Identification of antigenic sites on staphylococcal enterotoxin B and toxoid

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Abstract

The staphylococcal enterotoxins (SEs) are capable of causing both food poisoning and a toxic shock-like illness in man. In addition, SEs are known to act as superantigens, stimulating T-cells according to their T-cell receptor Vβ type. Relatively little is known of their antigenic determinants and how these may relate to the structure and function of the toxins. As a step in the study of these relationships, the entire molecule of SEB was synthesized in duplicate as a series of octapeptides overlapping by seven residues. This series thus represented all the potential linear epitopes of eight residues or less. The reactivity of the octapeptide series with antisera raised to purified SEB and to formaldehyde-inactivated SEB has been used to locate several antigenic sites on native SEB and to identify antigenic differences in the toxoid. Three antigenic peptides identified from the antigenic profile were synthesized and characterized. These represented amino acids 21–32, 93–107 and 202–217 of SEB. None of these peptides affected SEB-induced T-cell proliferation. However, the occurrence or absence of cross-reactivity of these peptides with antibodies to native SEB corresponds to the degree of exposure and/or the rigidity of these regions within SEB.

Keywords: Staphylococcal enterotoxin; Epitope mapping; Antigenic site; Toxoid

1. Introduction

The staphylococcal enterotoxins (SEs) are a family of serologically defined, low molecular mass proteins (26–30 kDa) produced by some strains of Staphylococcus aureus [1,2]. The SEs (designated SEA, SEB, SEC1, SEC2, SEC3, SED, SEE and SEH), commonly cause food poisoning (emesis and diarrhoea) in man, but are also implicated in the pathogenesis of a toxic shock-like illness [3–5]. Although the precise mechanisms of action of the SEs are unknown, their ability to induce T-cell activation is thought to be an important factor in toxic shock [6–8]. This interaction is dependent upon the T-cell receptor (TCR) Vβ type [6,9] and the presence of accessory cells expressing major histocompatibility complex class II molecules although the toxin does not need...
to be processed [7]. In this way, the SEs stimulate up to 20% of the total T-cell population; antigens that behave in such a way have been termed superantigens [6].

The mechanism of action of the SEs as superantigens has been clarified by the resolution of the three-dimensional structure of SEB [10] which enabled the localisation of putative MHC and TCR binding sites on the toxin [11]. This was refined by determining the three-dimensional structure of SEB bound to HLA-DR1 [12]. Similar data have been generated for toxic shock syndrome toxin-1 (TSST-1) [13] and its interaction with HLA-DR1 [14]. Evidence gained from previous studies has dissociated the emetic and mitogenic functional sites [15–17]. This includes data stating that formaldehyde-treated SEB loses its emetic and diarrhoea-inducing properties whilst retaining its mitogenic ability [18,19].

A method developed by Geysen et al. [20,21] enables the simultaneous synthesis of many peptides in a convenient form. Peptides are synthesized by the sequential attachment of F-moc N-terminally protected amino acid residues to chemically derivatized polyethylene pins. The resulting 96-pin blocks can be conveniently screened with antisera for peptide reactivity and, as the peptide-antibody complex can be...
disrupted, the peptides can be reused. It was anticipated that the definition of antigenic regions by such a method may offer clues to the whereabouts of functional domains and thus provide a basis for future studies.

Using this pin method and the published amino acid sequence [22], the entire SEB molecule was synthesized in duplicate as a series of octapeptides with seven residue overlap. In this report we outline our findings following initial mapping studies with characterized antisera raised against highly purified native SEB or formaldehyde-inactivated SEB (toxoid) (SEBT). The implications of these findings for the identification of functional sites on SEB are discussed.

2. Materials and methods

2.1. Toxin and toxoid

SEB was purified from *S. aureus* cultures as previously described [23]. Purified SEB was treated with formaldehyde at pH 7.5 for 30 days, as described by Warren et al. [19], to produce SEB toxoid.

2.2. Animals

Outbred New Zealand White rabbits (2–3 kg) were obtained from Froxfield SPF (Hampshire, UK). 6–8-week old BALB/c mice were obtained from Harlan-Olac (Bicester, UK).

