

Immunological Characterization of Papain-Induced Fragments of *Clostridium botulinum* Type A Neurotoxin and Interaction of the Fragments with Brain Synaptosomes

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After treatment of *Clostridium botulinum* type A neurotoxin with papain, three fragments (M_r s, 101,000, 45,000, and 43,000) were purified by hydrophobic and ion-exchange chromatography with a high-performance liquid chromatographic system. Immunoblotting analyses with monoclonal antibodies showed that the 101,000-dalton fragment consisted of the light chain and a part of the heavy chain (H-1 fragment) linked together by a disulfide bond, and the other two fragments were correlated to the remaining portion of the heavy chain (H-2 fragment). The 45,000- and 43,000-dalton fragments effectively competed for binding of the ^{125}I -labeled neurotoxin to synaptosomes, while no inhibition was observed with the 101,000-dalton fragment. The results indicate that the H-2 fragment interacts with the binding site on the neural membrane. The binding of the neurotoxin was impaired by treatment of synaptosomes with neuraminidase. Incorporation of gangliosides into neuraminidase-treated synaptosomes resulted in the restoration of binding. The results suggest that gangliosides are one of the components of the toxin-binding site.

Clostridium botulinum toxin has been classified into seven immunological types, A through G (22, 26). The toxin consists of a highly potent neurotoxin and a nontoxic component (22). The neurotoxin exerts its toxic action by inhibition of acetylcholine release from the nerve endings, causing neuromuscular paralysis (8, 24). The neurotoxin is made up of two chains, the heavy (M_r , about 100,000) and the light (M_r , about 50,000) chains, which are covalently linked together with at least one disulfide bond (6). The heavy chain is responsible for binding the neurotoxin to neural membranes (2, 4, 15, 21, 23). We obtained a fragment that was distinct from either the heavy or the light chain by treatment of type B and E neurotoxins with chymotrypsin or trypsin (16, 17). Immunological analyses with monoclonal antibodies (MAbs) revealed that the fragment that was obtained, the L H-1 fragment, consisted of the light chain and the amino-terminal portion of the heavy chain. The L H-1 fragment seemed to resemble in structure fragment B of the tetanus toxin (9), and it inhibited the binding of ^{125}I -labeled neurotoxin to synaptosomes to a lower extent than did unlabeled neurotoxin or the heavy chain (16). Moreover, an MAb that recognized an epitope on the carboxyl-terminal portion of the heavy chain of type E neurotoxin effectively competed with the neurotoxin for the binding to synaptosomes (16). Shone et al. (23) have also reported that such a type A neurotoxin lacking the carboxyl-terminal region of the heavy chain did not bind to synaptosomes. From those observations, the carboxyl-terminal portion of the heavy chain has been proposed to recognize the binding site, but there is still no direct evidence to show that the isolated carboxyl-terminal portion of the heavy chain interacts with the binding site on the neural membrane.

To clarify the role that the carboxyl-terminal portion of the heavy chain plays in the binding of the neurotoxin to synaptosomes, attempts were made to obtain a carboxyl-

terminal portion of the heavy chain by treating type A neurotoxin with papain. In addition, we scrutinized the antigenic structure of the neurotoxin and its fragments from epitopes recognized by MAbs. This report also describes the properties of the type A neurotoxin-binding site on the synaptosome membrane.

MATERIALS AND METHODS

Neurotoxin and fragments. Type A progenitor toxin was prepared by a previously described method (25). The neurotoxin was purified by DEAE-Sephadex A-50 chromatography (25). The purified neurotoxin was dialyzed against 0.05 M phosphate buffer (pH 7.5) and stored at 4°C. The heavy and light chains of type A neurotoxin were prepared by a method that has been described elsewhere (18).

