Group B Streptococcal β-Hemolysin/Cytolysin Promotes Invasion of Human Lung Epithelial Cells and the Release of Interleukin-8

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Pneumonia and lung injury are hallmarks of early-onset neonatal group B streptococcal (GBS) infections. Production of a β-hemolysin/cytolysin (β-h/c) encoded by the *cylE* gene is associated with GBS virulence in vivo. To elucidate the contribution of the β-h/c toxin to lung injury, the interactions of GBS wild-type strains and isogenic *cylE* mutants with A549 lung epithelial cells were examined. Compared with wild-type GBS strains, *cylE* mutants did not produce cytolytic injury, even at high inocula, and exhibited decreased cellular invasion. Additionally, *cylE* mutants induced less A549 cell release of the neutrophil chemoattractant interleukin (IL)–8. GBS invasion and IL-8 induction were significantly reduced in the presence of dipalmotyl phosphatidylcholine, a major constituent of lung surfactant and a known inhibitor of β-h/c activity. These data indicate that the GBS β-h/c contributes to invasion and immune activation of lung epithelial cells and may represent a multifunctional virulence factor in the early pulmonary stages of GBS infection.

Group B streptococcal (GBS) infection is the leading cause of serious bacterial infections in human newborn infants [1]. Early-onset GBS disease is heralded by respiratory signs, and 80% of autopsy cases show histological evidence of lobar or multilobar pneumonia [2, 3]. GBS pneumonia is characterized by evidence of severe lung injury, including dense bacterial and neutrophilic infiltrates, alveolar hemorrhage, proteinaceous exudate, and hyaline membrane formation [4, 5]. Premature, low–birth weight infants are at especially high risk for acquiring GBS pneumonia and invasive disease [6].

The lung is the apparent portal of entry for group B streptococci (GBS) into the bloodstream, after which septicemia may ensue [1, 7]. To access the circulation, the bacteria must first breach 2 host cellular barriers—the alveolar epithelium and the pulmonary capillary endothelium. A relevant virulence mechanism involves the ability of GBS to penetrate and to survive intracellularly within these host cells. GBS invasion of alveolar epithelial cells and pulmonary endothelial cells has been clearly demonstrated in human tissue culture lines and in the primate model of early-onset infection [8–10]. In severe pulmonary infections, direct cytotoxicity from bacterial factors, along with byproducts of the neutrophil inflammatory response, would likely further compromise the integrity of the neonatal mucosal barrier [11].

Most GBS clinical isolates exhibit a β-hemolytic phenotype when plated on blood agar [12]. The GBS β-hemolysin is a pore-forming membrane toxin capable of injuring a broad range of eukaryotic cell types [13, 14]. Because of the notable direct correlation between the level of GBS β-hemolysin production and cytolytic injury to human alveolar epithelial [15] and pulmonary capillary endothelial cells [16], the designation β-hemolysin/cytoysin (β-h/c) has been applied to the toxin. GBS β-h/c–induced injury of lung epithelial cells is blocked by the phospholipid dipalmotyl phosphatidylcholine (DPPC) [15], the major component, by weight, of human pulmonary surfactant [17]. This observation provides a theoretical rationale by which the β-h/c toxin may contribute to the increased susceptibility of premature surfactant-deficient neonates to severe GBS disease [14, 15]. Animal model studies that have used GBS transposon mutants have correlated GBS β-h/c expression with disease severity. When compared with the corresponding wild-type parent strains, β-h/c–negative mutants were less virulent in adult mice after intranasal [18] or intravenous [19] inoculation, in neonatal rats after transthoracic inoculation [20], and in adult rabbits after intravenous administration [21].

The genetic basis for the GBS β-h/c phenotype has very recently been elucidated [22, 23]. A single gene, *cylE*, which encodes a novel 78-kD predicted protein product, is both necessary for GBS β-h/c production and sufficient to confer a β-hemolytic phenotype to *Escherichia coli* [23]. Discovery of GBS β-h/c gene *cylE* facilitates precise in vitro and in vivo analysis of the specific virulence role(s) of the β-h/c toxin during key steps in the infectious process. In the present study, we used targeted GBS *cylE* knockout mutants to study the effects of β-h/c ex-

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pression on the bacterium's ability to invade lung epithelial cells and trigger release of the chemokine IL-8.

Materials and Methods

Bacterial strains. COH1 (serotype III), A909 (serotype Ia), and NCTC 10/84 (serotype V) are well-characterized GBS clinical isolates from the blood or cerebrospinal fluid of neonates with sepsis [24–26]. These strains are representative of the 3 most common GBS serotypes associated with early-onset infection [1]. The isogenic β-h/c–deficient COH1:*cylE*.KO, A909:*cylE*.KO, and NCTC:*cylE*.KO are products of targeted plasmid integrational mutagenesis of the β-h/c structural gene, *cylE* [23]. COH1-13, which lacks the type III surface polysaccharide capsule, is a Tn916ΔE mutant of parent strain COH1 [27]. By use of plasmid integration [23], COH1-13:*cylE*.KO was created for these studies, to provide an unencapsulated, β-h/c–deficient double mutant. Bacteria were grown in Todd-Hewitt broth to log-phase (OD_{600}, 0.4, ~10^8 cfu/mL), washed twice in PBS, and then resuspended and diluted in cell culture medium for use in the cytotoxicity, cellular invasion, and IL-8 release assays.

Lung epithelial cell culture. A549 cells (American Type Culture Collection), a human type II alveolar epithelial carcinoma cell line, were maintained and passaged in RPMI 1640 tissue culture medium that contained 10% fetal calf serum. Monolayers were seeded into 96- or 24-well tissue culture plates (Corning) and were used experimentally within 24 h of establishing confluence. Monolayers were washed 3 times with PBS, and fresh culture medium was added immediately prior to assays.

Cytotoxicity assay. Cytotoxicity of GBS strains was determined by measuring release of the intracellular enzyme lactate dehydrogenase (LDH) from A549 monolayers in a 96-well microtiter plate assay, as described elsewhere [15].

