CROSS REACTIONS BETWEEN TRYPTIC POLYPEPTIDES OF STAPHYLOCOCCAL --ETC(U)

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CROSS-REACTIONS BETWEEN TRYPsic POLYPEPTIDES OF STAPHYLOCOCCAL
ENTEROTOXINS B AND C

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**Abstract:**
See other side.
The strong cross-reactions demonstrated for staphylococcal enterotoxins B (SEB) and C (SEC) by measurement of antigen-binding capacity (J. Immunol. 120:86, 1978) were reflected in well-defined polypeptides obtained by limited tryptic digestion from SEB and SEC (J. Biol. Chem. 248:7289, 1973; 251:5580, 1976). Two antigenic determinants on each enterotoxin were capable of reacting with heterologous antibody, one on the first 57 amino acids and one on the last 150 residues of the polypeptide backbone. The larger, carboxyl terminal polypeptides bound efficiently to homologous antiserum but about two orders of magnitude less efficiently to heterologous antibody. The amino terminal peptides showed only weak homologous binding but nearly comparable heterologous binding.

It is proposed that the determinant on the amino terminal polypeptides is largely responsible for the strong reciprocal binding of the intact enterotoxins and that their low antigen-binding capacity is due to a random or a structurally distorted conformation in solution.
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SUMMARY

The strong cross-reactions demonstrated for staphylococcal enterotoxins B (SEB) and C1 (SEC1) by measurement of antigen-binding capacity (J. Immunol. 120:86, 1978) were reflected in well-defined polypeptides obtained by limited tryptic digestion from SEB and SEC1 (J. Biol. Chem. 248:7289, 1973; 251:5580, 1976). Two antigenic determinants on each enterotoxin were capable of reacting with heterologous antibody, one on the first 57 amino acids and one on the last 150 residues of the polypeptide backbone. The larger, carboxyl terminal polypeptides bound efficiently to homologous antiserum but about two orders of magnitude less efficiently to heterologous antibody. The amino terminal peptides showed only weak homologous binding but nearly comparable heterologous binding. It is proposed that the determinant on the amino terminal polypeptides is largely responsible for the strong reciprocal binding of the intact enterotoxins and that their low antigen-binding capacity is due to a random or a structurally distorted conformation in solution.
The staphylococcal enterotoxins have been classified on the basis of serologic individuality. However, cross-reaction between types A and E has been demonstrated (1) and clearly the lack of cross-reaction does not preclude the presence of common antigenic determinants among the various types. We have described a strong reciprocal reaction between SEB² and SEC₁ with each other's antibody by measurement of antigen-binding capacity (2).

SEB and SEC₁ undergo limited specific digestion by trypsin (3, 4). The primary cleavage in both instances occurs at a site on the polypeptide chain interior to the single disulfide loop of these proteins. A secondary cleavage also occurs, which for SEC₁ goes rapidly to completion. Singly cleaved enterotoxin B, SEB-T, and the doubly cleaved enterotoxin C₁, SEC₁-T₂, may be represented in linear form. The indicated molecular weights of the SEC₁-T₂ polypeptides are known precisely (3) but since the amino acid sequence of SEC₁ has not been established, the molecular weights associated with the SEC₁-T₂ polypeptides are the values calculated from amino acid analysis (4):

SEB-T

```
SEB-11,500
```

```
Glu Lys S-S Thr Lys
```

```
SEB-17,000
```

```
SEC₁-6,500
```

```
Glu Lys S-S Asx Gly
```

```
SEC₁-4,000
```

```
SEC₁-2,000
```

```
SEC₁-19,000
```

```
Val Gly
```

```
SEC₁-19,000
```

```
SEC₁-22,000
```

```
SEC₁-2,000
```
SEB-11,500 and SEB-17,000 have been shown to contain antigenic sites for antibody to SEB (5). Similarly SEC₁-6,500 and SEC₁-22,000 (and its constituent fragment, SEC₁-19,000) possess determinants for antibody to SEC₁ (6). SEC₁-4,000 does not react with antibody to native SEC₁ (6). We have now investigated the cross-reaction of these peptides with antiserum to the heterologous enterotoxin; the presence of two common binding sites on structurally analogous polypeptides from widely separated regions of the molecules is reported here.