Isolation of papain-induced fragments. Enzyme treatment was carried out in 0.05 M phosphate buffer (pH 7.5) at 37°C. The neurotoxin (1 mg/ml) was treated for 1 h with papain (twice crystallized; Sigma Chemical Co., St. Louis, Mo.) at a toxin-to-enzyme ratio of 40:1. Antipain (Peptide Institute Inc., Osaka, Japan) was used as an inhibitor. After digestion, high-performance liquid chromatography (HPLC) was run with an HPLC system (Japan Spectroscopic Co., Tokyo, Japan) to separate the papain-induced fragments. The mixture was loaded onto a column of PROTEIN PAK G-ether (Nihon Waters Ltd., Tokyo, Japan) that was equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1.7 M $(\text{NH}_4)_2\text{SO}_4$ and eluted with a linear $(\text{NH}_4)_2\text{SO}_4$ gradient from 1.7 to 0.34 M at a flow rate of 0.5 ml/min. The eluate was rechromatographed with a column of PROTEIN PAK G-ether or TSK gel DEAE-5PW (Toyo Soda Manufacturing Co., Tokyo, Japan). Finally, the fragment was dialyzed against 0.05 M phosphate buffer (pH 7.5) containing 0.5 M NaCl.

Production of MAbs. BALB/c mice were immunized as described previously (11). 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 Ger-

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many), and the resulting hybridomas were obtained by limiting dilution. The MAb was purified from ascitic fluid by Affi-Gel-protein A (Bio-Rad Laboratories, Richmond, Calif.) or DEAE-Affi-Gel blue (Bio-Rad) chromatography. The subclass and light chain of each MAb were determined by a method reported previously (16).

Production of MAbs from hybridomas was examined by enzyme-linked immunosorbent assay (ELISA) techniques, as follows. Neurotoxin or fragment (1 µg/0.1 ml), in 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl (phosphate-buffered saline [PBS]), was added to each well of a 96-well assay plate (Falcon; Becton Dickinson Labware, Oxnard, Calif.). 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 well. After 2 h at 37°C, the wells were washed, and 0.1 ml of rabbit anti-mouse immunoglobulin G (IgG) conjugated with peroxidase (Cooper Biomedical, Inc., West Chester, Pa.) diluted 1,000-fold was added to each well. After 2 h at 37°C, the wells were washed and 0.15 ml of a substrate solution (0.8 mg of 5-aminosalicylic acid per ml and 0.05% H₂O₂; 9:1) was added to each well. After 45 min at 37°C, the reaction was terminated by adding 20 µl of 0.1 N NaOH. The developed color intensities were read with an enzyme immunoassay reader (model 2550; Bio-Rad). To determine the epitope specificity of the MAB, a competitive ELISA was performed with peroxidase-conjugated MAB (11); conjugated MAB was mixed with graded amounts of homologous or heterologous nonconjugated MAB, and ELISA was carried out as described above.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in a 10% gel as described previously (19). The sample was boiled for 3 min with 1% SDS in the presence or absence of 50 mM dithiothreitol. A 20-µl sample containing approximately 3 µg was applied to the gels. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue for 30 min. Molecular weights were estimated with standard molecular weight markers (Sigma).

For immunoblotting (5), the neurotoxin and the fragments were transferred electrophoretically to nitrocellulose paper (TM-2; Toyo Roshi, Tokyo, Japan). The nitrocellulose paper was treated with 3% bovine serum albumin in PBS. The paper was then incubated for 30 min with the respective MAbs diluted to 20 µg/ml in PBS-bovine serum albumin. It was washed with PBS and then treated for 30 min with peroxidase-conjugated anti-mouse IgG diluted with PBS-bovine serum albumin. After washing, the nitrocellulose paper was exposed to the substrate solution (0.05% 3,3'-diaminobenzidine and 0.003% H₂O₂ in PBS). The reaction was stopped by a rinse with distilled water.

