INFECTION AND IMMUNITY, Dec. 1980, p. 656-660
0019-9567/80/12-0656/05$02.00/0

Vol. 30, No. 3

Antigenic Similarity of Toxins Produced by *Clostridium botulinum* Type C and D Strains

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Antisera against purified type C_1 toxin of *Clostridium botulinum* and its heavy-chain component cross-neutralized type D toxin. Antisera against partially purified type D toxin cross-neutralized type C_1 toxin. From the latter serum, a component which neutralized only type D toxin and a component which equally neutralized both type C_1 and D toxins were obtained. We concluded that the cross-neutralization was not due to the fact that type C and D strains produce both C_1 and D toxins but rather to the fact that the toxins have an antigen(s) common to their molecules. The results of the agar gel-double-diffusion test also supported this conclusion.

Toxins produced by *Clostridium botulinum* type C and D strains are complex. Type C and D strains have been reported to produce three different toxins, types C_1, C_2 and D, in the proper medium (1, 5). Strains which produce predominantly C_1 and C_2 toxins are designated C_α and C_β, respectively; type D strains produce D toxin in the greatest amount. These conclusions were based on the cross-neutralization between each toxin type and antisera raised with detoxified culture fluids of types C and D. Type C_1 and D toxins exist in culture supernatants as molecules, with sedimentation constants of 12 to 16S (7, 10, 11), formed by the association of the neurotoxin (7S) and a nontoxic nonhemagglutinating or a nontoxic hemagglutinating component. This association is dissociated in an alkaline solution. The molecular weights of purified C_1 and D neurotoxins were reported previously as 141,000 and 170,000, respectively (7, 13).

A nontoxic hemagglutinating component was estimated to consist of a nontoxic nonhemagglutinating component with the other subcomponent(s) possessing hemagglutinating activity (6, 7). In this paper, whole nontoxic components and purified C_1 toxin are referred to as hemagglutinin (HA) and C_1 neurotoxin or 7S toxin, respectively. It was reported that the antigenicity of HA from type C and D toxin preparations was identical, but the antigenicity of the neurotoxins in these preparations was different (7, 12).

Production of C_1 and D toxins and HA is governed by bacteriophages (3, 4, 8, 9). Cultures that are cured of their prophages cease to produce C_1 and D toxins and HA, but are again converted to toxin and HA producers when reinfected with the phages. C_2 toxin production is not related to these phages since some cured strains still continue to produce C_2 toxin when incubated in the proper medium (2, 8). C_2 toxin is produced as a precursor so that its presence becomes apparent only after the cultures are activated with trypsin. Therefore, we call the cured strains nontoxigenic strains even though they produce C_2 toxin.

In this report, we present neutralization and gel diffusion data indicating that sufficient homology exists among the molecules of type C_1 and D toxins and toxins produced by the organisms which reconverted from nontoxigenic to toxin production by bacteriophages.

MATERIALS AND METHODS

Bacterial strains. The following *C. botulinum* strains were used: C-Stockholm (C-ST), C-CB19, D-South African toxigenic (D-SA TOX), D-1873, and D-CB16. Strains C-CB19 and D-CB16 were obtained from I. Ohishi (College of Agriculture, University of Osaka Prefecture, Osaka, Japan). The other strains were obtained from our laboratory. Converted toxigenic strains C-A02 NT(c-st) and D-SA NT(d-sa) were obtained from plaques made on nontoxigenic cells by phages (9). Cultures were maintained in cooked meat medium at 4°C. Media used for toxin production were reported in previous articles (8, 13).

Chemicals. Sephadex G-200 (medium and superfine), sulphopropyl Sephadex C-50, quaternary aminoethyl Sephadex A-50, and CNBr-activated Sepharose 4B were products of Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (Brown), sodium dodecyl sulfate, 2-mercaptoethanol, and agarose were purchased from Nakarai Chemicals, Kyoto, Japan. Freund incomplete adjuvant was purchased from Difco Laboratories, Detroit, Mich.

