Protection against *Staphylococcus aureus* and tetanus infections by a combined vaccine containing SasA and TeNT-Hc in mice

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Abstract. In developing countries, trauma patients and neonates are vulnerable to *Staphylococcus aureus* (*S. aureus*) and *Clostridium tetani* infections. It has been suggested that a combined vaccine against the two infections may be a reliable and cost-effective strategy. Previous studies have indicated that the *S. aureus* surface protein A (SasA) and the C fragment of tetanus neurotoxin (TeNT-Hc) may be suitable candidates for a vaccine against *S. aureus* and tetanus infections, respectively. In the present study, mice were immunized with a combined vaccine containing SasA and TeNT-Hc, which induced a robust immune response to both antigens, and mutual interference between SasA and TeNT-Hc was not observed. In the *S.aureus* challenge model, the combined vaccine fully protected BALB/c mice against lethal intraperitoneal challenges with 3×10^9 colony-forming units of a methicillin-resistant *S. aureus* USA300 strain. In the TeNT challenge model, the combined vaccine conferred complete protection against a lethal dose of (2×10^3) xLD50 tetanus toxin. These results implied that SasA and TeNT-Hc promising components for a combined vaccine against *S. aureus* and tetanus infections.

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Abbreviations: S. aureus, *Staphylococcus aureus*; MRSA, Methicillin-resistant *S. aureus*; IsdB, iron-responsive surface determinant B; TeNT-Hc, C fragment of tetanus neurotoxin; SasA, *Staphylococcus aureus* surface protein A; ELISA, enzyme-linked immunosorbent assay; TMB, 3,3',5,5'-tetramethylbenzidine dihydrochloride

Key words: vaccine, *Staphylococcus aureus*, tetanus neurotoxin, protective immunity trauma

Introduction

Clinical trauma patients are frequently diagnosed with *Staphylococcus aureus* (*S. aureus*) and tetanus infections, particularly those in developing countries (1,2). These infections have contributed greatly to the morbidity and mortality rates of neonates in developing countries such as Nigeria (3). *S. aureus* colonizes in approximately one-third of the human population (4), and is responsible for various diseases ranging from skin and soft tissue infections to life-threatening septicemia with metastatic complications (5). Tetanus is caused by tetanospasmin, a neurotoxin produced by the obligate anaerobic bacterium *Clostridium tetani*. Although the incidence of tetanus is low in developed countries, the worldwide mortality rates range from 6-72% (6), depending on the medical condition. In 2013, the World Health Organization estimated that ~49,000 newborns died from neonatal tetanus (7).

Combination vaccines are a reliable strategy to protect against two or more pathogens, and may reduce costs of mass vaccination and disposables (8). Those susceptible to trauma, including athletes, soldiers and the police, as well as women of childbearing age in developing countries, may benefit from the development of a combined vaccine against *S. aureus* and tetanus infections. *S. aureus* surface protein A (SasA), a cell wall-anchored protein of *S. aureus*, consisting of 2,271 amino acid residues, is a potential vaccine candidate for *S. aureus* infections (9). SasA has been reported to be a virulence determinant in endovascular infections, as the SasA mutant strain was observed to decrease the ability of catheterized rabbits to form vegetative plaques on heart valves (10). In addition, SasA is prevalent in *S. aureus* clinical isolates, and is expressed during *in vivo* growth of the bacteria (11). Notably, immunization with SasA was able to protect mice against a lethal *S. aureus* challenge (9). The tetanus toxin is a 150 kDa protein that has three domains (12), including the N-terminal zinc endopeptidase domain (13), the internal heavy chain translocation domain and the C-terminal heavy chain receptor-binding domain known as the C-fragment of tetanus neurotoxin (TeNT-Hc) (14). The genetic recombinant TeNT-Hc, which is nontoxic but has ganglioside binding activities, has been proposed as a possible replacement for the tetanus toxoid vaccine (15). TeNT-Hc is expected to demonstrate clear advantages over the existing tetanus toxoid vaccine with regard to ease of production, homogeneity and characterization.

