Immunization with a Nontoxic Mutant of Staphylococcal Enterotoxin A, SEAD227A, Protects against Enterotoxin-Induced Emesis in House Musk Shrews

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Background. Staphylococcal enterotoxins (SEs) are the most common cause of foodborne diseases and toxic shock throughout the world. However, no vaccine that prevents emesis induced by SEs has been described.

Methods. A nontoxic mutant of SEA, SEAD227A, was constructed by site-directed mutagenesis and was purified by means of the Escherichia coli expression system. House musk shrews, a small emetic animal model, were immunized with SEAD227A and then challenged with wild-type SEA. SEA-induced emesis was recorded for 3 h. Antibody production was analyzed by gel double-immunodiffusion assay. Neutralizing activities of the antibodies with respect to superantigenic and emetic activities were analyzed in vitro and in vivo.

Results. SEAD227A was devoid of both superantigenic and emetic activities but still retained its immunological activity. Immunization with SEAD227A strongly induced specific antibody production and provided significant protection against SEA-induced emesis. Antibodies from immunized shrews markedly inhibited the SEA-induced proliferation of spleen cells and also significantly ablated SEA-induced vomiting in the animals.

Conclusions. These results suggest that vaccination with SEAD227A, which is devoid of toxic properties, provides protection against SEA-induced emesis. This nontoxic mutant and its specific antibodies might be useful in the prevention and treatment of staphylococcal food poisoning.

Staphylococcal enterotoxins (SEs) produced by Staphylococcus aureus are the most recognizable bacterial superantigenic toxins that cause food poisoning and toxic shock syndrome in humans throughout the world [1–3]. The primary symptoms of food poisoning are nausea, vomiting, abdominal cramping, and diarrhea occurring within 1–4 h after ingestion of the contaminated food. In addition to causing emesis, SEs are pyrogenic superantigens that stimulate the massive proliferation of large populations of T cells and the uncontrolled release of proinflammatory cytokines, which cause life-threatening toxic shock syndrome [4–6].

SEs have been divided into 5 serological types (SEA to SEE) on the basis of their antigenicity. In recent years, new types of SEs and SE-like toxins (SEG to SElV) have been reported [7–9]. SEs can directly bind to major histocompatibility complex (MHC) class II molecules and to T cell receptors (TCRs) that bear specific Vβ elements [10, 11], subsequently leading to the massive proliferation of T cells and the release of proinflammatory cytokines—including interleukin (IL)–1, IL-2, IL-6, interferon (IFN)–γ, and tumor necrosis factor-α (TNF-α)—which accounts for their toxicity [7, 12, 13]. Mutagenesis of potential TCR and MHC class II binding sites is a central effort of modern vaccine strategies and has been extensively used in the past 10 years. Previous studies have described the toxicity and biological activity of wild-type and mutant SEs and showed that genetically altered SEs that had been inactivated by means of the site-directed mutagenesis strategy and that lacked su-
perantigenic effects were highly immunogenic in mice [14–16]. Several investigations have shown that immunization with recombineant and/or mutant SEB and SEC could protect mice and rabbits against lethal shock induced by the wild type of their superantigenic toxins [17, 18]. Recently, we demonstrated that immunization with a mutant SEC could protect against S. aureus infection in a mouse model [19].

Over the last few decades, a number of studies of the nature of SEs have been conducted, and the molecular basis of the superantigenic activities of SEs has been extensively studied. However, little is known about the mechanism by which the toxins could induce symptoms of food poisoning, which in turn hampers the design of protective measures and anticotoxic drugs [20, 21]. The lack of progress in identifying a mechanism and studying the protective measures of the emetic activity of SEs is partially attributed to the lack of a rodent model for toxin-mediated food poisoning [20, 22, 23]. Our previous studies have demonstrated that house musk shrew (Suncus murinus) has an emetic response to the peroral and intraperitoneal administration of SEs and that it is a suitable animal model for studying the emetic activity of SEs [22, 24]. In the present study, to shed some light on the problem of the protective measures of SE-induced emesis, we prepared a nontoxic mutant of SEA, SEAD227A, and investigated whether vaccination with it could protect against SE-induced vomiting in house musk shrews. Our results showed that SEAD227A is devoid of both superantigenic and emetic activities but still retains immunoreactivity. Immunization with SEAD227A significantly protected against SEA-induced emesis in house musk shrews, and the antiserum from the immunized shrews showed strong neutralizing activity against both the superantigenic and emetic activities of SEA in vitro and in vivo.