The binding of ¹²⁵I-labeled neurotoxin to synaptosomes. The neurotoxin (50 µg) was radioiodinated with Na¹²⁵I (0.5 mCi; Dupont, NEN Research Products, Boston, Mass.) by the method reported previously (15). About 90% of the toxicity of unlabeled neurotoxin was retained after labeling. Before each experiment, synaptosomes were prepared from mouse brain (28) and suspended in a physiological salt solution (120 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 4 mM MgCl₂, 5 mM Tris [pH 7.0]) with or without added 0.1% bovine serum albumin. In binding experiments, synaptosomes (25 µg of protein) were incubated with ¹²⁵I-labeled neurotoxin in 0.2 ml of the salt solution-bovine serum albumin in a well of a plate (HA; Millipore Corp., Bedford,

TABLE 1. Properties of MAbs against type A neurotoxin

MAb	ELISA value (OD ₄₅₀) in well coated with ^a :			Neutralizing activity (%) ^b
	Toxin	H chain	L chain	
A101	>2.000	>2.000	0.281	81.7
A102	0.173	1.275	0.094	100
A107	1.320	1.780	0.204	1.3
A108	1.244	1.864	0.158	12.9
A109	1.259	0.063	0.088	20.5
A113	0.610	0.090	1.902	95.2
A115	1.522	0.180	>2.000	95.2

^a Values were obtained with each antibody at 1 µg/ml. OD₄₅₀, Optical density at 450 nm. H chain, Heavy chain; L chain, light chain.

^b Values indicate the remaining toxicities.

Mass.) for 15 min at 37°C. For testing the inhibition of binding of the ¹²⁵I-labeled neurotoxin, synaptosomes were preincubated with unlabeled neurotoxin or the fragment at various concentrations. After 15 min at 37°C, ¹²⁵I-labeled neurotoxin was added to a final concentration of 0.5 nM and the mixture was incubated for an additional 15 min at 37°C. After incubation the synaptosomes were separated by suction through the filter membrane. The filter membrane was then washed five times with the salt solution-bovine serum albumin. The membrane was punched out, each disk was placed in a polystyrene tube, and the radioactivity was determined with a NaI well-type scintillation counter.

Treatment of synaptosomes with enzymes and gangliosides. Treatment of synaptosomes with enzymes and gangliosides was carried out in the salt solution at 37°C. Synaptosomes (2 mg of protein per ml) were treated for 30 min with neuraminidase (from *Streptococcus* sp.; Seikagaku Kogyo Co., Ltd., Tokyo, Japan) at various concentrations up to 0.1 U/ml or lysyl endopeptidase (Wako Pure Chemical Industries, Osaka, Japan) at various concentrations up to 100 µg/ml. Before and after neuraminidase treatment at 0.05 U/ml, synaptosomes were incubated for 30 min with a ganglioside mixture isolated by the method of Iwamori and Nagai (10). After treatment, the synaptosomes were washed twice with the salt solution and finally suspended to the original volume of the salt solution-bovine serum albumin.

Other methods. For determining the neutralizing activity of MAbs, the neurotoxin was diluted to 10 µg/ml with PBS, and the dilution was mixed with an equal volume of each MAb (200 µg/ml). After incubation for 30 min at 37°C, the remaining toxicity was determined by the time-to-death method by intravenous injection of neurotoxin into mice (14). Protein contents were determined by the method of Lowry et al. (20). *N*-Acetylneuraminic acid was determined by the method of Aminoff (1). Ganglioside contents were expressed in *N*-acetylneuraminic acid equivalents.

RESULTS

Properties of MAbs. A total of seven cell lines were established, and each MAB was purified from ascitic fluid. All MAbs were of the IgG1 class and contained a kappa light chain. In the ELISA, four MAbs reacted to the heavy chain and two other MAbs reacted to the light chain (Table 1). The results corresponded to those of immunoblotting analyses (data not shown). The remaining MAB (A109) did not react with either fragment but bound to the light chain in the immunoblots. This may suggest that the epitope recognizing MAB A109 is masked on the surface of the ELISA plate when the light chain isolated was coated on the plate. By

