SHORT COMMUNICATION

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF CLOSTRIDIUM BOTULINUM TOXIN TYPE A

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SUMMARY: The enzyme linked immunosorbent assay using the so-called "double-sandwich technique" has been applied to determine botulinum toxin type A. By this assay, 50-100 mouse ip LD\textsubscript{50} of toxin type A can be detected. No cross-reaction occurs with botulinum toxins of other types tested. In all probability this is due to the high specificity of the antiserum prepared against the toxic component of type A toxin.

Enzyme-linked immunosorbent assays (ELISA) have found wide application, especially for determination of proteins such as hormones and immunoglobulins (Ruitenberg et al., 1974; Veldkamp and Visser, 1975; Engvall and Perlmann, 1972). By this assay, 1-50 ng of proteins per ml can be detected (Van Weemen and Schuurs, 1971).

In the present publication, we report preliminary results of the application of the ELISA technique for quantification of Clostridium botulinum toxin type A. For the determination of this toxin, the so-called "double-sandwich technique" has been found to be suitable. For this purpose polystyrene tubes are coated with horse serum against botulinum toxin type A. After incubation with the toxin, the adsorbed toxin is labelled with rabbit serum against botulinum toxin type A. The amount of rabbit serum adsorbed is measured with sheep anti-rabbit serum conjugated with enzyme. The amount of enzyme adsorbed is determined spectrophotometrically with a specific substrate for the enzyme.

Antisera against botulinum toxin type A: Horse serum against botulinum type A toxin was supplied by Dr. J. Keppie, Microbiological Research Establishment, Porton, Salisbury. One milligram of this freeze-dried material neutralized 98,000 mouse ip LD\textsubscript{50} of toxin type A.

Rabbit serum against botulinum type A toxin was prepared by immunization of rabbits with toxoid of the 7s toxic component (Sugii and Sakaguchi, 1975). The toxoid of the 7s toxic component was supplied by Dr. G. Sakaguchi,
University of Osaka Prefecture, Japan. The anti-toxic component was prepared by injecting rabbits subcutaneously each with 0.1, 0.1 and 0.2-mg toxoid with Freund's complete adjuvants on days 0, 30 and 60, respectively. On day 70 the rabbits were bled. The IgG fraction was isolated from the sera by the technique described by Steinbuch and Audran (1969), and freeze-dried. One milligram of this material neutralized 175,000 mouse ip LD$_{50}$ of toxin A.

Sheep-anti-rabbit serum-enzyme conjugate: Horse radish peroxidase (HRP) Sigma type V1 (Sigma Chemical Company, St. Louis, MO., U.S.A.) was conjugated to sheep-anti-rabbit serum according to the method described by Nakane and Kawaoi (1974). Unlabelled material was separated with a Sephadex G-150 column (400×38 mm), equilibrated with 0.01 M phosphate buffer, pH 7.2, in physiological saline solution (PBS). The same solution was used as elution fluid. The effluent fluid was continuously monitored for protein at 280 nm and for the presence of HRP at 403 nm. The fraction with an $R_z$-value (E$_{403}$nm / E$_{278}$nm) of 0.6–0.8 was used.

Determination of HRP activity: A substrate solution was prepared by dissolving 80 mg of 5-amino-salicylic acid (syn. 5-amino-2-hydroxy benzoic acid) (Merck) in 100 ml of hot distilled water at 70°C. Prior to use the pH of this stock solution was adjusted to 6.0 with 1 n NaOH. An aliquot of 10 ml of 0.05% H$_2$O$_2$ was added to 90 ml of the solution.

Botulinum toxins: A freeze-dried crude toxin A, 1.3×10$^5$ mouse ip LD$_{50}$ per mg, was supplied by Dr. J. Keppie. To obtain other toxins, liver broth as described by Haagsma (1973) was incubated with spores of each of C. botulinum types A, B, C and E. The cultures were incubated anaerobically at 30°C for 6 days. The titer of toxin was determined by mouse ip injection of 0.5 ml of serial 3-fold dilutions of the supernatant into four mice per dilution (Reed and Muench, 1938), and by the ELISA technique with type A antiserum.

ELISA: The ELISA technique described by Engvall and Perlmann (1971, 1972) with some modifications was employed.

