INFECTION AND IMMUNITY, Apr. 1988, p. 898–902
0019-9567/88/040898-05$02.00/0

Vol. 56, No. 4

Establishment of a Monoclonal Antibody Recognizing an Antigenic Site Common to *Clostridium botulinum* Type B, C_1, D, and E Toxins and Tetanus Toxin

KAYO TSUZUKI,[1]* NORIKO YOKOSAWA,[1] BUNEI SYUTO,[2] IWAO OHISHI,[3] NOBUHIRO FUJII,[1] KOUICHI KIMURA,[1] AND KEIJI OGUMA[1]

Department of Microbiology, Sapporo Medical College,[1] and Department of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University,[2] Sapporo 060, and College of Agriculture, University of Osaka Prefecture, Sakai-shi, Osaka 591,[3] Japan

Received 1 September 1987/Accepted 31 December 1987

The partial amino acid sequence of the light-chain (Lc) component of *Clostridium botulinum* type C_1 toxin was determined. The sequence was quite similar to those of the other types of botulinum and tetanus toxins. Nine monoclonal antibodies against botulinum type E toxin were established by immunizing BALB/c mice with type E toxoid or its Lc component. Six antibodies reacted with the heavy-chain component and three reacted with the Lc component of the toxin. One of the latter three antibodies reacted with botulinum type B, C_1, and D toxins and tetanus toxin, as well as botulinum type E toxin. This antibody recognized the Lc components of these toxins, indicating that there exists one common antigenic determinant on the Lc regions of these toxins.

The protein neurotoxins of *Clostridium botulinum* cause fatal intoxication in both animals and humans. The toxins are classified into seven groups (types A through G) based on their antigenicity. All toxins except type G have been purified. The M_rs of all toxin types are approximately 150,000. They are produced as a single polypeptide chain, and are nicked by proteases produced by the organisms or by artificially added trypsin. The nicked toxins can be separated into two fragments, designated heavy chain (Hc; M_r, 100,000) and light chain (Lc; M_r, 50,000), following reduction of a disulfide bond which connects the fragments. All toxins seem to attach to cholinergic nerve endings and block the release of transmitter (acetylcholine). The detailed mechanism of action, however, has not been determined.

We previously prepared monoclonal antibodies against type C_1 and D toxins to clarify the mechanism of toxin action and the antigenic structures of the toxins (8, 9). Antibodies which reacted with both C_1 and D toxins, but not with any other types of toxins, were obtained. Recently, Hambleton et al. (3) have reported that one monoclonal antibody obtained by immunizing BALB/c mice with type A toxoid reacted with botulinum type A, B, and E toxins and tetanus toxin, as well as the enterotoxins of *Clostridium difficile* and *Clostridium perfringens* (3).

Here, we describe the establishment of a monoclonal antibody which reacts with botulinum type B, C_1, D, and E toxins and tetanus toxin, but not with botulinum type A and F toxins or the enterotoxin of *C. perfringens*.

MATERIALS AND METHODS

Preparation of toxins and their components. The toxins purified from the following botulinum strains were used: A-97, B-Okra, C-Stockholm (C-ST), C-6813, D-1873, E-Iwanai, and F-Langeland. Also, components I (M_r, 55,000) and II (M_r, 105,000) of botulinum C_2 toxin (strain 92-13) and *C. perfringens* enterotoxin (M_r, 35,000) were used. All these toxins were purified as described previously (10–13, 15, 17–19). Tetanus toxin was kindly provided by M. Matsuda (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). Hc and Lc components of C-ST toxin were purified as reported previously (16), and those of type E toxin were obtained by the same procedure with slight modifications. The type E toxin preparation (10 mg in 4.3 ml) was dialyzed against 0.06 M phosphate buffer (PB; pH 6.0) and treated with trypsin at a final concentration of 25 μg/ml for 30 min at 37°C. The reaction was terminated by the addition of soybean trypsin inhibitor (100 μg/ml). The nicked toxin was dialyzed against 0.01 M PB (pH 7.4) and applied to a quarternary aminoethyl Sephadex A-50 column (1.2 by 6.5 cm; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. After the column was reacted at 0°C for 1 h, it was treated with 2 ml of 0.05 M dithiothreitol for 3 h and then with 2 ml of 5% 2-mercaptoethanol (2-ME) in 0.06 M PB overnight. The Lc component was eluted with 20 ml of 5% 2-ME in 0.06 M PB containing no NaCl; the Hc component was eluted with 30 ml of 5% 2-ME in 0.06 M PB containing 0.24 M NaCl. The treatment of dithiothreitol and the elution of each component were carried out at 0°C.

