, 4 ml/kg, n = 8), and for nontreated controls (n = 8). Levels of statistical significance are indicated for final recordings only: **P < 0.01 vs. control animals.

Finally, the content of acidic phospholipids was varied by comparing SP-C33 in DPPC-POPG (68:31) to SP-C33 in DPPC-POPC-POPG (68:16:15) (Fig. 7).

taining PA during the initial 15 min of ventilation (Fig. 6).
These experiments showed equal effects of the two surfactants.

**Histological findings in animals treated with different types of surfactant (series I–VI).** Results of histological examination of animals treated without PEEP are summarized in Table 1. As expected, animals treated with Curosurf had an improved grade of alveolar air expansion compared with nontreated controls (median, 2–2.5 vs. 0–0.5). The effects of Survanta were less prominent (median, 1.5). Only modest effects were seen in animals receiving SP-C33-based surfactant (median, 0–1), irrespective of the composition of the lipid mixture. In most of the animals treated with SP-C33-based artificial surfactant, alveolar air expansion was patchy, involving <25% of the parenchyma (grade I). Palmitoylation of SP-C33 did not seem to improve the therapeutic effect. Data from series I, IV, and V allow evaluation of lung morphology in relation to the distending pressure applied during the fixation procedure. In animals treated with Curosurf or artificial surfactant made from SP-C33 reconstituted with DPPC-PG-PA (68:22:9), the grade of alveolar air expansion tended to be higher if the lungs were fixed at a distending pressure of 25 cmH₂O (series IV and V) rather than 10 cmH₂O (series I). Airway epithelial necrosis tended to be less prominent in animals receiving any type of modified natural or artificial surfactant (median, 1–1.5) than in controls (median, 2), without significant differences between Curosurf, Survanta, or SP-C33-based material.

**Effects of SP-C33 surfactant in animals ventilated with PEEP (series VII).** VT during artificial ventilation without PEEP were increased significantly in surfactant-treated animals compared with nontreated controls (P < 0.01) and were larger in animals receiving Curosurf than in those treated with SP-C33 in DPPC-POPC-POPG (68:11:20) (P < 0.05) (Table 2). Lung gas volumes measured at the end of the experiment were more than three times larger in Curosurf-treated animals than in littermates receiving SP-C33-based surfactant (P < 0.01), which were not significantly different from controls. During ventilation with PEEP (3–4 cmH₂O), VT were slightly decreased in Curosurf-treated animals compared with volumes recorded with the same peak pressure without PEEP (this was expected because the pressure gradient, i.e., peak pressure minus PEEP, was reduced) and were nearly the same in both groups of surfactant-treated animals. Notably, lung gas volumes in animals receiving artificial surfactant were nearly three times larger after ventilation with PEEP vs. no PEEP. The sum of median values for VT and end-expiratory lung gas volume in Curosurf-treated animals ventilated with PEEP was ~50 ml/kg, which was associated with a high incidence of pneumothorax (6 of 11, 55%). Ventilation with PEEP also had an impact on the histological findings, improving the alveolar Vv assessed by computerized image analysis (Table 2). In series VII, as a whole, there was a significant correlation between lung gas volumes measured by the water displacement technique and alveolar Vv in histological sections (r = 0.79; P < 0.0001).

### Table 1. Histological observations in 6 series of experiments on ventilated preterm newborn rabbits receiving modified natural surfactants, artificial surfactant based on SP-C33 and different mixtures of synthetic lipids, or no material via the airways.

