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Lysis of Red Blood Cells and Alveolar Epithelial Toxicity by Therapeutic Pulmonary Surfactants

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ABSTRACT

The risk of pulmonary hemorrhage is increased in extremely low birth weight infants treated with surfactant. The pathogenesis of this increased risk is far from clear. We tested whether exposure of cell membranes to surfactant may lead to increased membrane permeability, hypothesizing that this process may contribute to the occurrence of alveolar hemorrhage after surfactant treatment. Aliquots of washed packed red blood cells (used as membrane model) were suspended in 0.9% NaCl with various concentrations of Survanta or Exosurf for either 2 or 24 h at 37°C. Cytolysis was measured by spectrophotometric determination of free Hb after centrifugation. Red cells suspended in 0.9% NaCl alone, distilled water, or various concentrations of melittin were used as negative and positive controls. Both surfactants were associated with increased hemolysis to 35% of maximum at concentrations of 1.25 mg/2 mL. Above these concentrations, Survanta was associated with no increase in hemolysis, whereas Exosurf increased hemolysis to 60% of maximum at concentrations of 12.5 mg/2 mL. In additional experiments, primary cultures of alveolar type II cells from adult rats were treated with Survanta, Exosurf, the Exosurf components tyloxapol and hexadecanol, melittin, or culture medium alone. After 24 h of incubation, lactate dehydrogenase release into the media was measured as a percent of total lactate dehydrogenase activity to indicate cytotoxicity. Lactate dehydrogenase release was <10% for control experiments but increased sharply with Exosurf and its components tyloxapol and hexadecanol. These results indicate that surfactant may be associated with *in vitro* cytotoxicity and that this property differs for different surfactants and different dosages. (***Pediatr Res* 37: 26–30, 1995**)

Abbreviations

DMEM, Dulbecco's modified Eagle's medium
DPPC, dipalmitoyl phosphatidylcholine
LDH, lactate dehydrogenase
RBC, red blood cell
RDS, respiratory distress syndrome

In extremely low birth weight infants with RDS, the risk of pulmonary hemorrhage may be increased after intratracheal treatment with surfactant. In the only surfactant study conducted exclusively in infants weighing <700 g at birth (1), the incidence of pulmonary hemorrhage was significantly increased from 2 to 12% ($p = 0.006$) in infants treated with synthetic surfactant (Exosurf Neonatal). Van Houten *et al.* (2) and Long *et al.* (3) reported a much smaller increase in the incidence of pulmonary hemorrhage from 1.0 to 1.9% in preterm infants weighing >700 g at birth treated with synthetic surfactant. Reporting on a multicenter Survanta trial with 798 preterm infants weighing 600–1750 g at birth, Liechty *et al.* (4) described a slight increase in the incidence of pulmonary hemorrhage from 5.4 to 7.1% ($p = 0.319$) in infants treated with natural surfactant. Interestingly, the incidence of pulmonary hemorrhage was significantly higher in both the treated and the control groups of natural surfactant trials (5.9 and 5.4%) than in synthetic surfactant trials (2.5 and 1.0%) (5). Various explanations for the possible increased risk of pulmonary hemorrhage after surfactant treatment in very preterm infants have been proposed. Stress failure in the capillary walls in the lung may have a role in some types of pulmonary diseases, resulting not only in protein leakage, but also in frank hemorrhage into the alveolar spaces (6). Pressure changes in alveolar blood vessels, associated with the sudden increase in aeration produced with surfactant therapy, may produce intraalveolar hemorrhage (7). Retrospective studies looking for the possible association between pulmonary hemorrhage and a bleeding diathesis found no evidence that surfactant treatment increased the incidence of generalized bleeding (8).

The leading clinical belief concerning the pathogenesis of pulmonary hemorrhage after surfactant therapy in extremely low birth weight infants with RDS is increased alveolar ventilation and increased Po_2 in pulmonary arterioles resulting in

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decreased pulmonary vascular resistance and left-to-right shunting across the ductus arteriosus. Increased pulmonary blood flow subsequently results in endothelial and epithelial leakage and hemorrhagic pulmonary edema. Autopsy data on preterm infants dying after surfactant therapy show that surfactant-treated infants are more likely to have extensive intraalveolar hemorrhage with clinically significant pulmonary hemorrhage and that untreated preterm infants have higher incidences of interstitial hemorrhage and lung hematomas and significantly more large interstitial hemorrhages (9). This observation suggests that surfactant therapy may be instrumental in converting interstitial into intraalveolar blood loss or may exert a direct meltdown effect on the alveolar epithelium and endothelium.

