Hirosaki Med. J. 59 (Suppl.) : S210—S218, 2007

VACCINATION WITH A NONTOXIC MUTANT OF STAPHYLOCOCCAL ENTEROTOXIN C ELICITS TH2 IMMUNE RESPONSE FOR PROTECTION AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION

Dong-Liang Hu[1], Katsuhiko Omoe[2], Kouji Narita[1], Kunihiro Shinagawa[2] and Akio Nakane[1]

Abstract To develop a *Staphylococcus aureus* vaccine, we constructed and expressed a non-toxic mutant staphylococcal enterotoxin C (mSEC) and investigated whether immunization with mSEC can protect against *S. aureus* infection. Mice were immunized with mSEC and challenged with viable *S. aureus*. Bacteria counts in the organs in mSEC-immunized mice were significantly lower and the survival rate was higher than those of the control group. mSEC vaccination induced the production of T-helper 2 type antibodies. The production of interleukin-10 (IL-10) and IL-4 in vaccinated mice was significantly higher compared with the control group, whereas the production of gamma interferon (IFN-γ) was significantly decreased in the vaccinated mice. Furthermore, IFN-γ and tumor necrosis factor-α production in vitro was significantly inhibited by the sera from mSEC-vaccinated mice but not by those from control mice. These results suggest that vaccination with mSEC provides protection against *S. aureus* infection. SEC neutralizing antibodies and the IL-10 and IL-4 induction might play important role for the protection.

Hirosaki Med. J. **59**, **Supplement** : S210—S218, 2007

Staphylococcal enterotoxins (SEs), bacterial superantigenic proteins, produced by *S. aureus* play important roles in establishing and maintaining infections[1]. SEC is commonly produced among invasive *S. aureus* isolates, especially methicillin-resistant *S. aureus* (MRSA) strains, and can cause severe pathologies. Previous studies have reported that the majority of MRSA in the United States produce SEC or SEB in very high concentrations. The majority of *S. aureus* isolates from bovine mastitis also produce large amounts of SEC[2-4]. It has a significant economic impact on health care and dairy industry. To prevent *S. aureus* infection, there is considerable need to develop vaccines and therapeutic approaches capable of eliminating their toxicity.

Fields et al.[5] have reported the crystal structure of SEC complex with a T-cell receptor (TCR) β-chain, and showed that SEC2 and SEC3 bind in an identical way to the TCR β-chain. Recent studies demonstrated that several residues of SEC, including T20, N23, Y90, K103 and Q210, for binding to TCR and are important for superantigenicity[5-8]. Several reports have described toxicity and biological activity of wild type and mutant SEA, SEB and toxic shock syndrome toxin 1 (TSST-1), and showed that genetically altered SEA and SEB were immunogenic in mice and rhesus monkeys[9-12]. Immunization with recombinant or mutant SEA, SEB and TSST-1 could elicited neutralizing antibodies against wild type of SEs and protect mice or rabbits against lethal shock induced by the wild type of their superantigenic toxins[9,11,13-15].

In this study, we prepared a single mutant SEC (mSEC) that was devoid of superantigenic activity, and investigated whether vaccination with mSEC could protect against S. aureus infection in a mouse model. The results

[1] Department of Microbiology and Immunology, Hirosaki University Graduate School of Medicine, Hirosaki, 036-8562, Japan

[2] Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka, 020-8550, Japan

demonstrate that immunization with mSEC gives rise to protection against the bacterial infection. In addition, our studies show that the protection is mediated by SEC-specific neutralizing antibody, induction of IL-10 and IL-4, and down-regulation of IFN-γ production.

S. aureus strain 834, a clinical septic isolate that expresses SEC2 and TSST-1[16], was cultured at 37 ℃ in tryptic soy broth for 15 h, then collected by centrifugation and washed with sterile 0.01 M phosphate-buffered saline (PBS). The washed bacteria were diluted with PBS, and adjusted spectrophotometrically at 550 nm to the appropriate concentration. *S. aureus* FRI 361 expressing SEC2 was inoculated to 5 ml of soybean-casein-digest broth, and grown overnight at 37 ℃ with shaking (110 rpm). *Escherichia coli* DH5α (Toyobo Biochem.) and *E. coli* NM522 mutS (Amersham Pharmacia Biotech., Inc.) strains were routinely grown in Luria broth (Becton) at 37 ℃ with shaking (110 rpm).