Cellular invasion and adherence assays. Intracellular invasion of A549 monolayers GBS was measured by using an antibiotic protection method developed by Rubens et al. [9]. Companion assays were performed to measure total A549 cell–associated bacteria (invading and surface adherent) [28]. In these assays, the initial incubation period was shortened from 2 h to 30 min. Then, in place of the 2-h exposure to extracellular antibiotics, monolayers were washed 6 times with PBS before monolayer lysis and release of bacteria. In both cellular invasion and adherence assays, dilutions of the lysate were plated for counts of bacteria. Assays were performed in triplicate and were repeated 4 times. In phospholipid inhibition experiments, A549 medium with 500 μg/mL of DPPC (Sigma P-0763) was prepared by sonication and was used for the duration of the assay.

IL-8 induction assay. Log-phase GBS (1×10^4 to 1×10^8 cfu) were added to A549 cell monolayers in 24-well plates, which were centrifuged at 800 *g* for 10 min to place bacteria on the monolayer surface and then were incubated at 37°C in 5% CO_2 for the duration of the assay. For comparison of wild-type versus mutant bacteria, a standard inoculum of 1×10^6 cfu (MOI, 10:1) and an assay time of 4 h were used. The nonhemolytic, noninvasive *Lactococcus lactis* strain NZ9000 [29] and medium alone were used as negative controls; tumor necrosis factor (TNF)–α at 5 ng/mL was used as a positive control. In phospholipid inhibition experiments, 500 μg/mL of DPPC was added to the A549 cell medium. At the end of the exposure period, cell culture supernatants were collected, and bacteria were removed by centrifugation and stored at 4°C until IL-8 measurement. IL-8 from cell culture supernatants was measured by ELISA, as described elsewhere by Eckmann et al. [30]. IL-8 induction assays were performed in triplicate and were repeated 3 times.

Reverse-transcription polymerase chain reaction (RT-PCR) for IL-8 transcript. The standard IL-8 induction assay was performed as described above, with use of strain A909 and TNF-α as a positive control. At 0, 1, and 4 h, the supernatant was removed, and the A549 cell monolayers from each of 3 replicate wells were solubilized in 300 μL of Trizol reagent (Gibco/BRL) for RNA extraction, according to the manufacturer's protocol. Total cellular RNA (1 μg) was reverse transcribed at 42°C for 50 min in 20 μL of buffer that contained 50 m*M* Tris-HCl (pH 8.3); 75 m*M* KCl; 3 m*M* $MgCl_2$; dATP, dCTP, dGTP, and dTTP, each 1 m*M;* 10 m*M* dithiothreitol; 330 ng of random primers (pd[N]6; Amersham Pharmacia Biotech); and 200 U of SuperScript II (Gibco/BRL). Reactions were stopped by heating at 70°C for 15 min. The cDNA products (2 μL) were amplified by PCR in 50 μL of 20 m*M* Tris (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, each dNTP at 200 μ*M,* and each primer at 0.5 μ*M*. The reactions were heated to 95°C for 5 min and 55°C for 3 min before the addition of 2.5 U of Taq DNA polymerase (AmpliTaq Gold; Perkin Elmer). Cycling parameters were 72°C for 30 s, 94°C for 30 s, and 58°C for 1 min, for a total of 30 cycles. The primers and resulting PCR products for IL-8 (289 bp) and β-actin (661 bp) have been described elsewhere [31]. PCR products were analyzed by running reactions on 1% agarose/ethidium bromide gels. Negative controls for RT-PCR detection were performed by omission of reverse transcriptase from the cDNA synthesis reaction, to exclude amplification of contaminating DNA.

Statistical analyses. Percentage of invasion, percentage of total cell-associated bacteria, and IL-8 measurements are reported as the mean of all observations ± SE. To compare among strains or culture conditions, *t* tests were performed, using a statistical software package (Excel; Microsoft). $P < .05$ was considered to be significant.

Results

Cytolytic activity of GBS against A549 cells. The 3 wild-type GBS clinical isolates showed significant differences in A549 cytolytic activity, as measured by LDH release. After 4 h of incubation, complete monolayer lysis followed the initial MOI of 0.25:1 for the strongly hemolytic strain NCTC 10/84 (serotype V), 16–32:1 for the moderately hemolytic strain A909 (serotype Ia), and 128:1 for the weakly hemolytic strain COH1 (serotype III; figure 1). In contrast, no LDH release above baseline was detected for the nonhemolytic *cylE* mutants of each strain up to an MOI of 1028:1. These data confirmed the principal role of the β-h/c in GBS cytolytic activity [15] and provided parameters for subcytolytic bacterial inocula used in the quantitative cellular invasion and IL-8 assays to follow.

β-h/c expression contributes to GBS invasion of and adherence to A549 cells. The effect of eliminating β-h/c production

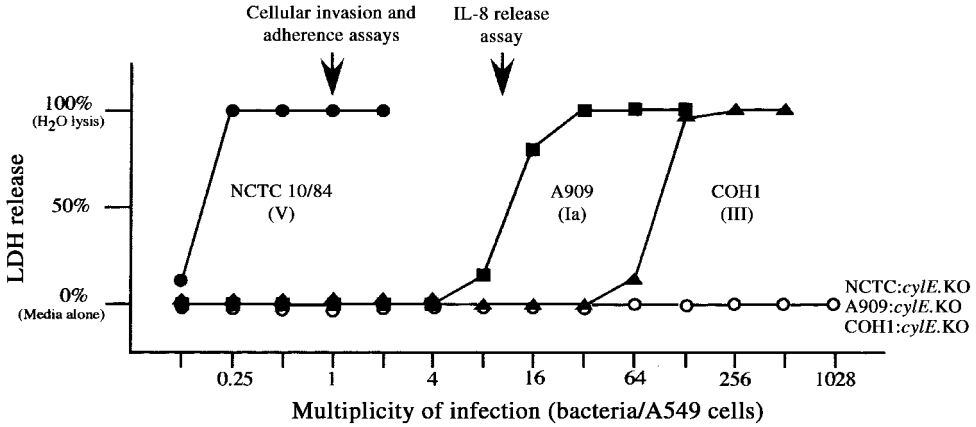

Figure 1. Group B streptococcal (GBS) injury to A549 lung epithelial cells, as measured by lactate dehydrogenase (LDH) release. Three different wild-type GBS strains of varying hemolytic activity were tested, along with their corresponding isogenic β-hemolysin/cytolysin mutants, by increasing MOI. Monolayers were exposed to the bacterial inoculum for 4 h before collection of supernatants for LDH determination by colorimetric assay. Arrows depict which MOI was used for subsequent experiments. IL, interleukin.

