Resistance of Different Surfactant Preparations to Inactivation by Meconium

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ABSTRACT

A disease similar to acute respiratory distress syndrome may occur in neonates after aspiration of meconium. The aim of the study was to compare the inhibitory effects of human meconium on the following surfactant preparations suspended at a concentration of 2.5 mg/mL: Curosurf, Alveofact, Survanta, Exosurf, Pumactant, rabbit natural surfactant from bronchoalveolar lavage, and two synthetic surfactants based on recombinant surfactant protein-C (Venticute) or a leucine/lysine polypeptide. Minimum surface tension, determined with a pulsating bubble surfactometer, was increased >10 mN/m at meconium concentrations ≥0.04 mg/mL for Curosurf, Alveofact, or Survanta, ≥0.32 mg/mL for recombinant surfactant protein-C, ≥1.25 mg/mL for leucine/lysine polypeptide, and ≥20 mg/mL for rabbit natural surfactant. The protein-free synthetic surfactants Exosurf and Pumactant did not reach minimum surface tension <10 mN/m even in the absence of meconium. We conclude that surfactant activity is inhibited by meconium in a dose-dependent manner. Recombinant surfactant protein-C and leucine/lysine polypeptide surfactant were more resistant to inhibition than the modified natural surfactants Curosurf, Alveofact, or Survanta but less resistant than natural lavage surfactant containing surfactant protein-A. We speculate that recombinant hydrophobic surfactant proteins or synthetic analogs of these proteins can be used for the design of new surfactant preparations that are relatively resistant to inactivation and therefore suitable for treatment of acute respiratory distress syndrome. (*Pediatr Res* 50: 44–49, 2001)

Abbreviations
(A)RDS, (acute) respiratory distress syndrome
MAS, meconium aspiration syndrome
SP, surfactant protein
rSP-C, recombinant surfactant protein C
KL_4 SP-B–like peptide
FFA, free fatty acids
DPPC, dipalmitoylphosphatidylcholine
PG, phosphatidylglycerol

Surfactant deficiency has been recognized as the cause of RDS in premature infants, and treatment with modified natural surfactant preparations has considerably improved the prognosis of this disease (1). However, it has been realized that secondary surfactant deficiency caused by inactivation of the surfactant system may occur in patients with mature lungs. In ARDS (acute (adult) RDS), surfactant inhibitors may reach the alveolar space by inhalation or aspiration, and proteins such as albumin or fibrinogen may leak into the airways as a consequence of increased vascular permeability caused by pneumonia, for example (2–4). To treat ARDS in such a patient,

relatively large doses of surfactant need to be instilled to overcome the amount of surfactant inhibitors present in the airways (5). Thus, surfactant preparations used for treatment of ARDS should be relatively resistant to inactivation (6). Recent advances in the synthesis and heterologous expression of lung SPs or their analogs might allow the production of designer surfactants (7) that are highly resistant to surfactant inhibitors. Under ARDS-like conditions, such preparations may be superior to currently available modified natural surfactants.

Aspiration of meconium can result in severe respiratory failure in term neonates (8–10). Surfactant inactivation is believed to play a key role in the pathophysiology of MAS, and inhibition of the surface tension–lowering activity of surfactant by meconium has been demonstrated *in vitro* (11, 12). In addition, *in utero* events, such as hypoxia, that trigger meconium passage into the amniotic fluid may interfere with surfactant synthesis and secretion by alveolar type II cells (9). In MAS, an early phase of airway obstruction and surfactant

dysfunction is observed, which is followed by a second phase with a marked inflammatory response in the airways (13). The liberation of cytokines, oxygen radicals, and enzymes (*e.g.* neutrophil elastase) from phagocytic cells may enhance surfactant inactivation. Apart from its biophysical effects at the air–liquid interface, surfactant has important immunomodulatory functions (14). The surfactant film is also important for fluid homeostasis and mucociliary clearance of aspirated particles. Both these mechanisms may be disturbed in MAS.

Animal models (15, 16) and early clinical studies (17–21) demonstrate that lung function and oxygenation in MAS can be improved by instillation of large doses of modified natural surfactants containing phospholipids and SP-B and SP-C.

