Analysis of the Epitopes on Staphylococcal Enterotoxin A Responsible for Emetic Activity

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ABSTRACT. To identify which region of staphylococcal enterotoxin A (SEA) is responsible for the emetic activity, twelve synthetic peptides corresponding to the entire SEA amino acid sequence and their respective anti-peptide antibodies were prepared and tested. The anti-peptide antibodies were tested for neutralization of SEA-induced emesis in Suncus murinus (Shrew mouse). The results indicate that SEA-induced emesis was neutralized by the mixture of three anti-peptide antibodies to A-7 (corresponding to amino acid residues 121–140), A-8 (141–160) and A-9 (160–180). These findings suggest that the regions corresponding to residues 121–180 may be the epitopes responsible for the emetic activity of SEA.

KEY WORDS: emetic activity, epitope, staphylococcal enterotoxin, Suncus murinus, synthetic peptide.

Staphylococcal enterotoxins (SEs), consisting of a single polypeptide chain, are emetic toxins that cause staphylococcal food poisoning syndrome. They are serologically classified into eight distinct types such as A (SEA), B (SEB), C (SEC), D (SED), E (SEE), G (SEG), H (SEH) and I (SEI) with varying molecular mass ranging from 25 to 30 kDa [14, 15, 18, 25]. Genes encoding for SEA through SEI have been cloned and their nucleotide sequences and amino acid sequences have been analyzed [2, 15, 18, 25]. In addition to the T-cell-stimulatory activities of SEs, the results suggest that these two activities may be incomplete correlation [5, 7]. However, the relationship between the molecular structure and emetic activity of SEA has not been fully understood. In this study, therefore, we utilized anti-peptide antibodies against various synthetic peptides corresponding to the entire SEA molecule to neutralize the SEA-induced emesis in Suncus murinus (shrew mouse) to identify which structural region is associated with the emetic activity of SEA.

MATERIALS AND METHODS

Purified SEA and rabbit anti-SEA antiserum: Purified SEA was prepared from culture supernatants of Staphylococcus aureus strain FRI-722 by methods described previously [21]. Briefly, the S. aureus strain was cultured in 4% NZ-amine type A medium (Wako Pure Chemical, Osaka, Japan) for production of SEA. The purification of SEA was performed using the CM-Sephadex C-25 (Pharmacia, Sweden) and DEAE-Sephadex A-25 chromatography (Pharmacia, Sweden). After the eluate was concentrated by lyophilization, the concentrated SEA was further purified by gel filtration on Sephadex G-75 superfine (Pharmacia, Sweden) as described previously [21]. Purified SEA was used for the preparation of anti-SEA serum in rabbits by the methods described previously [21]. Synthetic peptides and rabbit anti-peptide antibodies: According to the amino acid sequence of SEA reported previously [11], peptides corresponding to the entire sequence of SEA were synthesized with a Shimadzu PSSM-8 automated peptide synthesizer (Shimadzu Co., Ltd., Kyoto, Japan) using N- (9-fluorenyl) methoxycarbonyl chemistry [8, 26]. Twenty-mer representing the following amino acid residues of the protein with a molecular weight of 27,078 dalton was synthesized. They were A-1 (corresponding to amino acid residues 1-20), A-2 (21–40), A-3 (41–60), A-4 (61–80), A-5 (81–100), A-6 (101–120), A-7 (121–140), A-8 (141–160), A-9 (161–180), A-10 (181–200), and A-11 (201–220). A thirteen-mer was also synthesized, which was named as A-12 (221–233). Peptides were measured aseptically, dissolved in distilled water, mixed, and allowed to stand at 4°C for overnight before use. Antibodies against various synthetic peptides were produced by albino female Suncus murinus (Shrew mouse) to identify which structural region is associated with the emetic activity of SEA.
rabbits as described previously [8, 9]. The IgG fractions in these antisera were isolated by affinity chromatography on Affi-Gel Protein A (Bio-Rad, Laboratories, U.S.A.).

