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Comparison of Antigenicity of Toxins Produced by *Clostridium botulinum* Type C and D Strains

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C_1 neurotoxin of *Clostridium botulinum* strains C-Stockholm (C-ST), Cβ-Yoichi, C-468, CD6F, and C-CB19 and type D toxin of strains D-1873 and D-CB16 were purified by gel filtration, ion exchange, and affinity chromatographies. The purified toxins had di-chain structure made of heavy and light chains. The toxins of Cβ-Yoichi, C-468, CD6F, and C-CB19 reacted with anti-C-ST heavy chain and anti-C-ST light chain in immunodiffusion tests and enzyme-linked immunosorbent assay, whereas D-CB16 toxin reacted with anti-D-1873 heavy chain and anti-D-1873 light chain. However, C-6813 toxin reacted with anti-D-1873 heavy chain and anti-C-ST light chain but not with anti-C-ST heavy chain or anti-D-1873 light chain immunoglobulin G. These results indicate common antigens in the heavy chains of C-6813 and D-1873 toxins and in the light chains of C-6813 and C-ST toxins. Further, they provide evidence for heterogeneity within type C_1 toxin subunits.

Toxins produced by *Clostridium botulinum* type C and D strains are complex. Type C and D strains have been reported to produce three toxins of different antigenicity, designated C_1, C_2, and D, in the proper medium (2, 5). Type C strains produce predominantly C_1 toxin and minor amounts of C_2 and D toxins. In contrast, type D strains produce predominantly D toxin and minor amounts of C_1 and C_2 toxins. Although type C toxin was classified into two groups, C_1 and C_2, the relationships between C_1 and C_2 toxins and Cα and Cβ toxins (2, 11) is still obscure. In recent experiments, common antigens in the molecules of type C_1 and D toxins were observed (9, 10). Those complex findings lead us to presume that antigenic diversities exist within type C_1 toxin (9). Since the type C_1 and D toxins are composed of disulfide-linked heavy and light chains like type A, B, and E (trypsinized) toxins (3, 12, 13), the antigenic differences were analyzed by immunological methods. In this paper, the purification procedures are described, and the existence of two subtypes of C_1 toxin is discussed.

MATERIALS AND METHODS

Organisms and toxin production. Eight strains of *C. botulinum* were used: C-Stockholm (C-ST), Cβ-Yoichi, C-468, C-6813, CD6F, and C-CB19 as C type and D-1873 and D-CB16 as D type. The toxins were produced by the dialyzing cultivation method reported previously (12).

Purification of toxins. The toxins and heavy and light chains of strains C-ST and D-1873 were purified as reported previously (9, 13). Toxins of C-ST and D-1873 were homogeneous by disc gel electrophoresis and sodium dodecyl sulfate (SDS)-gel electrophoresis. The M_r of D-1873 toxin was 140,000, as determined in triplicate SDS-gel electrophoresis with 8% polyacrylamide gel, and the toxin was separated into heavy chain (M_r, 85,000) and light chain (M_r, 55,000) in the presence of 2-mercaptoethanol. The toxins of other strains were purified by the steps shown in Table 1. The crude toxin in the culture supernatant was precipitated by adding saturated ammonium sulfate solution to 35% saturation. The crude toxin, dissolved in 10 to 15 ml of 20 mM Tris-glycine buffer (pH 8.0) containing 0.5 M NaCl, was loaded on a column (3.5 by 120 cm) of Sephacryl S-300 (superfine; Pharmacia Chemicals) equilibrated previously with the same buffer and eluted with the buffer at a flow rate of 40 ml/h. The pool of toxin fractions was dialyzed against 72.5 mM borax-sodium dihydrogen phosphate buffer (pH 8.1) (buffer A), and the dialysate was applied to a column (2.2 by 15 cm) of quaternary aminoethyl-Sephadex A-50 (Pharmacia) equilibrated with buffer A. After the unbound protein was washed out with a one-column volume of buffer A, the toxin was eluted with a linear gradient of buffer A to buffer A plus 0.15 M NaCl. A pool of toxin fractions was concentrated by dialysis against saturated ammonium sulfate solution. The centrifuged toxin pellet was dissolved and passed through a column (3.3 by 50 cm) of Sephadex G-200 (superfine; Pharmacia), and the collected fractions were then applied to a column (1.2 by 10 cm) of anti-hemagglutinin-binding Sepharose 4B (9, 13) equilibrated with buffer A to remove remaining hemagglutinin and hemagglutinin-like proteins.

