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Invasion of Brain Microvascular Endothelial Cells by Group B Streptococci

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Group B streptococci (GBS) are the leading cause of meningitis in newborns. Although meningitis develops following bacteremia, the precise mechanism or mechanisms whereby GBS leave the bloodstream and gain access to the central nervous system (CNS) are not known. We hypothesized that GBS produce meningitis because of a unique capacity to invade human brain microvascular endothelial cells (BMEC), the single-cell layer which constitutes the blood-brain barrier. In order to test this hypothesis, we developed an in vitro model with BMEC isolated from a human, immortalized by simian virus 40 transformation, and propagated in tissue culture monolayers. GBS invasion of BMEC monolayers was demonstrated by electron microscopy. Intracellular GBS were found within membrane-bound vacuoles, suggesting the organism induced its own endocytic uptake. GBS invasion of BMEC was quantified with a gentamicin protection assay. Serotype III strains, which account for the majority of CNS isolates, invaded BMEC more efficiently than strains from other common GBS serotypes. GBS survived within BMEC for up to 20 h without significant intracellular replication. GBS invasion of BMEC required active bacterial DNA, RNA, and protein synthesis, as well as microfilament and microtubule elements of the eukaryotic cytoskeleton. The polysaccharide capsule of GBS attenuated the invasive ability of the organism. At high bacterial densities, GBS invasion of BMEC was accompanied by evidence of cellular injury; this cytotoxicity was correlated to beta-hemolysin production by the bacterium. Finally, GBS demonstrated transcytosis across intact, polar BMEC monolayers grown on Transwell membranes. GBS invasion of BMEC may be a primary step in the pathogenesis of meningitis, allowing bacteria access to the CNS by transcytosis or by injury and disruption of the endothelial blood-brain barrier.

Group B streptococci (GBS) are the most common cause of meningitis in human newborns. Mortality is high despite antibiotic therapy, and 25 to 50% of surviving infants are left with permanent neurological sequelae, including cognitive deficits, spastic quadriplegia, cortical blindness, deafness, and seizures (8, 15). Although neonatal meningitis develops as a consequence of hematogenous spread of the organism, the factors responsible for GBS entry into the central nervous system (CNS) have not been determined. GBS capsular serotypes commonly associated with bloodstream infections in newborns are Ia, Ib, II, III, and V (1, 37). All serotypes may produce meningitis; however, type III strains account for a disproportionate share of CNS isolates (1, 51).

The blood-brain barrier, responsible for maintaining biochemical homeostasis within the CNS, is a single layer of specialized brain microvascular endothelial cells (BMEC) which exhibit continuous tight junctions and a conspicuous absence of pinocytosis (3, 33). The association of GBS with meningeal infection implies a capacity for the organism to breach this endothelial blood-brain barrier. A common mechanism by which pathogenic microorganisms penetrate host barriers exploits eukaryotic endocytic pathways: the bacterium invades the host cell within a membrane-bound vacuole. In previous in vivo and tissue culture studies, we have demonstrated GBS invasion of alveolar epithelial and pulmonary endothelial cells (24, 41, 42), each an important step in the pathogenesis of systemic disease. In a separate line of investigation, we found that *Escherichia coli* K1, the second leading cause of neonatal meningitis, invaded cultured BMEC (25, 35, 36) and that a noninvasive Tn*phoA* mutant identified in vitro was significantly less able to penetrate the CNS in newborn rats challenged hematogenously (25).

We hypothesized that GBS, like *E. coli* K1 strains, are neurotropic, based on a unique ability to invade and survive with human BMEC, the single-cell layer which comprises the blood-brain barrier. To test this hypothesis, we developed an in vitro model using BMEC isolated from a human, immortalized by simian virus 40 transformation, and propagated in tissue culture monolayers. Electron microscopy was used to examine the ultrastructural characteristics of GBS interaction with BMEC. Next, the invasive abilities of various GBS strains and the contributions of selected bacterial or host cell processes to BMEC invasion were quantified in antibiotic protection assays. Finally, the ability of GBS to transcytose intact, polar BMEC monolayers was examined by means of a Transwell filter system.

MATERIALS AND METHODS

Bacterial strains and mutants. Six clinical isolates of GBS were used in this study: COH1, a highly-encapsulated type III strain (50); K79, a type III strain (27); B523, a type Ia strain; M709, a type Ib/c strain; DK23, a type II strain; and NCTC 10/84 (1169-NT1), a type V strain (52) (strains B523, M709, and DK23 were provided courtesy of C. J. Baker). All strains were isolated from the blood or spinal fluid of septic neonates. The Challis strain of *Streptococcus gordonii* was used as a control. GBS mutants COH1-13 (nonencapsulated), COH1-20 (nonhemolytic), and IN40 (hyperhemolytic) are previously described isogenic derivatives of strain COH1, each containing a single insertion of Tn*916*ΔE into their chromosome (30, 40). Bacteria were grown to mid-log phase in Todd-Hewitt

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broth to an optical density at 600 nm of 0.4 (equal to ~10^8 CFU/ml) for use in all assays.

BMEC cultures. Human BMEC were isolated from a brain biopsy of an adult female with epilepsy by methods previously described (45). These cells were positive for factor VIII-Rag, carbonic anhydrase IV, and *Ulex europaeus* agglutinin I; took up fluorescently labeled acetylated low-density lipoprotein; and expressed gamma glutamyl transpeptidase, demonstrating their brain endothelial cell properties (47). BMEC were subsequently immortalized by transfection with simian virus 40 large T antigen and maintained their morphologic and functional characteristics (46). BMEC were cultured in RPMI 1640, supplemented with 10% fetal calf serum, 10% NuSerum (Becton Dickinson, Bedford, Mass.), modified Eagle's medium nonessential amino acids, L-glutamine, and penicillin-streptomycin. Twenty-four- or 96-well tissue culture plates (Corning) were precoated with rat tail collagen to support the BMEC monolayers. Cultures were incubated at 37°C in a humid atmosphere of 5% CO_2. BMEC were split in a ratio of 1:4 twice a week with trypsin-EDTA. Immediately prior to each assay, the monolayers were washed three times with phosphate-buffered saline (PBS), and fresh BMEC medium without antibiotics (invasion assays) or RPMI 1640 alone (cellular injury assay) was added.

