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Penetration of the blood-brain barrier by *Staphylococcus aureus*: contribution of membrane anchored lipoteichoic acid

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Abstract

Staphylococcus aureus is one of the most prevalent organisms responsible for nosocomial infections, and cases of community-acquired *S. aureus* infection have continued to increase despite wide-spread preventative measures. Pathologies attributed to *S. aureus* infection are diverse; ranging from dermal lesions to bacteremia, abscesses, and endocarditis. Reported cases of *S. aureus*-associated meningitis and brain abscesses have also increased in recent years, however the precise mechanism whereby *S. aureus* leave the bloodstream and gain access to the central nervous system (CNS) are not known. Here we demonstrate for the first time that *S. aureus* efficiently adheres to and invades human brain microvascular endothelial cells (hBMEC), the single-cell layer which constitutes the blood-brain barrier (BBB). The addition of cytochalasin D, an actin microfilament aggregation inhibitor, strongly reduced bacterial invasion, suggesting an active hBMEC process is required for efficient staphylococcal uptake. Furthermore, mice injected with *S. aureus* exhibited significant levels of brain bacterial counts and histopathologic evidence of meningeal inflammation and brain abscess formation, indicating that *S. aureus* was able to breach the BBB in an experimental model of hematogenous meningitis. We found that a YpfP-deficient mutant, defective in lipoteichoic acid (LTA) membrane anchoring, exhibited a decreased ability to invade hBMEC and correlated to a reduced risk for the development of meningitis *in vivo*. Our results demonstrate that LTA mediated penetration of the BBB may be a primary step in the pathogenesis of staphylococcal CNS disease.

Keywords

S. aureus; MRSA; blood-brain barrier; LTA; meningitis; brain abscess

Introduction

The Gram positive pathogen *Staphylococcus aureus* is responsible for a diverse range of disease pathologies, ranging from relatively minor dermal lesions to severe invasive sepsis disorders, pneumonia, and deep tissue abscess [1-3]. *S. aureus* is the causative agent of the majority of hospital-acquired bacterial infections throughout the developed world [4,5], and community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is now the number one

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bacterial cause of death in the United States [6]. Contributing to the widespread pathogenesis of *S. aureus* is its presence as a persistent commensal microorganism in approximately 20% of the population, and intermittently in a further 60%; primarily in the anterior nares but also the axillae, groin, and gastrointestinal tract [7]. Because of its ubiquitous presence, a breach of host defenses can lead to invasive and potentially fatal *S. aureus* infection [3].

Most clinical isolates of *S. aureus* possess an array of virulence factors which allow invasion and blood stream dissemination even in the absence of significant tissue trauma [8]. The incidence of *S. aureus* bacteremia has risen considerably since the emergence of CA-MRSA and the prevalence of *S. aureus* infection in the hospital environment [9]. Endocarditis, vertebral osteomyelitis and deep tissue abscesses account for more than 50% of secondary *S. aureus* infections [10]. *S. aureus* is the organism most commonly associated with bacterial brain abscesses [11]. Brain abscesses are a complication that may arise following surgery, head injury or from inadequately treated *S. aureus* sepsis or meningitis [11]. Although meningitis is considered a rare complication of *S. aureus* infection, there are an increasing number of clinical reports detailing meningitis resulting from *S. aureus* infection of an unknown source [12,13]. In these cases, hematogenous spread of bacteria from a primary site of infection implies that *S. aureus* has the ability to cross the BBB to penetrate the CNS. While several other Gram positive pathogens, including *Streptococcus agalactiae* (group B Streptococcus, GBS) and *Streptococcus pneumoniae* (SPN), are well known for their ability to gain access to the CNS, staphylococcal BBB penetration has not been investigated.

