Localisation of the mitogenic epitope of staphylococcal enterotoxin B

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Summary. Limited digestion of staphylococcal enterotoxin B (SEB) with trypsin resulted in the generation of a 12-Kda amino-terminal fragment and a 17-Kda carboxy-terminal fragment which were isolated by preparative iso-electric focusing. The carboxy-terminal fragment exhibited significant mitogenic activity for murine splenocytes, whereas the isolated amino-terminal fragment possessed little detectable mitogenic activity. Monoclonal antibodies (MAbs) specific for the carboxy-terminal fragment neutralised most of the mitogenic activity of both the intact toxin and the carboxy-terminal fragment. MAbs specific for the amino-terminal fragment had no detectable neutralising activity. These results support the hypothesis that the epitope(s) responsible for mitogenic activity is located in the carboxy-terminal region of SEB.

Introduction

The staphylococcal enterotoxins are responsible for staphylococcal food poisoning. Staphylococcal enterotoxin B (SEB) is one of a family of pyrogenic exotoxins from gram-positive organisms that are thought to contribute to toxic shock syndrome, are immunosuppressive, and exhibit significant mitogenic activity for human and murine lymphoid cells.1-8

With the possible exception of toxic shock syndrome toxin 1, these exotoxins share several structural similarities. Staphylococcal enterotoxins (SE) A-E and streptococcal exotoxin A (SpEA) have similar M, values (26-30 Kda), are basic proteins, and possess a single intrachain disulphide bridge.9-10 This disulphide bridge creates a loop structure which is common to each of these toxins. Several of these pyrogenic exotoxins have been cloned and sequenced; based on the predicted amino-acid sequence, SEC1, SEB, and SpEA appear to be the most highly conserved—68% homology for SEB and SEC1, 46% homology for SEC1, and SpEA.11-19

Previous studies directed toward the location of the active epitopes responsible for the biological activity of these exotoxins have yielded conflicting results.20-24 Most recently, amino-terminal trypsin fragments of SEA and SEC2 have been reported to possess the epitope(s) responsible for mitogenic activity.22 These results are consistent with those of a study which showed that a synthetic peptide corresponding to the 27 amino-terminal amino acids of SEA blocked the mitogenic response to this enterotoxin.23 On the other hand, a carboxy-terminal trypsin fragment of SEC1 has been reported to possess the epitope(s) responsible for both pyrogenic and mitogenic activity.24 In the present study we examined the amino- and carboxy-terminal trypsin fragments of SEB.

Materials and methods

Limited trypsin-digestion of SEB

SEB (Sigma Chemical Co., St Louis, MO, USA) 2 mg in 3 ml of 0·01 M phosphate buffer, pH 8·05, was mixed with 130 µg of N-tosyl-L-phenylalanine chloromethyl ketone (Sigma) treated trypsin and digestion was allowed to proceed at 30°C for 4 h. The reaction mixture was then passed over a trypsin inhibitor affinity column prepared with Reacti-Gel 6 × (Pierce Chemical Co., Rockford, IL, USA). The digested material was treated with 1 M dithiothreitol for 1 h at 37°C, 140 µg of iodoacetic acid was added, and the mixture was incubated under nitrogen for 1 h at 37°C. The reduced and acetylated trypsin-digested SEB (referred to as SEB-TRa) was lyophilised and stored dry at 20°C.

Isolation of trypsin fragments

The SEB-TRa was resuspended in distilled water and subjected to iso-electric focusing (IEF) in a polyacrylamide gel containing 0·63 M sucrose, 5 M urea, and ampholytes in the iso-electric point (pI) range of 3–10 (Pharmacia AB Biotechnology, Uppsala, Sweden). Focusing was done with a BioRad Model...
111 Mini IEF Cell (BioRad, Richmond, CA, USA) for 30 min at 100 V, followed by 30 min at 200 V, and then 45 min at 450 V. IEF gels were either silver stained by the method of Merrill and Goldman or gel slices were made with a razor blade and placed into 1.5-ml Eppendorf tubes in 1 ml of distilled H₂O for protein elution. Protein was eluted by rotating the tubes overnight at 4°C, separated from IEF gel material and dialysed against distilled water, and then lyophilised and stored at 20°C.