Preparation of polyclonal antibodies. Polyclonal rabbit sera against components I and II of botulinum C_2 toxin and *C. perfringens* enterotoxin were those prepared previously (12, 18).

Preparation of monoclonal antibodies. Type E toxoid was prepared by dialyzing the purified toxin against 0.05 M PB (pH 6.0) containing formaldehyde at 0.4% for 1 week at room temperature. Each 50 μg of type E toxoid or Lc component of the toxin, the latter of which was mixed with an equal amount of adjuvant, was injected intraperitoneally into BALB/c mice twice at 2-week intervals. As adjuvant, the water-soluble cell wall fractions of *Mycobacterium tuberculosis*, which was provided by K. Yamamoto (Institute of Immunological Science, Hokkaido University), was used. After more than 2 weeks a booster dose of 30 μg without adjuvant was given by an intravenous injection. The spleen cells from immunized mice were fused by polyethylene glycol with P3-NS1-1-Ag4-1 myeloma cells which were incubated in Dulbecco modified Eagle medium (Flow Labora-

* Corresponding author.

tories, Irvine, Scotland) supplemented with 20% fetal bovine serum. The resulting hybrid cells were selected by treatment with Dulbecco modified Eagle medium supplemented with 20% fetal bovine serum and containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine. Antibody production by the hybridomas was screened by enzyme-linked immunosorbent assay (ELISA) with alkaline phosphatase-labeled rabbit anti-mouse serum which was prepared previously (8). The cell lines producing antibody were expanded into 48-well plates. After the toxin-neutralizing activities of the antibodies were examined, nine cell lines were selected and cloned by the limiting dilution method. Thereafter, the ascites fluids were obtained by injecting intraperitoneally more than 10^7 cells into BALB/c mice which were treated with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane; Wako Pure Chemical Co., Osaka, Japan).

Purification of monoclonal antibodies. Immunoglobulin was purified from ascites fluids as described previously (7). Ascites fluids (4 ml) were dialyzed against 0.1 M Tris hydrochloride (pH 7.4) and subjected to column chromatography on DEAE-cellulose (1.6 by 10 cm; DE52; Whatman, Inc., Maidstone, Kent, England) equilibrated with the buffer described above. The preparation was run at 40 ml/h, and the fractions that did not bind to the column were collected. The immunoglobulins that were thus obtained were precipitated by the addition of an equal volume of saturated ammonium sulfate and dialyzed against 10 mM phosphate-buffered saline (PBS; pH 7.2).

Type of immunoglobulin. The subclass of the antibodies was determined by the Ouchterlony immunodiffusion test with rabbit immunoglobulin G (IgG) against different mouse immunoglobulin subclasses (IgG1, IgG2a, IgG2b, IgG3, and IgM; κ and λ light chains) which were purchased from Miles Laboratories, Inc. (Elkhart, Ind.).

