The accessory gene regulator (agr) controls Staphylococcus aureus virulence in a murine intracranial abscesses model

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ABSTRACT

Background: Intracranial abscesses are associated with high mortality. Staphylococcus aureus is one of the main pathogens that cause intracranial infection. Until now, there is no report to identify the key effectors of S. aureus during the intracranial infection.

Methods: The murine intracranial abscesses model induced by S. aureus was constructed. The vital sign and survival rate of mice were observed to evaluate the infection. Histological examination was used to diagnose the pathological alterations of mouse tissues. The sensitivity of S. aureus to whole blood was evaluated by whole-blood killing assay.

Results: In murine intracranial abscesses model, it was shown that the mortality caused by the accessory gene regulator (agr) locus deficient strain was significant decreased compared with its parent strain. Moreover, we found that RNAIII, the effector of agr system, was essential for the intracranial infection caused by S. aureus. In the further investigation, it was shown that restoration the expression of α-toxin in agr deficient strain could partially recover the mortality in the murine intracranial abscesses model.

Conclusion: Our data suggested that the agr system of S. aureus is an important virulence determinant in the induction and mortality of intracranial abscesses in mice.

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Introduction

Intracranial abscesses frequently occur as a complication of sinusitis or neurosurgery, which are associated with high mortality (19–43%).1 Until now, the incidence, etiology, management and morbidity of intracranial abscesses are not well understood. Staphylococcus aureus is one of the main pathogens that cause intracranial abscesses.1–8

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The accessory gene regulator (agr) was identified as a quorum sensing system in S. aureus. RNAIII is the major effector of agr system. It acts as a small RNA regulating the expression of many virulence factors, including most of those encoding cell-wall-associated and extra-cellular proteins.\textsuperscript{9,10} Exotoxins expressed by S. aureus play important roles in the infection. α-Toxin is one of exotoxins, which is regulated by RNAIII. As a pore forming toxin, α-toxin can cause hemolysis and tissue damage. Recently, it has been found that α-toxin can also interact with its receptor ADAM10 to aggravate S. aureus infection.\textsuperscript{11} Herein we describe an animal model of intracranial abscesses caused by S. aureus that we have constructed. The pathogenic role of agr system is investigated in this model.

### Materials and methods

#### Mice

Adult female C57BL/6J mice (6–8 weeks old), obtained from Vital River Laboratories, were used for all experiments. Mice were housed in group cages, maintained on a 12:12 hour light-dark cycle, in a controlled environment (25 °C) and given unrestricted access to food and water. All animal experimental protocols of the study are in accordance with the national guidelines for the use of animals in scientific research “Regulations for the Administration of Affairs Concerning Experimental Animals”. It is also approved by Animal Care and Use Committee of Beijing Institute of Basic Medical Sciences with the approval number BMS-130112.

#### Staphylococcus aureus strains and culture conditions

S. aureus cells were grown in 5 mL of brain heart infusion (BHI), and Escherichia coli cells were grown in Luria-Bertani (LB) medium. Bacteria were cultured at 37 °C for 12 h with shaking at 200 rpm in a 25-mL test tube. Cells were transferred from 1 mL of pre-culture to 100 mL of BHI or LB medium in a 500-mL flask. S. aureus cells were routinely grown in BHI and E. coli cells were grown in LB medium either with no antibiotics, or with 40 μg/mL Chloramphenicol, 50 μg/mL tetracycline, 100 μg/mL ampicillin, and 50 μg/mL kanamycin.

#### Table 1 – Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Comments</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>S. aureus 8325-4</td>
<td>Wild-type, rsbU&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30</td>
</tr>
<tr>
<td>RN6910</td>
<td>Laboratory virulent strain derived from 8325</td>
<td>31</td>
</tr>
<tr>
<td>RN6911</td>
<td>RN6911 with an agr mutation, tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>RN6911-hla</td>
<td>RN6911 restoring HLA activity, cmr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>ΔRNAIII</td>
<td>Restriction-negative strain, 8325 derivative, kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>ΔRNAIII-R</td>
<td>ΔRNAIII restoring RNAIII activity cmr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>E. coli</td>
<td>A host strain for cloning</td>
<td>Transgene</td>
</tr>
</tbody>
</table>

