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# Antigenic Structure of *Clostridium botulinum* Type B Neurotoxin and Its Interaction with Gangliosides, Cerebroside, and Free Fatty Acids

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**A fragment distinct from the heavy and light chains was obtained by treatment of *Clostridium botulinum* type B neurotoxin with chymotrypsin. Enzyme-linked immunosorbent assay and immunoblotting analysis with monoclonal antibodies showed that the fragment consisted of the light chain and part of the heavy chain (H-1 fragment) linked together by a disulfide bond. Monoclonal antibodies reacting to the heavy chain but not to the fragment were thought to recognize the epitopes on the remaining portion (H-2 fragment) of the heavy chain, being easily digested by chymotrypsin. Thus, the antigenic structure of type B neurotoxin resembles those of type A and E neurotoxins. The chymotrypsin-induced fragment bound to cerebroside and free fatty acids but not to gangliosides. The manner of binding of type B neurotoxin to gangliosides and free fatty acids was different from those of type A and E neurotoxins. Such differences in the reactivities to lipids may be related to the finding that each neurotoxin binds to a type-specific site on the neural membrane.**

*Clostridium botulinum* toxin has been classified into seven immunological types (A through G) (20, 27). The toxin consists of a highly potent neurotoxin and nontoxic components (20). The neurotoxin specifically inhibits acetylcholine release from nerve endings (5, 24). The neurotoxin is primordially synthesized as a single-chain peptide ($M_r$, ca. 150,000), which is cleaved (nicked) endogenously in the culture or exogenously with trypsin or a lysine-specific endoprotease to a dichain protein (3, 15). The two chains, called the heavy ($M_r$, ca. 100,000) and light ($M_r$, ca. 50,000) chains, are covalently linked with at least one disulfide bond (3, 20, 27). The two chains differ antigenically from each other (14, 20). The heavy chain is responsible for binding the neurotoxin to neural membranes. Shone et al. (23) reported that the binding of type A neurotoxin to synaptosome membranes depends upon the carboxyl-terminal region of the heavy chain. We, using monoclonal antibodies (MAbs), also demonstrated that type E neurotoxin is composed of at least three moieties different antigenically and probably functionally and that a MAb recognizing the epitope on the carboxyl-terminal portion of the heavy chain effectively competes with the neurotoxin for the binding to synaptosomes (13). The structure of type B neurotoxin seems to be similar to those of type A and E neurotoxins, but it is not as yet fully understood. There are differences among toxin types in the binding site on neural membranes (1, 2, 4, 12).

To clarify the structure of type B neurotoxin, a fragment distinct from the heavy and light chains was obtained by limited treatment of type B neurotoxin with chymotrypsin. We attempted to prepare MAbs against type B neurotoxin and to use them for analyzing the antigenic structure of the neurotoxin and its fragments from the epitopes which monoclonal antibodies recognize. A membrane constituent containing sialic acids, probably gangliosides, has been a candidate for the receptor of neurotoxin (4, 5, 24). This report describes also the interaction of type B neurotoxin and its fragments with gangliosides and their components in comparison with that of type A and E neurotoxins.

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## MATERIALS AND METHODS

**Neurotoxin and fragments.** Type B progenitor toxin was prepared by a previously published method (16). The neurotoxin was purified by DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) chromatography and converted to toxoid by dialysis against 0.1 M phosphate buffer, pH 7.0, containing 0.4% Formalin (14, 17). The purified neurotoxin proved to be unnicked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). The neurotoxin was activated with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma Chemical Co., St. Louis, Mo.) at a toxin/-enzyme ratio of 100:1 for 15 min at pH 7.5 and 37°C. The heavy and light chains ($M_r$s, 105,000 and 54,000, respectively) of type B neurotoxin were prepared by methods described elsewhere (14, 21). Antisera against the neurotoxin and the heavy chain were obtained from rabbits immunized by a method described previously (14). An immunoglobulin G (IgG) fraction was isolated by the method of Steinbuch and Audran (26).