Electron microscopic studies. To monolayers of ~2×10^4 BMEC cells in 24-well tissue culture plates, 10^5 (multiplicity of infection [MOI], 5 bacteria/cell) or 10^7 (MOI, 500 bacteria/cell) CFU of log-phase GBS strain COH1 were added in tissue culture medium, centrifuged at $800 \times g$ for 10 min to place GBS at the surface of the BMEC monolayer, and then incubated for 2 h at 37°C with 5% CO_2. The supernatants were removed by gentle aspiration, and the monolayers were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and then postfixed in 2% osmium tetroxide in double-distilled water as described previously (6, 7). Following dehydration of samples through a graded alcohol series, the cells were embedded in Medcast (Ted Pella, Inc., Redding, Calif.). Thin sections were cut with a diamond knife on an LKB Nova ultramicrotome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate, and then examined with a JEOL-1200EX electron microscope at 80 kV at magnifications of ×4,000 and ×10,000.

Cellular invasion assay. Log-phase GBS (10^8 CFU/ml) were pelleted, washed, and resuspended in RPMI medium. Dilutions in RPMI 1640 were performed such that inocula of between 10^3 and 10^7 CFU were added to a well of a 24-well tissue culture plate containing a monolayer of BMEC in 0.5 ml of medium (MOI of 0.05 to 500 bacteria/cell). The plates were centrifuged at $800 \times g$ for 10 min to place GBS at the surface of the BMEC monolayer, and then they were incubated for 2 h at 37°C with 5% CO_2 to allow cellular invasion by the bacteria. The monolayers were washed three times with PBS, 1 ml of BMEC medium containing 100 μg of gentamicin and 5 μg of penicillin G per ml was added to each well, and the plates were incubated for 2 h at 37°C with 5% CO_2 to kill extracellular and surface-adherent bacteria. The monolayers were washed three times with PBS, 0.1 ml of trypsin-EDTA solution was added, the mixture was incubated for 10 min at 37°C, and then 0.4 ml of 0.025% Triton X-100 was added, and each BMEC monolayer was disrupted by repeated pipetting to liberate intracellular bacteria. One-tenth of the lysate (50 μl) from each well was added to 3.5 ml of Todd-Hewitt soft (0.7%) agar maintained at 50°C, and then the sample was vortexed briefly, pour plated onto standard Todd-Hewitt agar, and incubated overnight at 37°C. The percent invasion of BMEC by GBS was calculated as [10 × (CFU on plate count)/CFU in original inoculum] × 100%. In one set of experiments, total BMEC-associated (invading plus surface-adherent) GBS were quantified as follows. BMEC monolayers were exposed for 2 h at 37°C to identical inocula of GBS as described above, but in place of the 2-h exposure to extracellular antibiotics, the monolayers were washed six times with PBS, then lysis was performed with trypsin-EDTA plus Triton X-100 as described above, and a 50-μl aliquot of the lysate was used for quantitative plating. Lysis conditions do not affect GBS viability (42). All cellular invasion assays were performed in triplicate and repeated twice.

Invasion inhibition studies. Invasion assays were performed as described above, except that the BMEC medium contained the indicated concentration of the inhibitor throughout the initial 2 h of incubation. When antibiotic inhibitors of bacterial cell functions were tested, concentrations corresponding to 0.5, 1.0, and 2.0 times the MIC of the inhibitor for test strain COH1 were used (42). For test strain COH1, the MICs were as follows: ciprofloxacin, 0.78 μg/ml; rifampin and minocycline, 25.0 μg/ml (each). When inhibitors of eukaryotic cell function were tested, the BMEC were additionally preincubated in the presence of the inhibitor for 30 min at 37°C. One exception was that BMEC cells were preincubated with nocodazole for 1 h on ice, as required for complete microtubule disruption (39), and then warmed to 37°C for 30 min prior to the assay. We had earlier shown that de novo protein synthesis by eukaryotic cells, as measured by [^{35}S]methionine incorporation, is present during the 2-h invasion assay but can be inhibited by cycloheximide (42). All cellular invasion assays were performed in triplicate and repeated twice. Data are expressed as percent invasion relative to that in assays performed concurrently without inhibitor.

Assay for cytolytic activity. A microtiter plate assay was used to determine the BMEC cytolytic activity of GBS. Briefly, 10^8 CFU of log-phase GBS were pelleted, washed, and 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 BMEC (~4×10^3 cells; MOI, 2,500 bacteria/cell), and serial twofold dilutions in RPMI were added onto other monolayers across the plate. RPMI medium alone and bacteria in RPMI without a BMEC monolayer were used as negative controls; complete lysis of a BMEC monolayer with 100 μl of distilled H_2O 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 with a miniaturized version of the Sigma colorimetric assay as previously described (30). The BMEC cytolytic titer of a GBS strain was calculated as the reciprocal of the greatest dilution producing 50% LDH release versus that of the positive control. The assay was performed in duplicate and repeated four times.

Transcytosis assay. An assay was developed to examine the ability of GBS to transcytose polarized BMEC monolayers. BMEC were seeded onto the apical side of a 4.7-cm^2 collagen-coated polytetrafluoroethylene membrane with a pore size of 0.4 μm (Transwell-COL; Millipore). The basolateral chamber of a six-well cluster plate contained 2.6 ml and the apical chamber of the Transwell contained 1.5 ml of BMEC medium. The cells required 10 to 12 days to form intact polarized monolayers based on electrical resistance (ohms per square centimeter) measured with a Millicell-ERS resistance system (Millipore). For the transcytosis assay, the BMEC monolayers (~4×10^4 cells per Transwell) were washed, and fresh BMEC medium without antibiotics was added. Log-phase (10^5 CFU; MOI, 2.5 bacteria/cell) GBS strain COH1 and/or *S. gordonii* (control) cells were applied to the apical chamber, and the monolayers were incubated at 37°C in 5% CO_2. The electrical resistance was measured as the membranes were transferred to new six-well cluster plates containing fresh medium in the basolateral chamber. Fifty-microliter samples from the lower chamber were obtained at 1, 2, and 4 h of incubation and plated on Todd-Hewitt agar for quantitation. For coinfection experiments, aliquots were plated on New Granada medium (11), which allowed discrimination of GBS from *S. gordonii* on the basis of pigment production by GBS. Transcytosis assays were performed in quadruplicate and repeated three times.

