Evaluation of Type A Botulinal Toxin Assays that Use Antitoxin to Crystalline Toxin

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The type A botulinal toxin assay by the reverse passive hemagglutination procedure which uses antitoxin to crystalline toxin was examined for specificity. The analysis was based on the fact that crystalline type A toxin is a complex of neurotoxic protein (Aα) and a nontoxic protein (Aβ). By using these components, obtained in essentially pure forms, it was shown that the antitoxin to crystalline toxin has a significantly higher titer to Aβ than to Aα. When Formalin-treated red blood cells were sensitized with this antitoxin, the antibodies coupled to the cells were, for practical results, only anti-Aβ. When the suspension is reacted with dilutions of type A toxic solutions, the limiting dilutions are determined by Aβ and not by the neurotoxin, which should be the determinant if the assay is to measure toxicity. These observations may be pertinent to the development of serological assays for other botulinal toxin types.

The several studies on the assay of botulinal toxin by in vitro serological procedures are predicated on the specific reaction of toxin with its antitoxin (9, 14, 17). Although there was early recognition that toxin neutralizing antibodies seemed unrelated to the end points of some of these tests (10), recent reports do not always present convincing evidence that the procedure being studied is specific for the neurotoxic antigen.

The latest example is the assay of type A toxin by a reverse passive hemagglutination (RPH) test which used antitoxin to crystalline type A toxin (8). In the procedure a suspension of red blood cells, to which antitoxin had been coupled, was added to dilutions of toxin. Toxin concentration in the test sample was calculated from a comparison of the limiting dilutions of the sample and of the standard crystalline toxin solution of known concentration.

The purpose of the present communication is to present evidence that the test is based on reaction of a nontoxic protein instead of the neurotoxic protein. The approach can be understood best from a summary of what is known about crystalline type A botulinal toxin. Acidic solutions of this toxic preparation behave in certain physicochemical tests as a homogeneous preparation (15). Nevertheless, in addition to botulinal toxicity it possesses hemagglutinat-}

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ing activity. The two activities can be separated when crystalline toxin as alkaline solutions is absorbed with erythrocytes (13) or subjected to ultracentrifugation (18) or ion exchange chromatography (4). The isolated neurotoxic protein of 150,000 molecular weight has greater specific toxicity than the starting crystalline toxin of 900,000 molecular weight. Crystalline toxin is a complex of neurotoxin (α component) and hemagglutinin (β component) which are held together by means other than covalent bonds. The Aα to Aβ ratio (wt/wt) in crystalline toxin is 1 to 4 (4) or, possibly, 1 to 9 (1).

The two proteins constituting the crystalline toxin have different amino acid compositions (1) and are antigenically distinct (13); antitoxin resulting from immunization with toxoided crystalline toxin gives two immune precipitate lines when it is reacted against crystalline toxin in Ouchterlony double-diffusion serology (4). It follows that when anticerulline type A serum is used in a toxin assay procedure (8), either neurotoxin or Aβ or the combination could determine the end points. The correct alternative can be determined by using purified Aα and Aβ preparations in the RPH test.

MATERIALS AND METHODS

Antigens. Crystalline type A toxin was prepared with the Hall strain as described (8). Neurotoxin and hemagglutinin were obtained from this preparation by two-step procedures.

A solution of the crystals was chromatographed, as described for diethylaminoethyl (DEAE)-cellulose
(3), on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) with 0.025 M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.9. Residual Aα in the recovered Aα pool was removed during chromatography of the pool on SP-Sephadex C-50 (Pharmacia Fine Chemicals) with 0.02 M sodium phosphate buffer, pH 6.3. The hemagglutinin came through unretarded. A linear NaCl gradient of 0.2 M maximum in the phosphate buffer eluted Aα in a distinct protein peak. The several lots prepared were pure by electrophoresis in polyacrylamide-sodium dodecyl sulfate (6) and had specific toxicities of 1 x 10⁴ mean lethal dose/mg of protein.

Hemagglutinin was obtained from the second of the two protein peaks eluted in the chromatography of crystalline toxin on DEAE-Sephadex. The pool was chromatographed on SP-Sephadex C-50 with 0.02 M sodium phosphate buffer, pH 7.2. Void volume fractions with absorbance at 278 nm were pooled as the Aβ sample, which had a trace amount of toxin (about 0.02 μg of Aα per 1.0 mg of total protein).

**Protein measurements.** Absorbance at 278 nm was used. The E₁%₂₇₈ for Aα was 16.3 and for Aβ and crystalline toxin was 16.6 (12).

