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SURFACTANT TREATMENT IN NEONATAL GROUP B STREPTOCOCCAL PNEUMONIA

EXPERIMENTAL AND CLINICAL STUDIES

EGBERT HERTING

STOCKHOLM 1999

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ABSTRACT

Surfactant dysfunction is probably involved in the pathophysiology of neonatal group B streptococcal (GBS) pneumonia. Aim of the present studies was to evaluate efficacy and safety of surfactant replacement therapy for term and preterm neonates with severe respiratory failure due to GBS infection. We investigated the effects of surfactant on growth of GBS and on oxidative metabolism of polymorphonuclear neutrophilic granulocytes (PMN) stimulated with GBS, and developed an animal model of GBS pneumonia for studies on lung function, inflammatory response and bacterial proliferation in lung tissue.

Surfactant reduced nitroblue tetrazolium-reduction of resting PMN. In contrast, when PMN were stimulated with encapsulated GBS and a specific antibody no suppression in the release of reactive oxygen species from PMN could be observed following incubation with surfactant.

We tested the effects of surfactant treatment in tracheotomized, ventilated, near-term newborn rabbits with experimental GBS pneumonia. In this model we found reduced bacterial proliferation following instillation of exogenous surfactant as compared to controls receiving saline. In addition, lung function was significantly improved in preterm rabbits with GBS pneumonia receiving surfactant. The surfactant associated protein A (SP-A) stimulates the phagocytosis of bacteria by alveolar macrophages and is believed to play an important role in the pulmonary antimicrobial defense system. However, inactivation of SP-A by a monoclonal antibody did not influence bacterial proliferation in our model. Furthermore, we could demonstrate that simultaneous instillation of surfactant and specific antibodies against the polysaccharide capsule of GBS reduced bacterial growth in the lungs of GBS infected newborn rabbits more effectively than either treatment alone. We speculate that this might be due to a more homogenous and rapid distribution of the antibodies within the lung.

To study the effectiveness of surfactant treatment in neonatal GBS pneumonia, we assessed oxygen requirements and complication rates in 118 neonates with severe respiratory failure due to GBS infection. We could demonstrate a significant improvement in oxygenation within 1 h following surfactant instillation. However, the response to exogenous surfactant was slower than in non-infected infants with respiratory distress syndrome.

The use of surfactant for treatment of bacterial pneumonia deserves further experimental evaluation not only in neonates, but also in children and adults.

Key words: group B streptococci-infants-neonates-pneumonia-pulmonary surfactant

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"Even thousand miles begin with the first step..."

Chinese saying

TO

ANNA, TOBIAS, JONAS, JAN

AND MECHTHILD

The present thesis is based on the following papers referred to in the text by their respective Roman numbers:

I. Herting E, Jarstrand C, Rasool O, Curstedt T, Håkansson S, Robertson B: Effect of surfactant on nitroblue tetrazolium (NBT) reduction of polymorphonuclear leucocytes stimulated with type Ia group B streptococci. Acta Paediatr 1995;84:922-926

II. Herting E, Jarstrand C, Rasool O, Curstedt T, Sun B, Robertson B: Experimental neonatal group B streptococcal pneumonia: effect of a modified porcine surfactant on bacterial proliferation in ventilated near-term rabbits.
Pediatr Res 1994;36:784-791

III. Herting E, Sun B, Jarstrand C, Curstedt T, Robertson B: Surfactant improves lung function and mitigates bacterial growth in immature ventilated rabbits with experimentally induced neonatal group B streptococcal pneumonia.
Arch Dis Child 1997;76:F3-F8

IV. Herting E, Strayer DS, Jarstrand C, Sun B, Robertson B: Lung function and bacterial proliferation in experimental neonatal pneumonia in ventilated rabbits exposed to monoclonal antibody to surfactant protein A. Lung 1998;176:123-131

V. Herting E, Gan X, Rauprich P, Jarstrand C, Robertson B: Combined treatment with surfactant and specific immunoglobulin reduces bacterial proliferation in experimental neonatal group B streptococcal pneumonia.
Am J Respir Crit Care Med 1999;159:1862-1867

VI. Herting E, Gefeller O, Land M, van Sonderen L, Harms K, Robertson B and members of the Collaborative European Multicenter Study Group.
Surfactant treatment of neonates with respiratory failure and group B streptococcal infection.
Manuscript (1999) submitted

Abbreviations

ARDS	acute (adult) respiratory distress syndrome
BW	body weight
CFU	colony forming units
CRP	C-reactive protein
DPPC	Dipalmitoylphosphatidylcholine
FiO_2	fraction of inspiratory oxygen
GBS	group B streptococci
GBS LD	low density (= encapsulated) phase variant of GBS
GBS HD	high density (= non-encapsulated) phase variant of GBS
I/T-ratio	ratio of immature to total neutrophil count
NBT	nitroblue tetrazolium
PEEP	positive end-expiratory pressure
PBS	pulsating bubble surfactometer
PIP	peak inspiratory pressure
PMN	polymorphonuclear neutrophilic granulocytes
RDS	respiratory distress syndrome
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D

Contents

1. INTRODUCTION

1.1 PULMONARY SURFACTANT

Milestones from history. Pulmonary surfactant is a film of **surf**ace **act**ive **a**ge**nts** that coats the alveolar surface and the small conducting airways preventing lung collapse at the end of expiration. Laplace described the physical principles of surface tension more than 200 years ago. However, it was not until the 1920s that the physiological significance of air/liquid interfaces was clearly understood. On the basis of recordings from excised lungs filled with air or liquid, von Neergaard concluded in 1929 that "in all states of expansion surface tension was responsible for a greater part of lung elastic recoil than was tissue elasticity". His concept was forgotten until 1947 when Gruenwald described surface tension as a factor in the "resistance of neonatal lungs to aeration". It was not until 10 years later that systematic research on surface activity of alveolar lining material was initiated and Pattle (1955) and Clements (1956) described the role of surfactant for lung stability during the respiratory cycle (for a review of the history of surfactant research see: Obladen 1992).

In 1959 Avery und Mead discovered that surfactant deficiency was the cause for neonatal respiratory distress syndrome (RDS). In the following years the surfactant system was characterized and successful experiments of surfactant replacement in preterm newborn animals were performed by Enhörning and Robertson in 1972. The initial clinical trials using isolated synthetic phospholipids for treatment of babies with RDS were disappointing (Robillard *et al.* 1964). However, in 1980 Fujiwara and coworkers reported the successful use of a modified bovine surfactant for treatment of neonatal RDS. In this and many subsequent trials surfactant was shown to improve pulmonary gas exchange and reduce morbidity and mortality of neonates with severe RDS. Up to now more than 6000 infants have been studied in 33 trials. Surfactant replacement therapy has such a distinct effect on the mortality of premature infants that a remarkable reduction in overall infant mortality has been observed in several countries after the introduction of commercially available surfactant preparations (for review see: Soll 1998).

Composition, function. Natural surfactant is a mixture of lipids, proteins (about 10%), and a small proportion (less than 1%) of carbohydrates. The major lipid component is dipalmitoylphosphatidylcholine (DPPC). The phospholipids form a film on the alveolar surface reducing surface tension close to zero at end-expiration thereby preventing alveolar collapse. Adequate surface activity in vivo is only found in surfactant preparations that

contain phosholipids and specific, surfactant associated proteins (SP = surfactant protein). Four surfactant proteins (A - D) have been described to date (Johansson *et al.* 1994).

The hydrophobic surfactant proteins SP-B und SP-C play a key role in the adsorption and spreading of the lipids on the alveolar surface (Whitsett und Baatz 1992). Inactivation of SP-B in immature ventilated newborn rabbits by a monoclonal antibody causes immediate severe respiratory failure (Robertson *et al.* 1991). SP-B defiency was recently described as the underlying molecular defect of congenital alveolar proteinosis, a rare cause of lethal respiratory failure in infants and neonates (Nogee *et al.* 1994). Experiments on animals that are genetically deficient in surfactant proteins ("knock out mice") have greatly contributed to the understanding of the role of surfactant proteins in recent years (LeVine *et al.* 1997, LeVine *et al.* 1998).

SP-A is the most abundant hydrophilic surfactant protein. In its monomeric form it has a molecular mass of 28-36 kDa. The functional form of SP-A probably consists of 18 monomeric subunits. The molecule has structural similarities to the complement factor C1q and the mannose binding protein, both of which are involved in host defence. SP-A is believed to play a pivotal role in surfactant metabolism, regulating secretion and uptake of surfactant (Jobe und Rider 1992). In addition, SP-A seems to enhance the resistance of surfactant to inactivation by plasma proteins (Cockshutt *et al.* 1990). However, SP-A deficient (”SP-A knock out”) newborn mice do not suffer from respiratory failure, indicating that this protein is not required for normal respiratory function (LeVine *et al.* 1997). On the other hand SP-A and SP-D stimulate alveolar macrophages and are important factors in the pulmonary immune defence system (van Iwaarden 1992, van Iwaarden and van Golde 1995). The importance of SP-A for host defence, has recently been underlined by the observation of increased susceptibility to infections with group B streptococci in SP-A deficient mice (LeVine *et al.* 1997).

1.2 GROUP B STREPTOCOCCI

Milestones from history. Group B streptococci (GBS) are Gram-positive microorganisms with an average diameter of 0.8 to 1 µm. Under the microscope the organism can be recognized by their ability to form characteristic chains (Figure 1). On blood agar plates a typical ß-haemolysis can be seen. In 1893 Kitt described bacteria causing mastitis in cows that failed to give milk and named them *Streptococcus agalactiae contagiosae* (for review of historical aspects of GBS infections see: Dillon 1985). For many years these bacteria were considered

non-pathogenic for humans (Ayers and Rupp 1922). GBS were recognized as a cause for human infections in 1938 when Fry described 3 cases of fatal puerperal sepsis. The first cases of neonatal GBS infections were reported 60 years ago (Brown 1939). In the sixties GBS were identified as a major cause for neontal infections (Hood *et al.* 1961). During the seventies there was a dramatic increase in the incidence of septicaemia due to GBS, and GBS replaced E. coli and/or S. aureus as the most common organism associated with bacteraemia in the first 2 months of life (Baker and Edwards 1995).

Lancefield classified streptococci in groups (A - E) on the basis of their haemolytic capacity and their antigenic structure (Lancefield 1933). For GBS he described the 3 specific antigens I - III (Lancefield 1934). This classification is still valid but new types and subtypes have been added as other antigenic structures have been discovered. Subtype I and III cause the majority of neonatal infections, and type III is especially encountered in infants with GBS meningitis.

