CROSS REACTION IN REVERSED PASSIVE HEMAGGLUTINATION BETWEEN CLOSTRIDIUM BOTULINUM TYPE A AND B TOXINS AND ITS AVOIDANCE BY THE USE OF ANTI-TOXIC COMPONENT IMMUNOGLOBULIN ISOLATED BY AFFINITY CHROMATOGRAPHY

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SUMMARY: Passive hemagglutination and agar gel double diffusion tests demonstrated cross reactions between Clostridium botulinum type A and B toxins to a high level and between types E and F and types A and F to a much lower level. The cross reactions between types A and B and types A and F were due to the nontoxic component; that between types E and F to both the toxic and nontoxic components.

Anti-toxic component immunoglobulins types A and B were isolated from anti-progenitor toxin of the corresponding types by affinity chromatography. Reversed passive hemagglutination tests with formalinized sheep red blood cells coupled with anti-toxic component immunoglobulins type A or B caused no cross reaction between these types and detected 8-89 LD50/ml of type A and 10-59 LD50/ml of type B toxins in either a crude or purified form.

DIRECT HEMAGGLUTININ possessed by type A and some type B toxins did not disturb the reversed passive hemagglutination tests.

INTRODUCTION

Besides the mouse injection method, attempts have been made to detect Clostridium botulinum toxins in food and other specimens by various immunological methods including passive (PHA) (Yafaev and Chepelev, 1961) and reversed passive hemagglutination (RPHA) (Johnson et al., 1966). Although little or no cross reaction was observed between type E and any other types (unpublished data), considerable cross reactions were noticed between types A and B (Yafaev and Chepelev, 1961; Johnson et al., 1966).

By using the dissociated toxic and nontoxic components (abbreviated to TC and NC, respectively) (Lamanna and Sakaguchi, 1972), we re-examined cross reactions among type A, B, E and F toxins by agar gel double diffusion (AGD) and PHA tests with highly potent antitoxins prepared with highly purified progenitor toxin (PT) of each type. These tests demonstrated common antigenicity possessed by NC of types A and B and also that to a far less extent possessed by NC of types A and B.
E and types A and F, and by both TC and NC of types E and F. To avoid such cross reactions in RPHA, it appeared essential to use sheep red blood cells (SRBC) coupled with monospecific anti-TC of at least types A and B. This could be accomplished either by immunizing the animal with completely pure TC of each type, or by removing anti-NC from anti-PT, which may always contain both antibodies, anti-TC and anti-NC, in comparable amounts (Sakaguchi and Sakaguchi, 1973). The second alternative seemed more feasible for us, since highly potent horse anti-PT against highly purified PT of each type was being produced commercially.

The present report deals with the cross reactions occurring among type A, B, E and F toxins in AGD, PHA and RPHA tests, isolation of anti-TC from anti-PT of types A and B by affinity chromatography, and the specificity and sensitivity of the RPHA test using SRBC coupled with purified anti-TC of type A or B.

**MATERIALS AND METHODS**

*Toxins:* Type A crystalline toxin was provided by courtesy of Dr. E. J. Schantz, Food Research Institute, University of Wisconsin, Madison, Wisconsin.

Highly purified type A toxin was obtained from cultures of *C. botulinum* type A strain Hall (provided by Dr. E. J. Schantz) and strain 97 (given from the Institute of Medical Science, Tokyo University, Tokyo, in 1951). The organisms were grown in glucose-yeast extract-peptone medium (pH 7.0) for 4 days at 30°C. The toxin was purified by the following sequential steps: acid precipitation at pH 3.5; extraction with 0.075 M acetate buffer (pH 6.5) containing 1.0 M sodium chloride (Lamanna, Eklund and McElroy, 1946); protamine treatment to remove RNA contained in such a large quantity; precipitation at 40% saturation of ammonium sulfate; SP-Sephadex (C-50) (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography at pH 3.8; and Sephadex G-200 (Pharmacia Fine Chemicals) gel filtration at pH 6.8 or 3.8. In gel filtration, type A toxin was eluted in two peaks; the early eluted toxin, containing 1–2 × 10⁸ LD₅₀/mg N, was named large toxin (L-toxin) and the late eluted toxin, containing 7 × 10⁸ LD₅₀/mg N, medium toxin (M-toxin). The elution position of M-toxin corresponded to that of about 12 S. The term "PT" in this report refers to a preparation containing both L- and M-toxins for both types A and B.