In the present study, mice were immunized with a combined vaccine consisting of SasA (9) and TeNT-Hc (16) formulated by adsorption to Alhydrogel. The antibody titers and protective efficacy were measured for individual and combined vaccine administrations. The results demonstrated that the combined vaccine was able to induce protective immunity against *S. aureus* and tetanus neurotoxin challenges.

Materials and methods

Bacterial strains and growth conditions. The *S. aureus* strain USA300 (BAA-1556™; American Type Culture Collection, Manassas, VA, USA) was cultivated in tryptic soy broth (TSB; tryptone 15 g/l, soybean peptone 5 g/l, NaCl 5 g/l, pH 7.2, used after 121°C, 20 min) at 37°C. The *Escherichia coli (E. coli)* strain BL21 (DE3; Merck Millipore, Darmstadt, Germany) were cultivated in Luria-Bertani broth (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, pH 7.2, used after 121°C, 20 min) at 37°C and 100 μg/ml ampicillin (10 g ampicillin powder dissolved in 100 ml H_2O, used after filtration and diluted 1:1,000) was used for plasmid selection.

Subunit antigens. The antigens used in the current study were expressed in *E. coli* BL21 (DE3) and purified to >90%. The purification protocols were the same as described previously (9,16). Briefly, the polyhistidine-tagged recombinant SasA (rSasA) was purified by anion exchange, HisTrap and gel filtered chromatography (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The non-tagged TeNT-Hc antigen was purified by anion exchange, hydrophobic interactions and gel-filtered chromatography (GE Healthcare Bio-Sciences).

Active immunization. A total of 40 female BALB/c mice (age, 6-8 weeks; weight, 18-20 g) were purchased from The Beijing Laboratory Animal Center (Beijing, China), and were divided into 4 groups of 10 mice each. All mice had free access to food/water and were raised under standard conditions (temperature 25±2°C, relative humidity 50±10%) with a dark/light cycle (14/10 h). The mice were immunized intraperitoneally with 10 μg rSasA, 10 μg TeNT-Hc, 10 μg rSasA + 10 μg TeNT-Hc adsorbed to 0.75 mg aluminum hydroxide adjuvant (Brenntag Biosector A/S, Frederikssund, Denmark). The 0.75 mg aluminum hydroxide adjuvant was used as a negative control. Mice were injected at weeks 0, 2 and 4. Blood samples were drawn at weeks 2, 4 and 6 and every 4 weeks thereafter until week 26 through the tail vein and screened for reactivity to rSasA or TeNT-Hc antigens. Subsequent to the experiments, all mice were sacrificed by CO_2 asphyxiation. All experiments were performed in agreement with the institutional guidelines approved by the Laboratory Animal Care and Use Committee of the Beijing Institute of Biotechnology (IACUC of AMMS-08-2014-006).

Serological analysis of antibodies. Serum antibody titers of the antigen-specific total IgG, IgG1 and IgG2a were determined by enzyme-linked immunosorbent assay (ELISA). Microplates (96-well) were coated overnight with rSasA or TeNT-Hc (2 μg/ml) in coating buffer (50 mM carbonate buffer, pH 9.6) at 4°C. The plates were blocked with 2% (w/v) bovine serum albumin (2 g dissolved in 100 ml H_2O; Sigma-Aldrich; Merck

Millipore) in PBS at 37°C for 1 h. Duplicate two-fold serial dilutions of serum in an appropriate range (1:100~1:409,600) were incubated in the plates at 37°C for 1 h followed by washing with PBS with 3% Tween 20 (PBST). Then horseradish peroxidase-labelled goat anti-mouse total IgG, IgG1 (ab97240; 1:10,000) or IgG2a (ab97245; 1:10,000) antibodies (Abcam, Cambridge, MA, USA) were applied for 1 h at 37°C and then washed with PBST. The plates were incubated with 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate (Sigma-Aldrich; Merck Millipore) at room temperature for 10 min in the dark. The colorimetric reaction was stopped with 2 M sulfuric acid, and the optical density at 450 nm (OD_{450}) was read in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each step was followed by washing three times with PBST. The antibody-positive cut-off values were set as two times greater than the OD_{450} means of the pre-immunized sera. The ELISA antibody titer was expressed as the highest serum dilution showing a positive reaction.

