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Citation	弘前医学. 59(Suppl.), 2007, p.S227-S234
Issue Date	2007-11-29
URL	http://hdl.handle.net/10129/2240
Rights	
Text version	publisher



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PROTECTIVE EFFECT OF INTRANASAL VACCINATION WITH NONTOXIC MUTANT TSST-1 AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION

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Abstract Infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been the most commonly acquired types of nosocomial infections. It was reported that anterior nares are the major reservoir of *S. aureus* and the source of 80% of *S. aureus* bacteremia is endogenous. Considering these facts, elimination and reduction of nasal carriage are thought to be effective protection against systemic *S. aureus* infection and nosocomial infection. Toxic shock syndrome toxin 1 (TSST-1) is one of superantigens secreted by *S. aureus*. Previously, it was reported that mutant form (H135A) of TSST-1 (mTSST-1) was shown to be nontoxic, and subcutaneous vaccination with mTSST-1 could protect against systemic *S. aureus* infection in a mouse model. In this study, we investigated the protective effect of intranasal vaccination with mTSST-1 supplemented with non-toxic mutant (H44A) *Escherichia coli* heat labile toxin (mLT) as a mucosal adjuvant. The results demonstrated that intranasal immunization with mTSST-1 plus mLT could efficiently induce production of anti-TSST-1 antibodies in sera and also induce anti-TSST-1 IgA production in bronchoalveolar lavage fluids (BALF) of vaccinated mice. In nasal-associated lymphoid tissues (NALT) of vaccinated mice, anti-TSST-1 IgA secreting cells were significantly increased. To evaluate of the protective effect of this vaccine against systemic *S. aureus* infection, BALB/c mice were vaccinated with mTSST-1 plus mLT and challenged with clinical isolated *S. aureus* 834 intravenously. Bacterial numbers in spleen and liver, and cumulative mortality rate of vaccinated mice were lower than those of control mice. We further developed a mouse model of nasal *S. aureus* colonization. *S. aureus* bacterial numbers in nasal cavity of vaccinated mice were significantly reduced compared with those of control mice. These results indicate that intranasal immunization with mTSST-1 plus mLT is able to induce systemic and mucous immune responses and of provide protection against systemic *S. aureus* infection and nasal colonization.

Hirosaki Med. J. 59, Supplement : S227—S234, 2007

Staphylococcus aureus is an important opportunistic human pathogen, and causes a wide range of infectious disease from superficial infections to more life-threatening diseases. Infections caused by methicillin-resistant *S. aureus* (MRSA) strains have been the most commonly nosocomial infections, resulting in increased morbidity, mortality, length of hospital stay, and health care costs¹⁾. In serious infections caused by MRSA, vancomycin is used currently, but MRSA strains that have reduced susceptibility to vancomycin [vancomycin intermediately susceptible *S. aureus* (VISA)] were isolated from patients with serious infections, these isolates are also resistant to many antimicrobials, leaving few options for

effective antimicrobial therapy²⁾. Consequently, there is a need for effective treatment and prevention strategies against MRSA infections, such as immunotherapy.

To design vaccines to protect against *S. aureus* infection, various virulence factors of this microorganism have been targeted, including capsular polysaccharides^{3,4)}, cell wall-associated proteins⁵⁻⁷⁾ and toxins⁸⁻¹⁰⁾. Toxic shock syndrome toxin 1 (TSST-1) is one of superantigenic exotoxins secreted by *S. aureus*, especially MRSA and a major virulence factor in toxic shock syndrome (TSS), staphylococcal scarlet fever, and neonatal toxic shock-like exanthematous diseases¹¹⁻¹³⁾. Previously, it was reported that mutant form (H135A) of TSST-1 (mTSST-1)

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was shown to be less toxic and still have the same levels of antigenicity, and the vaccination with mTSST-1 via subcutaneous route could protect against systemic *S. aureus* infection in a mice model¹⁴.