2.3. Production of polyclonal antisera

Antisera to SEB were raised in rabbits by a series of 5–8 subcutaneous injections of increasing doses of toxin (2–600 μg per rabbit) over a period of approx. 2 months [24]. Freund’s complete adjuvant was used for the initial injections, booster injections of toxin were given in phosphate-buffered saline (PBS). Antisera were harvested 3–8 weeks after the final injection and stored at –20°C. Anti-toxoid sera were raised in a similar manner. The above antisera were purified using the method of Oi and Herzenberg [25]. Rabbit antiserum to SEB obtained commercially (Sigma) was employed in the experiments investigating antigenic cross-reactivity between native SEB and peptides.

2.4. Standard ELISA procedure

96-well plates (Immulon, USA) were coated with toxin or peptides synthesised in bulk (as described in Section 2.9) at a concentration of 20 μg/ml overnight at 4°C and washed once with 0.1% Tween 20 in PBS (PBS-T). The plates were blocked for 2 h with 10% FCS, 1% BSA in RPMI 1640 and washed once with PBS-T. 5-fold serial dilutions of each antiserum were prepared in PBS and added to the plate. The plates were shaken for 2 h at room temperature and washed three times with PBS-T. Horseradish peroxidase conjugated goat anti-rabbit antibody (Sigma) was added at the recommended dilution and the plates were shaken for a further 2 h, before washing three times with PBS-T. Substrate (1.3 mM H₂O₂, 0.01% 3,3’,5,5’-tetramethylbenzidine dihydrochloride (TMB), 1% dimethylsulfoxide, 0.1 M citric acid, 0.1 M sodium acetate) was added and the reaction stopped after 15 min by the addition of 50 μl of 1 M sulfuric acid. The absorbance was read at 450 nm.

To investigate the cross-reactivity of anti-SEB with synthetic peptides, anti-SEB (1/80) was preincubated with soluble native SEB (100 μg/ml) for 1 h at 37°C
prior to addition to ELISA wells coated with native SEB or with peptide.

2.5. T-cell proliferation assay

Mesenteric lymph nodes from BALB/c mice were teased apart and used as a cell population rich in T-lymphocytes. Additional antigen presenting cells (APC) were obtained from BALB/c spleens by injecting tissue culture medium and teasing them apart. To prevent the APC from proliferating, spleen cells were incubated with mitomycin C at 37°C for 30 min (25 μg per ml of cells at a density of 5–10 × 10⁶ per ml) and washed five times with medium. T-cells were incubated in 96-well flat-bottomed culture plates at a density of 2.10⁵ cells per well in complete medium (2 mM L-glutamine; 10 mM HEPES; 1% Hybri-Max antibiotic-antimycotic (Sigma); 10% foetal calf serum; 5 × 10⁻³M 2-mercaptoethanol in RPMI 1640) together with an equal density of APC. The T-cell cultures were treated with varying combinations of toxin (at a concentration of 0.5 μg/ml) and antiserum (at a final dilution of 1/40). The resulting cultures were incubated for 72 h at 37°C in 5% CO₂ in air and then pulsed with 1 μCi per well of [³H]thymidine for 18 h. The cells were harvested onto glass-fibre filter paper using an automated Skatron cell harvester and counted using an Intertechnique liquid scintillation counter.

2.6. Octapeptide synthesis

Octapeptides were synthesized on polyethylene pins according to the methods of Geyesen et al. [20,21], using an epitope mapping kit available commercially (Cambridge Research Biochemicals, Northwich, Cheshire, UK). A duplicate series of octapeptides with seven-residue overlap was synthe-
sized to encompass the entire amino acid sequence of SEB. The supplied pins incorporated a flexible protected β-alanine residue attached to the pin via hexamethylene diamine and acryl acid. Following deprotection of the β-alanine, peptide synthesis involved sequential ester coupling to the pins of Fmoc (fluorenyl methoxycarbonyl) protected L-amino acids with t-butyl side-chain protection. The amino-terminal Fmoc protection was removed after each round of addition. The completed peptides were subjected to amino-terminal acetylation and deprotection of the side-chains.

