The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion

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In humans, Streptococcus pneumoniae (SPN) is the leading cause of bacterial meningitis, a disease with high attributable mortality and frequent permanent neurological sequelae. The molecular mechanisms underlying the central nervous system tropism of SPN are incompletely understood, but include a primary interaction of the pathogen with the blood–brain barrier (BBB) endothelium. All SPN strains possess a gene encoding the surface–anchored sialidase (neuraminidase) NanA, which cleaves sialic acid on host cells and proteins. Here, we use an isogenic SPN NanA–deficient mutant and heterologous expression of the protein to show that NanA is both necessary and sufficient to promote SPN adherence to and invasion of human brain microvascular endothelial cells (hBMECs). NanA–mediated hBMEC invasion depends only partially on sialidase activity, whereas the N-terminal lectinlike domain of the protein plays a critical role. NanA promotes SPN–BBB interaction in a murine infection model, identifying the protein as proximal mediator of CNS entry by the pathogen.

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RESULTS AND DISCUSSION

SPN NanA mutant and heterologous expression of NanA in Lactococcus lactis

NanA is a molecule of 1,035 amino acids and ~107 kD molecular mass containing a C-terminal LP(X)TG anchor motif for sortase-mediated anchoring to the SPN cell wall.
NanA is necessary and sufficient to promote hBMEC adherence and invasion

Studies of microbial interactions with the human BBB have been greatly facilitated by the development of an immortalized hBMEC line retaining the morphological and functional characteristics of primary endothelium (Kim, 2006). Adherence and intracellular invasion of hBMEC within a membrane-bound vacuole appears to be a common phenotypic property of several CNS bacterial pathogens, including SPN (Ring et al., 1998), group B Streptococcus (Nizet et al., 1997), Escherichia coli K1 (Huang et al., 1995), and Haemophilus influenzae (Orihuela et al., 2009). Using an in vitro assay, we found the SPN \( \Delta \text{NanA} \) mutant to exhibit a >90% decrease in hBMEC adherence and invasion compared with the WT parent strain D39, a defect that was corrected upon complementation of the mutant with NanA on a plasmid vector (Fig. 1 D; \( P < 0.001 \)). Conversely, heterologous expression of SPN NanA in L. lactis conferred a >10-fold increase in the bacterium’s ability to adhere to and invade the cultured hBMEC (Fig. 1 E; \( P < 0.001 \)).

A sialidase inhibitor does not fully block NanA–dependent hBMEC invasion

To further dissect the roles of SPN NanA and its enzymatic activity in mediating pneumococcal interactions

**Figure 1.** SPN NanA is necessary and sufficient to promote hBMEC invasion and adherence in vitro. (A) Loss of sialidase activity in isogenic SPN \( \Delta \text{NanA} \) mutant, which is restored by plasmid complementation. (B) Heterologous expression of NanA on the Lactococcus lactis surface as detected by flow cytometry. (C) Gain of neuraminidase activity in L. lactis transformed with plasmid containing SPN NanA. (D) Decreased hBMEC adherence and invasion by \( \Delta \text{NanA} \) mutant compared with WT parent SPN strain, restored by complementation with a NanA-expressing plasmid in trans. (E) Increased hBMEC adherence and invasion by L. lactis expressing SPN NanA. Sialidase assay performed three times with similar results; representative experiment shown. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by Student’s t test.
Exogenous sialidase treatment modestly enhances NanA-dependent hBMEC invasion

Addition of exogenous sialidase (from *Arthrobacter ureafaciens*) failed to rescue the adherence and invasion defects of the SPN ΔNanA mutant, and did not stimulate *L. lactis* hBMEC interactions in the absence of NanA (Fig. 2, A and B). However, for SPN- or *L. lactis*-expressing NanA, exogenous sialidase stimulated a modest (20–30%), but statistically significant, increase in hBMEC adherence and invasion (Fig. 2, A and B). These results indicate that SPN NanA is both necessary and sufficient to promote efficient bacterial hBMEC adherence and invasion, and suggest that this phenotype is enhanced by, but not dependent on, the sialidase activity of the surface-bound protein.