S. aureus has been known to adhere and colonize the mucosal epithelium, and anterior nares were known the major reservoir of *S. aureus*. It was reported that the source of 80% of *S. aureus* bacteremias has been shown endogenous since infecting bacteria have been shown by genotypic analysis to be identical to organisms recovered from the nasal mucosa^{15,16}. Considering these facts, elimination and reduction of nasal carriage are thought to be effective protection against systemic *S. aureus* infection and for the suppression *S. aureus* nosocomial or community infection. Currently, there is much interest in the mucosal route of immunization to protect against various pathogens that gain entry into the host via mucosal tissue. Mucosal antibodies generated at the point of entry might play an important role in blocking bacterial colonization, enhancing clearance, and prevention of systemic infection¹⁷. In this study, we investigated the protective effect of intranasal vaccination with mTSST-1 supplemented with non-toxic mutant *Escherichia coli* heat labile toxin (mLT, His to Arg substitution at position 44 from the N-terminus of the A1 fragment of the A subunit)¹⁸, as a mucosal adjuvant.

To prepare the inoculum for infection and colonization experiments, a clinical isolate *S. aureus* 834 strain, which expresses TSST-1 and staphylococcal enterotoxin C2 (SEC2)¹⁹, was used. The bacteria were cultured at 37 °C in tryptic soy broth (BD Bioscience, Sparks, MD, USA) for 15 h, collected by centrifugation, washed with sterile PBS, and then resuspended with sterile PBS. The bacterial numbers were adjusted spectrophotometrically at 550 nm to an appropriate value. To prepare mTSST-1 and recombinant TSST-1 (rTSST-1), The *E. coli* DH5a strain

(Toyobo Biochemicals, Osaka, Japan) containing pGXmTST and pGXrTST that encodes mTSST-1 and rTSST-1 respectively, was used in this study¹⁴. The bacteria were routinely grown in Luria-Bertani broth (Invitrogen, Carlsbad, CA, USA) at 37°C with shaking (110 rpm). To maintain plasmid in *E. coli*, 100 µg/ml ampicillin was added. The *E. coli* DH5a derivatives were grown in 2×YTA medium containing 100 µg/ml ampicillin at 37 °C with shaking. Expression and purification of rTSST-1 and mTSST-1 were performed as described by Hu et al¹⁴. Purified mTSST-1 or rTSST-1 was dissolved in PBS. A mucosal adjuvant, the mutant form (H44A) of the heat labile toxin (mLT) was used¹⁸. For initial immunization, 10 µg mTSST-1 was mixed with 5 µg mLT in 20 µl of PBS. Prepared each mixture was administered to external nares of mice intranasally. Booster immunizations were performed 2 and 4 weeks after initial immunization with the same manner. The control mice were administered 5 µg mLT dissolved in 20 µl PBS or 20 µl PBS alone. Seven days after last booster, for systemic infection, mice were challenged with 5×10⁷ CFU of *S. aureus* by intravenous injection. To prepare the mouse nasal colonization model, the inoculum, which contained a dose of *S. aureus* 1×10⁹ CFU in 20 µl of PBS, was pipetted slowly onto the nares of the anesthetized mice²⁰. Three days after intravenous challenge with *S. aureus*, blood samples and bronchoalveolar lavage fluids (BALF) were prepared, fecal pellets were obtained 6 h after challenge. The production of anti-TSST-1 antibody and anti-*S. aureus* cell protein antibody in serum, BALF and fecal samples were measured by enzyme-linked immunosorbent assays (ELISAs) as described previously¹⁴. Serum samples were diluted with 10% Blockace in PBS (1:100), and then serial two-fold dilutions were prepared. As a control, 10% Blockace in PBS was substituted for prepared samples. High levels of anti-TSST-1 IgG1, IgG2a, IgG2b and IgA antibodies were

