## Virulence Role of Group B Streptococcus β-Hemolysin/Cytolysin in a Neonatal Rabbit Model of Early-Onset Pulmonary Infection

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We examined the virulence role of group B Streptococcus (GBS)  $\beta$ -hemolysin/cytolysin ( $\beta$ h/c) in a neonatal-rabbit model of GBS pulmonary infection. Rabbits infected intratracheally with wild-type (wt) GBS developed focal pneumonia and, by 18 h after infection, had 100-fold more bacteria in lung tissue than did rabbits infected with a  $\Delta\beta$ h/c mutant. Mortality (40% vs. 0%), development of bacteremia, and mean bacterial blood counts were all significantly higher in the rabbits challenged with wt GBS than in those challenged with the  $\Delta\beta$ h/c mutant. Lung compliance during mechanical ventilation was impaired after injection of wt GBS but not after injection of the  $\Delta\beta$ h/c mutant strain. This work, to our knowledge, provides the first in vivo evidence for a critical role of the  $\beta$ h/c toxin in GBS neonatal pneumonia and in the breakdown of the pulmonary barrier to systemic infection.

Group B *Streptococcus* (GBS) is the leading cause of serious bacterial infections in human newborns. Between 15% and 30% of healthy women are colonized with GBS in the genitourinary or lower gastrointestinal tract [1]. Vertical transmission of GBS from mother to infant can occur after ascending infection of the placental membranes or aspiration of contaminated vaginal fluids during delivery. Early-onset GBS disease is typically heralded by the development of respiratory symptoms in the first

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several hours of life, reflecting an initial pulmonary focus of infection. Pathologic changes in neonatal GBS pneumonia include bacterial and neutrophilic infiltrates, intraalveolar edema, hyaline membranes, focal atelectasis, and evidence of pulmonary epithelial and endothelial cell infiltrates [2]. In nearly all cases, the bacterium penetrates lung barriers to reach the blood-stream, which frequently results in complications such as septicemia and meningitis.

The specific molecular determinants that allow GBS to breach the lung barrier and produce systemic infection are unknown. A candidate virulence factor that may play a role in this process is the GBS  $\beta$ -hemolysin/cytolysin ( $\beta$ h/c). This surface-associated toxin is produced by the vast majority of GBS clinical isolates and generates the zone of  $\beta$  hemolysis surrounding colonies plated on blood agar media. The cylE gene in the GBS chromosome is required for  $\beta$ h/c production and is sufficient to confer  $\beta$  hemolysis when cloned in *Escherichia coli* [3]. The association between cylE and βh/c production has been confirmed in multiple GBS strains of varying serotypes and by single-gene complementation to restore wild-type (wt) toxin production levels [3]. In tissue culture studies, GBS  $\beta$ h/c has been shown to produce direct cytolytic injury to human pulmonary epithelium and endothelium by forming pores in the target cell membrane [4]. Subcytolytic concentrations of  $\beta$ h/c can also promote GBS invasion of cultured lung epithelial cells and trigger the release of the neutrophil chemokine interleukin-8 (IL-8) [5]. The phospholipid dipalmotyl phosphatidylcholine (DPPC), a major component of human surfactant, inhibits GBS βh/c-mediated cytotoxicity, invasion, and IL-8 activation, providing a potential link to the increased susceptibility of premature, surfactant-deficient neonates to lung damage and sepsis from GBS infection [4, 5]. GBS  $\beta$ h/c production has been clearly associated with increased virulence in intravenous challenge of mice and rabbits [6, 7], but these experiments have bypassed the lung and, thereby, the natural route of neonatal infection.

In this present study, we have developed a novel neonatal-rabbit model of early-onset GBS pneumonia and sepsis through direct transcutaneous instillation into the trachea. Using a wt strain and its isogenic cylE knockout  $\beta$ h/c-deficient ( $\Delta\beta$ h/c) mutant, we specifically examine the contribution of the  $\beta$ h/c toxin to GBS pneumonia, lung function, and disruption of the pulmonary barrier to bloodstream infection.