Statistical analysis. All data in figures are presented as means ± standard deviations (error bars). The effect of inhibitors on GBS invasion of BMEC was tested with a one-tailed, unpaired *t* test. The effect of capsule phenotype on invasion was assessed with a two-tailed, unpaired *t* test. Changes in electrical resistance across polar BMEC monolayers over time were compared by single-factor analysis of variance.

RESULTS

Electron microscopy. GBS interactions with BMEC were examined by electron microscopy. Figure 1 shows low- and high-magnification views of a BMEC exposed for 2 h to 10^5 CFU of type III GBS strain COH1 (MOI, 5 bacteria/cell). Invading GBS are observed intracellularly within membrane-bound vacuoles. At the cytoplasmic surface, a GBS is found enveloped by microvillus structures of the BMEC. The bacterium is closely contacting the cell membrane, appearing to elicit its own endocytic uptake. In Fig. 2, BMEC invasion at low (MOI, 5 bacteria/cell) and high (MOI, 500 bacteria/cell) inocula of GBS strain COH1 are compared. At the lower inoculum (Fig. 2A), normal BMEC morphology, including dense regular cytoplasmic contents, a paucity of pinocytotic vesicles, and evenly distributed nuclear chromatin, is observed. GBS are found in close proximity to a microvillus projection at the cell surface, and invading GBS, including a dividing form, are seen within a membrane-bound intracellular vacuole. At the higher inoculum (Fig. 2B and C), invading GBS are also observed, but only in association with signs of BMEC injury, including disruption of the endocytic vacuole, loss of cytoplasmic density, splitting, and discontinuity of the cytoplasmic membrane, dilation of the endoplasmic reticulum, and clumping of nuclear chromatin.

Standardization of invasion assay. In order to optimize the 2-h antibiotic protection assay, a standard curve was developed (data not shown). GBS invasion of BMEC was found to be roughly linear for bacterial inocula of 1×10^3 to 2×10^5 CFU per well but began to plateau at higher inocula, at which point morphologic evidence of BMEC injury was sometimes observed. A standard inoculum of 10^5 CFU of log-phase GBS (MOI, 5 bacteria/cell) was selected for assays which compared the levels of invasiveness of different strains or tested the effect of inhibitors on the invasion process.

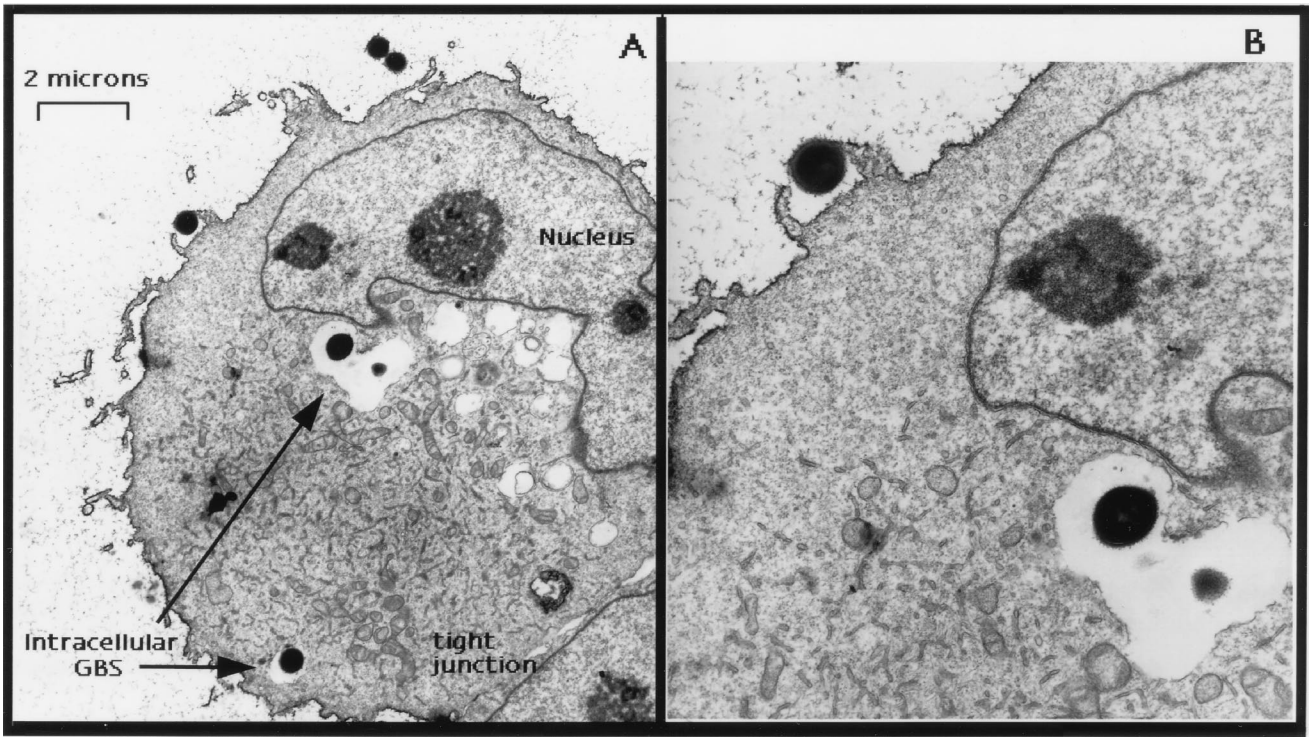

FIG. 1. Transmission electron micrograph demonstrating GBS invasion of BMEC at magnifications of ×3,000 (A) and ×7,500 (B). Intracellular GBS are found within membrane-bound vacuoles. At the cell surface, a GBS appears to induce BMEC structural rearrangements consistent with an endocytic mechanism. Composite image was constructed with Adobe Photoshop 3.0.

