Accessory Gene Regulator Controls *Staphylococcus aureus* Virulence in Endophthalmitis

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**Purpose.** To evaluate the contribution of toxins to the severity of *Staphylococcus aureus* endophthalmitis.

**Methods.** Experimental endophthalmitis was established by injecting rabbit eyes with wild type *S. aureus* ISP479 and the isogenic attenuated strain, ISP546, defective in expression of the global regulator locus *agr*. *agr* regulates expression of at least 19 exoproteins that are potentially important in the pathogenesis of endophthalmitis. Infections were evaluated using electroretinography, slit lamp biomicroscopy, and histology. Two concentrations (approximately 10 and 1000 organisms) of bacteria were injected.

**Results.** The *agr−* strain consistently resulted in slower loss of b-wave response when compared to the wild type strain, irrespective of inoculum size. Clinical signs were less severe among the *agr−* group at 24 and 48 hours when 10 organisms were injected. However, when the number of bacteria injected was increased to 1000, earlier onset of clinical signs was observed, with both groups showing maximum cell and flare and a white fundal reflex at 48 hours after infection. Histologic examination of eyes enucleated 36 hours after inoculation revealed that the wild type strain induced focal retinal destruction and mild vitritis, whereas eyes infected with the *agr−* strain remained completely normal. Histologic examination carried out when loss of B-wave response was 100% revealed that retinal changes for both groups could not be distinguished.

**Conclusions.** These data indicate that toxin production by *S. aureus* contributes to severity of endophthalmitis by accelerating the rate of onset of retinal damage. Therefore, toxin-targeting therapies instituted early in the course of infection could preserve retinal function.


*Staphylococcus aureus* is among the leading causes of postoperative and posttraumatic endophthalmitis. A survey of the literature reveals that *S. aureus* endophthalmitis is associated with poor visual outcome (defined here as best-corrected visual acuity of 20/400 or worse) in more than 52% of reported cases.1-8 The recent emergence of increased antibiotic resistance among staphylococcal strains threatens to further increase the rate of treatment failure for this organism.9 The severity of *S. aureus* endophthalmitis is likely the result of one or a number of the myriad toxins that are expressed by strains of *S. aureus*. α-toxin has been reported to be involved in the pathogenesis of *S. aureus* keratitis10; however, little is known concerning the virulence traits expressed by *S. aureus* intraocularly. The same can be said for most other commonly isolated intraocular pathogens. One exception to this is *Enterococcus faecalis*, which is also an important cause of postoperative and posttraumatic endophthalmitis. By comparing the course of endophthalmitis caused by an isogenic mutant of *E. faecalis*, defective in expression of the cytolysin, with a cytolytic parental strain in a rabbit eye model, Jett et al11 demonstrated the involvement of a cytolysin in the pathogenesis of *E. faecalis* endophthalmitis.
**agr Controls Staphylococcus aureus Virulence in Endophthalmitis**

S. aureus is known to express an estimated 35 extracellular products, many of which have been implicated as virulence factors. The expression of these exoproteins, as well as certain cell surface proteins in S. aureus, has been shown to be coordinately controlled by a number of global regulatory elements, designated agr, sar, and xpr, that act at the transcriptional level. Mutant strains of S. aureus defective at these loci have been characterized at the phenotypic level. Expression of α-, β-, γ-, and δ-toxins, toxic shock syndrome toxin-1 (TSST-1), and staphylococcal enterotoxins B and C are decreased in agr mutants, whereas coagulase, protein A, and fibronectin-binding protein are elevated relative to the parental strain. The xpr locus appears to regulate exoprotein expression in a manner similar to agr. sar may act as a counterregulatory system to agr because the pattern of exoprotein expression governed by these elements is diametrically opposed (with the exception of α-, β-, and δ-toxin, which are reduced in both agr and sar mutants).