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

#### Mouse brain infection model

Mice were anesthetized with 1% pentobarbital sodium (3–5 mg/100 gbw i.p.). While under anesthesia, mice were prepared for injection by securing the head in a stereotactic frame and quickly locating appropriate coordinates in the frontal cortex region on the skull. The general location for injection is 1 mm anterior to bregma and 1 mm lateral to the sagittal suture on the right side.\textsuperscript{12} Parting the fur with a needlepoint along both axes is appropriate for injection. To make an injection, orient the needle perpendicular to the skull and press through the calvarium to approximately 2 mm depth. Inject 5 μL bead suspension (10<sup>6</sup> CFU) into the brain by pressing the syringe button down and waiting 2 s for the bacteria to enter the brain. Control mice received injections of sterile saline. Carefully remove the needle and place the mouse in a clean, warm cage. All mice survived the injection procedure. After recovery from anesthesia, mice were kept at room temperature (25 °C) and provided with standard chow and watered ad libitum. 48-hour mortality, average time of death and rectal temperature were monitored.

#### Histological examination of mouse tissues

Mice were killed 12 h after injection and brains were removed immediately. Mouse brain was fixed in 10% neutral-buffered formalin for 48 h. The fixed tissue was processed through graded series of ethanol, followed by xylene, and embedded in paraffin. Tissue blocks through the entire abscess were sectioned at 5 μm and slides stained with hematoxylin–eosin.

#### Whole-blood killing assays

Bacteria cultures were washed twice in PBS, diluted to an inoculum of 10<sup>5</sup> CFU in 25 μL PBS, and mixed with 75 μL of freshly drawn mouse blood in heparinized tubes. The tubes were incubated at 37 °C for 4 h with agitation, at which time dilutions were plated on BHI agar for enumeration of surviving CFU.\textsuperscript{13}
Restoring HLA activity in RN6911

The whole HLA coding region was amplified by PCR and cloned into pOS1 vector. The resulting plasmid (pOS1-hla) was transformed into E. coli DH5α. Positive clones were selected on LB plates containing 100 μg/mL ampicillin. The recombinant plasmid was isolated from positive clones and transformed to S. aureus RN4220 cells. Transformants were selected on tryptic soy agar plates containing 40 μg/mL chloramphenicol at 37°C. The positive clone was selected by colony PCR. Then the plasmid was isolated and transformed to RN6911. The transformants were selected on tryptic soy agar plates containing 40 μg/mL chloramphenicol at 37°C for 20 h. Plasmids of positive restoring colonies were extracted and confirmed by PCR.

Statistical analysis

Data are expressed as mean ± SEM. The significance of differences between experimental groups was determined by two-tailed t-test assuming a 95% confidence interval.

Results

Construction of murine intracranial abscesses model caused by S. aureus

Injection of S. aureus 8325-4 into murine frontal cortex could produce a focal brain abscess with a clinical course similar to that seen in clinical patients with intracranial infection. Within 48 h post bacterial challenge, mice demonstrated clinical signs of illness including hunched posture, ruffled fur, lethargy, spasm, and finally expired. In contrast, any overt changes in posture, grooming, or physical activity of mice injected with sterile saline were not observed. However, challenge with S. aureus could not induce significant changes of rectal temperature (Fig. 1A). More than 50% of mice were dead after 48 h (Fig. 1B). Brains were collected 8 h after injection of Bacteria. A well-circumscribed abscess containing neutrophil and macrophage infiltrate, with significant mass effect was observed in histological examination of brains injected with S. aureus (Fig. 1C). Meanwhile, we also evaluate the hemogram
Fig. 2 – The role of agr system in murine intracranial abscesses model. (A) Survive curve of mice challenged with different strains (n = 16 mice in each group). Mice were challenged with RN6390B, RN6911, and saline, respectively. The number of dead mice was recorded. The survival rate of the two groups was calculated individually. (B) Histological examination of mouse brain tissue. Brain tissues from RN6390B and RN6911 groups were collected at 12 h post-challenge with bacteria and fixed. The fixed tissues were stained by H&E. (C) Percentage of neutrophils in the three groups (RN6390B, RN6911 and saline) at different time points (0 h, 4 h and 8 h) post-challenge with S. aureus (n = 10 in each group). (D) Survive curve of mice challenged with different strains (n = 22 mice in each group). Mice were challenged with S. aureus 8325-4, RNAIII, ΔRNAIII-R, and saline, respectively. The number of dead mice was recorded. The survival rate of the two groups was calculated individually. (E) Survival percentage of the different strains (RN6390B and RN6911) in murine whole blood. Bacteria (1 × 10^5 CFU) were incubated with murine whole blood for 4 h. Survival rate of bacteria were determined at BHI plate.