MATERIALS AND METHODS

Preparation of enterotoxins and tryptic peptides. The enterotoxins were produced and isolated by published methods as modified in our laboratory (3, 4). All the polypeptides were isolated by repeated gel chromatography on Sepharose 6B in 6 M guanidine hydrochloride, SEC₁-6,500 and SEC₁-22,000 directly from SEC₁-T₂, and SEB-11,500 and SEB-17,000 from SEB-T and SEC₁-4,000 and SEC₁-19,000 from SEC₁-22,000 after reduction and alklylation with iodoacetamide. By polyacrylamide gel electrophoresis in sodium dodecyl sulfate all the polypeptides were essentially homogeneous. Stable solutions of all the peptides were obtained by dilution of their concentrated solutions in 6 M guanidine hydrochloride into 10% bovine serum albumin.

Preparation of antisera. Antisera to SEB and SEC₁ were prepared by intracutaneous injection without adjuvant in New Zealand white rabbits.³ A regimen based on that developed by Silverman was employed (7). Pools were made from those sera giving identical Ouchterlony titers. Rabbit antiserum for SEC₁-22,000 was produced by intramuscular inoculation of three 100-μg doses of polypeptide at weekly intervals.
in 10% rabbit serum albumin. Most of the serum used in this study was from a bleeding of a single rabbit made one week after a second course of immunization administered 3 months after the first series of injections. The specific immunoglobulin content of these antisera was determined by the antigen-binding capacity assay assuming that in antigen excess all of the antigen was present as $A_gA_b$: anti-SEC, 0.66 mg/ml; anti-SEC$_1$, 0.46 mg/ml; anti-SEC$_2$, 22,000, 0.073 mg/ml.

**Labeling of enterotoxins and tryptic polypeptides.** All labeling was carried out by the gaseous diffusion method of Cruber and Wright (8) with $^{125}$I. Because some of the polypeptides had very poor solubility in aqueous buffers all of them were dissolved in 6 M guanidine hydrochloride during labeling. Unbound radioisotope was removed by dialysis against 6 M guanidine hydrochloride. The concentrations of the labeled enterotoxins and polypeptides were determined from their absorbance at 277 nm. Where extinction coefficients have not been determined experimentally they were calculated from the tyrosine and tryptophan content of the peptides.

**Antigen-binding capacity assay.** A modification of the Farr technique (9) based on the ability of protein A-containing strains of *Staphylococcus aureus* to react specifically and with high affinity with the Fc portions of IgG was employed (10). Labeled antigen (20 to 100 ng in 500 μl) was added to 500-μl volumes of twofold serially diluted antiserum in phosphate buffered saline containing 0.5% bovine serum albumin. Dilution of the labeled enterotoxins was made in the same diluent but the 6 M guanidine hydrochloride solutions of the labeled polypeptides were diluted with a 10% albumin solution in buffered saline. To separate bound from unbound antigen a 10% suspension
of the Cowan I strain of *S. aureus* was added. For serum dilutions of 1:40 and above, 100 μl of the 10% suspension were adequate to precipitate all the immunoglobulin but for dilutions of 1:10 and 1:20, 500 μl were required and for 1:5 dilutions of serum a full milliliter was needed. The reaction tubes were treated and the calculation of percentage of antigen bound to antibody was made as previously described (2). In the calculations a correction was made for nonspecific precipitation by the bacterial cell suspension.

Fifty percent endpoints were estimated from log-log plots of the volume of antibody against the percent of antigen bound. These graphs were linear from about 20 to 80% bound and had nearly identical slopes. This latter property facilitated the endpoint estimation when an extrapolation from the data was required.

**Circular dichroic spectra.** A Jasco J-40 instrument equipped with a data processor was used to obtain circular dichroic spectra. The polypeptides were prepared for analysis by dialysis of concentrated solutions in 6 M guanidine hydrochloride against repeated changes of large volumes of phosphate-buffered saline. Sixty-four repetitive scans were run using the data processor at a time constant of 4 s and with a resolution of 0.2 nm/step.