*Materials and methods.* The *wt* GBS strain used in these studies was NCTC10/84 (1169-NT1), a serotype V isolate from the blood of a septic neonate [8]. The corresponding  $\Delta\beta$ h/c mutant NCTC:*cyl*ΕΔ*cat* was generated by precise in-frame allelic

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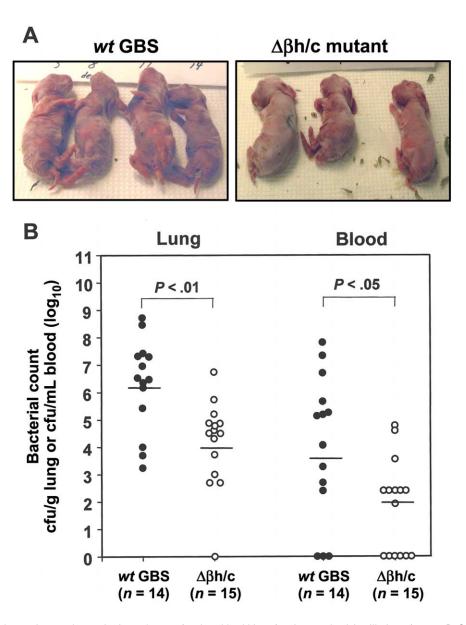
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exchange mutagenesis [3]. GBS strains were grown to logarithmic phase (OD<sub>600</sub> = 0.4, or  $\sim$ 1 × 10<sup>8</sup> cfu/mL) in Todd-Hewitt broth (THB), washed in PBS, then resuspended in PBS at the appropriate concentration for use in the infectious challenges.

Pregnant New Zealand white rabbit does were obtained from Irish Farms and were acclimated for 5-7 days before term (day 31). Does were allowed to deliver litters normally and to nurse pups for up to 24 h. Pups were then removed, kept under a radiant warmer bed, and sedated with ketamine/xylazine (25/ 8 mg/kg) subcutaneously. The mean  $\pm$  SD weight of rabbit pups used for these studies was  $0.059 \pm 0.017$  kg, with a range of 0.025-0.106 kg. Skin was sterilized with isopropyl alcohol, and the GBS suspension (5 mL/kg) was injected percutaneously into the trachea. The standard dose was  $\sim 3 \times 10^7$  cfu/kg (range,  $2.5 \times 10^7 - 5.0 \times 10^7$  cfu/kg); a higher inoculum of  $\sim 3 \times 10^9$  cfu/ kg was used for a small number of additional histopathologic studies. Treated rabbits were immediately provided with supplemental oxygen, allowed to recover on the warmer bed, and then returned to the doe for 18 h. Rabbit pups were observed for respiratory function, color, and signs of distress, and any that appeared to be in extremis before the end of the study were euthanized. At the 18-h end point, surviving rabbit pups were sedated with ketamine/xylazine and anesthetized with pentobarbital (10 mg/kg) intraperitoneally before placement of a blunt 18-gauge needle as a tracheostomy tube. Rabbits were then transferred to a specially built Plexiglas plethysmograph (Ridout Plastics) warmed to 39°C and were ventilated in parallel (InfantStar 950; Infrasonics) under 100% O2. Ventilatory settings were as follows: rate, 30 breaths/min; maximum inspiratory pressure, 15 cm H<sub>2</sub>O; and end expiratory pressure, 2 cm H<sub>2</sub>O. Flow into and out of the plethysmograph was measured with a pneumotachograph and pressure transducer (model LCVR ± 2 cm H<sub>2</sub>O; Celesco). Airway pressure was measured with a pressure transducer (model LCVR ± 50 cm H<sub>2</sub>O; Celesco). Respiratory system compliance  $(C_{rs})$  was calculated by integrating the flow data and dividing the integrated flow by the change in airway pressure. After 25 min of ventilation, rabbits were euthanized with an overdose of pentobarbital. The chest cavity was opened, the right bronchus was ligated with a suture, and the right lung was excised and homogenized using a Tissue Tearor (BioSpec Products). A cardiac puncture was performed for blood collection. Bacterial counts were determined by plating serial dilutions of lung homogenate or blood on THB agar for overnight incubation and enumeration of colony-forming units. The left lung was fixed at 20 cm H<sub>2</sub>O of pressure in 3.7% formalin and embedded in paraffin, and the sections were stained with hematoxylin-eosin for histologic analysis. All aspects of this protocol were reviewed and approved by the Animal Subjects Committee at the University of California at San Diego. Statistical analysis was performed by nonparametric Mann-Whitney U test, with P < .05 considered to be significant.

