Diminished Virulence of an Alpha-Toxin Mutant of 
*Staphylococcus aureus* in Experimental Brain Abscesses

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*Staphylococcus aureus* is one of the major etiologic agents of brain abscesses in humans, occasionally leading to focal neurological deficits and even death. The objective of the present study was to identify key virulence determinants contributing to the pathogenesis of *S. aureus* in the brain using a murine brain abscess model. The importance of virulence factor production in disease development was demonstrated by the inability of heat-inactivated *S. aureus* to induce proinflammatory cytokine or chemokine expression or brain abscess formation in vivo. To directly address the contribution of virulence determinants in brain abscess development, the abilities of *S. aureus* strains with mutations in the global regulatory loci *sae* and *agr* were examined. An *S. aureus* *sae* *agr* double mutant exhibited reduced virulence in vivo, as demonstrated by attenuated proinflammatory cytokine and chemokine expression and bacterial replication. Subsequent studies focused on the expression of factors that are altered in the *sae* *agr* double mutant. Evaluation of an alpha-toxin mutant revealed a phenotype similar to that of the *sae* *agr* mutant in vivo, as evidenced by lower bacterial burdens and attenuation of cytokine and chemokine expression in the brain. This suggested that alpha-toxin is a central virulence determinant in brain abscess development. Another virulence mechanism utilized by *staphylococci* is intracellular survival. Cells recovered from brain abscesses were shown to harbor *S. aureus* intracellularly, providing a means by which the organism may establish chronic infections in the brain. Together, these data identify alpha-toxin as a key virulence determinant for the survival of *S. aureus* in the brain.

**Staphylococcus aureus** is a potent and versatile pathogen of humans. The frequencies of both nosocomial and community-acquired staphylococcal infections have increased steadily over the years (22). In addition, treatment of these infections has become more challenging due to the emergence of multidrug-resistant strains (8, 29). *S. aureus* infection may be manifested in a wide variety of forms, including focal abscesses, arthritis, endocarditis, and sepsisemia. Moreover, *S. aureus* has a diverse arsenal of virulence factors that contribute to the pathogenesis of disease. These can be broadly subdivided into surface and extracellular secreted proteins. Surface proteins include both structural components of the bacterial cell wall, such as peptidoglycan and lipoteichoic acid, and surface proteins preferentially expressed during exponential growth, including protein A, fibronectin-binding protein, and clumping factor. Secreted proteins are generally elaborated during the stationary phase of bacterial growth and include such proteins as alpha-toxin, enterotoxin B, lipase, and V8 protease.

The differential regulation of surface and extracellular virulence factors during the growth of *S. aureus* is controlled by at least three global regulatory systems, including *sae*, *agr*, and *sae* (4, 10, 20). The *sae* locus is involved in the expression of exoproteins and cell wall proteins that are potential virulence determinants in experimental infections (4, 6, 13). The *agr* locus up-regulates the production of extracellular proteins while repressing the synthesis of surface proteins (20, 24, 27, 28). The *sae* regulatory locus activates the production of several exoproteins, including alpha- and beta-toxin, coagulase, and protein A (10). As an alternative to dealing with antibiotic-resistant strains, the effective targeting and inactivation of these global regulatory loci could have a profound impact on disease therapy. Therefore, an understanding of the host response to *S. aureus* global regulatory mutants in complex disease models may reveal the importance of key virulence determinants in disease progression.

One virulence mechanism utilized by *staphylococci* is intracellular survival (21). The intracellular environment protects *staphylococci* from host defense mechanisms as well as the bactericidal effects of antibiotics. Intracellular survival of *S. aureus* has been demonstrated in both epithelial cells and neutrophils (12, 16). Staphylococci also produce cytotoxins, such as alpha-toxin, which cause pore formation and induce proinflammatory changes in mammalian cells (11, 30). Both the intracellular survival of *S. aureus* and the production of virulence factors, such as alpha-toxin, most probably play an important role in the complex response to *S. aureus* in the host.

