

Lung Function and Bacterial Proliferation in Experimental Neonatal Pneumonia in Ventilated Rabbits Exposed to Monoclonal Antibody to Surfactant Protein A

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Abstract. Surfactant protein A (SP-A) increases the resistance of surfactant to inhibition by plasma and other proteins. In a previous study we found that a monoclonal anti-SP-A antibody (R 5) increased the sensitivity of surfactant to inhibition by fibrinogen *in vivo* and *in vitro*. SP-A has been shown to stimulate microbial phagocytosis and killing by alveolar macrophages. We hypothesized that using R 5 to inactivate SP-A in an animal model mimicking congenital group B streptococcal (GBS) pneumonia might result in increased bacterial proliferation and a deterioration in lung function. Newborn near term rabbits were delivered by Cesarean section, anesthetized, tracheotomized, and ventilated for 5 h in a plethysmograph system allowing measurement of dynamic lung-thorax compliance. Postnatally the animals received one intratracheal injection (5 ml/kg) of R 5, nonspecific IgG, or normal saline. At 30 min all animals received a standard dose of an encapsulated GBS strain by intratracheal injection. The number of bacteria (mean log₁₀ CFU/g lung ± S.D.; CFU = colony forming unit) was evaluated in lung homogenates. Histologic lung sections were judged by light microscopy. Bacterial proliferation was similar in rabbits treated with the monoclonal antibody (9.33 ± 0.39; *n* = 14) and in control animals receiving saline (9.16 ± 0.35; *n* = 14) or nonspecific IgG (9.26 ± 0.31; *n* = 11). No significant differences were noted on the histologic analysis or in measurements of lung function. We conclude that intratracheal instillation of a monoclonal anti-SP-A antibody did not increase bacterial proliferation

in GBS-infected newborn rabbits. These findings suggest that SP-A does not play an important role in protection against encapsulated GBS strains in the neonatal period.

Key words: Antibody—Surfactant protein A—Group B streptococci—Pneumonia.

Introduction

Surfactant therapy has markedly improved the prognosis of premature newborn infants with respiratory distress syndrome (RDS). New targets for surfactant replacement therapy are currently being identified. Possible new indications include meconium aspiration syndrome, congenital pneumonia, pulmonary hemorrhage, early chronic lung disease, and acute respiratory distress syndrome (ARDS) in children and adults. In these conditions unlike neonatal RDS there is no primary surfactant deficiency caused by immaturity of the surfactant system [for review, see Ref. 27]. Substances present in the airways under pathologic conditions (like free fatty acids in meconium aspiration syndrome) or endogenous proteins (e.g. fibrinogen or albumin entering the airspaces because of capillary leakage in ARDS) may cause secondary surfactant dysfunction via inhibitory mechanisms [3, 12, 17].

In the commercially available modified natural surfactant formulations the large, relatively hydrophilic, surfactant proteins SP-A and SP-D are removed during preparation. In vitro, SP-A increases surfactant resistance to inhibition [1]. SP-A and SP-D stimulate alveolar macrophages in vitro and seem to be important factors in the lung defense system [24, 29]. In a recent study [20], we showed that a monoclonal antibody to surfactant protein A (R 5) increased the sensitivity of endogenous pulmonary surfactant to inactivation by fibrinogen not only in vitro but also in vivo.

We used a monoclonal antibody to inactivate SP-A to test the participation of SP-A in the handling of bacterial infections in the neonatal lung. Our hypothesis was that the blocking of endogenous SP-A by this antibody in ventilated near term rabbit fetuses with experimental neonatal group B streptococcal (GBS) pneumonia [8] would promote bacterial growth and impair lung function.

Materials and Methods

Animals

Pregnant New Zealand White rabbits were obtained from local suppliers. Rabbit fetuses were delivered by Cesarean section at a gestational age of 29.5 days. Term gestation for rabbits is 30–31 days. At 29.5 days the animals have mature lung function [22].