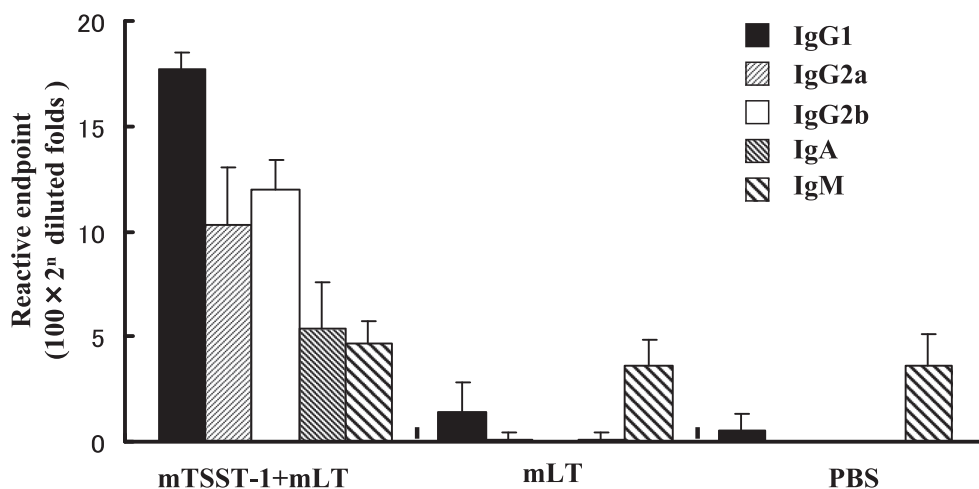


Figure 1 Serum antibody responses of vaccinated mice and control mice. Mice were intranasally vaccinated with mTSST-1 plus mLT, mLT or PBS alone, and then challenged with 5×10^7 CFU of *S. aureus* on day 7 after last booster. Serum samples were obtained 3 days after infection. Anti-TSST-1 antibody titers were determined by ELISAs. The data are mean endpoint titers \pm SD.

detected in sera of mTSST-1 vaccinated mice (Figure 1).

In contrast, except for IgM, only low levels of anti-TSST-1 antibodies were detected in serum samples from the control mice. Previous studies indicated that the neutralizing activities of antibodies to superantigen, such as SEA, SEB or TSST-1, could play important role in protective effects against systemic *S. aureus* infection or lethal shock induced by these toxins^{10,21-22}. Intranasal vaccination with mTSST-1 plus mLT could induce high levels of anti-TSST-1 specific antibodies in the sera of vaccinated mice, and the ability to induce antibodies of this intranasal vaccination could be comparable to that of intraperitoneal or subcutaneous vaccination.

mLT have been reported to be a potent adjuvant capable to induce antibodies both in serum and mucosal exudate, and shown less toxic than native LT in vitro or in vivo. Next, we evaluated whether intranasal vaccination with mTSST-1 plus mLT could effectively induce anti-TSST-1 IgA production in mucosal exudates. Seven days after last booster, vaccinated mice and control mice were challenged with 5×10^7

CFU of *S. aureus* by intravenous injection, fecal pellets and BALF samples were obtained after 6 h and 3 days after challenge respectively. Significantly high levels of anti-TSST-1 IgA were detectable in the BALF obtained from vaccinated mice (Figure 2A). Although anti-TSST-1 IgA production in fecal pellets was low levels compared with that in BALF, slightly higher levels anti-TSST-1 IgA were detected in fecal pellets obtained from immunized mice than those of control mice (Figure 2B). These IgA production manner was correspondence with the data previously described that nasal immunizations could stimulate an immune response in the respiratory tract effectively, slightly evoking an immune response in the gut²³. On the other hand, in BALF and fecal pellets, anti-*S. aureus* cell protein antibody production was low levels and significant differences were not detected on comparison of IgA production levels between vaccinated mice and control mice. (Figure 2A, B).

To evaluate anti-TSST-1 IgA production in nasal cavity, the numbers of anti-TSST-1 specific IgA secreting cells in nasal-associated lymphoid

