**Contribution of the flexible loop region to the function of staphylococcal enterotoxin B**

Saeuko Yanaka¹, Motonori Kudou¹, Yoshikazu Tanaka¹, Takumi Sasaki², Sumiyu Takemoto², Atsuko Sakata², Yukio Hatatori³, Tomoyuki Koshi³, Shiro Futaki⁴, Kouhei Tsumoto¹,⁵ and Toshihiro Nakashima²,⁵

¹Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan, ²First Research Department, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan, ³Tokyo New Drug Research Laboratories, Kowa Company, Ltd, Tokyo, Japan and ⁴Institute for Chemical Research, Kyoto University, Kyoto, Japan

To whom correspondence should be addressed.
E-mail: tsumoto@k.u.tokyo.ac.jp (K.T.); nakashima-to@kaketsuken.or.jp (T.N.)

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Staphylococcal enterotoxin B (SEB), a toxin produced by *Staphylococcus aureus*, causes food poisoning and other fatal diseases by inducing high levels of pro-inflammatory cytokines. These cytokines are released from CD4⁺ T cells and major histocompatibility complex (MHC) class II antigen-presenting cells, which are activated through binding of wild-type (WT) SEB to both the MHC class II molecule and specific T-cell receptor Vβ chains. Here, we focused on a trypsin/cathepsin cleavage site of WT SEB, which is known to be cleaved *in vivo* between Lys97 and Lys98, located within the loop region. To know the function of the cleavage, an SEB mutant, in which both of these Lys residues have been changed to Ser, was examined. This mutant showed prolonged tolerance to protease cleavage at a different site compared to Thr107 and Asp108, and structural analyses revealed no major conformational differences between WT SEB and the mutant protein. However, differential scanning calorimetric analysis showed an increase in enthalpy upon thermal denaturation of the mutant protein, which correlated with the speed of cleavage between Thr107 and Asp108. The mutant protein also had slightly increased affinity for MHC. In the *in vivo* experiment, the SEB mutant showed lower proliferative response in peripheral blood mononuclear cells and had lower cytokine-induction activity, compared with WT SEB. These results highlight the importance of the flexible loop region for the functional, physical and chemical properties of WT SEB, thus providing insight into the nature of WT SEB that was unrevealed previously.

**Keywords:** differential scanning calorimetry/ flexible loop/ protease cleavage/staphylococcal enterotoxin B

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**Introduction**

*Staphylococcus aureus* is a common bacterium that resides on human skin. It is an opportunistic bacterial pathogen that causes life-threatening conditions and also easily acquires resistance to antibiotics (Holden et al., 2004; Lindsay and Holden, 2006). Staphylococcal enterotoxin B (SEB) is a protein toxin produced by *S. aureus* that is frequently associated with food poisoning and toxic shock syndrome (Dinges et al., 2000). The pathogenic activity of SEB results from its ability to induce a superantigenic response, which is due to its binding to both the specific T-cell receptor (TCR) Vβ and major histocompatibility complex (MHC) class II molecules, i.e. human leukocyte antigen-DR (HLA-DR) of antigen-presenting cells (APCs) (Dinges et al., 2000). This binding event forms a molecular bridge between the T cell and MHC II-expressing APCs. These activated T cells release a high level of pro-inflammatory cytokines (Micsun and Thibodeau, 1993; Dinges et al., 2000). Because *S. aureus* has this pathogenicity and usually resides on human skin, it is difficult to protect against *S. aureus* infections. Moreover, SEB is resistant towards heat and acid, and its potential as a biological weapon has been discussed (Alouf and Müller-Alouf, 2003). Several groups have developed attenuated vaccines for SEB by creating non-toxic SEB mutants, and the toxicity of SEB vaccines has been measured (Woody et al., 1997; Ulrich et al., 1998; Boles et al., 2003; Korolev et al., 2003; Massey et al., 2006). The aim of developing these SEB mutants was to reduce the superantigenicity of SEB by modifying the strength of binding between SEB and either the TCR Vβ chain or the HLA-DR molecules. Previous studies comparing wild-type (WT) SEB with another strong enterotoxin derived from *S. aureus*, which is staphylococcal enterotoxin C3 (SEC), suggest that the N23Y mutation may also reduce the binding affinity of WT SEB for the TCR (Leder et al., 1998). Therefore, we generated SEB with an N23Y mutation in the TCR Vβ-binding site, and N23Y had reduced activity in eliciting pro-inflammatory cytokine production (Patent WO 2005/023853 A1). However, in this study, it was revealed that N23Y-modified SEB (designated as N23Y) is sensitive to proteases, including digestive enzymes, indicating that this mutant would not be useful as a vaccine against WT SEB. Thus, it is necessary to search for other mutant candidates to develop a safe vaccine against WT SEB.

