

IMMUNIZATION WITH NONTOXIC MUTANT STAPHYLOCOCCAL ENTEROTOXIN A PROTECTS AGAINST ENTEROTOXIN-INDUCED EMESIS IN HOUSE MUSK SHREWS

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Abstract Background. Staphylococcal enterotoxins (SEs) are the most common cause of food-borne diseases and toxic shock syndrome throughout the world. However, little is known about the mechanism of emesis induced by SEs and no vaccine that prevents SE-induced emesis has been described. **Methods.** A nontoxic mutant SEA, SEAD227A, was constructed by site-directed mutagenesis and purified from *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 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 significantly provided the protection against SEA-induced emesis. The antibodies from immunized shrews markedly inhibited the SEA-induced proliferation of spleen cells and also significantly ablated the SEA-induced vomiting in the animals. **Conclusions.** These results suggest that vaccination with SEAD227A 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

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Introduction

Staphylococcal enterotoxins (SEs) are the most recognizable bacterial superantigenic toxins causing food poisoning and toxic shock syndrome in humans throughout the world every year¹⁻³⁾. The primary symptoms of food poisoning are nausea, vomiting, abdominal cramping and diarrhea occurring within 1 to 4 h after ingestion of the contaminated food. In addition to causing emesis, SEs are pyrogenic superantigens that stimulate large populations of T cells to massive proliferation and uncontrolled release of proinflammatory cytokines, which cause life-threatening toxic shock syndrome⁴⁻⁶⁾.

SEs have been divided into five serological

types (SEA though to SEE) based on their antigenicity. In recent years, new types of SEs and SE-like toxins (SEG to SEIV) have been reported⁷⁻⁹⁾. SEs can directly bind to major histocompatibility complex (MHC) class II and to the T-cell receptor (TCR) bearing specific V β elements^{10,11)}, and subsequently lead to massive proliferation of T cells and release of proinflammatory cytokines^{7,12,13)}. Previous studies described toxicity and biological activity of wild type and mutant SEs, and showed that genetically altered SEs inactivated by site-directed mutagenesis strategy and lacking superantigenic effects were highly immunogenic in mice¹⁴⁻¹⁸⁾. Recently, we demonstrated that immunization with mutant SEC could protect

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against *S. aureus* infection in a mouse model¹⁹.

Over the past few decades, a number of studies have been conducted on the nature of SEs and the molecular basis of the superantigen activities of SEs has been extensively studied. However, little is known about a mechanism by which the toxins can induce symptoms of food poisoning, which in turn hampers the design of protective measures and antitoxic drugs^{20,21}. The lack of progress in studying a mechanism and 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 shows the 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 this study, to shed some light on the problem of protective measures of SE-induced emesis, we prepared a nontoxic mutant SEA, SEAD227A, and investigated whether vaccination with SEAD227A could protect against SEA-induced vomiting in house musk shrews.

Materials and Methods

Animals

House musk shrews 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. All animal experiments were carried out 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* DH5 α (Toyobo Biochem) and *E. coli* NM522 *mutS* (Amersham Pharmacia

Biotech) were routinely grown in Luria broth (Becton) at 37°C with shaking (110 rpm). *E. coli* DH5 α derivatives were grown in 2x YTA medium containing 100 μ g of ampicillin per mL at 37°C with shaking.

Expression and purification of SEA and mutant SEAD227A

To construct the SEA expression plasmid, the DNA fragments were digested with *Eco*RI and *Bam* HI. The fragments were cloned into the pGEX-6p-1 (Amersham Pharmacia Biotech), and then transformed into *E. coli* DH5 α cells. To construct and express mutant SEA, a selection primer (5'-GCGTGACACCACGATGCCCCG GGCAATGGCAACAACG) and a mutagenic primer (5'-CTGGTACGATGGGTGCTATGA AATATTTATATG) were designed to change oligonucleotide GAT (coding for aspartic acid 227 in the C terminus of the SEA molecule) to GCT (coding 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* DH5 α . Expression of GST-fused SEA or GST-fused SEAD227A, cleavage and removal of the GST tag from SEA or SEAD227A were performed as described previously²².