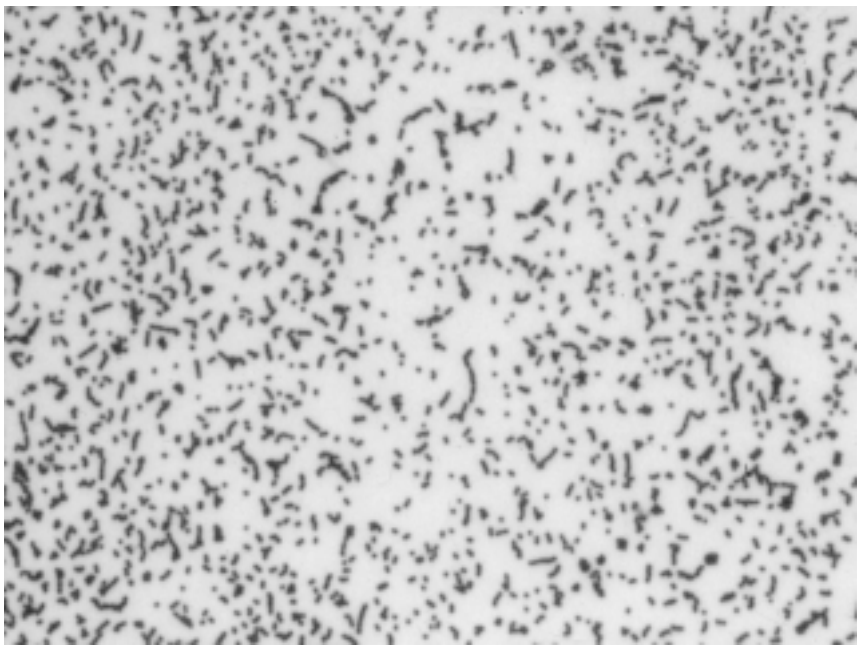

Figure 1:
Gram staining of GBS strain 090 Ia LD, x 200

Neonatal GBS infections. Infections with GBS are most commonly observed in the first days of life (Baker and Edwards 1995). About 2/3 of all cases are diagnosed in the first week of life ("early onset" septicaemia). Haemolysins and the polysaccharide capsule of GBS seem to be important virulence factor. If mothers lack opsonizing antibodies against the polysaccharide capsule, neonates are especially prone to severe infections (Baker *et al.* 1976). Infection rate and mortality are substantially higher in premature infants, and low levels of

specific immunoglobulins, as well as insufficiency/immaturity of the complement system and defects in phagocytosis by neutrophilic granulocytes have been described in affected premature neonates (Baker 1997).

Vaginal GBS colonization is found in between 10 and 30% of pregnant women. However, less than 50% of the infants born to colonized mother demonstrate GBS in cultures taken from the skin surface. Only 1 of 100 infants with GBS colonization develops severe systemic infection (American Academy of Pediatrics, Committee on Infectious Diseases and Committee on Fetus and Newborn 1992 and 1997). Nevertheless, as colonization is so common, the organism is still a leading cause of early onset septicaemia (1 to 4 cases per 1000 liveborn infants) in neonates in Northern Europe (Källmann 1997) and the USA (Philip 1994). The majority of neonates with GBS infections develop respiratory symptoms (Weisman *et al.* 1992). In premature neonates GBS pneumonia and RDS may coexist (Ablow *et al.* 1976).

1.3 NEONATAL PNEUMONIA

Connatal infections constitute a frequent problem in neonatology. The incidence of severe bacterial infections that need antibiotic treatment is estimated between 10 and 20 cases per 1000 newborn infants in Western countries. Premature and/or prolonged rupture of membranes is the most important risk factor for amnion infection syndrome. The lung is commonly the entry site for systemic bacterial infections, as infected amniotic fluid may be aspirated prenatally or during the delivery.

The invasion of microorganisms into the lungs evokes an inflammatory response, attracting phagocytes to the airways. As newborns are relatively deficient in alveolar macrophages during the first days of life (Zeligs *et al.* 1984), neutrophilic polymorphonuclear granulocytes (PMN) play a key role in antibacterial defence. The leucocytes release a variety of cytokines and reactive oxygen metabolites. These products cause damage to the alveolar-capillary barrier with a resultant influx of plasma proteins (Figure 2). Proteins like albumin and fibrinogen can directly inhibit surfactant function (Seeger *et al.* 1985) causing severe respiratory distress (Somerson *et al.* 1971). In addition, enzymes released from the granules of PMN, e.g. neutrophil elastase, may cause proteolytic damage to surfactant proteins (Pison *et al.* 1989). Surfactant synthesis is decreased during acidosis and hypoxia and a direct degrading effect of bacterial enzymes (e.g. phospholipases) on surfactant lipids has been described (Holm *et al.* 1991). Lysophospholipids generated by phospholipase A_2 not only

inhibit surfactant, but make it more susceptible to inhibition by serum proteins (Cockshutt and Possmayer 1991).

As hypoxaemia and hypercarbia may ensue, mechanical ventilation has to be initiated in severely affected infants. Inhomogenous aeration with overextended and atelectatic regions within the lungs may be the consequence and shear forces may lead to mechanical disruption of airway epithelium with further leakage of plasma proteins. Thus, in theory, early replacement therapy with exogenous surfactant may be beneficial in infants with severe pneumonia in order to prevent ventilator induced lung injury and the consequent leakage of plasma proteins into the bronchoalveolar space.

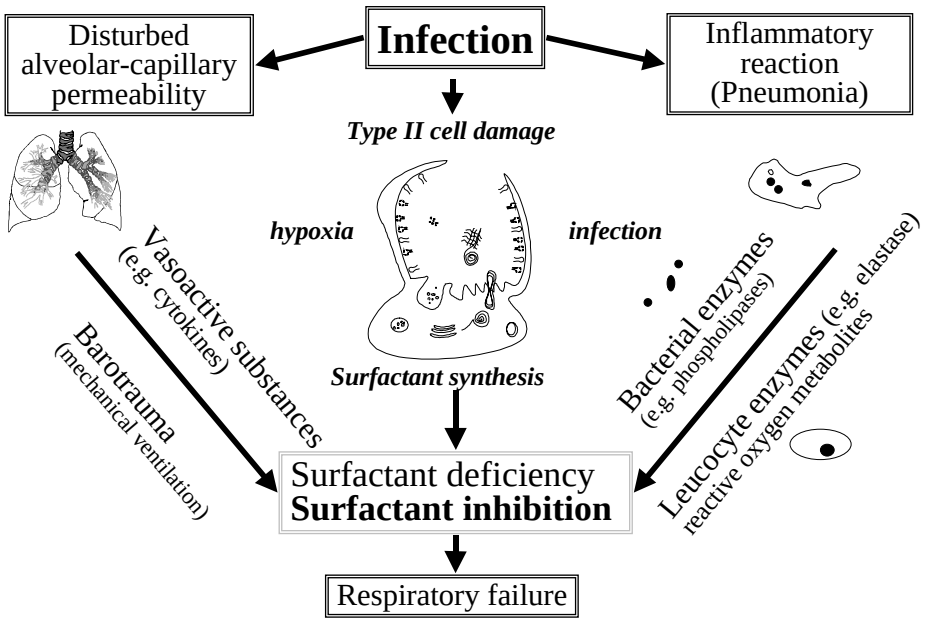

Figure 2: Pathophysiology of neonatal pneumonia

Clinical, laboratory and radiological signs cannot differentiate with certainty between "idiopathic" respiratory distress syndrome and GBS pneumonia in the early course of the disease (Ablow *et al.* 1976).

Soon after the introduction of surfactant therapy for neonatal RDS, improvement of gas exchange was described in case reports on infants with neonatal pneumonia treated with surfactant (Herting *et al.* 1989, Gortner *et al.* 1993, Khammash *et al.* 1993, Auten *et al.* 1991, Fetter *et al.* 1995). However, no systematic studies were available and concern was expressed on the basis of animal and in vitro experiments that surfactant treatment might promote bacterial growth in the neonatal lung (Sherman *et al.* 1988).

2. AIMS OF THE STUDIES

The specific aims of the studies reported in this thesis were:

1. To study the effects of surfactant on the oxidative metabolism of polymorphonuclear neutrophilic granulocytes stimulated by group B streptococci (GBS) opsonized with a specific antiserum (paper I).

2. To develop a new model of GBS pneumonia in ventilated near-term newborn rabbits, and to study the effects of surfactant on bacterial proliferation and lung histology in this model (paper II).

3. To study the effects of surfactant replacement on lung function and bacterial proliferation in experimental GBS pneumonia in ventilated immature newborn rabbits (paper III).

4. To investigate whether bacterial growth in experimental neonatal GBS pneumonia is modified by blockage of endogenous surfactant protein A with a monoclonal antibody (paper IV).

5. To evaluate the effects of combined treatment with surfactant and specific anti-bacterial antibodies on lung function and bacterial proliferation in experimental neonatal GBS pneumonia (paper V).

6. To study the clinical effects of surfactant treatment on gas exchange in term and preterm neonates with respiratory failure and GBS infection (paper VI).

3. MATERIAL AND METHODS

3.1 SURFACTANT

Modified natural surfactant preparations are produced by organic solvent extraction of phospholipids and associated hydrophobic proteins from lung lavage fluid or tissue homogenate. They contain only about 1% of surfactant proteins (SP-B and SP-C). The hydrophilic proteins SP-A and SP-D are removed by the extraction procedure.

Curosurf . Curosurf, the modified natural surfactant used in the present experiments, is isolated from minced pig lungs (Wiseman and Bryson 1994). Neutral lipids, cholesterol and lipid esters are removed by a combination of chloroform-methanol extraction and liquid-gel chromatography. It is sterilized by high pressure filtration through a micropore filter system and suspended at a concentration of 80 mg/ml of phospholipids. Apart from 99% polar lipids (~35% DPPC) it contains only SP-B and SP-C in approximate molar proportions 1:2 (Curstedt *et al.* 1992). Curosurf has been shown to be effective in animal models of surfactant deficiency or dysfunction and in randomized controlled clinical trials for treatment of neonatal RDS (Collaborative European Multicenter Study Group 1988).

Natural surfactant. Natural pig and rabbit surfactants were prepared by lung lavage and sucrose-gradient centrifugation. The material was dissolved in saline at a phospholipid concentration of 40 mg/ml. The DPPC content was about 45%, the protein content ~ 10%. In contrast to modified surfactant preparations like Curosurf, SP-A (~ 50% of total proteins) and SP-D are present in natural surfactant prepared by this technique.

3.2 BACTERIA

We have identified a GBS strain pathogenic to rabbits and developed an animal model to study the effects of exogenous surfactant on ventilated near-term newborn rabbits following intratracheal infection with GBS. An abundantly encapsulated low density (LD) phase variant of GBS was processed from the reference strain 090 Ia Colindale by repeated gradient centrifugation (Håkansson *et al.* 1988, Håkansson *et al.* 1990). The strain was kindly provided by Stellan Håkansson, University of Umeå, Sweden. It was stored in aliquots at -70° C, precultured, washed, centrifuged and suspended in normal saline at a concentration of 10^9 live bacteria per ml. Using an overnight culture (16 h) and a second shorter preincubation period (3 h) the bacteria were brought to the mid-logarithmic growth phase. Using a calibration

curve of the optical density at 595 nm, nearly identical bacterial numbers could be obtained at the beginning of the experiments. The number of colony forming units (CFU) in the stock suspension was determined for each individual experiment by serial dilution and bacterial counting on blood agar plates following a 24-h incubation period at 37° C in an atmosphere with 5% CO_2. For comparative in vitro studies *Escherichia coli (ATTC 25922)* and *S. aureus (ATTC 25923)* were used. As live bacteria may release reactive oxygen metabolites, heat-killed microorganisms were used for the NBT tests. In addition to the GBS strains we used *Candida albicans (ATCC 10231)* and *E. coli (K12)*, obtained from the National Institute of Microbiology in Stockholm.