**Antitoxin.** A solution of crystalline toxin in phosphate buffer of 0.075 M, pH 6.4, was incubated for 14 days at 37 C in the presence of 0.4% Formalin. The toxoid was emulsified with an equal volume of complete Freund adjuvant, and a mixture volume containing 1.0 mg of antigen protein was injected intramuscularly into a rabbit. One month later a dose of 3.0 mg of antigen was given in incomplete Freund adjuvant. The serum, obtained by bleeding out the animal 14 days later, had 800 IU of type A antitoxin per ml. It gave the expected two precipitate lines in Ouchterlony gel diffusion tests when crystalline toxin was used as antigen.

Globulins in the antitoxin were precipitated by diluting the serum 1:1 (vol/vol) with 0.075 M, pH 7.2, sodium phosphate buffer containing 0.075 M NaCl (PBS) and by adding (NH₄)₂SO₄ to final 50% saturation. The precipitate, which developed during holding at room temperature for 1 h, was collected by centrifugation and dissolved in a small volume of PBS. The resulting solution was chromatographed on Sephadex G-200 equilibrated with PBS. A sample, up to 5 ml in volume, was applied to a column (2.5 by 40 cm). The second of the two protein peaks eluted by PBS contained most of the antitoxic activity and was used as the immunoglobulin G (IgG) antitoxin sample.

**Sensitization of Formalized sheep red blood cells.** Sheep blood was collected in Alsever solution. Sheep red blood cells (RBC) were washed, treated with Formalin, and conjugated to antitoxin or antigen with bis-diazotized benzidine (BDB) by a procedure which was essentially that used by Johnson et al. (9). After the coupling reaction, the cells were washed with 0.15 M sodium phosphate buffer, pH 7.3, and made into a 0.5% suspension in PBS containing 0.25% bovine serum albumin, fraction V (Nutritional Biochemicals, Cleveland, Ohio).

The IgG solution to be used for coupling was diluted to contain 800 hemagglutinating units per ml. The arbitrary hemagglutinating unit is the minimum antitoxin amount which is required to agglutinate a Aα-sensitized cell suspension (see below). For the particular IgG preparation being used, about 1 antitoxin IU equaled 400 hemagglutinating units. Use of this antitoxin concentration for the coupling step gave cell suspensions which were maximally sensitized for agglutination by the neurotoxin antigen.

Purified Aα could be used directly, but crystalline toxin and Aβ had to be prepared (incubation in final 0.4% Formalin) for 1 week at 37 C, followed by dialysis against PBS) for coupling to Formalized RBC. Without the treatment, the sensitized cell suspensions were not suitable for the tests. Antigen solutions used in the coupling reaction had protein concentrations of 10 to 20 μg/ml.

**RPH test.** A microtitration procedure was used. Serial, twofold antigen dilutions of 50-μliter volumes were prepared in U-bottomed wells of titration plates by using microdiluters (Cooke Engineering Co., Alexandria, Va.). Diluent was PBS-serum albumin. To the dilutions was added 50 μlitters of the antitoxin-sensitized cell suspension. Tests were read after 2 h at room temperature (about 22 C).

**Passive hemagglutination test.** In the passive hemagglutination (PH) test, the procedure was the reverse of RPH tests in that RBC sensitized with antigen were added to dilutions of antitoxin IgG fractions.

**RESULTS**

RPH tests were done by adding antitoxin-sensitized cell suspensions to dilutions of antigens (Table 1). Titers of tests where Aβ was present were due to this protein reacting with its antibody; botulinial hemagglutinin of itself does not agglutinate RBC which have been treated with Formalin (Lamanna and Aragon, Bacteriol. Proc., p. 94, 1956).

The antitoxin-sensitized cell suspension was agglutinated by low concentrations of Aβ antigen. This contrasted to the poor agglutinability of the suspension by Aα. The protein concentrations calculated to be in the titer dilutions

**Table 1. Reverse passive hemagglutination tests in which cells sensitized with antiserum to crystalline type A toxin are reacted with crystalline toxin or its components**

| Antigen | Starting concn (μg/ml) | Dilution* | Titer (μg/ml) |
|---------|-----------------------|-----------|--------------|
| Aα      | 250                   | 1         | 25.0         |
| Aβ      | 200                   | 10        | 0.0015       |
| Aα + Aβ | 250                   | 18        | 0.00095      |
| Crystalline | 250                  | 18        | 0.00095      |

* Reciprocal of highest dilution giving hemagglutination; figures are logarithms to base 2.

* Protein concentration of titer dilution.

50 μg of Aα + 200 μg of Aβ; based on ratio of antigens in crystalline toxin (4).
illustrate this great difference (16,670 fold) in the sensitivity to the two antigens.

