INFECTION AND IMMUNITY, June 1986, p. 786–791
0019-9567/86/060786-06$02.00/0

Vol. 52, No. 3

The Use of Monoclonal Antibodies to Analyze the Structure of *Clostridium botulinum* Type E Derivative Toxin

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Received 23 December 1985/Accepted 7 March 1986

Six monoclonal antibodies against *Clostridium botulinum* type E derivative toxin were prepared. Three of the five binding to the heavy chain neutralized the derivative toxin; the other one binding to the light chain did not. Immunoblotting analysis with the monoclonal antibodies showed that the fragment obtained by tryptic digestion consisted of the light chain and part of the heavy chain (H-1 fragment) linked together by a disulfide bond(s) and that the antigenic determinants common between type E and F derivative toxins were located on both the heavy and light chains. The fragment induced by chymotrypsin treatment, like the tryptic fragment, bound to four monoclonal antibodies. The mild tryptic treatment and reduction resulted in separation of the chymotryptic fragment into two smaller fragments corresponding to the light chain and H-1 fragment. These results indicate that H-1 fragment contains the amino-terminal portion of the heavy chain. The monoclonal antibody neutralizing the toxin and probably recognizing the epitope on the carboxyl-terminal portion (H-2 fragment) of the heavy chain effectively competed for binding of ^{125}I-labeled derivative toxin to synaptosomes. Of the two monoclonal antibodies neutralizing the toxin and recognizing the epitopes on H-1 fragment, one partially inhibited binding, but the other did not. This suggests that the binding of ^{125}I-labeled derivative toxin depends mainly on the carboxyl-terminal region of the heavy chain and that interference with binding is not the only means of toxin neutralization.

Clostridium botulinum progenitor toxin consists of a toxic component and nontoxic components (18), and the former specifically inhibits acetylcholine release from nerve endings (24). The neurotoxic component or derivative toxin is synthesized as a single-chain polypeptide (M_r, about 150,000) (18, 25). It undergoes proteolytic cleavage (nicking) by an endogenous enzyme to yield the dichain molecule, in which the heavy chain (M_r, about 100,000) is covalently linked by at least one disulfide bond to the light chain (M_r, about 50,000). Treatment with trypsin or a lysine-specific endoprotease nicks the single-chain derivative toxin into the dichain molecule (3, 10). The two chains of the derivative toxin of types A through C possess antigenicities distinct from each other (9, 12, 26). The heavy chain is responsible for binding to brain synaptosomes (8, 15). The two chains of type E derivative toxin have recently been purified (20), but little has been learned about the antigenic structures or functions of the chains.

Monoclonal antibodies, with their unique specificities, have provided advanced tools for analysis of antigenic structure. This report describes the properties of several monoclonal antibodies against type E derivative toxin and their use for understanding the antigenic structure-function relationship of their epitopes on the toxin molecule. Some workers indicated cross-reaction occurring between type E and F derivative toxins (2, 4). No attempt, however, has been made to elucidate the antigenic similarity on the molecular level. We demonstrate here that the antigenic similarity between derivative toxins of the two types is due to common antigenic determinants found in both the heavy and light chains.

MATERIALS AND METHODS

Derivative toxin and toxoid. Type E progenitor toxin was prepared by a previously published method (6). The derivative toxin was purified by DEAE–Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography and converted to a toxoid by dialysis against 0.4% Formalin in 0.1 M phosphate buffer, pH 7.0, for 5 days at 30°C (5, 6). The derivative toxin was activated with trypsin (L-tosylamido-2-phenylethyl chloromethyl ketone treated; Sigma Chemical Co., St. Louis, Mo.) at a toxin-to-enzyme ratio of 200:1 for 30 min at 30°C in 0.05 M phosphate buffer, pH 7.5. Type F derivative toxin was purified by a method described elsewhere (16, 17).

Production of hybridoma cell lines. BALB/c mice were immunized by a method described previously (5). Spleen cells were fused with myeloma cells (Sp2/0-Ag14) with polyethylene glycol (M_r, 1,300 to 1,600; Sigma), and the resulting hybridomas were obtained by limiting dilution (5). To obtain monoclonal antibodies, approximately 10^7 hybridoma cells were inoculated into the peritoneal cavity of BALB/c mice primed with pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.); ascitic fluid was collected in about 2 weeks. The monoclonal antibody was purified from ascitic fluid by DEAE–Affi-Gel blue (Bio-Rad Laboratories, Richmond, Calif.) chromatography (1). Agar gel diffusion tests were carried out with anti-mouse immunoglobulin G (IgG; Nordic Immunologic Laboratories, Tilburg, The Netherlands) to determine the immunoglobulin class and subclass of each monoclonal antibody. The light chain of the monoclonal antibody was identified by enzyme-linked immunosorbent assay (ELISA) with anti-mouse kappa and lambda chains (Nordic). The polyclonal antibody was purified from mouse immune serum by affinity chromatography with CNBr-Sepharose 4B (Pharmacia) coupled with type E derivative toxin (19).