Figure 2. Influence of exogenous sialidase or sialidase inhibition on the NanA-mediated hBMEC invasion phenotype. (A) Dose-dependent inhibition of the SPN NanA sialidase activity by the inhibitor Neu5Ac2en. (B) Dose-dependent partial inhibition of SPN invasion of hBMEC by Neu5Ac2en. (C) Addition of the sialidase inhibitor Neu5Ac2en reduces, but does not eliminate, hBMEC adherence and invasion by WT SPN. (D) Sialidase inhibition reduces, but does not eliminate, the ability of NanA to confer an hBMEC adherence and invasion phenotype to *L. lactis*. (E) Exogenous *A. ureafaciens* sialidase does not rescue hBMEC adherence and invasion defects of the SPN ΔNanA mutant. (F) Exogenous sialidase is not sufficient to promote increased hBMEC adherence and invasion by *L. lactis* lacking NanA. Sialidase assay performed three times with similar results; representative experiment shown. Adherence and invasion assays performed in triplicate and repeated three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by Student’s t test.
Relative contributions of NanA enzyme active site and N-terminal lectinlike domain in pneumococcal hBMEC invasion

Two targeted deletions were constructed in the recombinant NanA protein and cloned into expression plasmids (Fig. 3 A). These plasmids were then reintroduced by complementation into the SPN ΔNanA mutant to determine the consequences of each deletion on NanA-mediated hBMEC interactions. First, active site residues essential for the sialidase activity of SPN NanA have recently been identified (Yesilkaya et al., 2006), and we deleted a region containing two of these residues (Glu647 and Arg663) from pNanA to yield the derivative plasmid, pNanAΔenz (Fig. 3 A). When pNanAΔenz was used to complement the ΔNanA mutant, surface expression of the protein was restored to WT levels as assessed by flow cytometry (Fig. 3 B), but, as expected, this expression was not associated with detectable sialidase activity (Fig. 3 C).

Second, SPN NanA belongs to a superfamily of concavalin A–like glucanases, including the *Vibrio cholerae* sialidase (Crennell et al., 1994) and the *Trypanosoma cruzi* trans-sialidase (Buschiazzo et al., 2002), which contain a sialic acid cleavage domain and a second “lectinlike domain.” Because the sialidase activity of NanA is hypothesized to promote SPN colonization by modulating carbohydrate moieties on epithelial cell surfaces and exposing higher affinity receptors (Tong et al., 2002; King et al., 2006), it is conceivable that a NanA lectinlike domain, if indeed functional in promoting binding, could work together with its enzymatic activity in mediating host cell interactions. This laminin G-like domain of NanA is situated toward the N terminus, and we generated a deletion derivative of pNanA lacking this domain, pNanAΔlect (Fig. 3 A). When pNanAΔlect was used to complement the SPN ΔNanA mutant, WT levels of both surface protein...
expression (Fig. 3 B) and sialidase enzymatic activity (Fig. 3 C) were restored.

The ΔNanA mutant strains complemented with pNanA, pNanAΔEnz or pNanAΔLec were tested in the hBMEC invasion and adherence assays, as well as for their ability to transcytose across a polar hBMEC monolayer from the luminal to basolateral side (Fig. 3 D). In contrast to the full restoration of WT levels of adherence and invasion achieved by complementation with a WT NanA expression plasmid (Fig. 1 D), the pNanAΔEnz yielded only partial complementation of the phenotypes, to ~50% WT levels (Fig. 3 D). On statistical analysis, these intermediate levels of hBMEC adherence, invasion, and transcytosis were significantly greater than those observed for the ΔNanA mutant, although significantly less than those observed for the WT parent strain or the mutant complemented with the intact NanA protein (Fig. 3 D). Thus, NanA sialidase enzymatic activity makes a small, but still demonstrable, contribution to these pneumococcal–hBMEC interactions, which is consistent with the modest effects of sialidase inhibition or exogenous sialidase treatment (Fig. 2, A–F). In contrast, only a very small increase in adherence, invasion, or transcytosis was detected when the ΔNanA mutant was complemented with pNanAΔLec (to ~20% WT levels), and this did not achieve statistical significance when compared with the same mutant transformed with empty vector control. Moreover, the pNanAΔLec–complemented mutant was significantly less adherent and invasive than the pNanAΔEnz–complemented strain (P < 0.001; Fig. 3 D). These data reveal a clear role of the N-terminal lectinlike domain in NanA-mediated pneumococcal hBMEC interactions. The dual functionality of pneumococcal NanA in steric acid cleavage and promotion of host cell binding draws analogy to the activities of *T. cruzi* trans-sialidase, a virulence factor that can bind to lymphocytes, neurons, and heart endothelial cells to stimulate important host cell activation and proliferation phenotypes (Saavedra et al., 1999; Chuenkova and Pereira, 2000; Gao et al., 2002; Chuenkova and Pereira-Perrin, 2004).

