Emetic Potentials of Newly Identified Staphylococcal Enterotoxin-Like Toxins

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Staphylococcal enterotoxins (SEs) are a common causative agent of food poisoning. Recently, many new SE-like (SEl) toxins have been reported, although the role of SEls in food poisoning remains unclear. In this study, the emetic potentials of SElK, SElL, SElM, SElN, SElO, and SElQ were assessed using a monkey-feeding assay. All the SEls that were tested induced emetic reactions in monkeys at a dose of 100 μg/kg, although the numbers of affected monkeys were significantly smaller than the numbers that were affected after consuming SEA or SEB. This result suggests that these new SEls may play some role in staphylococcal food poisoning.

Staphylococcal enterotoxins (SEs) are exotoxins that cause staphylococcal food poisoning in humans worldwide (1–4). Several classical types (SEA, SEB, SEC, SED, and SEE) have been characterized (1, 2, 5). Recently, SEG, SEH, SEI, SER, SES, and SET were identified as potential agents of food poisoning (6–10). In addition, new proteins (SE-like toxin J [SElJ], SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SElR, SElS, and SElX) with amino acid sequences that are similar to those of the above-mentioned SEs have been identified (11–19). SEls are also known to be members of the superantigen family (3, 20). During the last few decades, numerous studies have been conducted on the nature of SEs and their superantigenic activities (3, 20). However, the emetic activities of these toxins have not been studied. To better understand the etiologic nature of staphylococcal food poisoning, the emetic potentials of SEls should be evaluated in a primate model.

We demonstrated the emetic activities of SElK, SElL, SElM, SElN, SElO, SElP, and SElQ using a primate model ( cynomolgus monkeys); the number of vomiting events, the time until the first vomiting event (latency period), and behavioral changes were recorded for each animal. We compared the emetic activities of classical and new SEs as well as the activities of three large groups of SEs that were grouped according to the similarity of their amino acid sequences.

MATERIALS AND METHODS
Preparation of SEls. To construct the SElK, SElL, SElM, SElN, SElO, and SElQ expression plasmids, PCR primers were designed to amplify the gene fragment corresponding to the mature forms of these SEls (Table 1). The selk and selq genes were amplified from genomic DNA of the Staphylococcus aureus S6 strain, which harbors the sea, seb, selk, and selq genes (21). The selk gene was amplified from genomic DNA of the S. aureus bov1117 strain (harboring sea, sec, sel, sed, selj, selr, and tst-1), isolated from cow’s milk, and the selm, seln, and sele genes were amplified from genomic DNA of the S. aureus Fukuo 2 strain (21, 22). The PCR products were digested with BamHI and EcoRI or Sall and then subcloned into a pGEX6P-1 glutathione S-transferase (GST) fusion expression vector. These clones were designated pKXX (including the selk gene), pKX (including the selq gene), pKOX (including the selq gene), and pKQX (including the selq gene). The expression and purification of the GST-fused recombinant proteins and the cleavage and removal of the GST tag from recombinant SEs (rSEs) were performed by methods described elsewhere (22). Each of the resulting mature SEs had five additional amino acid residues, GPLGS, at its N terminus. The recombinant SEA with the N-terminal GPLGS has almost the same biological activity as the natural SEA, as demonstrated in our previous results on superantigenicity in mice (23, 24) and emetic activities in house musk shrews (25, 26). The protein concentration was determined using the Bradford method, as modified by Bio-Rad Laboratories (Richmond, CA), using bovine serum albumin (Bio-Rad Laboratories) as a standard. The preparation of the recombinant SEA, SEB, and SEIP has been described elsewhere (11, 21). To confirm that these SEs and SEls maintain their superantigenic activities, the inductions of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) production by rSEs and rSels in murine splenocytes were assessed using a sandwich enzyme-linked immunosorbent assay (ELISA) (27). All SEs and SEls induced the production of significant amounts of IFN-γ and TNF-α in murine splenocytes. We also examined mitogenic assay of SEs as assessed by measuring proliferation of monkey peripheral blood mononuclear cells (PBMC). All SEs were mitogenic to monkey PBMC, while no correlation between mitogenicity and the emetic activity of each SE was observed (data not shown).

