Detection of *Clostridium botulinum* Toxin by Local Paralysis Elicited with Intramuscular Challenge

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*Clostridium botulinum* toxin can be identified by a characteristic, acute local paralysis that follows its injection into the gastrocnemius ("calf" muscle) of mice. The local botulism can be elicited with slightly less than one-tenth the toxin amount that is needed to kill mice by the intraperitoneal (i.p.) challenge route. The practical sensitivities of the intramuscular (i.m.) versus i.p. tests are about equal, however, because maximum sample volume injectable i.m. is 0.1 ml as compared to the 0.5-ml range that can be given i.p. i.m. injection of 10 or more mouse i.p. mean lethal doses causes paralysis in about 1 h, and an i.m. injection of about 0.5 i.p. mean lethal doses causes paralysis in 3 to 4 h. Toxin neutralization by homologous type of antitoxin only can be demonstrated with an incubated mixture of toxin and antitoxin. Although not as convenient as the i.p. method for routine use to detect botulinum toxin, the i.m. method has characteristics which could make it a useful supplement to the presently accepted i.p. procedure.

The paralytic syndrome of botulism is the result of toxin blocking the acetylcholine-mediated transmission of nervous stimuli across peripheral synapses and nerve-effector junctions (2). When botulinum toxin is applied to a rat skeletal muscle so that the amount diffusing systemically is less than one lethal dose, a local paralysis of several months duration results (6). Injection of botulinum toxin directly into the gastrocnemius and hamstring muscles of mice also leads to chronic local botulism (3).

The accepted method for identifying botulinum toxin depends on death of mice given an intraperitoneal (i.p.) injection of the sample and the neutralization of the lethal agent by one of the antibotulinum typing sera. This report describes an intramuscular (i.m.) test for botulinum toxin in which toxin injected into the "calf" muscle of mice cause flaccid paralysis of the foot of the injected leg.

**MATERIALS AND METHODS**

Toxins and antitoxins. *Clostridium botulinum* toxin types A, B, C, D, E, and F were tested. Several different lots of type A toxic crystals (4) were tried in the initial developmental studies. Toxic preparations eventually used included supernatant fluids obtained by centrifuging cultures grown in cooked meat medium (Difco, Detroit, Mich.) or toxins in different stages of purification. Cultures were those from different sources and had been maintained in this laboratory for many years.

Activation of type E toxic preparations was by adding crystalline trypsin to 0.01% and incubating the mixture for 30 min at 37 C. Toxicity of these samples increased up to 50-fold when tryps inized.

Antitoxins were those obtained from the Center for Disease Control, Atlanta, Ga., or the higher titered sera kindly supplied by J. Keppie, Microbiological Research Establishment, Porton, England.

i.m. injections. HA/IRC Swiss mice of 20 to 25 g body weight were used. Toxin was injected into the gastrocnemius-soleus muscle complex with a 27-gauge hypodermic needle fitted with a 0.5- or 1.0-ml tuberculin syringe.

When many mice were to be tested, the most expeditious injection procedure was to have one person immobilize the animals; a second worker could then gently stretch a hind leg of the mouse with one hand and inject with the other. A slight jab was used to penetrate the skin and enter about halfway into the muscle complex. Two injections of 0.05 ml each were made, one from the lateral and the second from the medial aspect. Larger volumes were not used because of inoculum spread; even with 0.1 ml some of the injected fluid could be often seen spreading into the thigh.

Sensitivity of the i.m. test. The amount of toxin needed to elicit local botulism in one-half the tested number of animals (ED50) was determined by parallel titrations of toxin by the i.p. and i.m. procedures. Toxicity of stock toxin solution was first estimated by the intravenous assay procedure (1) and a dilution of an estimated 400 to 500 mouse i.p. mean lethal doses (LD50)/ml prepared with M/15 phosphate buffer, pH 6.4, and containing 0.1% gelatin. This working dilution was further diluted serially in two-
fold increments, and the successive dilutions expected to span the critical 100 to 0% response range were injected i.p. (0.5 ml/animal) into separate groups of 8 or 10 mice each. Deaths during 96 h were tabulated by dilutions, and the i.p. LD₅₀/ml of the working dilution was calculated from this data by the Reed and Muench procedure (5).

The same and further dilutions were tested i.m. in 0.1-ml volumes in other groups of mice. Animals were examined for paralysis at 2, 4, 18, 48 and 72 h. The first time local paralysis was seen the mouse was given an identifying mark; these animals are included with the reactors although paralysis might have disappeared by the end of the observation period. The percentages of positives among the test groups of mice were tabulated, not by dilutions, but by the i.p. toxicities in the dilutions as calculated from the result of the i.p. titration. Use of this data in the Reed and Muench method gave i.m. ED₅₀ doses expressed in i.p. LD₅₀ units.