Antibody

R 5 rat monoclonal antibody to rabbit SP-A was generated and purified as reported elsewhere [19, 21]. The final product contained >99% IgG as judged by sodium dodecyl sulfate-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 [see Ref. 20]. In vitro measurements of surface activity of a "complete," SP-A containing natural rabbit surfactant preparation (produced by lavage and sucrose gradient centrifugation [30]) in the pulsating bubble surfactometer demonstrated a significant increase in surface tension after the addition of R 5 anti-SP-A antibody [20]. We estimated the pool size of endogenous SP-A in term newborn rabbits to be 0.5 $\mu\text{g/g}$ body weight, based on the data of Stevens and colleagues [18]. 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 18-mer form, 100 μg of the antibody is a very large molar excess.

Bacteria

An abundantly encapsulated low density (LD) phase variant of GBS was processed from the reference strain GBS 090 Ia Colindale by repeated gradient centrifugation [4]. The strain is a kind gift from Stellan Håkansson, University of Umeå, Sweden. The strain was stored in aliquots at -70°C , precultured, washed, centrifuged, and suspended in saline at a concentration of 10^9 live bacteria/ml. 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 after a 24-h incubation period at 37°C in an atmosphere with 5% CO_2 . Details have been described elsewhere [8].

Experimental Protocol

Ventilator System. Near term rabbit fetuses were delivered, anesthetized, and tracheotomized at birth and transferred to a warmed plethysmograph system [22] as described previously [8]. They were ventilated in parallel in sealed Plexiglas chambers with a common ventilator system (Servo 900 B) delivering 100% oxygen. The working (= maximum) pressure was set at 50 cmH_2O . The frequency was 40/min, the inspiration/expiration time ratio was 1:1. No positive end-expiratory pressure (PEEP) was applied, as PEEP might mask differences in compliance because of variations in surfactant function. The peak inspiratory pressure was recorded with a pressure transducer (EMT 34) and adjusted individually for each animal to obtain a tidal volume of 8–10 ml/kg of body weight. Tidal volume was recorded with a specially designed "Fleisch-tube," a differential pressure transducer (EMT 31), an integrator (EMT 32), an amplifier (EMT 41) and a recording system (Mingograf 81; all equipment, Siemens-Elementa, Solna, Sweden). Lung-thorax compliance ($\text{ml/kg} \times \text{cmH}_2\text{O}^{-1}$) was calculated from the quotient of tidal volume (expressed in ml/kg) and peak inspiratory pressure (expressed in cmH_2O). All 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 $>100/\text{min}$ without evidence of arrhythmia or atrioventricular block.

Experimental Groups. At birth the animals were allocated in random order to three different treatment groups. Using a specially designed tracheotomy tube with an indwelling wedged plastic catheter tubing (inner diameter, 0.75 mm, Portex®, Hythe, Kent, UK) 5 ml/kg body weight of the antibody preparation (corresponding to 75 mg/kg IgG) was injected immediately postnatally into the liquid-filled lungs of the animals. Controls received the same dose and volume of a nonspecific rat IgG preparation (Sigma Chemicals, St. Louis, MO, USA) or normal sterile saline. The animals were connected to the ventilator system. At 30 min all experimental groups received an intratracheal bolus injection of 5 ml/kg 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 three times 10 ml/kg body weight of air with a microsyringe. The three experimental groups that originated from this procedure were as follows:

1. Antibody-treated infected animals (anti-SP-A/GBS)
2. IgG (nonspecific)-treated infected rabbits (IgG/GBS)
3. NaCl-treated infected controls (NaCl/GBS)

The animals were ventilated for 5 h. At the end of the experiments the animals were killed, and the chest was opened with sterile instruments after the diaphragm had been examined for the evidence of pneumothorax.