The titer obtained with the antigen solution which was constituted to simulate the composition of crystalline toxin is, within experimental variation, the same as that obtained with $A\beta$ alone. These titers are not significantly different from that obtained with a solution made of crystalline toxin. When crystalline toxin is the antigen in the RPH tests, the titer is due primarily, probably exclusively, to $A\beta$ antigen.

Table 2 shows the results of PH tests done with cell suspensions separately sensitized with different antigens. Results are those obtained with maximally sensitized suspensions. That the antitoxin should have ability to neutralize type $A$ toxin is shown by the high IgG dilution which agglutinated $A\alpha$-sensitized cell suspensions. However, the $A\beta$-sensitized suspension was agglutinated by an antitoxin concentration 2$^\times$ times less than that needed to agglutinate $A\alpha$-sensitized suspension. In PH, as in RPH, tests using crystalline toxin, the observed titers are indicated to be determined by $A\beta$ antigen with $A\alpha$ playing a subordinate role.

**DISCUSSION**

Antitoxin used in the present experiments was obtained from a rabbit immunized with a toxoid of crystalline type $A$ toxin. PH tests with this particular serum, and confirmed with a different lot, show such antitoxins to have anti-$A\beta$ titer which is significantly higher than anti-$A\alpha$. Such a difference is not surprising, because the immunizing antigen has a greater quantitative amount of $A\beta$ protein than $A\alpha$ (1, 4).

Sites on the RBC to which antibody molecules can be conjugated with BDB are finite in number. When anti-crystalline type $A$ serum is used to sensitize RBC, antibodies to the two antigens compete for these attachment sites. The resulting suspension need not be optimally sensitized with both; indeed such an occurrence would be surprising. With the preponderance of anti-$A\beta$ over anti-$A\alpha$ in the antitoxin, the cell suspension should be more optimally sensitized to react with $A\beta$ antigen. This interpretation is consistent with the experimental observations. Furthermore, the extremely low sensitivity of the antitoxin-sensitized suspension to $A\alpha$ antigen indicates that the anti-$A\alpha$ molecules on the cells are too few to be meaningful.

With the RBC suspension sensitized to agglutinate only with $A\beta$, the nontoxic protein determines the end points of RPH tests with type $A$ toxic solutions. The neurotoxin, which should be the effective antigen if the purpose of the procedure is assessment of toxicity, does not play a significant role. The fact that neurotoxin can be calculated or shown by mouse tests to be present in the titer dilution is not proof that it is the determinant of the titer. Similarly, the reported correlation between RPH titers and toxicities (8) is not evidence that the test measures neurotoxin; the titer would change proportionately with the $A\beta$ concentration.

Indirect assay of neurotoxin in crystalline toxin preparations seems possible. Crystalline toxin preparations, routinely prepared with a particular culture strain, have the same $A\alpha$ to $A\beta$ ratio, because they all have the same specific toxicities. Thus, the $A\beta$ titer could be obtained by the RPH test and then be converted into $A\alpha$ titer by using this ratio.

It is not known if all type $A$ cultures produce the same $A\alpha$ to $A\beta$ ratio or if the ratio in crystalline toxin is the same as in the culture fluid from which the crystals are derived. If not, quantitation of neurotoxin in toxic culture fluids with the indirect approach could give significant errors.

When antitoxin to crystalline toxin is used in serological procedures other than RPH tests, the same specificity problem must be resolved. The obvious solution would be use of antiserum which is monospecific for $A\alpha$. The recently reported radioimmunoassay procedure (2) utilized such an antitoxin.

The botulinogenic entities in culture fluids of the several *Clostridium botulinum* types tested (A through F) behaved during ultracentrifugation as molecules of at least several hundred thousand molecular weight (16). Type B and E toxin preparations, which are essentially homogeneous in acidic conditions, have been prepared and shown to be composed of units of 500,000 (7) and 350,000 (11) molecular weight, respectively. However, these are molecular complexes similar to crystalline type $A$ toxin in that a neurotoxic protein is associated with a nontoxic protein. The purified type B neurotoxin is of 165,000 molecular weight (5) and type

**Table 2. Passive hemagglutination titers when cells sensitized with different antigens were reacted in dilutions of antiserum to crystalline type $A$ toxin**

| Antigen             | Titer* |
|---------------------|--------|
| $A\alpha$           | 14     |
| $A\beta$            | 18     |
| Crystalline toxin   | 19     |

*Reciprocal of highest antitoxin dilution causing hemagglutination; numbers are logarithms to base 2.
E neurotoxin of 150,000 molecular weight (11). It follows that the present discussion on type A toxin assay is relevant to in vitro serological assay procedures for type B and E toxins when antitoxins to the large toxic complexes are used.

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