Hirosaki Med. J. 59 : 71—82, 2008

ORIGINAL ARTICLE

PROTECTIVE EFFECT OF INTRANASAL VACCINATION WITH NONTOXIC MUTANT TOXIC SHOCK SYNDROME TOXIN 1 AGAINST *STAPHYLOCOCCUS AUREUS* INFECTIONS

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Abstract Toxic shock syndrome toxin 1 (TSST-1) produced by *Staphylococcus aureus* is one of superantigenic exotoxins and is best known for its involvement in toxic shock syndrome. To investigate whether an intranasal vaccination with a nontoxic mutant TSST-1 (mTSST-1) can protect against systemic infection and nasal colonization of *S. aureus*, mice were immunized with mTSST-1 plus a nontoxic mutant heat labile toxin (mLT) of *Escherichia coli*. After an intravenous challenge with *S. aureus*, bacterial growth in the organs of immunized mice reduced and the survival rate was significantly higher, compared with the naive mice. To evaluate the effect of vaccination with mTSST-1 against nasal *S. aureus* colonization, mice were challenged with *S. aureus* intranasally. The bacterial counts in the nasal cavity of immunized mice were significantly reduced. Vaccination with mTSST-1 plus mLT efficiently induced anti-TSST-1 antibodies in the sera and mucosal exudates, and increased anti-TSST-1 IgA-secreting cells in nasal-associated lymphoid tissues. These results indicate that an intranasal vaccination with mTSST-1 plus mLT could induce systemic and mucosal immune responses and provide protection against not only systemic infection but also nasal colonization of *S. aureus*.

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Key words: Mutant toxic shock syndrome toxin-1; Intranasal vaccination; Nasal *S. aureus* colonization.

原 著

無毒変異 TSST-1 経鼻粘膜ワクチンによる黄色ブドウ球菌感染防御効果

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抄録 毒素性ショック症候群毒素1 (TSST-1) は黄色ブドウ球菌の産生するスーパー抗原の1種である．本研究では無毒変異 TSST-1 (mTSST-1) による経鼻粘膜免疫の黄色ブドウ球菌全身感染，鼻腔内定着に対する防御効果を検討した．マウスを mTSST-1 で経鼻粘膜免疫をした後，黄色ブドウ球菌を経静脈感染させた，免疫群マウスでは非免疫群マウスに比べ臓器内生菌数は低下し，高い生存率を示した．また黄色ブドウ球菌を鼻腔内に投与した場合，免疫群マウスの鼻腔内黄色ブドウ球菌生菌数は減少した．mTSST-1 経鼻粘膜免疫は血中，粘膜分泌液中の抗 TSST-1 抗体，さらに鼻関連リンパ組織における抗 TSST-1 IgA 産生細胞を誘導した．これらの結果から，mTSST-1 経鼻粘膜免疫は全身免疫，粘膜免疫を誘導し，黄色ブドウ球菌全身感染だけではなく鼻腔内定着に対しても防御効果を示すことが示唆された．

弘前医学 59 : 71—82, 2008

キーワード：無毒変異 TSST-1；経鼻粘膜免疫；黄色ブドウ球菌鼻腔内定着．

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Received for publication, November 26, 2007
Accepted for publication, December 21, 2007

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別刷請求先：成田浩司
平成19年11月26日受付
平成19年12月21日受理

INTRODUCTION

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have been the most common nosocomial infections, resulting in increased morbidity, mortality, length of hospital stay, and health care costs[1]. The growing prevalence of antibiotic resistant *S. aureus* strains threatens the effectiveness of current strategies for managing *S. aureus* infection and demonstrates the need for other means of controlling and preventing staphylococcal infections such as immunotherapy.

S. aureus has been known to adhere and colonize the mucosal epithelium, and anterior nares are known as the major reservoir of *S. aureus*. Previous studies showed that 80% of *S. aureus* bacteremias were endogenous since infecting bacteria were identical to organisms recovered from the nasal mucosa[2,3]. Elimination and reduction of nasal carriage are thought to be effective protection against systemic *S. aureus* infection and be useful for the control of *S. aureus* nosocomial infection. Mucosal antibodies generated at the entry sites of bacteria might play an important role in blocking bacterial colonization, enhancing clearance, and preventing systemic infection[4]. Currently, there is much interest in the route of mucosal immunization to protect against various pathogens that gain entry into the host via mucosal tissues.

To design vaccines to protect against *S. aureus* infection, various virulence factors of this microorganism including capsular polysaccharides[5], cell wall-associated proteins[6] and toxins[7,8] have been targeted. Toxic shock syndrome toxin 1 (TSST-1) is one of superantigenic exotoxins secreted by *S. aureus*, and a major virulence factor in toxic shock syndrome, staphylococcal scarlet fever and neonatal toxic shock-like exanthematous diseases[9]. Several papers have shown that a majority (88-95.8%) of *S. aureus* strains isolated in Japanese hospitals carry the TSST-1 (*tst*) gene[10-13]. Previous studies have reported that a mutant TSST-1 (mTSST-1), the histidine at position 135 was changed to alanine, negatively affects the binding of TSST-1 to the T cell receptors, shows less toxicity and still has antigenicity[14]. Vaccination with mTSST-1 by intraperitoneal injection could protect mice against challenge with lethal doses of TSST-1 potentiated with lipopolysaccharide[15]. Subcutaneous immunization with mTSST-1 could reduce the bacterial number in the organs and increase survival rates of the mice challenged with lethal doses of *S. aureus*[14].