Partial purification of toxins. Strains C-ST and D-SA TOX and converted toxigenic strains C-A02 NT(c-st) and D-SA NT(d-sa) were incubated by the cellophane tube procedure (8). Each 2 liters of culture (2×10^5 to 4×10^4 LD_{50}/ml) was centrifuged (6,000 × *g*, 30 min), and the toxins in the supernatants were

precipitated with saturated ammonium sulfate. The precipitates were suspended in 10 ml of buffer, dialyzed, and subjected to column chromatography on Sephadex G-200 (medium, 2.5 by 90 cm) and sulphopropyl Sephadex C-50 (1.5 by 25 cm) columns. Sodium acetate-acetic acid buffer, 0.1 M, pH 4.8, was used throughout the experiments. Toxins eluted from a sulphopropyl Sephadex C-50 column with a linear gradient of NaCl in buffer from 0 to 0.3 M were concentrated by ultrafiltration to 8 ml (1×10^7 to 3×10^6 LD_{50}/ml).

Purification of C_1 neurotoxin and its separation. C_1 neurotoxin was purified from the C-ST strain by Sephadex G-75, DEAE-cellulose, and quaternary aminoethyl Sephadex A-50 (13) column chromatography. All experiments were done in an alkaline solution of 0.064 M ($\mu = 0.12$) borax-phosphate buffer, pH 8.0, to separate the neurotoxin and HA. However, the preparation eluted from quaternary aminoethyl Sephadex A-50 still possessed a trace of HA. Contaminating HA was removed by applying it to a Sepharose 4B column bound with anti-HA globulin obtained from anti-C crude toxin horse serum (13). Heavy-chain (Hc) and light-chain (Lc) components of this purified 7S toxin were obtained as follows. A 10-mg amount of toxin was adsorbed on a column of quaternary aminoethyl Sephadex A-50 (1.2 by 8 cm) equilibrated with 0.064 M borax-phosphate buffer, pH 8.0. From this column, Lc component was first eluted with 5% 2-mercaptoethanol in the same buffer, and then undissociated toxin was eluted with 0.1 M NaCl in buffer with 2-mercaptoethanol. Finally, Hc component was eluted with 0.2 M NaCl in buffer with 2-mercaptoethanol.

Partial purification of HA. As reported previously, DEAE-cellulose (Brown) equilibrated with 0.064 M borax-phosphate buffer does not absorb C_1 neurotoxin, but does bind HA (13). HA was eluted from this column with 0.3 M NaCl in the same buffer. This HA fraction was contaminated with toxin, and it was removed by applying it to a Sephadex G-200 column (superfine, 3.2 by 45 cm) and then to a Sepharose 4B column which was conjugated with anti-C-ST 7S globulin as described below. The final HA preparation had no toxigenicity in mice but showed an HA titer of more than 1×10^4 by the tube method with 1% human erythrocytes (8).

Preparation of antisera. Antisera against partially purified toxins, HA, purified C-ST 7S toxin, and Hc and Lc components were made in rabbits. Toxins were made toxoid by dialyzing against 0.01 M sodium-phosphate buffer, pH 8.0, containing Formalin at 0.4% for 7 days at 30°C (7). After mixing 6 ml of each of the preparations with an equal volume of Freund incomplete adjuvant, three 2-ml aliquots of each emulsion were injected subcutaneously into two rabbits at 3-week intervals. The sera were harvested 3 weeks after the last injection.

Affinity chromatography. A 5-mg portion of C-ST 7S toxin and 15 mg of HA in 10 ml of 0.064 M borax-phosphate buffer, pH 8.0, were separately coupled with CNBr-activated Sepharose 4B at 4°C overnight with gentle mixing, and toxin and HA columns were made. Antisera against C-ST 7S toxin and HA were loaded on these toxin and HA columns, respectively. Columns were kept overnight at 4°C and then washed with borax-phosphate buffer, pH 8.0; immunoglobulins were eluted next with 0.2 M glycine-HCl buffer, pH 2.5, at a flow rate of 5 ml/h. The pH of the eluted fractions was immediately adjusted to 7.2 by adding several drops of 1.0 M glycine-NaOH buffer, pH 11.0. Immunoglobulins thus obtained neutralized 10 LD_{50} of C_1 toxin per ml and 8 U of HA to 8×10^3 and 1×10^4, respectively. Immunoglobulins (10 ml of each) were mixed with 3 g of activated Sepharose 4B, and anti-C-ST 7S toxin and anti-HA columns were made.