ELISA. The reaction of monoclonal antibodies with different types of toxins and the subcomponents of the toxins was studied by ELISA. The ELISA was carried out by the procedures reported previously (8, 9), with slight modifications. The 96-well microtiter plates (Immunoplate II; Nunc, Roskilde, Denmark) were coated with 100 μl of toxins or their components (2.5 μg/ml). After the wells were washed with 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.05% Tween 20 and 0.5 M NaCl (T-TBS), they were blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. Then, each 100 μl of diluted antibody solution and alkaline phosphatase-conjugated rabbit anti-mouse serum were subsequently reacted for 2 h at 37°C. After this solution was washed with T-TBS, 100 μl of 2.5 mM *p*-nitrophenyl phosphate dissolved in 50 mM carbonate-bicarbonate buffer (pH 9.8) with 1 mM $MgCl_2$ was reacted for 1 h at 37°C. The reaction was terminated by the addition of 25 μl of 3 N NaOH, and the A_{410} was measured with a microELISA minireader (MR590; Dynatech Laboratories, Inc., Alexandria, Va.).

Toxin neutralization test. The toxin-neutralizing activities of immunoglobulins obtained from ascites fluids were examined as reported previously (8, 9). Antibodies were diluted in serial twofold steps with PBS, mixed with an equal volume of ten times the 50% lethal dose (LD_{50}) of toxin per ml, and incubated at 37°C for 2 h; and then each 0.5 ml of the mixture was injected intraperitoneally into three mice. The neutralizing activities of the hybridomas formed in the microplates were checked as follows. The hybridomas were expanded into 48-well plates. The culture fluids (0.3 ml) were diluted twice with PBS and mixed with an equal volume of 10 LD_{50}s of toxin per ml, and then 0.5 ml of the mixture was injected intraperitoneally into two mice.

Dot blot analysis. A total of 1 μg of each of the purified toxins and their components in 10 μl of 50 mM carbonate-bicarbonate buffer (pH 9.6) were spotted onto nitrocellulose membranes, and the membranes were assembled in a microfiltration apparatus (Bio-Dot; Bio-Rad Laboratories, Richmond, Calif.), as described by the manufacturer (1). After filtration, the membrane was removed from the apparatus and washed with T-TBS. The membrane was then cut into strips, and each strip was placed in an incubation tray containing 2 ml of 10% skim milk in PBS for 2 h at 4°C. After the solutions were washed, 1 μg of each antibody solution in 2 ml of 10% skim milk in PBS was reacted overnight at 4°C and sufficiently washed with T-TBS. Thereafter, 2 ml of peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit serum (Dakopatts a/s, Glostrup, Denmark), which was diluted 1:1,000 with 10% skim milk in PBS, was reacted for 2 h at room temperature. After the solution was washed, the peroxidase activity was detected by exposing the membrane to 0.2 mg of 3,3-diaminobenzidine per ml–0.01% H_2O_2 in 50 mM Tris hydrochloride (pH 7.6) for 10 to 30 min.

Western blot analysis. Botulinum type C_1, D, and E and tetanus toxins were employed for Western blot analysis. Botulinum type E and tetanus toxins were first treated with trypsin in an attempt to form a nick in the toxin molecules. A total of 100 μg of each of the toxins and 2.5 μg of trypsin were suspended into 90 and 10 μl of 0.06 M PB (pH 6.0), respectively, and then reacted for 30 min at 37°C. The other toxins (100 μg) were suspended into 100 μl of 0.06 M PB (pH 6.0). All toxin preparations were diluted 1:25 with distilled water and mixed with an equal volume of 2-ME and sodium dodecyl sulfate solution. After the preparations were heated for 5 min at 100°C, 50 μl of the preparations (containing 1 μg of protein) were electrophoresed, as described by Laemmli (5), by using a 3% stacking gel and an 8% running gel. The separated proteins were transferred to a nitrocellulose membrane (50 V, overnight). The membrane was cut into strips and placed into an incubation tray containing 2 ml of 10% skim milk in PBS for 2 h at 4°C. Thereafter, the antibody solution, peroxidase-labeled antibody, and its substrate were reacted as described above for the dot blot analysis.

Amino acid sequence. The partial amino acid sequence of the N terminus of the Lc component of C-ST toxin was determined by applying it onto a gas-phase sequencer (model 470A; Applied Biosystems, Inc.) coupled to an automated phenylthiohydantoin derivatizer-analyzer (model 4204; Applied Biosystems, Inc.) and a high-pressure liquid chromatographic on-line system (120A; Applied Biosystems, Inc.).