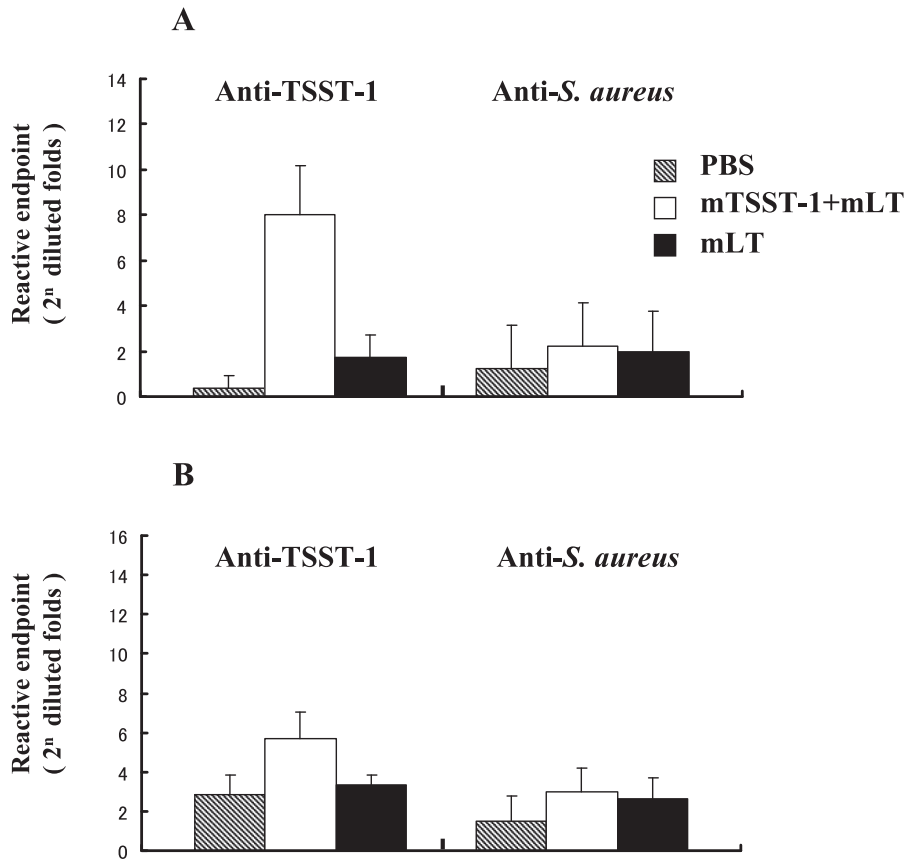


Figure 2 Antibody responses in BALF (A) and fecal pellets (B). Mice were intranasally vaccinated with mTSST-1 plus mLT, mLT or PBS alone, and then challenged with 5×10^7 CFU of *S. aureus* intravenously on day 7 after last booster. Anti-TSST-1 antibody and anti-*S. aureus* cell protein antibody titers were determined by ELISAs. The data are mean endpoint titers \pm SD.

tissue (NALT) of vaccinated mice and control mice were analyzed 7 days after last booster as previously described²⁴. After sacrificed by cervical dislocation, the head of mouse was cut off along the line between upper and lower jaw. The facial skin, excess soft tissue and cheek bone were removed, and the nose part was separated from the rest of the head along the line of the eyeballs. The tip of the nose containing the incisor was cut off. The palate was separated from the rest of the nasal tissue by peeling. NALT cells were released by gently teasing the obtained palate between frosted glass slides in RPMI 1640 medium (Nissui pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 2% fetal calf serum. The cells were washed two

times and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, and 100 μ g/ml of streptomycin, and then prepared NALT cells were analyzed for their ability to secrete anti-TSST-1 IgA production by ELISPOT (Protein DetectorTM; KPL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, a 96-well plate with PVDF base was coated overnight with 5 μ g/ml rTSST-1 in coating solution, all wells were then blocked 1 h at room temperature, the prepared NALT cells described above were added to each well at concentration of 2×10^5 cells/well and incubated for 24 h at 37°C in 5% CO₂ in air. The plate was washed and then incubated with 100 μ l biotinylated goat anti-mouse IgA (Santa

Table 1. Protective effect of vaccination with mTSST-1 plus mLT on host resistance against systemic *S. aureus* infection

Immunization	Bacterial number (log bacteria / organ)	
	Spleen	Liver
mTSST-1+mLT	3.6 ± 0.5 ^b	5.1 ± 0.4 ^b
mLT	4.5 ± 0.6	6.1 ± 1.0
PBS	4.8 ± 0.8	6.4 ± 0.9

One week after last booster, mice were infected with 5×10^7 CFU of *S. aureus* intravenously. The bacterial numbers of spleen and liver were determined on day 3 after challenge. The data are means \pm SD. ^bStatistically significant difference from a control (mLT alone) at $P < 0.05$.

Cruz Biotechnology, Santa Cruz, CA, USA). Streptavidin–horseradish peroxidase was added to the washed plate and spots were developed with True blue substrate. Spots were counted with the aid of a dissecting microscope. The numbers of anti-TSST-1 IgA antibody secreting cells per 2×10^5 NALT cells from vaccinated mice were significantly increased compared with those of control mice (data not shown). These results indicate that mLT would be an effective mucosal adjuvant and intranasal vaccination with mTSST-1 supplemented with mLT is able to induce anti-TSST-1 antibody not only in sera but also in mucosal exudates effectively.