METHODS

Animals. House musk shrews (Suncus murinus, Jic-SUN) were purchased from Clea Japan. The shrews were housed in plastic cages under specific pathogen–free conditions at the Institute for Experimentation, Hirosaki University Graduate School of Medicine. The daily cycle consisted of 12 h of light and 12 h of darkness; animals were fed on commercial Suncus murinus formula (Clea Japan) and were provided water ad libitum. All animal experiments were done in accordance with the guidelines for animal experimentation of Hirosaki University.

Bacterial strains and culture condition. For genomic DNA preparation, S. aureus FRI 722 expressing SEA was inoculated into 5 mL of soybean-casein digest broth (Nissui) and grown overnight at 37°C with shaking (110 rpm). Escherichia coli DH5α (Toyobo Biochem) and E. coli NM522 mutS (Amersham Pharmacia Biotech) were routinely grown in Luria broth (Becton Dickinson) at 37°C with shaking (110 rpm). The antibiotic concentration used to maintain plasmids in E. coli was 100 μg/mL ampicillin. E. coli DH5α derivatives were grown in 2X YTA medium containing 100 μg/mL ampicillin at 37°C with shaking.

Expression and purification of SEA and the mutant SEAD227A. Genomic DNA containing the sea gene was isolated from S. aureus FRI 722, as described elsewhere [22]. To construct the SEA expression plasmid, the DNA fragments were digested with EcoRI and BamHI. The fragments were cloned into pGEX-6p-1 (Amersham Pharmacia Biotech), a glutathione S-transferase (GST) fusion expression vector, and then transformed into E. coli DH5α cells. The resultant plasmid was named pKAXI. To construct and express mutant SEA, a selection primer (5’-GGTGACACCACGATGCCCGCGCAATGGCAACAACG-3’) and a mutagenic primer (5’-CTGGTACGATGGGTGCTATGAAATATTTATG-3’) were designed to change oligonucleotide GAT (which codes for aspartic acid 227 in the C terminus of the SEA molecule) to GCT (which codes for alanine). Site-directed mutagenesis was performed as described by Hu et al. [19]. The aspartic-acid-to-alanine mutant plasmid was designated pGXD227A and transformed into E. coli DH5α. Expression of GST-fused SEA or GST-fused SEAD227A and cleavage and removal of the GST tag from SEA or SEAD227A were performed as described elsewhere [22].

Immunoreactivity of SEAD227A. The immunoreactivity of SEAD227A with anti-SEA antibody was measured by ELISA. Microplates (96 well; Nunc) were coated overnight at 4°C with mouse anti-SEA antibody (10 μg/mL) in carbonate buffer (pH 9.5). The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked for 2 h at 37°C with 10% Blockace (Dai-nippon Pharmaceutical) in PBS (PBSB). SEAD227A samples were diluted in PBSB and added to washed wells for 2 h at 37°C. Wells were washed with PBST, and rabbit anti-SEA antibody (5 μg/mL) was added for 2 h at 37°C. Wells were again washed with PBST, and goat anti–rabbit IgG (Southern Biotechnology Associates) was diluted in PBSB and added for 2 h at 37°C. Wells were washed, and a substrate solution containing o-phenylenediamine and H2O2 was added to each well for color development. The reaction was terminated by the addition of 25 μL of 8N H2SO4. The reaction was measured at 490 nm in an ELISA plate reader.

Cell proliferation assay. To investigate SEA and SEAD227A responses to house musk shrew cells, spleen cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 μmol/L sodium pyruvate (Wako Pure Chemical), and 50 μmol/L 2-mercaptoethanol (Wako). One million spleen cells per milliliter were incubated with various amounts of SEA or SEAD227A in round-bottomed microplates at 37°C for 48 h. The cultures were pulsed for 24 h with 20 kBq/well [3H]-thyidine (ICN Biomedicals) and then harvested on glass-fiber filters. The amount of incorporated [3H]-thyidine was measured using liquid scintillation counting. To investigate SEA and SEAD227A responses to human peripheral blood mononuclear cells (PBMCs), human PBMCs by healthy volunteers were prepared by Lymphoprep Tube (Nyc-comed) centrifugation, and 1 × 10^5 cells were added to micro-
plate wells containing various dilutions of SEA or SEAD227A in 0.1 mL of culture medium, as described above. Cell proliferation was measured as described above.

**Assays of cytokines.** Titers of IFN-γ, TNF-α, and IL-2 in cultures of human PBMCs were determined by double-sandwich ELISAs. PBMCs were placed in a 24-well tissue culture plate (Greiner) at a density of 1 × 10^5 cells/well in the presence of SEA or SEAD227A. The supernatants of cell culture were collected after 72 h of incubation at 37°C in a 5% CO2 incubator. Cytokine titers were determined by use of human IFN-γ and TNF-α ELISA kits (Biosource International) and an IL-2 ELISA kit (Biosource Europe).

