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Group B Streptococcal Beta-Hemolysin Expression Is Associated with Injury of Lung Epithelial Cells

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Group B streptococci (GBS) are the leading cause of serious bacterial infection in newborns. Early-onset disease is heralded by pneumonia and lung injury, and the lung may serve as a portal of entry for GBS into the bloodstream. To examine a potential role for GBS beta-hemolysin in lung epithelial injury, five wild-type strains varying in beta-hemolysin expression were chosen, along with five nonhemolytic (NH) and five hyperhemolytic (HH) variants of these strains derived by chemical or transposon mutagenesis. Monolayers of A549 alveolar epithelial cells were exposed to log-phase GBS or stabilized hemolysin extracts of GBS cultures, and cellular injury was assessed by lactate dehydrogenase (LDH) release and trypan blue nuclear staining. Whereas NH strains produced no detectable injury beyond baseline (medium alone), hemolysin-producing strains induced LDH release from A549 cells in direct correlation to their ability to lyse sheep erythrocytes. HH strains were also associated with marked increases in trypan blue nuclear staining of A549 monolayers. The extent of LDH release produced by HH strains was significantly reduced in the presence of dipalmitoyl phosphatidylcholine, a known inhibitor of hemolysin and the major phospholipid component of human surfactant. Electron microscopic studies of A549 cell monolayers exposed to HH GBS mutants revealed global loss of microvillus architecture, disruption of cytoplasmic and nuclear membranes, and marked swelling of the cytoplasm and organelles. We conclude that GBS hemolysin expression correlates with lung epithelial cell injury and may be important in the initial pathogenesis of early-onset disease, particularly when pulmonary surfactant is deficient.

Group B streptococci (GBS) are the leading cause of serious infection in human newborns (4). Early-onset disease is heralded by respiratory symptoms, caused by pneumonia with dense bacterial infiltration, alveolar hemorrhage, and inflammatory exudate as characteristic histologic features (1, 19). Premature, low-birth-weight infants are at greatly increased risk for acquiring GBS pneumonia and invasive disease (9).

The lung is a likely portal of entry for GBS into the bloodstream, following aspiration of infected amniotic or vaginal fluids. Our laboratory has demonstrated that GBS are capable of invading alveolar epithelial (34) and endothelial cells (16), remaining within membrane-bound vacuoles. This process may represent an initial step in the pathogenesis of invasive disease. In tissue culture, cellular invasion by GBS can occur at low inocula without apparent damage to the host cells. It is therefore likely that additional GBS virulence attributes play a role in producing or eliciting the diffuse tissue injury seen in newborn pneumonia.

The vast majority of GBS clinical isolates demonstrate beta-hemolysis when plated on sheep blood agar (13). The degree of beta-hemolytic activity appears to correlate with the amount of an orange carotenoid like-pigment produced by the organism (41). The GBS beta-hemolysin(s) itself has never been isolated, largely because it is unstable. High-molecular-weight carrier molecules such as starch, albumin, or Tween 80 are required to preserve hemolytic activity in GBS culture supernatants (25). Interestingly, the hemolytic activity of such preparations is inhibited by certain phospholipids (25) such as dipalmotyl phosphatidylcholine (DPPC), the major component of human surfactant (31).

More recently, stabilized extracts containing the GBS hemolysin have been shown to alter the normal morphology of McCoy cells (mouse fibroblasts), suggesting the beta-hemolysin may affect a broader range of eukaryotic cell membranes (42). We sought to test the hypothesis that beta-hemolysin expression might therefore contribute directly to injury of human lung epithelial cells in vitro.

In this report, we correlate the association of GBS beta-hemolysin expression with injury of human alveolar epithelial cells in a tissue culture model. Chemical and transposon mutagenesis were used to produce nonhemolytic (NH) and hyperhemolytic (HH) GBS variants, which were noninjurious and hyperinjurious to epithelial cell membranes, respectively.

MATERIALS AND METHODS

Bacterial strains. Five clinical isolates of GBS were chosen for study: COH1, a highly encapsulated type III strain (47); COH31, a weakly encapsulated type III strain (35); A909, a type Ia strain (24); UAB (also called Bib 501 Sm1), a type Ib strain (17), and NCTC 10/84 (also called 1169-NT1), a type V strain (48). All strains were isolated from the blood of septic neonates except for COH31, which was isolated from a diabetic foot ulcer in an adult. Bacteria were maintained and grown to mid-log phase in Todd-Hewitt broth (THB) to an optical density at 600 nm of either 0.4 or 0.8 (equivalent to $\sim 10^8$ or $\sim 10^9$ CFU/ml, respectively) for use in hemolysin or cellular injury experiments.

Chemical mutagenesis. $NaNO_2$ mutagenesis of strain COH1 was performed by using a modification of the technique described by Alper and Ames (2) in a protocol biased to produce nucleotide deletions (36). Approximately 5×10^9 log-phase organisms in THB were pelleted by centrifugation and resuspended in 300 μl of 0.1 M sodium acetate buffer (control) or 300 μl of the same buffer containing 0.1 M $NaNO_2$. The resuspended bacteria were incubated at 37°C for various durations (between 1 and 20 min), and the reaction was stopped by adding 10 ml of THB on ice. The bacteria were once again pelleted by centrif-

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TABLE 1. Isolation of GBS mutants with alterations in beta-hemolysin phenotype

Phenotype	Parent strain (capsule type)	Mutagenesis	Frequency of mutants	Mutant chosen (no. of transposon insertions)
NH	COH1 (III)	$NaNO_2$	1 of 2,500	CM153
		Tn*916*ΔE	2 of 2,000	COH1-20 (1)
	COH31 (III)	Tn*916*	2 of 2,000	COH31c12 (2)
	A909 (Ia)	Tn*916*ΔE	0 of 2,000	
		Tn*917*	3 of 3,000	A909-NH2 (1)
	UAB (Ib)	Tn*916*ΔE	—[a]	UABH- (2)
HH	COH1 (III)	$NaNO_2$	20 of 2,500	CM48
		Tn*916*ΔE	13 of 2,000	IN40 (1)
	COH31 (III)	Tn*916*	3 of 2,000	COH31c35 (1)
	A909 (Ia)	Tn*916*ΔE	3 of 2,000	A909-HHA (2)
		Tn*917*	4 of 3,000	A909-HH4 (1)

[a] Mutant kindly provided by D. G. Pritchard, Birmingham, Ala.

ugation, resuspended in 5 ml of fresh THB, and incubated at 37°C for 1 h. Dilutions of these cultures were plated on Todd-Hewitt agar overnight, and the culture which resulted in 99% killing of COH1 compared with the control (10-min $NaNO_2$ exposure) was selected for beta-hemolysin screening on tryptic soy agar–5% sheep blood agar.