Preparation of anti-toxin subunit IgG. The purified toxin of strain C-ST or D-1873 was detoxified with 0.4% formaldehyde (9, 10) for 4 days at room temperature and was used to immunize rabbits. The obtained antiserum was applied to a column (1 by 4 cm) of C-ST (heavy chain)- or D-1873 (heavy chain)-binding Sepharose 4B (13). The column was washed with 10 column volumes of 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl (phosphate-buffered saline) to remove unbound nonspecific serum proteins. Then, anti-C-ST heavy chain (or anti-D-1873 heavy chain) immunoglobulin G (IgG) was eluted with 3 M KSCN and dialyzed against phosphate-buffered saline. Anti-C-ST light chain (or anti-D-1873 light chain) IgG was similarly prepared from the rabbit antiserum by affinity chromatography with light chain-binding Sepharose 4B.

Immunodiffusion test. The Ouchterlony double diffusion test was performed in 1% special agar B (Wako). Wells (5.0 mm in diameter) were spaced 5.0 mm apart. The center well

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TABLE 1. Purification procedure, purity, and toxicity of toxins

Toxin	Purification procedure				Purity (%)[a]	Toxicity (MLD/A_{278})[b] ($\times 10^7$)
	Sephacryl S-300 gel filtration	Quaternary aminoethyl-Sephadex A-50 ion exchange	Sephadex G-200 gel filtration	Anti-hemagglutinin affinity chromatography		
Cβ-Yoichi	+[c]	+		+	71.2	6.0–11.0
C-468	+		+	+	89.5	3.0–4.0
C-6813	+		+	+	57.0	6.0–10.0
CD6F	+		+	+	50.0	2.0–2.5
C-CB19	+		+	+	50.0	5.6–10.0
D-CB16	+	+		+	85.8	1.0–1.2

[a] Purity was determined by a densitometer with the electrophoretogram of the toxin in polyacrylamide gel with SDS.
[b] MLD/A_{278}, Minimum lethal dose/absorbance at 278 nm.
[c] +, Purification step used for each toxin.

was filled with 20 μl of anti-heavy chain IgG (0.78 mg/ml) or anti-light chain IgG (1.53 mg/ml). The peripheral wells were filled with 20 μl of various purified toxins (0.4 mg/ml). The charged plates were incubated for 4 days at room temperature.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed with microtiter plates (model 239454; Nunc) by the sandwich method (7) with modification. A 100-μl volume of rabbit antitoxin heavy chain (or light chain) IgG in buffer A (36 μg/ml) was added to each well, and the microtiter plate was refrigerated (5°C) overnight. Wells were rinsed twice with phosphate-buffered saline buffer containing 0.5% bovine serum albumin, 0.05% Tween 20, and 0.02% NaN_3 (pH 7.2) (buffer B). A 100-μl sample of antigen was added to each well (1.0 to 100 ng of toxin per well), the plate was incubated at 37°C for 2 h, and the unbound toxin was removed with four rinses with buffer B. Then, a 200-μl portion of alkaline phosphatase (900 U/mg; Sigma Chemical Co.)-horse antitoxin IgG conjugate was added to the wells. The plate was again incubated at 37°C for 2 h and then washed four times with buffer B. The enzymatic reaction was started by adding 200 μl of 2.5 mM *p*-nitrophenyl phosphate dissolved in 50 mM carbonate-bicarbonate buffer containing 1 mM $MgCl_2$ (pH 9.8). After 1 h of incubation at 37°C, the reaction was terminated by the addition of 25 μl of 3 M NaOH. The absorbance of the reaction mixture was determined at 405 nm by a Titertek Multiskan (Flow Laboratories, Inc.)