**RESULTS AND DISCUSSION**

Binding curves for the tryptic polypeptides with antiserum to SEB are presented in Figure 1 and with antiserum to SEC in Figure 2. It is quite evident by comparison with the binding curves of the intact enterotoxins that the binding capacity of the larger fragments, SEC – 22,000, SEC – 19,000 and SEB – 17,000, for each homologous antiserum is
very strong. Nearly complete precipitation of label was readily achieved at high dilutions of the antisera. Homologous reactions of the smaller polypeptides and heterologous reactions of all the polypeptides are considerably weaker. Indeed, to demonstrate binding it was necessary in several instances to reduce the concentration of labeled antigen some four- to fivefold and to increase the volume of antiserum added up to 100 μl/ml. The data in the figures have been normalized to equal amounts of labeled antigen; this is based on the assumption that over the range of antigen employed the ratio of antibody to antigen at the endpoint is independent of antigen level.

In addition to the binding curves shown in the figures, binding capacity was measured for all the tryptic polypeptides and for the two intact enterotoxins to antisera to SEB to SEC1 and to SEC1-22,000. When the 50% endpoint values were converted to molar antibody to antigen ratios the values shown in Table I were obtained. In these calculations the simplifying assumption was made that all the antigen bound at the endpoint was in the form Ag₂Ab (11) so that for bivalent antibody the molar ratio of antigen to antibody was 4. Efficacy of binding is inversely proportional to the tabular values. The most striking observation is that there are significant heterologous interactions and that the reciprocal reaction between the whole enterotoxins with each other's antiserum is reflected in their constituent polypeptides. Consider first the large polypeptides from the carboxyl terminal end of the amino acid backbone and comprising more than half the enterotoxin molecules. The 17,000 Mr polypeptide from SEB binds to anti-SEC₁ and the 22,000 Mr polypeptide and the 19,000 Mr polypeptide from SEC₁ bind to anti-SEB. The molar ratios of antibody to antigen of the
latter two polypeptides at the 50% endpoint are in the same ratio to
each other for all three antisera suggesting that the cross-reacting
determinant of SEC<sub>1</sub>-22,000 is contained totally within SEC<sub>1</sub>-19,000.
This is supported by the lack of binding of SEC<sub>1</sub>-4,000 to either
anti-SEB or to anti-SEC<sub>1</sub>. (The binding of SEC<sub>1</sub>-4,000 to anti-SEC<sub>1</sub>-
22,000 is attributed to a response to a non-native determinant in that
antiserum [6]). The superior binding capacity of SEC<sub>1</sub>-22,000 may be
ascribed to a higher degree of refolding to a native-like conformation.

Each of these three carboxyl terminal polypeptides binds about
two orders of magnitude more efficiently to its homologous antiserum
than to the heterologous antiserum. Thus the relative values were:
214 for SEC<sub>1</sub>-22,000 (96/0.44); 191 for SEC<sub>1</sub>-19,000 (287/1.5); and
102 for SEB-17,000 (224/2.2). A comparison of the binding of the
structurally analogous polypeptides from the two enterotoxins, SEC<sub>1</sub>-
19,000 and SEB-17,000, with the same antiserum yields similar estimates
of binding efficacy. With anti-SEC<sub>1</sub>, SEB-17,000/SEC<sub>1</sub>-19,000 was 149
(224/1.5) and with anti-SEB, SEC<sub>1</sub>-19,000/SEB-17,000 was 130 (287/2.2).
These crude estimates show quite clearly that an antigenic determinant
exists on SEC<sub>1</sub>-19,000 and SEB-17,000 which, although capable of reacting
with heterologous antiserum, is not functionally identical. It is
probably also reasonable to infer that they differ structurally in the
native enterotoxins.

The very excellent binding of SEB-17,000 to the antiserum to
SEC<sub>1</sub>-22,000 was unexpected. The molar ratio was even lower than with
the homologous antiserum (1.6 with anti-SEC<sub>1</sub>-22,000 and 2.2 with anti-
SEB). This must reflect reaction with an antibody population arising
from non-native determinants on SEC<sub>1</sub>-22,000. It also suggests that
SEB-17,000 and SEC₁-22,000 possess regions of common structure in addition to that responsible for the common determinant described above.