S. aureus challenge model. Overnight cultures of *S. aureus* strain USA300 were diluted 1:100 in fresh TSB and cultivated at 37°C until reaching the mid-late logarithmic phase. *S. aureus* challenge was performed as described previously (17). Immunized mice were challenged by intraperitoneal injection with a lethal dose of *S. aureus* USA300 ($3x10^9$ colony-forming units in 100 μl/mouse), at 6 weeks after the primary immunization. Infected animals were monitored for survival for 5 days.

TeNT challenge model. At 6 weeks following primary immunization, the mice were challenged with ($2x10^3$)xLD50s (the LD50 in mice was determined by the improved Karber method (18). The LD50 of tetanus neurotoxin was ~15.8 ng/kg in mice) of tetanus neurotoxin [in 0.5 ml borate-buffered saline (0.5 g borax, 4.5 g boric acid and 8.5 g sodium chloride in 1 l distilled water)] by subcutaneous injection. Survival was monitored for five days.

Statistical analysis. Unpaired Student's two-tailed *t*-test was used to analyze the differences in ELISA titers between the groups. To compare the survival rates in the challenge models, experiments were analyzed using the Gehan-Breslow-Wilcoxon test with GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). P≤0.05 was considered to indicate a statistically significant difference.

Results

Antibody titer following immunization. A total of 3 groups of 10 mice were intraperitoneally immunized three times (at weeks 0, 2 and 4) with 10 μg rSasA, 10 μg TeNT-Hc or 10 μg rSasA + 10 μg TeNT-Hc. Blood samples were collected periodically through the tail vein to assay antigen-specific antibody responses by ELISA. The responses were compared between the groups over 26 weeks (Fig. 1). Detectable levels of IgG against rSasA and TeNT-Hc antigens were observed at 2 weeks following primary immunization, and the titers were maintained over 6 months.

No significant differences in TeNT-Hc specific IgG titers were observed between the TeNT-Hc immunized group and TeNT-Hc + SasA immunized group over the
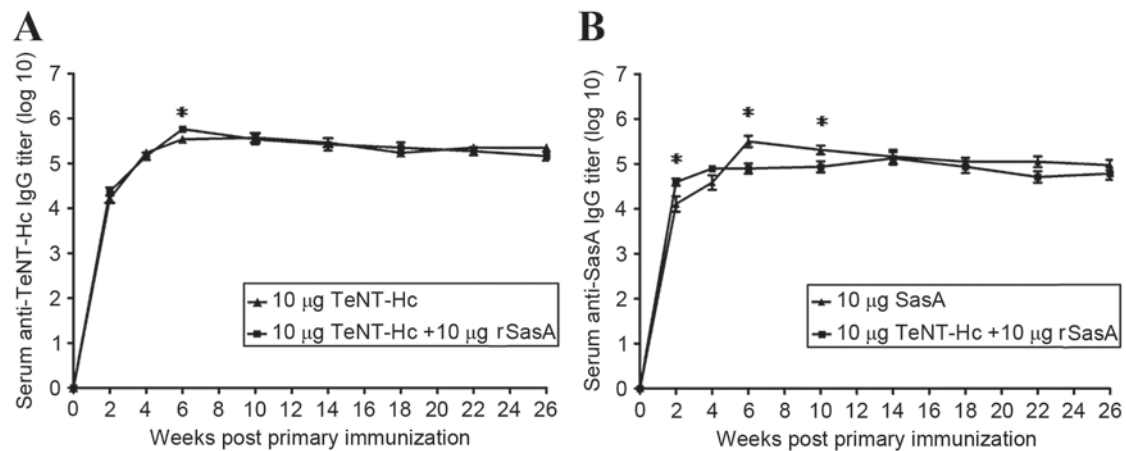
Figure 1. Kinetics of the IgG response in BALB/c mice following vaccination. A total of 3 groups of 10 mice were immunized three times (at weeks 0, 2 and 4) intraperitoneally with 10 µg rSasA, 10 µg TeNT-Hc or 10 µg rSasA + 10 µg TeNT-Hc. Serum of immunized mice were collected to monitor antigen-specific IgG response by ELISA; (A) Serum anti-TeNT-Hc IgG and (B) serum anti-SasA IgG titers. Data are presented as the log10 mean antibody titers for each group. The error bars represent the significant error. Unpaired two-sided Student's *t*-test was employed to assess the significant difference between groups. *P<0.05 vs. 10 mg TeNT-Hc + 10 SasA. IgG, immunoglobulin G; rSasA, recombinant *Staphylococcus aureus* surface protein; TeNT-Hc, C fragment of tetanus neurotoxin; ELISA, enzyme-linked immunosorbent assay.