ELISA for detecting monoclonal antibodies. Monoclonal antibodies were detected by an ELISA technique described previously (5). In brief, 1 μg of derivative toxin in 0.1 ml of 0.08 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) was added to each well of a 96-well Falcon assay plate

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TABLE 1. Some properties of monoclonal antibodies against type E derivative toxins

Antibody	Subclass	Fragment	ELISA value (OD_{450}) in well coated with derivative toxin type[a]:		Neutralizing activity (%)[b]	^{125}I-labeled toxin bound to synaptosome (cpm)[c]
			E	F		
E14	G1	H	1.121	0	2.0	757 ± 81
E16	G2b	H	0.798	0.720	100	ND[d]
E17	G1	H	1.140	0	31.4	95 ± 48
E19	G1	H	0.682	0.020	92.7	ND
E22	G1	H	0.964	0.005	36.3	3,930 ± 155
E30	G1	L	1.567	1.641	100	ND

[a] The values were obtained with each antibody at 1 μg/ml. OD_{450}, Optical density at 450 nm.
[b] The data express remaining toxicity. When mixed with polyclonal antibody, the remaining toxicity was less than 0.3%.
[c] ^{125}I-labeled derivative toxin (100,000 cpm, 0.9 nM) was incubated with each monoclonal antibody (10 μg/ml) before the binding experiment. In the absence of monoclonal antibody, the ^{125}I-labeled toxin bound was 4,450 ± 216 cpm. Nonspecific binding was measured in the presence of 1 μM unlabeled derivative toxin. Each point represents the mean ± the standard error of four determinations.
[d] ND, Not determined.

(Becton Dickinson Labware, Oxnard, Calif.). After 3 h at 37°C, the wells were washed once with PBS–0.05% Tween 20. Each well then received 0.2 ml of 0.2% bovine serum albumin (BSA; 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 monoclonal antibody, 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.) was added to each. After 2 h, the wells were washed and 0.2 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 developed color intensities were read with an MTP 12 microplate photometer (Corona Electric Co., Ibaraki, Japan). To determine the epitope specificity of the monoclonal antibody, competitive ELISA was performed with the peroxidase-conjugated monoclonal antibody (5); the conjugated monoclonal antibody was mixed with graded amounts of homologous or heterologous nonconjugated monoclonal antibody, and ELISA was carried out as described above.

Digestion of derivative toxin with trypsin and chymotrypsin. Treatment with either enzyme was carried out in 0.05 M phosphate buffer, pH 7.5, at 37°C. The derivative toxin (1 mg/ml) was digested for 8 h with trypsin (Sigma) at a toxin-to-enzyme ratio of 40:1. Leupeptin (Peptide Institute Inc., Osaka, Japan) was used as an inhibitor. After digestion, high-performance liquid chromatography (HPLC) was run with a Jasco HPLC System (Japan Spectroscopic Co., Tokyo, Japan) to separate the tryptic fragment. A portion of a sample (100 μg) was loaded on a column of Finepak SIL, AF 102, and eluted with 0.05 M phosphate buffer, pH 7.0, containing 0.1 M sodium citrate at a flow rate of 1 ml/min. The eluate was collected and concentrated with Centricon 30 (Amicon Corp., Lexington, Mass.).