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 μ M sodium pyruvate (Wako Pure Chemical) and 50 μ M 2-mercaptoethanol (Wako). One million spleen cells per mL 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 of [³H] thymidine (ICN Biomedicals) and then harvested on glass fiber filters. The amount of incorporated [³H] thymidine 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 (Nycomed) centrifugation, and 1×10^5 cells were added to microplate wells containing various dilutions of SEA or SEAD227A in 0.1 mL culture medium as above. Cell proliferation was measured as described as above.

Assays of cytokines

Titers of IFN- γ , TNF- α , and IL-2 in the cell culture of human PBMCs were determined by double-sandwich ELISAs. PBMCs were placed in a 24-well tissue culture plate (Greiner) at a density of 1×10^6 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 the human IFN- γ , TNF- α ELISA kits (Biosource International), and IL-2 ELISA kit (Biosource Europe S. A.).

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 μ g of SEAD227A or alum alone were injected at two subcutaneous sites on the backs of the shrews. Booster immunizations were carried out 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 bellow.

Assay of emetic activity

Purified SEA was diluted in PBS and 200 μ L at an appropriate dilution were 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 vomits (frequency of vomiting), the time to the first vomiting episode, and any behavioral changes during 3 h of the observation were recorded by video camera.

Assays of specific antibodies

The production of anti-SEA antibodies in serum samples of immunized shrews was measured by gel double-immunodiffusion assay basically by the method described by Robbins et al.²⁶⁾ with minor modification. 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 sera or control sera were pre- incubated 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 sera against spleen cell proliferation induced by SEA, anti-SEAD227A sera or control sera were pre-incubated 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 of [³H] thymidine and then harvested on glass fiber filters. The amount of incorporated [³H] thymidine was measured using liquid scintillation counting as described above.

Results

Characteristics and immunological reactivity of SEAD227A

It has been suggested that several residues of SEA, including F47, H187, H225 and D227,

