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Immunological Characterization of *Clostridium butyricum* Neurotoxin and Its Trypsin-Induced Fragment by Use of Monoclonal Antibodies against *Clostridium botulinum* Type E Neurotoxin

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We examined the reactivities of *Clostridium butyricum* neurotoxin to nine monoclonal antibodies against *Clostridium botulinum* type E neurotoxin which recognize the light chain or the amino-terminal half (H-1 fragment) or the carboxyl-terminal half (H-2 fragment) of the heavy chain of botulinum neurotoxin. Butyricum neurotoxin and its derived chains did not react to two of four monoclonal antibodies recognizing the light chain, one of three recognizing the H-1 fragment, and one of two recognizing the H-2 fragment. The results indicate that the immunological difference between the two neurotoxins is not attributable to a particular portion of the toxin molecule. The fragment of butyricum neurotoxin obtained by prolonged tryptic treatment was found to comprise the light chain and H-1 fragment linked together by a disulfide bond.

Infant botulism is caused by neuromuscular paralysis due to the toxin produced in the intestines by ingested spores (13, 14). Most cases have been caused by *Clostridium botulinum* type A or B. The organism isolated from a case of type E infant botulism in Italy was identified as *Clostridium butyricum*, but it produced a neurotoxin that was neutralized by botulinum type E antitoxin (1, 12). Giménez and Sugiyama (6) purified the neurotoxin from the culture of the isolate and demonstrated that *C. butyricum* neurotoxin is antigenically similar but not identical to type E neurotoxin. Botulinum neurotoxin (M_r, 150,000) is composed of light (M_r, 50,000) and heavy (M_r, 100,000) chains linked together by at least one disulfide bond. We have prepared monoclonal antibodies (MAbs) for botulinum neurotoxin and analyzed the neurotoxin molecule for its immunological, structural, and functional properties (8–10). Botulinum neurotoxin consists of at least three domains (L, H-1, and H-2) which possess probably functionally different properties. By using MAbs against type E neurotoxin, we expected that butyricum neurotoxin could be characterized and compared with type E neurotoxin. Our experiments provided basic information on the structure, function, and immunological properties of butyricum neurotoxin.

Toxigenic *C. butyricum* 5262 was provided by C. L. Hatheway, Centers for Disease Control, Atlanta, Ga. *C. botulinum* type E strain German sprats, stocked in our laboratory, was used for preparation of type E toxin. Type E progenitor toxin was purified by a previously described method (7). Type E neurotoxin was isolated from the progenitor toxin by DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography at pH 8.0. Butyricum toxin was purified according to the method of Giménez and Sugiyama (5, 6). Butyricum neurotoxin free of the nontoxic component was isolated by DEAE-Sephacel (Pharmacia) chromatography (5). Each neurotoxin (500 μg/ml) was activated at 30°C for 10 min with trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone treated; Sigma Chemical Co., St. Louis, Mo.) at a toxin-to-enzyme ratio of 200:1 in 0.05 M phosphate buffer, pH 7.5. Butyricum neurotoxin (1 mg/ml) was also digested at 37°C for 6 h with trypsin at a toxin-to-enzyme ratio of 40:1 at pH 7.5 in the absence of dithiothreitol (DTT). After incubation, leupeptin (Peptide Institute Inc., Osaka, Japan) was added to stop hydrolysis, and the mixture was applied to a PD-10 column equilibrated with 0.02 M phosphate buffer, pH 5.0. The eluted protein fraction was immediately applied to a Mono S HR 5/5 column (Pharmacia) equilibrated with the same buffer. The fragment was eluted with a linear gradient from 0 to 0.2 M NaCl at a flow rate of 0.5 ml/min.

