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# NOTES

## Comparison of Progenitor Toxins of Nonproteolytic with Those of Proteolytic *Clostridium botulinum* Type B

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A nonproteolytic strain of *Clostridium botulinum* type B produces two toxins of different molecular weight (16*S* and 12*S*) that are indistinguishable from the corresponding toxins of a proteolytic strain in molecular weight and construction but differ in potential toxicity, activation ratio, and hemagglutinability. Successful hybridization between the toxic and nontoxic components (both 7*S*) of 12*S* toxins of biologically heterologous type B strains confirmed the physicochemical similarity between the toxic as well as the nontoxic components.

All *Clostridium botulinum* type A and some type B and F strains are proteolytic; all type C, D, and E strains and some type B and F strains are nonproteolytic. Nonproteolytic strains of types B, E, and F are known to produce toxins that are activated upon trypsinization (2, 3, 5). The progenitor toxin of a proteolytic type B strain Okra was obtained in two different molecular forms, 16*S* (L toxin) and 12*S* (M toxin) (9). Both L and M toxins consist of neurotoxic and nontoxic components. The neurotoxic component of either toxin has a molecular weight of 170,000 (7*S*) (1, 8). The nontoxic component of L toxin is about 12*S* and contains hemagglutinin activity, whereas that of M toxin is 7*S* and contains no hemagglutinin activity (9).

The toxins of proteolytic and nonproteolytic type B strains are slightly different antigenically (11, 12). The cultural and biological properties of nonproteolytic type B strains are more like those of type E and therefore different from those of proteolytic type B strains in that gas formation in carbohydrate fermentation is strong, the minimum growth temperature is 3 C, and the maximum heat resistance of spores is about 30 min at 60 C (13). The binding and competition of deoxyribonucleic acid isolated from cultures showed that a nonproteolytic type B strain was 100% homologous, whereas a proteolytic type B strain was only 11% homologous to a type E strain (10).

The toxin of a nonproteolytic type B strain has never been purified. We attempted to purify the progenitor toxin(s) of a nonproteolytic type B strain QC and compared their molecular construction and other properties with those of a proteolytic type B strain Okra. Kitamura and Sakaguchi (6) succeeded in reconstructing type E 12*S* toxin molecules with the dissociated 7*S* toxic and 7*S* nontoxic components, but failed to achieve hybridization between components of type E and A progenitor toxins. We also attempted to make hybrids with each component of M toxin of the nonproteolytic strain and the matching components of the M toxin of a proteolytic strain of *C. botulinum* type B and succeeded in achieving hybridization in any combination.

Nonproteolytic *C. botulinum* type B strain QC was grown in peptone-yeast extract-glucose medium for 4 days at 30 C. Progenitor toxins were purified by procedures similar to those used for purifying toxins of proteolytic type B strain Okra (9). Determinations of toxicity, protein, ribonucleic acid, and direct hemagglutinin, polyacrylamide gel electrophoresis, and agar gel diffusion were performed as reported previously (9). Horse antitoxic plasma against type B QC toxins (710 IU/ml), given by H. Kondo, Chiba Serum Institute, Ichikawa-shi, Chiba, was used for agar gel diffusion tests.

As was the case with proteolytic type B, two toxins of different molecular weight were eluted from Sephadex G-200 at the final step of purification. The early-eluted toxin was called L (large) toxin and the late-eluted was called toxin M (medium) toxin. The yield of purified type B QC L and M toxins was only 3.0 mg from a 30-liter culture. The recovery in potential toxicity (mouse intraperitoneal toxicity attained after trypsinization at pH 6.0) was only 3.4% of that found in culture. The specific potential toxicity (the potential toxicity per milligram of protein nitrogen) increased 600-fold by

the purification procedures. The corresponding figure for type B Okra toxin was about 10-fold (9). The specific potential toxicity of purified type B QC M toxin was $8 \times 10^8$ to $9 \times 10^8$ mean lethal doses per mg of N, and that of L toxin was $5 \times 10^8$ to $6 \times 10^8$ mean lethal doses per mg of N. Although the preparations were still not completely pure as judged by polyacrylamide gel electrophoresis, these toxicities were about twice those of type B Okra L and M toxins (9). Because of such high toxicities and relatively low toxin levels found in cultures, it was not easy to purify type B QC toxin. Hemagglutinin activity was not found in M toxin but was found in L toxin at 30 $\mu$g/ml or higher concentrations. The activity was considerably higher than that of Okra L toxin (9).

Potentiation results from trypsinization of crude culture of a nonproteolytic type B strain (2). Since trypsinization of highly purified QC L and M toxins increased the toxicities by about 100-fold, the phenomenon can be regarded as true activation. The activation ratio was considerably higher than that of type B Okra L and M toxins (9).