BALB/c mice were purchased from Clea Japan, Inc., Tokyo, Japan. The mice were housed in plastic cages under specific-pathogen-free conditions at the Institute for Experiments, Hirosaki University School of Medicine. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

In order to construct the recombinant SEC (rSEC) expression plasmids, PCR primers were designed to amplify the gene fragment corresponding to mature form of SEC (SEC2/GST+, the sequence includes a 5' BamHI site; 5'-CCCCGGATTCGAGAGCCAACCAGACCCTACG: and SEC2/GST-, the sequence includes a 5' EcoRI site; 5'-CCCCGAATTCTTATCCATTCTTTGTTGTAAGGT GG). The sec2 gene was amplified by PCR using Pyrobest DNA polymerase (Takara), and sec2 fragment was then subcloned to pGEM3Zf (+), and designated as pKOC2. The sec2 fragment was then digested from pKOC2 with BamHI and EcoRI and was subcloned into the pGEX-6P-1 glutathione S-transferase (GST) fusion expression vector. The resultant plasmid containing sec2 was named pKC2X1. Expression, purification of GST-fused rSEC, and the cleavage and removal of the GST tag from rSEC were performed according to Omoe et al.[17]

For construction and expression of mSEC, a selection primer (5'-GCGTGACACCACGATGCCCGCGGCAATGGCAACAACG) and a mutagenic primer (5'-CTGGTACGATGGGTGCTATGAAATATTTATATG) were designed to change two oligonucleotides AAT (encoding for 23 asparagine in the N-terminal of SEC2 molecule) to GCT (alanine). Site-directed mutagenesis was performed according to Hu et al.[18] Mutant DNA sequence was confirmed as described above. The asparagine-to-alanine mutant plasmid was identified as pGXmSEC, and transformed into *E. coli* DH5α. Expression and purification of GST-fused mSEC, and the cleavage and removal of the GST tag from mSEC were performed as the method as rSEC.

Purified mSEC was compared on Coomassie blue-stained SDS-PAGE with wild-type rSEC and showed the presence of a readily detectable purified protein band that co-migrated with purified rSEC. The immunological reactivity of mSEC and rSEC with polyclonal rabbit anti-rSEC antibody was assayed by using gel immunodiffusion. The antibody reacted readily with purified mSEC and rSEC, and the precipitation lines between mSEC and rSEC were united each other (data not shown). These results indicate that mSEC retains the matched antibody-binding epitopes as wild type rSEC. To confirm whether the superantigenic activity of the mSEC is deleted, the proliferation and cytokine production induced by mSEC and rSEC in mouse spleen cells were determined. Substantial amounts of cytokines were induced in the cell cultures at all concentrations of rSEC treated. In contrast, mSEC had no detectable