The aim of the present study was to compare the relative resistance of different surfactant preparations to inactivation by meconium. Three modified natural surfactants (Curosurf, Alveofact, and Survanta) in clinical use in Europe and the United States, two synthetic protein-free surfactants (Exosurf and Pumactant), and two recently described surfactants based on rSP-C or KL$_4$ surfactant were incubated with increasing concentrations of meconium. Minimum surface tension was determined with a pulsating bubble surfactometer (22). Complete natural surfactant containing both the hydrophobic proteins SP-B and SP-C and the hydrophilic proteins SP-A and SP-D was used for comparative studies.

METHODS

Meconium

The first meconium of healthy term newborn infants was collected with parental consent. The project was approved by the institutional review board of the Medical University of Göttingen.

Samples from 16 infants were pooled [entry criteria: gestational age, 37–41 wk; birth weight, between 3rd and 97th percentiles; no signs of maternal infection; clear amniotic fluid; no signs of asphyxia (umbilical cord pH >7.25); no bacterial contamination of meconium (<10^4 bacteria/mL)]. The water content of the original meconium pool was 72%. The material was lyophilized and stored at $-70°C$ until use. The dry meconium was resuspended under sonication in saline 0.9% with 1.5 mM CaCl$_2$ (23) to reach meconium concentrations from 0.04 to 40 mg/mL. As equal parts of surfactant preparations and meconium suspensions were mixed, the final meconium concentration varied from 0.02 to 20 mg/mL. As up to 200 g of meconium may be passed by a full-term neonate at birth (9) and the volume of amniotic fluid at birth rarely exceeds 2000 mL, meconium concentrations >20 mg/mL can certainly be reached even assuming uniform distribution.

Surfactant

Natural surfactant. Natural surfactant was obtained by bronchoalveolar lavage of adult rabbit lungs. The surface-active material was harvested by sucrose gradient centrifugation (12). As it is not exposed to treatment with organic solvents, complete natural lavage surfactant contains up to 10% of proteins, including SP-A, SP-B, SP-C, and SP-D.

SP-A represents approximately 50% (by weight) of the total protein (24).

Modified natural surfactant preparations. Curosurf (batch no. 96/0052, Chiesi Farmaceutici, Parma, Italy) is produced from minced pig lungs and consists of 99% phospholipids and 1% hydrophobic surfactant proteins (SP-B and SP-C). Alveofact (SF-RI 1, July 1997, Dr. Karl Thomae Ltd, Biberach, Germany), a compound obtained from bovine lung lavage, is composed of 90% phospholipids, approximately 1% hydrophobic proteins, 3% cholesterol, 0.5% FFA, and other components such as triglycerides. Survanta (batch no. 95–896Z7, Abbott Ltd, Wiesbaden, Germany) is prepared by lipid extraction of minced bovine lungs and contains approximately 84% phospholipids, 1% hydrophobic proteins, and 6% FFA. To this preparation DPPC mixed with palmitic acid and tripalmitin are added to standardize the composition.

Protein-free synthetic surfactant preparations. Exosurf (batch no. T4798A, Wellcome, Burgwedel, Germany), a synthetic, protein-free surfactant, is composed of approximately 85% DPPC mixed with cetyl alcohol and tyloxapol. Pumactant [ALEC (artificial lung-expanding compound), batch no. 82, Britannia Pharmaceuticals, Redhill, Surrey, U.K.] is an artificial, protein-free compound composed of a mixture of DPPC and PG at a weight ratio of 7:3.

Synthetic surfactant preparations containing peptides/recombinant proteins

KL$_4$, a 21-residue leucine/lysine peptide (Sinapultide), was synthesized by one of us (J.J.) and combined with DPPC and PG at a weight ratio of 3:1 and palmitic acid (15%) in an isotonic aqueous suspension. The KL$_4$ peptide, added to the lipids at a concentration of 3%, mimics the alternating hydrophobic and hydrophilic pattern of SP-B (25, 26). KL$_4$ has been tested *in vitro*, in animal experiments, and in clinical trials for treatment of RDS, MAS, and ARDS (27, 28). Larger trials with a commercial product (Surfaxin, Discovery Laboratories, Doylestown, PA, U.S.A.) are under way.