Transposon mutagenesis. Transposon Tn*916* or Tn*916*ΔE mutagenesis of GBS strains was performed as previously described, by conjugation with the high-frequency *Enterococcus faecalis* donor CG110 (47) or RH110 (32), respectively. Transposon Tn*917* mutagenesis of strain A909 was accomplished by introduction of Tn*917* on a conditional pWV01 family vector, pTV_1OK, by electroporation. Transformants were amplified at the permissive temperature (30°C) under erythromycin selection, and chromosomal transposition events were selected for by a shift to the nonpermissive temperature (39°C) while maintaining erythromycin selection (15). Transposon mutants were screened for variations in hemolysin phenotype by plating on tryptic soy agar–5% sheep blood agar and overnight incubation at 37°C.

Southern blot analysis. Total cellular DNA was isolated from GBS by a modification of the Hull protocol (21) in which 50 μg of mutanolysin per ml was substituted for lysozyme (23). DNA was digested to completion with the restriction enzyme *Eco*RI or *Hin*dIII, separated by 0.8% agarose gel electrophoresis, and transferred to nylon filters by the Southern method (38). Digoxigenin-labeled probes of (i) the Tn*916*ΔE-containing *Eco*RI fragment of plasmid pCER110 and (ii) the Tn*917*-containing *Eco*RI-*Pst*I fragment of plasmid pTV_1OK were prepared by using a Genius kit (Boehringer Mannheim). The appropriate probe was hybridized to the target filter under standard conditions, and the probe-positive bands were visualized by chemiluminescence as recommended by the manufacturer.

Phenotypic analysis of mutants. Chemical and transposon mutants were compared with the wild-type parent strains for logarithmic growth in THB and RPMI 1640 by optical density measurement. Production of GBS surface antigen was tested by latex agglutination (Streptex; Wellcome) and production of type-specific capsule by immunoblot assay. Production of CAMP factor was assessed by streaking each strain on Todd-Hewitt agar–5% sheep blood agar to within 3 mm of a perpendicular linear growth of *Staphylococcus aureus*. Acetoin production, the enzymatic activities of hippuricase, β-glucuronidase, alkaline phosphatase, leucine arylamidase, and arginine dehydrolase, and the ability to ferment 10 sugar substrates were tested by using the API 20 Strep identification system for streptococci (bioMerieux).

Preparation of GBS hemolysin extracts. From logarithmic growth in THB, 10^{10} CFU of GBS was pelleted by centrifugation at 3,000 × *g* (Jouan GR412) for 10 min, washed once in phosphate-buffered saline (PBS), and then resuspended in 1 ml of PBS containing 0.2% glucose, 1% dextran, and 3% Tween 80 (25). The suspension was incubated at 37°C in 5% CO_2 for 30 min and centrifuged at 3,000 × *g* for 10 min, and the supernatant (hemolysin extract) was removed from the bacterial pellet and stored on ice for not more than 1 h prior to use.

Assay for hemolytic activity. A modification of the method of Marchlewicz and Duncan (25) was used to quantify GBS hemolysin activity, using whole bacteria and the dextran-Tween 80 hemolysin extracts. From logarithmic growth in THB, 10^8 CFU of GBS was pelleted by centrifugation at 3,000 × *g*, washed once with PBS, and resuspended in 1 ml of PBS with 0.2% glucose. In a 96-well conical-bottom microtiter plate, 100 μl (10^7 CFU) of the bacterial resuspension was placed in the first well, and serial twofold dilutions in PBS-glucose were performed across the plate, each in a final volume of 100 μl. An equal volume of 1% sheep erythrocytes (RBC) in PBS-glucose was then added to each well, and the plate was incubated at 37°C in 5% CO_2 for 1 h. PBS-glucose alone and RBC lysis with 0.1% sodium dodecyl sulfate (SDS) were used as negative and positive controls, respectively. After incubation, the plates were spun at 3,000 × *g* for 10 min to pellet unlysed RBC and bacteria, and 100 μl of the supernatant was transferred to a replica plate. Hemoglobin release was assessed by measuring A_{420}, and the hemolytic titer of a given strain was determined as the reciprocal of the greatest dilution producing 50% hemoglobin release compared with the SDS control. For determining the hemolytic titers of hemolysin extracts, 100 μl of extract was subjected to serial twofold dilutions in PBS-glucose and assayed in an identical fashion. The size of bacterial inoculum and duration of the assay were standardized such that the weakly hemolytic wild-type strain COH1 and its hemolysin extract possessed a hemolytic titer of 1. All assays were performed in duplicate and repeated three times.

Epithelial cell cultures. 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 containing 10% fetal calf serum. Confluent monolayers of A549 cells in 96- or 24-well tissue culture plates (Corning) or 8-well chamber slides (Nunc) were washed three times with PBS, and RPMI 1640 without fetal calf serum was added immediately prior to inoculation with bacteria or hemolysin extracts in all experiments.

LDH cytotoxicity assay. As in the assay for hemolytic activity, 10^8 CFU of log-phase GBS was pelleted and washed but then was resuspended in 1 ml of RPMI medium without fetal calf serum. A 100-μl aliquot (10^7 CFU) was added to the first well of a 96-well culture plate containing a monolayer of A549 cells (~2 × 10^4 cells; multiplicity of infection of 500:1), and serial twofold dilutions were performed in RPMI 1640 onto other monolayers across the plate. RPMI 1640 alone and bacteria in RPMI 1640 without an A549 cell monolayer were used as negative controls; distilled H_2O lysis of a complete A549 cell monolayer was used as a positive control. The plate was incubated at 37°C in 5% CO_2 for 4 h, at which time a 20-μl aliquot of each supernatant was transferred to a replica plate for lactate dehydrogenase (LDH) measurement using a miniaturized version of the Sigma colorimetric assay (catalog no. 500-C). To each well, 100 μl of 0.1% NADH in standardized pyruvate substrate was added, and the plate was incubated for 30 min at 37°C. Sigma color reagent (100 μl) was then added to each well for 20 min at room temperature. The A_{420} of each well was used to calculate the residual pyruvic acid activity, which is inversely proportional to LDH activity. The cytotoxic titer of a bacterial strain was calculated as the reciprocal of the greatest dilution producing 50% LDH release compared with the H_2O lysis control. Cytotoxic titers for hemolysin extracts were determined in a similar fashion, and the duration of the LDH assay was again standardized such that COH1 and its extract possessed a cytotoxic titer of 1. All assays were performed in duplicate and repeated three times.