3.3 ANTIBODIES

Antibody to SP-A. R 5 rat monoclonal antibody to rabbit SP-A was generated and purified as reported elsewhere (Strayer *et al.* 1996). The final product contained >99% IgG as judged by sodium dodecyl polyacrylamide gel electrophoresis. No cross reactivity of this antibody with SP-D or other proteins has been detected to date. The antibody was dissolved in normal saline at a concentration of 15 mg/ml and stored in aliquots at −70° C until use. The binding of R 5 to a 30 kDa protein, consistent with SP-A in a natural rabbit surfactant preparation, was demonstrated by Western blotting (Strayer *et al.* 1996). In vitro measurements of surface activity of a "complete", SP-A containing, natural rabbit surfactant preparation (produced by lung lavage and sucrose gradient centrifugation) in a pulsating bubble system (see below) demonstrated a significant increase in surface tension after addition of R 5 anti-SP-A antibody. We estimated the pool size of endogenous SP-A in term newborn rabbits to be 0.5 μg/g body weight, based on the data of Stevens and colleagues (1987). In a fetus weighing 40 g, this corresponds to 20 μg. If all of the SP-A were in monomeric form, an equimolar amount of antibody would thus be 100 μg. If SP-A is in its natural 18mer form, 100 μg of the antibody is a very large molar excess.

Antibody to GBS. A polyclonal antibody to GBS 090 Ia LD was generated in adult rabbits by repeated intravenous injection of heat-killed bacteria (Jelinkovà 1977). The IgG fraction was purified by affinity chromatography (Protein A, Sephadex 6 MB, Kabi Pharmacia, Stockholm, Sweden) and ultrafiltration (Amicon Centriplus 100, Amicon, Witten, Germany). The final product contained >99% IgG as judged by cellulose/acetate foil serum-electrophoresis (LMB, Bad Godesberg, Germany). The capacity of this antibody to mediate binding of GBS to PMN thereby stimulating the phagocytes to an increased oxidative

metabolism was tested using the nitroblue tetrazolium (NBT) test (see below). Curosurf and the suspension of the specific antibody in saline were mixed in proportion 1 : 1 and incubated for 60 min at 37° C before use. The final concentrations of Curosurf and IgG in the suspension given to the animals were 40 mg/ml and 2.5 mg/ml, respectively. The antibody was dissolved in normal saline at a concentration of 5 mg/ml and stored in aliquots at -70° C until use.

3.4 SURFACE TENSION MEASUREMENTS

The Pulsating Bubble Surfactometer (PBS) (Electronetics Corporation, Buffalo, USA) measures surface tension by generating a bubble in a sample chamber filled with fluid. A bubble that is communicating with ambient air is kept at preset maximum and minimum diameters during pulsation with a preset rate, mimicking in- and expiration. Surface tension (γ) in the bubble is determined during cyclic compression from the pressure gradient across the bubble wall (P) and its radius (r) according to the law of Laplace $P = 2\gamma/r$ (Enhörning *et al.* 1992). Daily calibration of the system was performed with 50% methanol ($\gamma = 34 \pm 2$ mN/m) and distilled water ($\gamma = 70 \pm 2$ mN/m).

Influence of IgG on surface tension. The influence of rabbit IgG (Sigma Chemicals, St. Louis, Missouri, USA) on the surface activity of Curosurf, suspended in normal saline at a concentration of 2 mg/ml or 5 mg/ml, was tested in the PBS. Measurements were made at 37° C during 50% cyclic area compression at a rate of 40 min^{-1}. Surface tension at minimum bubble size (γ_{min}) was recorded after 5 min of pulsation. IgG was added at concentrations ranging from 1 to 40 mg/ml and the sample was incubated at 37° C for 30 min before analysis.

3.5 INFLUENCE OF SURFACTANT AND ANTIBODY ON RELEASE OF REACTIVE OXYGEN METABOLITES BY POLYMORPHONUCLEAR LEUCOCYTES (PMN)

The production of reactive oxygen metabolites by stimulated phagocytes contributes to the killing of ingested bacteria. PMN were isolated and purified from healthy adult donors as described before (Bøyum 1968). Both the encapsulated (GBS LD) and the non-encapsulated phase variant (GBS HD) of the GBS strain were used for these studies. The bacteria (3×10^8) were heat-killed and incubated with 5×10^5 PMN, specific IgG-antibody (8.5 mg/ml) and/or Curosurf (4 mg/ml) on ELISA plates. Commercially available non-specific rabbit IgG and normal saline were used as controls. Oxygen metabolite production was estimated by the

nitroblue tetrazolium reduction test. Colourless NBT is reduced by the superoxide anion to dark blue formazan, which can be measured spectrophotometrically (for details see paper I).

3.6 INFLUENCE OF SURFACTANT ON IN VITRO BACTERIAL GROWTH

Bacteria were precultured and washed in saline as described in Figure 3 and inoculated in sterile saline or a nutrient medium (Standard 1-Nährbouillon, Merck, Darmstadt, Germany). Proliferation was determined without and with 1 mg, 10 or 20 mg/ml of surfactant. The samples were incubated under agitation (Shaking incubator, New Brunswick Scientific, Edison, NJ, USA) for 5 h. Aliquots (0.5 ml) were taken, serially diluted and spread on blood agar plates. After 24 h incubation colony counting was performed (Figure 3).

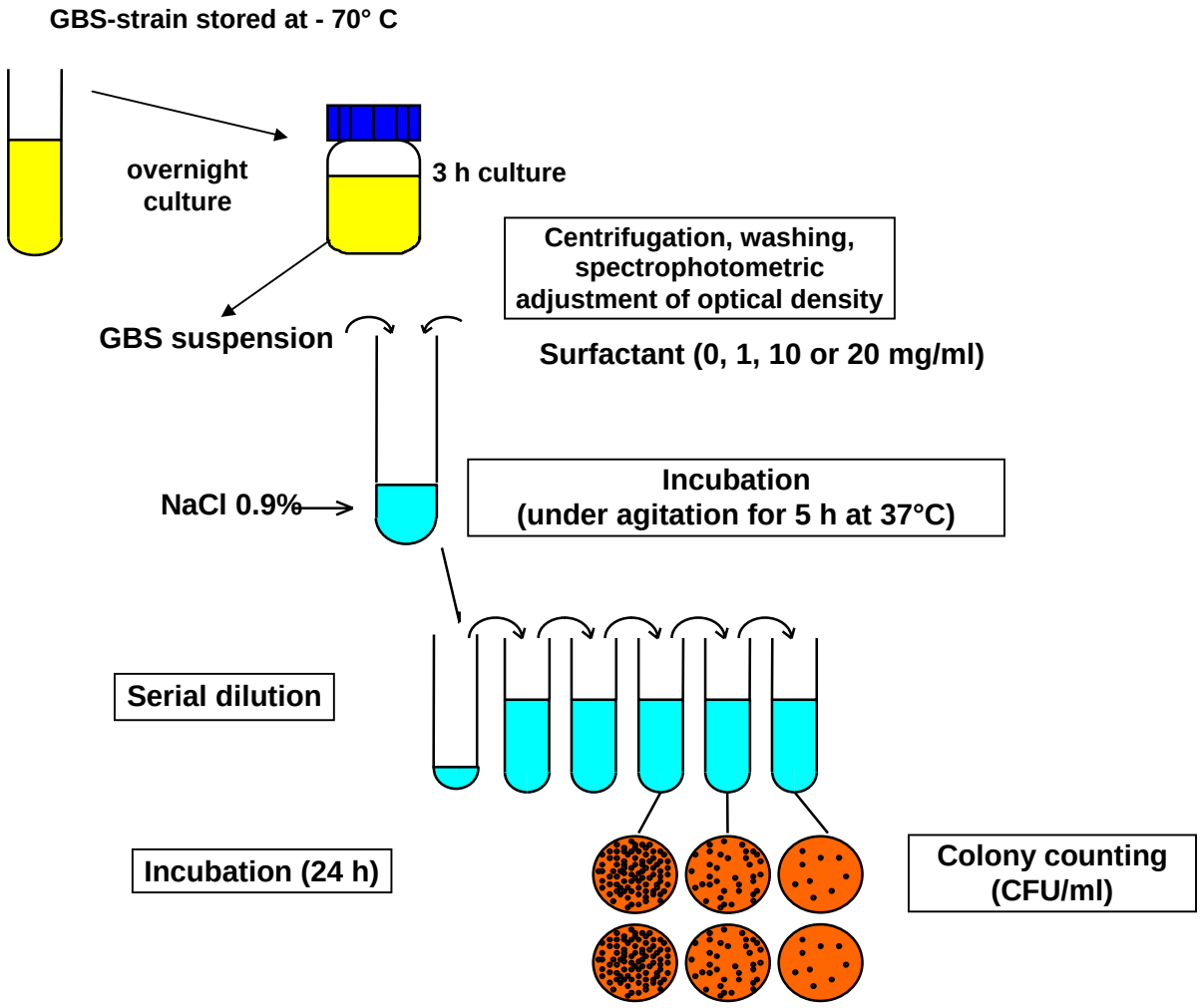

Figure 3: Schematic drawing of culturing of GBS and determination of bacterial growth

3.7 ANIMAL EXPERIMENTS

3.7.1 Animals

Pregnant New Zealand White rabbits were obtained from local suppliers. Rabbit fetuses were delivered by Caesarian section at a gestational age of 29.5 days (papers II, IV and V) or 28 days (paper III). Term gestation for rabbits is 30 to 31 days. At 29.5 days the animals have nearly mature lung function, as measured by dynamic compliance (Sun *et al.* 1991).

3.7.2 Experimental protocols

The rabbit fetuses were anaesthetized and tracheotomized at birth and allocated in a random order to the different treatment groups. In studies II and III an empty cannula was inserted into the trachea, the newborn rabbits were immediately connected to the ventilator circuit and a muscle relaxant was injected intraperitoneally. GBS or saline was injected intratracheally at 15 min (dose volume: 5 ml/kg body weight (bw)), and in these experiments a subgroup of animals was sacrificed 1 min following infection to study the number of viable GBS recovered from the lungs immediately following the inoculation (*GBS 1 min*). At 30 min the other animals were given Curosurf (*GBS/Curosurf*) or saline (*GBS/Saline*) at a dose volume of 2.5 ml/kg bw (surfactant dose: 200 mg/kg bw).

In the studies reported in papers IV and V antibody/surfactant mixtures were instilled immediately into the liquid filled lungs before the onset of breathing. Controls received the same volume of non-specific rabbit IgG, surfactant or normal sterile saline. At 30 min all experimental groups received an intratracheal bolus injection of 5 ml/kg bw of the GBS suspension. Before reconnecting the animals to the ventilator system, the instilled liquid was moved from the central airways to peripheral airspaces by injecting 3 times 10 ml/kg bw of air with a microsyringe.

All animals were then transferred to a warmed multi-plethysmograph system as previously described (Sun *et al.* 1991). They were ventilated in parallel in sealed plexiglass chambers with a common ventilator (Servo 900 B) delivering 100% oxygen (Figure 4). The working (= maximum inspiratory) pressure was set at 50 cm H_2O. The frequency was 40 per min, the inspiration/expiration time ratio 1:1. No positive end-expiratory pressure (PEEP) was applied, as PEEP might mask differences in compliance due to variations in surfactant function (Rider

et al. 1992). The peak inspiratory pressure was recorded with a pressure transducer (EMT 34) and individually adjusted for each animal to obtain a tidal volume of 6-10 ml/kg bw. Tidal volume was recorded with a specially designed "Fleisch-tube", a differential pressure transducer (EMT 31), an integrator unit (EMT 32), an amplifier (EMT 41) and a recorder (Mingograf 81; all equipment, Siemens-Elema, Solna, Sweden). Lung-thorax compliance (ml x kg^{-1} x cm H_2O^{-1}) was calculated from the quotient of tidal volume and peak inspiratory pressure. The animals were ventilated for 5 h. Recordings were obtained at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min. ECG was recorded at the same intervals and animals were counted as survivors if the heart rate was >100 beats per min without evidence of arrhythmia or atrioventricular block. At the end of the experiments the rabbits were killed by intracerebral injection of lidocaine and the chest was opened with sterile instruments after the diaphragm had been examined for evidence of pneumothorax. Blood from the right cardiac ventricle was aspirated for blood cultures (Bactec Plus Blood Culture System, Becton Dickinson, Sparks, MD, USA). A heparinized sample was taken for blood gas analysis.