The binding capacity of the amino terminal polypeptides, the 6,500 $M_r$ polypeptide from SEC₁ and the 11,500 $M_r$ polypeptide from SEB was very much weaker than the larger carboxyl terminal polypeptides even to the homologous antiserum. However, the relative binding efficacy with heterologous antiserum was much greater than that observed with the larger polypeptides. The value for SEC₁-6,500 was only 4.1 (329/81). Similarly a comparison of the binding of the two smaller polypeptides with anti-SEB, SEC₁-6,500/SEB-11,500 was 1.7 (329/189). This suggests a high degree of similarity in the structure of this determinant in SEB and SEC₁, which is presumably contained in the first 57 amino acid residues of the amino acid sequence.

There was one apparent anomaly in these observations, the failure to find binding of SEB-11,500 to anti-SEC₁. To test the possibility that this was due to inadequate or improper folding of labeled SEB-11,500 upon dilution into 10% bovine serum albumin, the technique used for all the peptides in this study, several other methods of renaturation were attempted. In all but one there was no significant change. However, when the labeled antigen in 6 M guanidine hydrochloride was dialyzed directly against phosphate buffered saline a remarkable result was obtained (Table II). The affinity to anti-SEB was increased 80-fold and, where no reaction had previously been found with the anti-SEC₁-22,000 or with anti-SEC₁, excellent binding was now seen. We attribute this enormous change largely to aggregation of the antigen. This effect was reported earlier (5) and is attested to here by an increase in nonspecific precipitation with the $S. aureus$ cells to about 40% of
the total radioactivity. The binding to the antiserum to the 22,000 M
polypeptide may again be due to the presence of an unfolded determinant
in SEB-11,500. A similar effect was noted with SEC₁-4,000 (6) and it
should be added that the carboxyl terminal sequence of SEB-11,500 is
probably homologous with SEC₁-4,000. The essential point is that this
experiment demonstrates the heterologous binding of SEB-11,500 to
enterotoxin C₁ antiserum.

Thus we have demonstrated the presence of two antigenic determinants
on SEB and SEC₁ capable of reacting with heterologous antibody. It is,
however, difficult to reconcile the weak heterologous binding of the
larger polypeptides and the weak homologous binding of the smaller amino
terminal peptides with the extremely high binding capacity of the intact
enterotoxins for heterologous antibody. Only four times the amount of
antibody is required to give equivalent binding with the heterologous
antigen as with the homologous antigen for both enterotoxins (2).

One may invoke the concept of a native format determinant proposed
by Sachs et al. (12). All the components of the determinant may be
present in a limited length of the polypeptide chain but may exist in
solution in equilibrium among a variety of random conformations. One
factor in the overall binding constant is a conformational equilibrium
constant. The larger polypeptides would appear to have a high
conformational constant. They bound very well to the homologous antibody;
the strong binding of SEC₁ to antiserum raised against SEC₁-22,000 is
also supportive of this contention, it being well established that
antibodies elicited by immunization with denatured proteins either
fail to react, or do not react extensively, with the native protein (13).
Moreover, we have shown that the circular dichroic spectra in the far
ultraviolet of the 22,000 $M_r$ polypeptide and the 19,000 $M_r$ polypeptide from SEC$_1$ are similar to that of the native enterotoxin (6) indicating significant refolding to a native-like conformation. Conversely the CD spectrum of SEC$_1$-6,500 indicated it to be in a random chain conformation (6) and this fragment would therefore have a comparatively low conformational constant accounting for its weak antigen-binding capacity.

The CD spectra of the SEB tryptic polypeptides (Figure 3) are not, however, consistent with this simple explanation. Both SEB-11,500 and SEB-17,000 have a sufficiently stable folding to provide spectra indicative of significant secondary structure. Furthermore, these spectra differ from those of the parent enterotoxin. The weak binding of SEB-11,500 and the good binding of SEB-17,000 may not then be interpreted in terms of conformational constants but perhaps as a reflection of the correctness of folding, the determinant of the larger polypeptide being in a relatively native conformation and SEB-11,500 having its determinant in a distorted conformation.