rSP-C surfactant (kind gift from D. Häfner, Byk Gulden, Konstanz, Germany) is prepared as an amorphous dry powder and is resuspended in 0.9% NaCl. The final suspension contains (by weight) 1.8% rSP-C, 63% DPPC, 28% PG, 4.5% palmitic acid, and 2.5% CaCl$_2$. *In vitro* studies and animal experiments indicate excellent biophysical function (29). A clinical study enrolling ARDS patients in Europe, South Africa, the United States, and Canada (30) has recently been started with a commercial product (Venticute, Byln Gulden, Kinslum, Germany). Figure 1 demonstrates the relative differences in composition of the commercially available modified natural surfactant preparations (Alveofact, Curosurf, and Survanta) in comparison with KL$_4$, rSP-C, and natural rabbit surfactant (31, 32).

Surface tension measurements

The influence of meconium on the surface activity of the different surfactant preparations was tested in a pulsating bubble surfactometer (Electronetics Corporation, Buffalo, NY, U.S.A.). In all samples the concentration of surfactant was 2.5
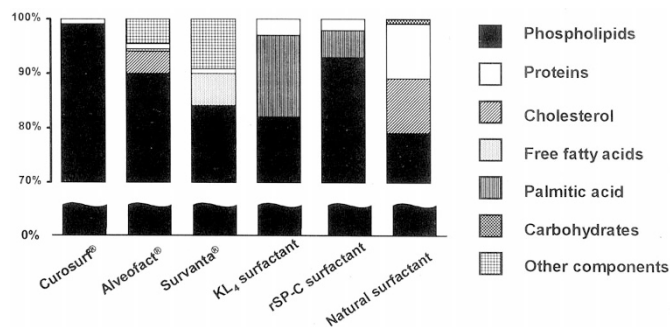
Figure 1. Composition of different surfactant preparations. Modified from Wauer (31) and Seeger *et al.* (32). All preparations contain >70% phospholipids.

mg/mL, which is close to the estimated critical concentration of surfactant in fetal lung liquid at birth (33). Measurements were made at 37°C during 50% cyclic area compression at a rate of 20 per minute. Surface tension at minimum bubble size (γ_{min}) was recorded after 5 min of pulsation. Meconium at concentrations ranging from 0.02 to 20 mg/mL was incubated with the surfactant at 37°C for 30 min under gentle agitation before analysis.

pH values have been shown to influence minimum surface tension of surfactant (34). pH values of the meconium and surfactant samples at 2.5 mg/mL ranged from 6.0 (KL_4) to 6.7 (rSP-C), with intermediate values for all other surfactants. According to Amirkhanian and Merritt (34), the "pH-range 5.0–7.4 is considered the optimal range for minimum surface tension of surfactants used for *in vitro* surface tension measurements when evaluating inhibitors of surfactant function."

Statistical analysis

All data represent the mean ±SD of six repeated experiments. Statistical differences were evaluated by ANOVA and Dunnett's multiple comparison test using GraphPad software (Graph Pad Software Inc., San Diego, CA, U.S.A.). Statistical significance was accepted at $p < 0.05$.

RESULTS

Meconium had a dose-dependent inhibitory effect on all examined surfactant preparations. Minimum surface tension was increased >10 mN/m at meconium concentrations >0.04 mg/mL for Curosurf, Alveofact, and Survanta. Survanta seemed to be slightly more resistant to inhibition than Curosurf or Alveofact, but these differences did not reach statistical significance (Fig. 2).

KL_4 and rSP-C surfactant were more resistant to the inhibitory effects of meconium. An increase of $\gamma_{min} > 10$ mN/m was observed for rSP-C surfactant at concentrations >0.16 mg/mL and for KL_4 at meconium concentrations >0.64 mg/mL (Fig. 3).