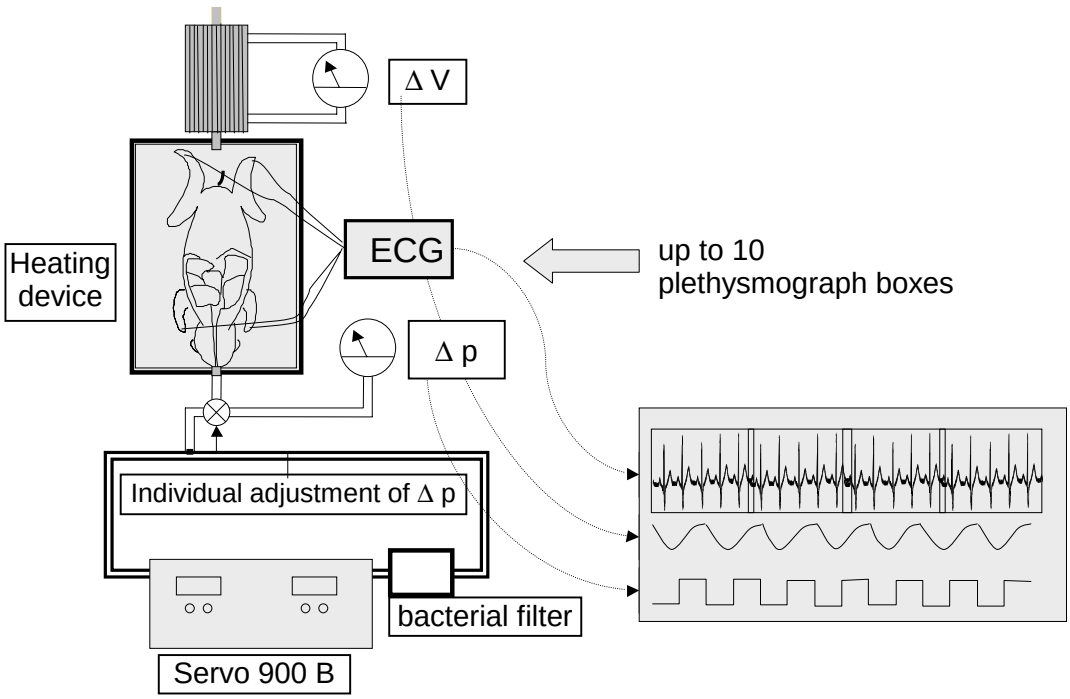

Figure 4: Schematic drawing of the set up of the animal experiments

Histologic examination. In study II, the whole left lung was used for bacterial counting whereas the right was lung was fixed by immersion for histology. In studies III and V, the left main bronchus was tied and the left lung was excised, divided in two parts of approximately equal size by a frontal section and weighed. The peripheral part was placed immediately into the sterilized tube of a tissue homogenizer (Kontes Scientific Glasware Instruments,

Vineland, NJ, USA) and stored on ice until further processing, the central part was fixed for histological examination.

In study IV a different approach was taken that allows to evaluation of lung expansion under standardized conditions. At the end of the experiment a catheter was tied into the pulmonary artery. The lungs were opened by inflating them with a transpulmonary pressure of 30 cm H_2O via the tracheal tube. After 60 sec, this pressure was lowered to 10 cm H_2O and maintained throughout the fixation procedure. The right lung was fixed with a mixture of 4% formaldehyde and 1% glutaraldehyde infused for 30 min into the main pulmonary artery at a pressure of 65 cm H_2O. The lungs were stored in 4% formaldehyde and subsequently embedded in paraffin.

Transverse sections, stained with haematoxylin-eosin or Gram stain, were examined by light microscopy with special reference to the presence of intra-alveolar edema, hyaline membranes, epithelial necrosis, bacterial proliferation and recruitment of inflammatory cells to the airspaces. Volume density of alveolar gas (Vv) in histologic sections was evaluated with conventional point counting using total parenchyma as reference volume (Robertson and Lachmann 1988). The coefficient of variation (CV) of alveolar Vv was calculated by the standard formula from the mean and the SD. CVVv is a measure of the field-to-field variability of alveolar expansion.

In all studies the influx of inflammatory cells was estimated using a semiquantative grading system (paper I). Severe pneumonia was defined as an inflammatory reaction involving more than 30% of total lung parenchyma.

For all histological examinations the slides were coded so that the investigator was unaware of the experimental conditions of the individual animals.

Broncho-alveolar lavage (paper V). The right lung remained in situ and was lavaged via the tracheal cannula. 0.9% saline with heparin (2 U/ml) at room temperature was instilled into the lungs via the tracheal cannula at a volume of 20 ml/kg bw. This volume was washed in and out three times, and the procedure was repeated four times with an average recovery of 83 ± 9% (no significant differences between the groups). The lavage fluid from each animal was pooled and centrifuged at 150 g for 10 min. The resulting pellet was collected and saline was added to obtain a final volume of 1 ml of cell suspension. One hundred µl of this suspension was stained with Giemsa-Romanowski stain and the cells were counted in a Bürker chamber

and in a flow cytometer. Cytospin preparations from the rest of the suspension were stained with May-Grünwald-Giemsa stain and used for differential counting of inflammatory cells (see cover illustration). The supernatant was stored at –70° C and its protein content was analyzed (Protein Assay, Bio-Rad Laboratories, Munich, Germany).

Bacterial counting. The weight of the lung specimens was adjusted to 1 g with sterile normal saline. The samples were homogenized with a high speed (15,000 rpm) nylon microchamber tissue homogenizer (Sorval® Omnimix, Dupont Instruments, Newton, CT, USA). A serial dilution was performed and the diluted supensions were spread on blood agar plates. Colony counting was performed after 24 h incubation (see also Figure 3). As bacterial proliferation follows a logarithmic growth curve the results were expressed as mean $\log_{10}$ CFU/g lung (wet weight). The calculated number of CFU given to the rabbit (CFU/rabbit) represents a good estimate of the number of CFU in the lung of the rabbit (CFU/g lung) at the beginning of the experiments, as the bacteria were administered intratracheally and the total lung weight of a term rabbit is about 1 g. We observed a very close correlation between the number of GBS instilled into the airways and the number of live bacteria detected in the left lung of animals sacrificed 1 min following intratracheal infection (see Figure 1 in paper II). A positive difference between CFU/g lung after 5 h of ventilation and CFU/rabbit at the beginning of the experiment thus indicates bacterial growth, a negative difference a decrease in the number of viable bacteria in lung homogenates.

3.7.3 Ethical approval

The study design and the management of the animals complied with national legislation and the rules of the Karolinska Institute. The trial protocols were approved by the local ethics committee for animal research (Stockholm Norra Försöksdjursnämnd).

3.8 STATISTICAL ANALYSIS

Experimental data are given as mean ± SD or median and range. Values for lung weight and physiological data were subjected to analysis of variance (ANOVA) using the CRISP software programme (Crunch Software, San Francisco, CA, USA). Between-group differences were evaluated by Student-Newman-Keuls´ test. Differences in the incidence of complications between the groups were analysed with the χ^2 test. The limit level of statistical

significance was defined as p = 0.05. Graphs were produced with the software programs Graph Pad Prism, Microsoft Graph and Microsoft Excel.

3.9 CLINICAL STUDY ON THE EFFECTS OF SURFACTANT TREATMENT IN GBS INFECTED NEONATES

The prospective study started in May 1993 and was continued until April 1998. In addition, we asked for retrospective evaluation of surfactant treated, GBS infected babies from 1987 onwards. Data from 28 European centers (see Appendix of paper VI) were collected using a standardized case record form requesting information on pregnancy complications, laboratory and radiological findings, gas exchange and ventilatory parameters before and after surfactant replacement as well as outcome parameters. For definition of outcome variables and disease severity we used the same diagnostic criteria as previously applied in trials organized by the Collaborative European Multicenter Study Group (Collaborative European Multicenter Study Group 1988, Speer *et al.* 1990, Speer *et al.* 1992, Speer *et al.* 1995).

Study group. Infants eligible for the study had to fulfill the following criteria:

- GBS infection verified by bacterial culture
- Clinical and/or laboratory signs of acute inflammatory disease
- Respiratory failure requiring mechanical ventilation
- Surfactant treatment

Infants with severe malformations or evidence of GBS infection only by immunological methods (latex agglutination tests) were not included.

Control group. From the data base of previous clinical trials of surfactant replacement therapy organized by the Collaborative European Multicenter Study Group a non-infected control group of 236 neonates was recruited. The selected infants were treated with surfactant for severe RDS, but did not demonstrate signs of pneumonia or septicaemia in the first week of life. Main parameters evaluated were oxygen requirement, ventilator settings and incidence of complications.

Statistical analysis. The documentation of study variables (i.e. characterization of infants, ventilatory parameters, and outcome) in the previous studies was similar to that in the present

investigation which enabled us to pool the data. Descriptive statistical methods were used to characterize the distribution of relevant variables in the GBS group. Imbalances between the GBS and the non-infected control group with respect to the distribution of some variables - resulting from the eligibility criteria for recruiting infants to the clinical trials - were accounted for in the statistical analysis. The comparison of outcome data between the GBS and the non-infected control group was performed using the recently described propensity score adjustment technique (Roosenbaum *et al.* 1994, D´ Agostino 1998). This statistical method allows efficient control for imbalances in prognostic variables between two non-randomized groups. In our situation, the propensity score adjustment was applied to account for the effects of different distribution of confounding variables. Adjustments were made for sex, gestational age, birthweight, FiO_2 before therapy, and Apgar score at 5 min. All of these variables have been shown to influence the outcome of infants in previous surfactant trials (Herting *et al.* 1992).

Results of this analysis are reported as estimated odds ratios (OR) that indicate in our case the relative increase in outcome risk for GBS infected infants as compared to non-infected controls receiving surfactant treatment for RDS. Asymptotic 95% confidence intervals accompany the OR to give an impression about the low precision of our risk estimates due to the relatively small sample size. All statistical analyses were performed with the statistical program SAS (Version 6.12, SAS Institute Inc., Cary, NC, USA).

4. RESULTS AND COMMENTS

4.1 Studies on neutrophil function

Influence of surfactant on oxygen metabolite release by PMN. Surfactant (Curosurf) at a concentration of 4 mg/ml suppressed the resting activity of unstimulated PMN significantly (Figure 5). The non-encapsulated strain GBS HD significantly stimulated PMN in the absence of specific antiserum, whereas the encapsulated phase variant (GBS LD) needed opsonization by a specific antiserum. However, when the PMN were stimulated by GBS and the polyclonal anti-GBS antibody the increase in oxygen metabolite release was not inhibited by surfactant (Figure 5).