Trypan blue dye exclusion assay. To monolayers of ~4 × 10^4 A549 cells in eight-well chamber slides, 10^7 CFU of GBS resuspended in RPMI 1640 (multiplicity of infection of 250) was added to a final well volume of 200 μl and incubated for 1 h at 37°C in 5% CO_2. After incubation, the supernatant was removed by gentle aspiration, 100 μl of 0.04% trypan blue in RPMI 1640 was added to each well, and the slides were returned to 37°C for an additional 5 min. The supernatant was again removed, the monolayers washed once with PBS, and the cells were fixed with 100 μl of 3% glutaraldehyde. Eosin (0.5%) was used as

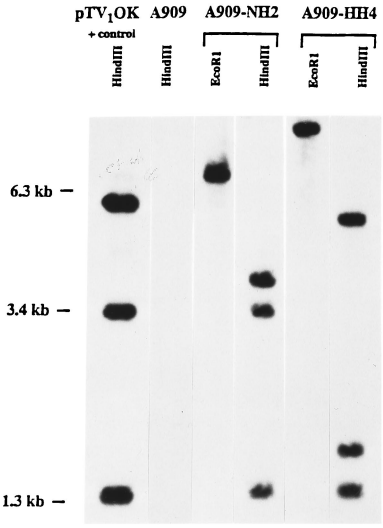


FIG. 1. Southern blot analysis of *Eco*RI or *Hin*dIII chromosomal digests of GBS type Ia strain A909, the NH Tn*917* mutant A909-NH2, and the HH Tn*917* mutant A909-HH4. The probe is digoxigenin-labeled Tn*917*. As *Eco*RI does not cut within the transposon and *Hin*dIII cuts twice (liberating a 1.3-kb internal fragment), the pattern of one hybridizing band upon *Eco*RI digestion and three hybridizing bands upon *Hin*dIII digestion indicates that a single Tn*917* insertion into the A909 chromosome is associated with either the NH or the HH phenotype.

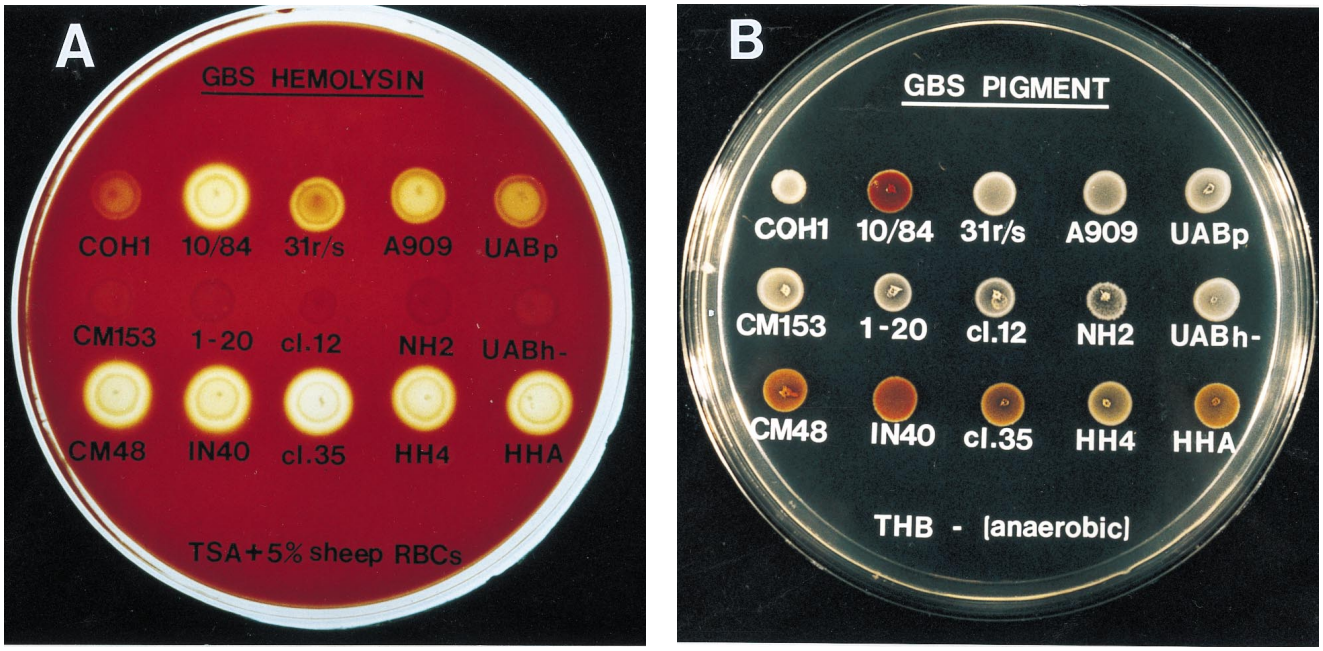


FIG. 2. Correlation of GBS beta-hemolysin expression with production of an orange pigment in GBS clinical isolates (top row), NH mutants (middle row), and HH mutants (bottom row) after overnight growth on tryptic soy agar–5% sheep blood agar (A) or overnight growth under anaerobic conditions on Todd-Hewitt agar (B).

a counterstain, and the monolayers were examined under high-power light microscopy. The percentage of trypan blue-stained nuclei in three high-power fields was determined. Each sample was run in duplicate wells, and the assay was repeated twice.

Electron microscopic studies. To monolayers of $\sim 10^5$ A549 cells in 24-well tissue culture plates, 4×10^7 CFU of GBS resuspended in RPMI 1640 (multiplicity of infection of 400) was added to a final well volume of 400 μl and incubated for 15 min at 37°C. The supernatants were removed by gentle aspiration, and the monolayers were fixed with 3% glutaraldehyde and prepared for transmission electron microscopy as described before (7).

Inhibition of cellular injury by phospholipid. A549 cell monolayers in 24-well tissue culture plates ($\sim 10^5$ cells per well) were exposed to 10^7 CFU of HH wild-type strain NCTC 10/84 or HH Tn*916*ΔE mutant strain IN40 or to 100 μl of hemolysin extract prepared from each strain. Experimental wells contained 500 μl of RPMI or RPMI plus 100, 300, or 500 μg of DPPC (Sigma) per ml solubilized by sonication for 1 min at 30 W. The monolayers were incubated at 37°C with 5% CO_2 for a total of 4 h. At hourly intervals, a 20-μl aliquot from each well was removed and assayed for LDH in the microtiter plate assay described above. Monolayers incubated with RPMI 1640 at each concentration of DPPC were used as negative controls; distilled H_2O lysis of a complete A549 cell monolayer was used as a positive control. Each condition was performed in triplicate and repeated two times. Data were expressed as percent LDH release compared with the positive control.