The sedimentation constants of QC L and M toxins were estimated to be 16*S* and 12*S*, respectively, since they emerged at the same positions in Sephadex G-200 gel filtration at pH 4.0 and sedimented to the same relative positions in sucrose density gradient centrifugation at pH 6.0, as did Okra L and M toxins.

The M toxin molecule (12*S*) appeared to consist of one molecule each of toxic and nontoxic components of the same molecular size of 7*S*, as was the case with Okra M toxin (9). In diethylaminoethyl-Sephadex chromatography at pH 8.0 (7), it behaved exactly like Okra M toxin; it was resolved into two protein peaks, the first one being toxic and the second one nontoxic, of the same areas. The elution positions of the two components were identical to those of the corresponding components of Okra M toxin. QC L toxin was eluted also in toxic and nontoxic peaks under the same conditions, but the area of the second peak was about three times as large as that of the first one. The elution positions and the relative areas of the two components were the same as those of the corresponding components of Okra L toxin (9).

Reconstruction of the 12*S* toxin molecule was attempted with the toxic and nontoxic components dissociated from M toxins of strains QC and Okra. The same quantities on a protein basis of the toxic and nontoxic components of the same or different origin were mixed together. The mixtures were dialyzed overnight against 0.05 M acetate buffer, pH 6.0, at 4 C. A 0.2-ml portion of each mixture was centrifuged in a 5 to 20% sucrose density gradient made in 0.05 M acetate buffer, pH 6.0, at 130,000 $\times$ *g* for 8 h at 4 C. Fractions were collected and analyzed for protein content and potential toxicity.

The 12*S* toxin molecule was reconstructed with the two dissociated components originating from the same M toxin (Fig. 1 a, b). Hybridization of the toxic component of either strain with the nontoxic component of the other strain was also successful (Fig. 1c, d). Each reconstructed or hybridized 12*S* toxin molecule proved to contain both the toxic and nontoxic components by agar gel double-diffusion tests (Fig. 2). Successful reconstruction of the 12*S* toxin molecule with toxic and nontoxic components of M toxin of the same as well as different origin confirmed the physicochemical similarity between the corresponding components of M toxins of proteolytic and nonproteolytic type B strains.

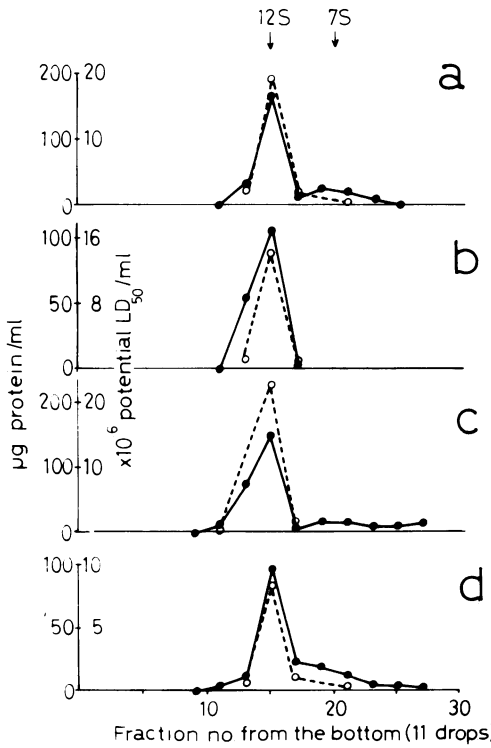


FIG. 1. *Ultracentrifugal analysis for reconstruction of 12S molecules in a sucrose density gradient (5 to 20%) at pH 6.0. (a) Toxic component (TC) plus nontoxic component (NC) of Okra M toxin; (b) TC plus NC of QC M toxin; (c) TC of Okra M toxin plus NC of QC M toxin; (d). TC of QC M toxin plus NC of Okra M toxin. Symbols:* ●, *protein;* ○, *potential toxicity.*