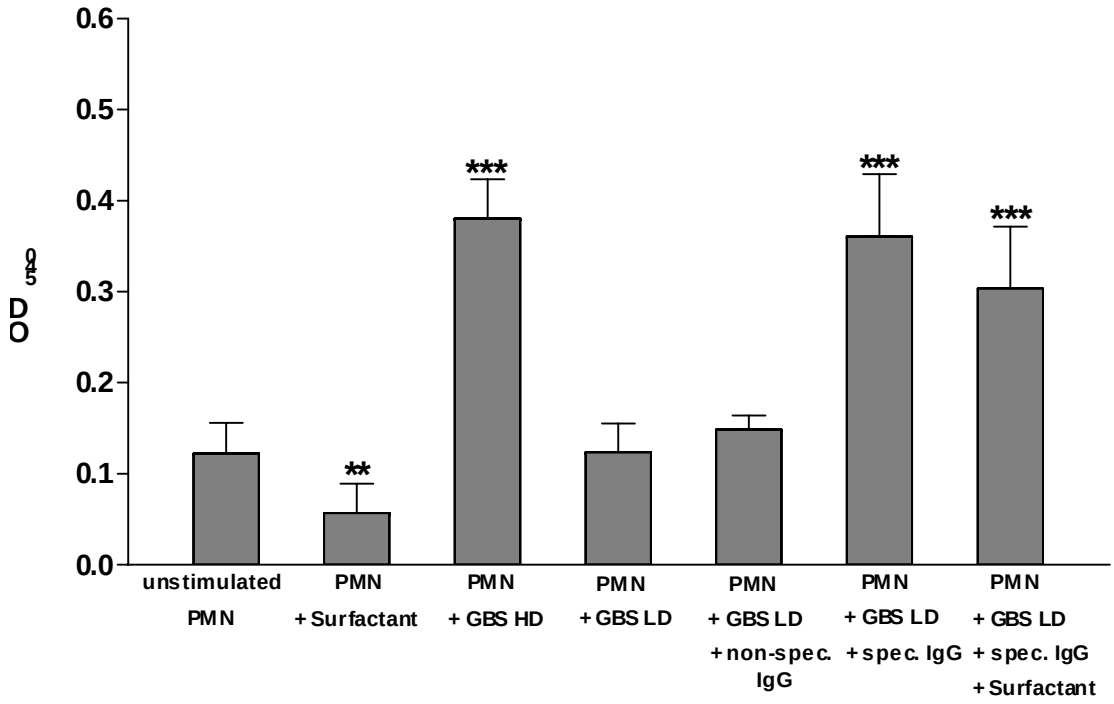

Figure 5: Oxygen metabolite release from polymorphonuclear neutrophilic granulocytes (PMN) stimulated by encapsulated (GBS LD) or non-encapsulated group B streptococci (GBS HD) as measured spectrophotometrically (OD_{540} = optical density at 540 nm) by NBT-reduction test. GBS LD needed opsonization by specific antibodies to elicit PMN stimulation. Surfactant does not suppress the antibody mediated response. The bars represent mean and SD. ** p <0.01; *** p <0.001 vs. unstimulated PMN.

4.1.1 Comments

These findings demonstrate that that the polysaccharide capsule is an important virulence factor that protects GBS from phagocytosis in the absence of specific antibodies. Significant stimulation of oxygen metabolite release from PMN occurred only after the encapsulated

bacteria had been opsonized with specific IgG. In further studies using live bacteria we found that the observed stimulation of NBT-reduction correlates to bacterial killing of GBS by isolated PMN. Again Curosurf did not suppress the phagocytosis of opsonized GBS LD by PMN (Rauprich *et al.* 1997).

These in vitro studies provided the basis for the animal experimental work in which we mixed a specific antibody with surfactant in order to facilitate spreading of the antibody in the lungs. Together with the in vitro studies in the pulsating bubble surfactometer (Figure 1 in paper V) these results indicate that surfactant can be mixed with a polyclonal antibody without loss of activity of the antibody as tested by the NBT-reduction test. We were able to demonstrate that IgG at low concentrations did not interfere with the surface activity of Curosurf. In consequence, it seems possible to use surfactant as a "carrier" for an anti-GBS immunoglobulin (paper V).

4.2 In vitro studies on bacterial growth

Effects of Curosurf on the growth of GBS. Curosurf decreased bacterial growth significantly in a dose dependent manner. The effects of Curosurf on the encapsulated GBS LD variant were less prominent than on the non-encapsulated GBS HD strain (Figure 6). At concentrations ≥10 mg/ml a highly significant reduction in the number of live bacteria was observed after 5 h incubation with Curosurf.

Effects of Curosurf on the growth of E. coli and S. aureus. In constrast to the findings with GBS, we observed no distinct effects of Curosurf on the in vitro growth of E. coli. We found slight promotion of the growth of S. aureus in Curosurf (Figure 6).

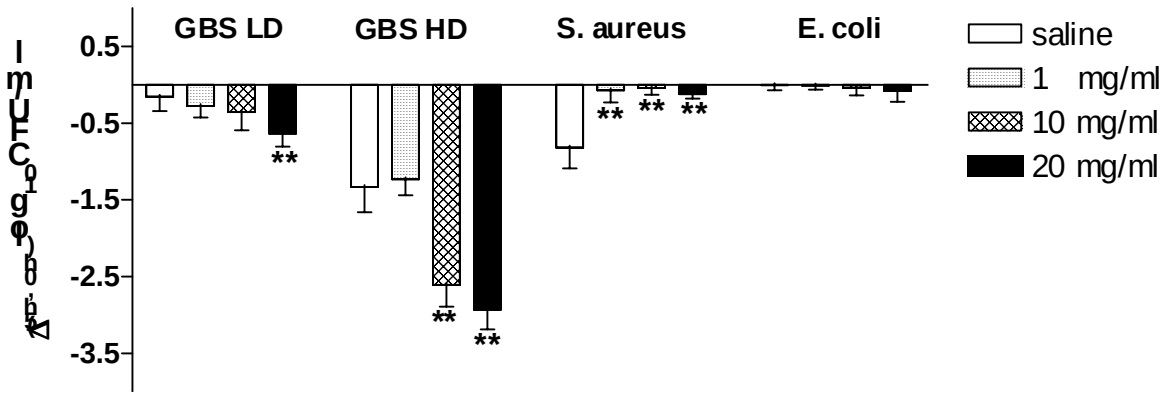

Figure 6: Effects of Curosurf on bacterial growth (p <0.01 vs. saline)**

4.2.1 Comments

Bacterial growth in the presence of surfactant is strongly influenced by the microbial species used. Although we observed strong effects of Curosurf on GBS, no bactericidal effects were seen with S. aureus or E. coli. When comparing different surfactant preparations (Alveofact (bovine), Survanta (bovine), Exosurf (synthethic, protein-free) and Pumactant (synthetic, protein-free)) we found significant promotion of bacterial growth only for E. coli in the presence of Survanta. No other surfactant reduced the growth of GBS to a similar extent as Curosurf (Herting *et al.* 1998). The observed differences may be explained by differences in the compsoition of the surfactant preparations. In addition, bacteria surrounded by a capsule (GBS LD and E. coli) were more resistant to growth inhibition by surfactant, indicating that the capsule might protect the bacterial cell wall from the influences of the phospholipids. However, surfactant promoted the growth of S. aureus, a bacterium that is able to catabolize surfactant lipids by means of phospholipases.

4.3 ANIMAL STUDIES

4.3.1 Rabbit model of experimental neonatal GBS pneumonia

Using intratracheal injection of the encapsulated GBS strain 090 Ia LD we were able to elicit severe pneumonia and septicaemia in ventilated rabbits within a period of 5 h (Figure 7). Several other wild types of GBS, isolated from infants with septicaemia were found to be less pathogenic. The non-encapsulated variant did not provoke pneumonia or bacterial proliferation in the rabbit model. To induce pneumonia within 5 h more than 10^7 CFU/ml had to be injected intratracheally (Herting *et al.* 1995).

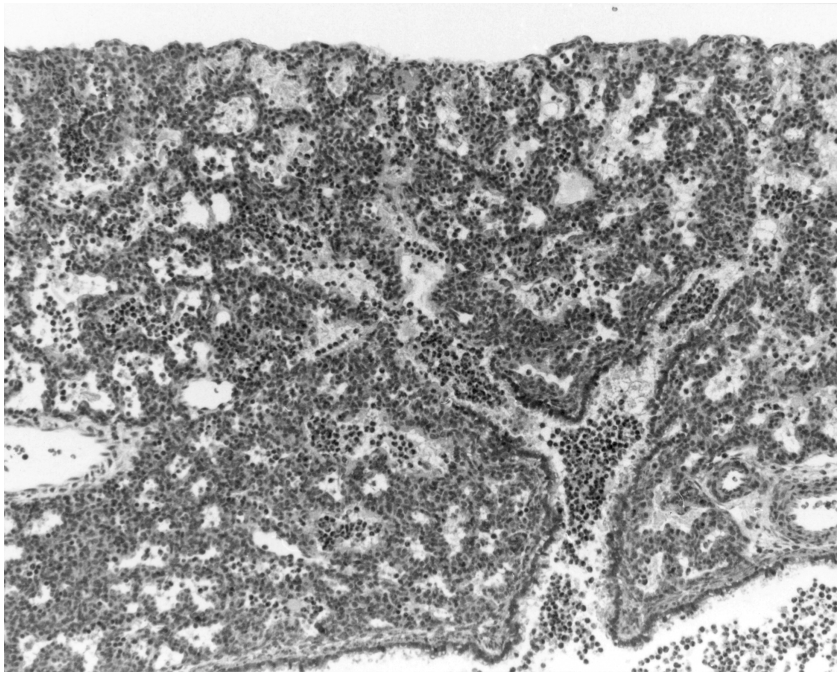

Figure 7:
Severe pneumonia with atelectasis, oedema and hyaline membrane formation in a GBS infected rabbit, ventilated for 5 h. Note the influx of inflammatory cells, especially PMN, into the airways. Haematoxylin-eosin staining. x 130.

4.3.1.1 Comments

The intratracheal injection closely mimics an ascending infection, a situation that is commonly found in GBS infected neonates. Other authors have used aerosol administration (Sherman *et al.* 1988) or injected bacteria into the trachea with a fine needle (van t´Veen *et al.* 1996). As these other studies were conducted in less sick, spontaneously breathing animals, evaluation of lung function was not possible. On the other hand, non-ventilated animals can be observed for longer periods. Our model mimics the situation of severe infection in a ventilated patient receiving surfactant treatment.

4.3.2 Effects of surfactant on bacterial growth, lung function and inflammatory reaction in experimental GBS pneumonia

When the number of CFU in lung homogenates from animals that were killed one minute following the intratracheal injection (GBS/1min) was compared with the number of CFU/g lung after 5 h of ventilation significant bacterial proliferation was observed both in term and preterm rabbits. As a logarithmic scale was used, the increase in number of CFU/g lung was more than tenfold during the period of observation (Figure 8). In surfactant treated animals we found significantly reduced bacterial numbers in lung homogenates as compared to infected animals treated with saline. This was true for rabbits both with gestational age of 28 and 29.5 days. Bacterial growth was more rapid in the 28 d gestation rabbits, especially when considering the fact that not all of the immature animals survived for 5 h. Mean survival time was only about 3 h for these animals. No significant differences in survival time were observed between the groups.

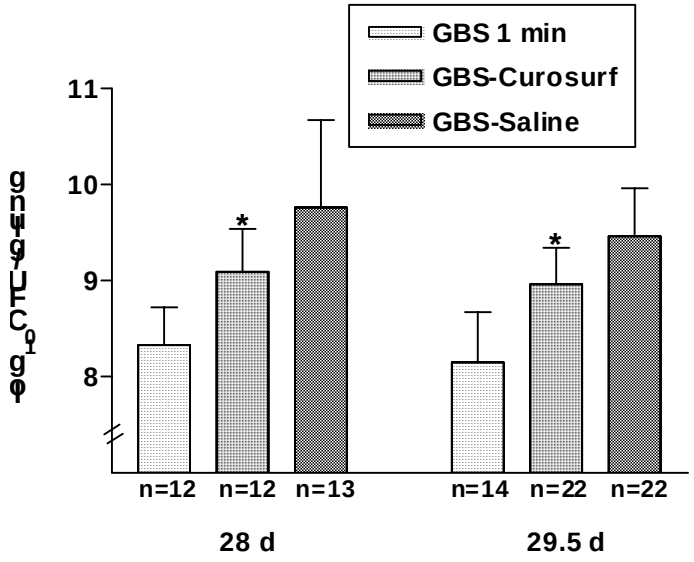

Figure 8:
Bacterial proliferation in lung homogenates in preterm (28 d) and near-term (29.5 d) newborn rabbits (mean + SD).
Significant bacterial proliferation was observed in all GBS infected ventilated rabbits as compared to the beginning of the experiments (GBS 1 min).
Significantly less bacteria were found in lung homogenates of surfactant treated animals (* p <0.05 vs. GBS-Saline).