The Gram positive cell wall is composed of a thick layer of peptidoglycan as well as lipid-linked teichoic acids, known as lipoteichoic acids (LTA). LTA is a glycerol phosphate polymer that extends through the peptidoglycan cell wall and is thought to be involved in host cell attachment by various pathogens [14,15]. In staphylococci, streptococci and bacilli, LTA is attached to the cytoplasmic membrane via a glycolipid anchor; specifically β -gentiobiosyldiacylglycerol (diglucosyl-diacylglycerol [DGlcDAG]) [16,17]. YpfP is a glycosyltransferase required for DGlcDAG synthesis in both *S. aureus* and *Bacillus subtilis* [16]. Deletion of *ypfP* in *S. aureus* results in an 87% reduction in cellular-associated LTA [17]. In the present study we demonstrate for the first time the ability of *S. aureus* to directly invade and penetrate BBB endothelium. Resulting tissue damage, brain abscesses and meningeal inflammation were visualized following infection in an *in vivo* model of hematogenous staphylococcal meningitis. Furthermore, a Δ *ypfP* mutant strain exhibited a significant decrease in BBB penetration, indicating that LTA surface anchoring contributes to the development of *S. aureus* CNS disease arising from hematogenous dissemination.

Materials and Methods

Bacterial strains and culture conditions

S. aureus wild-type strains used were ISP479C [18], SA113 [19], USA 300 strain TCH1516 [20] and MRSA strain ATCC 33591. The *ypfP::ermB* (Δ *ypfP*) mutant was constructed by gene replacement of SA113 *ypfP* with *ermB* [17]. The Δ *ypfP*(pRB473ypfP) complemented mutant strain (Δ *ypfP* compl.) contains the *ypfP* gene and upstream promoter expressed on shuttle vector pRB473 [17]. All *S. aureus* strains were grown in Luria-Bertani (LB) medium or Tryptic Soy medium (TS), and the Δ *ypfP* mutant and complementation plasmid maintained in medium containing erythromycin ($15 \mu\text{g.mL}^{-1}$) and chloramphenicol ($10 \mu\text{g.mL}^{-1}$) respectively. *S. agalactiae* (group B streptococcus, GBS) wild-type strain COHI [21] and its corresponding glycosyltransferase mutant, Δ *iagA* [22], were grown in Todd-Hewitt Broth (THB) medium and THB supplemented with chloramphenicol ($2 \mu\text{g.mL}^{-1}$) respectively. *Streptococcus pneumoniae* (SPN) wild-type strain D39 [23] and glycosyltransferase mutant Δ *GTG* were grown in THB supplemented with 0.5% yeast

extract (THY) under microaerophilic conditions (37°C, 5% CO₂), the ΔGTG strain was maintained with erythromycin (2 $\mu\text{g}\cdot\text{mL}^{-1}$).

Human BBB model and bacterial assays

The human brain microvascular endothelial cell line, hBMEC [24], was obtained from Kwang Sik Kim (Johns Hopkins University, Baltimore, Maryland). Cells were maintained in RPMI cell culture medium (Invitrogen) 10% fetal bovine serum (FBS), at 37°C with 5% CO₂ as described elsewhere [24,25]. Human umbilical vein endothelial cells (HUVEC) were maintained in Endothelial cell Growth Medium (EGM) as per manufacturer's instructions (Lonza). *S. aureus* adherence and invasion assays were performed as described previously for other Gram positive meningeal pathogens [22,26,27], with minor modifications as described in figure legend. Experiments involving cytochalasin D (Sigma), an actin polymerization inhibitor, were carried out as described previously [28]. Comparative hBMEC adherence and invasion assays were carried out with meningeal pathogens GBS (MOI of 1.0) and SPN (MOI of 10) as described previously [22,27].

Microscopy

To visualize cell-associated and invasive *S. aureus*, hBMEC were propagated on collagen coated glass cover slips. *S. aureus* were added to confluent hBMEC monolayers at an MOI of 10 and incubated for 60 min. Monolayers were washed and heat-fixed prior to Gram staining and mounted using Cytoseal-60 (Thermo Scientific). Images were digitally captured using a Zeiss Axio inverted microscope equipped with a charge-coupled device camera. For transmission electron microscopy (TEM) hBMEC were propagated in Permanox plastic chamber slides (Thermo Scientific). *S. aureus* were added to confluent hBMEC monolayers as described above. Following incubation monolayers were washed and samples were preserved in one of two ways: 1) 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 90 minutes at room temperature, rinsed in cacodylate buffer, then post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 90 minutes. 2) 2.0% glutaraldehyde and 2.5% sucrose in 0.1 M cacodylate buffer, pH 7.4 for 10 minutes at room temperature followed by 70 minutes on ice, rinsed in cacodylate-sucrose buffer, then post fixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide in 0.1 M cacodylate buffer for 90 minutes on ice. Samples were dehydrated in ethanol, embedded in epoxy resin, sectioned at 60 to 70 nm, and picked up on formvar-coated grids. Grids were stained with uranyl acetate and lead citrate, viewed using a Tecnai-12 (FEI) transmission electron microscope and photographed using a Teitz 215 bottom mount digital camera.

