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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Abbreviations used: BBB, blood–brain barrier; CbpA, choline-binding protein A; CNS, central nervous system; hBMEC, human brain microvascular endothelial cell; Neu5Ac2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; SPN, *Streptococcus pneumoniae*.

Streptococcus pneumoniae (SPN; pneumococcus) accounts for 50% of bacterial meningitis in humans. This often devastating infection carries a 30% mortality rate, and up to half of survivors experience neurological sequelae reflecting a wide spectrum of brain injuries, including cortical neuronal necrosis and hippocampal neuronal apoptosis (Koedel et al., 2002; van de Beek et al., 2004; Weisfelt et al., 2006; Weber and Tuomanen, 2007). To cause meningitis, bloodborne bacteria must first attach to and penetrate human brain microvascular endothelial cells (hBMECs), which is the single-cell layer comprising the majority of the blood–brain barrier (BBB; Tuomanen, 1996; Kim, 2003). The molecular mechanisms underlying the distinct central nervous system (CNS) tropism of SPN are poorly understood. All SPN clinical isolates express the surface-anchored sialidase (neuraminidase) NanA (Cámara et al., 1994; Pettigrew et al., 2006) that targets sialic acid residues on host cells and bacterial flora to promote SPN mucosal colonization (Shakhnovich et al., 2002; Tong et al., 2002). Here, we use an isogenic SPN NanA-deficient mutant and heterologous expression of the NanA constructs to probe the contribution of this protein to SPN-hBMEC interactions in vitro and BBB penetration in vivo.

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

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 206 No. 9 1845-1852
www.jem.org/cgi/doi/10.1084/jem.20090386

The Journal of Experimental Medicine

(Lock et al., 1988; Cámara et al., 1994). NanA sialidase activity targets at least three linkage classes of substrates: the α-2,6 and α-2,3 linkages of *N*-acetyl-neuraminic acid to galactose and the α-2,6 linkages of *N*-acetyl-neuraminic acid to *N*-acetyl-galactosamine (Scanlon et al., 1989). To investigate the potential contribution of NanA to SPN interactions with the BBB endothelium, we compared the well-characterized serotype 2 strain clinical isolate D39, which was originally isolated by Avery in 1916 (Avery et al., 1944) and is highly virulent in animal infection models (Lanie et al., 2007), to its isogenic mutant ΔNanA, which is confirmed to possess a targeted nonpolar mutation that eliminates NanA expression (Winter et al., 1997). Using a fluorescent assay, we found the ΔNanA mutant to lack sialidase activity present in the WT parent strain D39 (Fig. 1 A). For complementation and gain-of-function analyses, we amplified the NanA gene from the D39 chromosome, cloned it into an expression vector to generate the recombinant plasmid pNanA, and used this to transform the SPN ΔNanA mutant and nonpathogenic Gram-positive bacterium *L. lactis*, confirming NanA surface expression by flow cytometry in the latter (Fig. 1 B). Complementation in trans with NanA restored sialidase activity to the SPN ΔNanA mutant (Fig. 1 A). Furthermore, compared with the empty vector control, pNanA conferred significant sialidase activity to *L. lactis* (Fig. 1 C).

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 ΔNanA 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