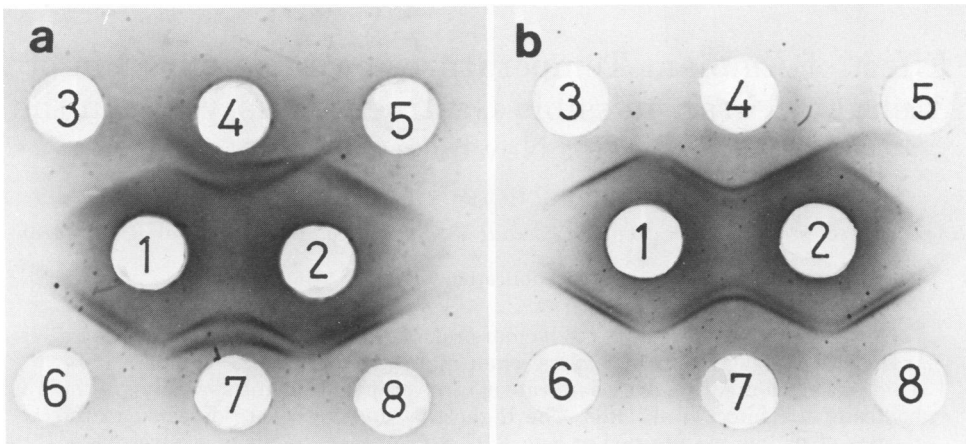


FIG. 2. *Agar gel double-diffusion tests with hybrid toxins. (a) Toxic component (TC) of Okra M toxin plus nontoxic component (NC) of QC M toxin. (1, 2) Antitoxin type B; (3) NC of QC M toxin; (4, 7) hybrid toxin; (5) TC of Okra M toxin; (6) QC M toxin; (8) Okra M toxin. (b) NC of Okra M toxin plus TC of QC M toxin. (1, 2) Antitoxin type B; (3) TC of QC M toxin; (4, 7) hybrid toxin; (5) NC of Okra M toxin; (6) QC M toxin; (8) Okra M toxin. The antitoxin was used at 355 IU/ml, and the antigens were used at concentrations of 176 to 282 μg/ml.*

This study demonstrated that not only the toxic but also the nontoxic components produced by genetically and biologically distinct organisms are immunologically and physicochemically similar. Bacteriophages isolated from proteolytic and nonproteolytic type B strains were morphologically indistinguishable and classified into the same group (4). Although the role of the bacteriophage in toxigenicity of *C. botulinum* type B has not been elucidated, the present findings could be explained by the infection of entirely different host organisms with identical pairs of bacteriophage(s) controlling formation of the toxic and nontoxic components.

## LITERATURE CITED

1. **DasGupta, B. R., D. A. Boroff, and K. Cheong.** 1968. Isolation of chromatographically pure toxin of *Clostridium botulinum* type B. Biochem. Biophys. Res. Commun. 32:1057-1063.
2. **Dolman, C. E., M. Tomsich, C. C. R. Campbell, and W. B. Laing.** 1960. Fish eggs as a cause of human botulism. J. Infect. Dis. 106:5-19.
3. **Duff, J. T., G. G. Wright, and A. Yarinsky.** 1956. Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol. 72:455-460.
4. **Eklund, M. W., E. T. Poysky, and E. S. Boatman.** 1969. Bacteriophages of *Clostridium botulinum* types A, B, E, and F and nontoxigenic strains resembling type E. J. Virol. 3:270-274.
5. **Holdeman, L. V.** 1967. Growth and toxin production of *Cl. botulinum* type F, p. 176-184. *In* M. Ingram and T. A. Roberts (ed.), Botulism 1966. Chapman and Hall, London.
6. **Kitamura, M., and G. Sakaguchi.** 1969. Dissociation and reconstruction of 12-S toxin of *Clostridium botulinum* type E. Biochim. Biophys. Acta 194:564-571.
7. **Kitamura, M., S. Sakaguchi, and G. Sakaguchi.** 1969. Significance of 12S toxin of *Clostridium botulinum* type E. J. Bacteriol. 98:1173-1178.
8. **Kozaki, S., and G. Sakaguchi.** 1975. Antigenicities of fragments of *Clostridium botulinum* type B derivative toxin. Infect. Immun. 11:932-936.
9. **Kozaki, S., S. Sakaguchi, and G. Sakaguchi.** 1974. Purification and some properties of progenitor toxins of *Clostridium botulinum* type B. Infect. Immun. 10:750-756.
10. **Lee, W. H., and H. Riemann.** 1970. Correlation of toxic and nontoxic strains of *Clostridium botulinum* by DNA composition and homology. J. Gen. Microbiol. 60:117-123.
11. **Sakaguchi, G., S. Sakaguchi, S. Kozaki, S. Sugii, and I. Ohishi.** 1974. 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. Jpn. J. Med. Sci. Biol. 27:161-172.
12. **Shimizu, T., and H. Kondo.** 1973. Immunological difference between the toxin of a proteolytic strain and that of a nonproteolytic strain of *Clostridium botulinum* type B. Jpn. J. Med. Sci. Biol. 26:269-271.
13. **Smith, L. Ds., and L. V. Holdeman.** 1968. The pathogenic anaerobic bacteria. Charles C Thomas, Springfield, Ill.