Neutralization test. Antisera were diluted in serial 10-fold steps and then 2-fold steps with 0.01 M sodium phosphate-buffered saline, pH 7.2. Each 100 LD_{50}/ml of toxins was made with 0.02 M phosphate buffer, pH 6.0, containing gelatin at 0.2%, and kept frozen at −80°C until use. The stock toxins were diluted 10 times with 0.01 M phosphate-buffered saline, pH 7.2, and then mixed with an equal volume (1 ml) of each diluted antiserum. After incubation at 37°C for 2 h, 0.5 ml of mixture was injected intraperitoneally into three mice. The mice were observed for 6 days. The highest dilution which neutralized toxin was reported as the neutralization titer of antisera.

Agar gel-double-diffusion test. A 7-ml portion of 1% agarose gel was poured into petri dishes (9 cm in diameter). Wells (5 mm in diameter) were made with a distance of 4 mm. Each well was filled with a 15-μl sample and incubated in a moist chamber for 3 days at 20°C. Purified C-ST 7S toxin, Hc and Lc components containing 300 μg of protein per ml and partially purified type D toxins showing 5×10^6 LD_{50}/ml were used.

RESULTS

Characteristics of purified C_1 toxin and Hc and Lc components. Purified C-ST 7S toxin showed no HA activity, but a toxicity of 5×10^7 LD_{50}/mg of protein (13) was observed. A 50-μg portion of purified toxin showed a single band of molecular weight 141,000 in sodium dodecyl sulfate-polyacrylamide electrophoresis performed by the method of Weber et al. (14). In the presence of 2-mercaptoethanol at a concentration of 1%, toxin dissociated into two components (Fig. 1) with molecular weights of 98,000 (Hc) and 53,000 (Lc).

Purified Hc and Lc components which had no lethal activity in mice were obtained from C-ST 7S toxin as described above. Upon electrophoresis with 0.1% sodium dodecyl sulfate, each 50-μg portion of these purified Hc and Lc preparations migrated to the same positions of Hc and Lc components observed above. (Detailed characteristics of Hc and Lc components will be published in a separate article.)

Cross-neutralization of type C_1 and D toxins. The toxin-neutralizing ability of antisera was determined by mixing an equal volume of diluted serum with 10 LD_{50}/ml of toxins, C-ST, C-A02 NT(c-st), C-CB19, D-SA TOX, D-SA NT(d-sa), D-1873, and D-CB16. Antisera pre-

pared with D-SA and D-SA NT(d-sa) toxoids cross-neutralized C-ST, C-A02 NT(c-st) and C-CB19 toxins (Table 1). Antisera prepared with partially purified C-ST and C-A02 NT(c-st) toxoids cross-neutralized D-SA and D-SA NT(d-sa) toxins, but not D-1873 and D-CB16 toxins. Antisera against C-ST 7S toxin and Hc component cross-neutralized these four different type D toxins, although the neutralization titer to D-SA and D-SA NT(d-sa) toxins was much higher than that to D-1873 and D-CB16 toxins. Antisera against C_1 and D toxins neutralized three C_1 toxins and four D toxins, respectively, to a similar extent. The neutralization titer of the anti-Lc component serum to C_1 toxin was very low, although the serum formed a clear precipitin line with both Lc component and C-ST toxin in the Ouchterlony immunodiffusion test.

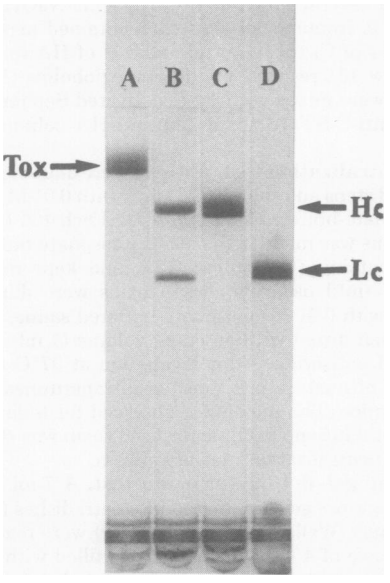

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide electrophoresis of purified C-ST toxin and Hc and Lc components. C-ST 7S toxin was treated with 1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer, pH 7.2, at 100°C for 2 min in the presence (B) or absence (A) of 1% 2-mercaptoethanol. Hc (C) and Lc (D) components obtained as described in the text were treated with 1% sodium dodecyl sulfate at 100°C for 2 min. Preparations (50 μg of each) were loaded onto a gel. Electrophoresis was performed with 8% polyacrylamide gels containing 0.1% sodium dodecyl sulfate at 8 mA per gel.*