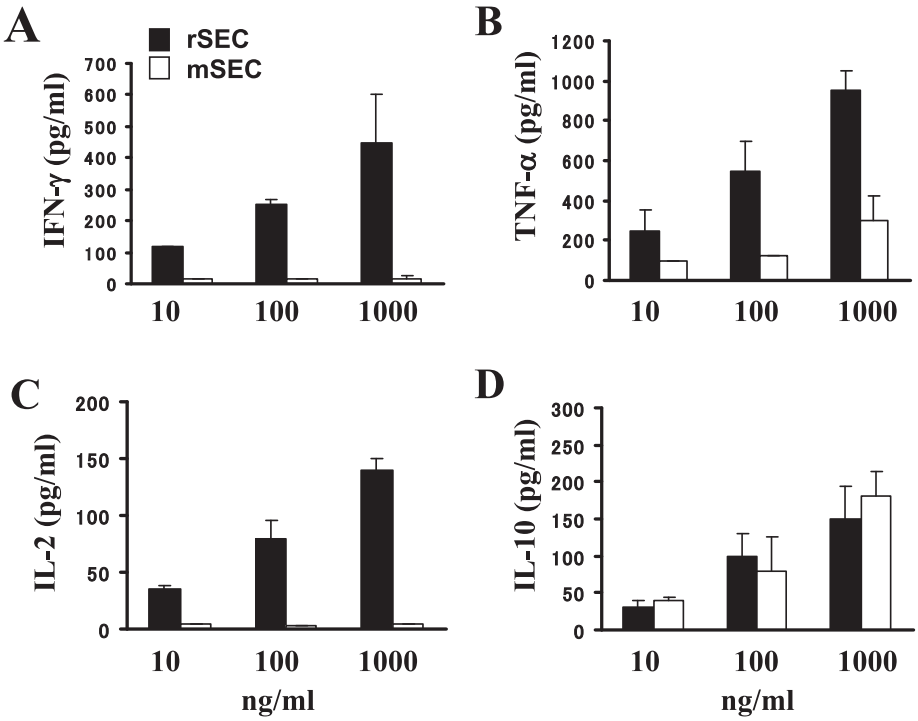

Figure 1 Superantigenic activity of mSEC. Titers of IFN-γ (A), TNF-α (B), IL-2 (C) and IL-10 (D) in supernatants of splenic cell cultures stimulated with rSEC or mSEC were measured by ELISAs. Each result is the mean ± SD based on samples obtained from three to five mice.

or the lower IFN-γ, TNF-α and IL-2 production (Figure 1). These results indicated that the mSEC is significantly devoid of superantigenic activity.

To further examine whether the toxicity of mSEC protein is also detected in vivo, the toxic effect of mSEC was tested in lipopolysaccharide (LPS)-potentiated mouse lethality model. Mice were first injected intraperitoneally with rSEC or mSEC diluted in PBS (0.1 to 20 μg per mouse). The mice were injected again with 75 μg of LPS from E. coli O55: B5 (Sigma) 4 h later, and lethality was recorded over 72 h. The controls included animals given either SEC or LPS alone, and the survival was observed. In striking contrast to the 78% mortality rate of mice injected with 15 μg rSEC plus LPS, none of the mice died when they were given an equivalent dose of mSEC plus LPS (data not shown).

We next investigated the protective effect of mSEC-vaccination against *S. aureus* infection. Purified rSEC or mSEC was dissolved in PBS and emulsified 1:1 in alum adjuvant (Pierce). Two hundred microliters of the emulsion containing 10 μg of rSEC or mSEC, or alum alone, were injected at two subcutaneous sites on their backs. Booster immunizations were performed 2 and 4 weeks after the initial vaccination. The mice were challenged on day 7 after last booster with a lethal dose of *S. aureus* 834 by intravenous injection. Blood samples were obtained before and after bacterial challenge. Bacteria in the spleen and liver were enumerated on day 3 after infection by preparing homogenates of these organs in PBS and by plating 10-fold serial dilutions on tryptic soy agar (Becton). Colonies were counted after 24 h of incubation at 37 ℃. There were significantly fewer bacterial cells in the spleens and livers of mSEC-immunized mice than in the organs of control mice[2]. For the