Here, we generated mutations in the loop region (Tyr94-Thr113) of WT SEB, which is normally cleaved by the digestive enzymes trypsin and cathepsin between K97 and K98 and between K238 and K239 (Spero et al., 1973). The loop region of SEB is adjacent to the HLA-DR-binding site, but has no direct contact with HLA-DR molecules (Bolin et al., 2000). We introduced mutations in the K97/K98 protease cleavage site to achieve resistance to proteases aiming not to affect the HLA-DR-binding activity, which is necessary for anti-SEB antibody production. In this process, we gained N23YK97SK98S-modified SEB (the atomic coordinates for N23YK97SK98S are available in the Protein Data Bank under accession number 3GP7), designated as N23YK97SK98S, which shows resistance to trypsin and cathepsin without loss of binding activity for HLA-DR.
In the present study, we describe the biochemical and physical properties of SEB mutants and discuss the properties of the SEB molecule that was revealed by examining this mutant.

Results

Protease resistance of N23YK97SK98S

WT SEB is cleaved by trypsin between Lys97 and Lys98 and between Lys238 and Lys239 (Spero et al., 1973). We examined the resistance of N23YK97SK98S to proteolysis by trypsin and cathepsin. Compared with WT SEB, N23YK97SK98S exhibited resistance to proteolysis between Ser97 and Ser98 (Fig. 1). Exposure to trypsin for 3 h completely digested WT SEB, whereas N23YK97SK98S remained intact. N-terminal sequence analysis revealed the trypsin cleavage site of WT SEB to be between Lys97 and Lys98. N23YK97SK98S showed similar resistance to proteolysis by cathepsin B. It is previously revealed that N23Y is resistant to trypsin (Patent WO 2005/023853 A1).

Crystal structure analysis of N23YK97SK98S

Crystal structure analysis of N23YK97SK98S (Fig. 2) was determined and compared with the crystal structure previously reported for WT SEB (Papageorgiou et al., 1998). There was no significant difference in the overall structure of WT SEB and N23YK97SK98S. Looking into the loop region near the binding site of HLA, the loop was greatly disordered in the mutant, showing its flexibility. On the other hand, the reported structure of WT SEB contains the model of the loop region. However, the residues are replaced with alanine, suggesting disorder in the electron density map. Also, in SEB–HLA complex crystal structures (Bolin et al., 2000), the loop region is disordered, and we can see that the loop region of WT is also flexible. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the crystallized sample showed that both WT SEB and N23YK97SK98S were digested into two fragments. N-terminal sequence analysis showed that the cleavage site was between Thr107 and Asp108 (data not shown).

Cleavage analysis of SEB mutants

To further examine the cleavage site in SEB between Thr107 and Asp108, we introduced the mutations T107A and D108A into N23YK97SK98S to generate N23YK97SK98ST107AD108A. We also generated the mutants H32AS34A and S34A. His32 and Ser34 are located between the N-domain (Asn31–Asp127) and C-domain (the rest of the SEB) of SEB, which might be the core residues for the stability of domains. These residues are distant from the loop region of SEB, whereas Thr107 and Asp108 are within the loop region. Together with these mutants, we also prepared WT SEB as well as the N23Y for cleavage analysis. Degradation occurred at a faster rate for the mutants H32AS34A, S34A and N23Y than for WT SEB and the mutants N23YK97SK98S and N23YK97SK98ST107AD108A (Fig. 3). The mutants H32AS34A, S34A and N23Y were almost completely degraded after 5 days at 37°C. By comparison, degradation was only slight for the mutants N23YK97SK98S and N23YK97SK98ST107AD108A under the same conditions and when kept for more than 5 days at 37°C.
Differential scanning calorimetry analysis

WT SEB and mutants were analyzed by differential scanning calorimetry (DSC; Fig. 4). Our results show that the enthalpy required for the unfolding is less for the mutants H32AS34A, S34A and N23Y, in which the core structure of SEB is modified, than for WT SEB or the mutants N23YK97SK98S and N23YK97SK98ST107AD108A, which have mutations in the loop region. The enthalpy required for the unfolding of each mutant is listed in Table I.