2.7. ELISA for octapeptide screening

The octapeptides bound to the pins were screened with antisera using the manufacturer’s recommended ELISA procedure. 96-well reaction trays were pre-blocked with blocking buffer (1% ovalbumin, 1% bovine serum albumin, 0.1% Tween 20, 0.01% sodium azide in phosphate-buffered saline). The pin-bound octapeptides were then placed in the reaction trays containing appropriate dilutions of test antisera (1/200 or 1/500 in blocking buffer) and incubated at 4°C overnight. The pins were washed four times in PBS-T before transfer to reaction trays containing Fab fragments of donkey anti-rabbit detection antibody conjugated to horseradish peroxidase (Amersham International) diluted in PBS-T. The pins were incubated for 1 h at room temperature on a rotary shaker and washed in PBS-T as above before transfer to reaction trays containing substrate solution consisting of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) dissolved in 0.1 M disodium hydrogen orthophosphate and 0.1 M citric acid, adjusted to pH 4. The trays were shaken at room temperature for up to 30 min to allow colour development. The reaction was halted by removal of the pins and the absorbance read at 405 nm.

Peptide-antibody complexes were disrupted between each antiserum screening using a sonication bath containing approx. 500 ml of the manufacturer’s recommended disruption buffer (1% sodium dodecyl sulfate and 0.1 M sodium dihydrogen orthophosphate adjusted to pH 7 to which 0.1% 2-mercaptoethanol was added after pH adjustment) at an initial temperature of 65°C. The pins were then washed twice for 1 min in hot distilled water (60–70°C) and submerged in gently boiling methanol for 2 min. The pins were then allowed to air dry before reuse or storage over silica gel.

2.8. Quality control

The octapeptide screening assay was tested each time by including two controls provided by the kit manufacturers. These consisted of two pins of pre-synthesized tetramers, one tetramer (PLAQ) reacts with a mouse monoclonal antibody (also provided by the manufacturers), the second tetramer (GLAQ) does not react with the mouse monoclonal antibody. In this study we also synthesized both control tetramers alongside the SEB octamers to monitor our peptide synthesis. Finally, the SEB molecule

![Graph](image-url)

Fig. 4. Reactivity of absorbed versus unabsorbed anti-SEB against the three antigenic peptides. Polyclonal anti-SEB (1/80) was preabsorbed with SEB (100 µg/ml) for 1 h at 37°C. The preabsorbed serum was then used in an ELISA with either peptide 21-32, 93-107 or 202-217 as appropriate as the coating antigen. Unabsorbed polyclonal anti-SEB was used as a control. The reactivity of anti-SEB serum against the peptides could only be absorbed by SEB in the case of peptide 93-107.
was synthesized in duplicate as octamers to reduce the risk of false positive or negative results.

The integrity of the peptide reactivity was monitored by comparing the initial screening assay with a final assay, performed after all other screening, using the same anti-SEB antiserum sample. The two profiles obtained were identical (data not shown).
2.9. Bulk peptide synthesis

Three regions of SEB (amino acids 21–32, 93–107 and 202–217) were synthesized as free peptides using an automated Applied Biosystems 431A peptide synthesizer on a 0.25 mmol scale following the manufacturer's instructions. They were synthesized onto Rink resin (4-[2',4'-dimethoxyphenyl-Fmoc-amino-methyl]phenoxy) (Novabiochem), from C- to N-terminal. A cysteine residue was attached to the N-terminal end of peptides 21–32 and 202–217 to facilitate coupling of the completed peptide to a carrier protein: peptide 93–107 had an N-terminal cysteine naturally. The peptide sequences were:

21–32: C-MENMKVLVDNHI
93–107: CYFSKKTNDNSHQT
202–217: C-PAPGDKFDQSKYLMY

The peptides were cleaved from the resin before use.

2.10. Peptide cleavage from Rink resin

The resin-attached peptides were placed on ice and the cleavage mixture added. The composition of the cleavage solution was dependent upon the amino acid content of the peptides. The cleavage solution used for peptides 21–32 and 202–217 consisted of 0.75 g crystalline phenol, 0.25 ml 1,2-ethanediol (EDT), 0.5 ml thioanisole, 0.5 ml distilled water and 10 ml trifluoroacetic acid (TFA). Peptide 93–107 was cleaved using a solution of 0.5 ml distilled water and 9.5 ml of TFA. The resin was stirred at room temperature for 90 min. The resin and peptide was filtered using TFA to rinse and the volume reduced by vacuum in a rotary evaporator. The peptide was precipitated with ether and dried. The integrity of the peptides was checked by high performance liquid chromatography, kindly carried out by Dr. C. Shone.

