Alveolar metabolism of natural vs. synthetic surfactants in preterm newborn rabbits

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Numerous studies have addressed the response of the premature newborn to different preparations of surfactant, both natural and synthetic, in rabbit (4, 9, 18, 19, 26–28, 30, 31) and sheep (2, 9, 11, 13) animal models. For the most part, these studies have addressed the short-term response; there is not an abundance of information available on the long-term effects of various surfactant treatments. It is becoming increasingly apparent that the acute effects of a surfactant on the preterm lung may not necessarily reflect its long-term effect (9). An important consideration in this regard is the bioavailability of the instilled surfactant preparation.

Preterm rabbits at 27 days of gestation (term = 31 days) have almost no alveolar surfactant, as evaluated by alveolar lavage, and very little intracellularly stored surfactant (21, 22); therefore, they are widely used for studying several aspects of lung development (4, 9, 18, 19, 26–28, 30, 31). We recently used this model to examine the effects of the intratracheal administration of different surfactant preparations on lung water clearance (16, 24) in preterm rabbits ventilated for periods of up to 6 h. A surfactant preparation obtained from term fetal rabbits (FRS) was associated with longer survival and improved lung water clearance compared with an adult rabbit surfactant preparation (ARS) and two commercially available preparations, Exosurf and Survanta. In vitro studies on FRS in our laboratory (23) have shown that, after 3 h of surface area cycling (7), FRS converted from the biologically active heavy-aggregate form (H) to the inactive light-aggregate (L) form at a significantly slower rate than ARS (i.e., <20% vs. ~60%). If FRS functions in a similar manner when instilled into lungs, the bioavailability of its active (H) form would be expected to be longer compared with ARS and other surfactants.

The purpose of the present investigation was to study and compare the metabolic fate of the two natural surfactants (FRS and ARS) and the two commercially available surfactants (Exosurf and Survanta) using the preterm rabbit model.

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METHODS

Preparation of surfactant for instillation. Survanta, a modified natural surfactant prepared by lipid extraction of minced bovine lungs followed by enrichment with dipalmitylphosphatidylcholine (DPPC), palmatic acid, and tripalmitin, was purchased from Ross Laboratories (Columbus, OH). This preparation contains surfactant protein (SP)-B and SP-C but not SP-A.

Exosurf, a totally synthetic surfactant that contains DPPC, hexadecanol, and tyloxapol and none of the surfactant-associated proteins, was purchased from Burroughs-Wellcome (Research Triangle Park, NC).

The natural surfactants were isolated from adult and fetal rabbit lungs using differential and density gradient procedures, which have been described in detail in previous reports (21, 22, 23). We have previously reported that the surfactant preparation isolated by this procedure consists entirely of characteristic lamellar body (LB)-like structures, as evidenced by electron microscopic analysis (22), and is virtually free from contamination by other nonsurfactant membrane components, as determined by marker enzyme assays (22). Also, as previously reported (21, 22), this preparation has the chemical composition characteristic of surfactants isolated from a wide variety of mammalian species, is highly surface active, as determined in vitro by the pulsating bubble surfactometer (22), and is biologically active, as assessed in vivo in the preterm rabbit model (16, 24). We also found that surfactants isolated separately from the extracellular and intracellular pools were identical in these properties (Oulton, unpublished observations). Therefore, to conserve material, especially that obtained from the term fetal rabbit pup, in which the quantities were limited, we isolated the total lung surfactant pool and used it in this study.

Two adults and three litters of term fetal pups were used for the natural rabbit surfactant preparations used in this study. The washed surfactant pellets were suspended in sterile 0.85% NaCl, and an aliquot was removed for phosphorus analysis. The natural surfactants, ARS and FRS, contain sterile 0.85% NaCl, and an aliquot was removed for phospholipid content and composition (22).

Preparation of surfactant for instillation. The neonatal lungs were lavaged in situ five times with 0.8 ml of sterile 0.85% NaCl. The lavage was centrifuged at 10,000 g for 30 min, and the proportion in the heavy aggregate form (H) was determined by lipid phosphorus analysis. Approximately 97% of each surfactant preparation, including Survanta and Exosurf, was in the H form.

Study protocol. This study protocol was reviewed and approved by the University Committee on Laboratory Animals, Dalhousie University, Halifax, Nova Scotia.