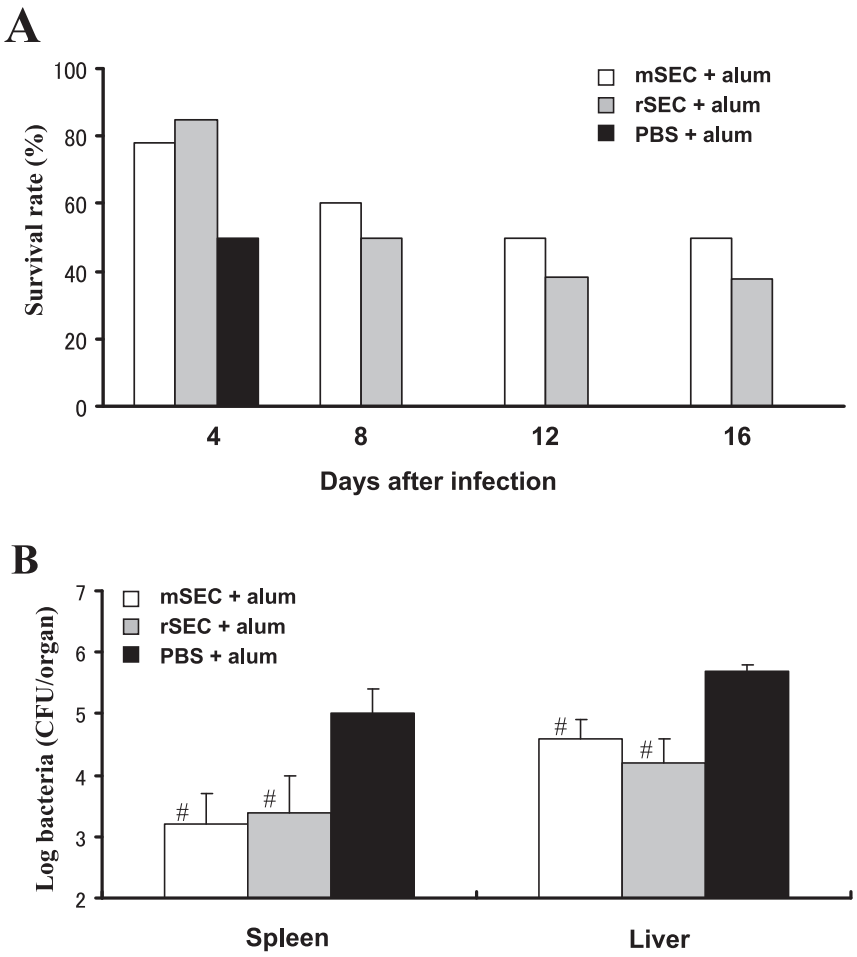

Figure 2 Protective effect of vaccination with mSEC on host resistance against *S. aureus* infection. (A) Survival rates of immunized mice and control mice. Animals were immunized with mSEC or rSEC with alum or alum alone and were infected with 5×10^7 CFU of *S. aureus* on day 7 after last boost. The animals were monitored for 16 days postinfection. Data are mean ± SD (n=16) of three independent experiments. (B) Bacteria numbers in the organs of immunized mice and control mice. Animals were immunized with rSEC or mSEC with alum or alum alone and then were infected with 5×10^7 CFU S. aureus on day 7 after last boost. The numbers of bacteria in the spleens and livers were determined on day 3 after infection. Results are representative of three experiments and data are mean ± SD based on five mice.

survival of mice, on day 8 after challenge, 62.5% of the mice immunized with mSEC survived, and 50% of mSEC-immunized group still survived on day 15 after infection. Conversely all of the mice injected with alum alone died 8 days after bacterial challenge (Figure 2). In addition, we immunized with rSEC in the same way. The result also showed protective effect, while on day 12, only 37.5% of the mice survived. These results indicated that vaccination with mSEC provides protection against *S. aureus* infection.

Anti-SEC antibodies were measured by enzyme-linked immunosorbent assays (ELISAs) as described previously[18]. Serum samples were diluted with PBS (1:100) and then serial two-fold dilution was performed. The levels of Immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, and IgM were expressed as maximal dilution

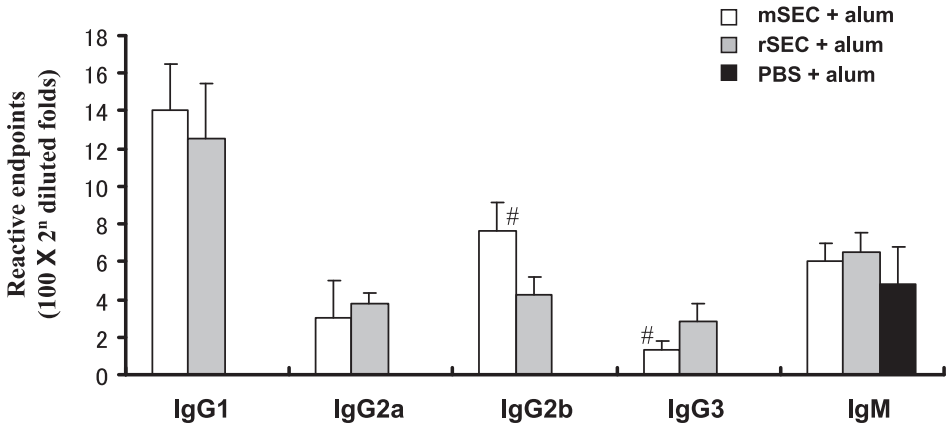

Figure 3 Antibody responses of mSEC-vaccinated mice. Animals were vaccinated with rSEC or mSEC with alum or alum alone and then were infected with 5×10^7 CFU of S. aureus on day 7 after last boost. The serum samples were obtained on day 3 after infection. Anti-SEC-specific antibody titers were determined by ELISAs. Results are representative of three experiments and data are mean endpoint titers ± SD of samples from five mice. The # marker indicates that a value is significantly different from the value obtained for the rSEC-immunized mice at $P<0.05$.

of sera. A strong IgG1 antibody response to SEC was seen in the sera obtained from mice immunized with mSEC or rSEC (Figure 3).