Invasion of BMEC by GBS of various serotypes. The GBS capsular serotypes commonly associated with bloodstream infections in newborns are Ia, Ib, II, III, and V; however, type III accounts for a disproportionate amount of meningeal isolates. Invasion of BMEC by two type III GBS clinical isolates was compared to that by clinical isolates belonging to other capsular serotypes (Fig. 3). All GBS strains invaded BMEC, whereas the control *S. gordonii* strain was noninvasive. The two type III isolates invaded BMEC more efficiently (5 to 6% of the original inoculum) than strains belonging to the other common capsule serotypes (1 to 2.5%).

Assay for intracellular replication. On certain electron micrographs, dividing forms of GBS were observed inside BMEC within large endocytic vacuoles (Fig. 2). We hypothesized that GBS may replicate intracellularly after invasion of BMEC. To test this hypothesis, we performed an invasion assay in which the time between addition of extracellular antibiotics and lysis of the BMEC monolayer to enumerate intracellular GBS was lengthened from 2 h to 4 or 20 h (Fig. 4). With both type III strains tested, the number of intracellular GBS did not change significantly at 2, 4, and 20 h after addition of antibiotics. These data indicated that GBS survived within BMEC for an extended period of time following invasion, but that significant intracellular replication of GBS did not occur.

GBS invasion relative to adherence of BMEC. We hypothesized that GBS adherence to the BMEC surface may be a preliminary step in the invasion process. To assess the degree of GBS adherence to BMEC relative to cellular invasion, total cell-associated (surface-adherent plus intracellular) GBS were quantified from BMEC monolayer lysates prepared after the initial 2-h incubation and wash steps, but prior to exposure to extracellular antibiotics. A standard invasion assay was performed with replicate BMEC monolayers for each inoculum of the test strain COH1. Data are expressed as percent invasion at each inoculum and are shown in Fig. 5. At a very low inoculum (10^3 CFU; MOI, 0.05 bacteria/cell), the number of intracellular GBS was statistically equivalent to the total number of BMEC-associated GBS, suggesting highly efficient endocytic uptake of any surface-adherent organisms. As the bacterial inoculum was increased 10- and 100-fold, the absolute number of intracellular GBS recovered increased. However, the percentage of intracellular GBS recovered relative to (i) the original inoculum or (ii) the percentage of BMEC-associated GBS dropped in a stepwise fashion, indicating that the efficiency of the endocytic uptake mechanism was saturable (Fig. 5). At the standard inoculum used for comparative assays (10^5 CFU; MOI, 5 bacteria/cell), approximately 30% of total BMEC-associated GBS had invaded the intracellular compartment within the 2-h incubation period.

Effect of bacterial inhibitors on BMEC invasion. Inhibition of bacterial DNA, RNA, or protein synthesis was achieved with the antibiotics ciprofloxacin, rifampin, and minocycline, respectively. BMEC invasion assays were performed in the presence of each antibiotic at 0.5, 1.0, and 2.0 times the MIC for GBS strain COH1 (42). Each antibiotic resulted in dose-related decreases in GBS invasion of BMEC (Fig. 6). The protein synthesis inhibitor minocycline produced the most marked effect. These data demonstrated that active synthesis of bacterial DNA, RNA, and protein was important for BMEC invasion.

Effect of eukaryotic inhibitors on BMEC invasion. We tested the effects of various inhibitors of eukaryotic cell function on GBS invasion of BMEC. The invasion assay was performed on BMEC monolayers preincubated with cycloheximide (protein synthesis inhibitor), cytochalasin D (actin microfilament aggregation inhibitor), colchicine, or nocodazole (mi-

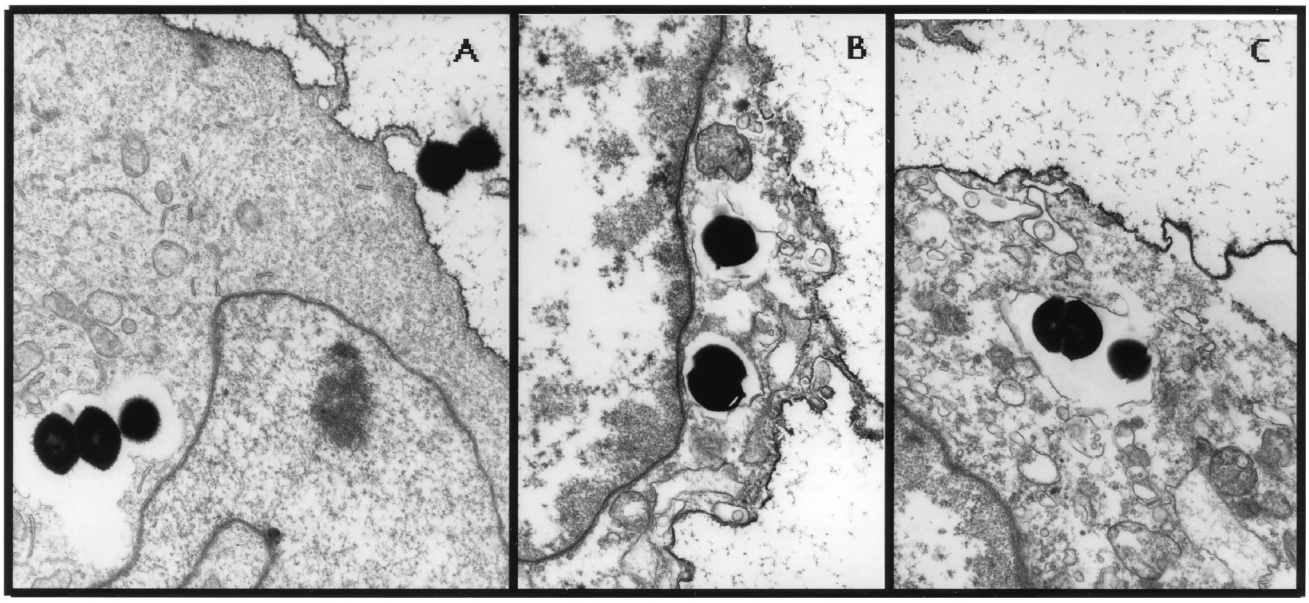

FIG. 2. Transmission electron micrographs (magnification, ×7,000) demonstrating BMEC invasion by GBS at low (MOI, 5 bacteria/cell [A]) and high (MOI, 500 bacteria/cell [B and C]) inocula. Although intracellular GBS are identified in each case, higher inocula are associated with evidence of significant BMEC injury, including disruption of the endocytic vacuole, loss of cytoplasmic density, and clumping of nuclear chromatin. Composite image was constructed with Adobe Photoshop 3.0.