Agar gel-double-diffusion test. The Ouchterlony immunodiffusion test was carried out with the toxins and antisera obtained. Antiserum against partially purified D-SA toxin contained an antibody against HA. Therefore, this serum was subjected to an HA column to remove it before use. From partially purified D-SA and D-SA NT(d-sa) toxins, HA was also removed by applying them to a column of anti-HA at pH 8.0. The precipitin lines were formed between anti-C-ST 7S toxin serum and D-SA and D-SA NT(d-sa) toxins, and both lines spurred to the line formed with Hc component. Hc and Lc components showed the different antigenicity (Fig. 2A). Lines were also formed between anti-D-SA serum and both C-ST 7S toxin and Hc component, although no line appeared with Lc component. These lines fused to each other and both spurred to the lines formed between anti-D-SA toxin serum and both D-SA and D-SA NT(d-sa) toxins (Fig. 2B). When HA was put in a center well, lines appeared only with anti-HA

TABLE 1. *Neutralization test with antisera and toxins*[a]

Antiserum	Toxin				
	C-ST	C-CB19	D-SA	D-1873	D-CB16
Anti-D-SA	256	128	4×10^4	2×10^4	2×10^4
Anti-D-SA NT(d-sa)	16	8	4×10^3	2×10^3	2×10^3
Anti-C-ST[b]	4×10^3	4×10^3	8	<4	<4
Anti-C-AO2 NT(c-st)	8×10^3	8×10^3	32	<4	<4
Anti-C-ST 7S[c]	8×10^4	4×10^4	512	16	32
Anti-C-ST Hc	2×10^4	2×10^4	128	8	8
Anti-C-ST Lc	4×10^2	2×10^2	<4	<4	<4
Anti-HA[d]	<4	<4	<4	<4	<4
Anti-D-CDC[e]	<4	<4	32	32	32
Anti-D-SA specific	<4	<4	2×10^4	8×10^3	8×10^3
Anti-D-SA common concn[f]	1,024	1,024	1,024	128	256

[a] The neutralization test was performed by mixing diluted sera with 10 LD_{50}/ml of toxins. Numbers in the table are reciprocals of the final highest dilutions of sera to neutralize toxins. The same results were obtained by using C-AO2 NT(c-st) and D-SA NT(d-sa) toxins instead of C-ST and D-SA toxins, respectively.

[b] Serum against partially purified C-ST toxin.

[c] Serum against purified C-ST 7S toxin.

[d] Serum against HA or nontoxic components.

[e] Serum was obtained from the Center for Disease Control, Atlanta, Ga.

[f] Serum was concentrated by ultrafiltration.