Murine model of hematogenous *S. aureus* CNS infection

All animal experiments were approved by the Office of Lab Animal Care at San Diego State University and performed using accepted veterinary standards. Out bred 8-week old CD-1 male mice were used for all experiments (Charles River Laboratories). Pilot studies were performed to first identify the optimal bacterial dose and growth phase used. Mice were injected intravenously, via tail vein, with *S. aureus* or the $\Delta ypfP$ mutant as indicated. At various time points, mice were euthanized and blood, brains and kidneys collected, homogenized and plated to determine bacterial counts in each tissue. Half of the brain was fixed in 4% paraformaldehyde and embedded in paraffin for sectioning and histopathologic analysis. Competition assays were carried out by mixing prepared *S. aureus* (SA113) and isogenic $\Delta ypfP$ mutant cultures 1:1 prior to injection.

Statistical analyses

GraphPad Prism 5.0 was used for graphing and statistical analyses of all data. Two-tailed *t*-tests were used to analyze all comparative data sets. Two-sided chi-square analysis was

carried out to determine significance for frequency of infection models. Significance was accepted at $P < 0.05$.

Results and Discussion

S. aureus adheres to and invades brain endothelium

All strains of *S. aureus* are capable of causing serious disease, and the recent rise in numbers of patients presenting with CA-MRSA infections has led to an increase in the overall number of deaths attributed to invasive and disseminated *S. aureus* infection. *S. aureus* meningitis, although less common than that caused by traditional Gram positive meningeal pathogens such as SPN and GBS, is associated with a mortality rate greater than 50% [12]. We used immortalized hBMEC, the endothelial cell layer which constitutes the BBB [24], to examine the physical interaction of *S. aureus* with the human BBB. After a 60 min infection period, adherent staphylococci were visualized by microscopy (Fig. 1A). Subsequent analysis using TEM revealed bacteria in close proximity to cell surface microvillus projections and inside endocytic vacuoles (Fig. 1B-C), confirming the ability of *S. aureus* to adhere to and invade brain endothelial cells. To quantify the number of adherent and invasive organisms, we optimized our previously established hBMEC assays [22]. All *S. aureus* strains examined exhibited the ability to attach to and invade hBMEC (Fig. 1D).

The presence of intracellular staphylococci observed by EM analysis suggested that *S. aureus* may alter the host cytoskeleton to initiate its own uptake. To determine whether the observed *S. aureus* invasion required elements of the hBMEC cytoskeleton, we performed invasion assays using hBMEC monolayers preincubated with cytochalasin D, a potent actin microfilament aggregation inhibitor. As shown in Fig. 1E, the addition of cytochalasin D resulted in a marked decrease in *S. aureus* invasion compared to the DMSO treated control ($P < 0.001$), suggesting that *S. aureus* modulates host cell cytoskeleton rearrangement during cellular invasion. When assessing *S. aureus* invasion relative to adherence of hBMEC, an average of 34% of total hBMEC-associated *S. aureus* had invaded the intracellular compartment within the incubation period. This relative rate of invasion is comparable to that reported for GBS (30%) [26], and *S. pneumoniae* (7%) [29].

S. aureus penetration of the BBB *in vivo*

Previous animal models of *S. aureus* CNS disease and brain abscess formation have relied on the introduction of bacteria directly to the brain via surgical implantation of coated beads [11,30], and do not allow investigation of direct BBB penetration. We therefore sought to corroborate our findings *in vivo* in a murine model of hematogenous meningitis. Mice were infected with *S. aureus* intravenously via tail vein injection and monitored for 96 h post-injection (p.i.). In this model, the number of bacteria recovered from the bloodstream decreased over time (Fig. 2A) while *S. aureus* penetrated the BBB and persisted in the brain (Fig. 2B). Consistent with previous studies [31], bacteria were recovered from the kidneys of all mice, with the bacterial load increasing throughout the course of the experiment (Fig. 2C). Histopathology of brains from representative mice infected for 96 hr with *S. aureus* displayed meningeal thickening and hemorrhage, leukocytic infiltration and areas of micro-abscess formation in the brain parenchyma (Fig. 2D-F). Clusters of *S. aureus* were observed at the center of the brain abscess (Figure 2G). Our results clearly show that not only does *S. aureus* penetrate the BBB, it persists in the brain for up to 4 days post-infection and is capable of causing clinical signs of meningitis and abscesses formation.