Type B toxin was purified from cultures of *C. botulinum* type B strains Okra and QC (both given by Dr. H. Iida, Hokkaido University School of Medicine, Sapporo, in 1969) grown under similar conditions as for type A cultures by the following sequential steps: acid precipitation at pH 4.0; extraction with 0.2 M phosphate buffer, pH 6.0; precipitation again by dialysis against 0.05 M acetate buffer, pH 4.2; extraction with the same buffer containing 0.5 M NaCl; protamine treatment to remove RNA, percolation through an SP-Sephadex column equilibrated with the same buffer to remove the excess protamine; SP-Sephadex chromatography at pH 4.2; and Sephadex G-200 gel filtration. The final gel filtration also gave two toxin peaks for either strain. Both toxins of either strain were activable with trypsin at pH 6.0; the activation ratio was much larger for QC toxin than for Okra toxin. The early eluted toxin, named L-toxin, contained 4 × 10⁸ potential LD₅₀/mg N and the late eluted one, named M-toxin, 7–9 × 10⁸ potential LD₅₀/mg N. The elution position of M-toxin coincided to that of type A M-toxin and also of type E PT, whose molecular weight was estimated to be 350,000 and sedimentation constant 11.6 S (Kitamura, Sakaguchi...
Type E toxin was purified from the bacterial cells of *C. botulinum* type E strain 35396 (given by Dr. K. F. Meyer, G. W. Hooper Foundation, University of California, San Francisco, California, in 1951) by the methods reported by Kitamura et al. (1968). The preparation contained 0.5–0.8×10^8 potential LD₅₀/mg N.

Type F toxin was purified from *C. botulinum* type F strain Langeland (given by Dr. V. Møller, Statens Seruminstitut, Copenhagen, in 1962) grown under similar conditions as for type A and B strains by the following sequential steps: acid precipitation at pH 4.0; extraction with 0.2 M phosphate buffer, pH 6.0; precipitation at 25–65% saturation of ammonium sulfate; precipitation by dialysis against 0.05 M acetate buffer, pH 4.5; extraction with the same buffer containing 0.5 M sodium chloride; protamine treatment; SP-Sephadex chromatography at pH 4.2; and gel filtration on Sephadex G-200. The purified toxin, eluted in a single peak from Sephadex like type E toxin, contained 2×10^8 LD₅₀/mg N. Further details of purification and properties of type A, B, and F toxins will be described in separate communications.

The TC of each type was isolated by applying a portion of PT of each type or L or M-toxin of type A or B dialyzed against 0.01 M phosphate buffer, pH 8.0, to chromatography on DEAE-Sephadex (A-50) (Pharmacia Fine Chemicals) equilibrated with the same buffer and eluting by linear gradient increase in NaCl concentration from 0 to 0.5 M (Kitamura, Sakaguchi and Sakaguchi, 1969).

Less purified preparations of type A and B toxins, and crude culture supernatant of strains Hall, Okra and QC were also used for certain experiments.

Antitoxins: Anti-PT used were crude horse plasma given by courtesy of Dr. H. Kondo, Chiba Serum Institute, Ichikawa-shi, Chiba. The horses were hyperimmunized initially with aluminum phosphate gel-precipitated toxoid and then with toxin without adjuvant. Highly purified PT (containing both L- and M-toxins for types A and B) of each type was used for immunization. Anti-type A (97) PT contained 1250 international units (IU)/ml; anti-type B (Okra) toxin, 800 IU/ml; anti-type B (QC) toxin, 710 IU/ml; anti-type E (35396) toxin, 900 IU/ml; and anti-type F (Langeland) toxin, 160 IU/ml.