Time-dated New Zealand rabbit pups were delivered at 27 days of gestation by cesarean section under intravenous pentobarbital sodium anesthesia. A number of pups from various litters were killed at birth and served as controls. The treated pups had a tracheotomy and insertion of a 20-gauge angiocatheter into the trachea, through which one of the four surfactants was immediately instilled. Although the average litter size was 7 or 8 pups, the litters varied from 2 to 14, making it impossible to randomize treatment with all four surfactants to each litter. Thus we administered two different surfactants per experiment and varied the treatment protocol so that each of the four preparations was studied with each of the others.

Survanta and Exosurf were administered at the clinically recommended dose (100 and 67.5 mg phospholipid/kg body wt, respectively), and the natural surfactants were administered at a dose of 50 mg phospholipid/kg body wt followed by two puffs of air. In a preliminary study (unpublished observations) in which we compared the survival rates, lung water clearance, and retention of the alveolar H form of surfactant of 27-day gestation pups after administration of either 50 or 75 mg of natural surfactant phospholipid/kg body wt, we found no difference. To conserve this surfactant preparation, we used the lower dose for these experiments.

After instillation of surfactant, the pups were attached to a Harvard Apparatus respirator for small animals and ventilated at a rate of 50 breaths/min with an inspiration-expiration ratio of 1:1 in 100% oxygen. The ventilator was initially set at a pressure of 25–30 cmH2O, with an end-expiratory pressure of 2–3 cmH2O. The inspiratory pressure was decreased to 20–22 cmH2O as chest movement allowed. In a separate study, we calculated that these ventilatory conditions correlated with tidal volumes in the range of 7–8 ml/kg (data not shown).

Pups were randomly assigned to a surfactant treatment group and to ventilation times of 0, 5, 60, or 120 min. Pups that died during ventilation were removed from the ventilator and excluded from the study.

Two hours was selected for the study duration as this was the time at which deterioration of the pups was first observed in previous studies (16, 24). The time from birth to attachment to the ventilator was typically 4–5 min. The pups were covered with a plastic sheet and warmed with a heating pad and heating lamps to keep oropharyngeal temperature at 37.5°C, as intermittently measured by a thermistor probe. Fine-needle electrodes were used to monitor heart rate (HR) by electrocardiogram wave form at 30-min intervals or more frequently if a pup was pale or cyanotic. If bradycardia (HR <100 beats/min) or pneumothorax was observed, the pup was considered to be dead and excluded from the study. At the end of each prescribed ventilation period, the pups were killed with an intraperitoneal injection of pentobarbital sodium.

Biochemical analysis. The neonatal lungs were lavaged in situ five times with 0.8 ml of sterile 0.85% NaCl. The lavage was centrifuged at 10,000 g for 30 min to obtain the H form of surfactant (pellet) and L form (supernatant). We have previously shown that centrifugation at 10,000 g for 30 min is the maximum that is required to entirely sediment the H form of alveolar surfactant in fetal and newborn lungs (22).

We have also shown that a prior low-speed centrifugation is not only unnecessary because of the negligible alveolar macrophage population in these immature pups (22) but, in fact, is undesirable. Forces as low as 140 g for 5 min have been shown to result in substantial losses of H (22). We found that the 10,000-g supernatant is composed of small univesicular particles that sediment on centrifugation at 100,000 g for 15 h with a soluble supernate (data not shown). For routine study, we did not fractionate the 10,000-g supernatant and thus refer to the whole fraction as L.

The postlavaged lung tissue was removed, weighed, and homogenized, and the intracellularly stored surfactant (LB fraction) was isolated using the differential and density gradient procedure referred to above for preparations of the natural surfactants (21–23).

Aliquots of each H, L, and LB fraction were removed for determination of phospholipid content and composition (22).
For the untreated, unventilated control group, lavage returns and the corresponding lung tissue from 15 pups were separately pooled in groups of three or four for isolation of H, L, and LB. This pooling was required to obtain enough material for phospholipid analysis of these fractions. For all treated pups, lavage samples were analyzed individually for H and L fractions, and postlavaged lung tissue was pooled from groups of two or three pups for isolation of LB at each study time.

Statistical analysis. Each data point is expressed as the mean ± SD for the number of observations made. Significant differences (P < 0.05) between groups were assessed by ANOVA followed by Duncan’s multiple range test (20).