Deterioration of dynamic compliance was observed in infected premature rabbits not receiving surfactant. At 180 min (the mean survival time) compliance was significantly higher in the GBS infected surfactant treated rabbits than in saline treated controls (Figure 9).

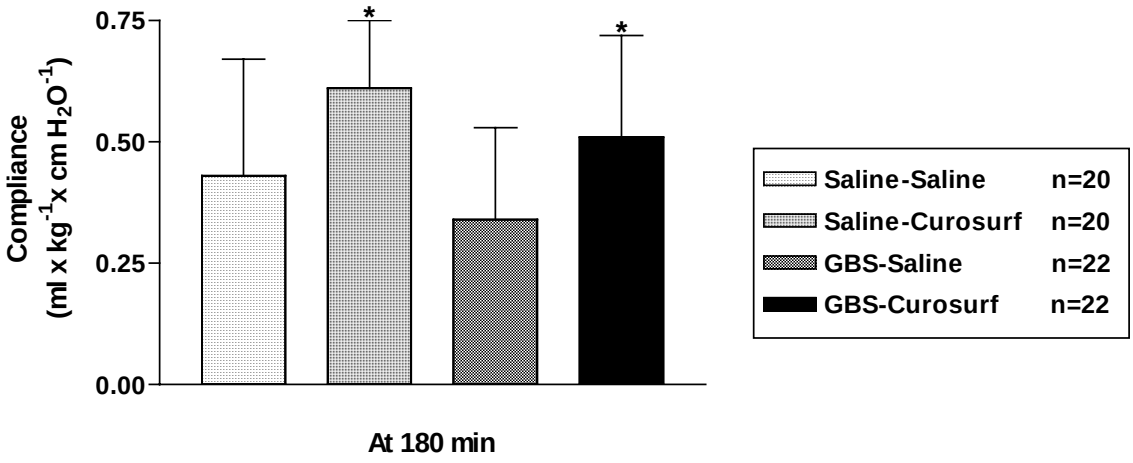

Figure 9: Lung-thorax compliance (mean + SD) of premature newborn rabbits (28 d) in different experimental groups at 180 min after the onset of ventilation. Surfactant-treated animals had significantly higher compliance values than the other groups (* p <0.05 vs. GBS-Saline).

On histological examination inflammatory changes tended to be less prominent in surfactant treated animals (see Table 3 in paper III). However, this difference did not reach the limit level of statististical significance.

4.3.2.1 Comments

Surfactant treatment of GBS infected immature rabbits improved lung function and mitigated bacterial growth in our experiments. These findings illustrate that secondary surfactant deficiency is involved in the pathophysiology of GBS pneumonia, especially in preterm neonates. Although the mechanisms responsible for the reduced bacterial proliferation following surfactant treatment are as yet unclear, it seems likely that improved lung function and prevention of atelectasis are important factors permitting effective mucociliary clearance of inhaled bacteria from the airways. Based on the in vitro observations, we furthermore speculate that surfactant lipids exert a direct bacteriostatic effect at high phospholipid concentrations. Surfactant probably also acts by prevention of oedema formation caused by epithelial disruption.

In a separate study, not included in this thesis, we found that tissue activity of free elastase, a key proteolytic enzyme of PMN, was reduced in lung homogenates from surfactant treated GBS infected rabbits as compared to saline treated controls (Herting *et al.* 1996). This corollary finding might indicate a "down-regulation" of the inflammatory response in the

lung, and is consistent with the data from our in-vitro experiments on isolated PMN exposed to GBS and surfactant (paper I).

4.3.3 Effects of surfactant protein A

Although SP-A is clearly less important for adequate surface activity than SP-B and SP-C, the protein seems to play an essential role in host defence, regulation of surfactant recycling and as a factor increasing resistance of surfactant to inhibition by plasma proteins (Cockshutt *et al.* 1990). Data from our previous animal studies on non-infected rabbits indicate that inactivation of endogenous SP-A with the same monoclonal antibody used in the present experiments increases the sensitivity of surfactant to inhibition by fibrinogen (Strayer *et al.* 1996).

In the present experiments, administration of anti-SP-A antibody to GBS infected rabbits had no effect on lung function (see Figure 2 in paper IV) or bacterial growth (Figure 10).

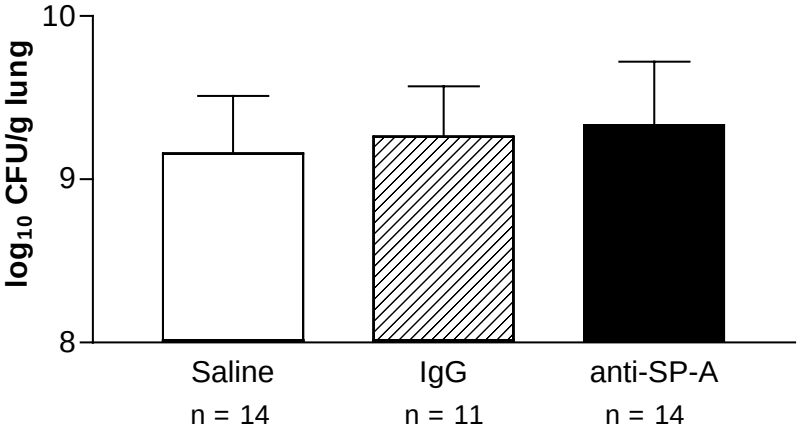

Figure 10:
Bacterial proliferation (mean + SD) in lung homogenates from GBS infected near-term newborn rabbits exposed to saline, non-specific IgG or monoclonal anti-SP-A antibody.

4.3.3.1 Comments

Blockage of endogenous SP-A with the monoclonal antibody had no effect on bacterial proliferation in GBS infected aninals. We have also tested surfactant preparations containing SP-A in our GBS-pneumonia model and found that the presence of SP-A did not further mitigate bacterial proliferation. Animals treated with a natural porcine surfactant containing SP-A or Curosurf enriched with 2% of SP-A had a similar number of bacteria in lung homogenates as those treated with Curosurf containing no SP-A (Herting *et al.* 1995). It has been shown that SP-A stimulates a variety of functions of alveolar macrophages (for review see: Wright 1998). In the normal adult lung the alveolar macrophage is the predominant

inflammatory cell in bronchoalveolar lavage fluid. Under our experimental conditions we observed a strong influx of PMN (see Table 2 in paper V). As newborn rabbits are relatively deficient in alveolar macrophages during the first few days of life (Zeligs *et al.* 1984), PMN may act as ”the second line of defence” (Hall and Sherman 1992). Our negative findings using the monoclonal anti-SP-A antibody may indicate that normal host defence mechanisms were ”overwhelmed” by the large number of bacteria administered into the airways in our experiments.

4.3.4 Combined treatment with surfactant and specific immunoglobulin

Neonates suffering from GBS infections often lack type specific opsonising antibodies. We therefore studied the influence of combined intratracheal treatment with surfactant and a specific antibacterial polyclonal antibody (IgG fraction) on bacterial proliferation and lung function in experimental GBS pneumonia. After 5 h of ventilation the mean estimated increase in bacterial number (log_{10} colonies per g) in lung homogenates was 0.76 in the Antibody-, 0.92 in the Non-specific IgG-, 0.55 in the Surfactant- and 1.29 in the Saline-group (Figure 11). A mean decrease in bacterial number (- 0.05) was observed in the group that received combined treatment with surfactant and antibody ($p < 0.05$ vs. all other groups). In addition, lung-thorax compliance was significantly higher in surfactant treated animals (see Figure 4 in paper V).

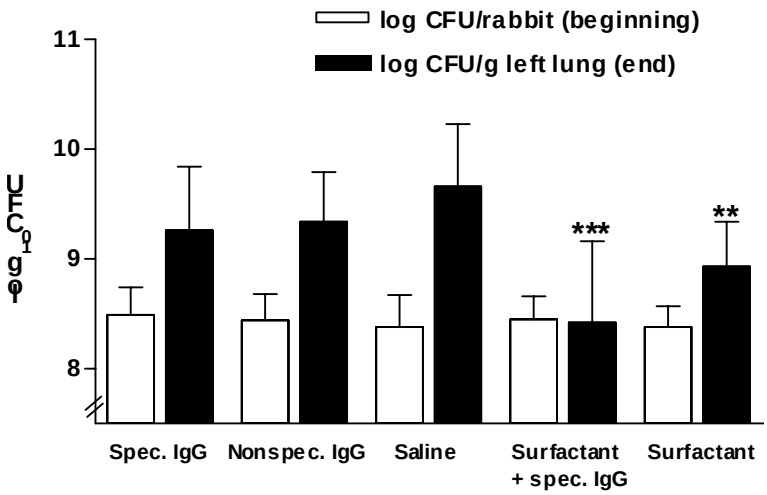

Figure 11:
Bacterial proliferation in left lung homogenates expressed as log_{10} colony forming units (CFU) per g lung tissue sampled from different groups of animals at the end of the experiments (black bars). There is significant reduction in bacterial growth in the Surfactant group (p < 0.01 vs. Saline group) and in the Surfacant + Specific-IgG group (*** p <0.001 vs. Saline group). A similar number of CFU was given to all animals at the beginning of the experiment (white bars). Values are mean and SD.**

4.3.4.1 Comments

We conclude that in experimental neonatal GBS pneumonia combined treatment with surfactant and a specific immunoglobulin against GBS reduced bacterial proliferation more

effectively than either treatment alone. The design of our experiments did not allow distribution studies on either surfactant apoproteins or antibacterial antibodies. However, Kharasch *et al.* (1991) demonstrated a more even distribution of radioactive sulfur colloids in hamster lungs when the material was mixed with surfactant. We therefore suggest that in our experiments surfactant may have served as a carrier, promoting distribution of immunoglobulins (Hill *et al.* 1992, Weisman *et al.* 1992, Weisman *et al.* 1993) to the sites of bacterial proliferation and inflammation in terminal air spaces.

4.4 SURFACTANT TREATMENT OF NEONATES WITH RESPIRATORY FAILURE AND GBS INFECTION

The study comprised 118 babies with respiratory failure, clinical and/or laboratory signs of acute inflammatory disease, and GBS infection proven by culture results. Median birth weight in the study group was 1468 g (25th - 75th percentile: 1015 - 2170 g), median gestational age 30 weeks (27 - 33 weeks). Thirty-one percent of the infants weighed >2000 g. Median age at surfactant treatment was 6 h. The mean initial surfactant dose was 142 mg/kg (SD 53). Ninety of the infants were treated with Curosurf, 13 with Survanta, 12 with Alveofact and 3 with Exosurf. 52 % of infants received more than one dose of surfactant.