In this study, we explored an alternative mechanism for pulmonary hemorrhage in extremely low birth weight infants after surfactant treatment. If surfactant constituents remain in contiguity with cell membranes in sufficient concentrations for long enough periods, membrane leakage or damage may occur, allowing for eventual hemorrhage across capillary alveolar membranes. To test this possibility, we incubated surfactants in current clinical use (Exosurf and Survanta) with RBC or alveolar type II cells and measured cytolysis or cell leakage.

METHODS

Materials. The surfactants used were commercially available Survanta (Ross Laboratories, Columbus, OH) and Exosurf (Burroughs Wellcome Co., Research Triangle Park, NC). Elastase was obtained from Worthington Biochemical Corp. (Freehold, NJ), FCS from Hyclone Laboratories (Logan, UT), DPPC from Avanti Polar Lipids (Birmingham, AL), tissue culture media from Gibco Laboratories (Grand Island, NY), plasticware from Falcon (Oxnard, CA), and pathogen-free rats from Harlan Sprague Dawley (San Diego, CA). Melittin from honey bee venom and DMSO and all other biochemicals (of the highest grade available) were obtained from Sigma Chemical Co. (St. Louis, MO).

Hemolysis. Hemolysis experiments were carried out using out-dated blood from our institution's blood bank with approval of the institutional research committee. All units were hepatitis B virus and human immunodeficiency virus negative. Fresh blood, anticoagulated with EDTA, from laboratory volunteers, was used to check results. Samples were washed in 0.9% unbuffered NaCl five times, and spectrophotometric assessment of supernatants was carried out at wavelength 540 nm (UV-VIS 1201 spectrophotometer, Shimadzu Co., Kyoto, Japan) to determine the presence of free Hb. Readings did not change significantly after the fourth and fifth washings. Aliquots of 30 μL of washed packed RBC were resuspended in 2 mL of 0.9% NaCl; surfactant or DPPC was added in concentrations of 0.125, 0.250, 0.625, 1.25, 2.5, 6.25, 12.5, 18.75, and 25 mg/2 mL; and these suspensions were incubated for 2 or 24 h at 37°C with intermittent mixing. The highest concentration of surfactant or DPPC used in these experiments corresponds with the amount that may be present at the alveolar lung layer after surfactant therapy. RBC suspended in 0.9% NaCl and in distilled water only were used as negative and positive controls, respectively. After the 2- or 24-h incubation period, suspensions were centrifuged at 5000 $\times$ g for 30 min and the supernatants analyzed spectrophotometrically for the presence of free Hb. The two surfactants were suspended according to the supplier's recommendation. Osmolarity of Survanta and Exosurf was measured by freezing point depression and found to be 293 and 152 mosmol/L, respectively (n = 4).

Melittin-induced hemolysis (10) was conducted by adding 1, 2, 5, 10, and 20 μM of this peptide in 2% DMSO/isotonic buffer solution to 30 μL of RBC, followed by incubation at 37°C for 30 min. The suspension was then centrifuged at 5000 $\times$ g for 2 min, and the absorbance of the supernatant was measured at 540 nm. RBC suspended in 2% DMSO alone did not show increased hemolysis.

Alveolar type II cell effects. Alveolar type II cells were harvested from male, 250- to 300-g, pathogen-free rats following the lung lavage, elastase digestion, mechanical dissociation, and rat IgG differential adherence protocol of Dobbs *et al.* (11). This methodology yields 2×10^7 to 3×10^7 alveolar type II cells per rat with >90% viability by trypan blue staining and >90% purity by phosphene 3R fluorescent staining (12). The protocol was approved by the Drew University Animal Care and Use Committee.