Lipoteichoic acid cell wall anchoring contributes to *S. aureus* BBB penetration

Deletion of the GBS glycosyltransferase, *iagA*, resulting in defective LTA surface-anchoring, was shown to affect the ability of GBS to invade hBMEC and penetrate the BBB

in vivo [22]. Although not homologous, the functional similarities between GBS *iagA* and the *S. aureus* *ypfP*-encoded glycosyltransferase led us to hypothesize that LTA membrane anchoring may also play a role in *S. aureus* interaction with BBB endothelium. *S. aureus* strain SA113 and isogenic $\Delta ypfP$ mutant [17] were used to investigate the role of LTA surface anchoring in *S. aureus* BBB penetration. Adherence and invasion assays using our *in vitro* hBMEC model showed that the $\Delta ypfP$ mutant strain maintained hBMEC adherence capability but exhibited a significant decrease in hBMEC invasion compared to the wild-type strain ($p < 0.01$) (Fig. 3A). Similar results were obtained in another endothelial, HUVEC, cell line (Supplemental Fig. 1). Complementation of the mutation with the *ypfP* gene restored invasion to WT levels (Fig. 3A). In SPN, glycosyltransferase homologues responsible for synthesis of diglycosyldiacylglycerols have been described [32,33] but have not been studied in the context of bacterial invasion of host cell barriers. We examined hBMEC invasion of an SPN mutant, ΔGTG , lacking a glycosyltransferase required for MGlcDAG synthesis (a precursor to DGlcDAG) and observed a similar reduction in invasive capability comparable to *S. aureus* $\Delta ypfP$ and GBS $\Delta iagA$ (Fig. 3B). These results suggest that the role of LTA surface-anchoring in brain endothelial cell invasion is conserved amongst other meningeal pathogens, and may extend to other endothelial cell types.

We hypothesized that the decreased invasion by the *S. aureus* $\Delta ypfP$ mutant would translate into a diminished ability to penetrate the BBB and produce meningitis *in vivo*. We therefore infected groups of mice with the WT strain or the isogenic $\Delta ypfP$ mutant. Seventy-two hours post infection the levels of *S. aureus* detected in the blood of each group were essentially identical (Fig. 3C). However, significantly fewer animals infected with the $\Delta ypfP$ mutant had bacteria in the brain compared to animals infected with the WT strain (Figure 3D). Interestingly, there was no difference in the ability of the $\Delta ypfP$ mutant to enter the kidney (Supplemental Fig. 2). To further assess the contribution of YpfP to *S. aureus* BBB penetration and bacterial fitness, we performed a more sensitive *in vivo* competition assay. Mice were challenged with equal amounts of WT *S. aureus* and the $\Delta ypfP$ mutant. At the experimental end point (96 h), brains were collected for the enumeration of surviving bacteria. Significantly more WT than $\Delta ypfP$ mutant bacteria were recovered from the brain, $P < 0.001$, as demonstrated by a WT:mutant ratio > 1 in 10 out of 12 animals (Fig. 3E). This finding demonstrates a crucial role for *ypfP* in bacterial fitness and further corroborates the contribution of anchored LTA to *S. aureus* CNS disease. Histopathology of brains from representative WT or mutant-infected animals in Fig. 3D revealed characteristic meningeal inflammation and hemorrhage in animals with a high bacterial load, regardless of the presence of anchored LTA, and normal brain architecture in animals infected with the *ypfP* mutant with little or no recovered CFU (Figs. 3F-H). While our studies suggest that LTA significantly impacts BBB penetration, likely other staphylococcal factors also contribute to CNS invasion, as bacterial CFU were recovered from the brains of 50% of mice infected with the $\Delta ypfP$ mutant. Additionally our data demonstrate that LTA anchoring contributed to bacterial invasion of multiple endothelial cell lines but not to penetration of the kidney *in vivo*, indicating that *ypfP*-mediated invasion applies to some but not all endothelial barriers.