In this study we have utilized a murine experimental brain abscess model using *S. aureus*, one of the major etiologic agents of brain abscesses in humans (23, 31). The course of brain abscess progression in the rodent model closely parallels what is observed in human disease in terms of histological appearance, infiltrating leukocytes, and chronicity (7, 19). Therefore, evaluating the role of bacterial virulence determinants and the host immune response to *S. aureus* in this model system should approximate conditions encountered during human disease. We have previously demonstrated that *S. aureus* induces rapid and sustained expression of numerous proinflammatory cytokines and chemokines in both the rat (18) and
mice (19) brain abscess models. However, the role of bacterial virulence factors in the expression of these mediators remains to be defined.

The present study was designed to examine the role of S. aureus virulence determinants in brain abscess development. The results demonstrate that staphylococcal strains that lack both the sarA and agr global regulatory loci or alpha-toxin exhibit reduced virulence in vivo. Examination of proinflammatory cytokine and chemokine expression revealed that although both sarA agr and alpha-toxin mutants are capable of inducing mediator expression during the acute phase of infection, this response is rapidly attenuated compared to the strong response, which is rapidly attenuated compared to the strong

**TABLE 1. S. aureus strains used in this study**

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Genotype</th>
<th>Antibiotic resistance</th>
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<tbody>
<tr>
<td>ALC132</td>
<td>RN6390 isogenic strain</td>
<td>None</td>
</tr>
<tr>
<td>ALC136</td>
<td>sarA mutant</td>
<td>Erm&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALC134</td>
<td>agr mutant</td>
<td>Tet&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALC135</td>
<td>sarA agr double mutant</td>
<td>Erm&lt;sup&gt;+&lt;/sup&gt; Tet&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALC837</td>
<td>Alpha-toxin</td>
<td>Erm&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALC8112</td>
<td>Lipase-negative</td>
<td>Erm&lt;sup&gt;+&lt;/sup&gt; Tet&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
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All of the mutants utilized were derived from the S. aureus strain RN6390.

**Preparation of S. aureus-derived brain abscesses.** Live S. aureus-derived virulence factors, in particular alpha-toxin, in brain abscess induction was minimal, with inflammatory changes in the brain (18, 19). The mortality rate associated with brain abscess induction was minimal, with >95% of animals surviving the procedure. To quantify the numbers of viable bacteria associated with brain abscesses in vivo, homogenates were prepared by disrupting brain abscess tissues (consisting of both solid tissue and purulent material) in 0.5 ml of DPBS supplemented with a complete protease inhibitor cocktail tablet (Roche, Indianapolis, Ind.).Serial 10-fold dilutions of homogenates were plated onto blood agar plates (Becton Dickinson, Franklin Lakes, N.J.)

**Induction of experimental brain abscesses.** Mice were anesthetized with averitin (2,2,2-tribromoethanol) intraperitoneally, and a 1-cm longitudinal incision was made along the vertex of the skull extending from the ear to the eye, exposing the frontal sutures. A burr hole was drilled 1 mm anterior and 1 mm lateral to the frontal suture of the calvarium. A Hamilton syringe fitted with a flexible tubing and a pulled, fine-tipped glass micropipette (diameter < 0.1 mm) was used to deliver beads into the brain parenchyma. A total of 3 μl of beads (10<sup>6</sup> CFU) was slowly infused 3 mm deep from the external surface of the calvarium to prevent reflux during injection. Using this approach, bacteria were reproducibly deposited into the head of the caudate or adjacent frontal lobe white matter. To collect brain abscess tissues for analysis, lesion sites were demarcated by the stab wound created during injections. Brain tissues were sectioned 0.5 mm on all sides of the stab wound, and cortical material was removed in order to focus on changes occurring in the white matter. Previous studies have established that implantation of agarose beads alone induces minimal proinflammatory cytokine or chemokine expression or cellular infiltration, indicating that neither the stab wound nor the deposition of foreign material (agarose beads) induces inflammatory changes in the brain (18, 19). The mortality rate associated with brain abscess induction was minimal, with >95% of animals surviving the procedure.