The sequence of the isolated fragments was verified over eight residues by means of the gas-phase protein sequencing facility of the Temple University School of Medicine. The sequencing suggested purity of >90%. The sequencing facility utilises the standard Edman degradation technique with the model 470A gas-phase automated sequencer (Applied Biosystems, Foster City, CA, USA) with an on-line model 120A HPLC PTH analyser.

**SDS-PAGE and Western blot analysis**

Samples were subjected to standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in polyacrylamide 15% gels according to the method of Laemmli. After electrophoresis, the gels were silver stained for protein by the method of Merril and Goldman, or the gel was used for electrophoretic transfer to nitrocellulose paper according to the method of Towbin et al., as described previously. Briefly, selected lanes of the nitrocellulose paper were used for direct protein staining with methyl green, or were probed with monoclonal or polyclonal antibody as needed. The reactivity of antibodies with bands on the nitrocellulose paper was detected by the sequential use of peroxidase-labelled anti-mouse antisera, followed by peroxidase-anti-peroxidase complex. The peroxidase activity was detected with 3,3-dimethoxybenzidine dihydrochloride (Sigma).

**Assay for mitogenic activity**

Mitogenic activity of SEB, SEB-T₁₆₀, or isolated fragments was assayed as described previously, except that the tissue culture medium used consisted of Eagle's suspension MEM supplemented with (final concentration) 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, gentamicin 50 μg/ml, 2 mM L-glutamine, FCS 10%, 0.05 mM 2-mercaptoethanol, and adenosine, uridine, cytosine and guanosine 10 μg/ml each. The assay was performed with splenocytes obtained from BALB/cAnSKH mice obtained from the breeding facility at Temple University School of Medicine. Splenocytes were cultured at a concentration of 8 x 10⁵ cells/well in a 0.2-ml final volume.

**Generation and characterisation of MAbs**

The generation and characterisation of carboxy-terminal-specific MAb 1FD7 was reported previously. This MAb is one of a group of MAbs which were generated from hybridomas produced with cells from mice immunised with native SEB. Additional MAbs were produced in the same way except for the immunising antigen. The immunising agent for each of the hybridomas used in this study is shown in table I. In each case, mice were first immunised with 15-20 μg of antigen in complete Freund's adjuvant by intraperitoneal (i.p.) injection. Mice were given booster doses of 15-25 μg of antigen in incomplete Freund's adjuvant at 4-5-week intervals. A month after the last injection, mice were given 25 μg of antigen mixed with 15 μg of LPS by i.p. injection, 4-7 days before fusion. The fusion, hybridoma cloning, selection by ELISA, isotope analysis, and generation of ascites were done as described for the previously generated carboxy-terminal-specific MAbs. All antibodies used in this study were found to be free of non-specific inhibitory activity as assessed by the response of splenocytes to stimulation by phorbol myristate acetate (PMA) in the presence of ionomycin.

One hybridoma (SP1C4G9) was obtained from mice immunised with a 15-amino-acid peptide (ESQPDPIKDELHKS) corresponding to the amino-terminal 15 amino acids of SEB. Mice were immunised with the peptide linked to ovalbumin. The peptide was synthesised in a RaMPS manual solid-phase peptide synthesising unit according to the instructions of the manufacturer (E. I. du Pont de Nemours and Co., Wilmington, DE, USA) by means of N-(9-fluorenylmethoxycarbonyl)-amino acid chemistry. The sequence of the peptide was verified by automated gas-phase protein sequencing as described above. The peptide was linked to ovalbumin by the glutaraldehyde cross-linker method as described by Maelicke et al., with a peptide:carrier ratio of 6:1.

**Neutralisation of mitogenic activity by MAbs**

The ability of various MAbs and polyclonal antisera to neutralise the mitogenic activity of SEB, SEB-T₁₆₀, or isolated fragments was determined by mixing various dilutions of antibody with designated concentrations of toxin in a final volume of 0.1 ml at 4°C. The antibodies and toxin (or toxin fragments) were dialysed against the tissue culture medium described above before use. After incubation for 2 h, the antibody and toxin mixture was added to splenocytes and the mitogenesis assay described above was initiated.

**Results**

**Limited digestion of SEB by trypsin**

Previous results from our laboratory and others showed that limited digestion of SEB with trypsin resulted primarily in the cleavage of the protein at a single site. In reducing conditions, the trypsin diges-
Fig. 1. IEF of SEB and SEB-TRA (3 µg) in water. Bands corresponding to pIs 6.5 and 5.8 were removed from a similar IEF experiment, the proteins were eluted in water and re-focused. The locations of IEF standards (cytochrome C, pI 9.6; whale myoglobin, pI 8.05; human carbonic anhydrase, pI 6.5; and β-lactoglobulin B, pI 5.1) are indicated.