RESULTS

Selection of strains and isolation of mutants. To demonstrate that the association of beta-hemolysin production and epithelial cell injury is present across various GBS serotypes, five wild-type strains representing four capsular serotypes (Ia,

TABLE 2. Hemolytic and cytolytic titers of GBS clinical isolates, chemical and transposon mutants with altered beta-hemolysin phenotype, and starch-Tween 80 hemolysin extracts from each strain

Strain	Description	Titer[a]			
		Hemolytic		Cytolytic	
		Organism	Extract	Organism	Extract
COH1	Type III clinical isolate	1	1	1	1
CM153	$NaNO_2$ mutant	0	0	0	0
COH1-20	Tn*916*ΔE mutant	0	0	0	0
CM48	$NaNO_2$ mutant	32	32–64	64–128	128–256
IN40	Tn*916*ΔE mutant	16–32	32	64	64–128
COH31	Type III clinical isolate	4	4	8–16	4–8
COH31c12	Tn*916* mutant	0	0	0	0
COH31c35	Tn*916* mutant	16	32	64–128	32–64
A909	Type Ia clinical isolate	4	4	8–16	4–8
A909-NH2	Tn*917* mutant	0	0	0	0
A909-HH4	Tn*917* mutant	16	16–32	32–64	64
A909-HHA	Tn*916*ΔE mutant	16	32	32–64	64–128
UAB	Type Ib clinical isolate	8	4	16–32	8
UABH-	Tn*916*ΔE mutant	0	0	0	0
NCTC 10/84	Type V clinical isolate	32–64	32–64	512–1,024	128–256

[a] Inverse of the greatest dilution producing 50% lysis of sheep RBC (hemolytic titer) or 50% release of LDH from a monolayer of A549 alveolar epithelial cells (cytolytic titer), as determined in a microtiter plate assay. The initial inoculum in each assay was standardized such that the weakly hemolytic clinical isolate COH1 possessed a titer of 1.

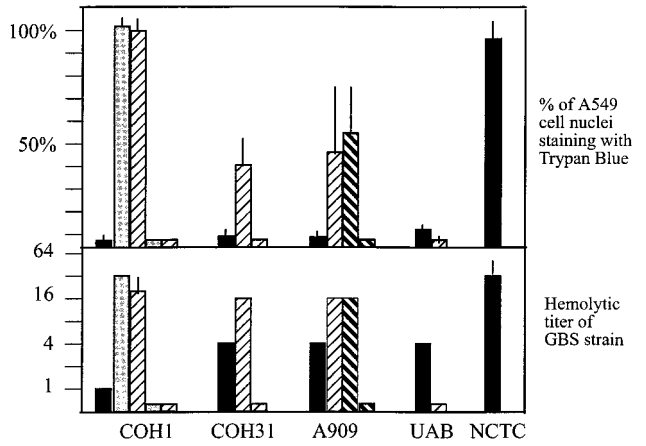


FIG. 3. Correlation of hemolytic titers of GBS clinical isolates and hemolysin mutants with trypan blue staining of A549 alveolar epithelial cell nuclei in tissue culture monolayers exposed to each strain. ■, wild-type strain; ▨, $NaNO_2$ mutant; ▨, Tn*916* mutant; ▧, Tn*917* mutant.

Ib, III, and V) were selected for study. Furthermore, to demonstrate that the association of altered beta-hemolysin production with changes in cytotoxicity was independent of the method of mutagenesis, we studied NH and HH mutants created by using three different techniques: $NaNO_2$ chemical mutagenesis, transposon Tn*916* (Tn*916*ΔE) mutagenesis, and transposon Tn*917* mutagenesis. The frequencies of altered hemolysin phenotypes among the survivors of the $NaNO_2$ mutagenesis protocol, transconjugates expressing the antibiotic resistance marker of transposon Tn*916* or Tn*916*ΔE, or Tn*917* transformants expressing erythromycin resistance are summarized in Table 1.