26 weeks (Fig. 1A), except for the week 6, when the combined vaccine induced anti-TeNT-Hc IgG titers 1.683-fold higher than TeNT-Hc (P=0.0313). Significant differences in rSasA specific IgG titers were observed between the rSasA immunized group and the TeNT-Hc + SasA immunized group in weeks 2, 6 and 10 (Fig. 1B). The combined vaccine induced rSasA specific IgG titers 3.083-fold higher than rSasA alone at week 2 (P=0.0210). However, at weeks 6 and 10, the combined vaccine induced rSasA specific IgG titers 3.999 times (P=0.0032) and 2.377 times (P=0.0323) lower than rSasA alone, respectively.

IgG isotyping. Serum samples from immunized mice at week 6 were assayed for the presence of antigen-specific IgG1 and IgG2a antibodies by ELISA. rSasA and the combined vaccine induced the robust production of IgG1 and IgG2a antibodies specific to rSasA (Fig. 2). No significant differences in rSasA IgG1 and IgG2a titers were observed between the rSasA and rSasA + TeNT-Hc groups. Similar results were observed from TeNT-Hc-immunized mice and the combined vaccine-immunized mice (Fig. 2). The combined vaccine induced specific IgG1 and IgG2a titers comparable to rSasA or TeNT-Hc vaccines. Additionally, the antigen-specific IgG1/IgG2a ratio was not influenced by co-administration of the two antigens (data not shown).

In vivo protection against S. aureus challenge. Immunized mice were challenged by intraperitoneal inoculation with 3x10⁹ colony-forming units of *S. aureus* USA300, 6 weeks following primary immunization. Approximately 90% of mice in the control group died within 24 h (Fig. 3A). By contrast, immunized mice survived for 120 h post challenge. All mice immunized with 10 µg rSasA or 10 µg rSasA+10 µg TeNT-HC survived the challenge with symptoms of infection such as temporary leg paralysis.