Animals. Eleven cynomolgus monkeys ( Macaca fascicularis ) (females; 4 years old; body weight, 2.1 to 3.0 kg) bred at the Tsukuba Primate Research Center (Tsukuba, Japan) were used in this study. All the monkeys were individually housed in stainless steel cages at 23°C to 27°C and...
RESULTS AND DISCUSSION

Eleven cynomolgus monkeys were used for a feeding assay to assess the emetic potentials of SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, and SEIQ. We did not administer the same SE to an individual monkey to exclude influences that may be caused by giving the same toxin more than twice, except for SEA, where responses to various doses were compared. First, the monkeys were given 10 or 100 µg/kg of one of the 7 SEls (SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, or SEIQ). The results of the tests are summarized in Table 2. All 7 SEls induced vomiting responses within 1 to 4.5 h after the challenge. Vomiting responses occurred 2 to 36 times. All the SEls that were tested were confirmed to have emetic activities, and this fact suggests that these toxins may play some role in staphylococcal food poisoning. We propose that these toxins should be renamed SEs instead of SEls to toxins; the SEs were administered via a nasogastric tube without the use of an anesthetic. For most of the responses, a projectile vomiting pattern was seen. These results showed that the monkeys used in the test were susceptible to the emetic activities of SEs. Using these monkeys, the emetic potentials of the 7 SEls were then examined.

Six to eight monkeys were challenged with 100 µg/kg of one of the 7 SEls (SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, or SEIQ). The results of the tests are summarized in Table 2. All 7 SEls induced vomiting responses within 0.8 to 2.5 h after the challenge. Vomiting responses occurred 3 to 48 times (Table 2). For most of the responses, a projectile vomiting pattern was seen. These results showed that the monkeys used in the test were susceptible to the emetic activities of SEs. Using these monkeys, the emetic potentials of the 7 SEls were then examined.

The number of vomiting events, the time until the first vomiting event (latency period), and behavioral changes were recorded. To minimize the effect of previous intoxication, there was at least a 2-week interval between the intoxication experiments. Statistical analysis. All the statistical analyses were performed using JMP, version 10.0.0 (SAS Institute, Cary, NC). Differences among the groups were statistically analyzed using a one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered significant. Regression curves were drawn to visualize the relationship between the number of vomiting events and the latency period. The goodness of fit of the regression curve was evaluated using R².

Emetic assay. An emetic assay using a primate model was performed as described by Bergdoll (29) with slight modifications. Briefly, each toxin (SEA, SEB, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, or SEIQ) was dissolved in 10 ml of sterile distilled water for injection (Otsuka Seiyaku, Tokushima, Japan) and fed to cynomolgus monkeys at a dose of 10 or 100 µg/kg by nasogastric intubation without the use of an anesthetic. The monkeys were kept under continuous observation for 5 h after the oral administration of the toxin in parallel with real-time recording using a video camera. The number of vomiting events, the time until the first vomiting event (latency period), and behavioral changes were recorded. To minimize the effect of previous intoxication, there was at least a 2-week interval between the intoxication experiments.

Statistical analysis. All the statistical analyses were performed using JMP, version 10.0.0 (SAS Institute, Cary, NC). Differences among the groups were statistically analyzed using a one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered significant. Regression curves were drawn to visualize the relationship between the number of vomiting events and the latency period. The goodness of fit of the regression curve was evaluated using R².

RESULTS AND DISCUSSION

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During the present studies, emesis was observed at around 1 h times in the 10-µg/kg SEA group and a wide range of times (2 to 48) in the 100-µg/kg SEA group (Table 2). The monkey that vomited in response to the 10-µg/kg SEA dosage also vomited in response to the 100-µg/kg SEA dosage. All 4 monkeys in the SEB group exhibited vomiting responses within 0.8 to 2.5 h after the challenge. Vomiting responses occurred 3 to 48 times (Table 2). For most of the responses, a projectile vomiting pattern was seen. These results showed that the monkeys used in the test were susceptible to the emetic activities of SEs. Using these monkeys, the emetic potentials of the 7 SEls were then examined.