Surface plasmon resonance analyses

To confirm whether the SEB mutants can bind to HLA-DR, the binding affinity of WT SEB and N23YK97SK98S for HLA-DR strain B*4501 was measured by surface plasmon resonance (SPR) analysis. The calculated dissociation constants are shown in Fig. 5 (WT: $K_d = 1.00 \times 10^{-6}$ M, N23Y: $K_d = 8.55 \times 10^{-7}$ M and N23YK97SK98S: $K_d = 0.31 \times 10^{-6}$ M). The SEB N23YK97SK98S had a 3-fold higher binding affinity for HLA*B4501 than WT, and N23Y, despite the lack of mutation in the HLA binding site of this mutant.

The resonance units (RUs) are rather low, perhaps due to the sensor chip type we used. CM5 sensor chip immobilizes proteins at either Lys residue or N-terminal. SEB is rich in Lys residues near the binding area of HLA-DR. When SEB is immobilized at the Lys-rich area, this causes steric effects for HLA-DR to bind to SEB, which will decrease the amount of response achieved.

Peripheral blood mononuclear cell proliferation assay

We measured the proliferative responses of peripheral blood mononuclear cell (PBMC) to each mutant and WT SEB by analyzing [$^3$H]-thymidine uptake. All SEB molecules induced a proliferative response of PBMC in a concentration-dependent manner. When PBMCs were treated with N23Y, N23YK97SK98S and the N23YK97SK98ST107AD108A mutant SEB, proliferative responses were lower than those treated with WT SEB (Fig. 6B and data not shown). In contrast, the mutants H32AS34A and S34A induced a slightly higher proliferative response than that induced by WT SEB (Fig. 6A).

Cytokine secretion assay

We used a cytokine secretion assay to investigate the ability of WT SEB and SEB mutants to directly induce cytokine secretion from PBMC. Stimulatory activity of cytokine secretion of SEB was not completely reduced by the N23Y modification alone; however, the combination of the K97SK98S and N23Y modifications drastically reduced the secretion of the inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-12, IFN-γ, IL1-ra and GM-CSF (Fig. 7). Stimulatory activity of cytokine secretion of N23YK97SK98S and N23Y was the same level for the anti-inflammatory cytokine IL-4. Additional mutations at Thr107 and Asp108 did not alter the cytokine-inducing activity of SEB (data not shown).

Effect of SEB modifications on the production of anti-SEB antibodies

We compared the effects of different SEB mutations on the production of anti-SEB antibodies in mice exposed to SEB mutants by oral administration (Fig. 8). SEB mutants induced a higher antibody titer than did WT SEB. The mice administrated with SEB N23YK97SK98S had a higher anti-SEB antibody titer than those administrated with SEB N23Y.
Discussion

Structural analysis highlights the importance of the loop region for SEB

Structural analysis revealed no notable differences between the overall structure of N23YK97SK98S and WT SEB at the molecular level (Fig. 2). However, through crystallization and analysis of the crystals we found that the loop region of the mutant molecule was disordered and cleaved between Thr107 and Asp108. This cleavage site is different from the trypsin and cathepsin cleavage site. Cleavage of the loop region at this newly identified site does not affect the overall structure of WT SEB, because the two ends of the loops are covalently linked by disulfide bond between two cysteine residues (Papageorgiou et al., 1998). The disorder of the loop region that we observed was most likely because of structural mobility of the digested loop region. Since loop cleavage by trypsin and cathepsin changes the activity of SEB, it is possible that cleavage between Thr107 and Asp108 also changes the WT SEB activity. We crystallized WT SEB as a reference, and analysis of the crystals by SDS-PAGE revealed that the disordered loop region of WT SEB, like that of N23YK97SK98S, is also cleaved between Thr107 and Asp108. Although the mechanism underlying the cleavage of SEB between Thr107 and Asp108 is not clear, His32 and Ser34, which are in a structurally distant region, affect the susceptibility of the loop region to cleavage more than changes in the sequence of the loop region itself. The importance of the loop region for staphylococcal enterotoxins has been pointed out previously, and mutants were made to examine the effect of cysteine disulfide bond stabilizing the loop region (Grossman et al., 1991). However, the state of loop itself has not been examined so much, and further inspection is required. These results provide an insight into the condition of SEB loop region, and later experiments were conducted focusing on the state of the loop region.