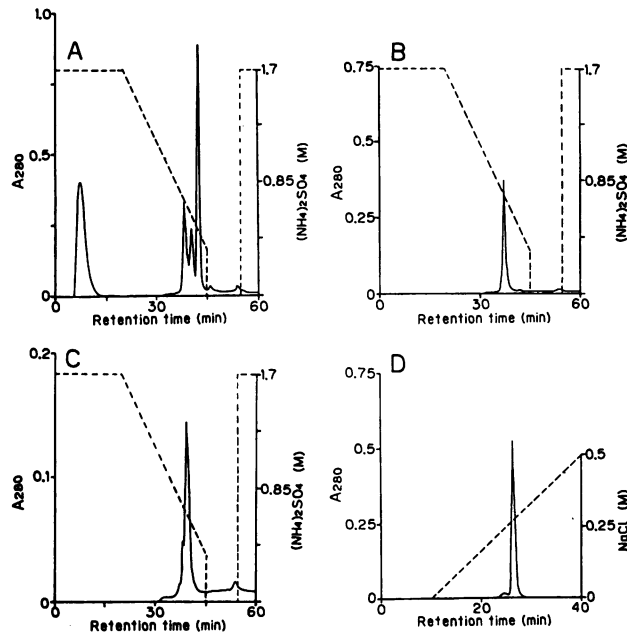


FIG. 1. HPLC elution profiles of type A neurotoxin treated with papain and the induced fragments. (A) Isolation of papain-induced fragments with a column of PROTEIN PAK G-ether. Type A neurotoxin (950 µg) treated with papain was applied to a column equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1.7 M $(\text{NH}_4)_2\text{SO}_4$. The broken line shows the $(\text{NH}_4)_2\text{SO}_4$ concentration gradient. (B) Rechromatography of the first eluted fraction (70 µg) on a column of PROTEIN PAK G-ether. (C) Rechromatography of the second eluted fraction (70 µg) on a column of PROTEIN PAK G-ether. (D) Rechromatography of the third eluted fraction (385 µg) with a column of TSK gel DEAE-5PW equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5). The fragment was eluted with a gradient from 0 to 0.5 M NaCl. The broken line shows the NaCl concentration gradient.

competitive ELISA, the binding sites of MAbs A107 and A108 were similar. The other MAbs were found to recognize distinct sites on the toxin molecule. Three MAbs (A107, A108, and A109) were capable of neutralizing the neurotoxin (Table 1).

Properties of papain-induced fragments. After treatment of the neurotoxin with papain, hydrophobic chromatography was performed with a column of PROTEIN PAK G-ether. Three peaks were eluted at $(\text{NH}_4)_2\text{SO}_4$ concentrations from 0.85 to 0.4 M (Fig. 1A). The first and second peaks were rechromatographed separately under the same conditions. The protein in the first fraction eluted as a single peak, while that in the second fraction eluted as two peaks located close to each other, probably because of the overlap of the first peak (Fig. 1B and C). When the third fraction was applied to a column of TSK gel DEAE-5PW, a major peak that eluted at an NaCl concentration of 0.27 M was separated from a minor peak at an elution position identical to that of the neurotoxin (Fig. 1D). After rechromatography, the first fraction contained no toxicity, whereas the second and third ones did, recovering, respectively, 2 and 0.01% of the original toxicity. Each major peak was pooled and subjected to SDS-PAGE (Fig. 2). The first and second fractions each migrated as a single band (M_r s, 45,000 and 43,000, respectively) with or without reduction. The third fraction also migrated as a single band (M_r , 101,000) without reduction; however, reduction resulted in separation into two bands

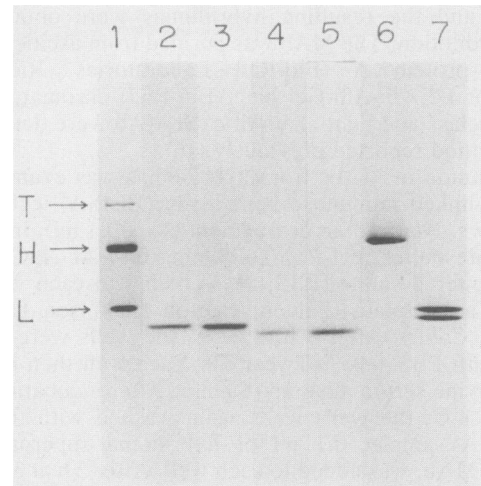


FIG. 2. SDS-PAGE of type A neurotoxin and the fragments. Lanes: 1, neurotoxin; 2 and 3, the first peak; 4 and 5, the second peak; 6 and 7, the third peak. Samples in lanes 1, 3, 5, and 7 were electrophoresed in the presence of dithiothreitol. Abbreviations: T, neurotoxin; H, heavy chain; L, light chain.

