Rapid Detection and Quantitative Estimation of Type A Botulinum Toxin by Electroimmunodiffusion

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Received for publication 16 April 1971

An experimental system is described for the detection and quantitative estimation of type A botulinum toxin by electroimmunodiffusion. The method is shown to be rapid, specific, and quantitative. As little as 14 mouse LD50 per 0.1 ml of type A toxin was detected within 2 hr. When applied to experimentally contaminated foods such as canned tuna, pumpkin, spinach, green beans, and sausage, the technique detected botulinum toxin rapidly and identified it as to type and quantity. A specific rabbit type A antitoxin was produced for this in vitro system since the equine antitoxic (Center for Disease Control) tested in this experiment was found to be unsuitable.

The presently accepted standard procedure for detection of botulinum toxins in foods, gastric contents, or blood serum is the mouse protection test. Death of unprotected mice and survival of mice protected by specific antitoxin constitute a positive test for the presence of the botulinum toxin (5). However, the test is found to be imperfect for the examination of foods, since nonspecific deaths occur from the presence of toxic amines and ammonia produced in foods as a result of microbial degradation (4). Also, the mouse bioassay should be improved upon since the length of time required to obtain results may extend from 10 to 24 hr.

The fluorescent-antibody technique has been employed by several investigators (2, 9, 11) to detect and differentiate spores and intact cells, but this method is of little value in detection of the soluble toxin which may remain long after the germination or disintegration of the spores or autolysis of the cells. Passive hemagglutination (7) and immunodiffusion (1, 10) have also been suggested, but these techniques have limited value. Preparation of sensitized erythrocytes which will not give nonspecific hemagglutination in dilutions of complex food mixtures is a demanding task, if not impossible, and, in the case of immunodiffusion, 16 to 24 hr may elapse before results can be obtained.

The present study was initiated to adapt an existing specific and rapid serological technique,

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electroimmunodiffusion, for detection and quantitative estimation of botulinum toxin in foods. The technique eliminates nonspecific results, and its rapidity makes it of practical value as a detection device.

MATERIALS AND METHODS

Organism. Clostridium botulinum type A (78A), obtained from the National Canners Association Western Research Laboratory, Berkeley, Calif., was used for the preparation of type A toxin. Other botulinum strains used for testing cross-reactivity of the antitoxin were type A (33A), type B (115B and 213B), type C (11772), type E (VH), and type F (Langeland Strain).

Toxin preparation. Toxin to be used for the production of specific antitoxin was isolated by the method of Duff et al. (6) from 3,200 ml of a 96-hr culture of C. botulinum 78A. A volume (0.1 ml) of this isolate was further purified by the use of flat gel polyacrylamide electrophoresis (C. A. Miller, Ph.D. Thesis, Oregon State Univ., Corvallis, 1970). The latter purification method separates the toxin isolate into at least four discrete migrating bands which can be easily stained by Coomassie Brilliant Blue R 250. One of these independent migrating bands in the unstained gel which exhibited a toxicity of $3.8 \times 10^8$ LD50 per ml of eluate was used for the immunization of rabbits.

Antitoxin preparation. The polyacrylamide gel containing the toxic band was sectioned into three equal pieces (4 mm in width, 25 mm in length). A small volume (0.5 ml) of gelatin phosphate buffer was added, and the gel was macerated. Equal volumes of saline and Freund complete adjuvant were added to each sample to effect a final volume of 10 ml. After base bleedings, three rabbits were injected intramuscularly and subcutaneously with 1-ml volumes of
the emulsion on days 1, 14, and 43. Pooled antitoxin obtained 2 and 3 weeks subsequent to the final toxin injection was tested for its specificity by immunodiffusion and immunoelectrophoresis and for its neutralizing capacity by the mouse protection test.

Types A and B equine antitoxin obtained from the Center for Disease Control, Atlanta, Ga., were also used in this study. Each equine antitoxin is standardized to contain 10 international units/ml.

For electroimmunodiffusion, sera were dialyzed against a 0.025-μm barbital buffer (pH 8.4) for 24 hr at 4 C. To determine the desirable concentration of each serum to incorporate into the gel medium for maximal cone length, several dilutions of the rabbit antitoxin were tested.