**Preparation of S. aureus-derived brain abscesses.** Live S. aureus-encapsulated organisms as described in Materials and Methods. Animals were euthanized 7 days later, and brain lesions were collected for histological analysis. Brain tissues (5-μm sections) were stained using hematoxylin and eosin to reveal changes in tissue architecture. (A) Note the formation of a large, well-demarcated abscess in the animal which received live S. aureus. The arrows delineate the margin between surrounding brain parenchyma and the abscess, which is denoted by an asterisk. (B) The arrow denotes a small inflammatory focus associated with the stab wound created during the injection of heat-inactivated organisms. Results presented in both panels are representative of three independent experiments. Original magnification, ×22.5.
Brain abscesses harbored viable *S. aureus* and myelin debris from cells. Cells were washed twice with 1% Percoll gradient (Amersham Pharmacia Biotech, Piscataway, N.J.) to separate *Gentamicin kills extracellular* is sensitive to gentamicin and rifampin (T. Kielian, unpublished observations). *S. aureus* assays were performed. The number was determined by using trypan blue exclusion dye analysis.

To determine whether brain abscess formation requires ongoing bacterial replication and/or the production of extracellular virulence factors, the ability of heat-inactivated bacteria to induce abscesses was examined. An advantage of using heat-inactivated organisms as the inflammatory stimulus is that the contribution of an evolving or resolving infectious process can be eliminated. In addition, virulence factors are not produced by heat-inactivated organisms, allowing the direct assessment of the importance of these mediators in abscess pathogenesis. Heat inactivation of *S. aureus* was con-

**RESULTS**

**Immunohistochemistry.** To prepare tissues for immunohistochemistry, animals were perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed, postfixed in paraformaldehyde for 30 min, and washed in 0.2 M phosphate buffer, pH 7.4, overnight. Tissues were cryoprotected in 30% sucrose for 24 h and then snap frozen at the optimal cutting temperature for immunohistochemistry.

Frozen sections of fixed tissues were processed for immunohistochemistry using the avidin-peroxidase method as previously described (14). The following antibodies were used for analysis: anti-GR-1 (neutrophil-specific); anti-CD11b, which reacts with the beta-integrin subunit expressed on neutrophils, monocytes/macrophages, and microglia; and the isotype control antibody rat immunoglobulin G2b (all from BD PharMingen). Sections were then incubated with a biotinylated secondary anti-rat immunoglobulin G antibody (Vector Laboratories) and developed using the substrate 3,3'-diaminobenzidine.

**Isolation of cells from brain abscesses.** Brain abscesses were collected from animals at days 5 and 7 following bacterial exposure to recover infiltrating and resident cells for gentamicin protection assays. Briefly, mice were perfused transcardially with DPBS to eliminate intravascular leukocytes. Tissue blocks containing the brain abscesses were pooled, minced into fine pieces using forceps, and incubated with collagenase type II (final concentration, 1 mg/ml; Sigma) for 20 min at 37°C. The resulting cell suspension was layered onto a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, N.J.) to separate myelin debris from cells. Cells were washed twice with 1× DPBS, and viability was determined by using trypan blue exclusion dye analysis.