<table>
<thead>
<tr>
<th>Series</th>
<th>Treatment a</th>
<th>n</th>
<th>Alveolar Expansion b</th>
<th>Airway Epithelial Necrosis c, Grade</th>
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<tr>
<td>II</td>
<td>Survanta</td>
<td>8</td>
<td>1.5 (0–2) a</td>
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<td>Controls</td>
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Values are shown as median (range); n, no. of animals. All animals were ventilated for 30 min with a standardized sequence of insufflation pressures (see Figs. 4–7). In series I, lungs were fixed by vascular perfusion while being expanded with a transpulmonary pressure of 10 cmH₂O. In series II–VI the lungs were expanded with a transpulmonary pressure of 25 cmH₂O during perfusion fixation.


b P = no alveolar air expansion (collapsed or liquid-filled alveoli); 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100% of alveoli air expanded. *P = absent; 1 = mild; 2 = moderate; 3 = prominent. aP < 0.001 vs. controls. aP < 0.01 vs. controls. bP < 0.05 vs. controls. cP < 0.01 vs. SP-C33 (A).
Table 2. *V*\textsubscript{T} with an insufflation pressure of 25 cmH\textsubscript{2}O, incidence of pneumothorax, lung gas volumes, and alveolar V\textsubscript{V}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PEEP, cmH\textsubscript{2}O</th>
<th>n</th>
<th>V\textsubscript{T}, ml/kg</th>
<th>Lung Gas Volume,\textsuperscript{a} ml/kg</th>
<th>Pneumothorax, no.</th>
<th>V\textsubscript{V}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curosurf</td>
<td>0</td>
<td>8</td>
<td>28 (16–35)\textsuperscript{†∥}</td>
<td>21 (15–47)\textsuperscript{†∥}</td>
<td>3</td>
<td>0.66 ± 0.05∥</td>
</tr>
<tr>
<td>SP-C33</td>
<td>0</td>
<td>11</td>
<td>20 (7–33)\textsuperscript{†}</td>
<td>6 (3–12)\textsuperscript{†}</td>
<td>1</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>12</td>
<td>2 (1–21)</td>
<td>2 (0–6)</td>
<td>1</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>Curosurf</td>
<td>3–4</td>
<td>11</td>
<td>21 (14–25)\textsuperscript{†}</td>
<td>30 (23–36)\textsuperscript{†∥}</td>
<td>6</td>
<td>0.61 ± 0.06∥</td>
</tr>
<tr>
<td>SP-C33</td>
<td>3–4</td>
<td>11</td>
<td>19 (9–26)\textsuperscript{†}</td>
<td>17 (5–33)\textsuperscript{†}</td>
<td>3</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>Controls</td>
<td>3–4</td>
<td>12</td>
<td>3 (0–23)</td>
<td>3 (0–5)</td>
<td>0</td>
<td>0.41 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SD or median (range); n, no. of animals. V\textsubscript{T}, tidal volume; V\textsubscript{V}, volume density; PEEP, positive end-expiratory pressure.

\textsuperscript{a}Excluding animals with pneumothorax. \textsuperscript{†}P < 0.01 vs. controls. \textsuperscript{∥}P < 0.05 vs. SP-C33 surfactant.

**DISCUSSION**

Suspensions (80 mg/ml) of a poly-Val→poly-Leu substituted SP-C analog, SP-C33, mixed with DPPC-POPC-POPG (68:16:15) show physiological activity in the present animal model of neonatal RDS. SP-C33 is active also when combined with other lipid mixtures, although the effect then appears to be lower. When the animals were ventilated without PEEP, improvements in V\textsubscript{T} obtained with SP-C33-based surfactant were not associated with augmented alveolar expansion in histological lung sections or increased lung gas volume, indicating poor alveolar stability at end expiration. This relative treatment failure can apparently be compensated for by application of PEEP, which in the present study led to a nearly threefold increase in lung gas volume associated with a significant improvement of alveolar expansion also in histological sections. Alveolar V\textsubscript{V} of animals ventilated with PEEP after receiving SPC-33 surfactant was improved by 34% compared with controls yet did not quite reach the level obtained by treatment with Curosurf. In contrast, animals treated with Curosurf do not seem to need ventilation with PEEP to maintain adequate lung gas volume. In these animals, the combination of large V\textsubscript{T} and large lung gas volumes may in fact be harmful, as indicated in the present study by a high incidence of pneumothorax.