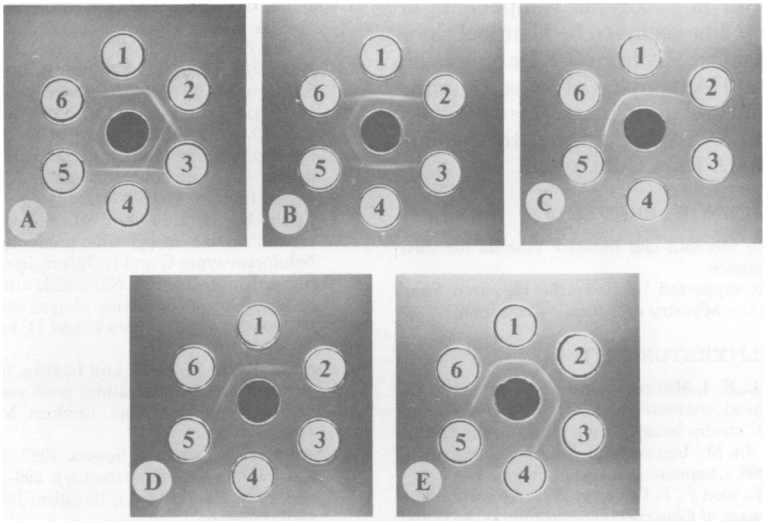

FIG. 2. *Agar gel-double-diffusion tests. (A) Center well, anti-C-ST 7S toxin serum; (1, 4) Hc component; (2) C-ST 7S toxin; (3) Lc component; (5) D-SA toxin; and (6) D-SA NT(d-sa) toxin. (B) Center well, anti-D-SA toxin serum; (1) D-SA toxin; (2) Lc component; (3) HA; (4) D-SA NT(d-sa) toxin; (5) Hc component; and (6) C-ST 7S toxin. (C) Center well, HA; (1) anti-D-SA toxin serum which was not applied to an HA column; (2) anti-D-SA toxin serum passed through an HA column; (3) anti-C-ST 7S toxin serum; (4) anti-Hc component serum; (5) anti-D-SA-common serum; and (6) anti-HA serum. (D) Center well, anti-D-SA-specific serum; (1) D-SA toxin; (2) C-ST 7S toxin; (3) Hc component; (4) Lc component; (5) HA; and (6) D-SA NT(d-sa) toxin. (E) Center well, anti-D-SA-common serum; (1) D-SA toxin; (2) D-SA NT(d-sa) toxin; (3) Hc component; (4) Lc component; (5) HA; and (6) C-ST 7S toxin.*

serum and anti-D-SA toxin serum, which was not applied to an HA column (Fig. 2C).

Preparation of anti-D-SA-specific and anti-D-SA-common sera. An antibody which neutralized only type D toxin and an antibody which neutraized both type C_1 and D toxins were obtained from whole anti-D-SA toxin serum by using affinity chromatography. Anti-D-SA toxin serum was first applied to an HA column and the pass-through fractions were applied to a C-ST 7S toxin column. The pass-through fractions from the latter column neutralized only type D toxin and were named anti-D-SA-specific serum (Table 1). This serum formed a precipitin line with only type D toxins (Fig. 2D). The fractions eluted from a C-ST 7S toxin column with 0.2 M glycine-HCl buffer, pH 2.5, were concentrated by ultrafiltration and then used for both neutralization and immunodiffusion tests. The serum equally neutralized C-ST, C-CB19 and D-SA toxins; it also neutralized D-1873 and D-CB16 toxins, but to a lesser extent (Table 1). In Ouchterlony tests, a line appeared with D-SA, D-SA NT(d-sa), and C-ST 7S toxins and the Hc component. All these lines fused to each other (Fig. 2E). This immunoglobulin was designated anti-D-SA-common serum.

DISCUSSION

Anti-D and anti-C toxin sera cross-neutralized C_1 and D toxins, respectively, even though purified C-ST 7S toxin was used as an immunogen. Titers of three anti-D toxin sera to neutralize four different type D toxins were similar, but those of anti-C toxin sera were different depending on the type D toxins used. These results suggest that cross-neutralization is not due to the fact that type C and D strains produce both C_1 and D toxins, but to the fact that there exists sufficient homology among the molecules of type C_1 and D toxins to permit partial neutralization by a serum against a single toxin type. Similarity of C-ST and C-CB19 toxins to D-SA toxin may be greater than to D-1873 and D-CB16 toxins.

This hypothesis was also supported by agar gel-double-diffusion testing. The data indicate that C-ST toxin has a common part(s) to D-SA toxin in its Hc portion. Furthermore, anti-D-SA-specific and anti-D-SA-common sera were obtained. The conclusion that type C and D strains produce three different toxins should be reconsidered.

Our data also pointed out that the Hc portion can be used as a vaccine because it has no lethal activity but is immunogenic.

The same reuslts were obtained with toxins from the converted strains and their antisera instead of the original toxins and their antisera. This suggests that one phage governs the production of only a single toxin type.

Antigenicity of nontoxigenic components of type C_1 and D toxins seems to be identical since (i) antiserum against HA from C-ST crude toxin neutralized the HA activity of type D crude toxin and (ii) the precipitin line formed between this serum and D-SA toxin fused to the line made with the HA from C-ST toxin.

ACKNOWLEDGMENTS

We thank Eriko Momota and Masako Yoshida for their fine technical assistance.

This study was supported by Scientific Research Fund grant 477223 from the Ministry of Education of Japan.

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