Using electron microscopy and an established *in vitro* model of the BBB, we demonstrate here for the first time that *S. aureus* is capable of invading hBMEC, the single cell layer that comprises the BBB. These results were corroborated *in vivo* in a model of hematogenous staphylococcal meningitis and CNS infection, whereby *S. aureus* penetrated the BBB within 24 h of initial infection, and persisted in the brain despite clearance from the bloodstream. Our results further suggest an important role for staphylococcal LTA in BBB penetration and CNS disease. While *S. aureus* BBB invasion requires further investigation, the results presented here represent an important development in our understanding of the ability of this formidable pathogen to penetrate the CNS. Furthermore, therapies directed at disrupting the

LTA-glycolipid linkage may also be a novel approach for the prevention of CNS infection by *S. aureus* and other meningeal pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *S. aureus* adheres to and invades human brain microvascular endothelial cells (A) Micrograph of Gram-stained hBMEC infected with *S. aureus* strain ISP479C (MOI=10). Transmission electron micrographs demonstrating hBMEC invasion by *S. aureus* strains ISP479C (B) and SA113 (C) (MOI=10). (D) Adherence and invasion of hBMEC by *S. aureus* ISP479C, SA113, ATCC 33591 and USA 300 strain TCH1516 following a 60 min incubation. Data are expressed as the total adherent or intracellular CFU recovered compared to the input inoculum (MOI=0.1). (E) *S. aureus* ISP479C hBMEC invasion following treatment with cytochalasin D or equivalent concentrations of DMSO. All experiments were repeated at least three times in triplicate; data from a representative experiment are shown. Error bars indicate 95% confidence intervals of mean values from three wells. ***, $P < 0.001$.

Figure 2. *S. aureus* BBB penetration *in vivo* using a mouse model of hematogenous meningitis Male CD-1 mice (n=32) were injected intravenously via tail vein with 2×10^8 cfu *S. aureus* ISP479C. Infection experiments were performed three times, a representative experiment is shown. Cohorts of 8 individuals were sacrificed at 24 h intervals and bacteria enumerated from (A) blood, (B) brain and (C) kidney. Histopathology of H&E (D-F) or Gram stained (F) brain tissue 96 h post-infection. Representative samples showed (D) meningeal thickening and hemorrhage and (E) abscesses formation and cellular infiltration. (F-G) close up of the abscess in (E), (G) depicts presence of staphylococci in abscess (arrow).

Figure 3. Contribution of membrane-anchored LTA to *S. aureus* BBB penetration

(A) Relative hBMEC adherence and invasion by *S. aureus* SA113, isogenic mutant strain $\Delta ypfP$ and complemented mutant strain $\Delta ypfP$ compl. (MOI=0.1). (B) Invasion of hBMEC by parental *S. aureus* SA113 (MOI=0.1), group B *Streptococcus* (GBS) (MOI=1.0) and *Streptococcus pneumoniae* (SPN) (MOI=10) and their respective glycosyltransferase mutants $\Delta ypfP$, Δlag and ΔGTG . All experiments were repeated at least three times in triplicate; data from a representative experiment are shown. *, $p < 0.05$, **, $p < 0.01$. *S. aureus* SA113 and $\Delta ypfP$ CFU recovered from blood (C) and brain (D) tissue of CD-1 male mice 72 h post- i.v. infection ($\sim 3 \times 10^6$ cfu). (E) Analysis of bacterial counts recovered from the brain 96 h post- i.v. injection with equal amounts of WT and $\Delta ypfP$ mutant *S. aureus* SA113 strains (1×10^7 total cfu). CFU were enumerated on LB plates or on LB supplemented with erythromycin to distinguish between WT and mutant bacteria. An overall ratio of 1 indicates equal numbers of WT and $\Delta ypfP$ CFU, a ratio greater than 1 indicates a higher number of WT bacteria were recovered. (F-H). Histopathology of H&E-stained brain tissue from representative mice infected with SA113 or $\Delta ypfP$ in (D). (F) Hemorrhage and meningeal thickening observed in a WT-infected mouse with a high recovered CFU. (G) Normal brain architecture was observed in a mouse infected with the $\Delta ypfP$ mutant with little or no recovered CFU. (H) Meningeal thickening was evident in a $\Delta ypfP$ -infected mouse with a high recovered CFU.