In this study, we investigated whether an intranasal vaccination with nontoxic mTSST-1 plus nontoxic mLT, a mutant heat labile toxin (mLT) of *Escherichia coli*[16], could elicit the protective effect against nasal and systemic *S. aureus* infections. Our results showed that an intranasal vaccination with mTSST-1 plus mLT induced systemic and mucous immune responses and provided protection against not only systemic infection but also nasal colonization of *S. aureus*.

MATERIALS and METHODS

Animals

BALB/c mice, 5 to 8 weeks old, were purchased from Clea Japan, Tokyo, Japan. Mice were maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine. The daily cycle consisted of 12 h light and 12 h darkness, and food and water were available at all times. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

Bacterial strains and culture condition

To prepare the inocula for infection and colonization experiments, a clinically isolated *S.*

aureus 834 strain, which expresses TSST-1 and staphylococcal enterotoxin C (SEC) [17], was used, and a non TSST-1 producing *S. aureus* clinical isolate, 524 strain was also used to determine bacterial counts in the organs after intravenous infection. The bacteria were cultured at 37℃ in tryptic soy broth (BD Bioscience, Sparks, MD) for 15 h, collected by centrifugation, washed with sterile phosphate-buffered saline (PBS), and then resuspended with sterile PBS. The bacterial numbers were adjusted spectrophotometically at 550 nm to an appropriate value. The *E. coli* DH5α strains containing pGXmTST or pGXTST plasmid[14] were used for preparing mTSST-1 and recombinant TSST-1 (rTSST-1), respectively.

Expression and purification of rTSST-1, mTSST-1 and mLT

Expression and purification of rTSST-1 and mTSST-1 were performed as described by Hu et al[14]. Briefly, *E. coli* DH5α cells containing pGXmTST or pGXTST were cultured in 2 × YTA medium containing 100 μg/ml of ampicillin at 37℃ with shaking. Then 0.5 mM isopropyl-1-thio-β-D-galactopyranoside was added for inducing the expression of glutathione-S-transferase (GST)-mTSST-1 and GST-TSST-1 fusion protein. The bacteria were cultured for additional 3 h and collected by centrifugation, the fusion proteins were extracted from bacterial pellets by B-PER bacterial protein extraction reagent (Piece, Rockford, IL). Purification of fusion proteins and removal of the GST tag were performed by using bulk GST purification modules (Amersham Pharmacia Biotech, Piscataway, NJ). The concentrations of the resultant proteins were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), and the purity was confirmed by SDS-PAGE. mLT (LT H44A) was constructed by site-directed mutagenesis, and purified from *E. coli* containing plasmid encording mLT[18].

Intranasal immunization and *S. aureus* infection

Mice were anesthetized by an intraperitoneal injection of Nembutal (Dainippon Pharmaceutical Co., Osaka, Japan). For initial immunization, 10 μg of mTSST-1 was mixed with 5 μg of mLT in 20 μl of PBS, and then was administered intranasally to external nares of a mouse. Boost immunizations were performed 2 and 4 weeks after initial immunization with the same manner. Control mice were injected with 5 μg of mLT or PBS alone. For systemic infection, mice were challenged with 5×10^7 CFU of TSST-1 producing or non-producing *S. aureus* by intravenous injection on day 7 after the last booster. For nasal colonization of *S. aureus*, mice were anesthetized by Nembutal, and then 20 μl of inoculum which contained 10^9 CFU of *S. aureus* was pipetted slowly onto the nares of a mouse[19] on day 7 after the last booster.

Preparation of fecal extracts and bronchoalveolar lavage fluids (BALF)

Fecal pellets (100 mg) were collected at 6 h after challenge with 5×10^7 CFU of *S. aureus* by intravenous injection and suspended in 1 ml of PBS containing 0.1% sodium azide. Fecal pellets were broken by sterile toothpick and vortexed for 10 min, and then centrifuged at 2000 g for 5 min. The supernatants were collected as fecal extracts[20]. BALF was obtained 3 days after challenge with 5×10^7 CFU of *S. aureus* by intravenous infection or 10^9 CFU of *S. aureus* by intranasal inoculation. For preparation of BALF, a flexible polyethylene cannula was inserted into the trachea and fixed with a ligature. BALF was obtained by irrigation with 0.6 ml of PBS twice, and to remove cellular debris, the recovered wash was centrifuged at 300 g for 10 min.

Determination of bacterial numbers

On day 3 after an intravenous infection with *S. aureus*, the bacterial numbers in the spleens and livers were enumerated. An aliquot of organ

was homogenized and diluted in serial 10-folds with sterile PBS. One hundred μl of dilutions were plated on tryptic soy agar (BD Bioscience). Colonies were counted after 24 h of incubation at 37°C, and the bacterial number per organ was calculated with the number of colony, dilution and weight of organ. To evaluate *S. aureus* nasal carriage, the nose areas were cleansed with 70 % ethanol to eliminate external skin colonization by *S. aureus*, and the noses were surgically removed. The excised nose was placed in 500 μl of PBS and then homogenized. The numbers of *S. aureus* in nasal cavities were evaluated by plating 100 μl of nasal suspension on mannitol salt agar (Eiken Chemical Co., Tokyo, Japan), whereas total bacteria in the nasal cavities were evaluated by plating 100 μl of nasal suspension on tryptic soy agar.