Rabbit natural surfactant prepared by lavage and sucrose gradient centrifugation, containing all SPs including approximately 5% of SP-A, was far more resistant to inhibition than all other surfactant preparations tested. Addition of 20 mg/mL meconium was necessary to induce a significant increase in
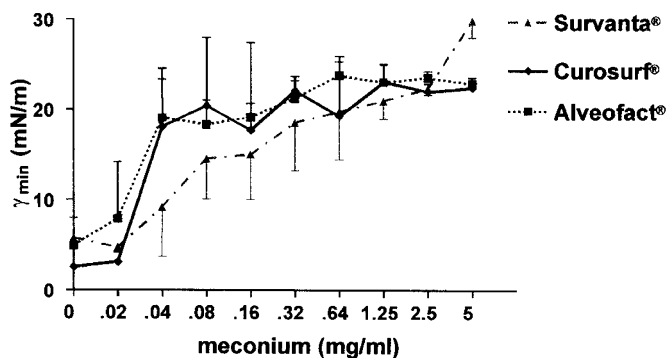

Figure 2. Minimum surface tension (γ_{min}) of three modified natural surfactant preparations (phospholipid concentration, 2.5 mg/mL) exposed to increasing concentrations of human meconium. Values are mean ±SD from six repeated experiments. A significant increase in γ_{min} ($p < 0.01$ *vs* surfactant without meconium, *i.e.* 0 mg/mL) occurred at meconium concentrations ≥0.02 mg/mL for Alveofact and at ≥0.04 mg/mL for Curosurf and Survanta.
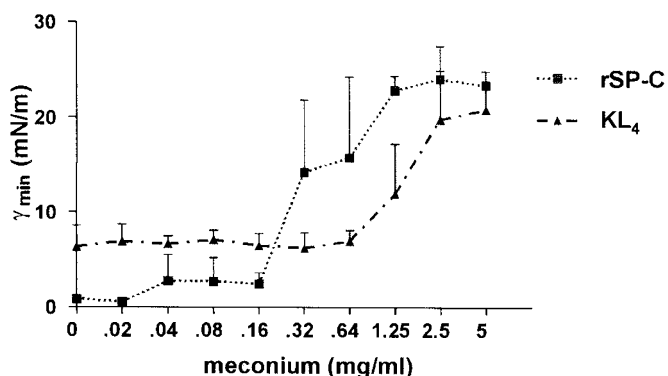

Figure 3. Minimum surface tension (γ_{min}) of KL_4 and rSP-C surfactant (phospholipid concentration, 2.5 mg/mL) exposed to increasing concentrations of human meconium. Values are mean ±SD from six repeated experiments. A significant increase in γ_{min} ($p < 0.01$ *vs* surfactant without meconium, *i.e.* 0 mg/mL) occurred at meconium concentrations ≥0.32 mg/mL for rSP-C surfactant and at ≥1.25 mg/mL for KL_4 surfactant.

minimum surface tension. Figure 4 illustrates the differences in the capacity to resist inactivation by increasing meconium concentrations for modified natural surfactant (Curosurf) containing the hydrophobic surfactant proteins SP-B and SP-C, the two synthetic preparations containing KL_4 or rSP-C, and a complete natural surfactant, containing all surfactant proteins including the hydrophilic SP-A and SP-D.

The inhibition observed by meconium was reversible. For these studies we incubated surfactant (2.5 mg/mL) with meconium at a concentration of 2.5 mg/mL, a ratio that increased minimum surface tension to ≥20 mN/m for all protein- and peptide-containing surfactants (Fig. 4). When an identical volume of surfactant at a concentration of 7.5 mg/mL (*i.e.* final total surfactant concentration, 5 mg/mL) was added to the inhibited samples, mean minimum surface tension was reduced again to 2.8 ± 3.1 mN/m for Curosurf, 4.6 ± 5.3 mN/m for Alveofact, 12.4 ± 2.3 mN/m for Survanta, 10.5 ± 5.0 mN/m for KL_4, and 1.6 ± 1.0 mN/m for rSP-C surfactant.