In contrast, sera from mice injected with alum only failed to react significantly to SEC. Interestingly, there were the higher level of IgG2b in the sera obtained from mSEC-vaccinated mice than that from rSEC-immunized group. In contrast, the sera from mSEC-immunized mice showed the lower level of IgG3 than that from rSEC-immunized mice (Figure 3).

To determine the neutralizing activity of anti-mSEC serum against cytokine production induced by rSEC in vitro, anti-mSEC serum or control serum was pre-incubated with rSEC at 37 ℃ for 1 h before the rSEC was added to the spleen cell cultures. After 72 h of incubation at 37 ℃ in a 5% CO_2 incubator, the supernatants were collected and IFN-γ and TNF-α, which are commonly associated with superantigenic activity. Serum samples from the mice immunized with mSEC plus alum effectively inhibited IFN-γ (56%) and TNF-α (52%) production from murine spleen cells by SEC, relative to serum samples from the controls (18% and 25%). These results suggest that the inhibition of inflammatory cytokine production may be involved in the protective effect obtained from immunization with mSEC.

The amounts of IFN-γ, TNF-α, IL-4 and IL-10 in the supernatant of cell culture and sera of mice were determined by double-sandwich ELISAs as described previously[16,19]. Organ extracts were prepared by centrifuging 10% (wt/vol) spleen and homogenized in RPMI 1640 medium containing 1% (wt/vol) 3-[(cholamidopropyl)-demithylammonio]-1-propanesulfate (CHAPS; Wako) at 2,000 g for 20 min. IFN-γ production in the sera of mSEC-vaccinated mice peaked at 12 h and decreased at 24 h of infection (Figure 4). The IFN-γ production in the sera from mSEC-vaccinated mice showed significant lower levels than that in the sera from control mice. On the other hand, the titers of IL-10 and IL-4 in the sera of mSEC-vaccinated mice increased from 4 h and 6 h, and were significantly higher than those of control mice (Figure 4). These results indicated that mSEC-vaccination inhibited