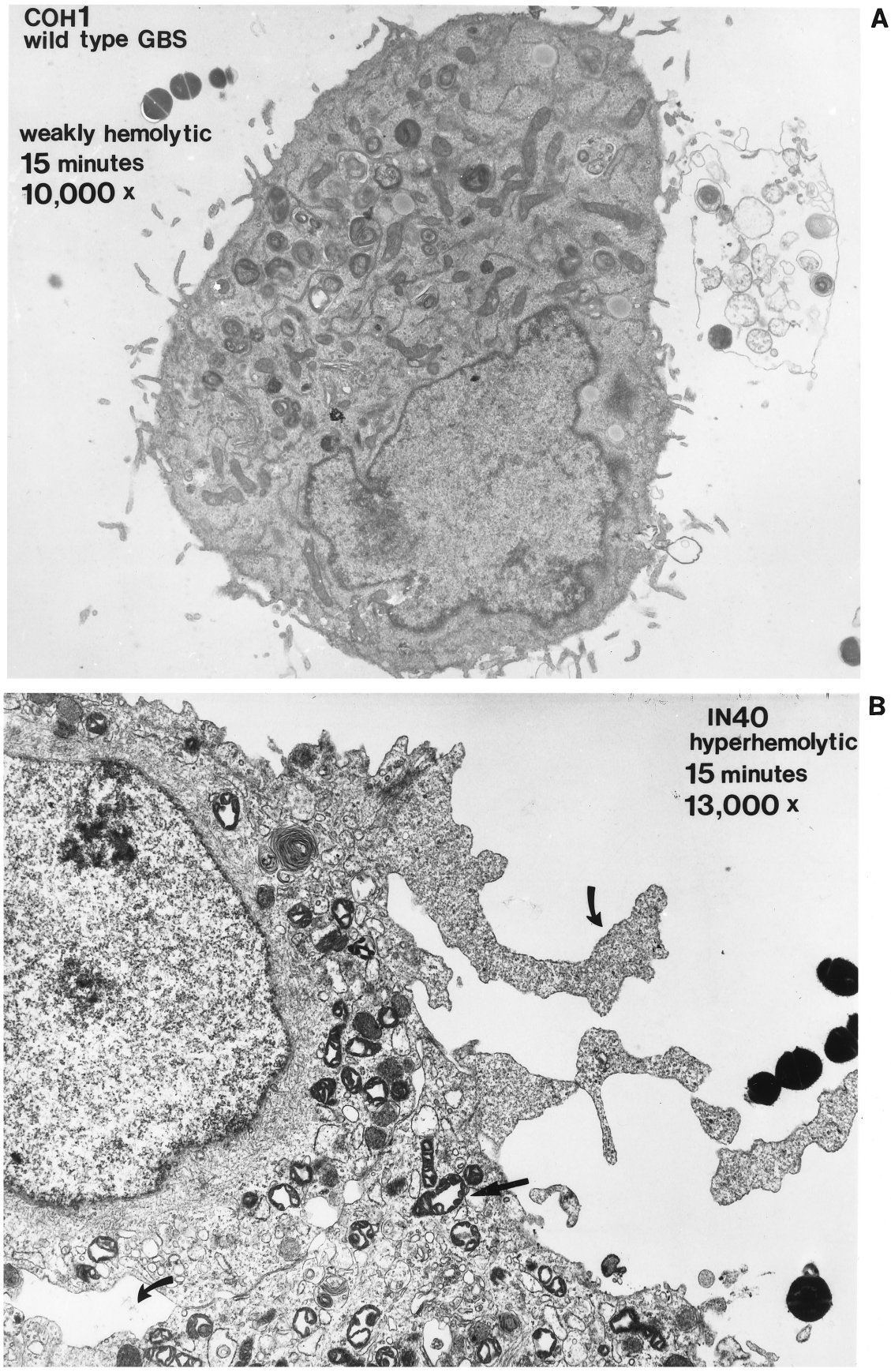
Southern blot analysis of transposon mutants. To quantify the number of transposon insertions, Southern blot analysis was performed on chromosomal digests of each transposon mutant by using the appropriate transposon-specific digoxigenin-labeled DNA probe. These studies revealed that transconjugate strains COH1-20 (NH) and IN40 (HH) contain one copy of transposon Tn*916*ΔE, whereas strains A909-HHA (HH) and UABH-(NH) have two copies of Tn*916*ΔE (data not shown). NH transconjugate strain COH31c12 has two copies of transposon Tn*916* but has one insertion in common with an independently isolated NH transconjugate, COH31c5 (Southern analysis previously described [45]). HH strain COH31c35 has a single copy of Tn*916* (data not shown). Southern blot analysis of Tn*917* mutants A909-NH2 and A909-HH2 reveals a single band hybridizing to the Tn*917* probe with *Eco*RI digestion (*Eco*RI does not cut Tn*917*) and three bands on *Hin*dIII digestion (*Hin*dIII cuts Tn*917* twice, liberating a 1.3-kb internal fragment), indicating that a single transposon insertion can be associated with either an NH or an HH phenotype (Fig. 1). Two other independently isolated NH Tn*917* mutants of strain A909 possessed the same *Eco*RI and *Hin*dIII hybridization pattern as A909-NH2. Of four independently isolated HH Tn*917* mutants of strain A909, one double-insertion mutant shared the hybridizing bands of A909-HH4, whereas a distinct hybridization pattern was found in the remaining two (data not shown). To summarize, in five of the eight transposon mutants used in this study, a single chromosomal insertion of the transposon was associated with alteration of hemolysin phenotype.

Phenotypic comparison of mutants with the wild type. Chemical and transposon mutants with altered beta-hemolysin phenotype were tested for changes in other phenotypic traits to look for possible pleiotropic mutations. All mutants expressed the carbohydrate group B antigen, produced the corresponding type-specific capsular polysaccharide, and exhibited comparable logarithmic growth in THB and RPMI 1640 compared with the wild-type parent strains (data not shown). In addition, all mutant strains possessed identical enzymatic profiles and sugar fermentation patterns on the API 20 Strep identification system for streptococci as their respective parent strains. All HH mutants, whether produced by chemical or by transposon mutagenesis, exhibited diminished, but not absent, CAMP factor expression compared with parent strains. The HH wild-type strain NCTC 10/84 also had significantly diminished CAMP factor expression. NH mutants were identical to the parent in CAMP factor expression.

Correlation of hemolytic activity and pigment production. A close link between GBS expression of beta-hemolysin and production of an orange pigment has been suggested on both epidemiological (28) and experimental (41) grounds. Figure 2 compares the variation in beta-hemolysin activity among the wild-type and mutant strains after overnight growth on blood agar plates with the variation in pigment production on Todd-Hewitt agar after overnight growth under anaerobic conditions, known to favor pigment production (27). Increased beta-hemolysin activity among the five wild-type strains was associated with increased expression of an orange pigment. NH mutants were associated with loss of pigment expression. HH mutants expressed increased pigment compared with the parent strains. The link between the degree of expression of GBS beta-hemolysin and orange pigment was therefore maintained following mutagenesis of the beta-hemolysin phenotype.

Quantification of hemolytic activities of wild-type GBS strains and hemolysin mutants. A liquid-phase microtiter dilution assay in PBS-glucose was used to quantify the hemolysin production of each wild-type strain and mutant. The hemolytic titers of the five wild-type GBS strains studied are shown in Table 2. Strains COH31 and A909 produced 4 times, strain UAB parent produced 8 times, and strain NCTC 10/84 produced 32 to 64 times as much hemolytic activity in the microtiter well assay as the weakly hemolytic strain COH1. Standardized such that the COH1 titer equaled 1, the hemolytic titer of starch-Tween 80-stabilized extracts of each strain corresponded to the hemolytic titer of the intact organism. NH mutants, whether created by chemical or by transposon mutagenesis, produced no detectable hemolytic activity in the microtiter assay, nor was hemolytic activity extractable with starch-Tween 80. HH mutants, whether created by chemical or by transposon mutagenesis, produced 4 to 32 times as much hemolytic activity as the parent strain, an increase which was preserved in starch-Tween 80 extracts. Alteration of beta-hemolysin expression by intact organisms was thus accompanied by a consistent alteration in the ability of the hemolysin to be stabilized or extracted by the starch-Tween 80 carrier. PBS-glucose alone did not produce RBC lysis. The hemolytic titers of each strain or starch-Tween 80 extract were consistent and reproducible within 1 or 2 dilutions.