RESULTS

Condition of animals. Fifteen pups served as non-treated, nonventilated controls; 133 pups were intubated, treated, and ventilated for 0–120 min, and 25 of these were treated and not ventilated (0 min). Thirteen pups (2 or 3 from each treatment group) had to be removed from the ventilator and excluded from the study because of deteriorating conditions during ventilation; of these, seven had tracheal tears, but the cause of deterioration of the other six was not determined. The remaining pups (n = 95) survived the treatment without incident; none developed bradycardia or pneumothorax. The body weights at delivery ranged from 26.2 to 34.0 g, and lung weights at autopsy ranged from 0.66 to 0.81 g. There were no significant differences in either the body or lung weights among the different study groups (results not shown).

Recovery of H phospholipid from alveolar lavage immediately after surfactant instillation. Lung lavage fluid from pups that were killed immediately after surfactant instillation contained very little H phospholipid, i.e., <60 µg/pup (see Table 1). After treatment with each surfactant preparation, H phospholipid significantly increased to ~600 µg per pup with Exosurf and Survanta and 300 µg per pup with ARS and FRS.

Phospholipid compositions of representative H samples obtained immediately after each treatment are shown in Table 1. Not enough samples were available for statistical analysis, but differences were observed among the various groups. In the untreated control pups, PC was the predominant phospholipid (52% of the total lipid phosphorus) and PG was absent. After treatment, the composition of the recovered H in each treatment group approximated that of the surfactant preparation instilled. After treatment with Exosurf, the H fraction was comprised almost entirely of PC (97%) and contained no PG. With the other surfactants, the H fraction contained 75–85% PC and PG was present. With Survanta and ARS, the relative proportion of PG was greater than that for PI, whereas the reverse was found after treatment with FRS.

Table 1. Alveolar H phospholipid content and composition in unventilated preterm rabbits immediately after the various surfactant treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals</th>
<th>Content, µg/pup</th>
<th>PC</th>
<th>PG</th>
<th>PI</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>55 ± 22</td>
<td>52 ± 3</td>
<td>0</td>
<td>9 ± 3</td>
<td>39 ± 10</td>
</tr>
<tr>
<td>Exosurf</td>
<td>4</td>
<td>587 ± 126*</td>
<td>98 ± 2*</td>
<td>0</td>
<td>1 ± 1*</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>Survanta</td>
<td>3</td>
<td>614 ± 80*</td>
<td>81 ± 2*</td>
<td>5 ± 1</td>
<td>2 ± 1*</td>
<td>12 ± 1*</td>
</tr>
<tr>
<td>Adult rabbit surfactant</td>
<td>6</td>
<td>337 ± 126*</td>
<td>77 ± 7*</td>
<td>7 ± 1</td>
<td>4 ± 1*</td>
<td>12 ± 5*</td>
</tr>
<tr>
<td>Fetal rabbit surfactant</td>
<td>12</td>
<td>307 ± 117*</td>
<td>77 ± 1*</td>
<td>3 ± 2</td>
<td>10 ± 1</td>
<td>10 ± 2*</td>
</tr>
</tbody>
</table>

Composition, %total lipid phosphorus

Values are means ± SD of no. of animals shown. The untreated control pups were killed immediately after lavage, and the treated pups were killed immediately after surfactant instillation. Composition is of representative phospholipids of the biologically active heavy-aggregate form (H) from each treatment group. PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; other includes, phosphatidylserine, sphingomyelin, phosphatidylethanolamine, and other minor phospholipids. *Significantly different from untreated control [P < 0.05 by Duncan’s multiple range test (DMRT)].
between 5 and 60 min. At 120 minutes, the L phospholipid pool was approximately the same size after each treatment; however, it comprised different proportions of the total alveolar surfactant pool: 19% (mean) for the FRS-treated pups and closer to 30% for the other groups. Not enough phospholipid was recovered in any of the L preparations for compositional analysis.

With the exception of FRS, the increase in L did not account for the total loss of H phospholipid or follow the pattern of this loss. Because the H pool was approximately 10 times larger than the L pool during the time period studied, the small increases in the L pool did not necessarily reflect changes in the larger H pool.

Recovery of LB phospholipid after surfactant instillation. There was no significant change in LB total phospholipid content per pup at any of the time intervals after treatment with any of the surfactants, with the exception of a small but significant increase at 120 min after treatment with Exosurf (Table 2). Representative phospholipid compositions are shown for LB fractions isolated 120 min after the various surfactant treatments. We did not have sufficient samples for statistical analysis, but the data showed that, in the untreated controls, PC accounted for ~60% of the total LB lipid phosphorus, increasing to ~80% after treatment with Exosurf. The overall phospholipid composition of LB did not appear to change after treatment with the other surfactants. PG was not present in LB from untreated controls and did not appear by 120 min after any of the surfactant treatments, even for those that contain PG (i.e., Survanta, ARS, FRS).