Blood from the right cardiac ventricle was aspirated for a blood culture (Bactec Plus blood culture system, Becton Dickinson, Sparks, MD, USA). A heparinized sample was taken for blood gas analysis. The left lung was excised, weighed, placed immediately into the sterilized tube of a tissue homogenizer (Kontes Scientific Glassware Instruments, Vineland, NJ, USA), and stored on ice until further processing.

Bacterial Counting. The weight of the lung specimens was adjusted to 1 g with sterile 0.9% NaCl. 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 suspensions were spread on blood agar plates. Colony counting was performed after a 24-h incubation. Because bacterial proliferation follows a logarithmic growth curve, the results were expressed as mean \log_{10} CFU/g of lung (wet weight).

Histologic Examination of the Lungs. 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 cmH₂O via the tracheotomy. After 60 s this pressure was lowered to 10 cmH₂O 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 cmH₂O. The lungs were stored in 4% formaldehyde and subsequently embedded in paraffin. Transverse sections, stained with hematoxylin-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 (V_v) in histologic sections was evaluated by a conventional point-counting method with total parenchyma as the reference volume as described previously [16]. The coefficient of variation (CV) of alveolar V_v was calculated by the standard formula from the mean and the S.D. CVV_v is a measure of the field-to-field variability of alveolar expansion. The slides were coded so that the evaluator was unaware of the experimental conditions of the individual animals.

Ethical Approval. The study design and the management of the animals complied with national legislation. The trial protocol was approved by the local committee for animal research.

Statistical Analysis

Data are given as mean \pm S.D. Values for lung weight and physiologic data were subjected to analysis of variance (ANOVA) using the CRISP software program (Crunch Software, San Francisco, CA, USA). Between-group differences were evaluated by *t*-test. Differences in the incidence of complications between the groups were analyzed with the χ^2 test. The limit level of statistical significance was defined as $p = 0.05$.

Results

Characterization of the Experimental Animals

Thirty-nine rabbits from six litters were included in the final data analysis. Fourteen animals received the SP-A antibody (anti-SP-A/GBS), 11 nonspecific rat IgG (IgG/GBS), and 14 saline (NaCl/GBS). Eight fetuses (two in the anti-SP-A/GBS group, four in the IgG/GBS group, and two in the NaCl/GBS group) were not included because they demonstrated ECG abnormalities already at 30 min ($n = 2$) or did not survive the 5-h ventilation period. There were no significant differences in body weight, lung weight, and other physiologic measures in survivors at the end of the experiment (Table 1).

Table 1. Characterization of animals in the experimental groups

Parameter	Anti-SP-A/GBS (<i>n</i> = 14)	Rat IgG/GBS (<i>n</i> = 11)	NaCl/GBS (<i>n</i> = 14)
Treatment at birth	Monoclonal anti-SP-A, 5 ml/kg	Nonspecific rat IgG, 5 ml/kg	Sterile 0.9% NaCl, 5 ml/kg
Treatment at 30 min	GBS, 5 ml/kg	GBS, 5 ml/kg	GBS, 5 ml/kg
Body weight (g)	48 ± 8	46 ± 7	43 ± 12
Left lung weight (g)	0.45 ± 0.12	0.44 ± 0.11	0.42 ± 0.15
Final heart rate (min ⁻¹)	209 ± 65	223 ± 33	221 ± 57
Final pCO ₂ (kPa)	7.0 ± 1.8	8.2 ± 1.8	6.7 ± 0.8

Values are the mean ± S.D.

Bacterial Proliferation

All animals were infected with a similar number of bacteria. The number of bacteria applied was 8.79 ± 0.28 (mean ± S.D. log₁₀ CFU/rabbit) in the anti-SP-A/GBS group, 8.71 ± 0.35 in the IgG/GBS group, and 8.72 ± 0.30 in the NaCl/GBS animals. A significant proliferation of bacteria occurred during the experimental period (Fig. 1). Assuming a uniform distribution of the instilled bacteria between the right and the left lung and a total lung weight of 1 g, the estimated average increase in bacterial numbers was 845% for anti-SP-A/GBS animals, 653% for the IgG/GBS and 585% for the NaCl/GBS group. After logarithmic transformation these differences in bacterial proliferation did not reach the limit level of statistical significance (Fig. 1). With two exceptions (one in the anti-SP-A/GBS group and one in the IgG/GBS group) all blood cultures were GBS positive at the end of the experiment.