Protein concentration. The protein contents of purified toxins and their Hc and Lc components and immunoglobulins were determined by the method of Lowry et al. (6). The amount of protein in the toxin preparation just before treatment with formaldehyde was expressed as the concentration of protein in the toxoid preparation.

RESULTS

Establishment of hybridomas against type E toxin. BALB/c mice were immunized with type E toxoid without adjuvant. Fusion experiments were performed four times by using polyethylene glycol 1540 from Wako Pure Chemical Co. The fusion rate was about 10 to 60%, and 88 hybridomas were obtained. Of the 88 hybridomas, only 17 produced antibody against type E toxin, and 5 of these 17 showed toxin-neutralizing activity in mice. All of these 17 antibodies

TABLE 1. Characterization of monoclonal antibodies against type E toxin

Antibody	Reactive component	Toxin neutralization (μg/ml)[a]	ELISA results for the following toxin[b]:										
			Botulinum									Tetanus	PET
			A	B	C_1-a	C_1-b	C_2-I	C_2-II	D	E	F		
LE15-5	Hc	0.1	−	−	−	−	−	−	−	+	−	−	−
LE34-6	Hc	1.0	−	−	−	−	−	−	−	+	−	−	−
EK18-2	Hc		−	−	−	−	−	−	−	+	−	−	−
EK19-7	Hc	20	−	−	−	−	−	−	−	+	−	−	−
EK21-4	Hc	0.4	−	−	−	−	−	−	−	+	−	−	−
AE27-9	Hc	10	−	−	−	−	−	−	−	+	−	−	−
EL161-38	Lc		−	+	+	+	−	−	+	+	−	+	−
EL211-3	Lc		−	−	−	−	−	−	−	+	−	−	−
EL219-15	Lc		−	−	−	−	−	−	−	+	−	−	−

[a] Minimum amount of IgG needed to neutralize 10 LD_{50}s per ml of toxin.
[b] Abbreviations: C_1-a, C_1 toxin purified from C-ST: C_1-b, C_1 toxin purified from C-6813; C_2-I, component I of C_2 toxin; C_2-II, component II of C_2 toxin; PET, *C. perfringens* enterotoxin.

reacted with the Hc component. Therefore, to obtain antibodies that reacted with the Lc component, mice were immunized with an adjuvant-Lc component preparation. Fusion experiments were performed two times with polyethylene glycol 1500 (Boehringer GmbH, Mannheim, Federal Republic of Germany) instead of polyethylene glycol 1540. The fusion rate was increased to about 90%, and 512 hybridomas were formed. Of these 512 hybridomas, 306 were positive in ELISA with the Lc component of type E toxin. None of the 306 hybridomas, however, had toxin-neutralizing activity. Six hybridomas (five toxin-neutralizing [LE15-5, LE34-6, EK19-7, EK21-4, and AE27-9] and one nonneutralizing [EK18-2]) and three hybridomas (EL161-38, EL211-3, and EL219-5), which reacted strongly with Hc and Lc portions in ELISA, respectively, were selected and cloned by the limiting dilution method. The cloned cells were injected into BALB/c mice, and ascites fluids were obtained. Immunoglobulins were purified from the ascites fluids that were thus obtained.

Characterization of monoclonal antibodies. The subclasses of the nine antibodies were determined by the double immunodiffusion test. All the antibodies formed precipitin lines only with the sera against IgG1 and the κ light chain. Five antibodies that reacted with the Hc component neutralized the type E toxin. The minimum amount of IgG needed to neutralize 10 LD_{50}s of toxin per ml was determined (Table 1). LE15-5 and EK21-4 showed much higher neutralizing activities than did the other three antibodies. In the titration curves with ELISA, three antibodies (EL161-38, EL211-3, and EL219-15) which reacted with the Lc component showed higher optical density values than the six antibodies that reacted with the Hc component. However, these three antibodies possessed no toxin-neutralizing activity in mice.