Affinity chromatography: One gram of CNBr-activated Sepharose 4 B (Pharmacia Fine Chemicals) was allowed to swell and washed repeatedly with 0.001 M HCl and finally with 0.1 M carbonate-bicarbonate buffer, pH 9.0. To this added was 5–8 mg type A or B TC or PT dialyzed overnight against the same buffer. The mixture was allowed to stand for 16 hr at 4°C, washed twice with the same buffer, treated with 1 M ethanolamine at pH 8.0 for 2 hr at 4°C, and then washed alternately with 40-m1 portions of 0.1 M Tris-HCl buffer, pH 8.0, and 0.1 M acetate buffer, pH 4.0. The coupled Sepharose was packed in a column to a size of 0.9×5.5 cm, washed with 0.2 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. A 1- to 3-ml portion of type A or B anti-PT dialyzed against the same buffer was applied to the column. The column was washed with 35 ml of the buffer; the adsorbed specific immunoglobulin (lg) eluted with 0.2 M glycine-HCl buffer, pH 2.3, containing 0.5 M NaCl. One-milliliter fractions were collected; protein content of each fraction and PHA titers of protein-containing fractions were determined. The PHA-active fractions were pooled and dialyzed against 0.2 M Tris-HCl buffer, pH 8.0. The anti-TC-lg fractions obtained by several runs of affinity chromatography were pooled and concentrated by ultrafil-
tration through PM-10 membrane (Amicon Co., Lexington, Massachusetts).

Passive and reversed passive hemagglutination: The procedures reported by Johnson et al. (1966) were followed in principle. Washed SRBC were treated with 1.5% formalin at pH 7.2 for 18 hr at 37°C (Butler, 1963). The formalinized sheep red blood cells (FSRBC) were stored at 4°C in a 50% suspension in PBS containing merthiolate at 0.01%.

Coupling FSRBC with toxin or antitoxic Ig was performed by a modification of the procedures reported by Gordon, Rose and Sehon (1958). To one volume of a 50% suspension of FSRBC, added were 30 volumes of PT, TC or NC of each type at a concentration of 13-25 μg/ml or anti-PT or anti-TC-Ig at 50-100 μg/ml in 0.15 M phosphate buffer, pH 7.3, and 15 volumes of a 10-fold dilution of bis-diazotized benzidine prepared according to Gordon et al. (1958). The mixture was allowed to stand for 18 min at room temperature. The coupled FSRBC were finally suspended to 2.5% in PBS containing bovine serum albumin (BSA) at 0.25%. Uncoupled FSRBC were treated in the same way without added toxin or antitoxin.

The PHA and RPHA tests were performed in the same way as reported (Sakaguchi and Sakaguchi, 1973) in wells in plastic trays (Tomy Seiko, Tokyo) with 0.5-ml portions of twofold serial dilutions of toxin or antitoxin in PBS containing BSA at 0.25% and 0.05-ml portions of a 2.5% suspension of toxin or antitoxic-Ig-coupled FSRBC. Agglutination was read in 2 and 18 hr of incubation at room temperature.

Agar gel double diffusion: The AGD test was performed in 1% agar gel (Special Agar, Wako Pure Chemical Co., Osaka) in 0.05 M acetate buffer, pH 6.0, containing merthiolate at 0.01%. Each well in agar gel on a 5×5 cm glass plate had a 3-mm diameter and a 7-mm distance between every two wells. After incubation of the plate in a moist chamber for 3 days at 20°C, the precipitin bands developed were stained with thiazine red. Dilutions of anti-PT containing 160-300 IU/ml and toxin solutions containing 0.19-0.36 mg protein/ml were used.

Protein contents: Protein contents of toxin and antitoxin preparations were determined by the method of Lowry et al. (1951) and expressed in BSA equivalent.

Toxin potencies: The time-to-death method (Boroff and Fleck, 1966; Sakaguchi, Sakaguchi and Kondo, 1968) was used. A standard curve was prepared with each PT of types A and F and each activated PT of types B and E. The toxin potency was expressed in ip LD₅₀ per ml or per mg N.

RESULTS

Cross Reactions among Type A, B, E and F PT in PHA and AGD Tests

The results of PHA tests with FSRBC coupled with each of TC and NC of types A, B, E and F and anti-PT of each type so diluted as to contain 1.0 IU/ml are summarized in Table I.