crotubule polymerization inhibitors). Each inhibitor was also present for the initial 2-h incubation period. Over the dose ranges tested, decreases in BMEC invasion by GBS strain COH1 were observed with each inhibitor (Fig. 7). These data indicated that active BMEC protein synthesis, as well as microfilament and microtubule elements of the BMEC cytoskeleton, was required for efficient uptake of GBS within endocytic vacuoles.

Effect of GBS polysaccharide capsule on BMEC invasion. The polysaccharide capsule of type III GBS is a proven virulence factor important in resistance to phagocytic clearance (40, 50). Because of the predominance of type III strains among isolates from infants with meningitis, we sought to examine the contribution of the type III capsule itself to GBS invasion of BMEC. This was accomplished by comparing the invasive ability of type III strain COH1 to that of its isogenic, capsule-deficient Tn*916* derivative, COH1-13. The capsule-deficient mutant was found to invade BMEC more efficiently (17.2% ± 1.4% of input inoculum) than the parent strain (9.9% ± 1.2% of input inoculum) ($P < 0.001$). Thus, the presence of the type III polysaccharide capsule appeared to attenuate the ability of GBS to invade BMEC.

Effect of GBS beta-hemolysin phenotype on BMEC invasion. The vast majority of GBS isolates, regardless of capsule serotype, exhibit beta-hemolytic activity on blood agar. We previously identified a potential virulence role for GBS beta-hemolysin expression in lung epithelial cell injury characteristic of early-onset pneumonia (30). We examined a possible role for GBS beta-hemolysin expression in BMEC invasion by comparing the invasive ability of type III strain COH1 to those of the isogenic Tn*916* mutants COH1-20 and IN40, which exhibit nonhemolytic and hyperhemolytic phenotypes, respectively. Studies were performed with three different initial inocula (10^4, 10^5, and 10^6 CFU), and the results are expressed as total intracellular CFU rather than percent invasion (Fig. 8). The increase in intracellular CFU associated with a higher inoculum was more pronounced for the nonhemolytic mutant than for the parent strain. The amount of intracellular CFU recovered from the hyperhemolytic mutant actually dropped off as the initial inoculum was increased from 10^5 to 10^6 CFU. At higher bacterial inocula, greater beta-hemolysin expression by GBS was associated with an apparent decrease in BMEC invasion.

GBS beta-hemolysin-associated injury to BMEC. We hypothesized that the apparent decrease in BMEC invasion associated with (i) higher bacterial inocula and (ii) greater beta-hemolysin expression was attributable to injury to the BMEC monolayer, with resultant lysis of BMEC and exposure of intracellular GBS to killing by gentamicin and penicillin. To quantify cellular injury by GBS, release of the intracellular enzyme LDH from the BMEC monolayers was measured in a microtiter plate dilution assay (Table 1). No LDH release beyond baseline (medium alone) was detected from BMEC monolayers exposed for 4 h to 10^7 CFU of the nonhemolytic mutant strain COH1-20 (MOI, 2,500 bacteria per cell). In contrast, exposure to the weakly hemolytic parent strain COH1 released 50% of total LDH from BMEC monolayers at an

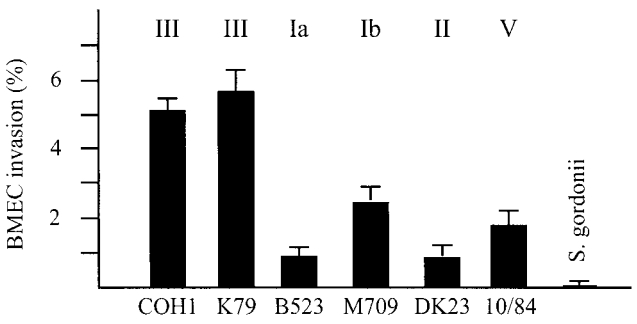

FIG. 3. BMEC invasion by GBS strains representing the capsular serotypes (Ia, Ib, II, III, and V) commonly associated with neonatal infection.

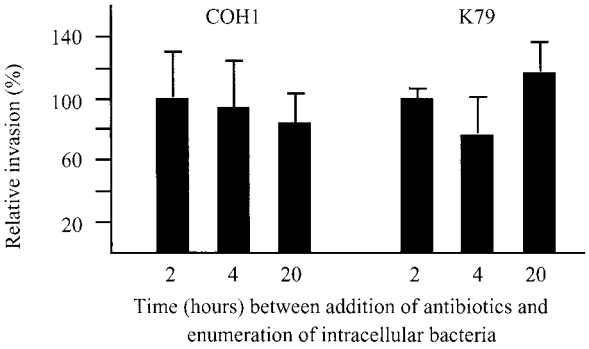

FIG. 4. Time course assay to determine whether serotype III GBS multiply intracellularly after invasion of BMEC.

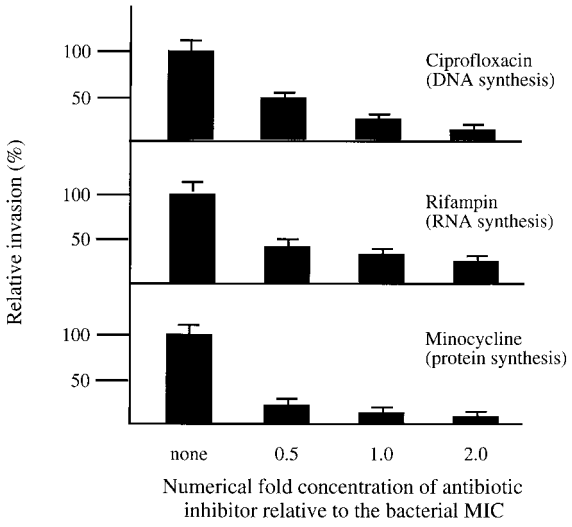

FIG. 6. Effect of various antibiotic inhibitors of bacterial cell functions on the ability of GBS to invade BMEC ($P < 0.001$ at each concentration of each antibiotic versus control). Each antibiotic inhibitor was tested at concentrations equal to 0.5, 1.0, and 2.0 times the MIC of that antibiotic for GBS test strain COH1.