**Toxicity assay of SEA and SEAD227A.** The toxic effect of SEA and SEAD227A in BALB/c mice was tested in a lipopolysaccharide (LPS)–potentiated mouse lethality model [25]. Mice were first injected intraperitoneally with SEA or SEAD227A diluted in PBS (0.1–10 μg per mouse). The mice were injected again with 80 μg of LPS from *E. coli* O55:B5 (Sigma) 4 h later. The controls included animals given either SEA or LPS alone, and lethality was recorded over 72 h.

**Immunization with SEAD227A and administration of SEA.** For vaccination of house musk shrews, purified mutant SEAD227A was dissolved in PBS and emulsified 1:1 in alum adjuvant (Pierce). Two-hundred-microliter portions of the emulsion containing 10 μg of SEAD227A or alum alone were injected at 2 subcutaneous sites on the backs of the shrews. Booster immunizations were done 2 and 4 weeks after the initial vaccination. The animals were challenged with SEA on day 7 after the last booster by intraperitoneal injection or oral administration, and the emetic responses were assayed as described below.

**Assay of emetic activity.** Purified SEA was diluted in PBS, and 200 μL at an appropriate dilution was administered intraperitoneally into house musk shrews. Animals were not starved beforehand. The animals were observed for emetic responses for 3 h after the intraperitoneal or oral administration of SEA. The number of vomiting shrews and the number of times an animal vomited (frequency of vomiting), the time to the first vomiting episode, and any behavioral changes during the 3-h observation were monitored by use of a video camera and recorded.

**Assays of specific antibodies.** The production of anti-SEA antibodies in serum samples of immunized shrews was measured by gel double-immunodiffusion assay, basically using the method described by Robbins et al. [26] with minor modifications. Briefly, 1.2% Noble agar (Becton Dickinson) in PBS was poured into plastic petri dishes. Wells of the same size (diameter, 8.0 mm) were cut, and the agar plugs were removed. Serum (at 1:10, 1:20, and 1:40 dilution) from immunized or control animals and SEA were added to wells (75 μL/well) and incubated in a humidified box at 37°C for 24 h. Nonprecipitated proteins were removed by soaking the gel in PBS for 48 h with frequent changes. The gel was stained with 0.1% Coomassie brilliant blue R-250 in 10% acetic acid–40% methanol in distilled water, followed by destaining in the solvent.

**Neutralization assay.** For determination of neutralizing activities of anti-SEA sera against vomiting, anti-SEAD227A serum or control serum was preincubated with SEA at 37°C for 1 h before SEA was administrated to shrews. The emetic responses of animals were recorded and analyzed. For determination of neutralizing activities of anti-SEA serum against spleen cell proliferation induced by SEA, anti-SEAD227A serum or control serum was preincubated with SEA at 37°C for 1 h before SEA was added to spleen cell cultures. After 48 h of incubation at 37°C in a 5% CO2 incubator, the cultures were pulsed for 24 h with 20 kBq/well [3H]-thymidine and then harvested on glass-fiber filters. The amount of incorporated [3H]-thymidine was measured using liquid scintillation counting, as described above.

**Statistical analysis.** Data were expressed as means ± SDs, and the Mann-Whitney *U* test was used to determine the significance of the differences in proliferation, cytokine production in cell cultures, and emetic responses in house musk shrews between vaccinated and control groups. The percentages of emesis in immunized and control shrews were compared using Fisher’s exact test (2-tailed).

### RESULTS

**Characteristics and immunological reactivity of SEAD227A.** It has been suggested that several residues of SEA, including F47, H187, H225, and D227, are important for binding to MHC class II molecules and for superantigenicity [27, 28]. In the present study, we replaced the D227 residue of the SEA molecule with A227 (figure 1A), and we designated the mutant gene product SEAD227A. Purified SEAD227A was compared with wild-type SEA on a Coomassie blue-stained SDS-PAGE gel, and the results revealed the presence of a readily detectable purified protein band that comigrated with purified SEA (figure 1B). The immunological reactivities of SEAD227A and SEA with polyclonal rabbit anti-SEA antibodies were assayed by ELISA. The antibodies readily reacted with purified SEAD227A and SEA (figure 1C).