**Gentamicin protection assay.** To determine whether cells recovered from brain abscesses harbored viable *S. aureus* intracellularly, gentamicin protection assays were performed. The *S. aureus* parental strain used in this study, RN6390, is sensitive to gentamicin and rifampin (T. Kielian, unpublished observations). Gentamicin kills extracellular *S. aureus*, but because its ability to permeate the eukaryotic cell membrane is limited, intracellular organisms are protected from its bactericidal activity. Following the isolation of cells from brain abscesses, an aliquot of cells was taken to determine the total bacterial titer (extra- plus intracellular organisms). In addition, cells were treated with gentamicin (100 μg/ml) for 2 h at 37°C to kill extracellular organisms. After 2 h, cells were washed twice to remove the gentamicin and serial dilutions of each treatment were performed to determine bacterial titers (log_{10} CFU). To ensure that intracellular bacteria were sensitive to an antibiotic which can penetrate mammalian cell membranes, cells were incubated with rifampin (1 mg/ml), which effectively reduced the number of intracellular CFU.

**RPA.** Cytokine and chemokine mRNA expression in brain abscess tissues was examined by RNase protection assay (RPA) using the RiboQuant RPA kit (BD PharMingen). The multiprobe template sets used for analysis include mCK-2, mCK-3b, and mCK-5. All template sets contain probes for the housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase, which serve as internal controls for the assay. Probes were synthesized using [α-32P]UTP, resulting in an average specific activity of 4 × 10^6 cpm/μl. RPA was performed according to the manufacturer’s instructions, using 10 μg of total RNA per sample. Products were resolved on a 6% acrylamide gel, dried, and exposed to film (BioMax MR; Kodak, Rochester, N.Y.).

**Results.** Significant differences between experimental groups were determined by using the Mann-Whitney rank sum test at the 95% confidence interval.

**FIG. 2. Live, but not heat-inactivated, *S. aureus* induces potent proinflammatory cytokine and chemokine expression in the brain. Mice were implanted with live or heat-inactivated (H-I) encapsulated organisms as described in Materials and Methods. Animals were euthanized at the indicated time points, and inoculation sites were collected for RNA extraction and analysis by RPA. The identity of each experimental mRNA is denoted at the left. Results presented are representative of three independent experiments.**
firmed by the inability of organisms to grow in BHI medium or on blood agar plates (data not shown). As shown in Fig. 1A, animals receiving live S. aureus developed large brain abscesses associated with a significant neutrophil and mononuclear cell infiltrate. In contrast, there was no evidence of abscess formation or any cellular infiltrates in those animals receiving heat-inactivated S. aureus (Fig. 1B). To determine whether the inability of heat-inactivated organisms to induce abscess formation was related to the number of bacteria inoculated into the brain, animals were challenged with 1-log-greater numbers of heat-inactivated S. aureus. Even increasing the number of heat-inactivated bacteria by 1 log was not sufficient to induce abscess formation (data not shown).

We have previously demonstrated that live S. aureus induces the rapid and sustained expression of proinflammatory cytokines and chemokines in the brain (18, 19). The inability of heat-inactivated organisms to induce abscess formation suggested that their ability to initiate proinflammatory cytokine and chemokine production might be impaired. Indeed, both proinflammatory cytokine and chemokine induction in the brain was significantly attenuated in those animals receiving heat-inactivated compared to live organisms (Fig. 2). Increasing the number of heat-inactivated organisms inoculated into the brain by 1 log was not sufficient to attain the levels of proinflammatory cytokine and chemokine expression observed in response to live bacteria (data not shown). These results suggest that some factor(s) produced by viable organisms is critical to the induction of proinflammatory cytokine and chemokine expression and brain abscess development.