Assay of specific antibodies

Fecal samples and BALF were obtained as described above, sera were obtained 3 days after challenge with *S. aureus* by intravenous injection. Antibody titers against TSST-1 and *S. aureus* cell protein antibody in BALF, sera and fecal samples were measured by enzyme-linked immunosorbent assays (ELISAs) as described previously[14]. The samples of serum, BALF and fecal extracts were serially two-fold diluted with 10% Blockace (Dainippon Pharmaceutical) in PBS. Antibody titers were defined as reciprocal last dilutions that gave an optical density reading at 490 nm of more than twice above the mean OD of control wells containing 10% Blockace in PBS.

Spleen cell culture

The spleens of immunized mice and control mice were aseptically removed on day 7 after the last booster, and the spleen cells were obtained by squeezing organs in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan). Each cell suspension was filtered through stainless steel mesh (size 100), and erythrocytes were lysed with 0.85% NH_4Cl. After washing 3 times with RPMI 1640 medium, 1×10^6 cells were suspended in 1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 3% L-glutamine (Wako Pure Chemical Industries, Osaka, Japan), 100 U/ml of penicillin G, and 100 μg/ml streptomycin in a 24 well culture plate, and the culture were stimulated by addition of 100 ng/ml of rTSST-1. After 72 h incubation at 37℃ in 5% CO_2, the supernatants were collected by centrifugation at 1500 g for 20 min, and stored at −80℃ until the cytokine assays were preformed.

Cytokine assays

The amounts of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in the supernatants of cell cultures were determined by double-sandwich ELISAs as described previously[17, 21].

Blood killing assay

Heparinized whole blood was obtained from immunized and control mice on day 7 after the last immunization. Whole blood killing assay was performed as described previously[22]. Briefly, 200 μl of whole blood and 100 μl of *S. aureus* suspension containing 3×10^4 CFU were mixed in polyethylene tubes and incubated on a rotator at 37℃. Samples were taken at 0 and 120 min after incubation and serial 10 fold dilutions of samples were made in PBS and plated onto tryptic soy agar plates.

Preparation of nasal-associated lymphoid tissue (NALT) cells

NALT cells were isolated from immunized and control mice on day 7 after the last booster as previously described[23]. Prepared NALT cells were released in RPMI 1640 medium supplemented with 2% fatal calf serum and filtered through stainless steel mesh (size 100).

The cells were washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 3% L-glutamine, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. NALT cells were used for analyzing anti-TSST-1 IgA production by ELISPOT assay.

ELISPOT assay

ELISPOT assay was performed according to the manufacturer's instructions (Protein Detector, KPL, Gaithersburg, MD). Briefly, a 96-well plate with PVDF base was coated overnight with 5 μg/ml of rTSST-1 in coating solution. Prepared NALT cells were added to each well at a concentration of 2×10^5 cells/well and incubated for 24 h at 37℃ in 5% CO_2. The plate was washed and then incubated with 100 μl of biotinylated goat anti-mouse IgA (Santa Cruz Biotechnology, Santa Cruz, CA). Streptavidin-horseradish peroxidase was added to the washed plate and spots were developed with True blue substrate. Positive spots were counted with the aid of a dissecting microscope.

Statistical analysis

Statistical analysis of bacterial counts in the organs, cytokine titers and blood killing assay were made by Student's *t*-test. For survival experiments, the Kaplan-Meier method was used to obtain the survival fractions, and significance was determined by a log rank test. $P < 0.05$ was considered significant.

RESULTS

Effect of an intranasal immunization with mTSST-1 against systemic *S. aureus* infection

To confirm the protective effect against systemic *S. aureus* infection, the immunized and control mice were challenged with TSST-1 producing or non-producing *S. aureus* intravenously, and the bacterial counts in spleen and liver were determined. In the challenge with a TSST-1 producing *S. aureus* strain, the bacterial numbers in the spleens and livers of immunized mice were significantly fewer than those in the organs of control mice, but in the challenge with a non-TSST-1 producing strain, significant differences were not shown between the immunized and control mice (Fig 1A). We further monitored the survival rates of