The protein-free synthetic surfactants Exosurf and Pumactant did not reach $\gamma_{min} < 10$ mN/m even in the absence of meconium, so that further studies with increasing meconium concentrations were not performed.
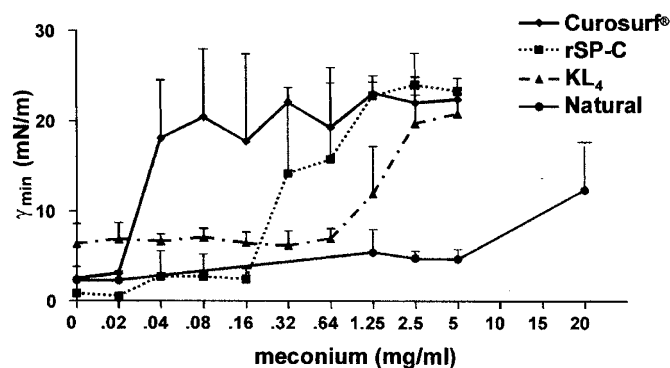
Figure 4. Minimum surface tension (γ_{min}) of different surfactant preparations (phospholipid concentration, 2.5 mg/mL) exposed to increasing concentrations of human meconium. Curosurf, a modified porcine surfactant, and the new synthetic preparations, KL$_4$ and rSP-C surfactant, were less resistant to inactivation than complete natural rabbit surfactant obtained by bronchoalveolar lavage. Values are mean ±SD from six repeated experiments. An increase in γ_{min} >10 mN/m was noted for Curosurf at meconium concentrations ≥0.04 mg/mL, for rSP-C surfactant ≥0.32 mg/mL, and for KL$_4$ surfactant at ≥1.25 mg/mL, whereas 20 mg/mL of meconium was required to inhibit the natural surfactant.

DISCUSSION

At a phospholipid concentration of 2.5 mg/mL, all surfactants containing proteins or peptides reduced minimum surface tension to <10 mN/m in the absence of meconium. Meconium is a potent inhibitor as an increase in minimum surface tension >10mN/m was observed for all modified natural surfactant preparations already at meconium concentrations between 0.02 and 0.04 mg/mL, *i.e.* at a ratio of about 1:100 between meconium and surfactant. The concentration of 2.5 mg/mL makes surfactant more sensitive to inactivation than concentrations ≥5 mg/mL that were used by other investigators (12). The inactivation of surfactant is reversible, as we could demonstrate that addition of extra surfactant to the samples inhibited by meconium restored minimum surface tension to values approximately ≤10 mN/m. Thus the differences observed in sensitivity to inactivation in our study may be counteracted to a large extent by modification of the dose of the individual surfactant preparation used. However, it has to be noted that low surface tension *in vitro* does not necessarily reflect good *in vivo* activity.

With the protein-free preparations Exosurf and Pumactant, such low values in surface tension were not achieved in the absence of meconium even when the phospholipid concentration was increased to 10 mg/mL. The slow reduction in oxygen demand observed in neonatal RDS after treatment with Exosurf (35) or Pumactant suggests that the exogenous synthetic phospholipids may need to be activated by entering the recycling machinery of the alveolar type II cells to exert a therapeutic effect in the airspaces (36). In clinical trials of RDS and ARDS, Exosurf and Pumactant demonstrated relatively little effects compared with natural modified surfactants (35, 37, 38).

The exact mechanisms by which meconium interferes with the surfactant layer at the air–liquid interface are as yet unknown. Using electron microscopy, Bae *et al.* (39) noticed that incubation of Survanta with meconium resulted in a transformation from loosely stacked layers, representing active surfactant, to lamellar or folded linear structures representing inactivated material. These findings are reminiscent of the conversion of the highly surface-active subtype of large surfactant aggregates to small aggregates with poor surface activity observed in adults with ARDS (40). Meconium is a complex mixture containing proteins, cell debris, bile acids, Hb, and bilirubin metabolites, for example (9). All of these components are individually capable of inhibiting surfactant function (4). Both the chloroform-soluble hydrophobic components and the water-soluble components of meconium can interfere with the biophysical activity of surfactant, but the specific inhibitory activity is higher for the chloroform-soluble fraction (12). Probably the incubation of individual components of meconium with surfactant would follow a similar pattern of resistance to inhibition.

Our *in vitro* results are in keeping with the concept that relatively high surfactant doses are needed for treatment of secondary surfactant deficiency in MAS. Possibly, the presence of a large amount of inhibitors in the alveolar space needs to be overcome by the exogenous surfactant before an effect on lung function and oxygenation can be observed. In a controlled randomized trial using Survanta for early treatment (<6 h) of MAS, no acute effects on oxygenation were observed after a dose of 150 mg/kg body weight. When a second dose of 150 mg/kg body weight was instilled 6 h later, there was a significant drop in oxygen demand, and further improvement was seen after administration of a third dose (21). In adults with ARDS caused by sepsis or pneumonia, a dose of 300 mg/kg body weight of Alveofact was reported to improve gas exchange (5).