on the ability of GBS to intracellularly invade lung epithelial cells was studied in a quantitative assay. Invasion studies were performed at an MOI of 1:1 for 2 h, below the threshold for monolayer cytolysis by the GBS wild-type strains. All 3 wild-type strains grew equally well through the logarithmic phase in Todd-Hewitt broth (THB). However, after transfer to A549 cell tissue culture media under conditions mimicking those used in the invasion, adherence, and IL-8 assays, strain COH1 (III) grew at 125% of the rate of strain NCTC10/84 (V) and at 200% of the rate of strain A909 (Ia). The growth of the β-h/c mutants was similar to that of their respective parent strains, both in THB and after transfer to the tissue culture medium (data not shown). As shown in figure 2*A,* each isogenic-deficient β-h/c mutant exhibited a significant decrease in its ability to invade A549 cells, compared with that of the parent strain. To determine whether the decrease in intracellular invasion was due to a decrease in the ability of β-h/c mutants to adhere tightly to the A549 monolayer surface, a 30-min assay with stringent washing was performed to quantify total cell-associated (surface-adherent plus intracellular) bacteria. Figure 2*B* shows that significant decreases (20%–45%) in total cell-associated bacteria were observed with all β-h/c mutant cells, compared with the wild-type parent strains, which closely mirrored the intracellular invasion findings. As predicted by the LDH release assays, strain NCTC10/84 at an MOI of 1:1 produced evidence of monolayer cytotoxicity during the course of the adherence and invasion assays. We speculate that this cytotoxicity may have led to an underestimation of the wild-type NCTC10/84 invasion and adherence potential. Overall, we conclude that GBS strains that lacked β-h/c were attenuated in their ability to invade and adhere to A549 epithelial cells.

GBS stimulate IL-8 production by A549 cells in a time- and dose-dependent fashion. Because neutrophilic infiltration is a hallmark of GBS pneumonia, we hypothesized that exposure to GBS would trigger lung epithelial cells to release the neutrophil chemoattractant IL-8. Figure 3*A* shows the time course of IL-8 release from A549 cells exposed to 1×10^6 cfu of wild-type GBS strain A909 (MOI, 10:1). IL-8 release peaked at 4–6 h and then reached a plateau as bacterial cytoxicity to the mono-

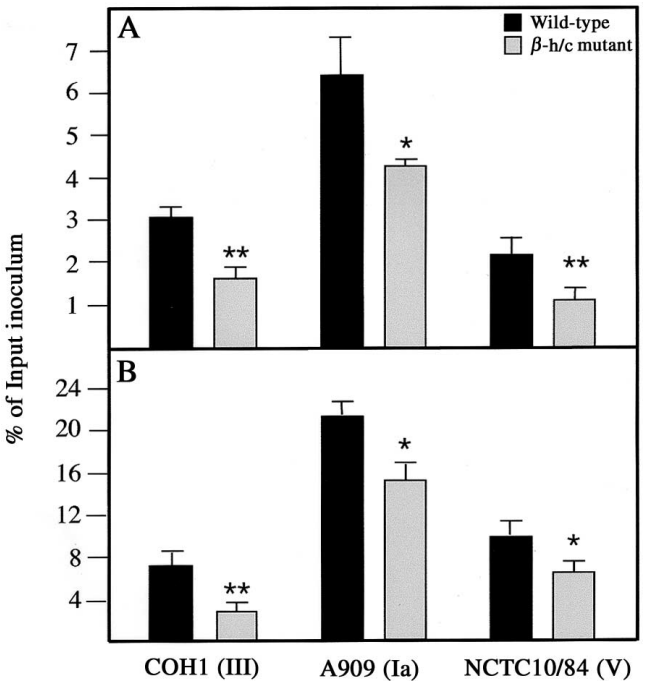

Figure 2. Effect of group B streptococcal β-hemolysin/cytolysin (β-h/c) expression on invasion of and adherence to A549 lung epithelial cells. *A,* Percentage of intracellular bacteria after 2 h of incubation followed by 2 h of antibiotic exposure to kill extracellular bacteria; *B,* percentage of total cell-associated bacteria after 30 min of incubation and stringent washing. MOI is 1:1 in both cases. Experiments were performed in triplicate wells. Data shown represent mean ± SE of 4 separate experiments. $*P < .05$; $**P < .01$.

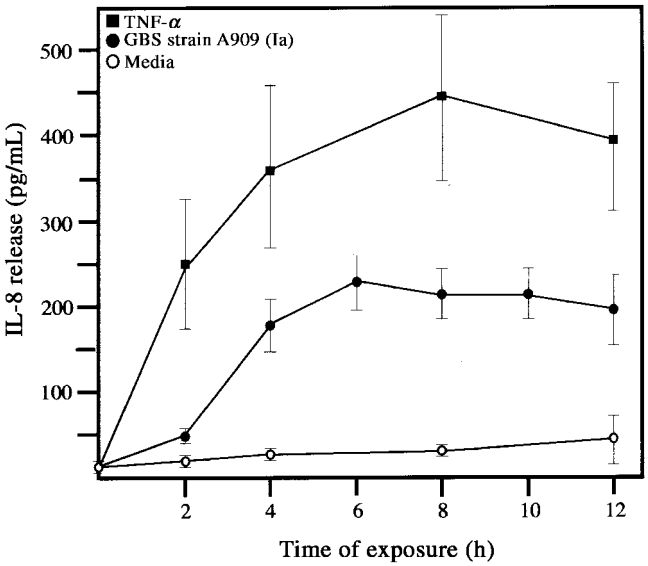

Figure 3. Time-dependent interleukin (IL)–8 release by A549 epithelial cells exposed to group B streptococcal (GBS) wild-type strain A909 (Ia) at an MOI of 10:1, as measured by ELISA. Supernatants were collected for IL-8 quantitation at the time points indicated. Tumor necrosis factor (TNF)–α at 5 ng/mL was used as a positive control, and tissue culture media served as a negative control.

