Influence of Modified Natural or Synthetic Surfactant Preparations on Growth of Bacteria Causing Infections in the Neonatal Period

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Connatal bacterial pneumonia is common in neonates. Animal studies and initial clinical reports indicate that surfactant dysfunction is involved in the pathophysiology of severe neonatal pneumonia. Since respiratory distress syndrome and connatal pneumonia may be difficult to differentiate in the first hours of life, neonates with respiratory failure due to bacterial infections might receive surfactant. Under such conditions surfactant components might be catabolized by bacteria and promote bacterial growth. We therefore investigated the influence of three modified natural (Curosurf, Alveofact, and Survanta) and two synthetic (Exosurf and Pumactant) surfactant preparations on the growth of bacteria frequently cultured from blood or tracheal aspirate fluid in the first days of life. Group B streptococci (GBS), Staphylococcus aureus, and Escherichia coli were incubated in a nutrient-free medium (normal saline) for 5 h at 37°C, together with different surfactants at concentrations of 0, 1, 10, and 20 mg/ml. With the exception of E. coli, incubation in saline alone led to a variable decrease in CFU. In the presence of Alveofact, Exosurf, and Pumactant the decline in bacterial numbers was less marked than in saline alone. Curosurf was bactericidal in a dose-dependent fashion for GBS and had a strong negative impact on the growth of a GBS subtype that lacked the polysaccharide capsule. In contrast, Survanta (10 and 20 mg/ml) significantly promoted the growth of E. coli, indicating that surfactant components may actually serve as nutrients. We conclude that bacterial growth in different surfactant preparations is influenced by microbial species and the composition and dose of the surfactant. Further studies are necessary to elucidate the mechanisms behind our findings and to evaluate the effects of surfactant on bacterial growth in vivo.

Pulmonary surfactant is a complex mixture of phospholipids and specific proteins that line the alveolar surface of the lung. Its major function is to reduce surface tension, thereby protecting the alveoli against collapse at the end of expiration (8, 11, 39). In addition, the role of surfactant in host defense against inhaled bacteria has been recognized over the last several years (for a review, see reference 38).

Since the observation that surfactant deficiency in lungs of premature newborns is responsible for the respiratory distress syndrome (RDS), exogenous surfactant therapy has become an established treatment of RDS (17). However, surfactant dysfunction has been described in other pulmonary diseases. Surfactant inactivation probably plays a key role in the pathophysiology of acute RDS due to pneumonia in neonates, infants, and adults (32). The infection provokes an influx of inflammatory cells with a resulting release of cytokines, enzymes, and reactive oxygen metabolites. Disruption of the epithelial-endothelial barrier leads to leakage of plasma proteins into the airspaces with consequent inhibition of surfactant function. The detrimental effects of plasma proteins on surfactant function can be overcome in vitro by increasing the surfactant concentration (7, 15).

Group B streptococci (GBS), Staphylococcus aureus, and Escherichia coli are responsible for most cases of early-onset infections in the neonatal period (1, 29). Neonates with severe respiratory failure due to pneumonia caused by these organisms may therefore receive exogenous surfactant. It has been speculated that surfactant given under such circumstances might serve as a nutrient for bacteria and thereby promote microbial growth (31). Only a few reports, with divergent results, have been published concerning the direct influence of surfactant on bacterial growth (3, 16, 19, 23). Either these studies evaluated the effects of low phospholipid concentrations (<1 mg/ml) or the authors did not specify the phospholipid concentration of the surfactant material as crude extracts of bronchoalveolar lavage fluid from animal sources were used. Only two studies have analyzed bacterial growth in the presence of commercially available surfactant preparations currently used for replacement therapy in newborns. Sherman et al. (31) reported that complete natural surfactant derived from human amniotic fluid or natural sheep bronchial lavage fluid promoted the growth of GBS, whereas Exosurf, a synthetic surfactant containing two alcohols as spreading agents, was bactericidal. Intermediate effects were observed for modified natural surfactants derived from bovine, porcine, or calf lungs. Neumeister et al. (27) observed that the modified bovine surfactant Survanta significantly promoted the growth of E. coli. However, these observations were either limited to one bacterial strain (31) and/or one phospholipid concentration (27, 31).