The reactions of the nine antibodies with different types of toxins were examined by both the neutralization test and ELISA. None of the antibodies neutralized the toxin except type E toxin; but one antibody (EL161-38) strongly reacted with botulinum type C_1 and D and tetanus toxins, as well as type E toxin, in ELISA (Fig. 1). The reaction of this antibody with botulinum type B toxin was weak; and there was no reaction with type A, C_2, or F toxin or *C. perfringens* enterotoxin.

Dot blot and Western blot analyses. The reaction of EL161-38 with different types of toxins was confirmed by dot blot and Western blot analyses. In the dot blot analysis (Fig. 2), EL161-38 reacted with botulinum type B, C_1, D, and E and tetanus toxins; but it did not react with botulinum type A, C_2, or F toxin or *C. perfringens* enterotoxin. This was the same result obtained by ELISA. As controls, EK18-2, which reacts with the Hc component of type E toxin; N-CDA5-6, which commonly reacts with Lc components both C_1 and D toxins (9); rabbit antisera against components I and II of C_2 toxin (12); and immunoglobulin against *C. perfringens* enterotoxin (18) were used. Dot blot analysis was also performed with the purified Hc and Lc components of type C-ST toxin (Fig. 3). EL161-38 reacted with the Lc component, but not with the Hc component.

In the Western blot analysis, EL161-38 reacted only with the Lc components of botulinum C-ST, C-6813, D-1873, and E-Iwanai and tetanus toxins (Fig. 4). As a control, N-CDA14-6, which reacted with the Hc component of both C_1 and D toxins, was used (9).

Comparison of partial amino acid sequences of botulinum and tetanus toxins. The partial amino acid sequences of the N terminus of the Lc component of botulinum C_1 (C-ST) toxin was determined and compared with those of botulinum type A, B, and E and tetanus toxins reported previously (2, 14).

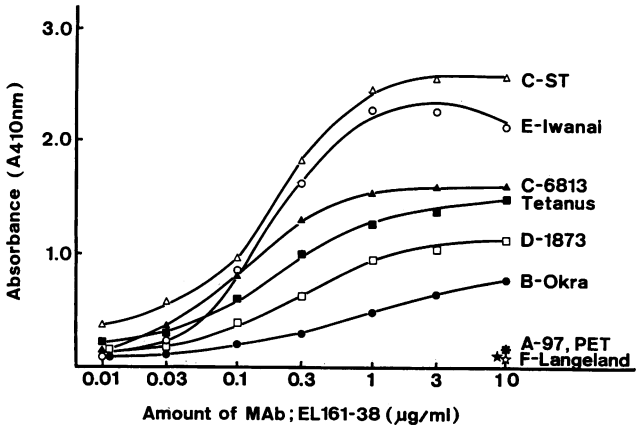

FIG. 1. Titration curves obtained by ELISA. A portion (250 ng) of different types of purified toxins was coated onto a well of a microtiter plate, and ELISA was performed with different amounts of monoclonal antibody EL161-38. In the case of botulinum type A and F toxins and *C. perfringens* enterotoxin (PET), the reaction was negative, even though 2.5 μg of toxin was used. Symbols: △, C-ST; ○, E-Iwanai; ▲, C-6813; ■, tetanus; □, D-1873; ●, B-Okra; ✱, A-97; ★, *C. perfringens* enterotoxin; ☆, F-Langeland.