layer became readily evident on microscopic examination. IL-8 released by A549 cells on exposure to GBS was ~50% the level observed with the TNF-α–positive control stimulus. Maximal IL-8 release was noted at an inoculum of 1×10^7 cfu (MOI, 100:1) for strain COH1 and at an inoculum of 1×10^6 cfu (MOI, 10:1) for strain A909 (data not shown). At a higher MOI for each strain, the level of IL-8 release decreased in association with β-h/c–induced monolayer injury. Our results indicate that subcytolytic inocula of GBS induce IL-8 production by A549 cells in a time- and dose-dependent fashion. RT-PCR was used to determine whether IL-8 was being induced at the mRNA expression level. Figure 4 shows that, although TNF-α induced immediate IL-8 mRNA expression, the specific increase in IL-8 mRNA levels after exposure to GBS was barely detectable at 1 h and maximal at 4 h.

GBS β-h/c promotes IL-8 induction. We sought to test the specific requirement of β-h/c expression in IL-8 induction by comparing isogenic β-h/c–deficient mutants with wild-type GBS parent strains. To avoid confounding effects of cytotoxicity on data interpretation, a standard bacterial inoculum (MOI, 10:1) and a 4-h exposure were selected for comparative analyses of IL-8 induction in response to GBS wild-type versus mutant strains. This inoculum resulted in complete monolayer destruction with the strongly hemolytic NCTC10/84 (figure 1); consequently, this strain was not used in IL-8 studies. As shown in figure 5, the β-h/c–deficient mutants A909:*cylE*.KO and COH1:*cylE*.KO elicited significantly lower levels of A549 cell IL-8 release than their respective β-h/c–producing parent strains. Because gram-positive bacterial cell wall components (e.g., lipotechoic acid and peptidoglycan) have been shown to activate host cytokine responses, we hypothesized that the polysaccharide capsule of GBS could itself be an activator (or, alternatively, could cloak more potent cell wall–stimulating factors). However, the isogenic capsule–deficient mutant COH1-13 did not differ from its parent strain COH1 in the level of IL-8 induction (figure 5). Consistent with earlier observations, the double capsule and β-h/c–deficient mutant COH1-13:*cylE*.KO induced markedly less IL-8 release than that induced by the capsule-deficient but β-h/c–producing COH1-13. Used as a control, the nonhemolytic and noninvasive *L. lactis* did not elicit significantly more IL-8 release than media alone. Thus, it appears that β-h/c production or bacterial invasion may be more important factors in the ability of GBS to induce IL-8 release from A549 cell monolayers than capsule or other cell wall components.

Surfactant phospholipid DPPC inhibits GBS A549 cell invasion and IL-8 induction. DPPC is the major constituent of human surfactant and is a known inhibitor of GBS hemolytic and cytolytic activity [14, 15]. Because we observed that GBS β-h/c activity was associated with increased lung epithelial cell invasion, adherence, and IL-8 release, we hypothesized that DPPC might inhibit these processes. Using a concentration (500 μg/mL) that approximates the DPPC level in the alveolar fluid of a term neonate, we added this phospholipid to standard cellular invasion and IL-8 induction assays, using wild-type

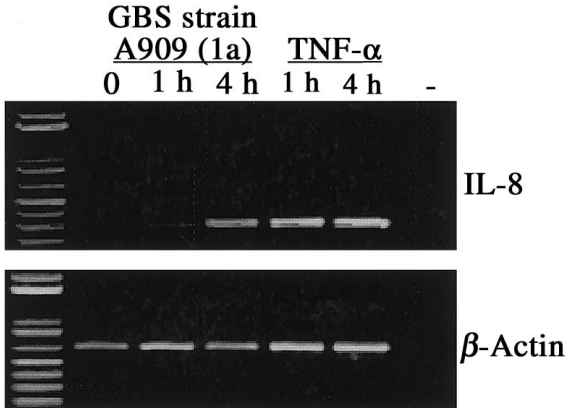

Figure 4. Production of interleukin (IL)–8 mRNA by A549 lung epithelial cells on exposure to wild-type group B streptococcal (GBS) strain A909 (Ia) and tumor necrosis factor (TNF)–α. Total cellular RNA was extracted at the time indicated and was reverse transcribed, and the resulting cDNA was amplified with IL-8– or β-actin–specific primers. The polymerase chain reaction (PCR) products for IL-8 (289 bp) and β-actin (661 bp) are shown. Negative controls for reverse-transcription PCR were performed on each RNA preparation by omitting reverse transcriptase from the amplification reaction to control for contamination of DNA. The control from the sample exposed to group B streptococci for 4 h is shown.

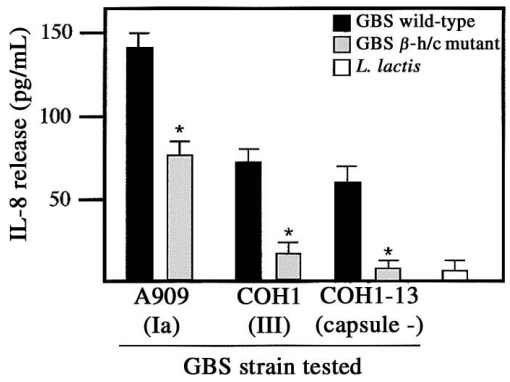

Figure 5. Effect of group B streptococcal (GBS) β-hemolysin/cytolysin (β-h/c) expression on induction of interleukin (IL)–8 release from A549 lung epithelial cells. A549 cells were exposed to a bacterial MOI of 10:1 for 4 h, at which time the supernatant was collected for IL-8 determination by ELISA. A nonhemolytic and noninvasive strain of *Lactococcus lactis* was used as a control. Experiments were performed in triplicate wells. Data shown represent the mean ± SE of 3 separate experiments. The media baseline IL-8 release 77.7 + 8.5 pg/mL was subtracted from all values. $*P < .0002$.