(M_r s, 53,000 and 48,000). The mobility of one of the two bands corresponded to that of the light chain (M_r , 53,000).

Immunoblotting with MAbs. Of the four MAbs that recognized the heavy chain (M_r , 93,000), MAb A101 reacted to the 43,000- and 45,000-dalton fragments. MAb A108 bound to a 101,000-dalton fragment and to the 48,000-dalton fragment derived from this fragment by reduction, as did MAbs A102 and A107. Three other MAbs (A109, A113, and A115) reacted with the 101,000-dalton fragment and another derivative with the same mobility as that of the light chain (Fig. 3).

Binding of ^{125}I -labeled neurotoxin to synaptosomes. Of the papain-induced fragments, 43,000- and 45,000-dalton fragments effectively inhibited the binding of ^{125}I -labeled neurotoxin, as did unlabeled neurotoxin, while no inhibition was observed with the 101,000-dalton fragment (Fig. 4). To characterize the binding components, synaptosomes were preincubated with lysyl endopeptidase or neuraminidase.

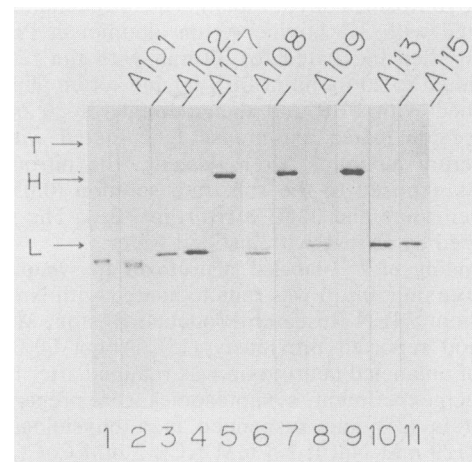


FIG. 3. Immunoblotting analyses of papain-induced fragments bound to MAbs. Lanes: 1, 45,000-dalton fragment; 2, 43,000-dalton fragment; 3 to 11, 101,000-dalton fragment. Samples in lanes 5, 7, and 9 were electrophoresed in the absence of dithiothreitol. The MAb used is shown on the top of the lane.

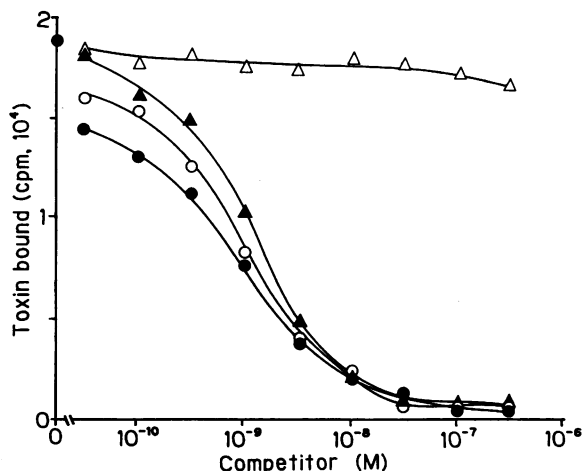


FIG. 4. Inhibition of binding of ¹²⁵I-labeled type A neurotoxin to synaptosomes with unlabeled neurotoxin and papain-induced fragments. The ¹²⁵I-labeled neurotoxin (71,000 cpm, 0.5 nM) was added to synaptosomes that were preincubated with unlabeled neurotoxin (●), the 45,000-dalton fragment (○), the 43,000-dalton fragment (▲), or the 101,000-dalton fragment (△). Nonspecific binding was measured in the presence of 1 μM unlabeled neurotoxin. Each point represents the mean of four determinations.