The control of expression of virulence factors has been described as a virulence determinant in itself. Smeltzer et al compared the lethality of isogenic S. aureus strains differing only in the agr locus in a mouse intraperitoneal model. At bacterial numbers used, the wild type organism was lethal in 100% of mice. In sharp contrast, identical inocula of agr− or xpr− deficient mutants resulted in no deaths or overt ill effects among any of the mice injected. These results clearly demonstrate that one or several of the toxins regulated by agr or xpr is responsible for the virulence of the organism in the mouse intraperitoneal model. Abdelnour et al similarly showed the involvement of virulence factors under the control of agr in the induction and progression of septic arthritis in mice. In a rabbit model of endocarditis, Cheung et al demonstrated diminished infectivity and adherence in a sar mutant when it was compared to a wild type parental strain.

To determine the role of extracellular and cell wall-associated proteins in the establishment and progression of S. aureus endophthalmitis, the relative severity of infections caused by an agr− strain of S. aureus and an isogenic agr+ strain were compared in a rabbit endophthalmitis model. The results suggest that exoproteins, whose expression is controlled by the agr locus, contribute to the severity of S. aureus endophthalmitis.

**METHODS**

**Bacterial Strains and Media**

S. aureus ISP479 was used as the reference wild type strain (agr+) in this study. S. aureus ISP479 is a derivative of NCTC 8325 (a human infection-derived isolate) that has been cured of prophages. ISP479 expresses α-, β-, γ-, and δ-hemolysins, enterotoxin, acid phosphatase, serine protease, metalloprotease, lipase, hyaluronate lyase and staphylokinase, and low levels of coagulase and protein A. ISP479 and ISP546 is a derivative of strain ISP479 with a chromosomal Tn551 insertion in the agr locus, resulting in an agr− phenotype. Phenotypically, this strain does not express α-hemolysin, it expresses low levels of β-hemolysin, enterotoxin C, serine protease, and nuclease, and it expresses elevated levels of coagulase and protein A. ISP479 and ISP546 can be differentiated by the presence of zones of clearing surrounding ISP479 (agr+) colonies on horse blood agar plates that are absent around ISP546 (agr−) colonies. All strains were propagated in Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI) at 37°C without aeration. Before intravitreal injection, cultures were serially diluted in phosphate buffered saline (PBS). Enumeration of organisms and confirmation of phenotype at the time of injection and recovery from the vitreous was accomplished by plating on BHI agar plates containing whole horse blood (5% vol/vol).

**Vertebrate Animals**

New Zealand White rabbits weighing 2 to 4 kg were used in this study. Animals were housed and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research at the Dean A. McGee Eye Institute animal care facility.

**Intraocular Injections**

Before intraocular injection, the animals underwent general anesthesia with intramuscular ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg of body weight). Once anesthetized, the animals were given topical ocular anesthesia (0.5% proparacaine hydrochloride), and the pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. To relieve intraocular pressure, 0.1 ml of aqueous humor was aspirated through a 27-gauge needle introduced through the limbus. A 25-gauge needle attached to a sterile disposable 1-ml syringe was then introduced through the pars plana approximately 3 mm posterior to the limbus, and 0.1 ml of S. aureus suspension in PBS (containing a predetermined number of organisms) was slowly infused into the center of the vitreous body.

**Electroretinography**

ISP479 (agr+) and ISP546 (agr−) strains of S. aureus were compared for the ability to affect neuroretinal responsiveness in infected eyes. Animals underwent general and topical anesthesia as described. After pharmacologic dilation and 30 minutes of dark adap-
titration, baseline b-wave amplitude was established by illuminating the retina with a single, low-intensity flash (1/second) and measuring the resulting b-wave response. The final b-wave response was the average of seven repeated measurements. After at least 48 hours of recovery, a suspension of *S. aureus* in PBS was injected into the right eye. The left eye served as an absolute, uninjected control. Electroretinography (ERG) was performed 24, 48, 72, and 96 hours after injection. Percent loss of retinal function (b-wave amplitude) was calculated as follows: \((1 - \frac{\text{experimental b-wave amplitude}}{\text{baseline b-wave amplitude}}) \times 100\). The Mann–Whitney test was used to analyze ERG results. \(P < 0.05\) was considered statistically significant.