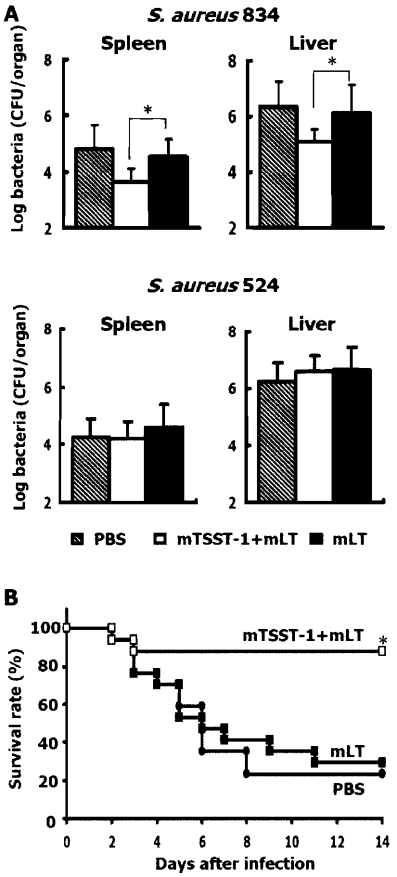

Fig. 1 Protective effect of immunization with mTSST-1 on host resistance against systemic *S. aureus* infection. (A) Immunized and control mice were infected with TSST-1-producing *S. aureus* 834 strain or non-producing *S. aureus* 524 strain intravenously. The bacterial numbers in the spleens and livers were determined. Data are means ± SD for 6 to 8 mice. The asterisk represents a statistically significant difference from a control (mLT alone) at $P < 0.05$. (B) Survival rates of immunized mice and control mice. Immunized and control mice were intravenously infected with *S. aureus*. The animals were monitored for 14 days after infection (the number of animals of each group was 16-17). The asterisk represents a statistically significant difference from the control (mLT alone) at $P < 0.05$.

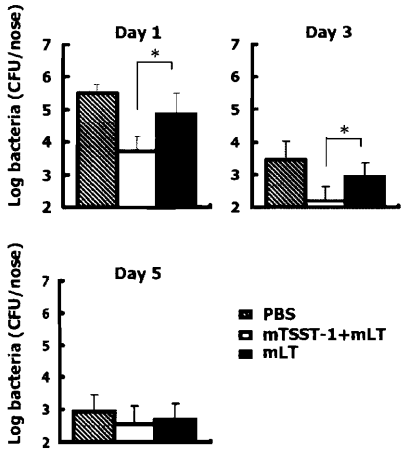

Fig. 2 Protective effect of immunization with mTSST-1 against nasal *S. aureus* colonization. Mice were intranasally immunized with mTSST-1 plus mLT, mLT or PBS alone and inoculated with *S. aureus* intranasally. The bacterial numbers per nose were determined on days 1, 3 and 5 after inoculation. Data are means ± SD for 5 to 8 mice. The asterisk represents a statistically significant difference from the control (mLT alone) at $P < 0.01$.

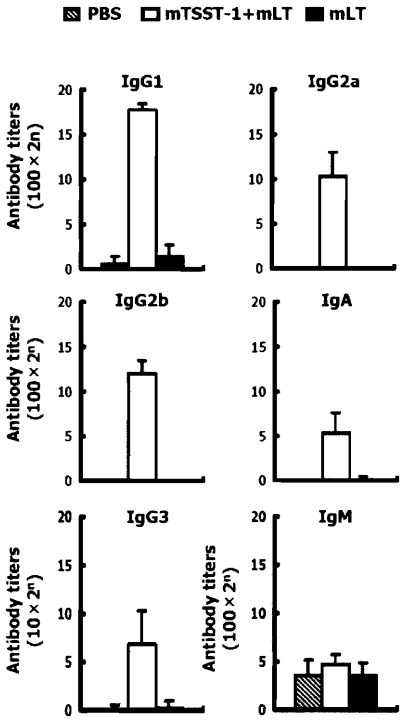

Fig. 3 Antibody responses in the sera of immunized and control mice. Serum samples from immunized and control mice were obtained on day 3 after infection. Anti-TSST-1 antibody titers were determined by ELISAs. Data are mean endpoint titers ± SD for 6 to 8 mice.

immunized and control mice for 14 days after challenge. The survival rate of immunized mice was 87.5% on day 3 after challenge and no change of the survival rate was observed until day 14 after challenge ($P < 0.05$). In contrast, the survival rates of control mice administered with mLT or PBS alone were 29.4%, 23.5% respectively (Fig. 1B).

Effect of an intranasal immunization with mTSST-1 against nasal *S. aureus* colonization

To evaluate the protective effect of vaccination with mTSST-1 against nasal colonization of *S. aureus* in mice, we established a nasal *S. aureus* colonization model by modifying the previous method[19]. mTSST-1-immunized and control mice were challenged with *S. aureus* intranasally, and the numbers of *S. aureus* in nasal cavities were determined on days 1, 3 and 5 after inoculation. The numbers of *S. aureus* per nose of the immunized mice were significantly reduced compared with those of control mice on days 1 and 3 ($P < 0.01$). However, there was no significant difference between the immunized and control mice on day 5 (Fig. 2).

Serum antibody responses

Serum samples were obtained from immunized and control mice on day 3 after challenge, and the titers of anti-TSST-1 antibody were determined. Significantly high titers of anti-TSST-1 IgG1, IgG2a, IgG2b, IgG3 and IgA antibodies were detected in the sera of mTSST-1-immunized mice compared with those of control mice (Fig. 3). There was no significant difference in IgM production between the immunized and control mice.