DISCUSSION

When treated with any one of the four surfactant preparations at delivery, 27-day gestation rabbit pups demonstrated an immediate 6- to 10-fold increase in the metabolically active H fraction of alveolar lavage fluid. The phospholipid composition of the H obtained immediately after treatment showed changes from nontreated pups that were indicative of the type of surfactant administered. The alveolar H composition of
each group then remained relatively constant over 2 h of ventilation, indicating the origin of H from the instillate at all study times rather than the endogenous pool.

Pups treated with either Exosurf or Survanta lost ~50% of the H form by 5 min, with a slower rate of continuing loss of up to 80% by 2 h. ARS- and FRS-treated pups showed no loss of H at 5 min. However, ARS-treated pups showed a >50% loss of H by 1 h, with no further loss over the second hour, whereas FRS-treated pups showed no significant loss of H over the entire study period. By 2 h, <15% of the H form of FRS was lost from the airway.

Other studies have reported rapid and increasing loss of administered surfactant, including E, S, and various natural preparations, from the airway of pre-term animals over time (2, 10, 11, 13). This is the first report of a natural surfactant, FRS, that was not lost and in which the H form was retained in the airway for 2 h after administration.

The high loss of H phospholipid from the airway lavage within 5 min of Exosurf or Survanta instillation was not explained by recovery in the L fraction. It is possible that the H form from these preparations became tissue associated. Rapid tissue association of a variety of instilled surfactants has been reported in several animal models, including adults (15, 35) and newborns (11, 13, 25, 29). Tissue association may also account for the later losses of the H form of Exosurf and Survanta observed in our study. Ikegami et al. (11) reported that, 5 h after ventilating 132-day gestation lambs that had been instilled with various surfactant

Table 2. Lamellar body phospholipid content and composition at 120 min after various surfactant treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals</th>
<th>Content, µg/pup</th>
<th>PC</th>
<th>PG</th>
<th>PI</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>15</td>
<td>29.0 ± 5.4</td>
<td>58</td>
<td>0</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>Exosurf</td>
<td>10</td>
<td>51.3 ± 16.5*</td>
<td>79</td>
<td>0</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Survanta</td>
<td>10</td>
<td>36.6 ± 18.0</td>
<td>70</td>
<td>0</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Adult rabbit surfactant</td>
<td>5</td>
<td>28.1 ± 5.5</td>
<td>64</td>
<td>0</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>Fetal rabbit surfactant</td>
<td>7</td>
<td>34.9 ± 7.1</td>
<td>61</td>
<td>0</td>
<td>6</td>
<td>33</td>
</tr>
</tbody>
</table>

Values are means ± SD of no. of animals shown. The untreated, unventilated control pups were killed immediately on delivery, and the treated pups were killed after 120 min of ventilation. Phospholipid compositions are of representative samples from each treatment group. Not enough material was available for statistical analysis of the compositional data. *Significantly different from control group (P < 0.05 by DMRT).
preparations, including Exosurf and Survanta, at delivery, ~80–90% of the administered surfactant was recovered in the tissue homogenate.

Other sources of H form removal from the alveoli include degradation (19), which could provide substrate for new LB synthesis or complete removal from the lung and the surfactant pathways (5). A portion of the H form of the Exosurf preparation administered in this study appears to have been cycled into the LB pool by 2 h; administration of the other surfactant preparations did not show a significant change in LB phospholipid content or any apparent change in LB phospholipid composition, indicating that there was no demonstrable recycling to the LB of these preparations for up to 2 h. The mechanisms for Exosurf recycling may be different from those for natural or modified natural surfactants; further studies with labeled instillates are required for clarification of this issue.

Without labeling, our data do not indicate whether LB synthesis or secretion is occurring. However, because the LB pool is very small in the 27-day rabbit pup (22) and 27-day pups are unable to survive more than 1 h without exogenous surfactant (16), LB secretion must be extremely small. During the 2-h time period of the present study, the composition of alveolar H obtained from each treatment group approximated that of the instillate and not that of the LB fraction, indicating minimal LB secretion. Similarly, Ikegami and Jobe (10) reported minimal LB secretion in 134–136 day gestation lambs that had been instilled with radiolabeled sheep surfactant and ventilated up to 10 h.