Results. Rabbit pups in their first day of life were infected intratracheally with an equivalent inoculum  $(3 \times 10^7 \text{ cfu/kg})$  of wt GBS (n = 23), the isogenic  $\Delta \beta$ h/c mutant (n = 15), or saline alone (control, n = 4). Within 6 h of injection, several rabbits infected with wt GBS showed tachypnea and labored breathing, clinical findings that were absent in the  $\Delta\beta$ h/c mutant–infected rabbits and controls. By 18 h after injection, 9/23 (39%) of the wt GBS-infected rabbits had died, and the remainder of the treatment group exhibited obvious cyanosis, lethargy, and respiratory distress (figure 1A). In contrast, no rabbits challenged with the  $\Delta\beta$ h/c mutant or saline alone died, and all were normal in appearance, with minimal or no respiratory symptoms at 18 h after injection (figure 1A). Quantitative cultures for GBS were performed on lung tissue; all surviving rabbits were euthanized, and blood was collected. Compared with wt GBS-infected rabbits, rabbits challenged with the GBS  $\Delta\beta$ h/c mutant had mean GBS counts in the lungs that were 100-fold lower (>1.0  $\times$  10<sup>6</sup> vs.  $1.0 \times 10^4$  cfu/g lung tissue; P < .01) and mean levels of bacteremia that were 100-fold lower (>1.0  $\times$  10<sup>4</sup> vs. <1.0  $\times$  10<sup>2</sup> cfu/ mL blood; P < .05) (figure 1B). Forty percent of rabbits challenged with the  $\Delta\beta$ h/c mutant did not have detectable (<20 cfu/ mL) GBS in the bloodstream. These results demonstrate that the  $\beta$ h/c toxin contributes to GBS proliferation in lung tissues, promotes penetration of the pulmonary and epithelial barriers, and produces high lethality in the neonatal infection model.

Sections of lung tissues from wt GBS-infected,  $\Delta\beta$ h/c mutant-infected, and control rabbits were examined at 18 h after injection for histopathologic changes. Scattered foci of neutrophil infiltration were observed throughout the alveolar epithelium of wt GBS-infected rabbits (figure 2A, lower inoculum), whereas such changes were extremely rare in the rabbits challenged with the  $\Delta\beta$ h/c mutant (figure 2A, lower inoculum) and in saline-treated controls (data not shown). On histologic examination, alveolar expansion was notably diminished in the wt GBS-infected rabbit lungs, compared with those from rabbits infected with the  $\Delta\beta$ h/c mutant (figure 2*A*, *lower inoculum*). However, diffuse or severe bronchopneumonia was not observed in any group, and the alveolar architecture appeared relatively intact. Because wt GBS-infected rabbits consistently developed high-grade bacteremia, our results demonstrate that severe epithelial and endothelial barrier injury is not a prerequisite for GBS systemic dissemination. Severe early-onset GBS pneumonia can be associated with very high bacterial densities  $(1 \times 10^9 - 1 \times 10^{11})$ cfu/g lung tissue) [9]. Therefore, for comparison, we infected an additional group of rabbit pups with a 100-fold higher GBS inoculum ( $\sim 3 \times 10^9$  cfu/kg body weight). In these experiments, wt GBS-infected rabbits developed severe respiratory distress and cyanosis by 6 h after injection, at which point rabbits were euthanized for lung histopathologic analysis. Numerous foci of bacterial infiltration were present (figure 2A, higher inoculum), with clear bronchopneumonia and dense neutrophilic infiltration

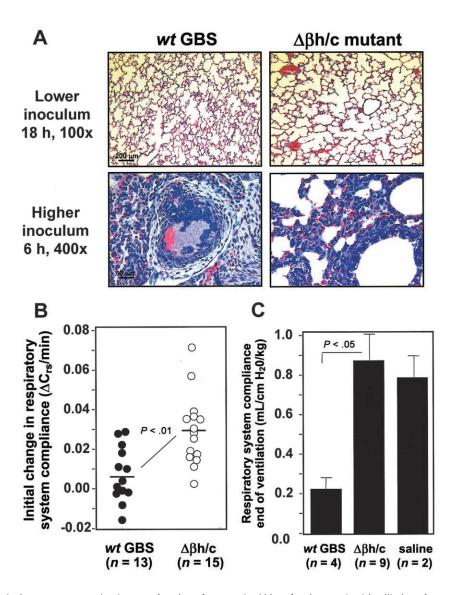


**Figure 1.** Clinical observations and quantitative cultures of 1-day-old rabbits after intratracheal instillation of group B *Streptococcus* (GBS). *A,* Appearance of rabbits 18 h after intratracheal instillation of wild-type (wt) or  $\beta$ -hemolysin/cytolysin-deficient ( $\Delta\beta$ h/c) mutant strains. *B,* Scatterplot of quantitative culture data collected from lung and blood of rabbits injected with either wt or  $\Delta\beta$ h/c mutant GBS for 18 h. Data are the logarithm of the mean bacterial counts in lung (cfu/g) or blood (cfu/mL); horizontal lines denote the geometric mean of the data points.

of the alveoli. Evidence of neutrophilic infiltration was also present in rabbits infected with the higher dose of the  $\Delta\beta$ h/c mutant (figure 2*A*), although at a markedly reduced severity compared with that in the *wt* GBS–infected rabbits.