**NanA peptide or anti-NanA antibody inhibits SPN hBMEC adherence and invasion**

The ability of NanA to promote a level of SPN hBMEC adherence and invasion even in the absence of its sialidase activity suggested the protein also serves directly as an adhesin/invasin. We found a dose-dependent inhibition of SPN adherence and invasion of hBMEC by the recombinant NanA protein fragment (Fig. 4 A), which is consistent with a competitive inhibition of cellular receptor(s), suggesting that SPN NanA can function in the capacity of an adhesin/invasin for the brain endothelium. Consistent with this model, addition of rabbit polyclonal antisera raised against the same recombinant NanA protein fragment also produced a concentration-dependent inhibition of SPN hBMEC adherence and invasion (Fig. 4 B).

**NanA promotes SPN–BBB interaction in vivo**

Lastly, we sought to determine whether the marked contribution of SPN NanA to hBMEC adherence and invasion in vitro translated to increased capacity of SPN to penetrate the BBB in vivo. Our choice of animal model had to exclude the confounding contribution of NanA to sustained bacteremia because a NanA-deficient SPN mutant was recently found to be cleared from the mouse circulation within 24 h of intravenous challenge, whereas the WT parent strain proliferated (Manco et al., 2006). To focus specifically on SPN–BBB interactions, we developed a short-term (4-h) murine intravenous challenge model that incorporated terminal exsanguination by saline perfusion (to eliminate blood contamination), allowing calculation of the ratio of SPN CFU in brain versus blood at the experimental endpoint. We found that at 4 h, the levels of WT D39 and isogenic ΔNanA mutant SPN in blood were similar, but the median ratio of brain/blood CFU in the WT strain (0.512%) was 16-fold greater than the ΔNanA mutant (0.031%; P < 0.01; Fig. 5 A). Histopathologic analysis by Student’s t test.
analysis and tissue Gram-stain could identify lancet-shaped diplococci associated with brain endothelium in sections from WT-infected mice; CNS-associated bacteria were scant or absent in those animals infected with the isogenic ΔNanA mutant (Fig. 5 B).

Role of NanA in SPN–BBB interactions and the development of meningitis

We conclude that NanA plays an important role in the SPN invasion of BBB endothelial cells and bacterial entry into the CNS, the critical first step in the pathogenesis of meningitis. The contribution of SPN NanA to hMBEC invasion involves an adhesin function, in which the N-terminal lectinlike domain plays a critical role, and a small but significant contribution of the sialidase activity, which may modify host cell receptors to reveal higher affinity ligands, as previously hypothesized for NanA in nasal colonization (King et al., 2006). After SPN breaches the BBB endothelium, subsequent brain injury is more likely the byproduct of different SPN virulence determinants (e.g., the pore-forming toxin pneumolysin; Braun et al., 2002) or host factors, as previous studies using direct intracerebral installation of SPN in mice or guinea pigs identified no differences between WT and ΔNanA mutant in meningeal inflammation, hearing loss, or neuronal injury scores (Winter et al., 1997; Wellmer et al., 2002). Nevertheless, a clinical correlation between the magnitude of free sialic acid levels in cerebrospinal fluid during pneumococcal meningitis and risk of adverse neurological sequelae including coma has been reported (O'Toole et al., 1971).

Loss of two additional SPN surface proteins has been previously associated with diminished invasion and transcytosis of cultured hMBEC cells—the fibronectin-binding protein PavA and choline-binding protein A (CbpA; Ring et al., 1998; Pracht et al., 2005). Operating in the context of the low capsule, “transparent” phase variant phenotype of SPN, CbpA binds the laminin receptor to promote hMBEC adhesion. In concert, CbpA presents SPN cell wall phosphorylcholine that attaches to platelet-activating factor receptor on the hMBEC surface, promoting invasion (Ring et al., 1998). Although the high-capsule, “opaque” phenotype of SPN predominates in the bloodstream (transparent forms are readily phagocytosed), the high-frequency switch between the two phase variants is sufficient to reveal a contribution of CbpA to BBB penetration in vivo (Orihuela et al., 2004). The studies of NanA reported in the present manuscript were all performed with inocula of SPN expressing the opaque phenotype. We propose that NanA and CbpA may function synergistically to promote passage of the phase-variant SPN bacterium across the BBB.