GBS and the corresponding isogenic β-h/c–negative mutant. The results of these studies are summarized in figure 6. We found that DPPC inhibited wild-type GBS invasion of A549 cells by 50% and IL-8 induction from A549 cells by 85%. In contrast, DPPC had no effect on initial GBS adherence to A549 cells, as measured by the total cell-associated bacteria after 30 min of exposure. DPPC also had significant inhibitory effects on invasion and IL-8 induction by the β-h/c mutant, although the absolute magnitude of these decreases was not as great as that seen with the more invasive and stimulatory parent strain. Control experiments indicated that the presence of DPPC did not affect growth of GBS in A549 culture media, the degree of IL-8 release stimulated in response to TNF-α, or the ability to detect IL-8 in the ELISA assay (data not shown). Thus, we found that DPPC inhibited GBS invasion of A549 cells and the amount of IL-8 released from these cells, but the magnitude of this inhibition was only partially explained by neutralization of β-h/c activity.

Discussion

GBS production of a β-h/c toxin has been correlated with cellular injury in vitro and with virulence after respiratory tract or bloodstream infection in vivo. In the present study, we probed mechanisms by which this toxin could contribute to the early pulmonary stages of GBS infection, using isogenic GBS *cylE* gene mutants that are β-h/c deficient. Our results confirmed that β-h/c is directly cytolytic to lung epithelial cells at a high bacterial MOI. At a lower bacterial MOI, we found that the β-h/c contributes to GBS intracellular invasion of lung epithelial cells and stimulates their release of the neutrophil chemoattractant IL-8. These findings are relevant to the pathogenesis of GBS pneumonia, which is characterized by diffuse lung cell injury, neutrophilic inflammation, and bacterial penetration into the bloodstream that produces septicemia.

GBS invasion of the neonatal lung epithelium is considered to be a key step in the establishment of systemic infection [11]. GBS isolates from the blood of infected neonates are significantly more invasive to respiratory epithelial cells than are isolates from vaginal carriers or from colonized neonates without clinical symptoms [32]. On microscopic examination, GBS are found in close association with the epithelial cell surface or intracellularly within membrane-bound vacuoles [8, 9]. This suggests that the organism first binds to the host cell surface and then, in a process resembling phagocytosis, elicits its own engulfment. Here we observed that GBS β-h/c expression promoted lung epithelial adherence and invasion. One hypothesis to explain this finding could be that the β-h/c, which normally associates with the GBS membrane [33], possesses adhesin domains for eukaryotic epithelial surface components within its native protein structure. Alternatively, or in addition, the lung epithelial cells may be "activated" in response to the effects of the hemolysin toxin to bind and to engulf more bacteria. The latter notion would be con-

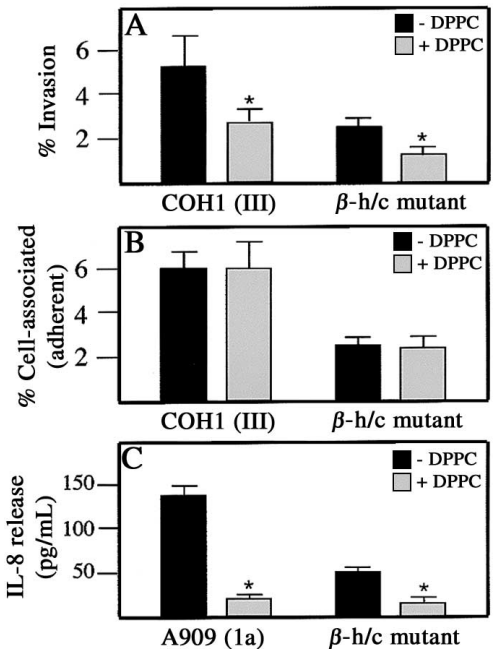

Figure 6. Effect of surfactant phospholipid dipalmotoyl phosphatidylcholine (DPPC) on interactions of group B streptococci with A549 lung epithelial cells. DPPC was added at 500 μg/mL (+DPPC). *A,* Two-hour cellular invasion assay that used strain COH1 and its isogenic β-hemolysin/cytolysin (β-h/c) mutant at an MOI of 1:1. *B,* Thirty-minute total cell-associated bacteria (adherence) assay that used strain COH1 and its isogenic β-h/c mutant at an MOI of 1:1. *C,* Four-hour interleukin (IL)–8 release assay that used strain A909 and its isogenic β-h/c mutant at an MOI of 10:1. All experiments were performed in triplicate wells. Data shown represent the mean ± SE of 3 separate experiments. The media baseline IL-8 release 49.9 ± 7.9 pg/mL was subtracted from all values. $*P < .001$. −DPPC, DPPC absent.

sistent with the observation that structurally diverse groups of hemolytic toxins from a variety of bacterial species have been shown to promote eukaryotic cell invasion—for example, *Proteus mirabilis* [34], *E. coli* [35], *Haemophilus ducreyi* [36], and *Streptococcus suis* [37].

IL-8 is a potent neutrophil chemotactic stimulus [38] that further acts to up-regulate the expression of adhesion molecules on the neutrophil surface [39], to promote their transendothelial migration [40], and to stimulate oxidative burst and release of lysosomal enzymes [41]. An elevated circulating IL-8 level is a strong predictor of bacterial infection in the newborn period [42], and marked increases in IL-8 levels have been detected in sputum or bronchoalveolar lavage fluid from patients with acute pulmonary infections [43, 44]. IL-8 is also the major neutrophil chemoattractant produced by lung epithelial cells after experimental infection [45]. In the present study, we found that GBS induced IL-8 release from A549 lung epithelial cells in a dose- and time-dependent fashion and that β-h/c activity contributed to this induction. The levels of IL-8 produced by the A549 cells in response to GBS were similar to those seen after live challenge with the related pulmonary pathogen *S. pneumoniae* [46].