**Thin Section Histopathology**

Infected eyes were enucleated for histopathologic analysis at 12, 24, and 36 hours after infection, and when loss of b-wave response was 100%. Eyes were fixed in 10% formalin for 24 hours and then sectioned and stained with hematoxylin and eosin following standard procedures. All histologic staining was performed with an automated slide stainer (Histomatic 172; Fisher Scientific, Pittsburgh, PA). Pathologic interpretation was made with the investigator blinded as to the identity of the infecting organism.

**Slit Lamp Biomicroscopy**

Infected eyes were examined by slit lamp biomicroscopy at 24, 48, 72, and 96 hours after infection to make comparative evaluations and follow the progression of endophthalmitis. Animals underwent general and topical ocular anesthesia as described. Clinical observations and evaluations included presence or absence of red reflex and anterior chamber reaction. The latter was graded on a scale of 0 to 4+ according to the severity of pathologic changes.

**Growth of *S. aureus* in Vitreous**

To ensure that differences observed were not attributable to a possible growth advantage for the wild type strain, the suitability of vitreous to support growth of ISP479\((agr^+)\) and ISP546\((agr^-)\), in vitro and in vivo was assessed. Vitreous was recovered by scleral incision into uninfected absolute control eyes that had been surgically removed and rinsed in sterile PBS. To determine growth rates in vitro, approximately 50 ISP479\((agr^+)\) or ISP546\((agr^-)\) were inoculated into 1 ml of recovered vitreous and allowed to incubate at 37°C for 24 hours. One-milliliter samples were taken at the time of inoculation, at 2-hour intervals for the first 8 to 10 hours, and at 24 hours after inoculation. To confirm that the vitreous tap was reflective of the total number of organisms present in the eye, one eye was enucleated at 24 hours after infection, and the entire vitreous was removed by scleral incision as described. The number of organisms in each sample was determined by plating 10-fold serial dilutions on BHI-horse blood agar. After 24 hours of incubation at 37°C, colonies were counted and concentrations were expressed as colony forming units (cfu)/ml of vitreous humor.

**RESULTS**

**Electroretinography**

Two concentrations of *S. aureus* ISP479 \((agr^+)\) and *S. aureus* ISP546 \((agr^-)\) were used to establish endophthalmitis in rabbit eyes: a low level (approximately 10 cfu [14.1 ± 9.4 for strain ISP479 and 8.5 ± 5.3 for strain ISP546]) and a high level (approximately 1000 cfu [988 ± 386 for strain ISP479 and 1105 ± 756 for strain ISP546]) suspended in 0.1 ml PBS. At the low inoculum level, infections caused by strain ISP479 \((n = 10)\) resulted in a significantly greater reduction in b-wave amplitude at postoperative day 1 when compared to infections caused by strain ISP546 \((n = 7)\) \((P < 0.02)\) (Fig. 1). Interestingly, strain ISP546 resulted in a supernormality in ERG response at this time in 3 of 7 rabbit eyes tested (data not shown). A
similar supernormality has been observed at postoperative day 1 in a rabbit model of *E. faecalis* endophthalmitis (inoculum = 10^4 organisms). The exact physiological basis for this observation is unknown; however, a release of vasoactive amines from mast cells in the choroid near the photoreceptor layer has been suggested to cause this phenomenon. On postoperative day 2, a significantly greater reduction in b-wave response was again observed for strain ISP479 when compared to ISP546 (57.5% versus 26.3%) (P < 0.001). At postoperative day 3, the reduction in b-wave response had reached 92.8% for ISP479 and 60.5% for ISP546. This difference was also found to be significant (P < 0.01). By postoperative day 4, no difference could be observed between the strains. It is noteworthy that, on average, complete loss of ERG response occurred earlier in the group infected with ISP479 (80 hours) than in the group infected with ISP546 (112 hours) (P < 0.02). When changes in ERG b-wave response were compared between infected and contralateral uninfected eyes for each strain, some interesting observations were made. For the group injected with the agr- strain, ISP546, the infected eyes were not significantly different from contralateral control eyes at the earlier times (postoperative days 1 and 2), but were significantly different at postoperative day 3 (P < 0.01) and postoperative day 4 (P < 0.001). However, for the group injected with the agr+ strain, the infected eyes were significantly different from the contralateral uninfected eye at all time points (postoperative day 1, P < 0.05; postoperative days 2, 3, and 4, P < 0.001).