A sarA agr S. aureus global regulatory mutant exhibits reduced virulence in an experimental brain abscess model. The findings obtained with heat-inactivated organisms suggested that S. aureus produces a virulence factor(s) which participates in brain abscess formation. The sarA and agr global regulatory loci are two major regulators of virulence factor expression in S. aureus. To determine what effect global regulatory loci play in brain abscess development, the ability of a sarA, agr, and sarA agr double mutant to induce disease was examined. Since each global regulatory loci mutant displays a particular virulence factor phenotype, analysis of each mutant would allow identification of a smaller subset of specific factors for further examination. The replication of a sarA agr double mutant was markedly attenuated compared to its isogenic control strain RN6390 at day 5 following bacterial exposure (Fig. 3). Interestingly, both sarA and agr single mutants replicated to the same extent as RN6390 in the brain parenchyma, suggesting an additive effect by the mutations in both regulatory loci. To determine whether the attenuated virulence of the sarA agr double mutant was related to an inability to replicate versus enhanced bacterial clearance, the kinetics of bacterial replication was evaluated for this mutant. As shown in Fig. 4, the number of viable organisms recovered from the brains of animals inoculated with the sarA agr double mutant was reduced as early as 24 h following bacterial exposure compared to its isogenic strain RN6390. However, the sarA agr double mutant was capable of replication to a limited extent, as evidenced by the small increase in bacterial titers observed at day 3 following bacterial exposure (Fig. 4). By day 5, the number of viable organisms in the brains of animals receiving the sarA agr double mutant was dramatically reduced compared to that in brains of animals receiving the wild type, with 3-log-fewer CFU in the former.

To determine whether the reduced virulence of the sarA agr double mutant correlated with an attenuated host immune response in the brain, proinflammatory cytokine and chemokine expression was evaluated in animals inoculated with either the sarA agr double mutant or RN6390. Initially, there were no observable differences in the amount of proinflammation...
tory cytokine and chemokine expression elicited by either strain (Fig. 5). However, within 48 h following bacterial exposure, proinflammatory cytokine and chemokine expression was undetectable in the brains of animals inoculated with the sarA agr double mutant compared to the continued induction in response to its isogenic strain RN6390 (Fig. 5). The cytokine and chemokine response to RN6390 was still detected at days 3 and 5 following bacterial exposure, whereas the response to the sarA agr mutant remained negative (data not shown). Even though bacterial burdens and mediator expression were attenuated in response to the sarA agr double mutant, these animals still developed rudimentary abscesses, albeit the lesions were dramatically smaller compared to those of animals receiving RN6390 (data not shown). This suggests that a virulence factor(s), whose expression is reduced or absent in the sarA agr mutant, must play an important role in the pathogenic response to *S. aureus* in the brain.

**Diminished virulence of an alpha-toxin mutant of *S. aureus* in experimental brain abscesses.** The reduced virulence of the sarA agr double mutant in the brain suggested that any one of a number of virulence factors could be involved in mediating tissue damage in this model. An important factor, the expression of which is greatly attenuated in the sarA agr double mutant, is alpha-toxin. Previous studies have demonstrated that alpha-toxin expression by the sarA agr double mutant is significantly lower compared to that by either sarA or agr single mutants (5). Alpha-toxin mediates its activity through forming pores in mammalian cell membranes, resulting in cell destruction by osmotic lysis. Because of its importance in other disease models (3, 15, 17), the role of alpha-toxin in brain abscess development was evaluated.

Replication of an *S. aureus* alpha-toxin mutant was markedly attenuated in the brain compared to that of RN6390, with the number of viable bacteria recovered approximately 3 to 4 logs lower in the former (Fig. 6). To determine whether the reduced virulence of the alpha-toxin mutant correlated with an attenuated host immune response in the brain, proinflammatory cytokine and chemokine expression was evaluated in animals inoculated with either the alpha-toxin mutant or...
RN6390. Similar to the results obtained with the sarA agr double mutant, the expression of proinflammatory cytokines and chemokines was attenuated in animals receiving the alpha-toxin mutant at 48 h following bacterial exposure (Fig. 7). However, the alpha-toxin mutant was capable of inducing mediator expression as demonstrated by the production of numerous mediators as early as 24 h, but this response was short-lived. The cytokine and chemokine response to RN6390 was still detected at days 3 and 5 following bacterial exposure, whereas the response to the alpha-toxin mutant remained negative (data not shown).