After isolation, type II cells were plated on plastic wells at a final density of 1 000 000 cells/2-mL well. The cell culture medium consisted of DMEM supplemented with 10% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2–95% air, and nonadherent cells were removed by washing three times with PBS. Surfactant suspensions or components in DMEM were added, and the plates were returned to the incubator for 24 h. Test solutions consisted of 2, 4, 6, and 8 mg of surfactant (Survanta or Exosurf) or DPPC per 10^6 cells and the two nonlipid components of Exosurf, the spreading agent hexadecanol and the dispersant tyloxapol. Hexadecanol and tyloxapol were tested at concentrations similar to their content in 2, 4, 6, and 8 mg of Exosurf (0.22, 0.44, 0.66, and 0.88 mg of hexadecanol per 10^6 cells and 0.15, 0.30, 0.44, and 0.59 mg of tyloxapol per 10^6 cells). The lowest concentrations of Survanta, Exosurf, hexadecanol, tyloxapol, and DPPC represent alveolar concentrations after surfactant therapy. DMEM and melittin (10, 20, and 30 μg per well) were used as negative and positive controls, respectively. After 24 h of incubation, medium and cells were harvested separately. Medium was harvested for LDH, protein, and DNA assays. Cells were washed three times with PBS and once with trypsin (0.05% wt/vol and 0.2% EDTA in DMEM) and then lysed with 0.1% Triton X-100. The suspension was centrifuged at 4°C at 1000 $\times$ g for 10 min and the supernatant used for LDH, protein, and DNA assays.

Cytotoxicity was estimated by measuring LDH release into the media as a percentage of total cellular LDH activity (LDH-20 kit, Sigma). The sensitivity of the LDH assay is 1 mU/mL.

The amount of protein in the samples was determined by a modification of the Lowry method (13), and the DNA content by a fluorometric method (14).

Statistical methods. All experiments were done in triplicate and replicated four times. Data from various groups are expressed as a mean ± SD of the four means per study. The significance of differences between multiple experimental groups was compared by analysis of variance followed by the Student-Newman-Keuls multiple comparison procedure. Statistical significance was defined as $p < 0.05$.

RESULTS

RBC. Concentration-dependent lysis of RBC suspended with Survanta and Exosurf for 2 or 24 h is shown in Figures 1 and 2. Similar results were found with fresh RBC obtained from laboratory personnel (data not shown). Minimal and maximal OD readings were observed in cells suspended in NaCl and distilled water, respectively. Hemolysis in DPPC samples was minimal and similar to that for NaCl. As shown in Figure 1 at 2 h, the amount of hemolysis present in Exosurf and Survanta samples was not different up to concentrations of 1.250 mg/2 mL ($p > 0.12$), whereas hemolysis was slightly higher for Exosurf at amounts greater than 1.250 mg/2 mL ($p < 0.0016$). This trend was also seen in the 24-h OD readings (Fig. 2), where more hemolysis was seen with Exosurf than Survanta above concentrations of 1.25 mg/2 mL. Both surfactants were associated with approximately 35% of maximal lysis at concentrations up to 1.25 mg/2 mL. Above this concentration Exosurf was associated with greater lysis, with no significant increases in cell lysis associated with Survanta. At a concentration of 12.5 mg/2 mL, Survanta was associated with 33% lysis and Exosurf with 60% lysis.

Maximal lysis of RBC was defined in incubations of RBC with melittin or distilled water and occurred at a concentration of 20 μM melittin in this assay (Fig. 3), with an absorbance of 2.022 at wavelength 540 nm. Figure 3 indicates a dose-dependent increase in hemolysis, with 100% hemolysis occurring at a concentration of 20 μM melittin.

Alveolar type 2 cells. All the control cell LDH activity was recovered when combining activities measured in both medium and cells remaining attached to tissue culture dishes. LDH release was <10% under control conditions, after exposure to various concentrations of DPPC, and after exposure of cells to the maximum Survanta dose (Fig. 4). Exposure of 10^6 cells to 6–8 mg of Exosurf led to a significant increase in LDH release from the cells into the medium compared with Survanta (Fig. 4). Exposure to melittin (10 μg per well) resulted in an almost complete release of cellular LDH. Cells exposed to hexadecanol or tyloxapol at doses corresponding to 6–8 mg of surfactant per 10^6 cells released LDH at a rate exceeding that of Survanta ($p < 0.05$ for hexadecanol and $p < 0.02$ for tyloxapol) but less than that of Exosurf itself ($p < 0.05$ for hexadecanol and $p < 0.05$ for the highest dose of tyloxapol) (Fig. 4).