It is suggested that the weak heterologous binding of the larger polypeptides is due to an actual compositional difference in the SEB and SEC$_1$ determinants. The weak homologous binding of the amino terminal polypeptides is considered to be due to a random conformation for SEC$_1$-6,500 and to an incorrect folding for SEB-11,500. Finally, since the ratio of binding capacity of SEC$_1$-6,500 with anti-SEB compared to anti-SEC$_1$ is only 1:4.1 and the ratio for SEB-11,500 with anti-SEC$_1$ compared to anti-SEB is only 1:7.1, it is proposed that the determinant on these fragments is primarily responsible for the excellent heterologous binding in the intact enterotoxins.
REFERENCES


### TABLE 1

**Binding of enterotoxin B and C₁ and tryptic peptides derived from them with antisera**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Anti-SEC₁-22,000</th>
<th>Anti-SEC₁</th>
<th>Anti-SEB</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC₁</td>
<td>0.57</td>
<td>0.25ᵇ</td>
<td>1.0</td>
</tr>
<tr>
<td>SEC₁-6,500</td>
<td>NBE</td>
<td>81ᵈ</td>
<td>329ᵈ</td>
</tr>
<tr>
<td>SEC₁-22,000</td>
<td>0.25ᵇ</td>
<td>0.44</td>
<td>94ᵈ</td>
</tr>
<tr>
<td>SEC₁-19,000</td>
<td>1.2</td>
<td>1.5</td>
<td>287ᵈ</td>
</tr>
<tr>
<td>SEC₁-4,000</td>
<td>4.3ᵈ</td>
<td>NBE</td>
<td>NBE</td>
</tr>
<tr>
<td>SEB</td>
<td>6.6</td>
<td>1.0</td>
<td>0.25ᵇ</td>
</tr>
<tr>
<td>SEB-11,500</td>
<td>NBE</td>
<td>NBE</td>
<td>189ᵈ</td>
</tr>
<tr>
<td>SEB-17,000</td>
<td>1.6</td>
<td>224ᵈ</td>
<td>2.2</td>
</tr>
</tbody>
</table>

ᵃData for SEC₁ peptides against anti-SEC₁-22,000 and anti-SEC₁ are from our report (6).

ᵇFixed by computational assumptions.

cNo binding evident.

dEndpoint estimated by linear extrapolation of log-log plot.
**TABLE II**

Effect of method of renaturation upon binding of SEB-11,500 to antisera

<table>
<thead>
<tr>
<th>Method of Renaturation</th>
<th>Anti-SEC&lt;sub&gt;1&lt;/sub&gt;-22,000</th>
<th>Anti-SEC&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Anti-SEB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution in 10% bovine serum albumin</td>
<td>NBE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NBE</td>
<td>189&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dialysis against phosphate buffered saline</td>
<td>1.8</td>
<td>17</td>
<td>2.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>No binding evident.

<sup>b</sup>Endpoint estimated by linear extrapolation of log-log plot.
FOOTNOTES

1 The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

2 Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; SEC, the variant of staphylococcal enterotoxin C with the more alkaline isoelectric point.

3 In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

4 The absence of cross-precipitation precludes the possibility of three cross-reacting determinants.
LEGENDS FOR FIGURES

Figure 1. Binding of $^{125}\text{I}$-labeled enterotoxin B and $^{125}\text{I}$-labeled polypeptides derived from enterotoxin B and enterotoxin C₁ by tryptic digestion to rabbit antiserum to enterotoxin B. The data have been normalized to equal amounts of labeled antigen. The curve for enterotoxin B is a composite of a large number of experiments.

- (◊) SEB-17,000;
- (□) SEC₁-22,000;
- (☐) SEC₁-19,000;
- (◇) SEB-11,500;
- (△) SEC₁-6,500.

Figure 2. Binding of $^{125}\text{I}$-labeled enterotoxin C₁ and $^{125}\text{I}$-labeled polypeptides derived from enterotoxin C₁ and enterotoxin B by tryptic digestion to rabbit antiserum to enterotoxin C₁. The data have been normalized to equal amounts of labeled antigen. The curve for enterotoxin C₁ is a composite of a large number of experiments.

- (□) SEC₁-22,000;
- (☐) SEC₁-19,000;
- (△) SEC₁-6,500;
- (◇) SEB-17,000.

Figure 3. Circular dichroic spectra in the far-ultraviolet of enterotoxin B and the polypeptides derived from it by trypsin digestion. SEB was analyzed in a 0.1-mm cell at 1.09 mg/ml; SEB-11,500 and SEB-17,000 were run in a 0.5-mm cell at concentrations of 0.25 mg/ml.