To assess the contribution of the  $\beta$ h/c toxin to lung dysfunction in early-onset GBS infection, rabbit pups surviving to 18 h after injection were sedated, tracheostomized, and ventilated in a warmed plethysmograph.  $C_{rs}$  was calculated from volume and pressure changes during ventilation and was analyzed by 2 methods. We first calculated the change in  $C_{rs}$  over time (slope) through the initial ventilation phase (0–10 min), during which a rapid increase in ventilator-induced alveolar

expansion should occur [10, 11]. We found that rabbits infected with wt GBS had a significantly reduced initial  $C_{rs}$  slope, compared with that measured for rabbits infected with the  $\Delta\beta$ h/c mutant (figure 2B). An additional compliance measurement was taken for several rabbits at the end of the 25-min ventilation period. Whereas rabbits challenged with the  $\Delta\beta$ h/c mutant were able to increase  $C_{rs}$  to a level comparable to that in saline-treated controls, rabbits infected with wt GBS failed to do so (figure 2C). These data indicate that, even though only limited foci of focal pneumonia were observed at the 18-h time point, the expected alveolar expansion with mechanical ventilation was distinctly impaired in the wt GBS-infected rabbits. Our



**Figure 2.** Histopathologic assessment and pulmonary function of neonatal rabbits after intratracheal instillation of group B *Streptococcus* (GBS). *A*, Histopathology of lungs, demonstrated by hematoxylin-eosin staining after intratracheal instillation of either a low (~  $3 \times 10^7$  cfu/kg; 18 h) or a high (~  $3 \times 10^9$  cfu/kg; 6 h) inoculum of wild-type (wt) or β-hemolysin/cytolysin–deficient ( $\Delta\beta$ h/c) mutant GBS strains. *B* and *C*, Respiratory system compliance ( $C_{rs}$ ) values of neonatal rabbits 18 h after injection with either wt or βh/c strains of GBS. *B*, Scatterplot of the slope of the  $C_{rs}$  values, presented as the change in  $C_{rs}$  per minute. Data were collected during the first 10 min of ventilation, from time 0 (<1 min of ventilation) to 5–10 min. Lines drawn among data points represent mean values for each group. *C*, Mean ± SE  $C_{rs}$  values of infected neonatal rabbits after 25 min of ventilation.

findings may reflect a direct effect of the  $\beta$ h/c toxin on pulmonary function or, alternatively, an indirect effect related to the increased numbers of wt versus mutant GBS resulting from  $\beta$ h/c-mediated resistance to immunological clearance.

**Discussion.** The pathogenesis of GBS pneumonia and the consequent breakdown of the lung barrier to systemic infection are not well understood. The histopathologic changes observed in autopsy studies and in a primate model of intraamniotic infection [9] reveal widespread injury to lung cells, which could be the direct result of bacterial toxins or the indirect result of the induced inflammatory response. Moreover, GBS can invade

alveolar epithelial and pulmonary endothelial cells, a process that could facilitate bloodstream entry even through intact cellular barriers [12, 13]. In premature infants, immature host defenses and the lack of pulmonary surfactant represent additional predisposing factors leading to a greatly increased risk of severe GBS pneumonia and systemic disease.

We believe that our neonatal model of GBS disease can add to our understanding of the common clinical scenario in which the neonate aspirates GBS-contaminated fluids in utero or during delivery and then gradually develops increasing respiratory symptomatology within the first several hours of life. Our results indicate that the  $\beta$ h/c toxin plays a critical role in GBS-induced pulmonary dysfunction and in the ability of the pathogen to penetrate the lung barrier, establish bacteremia, and produce high mortality. The deleterious effects of  $\beta$ h/c in the initial pulmonary stages of neonatal GBS infection likely reflect a combination of the toxin's cytolytic, proinvasive, and proinflammatory properties, with the ultimate balance of the individual virulence phenotypes dependent on the magnitude and tempo of bacterial exposure.

Our discovery of a key role for the  $\beta$ h/c mutant in the pathogenesis of early-onset pneumonia may explain the observed benefits of surfactant preparations in reducing GBS infection in ventilated animal models [14] and in retrospective studies of human infants receiving empiric replacement therapy [15]. Our model will facilitate future studies examining the pathophysiology of neonatal pneumonia and will help to determine whether DPPC or other anti- $\beta$ h/c therapies could benefit infants experiencing, or at high risk of developing, early-onset GBS infection.

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