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During the present studies, emesis was observed at around 1 h

TABLE 1 Nucleotide sequences and predicted sizes of PCR products for the staphylococcal enterotoxin-like toxin-specific oligonucleotide primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotides sequence (5’ → 3’)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>selk</td>
<td>SEKF1</td>
<td>GGGGGATCCCAAGGCGATGTAGGTCCA</td>
<td>678</td>
</tr>
<tr>
<td></td>
<td>SEKR1</td>
<td>GGGGAATCCTTATATCGTTTCTTTAAT</td>
<td></td>
</tr>
<tr>
<td>sell</td>
<td>SELF1</td>
<td>GGGGGATCCGAAGGCGATGTAGGTCCA</td>
<td>669</td>
</tr>
<tr>
<td></td>
<td>SELR1</td>
<td>GGGGTGCACATTTGGAATTCATCTTTTT</td>
<td></td>
</tr>
<tr>
<td>seln</td>
<td>SEMF1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td>687</td>
</tr>
<tr>
<td></td>
<td>SEMR1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td></td>
</tr>
<tr>
<td>selb</td>
<td>SENF1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td>702</td>
</tr>
<tr>
<td></td>
<td>SENR1</td>
<td>GGGGTGCACATTTGGAATTCATCTTTTT</td>
<td></td>
</tr>
<tr>
<td>selo</td>
<td>SEOF1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>SEOR1</td>
<td>GGGGTGCACATTTGGAATTCATCTTTTT</td>
<td></td>
</tr>
<tr>
<td>selq</td>
<td>SEQF1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td>669</td>
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<tr>
<td></td>
<td>SEQR1</td>
<td>GGGGGATCCGATGTCGGAGTTTTGAAT</td>
<td></td>
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</table>

TABLE 2 Emetic potentials of SEIs in cynomolgus monkeys

<table>
<thead>
<tr>
<th>SEL</th>
<th>Dose (µg/kg)</th>
<th>No. of cynomolgus monkeys:</th>
<th>Latency period, h (no. of vomiting events)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA</td>
<td>10</td>
<td>10</td>
<td>1.4 (7), 2.1 (2), 3.1 (2), 3.4 (4), 3.5 (4)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7</td>
<td>1.5 (22), 1.8 (23), 1.9 (14), 1.9 (48), 2.0 (2), 3.3 (6)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>0.8 (11), 2.0 (3), 2.0 (48), 2.5 (13)</td>
</tr>
<tr>
<td>SEB</td>
<td>100</td>
<td>6</td>
<td>1.0 (4), 2.3 (2)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6</td>
<td>3.0 (5)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7</td>
<td>1.9 (3)</td>
</tr>
<tr>
<td>SEIQ</td>
<td>100</td>
<td>6</td>
<td>2.1 (6), 2.4 (7)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>2.0 (18)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6</td>
<td>2.1 (8), 3.0 (3), 3.5 (4)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
<td>1.5 (36), 4.5 (3)</td>
</tr>
</tbody>
</table>
in one animal but was observed mostly at 2 to 3.5 h after the challenge. Emesis was not observed after 5 h. Thus, we think that the Bergdoll monkey-feeding test was sufficient for the emetic assay. We then analyzed the present data combined with previous data for SER and SES (11), since we performed these experiments in parallel using the same monkeys. Figure 1 shows the vomiting frequencies using two-dimensional data descriptions for individual monkeys and toxins. Some monkeys (no. 2, 3, 7, 8, 9, and 11) responded only to the classical SEs (SEA and SEB), while other monkeys (no. 4 and 10) responded to almost all the SEs, suggesting that the emetic responses of cynomolgus monkeys vary on an individual basis. Nevertheless, the classical SEs had a stronger emetic action than the new SEs, the range of which was 10% to 50%. The classical and new SEs have also been divided into three large groups based on the similarity of their amino acid sequences: the SEA group, the SEB group, and the SEI group (11, 20). In this study, we examined the emetic potentials of new SEs belong to the SEA group (SEN, SEO, and SEP) and the SEI group (SEK, SEL, SEM, and SEQ) (Fig. 1B). The intragastric administration of 150 μg/kg of SEI reportedly induced emetic reactions in one of four rhesus monkeys (6). Furthermore, SEL (14) and SEQ (16) lacked emetic activity in a monkey-feeding assay using two pigtail monkeys. Together, these reports suggest that in this classification, the SEI group may have lower emetic action than the others. The SEI group lacks the cystine loop structure that exists in both the SEA and SEB groups and was suggested to be weakly emetic or non-emetic (6, 13, 14, 16). As described above, the SEI group has emetic activity, suggesting that the lack of the loop structure does not cause loss of emetic activity. As shown in Fig. 1B, some members of SEA group showed weak emetic activity. These results suggest that there is another reason(s) for weak emetic activity other than the lack of the loop structure.