At the higher inoculum level (approximately 1000 cfu), the wild type strain (n = 5) again resulted in a significantly greater reduction in b-wave response compared to the agr- strain (n = 7) in the first 72 hours after inoculation (Fig. 2). Significant differences were observed between the strains at postoperative day 1 (P < 0.01), postoperative day 2 (P < 0.02), and postoperative day 3 (P < 0.01). Data were not analyzed after postoperative day 3 at the higher inoculum level because the majority of animals had already achieved complete loss of b-wave by this time. For the agr+ strain, at the higher inoculum level, significant differences were observed between infected and uninfected eyes at all times (P < 0.001); the same observation was made for this strain at the lower inoculum level. For the agr- strain at the high inoculum level, infected eyes were also significantly different from the contralateral uninfected eyes at all times (P < 0.001). This is in contrast to the lower inoculum level, where differences between infected and uninfected eyes could only be observed by postoperative day 3 for the agr- strain.

**Slit Lamp Examination**

When approximately 10 organisms were injected into rabbit eyes, the wild type strain ISP479 (n = 6) elicited an arithmetic mean of 2+ cell and 2.5+ flare in the anterior chamber at postoperative day 1. At the same time point, the agr- strain ISP546 (n = 5) resulted in an average of 0+ cell and 0.4+ flare. A normal red reflex was found for both groups. By postoperative day 2, rabbits injected with the strain ISP479 had an anterior chamber reaction of 4+ cell and flare, and red reflex was absent. At the same time point, ISP546 (agr-), had a slightly lower degree of anterior chamber reaction with 3+ cell and flare; however, unlike the agr+ group, this group retained significant red reflex. By postoperative days 3 and 4, both groups had maximum cell and flare as well as a white reflex.

In the high-level inoculum group (approximately 1000 organisms), slit lamp examination of eyes infected with the agr+ strain (n = 5) on postoperative day 1 revealed an average of 2+ cell and 3.4+ flare in the anterior chamber and a diminished red reflex. In contrast, eyes infected with the mutant strain ISP546 (n = 11) averaged 0.5+ cell and 2+ flare with a normal fundal glow at the same time point. At postoperative day 2, strain ISP546 resulted in 4+ cell and 4+ flare in the anterior chamber. Strain ISP546 was essentially the same as ISP479 on postoperative day 2 with 3.5 cell and 4+ flare. Moreover, at this time, both infections resulted in a white fundal reflex. Clinical findings on postoperative day 3 were similar to those on postoperative day 2.
Histopathologic Examination

Hematoxylin and eosin-stained sections through the entire globe of rabbit eyes infected with either ISP479 (agr+) or ISP546 (agr−) were examined for changes at 12, 24, and 36 hours after infection. The results are shown in Figure 3. At 12 hours after inoculation (panels A and D), eyes infected with agr+ and agr− strains showed normal histology. By 24 hours after inoculation (panels B and E), eyes infected with the agr+ strain showed iris engorgement (data not shown), but otherwise no abnormalities were apparent. Eyes injected with the agr− strain remained completely normal. However, by 36 hours after inoculation, eyes injected with wild type strain showed focal retinal destruction involving inner nuclear and ganglion cell layers but not the outer nuclear layer — also clusters of vitreous cells accumulated close to the focus of retinal destruction (panel C). The anterior chamber showed iris and ciliary vessel engorgement. A fibrinous coat was apparent on the anterior aspect of the iris. At the same time, eyes injected with the attenuated strain showed no discernible abnormalities (panel F). Histopathologic observations for eyes that had completely lost neuroretinal response, as determined by ERG, were also made (data not shown). Eyes infected with the wild type strain exhibited a severe inflammatory reaction in both the anterior segment and the vitreous, whereas the agr− strain exhibited only a mild to moderate inflammation in the anterior chamber and the vitreous. However, retinal changes for both groups could not be distinguished, and they included infiltration of numerous inflammatory cells into retinal layers and into the optic nerve head. Retinal detachment was also evident. Cystic changes and inflammatory cell infiltration resulted in near complete destruction of the inner limiting membrane, the nerve fiber, and the inner nuclear layers. The outer nuclear layer remained largely intact. The choriocapillaris also was moderately inflamed with vascular engorgement and dilation of choroidal vessels. The lack of observable difference between the two groups is not surprising because they were both examined when they no longer exhibited a b-wave response, which would of itself indicate that substantial retinal damage had occurred. However, even though retinal changes were similarly severe in both groups, the time taken to effect these changes was longer for the agr− strain (96 hours or greater) than for the agr+ (48 to 96 hours) strain (P < 0.02).