MOI of 1,250 bacteria per cell. The hyperhemolytic mutant strain IN40 produces 16 to 32 times more hemolysin than COH1 (30). Exposure of BMEC monolayers to the hyperhemolytic mutant resulted in release of 50% of the total LDH at an MOI of 10 to 20 bacteria per cell. Thus, there was a strong correlation between the amount of beta-hemolysin production by the test strain and injury to the BMEC monolayer. At high bacterial inocula, the actions of GBS beta-hemolysin may explain the electron microscopic findings of BMEC injury (Fig. 2) and confound interpretation of the cellular invasion assay (Fig. 8).

GBS transcytosis of a polar BMEC monolayer. The ability of cultured mammalian BMEC to form polar monolayers with tight junctions has been demonstrated by peripheral actin filament distribution on electron microscopy (33), impermeability to protein markers (14), asymmetric localization of the efflux peptide, P-glycoprotein, to the apical (luminal) surface (4), and transendothelial electrical resistance (12). In the present study, we noted morphologic evidence of tight junction formation between human BMEC by electron microscopy (Fig. 1). We found that BMEC grown to confluence on collagen-coated polytetrafluoroethylene Transwell membranes formed polar monolayers, as confirmed by the development of transendothelial electrical resistance equal to 500 to 600 $\Omega \cdot cm^2$.

We hypothesized that GBS enters the CNS by transcytosis (i.e., penetration through the intact polar BMEC monolayer which comprises the human blood-brain barrier). In this model, GBS would invade BMEC at the apical (luminal) side, survive intracellularly, and ultimately penetrate through the basolateral BMEC membrane to reach the cerebrospinal fluid within the subarachnoid space. To test this hypothesis in our cell culture model, we inoculated GBS in the apical chamber of the Transwell, allowed them to interact with the polar BMEC monolayer, and sampled the bottom (basolateral) chamber to identify bacteria which had penetrated through the monolayer. A noninvasive *S. gordonii* strain was used as a control, and coinfection experiments with both organisms were performed to assess the specificity of transcytosis.

The results of the Transwell experiments are summarized in Table 2. Because growth of bacteria in the bottom chamber following transcytosis would tend to skew the normal distribution, we report the median and range of CFU recovered in addition to the mean. Transcytosis of the BMEC monolayer by type III GBS (inoculum, 10^5 CFU) was detected in 6 of 11 wells by 1 h (mean, 273 CFU), 9 of 11 wells by 2 h (mean, 582

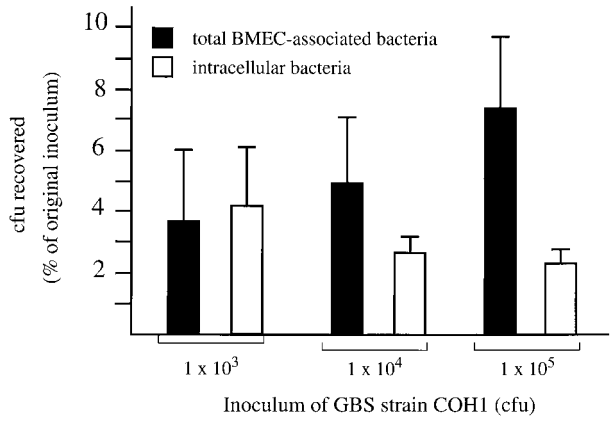

FIG. 5. Efficiency with which GBS invade BMEC monolayers relative to surface adherence.

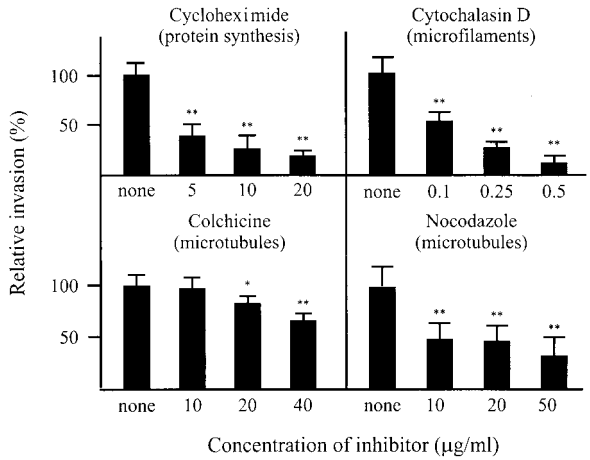

FIG. 7. Effect of specific inhibitors of various eukaryotic cell functions on GBS invasion of BMEC. *, $P < 0.05$ versus no inhibitor; **, $P < 0.001$ versus no inhibitor.

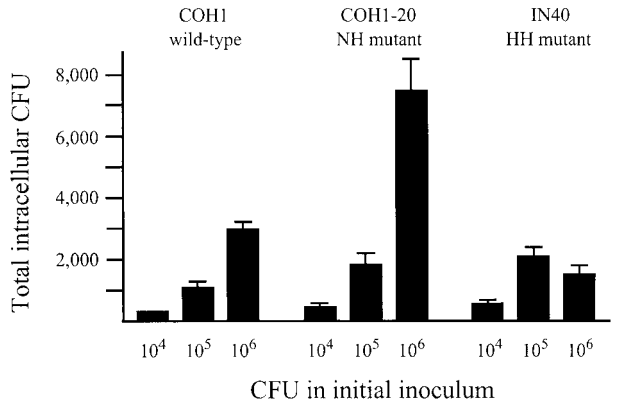

FIG. 8. Comparison of BMEC invasion by GBS strain COH1 and isogenic Tn*916*ΔE mutants with a nonhemolytic (NH) or hyperhemolytic (HH) phenotype. The percent invasion ranged from 2.70% at the low inoculum to 0.13% at the high inoculum.