Cross reactions at high levels occurred between NC of types A and B, and those at lower levels between NC of types A and F and both TC and NC of types E and F.

With type A (97) NC-coupled SRBC, the PHA titer of Anti-PT type A (97) and that of anti-PT type B (Okra) were the same; while that of Anti-PT type B (QC) was only 25% those of the other two antitoxins. With type B (Okra) TC-coupled SRBC,
TABLE I
Passive hemagglutination titers of anti-progenitor toxins types A, B, E, and F with sheep red blood cells coupled with each component of the toxins of the four types

<table>
<thead>
<tr>
<th>Antitoxin (1 IU/ml)</th>
<th>Type A (97)</th>
<th>Type B (Okra)</th>
<th>Type E (35396)</th>
<th>Type F (Langeland)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>NC</td>
<td>TC</td>
<td>NC</td>
</tr>
<tr>
<td>A (97)</td>
<td>800</td>
<td>800</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>B (QC)</td>
<td>1</td>
<td>200</td>
<td>400</td>
<td>200</td>
</tr>
<tr>
<td>B (Okra)</td>
<td>0</td>
<td>800</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>E (35396)</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>2,500</td>
</tr>
<tr>
<td>F (Langeland)</td>
<td>0</td>
<td>4</td>
<td>&lt;1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figures show the highest dilution of antitoxin causing positive hemagglutination.

1TC stands for toxic component; 2NC for nontoxic component.

the HA titer of anti-PT type B (QC) was 50% that of anti-PT type B (Okra). With type B (Okra) NC-coupled SRBC, the HA titer of anti-PT type A (97) was 50% that of anti-PT type B (Okra), but that of anti-PT type B (QC) was only 25%. These results and those obtained by Shimizu and Kondo (1973) suggest a closer antigenic relationship between NC of type A (97) and that of type B (Okra) than between those of type B (Okra) and (QC).

Cross reactions between type E and F toxins with respect to both TC and NC and between type A and F toxins with respect to NC were observed, but to much less extents. Also found were very minor unilateral cross reaction with respect to NC between types B and E and types A and E.

AGD tests confirmed the cross reactions between types A and B with respect to NC and between types E and F with respect to both TC and NC. The tests, however, indicated no antigenic difference between type B (Okra) and (QC) toxins. The results of AGD tests are shown in Fig. 1.

Purification of Type A and B Anti-TC-Ig by Affinity Chromatography

From the preceding experiments, it was anticipated that RPHA tests could distinguish between type A and B toxins only if anti-TC-coupled SRBC are used. Monospecific anti-TC-Ig was obtained from anti-PT horse plasma of each type by affinity chromatography on a column of CNBr-activated Sepharose 4B coupled with purified TC of the corresponding type. For comparison, CNBr-activated Sepharose coupled with PT of each type was also prepared.

No appreciable loss in toxic potency resulted from dialysis of TC overnight at 4°C against 0.1 M carbonate-bicarbonate buffer, pH 9.0, containing NaCl at 0.5 M. The efficiency of coupling to the gel was 95% for PT and 83 (77-90%) for TC on protein bases.

The results of affinity chromatography are shown in Table II. Although the recovery in HA titer was only a few percent, the specific activity increased by 11.4 to 14.7-fold.

In affinity chromatography of anti-PT type B (QC) on Sepharose coupled with
Fig. 1. Agar gel double diffusion tests with anti-progenitor toxin types A, B, E, and F. 
a. AT, anti-A (97); 1 and 4, A cryst.; 2, B (Okra) NC; 3, E (35396) PT; 5, B (Okra) 
PT; 6, B (QC) PT
b. AT, anti-B (Okra); 1 and 4, B (Okra) PT; 2, B (Okra) TC; 3 and 6, A cryst.; 5, B (Okra) NC
c. AT, anti-B (Okra); 1 and 4, B (Okra) PT; 2, B (Okra) TC; 3 and 6, A (97) PT; 5, B (Okra) NC
d. AT, anti-E; 1 and 4, E PT; 2, E TC; 3 and 6, F PT; 5, E NC
e. AT, anti-F; 1 and 4, F PT; 2, F TC; 3 and 6, E PT; 5, F NC
f. Left, anti-E; right, anti-F; top, E PT; bottom, F PT
AT stands for antitoxin; cryst. for crystalline toxin; PT for progenitor toxin; TC for toxic component; NC for nontoxic component.