Cleavage speed is related to the enthalpy required for unfolding: critical role of the flexible loop in the domain structure of SEB

To investigate the mechanism underlying the cleavage of SEB between Thr107 and Asp108, we performed cleavage and DSC analyses of several mutants including H32AS34A, S34A, N23Y, N23YK97SK98A and N23YK97SK98AT107AD108A (Fig. 1). We found that the speed of cleavage of the mutants was strongly related to the enthalpy required for unfolding. The mutants H32AS34A, S34A and N23Y, which required less enthalpy for unfolding, were cleaved faster than WT SEB and the mutants that required more enthalpy for unfolding. The mutations in H32AS34A, S34A and N23Y, which required less enthalpy for unfolding, were cleaved faster than WT SEB and the mutants that required more enthalpy for unfolding. The mutations in H32AS34A, S34A and N23Y, which are far from the loop, are at the groove of the two SEB domains. This area might be critical for WT SEB to maintain its thermal stability. Mutations at the groove of the two SEB domains may cause changes in the flexibility of the molecule and may be the reason underlying the drastic change in

Fig. 5. SPR analyses. The binding affinity of WT SEB, N23YK97SK98S, or N23Y for HLA was assessed using SPR and the $K_D$ was calculated from a Scatchard plot. See the ‘Materials and Methods’ section for detail. RU, resonance units.
thermal stability and susceptibility to cleavage of these mutants. Conversely, our results show that mutations in the loop region, which appear unrelated to the overall stability of the molecule, affect the thermal stability of SEB as a whole. N23Y and N23YK97SK98S only differ in the loop region, which would seem unlikely to change the overall stability of the molecule. However, when the K97SK98S modification was introduced into the loop region, the enthalpy change upon thermal denaturation increased and the speed of cleavage was markedly slowed. This result suggests that changing the flexibility of the loop region, which has no effect on the overall stability of WT SEB, might affect the flexibility of the two domains. Mutations in the loop regions of other proteins have also been reported to affect the protein stability, though to a less extent (Wang et al., 2005).

Correlation of thermal stability with activity

We assessed the relationship between thermal stability and activity by examining the effects of these mutants on the proliferative response of PBMC. H32AS34A and S34A do not have mutations in the MHC or TCR binding sites and induce a similar proliferative response to WT in PBMC. N23Y and N23YK97SK98S have the same mutation in the TCR binding region but have no mutations in other residues important for binding to receptors. Comparing between the mutants with no mutation in the binding site of TCR nor MHC, we assume that the similar response of WT SEB and H32AS34A, S34A is due to the activity of the cleavage between K97S and K98S. The cleaved SEBs induced similar proliferative response in PBMC. Likewise, comparing the mutants with mutation N23Y, N23YK97SK98S induced a lower proliferative response in PBMC than did the more thermally unstable N23Y. This difference might be due to the difference in the cleavage state of the loop. In summary, we assume that the proliferative response of PBMC might be related to differences in antigen activity of the SEB mutants, which was correlated with their thermal stability and, consequently, loop cleavage.

The flexible loop of staphylococcal enterotoxin B

The mutation in the loop region slightly increased the binding strength to HLA-DR

Binding affinity was 3-fold greater between N23YK97SK98S and HLA-DR than between WT, or N23Y, and HLA-DR despite the lack of mutation in the HLA-DR binding site of N23YK97SK98S. This stronger binding of N23YK97SK98S induced less proliferative response in PBMC or decreased cytokine secretion; i.e. those with greater thermal stability induce a lower proliferative response in PBMC and decreased
inflammatory cytokine production. Although many scientists have conducted mutation studies on SEBs with mutations in the TCR and HLA-DR binding sites and tried weakening their binding, there have been no studies of mutants that bind strongly to HLA-DR and bind weakly to TCR (Woody et al., 1997; Ulrich et al., 1998; Boles et al., 2003; Korolev et al., 2003; Massey et al., 2006). Our study suggests that with strong HLA-DR–SEB binding, and weakened TCR–SEB binding, cytokine production is reduced.