MAbs against type E neurotoxin were obtained by procedures described elsewhere (8). The MAbs were purified from ascitic fluid by Affi-gel protein A (Bio-Rad Laboratories, Richmond, Calif.) chromatography. Of nine MAbs used, four (E1, E2, E25, and E32) reacted to the light chain, three (E14, E20, and E22) reacted to the amino-terminal half of the heavy chain (H-1 fragment), and the other two (E16 and E28) reacted to the carboxyl-terminal half of the heavy chain (H-2 fragment). Four MAbs (E14, E22, E28, and E32) neutralized type E neurotoxin. The reactivities of MAbs to butyricum neurotoxin were examined by enzyme-linked immunosorbent assay (ELISA) according to a previously described method (8). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with a 10% gel or a 5 to 15% linear gradient gel as described previously (11). The sample was boiled for 3 min with SDS in the absence or presence of 50 mM DTT. 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, the toxins and the fragments were transferred electrophoretically to nitrocellulose paper (TM-2; Toyo Roshi, Tokyo, Japan) (8). The paper was treated with 10% newborn calf serum (GIBCO, Life Technologies Inc., Chagrin Falls, Ohio) and 0.1% Tween 20 in phosphate-buffered saline (PBS) to avoid nonspecific reactions. After being blocked, the paper was incubated with the respective MAbs diluted to 20 μg/ml and then

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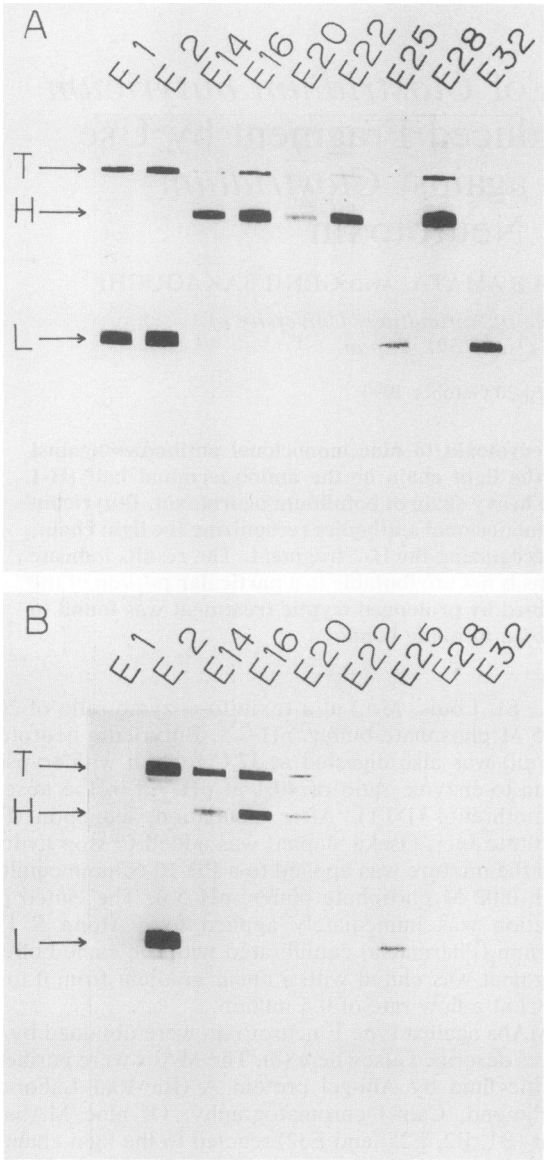

FIG. 1. Immunoblotting analyses of type E (A) and butyricum (B) neurotoxins with MAbs against type E neurotoxin. Partially nicked neurotoxin was used as sample to reveal the bands of neurotoxin (T), the heavy chain (H), and the light chain (L). Before being blotted, the samples were electrophoresed in the presence of DTT. The MAbs used are indicated above the lanes.