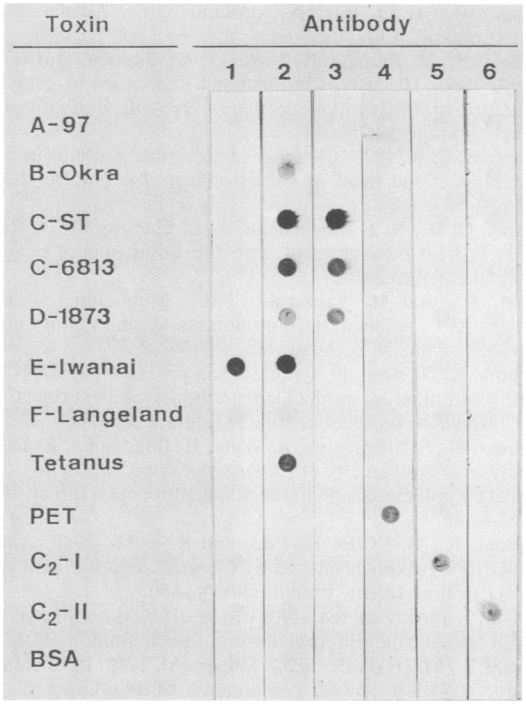

FIG. 2. Dot blot analysis with different monoclonal antibodies and toxins. Eleven toxin types were blotted onto a nitrocellulose membrane and reacted with six different antibodies. Lanes: 1, EK18-2; 2, EL161-38; 3, N-CDA5-6; 4, anti-*C. perfringens* enterotoxin (PET); 5, anti-component I of C_2 toxin; 6, anti-component II of C_2 toxin. BSA, Bovine serum albumin.

High homology was observed among the Lc components of these toxins (Table 2).

DISCUSSION

BALB/c mice were immunized with type E toxoid or with a mixture of its Lc portion and an adjuvant. When type E toxoid was used, 17 monoclonal antibodies that reacted with the Hc component, but no antibody against the Lc component, were obtained. Of these 17 monoclonal antibodies, 5 showed toxin-neutralizing activity in mice. When the mixture of the Lc component and adjuvant was used as the immunogen, 306 antibodies that reacted with the Lc component were obtained. However, 0 of 306 antibodies possessed toxin-neutralizing activity.

One antibody (EL161-38) reacted strongly with the Lc component of botulinum type C_1 and D and tetanus toxins,

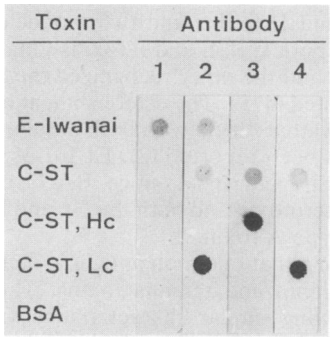

FIG. 3. Dot blot analysis with monoclonal antibodies and type E and C_1 toxins. Type E and C_1 toxins and the Hc and Lc components of C_1 toxin were blotted onto a nitrocellulose membrane and reacted with EK18-2 (lane 1), EL161-38 (lane 2), N-CDA14-6 (lane 3), and N-CDA5-6 (lane 4) antibodies, BSA, Bovine serum albumin.

as well as type E toxin, and weakly with type B toxin (but not with type A toxin or *C. perfringens* enterotoxin). This indicates that these toxins share a common antigenic determinant on their Lc components. Monoclonal antibodies that commonly react with the Hc and Lc components of botuli-

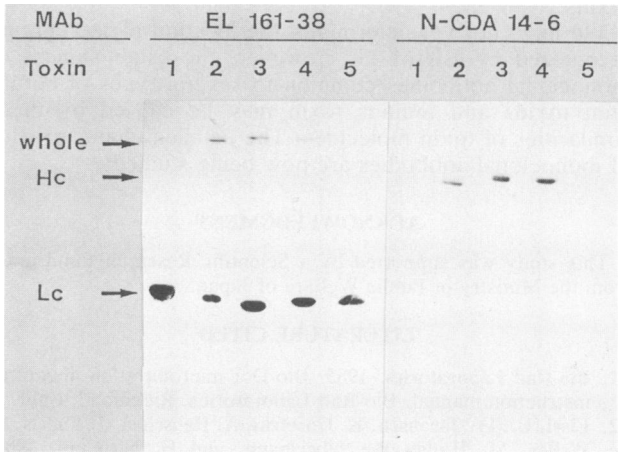

FIG. 4. Western blot analysis with EL161-38 and the different toxins. Botulinum type E and tetanus toxins were treated with trypsin. Thereafter, each toxin preparation was heated at 100°C for 5 min in the presence of 2-ME and sodium dodecyl sulfate and loaded onto sodium dodecyl sulfate-polyacrylamide gels, and then the Western blot analysis was performed with EL161-38 and N-CDA14-6 monoclonal antibodies (MAb). The toxins employed were tetanus (lanes 1), D-1873 (lanes 2), C-6813 (lanes 3), C-ST (lanes 4), and E-Iwanai (lanes 5).