**Digestion of neurotoxin with chymotrypsin.** Enzyme treatment was carried out in 0.05 M phosphate buffer, pH 7.5, at 37°C. The neurotoxin (2 mg/ml) was digested for 18 h with *p*-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (Sigma) at a toxin/-enzyme ratio of 200:1. *N*-Tosyl-L-phenylalanine chloromethyl ketone (Sigma) was used as an inhibitor. After digestion, the mixture was applied to a PD-10 column (Pharmacia) equilibrated with 0.05 M phosphate buffer, pH 5.0. The protein fraction eluted was then applied to a CM-Sephadex C-50 (Pharmacia) column (0.9 by 5 cm) equilibrated with the same buffer and eluted with a linear gradient of 0 to 0.5 M NaCl. The eluate was concentrated and dialyzed against 0.05 M phosphate buffer, pH 7.5. Treatment with lysyl endopeptidase (Wako Pure Chemical Industries, Osaka, Japan) was done for 5 min at 30°C at a protein/-enzyme ratio of 500:1. The reaction was stopped by adding *p*-tosyl-L-lysine chloromethyl ketone (Nakarai Chemicals, Ltd., Kyoto, Japan).

**Production of MAbs.** BALB/c mice were immunized as described previously (8). Spleen cells were fused with

myeloma cells (Sp2/0-Ag 14) in polyethylene glycol ($M_r$, 4,000; E. Merck AG, Darmstadt, Federal Republic of Germany), and the resulting hybridomas were obtained by limited dilution (8). MAb was purified from ascitic fluid by Affi-Gel–protein A (Bio-Rad Laboratories, Richmond, Calif.) chromatography. The subclass and light chain of each MAb were determined by a method reported previously (13).

Production of MAbs from hybridoma was examined by using an enzyme-linked immunosorbent assay (ELISA) technique as follows. Neurotoxin or fragment (1 μg per 0.1 ml) in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) was added to each well of a 96-well Falcon assay plate (Becton Dickinson Labware, Oxnard, Calif.). The fragment treated with 2 M urea and 50 mM dithiothreitol (DTT) for 60 min at 25°C was also coated to wells. After 3 h at 37°C, the wells were washed twice with PBS–0.05% Tween 20. Each well then received 0.2% bovine serum albumin (Sigma). After incubation overnight at 4°C, the wells were again washed with the same buffer. A sample, 0.1 ml of hybridoma supernatant or purified MAb, was added to each. After 2 h at 37°C, the wells were washed, and 0.1 ml of rabbit anti-mouse IgG conjugated with peroxidase (Cooper Biomedical, Inc., West Chester, Pa.) diluted 500-fold was added to each. After 2 h, the wells were washed and 0.15 ml of a substrate solution (0.8 mg of 5-aminosalicylic acid per ml–0.05% $H_2O_2$, 9:1) was added to each. After 45 min at 37°C, the reaction was stopped by adding 20 μl of 0.1 N NaOH. The developed color intensities were read with a model 2550 enzyme immunoassay reader (Bio-Rad). To determine the epitope specificity of MAb, a competitive ELISA was performed with peroxidase-conjugated MAb (8); conjugated MAb was mixed with graded amount of homologous or heterologous nonconjugated MAb, and an ELISA was carried out as described above.

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed with neurotoxin and fragments in a 10% gel as described previously (18). The sample was boiled for 3 min with 1% SDS in the presence or absence of 50 mM DTT. A 20-μl sample containing 3 to 5 μg was applied. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue for 30 min. Molecular weight was estimated with standard molecular weight markers (Sigma). For immunoblotting, the neurotoxin and the fragments were transferred electrophoretically to nitrocellulose paper (TM-2; Toyo Roshi, Tokyo, Japan) (29). The nitrocellulose paper was treated with 3% bovine serum albumin in PBS to block unoccupied sites. It was incubated with the respective MAb (20 μg/ml) or anti-heavy chain diluted 200-fold in PBS-bovine serum albumin for 30 min. It was washed with PBS and then treated for 30 min with peroxidase-conjugated anti-mouse IgG diluted 500-fold with PBS-bovine serum albumin. After being washed, the paper was exposed to the substrate solution (0.05% 3,3'-diaminobenzidine and 0.003% $H_2O_2$ in PBS). The reaction was stopped by a rinse with distilled water.