**Figure 5.** NanA contributes to SPN BBB penetration in vivo. (A) Comparison of bacterial counts (CFU) recovered from the blood and brain of mice 4 h after intravenous challenge with 5 × 10⁷ CFU of WT SPN strain D39 or its isogenic ΔNanA mutant. Results represent a single large experiment with n = 8 per group; all data shown. Because of the technically challenging complete saline perfusion before brain harvesting, individual mouse infections were initiated at staggered timed, with validation of bacterial inocula throughout by CFU counts. Although blood CFU recovered at 4 h did not differ between the two groups (P = 0.37), the ratio of brain CFU/blood CFU in individual mice was significantly higher in mice infected with the WT SPN strain than the ΔNanA mutant (P < 0.01). Statistics by Student's t test; red line shows median. A repeat experiment performed without saline perfusion and using a 6-h time point yielded similar results (Fig. S1). (B) Representative histopathological examination (100x oil immersion) of brain from WT SPN-infected mice showed Gram-positive cocci in association with brain microvascular endothelium or within the brain parenchyma (arrows); such findings were extremely rare or absent in animals infected with the ΔNanA mutant. Bar, 15 µM.
The conservation of NanA expression in all clinical isolates of SPN prompted interest in the recombinant protein as a universal vaccine antigen against this leading pathogen (Tong et al., 2005; Yesilkaya et al., 2006), as current conjugate polysaccharide vaccines only target a subset of the most common SPN serotypes. Our identification of NanA function in hBMEC adherence and invasion suggests such immunization strategies could have an added benefit in reducing the risk of meningitis as a secondary complication of invasive infection.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. SPN serotype 2 strain D39 (NCTC 7466) and its isogenic D39∆NanA mutant were used for these experiments. D39∆NanA was constructed by nonpolar insertion–duplication mutagenesis of the nanA gene, as previously described (Winter et al., 1997). SPN cultures were grown in Todd-Hewitt broth (THB) + 2% yeast extract (THY media). For complementation studies, the SPN ∆NanA mutant was transformed with empty vector control (pDC123), pNanA, or the deletion plasmids pNanΔlec and pNanΔenz, and grown in THY + 2 µg/ml chloramphenicol. L. lactis strain NZ9000 transformed with pDC123 or empty vector control (pDCern) were grown in THB + erythromycin (5 µg/ml). Surface expression of proteins was confirmed using a rabbit polyclonal antisera against NanA protein fragment (318–792), a fluorescent secondary antibody, and flow cytometry. After overnight incubation at 37°C, with 5% CO2, bacteria were reincubated into fresh media, grown to mid-logarithmic growth phase (OD600nm = 0.4), centrifuged at 4,000 rpm for 5 min, washed once with PBS, and then resuspended in the appropriate buffer for use in the assays described in the following paragraphs.

Generation of NanA expression plasmids. The NanA gene was amplified by PCR from the D39 chromosome using primers NanAUpF (5′-GATTGGAGAAAGGAGGGG-3′) and NanADwnR (5′-CGT-ATCACGCTGAAATGTCATC-3′), and the resulting amplicon directionally cloned into plasmid pDCern to yield the expression plasmid pNanA for gain-of-function studies in L. lactis. The amplicon was also subcloned into pDC123, which is identical to pDCern but bearing a chloramphenicol instead of erythromycin resistance marker, for complementation studies in the SPN ∆NanA mutant. Critical active site residues of NanA were eliminated from pNanA by inverse PCR using primers NanAenzUp (5′-TGTATTTTGGCAGCCTCTATGTC-3′) and NanAenzDwn (5′-GTTGACTGAGATCTCTTCAGTTG-3′). Blunt end ligation of the amplicon deleted residues 645–662, including 2 amino acids critical for sialidase activity, and the modified, enzyme-dead NanA was subcloned into vector pDC123 to produce pNanAΔenz, an expression plasmid for complementation analysis using the SPN ∆NanA mutant. To eliminate the N-terminal lectinlike domain of NanA, a similar process of inverse PCR followed by blunt end ligation was performed on pNanA using the primers NanAlecUp (5′-GGTTGACGTCCTCCCGAAGG-3′) and NanAlecDwn (5′-TGT-CCAGATGTAAGCGATGTC-3′). Subcloning of the modified peptide into pDC123 yielded pNanAΔlec, an expression/complementation plasmid containing NanA with a deletion of residues 74–229.