**PBMC proliferation assay and cytokine secretion assay**
One reason for the decreased activity of N23YK97SK98S as a superantigen is the Asn23 to Tyr modification, which was described earlier (Patent WO 2005/023853 A1; Leder et al., 1998). However, the other is the resistance to cathepsin, which was due to the K97SK98S modification, showing the importance of the loop region of SEB for the first time. It is known that cathepsin translation is induced when PBMCs are treated with WT SEB (Villadangos and Young, 2008). This implies that when PBMCs were treated with N23YK97SK98S, the cleavage pattern would be different from that of WT SEB, leading to a different reaction in vivo. Thus, cathepsin resistance appears to be important for the induction of antibody production (Mendis et al., 2005).

**Conclusion**
We have shown that N23YK97SK98S induces minimal cytokine production and blastogenesis of PBMC, while increasing antibody production. We suggest that the reduced ability of SEB mutants to induce cytokine secretion and blastogenesis of PBMC is related to their higher thermal stability, which in turn increases their resistance to cleavage. We also demonstrated that the mutation in the loop region slightly increased the binding strength to HLA-DR, which also seem to contribute to the less PBMC proliferation activity, cytokine production and antibody production. Our results suggest that unstable regions, like the loop region, are important for the thermal stability of SEB and are consistent with findings for other molecules with similar loop regions (Wang et al., 2005).

**Materials and methods**

**Mutagenesis**
The genes encoding N23Y-, H32AS34A-, S34A-, K97SK98S- and T107AD108A-modified SEBs were produced from the vector containing WT SEB using the QuikChange Multi Site-Directed Mutagenesis Kit (STRATAGENE) or KOD-Plus-Mutagenesis Kit (TOYOBO).

**Expression and purification of the mutants**
The gene encoding WT SEB or mutants constructed was cloned into the expression vector pTrc99A. This vector was transformed into BL21(DE3), which was then grown in 1 l of LB medium. When the OD 600 nm reached 0.6, gene expression was induced with 1 ml of 1 M isopropyl-β-thiogalactopyranoside. After 12 h, the culture medium was collected, and centrifuged at 8000 g for 30 min. The culture supernatant and the periplasmic fraction of the cells were collected and mixed with resin fixed with an anti-SEB antibody (SAS8-2-6 IgG; T.N., unpublished results) by stirring for 6 h. The resin was collected in an open column and then washed with 25 ml of 100 mM Tris–HCl (pH 8.0). After washing, N23YK97SK98S was eluted with 6 ml of 4 M MgCl2. N23YK97SK98S was dialyzed against 11 of 150 mM NaCl, 20 mM Tris–HCl (pH 8.0) and purified with SEC (HiLoad 26/60 superdex 75, GE healthcare Co.) using buffer consisting of 150 mM NaCl and 20 mM Tris–HCl (pH 8.0). The other mutants were expressed and purified by the same method, which is different from that described in previous reports (Coffman et al., 2002).

**Assay for resistance to proteases**
WT SEB and N23YK97SK98S were dialyzed against 0.1 M PBS (pH 7.2), and then concentrated to 0.2 mg/ml. Trypsin (0.2 μg) was added to 50-μl samples of these proteins, and the mixture was then incubated at 37°C for 3 h. The reaction mixtures were then subjected to SDS-PAGE.

**X-ray crystal structure analysis**
N23YK97SK98S was dialyzed against 0.01 M Tris–HCl (pH 8.0) and concentrated to 6.87 mg/ml. The crystallization buffer contained 10 mM Tris–HCl buffer (a mix of pH 7 and 9 at a ratio of 2:3), 18% (w/v) PEG 3350 and 0.02% sodium azide. The buffer and protein solutions were mixed at a ratio of 1:1, and then crystallized by the hanging drop method. X-ray diffraction experiments were performed at the Photon Factory (Tsukuba, Japan). The data for SEB crystals were collected on beam line BL-17A under cryogenic conditions (100 K). The structure of N23YK97SK98S was determined by the molecular replacement method using the program Phenix (Adams et al., 2002). WT SEB (Protein Data Bank code 3SEB) was used as search reference for the structure. The structural coordinates of N23YK97SK98S were modeled manually with the graphical program XtalView (McRee, 1999). The coordination file, the topology file and the parameter file were prepared with the CNS Server (available at http://cns.csb.yale.edu/v1.1/). To monitor the quality of refinement, a random 5% subset of all the reflections was set aside for the calculation of the R_free factor. The refinement of the structure was carried out with the program REFMAC5 (Murshudov et al., 1997). Finally, the water molecules were added automatically. The refinement statistics are summarized in Table S1. Supplementary data are available at PDBS online. The three-dimensional structure of N23YK97SK98S was superimposed on the structure of WT SEB with the LSQKAB program (part of the CCP4 suite of programs) (Kabsch et al., 1976). The root mean square deviations between the WT SEB and N23YK97SK98S were calculated. The resolution of SEB was 1.9 Å.