**TLC immunostaining.** Thin-layer chromatography (TLC) immunostaining was performed as described previously (9, 28). In brief, gangliosides were developed on a silica gel-coated plastic plate (Polygram, Sil G; Marcherey-Nagel, Doren, Federal Republic of Germany) with chloroform-methanol-2% $CaCl_2 \cdot H_2O$ (55:45:10), cerebroside was developed with chloroform-methanol-acetic acid (80:20:2), and free fatty acids were developed with *n*-hexane–diethyl ether–acetic acid (80:30:1). The plates were treated with 0.01 M phosphate buffer, pH 7.2, containing 1% egg albumen (Difco Laboratoreis, Detroit, Mich.) and 1% polyvinylpyrrolidone for 15 min at 37°C, and then the neurotoxin or fragment (10 nM) in 0.01 M phosphate buffer, pH 7.2, containing 3% polyvinylpyrrolidone was allowed to react overnight at 4°C. After washing, binding to lipids was detected with a rabbit IgG fraction against the neurotoxin (50 μg/ml) and peroxidase-conjugated anti-rabbit IgG (Tago Inc., Burlingame, Calif.) diluted 500-fold. The substrate solution contained 0.05% 4-chloro-1-naphthol and 0.01% $H_2O_2$ in 0.05 M Tris hydrochloride buffer, pH 7.4, containing 0.2 M NaCl.

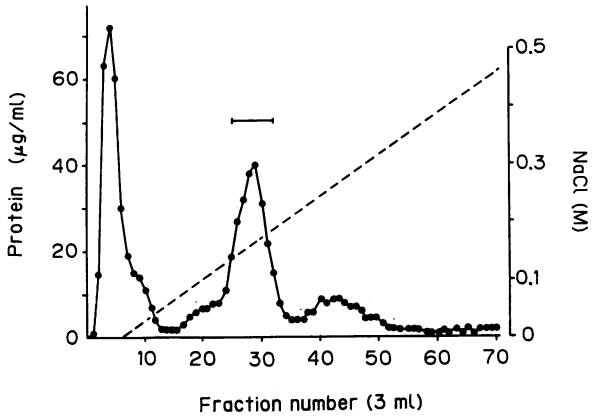


FIG. 1. Elution profile of type B neurotoxin (5 mg) digested with chymotrypsin from a carboxymethyl-Sephadex column.

**Other methods.** For determining the neutralizing activity of MAbs, the activated neurotoxin was diluted to 10 μg/ml ($1.3 \times 10^6$ 50% lethal doses per ml) with PBS, and the dilution was mixed with an equal volume of each MAb (200 μg/ml). After incubation of the mixture for 30 min at room temperature, the remaining toxicity was determined by the time-to-death method by intravenous injection into mice (11). The agar gel diffusion test was performed as described previously (14). Protein contents were determined by the method of Lowry et al. (19).

**Lipids.** Cerebroside was purchased from Serdary Research Laboratories, Ontario, Canada, and free fatty acids were from Wako Pure Chemical Industries. Gangliosides were given by M. Iwamori, Faculty of Medicine, University of Tokyo.

## RESULTS

**Properties of chymotrypsin-induced fragment.** After treatment of the neurotoxin with chymotrypsin, the sample was subjected to a carboxymethyl-Sephadex column. A major peak was eluted at an NaCl concentration of 0.18 M (Fig. 1). This peak contained no toxicity and migrated in SDS-PAGE as a single band ($M_r$, 115,000) with or without reduction (Fig. 2A, lanes 1 and 2). Treatment of the fraction with lysyl endopeptidase resulted in partial separation into two minor bands ($M_r$s, 84,000 and 27,000) (Fig. 2A, lane 3). When treated further with DTT, two major bands ($M_r$s, 59,000 and 54,000) and an additional minor band ($M_r$, 32,000) emerged (Fig. 2A, lane 4). The mobility of one of the major bands corresponded to that of the light chain. In the agar gel diffusion test, the chymotrypsin-induced fragment formed a single precipitin line against antineurotoxin, indicating that it was partly identical to that of the neurotoxin. From these findings, we proposed the probable structure of type B neurotoxin as illustrated in Fig. 3.