**Enzyme-linked immunosorbent assay (ELISA):** Reactivities of the antibodies to SEA or the synthetic peptides were determined in triplicate in ELISA as reported previously [8, 9]. One hundred µl of SEA (2.5 µg/ml) or the synthetic peptide (20 µg/ml) in 100 mM sodium carbonate buffer (pH 9.5) were adsorbed to each well of a 96-well ELISA plates (Nalge Nunc International). For color development, 200 µl of the substrate solution containing 0.02% H2O2 and 1 mg/ml of o-phenylenediamine (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M citrate-phosphate buffer (pH 5.0) were added to each well. The plates were held in the dark for 30 min at room temperature for color development. To stop the reaction, 50 µl of 5N H2SO4 were added to each well. The enzyme-substrate reaction was determined spectrophotometrically with Microplate reader Model 550 (Bio-Rad Laboratories, Inc., Hercules, CA., U.S.A.) at 492 nm.

**Animals and emetic assay:** Healthy adult (2–5 months old) *Suncus murinus* (Nihon Clea, Tokyo, Japan), weighing 50–70 g (male) and 40–50 g (female), were used. The animals were kept in a room at a temperature of 22–25°C. The room was lighted for 12 hr from 7:00 a.m. to 7:00 p.m. Each animal was placed in a separate cage and fed on commercial *Suncus murinus* formula (Nihon Clea) and provided water ad libitum. At the time of the experiment, each animal was transferred to a transparent cage. Ten min later, 200 µl of the test samples diluted in 0.01 M phosphate-buffered saline were administered intraperitoneally into *Suncus murinus*. The animals were observed for emesis for 3 hr after intraperitoneal administration of SEA. The number of vomiting and the latency to the first vomiting episode were recorded.

**Neutralization of SEA-induced emesis with anti-SEA antibody and anti-peptide antibodies:** To test the neutralizing activities of anti-SEA antibody and anti-peptide antibodies on SEA-induced emesis in *Suncus murinus*, these anti-peptide antibody solutions were prepared with the same antibody titers to native SEA as determined by ELISA. SEA was mixed with either each anti-peptide antibody or mixture of different anti-peptide antibodies at a final concentration of 5 µg/ml. After incubation at 37°C for 1 hr, 200 µl of the mixture were intraperitoneally injected into *Suncus murinus* (16.7 µg SEA/kg body weight). Normal rabbit serum and PBS were used as the negative control.

**RESULTS**

**Reactive end-points assay of anti-peptide antibodies with SEA:** The reactivity of the anti-peptide antibodies with their respective peptides and native SEA have been reported previously [8, 9]. In this work, in order to use these anti-peptide antisera to neutralize the SEA-induced emesis in *Suncus murinus*, we further determined the reactive endpoints of the antibodies with the native SEA by ELISA (Fig. 1). The antibodies against peptides A-2, A-3, A-5, A-6, A-7, A-8 and A-9 showed higher reactive endpoints with native SEA, whereas, the antibody to A-12 (221-233) showed the lowest endpoint. To test the neutralizing activities of anti-SEA antibody and anti-peptide antibodies on SEA-induced emesis in *Suncus murinus*, these anti-peptide antibody solutions were prepared with the same antibody titers to native SEA as determined by ELISA. Thus, antibodies to peptides A-1 to A-11 were diluted to 10 × 211 –fold, whereas antibody to peptide A-12 antibody was diluted to 10 × 26-fold.

**Neutralizing ability of anti-SEA antibody and anti-peptide antibodies to SEA-induced emesis in Suncus murinus:** Since 50% emetic dose (ED50) of SEA in *Suncus murinus* by

![Fig. 1. The reactivity endpoints (dilution fold) of anti-peptide antibodies with native SEA determined by ELISA. SEA was used at the concentration 25 µg/ml. Anti-peptide antibodies (1.0 mg/ml of IgG) were diluted from 20 to 10 × 215 folds respectively. Each results represents the mean value for three experiments.](image-url)
intraperitoneal administration has been reported to be 3 µg per kg body weight [10]. We intraperitoneally injected the 100% emetic dose (16.7 µg/kg) of SEA for neutralization tests. To study which anti-peptide antibody can neutralize the SEA-induced emesis in Suncus murinus, anti-SEA and a mixture of anti-peptide antibodies were used at first. The results are summarized in Table 1. As presented in Table 1, anti-SEA antiserum specifically neutralized SEA-induced emesis. The mixture of antibodies to twelve different peptides (A-1 to A-12) showed 66.7% neutralization (4 animals neutralized/6 animals tested). On the other hand, SEA-induced emesis was not neutralized by rabbit pre-immune serum (Table 1). These results suggest that the SEA-induced emesis in Suncus murinus were specifically neutralized by some of these 12 different anti-peptide antibodies.