Previous studies showed that vaccination with mTSST-1 by intraperitoneal injection could protect mice challenged with a lethal dose of TSST-1 potentiated with LPS²²⁾, and also shown that vaccination with mTSST-1 by subcutaneous injection could protect mice challenged with a lethal dose of *S. aureus*¹⁴⁾. To evaluate whether anti-TSST-1 antibodies induced by intranasal vaccination with mTSST-1 plus mLT could elicit protective effect against systemic *S. aureus* infection in vivo, mice were challenged with 5×10^7 CFU of *S. aureus* by intravenous injection 7 days after last booster. The bacterial numbers in the spleen and liver were enumerated 3 days after challenge by plating serial 10 fold dilutions of organ homogenates on triptic soy agar (BD

Bioscience). The numbers of bacterial cells in spleen and liver of vaccinated mice were significantly fewer than those in the organs of control mice (Table 1).

To further confirm the protective effect of intranasal vaccination with mTSST-1 plus mLT, cumulative mortality rates of vaccinated mice and control were monitored for 14 days after challenge. On day 3 after challenge, the cumulative mortality rate of vaccinated mice was 12.5% and the rate did not change until 14 days after challenge ($P < 0.05$). On the other hand, on day 12 after challenge, the cumulative mortality rates of mice administered mLT or PBS alone were 70.6% and 76.5% respectively. Our data showed that an intranasal vaccination with mTSST-1 plus mLT could significantly reduce mortality rates and decrease bacterial numbers in organs of vaccinated mice compared with those of the control mice. These results indicated that this intranasal vaccination could elicit the protective effect against systemic *S. aureus* infection with the same level as vaccination via intraperitoneal or subcutaneous route. A previous study has also shown that neutrophils play crucial protective role in early phase of *S. aureus* infection²⁵⁾. To evaluate the activity of phagocytes in blood of vaccinated mice and control mice, whole blood killing assay was performed²⁶⁾. Briefly, two hundred

microlitter whole blood and 100 μ l *S. aureus* to yield a final concentration of 1×10^5 CFU/ml were mixed and incubated on a rotator at 37°C. Samples were taken at time 0 and 120 min after incubation and serial 10 fold dilutions of samples were made in PBS and plated onto tryptic soy agar plates. Bacterial numbers at 2 h rotated whole blood samples obtained from vaccinated mice were significantly reduced compared with those in the control mice (data not shown). Although a protective mechanism of vaccination with mTSST-1 remains unclear, these results implicated that the neutralization activity of anti-TSST-1 antibody might play an important role in the enhanced phagocytic and bactericidal activities of neutrophils.

To evaluate the protective effect of vaccination with mTSST-1 plus mLT against nasal colonization in mice, mice were challenged with 1×10^9 CFU of *S. aureus* intranasally. One, three and five days after challenge, mice were sacrificed and the noses were surgically removed. The excised nose was placed in 500 μ l of PBS and then homogenized. The bacterial numbers of *S. aureus* in nasal cavity was evaluated by plating 100 μ l nasal suspension on mannit salt agar. On days 1 and 3, bacterial numbers in nasal cavity of vaccinated mice were significantly reduced compared with those of the control mice, mean bacterial number in nasal cavities of vaccinated mice and mLT alone administered mice were 5200 CFU per nose and 7800 CFU per nose respectively (on day 1), 160 CFU per nose and 870 CFU per nose, respectively (on day 3), but on day 5, the significant difference between the immunized mice and the control mice were not shown (data not shown). These results suggested that an intranasal vaccination with mTSST-1 plus mLT could elicit potential efficacy to protect against or prevent from *S. aureus* nasal colonization, and this efficacy might be due to the inhibition of *S. aureus* adhesion or attachment to nasal mucosa,

because reduced bacterial numbers in nasal cavity of the vaccinated mice were shown as early as day 1 after intranasal challenge, but not at day 5 after challenge.

In summary, our results demonstrated that a mucosal adjuvant mLT was able to effectively induce antibodies to TSST-1 both in sera and mucosal exudates, and that an intranasal vaccination with mTSST-1 supplemented with mLT elicited the protective effect against not only systemic *S. aureus* infection but also nasal *S. aureus* colonization. In case of systemic infection, the neutralization activity of anti-TSST-1 antibodies might play an important role in the enhanced phagocytic and bactericidal activities of neutrophils, and in case of nasal colonization, anti-TSST-1 IgA secreted into mucosal exudates might inhibit *S. aureus* adhesion or attachment to nasal mucosa. An intranasal vaccination with mTSST-1 plus mLT is useful in the control of *S. aureus* infection, especially nosocomial infection caused by MRSA.