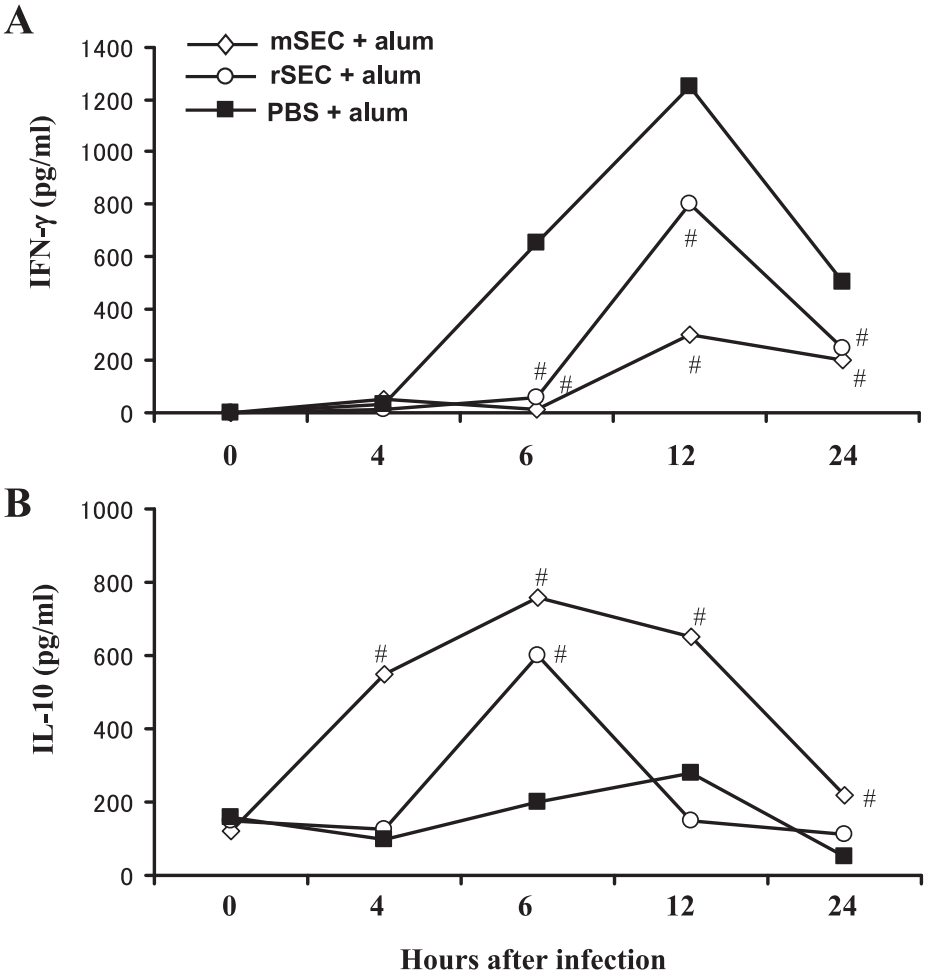

Figure 4 Endogenous cytokine production after infection with *S. aureus*. Mice were vaccinated with mSEC or rSEC plus alum or alum alone, and then were infected intravenously with 5×10^7 CFU S. aureus on day 7 after last boost. The titers of IFN-γ (A) and IL-10 (B) in the sera were determined by ELISAs. Results are representative of three experiments and the each point represents the mean±SD for a group of three to five mice. The # marker indicates that a value is significantly different from the value obtained for the control mice at $P<0.05$.

IFN-γ production and induced IL-10 and IL-4 production.

SEC, produced by pathogenic strains of *S. aureus*, especially clinic isolates of MRSA, is known for its involvement in TSS, persistent infection and causing bovine mastitis[2,4,20,21]. Several residues of SEC for binding to TCR and are important for superantigenicity have demonstrated[5-8]. In the present study, we designed to change the N23 of SEC molecule to A23, and expressed a single site mutant SEC devoid of superantigenic activity. Our results showed that this mutant toxin, mSEC, is highly effective in inducing toxin-specific antibodies capable of neutralizing superantigenicity, decreasing bacteria growth in organs and protecting animals from lethal *S. aureus* infection. Recent studies have undertaken the development of toxoid vaccines that may protect against the immunobiological effects of pyrogenic toxic superantigens, included SEs, TSST-1 and streptococcal pyrogenic exotoxin, presumably through neutralization by antibody[22-26]. Gampfer et al.[22] demonstrated that vaccination with

non-superantigenic SEB and TSST-1 developed antibody responses against these toxins and protected against challenge with lethal doses of superantigen potentiated with LPS. Nilsson et al.[24] and our previous study[18] demonstrated that immunization with non-superantigenic SEA and TSST-1 protected *S. aureus*-induced lethal septic shock. The protective mechanism of vaccination with these non-superantigenic toxins remains unclear.

mSEC-vaccinated mice produced high titers of SEC-specific IgG1 and significantly higher IgG2b antibodies compared with rSEC-immunized group, whereas the mSEC-vaccinated mice produced the lower level of IgG3 than that of the rSEC-immunized mice. The difference of induction of antibody subclass responses may be due to the lack of the superantigenic activity of mSEC. Furthermore, the serum samples from mSEC-immunized mice also significantly inhibited IFN-γ and TNF-α production from murine spleen cells by SEC. These results indicated that SEC-specific antibodies might play an important role in host resistance against *S. aureus* infection and neutralization of superantigenic activity. The mechanism of action of serum antibodies in *S. aureus* infections remains elusive. Recently, it was reported that anti-TSST-1 monoclonal antibody (mAb) cross-inhibited SEA-induced mitogenic activity and TNF-α production in vitro and protected against SEA-induced lethality in a mouse model[27]. The demonstrated effects of antibodies may be due to anti-inflammatory activity[28,29] and neutralization of the toxicity of *S. aureus* surface components and secreted products[24,28].