The purpose of this study was to examine the effects of different phospholipid concentrations of three modified natural (Curosurf, Alveofact, and Survanta) and two synthetic (Exosurf and Pumactant) surfactant preparations on the in vitro proliferation of GBS, S. aureus, and E. coli.
in milligrams per milliliter): peptone, 15.0; sodium chloride, 6.0; yeast extract, I; Merck, Darmstadt, Germany) composed of the following (concentrations are 8

with sterile isotonic saline, and resuspended in saline at a final concentration of 8

is more commonly observed in GBS-infected adults. The GBS LD subpopulation

serotypes Ia, Ib, and III account for most neonatal infections, whereas subtype II

field serotypes Ib and III) isolated from two neonates with GBS septicemia. The

University of Umeå, Umeå, Sweden, and was originally isolated from a neonate

GBS strain 090 Ia Colindale. The strain was a gift from Stellan Håkansson,

low-density and a high-density phase variant (LD and HD, respectively) from the

bacteria were harvested by centrifugation at 1,800

with fresh prewarmed broth and incubated for 3 h at 37°C. Subsequently, the

bacteria to the mid-logarithmic phase. For this, the starter culture was diluted 1:7

broth and cultured for 16 h at 37°C. Working cultures were made by growing the

proteins

and neutral lipids

Free fatty acids

DPPC, neutral lipids are added

Other components

<table>
<thead>
<tr>
<th>Component</th>
<th>Alveofact</th>
<th>Curosurf</th>
<th>Survanta</th>
<th>Exosurf</th>
<th>Pumactan</th>
<th>Natural surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids$^b$</td>
<td>88</td>
<td>99</td>
<td>84</td>
<td>82</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td>PC</td>
<td>72</td>
<td>78</td>
<td>62</td>
<td>82</td>
<td>70</td>
<td>63</td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>PG</td>
<td>8</td>
<td>3.5</td>
<td>2.5</td>
<td>0</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Cholesterol and neutral lipids</td>
<td>4</td>
<td>0</td>
<td>Not stated</td>
<td>0</td>
<td>0</td>
<td>5–10</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>Proteins</td>
<td>1.5 (only SP-B and SP-C)</td>
<td>1 (only SP-B and SP-C)</td>
<td>0.5–1 (only SP-B and SP-C)</td>
<td>0</td>
<td>0</td>
<td>5–10 (SP-A, SP-B, SP-C, SP-D)</td>
</tr>
<tr>
<td>Other components</td>
<td>Not stated$^c$</td>
<td>Not stated$^c$</td>
<td>DPPC, neutral lipids are added$^d$</td>
<td>11 (cetyl-alcohol), 7 (tyloxapol)$^e$</td>
<td>None</td>
<td>1 (carbohydrates, antioxidants, anionic peptides, etc.)</td>
</tr>
</tbody>
</table>

$^a$ Values are modified from references 30 and 37 and from product information. Source: Alveofact, bovine lung lavage; Curosurf, porcine lung homogenate; Survanta, bovine lung homogenate; Exosurf, synthetic; Pumactan, synthetic; natural surfactant, human amniotic fluid; SP, surfactant protein.

$^b$ PC, phosphatidylcholine; PG, phosphatidylglycerol.

$^c$ All preparations contain various amounts of sodium chloride and/or other stabilizing agents (e.g., sodium hydrogen carbonate).

MATERIALS AND METHODS

Bacterial strains. By repeated gradient centrifugation (9), we produced a low-density and a high-density phase variant (LD and HD, respectively) from the GBS strain 090 Ia Colindale. The strain was a gift from Stellan Häkkanson, University of Umeå, Umeå, Sweden, and was originally isolated from a neonate with early-onset sepsis. We also studied two GBS wild-type strains (Lancefield serotypes Ib and III) isolated from two neonates with GBS sepsisemia. The serotypes Ia, Ib, and III account for most neonatal infections, whereas subtype II

field serotypes Ib and III) isolated from two neonates with GBS septicemia. The

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bacteria to the mid-logarithmic phase. For this, the starter culture was diluted 1:7

broth and cultured for 16 h at 37°C. Working cultures were made by growing the

bacteria to the mid-logarithmic phase. For this, the starter culture was diluted 1:7

with fresh prewarmed broth and incubated for 3 h at 37°C. Subsequently, the

bacteria were harvested by centrifugation at 1,800 × g for 10 min, washed twice with sterile isotonic saline, and resuspended in saline at a final concentration of 8 × 10^8 CFU/ml. Bacterial suspensions were adjusted to the desired concentrations by determination of the optical density at 595 nm (Ultrospec III; Pharmacia

Materials and Methods

Growth and/or survival was measured in sterile saline in an electrolyte solution (20) mimicking alveolar fluid electrolyte content (sodium, 135 mmol/liter; chloride, 103 mmol/liter; potassium, 7.3 mmol/liter; calcium, 3.2 mmol/liter) and in a nutrient-rich medium (Standard 1 nutrient broth) containing 1, 10 or 20 mg of Curosurf per ml or no surfactant. Values are the mean CFU counts from four experiments.

