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 sarA and agr were examined. An S. aureus sarA 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 sarA agr double mutant. Evaluation of an alpha-toxin mutant revealed a phenotype similar to that of the sarA 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 sepsis. 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 sarA, agr, and sae (4, 10, 20). The sarA 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
TABLE 1. *S. aureus* strains used in this study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Genotype</th>
<th>Antibiotic resistance</th>
</tr>
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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>ALC812</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.

MATERIALS AND METHODS

Mice. Male AKR/J mice 6 to 8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, Maine). The animal use protocol has been approved by the Dartmouth College Institutional Animal Care and Use Committee and is in accord with National Institutes of Health guidelines for the use of rodents. 

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Preparation of *S. aureus*-laden agarose beads. Lactococcus innocuous, recipient cells, were encapsulated in agarose beads prior to implantation in the brain as previously described (18, 19). The use of agarose beads prevents bacterial dissemination or rapid wound sterilization by the host. Briefly, bacterial strains were grown to postexponential phase at 37°C in brain heart infusion (BHI) medium (Becton Dickinson, Sparks, Md.). A total of 10<sup>6</sup> bacteria were added to a solution of 1.4% low-melting-point agarose (type XII; Sigma, St. Louis, Mo.) at 40°C. The mixture was then added to rapidly swirling heavy mineral oil (Sigma) prewarmed to 37°C and quickly cooled to 0°C on crushed ice. Beads were washed four times in 1× Dulbecco's phosphate-buffered saline (DPBS) (Mediatech Cellgro, Herndon, Va.) to remove mineral oil. Beads with diameters between 50 and 100 μm, as determined by phase-contrast microscopy, were used for implantation into the brain. Heat-inactivated bacteria were prepared by incubating organisms for 1 h at 56°C prior to encapsulation. The bacterial viability or sterility of bead preparations was confirmed by overnight culture in BHI medium and quantitative culture on blood agar plates (Becton Dickinson, Franklin Lakes, N.J.).

Induction of experimental brain abscesses. Mice were anesthetized with avermectin (z-z-z-trimethoprim) 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 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.

Quantification of viable bacteria associated with brain abscesses in vivo. 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). Titers were calculated by enumerating colony growth and are expressed as the mean log<sub>10</sub> CFU per milliliter of homogenate.

FIG. 1. Brain abscesses are induced by live, but not heat-inactivated, *S. aureus*. Mice were implanted with live (A) or heat-inactivated (B) 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.
**RESULTS**

Brain abscesses are induced by live, but not heat-inactivated, *S. aureus*. 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-
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 proinflam-
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 abscess 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 demonstrated that inactivation of both the *sarA* and *agr* loci led to near-complete attenuation of virulence in experimental endophthalmitis (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 similar to wild-type strains. Only the inactivation of both regulatory loci effectively reduces virulence factor expression, effectively compromising bacterial replication and minimizing tissue damage in the brain.

Recently, *S. aureus* has been demonstrated to survive intracellularly within neutrophils and epithelial cells (12, 16). Therefore, we were interested in identifying whether viable organisms were associated with abscess-derived cells. This virulence mechanism could explain the phenomenon of daughter abscess formation in the brain which occurs in a small percentage 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 resembles “beads on a string” and is life-threatening if intraventricular rupture occurs, emptying purulent material into the cerebrospinal 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 currently not known. One possibility is that *S. aureus* is contained within neutrophils infiltrating the brain parenchyma. Previous studies have established that neutrophils constitute the majority 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 resident 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 importance 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 *V*8 protease and staphylococcal enterotoxin B contribute to CNS disease as they do in the periphery, or if this is where the similarities between these divergent compartments end.

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