GBS disease is uncommon in healthy adults, and $<1\%$ of newborns who are colonized at birth with GBS from vertical transmission go on to develop invasive infection [1]. Therefore, the ability of human lung epithelial cells to recognize and respond to GBS and GBS toxins with IL-8 production is likely to be adaptive in the majority of all host-pathogen encounters. In addition to promoting a potent and dose-dependent neutrophil recruitment [47], IL-8 augments the ability of neutrophils to kill a variety of bacterial and fungal pathogens [44, 48–50]. IL-8 release by A549 cells may be intimately linked to GBS intracellular invasion, given that both invasion and IL-8 release are correlated to β-h/c expression. Human intestinal and cervical epithelial cells secrete IL-8 in response to intracellular invasion by *Salmonella* species and *Listeria monocytogenes* [51]. This finding suggests a common pattern by which IL-8 secreted by epithelial cells may be the initiating signal for the innate immune response after bacterial invasion of mucosal surfaces [52]. The chemotactic signal could be augmented in vivo by alveolar macrophages, which generate factors, such as IL-1 and TNF-α, that are potent stimuli for the induction of IL-8 by the alveolar epithelial cells [45, 53]. The cascade may be further amplified as neutrophil adherence to lung epithelial cells stimulates IL-8 release [54] and nonopsonic binding of GBS induces the neutrophils themselves to produce IL-8 [55].

In the special circumstance of severe early-onset GBS infection, the overwhelming bacterial inoculum to the fetal lung might transform an adaptive IL-8–mediated neutrophil recruitment into a destructive acute inflammatory process. In utero infection appears to account for the 40%–60% of newborns with early-onset infection who have poor Apgar scores and develop pulmonary symptoms within a few hours of birth [56, 57]. Deficiencies in alveolar macrophage clearance by the newborn host allow multiplying GBS to attain very high concentrations within the alveolar spaces [5]. Indeed, when experimental GBS pneumonia is induced in newborn primates after intra-amniotic inoculation, bacterial densities reach 10^9–10^{11} cfu/g of lung tissue [8]. Under such circumstances, high levels of neutrophil recruitment and activation by IL-8 could aggravate pulmonary injury through release of proteases and reactive oxygen metabolites. High IL-8 concentrations in bronchoalveolar lavage fluid are, in fact, correlated with the incidence of respiratory failure in at-risk patients [58], and elevations of IL-8 have been observed in respiratory secretions of newborns who later developed chronic lung disease [59]. The high bacterial density of early-onset pneumonia may also well exceed the threshold for direct GBS β-h/c–mediated cytotoxicity to lung epithelium and endothelium, thus leading to extensive breakdown of the mucosal barrier and access for the bacteria into the bloodstream [10, 15].

In earlier studies, we have demonstrated that the phospholipid DPPC, the major component of human surfactant, inhibits GBS β-h/c–induced injury to A549 lung epithelial cells [15]. In the present study, we found that DPPC also inhibits the ability of GBS to intracellularly invade these cells and to stimulate their production of IL-8. Because GBS β-h/c appears to be a membrane pore–forming toxin, the protein would be expected to show affinity for the phospholipid components of the eukaryotic cellular membrane. Free extracellular phospholipid (i.e., DPPC) present in lung surfactant might act as a "sink," diminishing GBS injury of lung epithelial cells by competitive binding of the β-h/c toxin. Yet significant levels of DPPC inhibition of A549 cell invasion and IL-8 release were observed, even with β-h/c–negative *cylE* mutants. Thus, in addition to blocking β-h/c action, the presence of DPPC may interfere with other stimulatory GBS factors and/or modulate the pattern of host cellular response. DPPC inhibition of GBS induced lung cell injury, invasion, or inflammatory activation may be reflected in the increased incidence of severe GBS pneumonia and septicemia in premature surfactant-deficient neonates.

Our observations also provide in vitro data to suggest that administration of DPPC in surfactant preparations could be of therapeutic benefit in infants with severe early-onset GBS pneumonia. Indeed, in studies in which preterm rabbits undergoing mechanical ventilation were administered GBS intratracheally, surfactant treatment reduced histological evidence of lung inflammation, mitigated bacterial proliferation, and improved lung compliance [60–62]. Several clinical reports have also described improved gas exchange parameters or clinical outcome in preterm or full-term infants with GBS pneumonia who receive surfactant therapy [63–66]. The potential benefits of complete surfactant preparations against GBS lung infection are not limited to the β-h/c neutralizing effects of DPPC—for example, surfactant protein A is capable of binding to GBS and promoting opsonophagocytosis by alveolar macrophages [67].

In summary, the β-h/c toxin of GBS may contribute to neonatal disease pathogenesis by direct cytotoxicity, increased lung

epithelial cell invasion, and stimulation of IL-8 release. The availability of specific β-h/c mutants will facilitate further study of the role of this toxin in newborn infection and the potential therapeutic applications of blocking β-h/c activity in patients with invasive GBS disease.