Since both bacterial burdens and proinflammatory cytokine and chemokine responses were attenuated in animals receiving an alpha-toxin mutant, the ability of these organisms to produce brain abscesses was investigated. We were able to detect only small inflammatory foci in the brains of animals inoculated with the alpha-toxin mutant, compared to the large, well-formed abscesses in those mice receiving the isogenic strain RN6390 (Fig. 8). Immunohistochemical analysis revealed a paucity of neutrophils in the brains of animals injected with the alpha-toxin mutant, whereas these cells were the predominant type infiltrating abscesses in response to RN6390 (Fig. 8). Together, these data indicate that alpha-toxin is an important, and possibly pivotal, virulence determinant in CNS abscess formation.

**Lipase is not a critical virulence determinant in brain abscess formation.** In our experimental model, brain abscesses are induced in the white matter, which contains a large amount of lipid, namely, myelin. Therefore, we reasoned that bacterial lipase expression might be a key virulence determinant involved in the invasion and spread of bacteria throughout the brain parenchyma. To examine this possibility, the virulence of an S. aureus lipase mutant was evaluated. The growth kinetics of both the lipase mutant and RN6390 were identical (data not shown), suggesting that lipase does not contribute significantly to the virulence of S. aureus in the brain. **S. aureus survives intracellularly within cells recovered from brain abscesses.** S. aureus has the ability to survive and replicate intracellularly within neutrophils and epithelial cells (12, 16). Currently, it is not known whether bacteria persist within cells associated with brain abscesses. To determine whether cells isolated from abscesses harbor viable S. aureus, gentamicin protection assays were performed on cells recovered from abscesses 5 days following bacterial injection. This time point was selected for evaluation since it is when bacterial loads are maximal or on the decline. Viable bacteria were still detected in abscess-derived cells treated with gentamicin, despite the fact that the antibiotic effectively reduced the overall number of bacteria by elimination of extracellular organisms (Fig. 9). Treatment of cells with rifampin further reduced the number of viable S. aureus cells, demonstrating the sensitivity of bacteria to an antibiotic capable of penetrating mammalian cells, and thus supporting the argument for their probable intracellular location. These findings indicate that S. aureus can survive intracellularly within cells associated with brain abscesses in vivo, providing a mechanism by which this organism can establish chronic infections in the CNS.

**DISCUSSION**

Staphylococci produce a wide array of virulence determinants that play a role in the complex interactions between the
organism and its host. While the in vivo function of these virulence factors is incompletely understood, it is probable that the identification of key factors required for disease progression may lead to novel therapies in the treatment of staphylococcal infections. This study investigates the importance of virulence factors produced by *S. aureus* in experimental brain abscess development.

To establish whether ongoing bacterial replication and/or virulence factor production was required for brain abscess induction, the response to heat-inactivated organisms was examined. Heat-inactivated *S. aureus* itself was not sufficient to induce proinflammatory cytokine or chemokine expression or abscess formation in the brain. These findings suggested that the active secretion of a virulence factor(s) was important for disease induction. However, it was also conceivable that brain abscess formation was influenced by structural components of the bacterial cell wall which were limiting in these experiments. To ensure that the inability to induce cytokine or chemokine expression was not merely a result of suboptimal concentrations of cell wall products, we increased the amount of heat-inactivated organisms inoculated into the brain. We were unable to induce significant mediator expression or abscess formation following the introduction of a 1-log-greater number of heat-inactivated organisms, suggesting that virulence factor production is important for brain abscess formation. However, our results cannot discount a potential additive effect between virulence determinants and an increasing mass of cell wall products produced by viable organisms. We are currently evaluating the ability of purified peptidoglycan and lipoteichoic acid to induce pathology in the brain. Nonetheless, these data suggest an important role for virulence factor expression in the host response to *S. aureus* in the brain.