Our results showed that serum samples obtained from mSEC-vaccinated mice produced higher titers of IL-10 and IL-4, and lower titers of IFN-γ in response to *S. aureus* infection than did those obtained from the control mice. IL-10 is known to have anti-inflammatory actions in various inflammatory diseases[30-32]. Previous studies demonstrated that IL-10 plays a beneficial role in protecting the host from shock due to endotoxin[32], septic shock[30], and staphylococcal enterotoxin shock[31,33]. Our previous study showed that administration of anti-IL-10 mAb to mice inhibited the elimination of *S. aureus* from the organs, suggested that IL-10 might play a beneficial role in host resistance to *S. aureus* infection[34]. It is possible that this cytokine may regulate excess inflammatory responses in *S. aureus* infection. On the other hand, our previous study showed that IFN-γ plays a detrimental role in *S. aureus* infection in mice[16]. Administration of anti-IFN-γ mAb resulted in the suppression of bacterial growth in the organs and protected mice from the lethal effect of *S. aureus* infection. In addition, IFN-γ receptor-deficient mice developed severe sepsis with high mortality after *S. aureus* infection[35]. In the present study, the results suggest that mSEC vaccination may polarize Th0 toward Th2 in vivo, and that the regulation of cytokine production might be involved in acquisition of protection against in the mSEC-immunized mice. These results, together with previous data, indicated that the protection might be mediated by SEC-specific antibodies neutralizing the *S. aureus*-produced SEC, as well as by IL-10 and IL-4 induction, and down-regulating IFN-γ production induced by superantigenic toxins and *S. aureus*.

References

1) Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 2000;13: 16-34.

2) Edwards VM, Deringer JR, Callantine SD, Deobald CF, Berger PH, Kapur V, Stauffacher CV, Bohach GA. Characterization of the canine type C enterotoxin produced by *Staphylococcus intermedius* pyoderma isolates. Infect Immun 1997;65:2346-52.

3) Ferens WA, W. C. Davis, M. J. Hamilton, Y. H. Park, C. F. Deobald, L. Fox, and G. Bohach. Activation of bovine lymphocyte subpopulations

by staphylococcal enterotoxin C. Infect Immun 1998;66:573-80.

4) Fitzgerald JR, Monday SR, Foster TJ, Bohach GA, Hartigan PJ, Meaney WJ, Smyth CJ. Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. J Bacteriol 2001;183:63-70.

5) Fields BA, Malchiodi EL, Li H, Ysern X, Stauffacher CV, Schlievert PM, Karjalainen K, Mariuzza RA. Crystal structure of a T-cell receptor beta-chain complexed with a superantigen. Nature 1996;384:188-92.

6) Leder L, Llera A, Lavoie PM, Lebedeva MI, Li H, Sekaly RP, Bohach GA, Gahr PJ, Schlievert PM, Karjalainen K, Mariuzza RA. A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor β chain and major histocompatibility complex class II. J Exp Med 1998;187:823-33.

7) Lamphear JG, Bohach GA, Rich RR. Structural dichotomy of staphylococcal enterotoxin C superantigens leading to MHC class II-independent activation of T lymphocytes. J Immunol 1998;160: 2107-14.

8) Rossle SC, Bisch PM, Lone YC, Abastado JP, Kourilsky P, Bellio M. Mutational analysis and molecular modeling of the binding of *Staphylococcus aureus* enterotoxin C2 to a murine T cell receptor Vβ10 chain. Eur J Immunol 2002;32:2172-8.

9) Bavari S, Dyas B, Ulrich RG. Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. J Infect Dis 1996;174:338-45.

10) Neill RJ, Jett M, Crane R, Wootres J, Welch C, Hoover D, Gemski P. Mitogenic activities of amino acid substitution mutants of staphylococcal enterotoxin B in human and mouse lymphocyte cultures. Infect Immun 1996;64:3007-15.

11) Ulrich RG, Olson MA, Bavari S. Development of engineered vaccines effective against structurally related bacterial superantigens. Vaccine 1998;16: 1857-64.

12) Woody MA, Krakauer T, Ulrich RG, Stiles BG. Differential immune responses to staphylococcal enterotoxin B mutations in a hydrophobic loop dominating the interface with major histocompatibility complex class II receptors. J Infect Dis 1998;177:1013-22.