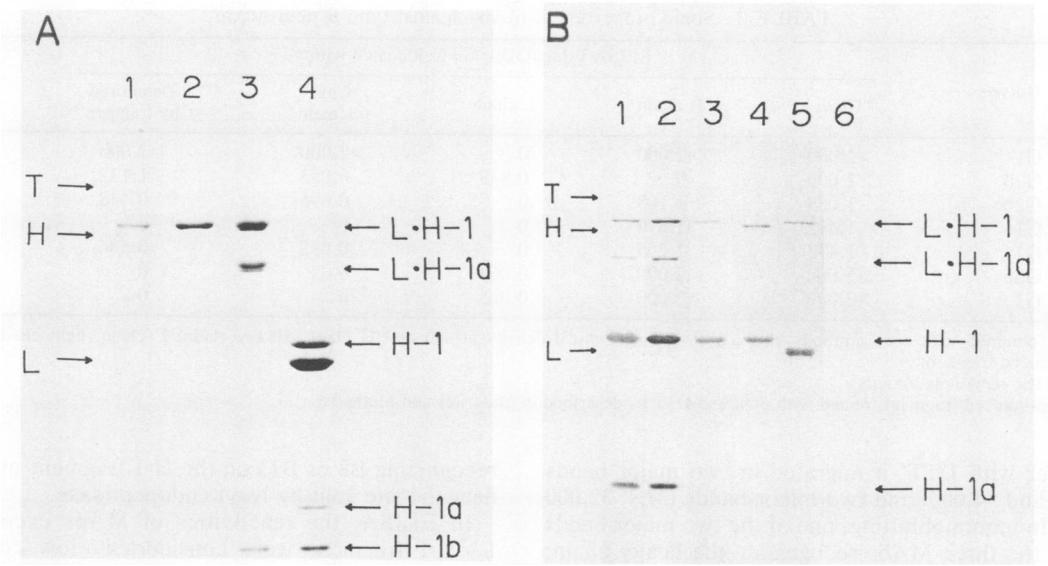


FIG. 2. SDS-PAGE (A) and immunoblotting analyses (B) of chymotrypsin-induced fragment. (A) Lanes: 1, untreated; 2, treated with DTT; 3, treated with lysyl endopeptidase; 4, treated with lysyl endopeptidase and DTT. (B) Bound to MAbs. Before blotting, the sample treated with lysyl endopeptidase was electrophoresed in the presence of DTT. Lanes: 1, anti-heavy chain; 2, B5; 3, B8; 4, B13; 5, B6; 6, B9. T, Neurotoxin; H, heavy chain; L, light chain.

**Properties of MAbs.** A total of seven lines were established, and each MAb was purified from the ascitic fluid (Table 1). Four MAbs were of the IgG1 subclass and three were of the IgG2b subclass. All MAbs contained a kappa light chain. Six MAbs reacted to the heavy chain and the seventh reacted to the light chain in ELISA. By competitive ELISA, the binding sites of B8 and B13 were similar. The other MAbs were found to recognize distinct epitopes on the toxin molecule. Four of them, B5, B6, B8, and B13, were found to react with the chymotrypsin-induced fragment in ELISA. When the fragment was further treated with urea and DTT, the reactivities in ELISA of three MAbs increased. The other three MAbs, B9, B14, and B17, did not bind to the chymotrypsin-induced fragment. Three MAbs reacting with both the heavy chain and the chymotrypsin-induced fragment and one MAb reacting with the light chain were capable of neutralizing the neurotoxin.