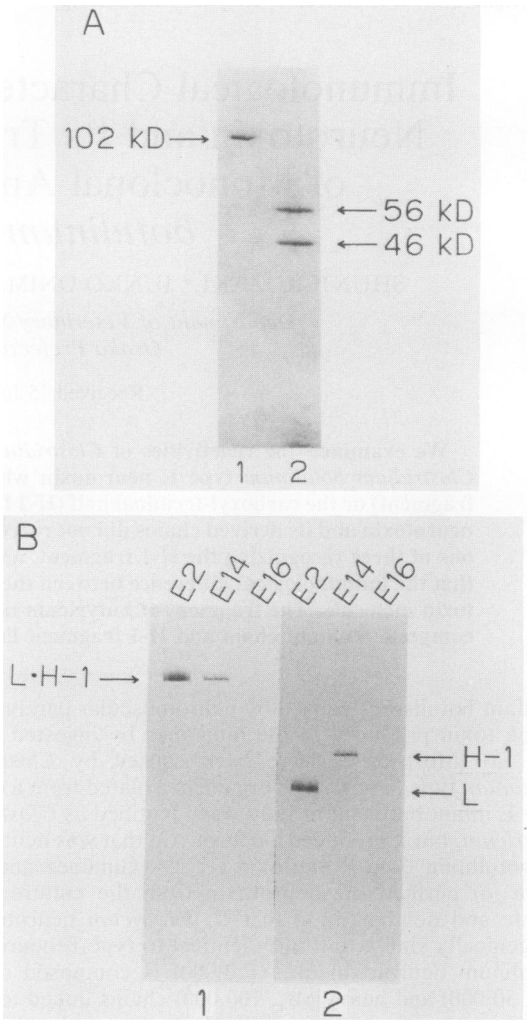

FIG. 2. (A) SDS-PAGE of the trypsin-induced fragment. The fragment was electrophoresed in the absence (lane 1) and presence (lane 2) of DTT. kD, Kilodaltons. (B) Immunoblotting analysis of the trypsin-induced fragment with MAbs against type E neurotoxin. Before being blotted, the samples were electrophoresed in the absence (panel 1) and presence (panel 2) of DTT. The MAbs used are indicated above the lanes.

treated with peroxidase-conjugated anti-mouse immunoglobulin G. After being washed, the nitrocellulose paper was exposed to a substrate solution (0.05% 3,3'-diaminobenzidine and 0.003% H_2O_2 in PBS).

By using SDS-PAGE with a linear gradient gel, activated butyricum neurotoxin was separated into two fragments in the presence of DTT; its heavy chain (M_r, 96,000) was comparable in molecular size to that of type E neurotoxin (M_r, 97,000), while the light chain of the former (M_r, 46,000) was found to be slightly smaller than that of the latter (M_r, 49,000). In an ELISA, five MAbs (E2, E14, E16, E20, and E25) reacted to butyricum neurotoxin, whereas the other four (E1, E22, E28, and E32) did not. MAb E14 neutralized type E neurotoxin (8) and butyricum neurotoxin as well. In immunoblotting, the five MAbs that reacted to butyricum neurotoxin in an ELISA also bound to the corresponding chain (Fig. 1). The results suggest that some epitopes different from those of type E neurotoxin are present in both chains of butyricum neurotoxin. The observation that MAb E32, which neutralized type E neurotoxin and recognized the light chain, did not react to butyricum neurotoxin may suggest that the toxic sites of the two neurotoxins, being located on the light chain (3), are antigenically different from each other.

After digestion with trypsin, a portion of butyricum neurotoxin was applied to a Mono S HR 5/5 column and eluted with NaCl linear gradient. A single peak, containing no toxicity, emerged at 0.1 M NaCl. The protein in this fraction migrated in SDS-PAGE as a single band (M_r, 102,000). This band was further separated into two fragments (M_rs, 56,000 and 46,000) under reducing conditions (Fig. 2A). The smaller fragment migrated to a position identical to that of the light

chain of the butyricum neurotoxin. In immunoblotting, MAbs E2 and E14 reacted respectively to the 46,000- and 56,000-Da fragments. MAb E16, which reacted to the heavy chain of butyricum neurotoxin, did not bind either band (Fig. 2B). We have reported that E16 recognizes the H-2 fragment of type E neurotoxin, while E2 and E14 react, respectively, to the light chain and H-1 fragment (8). The results described above indicate that tryptic digestion of butyricum neurotoxin resulted in destruction of the carboxyl-terminal portion of the heavy chain. This high susceptibility of butyricum neurotoxin to trypsin resembles that of type E neurotoxin (8). The induced fragment (M_r, 102,000) has characteristics similar to those of the L · H-1 fragments of types A, B, and E neurotoxins obtained by treatment with papain, trypsin, or chymotrypsin (8–10). The H-1 fragment (M_r, 56,000) of butyricum toxin had a molecular size very close to that of type E neurotoxin (M_r, 57,000) (8), suggesting that the heavy chain of butyricum toxin was split by trypsin at a site comparable to that of type E neurotoxin. From these observations, butyricum neurotoxin seems to have virtually the same structural domains as those of type E neurotoxin. The complete sequence of type A neurotoxin has recently been reported (2, 15), and partial sequences of other neurotoxins, including butyricum neurotoxin, have also been reported (2, 4). Therefore, comparison of the whole sequences of the two neurotoxins may lead to clarification of the different antigenic sites.

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