RESULTS

Local botulism. When signs of local botulism were studied with solutions made with differing amounts of type A toxic crystals, a gradation of responses was observed. Minimally effective toxin amounts caused a flaccid paralysis in which the toes of the injected leg become partially relaxed and curled and the digits come together in closer apposition than is normal. This minimal response is best seen by raising the animal by the tail so as to reduce the weight on the foot; it is also noticed when the animal is allowed to walk freely on a flat surface. When this first level of response occurs late (e.g., 18 h postchallenge), the animal may recover without the paralysis worsening.

With slightly higher doses, there develops what has been called "weakness of the ankle." The foot turns slightly outward, especially when it is not resting on a surface. Flaccid paralysis of the toes, which always is seen before this stage, is more marked when this second level of response becomes distinct.

With still higher doses, the two sequentially developing effects are followed by a severe paralysis involving the entire leg; the animal now drags the poisoned leg. With this challenge level, animals often die from toxin being absorbed systemically. Animals may become moribund in a few hours, but local paralysis is seen before death occurs.

Sensitivity of the i.m. test. Table 1 shows that for all toxin types tested, the killing of mice by challenges given i.p. requires at least 10 times more toxin than the minimum required to produce local botulism by the i.m. challenge route. The variations in results obtained with the same toxin type are not unexpected when one considers the generally acknowledged lack of precision of the i.p. titration and the variable loss of inoculum from the i.m. injection site. The available data indicate that the fraction of an i.p. LD₅₀ unit constituting 1 i.m. ED₅₀ dose is probably the same for all botulinum toxin types.

Time to appearance of paralysis. Because of prolonged latencies in some mice given minimal paralytic doses, the observation periods needed to detect all positively reacting animals ranged up to 72 h (Table 1). Especially with these low challenge doses, responses to type E toxin developed sooner than to the other toxin types and recovery from paralysis occurred more frequently.

An inverse relationship between doses and latencies was evident. All toxin types at levels of 10 or more i.p. LD₅₀/0.1 ml induced local botulism in about 60 min. There was an apparent minimal latent period which was not shortened by higher doses. Effect of 1 to 5 i.p. LD₅₀ was generally evident by h 3. The range of latencies in a group of mice injected with the same dose is illustrated by results obtained with solutions made with type A toxic crystals (Table 2).

Determination of toxin type. In the experiments described so far the known nature of the test materials made possible the assumption that the paralysis had been caused by botulinum toxin. Such assumption cannot be made in practical circumstances, and the paralyzing agent must be proven to be botulinum toxin by showing that the toxicity can be neutralized by one of the type-specific antitoxins.

Type homologous antitoxin given i.p. did not

| Table 1. Fraction of i.p. LD₅₀ unit in 1 paralytic ED₅₀ dose of C. botulinum toxins* |
|---------------------------------|-----------------|-----------------|-----------------|
| Toxin type*                      | No. of tests    | i.p. LD₅₀ in 1 ED₅₀ | Time to record |
|                                 | Range       | Avg   | all positives (h)     |
| A                               | 4           | 0.06–0.09 | 0.07 | 48 |
| B                               | 3           | 0.07–0.10 | 0.09 | 48 |
| C                               | 1           | 0.08   | 72  |
| D                               | 1           | 0.07   | 48  |
| E (activated)                   | 1           | 0.07   | 24  |
| E (not activated)               | 3           | 0.05–0.09 | 0.07 | 24 |
| F                               | 1           | 0.06   | 48  |

* Animals were examined at 2, 4, 18, 48, and 72 h.

A includes three crystalline and one crude toxin preparations; B includes one toxin of 50% purity and two crude preparations; C, D, and F are all culture fluid supernatants; E (activated) is essentially pure toxin that is trypsinized; E (not activated) includes two essentially pure and one crude preparation.
Table 2. Inverse relationship between challenge doses and times to development of local paralysis*  

| Toxin dose (i.p. LD₅₀/mouse) | Range of latencies (min) |
|-------------------------------|-------------------------|
| 20.6                          | 45–80                   |
| 6.86                          | 50–80                   |
| 3.43                          | 70–145                  |
| 0.69                          | 140–200                 |
| 0.34                          | 210–310                 |
| 0.069                         | —                       |

*Six mice were tested with each dose; toxin solutions were made with different amounts of type A toxic crystals.  
*None positive at 6 h; one positive at 18 h.

Prevent i.m.-injected toxin from causing local paralysis although it protected against the lethal action of toxin diffusing from the injection site. Even 10 IU of antitoxin given i.p. 2 h before injection of 10 i.p. LD₅₀ of toxin did not consistently prevent local botulism. Toxin typing was possible if antitoxin was added to the test sample and the mixture was held for 30 min at room temperature before being injected. Parallel did not develop when the injected mixture contained 1 IU of type A antitoxin and up to 100 i.p. LD₅₀ (maximum tested) of type A toxin. Specificity of neutralization was shown by the paralysis that followed i.m. injection of 0.1 ml of a mixture containing 1 i.p. LD₅₀ of type A toxin and 10 IU of type B or E antitoxin.