**Immunoblotting.** Anti-heavy chain reacted to 59,000- and 32,000-dalton fragments obtained from the chymotrypsin-induced fragment by treatment with lysyl endopeptidase and reduction (Fig. 2B, lane 1). B5 reacted with them in the same manner (Fig. 2B, lane 2). The other two MAbs, B8 and B13, recognizing the chymotrypsin-induced fragment, bound to the 59,000-dalton fragment but not to the 32,000-dalton fragment (Fig. 2B, lanes 3 and 4). Neither anti-heavy chain nor MAb bound to the 27,000-dalton fragment. B6 reacted to the 54,000-dalton fragment with a mobility corresponding to that of the light chain (Fig. 2B, lane 5). MAbs B9, B14, and B17 bound to the neurotoxin and heavy chain (data not shown) but not to any band of the chymotrypsin-induced fragment or its derivatives (Fig. 2B, lane 6).

**Binding of neurotoxin and fragments to lipids assessed by TLC immunostaining.** The neurotoxin and the heavy chain bound to ganglioside $G_{D1a}$ and $G_{T1b}$ but not to $G_{M1}$, $G_{D1b}$, or $G_{Q1b}$. The chymotrypsin-induced fragment and the light chain never bound to any ganglioside tested (Fig. 4). The chymotrypsin-induced fragment bound to cerebroside, as did the neurotoxin (Fig. 5). The binding of the neurotoxin and their fragments to free fatty acids with chain lengths of 14 to 22 carbons was also examined (Fig. 6). The free fatty acid most strongly bound to the neurotoxin was stearic acid, with 18 carbons in a saturated form. No binding of neurotoxin to oleic or linoleic acid, $C_{18}$ fatty acids with one and two unsaturated bonds, respectively, was observed. The heavy and light chains and the chymotrypsin-induced fragment were found to bind to the same free fatty acids to which neurotoxin bound.

## DISCUSSION

After treatment of type B neurotoxin with chymotrypsin, a 115,000-dalton fragment was purified by carboxymethyl-Sephadex chromatography. This fragment was found to have lost a part of the antigenicity of the neurotoxin in the agar gel diffusion test. This fragment, with or without reduction, migrated as a single band in SDS-PAGE. When treated mildly with lysyl endopeptidase, it dissociated partially into smaller fragments ($M_r$s, 84,000 and 27,000), and when

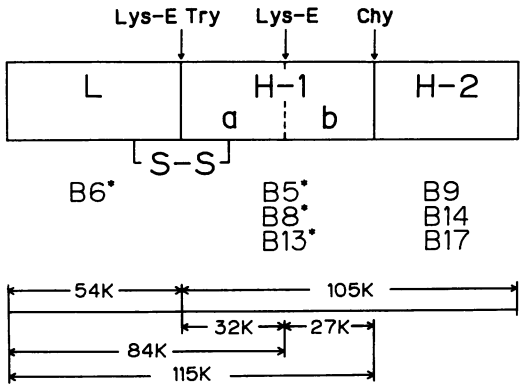


FIG. 3. Probable antigenic structure of type B neurotoxin. Arrows indicate the sites split by lysyl endopeptidase (Lys-E), trypsin (Try), and chymotrypsin (Chy). The MAb with an asterisk neutralizes the neurotoxin. Molecular weights ($10^3$) of the fragments are shown at the bottom.

TABLE 1. Some properties of MAbs against type B neurotoxin

| MAb | Isotype | ELISA value ($OD_{450}$) in well coated with[a]: | | | | | Neutralizing activity (%)[b] |
|---|---|---|---|---|---|---|---|
| | | Toxin | H chain | L chain | Chy fragment | Denatured Chy fragment[c] | |
| B5 | G1 | >2.000 | >2.000 | 0 | >2.000 | >2.000 | 25 |
| B6 | G2b | >2.000 | 0 | 0.839 | 0.053 | 1.132 | 5.3 |
| B8 | G2b | 1.089 | 1.108 | 0 | 0.096 | 0.448 | 15 |
| B9 | G1 | 1.412 | 1.670 | 0 | 0 | 0 | 76 |
| B13 | G1 | 1.473 | 1.255 | 0 | 0.032 | 0.586 | 0.2 |
| B14 | G2b | >2.000 | >2.000 | 0.064 | 0 | 0 | 62 |
| B17 | G1 | >2.000 | >2.000 | 0.065 | 0 | 0 | 85 |