Digestion yields a 17-Kda carboxy-terminal fragment and a 12-Kda amino-terminal fragment. Previous studies (verified in this laboratory) have demonstrated that the digestion takes place within the peptide loop structure following a lysine residue at position 98 in the intact toxin. The fragments continue to associate and separation appears to require denaturing reagents. Reduction and acetylation of the sulphydryl groups of the digested toxin results in the irreversible cleavage of the intrachain disulphide bridge.20,30

In an attempt to isolate the trypsin fragments, we subjected the SEB-TRA to IEF in the presence of 5 M urea. The results (fig. 1) show that native SEB focuses into a single dominant band (pI ~ 9.0), and additional minor bands that focus with apparent pIs of 8.5 and 9.4. The heterogeneity of SEB is consistent with the experience of others working with this toxin.31-34 The SEB-TRA exhibited much greater heterogeneity; after preparative IEF the individual bands were isolated and examined by SDS-PAGE (fig. 2). Most of the individual focused bands were composed of both fragments, suggesting that the fragments continue to associate to some extent even in the presence of 5 M urea. Similar heterogeneity has been observed in IEF analysis of trypsin-digested SEC.24 However, bands which focused with apparent pIs of 4.7, 5.1, 5.8 and 6.5 were found to be composed of either the 17-Kda carboxy-terminal fragment alone (pI 6.5), or of the 12-Kda amino-terminal fragment alone (pIs 4.7, 5.1 and 5.8). As expected, Western blot analysis of the fragments isolated by IEF showed that both the 17-Kda and 12-Kda fragments reacted with polyclonal anti-SEB antisera (fig. 2). Furthermore, only carboxy-terminal-specific MAbs reacted with the 17-Kda fragment isolated from the pI 6-5 band, whereas only amino-terminal-specific MAbs reacted with the 12-Kda fragment isolated from the pI 4-7, 5-1 and 5-8 bands.

Proliferative response of lymphocytes to the trypsin-digested SEB

When the capacity of SEB-TRA to induce a proliferative response in lymphocytes in vitro was

Fig. 2. Analysis of IEF-purified trypsin fragments (a) by SDS-PAGE or (b) after electrophoretic transfer to nitrocellulose, by Western blot with rabbit polyclonal anti-SEB as a probe. Lanes contained 5 µg of SEB or the non-fractionated SEB-TRA, or 2.5 µg of the IEF-purified fragments; carboxy-terminal (C) and amino-terminal (N) fragments are indicated.
examined, the mitogenic activity of SEB-T\textsubscript{RA} was roughly equivalent to that of native SEB (fig. 3). This is consistent with previously reported results\textsuperscript{20,30}.

The mitogenic activity of the carboxy-terminal and amino-terminal fragments isolated by preparative IEF was also evaluated. The mitogenic activity of the carboxy-terminal fragment was typically somewhat less than that obtained with the non-fractionated SEB-T\textsubscript{RA} (fig. 3). A modest degree of mitogenic activity was detected with the amino-terminal fragment. The weak degree of mitogenic activity could be due to contamination of the amino-terminal fragment with trace amounts of carboxy-terminal fragment or of native toxin.

Neutralisation of mitogenic activity by carboxy- and amino-terminal-specific MAbs

In previous studies with anti-SEB MAbs\textsuperscript{28}, several MAbs specific for the carboxy-terminal trypsin fragment were generated. In an attempt to generate MAbs specific for determinants on the amino-terminal fragment, mice were immunised with the amino-terminal fragment obtained by IEF. These mice were used as a source of B lymphocytes for the hybridoma fusion, and the antibody produced by the resulting hybridoma clones was assayed and characterised. Further mice were also immunised with the isolated carboxy-terminal fragment as a source of additional carboxy-terminal-specific antibodies.

Four hybridoma clones were obtained that produced antibody specific for the amino-terminal fragment by Western blotting analysis (fig. 4). Two of these, MAbs A4B6 and A3H7, typically give a strong reaction in the Western blotting assay, whereas two, A3H6 and A1G9, give a weaker reaction. Two further MAbs, (CB1B5B6 and H2G2), were also generated; they were specific for determinants present on the carboxy-terminal fragment. MAb CB1B5B6 was generated from mice immunised with the carboxy-terminal fragment and MAb H2G2 was generated from mice immunised with SEB-T\textsubscript{RA} (table I). Both MAbs typically gave a comparatively weak reaction by Western blot analysis (fig. 4).