In vivo protection against tetanus neurotoxin. The mice vaccinated three times with 10 µg TeNT-Hc or 10 µg rSasA + 10 µg
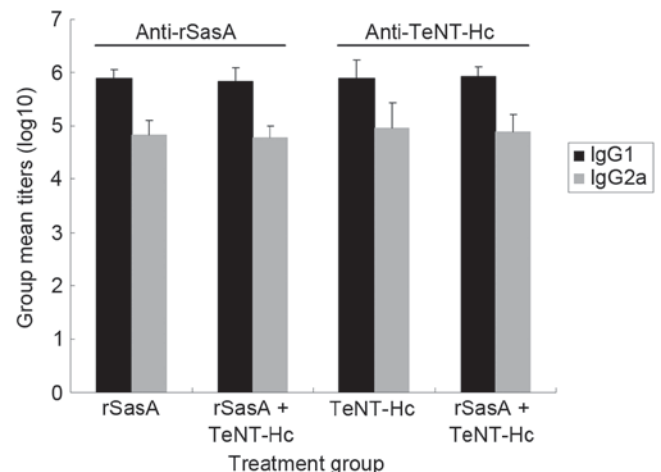

Figure 2. Antigen-specific IgG1 and IgG2a antibody titers induced by rSasA, TeNT-Hc and the combined vaccine. A total of 3 groups of 10 mice were immunized three times (at weeks 0, 2 and 4) intraperitoneally with 10 µg rSasA, 10 µg TeNT-Hc or 10 µg rSasA+10 µg TeNT-Hc. Serum of immunized mice were collected at 6 weeks following primary immunization. The antigen-specific IgG1 and IgG2a responses were determined by ELISA. No significant differences were observed. Data are presented as the log10 of the mean antibody titers for each group. The error bars represent the standard error. Unpaired two-sided Student's *t*-test was employed to assess the significant difference between groups. IgG, immunoglobulin G; rSasA, recombinant *Staphylococcus aureus* surface protein; TeNT-Hc, C fragment of tetanus neurotoxin; ELISA, enzyme-linked immunosorbent assay.

TeNT-Hc were challenged with tetanus neurotoxin, and its protective abilities were evaluated. When challenged with 2x10³ LD50s of tetanus neurotoxin, TeNT-Hc and the combined vaccine provided excellent protection; 9 mice vaccinated by TeNT-Hc survived and 1 mouse died from tetanus poisoning. All mice vaccinated by 10 µg rSasA + 10 µg TeNT-Hc survived without any observed symptoms (Fig. 3B). However, all mice immunized with aluminum hydroxide adjuvant alone did not survive at 24 h post-challenge.
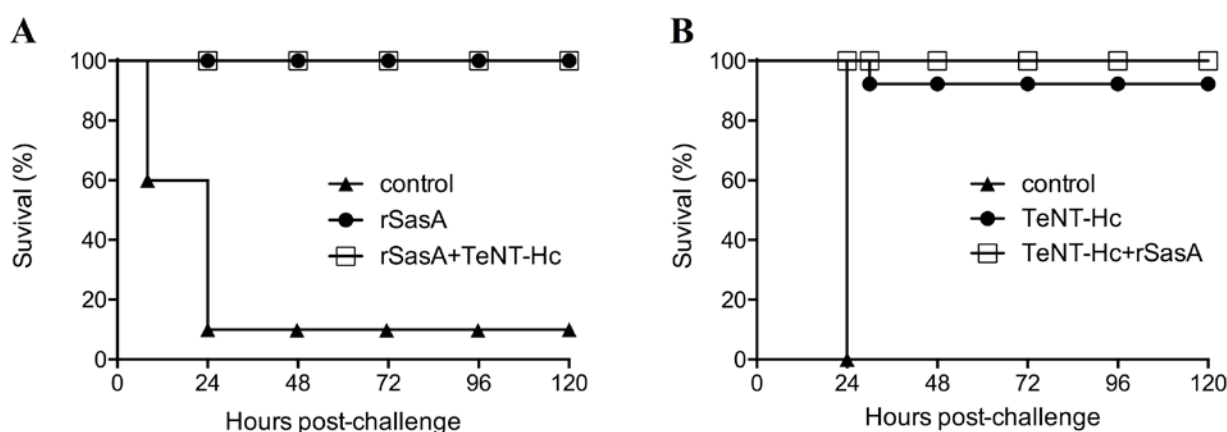
Figure 3. Survival of immunized mice after lethal challenge by *S. aureus* USA300 or TeNT. Groups of 10 female BALB/c (age, 6-8 weeks) mice were immunized three times (at weeks 0, 2 and 4) intraperitoneally with 10 µg rSasA, 10 µg TeNT-Hc, 10 µg rSasA+10 µg TeNT-Hc or 0.75 mg aluminum hydroxide adjuvant control. 6 weeks following primary immunization, mice were challenged by intraperitoneal injection with (A) a lethal dose ($3x10^9$ colony-forming units/mouse) *of Staphylococcus aureus* strain USA300 or (B) by subcutaneous injection with $2x10^3$ LD50s TeNT. Survival curves were compared using the Gehan-Breslow-Wilcoxon Test. *S. aureus, Staphylococcus aureus*; TeNT-Hc, C fragment of tetanus neurotoxin; rSasA, recombinant *S. aureus* surface protein; LD50, the lethal dose that causes mortality in 50% of subjects.