of mice at 4 h and 8 h after infection. The number of white blood cells was not significantly altered (Fig. 1D), but the percentage of neutrophil was significantly increased at different time points in this model (Fig. 1E).

The role of agr system in murine intracranial abscesses model

Given that agr system can regulate most of exotoxins expressed by S. aureus, we tried to determine whether agr system was involved in intracranial infection. Mice were injected with S. aureus RN6390B or RN6911 (agr-null strain). Injection with RN6911 could not induce death of mice (Fig. 2A). However, histological examination showed that RN6911 induced similar histological alteration of brain compared to RN6390B (Fig. 2B). The percentage of neutrophil was significantly increased in RN6390B and RN6911 groups, compared to sterile saline (Fig. 2C). It is well known that RNAIII is the key effector of agr system. Further investigating, we determined whether RNAIII deletion as well as agr-null strain was associated with decreased mortality of mice. The RNAIII deletion strains (ΔRNAIII) and the restored strain (ΔRNAIII-R) were previously constructed. Mice were challenged with wild-type S. aureus 8325-4, ΔRNAIII and ΔRNAIII-R, respectively. In line with expectation, ΔRNAIII could not induce the death of mice and challenge with the restoration RNAIII strain recovered virulence and the consequent mortality of mice (Fig. 2D). All these results suggested that agr system was essential for S. aureus induced mortality in the murine intracranial abscesses model.

As the alteration of hemogram induced by RN6911 was not different from that of RN6390B, we wondered if a deficient agr system could make the bacteria more sensitive to whole blood killing. RN6911 and RN6390B were incubated with murine whole blood respectively. The bacterial survival rate was assessed 4 h after incubation. The survival rate of RN6911
There are some constructed *S. aureus* infection models including arthritis, pneumonia, acute peritonitis, endocarditis, and skin infection. Until now, there are few reports about intracranial infection model caused by *S. aureus*. In this study, we constructed a murine model by injecting *S. aureus* 8325-4 into the frontal cortex. Mice challenged with *S. aureus* showed some clinical signs of illness. The number of white blood cells and the rectal temperature were not significant altered after injecting *S. aureus* suggesting that the sign of murine intracranial abscess was not exactly the same as human intracranial abscess. This may be due to difference in resistance to bacteria between mice and humans. However, the percentage of neutrophil in mice challenged with *S. aureus* was significantly higher than that of control group. This result is in accordance with the clinical sign seen in patients.

Agr system is well studied in *S. aureus* and is considered essential for infection. In line with expectations, we found that the mortality associated with agr-null strain was significantly decreased when compared to the wild type on intracranial abscesses model. Moreover, we also found that deletion of RNAIII suppressed virulence of *S. aureus* infection.

*S. aureus* can express various kinds of exotoxins which are necessary for causing infection. α-Toxin is the major exotoxin of *S. aureus*, regulated by RNAIIF. Our results showed that restoration of the level of α-toxin in agr-null strain partially recovered the mortality associated with *S. aureus* on the intracranial abscesses model. This result suggested that α-toxin should play an important role in intracranial infection. Moreover, some other molecules regulated by the agr system might also be involved in intracranial infection. The role of α-toxin and other molecules expressed by *S. aureus* in intracranial infection should be further investigated in the future.

Our study presents a murine intracranial abscesses model that can be used to investigate *S. aureus* infection in brain tissue. Further investigation on the role of agr system will increase our understanding of the intracranial abscesses caused by *S. aureus*.

**Conflicts of interest**

The authors declare no conflicts of interest.

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