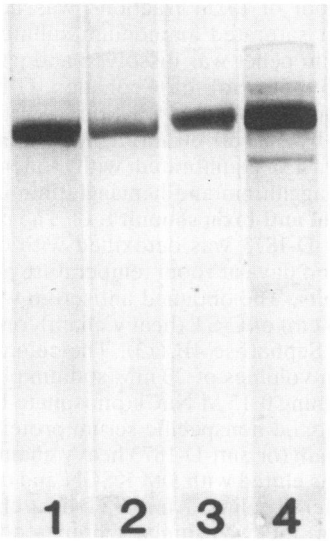

FIG. 1. SDS-polyacrylamide gel electrophoretic patterns of toxins. Lane 1, Cβ-Yoichi; lane 2, D-CB16; lane 3, C-468; lane 4, C-6813. Protein (50 μg) was loaded on a gel, and the electrophoresis in SDS was carried out by the method of Weber et al. (14) with 8% polyacrylamide gel at 8 mA per gel.

Toxicity assay. White mice (dd strain) weighing about 20 g were injected intravenously with 0.1-ml portions of the toxin in 0.1 M sodium phosphate buffer (pH 6.9). The time-to-death assay (1) used six mice for each determination.

Protein determination. Protein was routinely estimated by the method of Lowry et al. (6) with bovine serum albumin as standard. The concentrations of C-ST and D-1873 toxins were routinely estimated by absorbance at 278 nm and neutral pH by using $A_{cm}^{1\%} = 14.18$ and 11.40, respectively. Absorption coefficients were calculated from the weights of purified toxins.

RESULTS

Purification and molecular structure. The purification procedures, purities, and toxicities of six toxin preparations are summarized in Table 1. The acrylamide gel electrophoretic patterns of four toxins are shown in Fig. 1. The purity range was 50 to 90%, as determined by densitometry of protein bands. C-468 and D-CB16 toxins, recovered in three purification steps, were 85 to 90% pure (Table 1). All toxins had molecular weights within the range of 140,000 to 150,000 in polyacrylamide gel electrophoresis with SDS, and under reducing reagent they dissociated into heavy and light chains with molecular weights of about 100,000 and 50,000, respectively. When corrected for differences in purities, all toxin preparations had comparable specific toxicities.

Double-gel immunodiffusion test. Reactions of toxins with

TABLE 2. Double-diffusion test of toxin to anti-subunit IgG[a]

Toxin	C-ST		D-1873	
	Anti-heavy chain IgG	Anti-light chain IgG	Anti-heavy chain IgG	Anti-light chain IgG
Cβ-Yoichi	+	+	−	−
C-468	+	+	−	−
C-6813	−	+	+	−
CD6F	+	+	−	−
C-CB19	+	+	−	−
D-CB16	−	−	+	+

[a] +, Formation of precipitin line; −, no formation of precipitin line.