**Cleavage assay**
WT SEB and each SEB mutant were purified as described above, and then concentrated to 0.2 and 3 mg/ml in 10 mM Tris–HCl (pH 8.0). Samples (50 μl) of WT SEB and each SEB mutant were placed at 37°C for 1–5 days. After incubation, the samples were kept at −30°C to prevent further degradation. After 5 days, the samples were analyzed by SDS-PAGE.
**Differential scanning calorimetry**

We generated heat capacity curves for WT SEB and each mutant by using an ultrasensitive scanning micro-calorimeter (VP-DSC, MicroCal Inc., Northampton, MA, USA) at a heating rate of 1 K/min. The sample cell volume was ~0.4 ml. The data were analyzed using the ‘ORIGIN for DSC’ software package supplied by the manufacturer. To generate the heat capacity curves, the buffer base line was subtracted from the raw data and the concentration of each sample was standardized, using the same software package.

**Surface plasmon resonance**

The binding affinity of WT SEB and the mutants for HLA-DR strain B*4501 were investigated by SPR. HLA-DR was cloned into pET-22b (Novagen), expressed in E. coli and purified as described previously (Stöckel et al., 1994). All SPR measurements were performed at 25°C using the BIAcore-T100 apparatus (Pharmacia Biosensor AB, Uppsala, Sweden). The sensor chip used was CM5. For immobilization, we used the amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N’-3 (dimethylaminopropyl) carbodiimide and ethanolamine hydrochloride (Pharmacia Biosensor AB). The running buffer used was 10 mM HBS-EP (pH 7.4, GE Healthcare) containing 0.005% surfactant P20. The SPR signal for immobilized SEB was 10 mM HBS-EP (pH 7.4, GE Healthcare) containing 0.005% surfactant P20. The SPR signal for immobilized SEB was 10 mM HBS-EP (pH 7.4, GE Healthcare) containing 0.005% surfactant P20. The SPR signal for immobilized SEB was 10 mM HBS-EP (pH 7.4). The running buffer used was 10 mM HBS-EP (pH 7.4) containing 0.005% surfactant P20. The SPR signal for immobilized SEB was 10 mM HBS-EP (pH 7.4) containing 0.005% surfactant P20. The SPR signal for immobilized SEB was 10 mM HBS-EP (pH 7.4).

**Cell proliferation assay**

Human PBMCs were seeded into 96-well plates at 1 × 10^5 cells/well (Fig. 6A) or 2 × 10^5 cells/well (Fig. 6B). The cells were then treated with WT SEB and SEB mutants for 3 days at 0, 0.01, 0.1, 1, 10 and 100 ng/ml (Fig. 6A) or 0, 0.01, 1, 100 and 10 000 ng/ml (Fig. 6B). 1.85 kBq in Fig. 6A and 18.7 kBq/well Fig. 6B of [3H]-thymidine were added 16 h before harvest to examine the proliferative response activity. After 72 h of exposure to WT SEB and mutants, the cells were harvested with a cell harvester (Labomash) and radioactivity was measured by a liquid scintillation counter (Alokia). The proliferative response of PBMC to WT SEB and mutants was calculated as the mean values of triplicate samples from one experiment.

**Detection of cytokine secretion by PBMC**

PBMCs from healthy people were seeded onto 24-well plates at 1 × 10^6 cells/well. The cells were treated with WT SEB and mutants at 100 ng/ml for 2 days. The levels of several cytokines in the supernatant of the cultured medium, including TNF-α, IL-1β, IL-6, IL-8, IL-12, IFN-γ, IL1-ra, IL-4, IL-10 and GM-CSF, were quantified by using commercial ELISA kits (CytoSets, CytoFix, Asahi Technoglass Co.).

**Oral administration**

Female BALB/c mice (3–4 weeks old) were fed 10 μg of N23Y or N23YK97SK98S per mouse for 4 days to examine the effect of oral administration on antibody production. Blood was collected after 4 days of treatment and the anti-SEB antibody titer was measured.

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