The solvent chosen to dissolve the peptides was dependent upon the requirements of the experiment. Both peptides 21–32 and 93–107 were dissolved in 1% acetic acid before use in the T-cell assay whereas peptide 202–217 was water soluble. In order to conjugate the peptides to keyhole limpet haemocyanin (KLH) sodium acetate buffer was used for peptides 21–32 and 93–107 whereas peptide 202–217 was dissolved in water.

3. Results

3.1. Octapeptide screening with homologous antisera

The anti-SEB antisera recognised SEB in ELISA and inhibited SEB-induced T cell proliferation (data not shown). In the octapeptide screening, reactivity between duplicate peptides was consistent and reproducible for each serum tested. Normal rabbit serum (1/200 dilution) provided a negative control profile (Fig. 1a) and an absorbance of 0.4 (3-times the magnitude of mean absorbance of the normal rabbit serum control) was designated the cut-off for positive binding. Substantial correlation was evident between the peptide recognition profiles of the three rabbit anti-SEB polyclonal sera tested (from three different rabbits), although there was some minor individual variation (Fig. 1b–d). All polyclonal anti-SEB sera were used at a dilution of 1/500.

In total, 10 antigenic regions were defined (Figs. 1 and 2). Of particular note were the strongly reactive N- and C-terminal regions and the region that correlates with the cystine loop (peptides 95–100). The profiles produced by the antisera SEB (i) and SEB (ii) were virtually identical, the only qualitative variation between them being the number of reactive peptides in the 203–209 C-terminal region. The profile of SEB (ii) differed slightly in that peptides 10–20 and 71–73 were not recognized, although binding did occur with peptides 51–54, 64–70 and 183–189.

3.2. Octapeptide screening with anti-toxoid serum

The SEB-toxoid (SEBT) retained mitogenic activity for T-cells (about 50% of that shown by native holotoxin; data not shown). For ethical reasons it was not possible to test the emetic activity of the toxoid. The antisera raised against SEBT behaved very similarly to anti-SEB in binding to SEB in ELISA and inhibiting SEB-induced T cell proliferation (data not shown). This anti-SEBT antisem showed several differences in its profile of reactivity with the SEB octapeptides compared with the antisera to SEB native toxin (Fig. 3) (both anti-SEBT and anti-SEB were used at a dilution of 1/500). In particular, the anti-SEBT did not react with any peptides in the SEB series from number 64–74 (amino acid residues 64–81; region 1), but anti-SEBT reacted strongly
with peptides 108–112 (amino acid residues 108–119; region 2) of the SEB series, unlike the anti-SEB serum. Region 2 recognised by anti-SEBT overlaps with the SE conserved region around the second cysteine of the disulfide loop (see Fig. 2).

3.3. Antigenic peptide reactivity

Three peptides were synthesised representing parts of three antigenic regions of SEB as identified by screening the octapeptide series with anti-SEB sera. These consisted of amino acids 21–32, 93–107 and 202–217; their sequences are given in Section 2. None of these peptides were mitogenic, nor did they inhibit SEB-induced T-cell proliferation (data not shown).

Rabbit anti-SEB reacted with all three peptides in ELISA (Fig. 4). Pre-absorption of the anti-SEB serum with SEB removed the antiserum's reactivity with peptide 93–107, but did not remove the activity against peptides 21–32 or 202–217 (Fig. 4). This suggested that antibodies raised against SEB holotoxin and directed against the region containing amino acids 93–107 can cross-react with the peptide representing this region. This was confirmed by purifying antibodies from rabbit anti-SEB serum on an immunoadsorbent column of peptide 93–107. These antibodies were then shown to react with both native SEB holotoxin and peptide 93–107 in ELISA (data not shown).

4. Discussion

Until relatively recently, little was known concerning the relationship between the structure and function of the SEs. As previously stated, the definition of antigenic regions may offer clues to the whereabouts of functional domains. It was anticipated that antisera raised against purified toxin would contain antibodies capable of recognizing a range of epitopes, thus defining such antigenic regions. Secondly, it had been shown previously that after treatment of SEB with formaldehyde the toxin loses its emetic and diarrhoea-inducing properties but retains its mitogenic ability, albeit at a reduced level [18,19]. It was also hoped, therefore, to map antigenic changes in the functionally modified toxoid.