Our data showed no loss of the H form of ARS in the first 5 min but a major loss by 60 min, with low recovery in the L form. This indicates that ARS does not become tissue associated immediately after instillation but that it may be taken up by lung tissue during the first 60 min. In a previous study using radiolabeled ARS, we reported ~50% tissue association after 30 min of ventilation of 27-day gestation pups (36). It is possible that the tissue association occurs by rapid conversion to L, as observed in vitro (23), and immediate uptake of the L form by the type II cell.

The minimal loss of the H form of FRS was almost completely accounted for by the observed increase in the L form. The slow conversion of H to L is consistent with our in vitro surface-area cycling studies (23), which showed <20% H-to-L conversion of FRS, in contrast to the 60% conversion found for ARS. These data contrast with the report of Ueda et al. (33), who reported <20% H-to-L conversion for the surfactant subtypes from both term fetal lambs and adult sheep but a >50% conversion in surfactant from preterm fetal lambs. The different conclusions of the two studies, as described in detail elsewhere (23), may be partly explained by species differences, differences in centrifugation schemes used for subtype isolation, the amount of phospholipid used for cycling studies, and the developmental ages studied. In the in vitro study from our laboratory (23), we did not examine H-to-L conversion in preterm (27-day gestation) rabbit fetuses because the limited amount of secreted surfactant in these pups made such a study unfeasible.

Our laboratory identified the LB as a major source of the enzyme convertase, which is required for H-to-L conversion, and demonstrated developmental changes in activity after release of the contents of the LB to alveoli (23). Our group suggested that the differences in the rate of H-to-L conversion between FRS and ARS may be partly due to their differences in convertase activity (23). Because the enzyme has not been fully characterized, the mechanism responsible for these differences is not currently known.

Our study does not identify the unique properties of FRS that allow the major portion of it to remain in active form in the alveoli during the first 2 h after instillation. The possibilities include 1) developmental differences in convertase activity (23, 33); 2) developmental differences in SP-A, which plays a major role in the recycling of surfactant from the alveoli (12) and in H-to-L conversion (35); in ARS, SP-A is fully glycosylated and, in FRS, it is only partially or not glycosylated (1); 3) the nature of the structural forms present in H; FRS is a more homogenous preparation consisting entirely of newly secreted and unused lamellar structures (22), whereas the H form of ARS is more heterogenous, containing lamellar structures of varying sizes and tubular myelin forms (17); and 4) interaction with granulocyte-macrophage colony-stimulating factor, which increases with age in the alveoli and regulates alveolar pool size and the turnover of alveolar surfactant (34).

The effectiveness of alveolar surfactant depends not only on the amount of alveolar H and its rate of conversion to L but also on the balance between activation (apoprotein) and inactivation (soluble protein leak into the alveoli) (11). In the present report, all the surfactant preparations appeared to be equally effective in maintaining lung function during the 2-h study period. We did not examine specific lung function or pathological studies other than isolation of H, L, and LB fractions to delineate differences in the effect of each surfactant. The fact that ARS-, Exosurf-, and Survanta-treated pups survived to 2 h despite marked loss of H form is probably because there was still an adequate amount of active phospholipid in the alveoli for lung function at that time.

We have reported that, at delivery, the term neonatal rabbit pup yields an average of 269 ± 52 µg/pup (n = 5) (22). This approximates the amount of H obtained from alveolar lavage of Exosurf- and Survanta-treated pups but is more than that obtained from ARS-treated pups at 1 h; lesser amounts were obtained at 2 h. It approximates the amount of the H form of FRS obtained from alveolar lavage at 2 h. The continuing availability of the biologically active form of FRS in the alveoli at 2 h is consistent with our previous observation of better survival of ventilated 27-day gestation pups treated with FRS compared with ARS, Exosurf, and Survanta (16, 24).

In summary, our data indicate that, compared with natural surfactant obtained from adult rabbit lung (ARS) and two commercially available surfactant preparations...
(Exosurf and Survanta), a natural preparation obtained from term fetal rabbit lung (FRS) provided a biologically active form (H) of surfactant that could be recovered from the airway of the 27-day gestation rabbit pup without significant change up to 2 h after instillation. The other surfactants showed significant loss of the H form at varying times during the first 2 h. Some of the H form after treatment with Exosurf appeared to have been cycled into the LB pool. The longer retention of the active H form of FRS correlated with the longer survival of pups seen in previous studies (16, 24). These data suggest that a surfactant preparation with the unique properties of FRS may be useful in treating the premature human newborn, whose endogenous surfactant pool is slow to activate, and that this preparation merits further study.

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REFERENCES