Antibody responses in mucosal exudates

The BALF from immunized mice showed significantly higher levels of anti-TSST-1 IgA production. Furthermore, the production of

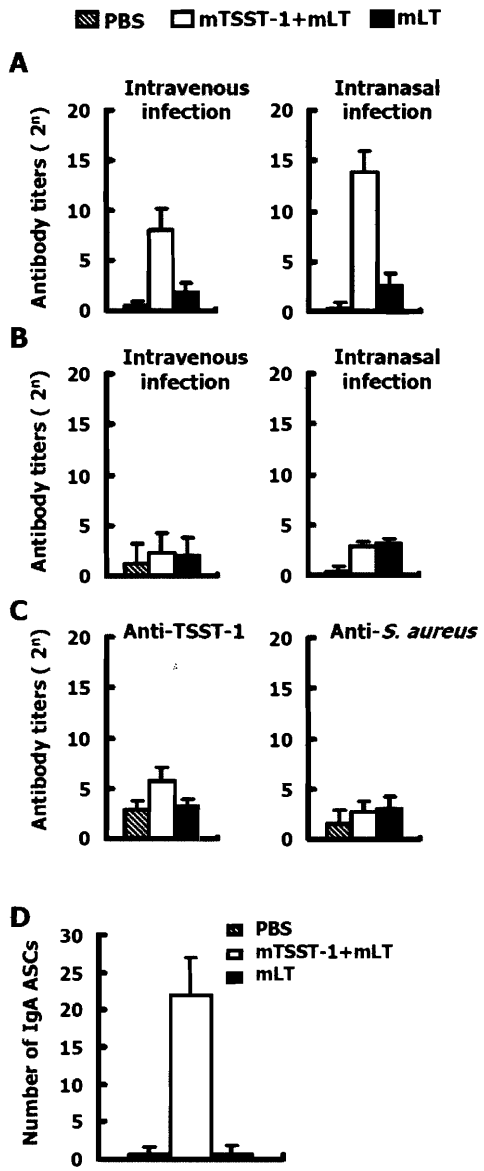

Fig. 4 Antibody responses in mucosal exudates of immunized and control mice. Mice were intranasally immunized with mTSST-1 plus mLT, mLT or PBS alone, and then infected with *S. aureus* intravenously or intranasally. The titers of anti-TSST-1 antibody and anti-*S. aureus* cell protein antibody were determined by ELISAs. (A) Anti-TSST-1 antibody titers in the BALF of mice infected intravenously or intranasally. (B) Anti-*S. aureus* cell protein antibody titers in the BALF. (C) Anti-TSST-1 antibody and anti-*S. aureus* cell protein antibody titers in fecal pellets obtained from the mice infected intravenously. Data are mean endpoint titers ± SD for 6 mice. (D). Detection of anti-TSST-1 IgA-secreting cells (ASCs) in NALT. NALT cells from immunized and control mice were analyzed for anti-TSST-1 IgA-ASCs by ELISPOT. Data are mean from a number of ASCs ± SD for 8 to 9 mice.

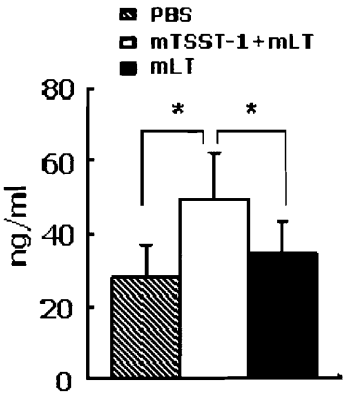

Fig. 5 IFN-γ production in spleen cells of immunized and control mice in vitro. Spleen cells were prepared from immunized and control mice and stimulated with rTSST-1. The amounts of IFN-γ in the supernatants of cell cultures were determined by double-sandwich ELISAs. Result represents the mean ± SD for 8 to 9 mice. The asterisk represents a statistically significant difference from the control (mLT alone) at $P < 0.05$.

IgA in the BALF from intranasally infected mice was higher than that from intravenously infected mice (Fig. 4A). Anti-*S. aureus* cell protein antibodies were substantially produced in neither the immunized mice nor the control mice (Fig. 4B). In fecal pellet extracts, the higher titers of anti-TSST-1 IgA were detected in the immunized mice compared with those of control mice, whereas the similar titers of anti-*S. aureus* IgA production was shown in both the immunized and control mice (Fig. 4C). We further analyzed the NALT cells secreting anti-TSST-1 specific IgA by ELISPOT. The numbers of cells secreting TSST-1 specific IgA in NALT from the vaccinated mice were significantly increased compared with those of control mice (Fig. 4D).

Cytokine production in the spleen cells of immunized and control mice in vitro

The IFN-γ titers in the supernatants of the spleen cell cultures from immunized and control mice were determined (Fig. 5). The significantly high titers of IFN-γ were detected in the cell cultures from the mice immunized

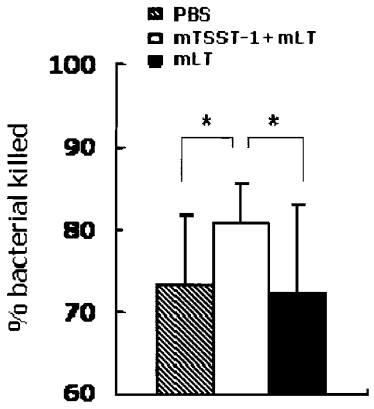

Fig. 6 Percentages of *S. aureus* killed by whole blood of immunized and control mice. Blood killing was represented by the percentage of killed bacterial counts in each sample between the 0 min and 120 min after incubation. Result represents the mean ± SD for 8 to 9 mice. The asterisk represents a statistically significant difference from the control (mLT alone) at $P < 0.05$.

with mTSST-1 intranasally compared with those from the control mice ($P < 0.05$). Although the titers of TNF-α in the cell cultures from the immunized mice were slightly higher than those in the cell cultures from control mice, the significant difference was not shown between the immunized and control mice (data not shown).