Within one hour of surfactant treatment median FiO_2 was reduced fr
percentile: 0.63 - 1.0) to 0.50 (0.35 - 0.80) ($p < 0.01$). The reduction i
airway pressure (MAP) was slower than in non-infected infants with RDS

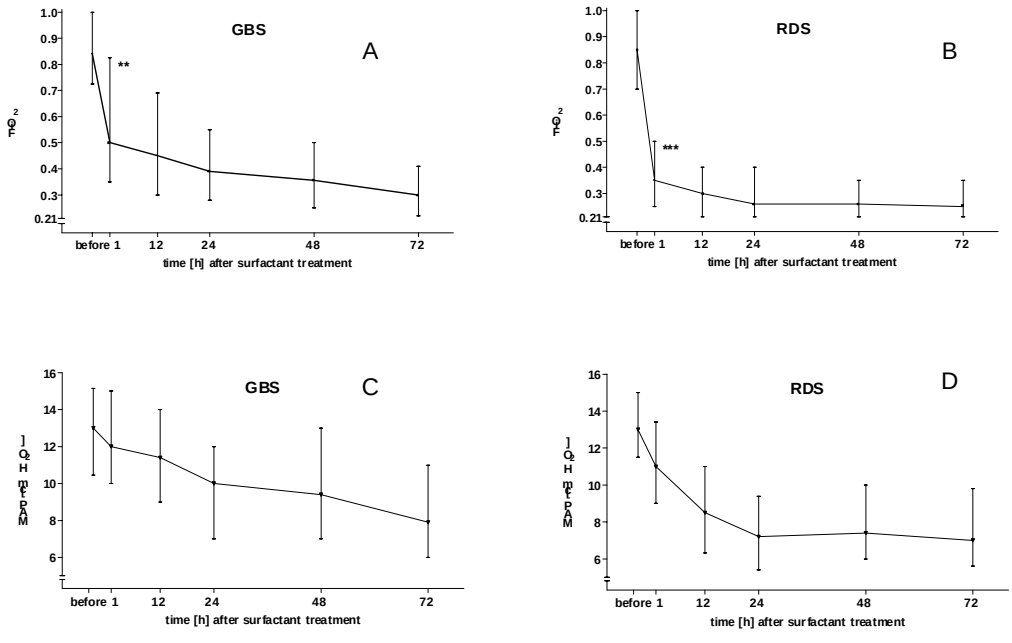

Figure 12 a - d: Fraction of inspiratory oxygen concentration (FiO_2) and mean airway pressure (MAP) following surfactant treatment in GBS infected neonates (Figures 12A and C) and in non-infected infants with RDS (Figures 12B and D).
Values are median (25^{th} - 75^{th} percentile). ** $p < 0.01$ and *** $p < 0.001$ vs. values before surfactant treatment.

The incidence of complications in the study group (mortality: 30%, pneumothorax: 16%, intracranial haemorrhage: 42%) was high compared to infants with RDS. Significantly fewer GBS infected neonates survived without intracranial haemorrhage or chronic lung disease (Figure 13).

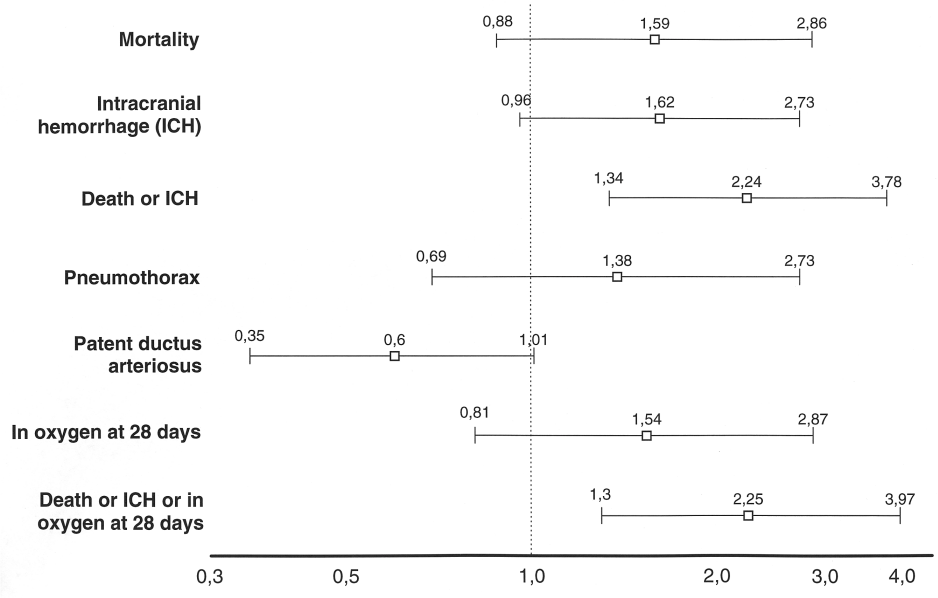

Figure 13: Adjusted risk (Odds ratio, 95% confidence interval) for selected outcome variables of GBS infected infants as compared to non-infected controls with RDS.

4.4.1 Comments

We conclude that surfactant therapy improves gas exchange in the majority of patients with GBS pneumonia. The reponse to surfactant is slower than in babies with RDS and repeated surfactant doses are often needed. The mortality and morbidity are substantial considering the relatively high mean birth weight of the treated infants.

5. GENERAL DISCUSSION

5.1 INFLUENCE OF SURFACTANT ON PMN FUNCTION

It has been shown that the inflammatory response to GBS in the neonatal lung is modulated by a variety of factors, including lung surfactant (Baker 1997). A depressing effect of phospholipids on various PMN functions has been observed by earlier investigators (Speer *et al.* 1991, Chao *et al.* 1995, Tegtmeyer *et al.* 1996). In our studies we found a decreased release of oxygen metabolites when non-stimulated PMN were incubated with Curosurf at a dose of 4 mg/ml. However, when the PMN were stimulated with GBS and a specific antibody no such inhibitory effect was found. These observations indicate that the surfactant lipids play an important role in the regulation of the inflammatory response in the mammalian lung. The enormous alveolar surface of the lung is exposed to contact with millions of inhaled particles and microorganisms each day. The coating of these particles with a lipid film might prevent an inflammatory response. Recent studies indicate that surfactant might indeed be of importance in the prevention of airway hyperreactivity (Wang *et al.* 1996).

Increased bacterial proliferation following surfactant treatment of pneumonia has been discussed previously as possible side effect of an elevated intraalveolar phospholipid load (Sherman *et al.* 1988), but our present data indicate no such interference with the bactericidal capacity of a stimulated immune system.

5.2 INFLUENCE OF SURFACTANT ON IN VITRO BACTERIAL GROWTH

Several investigators have evaluated the influence of lung lavage fluid, lipid extract or commercially available surfactant preparations on bacterial growth (for review see: Bartmann 1998). The results are conflicting, as might be expected since many different bacterial strains and various surfactant preparations at different concentrations were applied. Initial studies used complete bronchoalveolar lavage fluid rather than extracted surfactant (La Force *et al.* 1973, O´Neill *et al.* 1984, Jonsson *et al.* 1986). In most of these studies phospholipid concentrations below 10 mg/ml were investigated. In our studies we found a maximum inhibitory effect of surfactant on the growth of GBS at the highest concentration studied (20 mg/ml). In fact this finding might be of clinical relevance as surfactant accumulates in the alveolar hypophase at concentrations well above this level (Kobayashi and Robertson 1983). Assuming an endogenous surfactant pool size of 10 (premature infant with RDS) to 100 mg/kg bw (term infant) and a content of lung liquid of about 30 ml/kg bw at birth (Hallman *et*

al. 1986, Stevens *et al.* 1987, Kobayashi *et al.* 1990), the recommended replacement doses of surfactant (100-200 mg/kg bw) would result in an estimated average phospholipid concentration of 3–10 mg/ml already before resorption of fetal lung liquid. The mechanisms behind the bactericidal effects of Curosurf at high concentrations are yet unclear. The fact that Curosurf reduced bacterial growth especially in the non-encapsulated phase variant of GBS suggests that the phospholipids, possibly in conjunction with the hydrophobic proteins, might interact directly with the bacterial cell wall. The polysaccharide capsule of GBS LD might have protected the cell membrane to some extent. Recently, the possible role of antibacterial peptides (e.g. prophenin) probably also contained in modified porcine surfactant preparations has been discussed (Harwig *et al.* 1995). Significant differences in the effects on bacterial growth were observed between different surfactant preparations and different bacterial strains by other investigators (Sherman *et al.* 1994, Neumeister *et al.* 1996). Furthermore, amongst the recent studies there is no indication that surfactant replacement might promote bacterial growth thereby endangering infants who receive surfactant treatment for bacterial pneumonia.

5.3 INFLUENCE OF SURFACTANT TREATMENT IN EXPERIMENTAL NEONATAL GBS PNEUMONIA

We established a new animal model of neonatal GBS pneumonia in term and preterm rabbit newborn rabbits. Using a concentrated inoculum of precultured, abundantly encapsulated GBS injected into the trachea of experimental animals we were able to induce severe pneumonia, septicaemia and respiratory failure in ventilated newborn rabbits within 5 h following GBS infection. Surfactant treatment mitigated bacterial growth and improved lung function in immature ventilated GBS infected rabbits. These data seem to contradict earlier observations by Sherman *et al.* (1988) obtained with a different model, using aerolised GBS as the infectious inoculum. However, in a recent similar study by the same group there was no increased bacterial growth in surfactant treated animals (Sherman *et al.* 1994). Besides a direct effect of surfactant on bacteria, we speculate that improved lung function and prevention of atelectasis are important for adequate mucociliary clearance of microorganims invading the airways. In the recent literature improved lung function following surfactant replacement has been reported in a variety of ARDS-like inflammatory conditions, including bacterial (E. coli, Song *et al*. 1996), viral (Influenza A, Van Daal *et al.* 1991, Van Daal *et al.* 1992) and protozoal (Pneumocystis carinii) pneumonia (Eijking *et al.* 1991). In most of these animal studies only lung function was evaluated. In our studies we found a mitigated inflammatory response as indicated by milder inflammatory changes in histological lung

sections and lower lung tissue levels of free elastase in surfactant treated animals. These findings fit the concept that surfactant may "down-regulate" inflammatory processes in the lung (Speer *et al.* 1993), either by a direct effect or as a consequence of reduced bacterial proliferation.

5.4 INFLUENCE OF SURFACTANT PROTEIN A IN EXPERIMENTALLY INDUCED NEONATAL GBS PNEUMONIA

To study the importance of SP-A for lung defence mechanisms we administered a monoclonal anti-SP-A antibody to near-term newborn rabbits. Inactivation of endogenous SP-A did not result in any measurable acute deterioration in lung function. However, when fibrinogen was given to animals that were pretreated with the same monoclonal antibody a significant drop in compliance occurred (Strayer *et al.* 1996). These findings are consistent with the concept that SP-A increases resistance of surfactant to inhibition by plasma proteins as described previously (Cockshutt *et al.* 1990, Sun *et al.* 1997). After blockage of endogenous SP-A with this antibody we did not observe any significant differences in bacterial proliferation in GBS infected newborn rabbits, as compared to infected controls receiving saline or non-specific IgG. In agreement with our results, recent studies on gene targeted, SP-A deficient mice demonstrated largely normal lung function, although young adult SP-A knock-out mice were more susceptible to infections with GBS (LeVine *et al.* 1997) or Pseudomonas aeruginosa (LeVine *et al.* 1999). This seems to contradict our present finding that bacterial proliferation was unaffected in GBS infected animals pretreated with the monoclonal anti-SP-A antibody. However, in case of air-borne infections in adult, spontaneously breathing experimental animals alveolar macrophages are primarily attracted to the lungs. SP-A is a potent stimulator of alveolar macrophage function but no such effects have been reported for PMN. When a large dose of bacteria is inoculated in the airways of newborn rabbits as in the present studies, the acute inflammatory reaction is dominated by PMN and therefore relatively independent of SP-A.