Discussion

A combined vaccine may be a reliable and cost-effective strategy to prevent infections caused by two or more pathogens or a single pathogen with various serotypes. Several successful combined vaccines for children have been used for a number of years including, heterologous combinations such as diphtheria-tetanus-pertussis vaccine (19), the combined *Hemophilus influenzae* type B (HIB), the HIB/hepatitis B (HepB) vaccine (20), the combined HepA-HepB (21), in addition to homologous combinations such as multivalent pneumococcal vaccines (22). The results of the present study suggested that SasA and TeNT-Hc are promising candidates for a combined vaccine against *S. aureus* and tetanus infections.

Due to antibiotic resistance and a general lack of novel classes of antimicrobial agents against *S. aureus*, vaccines against this pathogen have been extensively investigated. A number of *S. aureus* antigens, including capsular polysaccharide (23), poly-N-acetylglucosamine (24), iron-responsive surface determinant B (25), clumping factor A (26), clumping factor B (27), protein A (28), fibronectin-binding protein (29), collagen adhesion (30), coagulase (31), α-hemolysin (32), Panton-Valentine leukocidin (33), staphylococcal enterotoxins (34) and toxic shock syndrome toxin 1 (35) have been studied in animal models. However, no vaccine developed thus far, has demonstrated efficacy in humans (36) for unknown reasons. In the present study, SasA induced a robust specific-antibody reaction and complete protection against the USA300 strain, which is a common source of methicillin-resistant *S. aureus* infections in the USA (37). In addition, SasA is prevalent in *S. aureus* clinical isolates and is immunogenic in humans, as titers of SasA-specific antibodies in the sera of convalescent patients infected by *S. aureus* were higher than that in the sera of healthy people (11). Taken together, SasA is a promising component for a combined vaccine against *S. aureus*. Recombinant TeNT-Hc has exhibited considerable promise as a next-generation subunit

vaccine against tetanus (15,16), particularly in formulations that can be administered orally (38) or by intranasal routes (39). Notably, the results of the present study demonstrated no mutual interference between TeNT-Hc and SasA in the antibody response, which is consistent with the fact that recombinant TeNT-Hc has been used as a vaccine carrier to enhance the immunogenicity of conjugate or fusion vaccines (40).

As *S. aureus* consists of a number of virulence factors, the development of a vaccine against this pathogen has proven challenging (41). Therefore, a multi-component vaccine has been suggested to achieve greater protective immunity against *S. aureus* (42). In future studies, more antigens of *S. aureus* may be added the combined vaccine, to determine whether it may confer protection against more clinical *S. aureus* strains and in different animal models.

In conclusion, the present study demonstrated that combined immunization with SasA and TeNT-Hc was as effective as individual immunizations for induction of a serological antibody response. The protective efficacy was not impaired when the antigens were administered in combination. The results provided preliminary evidence for the development of a combined vaccine against *S. aureus* and tetanus infections.

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