These results indicate that the 2 proteins share multiple epitopes. To confirm that the superantigenic activity of SEAD227A was deleted, the proliferation (figure 1D) and cytokine production (figure 1E–1G) induced by SEAD227A and SEA in human PBMCs were determined. Substantial amounts of cytokines were induced in the cell cultures at all concentrations of SEA used, and the results revealed higher proliferative activities. In contrast, for SEAD227A no detectable IFN-γ or TNF-α and significantly lower IL-2 production and proliferative activity were observed (figure 1). These results indicate that SEAD227A is significantly devoid of superantigenic activity.

To examine whether the nontoxicity of the SEAD227A protein could be shown in vivo, mice were inoculated with LPS plus either SEA or SEAD227A, and survival was observed. In striking
contrast to the 76% mortality rate observed among mice inoculated with 15 μg/H9262 of SEA plus LPS, none of the mice that were given an equivalent dose of SEAD227A plus LPS died (data not shown).

Superantigenic and emetic activities of SEAD227A in house musk shrews. To confirm whether the superantigenic activities of SEAD227A were also deleted for the emetic model animals, the proliferation induced by SEAD227A and SEA in the spleen cells of house musk shrews were determined. The results showed that SEAD227A is also significantly devoid of superantigenic activity for the animals (figure 2A). We further analyzed the emetic activity of SEAD227A on shrews. Animals were intraperitoneally administered SEA or SEAD227A at concentrations of 2.5, 5, 10, and 20 μg/kg, and emetic responses were recorded for 3 h. The results showed that the administration of SEA induced emesis in a dose-dependent manner and that a 20-μg/kg dose induced vomiting in all of the tested animals with a mean ± SD vomiting frequency of 11 ± 3.2 (figure 2B and 2C). However, SEAD227A showed no emetic activity at the doses of 2.5 and 5 μg/kg and significantly decreased the emetic activity even at the dose of 20 μg/kg. These results indicate that the mutant SEAD227A is devoid of both superantigenic and emetic activities.

Protective effect of immunization with SEAD227A against SEA-induced emesis. House musk shrews were immunized with SEAD227A plus alum or with alum alone 3 times and then challenged intraperitoneally with 5 or 10 μg/kg SEA on day 7 after the last booster immunization. SEAD227A-immunized shrews showed significantly lower emetic responses (figure 3A) and frequency of vomiting (figure 3B) than did the control animals immunized with alum alone. SEAD227A-immunized shrews also showed a significantly lower emetic response when challenged via oral inoculation of SEA (figure 3C and 3D). This experiment indicates that vaccination with the nontoxic mutant SEAD227A protects against SEA-induced emesis.
Antibody production in SEAD227A-immunized shrews. Because of the lack of isotype-specific reagents for shrews, antibody production in SEAD227A-immunized shrews was determined by gel double-immunodiffusion assay. A strong antibody response (1:40) to SEA was seen in the serum obtained from shrews immunized with SEAD227A (figure 4A). In contrast, serum from shrews immunized with alum only failed to react to SEA. In addition, SEAD227A-induced antibodies showed a specific reaction with SEA and SEAD227A but not with SEB, SEC, or toxic shock syndrome toxin (TSST)–1 (data not shown).

Neutralization of anti-SEAD227A antibodies on SEA-induced proliferation in vitro. The effect of serum from SEAD227A-vaccinated animals on SEA-induced proliferation of shrew spleen cells was determined in vitro. SEA was preincubated with anti-SEAD227A serum or control serum and then added to spleen cell cultures, and proliferation was analyzed. Serum from the shrews immunized with SEAD227A plus alum effectively inhibited the proliferation of spleen cells induced by SEA, compared with serum from the alum-injected control animals (figure 4B).

Neutralization by anti-D227A antibodies of the emetic responses induced by SEA in vivo. We further examined the effect of serum samples from SEAD227A-vaccinated shrews on SEA-induced emesis. SEA was preincubated with anti-SEAD227A serum or control serum and then administrated to house musk shrews. Serum from the SEAD227A-immunized animals effectively inhibited the emetic responses induced by SEA, compared with serum from the alum-injected control animals (figure 4C and 4D). These experiments indicate that serum from SEAD227A-vaccinated shrews provides efficient protection against SEA-induced vomiting.

DISCUSSION

Our results have demonstrated, for the first time, that immunization with a nontoxic mutant of SEA protects against an emetic response, a significant symptom of staphylococcal food poisoning in humans, in a small emetic animal model, house musk shrews. Furthermore, specific antibodies from the immunized shrews showed strong neutralizing activity against the superantigenic activity as well as the emetic activity of SEA in vitro and in vivo.