<table>
<thead>
<tr>
<th>Medium (mg/ml)</th>
<th>Mean CFU/ml (10^9) ± SD at time (h):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Electrolyte solution</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Nutrient-rich medium</td>
<td>6.4 ± 0.6 (Without surfactant)</td>
</tr>
<tr>
<td>With Curosurf (1)</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>With Curosurf (10)</td>
<td>6.1 ± 1.6</td>
</tr>
<tr>
<td>With Curosurf (20)</td>
<td>6.0 ± 0.8</td>
</tr>
</tbody>
</table>

$^d$ Growth and/or survival was measured in sterile saline in an electrolyte solution (28) mimicking alveolar fluid electrolyte content (sodium, 135 mmol/liter; chloride, 103 mmol/liter; potassium, 7.3 mmol/liter; calcium, 3.2 mmol/liter) and in a nutrient-rich medium (Standard 1 nutrient broth) containing 1, 10 or 20 mg of Curosurf per ml or no surfactant. Values are the mean CFU counts from four experiments.
analysis of variance using GraphPad software (GraphPad Software, Inc., San Diego, Calif.). Statistical significance was accepted at $P$ values of $<0.05$.

RESULTS

Effects of modified natural surfactants on bacterial survival.

(i) Curosurf. Curosurf in normal saline reduced the growth of both GBS with (LD) or without (HD) polysaccharide capsule (Fig. 1A). The number of viable HD GBS after 5 h of incubation was significantly decreased at Curosurf concentrations of 10 and 20 mg/ml compared to saline controls without Curosurf. No such decrease in bacterial numbers was observed for S. aureus or E. coli. Compared to S. aureus incubated in saline, the numbers of CFU per milliliter were slightly higher in solutions containing surfactant (Fig. 1A), but there was no increase in bacterial numbers compared to the number of CFU inoculated into the medium at the beginning of the experiments (0 h).

(ii) Alveofact. In the presence of Alveofact, all gram-positive bacteria demonstrated increased viability compared to GBS HD, GBS LD, or S. aureus incubated in saline alone (Fig. 1B). In contrast to the findings with Curosurf, no inhibitory effect was observed on the growth of both phase variants of GBS. No differences were observed between the effects of Curosurf and Alveofact when the surfactants were incubated with S. aureus. The survival of E. coli was not influenced by Alveofact (Fig. 1B).

(iii) Survanta. The addition of different concentrations of Survanta to GBS LD did not alter bacterial survival over the 5-h period, whereas Survanta significantly promoted the growth of E. coli. At phospholipid doses of $\geq 10$ mg/ml, the numbers of CFU per milliliter were increased 4.5 times compared to the bacterial count at the beginning of the experiments (0 h).

Effects of synthetic surfactants on bacterial survival: Exosurf and Pumactant. Incubation of bacteria in Exosurf (Fig. 1D) or Pumactant (Fig. 1E) resulted in a similar growth pattern. No effects were observed on GBS LD and E. coli. However, compared to the incubation in saline alone, both surfactants protected GBS HD and S. aureus from the negative impacts of incubation on cell viability in a nutrient-free medium.

Bacterial survival of different GBS subtypes in the presence of Curosurf. Figure 2 shows the survival of different GBS subtypes incubated in saline or in saline containing I, 10, or 20 mg of Curosurf per ml. The nonencapsulated HD variant of GBS was most sensitive to the negative impact of surfactant on survival. At Curosurf concentrations of 10 or 20 mg/ml, $<1\%$ of the initial number of bacteria were viable after 5 h of incubation. In contrast, the abundantly encapsulated strain GBS LD survived 5 h of incubation in saline without a significant decrease in the number of CFU. The effects observed following incubation of GBS LD with 20 mg of Curosurf per ml were rather moderate compared to incubation of GBS HD and the GBS wild-type strains with the same surfactant dose.

DISCUSSION

We evaluated bacterial proliferation under the influence of different natural and synthetic surfactant preparations that are in clinical use in Europe and/or the United States. For our studies we chose GBS, S. aureus, and E. coli since more than 75% of cases of early-onset neonatal septicemia are caused by these organisms. Connal infections often trigger premature birth and might be followed by respiratory failure within the first hours of life. Studies of surfactant treatment in infants with “idiopathic” RDS reveal that up to 20% of surfactant-treated neonates demonstrate signs of infection within the first days of life (for a review, see reference 12). All of the surfactant preparations investigated in this study have therefore been used in newborns with respiratory failure due to pneumonia.