Bactericidal activity of blood from the immunized and control mice

To determine whether leukocytes from mice immunized with mTSST-1 show the promoted bactericidal activity, *S. aureus* killing assay with whole blood was performed. The numbers of *S. aureus* in the mixtures with the blood of mTSST-1-immunized mice were significantly reduced compared with those of control mice at 2 h (Fig. 6).

DISCUSSION

S. aureus has been known to cause a diverse spectrum of severe infections, and the nose is regarded as the primary site of *S. aureus* carriage, and this asymptomatic carriage of *S. aureus* appears to play an important role in the epidemiology and pathogenesis of *S. aureus* infection[24]. Recently, there is great interest in developing mucosal vaccines against a variety of microbial pathogens[25]. Mucosal vaccination can elicit both systemic and mucosal immune responses, and prevent the infectious agent from attaching and colonizing at the mucosal epithelium. Mucosal immunization can also block microbial toxins binding to epithelial and other target organs[25]. Previous studies have shown that intranasal vaccination against superantigens produced by *S. aureus* could induce antibody production in sera and mucous exudates, and show protective efficacy against lethal dose of toxins[26]. However, only a few studies on protective effects of vaccination against mucosal *S. aureus* infection and nasal colonization have been reported[27].

Our results showed that an intranasal vaccination with mTSST-1 plus mLT significantly increased the survival rate of immunized mice and reduce bacterial counts in spleen and liver of these mice challenged with TSST-1 producing *S. aureus* but not non-producing *S. aureus*. Although mTSST-1 (H135A) was neither biologically toxic nor superantigenic when tested with mouse, rabbit splenocytes and human peripheral blood mononuclear cells, it still retain immunological activity[14]. Vaccination with mTSST-1 by intraperitoneal injections could protect mice against a challenge of a lethal dose of TSST-1 potentiated with lipopolysaccharide[28]. Vaccination with mTSST-1 by subcutaneous injections could protect mice against a lethal infection of *S. aureus*[14]. Our present results indicated that an intranasal vaccination with mTSST-1 plus mLT could protect against systemic *S. aureus* infection as the same level as the vaccination via the intraperitoneal or subcutaneous route.

The efficacy to reduce nasal colonization of intranasal vaccination with mTSST-1 plus mLT

was evaluated in a modified mouse model[19]. The bacterial numbers in the nasal cavities of vaccinated mice were significantly reduced compared with those of control mice on day 1 and 3, but not day 5 after challenge (Fig. 2). These results suggested that an intranasal vaccination with mTSST-1 plus mLT could elicit potential efficacy to prevent *S. aureus* nasal colonization. This efficacy might be due to inhibition of *S. aureus* adhesion or attachment to nasal mucosa, because the reduced bacterial numbers in the noses of immunized mice have been shown as early as next day after the intranasal challenge, but not day 5 after challenge.

E. coli LT is known to act as a strong mucosal adjuvant that enhances immune responses to mucosal co-administered bystander antigens. However, the use of LT as an adjuvant for mucosal vaccines is not feasible owing to its toxicity. Nontoxic mutants of LT reportedly retain the adjuvant property of LT[16]. A mLT (H44A) has been reported to be a potent adjuvant that can induce antibody in both serum and mucosal exudates, showing less toxic than native LT[16]. In this study, we used mLT as a mucosal adjuvant and evaluated the ability to induce systemic and mucosal immunity of an intranasal vaccination with mTSST-1. An intranasal vaccination with mTSST-1 plus mLT could induce high titers of anti-TSST-1 specific IgG1, IgG2a, IgG2b, IgG3 and IgA antibodies in the sera of immunized mice (Fig. 3), and the antibody production induced by the intranasal vaccination could be comparable to that induced by the intraperitoneal or subcutaneous vaccination. Previous studies indicated that the neutralizing activities of antibodies to staphylococcal superantigens could play an important role in protective effects against systemic *S. aureus* infection and lethal shock induced by these superantigenic toxins[8, 14, 15, 26]. In this study, an intranasal immunization with mTSST-1 plus mLT reduced bacterial counts in the organs of mice challenged with TSST-1 producing but not non-TSST-1 producing *S. aureus*. These results suggested that the anti-TSST-1-specific neutralizing activities of antibodies induced by vaccination are involved in protection against systemic infection caused by TSST-1 producing *S. aureus*. In addition, neutrophils are known to play a crucial protective role in the early phase of *S. aureus* infection[29]. In the present study, the higher titers of IFN-γ were detected in the supernatants of spleen cell cultures of immunized mice compared with those of the control mice when the cells were stimulated with rTSST-1 (Fig. 5). Previously it was reported that treatment of neutrophils with IFN-γ was shown to enhance phagocytosis and bacterial killing[30], to delay neutrophils apoptosis[31], and to increase the percentage of neutrophils able to phagocytize *S. aureus*[32]. It was also reported that the administration of anti-IFN-γ monoclonal antibody increased the bacterial numbers in the organs on day 1 but reduced on day 4 after infection with a lethal dose of *S. aureus*, indicating that IFN-γ might play a protective role in the early phase of *S. aureus* infection[17, 32].