Chymotrypsin digestion of the derivative toxin was done for 1 h at a toxin-to-enzyme ratio of 100:1. After adding chymostatin (Peptide Institute Inc.) to stop hydrolysis, the chymotrypsin-digested toxin was treated further with trypsin at a toxin-to-enzyme ratio of 200:1 for 30 min at 30°C.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of derivative toxin and fragments was performed in a 10% gel as described previously (13). The sample was boiled for 3 min with 1% SDS in the presence or absence of 100 mM dithiothreitol (DTT). A 10- to 20-μl portion of a sample containing approximately 10 μg was applied. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue for 30 min. The molecular weights of the protein bands were estimated by use of molecular weight markers (Sigma). For immunoblotting, the derivative toxin and the resulting fragments were transferred electrophoretically to nitrocellulose sheets (TM-2; Toyo Roshi, Tokyo, Japan) (27). After blotting, the sheet was soaked overnight in PBS containing 3% BSA (PBS-BSA). The sheet was then transferred to a fresh PBS-BSA solution containing the respective monoclonal antibody diluted to approximately 20 μg/ml and incubated for 30 min at room temperature. The sheet was washed with PBS and then treated for 30 min with peroxidase-conjugated anti-mouse IgG diluted 500-fold with PBS-BSA. After the sheet was washed, the bound peroxidase was allowed to react on 3,3'-diaminobenzidine (0.5 mg/ml)–0.003% H_2O_2 in PBS.

Inhibition of binding of ^{125}I-labeled derivative toxin to synaptosomes. The derivative toxin (50 μg) was radioiodinated with $Na^{125}I$ (0.5 mCi) (New England Nuclear Corp., Boston, Mass.) by a method reported previously (11). About 70% of the toxicity of unlabeled derivative toxin 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 $MgCl_2$, 2 mM $CaCl_2$, 5 mM Tris, pH 7.0, containing 0.1% BSA). Inhibition was examined with ^{125}I-labeled derivative toxin at a final concentration of 0.9 nM and a fragment or a monoclonal antibody at various concentrations. Synaptosomes (100 μg of protein) were incubated with ^{125}I-labeled derivative toxin in the presence or absence of a monoclonal antibody in 0.2 ml of salt solution for 15 min at 30°C. For testing inhibition of binding of the ^{125}I-labeled derivative toxin, synaptosomes were preincubated with unlabeled derivative toxin or the fragment for 5 min at 30°C. Monoclonal antibody was allowed to react with ^{125}I-labeled derivative toxin for 15 min at 30°C before incubation with synaptosomes. The synaptosomes after incubation were separated by filtration through a well of a Millititer HA plate (Millipore Corp., Bedford, Mass.). The filter membrane was then washed five times with 0.25 ml of salt solution by suction. The disk, obtained from the filter membrane with a punch, was placed in a polystyrene tube, and radioactivity was determined with an NaI-well-type scintillation counter.

Other methods. The neutralizing activity of each monoclonal antibody was determined in the following way. The derivative toxin activated with trypsin was diluted to 10 μg/ml (2×10^5 50% lethal doses per ml) with PBS, and the

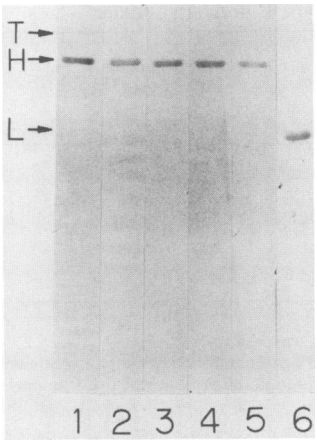

FIG. 1. Immunoblotting analyses of the heavy and light chains of type E derivative toxin bound to monoclonal antibodies. Lanes: 1, E14; 2, E16; 3, E17; 4, E19; 5, E22; 6, E30. T, Derivative toxin; H, heavy chain; L, light chain.

dilution was mixed with an equal volume containing 200 μg of each monoclonal antibody. After incubation of the mixture for 15 min at 37°C, the remaining toxicity was determined by the time-to-death method by intravenous injection into mice (7). Protein contents were determined by the method of Lowry et al. (14).

RESULTS

Properties of the monoclonal antibodies. Nine lines in all were established from 510 wells, and each monoclonal antibody was purified from the ascitic fluid. By competitive ELISA, six monoclonal antibodies were found to recognize distinct epitopes on the toxin molecule. The six each contained a kappa light chain. Two of them, E16 and E30, reacted with type F derivative toxin in an ELISA (Table 1). In immunoblotting, five bound to the heavy chain, and the other bound to the light chain (Fig. 1). Three monoclonal antibodies were capable of neutralizing the derivative toxin (Table 1). Their activities, however, were lower than that of polyclonal antibody purified from mouse immune serum.