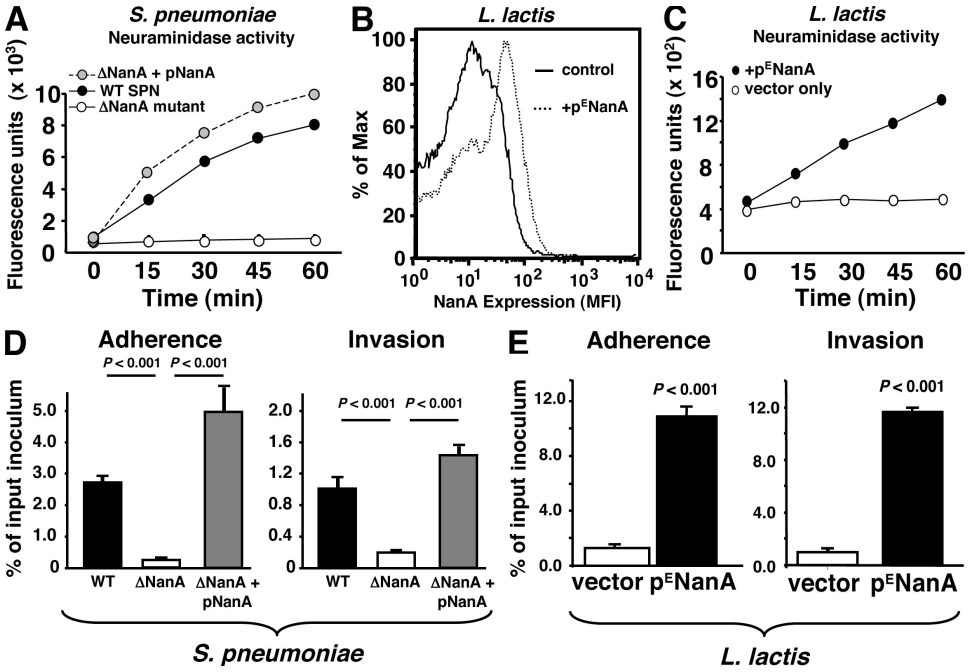

Figure 1. SPN NanA is necessary and sufficient to promote hBMEC invasion and adherence in vitro. (A) Loss of sialidase activity in isogenic SPN ΔNanA mutant, which is restored by plasmid complementation. (B) Heterologous expression of NanA on the *Lactococcus lactis* surface as detected by flow cytometry. (C) Gain of neuraminidase activity in *L. lactis* transformed with plasmid containing SPN NanA. (D) Decreased hBMEC adherence and invasion by ΔNanA 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.

with hBMEC, we used the broad-spectrum sialidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en; Holzer et al., 1993). Neu5Ac2en treatment resulted in a dose-dependent inhibition of NanA sialidase activity with maximal inhibition reaching a plateau between 2.5 and 10 mM (Fig. 2 A). Neu5Ac2en reduced, but did not eliminate, NanA-dependent adherence and invasion of hBMECs in both the SPN (Fig. 2, B and C) and *L. lactis* (Fig. 2 D) backgrounds. In contrast, the Neu5Ac2en inhibitor had no effect on the low-level baseline hBMEC interactions present in bacteria lacking NanA (Fig. 2, C and D). These findings support a significant contribution of NanA to hBMEC invasion that is independent of its enzymatic activity.

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.