Lung Function

After connection of the animals to the ventilator and an initial stabilization period the first compliance values were calculated at 15 min. At 15 and 30 min IgG and anti-SP-A-treated animals had similar compliance values. The values in the NaCl controls were considerably higher (Fig. 2). The difference approached statistical significance ($p = 0.07$ at 15 min, and $p = 0.06$ at 30 min vs the IgG/GBS group). Compliance values were similar in the different groups throughout the rest of the experimental period (Fig. 2).

Histology

The histologic examination did not detect differences among the experimental groups. Lung expansion patterns were similar among the different treatment groups. Volume density (V_v) of the alveolar spaces and the coefficient of variation of alveolar volume density (CVV_v) are demonstrated in Table 2. Hyaline membrane formation, the presence of epithelial necrosis, and the severity of the inflammatory reaction were similar in the different groups.

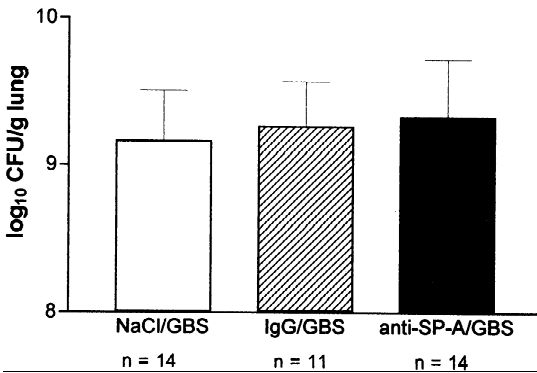


Fig. 1. Bacterial proliferation at the end of the experiment in the different groups. Values are mean \log_{10} CFU/g lung \pm S.D.

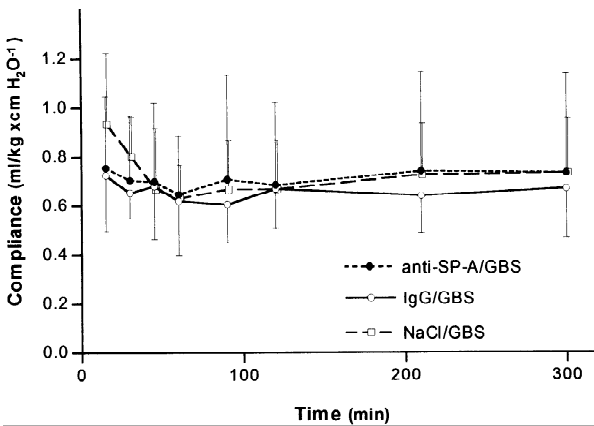


Fig. 2. Lung-thorax compliance in different experimental groups.

Table 2. Results of the histologic evaluation of alveolar expansion in the experimental groups

Parameter	Anti-SP-A/GBS, <i>n</i> = 14	Rat IgG/GBS, <i>n</i> = 11	NaCl/GBS, <i>n</i> = 14
V _v	0.57 \pm 0.09	0.54 \pm 0.10	0.59 \pm 0.08
CVV _v	0.23 \pm 0.07	0.25 \pm 0.08	0.21 \pm 0.08

Alveolar volume density (V_v) and coefficient of variation of V_v (CVV_v) were determined by a point counting method [16] with total lung parenchyma as a reference volume. Values are the mean \pm S.D.