Digestion of derivative toxin with trypsin and chymotrypsin. After digestion of the derivative toxin with trypsin for a prolonged period, a sample was subjected to HPLC. A single major peak emerged (Fig. 2). This peak contained about 0.1% of the original toxicity and migrated in SDS-PAGE as

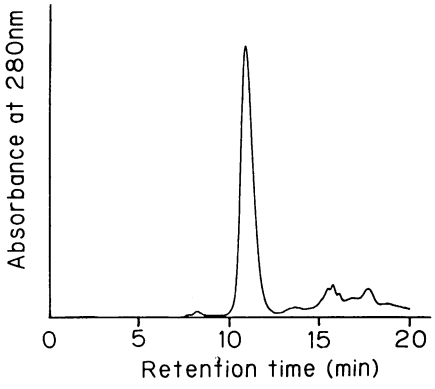

FIG. 2. HPLC elution profile of type E derivative toxin digested with trypsin.

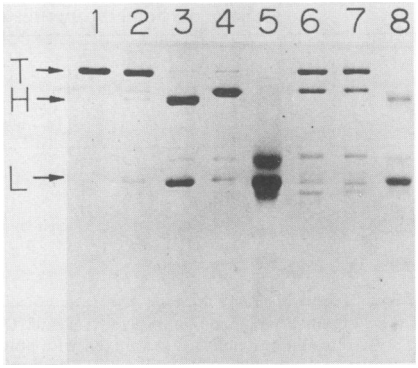

FIG. 3. SDS-PAGE of type E derivative toxin and fragments. Lanes: 1, derivative toxin; 2 and 3, activated derivative toxin; 4 and 5, trypsin-induced fragment; 6 and 7, chymotrypsin-digested toxin; 8, chymotrypsin- and trypsin-digested derivative toxin. Samples in lanes 1, 3, 5, 7, and 8 were electrophoresed in the presence of DTT.

a single major band (M_r, 105,000), the mobility of which was between those of the derivative toxin (M_r, 146,000) and the heavy chain (M_r, 97,000) (Fig. 3, lanes 1 to 4). Furthermore, reduction of the fraction with DTT resulted in separation into two bands (M_rs, 56,000 and 49,000). The mobility of one of the two bands corresponded to that of the light chain (Fig. 3, lane 5). The chymotrypsin-digested derivative toxin with or without reduction was also subjected to SDS-PAGE. Two major bands (M_rs, 146,000 and 110,000) and some minor bands were seen (Fig. 3, lanes 6 and 7). When the chymotrypsin-digested derivative toxin was further subjected to mild tryptic treatment and then reduced, the two major bands appeared to be separated into at least three different bands (M_rs, 97,000, 57,000, and 49,000), indicating that the two bands had the same mobility rates as the heavy and light chains (Fig. 3, lane 8).

Immunoblotting with monoclonal antibodies. By immunoblotting, the trypsin-induced fragment and chymotrypsin-digested derivative toxin were analyzed for binding to each monoclonal antibody. Of the five monoclonal antibodies recognizing the heavy chain, E14 reacted to the trypsin-induced fragment. It bound to the 56,000-dalton fragment derived from the trypsin-induced fragment by reduction (Fig. 4, lanes 1 and 2). E19 and E22 also reacted in the same

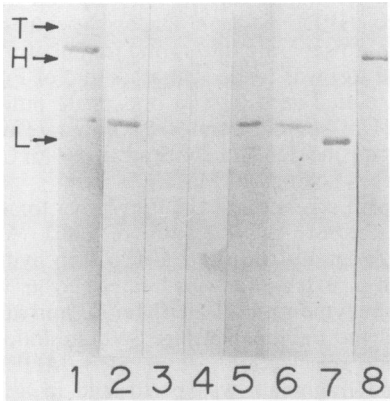

FIG. 4. Immunoblotting analyses of trypsin-induced fragment bound to monoclonal antibodies. Before blotting, samples in lanes 2 to 7 were electrophoresed in the presence of DTT. Lanes: 1 and 2, E14; 3, E16; 4, E17; 5, E19; 6, E22; 7 and 8, E30.

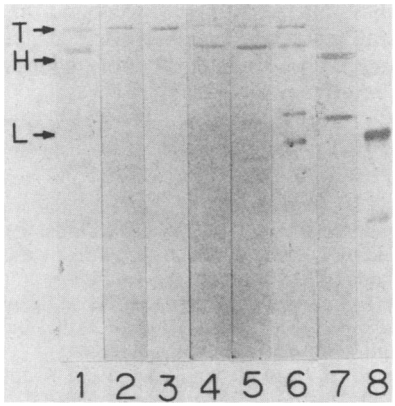

FIG. 5. Immunoblotting analyses of chymotrypsin-digested derivative toxin bound to monoclonal antibodies. Samples in lanes 7 and 8 were further treated with trypsin and then reduced with DTT. Lanes: 1 and 7, E14; 2, E16; 3, E17; 4, E19; 5, E22; 6 and 8, E30.

manner as did E14 (Fig. 4, lanes 5 and 6). The only monoclonal antibody recognizing the light chain bound to the 49,000-dalton fragment (Fig. 4, lanes 7 and 8). The other two, E16 and E17, did not react to any fragment (Fig. 4, lanes 3 and 4).