**Slide preparation.** Agarose (Calbiochem), 1.2%, was used as the gel medium for electroimmunodiffusion. The agarose was prepared with barbital buffer (0.025 μ, pH 8.4) and stabilized near 45 C before the addition of antitoxin. Large glass slides [projector slides, 4 by 3.5 inches (10.16 by 8.9 cm)] were coated with 2% Ionagar (Oxoid), and the agar was dried. The coated slides were then overlaid with 15 ml of the antitoxin-containing agarose. This volume was found to effect a gel depth of 2 to 3 mm. The slides were placed at room temperature in a moist chamber for 15 to 30 min before sample wells (3 mm in diameter) were pulled at the cathode end of the slide for introduction of samples containing toxin.

**Conditions of electroimmunodiffusion.** The agarose slides were placed in a water-cooled (4 C) electrophoretic chamber (Colab). Electrical bridges connecting the gel with the barbital buffer (0.1 μ, pH 8.4) reservoir were constructed carefully by anchoring the Whatman no. 4 paper wicks to the slides with molten agarose. This measure helps prevent drying of the gel under voltage stress. The sample wells were filled with the 78A toxin preparations, and a current of 5 ma/cm was applied for 1 to 4 hr. After removal from the electrophoretic chamber, the slides were observed for visible precipitation and were stained with either Amido Schwartz (3) or Thiazine Red R (8).

Measurements of the cones were taken, and a standard curve was prepared from known lethal dosages of 78A toxin. Test samples (supernatant fluids of food) containing known lethal dosages of 78A toxin were then subjected to electroimmunodiffusion, and cone lengths were plotted on the standard curve.

**RESULTS**

**Antitoxin specificity.** Rabbit antitoxin produced after three injections of the purified botulinum toxin exhibited a precipitin titer of 1:120, and it demonstrated a low neutralizing strength of 45 mouse LD₅₀ per ml.

Immunodiffusion conducted to test the rabbit antitoxin for cross-reactivity with toxin types other than A showed the rabbit antitoxin to be specific for type A botulinum toxin, since no precipitin arcs denoting cross-reactivity were observed between the rabbit antitoxin and culture supernatant fluids of *C. botulinum* types B, C, E, or F (Fig. 1). Immunelectrophoresis conducted by electrophoresing crude culture supernatant fluids of *C. botulinum* 78A in flat polyacrylamide gel and overlaying the acrylamide with Ionagar (Oxoid) containing the rabbit antitoxin showed only one precipitin arc which corresponded to the protein band previously shown to have a toxicity of 3.8 × 10⁶ LD₅₀ per ml of eluate.

Although neutralization tests conducted with mice confirmed the neutralizing specificity of the equine antitoxins (equine type A antitoxin was effective in neutralizing only type A toxin, whereas equine type B antitoxin neutralized only type B toxin), results of the in vitro immunodiffusion and immunoelectrophoresis tests with the crude culture supernatant fluids of *C. botulinum* 78A and the equine antitoxins were less satisfactory. No precipitin arcs or one faint disappearing arc occurred with the equine type A antitoxin, whereas at least two and sometimes three arcs occurred between the *C. botulinum* 78A crude culture supernatant fluid and the equine type B antitoxin. Because of the nonspecific results of these studies, the equine antitoxins were determined to be unsatisfactory for use in the electroimmunodiffusion toxin detection study.

**Electroimmunodiffusion.** Well-defined precipitin cones of specific length were obtained when experimental samples containing varied amounts of 78A toxin were electrophoresed into the antitoxin-containing agarose (Fig. 2). As shown in Fig. 3, the concentration of antitoxin also had an effect on the cone length. The cones obtained with the 1:30 antitoxin-agarose mixture were approximately twice the length of corresponding...
cones obtained with the 1:15 antitoxin-agarose mixture. The longer cones produced with the lower antitoxin concentration could therefore be measured more easily and with less attendant error.

**Fig. 2.** Electroimmunodiffusion of twofold dilutions of *Clostridium botulinum* 78A culture supernatant fluids into agarose containing the specific type A rabbit antitoxin. (The well at the far right contained 73 LD$_{50}$/5 μl.)