In this study, the results of a blood killing assay showed that whole blood obtained from the immunized mice significantly reduced the numbers of *S. aureus* compared with those from the control mice (Fig. 6). These results suggested that the increased IFN-γ production might enhance the phagocytic and bactericidal activities of neutrophils in the early phase of systemic *S. aureus* infection. The neutralizing activities of anti-TSST-1 specific antibodies and the up-regulated bactericidal activity of neutrophils induced by vaccination with mTSST-1 could play an important role in the protection against systemic *S. aureus* infection.

Anti-TSST-1 IgA antibody in BALF and IgA-secreting cells in the NALT of immunized mice were significantly increased. The IgA production

in fecal pellets was also slightly increased (Fig 4). These results are consistent with previous reports that the intranasal immunization could stimulate an immune response in respiratory tracts, slightly evoking an immune response in the gut[25]. IgA production in BALF was more effectively induced by the intranasal challenge than that by the intravenous challenge with *S. aureus* (Fig 4). Various studies on the effects of staphylococcal superantigens to mucosal epithelial tissues have been reported: staphylococcal enterotoxin A directly stimulated nasal epithelial cells to produce cytokines[33], TSST-1 caused increase in mucosal permeability and traversed the intact mucosa without disrupting tissue integrity in vitro with the use of porcine vaginal tissue[34], immune cell activation and cytokine release induced by superantigens can increase epithelial barrier permeability[34,35]. Although the relationship between TSST-1 produced onto nasal mucosa and the attachment or adhesion of *S. aureus* in nasal cavity is still unclear, it is possible that the promoted secretion of anti-TSST-1 IgA onto nasal mucosa could play an important role in inhibiting adhesion of *S. aureus* in nasal cavities of mice intranasally immunized with mTSST-1 plus mLT.

In summary, our results demonstrated that an intranasal vaccination with mTSST-1 plus mLT induces effective antibody production in serum and mucosal exudates, and elicits the protective effect against not only systemic infection, but also the nasal colonization of TSST-1 producing *S. aureus*. An intranasal vaccination with mTSST-1 plus mLT is considered to be useful for controlling *S. aureus* infection.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (17590381 to D.L.H and 18390132 to A.N.) and Grant-in Aid of Bovine Mastitis Project, Ministry of Agriculture, Forestry and Fisheries, Japan.

REFERENCES

1) Becker K, Pagnier I, Schuhen B, Wenzelburger F, Friedrich AW, Kipp F, Peters G, et al. Does nasal cocolonization by methicillin-resistant coagulase-negative staphylococci and methicillin-susceptible *Staphylococcus aureus* strains occur frequently enough to represent a risk of false-positive methicillin-resistant *S. aureus* determinations by molecular methods? J Clin Microbiol 2006;44:229-31.

2) von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N Engl J Med 2001;344:11-6.

3) Wertheim HF, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JA, van Keulen PH, et al. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet 2004;364:703-5.

4) Zhu D, Barniak V, Zhang Y, Green B, Zlotnick G. Intranasal immunization of mice with recombinant lipidated P2086 protein reduces nasal colonization of group B *Neisseria meningitidis*. Vaccine 2006;24:5420-5.

5) Fattom AI, Sarwar J, Basham L, Ennifar S, Naso R. Antigenic determinants of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharide vaccines. Infect Immun 1998;66:4588-92.

6) Hall AE, Domanski PJ, Patel PR, Vernachio JH, Syribeys PJ, Gorovits EL, Johnson MA, et al. Characterization of a protective monoclonal antibody recognizing *Staphylococcus aureus* MSCRAMM protein clumping factor A. Infect Immun 2003;71:6864-70.

7) Hu DL, Cui JC, Omoe K, Sashinami H, Yokomizo Y, Shinagawa K, Nakane A. A mutant of staphylococcal enterotoxin C devoid of bacterial superantigenic activity elicits a Th2 immune response for protection against *Staphylococcus aureus* infection. Infect Immun 2005;73:174-80.

8) Nilsson IM, Verdrengh M, Ulrich RG, Bavari S, Tarkowski A. Protection against *Staphylococcus*

aureus sepsis by vaccination with recombinant staphylococcal enterotoxin A devoid of superantigenicity. J Infect Dis 1999;180:1370-3.

9) Kikuchi K, Takahashi N, Piao C, Totsuka K, Nishida H, Uchiyama T. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* strains causing neonatal toxic shock syndrome-like exanthematous disease in neonatal and perinatal wards. J Clin Microbiol 2003;41:3001-6.

10) Ma XX, Ito T, Chongtrakool P, Hiramatsu K. Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant *Staphylococcus aureus* strains isolated in Japanese hospitals from 1979 to 1985. J Clin Microbiol 2006;44:4515-27.

11) Kawano F, Miyazaki H, Takami J, Fujino T, Sekiguchi J, Saruta K, Kuratsuji T, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a Kumamoto Hospital in 2002. Jpn J Infect Dis 2003;56:129-32.