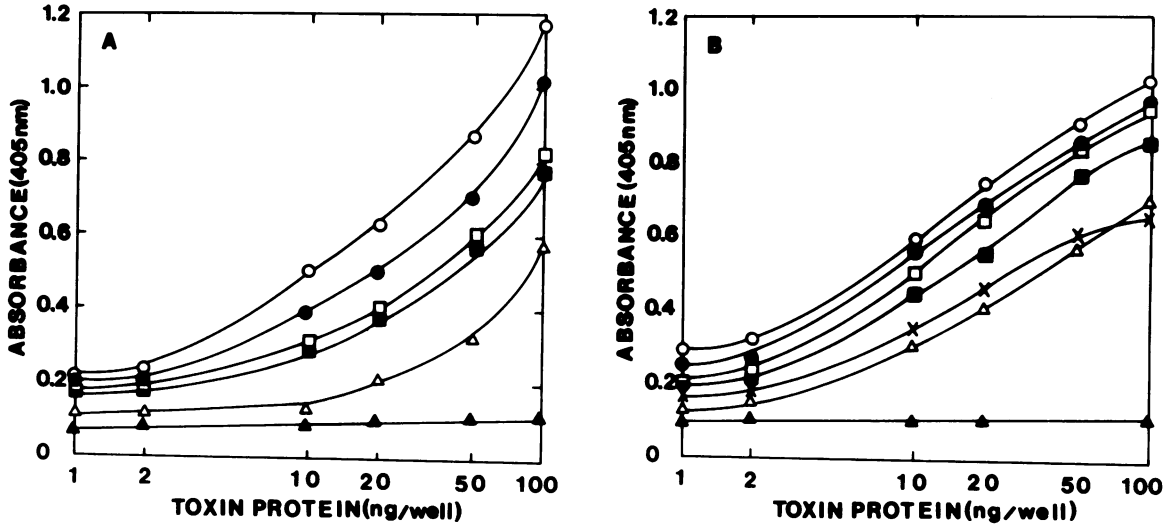

FIG. 2. ELISA titration curves of different toxins reacting with rabbit anti-C-ST subunit IgGs. Toxin dilutions were reacted with a constant amount of anti-C-ST heavy chain IgG (A) and anti-C-ST light chain IgG (B). Symbols: ○, C-ST; ●, Cβ-Yoichi; □, C-468; ■, C-CB19; △, CD6F; X, C-6813; ▲, D-CB16.

antibodies against light and heavy chains of C-ST and D-1873 are summarized in Table 2. When toxins precipitated with antibody preparations in agar gel, all reactions gave only one precipitin line. Type C toxins Cβ-Yoichi, C-468, CD6F, and C-CB19 reacted with both anti-C-ST subunit IgGs, and type D toxin D-CB16 reacted with both anti-D-1873 subunit IgGs. However, C-6813 toxin reacted with anti-D-1873 heavy chain IgG and anti-C-ST light chain IgG.

ELISA of toxins. ELISA titration curves of toxins reacting with anti-C-ST subunit IgGs and anti-D-1873 subunit IgGs are shown in Fig. 2 and 3. Confirming immunodiffusion test results show that Cβ-Yoichi, C-468, CD6F, and C-CB19 reacted with both of the anti-C-ST heavy chain and anti-C-ST light chain IgGs, whereas type D toxins D-CB16 and D-1873 (data not shown) did not react with anti-C-ST subunit IgGs. In contrast, type D toxin D-CB16 reacted with both anti-D-1873 heavy chain and anti-D-1873 light chain IgGs, but C-ST and other type C toxins (data not shown) did not react. However, type C toxin C-6813 reacted with anti-C-ST light chain and anti-D-1873 heavy chain IgGs but not with anti-C-ST heavy chain and anti-D-1873 light chain IgGs (data not shown). Quantitative response in ELISA was not related to purity (Fig. 2 and Table 1). Cβ-Yoichi (purity, 71.2%) was more reactive with both anti-C-ST subunit IgGs than C-468 (purity, 89.5%). Similarly, C-6813 (purity, 57%) was more reactive with anti-D-1873 heavy chain IgG than D-CB16 (purity, 85.5%). D-CB16 seems to have more antigenic determinants in common with the D-1873 light chain than the D-1873 heavy chain (Fig. 3).