Immunoblotting of the chymotrypsin-digested derivative toxin indicated that four monoclonal antibodies (E14, E19, E22, and E30) bound to the 110,000-dalton fragment and the derivative toxin (Fig. 5). The remaining two, E16 and E17, reacted only to the bands with mobilities corresponding to that of the derivative toxin. In addition, with the chymotrypsin-digested derivative toxin treated further with trypsin and then reduced with DTT, E14 reacted to the two bands of M_rs of 97,000 and 57,000, whereas E30 bound to the 49,000-dalton fragment with mobility corresponding to that of the light chain. The epitopes for monoclonal antibodies E19 and E22 were demonstrated on all of the fragments to which E14 reacted. Of the two monoclonal antibodies cross-reacting with type F derivative toxin, E16 bound to the heavy chain and E30 bound to the light chain (Fig. 6).

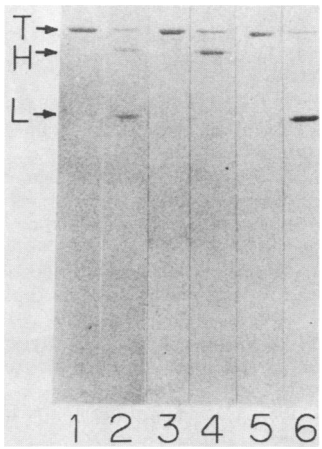

FIG. 6. Immunoblotting analyses of type F derivative toxin and its derived chains bound to monoclonal antibodies against type E derivative toxin. Samples in lanes 2, 4, and 6 were electrophoresed in the presence of DTT. After blotting, pieces of nitrocellulose sheet were stained with 0.1% amido black 10B (lanes 1 and 2). E16 was used for lanes 3 and 4; E30 was used for lanes 5 and 6.

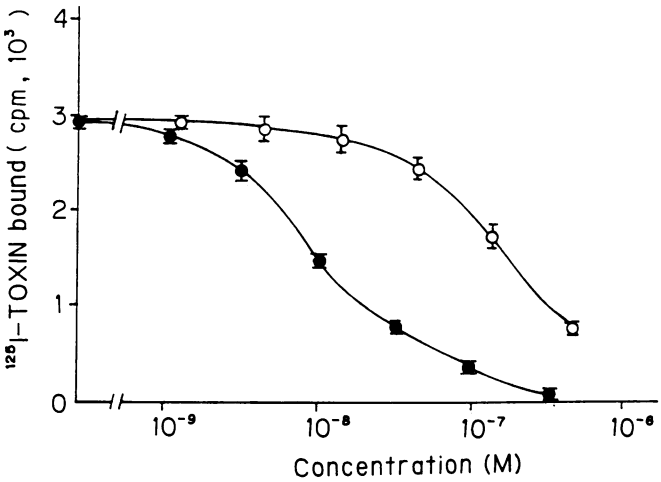

FIG. 7. Inhibition of binding of ^{125}I-labeled type E derivative toxin to synaptosomes with unlabeled derivative toxin and the trypsin-induced fragment. ^{125}I-labeled derivative toxin (60,000 cpm, 0.9 nM) was added to synaptosomes preincubated with unlabeled derivative toxin (●) or fragment (○). Nonspecific binding was measured as described in Table 1, footnote *c*. Each point represents the mean ± the standard error of three determinations.

Inhibition of binding of ^{125}I-labeled derivative toxin to synaptosomes by monoclonal antibody and fragment. Of the three monoclonal antibodies, E14, E17, and E22, capable of neutralizing the derivative toxin, E17 inhibited the binding of ^{125}I-labeled derivative toxin. E14 also inhibited binding but to a much lesser extent than E17 (Table 1). Even at a 10-fold concentration (100 μg/ml), inhibition of binding with E14 was lower than that attained with 10 μg of E17 per ml (data not shown).

The 105,000-dalton fragment obtained by tryptic digestion of the derivative toxin inhibited binding, but a 10-fold or higher concentration was required to attain the same level of inhibition as that attained with unlabeled derivative toxin (Fig. 7).