Another MAb, SP1C4G9, was generated with cells from mice immunised with a synthetic peptide corresponding to the amino-terminal 15 amino acids of SEB. MAb SP1C4G9 did not give a detectable reaction in the Western blot analysis. However, the reaction of this antibody with SEB in an enzyme-linked, immuno absorbent assay (ELISA) was inhibited by excess synthetic peptide.

In experiments to determine the capacity of these MAbs to neutralise the mitogenic activity of SEB, carboxy-terminal-specific antibody H2G2 significantly reduced the mitogenic activity of either SEB or SEB-T\textsubscript{RA} (figs. 5 and 6). An additional carboxy-terminal-specific MAb, 1FD7, described previously\textsuperscript{28}, also significantly inhibited the mitogenic activity of both the SEB and SEB-T\textsubscript{RA}. MAb CB1B5B6 was a particularly potent inhibitor of the mitogenic activity of SEB-T\textsubscript{RA}. However, all of the amino-terminal-specific MAbs failed to exhibit any detectable neutralising activity.

In experiments to determine the capacity of the MAbs to neutralise the mitogenic activity of the isolated carboxy- and amino-terminal fragments. As expected, only the carboxy-terminal-specific antibodies 1FD7 and CB1B5B6 exhibited significant neutralising activity for the carboxy-terminal fragment (fig. 7, left panel). On the other hand, the modest degree of amino-terminal fragment mitogenic activity was neutralised by the carboxy-terminal-specific antibody 1FD7. This result is consistent with the possibility.
Table I. MAbs used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Immunising agent</th>
<th>Isotope</th>
<th>Form</th>
<th>ELISA titre</th>
</tr>
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<tbody>
<tr>
<td>lFD7&lt;sup&gt;28&lt;/sup&gt;</td>
<td>SEB</td>
<td>IgG&lt;sub&gt;1&lt;/sub&gt;/k</td>
<td>Ascites</td>
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<tr>
<td>A4B6</td>
<td>N-Frag</td>
<td>IgG&lt;sub&gt;2a&lt;/sub&gt;/k</td>
<td>Supernatant</td>
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<td>IgM/k</td>
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<td>N-Pep</td>
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<td>Ascites</td>
<td>1024</td>
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<td>H2G2</td>
<td>SEB-T&lt;sub&gt;a&lt;/sub&gt;A</td>
<td>IgM/k</td>
<td>Ascites</td>
<td>3400</td>
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</table>

N-Frag, amino-terminal trypsin fragment of SEB.
C-Frag, carboxy-terminal trypsin fragment of SEB.
N-Pep, synthetic peptide corresponding to the amino-terminal 15-amino-acid residues of SEB linked to ovalbumin.

that the weak degree of amino-terminal fragment mitogenic activity is due to contamination by trace amounts of the carboxy-terminal fragment or of native toxin.

Discussion

This report describes studies designed to locate the epitope(s) of the SEB toxin responsible for mitogenic activity; two approaches to this analysis were used. In the first, amino- and carboxy-terminal trypsin fragments were isolated by preparative IEF in urea. The results from these experiments suggested that the carboxy-terminal fragment exhibited significant mitogenic activity. On the other hand, the amino-terminal fragment exhibited weak mitogenic activity. Because the isolation of the individual fragments required the use of a strong denaturing agent (5 M urea), we cannot rule out entirely the possibility that activity for the amino-terminal fragment was lost simply because of the nature of the isolation procedure. However, the experiments with the MAbs would appear to reduce the likelihood of this explanation.

In the second approach, the neutralisation of the mitogenic activity of the toxin by a battery of amino- or carboxy-terminal-specific MAbs was examined. The results (summarised in table II) showed that all carboxy-terminal-specific MAbs neutralised the mitogenic activity of the toxin. None of the amino-terminal-specific MAbs could neutralise the mitogenic activity of the toxin or of the trypsin-digested toxin and the individual fragments. The polyclonal antisera obtained from the amino-terminal fragment-immunised mice also failed to neutralise the mitogenic activity of the native or trypsin-digested toxin. The results provide further evidence that the mitogenic epitope(s) is contained within the 99–239 residue region defined by the carboxy-terminal trypsin fragment.