[a] Values were obtained with each antibody at 1 μg/ml. $OD_{450}$, Optical density at 450 nm. H chain, Heavy chain; L chain, light chain; Chy fragment, chymotrypsin-induced fragment.
[b] Value shows the remaining toxicity.
[c] Chymotrypsin-induced fragment treated with urea and DTT as described in Materials and Methods.

treated further with DTT, it migrated in two major bands ($M_r$s, 59,000 and 54,000) and two minor bands ($M_r$s, 32,000 and 27,000). In immunoblotting, one of the two major bands reacted with the three MAbs recognizing the heavy chain; the other major band, which migrated to the same position as the light chain, bound to MAb recognizing the light chain. Of three MAbs (B5, B8, and B13) reacting to the 59,000-dalton fragment, B5 alone bound to the 32,000-dalton fragment, as did anti-heavy chain. The two minor bands appeared to represent subfragments of the 59,000-dalton fragment having emerged by cleavage of an additional bond during the treatment with lysyl endopeptidase, as the sum of their molecular weights agreed with that of the 59,000-dalton fragment. These results indicate that the chymotrypsin-induced fragment consists of the light chain and a part of the heavy chain (the H-1 fragment) in the unnicked form, which are linked together with at least one disulfide bond. The L · H-1 fragment induced by chymotrypsin treatment resembles the corresponding fragments of type E neurotoxin obtained by tryptic treatment (13). By treatment solely with lysyl endopeptidase, the 27,000-dalton fragment (the H-1b fragment) was partially released from the H-1 fragment. This may indicate that half-cysteine in the heavy chain is located on the remaining 32,000-dalton fragment (the H-1a fragment). This suggests also that cleavage by lysyl endopeptidase at the junction of H-1a and H-1b fragments was incomplete, while that between the light and heavy chains was complete. The H-1b fragment seems to be less immunogenic from the results of immunoblotting. The epitope recognizing B8 or B13 on the H-1 fragment may be located near the site split by lysyl endopeptidase.

In ELISA, the reactivities of MAbs except B5 to the L · H-1 fragment were considerably low compared with those to neurotoxin or the two chains. Denaturation with urea and DTT, however, resulted in improving the reactivities of the L · H-1 fragment to such MAbs. The data suggest that some epitopes on the L · H-1 fragment are masked upon coating on the surface of the ELISA plate. The four MAbs bound to the L · H-1 fragment possessed neutralizing activity, whereas the other three MAbs, probably recognizing the remaining portion of the heavy chain (the H-2 fragment), did not. Schmidt et al. (22) reported that the light chain contains the amino-terminal end of the original single chain. Hence, the H-2 fragment must contain the carboxyl terminus and be digested easily by chymotrypsin. These results were consistent with the model proposed in Fig. 3. The probable antigenic structure of type B neurotoxin appears to be nearly the same as those of type A and E neurotoxins, as reported before (13, 23). The L · H-1 and H-2 fragments seem to respectively resemble fragments B and C induced from tetanus toxin by papain treatment (6).

Neurotoxin has been found to be inactivated with gangliosides, mainly $G_{T1b}$, which is a component of nerve cell membrane (10, 16, 25). These findings merely suggest that a

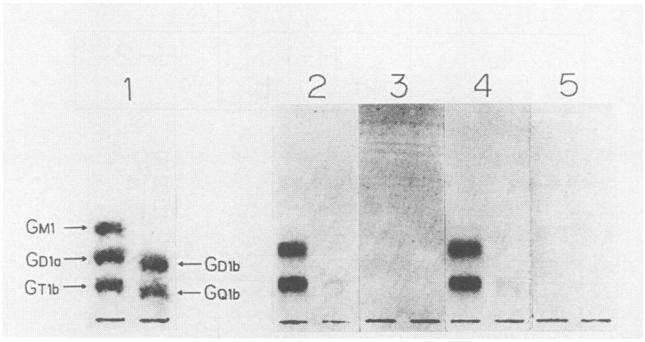


FIG. 4. Binding of neurotoxin and the fragments to gangliosides, detected by TLC immunostaining. Gangliosides (1 μg each) developed on a TLC plate were colored with resorcinol hydrochloride reagent (1) and stained by TLC immunostaining with neurotoxin (2), chymotrypsin-induced fragment (3), heavy chain (4), and light chain (5).