TABLE 2. Partial amino acid sequences of the N termini of botulinum and tetanus toxins

Lc	Amino acid sequence																								
	1				5					10				15				20							25
Type A[a]	**Pro**	Phe	Val	Asn	Lys	Gln	**Phe**	**Asn**	**Tyr**	Lys	**Asp**	**Pro**	**Val**	**Asn**	Gly	Val	Asp								
Type B[a]	**Pro**	Val	Thr	**Ile**	**Asn**	Asn	**Phe**	**Asn**	**Tyr**	**Asn**	**Asp**	**Pro**	Ile	Asp	Asn	Asn									
Type C[b]		Ile	Thr	**Ile**	**Asn**	Asn	**Phe**	**Asn**	**Tyr**	Ser	**Asp**	**Pro**	**Val**	Asp	Asn	Lys	Asn	**Ile**	**Leu**	**Tyr**	Leu	Asp	Thr	His	Leu
Type E[a]	**Pro**		Lys	**Ile**	**Asn**	Ser	**Phe**	**Asn**	**Tyr**	**Asn**	**Asp**	**Pro**	**Val**	**Asn**	Asp	Arg	**Thr**	**Ile**	**Leu**	**Tyr**	Ile				
Tetananus[c]	**Pro**	Ile	Thr	**Ile**	**Asn**	Asn	**Phe**	Arg	**Tyr**	Ser	**Asp**	**Pro**	**Val**	**Asn**	Asp	**Tyr**	**Ile**	Ile	Met	Met	Glu	Pro	Pro	Tyr	

[a] Data have been published previously by Sathyamoorthy and DasGupta (14). For type E toxin, a space was added between residues 1 and 2 (Pro and Lys), to facilitate comparison of the homology with the other sequences.

[b] Residue 1 could not be determined because the phenylthiohydantoin analysis for cycle 1 gave several peaks in addition to proline. Ile is considered residue 2.

[c] Data have been published previously by Eisel et al. (2).

num type C_1 and D toxins and two monoclonal antibodies that react with both type E and F toxins (one recognized the Hc component and the other recognized the Lc component) have been isolated (4, 9). The establishment of a monoclonal antibody (BA-1) that reacts with both botulinum and tetanus toxins has also been reported (2). EL161-38, however, was clearly different from BA-1 since BA-1 reacted with *C. perfringens* enterotoxin and both the Hc and Lc components of botulinum type A toxin.

It is interesting that common antigenic determinants exist between botulinum and tetanus toxins. These two toxins have the following similar characteristics. (i) Both are extremely powerful neurotoxins which inhibit the release of transmitters in synapses, and (ii) both consist of a single polypeptide chain with an M_r of about 150,000, and separate into Hc (M_r 100,000) and Lc (M_r 50,000) components by reducing a dilsulfide bond after mild trypsinization. It has been reported that a high level of homology exists in the N-terminal amino acid sequences of botulinum type A, B, and E and tetanus toxins (2, 14). The sequence of botulinum type C_1 toxin has not yet been determined. Therefore, we purified the Lc component of type C_1 toxin and clarified it. Type C_1 toxin also possesses a high level of homology with the toxins mentioned above, although the homogeneity to type A toxin seems to be slightly lower than that to the other toxins.

We have not yet determined the location of the epitope recognized by EL161-38. However, the establishment of monoclonal antibodies common to several types of botulinum toxins and tetanus toxin may be caused by these similarities of toxin molecules. The detailed characteristics of monoclonal antibodies are now being studied.