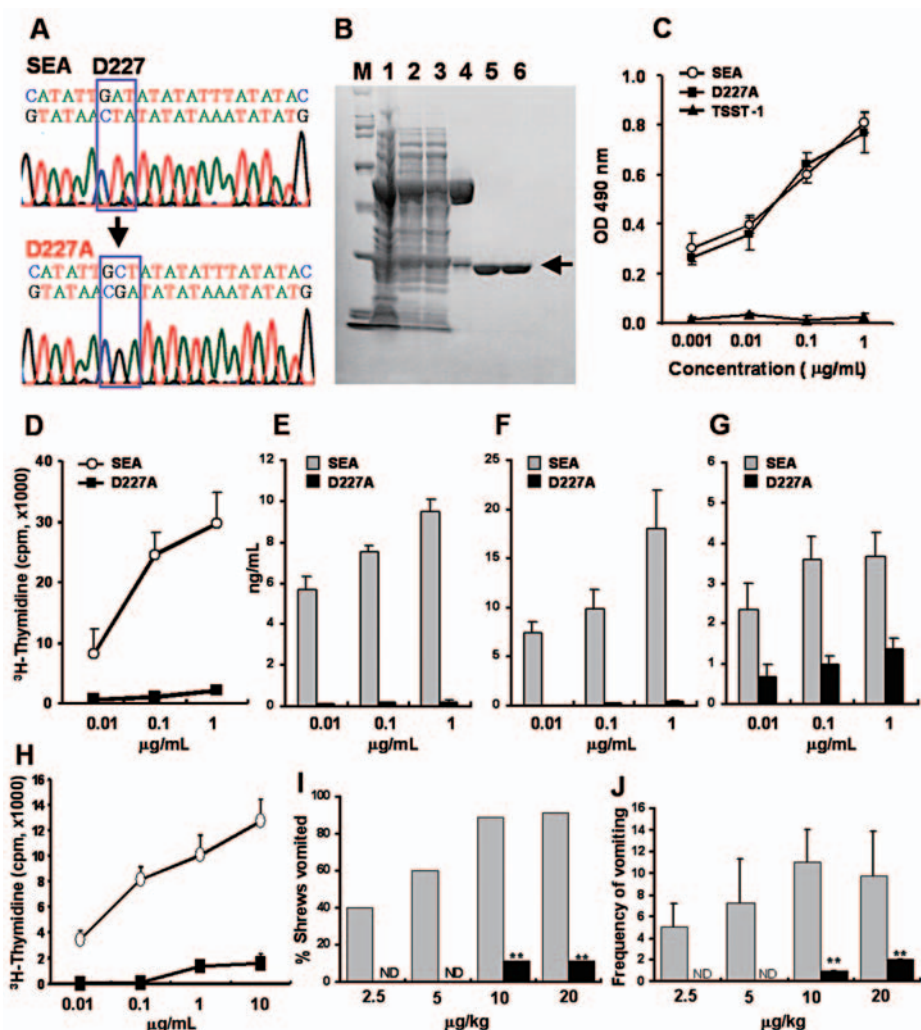


Figure 1 Characteristics of mutant SEAD227A. (A) Comparison of the sequences of SEA and SEAD227A. (B) SDS-PAGE, M, molecular size marker; lane 1, lysis of *E. coli* encoding pGXD227A; lane 2 and 3, supernatant of the lysis of *E. coli* encoding pGXD227A; lane 4, GST-SEAD227A; lane 5, SEAD227A; lane 6, SEA. (C) Reactions of SEAD227A and SEA with polyclonal rabbit anti-SEA antibodies as determined by ELISA. (D) Proliferation of human PBMCs induced by SEA and SEAD227A. Cell cultures were incubated with SEA or SEAD227A for 48 h and pulse-labeled with [³H] thymidine for 24 h. Results are mean cpm of triplicate wells \pm SD based on samples obtained from five donors. (E) to (G) Cytokine production in human PBMCs stimulated with SEA or SEAD227A. Titers of IFN- γ (E), TNF- α (F), and IL-2 (G) in supernatants that had been generated by stimulating human PBMC cultures with SEA or SEAD227A, as measured by ELISA. Each result is the mean \pm SD based on samples obtained from five donors. (H) Proliferation of shrew splenic cells induced by SEA and SEAD227A. Cultures were incubated with SEA or SEAD227A for 48 h and pulse-labeled with [³H] thymidine for 24 h. Results are mean cpm of triplicate wells \pm SD. (I) The dose-response effects of percentage of vomiting shrews and (J) Frequency of vomiting, the number of times an animal vomit, after intraperitoneal administration of SEA or SEAD227A. Each result is the mean \pm SD based on six to ten shrews. An asterisk indicates that a value is significantly different from the value obtained for the shrews immunized with alum alone (* P < .05, ** P < .01).

for binding to MHC class II are important for superantigenicity^{27,28}. In this study, we replaced the D227 residue of SEA molecule to A227 (figure 1A), and we designated the mutant gene product as SEAD227A. Purified SEAD227A was compared with wild-type SEA

on a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis 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 reacted readily with purified SEAD227A and SEA (figure 1C). These results indicated that the two proteins share multiple epitopes. To confirm that the superantigenic activity of SEAD227A was deleted, the proliferation (figure 1D) and cytokine production (figure 1E to G) 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. These results indicate that SEAD227A is significantly devoid of superantigenic activity.

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 1H). We further analyzed the emetic activity of SEAD227A on shrews. The results showed that the administration of SEA induced emesis in a dose-dependent manner and a 20 $\mu\text{g}/\text{kg}$ dose induced vomiting in all of the tested animals with a mean vomiting frequency of 11 ± 3.2 (figure 1I and J). However, SEAD227A showed no emetic activity at the doses of 2.5 and 5 $\mu\text{g}/\text{kg}$, and significantly decreased the emetic activity even at the dose of 20 $\mu\text{g}/\text{kg}$. These results indicated 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 for 3 times, and they were then challenged intraperitoneally with SEA on day 7 after the last booster immunization. SEAD227A-immunized shrews showed significantly lower emetic response (figure 2A) and frequency of vomiting (figure 2B) compared with those of the control animals that were immunized with alum alone. This experiment indicated that vaccination with the non-toxic mutant SEAD227A protected against SEA-induced emesis.