To delineate virulence factors which potentially participate in abscess pathogenesis, the growth of *S. aureus* global regulatory mutants was examined in the brain. Interestingly, an *S. aureus sarA agr* double mutant was markedly less virulent in vivo, whereas both single mutants behaved similarly to the parental strain RN6390 in terms of bacterial replication and

FIG. 8. An *S. aureus* alpha-toxin mutant fails to induce abscess formation in the brain. Mice were implanted with either the isogenic strain RN6390 (A and C) or an alpha-toxin mutant (B and D) as described in Materials and Methods. Animals were euthanized at day 7 following bacterial exposure to evaluate brain abscess formation and cellular infiltrates. Serial sections of brain lesions were stained with the neutrophil-specific antibody GR-1 (A and B) or anti-CD11b (C and D), which reacts with neutrophils, monocytes/macrophages, and resident microglia. Note the outer edge of an abscess in the animal receiving RN6390 (dense staining area in the corners of panels A and C), whereas a well-defined abscess was not detected in response to the alpha-toxin mutant. Results presented are representative of two independent experiments. Bars, 20 μm.
immune activation in the brain. This finding cannot be explained by impaired replication of the sarA agr double mutant, since previous studies have established that its growth rate is identical to that of its isogenic strain RN6390 (5). Additionally, this mutant replicated successfully during the first three days following implantation into the brain. This suggested that virulence factors, the expression of which are dramatically reduced in the sarA agr double mutant, are pivotal for brain abscess induction.

The findings obtained with the sarA agr mutant led us to examine the potential role of two virulence factors, alpha-toxin and lipase, in brain abscess development. Similar to the findings obtained with the sarA agr double mutant, the replication of an alpha-toxin mutant was significantly attenuated compared to that of its isogenic strain RN6390. Importantly, the virulence of a lipase mutant was equivalent to that of its isogenic strain RN6390, indicating that the results obtained with the alpha-toxin mutant were specific. In addition to its impaired replication and enhanced clearance, the alpha-toxin mutant did not induce well-defined abscesses in the brain; rather, minimal inflammation and few infiltrating cells were observed. This finding may be explained by the following. The rapid replication of wild-type S. aureus (RN6390) induces prolonged cytokine and chemokine expression and direct damage to the brain parenchyma by bacterial products, leading to abscess formation. RN6390 produces alpha-toxin, which forms small transmembrane pores spanning the plasma membrane of mammalian cells, leading to osmotic lysis. Secretion of alpha-toxin is an effective way to eliminate infiltrating neutrophils and other leukocytes, cells which play a pivotal role in containing bacterial burdens. The lack of toxin expression in the alpha-toxin mutant now allows more leukocytes to survive in the brain, rapidly reducing bacterial loads. The quick and effective containment of the alpha-toxin mutant in the brain prevents these immune responses and bacteria from persisting, which most likely explains the absence of well-defined abscesses. The striking reduction in virulence associated with the alpha-toxin mutant also indicates that alpha-toxin is the major virulence determinant in the brain and its activity cannot be substituted by the gamma- and delta-toxins which are still produced by this mutant. The finding that animals inoculated with the sarA agr double mutant develop microabscesses can also be explained on the basis of alpha-toxin expression. Although alpha-toxin production is significantly decreased in the sarA agr mutant, some protein is still detected due to induction by other regulatory systems (5). The small amount of alpha-toxin produced by the sarA agr double mutant may be sufficient to transiently compromise the host response, which eventually contains the infection without inducing much damage to the brain parenchyma, resulting in microscopic abscesses. However, it is likely that an additional factor(s) participates in S. aureus infection in the brain since the alpha-toxin mutant was not completely avirulent. Our findings demonstrating the importance of alpha-toxin in the brain are in agreement with others using various model systems (3, 15, 17, 26).