DISCUSSION

We obtained six monoclonal antibodies against *C. botulinum* type E derivative toxin that discriminated distinct epitopes on the toxin molecule. Five of them were of the IgG1 subclass, and the other was of the IgG2b subclass. Three of the five monoclonal antibodies binding to the heavy chain possessed derivative-toxin-neutralizing activity, whereas the one binding to the light chain did not. These findings agree with the previous observation that the anti-heavy-chain antibody plays a more important role in neutralization of the toxin (9).

When type E derivative toxin was treated with trypsin for a prolonged period, a 105,000-dalton fragment was obtained by HPLC. On reduction, this fragment migrated as two bands in SDS-PAGE. When this fragment was immunoblotted, one band reacted with three of the monoclonal antibodies recognizing the heavy chain, and the other, which migrated to the same position as did the light chain, bound to the monoclonal antibody recognizing the light chain. These results indicate that the fragment obtained by tryptic digestion of the derivative toxin was composed of the light chain and part of the heavy chain (named the H-1 fragment) linked together by at least one disulfide bond. It should be called the L · H-1 fragment.

On chymotrypsin treatment of the derivative toxin, an

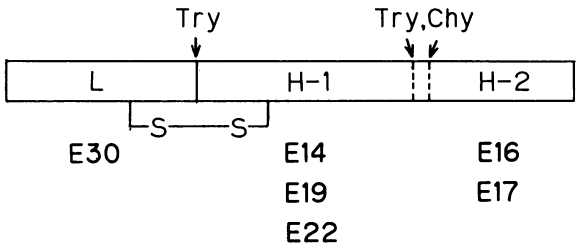

FIG. 8. Probable epitope locations on *C. botulinum* type E derivative toxin.

approximately 110,000-dalton fragment emerged in SDS-PAGE in the presence or absence of DTT, though some native toxin still remained after treatment. Four monoclonal antibodies (E14, E19, E22, and E30) reacted to the 110,000-dalton fragment. When subjected to additional tryptic cleavage and reduction, the fragment was separated into two smaller fragments (M_rs, 57,000 and 49,000), corresponding to the H-1 fragment and the light chain, respectively. The reactivities of the four monoclonal antibodies to these fragments were the same as those to the L · H-1 fragment. These results indicate that the 110,000-dalton fragment obtained by chymotrypsin treatment consists of the light chain and the H-1 fragment in the unnicked form. Sathyamoorthy and DasGupta (21) reported that the light chain contains the amino-terminal end of the original single chain and that nicking occurs at about one-third of the distance from the amino terminal.

The present data, therefore, demonstrate that the H-1 fragment contains the amino terminal of the heavy chain and that a disulfide bond links the light chain and H-1 fragment. The remaining portion of the heavy chain (named the H-2 fragment), which may have contained the carboxyl terminal easily digested by proteases, reacted with the other two monoclonal antibodies (E16 and E17). The most likely epitope locations on the derivative toxin are shown in Fig. 8.

Previous studies showed that binding of ^{125}I-labeled derived toxin to synaptosomes was mediated by the heavy chain (8, 15). Of the three monoclonal antibodies neutralizing the toxin and recognizing epitopes on the heavy chain, E14 and E17 inhibited the binding of ^{125}I-labeled derivative toxin to synaptosomes. Inhibition by E17 seems to have been stronger than that by E14, whereas E14 possessed higher neutralizing activity than E17. The observations suggest that the binding of ^{125}I-labeled derivative toxin to synaptosomes depends mainly on the H-2 fragment. Shone et al. (22) claimed that the fragment obtained from type A derivative toxin by prolonged tryptic digestion did not inhibit binding to synaptosomes. The L · H-1 fragment obtained in a similar way competed for binding of ^{125}I-labeled derivative toxin, and its inhibitory ability seemed to be about 10 times less than that of the derivative toxin. The present results indicate that the H-1 fragment is also partially related to binding, though the possibility cannot be excluded that such inhibition was due to the derivative toxin having contaminated the fragment preparation. The other monoclonal antibody, E22, hardly competed for binding. The epitope recognized by E22 might function for the translocation and following steps proposed by Simpson (23, 24).

The two monoclonal antibodies E16 and E30 were shown to react with type F derivative toxin. They bound to the corresponding chains of type F derivative toxin as those of type E derivative toxin; the antigenic determinants common to type E and F derivative toxins are located on both the heavy and light chains. Attempts to define the areas of the toxin molecule involved in binding, toxicity, and activation are under way to contribute to a better understanding of the function of botulinum toxins.

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