Discussion

In the last decade the structure and function of the proteins of the surfactant system have been well characterized [9]. The availability of recombinant surfactant preparations [14] with proteins or protein fragments has been connected with the hope of “designer surfactants” allowing treatment of different kinds of pulmonary diseases with surfactant preparations that are optimized in terms of surfactant protein and lipid

composition. In theory a surfactant suitable for treatment of RDS in a premature infant may not be optimal for treating a patient with ARDS caused by infectious lung disease. It has been shown that the surfactant proteins SP-B and SP-C are of prime importance for the physiologic function of the surfactant system [9]. Inactivation of SP-B by monoclonal antibodies results in immediate respiratory distress and destruction of a functioning surfactant film [15]. SP-B deficiency as a disease (congenital alveolar proteinosis) or as an experimental model (SP-B knockout mice) is a lethal condition. However, after lung transplantation in children with congenital alveolar proteinosis, anti-SP-B antibodies were detectable without impairment in lung function [6].

On the other hand, inactivation of SP-A in animal models by antibodies does not lead to changes in lung function or morphology [10, 20]. As reported recently, alveolar levels of surfactant seem to be normal in SP-A knockout mice [11]. Although SP-A seems to participate in the formation of tubular myelin and in regulating surfactant metabolism, application of an anti-SP-A antibody did not lead to deterioration of lung function in our model. Initial compliance values at 15 and 30 min tended to be lower both in the anti-SP-A and the controls receiving nonspecific IgG, indicating a slight inhibitory effect of the immunoglobulins on surface activity, which has been described by others before [3].

In vitro SP-A stimulates alveolar macrophages, and it increases opsonization, phagocytosis, chemotaxis, and killing [23, 25]. These effects can be blocked by applying anti-SP-A antibodies [24, 26]. However, the observed stimulation seems to be mediated by special receptors on alveolar macrophages [13], so that there is no stimulation of other immune-competent cells like monocytes, peritoneal macrophages, or neutrophilic granulocytes. Recent experiments indicate that SP-A is effective in defense against certain pathogens (e.g. influenza A) but less effective [25] against others (e.g. influenza B virus). The stimulatory effect seems to be dose dependent with considerable differences in the dose-response curves for different infectious agents [23, 25].

In our study we could not demonstrate any deleterious effect on lung function or bacterial proliferation by intratracheal application of a monoclonal anti-SP-A antibody. Although alveolar macrophages are the predominant phagocytic cell in the adult lung [2], newborn rabbit lungs are relatively deficient in alveolar macrophages so that neutrophils provide an important second line of defense in bacterial pneumonia [5]. However, SP-A does not exert a stimulatory effect on neutrophils [24].

The model used for the present experiments has certain limitations. The GBS strain used in our experiments is a "smooth strain," surrounded by a polysaccharide capsule that protects the bacteria from phagocytosis as long as opsonins are not present [7]. The capsule appears to be a major virulence factor [4] also in neonatal GBS infections. Therefore, these types of studies may not be extrapolated to conclusions regarding "rough" (unencapsulated) GBS strains or other bacteria. To induce reproducible pneumonia within a time span of 5 h of ventilation an infectious dose of 10^9 bacteria was applied intratracheally. This is undoubtedly somewhat different from the normal natural time course of an ascending infection. However, such an overwhelming infection is a well described feature of neonatal GBS infections. Despite antibiotic treatment and neonatal intensive care, mortality rates of up to 80% are described in premature babies with GBS infection [28]. Applying a lower infectious bacterial in-

oculum to spontaneously breathing animals might result in a localized pulmonary infection. Under such conditions more macrophages might be attracted to the airspaces. Therefore, other pneumonia models with different animals and/or different bacteria at different doses might produce different results.

We conclude that application of the monoclonal anti-SP-A antibody R 5 does not impair lung function or alter bacterial growth patterns in experimental neonatal group B streptococcal pneumonia. Our findings suggest that SP-A is only of limited importance in host defense against severe systemic neonatal infections with encapsulated group B streptococci.

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