Group B Streptococcal Pilus Proteins Contribute to Adherence to and Invasion of Brain Microvascular Endothelial Cells

Heather C. Maisey, Mary Hensler, Victor Nizet, and Kelly S. Doran*

Department of Pediatrics, Division of Pharmacology & Drug Discovery, University of California, San Diego School of Medicine, La Jolla, California 92093

Received 28 July 2006/Accepted 4 October 2006

Group B Streptococcus (GBS), a gram-positive bacterial pathogen, is a major cause of meningitis in newborns. In order to cause central nervous system infection, blood-borne GBS must interact with and breach the blood-brain barrier (BBB), comprised primarily of a single layer of brain microvascular endothelial cells. While GBS adheres to and invades human brain microvascular endothelial cells (hBMEC) (9), the specific GBS factors that contribute to this process are only beginning to be elucidated. Proper anchoring of lipoteichoic acid on the GBS surface facilitates hBMEC invasion, while the pore-forming β-hemolysin/cytolysin is cytolytic for hBMEC; each factor promotes BBB penetration and lethality in a mouse model of hematogenous meningitis (1, 2). A recent study has also demonstrated that GBS fibrinogen-binding protein FbsA contributes to hBMEC adherence and invasion in vitro (14).

Proteins targeted for cell surface expression in GBS are predicted to share a C-terminal sequence (L/IPXTG) for sortase recognition and anchoring to the gram-positive cell wall, and we have examined candidate genes encoding this motif for roles in the cellular pathogenesis of GBS meningitis. Serotype V GBS clinical isolate NCTC10/84 is highly virulent in the mouse model of hematogenous meningitis (1, 2). A recent study has also demonstrated that GBS fibrinogen-binding protein FbsA contributes to hBMEC adherence and invasion in vitro (14).

Surface filamentous structures known as pili have been discovered recently in the gram-positive streptococcal pathogens that cause invasive disease in humans, including group B Streptococcus (GBS). We show that two GBS proteins involved in pilus formation, encoded by pilA and pilB, also facilitate the interaction of this important agent of central nervous system infection with endothelial cells of the human blood-brain barrier.

To probe the functional role of PilA and PilB, we generated knockout mutants of GBS strain NCTC10/84 with mutations in each gene by precise, in-frame allelic replacement with a chloramphenicol resistance cassette using our established methods (6). These mutant strains exhibited growth kinetics equivalent to those of the wild-type (WT) GBS parent strain in Todd-Hewitt broth and the RPMI-based culture medium used in our in vitro assays. Additionally, no differences were observed between the WT and mutant strains with respect to β-hemolysin/cytolysin activity or sensitivity to the penicillin and gentamicin concentrations used in our invasion assays (data not shown).
The GBSΔpilA and GBSΔpilB mutants were analyzed using our well-characterized in vitro model of the BBB (2, 9), consisting of hBMEC that maintains the morphological and functional characteristics of primary brain endothelial cells (7, 12). Cell maintenance and quantitative assays for GBS hBMEC adherence and invasion were performed as described previously (9). Briefly, invasion of hBMEC by GBS was quantified using a standard antibiotic protection assay in which intracellular organisms were recovered and enumerated after selective killing of extracellular bacteria by antibiotic treatment. To assess the level of surface-adherent (total cell-associated) bacteria for the mutant and WT strains, bacteria were quantified from hBMEC monolayers prior to the addition of extracellular antibiotics. The data were expressed as percentages of adherence or invasion based on the original inoculum (10⁵ CFU; multiplicity of infection, 1 to 3 bacteria/cell) and then were normalized to levels calculated for the WT GBS strain. As shown in Fig. 2A, the GBSΔpilA mutant was 60% less adherent to hBMEC than the WT strain, while for the GBSΔpilB mutant there was not a significant change in adherence. Conversely, when strains were analyzed to determine their abilities to invade hBMEC, the GBSΔpilB mutant exhibited a significant reduction in invasion (40%) compared to the invasion by the WT and GBSΔpilA strains. Thus, it appears that the PilA protein contributes to the initial attachment of GBS to brain endothelium, while the PilB protein contributes to the process of bacterial internalization.

In order to further establish that the GBS pilA and pilB genes specifically contribute to hBMEC adherence and invasion, respectively, we performed single-gene complementation and heterologous expression analyses. Both pilA and pilB genes were amplified from GBS genomic DNA, cloned into the Escherichia coli-streptococcal shuttle expression vector pDCerm (6) to obtain pDCpilA and pDCpilB, and used to transform the corresponding GBSΔpilA and GBSΔpilB mutants. Complementation of the ΔpilA and ΔpilB mutant strains with the corresponding genes on plasmid vectors reversed the observed adherence (PilA) and invasion (PilB) defects (Fig. 2A and B). The presence of the vector-only control in the ΔpilA and ΔpilB mutant strains did not affect the recovery of adherent or intracellular bacteria (data not shown). To determine if the pilA and pilB genes are sufficient for hBMEC adherence and invasion, the pDCpilA and pDCpilB constructs were used to transform the nonpathogenic, noninvasive, gram-positive bacterium Lactococcus lactis. The empty pDCerm expression vector in L. lactis served as a control. Heterologous expression of the pilA gene in L. lactis resulted in a dramatic increase (~20-fold) in adherence to hBMEC (Fig. 2C), while similar expression of pilB increased the ability of this nonpathogen to invade hBMEC ~55-fold (Fig. 2D). Together, our results demonstrate that pilA is necessary and sufficient for hBMEC adherence, while pilB is necessary and sufficient for hBMEC invasion.

In summary, here we provide evidence that genes encoding surface-associated pilA play a role in the ability of GBS to adhere to and invade brain endothelium. Pili and pilus assembly have been best described in gram-negative bacteria, where
pili are known to mediate host-pathogen interactions important in colonization or the development of disease (11), and it is likely that they have a similar function in gram-positive pathogens (13). Our results suggest that the GBS PilA protein contributes to initial attachment to hBMEC as the GBS/H9004 pilA mutant was less adherent to host cells. These results are consistent with recent work demonstrating that the PilA homologue GBS 1478, but not the PilB homologue GBS 1477 (Fig. 1A), promoted adhesion to human pulmonary epithelial cells (3). The GBS pilA mutant did not exhibit a decrease in invasive ability compared to the WT strain. It is possible that the absence of PilA from the pilus structure results in increased interactions between PilB and hBMEC. Our results also demonstrate that PilB, a major pilus component, mediates GBS intracellular invasion of brain endothelium, a critical step in BBB penetration during GBS meningitis. We speculate that bacterial pilus components, including PilA and potentially PilC, promote the initial contact and attachment of GBS to host cell surfaces, which is followed by PilB-mediated initiation of bacterial internalization. The mechanism by which pili and pilus proteins facilitate bacterial invasion of host cells remains to be determined. Future examinations of the role of these specialized surface appendages in the pathogenesis of central nervous system infection by GBS and other pilus-expressing meningeval pathogens (e.g., *Streptococcus pneumoniae*) are merited.

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