LDH release from lung epithelial cells. To test the correlation of GBS beta-hemolysin expression with epithelial cell injury at a biochemical level, we measured release of the eukaryotic cytoplasmic enzyme LDH from A549 alveolar epithelial cells. Injury to cell monolayers exposed to serial dilutions of bacterial inocula was quantitated by LDH release in a microtiter plate assay and is reported as a cytolytic titer standardized such that the titer of wild-type strain COH1 equaled 1. Whereas NH strains produced no cellular injury beyond baseline (medium alone), hemolysin-producing strains induced


COH1
wild type GBS
weakly hemolytic
15 minutes
10,000 x
A
IN40
hyperhemolytic
15 minutes
13,000 x
B

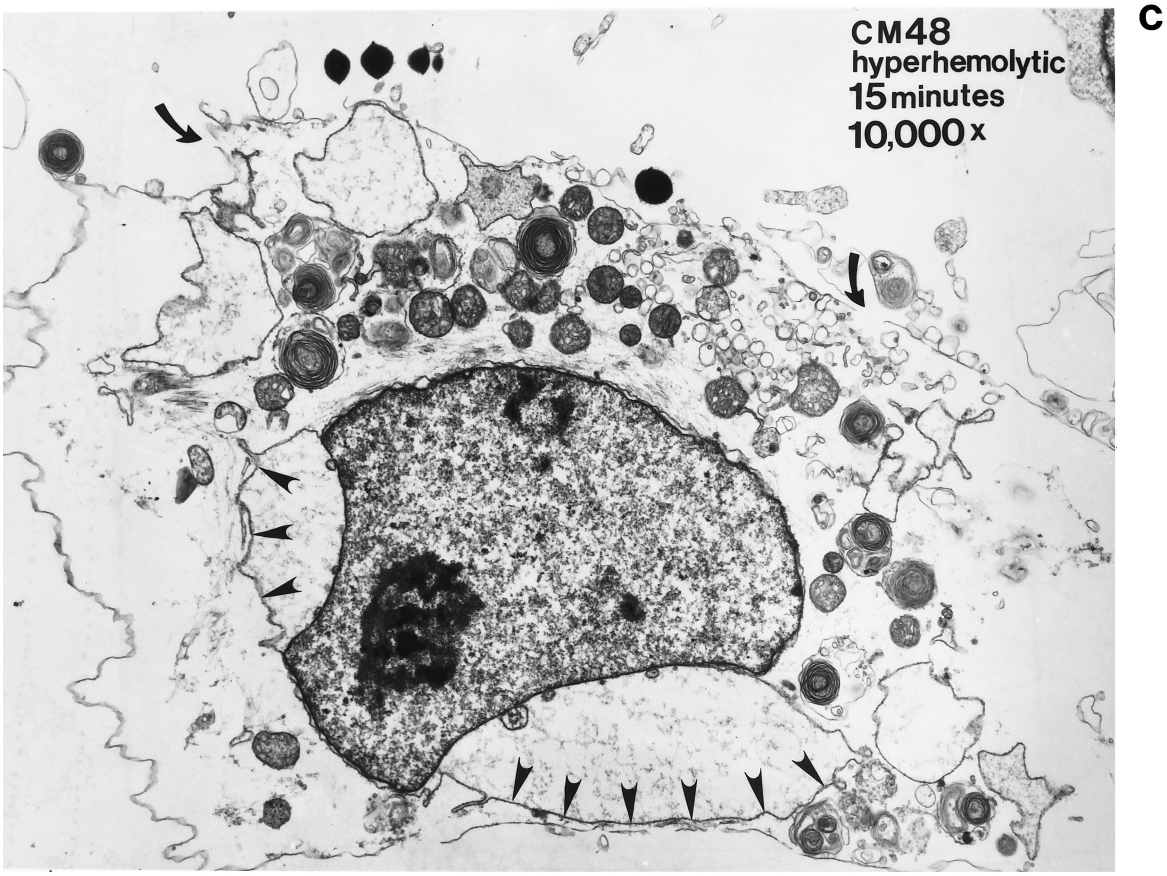


FIG. 4. Ultrastructural features of GBS hemolysin-associated epithelial cell injury. Transmission electron micrographs show A549 pneumocytes exposed to the weakly hemolytic parent strain COH1 (A), HH Tn*916*ΔE mutant IN40 (B), and HH chemical mutant CM48 (C). Arrows indicate surface bleb formation, dilated mitochondria, and intracytoplasmic vacuole formation (B) and splitting of the cytoplasmic and nuclear membranes (C). Loss of cytoplasmic density and clumping of nuclear chromatin are seen in panels B and C.

LDH release from A549 cells in direct correlation to their ability to lyse sheep RBC (Table 2). HH mutants produced equivalent LDH release at inocula 4- to 128-fold lower than those used for the parent strains from which they were derived. Cytolytic activity was stabilized in starch-Tween 80 extracts in proportion to the cytolytic titer of the intact organism. The cytolytic titers of each strain or starch-Tween 80 extract were consistent and reproducible within 1 or 2 dilutions. It therefore appears that the cytolytic activities of the GBS strains and their respective mutants tested correlated directly with their hemolytic activities and that the cytolytic factors were stabilized by the same carrier molecules as the hemolytic factors.

Trypan blue nuclear staining. The association of GBS hemolysin expression with A549 pneumocyte injury was also assessed histologically by using the vital dye trypan blue. This dye stains the nuclei of cells which have lost the membrane pump function to exclude it. Incubation of A549 cells with NH or weakly hemolytic strains was associated with little or no trypan blue staining, whereas incubation with HH mutants or the HH wild-type strain NCTC 10/84 resulted in positive staining of 35 to 100% of nuclei (Fig. 3). Trypan blue staining would appear to be less sensitive for low-level epithelial cell injury than LDH release but corroborates the biochemical evidence of cytotoxicity produced by the HH strains.

Electron microscopy. Electron microscopy was used to study the ultrastructural changes associated with GBS hemolysin-induced epithelial cell injury. Figure 4 compares transmission electron micrographs of A549 pneumocytes exposed for 15 min to identical inocula of the weakly hemolytic parent strain COH1, an HH Tn*916*ΔE mutant (IN40), and an HH chemical mutant (CM48). The pneumocyte exposed to strain COH1 exhibits an (i) intact cellular membrane with microvilli and (ii) dense regular cytoplasmic contents, organelles and chromatin. Pneumocytes exposed to the HH mutants exhibited dramatic loss of cytoplasmic density, breaks in the cytoplasmic membrane, dilated mitochondria, intracytoplasmic vacuole formation, frequent splitting of the nuclear membrane, and clumping of nuclear chromatin. Figure 5 compares scanning electron micrographs of A549 pneumocytes exposed to strain COH1 and the HH mutant CM48. Exposure to the HH mutant results in loss of microvillus architecture and bleb formation on the cell surface.