CFU), and 10 of 11 wells at 4 h (mean, 4,386 CFU). In contrast, transcytosis of BMEC by *S. gordonii* was negligible and was detected in 2 of 12 wells at 1 h (mean, 8 CFU), 0 of 12 wells at 2 h, and 2 of 12 wells at 4 h (mean, 17 CFU). Experiments in which equal numbers of GBS and *S. gordonii* cells were added to the apical chamber yielded results similar to those with GBS alone, and all bacteria which transcytosed the BMEC monolayer were identified as GBS by orange pigmentation of colonies on New Granada medium. The latter finding indicated that GBS transcytosis of the BMEC was not associated with disruption of monolayer integrity enough to allow passive transit of the noninvasive *S. gordonii* cells. Unexpectedly, the number of GBS which transcytosed BMEC was greater in monolayers coinfected with *S. gordonii* than in monolayers infected with GBS alone, especially at the 2- and 4-h time points. We speculate that certain nonspecific streptococcal factors (e.g., cell wall lipoteichoic acid) may act as stimuli for BMEC to engulf bacteria, but that only GBS (in contrast to *S. gordonii*) possesses the virulence attributes necessary to efficiently engage the BMEC cell surface, survive within the endocytotic vacuole, and ultimately exit by exocytosis through the basolateral cell membrane.

Transendothelial electrical resistance decreased by small amounts (8 to 14%) over the 4-h course of the transcytosis assay in monolayers exposed to GBS, *S. gordonii*, or both strains. Although this decrease in resistance over time reached statistical significance for BMEC monolayers exposed to GBS alone ($P < 0.05$), at no individual time point were significant differences noted among the monolayers exposed to the GBS strain versus the *S. gordonii* strain or both strains ($P > 0.2$). We conclude that GBS are capable of transcytosis across a polar BMEC monolayer and that such transit occurs principally through cells with intact tight junctions and not by passive diffusion across areas of monolayer disruption.

TABLE 1. Correlation of GBS beta-hemolysin production with injury of BMEC as measured by LDH release

Cell type	Titer		MOI resulting in 50% LDH release (no. of bacteria/cell)
	Hemolytic[a]	Cytolytic	
COH1 (parent)	1	2	1,250
COH1-20 (Tn*916*ΔE)	0	0	ND[b]
IN40 (Tn*916*ΔE)	16–32	128–256	10–20
S. gordonii	0	0	ND

[a] Obtained from previous studies (30).
[b] ND, not determined (no LDH release into medium detected at an MOI of 2,500 bacteria/cell).

TABLE 2. Transcytosis of GBS across polar BMEC monolayers grown on Transwell filters

Time checked	No. of monolayers in which transcytosis occurred/total monolayers	No. of CFU in bottom well		Electrical resistance ($\Omega \cdot cm^2$)[b]
		Mean (median)	Range[a]	
1 h				
GBS	6/11	273 (100)	0–1,050	599 ± 40
S. gordonii	2/12	8 (0)	0–50	571 ± 27
Both[c]	6/11	577 (100)	0–2,350	602 ± 25
2 h				
GBS	9/11	582 (350)	0–2,450	528 ± 4
S. gordonii	0/12	0 (0)		537 ± 16
Both	10/11	4,582 (400)	0–26,800	550 ± 22
4 h				
GBS	10/11	4,386 (2,200)	0–19,850	510 ± 21
S. gordonii	2/12	17 (0)	0–150	523 ± 26
Both	11/11	25,005 (8,700)	400–100,000	550 ± 37

[a] Limit of detection, 50 CFU.
[b] Baseline resistance (time 0) measurements: COH1 (GBS) monolayers, 590 ± 40 $\Omega \cdot cm^2$; *S. gordonii* monolayers, 558 ± 36 $\Omega \cdot cm^2$; coinfection (both) monolayers, 591 ± 36 $\Omega \cdot cm^2$.
[c] Both, coinfection experiment with GBS strain COH1 and *S. gordonii*. Note that all bacteria transcytosing the BMEC monolayer appeared to be GBS by pigmentation.

DISCUSSION

GBS are the leading cause of meningitis in human newborns. Inadequate understanding of the basic pathogenic mechanisms by which GBS penetrate the CNS contributes to the high morbidity and mortality associated with this infection. In the neonatal rat model, development of GBS meningitis is correlated to the magnitude and duration of bacteremia (17), an observation which holds for other bacterial agents of childhood meningitis (5, 43). The studies described herein demonstrate for the first time that GBS are capable of invading human BMEC, the single-cell layer which comprises the blood-brain barrier. GBS are able to transcytose polar BMEC monolayers, and at high bacterial concentrations, significant injury to BMEC can be correlated to beta-hemolysin expression by the organism. GBS invasion of BMEC may be a primary step in the pathogenesis of neonatal meningitis, allowing circulating bacteria access to the CNS by transcytosis or by injury and disruption of the endothelial blood-brain barrier.

We have previously demonstrated adhesion to and invasion of bovine and human BMEC by *E. coli* K1, the second leading cause of neonatal bacterial meningitis (25, 35, 36). Electron microscopic evidence of *Haemophilus influenzae* invasion of bovine BMEC has also been reported (33). Our present data suggest that GBS may be particularly proficient at entry into and survival within human BMEC. *E. coli* K1 cell invasion of human BMEC occurred at frequencies of 0.002 to 0.11% of the input inoculum (35), whereas GBS invaded at frequencies of 0.89 to 5.73% of the input inoculum. Strain differences in GBS invasion of BMEC were observed. Two GBS clinical isolates belonging to capsule serotype III were more invasive (5.08 and 5.73%) than isolates from serotypes Ia, Ib, II, and V (0.89 to

2.49%). These limited data are consistent with epidemiologic observations. Whereas cases of early-onset septicemia are rather evenly distributed among the five capsule serotypes we studied, a striking predilection for serotype III strains among infants with meningitis (up to 90% of isolates) is well documented (1, 51). It is interesting to speculate that type III GBS strains may possess qualitative or quantitative differences in specific virulence factors which facilitate BMEC invasion, thereby increasing the likelihood an infant will develop meningitis as a complication of bacteremia.