Fig. 2. Agar gel double diffusion tests with different preparations of type A and B toxins.
a. AT, anti-A (97); 1 and 4, A (Hall) M; 2, A cryst.; 3, A L; 5, B (Okra) L; 6, B (Okra) M
b. AT, anti-A (97); 1, A cryst.; 2, NC of A (Hall) L (direct hemagglutinin-positive); 3, B (Okra) NC; 4, A (Hall) M; 5, A (Hall) TC; 6, NC of A (Hall) M (direct hemagglutinin-negative)
c. AT, anti-B (Okra); 1 and 4, B (Okra) M; 2, A (Hall) M; 3, A cryst.; 5, A (Hall) L; 6, B (Okra)-L
d. AT, anti-B (QC); 1, B (QC) M; 2, A (Hall) M; 3, A cryst.; 4, B (Okra) M; 5, A (Hall) L; 6, B (QC) L
L stands for large-sized toxin; M for mediumsized toxin.
TABLE II

Purification of anti-progenitor toxin and anti-toxic component of types A and B by affinity chromatography

<table>
<thead>
<tr>
<th>Antitoxin</th>
<th>Recovery (%)</th>
<th>HA titer/mg protein to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td>in protein</td>
</tr>
<tr>
<td>A (97)</td>
<td>Anti-TC&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.1 7.3 0.01 ...</td>
</tr>
<tr>
<td></td>
<td>Anti-PT&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.49 ... 7.2 ...</td>
</tr>
<tr>
<td>B (Okra)</td>
<td>Anti-TC</td>
<td>0.6 6.2 0.08 ...</td>
</tr>
<tr>
<td></td>
<td>Anti-PT</td>
<td>0.8 ... 5.6 ...</td>
</tr>
</tbody>
</table>

<sup>1</sup>TC stands for toxic component; <sup>2</sup>PT for progenitor toxin; <sup>3</sup>NC for nontoxic component; <sup>4</sup>AC for affinity chromatography.

TC of type B (Okra), anti-TC adsorbed was about 33% on protein and 45% on HA-activity bases of those of anti-PT type B (Okra). Such diversity in affinity of anti-TC to TC of heterogenous type B toxin agreed well with those found in PHA. However, RPHA tests with SRBC coupled with anti-TC of type B (Okra) and those with the counterpart component of type B (QC) separated on the same column detected the same quantity of type B (Okra) PT or TC.

The minimum quantity of anti-PT-Ig and that of anti-TC-Ig needed for coupling SRBC were 63–126 µg/ml or 3,200–6,400 HA units/ml. Coupling SRBC with 31 µg/ml or lower concentrations of Ig resulted in lower sensitivities by 50% or more.

**RPHA Tests with Different Preparations of Type A and B Toxins and SRBC Coupled with Anti-TC or Anti-PT of Type A (97) or B (Okra)**

The RPHA titers of different preparations of type A and B toxins with SRBC coupled with anti-TC or anti-PT of types A (97) or B (Okra) purified by affinity chromatography are shown in Table III.

Anti-PT type A (97)-coupled SRBC were agglutinated with 8–50 LD<sub>50</sub>/ml of PT type A or 330–2,100 LD<sub>50</sub>/ml of PT type B; anti-PT type B (Okra)-coupled SRBC with 480–850 LD<sub>50</sub>/ml of PT type A and 21–130 LD<sub>50</sub>/ml of PT type B. Such cross reactions between types A and B were minimized to a negligible level by the use of anti-TC-coupled SRBC.

Type A crystalline and L-toxins of types A (Hall) and B (QC) contained direct hemagglutinin; however the specific RPHA titer was always higher by at least 300 times with anti-TC-coupled SRBC, thus direct HA and RPHA were always discernible.