12) Zaraket H, Otsuka T, Saito K, Dohmae S, Takano T, Higuchi W, Ohkubo T, et al. Molecular characterization of methicillin-resistant *Staphylococcus aureus* in hospitals in Niigata, Japan: divergence and transmission. Microbiol Immunol 2007;51:171-6.

13) Piao C, Karasawa T, Totsuka K, Uchiyama T, Kikuchi K. Prospective surveillance of community-onset and healthcare-associated methicillin-resistant *Staphylococcus aureus* isolated from a university-affiliated hospital in Japan. Microbiol Immunol 2005;49:959-70.

14) Hu DL, Omoe K, Sasaki S, Sashinami H, Sakuraba H, Yokomizo Y, Shinagawa K, et al. Vaccination with nontoxic mutant toxic shock syndrome toxin 1 protects against *Staphylococcus aureus* infection. J Infect Dis 2003;188:743-52.

15) Stiles BG, Krakauer T, Bonventre PF. Biological activity of toxic shock syndrome toxin 1 and a site-directed mutant, H135A, in a lipopolysaccharide-potentiated mouse lethality model. Infect Immun 1995;63:1229-34.

16) Hagiwar Y, Tsuji T, Iwasaki T, Kadowaki S, Asanuma H, Chen Z, Komase K, et al. Effectiveness and safety of mutant *Escherichia coli* heat-labile enterotoxin (LT H44A) as an adjuvant for nasal influenza vaccine. Vaccine 2001;19:2071-9.

17) Nakane A, Okamoto M, Asano M, Kohanawa M, Minagawa T. Endogenous gamma interferon, tumor necrosis factor, and interleukin-6 in *Staphylococcus aureus* infection in mice. Infect Immun 1995;63:1165-72.

18) Kato M, Imamura S, Kawase H, Miyama A, Tsuji T. Histidine-44 of the A subunit of *Escherichia coli* enterotoxin is involved in its enzymatic and biological activities. FEMS Microbiol Lett 1997;152:219-25.

19) Kiser KB, Cantey-Kiser JM, Lee JC. Development and characterization of a *Staphylococcus aureus* nasal colonization model in mice. Infect Immun 1999;67:5001-6.

20) Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, Noda M, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94:5267-72.

21) Nakane A, Nishikawa S, Sasaki S, Miura T, Asano M, Kohanawa M, Ishiwata K, et al. Endogenous interleukin-4, but not interleukin-10, is involved in suppression of host resistance against *Listeria monocytogenes* infection in gamma interferon-depleted mice. Infect Immun 1996;64:1252-8.

22) Rich J, Lee JC. The pathogenesis of *Staphylococcus aureus* infection in the diabetic NOD mouse. Diabetes 2005;54:2904-10.

23) Asanuma H, Thompson AH, Iwasaki T, Sato Y, Inaba Y, Aizawa C, Kurata T, et al. Isolation and characterization of mouse nasal-associated lymphoid tissue. J Immunol Methods 1997;202:123-31.

24) Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 1997;10:505-20.

25) Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11:S45-53.

26) Stiles BG, Garza AR, Ulrich RG, Boles JW. Mucosal vaccination with recombinantly attenuated staphylococcal enterotoxin B and protection in a murine model. Infect Immun 2001;69:2031-6.

27) Schaffer AC, Solinga RM, Cocchiaro J, Portoles M, Kiser KB, Risley A, Randall SM, et al. Immunization with *Staphylococcus aureus* clumping factor B, a major determinant in nasal carriage, reduces nasal colonization in a murine model. Infect Immun 2006;74:2145-53.

28) Gampfer J, Thon V, Gulle H, Wolf HM, Eibl MM. Double mutant and formaldehyde inactivated TSST-1 as vaccine candidates for TSST-1-induced toxic shock syndrome. Vaccine 2002;20:1354-64.

29) Verdrengh M, Tarkowski A. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. Infect Immun 1997;65:2517-21.

30) Ellis TN, Beaman BL. Interferon-gamma activation of polymorphonuclear neutrophil function. Immunology 2004;112:2-12.

31) Moulding DA, Walter C, Hart CA, Edwards SW. Effects of staphylococcal enterotoxins on human neutrophil functions and apoptosis. Infect Immun 1999;67:2312-8.

32) Zhao YX, Nilsson IM, Tarkowski A. The dual role of interferon-gamma in experimental *Staphylococcus aureus* septicaemia versus arthritis. Immunology 1998;93:80-5.

33) O'Brien GJ, Riddell G, Elborn JS, Ennis M, Skibinski G. *Staphylococcus aureus* enterotoxins induce IL-8 secretion by human nasal epithelial cells. Respir Res 2006;7:115.

34) Davis CC, Kremer MJ, Schlievert PM, Squier CA. Penetration of toxic shock syndrome toxin-1 across porcine vaginal mucosa ex vivo: permeability characteristics, toxin distribution, and tissue damage. Am J Obstet Gynecol 2003;189:1785-91.

35) Lu J, Philpott DJ, Saunders PR, Perdue MH, Yang PC, McKay DM. Epithelial ion transport and barrier abnormalities evoked by superantigen-activated immune cells are inhibited by interleukin-10 but not interleukin-4. J Pharmacol Exp Ther 1998;287:128-36.