13) Lowell GH, Colleton C, Frost D, Kaminski RW, Hughes M, Hatch J, Hooper C, Estep J, Pitt L, Topper M, Hunt RE, Baker W, Baze WB. Immunogenicity and efficacy against lethal aerosol staphylococcal enterotoxin B challenge in monkeys by intramuscular and respiratory delivery of proteosome-toxoid vaccines. Infect Immun 1996;64:4686-93.

14) Lowell GH, Kaminski RW, Grate S, Hunt RE, Charney C, Zimmer S, Colleton C. Intranasal and intramuscular proteosome-staphylococcal enterotoxin B (SEB) toxoid vaccines: immunogenicity and efficacy against lethal SEB intoxication in mice. Infect Immun 1996;64:1706-13.

15) Woody MA, Krakauer T, Stiles BG. Staphylococcal enterotoxin B mutants (N23K and F44S): biological effects and vaccine potential in a mouse model. Vaccine 1997;15:133-9.

16) 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.

17) Omoe K, Ishikawa M, Shimoda Y, Hu DL, Ueda S, Shinagawa K. Detection of seg, seh, and sei genes in Staphylococcus aureus isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg, seh*, or *sei* genes. J Clin Microbiol 2002;40:857-62.

18) 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.

19) Nakane A, Nishikawa S, Sasaki S, Miura T, Asano M, Kohanawa M, Ishiwata K, Minagawa T. 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.

20) Booth MC, Pence LM, Mahasreshti P, Callegan MC, Gilmore MS. Clonal associations among *Staphylococcus aureus* isolates from various sites of

infection. Infect Immun 2001;69:345-52.

21) Rasheed JK, Arko RJ, Feeley JC, Chandler FW, Thornsberry C, Gibson RJ, Cohen ML, Jeffries CD, Broome CV. Acquired ability of *Staphylococcus aureus* to produce toxic shock-associated protein and resulting illness in a rabbit model. Infect Immun 1985;47:598-604.

22) 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.

23) McCormick JK, Hirt H, Dunny GM, Schlievert PM. Pathogenic mechanisms of enterococcal endocarditis. Curr Infect Dis Rep 2000;2:315-21.

24) Nilsson IM, Patti JM, Bremell T, Hook M, Tarkowski A. Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. J Clin Invest 1998;101:2640-9.

25) Schlievert PM, Jablonski LM, Roggiani M, Sadler I, Callantine S, Mitchell DT, Ohlendorf DH, Bohach GA. Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals. Infect Immun 2000;68:3630-4.

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) Kum WW, Chow AW. Inhibition of staphylococcal enterotoxin A-induced superantigenic and lethal activities by a monoclonal antibody to toxic shock syndrome toxin-1. J Infect Dis 2001;183:1739-48.

28) Schlievert PM. Use of intravenous immunoglobulin in the treatment of staphylococcal and streptococcal toxic shock syndromes and related illnesses. J Allergy Clin Immunol 2001;108:S107-10.

29) LeClaire RD, Hunt RE, Bavari S. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. Infect Immun 2002;70:2278-1.

30) Berg DJ, Kuhn R, Rajewsky K, Muller W, Menon S, Davidson N, Grunig G, Rennick D. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. J Clin Invest 1995;96:2339-47.

31) Florquin S, Amraoui Z, Abramowicz D, Goldman M. Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. J Immunol 1994;153:2618-23.

32) Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. J Exp Med 1993;177:1205-8.

33) Hasko G, Virag L, Egnaczyk G, Salzman AL, Szabo C. The crucial role of IL-10 in the suppression of the immunological response in mice exposed to staphylococcal enterotoxin B. Eur J Immunol 1998;28:1417-25.

34) Sasaki S, Nishikawa S, Miura T, Mizuki M, Yamada K, Madarame H, Tagawa YI, Iwakura Y, Nakane A. Interleukin-4 and interleukin-10 are involved in host resistance to *Staphylococcus aureus* infection through regulation of gamma interferon. Infect Immun 2000;68:2424-30.

35) Zhao YX, Tarkowski A. Impact of interferon-gamma receptor deficiency on experimental *Staphylococcus aureus* septicemia and arthritis. J Immunol 1995;155:5736-42.