References

1. Edwards MS, Baker CJ. Group B streptococcal infections. In: Remington J, Klein JO, eds. Infectious diseases of the fetus and newborn infant. 5th ed. Philadelphia: WB Saunders, **2001**:1091–156.
2. Hemming VG, McCloskey DW, Hill HR. Pneumonia in the neonate associated with group B streptococcal septicemia. Am J Dis Child **1976**;130: 1231–3.
3. Vollman JH, Smith WL, Ballard ET, Light IJ. Early onset group B streptococcal disease: clinical, roentgenographic, and pathologic features. J Pediatr **1976**;89:199–203.
4. Ablow RC, Driscoll SG, Effmann EL, et al. A comparison of early-onset group B steptococcal neonatal infection and the respiratory-distress syndrome of the newborn. N Engl J Med **1976**;294:65–70.
5. Katzenstein AL, Davis C, Braude A. Pulmonary changes in neonatal sepsis to group B beta-hemolytic *Streptococcus:* relation of hyaline membrane disease. J Infect Dis **1976**;133:430–5.
6. Cochi SL, Feldman RA. Estimating national incidence of group B streptococcal disease: the effect of adjusting for birth weight. Pediatr Infect Dis **1983**;2:414–5.
7. Nizet V, Rubens CE. Pathogenic mechanisms and virulence factors of group B streptococci. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, eds. The gram-positive pathogens. Washington, DC: American Society for Microbiology Press, **2000**:123–36.
8. Rubens CE, Raff HV, Jackson JC, Chi EY, Bielitzki JT, Hillier SL. Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. J Infect Dis **1991**;164:320–30.
9. Rubens CE, Smith S, Hulse M, Chi EY, van Belle G. Respiratory epithelial cell invasion by group B streptococci. Infect Immun **1992**;60:5157–63.
10. Gibson RL, Lee MK, Soderland C, Chi EY, Rubens CE. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect Immun **1993**;61:478–85.
11. Nizet V, Ferrieri P, Rubens CE. Molecular pathogenesis of group B streptococcal disease in newborns. In: Stevens DL, Kaplan EL, eds. Streptococcal infections: clinical aspects, microbiology, and molecular pathogenesis. New York: Oxford University Press, **2000**:181–221.
12. Facklam RR, Padula JF, Thacker LG, Wortham EC, Sconyers BJ. Presumptive identification of group A, B, and D streptococci. Appl Microbiol **1974**;27:107–13.
13. Marchlewicz BA, Duncan JL. Lysis of erythrocytes by a hemolysin produced by a group B *Streptococcus* sp. Infect Immun **1981**;34:787–94.
14. Tapsall JW, Phillips EA. The hemolytic and cytolytic activity of group B streptococcal hemolysin and its possible role in early onset group B streptococcal disease. Pathology **1991**;23:139–44.
15. Nizet V, Gibson RL, Chi EY, Framson PE, Hulse M, Rubens CE. Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. Infect Immun **1996**;64:3818–26.
16. Gibson RL, Nizet V, Rubens CE. Group B streptococcal beta-hemolysin promotes injury of lung microvascular endothelial cells. Pediatr Res **1999**;45:626–34.
17. Rooney SA. The surfactant system and lung phospholipid biochemistry. Am Rev Respir Dis **1985**;131:439–60.
18. Wennerstrom DE, Tsaihong JC, Crawford JT. Evaluation of the role of hemolysin and pigment in the pathogenesis of early onset group B streptococcal infection. In: Kimura Y, Kotami S, Shiokawa Y, eds. Recent advances in streptococci and streptococcal diseases. Bracknell, UK: Reedbooks, **1985**:155–6.
19. Puliti M, Nizet V, von Hunolstein C, Bistoni F, Mosci P, Orefici G, Tissi L. Severity of group B streptococcal arthritis is correlated with beta-hemolysin expression. J Infect Dis **2000**;182:824–32.
20. Nizet V, Gibson RL, Rubens CE. The role of group B streptococci beta-hemolysin expression in newborn lung injury. Adv Exp Med Biol **1997**; 418:627–30.
21. Ring A, Nizet V, Shenep JL. Group B streptococcal β-hemolysin induces NO synthase in macrophages in vitro and multiorgan injury and death in vivo [abstract 26]. In: Programs and abstracts of the Septic Shock Caused by Gram-Positive Bacteria Conference (Vibo Valentia, Italy). Vibo Valentia: Amministrazione provinciale di Vibo Valencia and University of Messina, **1998**:6.
22. Spellerberg B, Pohl B, Haase G, Martin S, Weber-Heynemann J, Lutticken R. Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. J Bacteriol **1999**;181: 3212–9.
23. Pritzlaff CA, Chang JC, Kuo SP, Tamura GS, Rubens CE, Nizet V. Genetic basis for the beta-haemolytic/cytolytic activity of group B *Streptococcus.* Mol Microbiol **2001**;39:236–47.
24. Wessels MR, Benedi VJ, Kasper DL, Heggen LM, Rubens CE. The type III capsule and virulence of group B *Streptococcus.* In: Dunny GM, Cleary PP, McKay LL, eds. Genetics and molecular biology of streptococci, lactococci, and enterococci. Washington, DC: American Society for Microbiology Press, **1991**:219–23.
25. Madoff LC, Michel JL, Kasper DL. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun **1991**;59:204–10.
26. Wilkinson HW. Nontypable group B streptococci isolated from human sources. J Clin Microbiol **1977**;6:183–4.
27. Rubens CE, Wessels MR, Heggen LM, Kasper DL. Transposon mutagenesis of type III group B *Streptococcus:* correlation of capsule expression with virulence. Proc Natl Acad Sci USA **1987**;84:7208–12.
28. Nizet V, Kim KS, Stins M, et al. Invasion of brain microvascular endothelial cells by group B streptococci. Infect Immun **1997**;65: 5074–81.
29. Kuipers OP, Beerthuyzen MM, Siezen RJ, De Vos WM. Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis:* requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur J Biochem **1993**;216:281–91.
30. Eckmann L, Jung HC, Schurer-Maly C, Panja A, Morzycka-Wroblewska E, Kagnoff MF. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. Gastroenterology **1993**;105:1689–97.
31. Jung HC, Eckmann L, Yang SK, et al. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest **1995**;95:55–65.
32. Valentin-Weigand P, Chhatwal GS. Correlation of epithelial cell invasiveness of group B streptococci with clinical source of isolation. Microb Pathog **1995**;19:83–91.
33. Platt MW. In vivo hemolytic activity of group B streptococcus is dependent on erythrocyte-bacteria contact and independent of a carrier molecule. Curr Microbiol **1995**;31:5–9.
34. Peerbooms PG, Verweij AM, MacLaren DM. Vero cell invasiveness of *Proteus mirabilis.* Infect Immun **1984**;43:1068–71.
35. Straube E, Schmidt G, Marre R, Hacker J. Adhesion and internalization of *E. coli* strains expressing various pathogenicity determinants. Zentralbl Bakteriol **1993**;278:218–28.
36. Wood GE, Dutro SM, Totten PA. Target cell range of *Haemophilus ducreyi* hemolysin and its involvement in invasion of human epithelial cells. Infect Immun **1999**;67:3740–9.