For treatment of ARDS, it might be useful to remove surfactant inhibitors from the bronchoalveolar space by lavage with diluted surfactant suspensions. A pilot study has reported the successful use of this approach in neonates with MAS (41). In adults with ARDS, segmental lavage or surfactant instillation by means of a bronchoscope seems feasible (42). Preferably such techniques should be used early in the course of the disease before severe ventilator-induced lung injury has occurred (43, 44).

Our present findings together with data reported by others (45) indicate that peptide-containing synthetic surfactant preparations are more resistant to inhibitors than the modified natural surfactants currently used in clinical practice. Recent trials of rSP-C surfactant for treatment of ARDS in adult patients show that improvement in gas exchange can be achieved with doses ranging from 100 to 200 mg/kg body weight (30), which is in keeping with our *in vitro* results with KL$_4$ and rSP-C surfactant. However, larger trials are needed to find out whether the improvement in gas exchange is associated with a decrease in mortality. SPs are important factors increasing the resistance of surfactant preparations to inactivation. SP-B and SP-C play a key role for adequate surfactant function especially for rapid spreading and adsorption of the surfactant film (24). Our understanding has been recently enlarged by studies on humans and animals deficient in SPs (46–49). At the moment it seems that either SP-B or SP-C should be present in surfactant preparations to obtain *in vivo*

efficacy. Interestingly, Mbagwu *et al.* (50) observed that the combination of Survanta and two synthetic peptides (B1–78 and C1–35) led to a higher degree of resistance to surfactant inactivation by human albumin in ventilated preterm rabbits. Mizuno *et al.* (51) reported improved *in vivo* function of Survanta after SP-B supplementation. Consequently, protein content and composition of surfactant preparations may be critical issues in the design of an artificial surfactant for treatment of ARDS (6, 7).

Although KL$_4$ and rSP-C were less sensitive to surfactant inhibition than all other commercially available surfactant preparations, natural surfactant containing all SPs including SP-A and SP-D was even more resistant to loss of biophysical activity by exposure to increasing meconium concentrations. The role of SP-A in the resistance to surfactant inhibitors has been demonstrated in previous studies (52). Sun *et al.* (53) reported that in a rat model of MAS, natural porcine surfactant containing 5% SP-A was more effective than the same dose of Curosurf, which contains only polar lipids, SP-B, and SP-C. Addition of SP-A to Curosurf resulted in improved biophysical properties *in vitro* and better physiologic effects in a rabbit RDS model (53). Recently, the possibility that the carbohydrate components of SP-A might be important for resistance to inactivation led Taeusch *et al.* (54) to explore the effects of simple sugars, sugar polymers, and other nonionic polymers on surfactant inactivation by meconium. Interestingly, dextran and polyethylene glycol reversed the inactivation of Survanta by meconium. Similar data were recently reported by Tashiro *et al.* (55) for Curosurf fortified with dextran. Consequently, it might be possible to enhance the resistance of surfactant preparations by adding other components than naturally occurring or recombinant SPs. Differences in lipid composition, illustrated in Figure 1, also need to be considered in this context. For example, significant amounts of palmitic acid are added to Survanta and KL$_4$ surfactant. Further systematic studies are required to clarify how variations in the content of palmitic acid and other lipid constituents influence resistance to inactivation by meconium and plasma proteins. In the present study differences among modified natural surfactants used for treatment of neonatal RDS today were moderate and might only indicate the need for different dosage regimens for preparations of different origin.

We conclude that meconium is a potent inhibitor of all investigated surfactant preparations. The artificial rSP-C and KL$_4$ surfactants were more resistant to inhibition than the modified natural surfactants Curosurf, Alveofact, or Survanta, which are currently in clinical use. However, natural surfactant containing SP-A was still more resistant to inactivation. Recombinant proteins or synthetic analogs of these proteins can probably be used for making surfactants that are relatively resistant to inactivation and therefore suitable for treatment of ARDS and related conditions, including MAS.

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