The synaptosomes treated with lysyl endopeptidase were found to retain the ability to bind to ¹²⁵I-labeled neurotoxin. The treatment with neuraminidase decreased the amount of ¹²⁵I-labeled neurotoxin bound to the synaptosomes, and the *N*-acetylneuraminic acid contents of synaptosomes decreased by about 50% (Fig. 5). Gangliosides were incorporated into synaptosomes before neuraminidase treatment, which slightly facilitated the binding of ¹²⁵I-labeled neurotoxin. The amount of neurotoxin bound was restored on treatment of the neuraminidase-treated synaptosomes with gangliosides; however, the amount was lower than that bound to the native synaptosomes treated with gangliosides,

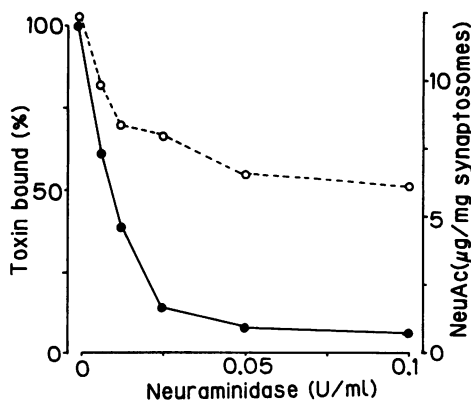


FIG. 5. Effect of neuraminidase on the binding of ¹²⁵I-labeled type A neurotoxin to synaptosomes. The ¹²⁵I-labeled neurotoxin (57,000 cpm, 0.5 nM) was added to synaptosomes that were treated with neuraminidase at various concentrations. The ¹²⁵I-labeled neurotoxin bound to untreated synaptosomes was 13,100 cpm, which was regarded as 100%. Each point represents the mean of three determinations. The broken line shows the *N*-acetylneuraminic acid (NeuAc) contents of the synaptosomes.

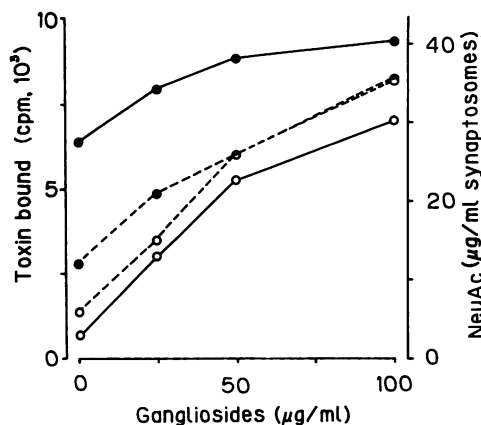


FIG. 6. Effect of incorporated gangliosides on the binding of ¹²⁵I-labeled type A neurotoxin to synaptosomes. Gangliosides were incorporated in synaptosomes that were untreated or treated with neuraminidase at a concentration of 0.05 U/ml. The ¹²⁵I-labeled neurotoxin (44,000 cpm, 0.5 nM) was then added to untreated (●) or neuraminidase-treated (○) synaptosomes. Each point represents the mean of three determinations. The broken lines show the *N*-acetylneuraminic acid (NeuAc) contents of the synaptosomes.

irrespective of the *N*-acetylneuraminic acid contents of the synaptosomes (Fig. 6).