Sialidase activity assay. A quantitative assay using 4-Methylumbelliferyl-N-acetyl-b-D-neuraminic acid sodium salt hydrate (4-MU; Fluca USA) was used to assay sialidase activity. SPN (WT D39 and isogenic ∆NanA mutant) and L. lactis–expressing NanA constructs were centrifuged, washed once, resuspended in 100 µl 0.1M phosphate citrate buffer (pH 6.5) to 105 CFU/ml. Bacterial suspensions were sonicated (Sonic Dismembrator 550, Thermo Fisher Scientific) on ice for 5 s, followed by 15-s rest intervals × 5 cycles. Sample aliquots of 50 µl were added to individuals wells of a 96-well microtiter plate and mixed with 50 µl of 4-MU diluted to 0.35% in phosphate citrate buffer. The plate was incubated at 37°C and fluorescence (excitation 360 nm, emission 460 nm) was recorded every 15 min from time 0 to 1 h, with reported values corrected for the reaction blank containing phosphate citrate buffer alone.

hBMEC assays. Immortalized hBMEC line hBMEC, a product of SV-40 large T antigen transformation maintaining the morphological and functional characteristics of primary endothelium, was obtained from K.S. Kim (John Hopkins University, Baltimore, MD). hBMECs were maintained in RPMI 1640 medium (Life Tech Technologies, Inc.) supplemented with 10% FBS, 10% NuSerum (BiD), and 1% MEM nonessential amino acids, and were incubated at 37°C in 5% CO2. Bacterial adherence and invasion assays were performed essentially as previously described (Doran et al., 2005) within 24 h of established confluence (~105 cells/well) at multiplicity of infection = 10 bacteria/cell and duration 30 min (adherence assays) or 2 h (invasion assays). For a transcytosis assay, hBMEC were grown to confluence on collagen-coated 3 µM pore Transwell plates (Corning-Costar); 5 × 105 CFU of log-phase SPNs were added to the upper (luminal) chamber, and CFU reaching the lower chamber enumerated at the 4-h time point. For inhibition studies, a recombinant NanA protein fragment (residues 318–792) at 2.5 or 5.0 µg/well or anti-NanA rabbit polyclonal antisera at 1:250 or 1:500 final dilution were added to the adherence and invasion assays as indicated; controls for these experiments were bovine serum albumin (5.0 µg/well) and rabbit polyclonal antihyaluronidase antisera (1:250 dilution), respectively. Murine model of SPN brain endothelial cell invasion. 8–10 wk old male BALB/c mice were injected intravenously with 5 × 106 CFU of SPN D39 or its isogenic D39∆NanA mutant. 4 h after injection, blood was collected and serial dilutions plated to enumerate bacterial CFU in the circulation. Mice were then deeply anesthetized using an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Their chests were opened, and blood was cleared by inserting a 23-gauge needle into the left ventricle, perfusing with sterile PBS for 7 min using a peristaltic perfusion pump (P-1; GE Healthcare), and allowing fluid to exit through the right atrium. After perfusion, brains were removed and the hemispheres were separated. One half of the brain was homogenized in PBS, diluted, and plated on THY agar plates to enumerate bacteria tightly adherent to the BBB; the other half was fixed in PBS + 4% PFA for histopathological analysis. A repeat mouse challenge experiment was performed as above, omitting the perfusion step, and using a 6-h rather than 4-h experimental endpoint.

Assurances. All animal experiments were approved by the University of California, San Diego (UCSD) Committee on the Use and Care of Animals and performed using accepted veterinary standards. The UCSD Biosafety Committee approved all protocols for bacteriologic experiments.

Online supplemental material. Fig. S1 is a repeat intravenous mouse challenge of WT SPN versus isogenic ∆NanA mutant to quantify bacterial entry into the brain compared with bacterial levels in the blood. This experiment represents a replicate of the study shown in Fig. 5, but omitting the saline perfusion step and using 6 h rather than 4 h endpoint. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090386/DC1.

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