SHORT COMMUNICATIONS

Activation of Some *Clostridium botulinum* Type D Toxin by Trypsin

By A. NAKANE, K. OGUMA AND H. IIDA

*Department of Bacteriology, Hokkaido University School of Medicine,
Sapporo 060, Japan*

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INTRODUCTION

Toxin produced by *Clostridium botulinum* types A, B, E and F may be activated by exposure to trypsin (Duff *et al.*, 1956; Bonventre & Kempe, 1959, 1960; Iida, 1970). In *C. botulinum* types C and D, the toxicity of C₂ toxin, which is a minor toxin produced by both types C and D, is increased when the culture supernatant is treated with trypsin (Jansen & Knoetze, 1971; Eklund & Poysky, 1972). Activation of the major toxins (C₁ and D), however, has never been reported. The present communication suggests that some strains of *C. botulinum* type D produce their major toxin in a form that may be activated by trypsin.

METHODS

Clostridium botulinum type C and D strains. The origins of the test cultures have been reported elsewhere (Oguma *et al.*, 1976). Strains C-Stockholm, C-468, C-6814, C-D6F, D-1873, D-South African and D-4947 produce the major toxins C₁ or D. Strains C-A02 and D-139 are 'cured' strains in that they have been deprived of the bacteriophages controlling the production of the major toxin by curing strains C-Stockholm and D-1873, respectively. These 'cured' strains produce only C₂ toxin (Nakane *et al.*, 1978).

Preparation of the toxins. Two different media were used for cultivation of the organisms. The major toxins were produced in LYG medium, in which C₂ toxin is not formed in detectable amounts (Oguma *et al.*, 1976, Nakane *et al.*, 1978). This medium contained (% w/v): lactalbumin, 1; yeast extract, 2; glucose, 0.5; and L-cysteine.HCl, 0.15; at pH 7.2. C₂ toxin of high titre was produced in TY/glycerol medium which contained (% w/v): trypticase (BBL), 2; yeast extract, 3; glycerol, 0.5; NH₄Cl, 0.5; and L-cysteine.HCl, 0.1; at pH 7.4 (Nakane *et al.*, 1978). Cultures were incubated in 20 ml media at 37 °C for 5 d. The culture supernatants, clarified by centrifuging at 1630 g for 10 min at 4 °C, were used as toxin preparations.

Toxicity assays and activation of the toxin. Toxicity was assayed by injecting intraperitoneally, into each of five mice, 0.5 ml samples of twofold serial dilutions in 0.02 M-KH₂PO₄/Na₂HPO₄ buffer, pH 6.0, containing 0.2% (w/v) gelatin. The LD₅₀ value was calculated by the method of Reed & Muench (1938).

Activation of the toxin was assessed by injecting a mixture of 0.5 ml toxin preparation and 0.5 ml 0.5% (w/v) trypsin (1:250; Difco) dissolved in 0.1 M-KH₂PO₄/Na₂HPO₄ buffer, pH 6.0. The mixture was incubated for 1 h at 37 °C and then cooled to 4 °C before use.

Antitoxin sera and toxin neutralization test. CDC anti-D serum was obtained from the Center for Disease Control, Atlanta, Ga., U.S.A. Rabbit antiserum against the major toxin of D-South African (anti-D-SA) was prepared in this laboratory. This strain was cultivated in LYG medium to avoid the production of C₂ toxin. A bacteria-free culture fluid, prepared from the culture obtained by the 'cellophane tube' method (Oguma *et al.*, 1976), was concentrated with saturated (NH₄)₂SO₄. The concentrated material was dialysed against 0.1 M-sodium acetate/acetic acid buffer, pH 4.8, at 4 °C for 16 h and then chromatographed on a Sephadex G-200 column (2.5 × 90 cm) which was equilibrated with the same buffer. The fractionated toxin was applied to an SP-Sephadex C-50 column (1.5 × 25 cm), equilibrated with the same buffer, and eluted with a linear gradient of equal volumes of equilibrating buffer and the same buffer containing 0.3 M-NaCl. The partially purified toxin, which was concentrated by ultrafiltration, was detoxified with formalin and the toxoid was used to immunize rabbits.

Rabbit antiserum against C₂ toxin (anti-D-139) was prepared with the toxin produced by strain D-139 in TY/glycerol medium during incubation at 37 °C for 7 d. The toxin from the culture supernatant was precipitated by (NH₄)₂SO₄ added to 60% saturation. The precipitate was dissolved in 0.05 M-KH₂PO₄/K₂HPO₄

Table 1. *Effect of trypsin treatment on the major toxins produced by C. botulinum types C and D*

The major toxin-producing strains were incubated in LYG medium. Toxicity was assayed in the untreated culture supernatants and in the culture supernatants after treatment with trypsin, as described in Methods.

Strain	$10^4 \times \text{Toxicity (LD}_{50} \text{ ml}^{-1})$	
	Untreated	Trypsin treated
C-Stockholm	15	15
C-468	0.63	0.63
C-6814	0.94	1.4
C-D6F	6.3	4.5
D-1873	150	150
D-South African	150	940
D-4947	1.5	9.4

buffer, pH 7.0, and dialysed against the same buffer at 4 °C for 16 h. The soluble fraction was passed through a column of DEAE-cellulose (2.5 × 30 cm) which was equilibrated with 0.05 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.0. The eluate was concentrated by ultrafiltration. Rabbits were immunized with the toxoid made from this toxin preparation.

In toxin neutralization tests, equal volumes of toxin preparation and antitoxin serum were mixed and incubated at 37 °C for 1 h. The mixture, containing 300 to 400 LD₅₀ of toxin and 5 international units of antitoxin serum, was titrated for active toxin.

RESULTS AND DISCUSSION

Activation of the major toxin

The effect of trypsin treatment on the toxicities of C₁ and D toxins was investigated. Four type C and three type D strains were incubated in LYG medium, in which no detectable C₂ toxin is produced (Oguma *et al.*, 1976; Nakane *et al.*, 1978). The toxicity of their clarified supernatants was determined with or without trypsin treatment. The toxicities of D-South African and D-4947 were significantly enhanced when the culture fluids were treated with trypsin, whereas the toxicities of D-1873 and the type C strains were little affected (Table 1). One possible explanation for this phenomenon is that C₂ toxin was also produced and was activated. However, this explanation may be discounted for the following reasons: (i) it is unlikely that such a high titre of C₂ toxin would be produced, since the toxicity of C₂ toxin was less than 10³ LD₅₀ ml⁻¹ in various media even after trypsin treatment (Nakane *et al.*, 1978); (ii) detectable amounts of C₂ toxin are not formed in LYG medium (Oguma *et al.*, 1976; Nakane *et al.*, 1978); and (iii) 'cured' strains of D-South African did not produce C₂ toxin (Nakane *et al.*, 1978).

Antigenicity of the trypsin-activatable toxin

We performed neutralization tests to obtain evidence that the major toxin (D toxin) but not C₂ toxin was activated by trypsin treatment in D-South African and D-4947. Anti-D toxin sera (CDC anti-D and anti-D-SA) and anti-C₂ toxin serum (anti-D-139) were used. The trypsin-activatable toxicity of D-South African and D-4947 and the non-activatable D toxin of D-1873 were all completely neutralized by CDC anti-D and anti-D-SA sera which had no ability to neutralize C₂ toxin. The toxicities of these samples were not due to C₂ toxin since the antiserum monospecific for it (anti-D-139) did not reduce the toxin titres. These results indicate that the D toxin (major toxin) produced by strains D-4947 and D-South African is activated by trypsin.

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