DISCUSSION

After treatment of type A neurotoxin with papain, three fragments were purified by hydrophobic and ion-exchange chromatography on the HPLC system. The first and third fractions contained no or little toxicity, whereas the second fraction was slightly toxic. On hydrophobic chromatography, the neurotoxin eluted at the position between the second and third peaks, so the toxicity may have originated from contamination with the neurotoxin. The third fraction was found to contain the 101,000-dalton fragment, which was separated on reduction into two fragments (*M_r*s, 53,000 and 48,000). The former fragment migrated to the same position as that of the light chain (*M_r*, 53,000) on SDS-PAGE and bound to the MAb that recognized the light chain in immunoblotting. The 48,000-dalton fragment reacted with the MABs that recognized the heavy chain. From these results, the 101,000-dalton fragment resembles the L H-1 fragments of type B and E neurotoxins obtained by treatment with chymotrypsin and trypsin, respectively (16, 17). Two fragments (*M_r*s, 43,000 and 45,000) that eluted in the first and second fractions reacted to the MAB that recognized the heavy chain but not to the L H-1 fragment, suggesting that both fragments may contain the remaining portion of the heavy chain. Shone et al. (23) have reported that trypsinization of type A neurotoxin induces the fragment that lacks the carboxyl-terminal half of the heavy chain. The trypsin-induced fragment seems to correspond to the L H-1 fragment obtained in our experiment, since neither one had an inhibitory effect on the binding of type A neurotoxin to synaptosomes (23). In the binding experiment, however, the 43,000- and 45,000-dalton fragments antagonized the specific binding of ¹²⁵I-labeled neurotoxin to the same extent as did unlabeled neurotoxin. The results indicate that the two fragments are related to the carboxyl half of the heavy chain, the H-2 fragment (16, 17). Moreover, the sum of the molecular weight of the H-1 fragment and that of the fragment that eluted in the first peak agreed with the

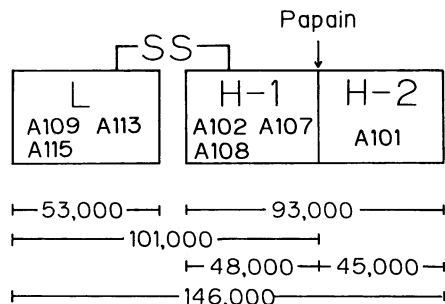


FIG. 7. Probable structure of type A neurotoxin. The molecular weights of the neurotoxin and the fragments are shown at the bottom. The neurotoxin was probably split by papain at another site 43,000 daltons from the carboxyl terminus. SS, Disulfide bond.

molecular weight of the heavy chain, which indicates that the latter fragment is the H-2 fragment. From these findings, we propose the probable structure of type A neurotoxin illustrated in Fig. 7. Shone et al. (23) mentioned that the binding region of type A neurotoxin is located close to the carboxyl terminus. If this is true, the 43,000-dalton fragment that eluted in the second fraction is the equivalent of the H-2 fragment, but it lacks about a 2,000-dalton portion from the amino terminus.

Some reports suggest that the site of botulinum neurotoxin binding to the neural membrane involves protein component(s) and sialic acid residues which may be gangliosides (3, 7, 12, 17). In the present investigation, the treatment of synaptosomes with neuraminidase resulted in reduced binding to ^{125}I -labeled neurotoxin, which is consistent with the results of previous reports (7, 29). Incorporation of ganglioside into neuraminidase-treated synaptosomes resulted in the effective restoration of the extent of binding, while gangliosides incorporated into untreated synaptosomes did not increase by much the amount of neurotoxin binding. These results provide evidence that gangliosides are, in fact, one of the components of the neurotoxin-binding site and may indicate that only a part of the ganglioside that is incorporated into synaptosomes participates in reconstitution of the binding site, as restoration of the neurotoxin binding may be dependent on the quantity of the specific gangliosides (such as G_{Q1b} , G_{T1b} , and G_{D1a}) to which type A neurotoxin binds that were used in the preparation (27). Lysyl endopeptidase treatment of synaptosomes had no effect on the binding of ^{125}I -labeled neurotoxin. This result is inconsistent with those of previous reports (7, 29), suggesting that both the binding sites for type A and B neurotoxins are trypsin sensitive. We have found, however, that ^{125}I -labeled type B neurotoxin does not bind to the synaptosomes that were treated with lysyl endopeptidase under the same conditions (unpublished data). Another report also showed that trypsinization of synaptosomes does not decrease their ability to bind type A neurotoxin (13). The different sensitivities to proteases may reflect distinct type-specific sites for type A and B neurotoxins, as it has been shown that each neurotoxin interacts with particular gangliosides and free fatty acids (17, 23).

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