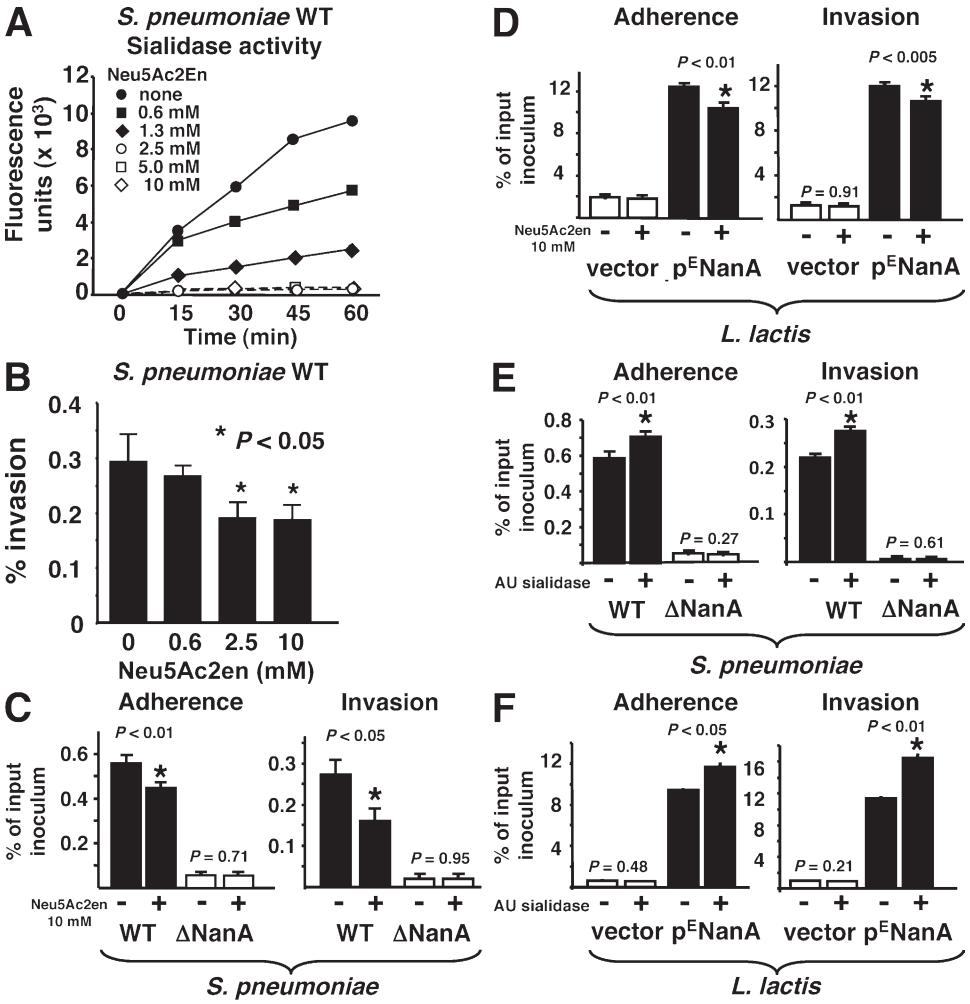

Figure 2. Influence of exogenous sialidase or sialidase inhibition on the NanA-mediated hBMEC invasion phenotype. (A) Dose-dependent inhibition of the SPN NanA sialidase activity by the inhibitor Neu5Ac2en. (B) Dose-dependent partial inhibition of SPN invasion of hBMEC by Neu5Ac2en. (C) Addition of the 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 *Trypanasoma 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