One milliliter of diluted horse serum against toxin type A (10 μg/ml dissolved in 0.01 M PBS, pH 7.2) was incubated with rotation overnight at room temperature in disposable polystyrene tubes (50×11 mm, LKB-produktor A.B., Stockholm, Sweden). The tubes were washed twice with tapwater containing 0.05% Tween 20. The 1 ml of the toxin sample, dissolved in 0.01 M PBS, pH 7.2, was incubated with rotation at 37°C for 90 min. After washing as described above, 1 ml of rabbit IgG against the 7s toxic component of toxin type A was added (20 μg/ml dissolved in 0.01 M PBS, pH 7.2). The tubes were incubated with rotation at 37°C for 90 min. After washing, 1 ml of a dilution of sheep-anti-rabbit serum-enzyme conjugate was added. After incubation with rotation at 37°C for 90 min and washing, 1 ml of the substrate solution was added. After incubation at 37°C for 30 min, the reaction was stopped by adding 0.1 ml n NaOH. The reaction product (brown color) was measured spectrophotometrically at 449 nm. The equipment was an in-line system based on a semi-automatic equipment for antigen analysis (O11i-system 3000, O11i Tuote, Kivenlathi, Finland).
In the first experiment, the minimum detectable amount of toxin of the freeze-dried crude toxin type A was determined by the ELISA and the mouse bioassay. ELISA tests were performed in triplicate and the results are presented in Fig. 1. The results showed that *Clostridium botulinum* toxin type A can be determined by ELISA. The reproducibility is high and the lowest detectable dose lies around 50–100 mouse ip LD$_{50}$.

The toxin production of the different *C. botulinum* types is presented in Table I. The amount of toxin present in the culture filtrate was measured by the mouse bioassay and ELISA. By ELISA, three dilutions of the culture filtrates were tested. From these results, it is clear that toxin type A is measurable by ELISA. No cross-reaction could be observed with culture filtrate of other toxin types. By the indirect hemagglutination test (Yafayef and Chepelev, 1961) and with the passive hemagglutination test (Johnson et al., 1966), cross-reactions occur between type A and type B toxins. Sakaguchi et al. (1974) demonstrated that cross-reactions are due to the nontoxic components of the botulinum toxin, because the nontoxic components of type A and type B toxins share common antigens. In the ELISA experiments described here no cross-reaction could be observed with toxins of other types. It is likely that this is due to the specificity of the IgG fraction of the rabbit serum against the toxic component (7s) of type A toxin (described and isolated by Sugii and Sakaguchi, 1976) used in these experiments. The ELISA requires purification of the rabbit serum by isolating the IgG-fraction. Due to this purification only small amounts of the IgG-fraction were needed which resulted in low extinction values of the blanks.
TABLE I

*Mouse LD$_{50}$ and ELISA extinction values of various culture filtrates of Clostridium botulinum*

<table>
<thead>
<tr>
<th>Clostridium botulinum type</th>
<th>log i.p. mouse LD$_{50}$</th>
<th>Extinction value ELISA dilution 1/8</th>
<th>1/32</th>
<th>1/128</th>
<th>1/512</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.30</td>
<td>n.d.*)</td>
<td>0.6</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>A1</td>
<td>4.37</td>
<td>n.d.</td>
<td>0.8</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>A2</td>
<td>4.81</td>
<td>n.d.</td>
<td>0.9</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>A3</td>
<td>4.08</td>
<td>n.d.</td>
<td>0.6</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>B</td>
<td>5.31</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 n.d.</td>
</tr>
<tr>
<td>C</td>
<td>4.83</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 n.d.</td>
</tr>
<tr>
<td>E</td>
<td>5.12</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0 n.d.</td>
</tr>
</tbody>
</table>

*) n.d. = not determined

The ELISA technique can be used for screening culture filtrates without the use of mice. Current research is focussed on the application of ELISA for determination of botulinum toxins of the other types.

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REFERENCES


SAKAGUCHI, G., SAKAGUCHI, S., KOZAKI, S., SUGIH, S. AND OHISHI, I. (1974): Cross-reaction in reversed passive hemagglutination between Clostridium botulinum type A and B toxins and