Therefore, comparable common antigenic determinants

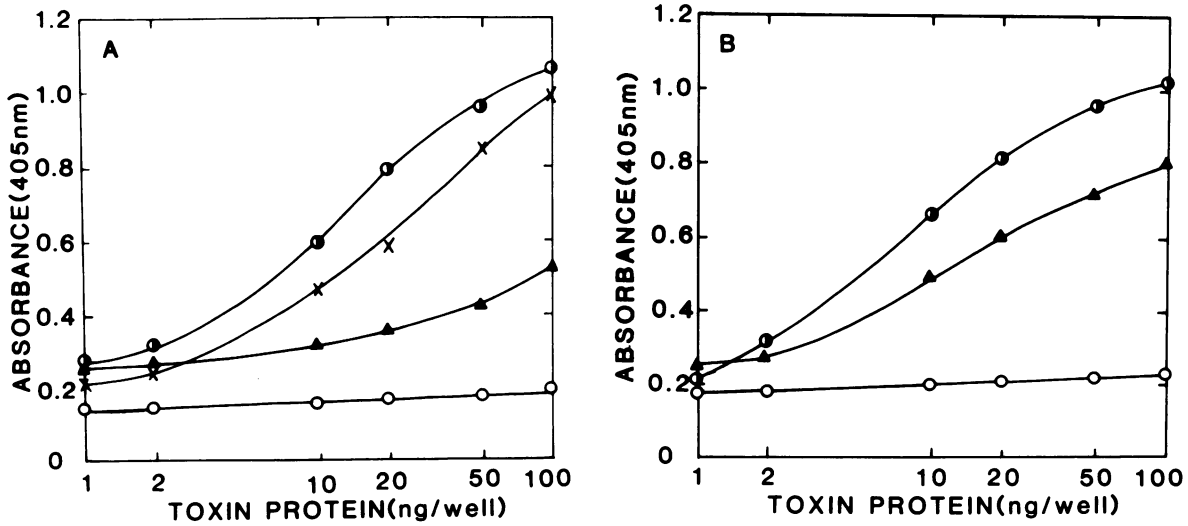

FIG. 3. ELISA titration curves of different toxins reacting with rabbit anti-D-1873 subunit IgGs. Toxin dilutions were reacted with a constant amount of anti-D-1873 heavy chain IgG (A) and anti-D-1873 light chain IgG (B). Symbols: ○, C-ST; ◑, D-1873; ▲, D-CB16; ×, C-6813.

for the C-ST heavy chain can be found in descending amounts in Cβ-Yoichi, C-468, C-CB19, and CD6F, and those for the light chain can be found in descending amounts in Cβ-Yoichi, C-468, C-CB19, C-6813, and CD6F. In contrast, type C toxin C-6813 has more comparable common antigenic determinants to the heavy chain of D-1873 than the heavy chain of D-CB16 and none to the heavy chain of C-ST.

DISCUSSION

In confirmation of other, earlier findings (9, 12, 13), the toxins described in this study have a common molecular structure in that they are composed of heavy chain (M_r, ca. 100,000) and light chain (M_r, ca. 50,000) linked by disulfide bond(s). This molecular similarity in types C_1 and D toxins is not limited to di-chain structure but extends to their subunit antigenic characteristics. Supporting evidence was obtained when antibodies against C-ST toxin cross-neutralized the D-South African toxin of a type D strain (9, 10) and when monoclonal antibody against C-ST toxin reacted with and neutralized D-South African toxin (8). The present results of the double-diffusion test and ELISA further demonstrated antigenic determinants common to the heavy chains of C-6813 (type C_1 toxin) and D-1873 (type D toxin). The composition of C-6813 toxin is that of a hybrid molecule composed of C_1 type light chain and D type heavy chain.

Further, since anti-subunit IgG coated was specific and the amount (3.6 μg per well) was in excess of the maximum toxin applied (100 ng per well), all the applied toxin should have been bound by the available IgG in all positive ELISAs, if the reactive antigens were identical. Thus, the rate of reaction in ELISA depends on the number of common antigenic sites and the avidity between antigen and IgG rather than on the purity of the sample applied. The distribution of antigenic determinants varies with the toxin sample.

Type C_1 toxin and type D toxin are di-chain proteins whose subunits have different antigenic determinants. The subunits themselves may differ among toxins of one type: they may be identical for toxins of the same type or may be antigenically related to the comparable subunits of the other type. Hence, these findings support the existence of Cα and Cβ toxins (11) and also indicate the existence of antigenic heterogeneity within type C_1 toxins. Since the determination of toxigenicity in type C and D toxins is controlled by the phage genetic code (4), the data lead us to speculate that phage recombination may occur in the process of phage infection.

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