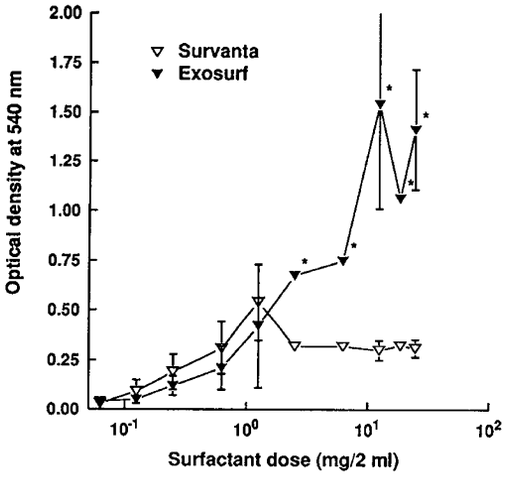

Figure 1. Hemolysis after 2 h of RBC exposure to Survanta or Exosurf. *, $p < 0.05$ *vs* Survanta.

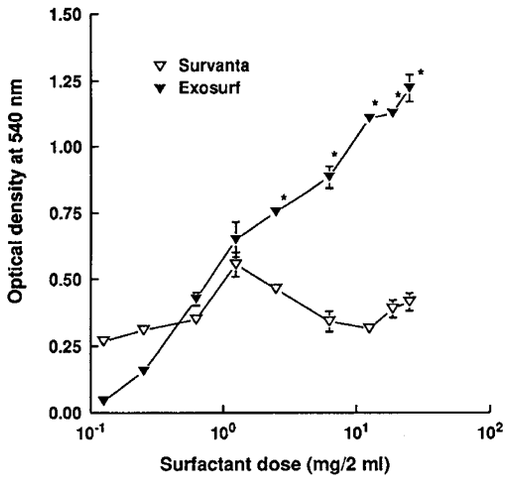

Figure 2. Hemolysis after 24 h of RBC exposure to Survanta or Exosurf. *, $p < 0.05$ *vs* Survanta.

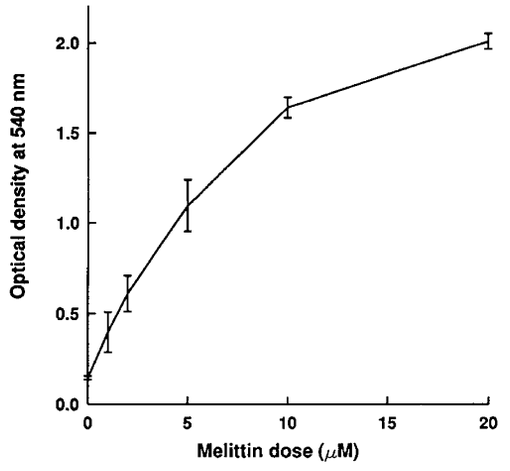

Figure 3. Dose-dependent effects of melittin on RBC hemolysis.

DISCUSSION

The results of this study indicate the possibility that high concentrations of surfactants used in the therapy of RDS may have untoward effects on epithelial cell membranes under some conditions. Cell lysis is indicated by the release of Hb into solution and the decrease in turbidity of the suspensions as indicated by increased OD readings (Fig. 1 and 2). Previous

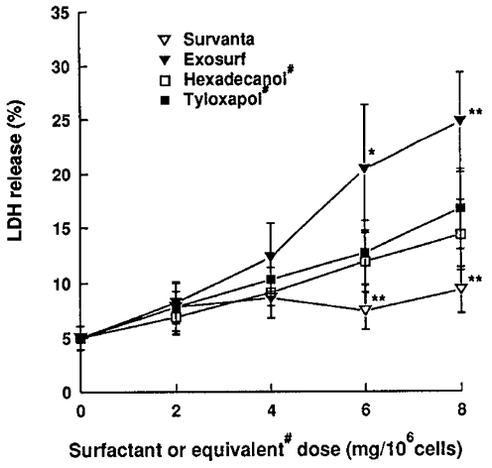

Figure 4. LDH release by alveolar type II cells after 24 h of exposure to Survanta, Exosurf, hexadecanol, and tyloxapol. For hexadecanol and tyloxapol, the values on the *x* axis reflect the amount of Exosurf that contains an equivalent amount of these substances. *, $p < 0.05$ *vs* Survanta and hexadecanol; **, $p < 0.05$ *vs* the three other substances.