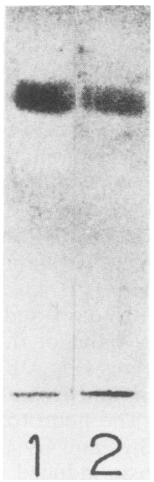


FIG. 5. Binding of neurotoxin and chymotrypsin-induced fragment to cerebroside, detected by TLC immunostaining. 1, Neurotoxin; 2, chymotrypsin-induced fragment. Cerebroside was developed in the amount of 10 μg.

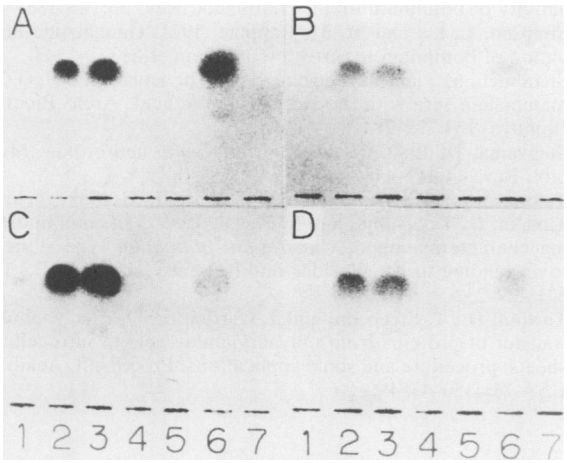


FIG. 6. Binding of neurotoxin and the fragments to free fatty acids, detected by TLC immunostaining. A, Neurotoxin; B, chymotrypsin-induced fragment; C, heavy chain; D, light chain. Free fatty acids (10 μg each) were developed. Lanes: 1, myristic acid ($C_{14:0}$); 2, palmitic acid ($C_{16:0}$); 3, stearic acid ($C_{18:0}$); 4, oleic acid ($C_{18:1}$); 5, linoleic acid ($C_{18:2}$); 6, arachidic acid ($C_{20:0}$); 7, behenic acid ($C_{22:0}$).

particular ganglioside or one of the other sialic acid-containing substances should be regarded as a receptor candidate (5, 24). In our previous report (9), we demonstrated that type E neurotoxin and heavy chain bind to gangliosides $G_{D1a}$, $G_{T1b}$, and $G_{Q1b}$ as assessed by the TLC immunostaining method. The reactivity of type B neurotoxin to gangliosides differs somewhat from those of type A and E neurotoxins. Type B neurotoxin bound to gangliosides $G_{D1a}$ and $G_{T1b}$ but not to $G_{Q1b}$, which reacts with type A and E neurotoxin (9, 28). The L · H-1 fragment of type B neurotoxin did not bind to gangliosides but to cerebroside, being free of the oligosaccharide portion of ganglioside. This fragment bound to free fatty acids, as did the L · H-1 fragment of type E neurotoxin (9). The reactivity of the L · H-1 fragment, and of the light chain as well, of type B to free fatty acids seemed to be weaker than those of the neurotoxin and the heavy chain. Such differences in reactivity in TLC immunostaining may be due to the amount of specific antibody bound to the neurotoxin and fragments. The manner of binding of type B neurotoxin and fragments to free fatty acids was different from that of type E neurotoxin and fragments; type B neurotoxin or fragments did not bind to free fatty acids with unsaturated bonds, which reacts with type E neurotoxin (9). Hoch et al. (7) reported that the heavy chain of type B neurotoxin caused channel formation in artificial lipid membrane. These observations may suggest that the H-2 fragment initially binds to the oligosaccharide portion of gangliosides and then the L · H-1 fragment interacts with cerebroside and free fatty acids, constituting the hydrophobic portion of gangliosides. The differences in the reactivity to lipids between type B and E neurotoxins may reflect the previous finding (1, 2, 4, 12) that each neurotoxin binds to a type-specific site on neural membranes. To validate the above hypothesis, a series of experiments are under way in this laboratory.

## ACKNOWLEDGMENTS

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