Inhibition by surfactant phospholipid. Phospholipid components of human surfactant are known to inhibit GBS beta-hemolysin-associated RBC lysis (25) and alterations of McCoy cell morphology (42). We sought to assess whether phospholipid might also be protective for lung epithelial cells exposed to high levels of GBS hemolysin. Using LDH release to measure injury of A549 cell monolayers, we added increasing concentrations of the phospholipid DPPC to the tissue culture media by sonication and exposed the monolayers to HH wild-type strain NCTC 10/84, HH transposon mutant IN40, and starch-Tween 80 hemolysin extracts from these strains (Fig. 6). Medium alone was used to define baseline (0%) LDH release,

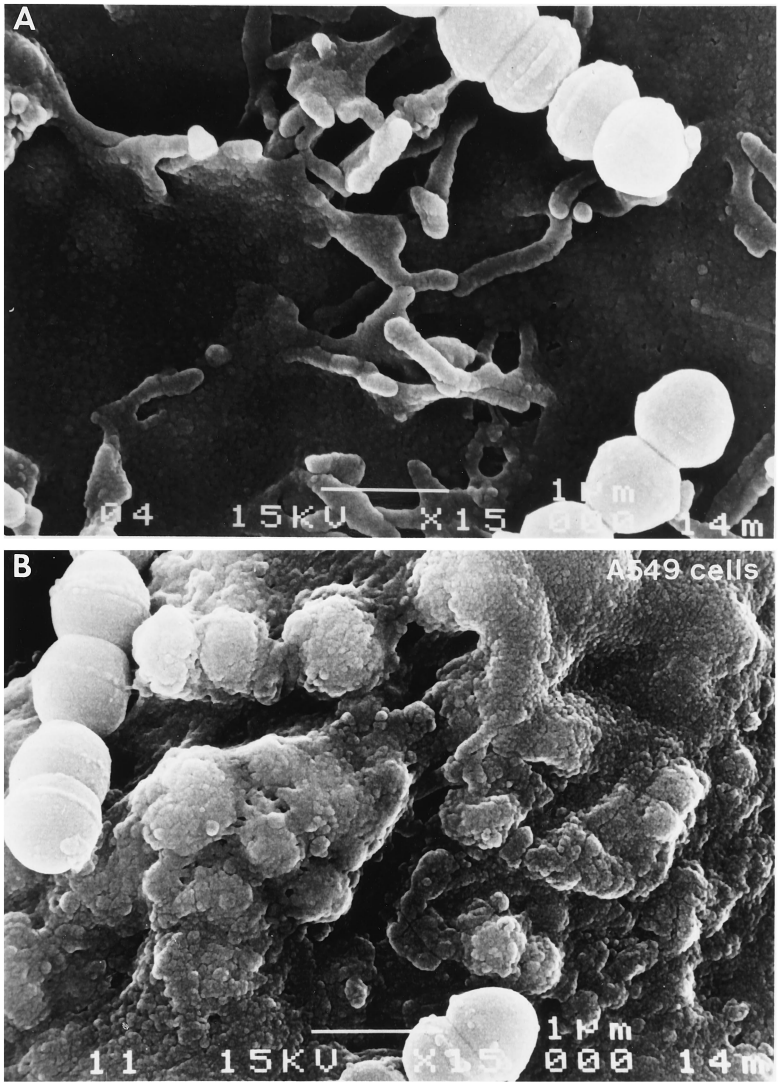


FIG. 5. Additional ultrastructural features of GBS hemolysin-associated epithelial cell injury. Scanning electron micrographs show A549 pneumocytes exposed to strain COH1 (A) and HH chemical mutant CM48 (B). Loss of microvillus architecture and bleb formation on the cell surface of the hyperhemolytic mutant are evident.

and H_2O lysis of the monolayer defined 100% LDH release; LDH quantitation itself was not affected by the range of concentrations of DPPC tested. A dose-related decrease in LDH release was found with increasing DPPC concentration following exposure of the epithelial cell monolayers to either intact HH GBS organisms or starch-Tween 80 hemolysin extracts. In the presence of 500 μg of DPPC per ml, there was >90% reduction in LDH release at 1 h of exposure in all cases. These differences were persistent over 4 h of exposure with the starch-Tween 80 extracts, but growth of either strain of intact bacteria ultimately overcame the DPPC inhibition and produced high-level injury by 4 h in both strains.

DISCUSSION

The studies described above demonstrate that the expression of beta-hemolytic activity of GBS can be correlated directly with injury of lung epithelial cells in vitro. Chemical and isogenic transposon mutants of GBS strains which are NH are noninjurious, and those which are HH are hyperinjurious, to lung epithelia. The GBS product(s) associated with epithelial cell injury is stabilized in culture extracts by the same compounds (starch-Tween 80) which stabilize activity against erythrocyte membranes. Thus, it appears that the GBS beta-hemolysin(s) possesses a broader range of host cell specificity and may be more appropriately classified as a membrane-disrupting cytolysin. Several other gram-positive hemolysins, e.g., the alpha-toxin of *S. aureus* (43), streptolysin O of *Streptococcus pyogenes* (6), and the plasmid pAD1-encoded hemolysin-bacteriocin of *E. faecalis* (22), are known to injure mammalian epithelial cells.

The GBS beta-hemolysin has yet to be isolated. Some evidence exists to suggest that it is normally attached to the bacterial surface membrane (30). Extracellular hemolytic activity is rapidly lost unless high-molecular-weight stabilizer molecules are present (25). Because the hemolysin does not alter the gel column elution behavior of various carrier molecules, it appears to be a small molecule (44). Sensitivity to the protease subtilisin suggests that it is a protein (26), but attempts to visualize it by gel electrophoresis or to raise antibody against crude preparations have been unsuccessful (11, 26).