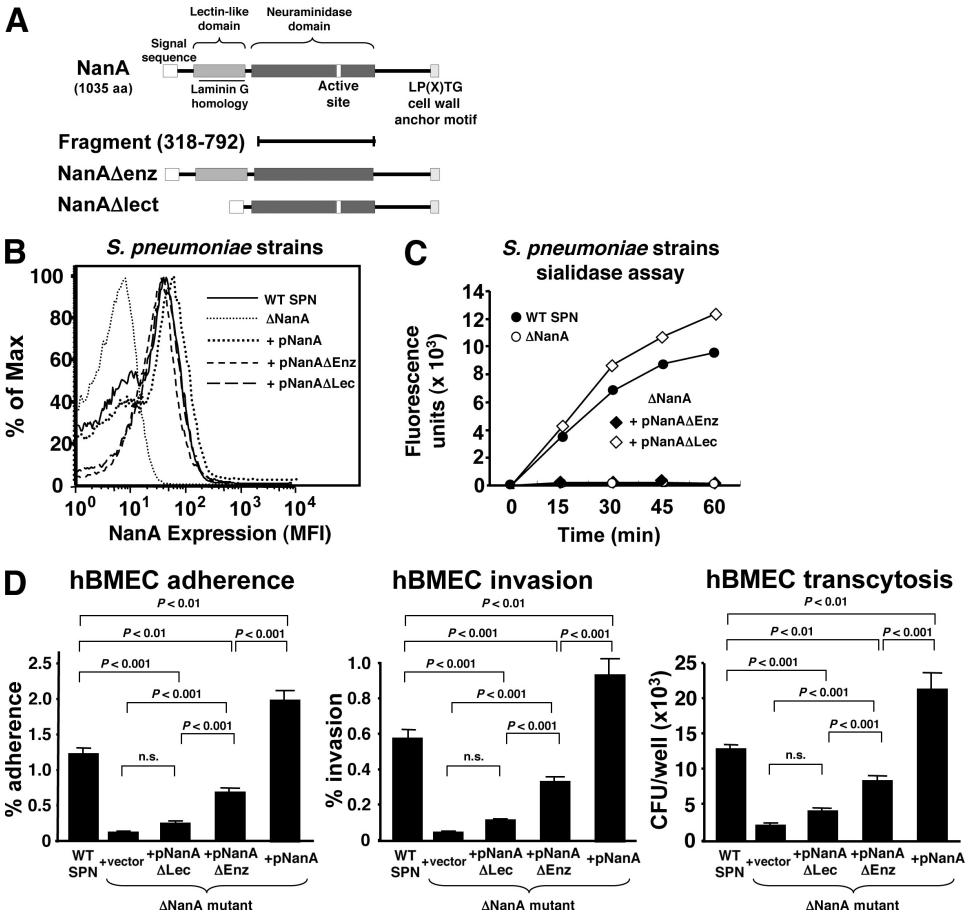

Figure 3. Relative contributions of the NanA enzymatic site and N-terminal "lectinlike" domains to the hBMEC invasion phenotype. (A) Scheme for deletion of active site residues and lectinlike domain from recombinant SPN NanA protein; position of protein fragment 318–792 used in subsequent studies. (B) Expression of mutant NanA protein constructs similar to that of NanA on the WT SPN surface as detected by flow cytometry using a rabbit polyclonal antisera against protein fragment 318–792. (C) Deletion of active site region eliminates NanA-induced sialidase activity in *L. lactis*, whereas deletion of the NanA lectinlike domain has no effect. (D) Targeted elimination of either the NanA enzymatic activity or the N-terminal lectinlike domain allows only partial complementation of the defect in hBMEC adherence, invasion, or transcytosis of the ΔNanA mutant versus WT SPN. Sialidase and transcytosis assays were performed three times with similar results; representative experiment shown. Adherence and invasion assays were performed in triplicate and repeated at least three times; graphs show cumulative data from all experiments. Error bars represent standard deviation, statistical analysis by analysis of variance with multiple pairwise comparisons.

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 sialic 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 PereiraPerrin, 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 moeity for the hBMEC surface or can stimulate an up-regulation of hBMEC processes that promote bacterial uptake independent of binding. To further probe these possibilities, an exogenous NanA protein fragment (amino acids 318–792, including key active site residues but lacking sialidase activity) was added to the adherence and invasion assays to determine if its presence blocked or stimulated SPN-hBMEC interactions. 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

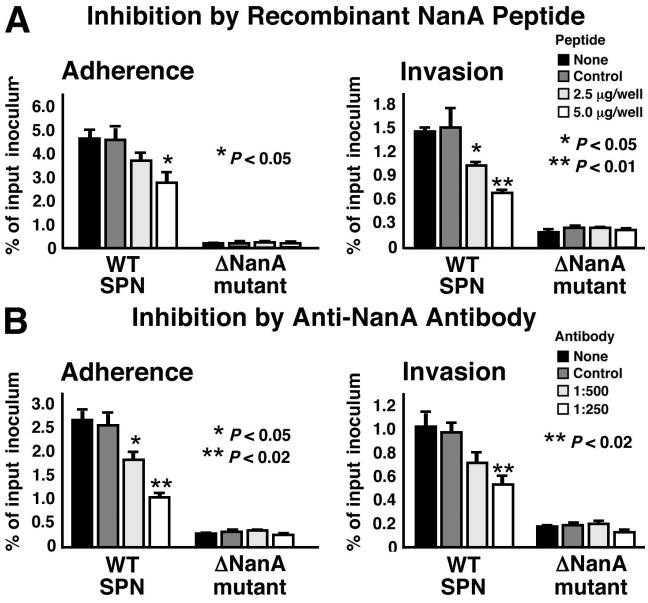

Figure 4. Inhibition of NanA-mediated SPN hBMEC adherence and invasion by recombinant protein or antisera. (A) Addition of a recombinant NanA protein fragment (amino acids 318–792) produces a dose-dependent inhibition of SPN hBMEC adherence and invasion. (B) Addition of rabbit polyclonal antisera against the same recombinant NanA protein fragment produces a concentration-dependent inhibition of SPN hBMEC adherence and invasion. Control peptide = bovine serum albumin 5.0 mg/ml; control antibody = polyclonal rabbit antihyaluronidase 1:250 final dilution. Assays were performed three times with similar results; representative assay shown. Error bars represent standard deviation, statistical 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 breeches 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 hBMEC 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 hBMEC adhesion. In concert, CpbA 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 CpbA may function synergistically to promote passage of the phase-variant SPN bacterium across the BBB.