Development of clinically active synthetic surfactants has turned out to be more complicated than initially anticipated. Several different peptides have been used to formulate synthetic surfactants. KL\textsubscript{4} surfactant contains 2–3% (wt/wt) peptide in phospholipids-PA mixtures and has been administered at a lipid concentration of 20–35 mg/ml (5–7, 32). As mentioned above, tracheal instillation of KL\textsubscript{4} surfactant improves arterial-to-alveolar oxygen tension ratio in experimental and clinical RDS (6, 28), and lung lavage with diluted KL\textsubscript{4} surfactant improves oxygenation in animal models of meconium aspiration syndrome (7). Airway lavage with KL\textsubscript{4} surfactant has also been tested in adult patients with acute RDS (40) and in infants with meconium aspiration syndrome (39). The latter study showed a trend (not significant) toward treatment effects in the lavaged infants, and further studies are underway. Another approach using 2% (wt/wt) of recombinant SP-C mixed with phospholipids/PA and suspended at 25 mg/ml is effective in animal models of RDS (8, 12) and acute lung injury (14–17). However, a recent clinical trial in patients with acute RDS indicates that mortality and requirement for mechanical ventilation are not significantly affected by treatment with recombinant SP-C surfactant (33). A third approach, explored in the present study, involves synthetic analogs of SP-C, in which the poly-Val region has been replaced with a poly-Leu region. SP-C33 surfactant has three properties that probably are important for formulation of a synthetic surfactant for clinical treatment of respiratory diseases. 1) It can be suspended at high concentration (80 mg/ml), 2) it exhibits significant activity without the presence of PA, and 3) it increases V\textsubscript{T} of immature newborn experimental animals to levels similar to those obtained with modified natural surfactant preparations currently used for treatment of neonatal RDS. Administration of surfactants at high concentration is likely important for optimal clinical effects, because surface activity, surfactant dose-response in animal models, as well as resistance to inhibition are increased at higher surfactant concentrations (4, 23, 31).

PA is present only in trace amounts in natural surfactant and in most surfactants currently used in clinical practice. The inclusion of PA in Survanta and in some synthetic surfactants stems from the observation that in vitro and in vivo properties of surfactant lipid/protein mixtures were seemingly improved in the presence of PA (38). In contrast, we found that omission of PA in SP-C33 surfactant increases the effects on V\textsubscript{T} in vivo and decreases the sensitivity to albumin inhibition in vitro. Resistance to inhibition by plasma proteins leaking into the airways is likely important for effective surfactant treatment, in particular in acute lung injury. PA also made the mixtures more difficult to suspend at high concentration, in particular when dipalmitoylated SP-C33 was used.

In animals treated with SP-C33 surfactant without PEEP, the degree of alveolar air expansion was lower than in littermates receiving modified natural surfactant, yet only mild epithelial damage was observed. The nontreated controls consistently showed more prominent alveolar damage than surfactant-treated animals, although the differences did not reach statistical significance (Table 1). In immature newborn rabbits, also relatively short periods (5–10 min) of me-
Mechanical ventilation induce significant damage to airway epithelium (25), with profuse protein leakage from the vascular compartment to the alveolar spaces (1). Our present findings suggest that SP-C33 surfactant promotes alveolar expansion during inspiration by lowering surface tension, thereby avoiding disruptive shear forces in the airway epithelium (24), but does not create a fully stable surfactant layer at end expiration. SP-C33-based surfactant obviously lacks SP-B. Curosurf has an SP-B content of ~30% compared with native porcine surfactant, and Survanta contains ~10% of the SP-B levels in bovine surfactant (3). It is conceivable that comparatively small amounts of SP-B also help to stabilize the surfactant layer at the air-water interface during expiration. Alveolar expansion and lung gas volumes were significantly improved when animals treated with SP-C33 surfactant were ventilated with PEEP. Similar effects were observed in premature lambs treated with recombinant SP-C surfactant (8), suggesting that ventilation with PEEP may be required to maintain adequate lung gas volume throughout the ventilatory cycle when synthetic surfactants based on SP-C are employed.

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DISCLOSURES

This work was supported by grants from the Swedish Research Council, Chiesi Farmaceutici, and Konung Oscar II:s Jubileumsfond.

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