The RPHA titer of M-toxin seemed to be always higher than that of L-toxin with respect to both types A and B. On the toxicity basis, however, the difference was not necessarily significant, while on protein basis, it appeared significant. The antigenic difference between type B (Okra) and (QC) with respect to TC and PT was apparent also in RPHA.
TABLE III
Reversed passive hemagglutination titers of different preparations of type A and B toxins with sheep red blood cells coupled with type A(97) or B(Okra) anti-progenitor toxin or anti-toxic component

<table>
<thead>
<tr>
<th>SRBC coupled with</th>
<th>Anti-TC</th>
<th>Type A (97)</th>
<th>Anti-PT</th>
<th>Type B (Okra)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin</td>
<td>Titer$^1$</td>
<td>LD$_{50}$/ml$^2$</td>
<td>ng/ml$^3$</td>
<td>Titer</td>
</tr>
<tr>
<td>A cryst.</td>
<td>64</td>
<td>59</td>
<td>5.2</td>
<td>64</td>
</tr>
<tr>
<td>A (Hall)-M</td>
<td>1,000</td>
<td>8</td>
<td>0.54</td>
<td>1,000</td>
</tr>
<tr>
<td>A (Hall)-L</td>
<td>130</td>
<td>53</td>
<td>3.8</td>
<td>130</td>
</tr>
<tr>
<td>B (QC)-M</td>
<td>&lt;0.2</td>
<td>...</td>
<td>...</td>
<td>16</td>
</tr>
<tr>
<td>B (QC)-L</td>
<td>&lt;0.2</td>
<td>...</td>
<td>...</td>
<td>8</td>
</tr>
<tr>
<td>B (Okra)-M</td>
<td>0.2</td>
<td>...</td>
<td>...</td>
<td>130</td>
</tr>
<tr>
<td>B (Okra)-L</td>
<td>0.2</td>
<td>...</td>
<td>...</td>
<td>32</td>
</tr>
</tbody>
</table>

1 The reciprocal of the highest dilution of toxin causing positive hemagglutination × 10$^{-3}$.
2 LD$_{50}$/ml of the highest dilution of toxin giving positive hemagglutination. Potential toxicities are given for type B toxin.
3 ng protein/ml of the highest dilution of toxin giving positive hemagglutination.
4 Uncoupled FSRBC; the figures indicate direct HA titers.

AGD Tests with Different Preparations of Type A and B Toxins and Their Components

In AGD tests with anti-PT type A (97), each of the two precipitin bands of type A crystalline, L- and M-toxins joined together, while only the one nearer to the antibody joined with the single band of L- and M-toxins type B (Fig. 2a). In Fig. 2b, it can be seen that the precipitin band of TC is nearer to the antigen and that of NC nearer to the antibody, and that NC of L-toxin (direct hemagglutinin-positive) and that of M-toxin type A (direct hemagglutinin-negative) formed a common
precipitin band, which joined together with that of NC of type B (Okra).

AGD tests with Anti-PT type B (Okra) and (QC) and with different preparations of type A and B toxins and their components are shown in Fig. 2c and d.

**RPHA Tests with SRBC Coupled with Anti-PT or Anti-TC of Type A or B and Crude Supernatants of Type A and B Cultures**

RPHA tests with four preparations of SRBC coupled with anti-PT or anti-TC of type A (97) or B (Okra) and crude culture supernatant of strains of type A (Hall), type B (Okra) and (QC) are shown in Table IV. The specificity of RPHA with anti-TC type A or B-coupled SRBC was proved. The lowest detectable level of toxin in culture was 14 LD50/ml for type A and 39-55 for type B with anti-TC-coupled SRBC.