**Fig. 3.** Comparison of precipitin cone lengths in gels containing 1:15 and 1:30 dilutions of rabbit type A antitoxin after 4 hr of electrophoresis. Symbols: ●, 1:15; ○, 1:30.

**Fig. 4.** Relationship between cone lengths and duration of electrophoresis for different concentrations of toxin. Symbols: ○——○, 2.5 LD$_{50}$; ●——●, 4.00 LD$_{50}$; ○——○, 7.15 LD$_{50}$; ●——●, 14.30 LD$_{50}$.

**Table 1.** Detection of type A *botulinum* toxin in foods by electroimmunodiffusion

<table>
<thead>
<tr>
<th>Food sample</th>
<th>LD$_{50}$ added/5 μl of whole food</th>
<th>LD$_{50}$ detected/5 μl of food supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sausage</td>
<td>15</td>
<td>12.9</td>
</tr>
<tr>
<td>Canned tuna</td>
<td>15</td>
<td>8.6</td>
</tr>
<tr>
<td>Canned pumpkin</td>
<td>15</td>
<td>4.1</td>
</tr>
<tr>
<td>Canned green beans</td>
<td>15</td>
<td>6.4</td>
</tr>
<tr>
<td>Canned spinach</td>
<td>15</td>
<td>6.1</td>
</tr>
</tbody>
</table>

The limit of sensitivity of the electroimmunodiffusion detection system for type A toxin with pooled rabbit antitoxin was 2.5 LD$_{50}$ per 5 μl of whole food LD$_{50}/0.1$ ml in the experimental system. This amount of type A toxin was detected after only 1 hr of electrophoresis (Fig. 4). It is certain that an increase in sensitivity can be obtained by selection of a specific and higher titered antitoxin, since an analysis of the independent sera composing the pooled rabbit antitoxin showed that one serum detected as little as 0.7 LD$_{50}$ per 5 μl (14 mouse LD$_{50}/0.1$ ml).

A preliminary investigation of the applicability of electroimmunodiffusion for detection of Type A
botulinum toxin in foods indicates that food components do not interfere with the formation of the precipitin cones and therefore with detection of the toxin. Low levels of type A botulinum toxin (Table 1) were detected in the unconcentrated supernatant fluids of foods to which the toxin had been added. The toxin was easily detected in such foods as sausage, canned tuna, pumpkin, green beans, and spinach.

**DISCUSSION**

The technique of electroimmunodiffusion discussed in this investigation has proven itself to be of value in the detection and quantitative estimation of botulinum type A toxin in foods as well as in the experimental system. The procedure is simple since no tedious, time-consuming preparation of reagents is necessary, and the method is also rapid since time of detection need not require more than 2 hr, including actual preparation and electrophoresis time.

The system was also found to be extremely sensitive; 0.7 LD₅₀ (14 LD₅₀ per 0.1 ml) was detected in the experimental system. Toxin was also detected in the unconcentrated supernatant fluids of foods contaminated experimentally with low levels of botulinum toxin.

The sensitivity was found to be primarily a function of the antitoxin concentration in the agarose medium, since minimal antibody concentration allowed for increased migration of the toxin. This point becomes of great importance when minute amounts of the toxin are to be detected. If antibody concentration is kept high, the result is precipitation of the toxin before it will migrate and no visible precipitin cone will appear. However, reduction of the antitoxin below the optimal level will also result in no visible precipitation. Therefore, some preliminary investigations must be conducted with the antitoxin to determine an optimal reaction concentration.

As can be concluded from the foregoing discussion, the sensitivity of electroimmunodiffusion is dependent upon the antiserum used in the system. For this reason, a monospecific antitoxin was prepared in rabbits because existing equine sera were found to be unsatisfactory as precipitating agents. The equine antitoxins presently available have been prepared primarily for in vivo neutralization experiments and unfortunately do not seem to be suitable for this in vitro serological system. The unsuitability of the equine types A and B antitoxins (Center for Disease Control) for other in vitro serological experiments has also been observed previously (7).

Research on the standardization of the technique and its applicability to botulinum toxins type B, C, D, E, and F is in progress in our laboratories.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service grant 5 TO1 GM00704-07 from the National Institute of General Medical Sciences.

**LITERATURE CITED**