5.5 COMBINED TREATMENT OF GBS PNEUMONIA WITH SURFACTANT AND A SPECIFIC IGG ANTIBODY

Infants of mothers who lack type specific antibodies to the GBS polysaccharide capsule are especially prone to severe infections (Baker *et al.* 1976). Thus active and passive immunization against GBS has been proposed (Baker *et al.* 1988, Schuchat *et al.* 1996, American Academy of Pediatrics: Committee on Infectious Diseases and Committee on Fetus and Newborn 1997). Our study (paper V) was designed to test the hypothesis that combined

intratracheal treatment with surfactant and a specific antibacterial immunoglobulin (IgG) would further improve lung function and mitigate bacterial proliferation in experimental neonatal GBS pneumonia. Combined treatment with surfactant and the antibody was shown to be more effective than either treatment alone.

It seems likely that simultaneous instillation of surfactant facilitates spreading of drugs in the peripheral airspaces, as previously shown for radio-labelled sulfur colloids administered via the airways together with surfactant (Kharash *et al.* 1991). Other investigators documented the effectiveness of surfactant as a vehicle for a variety of pharmacological substances including antibiotics (van t´Veen *et al.* 1995, van t´Veen *et al.* 1996), lysozyme (Sherman *et al.* 1993), antioxidants (Walther *et al.* 1995) and corticosteroids (Fajardo *et al.* 1998). A recent paper describing the use of surfactant as a carrier for an adenovirus vector (Katkin *et al.* 1997) investigated the possible use of surfactant in gene therapy.

Antibodies against proinflammatory cytokines (e.g. tumor necrosis factor alpha, Givner *et al.* 1995) or receptor antagonists (e.g. interleukin-1 receptor antagonist, Vallette *et al.* 1995) may be mixed with surfactant in the future. The protective role of interleukin-10 has recently been demonstrated in GBS infected mice (Cusomano *et al.* 1996).

Intravenous administration of antibiotics is standard treatment for neonates with respiratory distress until bacterial infection can be ruled out by means of negative cultures. However, with conventional treatment drug levels might be low in the alveolar spaces and bacterial resistence against antibiotics is a widespread clinical problem, especially in adults. Bacteria opsonized by specific IgG antibodies are phagocytized and consequently killed intracellularly. This is a potential advantage as bacterial toxins or cell wall components of extracellularly killed microorganisms can cause severe adverse systemic reactions such as endotoxin shock or pulmonary hypertension (Covert *et al.* 1993, Schreiber *et al.* 1992). In patients affected by severe pneumonia, topical immunoglobulins might therefore serve as an adjunct to standard antimicrobial therapy.

We conclude from our studies that combined treatment with surfactant and a specific anti-bacterial antibody improves lung function and mitigates bacterial growth in experimental GBS pneumonia in ventilated newborn rabbits. The possible role of surfactant as a "vehicle" for various drugs administered via the airways deserves further evaluation.

5.6 SURFACTANT TREATMENT OF NEONATES WITH RESPIRATORY FAILURE AND GBS INFECTION

Most neonates with systemic or pulmonary GBS infection have respiratory symptoms (Ablow *et al.* 1976, Weisman *et al.* 1992). Data from our animal experiments indicate that respiratory failure in neonatal GBS pneumonia may at least in part be due to surfactant deficiency (paper III). There is also anecdotal evidence from small uncontrolled clinical trials (Herting *et al.* 1989, Auten *et al.* 1991, Khammash *et al.* 1993, Gortner *et al.* 1993) and a recent multicenter study (Lotze *et al.* 1998) indicating that surfactant treatment can improve gas exchange in babies with neonatal pneumonia.

In our study on GBS infected neonates suffering from severe respiratory failure we were able to demonstrate a significant improvement in gas exchange following surfactant treatment. The design of the study did not allow conclusions about possible effects of surfactant therapy on mortality or rate of other complications. Unfortunately, it is not possible to study the effects of surfactant for treatment of GBS pneumonia in a prospective, randomized, controlled trial. Although leucocytopenia, increased number of immature neutrophils (I/T-ratio >0.17) and elevated serum C-reactive protein develop in the majority of cases within the first 24 h, no diagnostic features can distinguish with certainty beween "idiopathic" RDS and pneumonia in a premature baby within the first hours after birth, i.e. at the time when surfactant therapy should be considered. Moreover, in a premature baby both lung immaturity and pulmonary or systemic infection might contribute to the severity of respiratory failure. As surfactant improves gas exchange and survival in babies with immature lungs, it would be unethical to perform a controlled randomized study withholding surfactant treatment from the control group. In babies with RDS a good response to surfactant treatment corresponds to a low complication rate (Herting *et al.* 1992, Kuint *et al.* 1995).

When we compared the response to surfactant treatment of a group of non-infected neonates receiving surfactant treatment for severe RDS, a higher percentage of non-responders, a slower reduction in oxygen demand and an increased incidence of complications were observed with the GBS infected patients. Possibly a higher dose of surfactant and earlier treatment might have improved these results. In a recently published study of surfactant therapy for meconium aspiration syndrome initiated before the age of 6 h, a significant improvement in oxygenation was observed after a cumulative dose of 300 mg/kg bw (Findlay *et al.* 1996). An initial surfactant dose of 300 mg/kg bw was also used successfully in adult patients with ARDS triggered by septicaemia (Walmrath *et al.* 1996). Early treatment with

large doses of surfactant may be required to overcome the presence of intraalveolar surfactant inhibitors in this category of patients in order to prevent ventilator induced lung injury. Preliminary clinical data indicate that surfactant might also improve gas exchange in infants with ARDS-like disorders occurring after the neonatal period due to infection with pneumocystis carinii, chlamydia or respiratory syncytial (RS) virus (Harms and Herting 1995, Slater *et al.*1995).

We conclude that surfactant replacement, if necessary with high and repeated doses, improves gas exchange in term and preterm neonates with respiratory failure due to GBS infection. However, in view of the high mortality and morbidity of the disease, prevention strategies, in the future hopefully including vaccination of pregnant women against GBS (Baker 1997, Schuchat *et al.* 1996), remain of prime importance. In the coming years new synthetic surfactant preparations will probably be designed to enhance bacteriostatic effects and maximize resistance to inhibition by plasma proteins leaking into the airways during the course of bacterial pneumonia.

6. SUMMARY

Background. Surfactant dysfunction is probably involved in the pathophysiology of neonatal pneumonia. It has been known for years that neonatal pneumonia caused by group B streptococci (GBS) can mimic respiratory distress syndrome (RDS). Surfactant therapy has become standard treatment for RDS in the last decade.

The aim of the present studies was to evaluate efficacy and safety of surfactant replacement therapy for term and preterm neonates with severe respiratory failure due to GBS infection. We investigated the effects of surfactant on growth of GBS and on oxidative metabolism of polymorphonuclear neutrophilic granulocytes (PMN) stimulated with GBS, and developed an animal model of GBS pneumonia for studies on lung function, inflammatory response and bacterial proliferation in lung tissue.

Paper I. We evaluated the effects of modified porcine surfactant (Curosurf) on the oxygen radical release from PMN using the nitroblue tetrazolium (NBT) reduction test. Surfactant reduced NBT-reduction by resting PMN. In contrast, when PMN were stimulated with encapsulated GBS and a specific antibody no suppression in the release of reactive oxygen species from PMN could be observed following incubation with surfactant. It appears that surfactant might act in part by "down-regulating" the inflammatory response. These properties of surfactant are probably important as the enormous alveolar surface is exposed to millions of inhaled antigens each day. However, our data provide no evidence of impairment of PMN function by surfactant in an immune system stimulated by bacteria and specific antibodies. Thus it is likely that surfactant modulates the host defence system in such a manner as to permit a response during GBS infection while moderating the response of less serious invaders.

Paper II. As bacteriostatic properties of surfactant may be an important component of the non-specific host defence system of the mammalian lung, we designed an animal model of GBS pneumonia to test the effects of surfactant treatment in tracheotomized, ventilated, near-term newborn rabbits with a gestational age of 29.5 days. Intratracheal injection of a high dose of GBS led to an acute inflammatory lung injury mimicking the natural course of ascending infections in human neonates. In this model we found reduced bacterial proliferation following instillation of exogenous surfactant as compared to controls receiving saline. This indicates that surfactant may function to alter bacterial infectivity.

Paper III. A similar experimental model was applied to preterm newborn rabbits with a gestational age of 28 days. As in near-term animals we observed reduced bacterial proliferation in the surfactant-treated group. In addition, lung function was significantly improved in preterm rabbits with GBS pneumonia receiving surfactant treatment.

Paper IV. The surfactant associated protein A (SP-A) stimulates the phagocytosis of bacteria by alveolar macrophages and is believed to play an important role in the pulmonary antimicrobial defence system. However, inactivation of SP-A by a monoclonal antibody did not influence bacterial proliferation in our model. Our findings seem to contradict recent observations by others showing increased susceptibility of genetically SP-A deficient mice to GBS infection. However, it is thought that SP-A does not stimulate the phagocytosis of bacteria by PMN which in our study represented more than 80% of inflammatory cells in bronchoalveolar lavage fluid from animals with acute GBS pneumonia.

Paper V. A possible way to reduce mortality and morbidity in severe pneumonia might be through the use of surfactant as a vehicle for drugs, e.g. antibiotics or specific antibodies. We were able to demonstrate that simultaneous instillation of surfactant and specific antibodies against the polysaccharide capsule of GBS reduced bacterial growth in the lungs of GBS infected newborn rabbits more effectively than either treatment alone. We speculate that this might be due to a more homogeneous and rapid distribution of the antibodies within the lung. Admixture of IgG at low doses to surfactant did not influence its biophysical properties as measured with the pulsating bubble surfactometer or its capacity to improve lung function in our model.

Paper VI. To evaluate effectiveness of surfactant treatment in neonatal GBS pneumonia, we assessed oxygen requirements and complication rates in 118 neonates with severe respiratory failure due to GBS infection. We were able to demonstrate a significant improvement in oxygenation within 1 h following surfactant instillation. However, the response to exogenous surfactant was slower than in infants with RDS and more than 25% of infants showed no significant improvement in oxygenation within 1 h of surfactant treatment. Infants with GBS infection had a high incidence of complications when compared to neonates receiving surfactant replacement therapy for RDS. To date, we know of no larger studies on the use of surfactant to counteract inflammatory lung disease.

Conclusions. Besides its biophysical properties surfactant is an important constituent of the pulmonary host defence system. Bacterial pneumonia has recently been identified as a new potential target for surfactant replacement therapy in neonates, children and adults. In our animal model we found improved lung function and decreased intrapulmonary bacterial proliferation in premature GBS infected newborn rabbits following surfactant treatment. Blockage of endogenous SP-A by a monoclonal antibody did not promote bacterial growth in experimental neonatal GBS pneumonia. The use of surfactant as a carrier for a variety of drugs including antibiotics and antibodies for treatment of bacterial pneumonia deserves further experimental evaluation.

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Figure 14: "Friendly" subtype of GBS 090 Ia LD demonstrating a special form of ß-haemolysis

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” Nicht Kraft und Leidenschaft allein,

Geduld will bei dem Werke sein...”

Johann Wolfgang von Goethe

Cover illustration: Phagocytosis of encapsulated group B streptococci by human polymorphonuclear neutrophilic leucocytes