<table>
<thead>
<tr>
<th>TABLE IV</th>
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<tbody>
<tr>
<td><strong>Reversed passive hemagglutination titers of supernatant fluid of type A and B cultures with sheep red blood cells coupled with type A(97) or B(Okra) anti-progenitor toxin or anti-toxic component</strong></td>
</tr>
<tr>
<td>Culture of</td>
</tr>
<tr>
<td>Culture of</td>
</tr>
<tr>
<td>Type A (Hall)</td>
</tr>
<tr>
<td>Type B (QC)</td>
</tr>
<tr>
<td>Type B (Okra)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

1 The reciprocal of the highest dilution of culture supernatant giving positive hemagglutination.

2 LD50/ml of the highest dilution of the culture supernatant giving positive hemagglutination.

**DISCUSSION**

The RPHA test could be used for not merely the screening purpose but for typing and quantitation of a small amount of botulinum toxin in various specimens, such as food, vomitus, serum, feces, etc., although the sensitivity is lower than the mouse test. The RPHA test is simple and rapid to perform and does not require experimental animals nor tryptic activation to quantitate the potential toxicity. Before this method is used routinely, however, the following problems must be clarified: (1) cross reactions between types A and B and probably types C and D; (2) direct hemagglutinin possessed by toxins of types A, B, and some other types; and (3) nonspecific hemagglutinin possessed by various components of foodstuffs.

Cross reactions in RPHA tests between type A and B toxins were described (Johnson et al., 1966); whereas Evancho et al. (1973) described no cross reaction with anti-crystalline type A toxin-coupled SRBC and botulinum toxins of any other types. We demonstrated cross reactions occurring between these two types in PHA, AGD and RPHA tests to such an extent that type identification would become impossible. All the tests demonstrated that the cross reaction was due not to TC but to NC of
the PT molecules.

It seemed essential, therefore, to use monospecific anti-TC-Ig for sensitizing SRBC. This was easily obtainable by affinity chromatography of anti-PT type A or B on CNBr-activated Sephadex coupled with TC of either type separated chromatographically from PT. If a large quantity of highly potent anti-PT is available, this procedure may be more feasible than immunizing animals with purified TC; because the TC preparation must completely be pure, otherwise even a minute amount of NC contaminating TC would result in stimulation of the animal to produce anti-NC to a considerably high level. No appreciable impairment of the antigenicity of TC occurred during the processes of affinity chromatography including pH changes from 2.3 to 9.0. The low recovery of anti-TC encountered could be improved by scrutinizing further the optimum conditions for affinity chromatography.

Other minor cross reactions were also observed with respect to both TC and NC between types E and F and with respect to NC between types A and F, but these cross reactions were not so serious. Although no investigation was made with types C and D, much serious cross reaction is anticipated between those types as suggested by Jansen (1971) and Eklund and Poysky (1972).

By using anti-TC-coupled SRBC of types A and B, detection and quantitation of the toxin of either type can be made. The sensitivity was 8–59 LD₅₀/ml or 0.54–5.2 ng/ml for type A toxin and 10–59 LD₅₀/ml or 0.088–0.56 ng/ml for type B toxin.

Our purification procedures yielded two different-sized toxins, L- and M-toxins, of types A and B. These toxins can be demonstrated in culture (unpublished data) hence perhaps in foods. Type A crystalline and L-toxins of type A (Hall) and type B (QC) contained direct hemagglutinin activities, whereas neither M- nor L-toxin of type B (Okra) contained the activity and M-toxin of types A (Hall) and B (QC) little or no activity. Cross reactions between types A and B occurred regardless of the direct hemagglutinin activity. The specific RPHA titer always exceeded that of direct HA, therefore absorption or destruction of direct hemagglutinin in toxin materials does not seem necessary.

The above findings and the spur formation between L- and M-toxins in AGD tests suggest that the cross reaction is due to an NC shared by both L- and M-toxins. In addition to such NC, L-toxin may contain an additional NC, which possesses the direct HA activity in the cases of type A (Hall) and type B (QC) toxins, whereas no such activity in the case of type B (Okra) toxin. The molecular structures of L- and M-toxins and also of crystalline toxin will be a matter of separate communications.

Although no investigation was made on nonspecific hemagglutinin contained in various foodstuffs, our unpublished data demonstrated that peptic digestion at pH 4 of raw fish extract removes the nonspecific hemagglutinin without appreciable loss of the antigenicity of type E botulinum toxin. Enzymatic destruction of nonspecific hemagglutinin contained by various foods was reported also by Evancho et al. (1973).

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