Intravitreal Growth of S. aureus

To verify that differences in clinical findings after infection with wild type and mutant strains were not caused by differential growth rates for these organisms, growth rates in vitreous were analyzed (Fig. 4). It was found that in vitro both strains reached \(2 \times 10^8\) cfu per ml by 24 hours, with similar growth kinetics. In comparison, \(10^4\) cfu were recovered from the vitreous of enucleated eyes infected with either strain. Although this represents a substantial difference between the extent of growth of both strains in vitro and in vivo, no differences were observed between the wild type and the mutant strain when grown under either condition.

DISCUSSION

Generally, factors that contribute to severity of bacterial infections include secreted toxins and cell surface components that result in direct tissue damage, induction of host inflammatory response, or both. Intraocular infections are frequently rapid and severe because of the avascular nature of the intraocular space that allows the early stages of bacterial infection to proceed without significant host response. S. aureus expresses an estimated 34 extracellular proteins, many of which have been implicated as virulence factors. This, together with the vulnerability of the intraocular space to infection, makes S. aureus endophthalmitis a potentially devastating infection. This potential is usually realized because in 52% of patients with S. aureus endophthalmitis, there is severe loss of best-corrected visual acuity.

Two concentrations of bacteria, one low inoculum level of approximately 10 cfu and one high level of approximately 1000 cfu, were used to simulate closely the relatively small inocula that might complicate surgery. A high inoculum level of approximately 1000 cfu was used to simulate many previously published models of S. aureus endophthalmitis. At
both low and high inoculum levels, the agr+ strain ISP479 resulted in a greater loss of neuroretinal function in the first 3 days after infection compared to the agr− strain ISP546. Furthermore, when 10 cfu of the toxin producing wild type strain were injected, significant changes in b-wave response were observed as early postoperative day 1. In contrast, eyes infected with 10 cfu of the ISP546 could not be distinguished from uninfected eyes until postoperative day 3. These data support the suggestion that toxin production plays a key role in the severity of endophthalmitis, at least as it relates to the rate of onset of clinical symp-
When the number of organisms was increased, both groups by postoperative day 2. These results show that the wild type strain resulted in focal retinal damage and mild vitritis as early as 36 hours after infection, whereas the eyes infected with ISP479 resulted in a moderate anterior chamber reaction on postoperative day 1, whereas at the same time point the agr− strain ISP546 resulted in only a mild reaction. A normal red reflex was observed in both groups. By postoperative day 2, the inflammatory reaction had progressed to moderate and severe for agr− and agr+, respectively. When the number of organisms was increased, both groups exhibited a more rapid onset of clinical symptoms. The agr+ group continued to exhibit greater anterior chamber inflammatory reaction on postoperative day 1, whereas at the same time point the agr− strain ISP546 resulted in only a mild reaction. A normal red reflex was observed in both groups. By postoperative day 2, the inflammatory reaction had progressed to moderate and severe for agr− and agr+, respectively. When the number of organisms was increased, both groups exhibited a more rapid onset of clinical symptoms. The agr+ group continued to exhibit greater anterior chamber inflammatory reaction on postoperative day 1, whereas at the same time point the agr− strain ISP546 resulted in only a mild reaction. A normal red reflex was observed in both groups. By postoperative day 2, the inflammatory reaction had progressed to moderate and severe for agr− and agr+, respectively.