work done by others indicates that some surfactants have a toxic effect on cell membranes (15) ranging from decreased cell viability due to loss of solute-barrier properties of the cell membrane (16) to complete cell lysis (17, 18). These observations are surfactant concentration dependent, with low concentrations associated with decreased cell viability and higher concentrations associated with cell lysis. The lytic activities of nonpulmonary surfactants have been noted to be in descending order of activity: cationic, amphoteric, nonionic, and anionic surfactants (19). Tobler *et al.* (20) found that the surfactant concentration needed to cause a given effect depends on the detergent structure rather than on the type of cell being investigated. Our findings indicate a concentration effect of surfactant on cell membrane viability. At the lower concentrations of both Exosurf and Survanta, the lytic effects are 33% of maximum at 1.25 mg/2 mL ($p > 0.12$). Above these concentrations, Survanta is not associated with any significant increase in hemolysis, whereas Exosurf is associated with increased hemolysis up to 60% of maximum at concentrations of 25 mg/2 mL ($p = 0.0001$).

The lytic activity may be explained by the actual composition of Survanta and Exosurf. Exosurf is a purely synthetic surfactant composed of DPPC, hexadecanol, tyloxapol, and NaCl, whereas Survanta is extracted from cow lung with organic solvents and spiked with fatty acids, DPPC, and tripalmitin. The presented data show that DPPC is not lytic. Although we tested the two non-DPPC components of Exosurf for their effect on alveolar type II cells, we did not do so for the multitude of non-DPPC components of Survanta because of the clear differences in toxic effects of these two surfactant preparations. The negative effects of hexadecanol and tyloxapol on alveolar type II cells were found to be between those of Exosurf and Survanta. Their separate effects added up to the total Exosurf effect and may indicate that these additives, added to facilitate adsorption and spreading of DPPC, are responsible for the negative Exosurf effect.

We used both RBC and alveolar type II cells as models to test for surfactant-induced damage to membranes. By inference, these activities may be occurring at the two-cell interface at the level of the alveolar-capillary membrane barrier, with pulmonary hemorrhage the result of disruption of membrane integrity at this interface. An agent that lyses both cell types would, by analogy, be expected to also have cytotoxic effects on endothelial cells. Given the known difficulties in establishing endothelial cell cultures, RBC and alveolar type II cells were chosen as surrogates. The red cell membrane was our primary model for the possible lytic effect of pulmonary surfactants (21). We subsequently used type II cells to test whether lytic activity was specific to RBC. Exposure of type II cells to high doses of exogenous surfactants had an effect similar to that on RBC, leading to an increased LDH release in the case of exposure to Exosurf. Because Exosurf is the most potent in inducing lysis of RBC and type II cells, it would be expected to be similarly cytotoxic for endothelial cells. The mechanism by which protein-free Exosurf achieves this effect may be a generalized, detergent-like action in which the lipid components in Exosurf break down membrane permeability barriers. Given that Survanta is somewhat lytic for RBC, but not type II cells, any extrapolation that Survanta is similarly membrane-damaging for endothelial cells is less certain. A complete understanding of how Survanta induces hemolysis requires a systematic analysis of the lytic activity of the individual lipid and protein components, but such a study is clearly outside the scope of this study.

The connection between *in vitro* membrane lysis at high surfactant concentrations after 2–24 h of incubation and *in vivo* toxicity remains speculative. Alveolar type II cell membrane lysis is negotiable at therapeutic concentrations but increases sharply with increasing Exosurf concentrations, suggesting a toxic effect when supraphysiologic concentrations are reached by administering surfactant into an unevenly expanded or partially collapsed lung. RBC membrane lysis at therapeutic surfactant concentrations is only increased in the case of Exosurf treatment. Surfactant concentration and ionic strength may influence particle size. At higher surfactant concentrations and osmotic pressure, vesicle size would be expected to increase, thus reducing lytic activity, which is more typical of small vesicles.

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