Similar tests with other botulinum toxin types confirmed that only antitoxin of type homologous to the toxin would prevent development of local botulism. The toxin confirmation and typing procedure that was adopted was to mix 1 part antitoxin and 9 parts sample (vol/vol) so that the 0.1 ml to be injected contained 1 IU of antitoxin.

Toxin in foods. Different foods, with and without botulism toxin, were prepared. Fresh mushrooms were inoculated with C. botulinum spores and incubated under conditions where respiration of the vegetable could reduce the O₂ in the container. Canned foods were those to which toxin was added to about 10 or less i.p. LD₅₀/ml or in which toxin was produced in situ from an inoculum of 0.3 ml that was made by mixing equivolumes of 24-h-old cultures of Bacillus cereus, B. megaterium, and one (or no) C. botulinum. These cans of foods were resealed with solder; those inoculated with toxin were held at room temperature 24 to 48 days and those with bacteria at 30 C for up to 30 days. The food samples were tested by the i.m. procedure and an i.p. test which used 0.5-ml sample/mouse. When possible the tests were done with the supernatant fluid collected by centrifugation. When injectable fluid sample could not be obtained directly, the food was extracted with 1 or 2 parts (wt/vol) of phosphate-gelatin buffer.

Results are summarized in Table 3. Inability to detect toxin in some foods to which toxin had been added was not unexpected. The amount of toxin actually added could have differed from that calculated from the results of intravenous titrations of stock toxins, and some detoxification could have occurred during holding of the food.

When botulinum toxin was found, the proper toxin type was always identified. Except for two instances of conflict (mushroom inoculated with type B spores and a can of peas containing type B toxin at estimated 1 LD₅₀/ml), a positive or negative test for botulinum toxin by one method was duplicated by the other procedure. The two discrepant results were confirmed by repeat tests. Whatever the basis, results with these two samples cancel each other so that the data as a whole indicate the i.m. procedure to be as specific and sensitive as the i.p. test.

Effects of nonbotulinum lethal factors. Foods used in this study did not have mouse lethal factors which kill animals protected with homologous type of antitoxin and make difficult or impossible a conclusion as to the presence or absence of botulinum toxin. A limited series of tests were done to see whether or not the theoretically slower and reduced systemic absorption of injected solutes from the injection site might not make the i.m. method better than the i.p. test for examining such specimens.

Toxin sample (type A, about 10 i.p. LD₅₀/0.1 ml) was prepared in (NH₄)₂SO₄ solution of 10% saturation. Since one of two mice protected with type A antitoxin was killed by the toxic solution in less than 30 min, the botulinogenic nature of the sample could not be conclusively demonstrated by the i.p. test. i.m. injection of 0.1 ml resulted in an immediate tetanic response of the gastrocnemius, but this effect disappeared in less than 30 min. The true botulinum effect, which was prevented by homologous type of antitoxin, developed in the next 2 h. Essentially the same results were obtained with type B toxin in a solution of (NH₄)₂SO₄. Thus, the i.m. test identified the correct type of botulinum toxin in samples for which the i.p. test was unsuitable.

Discussion

Botulinum toxin injected into the gastrocnemius of mice elicits a local paralysis which is recognized by changes in the muscular control of the foot. The amount of toxin required to
produce this local botulism was slightly less than 1/10 that needed to kill mice by the i.p. challenge route. In practice, however, the sensitivities of the two tests were about equal, primarily because of volumes that can be used: 0.1 ml maximum i.m. versus about 0.5 ml i.p.

A disadvantage of the i.m. procedure is the effort needed for injecting mice and reading responses. Another possible disadvantage is the requirement for antitoxins of titer higher than generally available. If dilution of toxin in the sample is to be kept to a minimum, 1 volume of antitoxin should be mixed with 9 or more volumes of sample. If 0.1 ml of mixture is to contain 1 IU, antitoxin stock of at least 100 IU/ml are needed.

Although not as convenient for routine use as the i.p. procedure, the i.m. test could be useful as a supplementary procedure. Even with samples having as little as 5 i.p. LD₅₀/ml, the method generally gives definite results in 4 h or less. This is sooner than the deaths following i.p. challenge, although complete absence of illness in mice protected by one antitoxin type when all others show unmistakable botulism signs might sometimes justify the conclusion that the sample is botulogenic. Where sample amount is limited so that i.p. test cannot be done, the available sample might still be sufficient for the i.m. method. Although confirmation with natural samples is needed, results obtained with toxin solution of high (NH₄)₂SO₄ content suggest the possibility that nonbotulinum, mouse lethal factors might not interfere as much in the i.m. method as they can in the i.p. procedure.

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