The results of slit lamp examination, ERG, and histology demonstrate that one or more extracellular toxin produced by S. aureus and regulated by agr contributes to the severity of endophthalmitis. However, the contributing toxin(s) remains to be identified. In E. faecalis endophthalmitis, a pore-forming cytolsin has been shown to contribute to the pathogenesis of the disease. It was postulated in that study that the cytolsin may induce an early and uncontrolled release of inflammatory mediators from damaged tissue. The differences in clinical parameters observed for infections caused by the wild type and agr− strains do not appear to be the result of differences in growth rates either in vivo or in vitro. It was observed however, that when vitreous was recovered from enucleated eyes 24 hours after infection, only a fraction of the cfu observed in vitro were recovered (10⁴ versus 10⁸ cfu). Several investigators have shown that considerable variability occurs when attempting to quantify the number of organisms present in the globe after infection. Meredith et al suggested that host factors may contribute to this variability because such variability was seen even in eyes injected from the same preparation of organisms. The low number of organisms observed when growing in vivo may be caused by several factors. A recent study characterized the role of complement in the host inflammatory response in S. aureus endophthalmitis. It was found that the number of organisms recovered from eyes that had been decomplemented before infection was significantly elevated compared to normal control eyes, indicating that complement has a role in clearance of S. aureus from the eye. In addition, S. aureus may be adhering to intraocular tissues, possibly mediated by fibronectin binding protein located at the surface of S. aureus, and are, therefore, not recovered in a vitreous tap. Future experiments using cryosectioning of whole eyes and specific immunostaining techniques may be useful in identifying the distribution of S. aureus within the globe.

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FIGURE 4. Growth rate of Staphylococcus aureus ISP479 (agr+) and ISP546 (agr−) in vitreous in vitro when recovered from enucleated eyes and in vivo. □ = agr+ in vitro; ○ = agr− in vitro; ◇ = agr+ in vivo; Δ = agr− in vivo.
or phagocytic cells, thereby causing an exaggerated inflammatory reaction, and subsequently, indirect tissue damage. Alternatively, it was postulated that the toxin may directly lyse retinal cells. A functionally similar pore-forming toxin, α-toxin, is expressed by S. aureus ISP479 and has been the subject of extensive research. Cells that are especially sensitive to α-toxin include human endothelial cells, platelets, monocytes, and macrophages. α-toxin at sublytic levels may lead to the slow leakage of adenosine triphosphate and low molecular weight cellular components and influx of Ca²⁺ and Na⁺. Influx of Ca²⁺ into endothelial cells as a result of α-toxin pore formation has been shown to trigger the arachidonic acid cascade in endothelial cells. This leads to the production of leukotrienes, which mediate neutrophil accumulation and increased vascular permeability, and prostaglandins, which mediate inflammation and edema. Another effect of α-toxin includes triggering the release of the inflammatory mediator interleukin 1-β from human monocytes. These secondary events, induced after α-toxin pore formation, are consistent with the severe inflammatory response seen in S. aureus endophthalmitis, suggesting the possible involvement of α-toxin in the pathogenesis of S. aureus endophthalmitis.

The recent emergence of multidrug-resistant S. aureus strains has made many currently available antimicrobial agents ineffective and, as a result, poses a significant public health problem. The identification of toxins involved in S. aureus infections, including endophthalmitis, is essential to the development of novel therapeutic approaches. The finding here and by others that inactivation of global regulatory loci leads to diminished virulence highlights the prospect that these loci, or exoproteins under their control, may be potential targets for the development of novel antimicrobial agents. Future studies will be directed toward identifying the particular toxin(s) contributing to severity in endophthalmitis using mutant strains of S. aureus defective in the expression of individual toxins in the rabbit model.

Key Words
agr, endophthalmitis, Staphylococcus aureus, toxins, virulence

Acknowledgments
The authors thank Scottye Davis and Mark Ditmar for technical assistance.

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