Antibody production in SEAD227A-immunized shrews

Because of the lack of isotype specific reagents against the shrews, antibody production in SEAD227-immunized shrews was determined by gel-double immunodiffusion assay. A strong antibody response (1: 40) to SEA was seen in the sera obtained from shrews immunized with SEAD227A (figure 2C). In contrast, sera from shrew immunized with only alum failed to react to SEA.

Neutralization of anti-SEAD227A antibodies on SEA-induced proliferation in vitro

The effect of serum samples from SEAD227A-vaccinated animals on SEA-induced proliferation of spleen cells of shrews was determined in vitro. SEA was pre-incubated with anti-SEAD227A sera or control sera and then added to spleen cell cultures, and the proliferation was analyzed. Serum samples from the shrews immunized with SEAD227A plus alum effectively inhibited the proliferation of spleen cells induced by SEA compared to serum samples from the alum-injected controls (figure 2D).

Neutralization of anti-D227A antibodies on emetic responses induced by SEA in vivo

We further examined the effect of serum

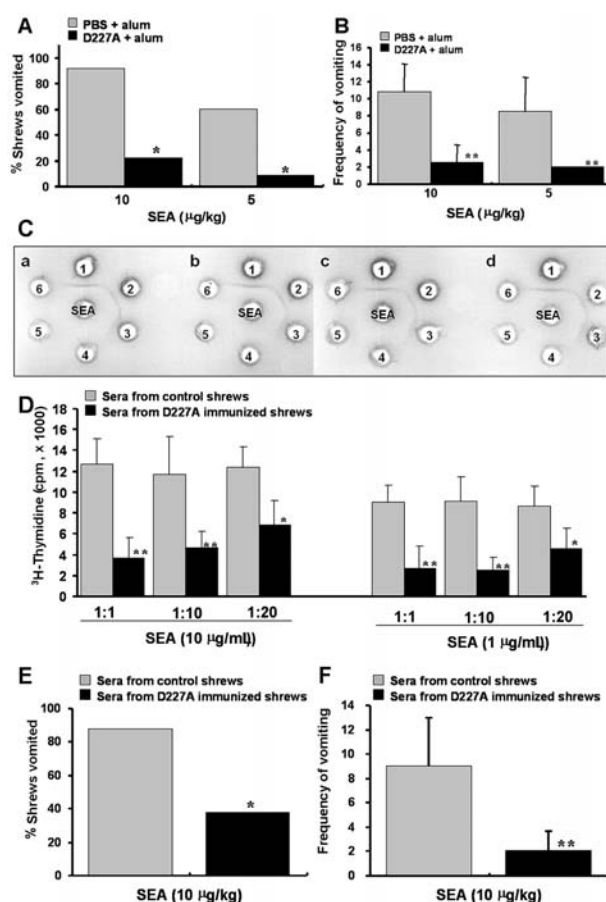


Figure 2 The protective effect of immunization with SEAD227A against SEA-induced emesis. (A) Percentage of vomiting animals after intraperitoneal administration of SEA in immunized and control house musk shrews. Animals were immunized with SEAD227A plus alum or with alum alone and then were administered with 10 and 5 µg/kg of SEA on day 7 after the last boost. The numbers of vomited animals were recorded. The data are average of three separate experiments included nine to twelve shrews for each group. (B) Frequency of vomiting after intraperitoneal administration of SEA for immunized and control house musk shrews. The animals were monitored for 3 h after SEA administration and times of vomiting episodes were recorded. (C) Serum antibody responses of SEAD227A-vaccinated shrews. Animals were immunized with SEAD227A plus alum or with alum alone. The serum samples were obtained on day 7 after the last boost. Anti-SEA-specific antibodies were determined by gel double-immunodiffusion assay. Center well was added with SEA (5 µg/mL), and around wells were added with the sera from immunized shrews (1, 2, 3, was diluted 1:10, 1:20, 1:40, respectively) and control shrews (4, 5, 6, was diluted 1:10, 1:20, 1:40, respectively). The results are shown by 4 representative samples of three experiments. (D) The inhibitory effect of anti-SEA227A sera on proliferation of spleen cell cultures induced by SEA. Spleen cells were obtained from naive shrews. SEA was incubated with the sera (diluted 1:1, 1:10 and 1:20) from shrews vaccinated with SEAD227A plus alum or alum alone at 37°C for 1 h and then was added to the spleen cell cultures. The cultures were incubated for 48 h and pulse-labeled with [³H] thymidine for 24 h. Results are mean cpm of triplicate wells ± SD. (E) and (F) The inhibitory effect of anti-SEA227A sera on emetic response in house musk shrews induced by SEA. SEA was incubated with the sera (diluted 1:1) from shrews vaccinated with SEAD227A plus alum or alum alone at 37°C for 1 h and then administered to shrews (10 µg/kg). The dose-response effects of percentage of vomiting shrews (E) and frequency of vomiting (F) after administration of SEA were recorded and analyzed. Each result is the mean ± SD based on five to eight shrews. An asterisk indicates that a value is significantly different from the value obtained for the shrews immunized with controls (**P* < .05, ** *P* < .01).