There are remarkable similarities in both structure and function among the various staphylococcal enterotoxins and SpEA; therefore, it is particularly surprising that conflicting data have been reported on the location of epitopes responsible for these functions. Using proteolytic fragment or synthetic peptide activity analysis, three independent research groups have found that amino-terminal regions of SEA, SEC₁
and SEC, possess epitopes involved in mitogenic activity. Spiero and Morlock have reported results which suggest that while the amino-terminal trypsin fragment of SEC exhibits mitogenic activity, a carboxy-terminal trypsin fragment exhibits emetic activity.

These results are in contrast to more recent studies, reported by Bohach et al., that have shown that a trypsin fragment composed of the 180 carboxy-terminal amino acids of SEC possessed both toxic and mitogenic activity. Furthermore, MAbs specific for the carboxy-terminal trypsin fragment were able to partially neutralise the mitogenic activity of intact SEC. The latter investigators were unable to isolate the amino-terminal trypsin fragment of SEC from the IEF gel; consequently, this fragment could not be analysed directly for biological activity. Moreover, amino-terminal-specific MAbs were apparently unavailable for determination of neutralisation activity in the latter study. Nevertheless, our results must be viewed as being consistent with the findings of the latter investigators, particularly in the light of the extensive homology of SEB and SEC in the carboxy-terminal region.

Purification of the individual trypsin fragments was based on IEF and considerable heterogeneity was found in both SEB and SEC-TKA preparations after IEF. The IEF heterogeneity of SEB has been observed by several other investigators. We have observed three major reproducible forms in our experiments, and other reports have suggested that a fourth form is common. Two major and two minor forms of SEB have been described, and both of the major as well as one of the minor forms were found to be toxic for monkeys. The primary differences in the multiple forms of SEB are believed to be caused by the variable number of amide groups in the enterotoxin molecule. It has been suggested that amide groups are removed during electrophoresis, during fermentation, or during purification procedures. Chesbro et al. found that heterogeneity in SEB arises during growth of the toxin-producing culture, in various culture conditions.

Table II. Neutralisation of mitogen activity of SEB by carboxy- or amino-terminal-specific MAbs and polyclonal antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody specificity</th>
<th>Neutralisation of mitogen activity</th>
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</thead>
<tbody>
<tr>
<td>MAb</td>
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<td></td>
</tr>
<tr>
<td>1FD7</td>
<td>COOH-Term</td>
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<td>2GD9</td>
<td>COOH-Term</td>
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</tr>
<tr>
<td>2DA3</td>
<td>COOH-Term†</td>
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</tr>
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<td>2HA10</td>
<td>COOH-Term†</td>
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</tr>
<tr>
<td>2EG5</td>
<td>COOH-Term†</td>
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</tr>
<tr>
<td>H2G2</td>
<td>COOH-Term</td>
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</tr>
<tr>
<td>CB1B5B6</td>
<td>COOH-Term</td>
<td>Yes</td>
</tr>
<tr>
<td>A4B6</td>
<td>NH2-Term</td>
<td>No</td>
</tr>
<tr>
<td>A1G9</td>
<td>NH2-Term</td>
<td>No</td>
</tr>
<tr>
<td>A3H6</td>
<td>NH2-Term</td>
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</tr>
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</tr>
<tr>
<td>SP1C4G9</td>
<td>NH2-Term†</td>
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<td>Anti-SEB</td>
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</tr>
<tr>
<td>Polyclonal</td>
<td>Anti-NH2-Term</td>
<td>No</td>
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</table>

† Specificity determined with partial-papain digestion products.
‡ Specificity is for N-terminal 15 amino acids.
in a pattern which correlated with the growth phase of the organism.

Recent reports indicate that the mitogenic activity of SEB is based on two functional activities. First, SEB (as well as other staphylococcal enterotoxins) is believed to bind specifically to MHC class II molecules in both mouse and man. Following this interaction, SEB stimulates virtually all TcR<sup>αβ</sup>-bearing T cells that express certain, but not all, V<sub>β</sub> alleles. It is possible that the binding of the class II molecule may be the function of one epitope, while the interaction with the TcR<sup>αβ</sup> heterodimer may be the function of a distinct epitope. Our results indicate that the carboxy-terminal trypsin fragment contains all essential epitopes required for mitogenic activity.

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References

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