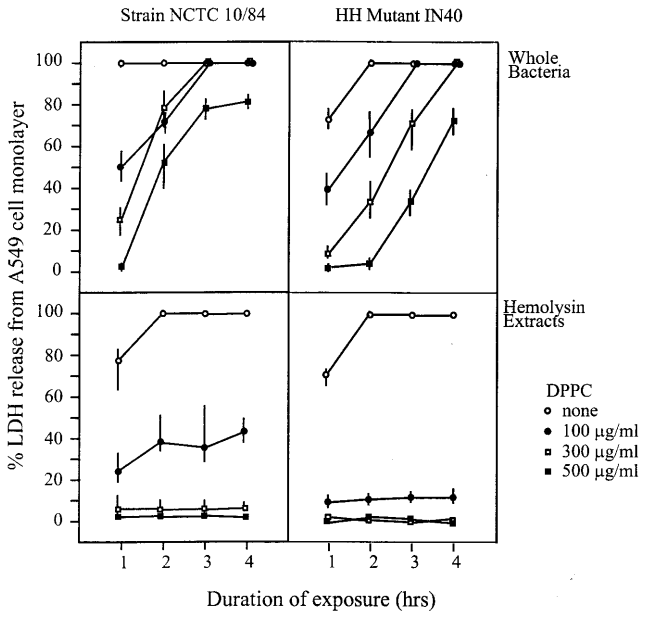


FIG. 6. Inhibition of GBS hemolysin-associated epithelial cell injury by the phospholipid DPPC. The graph depicts LDH release from the HH clinical isolate NCTC 10/84, the HH mutant IN40, and starch-Tween 80 hemolysin extracts from each strain. Increasing DPPC concentration in the medium is associated with decreased release of LDH and therefore diminished cellular injury.

The cloning of a GBS genetic determinant in a pUC8 vector which allowed *Escherichia coli* host strains to express beta-hemolysis on blood agar has been reported (10). An open reading frame with a deduced amino acid sequence of 230 residues, including a possible hydrophobic signal sequence of 19 amino acids at the N-terminal end, was identified. This putative GBS hemolysin gene had no homology with any known bacterial hemolysin or streptococcal protein.

The electron microscopic studies of GBS beta-hemolysin-induced injury to lung epithelial cells are suggestive of a pore-forming toxin. Discrete membrane disruptions, cellular swelling, loss of intracytoplasmic density, and changes in organelles and chromatin are consistent with entry of water into the cell and hypo-osmotic damage, analogous to changes seen with *S. aureus* alpha-toxin (5) or the terminal membrane attack complex (C5b-9) of human complement (29). Earlier work had shown that radiolabeled rubidium ($^{86}Rb^{+}$) and hemoglobin demonstrated identical efflux kinetics from sheep RBC following exposure to GBS beta-hemolysin (26), suggesting that the toxin produces membrane lesions of large size.

Injury of lung epithelial cells by GBS beta-hemolysin in vitro suggests a possible pathogenic role for this molecule in human disease. Direct damage to host cell membranes could contribute to the severe pneumonia characteristic of early-onset GBS infection. Disruption of the epithelial cell barrier might also facilitate access to the bloodstream and systemic spread by the organism. In the microtiter LDH release assay, injury of lung epithelial cells is detected at bacterial inocula of between 10^6 GBS per ml (most hemolytic clinical isolate NCTC 10/84) and 10^8 GBS per ml (least hemolytic clinical isolate COH1). When GBS pneumonia was induced in newborn primates, bacterial density reached 10^9 to 10^{11} organisms per g of lung tissue (33), indicating a large potential reservoir for beta-hemolysin production and epithelial cell injury.

There is precedent for pore-forming bacterial hemolysin-cytolysins as virulence factors involved in producing pneumonia or lung injury. *S. aureus* alpha-toxin (37) and *Escherichia coli* hemolysin (Hly) (12) produce thromboxane-mediated vasoconstriction and edema formation in isolated, perfused rabbit lungs and may be implicated in the development of septic lung failure. More recently, two RTX family hemolysins of the gram-negative bacterium *Actinobacillus pleuropneumoniae* were shown to be important virulence factors in the production of hemorrhagic and necrotic lung infections in swine (39).

Knowledge of the effects of GBS beta-hemolysin production on virulence in animal models is limited. NH mutant strain COH31 clone 12 did not show an increased 50% lethal dose compared with the parent strain following subcutaneous injection in neonatal rats (45). In contrast, intravenous administration of partially purified GBS hemolysin extracts in rabbits or rats produced dose-dependent hypotensive changes and a limited number of deaths due to shock, findings not produced by streptolysin S from *Streptococcus pyogenes* (18). Both models, however, bypass the initial interaction with the lung epithelial barrier critical to the pathogenesis of GBS pneumonia. In perhaps a more relevant model, Wennerstrom et al. (46) used NH and HH chemical mutants of a GBS isolate to inoculate adult mice intranasally and found that increased hemolysin production was associated with a decreased 50% lethal dose and earlier time to death for a given inoculum. Lung histopathology was not evaluated in that study.

GBS beta-hemolysin activity is inhibited by a number of phospholipid components of surfactant (25), and Tapsall and Phillips (42) have suggested that this inhibition may yield a clue to the increased susceptibility of premature infants, who are deficient in surfactant, to severe early-onset GBS pneumonia. Our observation that DPPC, the primary component of human surfactant, inhibits LDH release from lung epithelial cells exposed to beta-hemolysin is consistent with this hypothesis. The range of DPPC concentrations used in the inhibition experiments (100 to 500 μg/ml) corresponds roughly to the actual increase in concentration of DPPC found in human fetal alveolar fluid through the third trimester of pregnancy to delivery (8). In mechanically ventilated, preterm rabbits which received an intratracheal challenge of GBS, treatment with exogenous surfactant appeared to reduce inflammatory changes on histologic examination of lung tissue (20). It is noteworthy that pulmonary involvement is uncommon in late-onset GBS disease, which more often affects term infants (3), and recent reports of invasive GBS disease affecting immunocompromised adults (14).

The invariant link between the levels of beta-hemolysin expression and pigment production among the NH and HH GBS mutants produced in this study is consistent with the in vitro observations of others (40, 45, 46) and suggests a genetic linkage of the two phenotypes. Although it has been shown that the pigment is not a carrier for the hemolysin (41), we cannot exclude definitively the possibility that the pigment in some way contributes to GBS beta-hemolysin-induced cytotoxicity.

In summary, we have shown a direct correlation between GBS beta-hemolysin expression and lung epithelial cell injury, a correlation which holds across several serotypes and with a number of mutagenesis techniques. The isogenic, single-transposon-insertion GBS hemolysin mutants described in this study will allow future studies to (i) identify DNA sequences flanking the transposon insertion sites which may represent the beta-hemolysin structural gene or elements involved in its regulation, (ii) attempt to isolate the beta-hemolysin by comparative analysis of preparations from A909 and its NH and HH variants, (iii) investigate a possible genetic linkage between beta-hemolysin and pigment production, and (iv) directly test

the role of the GBS beta-hemolysin (cytolysin) in virulence in animal models of early-onset GBS pneumonia.

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