S. aureus induces potent proinflammatory cytokine and chemokine expression in the brain (18, 19). Since both sarA agr and alpha-toxin mutants exhibited a significant reduction in tissue damage in the brain, we were interested in determining whether this correlated with an attenuation in the host immune response. Both sarA agr and alpha-toxin mutants were initially capable of inducing proinflammatory cytokine and chemokine expression. However, this response was transient in that mediator production was undetectable 48 h following bacterial exposure, which correlated with a decrease in the numbers of viable organisms in the brain. This suggests that during the
initial stage of infection, sufficient organisms are present within
the brain parenchyma to trigger activation of host immune
responses. However, as the replication of these mutants is
rapidly held in check, the immune response begins to diminish.
Alternatively, there may be less of a quorum-sensing function
to activate hemolysin production as the number of organisms
begins to decline. This would, in effect, minimize damage to
the surrounding normal brain parenchyma resulting from an
overactive immune response which is thought to contribute to
abscission severity in response to fully virulent strains of S. aureus.

Work by others has demonstrated a critical role(s) for the
agr (1, 9) and sarA (25) loci in regulating S. aureus virulence in
vivo. However, our results differ from these studies in that we
observed a reduction in virulence only for an S. aureus strain in
which both regulatory loci were inactivated. Our findings are in
agreement with Cheung et al. who demonstrated diminished
virulence of an S. aureus sarA agr double mutant in a rabbit
model of endocarditis (5). In addition, Booth et al. also dem-
onstrated that inactivation of both the sarA and agr loci led to
near-complete attenuation of virulence in experimental en-
dophthalmitis (2). Our data in experimental brain abscesses
suggest that the residual virulence factor expression detected
in either the sarA or agr single mutants is sufficient to allow
these organisms to replicate and induce tissue pathology sim-
ilar to wild-type strains. Only the inactivation of both regula-
tory loci effectively reduces virulence factor expression, effec-
tively compromising bacterial replication and minimizing
the brain.

Recently, S. aureus has been demonstrated to survive intra-
cellularity within neutrophils and epithelial cells (12, 16).
Therefore, we were interested in identifying whether viable
organisms were associated with abscess-derived cells. This vir-
ulence mechanism could explain the phenomenon of daughter
abscission formation in the brain which occurs in a small percent-
age of affected individuals. As abscesses evolve, the fibrotic
wall surrounding the lesion may become weakened, allowing
contents to permeate neighboring tissue and establish a new
nidus of infection. This seeding of small microabscesses resem-
bles “beads on a string” and is life-threatening if intraventric-
ular rupture occurs, emptying purulent material into the cere-
brosplinal fluid. The survival of bacteria within the initial
abscess may be a prerequisite for daughter abscess formation.
The question remains whether bacteria survive extracellularly
or persist within cells associated with brain abscesses. Indeed,
we found that cells recovered from brain abscesses harbored
viable organisms. However, the identity of these cells is cur-
rently not known. One possibility is that S. aureus is contained
within neutrophils infiltrating the brain parenchyma. Previous
studies have established that neutrophils constitute the major-
ity of cells infiltrating acute brain abscesses (19). However, the
half-life of an activated neutrophil is relatively short, which
does not fit the profile of a cell that would persist, allowing a
daughter abscess to become established. Another possibility is
that S. aureus may survive within resident microglia, the resi-
dent macrophage population in the brain. We are currently
investigating the cellular localization of S. aureus in the brain
using a strain that constitutively expresses green fluorescent
protein. These studies should allow identification of cells that
are capable of supporting bacterial survival in the brain.

In summary, these studies have revealed the central impor-
tance of alpha-toxin in brain abscess development. It has been
well documented that many immune responses in the CNS are
distinct from those observed in peripheral tissues. However, as
shown here, the pivotal role of alpha-toxin in the CNS has also
been observed in other models of S. aureus infection in the
periphery. It will be interesting to determine whether other
virulence factors such as V8 protease and staphylococcal en-
terotoxin B contribute to CNS disease as they do in the
periphery, or if this is where the similarities between these diver-
gent compartments end.

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