Recommended doses for surfactant replacement therapy vary between 50 and 200 mg/kg of body weight for the initial treatment of babies with RDS. It has been recognized that doses of 300 mg of surfactant per kg (body weight) may be needed to overcome surfactant inhibition in pneumonia (35) or meconium aspiration syndrome (6). Such high doses would, even in babies devoid of endogenous surfactant, probably result in phospholipid concentrations well above 10 mg/ml in the alveolar lining layer, at least after resorption of fetal lung liquid (21). We speculate, on the basis of our present findings, that the surfactant layer on the alveolar surface might be an important part of the pulmonary defense system.

We found that Curosurf inhibited survival of GBS in saline in a dose-related fashion. For S. aureus, incubation in saline alone had a bactericidal effect. When Curosurf was added S. aureus was protected to some extent against the negative impact of the nutrient-free medium on microbial viability. In contrast, the viability of E. coli was unaffected by Curosurf, as well as by Alveofact, Exosurf, and Pumactant. These results probably reflect variations in the metabolic demands of different bacteria. In our in vitro assay system the bacteria were incubated in sterile saline, a nutrient-free medium. However, our own results of incubation of GBS and Curosurf in a culture medium containing glucose and protein (Table 2), as well as similar studies by Neumeister et al. (27), demonstrated that incubation of bacteria in nutrient-rich growth-promoting broth might mask the effects of surfactant that were observed when saline alone was used as a medium. Under normal physiological circumstances the alveolar lining fluid may be considered to be relatively poor in nutrient content. However, in the course of pneumonia and mechanical ventilation, serum components, including albumin and glucose, may leak into the bronchoalveolar space and increase the amount of nutrients available for bacterial proliferation.

Both the nonencapsulated GBS HD and S. aureus showed a slight decline in CFU during the 5-h incubation in sterile saline alone. GBS HD, the nonencapsulated phase variant, demonstrated a strong decline in viability when incubated with Curosurf, whereas GBS LD was clearly less susceptible under similar conditions, probably protected by the polysaccharide capsule. The capsule is an important virulence factor in GBS infections, and wild-type strains often contain a mixture of both encapsulated and nonencapsulated bacteria (9). These findings demonstrate that different subtypes of one bacterial species might differ in their interactions with surfactant. When we tested different clinical isolates from infants with GBS septicaemia, the observed variation was small compared to the differences between different bacterial species. A similar observation was made by Neumeister et al. (27), who compared the influence of surfactant on different reference strains and several clinical isolates of GBS, S. aureus, and E. coli.

Several years ago, Coonrod and Yoneda (3) demonstrated that the surfactant fraction of rat alveolar lining material caused lysis of Streptococcus pneumoniae and several other gram-positive bacteria (Streptococcus viridans, Streptococcus pyogenes, and Streptococcus bovis). These authors speculated that the observed bactericidal effect was due to free fatty acids contained in the lung lavage preparation (5). More recently, Brogden et al. (2) described an anionic bactericidal peptide in bovine pulmonary surfactant, and prophenin-1, an antibacterial peptide that might be associated with surfactant lipids, has
FIG. 1. Effects of Curosurf (A), Alveofact (B), Survanta (C), Exosurf (D), and Pumactant (E) on the in vitro growth of different bacterial strains. A total of $7 \times 10^7$ CFU of bacteria per ml were incubated with different concentrations of surfactant (1, 10, and 20 mg/ml) or without surfactant (saline) for 5 h at 37°C. Values are mean $[\log_{10}]$ CFU/ml ± the SD obtained from six experiments. *, $P < 0.01$ versus saline.
been isolated from porcine leukocytes (10). This antibacterial peptide has also been found in Curosurf, a surfactant extracted from porcine lung homogenate (36). Part of the observed effects may thus be due to direct negative effects of these bacterial peptides on the bacterial cell wall. The polarity of these peptides seems to be most important for their antibacterial activity. It has been shown that changes in, for example, the sodium, zinc, calcium, or phosphorus content of the incubation medium can modify the in vitro bactericidal activity of such peptides (2, 10, 36).