The type III polysaccharide capsule itself does not facilitate GBS invasion of BMEC. Rather, an acapsular transposon mutant invaded 70% more efficiently than the wild-type strain. It is possible that the polysaccharide capsule produces steric interference of certain receptor-ligand interactions important in the invasion process, or that repulsive forces are generated between negatively charged sialic acid residues on the capsule and the BMEC surface. One must also consider that the type III capsule confers an important survival advantage on GBS through inhibition of macrophage and neutrophil phagocytosis (40, 50). Should BMEC share rudimentary aspects of their endocytic uptake mechanism with these "professional" phagocytes, capsule attenuation of BMEC invasion may be a byproduct of the stronger selective pressure placed on GBS to avoid immunologic clearance.

Inhibition by capsular polysaccharide is among a number of features held in common when GBS invasion of BMEC is compared to GBS invasion of A549 alveolar epithelial cells (26, 42). Active synthesis of bacterial DNA, RNA, and protein was required for efficient invasion of either cell type, and decreased numbers of intracellular GBS were noted even at suboptimal MICs of each of the antibiotic inhibitors. Minocycline, a protein synthesis inhibitor, produced the most marked inhibition of GBS cellular invasion, as has been shown for other invasive bacterial pathogens (18). Invasion of BMEC and A549 cells by GBS was blocked by cytochalasin D, which inhibits actin microfilament polymerization, a finding consistent with the uptake of most bacteria into eukaryotic cells (10, 19, 21). In contrast to A549 cell invasion, however, GBS entry into BMEC was decreased by the eukaryotic protein synthesis inhibitor cycloheximide and the microtubule depolymerizing agents colchicine and nocodazole. These data indicate that either (i) more than one pathway exists for GBS entry into BMEC cells or (ii) the pathway for GBS entry into BMEC is more complex than that for A549 cell entry, involving de novo protein synthesis by the BMEC and both microfilament and microtubule components of the eukaryotic cytoskeleton. Invasion of epithelial cells by enteropathogenic or enterohemorrhagic *E. coli*, *Neisseria gonorrhoeae*, *Citrobacter freundii*, and *Haemophilus influenzae* (13, 31, 32, 38, 44) and invasion of human umbilical vein endothelial cells by *E. coli* K1 (29) are examples of pathogen-host cell interactions which are both microfilament and microtubule dependent.

As was found in the alveolar epithelial cell model, GBS did not replicate appreciably following invasion of BMEC, although the organisms were capable of surviving intracellularly for up to 20 h. Certain bacteria, for example *Shigella flexneri* or *Salmonella* spp., replicate following invasion of intestinal epithelial or reticuloendothelial cells (9, 20). For these enteric pathogens, intracellular parasitism appears to play a significant role in the clinical disease state, which may follow a protracted course. GBS, perhaps only marginally adapted to the intracellular environment of the host, is not associated with chronic systemic infections in human neonates. Rather, the pathogenic significance of cellular invasion by GBS may lie in transit of the bacterium through mucosal and endothelial barriers of the newborn host, with acute or fulminant septicemia and/or meningitis the potential consequence.

On electron microscopic examination, intracellular GBS were always observed within membrane-bound vacuoles and were never free within the BMEC cytoplasm. Our transcytosis experiments demonstrated that GBS translocated a polar BMEC monolayer on a Transwell filter within a few hours, without marked changes in transendothelial electrical resistance and without allowing passive diffusion of the noninvasive control strain of *S. gordonii*. Transwell experiments were performed with an inoculum of GBS strain COH1 (MOI, 2.5 bacteria per cell) considerably less than that associated with significant injury to BMEC (50% cytolytic titer, 1,250 bacteria per cell). Thus a potential mechanism by which GBS enter the central nervous system may involve (i) invasion of BMEC from the capillary lumen, (ii) transport within the membrane-bound endocytic vacuole to the basolateral side, and (iii) exocytosis onto the basement membrane of the subarachnoid space. A perivascular distribution of bacteria within the subarachnoid space is a characteristic histopathologic finding of GBS meningitis, preceding development of an inflammatory exudate in acute disease and in the youngest neonates (2, 17).

We observed injury of BMEC following exposure to GBS at high bacterial inocula. BMEC injury was correlated with beta-hemolysin production by the organism. Under electron microscopy, injury to BMEC resembled our earlier findings of GBS beta-hemolysin-associated lung epithelial cell injury (30), which we have attributed to an apparent pore-forming cytolytic activity of the GBS hemolysin. Characteristic features included disruption of the cytoplasmic membrane and loss of cytoplasmic density consistent with hyposmotic damage due to water influx (Fig. 2). Our in vitro findings may be of particular clinical relevance, because neonates with GBS meningitis have initial cerebrospinal fluid bacterial concentrations of 10^7 to 10^8 CFU per ml (16, 22). These densities are comparable to the density at which the weakly hemolytic type III clinical isolate COH1 was found to lyse 50% of a BMEC tissue culture monolayer (5×10^6 CFU in 0.2 ml = 2.5×10^7 CFU per ml).

In studies with polarized lung microvascular endothelial cell monolayers, we have shown that GBS hemolysin expression was associated with increased albumin flux across the endothelium (24). Damage to BMEC from the effects of GBS hemolysin could contribute to increased permeability of the blood-brain barrier. Leakage of plasma proteins into the subarachnoid space is an important pathogenic mechanism of bacterial meningitis, leading to development of cerebral edema, elevation of intracranial pressure, and impairment of cerebral blood flow (34, 48). Finally, endothelial cells injured or activated by bacterial products may release proinflammatory mediators such as cytokines or prostaglandins, promoting transendothelial migration of leukocytes and development of an inflammatory exudate (49).

In summary, we have provided the first evidence that GBS are capable of invading human BMEC, the single-cell layer comprising the blood-brain barrier. Our in vitro model should prove useful in further investigations of the earliest steps in the pathogenesis of GBS meningitis, including identification and characterization of specific virulence factors responsible for bacterial invasion, endothelial transcytosis, and entry into the CNS.

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