samples from SEAD227A-vaccinated shrews on SEA-induced emesis. SEA was pre-incubated with anti-SEAD227A sera or control sera and then administered to house musk shrews. Serum samples from the SEAD227A-immunized animal effectively inhibited the emetic responses induced by SEA compared to the serum samples

from the alum-injected controls (figure 2E and F). These experiments indicated that sera from SEAD227A-vaccinated shrews provide the efficient protection against SEA-induced vomiting.

Discussion

SEA is one of the most clinically important,

best-characterized bacterial superantigenic toxins and a major cause of symptoms of food poisoning in humans^{29,30)}. Despite their molecular characteristics and superantigenic activities have been elucidated, little is known about the mechanism how the toxins can induce the symptoms of food poisoning^{20,31)}. Several residues of SEA, including F47, H187, H225 and D227, have been suggested are important for binding to MHC class II and for superantigenicity^{5,27,28)}. In the present study, we designed to replace the D227 of SEA molecule to 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. Interestingly, this mutant protein, SEAD227A, is also devoid of emetic activity in house musk shrews, while it still retained immunological activities. Our results demonstrated that the residue of D227 at the carboxyl-terminal of SEA molecule is important for both superantigenic activities and emetic activities of the toxin.

SEs are known to act on host systems in three distinct ways: as enterotoxins that induce emesis and diarrhea in human and nonhuman primates³²⁾; as exotoxins that have been implicated in induction of toxic shock³³⁾; and as superantigens that induce extensive V β -specific T cell stimulation followed by anergy and apoptosis which result in immunosuppression^{6,34)}. Recent studies demonstrated that immunization with non-superantigenic SEC protected against *S. aureus*-induced lethal septic shock in mouse models¹⁹⁾. However, the mechanism of SEs-induced emesis remains unclear. The lack of progress is partially attributed 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 response³⁵⁻³⁷⁾. However, the use of monkeys in investigating SEs is severely

restricted by the high cost, the availability of the animals, and ethical considerations. Rodents cannot exhibit emesis due to lack of capability. Other experimental animals such as dogs, weanling pigs, and cats are less susceptible to SEs or their responses to SEs are not specific^{22,31,36)}. Recently, our studies showed that peroral and intraperitoneal administration of SEs induces a specific emetic response in house musk shrews, 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. Our results 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 the another is neutralization of the toxicity of the superantigenic toxins⁴⁰⁾. Our results in the emetic animal model indicated that SEA-specific antibodies might play an important role in neutralization of superantigenic activity as well as superantigenic toxin-induced emesis. Because the expression of SEs is common among invasive *S. aureus* strains and food-poisoning isolates, the nontoxic SEAD227A and its antibodies might be useful in the control of staphylococcal food-poisoning and treatment of food-borne diseases.

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