We found that all of the investigated surfactant preparations protected *S. aureus* from the negative effects of saline on bacterial growth. This might indicate that staphylococci can catabolize surfactant lipids to some extent. The production and release of phospholipases by *S. aureus* has been described (24). LaForce et al. (22) reported increased growth of *S. aureus* after incubation with complete natural rabbit surfactant. Apparently, the bacteria can use surfactant components as nutrients. Natural surfactant isolated by lung lavage and subsequent sucrose gradient centrifugation contains a small proportion of carbohydrates (<1%) and ca. 10% proteins, including the specific surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) (18). The hydrophilic proteins SP-A and SP-D are potent stimulators of macrophage function and are generally believed to serve as important components of the pulmonary host defense system against invading microorganisms (34). However, these proteins are removed by extraction with organic solvents and therefore absent in all of the industrially produced modified natural surfactants examined in the present study. SP-B, present in small amounts in all modified natural surfactants, may in itself have a bacteriostatic effect (see below).

The relative resistance of gram-negative *E. coli* to each of the investigated surfactants may reflect the failure of the surfactant molecules to penetrate the lipopolysaccharide layer. In fact, incubation of *E. coli* with Survanta significantly promoted *E. coli* growth in vitro, the effects of exogenous phospholipids on the proliferation of *S. aureus* after incubation with complete natural rabbit surfactant. Apparantly, the bacteria can use surfactant components as nutrients. Natural surfactant isolated by lung lavage and subsequent sucrose gradient centrifugation contains a small proportion of carbohydrates (<1%) and ca. 10% proteins, including the specific surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) (18). The hydrophilic proteins SP-A and SP-D are potent stimulators of macrophage function and are generally believed to serve as important components of the pulmonary host defense system against invading microorganisms (34). However, these proteins are removed by extraction with organic solvents and therefore absent in all of the industrially produced modified natural surfactants examined in the present study. SP-B, present in small amounts in all modified natural surfactants, may in itself have a bacteriostatic effect (see below).

The relative resistance of gram-negative *E. coli* to each of the investigated surfactants may reflect the failure of the surfactant molecules to penetrate the lipopolysaccharide layer. In fact, incubation of *E. coli* with Survanta significantly promoted bacterial growth. This has also been reported by other investigators (27). Recently, it has been shown that proliferation of *E. coli* is inhibited by mature human pulmonary SP-B or, more specifically, by residues 12 to 34 of SP-B (20). The reason for the observed proliferation of *E. coli* is unclear, but Survanta contains relatively little SP-B (25, 30) and, in contrast to the other modified natural surfactants examined, it is enriched with artificial lipids. Increased growth of *E. coli* has also been reported after exposure to a crude surfactant preparation obtained from dog lungs (16). In the present study, synthetic surfactants containing lipids only (Pumactant) or lipids plus spreading agents (Exosurf) had no effect on the proliferation of *E. coli*. Although differences in surfactant composition might explain some of these seemingly conflicting results, species differences may also play a role. For example, the clearing rate of inhaled pneumococci varies between different animals (4).

Our finding that some surfactant preparations may enhance bacterial survival or even promote bacterial proliferation (as observed for Survanta and *E. coli*) is certainly alarming and should be further studied in animal experiments. So far most studies on surfactant for treatment of inflammatory lung disease have focused on gas exchange. Song et al. demonstrated improved lung function in rats with *E. coli* pneumonia treated with Curosurf (33). Unfortunately, no attempts were made to examine bacterial growth in that study. Interestingly, the present in vitro data obtained with Curosurf and GBS are in keeping with our previous observations made with GBS-infected newborn rabbits, showing mitigation of bacterial proliferation in lung homogenate following treatment with this particular surfactant preparation (13).

Clinical and radiological signs do not differentiate with certainty between pneumonia and RDS in the first hours of life. Since we and others have observed that some surfactant preparations might promote bacterial growth, infants with severe respiratory failure treated with surfactant should receive antibiotic therapy until infection can be ruled out by culture and laboratory findings.

We conclude that bacterial growth in the presence of surfactant depends on the bacterial species and the origin and concentration of the applied surfactant preparation. Except for cultures of *E. coli* in Survanta, most surfactants do not seem to promote bacterial growth. However, *E. coli* is now rarely isolated from blood cultures or tracheal aspirate fluid in the neonatal period. Curosurf significantly diminished the proliferation of GBS, the organism that accounts for most cases of early-onset sepsis. Initial clinical observations give us no reason to believe that treatment with surfactant should have serious adverse effects in neonates with conntal pneumonia (14) but, clearly, further in vivo studies are necessary to clarify the relevance of the effects observed here. Even if exogenous surfactant obtained from animal lungs influences bacterial growth in vitro, the effects of exogenous phospholipids on the microbiology of the human lung remain unclear. Careful follow-up of babies with bacterial infections treated with surfactant therefore seems mandatory.

ACKNOWLEDGMENTS

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REFERENCES