References

- 1) Becker K, Pagnier I, Schuhen B, Wenzelburger F, Friedrich AW, Kipp F, Peters G, von Eiff C. 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) Finan JE, Archer GL, Pucci MJ, Climo MW. Role of penicillin-binding protein 4 in expression of vancomycin resistance among clinical isolates of oxacillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2001;45:3070-5.
- 3) Fattom AI, Horwith G, Fuller S, Propst M, Naso R. Development of StaphVAX, a polysaccharide conjugate vaccine against *S. aureus* infection: from the lab bench to phase III clinical trials. Vaccine 2004;22:880-7.
- 4) 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.
- 5) Hall AE, Domanski PJ, Patel PR, Vernachio JH, Syribeys PJ, Gorovits EL, Johnson MA, Ross JM, Hutchins JT, Patti JM. Characterization of a protective monoclonal antibody recognizing *Staphylococcus aureus* MSCRAMM protein clumping factor A. *Infect Immun* 2003;71:6864-70.
 - 6) Rennermalm A, Li YH, Bohaufs L, Jarstrand C, Brauner A, Brennan FR, Flock JI. Antibodies against a truncated *Staphylococcus aureus* fibronectin-binding protein protect against dissemination of infection in the rat. *Vaccine* 2001;19:3376-83.
 - 7) Visai L, Xu Y, Casolini F, Rindi S, Hook M, Speziale P. Monoclonal antibodies to CNA, a collagen-binding microbial surface component recognizing adhesive matrix molecules, detach *Staphylococcus aureus* from a collagen substrate. *J Biol Chem* 2000;275:39837-45.
 - 8) 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.
 - 9) Hume EB, Dajcs JJ, Moreau JM, O'Callaghan RJ. Immunization with alpha-toxin toxoid protects the cornea against tissue damage during experimental *Staphylococcus aureus* keratitis. *Infect Immun* 2000;68:6052-5.
 - 10) 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.
 - 11) Floret D. [Clinical aspects of streptococcal and staphylococcal toxic diseases]. *Arch Pediatr* 2001;8 Suppl 4:762s-8s.
 - 12) 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.
 - 13) van der Mee-Marquet N, Lina G, Quentin R, Yaouanc-Lapalle H, Fievre C, Takahashi N, Etienne J. Staphylococcal exanthematous disease in a newborn due to a virulent methicillin-resistant *Staphylococcus aureus* strain containing the TSST-1 gene in Europe: an alert for neonatologists. *J Clin Microbiol* 2003;41:4883-4.
 - 14) Hu DL, Omoe K, Sasaki S, Sashinami H, Sakuraba H, Yokomizo Y, Shinagawa K, Nakane A. Vaccination with nontoxic mutant toxic shock syndrome toxin 1 protects against *Staphylococcus aureus* infection. *J Infect Dis* 2003;188:743-52.
 - 15) 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.
 - 16) Wertheim HF, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JA, van Keulen PH, Vandenbroucke-Grauls CM, Meester MH, Verbrugh HA. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. *Lancet* 2004;364:703-5.
 - 17) 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.
 - 18) Hagiwar Y, Tsuji T, Iwasaki T, Kadowaki S, Asanuma H, Chen Z, Komase K, Suzuki Y, Aizawa C, Kurata T, Tamura S. Effectiveness and safety of mutant *Escherichia coli* heat-labile enterotoxin (LT H44A) as an adjuvant for nasal influenza vaccine. *Vaccine* 2001;19:2071-9.
 - 19) 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.
 - 20) 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.
 - 21) 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.

- 22) 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.
- 23) Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* 2005;11:S45-53.
- 24) Asanuma H, Thompson AH, Iwasaki T, Sato Y, Inaba Y, Aizawa C, Kurata T, Tamura S. Isolation and characterization of mouse nasal-associated lymphoid tissue. *J Immunol Methods* 1997;202:123-31.
- 25) Verdrengh M, Tarkowski A. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus*. *Infect Immun* 1997;65:2517-21.
- 26) Rich J, Lee JC. The pathogenesis of *Staphylococcus aureus* infection in the diabetic NOD mouse. *Diabetes* 2005;54:2904-10.