SEA is one of the most clinically important and best-characterized bacterial superantigenic toxins and is a major cause of symptoms of food poisoning in humans [29, 30]. Although their molecular characteristics and superantigenic activities have been elucidated, relatively little is known about the mechanism that allows the toxins to induce symptoms of food poisoning [20, 31]. SEA is a flat monomer composed of 233 amino acid residues, residing in 2 domains. Domain I consists of residues 31–116, and domain II consists of residues 117–233 together with the amino-terminal tail at 1–30 [5, 10]. Several residues of SEA, including F47, H187, H225, and D227, have been suggested to be important for binding to MHC class II molecules and for superantigenicity [5, 27, 28]. In the present study, we replaced the D227 of the SEA molecule with A227 and expressed a single-site mutant SEA devoid of superantigenic activity. We were unable to detect IFN-γ and TNF-α production and proliferative responses in human PBMCs with this mutant at concentrations up to 1000 ng/mL (figure 1). Interestingly, this
mutant protein, SEAD227A, is also devoid of emetic activity in house musk shrews and of lethal toxic activity in mice while still retaining immunological activity. Our results demonstrated that the residue of D227 at the carboxyl-terminal of the SEA molecule is important for both the superantigenic and emetic activities of the toxin. Additional changes and experiments may be required to confirm whether this single-amino-acid mutant could undergo reversion and whether such a protein could be used as a toxoid.

SEs are known to act on host systems in 3 distinct ways: as enterotoxins that induce emesis and diarrhea in human and nonhuman primates [32], as exotoxins that have been implicated in the induction of toxic shock [33], and as superantigens that induce extensive Vβ-specific T cell stimulation followed by anergy and apoptosis, which results in immunosuppression [6, 34]. Several reports have described the toxicities and the biological activities of wild-type and mutant SEA and SEB and showed that genetically altered SEs were immunogenic in mice and rhesus monkeys [15, 16, 35]. Immunization with recombinant or mutant SEA and SEB elicited neutralizing antibodies against wild types of SEs and protected mice or rabbits from lethal shock induced by the wild types of their superantigenic toxins [14, 17]. A recent study [19] demonstrated that immunization with non-supercient SEC protected against S. aureus–induced lethal septic shock in a mouse model. However, the mechanism of SE-induced emesis remains unclear. The lack of progress is partially attributable to the lack of convenient and appropriate animal models [20, 22, 23]. Monkeys have been considered to be a primary animal model because the administration of SEs elicits an emetic response [36, 37]; however, the use of monkeys to investigate SEs is severely restricted by the high cost, the availability of the animals, and ethical considerations. Rodents cannot exhibit emesis because of a lack of capability. Other experimental animals, such as dogs, weanling pigs, and cats, are either less susceptible to SEs or their responses to SEs are not specific [22, 31, 36]. Recently, our studies have demonstrated that peroral and intraperitoneal administration of SEs induces a specific emetic response in house musk shrews (which belongs to the
subfamily *Crocidurinae* of the family *Soricidae* of the order *Insectivora*), indicating that it is a suitable emetic animal model [22, 31]. In the present study, house musk shrews were immunized with the nontoxic mutant SEAD227A and then challenged with the wild-type SEA. The results showed that immunization with SEAD227A significantly increased the protection of shrews against SEA-induced emesis, suggesting that this nontoxic mutant vaccine can prevent staphylococcal food poisoning induced by *S. aureus*.

Our results also showed that SEAD227A is highly effective in inducing toxin-specific antibodies capable of neutralizing superantigenicity and protecting animals from SEA-induced emesis. The mechanism of action of serum antibodies in enterotoxin-induced vomiting in vivo remains elusive. One of the demonstrated
effects of antibodies is anti-inflammatory activity [38, 39], and another is neutralization of the toxicity of the superantigenic toxins [40]. Previous studies have identified cross-reactive antibodies between SEs and streptococcal pyrogenic exotoxin A [41, 42]. Recently, it was reported that anti-TSST-1 antibody also cross-inhibited SEA-induced mitogenic activity and TNF-α production in vitro and protected against SEA-induced lethality in a mouse model [43]. Our results in the emetic animal model indicate that SEA-specific antibodies might play an important role in the neutralization of superantigenic activity as well as in host resistance against superantigenic toxin-induced emesis. Because the expression of SEs is common among invasive S. aureus strains and food-poisoning isolates, the nontoxic SEAD227A and antibodies against it might be useful in the control of staphylococcal food poisoning and the treatment of foodborne diseases.

Acknowledgments

We thank Chika Maki, Yoshihiro Tomioka, and Yoshikazu Tanaka for assistance with the purification of SEA and SEAD227A and with animal experiments.

References