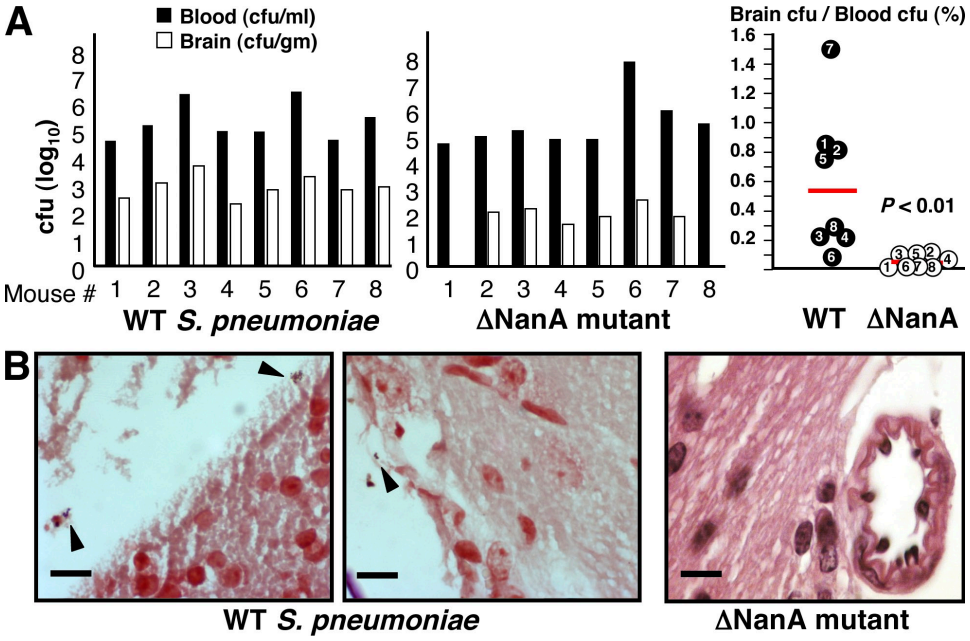

Figure 5. NanA contributes to SPN BBB penetration in vivo. (A) Comparison of bacterial counts (CFU) recovered from the blood and brain of mice 4 h after intravenous challenge with 5×10^7 CFU of WT SPN strain D39 or its isogenic ΔNanA mutant. Results represent a single large experiment with $n = 8$ per 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 (100× 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 D39ΔNanA mutant were used for these experiments. D39ΔNanA 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 ΔNanA mutant was transformed with empty vector control (pDC123), pNanA, or the deletion plasmids pNanAΔlect and pNanAΔenz, and grown in THY + 2 μg/ml chloramphenicol. *L. lactis* strain NZ9000 transformed with p^{E}NanA or empty vector control (pDCerm) 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% CO_2, bacteria were reinoculated into fresh media, grown to mid-logarithmic growth phase ($OD600_{nm}$ = 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 inthe following paragraphs.

Generation of NanA expression plasmids. The NanA gene was amplified by PCR from the D39 chromosome using primers NanAUpF (5′-GATTGGAGAAAGGAGAGGGG-3′) and NanADownR (5′-CGTATCAGCCTGAATGTCATC-3′), and the resulting amplicon directionally cloned into plasmid pDCerm to yield the expression plasmid p^{E}NanA for gain-of-function studies in *L. lactis*. The amplicon was also subcloned into pDC123, which is identical to pDCerm but bearing a chloramphenicol instead of erythromycin resistance marker, for complementation studies in the SPN ΔNanA mutant. Critical active site residues of NanA were eliminated from p^{E}NanA by inverse PCR using primers NanAenzUp (5′-TGTATTTTGCGCACGTCTATTGTTC-3′) and NanAenzDown (5′-GGTTGACTGGAGATCTTCAGGTTG-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 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 p^{E}NanA using the primers NanAlecUp (5′-GGTTGAGCTCTCCCCCGAAAG-3′) and NanAlecDown (5′-ATGCCAGATGTAACGCATGTGCA-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-α-D-neuraminic acid sodium salt hydrate (4-MU; Fluka USA) was used to assay sialidase activity. SPN (WT D39 and isogenic ΔNanA mutant) and *L. lactis*–expressing NanA constructs were centrifuged, washed once, resuspended in 100 μl 0.1M phosphate citrate buffer (pH 6.5) to 10^9 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 (Johns Hopkins University, Baltimore, MD). hBMECs were maintained in RPMI 1640 medium (Life Tech Technologies, Inc.) supplemented with 10% FBS, 10% NuSerum (BD), and 1% MEM nonessential amino acids, and were incubated at 37°C in 5% CO_2. Bacterial adherence and invasion assays were performed essentially as previously described (Doran et al., 2005) within 24 h of established confluence (~10^5 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 size 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 × 10^7 CFU of SPN D39 or its isogenic D39ΔNanA mutant. 4 h after injection, blood was collected and serial dilutions plated to enumerate bacterial CFU in the circulation. Mice were then deeply anesthesized 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 ΔNanA 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.

We gratefully thank Elaine Tuomanen and Justin Thornton for many useful suggestions on the manuscript.

This research was supported by National Institutes of Health grants AI077780 and HD051796 (V. Nizet) and NS051247 (K.S. Doran), an American Heart Association Career Investigator Award (V. Nizet), and a Burroughs-Wellcome Fund Career Award (K.S. Doran). Work in T.J. Mitchell's laboratory was supported by the Wellcome Trust and the Medical Research Council.

The authors have no conflicting financial interests.

Submitted: 18 February 2009
Accepted: 28 July 2009

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