ACKNOWLEDGMENT

This study was supported by a Scientific Research Fund grant from the Ministry of Public Welfare of Japan.

LITERATURE CITED

1. **Bio-Rad Laboratories.** 1985. Bio-Dot microfiltration apparatus instruction manual. Bio-Rad Laboratories, Richmond, Calif.
2. **Eisel, U., W. Jarausch, K. Goretzki, A. Henschen, J. Engels, U. Weller, M. Hudel, E. Habermann, and H. Niemann.** 1986. Tetanus toxin: primary structures, expression in *E. coli*, and homology with botulinum toxins. EMBO J. **5**:2495–2502.
3. **Hambleton, P., C. C. Shone, P. Wilton-Smith, and J. Melling.** 1984. A possible common antigen on clostridial toxins detected by monoclonal anti-botulinum neurotoxin antibodies, p. 449–450. *In* J. E. Alouf, F. J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, Inc. (London), Ltd., London.
4. **Kozaki, S., Y. Kamata, T. Nagai, J. Ogasawara, and G. Sakaguchi.** 1986. The use of monoclonal antibodies to analyze the structure of *Clostridium botulinum* type E derivative toxin. Infect. Immun. **52**:786–791.
5. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Nishi, S., and H. Yamazaki.** 1983. Monoclonal antibodies against AFP: application in immunoassay and affinity chromatography. Ann. N.Y. Acad. Sci. **417**:452–458.
8. **Oguma, K., T. Agui, B. Syuto, K. Kimura, H. Iida, and S. Kubo.** 1982. Four different monoclonal antibodies against type C_1 toxin of *Clostridium botulinum*. Infect. Immun. **38**:14–20.
9. **Oguma, K., S. Murayama, B. Syuto, H. Iida, and S. Kubo.** 1984. Analysis of antigenicity of *Clostridium botulinum* type C_1 and D toxins by polyclonal and monoclonal antibodies. Infect. Immun. **43**:584–588.
10. **Oguma, K., B. Syuto, H. Iida, and S. Kubo.** 1980. Antigenic similarity of toxins produced by *Clostridium botulinum* types C and D strains. Infect. Immun. **30**:656–660.
11. **Ohishi, I.** 1984. Oral toxicities of *Clostridium botulinum* type A and B toxins from different strains. Infect. Immun. **43**:487–490.
12. **Ohishi, I., M. Iwasaki, and G. Sakaguchi.** 1980. Purification and characterization of two components of botulinum C_2 toxin. Infect. Immun. **30**:668–673.
13. **Ohishi, I., and G. Sakaguchi.** 1974. Purification of *Clostridium botulinum* type F progenitor toxin. Appl. Microbiol. **28**:923–928.
14. **Sathyamoorthy, V., and B. R. DasGupta.** 1985. Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B, and E. J. Biol. Chem. **260**:10461–10466.
15. **Syuto, B., and S. Kubo.** 1977. Isolation and molecular size of *Clostridium botulinum* type C toxin. Appl. Environ. Microbiol. **33**:400–405.
16. **Syuto, B., and S. Kubo.** 1981. Separation and characterization of heavy and light chains from *Clostridium botulinum* type C toxin and their reconstitution. J. Biol. Chem. **256**:3712–3717.
17. **Terajima, J., B. Syuto, J. O. Ochanda, and S. Kubo.** 1985. Purification and characterization of neurotoxin produced by *Clostridium botulinum* type C6813. Infect. Immun. **48**:312–317.
18. **Yamamoto, K., I. Ohishi, and G. Sakaguchi.** 1979. Fluid accumulation in mouse ligated intestine inoculated with *Clostridium perfringens* enterotoxin. Appl. Environ. Microbiol. **37**:181–186.
19. **Yokosawa, N., K. Tsuzuki, B. Syuto, and K. Oguma.** 1986. Activation of *Clostridium botulinum* type E toxin purified by two different procedures. J. Gen. Microbiol. **132**:1981–1988.