To clarify the details of the vomiting response, we analyzed two indices (the number of vomiting events and the latency periods)

FIG 1 Vomiting frequencies using two-dimensional data description for each monkey and each SE. The monkey identification (ID) numbers are arranged vertically in order of vomiting sensitivity, and the SEs are displayed horizontally in the order of vomiting activity. (A) Classical and new SEs grouped together. (B) SEs grouped according to the similarity of their amino acid sequences. Each black square represents one individual positive for emesis. Data presented are results from a total of 11 monkeys. Data for SER and SES have been described previously (11).

FIG 2 Numbers of vomiting events induced by classical and new SEs. The data were analyzed using a one-way ANOVA. A P value of <0.05 was considered statistically significant. Data for SER and SES have been described previously (11).
according to the SE groups based on the similarity of the amino acid sequences (see Fig. S1 in the supplemental material), in individual monkeys (see Fig. S2 in the supplemental material), and according to classical and new SEs (see Fig. S3 in the supplemental material). Each SE group showed similar patterns for the two indices (Fig. S1). As discussed above, few monkeys vomited in response to SEI group toxins; however, the monkeys that vomited had similar results for the two indices (Fig. S1). The pattern of the two indices for the monkeys varied on an individual basis (Fig. S2). The patterns of the classical and new SEs also differed. The latency periods of the classical SEs seems to be shorter than those of the new SEs (Fig. S3). Furthermore, the numbers of vomiting events induced by the classical SEs were significantly higher than those for the new SEs (Fig. 2) (*P < 0.05).

During this study, we noticed that the number of vomiting events was higher if the latency period was relatively short. To visualize the relationship between the number of vomiting events and the latency period, we conducted a regression analysis between these parameters. As shown in Fig. 3a, the goodness of fit of the data supports our supposition to some degree ($R^2 = 0.143573$). Vomiting and diarrhea are known to help to remove pathogens from the digestive tract. We hypothesized that vomiting would also remove the SEs. Since the latency periods of around 1 h are exceptionally short, some of the SEs might have been removed during the vomiting (Fig. 3a, dotted circle). To test this assumption, we conducted a regression analysis after the removal of these data points, and a marked increase in the goodness of fit of the data was seen ($R^2 = 0.341961$) (Fig. 3b). These statistical analyses suggest the following two possibilities. First, if vomiting occurs relatively early, a large number of vomiting events is likely to occur. Second, early vomiting may remove the SEs from the digestive system. Further studies are needed to prove these suppositions.

Data concerning the prevalence of these new SE genes in food-derived and food poisoning-related *S. aureus* strains are growing. Many *S. aureus* strains harboring only new SE genes have been isolated from food poisoning cases (21, 31–33). This fact suggests that new SEs with relatively weak emetic activities have the ability to cause food poisoning in humans. The susceptibilities to the emetic activities of classical SEs differ among species, and humans are well known to be the species with the highest susceptibility to classical SEs (29). It seems that certain new SEs may induce emesis in humans more efficiently. Taken together, these findings suggest that the symptoms of staphylococcal food poisoning are induced by multiple classical and new SEs produced from a single *S. aureus* strain. To control staphylococcal food poisoning and to ensure food safety, the roles of new SEs as well as those of classical SEs must be considered.

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