To further identify which anti-peptide antibody can neutralize SEA-induced emesis, mixtures of antibodies to 6 different peptides such as A-1 to A-6, A-4 to A-9 and A-7 to A-12 were used for the neutralization tests (Table 2). The mixtures of antibodies to peptides A-4 to A-9 and to peptides A-7 to A-12 showed 66.7 and 50.0% neutralization, respectively, although the mixture of antibodies to peptides A-1 to A-6 did not neutralize the emesis (Table 2). The mixture of antibodies to 3 different peptides such as A-4 to A-6, A-7 to A-9 and A-10 to A-12 were also used for further studies. As summarized in Table 2, the mixture of antibodies to peptides A-7, A-8 and A-9 showed the highest neutralization (83.3%), whereas other two antibody mixtures did not at all. On the other hand, 50% neutralization was observed with antibodies to either two different peptides such as A-7 and A-8 or A-8 and A-9, or each peptide (Table 2). No neutralization was obtained with antibodies to each of other peptides.

From these findings, the mixture of antibodies to these peptides such as A-7 (121–140), A-8 (141–160) and A-9 (161–180) is found to be more effectively to neutralize SEA-induced emesis in Suncus murinus than the mixture of those to two peptides such as either A-7 and A-8 or A-8 and A-9. Since these three peptides (A-7, A-8, and A-9) correspond to amino acid residues 121–140, 141–160, and 161–180 of SEA, respectively, the region corresponding amino acid residues 121–180 may form the neutralizing epitopes responsible for the emetic activity of SEA.

**DISCUSSION**

*Suncus murinus* (shrew mouse), which belongs to the subfamily *Crocidurinae* of family *Soricidae* (order *Insectivora*), has been described as a new animal model for research on emesis in response to various drugs [3, 27]. Agata *et al.* [1] and Shinagawa *et al*. [22] reported that *Suncus murinus* has an emetic response to administration of the HEp-2 vacuolation factor produced by *Bacillus cereus*. Recently, we have reported that *Suncus murinus* serve as a suitable animal model to detect the enterotoxigenicity of SEA and to study the relationship between the molecule structure and emetic action of SEA [10]. In the present study, we are interested in how the structure of the SEA relates to the emetic activity. The neutralization epitope of
SEA for emetic activity were examined by using variety synthetic peptides and anti-peptide antibodies. The results showed that the mixture of the three anti-peptide antibodies to A-7 (121–140), A-8 (141–160) and A-9 (161–180) gave the highest neutralization than the others, suggests that the three anti-peptide antibodies are required for neutralizing the SEA-induced emesis in Suncus murinus. From these results, the neutralizing epitope of SEA responsible for emetic activity of the toxin may consist in the three peptides regions corresponding to amino acid residues 121–140, 141–160 and 161–180. Based on the three-dimensional structure of SEA molecule [19], these regions are all located on the same domain and the same side of SEA. On the other hand, mutation analysis in SEA and use of different recombinant SEA molecules suggested that residues 47, 61 and 225 are important for emetic activity in monkey [5,7]. Although the discrepancy between two different findings can not be accounted for at the present time, this may be due to difference in three-dimensional structure between native and recombinant SEA, or there are multiple sites for emetic activity on SEA.

This is the first report of native SEA structural regions associated with emetic activity in Suncus murinus by using anti-synthetic peptide antibodies. These findings are consistent with our previous report [9] that the region corresponding to amino acid residues 161-180 was the important functional site to induce murine IFN-γ production and that the residues 121-160 contained the neutralizing epitope responsible for IFN-γ production. This is also supported by the previous findings [6] that peptide SEA (121–149) induced TNF-α and IL-1 production, and T cell proliferation, suggesting that the region of SEA corresponding to residues 121–149 plays an important role in superantigen activity. Harris and Betley [5] have examined the emetic and superantigenic activities of a number of mutant SEAs, and suggested although there is not a strict correlation between activity in the murine T cell stimulation assays and in the simian emetic assay, activity in these assays appears to be somehow related. In the present study, the mixture of three antibodies (A-7, A-8 and A-9) neutralized the SEA-induced emesis in Suncus murinus. These results indicate the regions corresponding to amino acid residues 121–180 may be the neutralizing epitope of SEA responsible for emetic activity of the toxin. Further studies will be needed to establish the relationship of superantigen active site and emetic activity on SEA.

REFERENCES