The results of screening the peptide series with defined antisera were consistent and reproducible, successfully identifying the distribution of B-lymphocyte linear epitopes present on the SEB molecule. Based on our definition of reactivity we have defined 10 antigenic regions, throughout the SEB molecule, with each site potentially containing several epitopes (Fig. 2). Some of these sites may, in fact, be parts of non-linear epitopes after protein folding is taken into account. The most strongly reactive regions are present at the amino- and carboxy-terminal ends as well as the region between the cysteines.

The recent publication of the resolved three-dimensional structure of SEB [10] demonstrates that the cystine loop is exposed at the surface of the molecule and the conserved region which follows the cystine loop (amino acids 111–125 spanning the β-5 strand) forms part of the so-called α-4 groove (containing residues 113–166) on one side of the toxin (Fig. 5). This conserved region corresponds with the proposed active site for emesis (residues 113–126) [10,17]. The α-4 groove appears to be separate from the TCR and MHC-binding sites as proposed by Kappler et al. [11], and as indicated by the crystal structure of the SEB-DR1 complex [12]. This is consistent with the theory that the emetic and mitogenic functional sites on the SEB molecule are distinct. The data presented here are in agreement with the analysis of the three-dimensional structure [10], revealing both the antigenic potential of the cystine loop in SEB and identifying the conserved region on the C-terminal side of the loop as a putative emetic functional site on the toxin. Thus, the region 108–119 identified by our screening of the octapeptide series as showing differential reactivity with anti-SEBT compared with anti-SEB sera (Fig. 5a) overlaps the proposed active site for emesis [10,17]. Others have noted that the substitution of the cysteine at position 106 in SEA for alanine produced a non-emetic molecule [26].

Of the three extended synthetic peptides we analysed, peptide 202–217 represents part of the C-terminal domain of SEB, separate from the MHC and TCR binding sites, whereas peptide 21–32 contains a likely TCR binding residue and peptide 93–107 overlaps both MHC and TCR binding sites (Fig. 5b). However, none of the peptides either induced T-cell stimulation or inhibited SEB-induced T-cell stimula-
tion. This may be because these peptides, which were 12–16 amino acids in length, do not assume the correct three-dimensional conformations or, because they represent only parts of the MHC and TCR binding sites, do not interact with significant affinity. In other studies, longer peptides of SEA or SEB (27–33 amino acids) were able to block the binding of the corresponding holotoxins to MHC class II and/or inhibit SE-induced T cell proliferation [27–29]. However, some SEC1 peptides as short as 13 amino acids have been reported to have residual mitogenic activity and block T cell proliferation induced by the holotoxin [30].

The finding that antibodies raised to SEB holotoxin react with the peptides suggests that antigenic regions of the toxin can assume conformations of the peptides during the immunisation process. With regard to peptide 93–107, which represents part of the cystine loop (Fig. 5b), some of these antibodies can bind to both the synthetic peptide and to native SEB (Fig. 4). This suggests that the cystine loop in its native state and peptide 93–107 can assume similar antigenic conformations, which is consistent with the crystal structure of SEB [10] in which the cystine loop is exposed and appears relatively flexible (Fig. 5b). By contrast, antibodies in anti-SEB serum which bound to peptide 21–32 or peptide 202–217 did not bind to native (i.e. non-denatured) SEB (Fig. 4), suggesting that these regions are relatively rigid and/or concealed in the native structure which is, again, consistent with the crystal structure of SEB [10], in which parts of both of these regions have α-helical structures located between the two domains of the molecule (Fig. 5b).

In conclusion, by screening synthetic peptides with defined antisera, we have produced an antigenic map of the linear epitopes of SEB. We have provisionally defined 10 potentially important antigenic regions on the SEB molecule. Although we have not been able to identify conformational epitopes, we have provided a profile of the entire molecule of SEB, and have identified antigenic changes in the toxoid, which is known to be functionally modified. Synthetic peptides representing short sequences from within the antigenic regions designated 1, 5 and 10 were synthesized, but they failed to inhibit SEB-induced T-cell proliferation. However, the occurrence or absence of cross-reactivity of these peptides with antibodies to native SEB is consistent with their degree of exposure and/or rigidity within the three-dimensional structure of SEB.

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References