37. Norton PM, Rolph C, Ward PN, Bentley RW, Leigh JA. Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. FEMS Immunol Med Microbiol **1999**; 26:25–35.
38. Murphy PM. Neutrophil receptors for interleukin-8 and related CXC chemokines. Semin Hematol **1997**; 34:311–8.
39. Detmers PA, Lo SK, Olsen-Egbert E, Walz A, Baggiolini M, Cohn ZA. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J Exp Med **1990**; 171:1155–62.
40. Huber AR, Kunkel SL, Todd RF III, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science **1991**; 254:99–102.
41. Peveri P, Walz A, Dewald B, Baggiolini M. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J Exp Med **1988**; 167:1547–59.
42. Franz AR, Steinbach G, Kron M, Pohlandt F. Reduction of unnecessary antibiotic therapy in newborn infants using interleukin-8 and C-reactive protein as markers of bacterial infections. Pediatrics **1999**; 104:447–53.
43. Rodriguez JL, Miller CG, DeForge LE, et al. Local production of interleukin-8 is associated with nosocomial pneumonia. J Trauma **1992**; 33:74–82.
44. Standiford TJ, Kunkel SL, Greenberger MJ, Laichalk LL, Strieter RM. Expression and regulation of chemokines in bacterial pneumonia. J Leukoc Biol **1996**; 59:24–8.
45. Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. Exp Lung Res **1991**; 17: 17–23.
46. Madsen M, Lebenthal Y, Cheng Q, Smith BL, Hostetter MK. A pneumococcal protein that elicits interleukin-8 from pulmonary epithelial cells. J Infect Dis **2000**; 181:1330–6.
47. Leonard EJ, Yoshimura T, Tanaka S, Raffeld M. Neutrophil recruitment by intradermally injected neutrophil attractant/activation protein–1. J Invest Dermatol **1991**; 96:690–4.
48. Nibbering PH, Pos O, Stevenhagen A, Van Furth R. Interleukin-8 enhances nonoxidative intracellular killing of *Mycobacterium fortuitum* by human granulocytes. Infect Immun **1993**; 61:3111–6.
49. Djeu JY, Matsushima K, Oppenheim JJ, Shiotsuki K, Blanchard DK. Functional activation of human neutrophils by recombinant monocyte-derived neutrophil chemotactic factor/IL-8. J Immunol **1990**; 144:2205–10.
50. Ponglertnapagorn P, Oishi K, Iwagaki A, et al. Airway interleukin-8 in elderly patients with bacterial lower respiratory tract infections. Microbiol Immunol **1996**; 40:177–82
51. Eckmann L, Kagnoff MF, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun **1993**; 61: 4569–74.
52. Eckmann L, Kagnoff MF, Fierer J. Intestinal epithelial cells as watchdogs for the natural immune system. Trends Microbiol **1995**; 3: 118–20.
53. Kwon OJ, Au BT, Collins PD, et al. Inhibition of interleukin-8 expression by dexamethasone in human cultured airway epithelial cells. Immunology **1994**; 81:389–94.
54. Huang CD, Huang KH, Lin HC, Wang CH, Kuo HP. Neutrophil adherence to lung epithelial cells induce interleukin-8 release. Changgeng Yi Xue Za Zhi **1999**; 22:392–9.
55. Albanyan EA, Vallejo JG, Smith CW, Edwards MS. Nonopsonic binding of type III group B streptococci to human neutrophils induces interleukin-8 release mediated by the p38 mitogen–activated protein kinase pathway. Infect Immun **2000**; 68:2053–60.
56. Baker CJ. Early onset group B streptococcal disease. J Pediatr **1978**; 93: 124–5.
57. Stewardson-Krieger PB, Gotoff SP. Risk factors in early-onset neonatal group B streptococcal infections. Infection **1978**; 6:50–3
58. Donnelly SC, Strieter RM, Kunkel SL, et al. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. Lancet **1993**; 341:643–7.
59. Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ. Increase in interleukin-8 and soluble intercellular adhesion molecule–1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child Fetal Neonatal Ed **1995**; 72:F90–6.
60. 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**; 36:784–91.
61. Sherman MP, Campbell LA, Merritt TA, et al. Effect of different surfactants on pulmonary group B streptococcal infection in premature rabbits. J Pediatr **1994**; 125:939–47.
62. 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 Fetal Neonatal Ed **1997**; 76:F3–8.
63. Khammash H, Perlman M, Wojtulewicz J, Dunn M. Surfactant therapy in full-term neonates with severe respiratory failure. Pediatrics **1993**; 92: 135–9.
64. Auten RL, Notter RH, Kendig JW, Davis JM, Shapiro DL. Surfactant treatment of full-term newborns with respiratory failure. Pediatrics **1991**; 87: 101–7.
65. Gortner L, Pohlandt F, Bartmann P. Bovine surfactant in full-term neonates with adult respiratory distress syndrome–like disorders [letter]. Pediatrics **1994**; 93:538.
66. Herting E, Gefeller O, Land M, van Sonderen L, Harms K, Robertson B. Surfactant treatment of neonates with respiratory failure and group B streptococcal infection. Members of the Collaborative European Multicenter Study Group. Pediatrics **2000**; 106:957–64,1135.
67. LeVine AM, Kurak KE, Wright JR, et al. Surfactant protein–A binds group B streptococcus enhancing phagocytosis and clearance from lungs of surfactant protein–A–deficient mice. Am J Respir Cell Mol Biol **1999**; 20:279–86.