INFECTION AND IMMUNITY, Feb. 1984, p. 584–588
0019-9567/84/020584-05$02.00/0

Vol. 43, No. 2

Analysis of Antigenicity of *Clostridium botulinum* Type C_1 and D Toxins by Polyclonal and Monoclonal Antibodies

KEIJI OGUMA,[1]* SATORU MURAYAMA,[2] BUNEI SYUTO,[2] HIROO IIDA,[3] AND SHUICHIRO KUBO[2]

Department of Microbiology, Sapporo Medical College,[1] and Department of Biochemistry, Faculty of Veterinary Medicine,[2] and Department of Bacteriology, School of Medicine,[3] Hokkaido University, Sapporo, Hokkaido 060, Japan

Received 13 July 1983/Accepted 18 October 1983

Clostridium botulinum type C_1 toxin was purified from C-Stockholm (C-ST), and D toxin was purified from D-1873 and D-South African. Polyclonal antibodies against these toxins were prepared in rabbits. Twenty-eight monoclonal antibodies to these toxins were also prepared with BALB/c myeloma cells. The antibodies were analyzed by both enzyme-linked immunosorbent assay (ELISA) and a toxin neutralization test. ELISA was performed with the three purified toxins and heavy-chain (Hc) and light-chain (Lc) components derived from C-ST and D-1873 toxins. A neutralization test was carried out with 11 toxin preparations (7 from type C and 4 from type D cultures). ELISA results indicated that there exists at least one common antigenic determinant on each of the Hc and Lc components of the three purified toxins. The results of the neutralization test also indicated that type C_1 and D toxin preparations contain several common antigenic sites in their molecules. Some are common to toxins from several specific cultures, whereas others are common to toxins from a large number of cultures. It was speculated that toxins from two type C strains are composed of Hc and Lc components which are somewhat similar to those of D-1873 and C-ST toxins, respectively.

The classification of type C and D strains of *Clostridium botulinum* seems to be slightly confusing because they produce three different types of toxins, C_1, C_2, and D. Type C_2 toxin has exhibited characteristics different from those of typical botulinum neurotoxins (9, 10), and the antigenicity of these three types of toxins has been reported to be different (4). Cross-neutralizations with antisera raised with detoxified culture fluids were interpreted to indicate that a single culture produces C_1, C_2, and D toxins in different amounts (1, 2).

The use of pure toxins isolated from C-Stockholm (C-ST), D-1873, and D-South African (D-SA) and the corresponding antisera demonstrated that C_1 and D toxins have common antigenic parts, and these common parts cause cross-neutralization (7, 8). We also demonstrated that the antigenicities of D-1873 and D-SA toxins were not identical. Recently, four different monoclonal antibodies to C-ST toxins were obtained (5). Three of the antibodies reacted with only C-ST toxin, but one reacted with both C-ST and D-SA toxins on enzyme-linked immunosorbent assay (ELISA). This communication reports the preparation of many different monoclonal antibodies and their reactions in ELISA and toxin neutralization tests. These studies clarify the nature of the antigenic structures of C_1 and D toxins.

MATERIALS AND METHODS

Bacterial strains and myeloma cells. *C. botulinum* type C strains (ST, 468, 203, D6F, CB19, 6812, and 6813) and type D strains (1873, CB16, SA, and 4947) were used. The origin of these cultures has been reported previously (6, 8). Cultures were incubated and maintained in cooked meat medium (Difco Laboratories, Detroit, Mich.). For the formation of hybridomas, secreting type (P3X63-Ag8) and nonsecreting type (P3X63-Ag8-653) BALB/c myeloma cell lines were kindly provided by T. Togashi (School of Medicine, Hokkaido University, Hokkaido, Japan) and maintained in Dulbecco modified Eagle medium from GIBCO Laboratories, Grand Island, N.Y., supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and streptomycin.

Chemicals. CNBr-activated Sepharose 4B and quaternary aminoethyl Sephadex A-50 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Hypoxanthine, aminopterin, thymidine, and alkaline phosphatase were from Sigma Chemical. Co., St. Louis, Mo. Polyethylene glycol 1540 and *p*-nitrophenyl phosphate were from Wako Pure Chemical Co., Osaka, Japan.

Purification of toxins and their components. The C_1 toxin (C-ST) and D toxins (D-1873 and D-SA) were purified as reported previously (7, 11). Purified C-ST and D-1873 toxins, but not D-SA toxin, were dissociated into heavy-chain (Hc) and light-chain (Lc) components with reducing agents. Large amounts of Hc and Lc components were obtained from C-ST intact toxin by using a quaternary aminoethyl Sephadex A-50 column as reported previously (8, 12). The Hc and Lc components of D-1873 toxin could also be separated by the same procedure. A detailed description of the characteristics of purified D-1873 toxin and its components will be published in the near future by one of us.

Preparation of polyclonal sera. Rabbit antisera against C-ST, D-1873, and D-SA toxins were those previously prepared (7, 8). Immunoglobulins specific for C-ST and D-1873 toxins were obtained by affinity chromatography with C-ST and D-1873 toxin columns (5, 7).

Immunoglobulins reacting with the Hc and Lc components of these two toxins were also obtained by affinity chromatography. These components (3 mg) were separately conjugated with 2 g of CNBr-activated Sepharose 4B and poured into chromatographic columns (1.0 by 12 cm). Anti-C-ST serum (3 ml) was applied to separate columns with C-ST-Hc and Lc ligands, and anti-D-1873 serum was applied to columns with the ligand of D-1873 components. Specific immunoglobulins were eluted with 3 M KSCN (10 ml/h; 4°C) and then dialyzed against 0.01 M phosphate-buffered saline (pH 7.2).

Formation of hybridoma cell lines. Hybridomas with se-

* Corresponding author.

creting and nonsecreting types of mouse myeloma cells were produced as reported previously (5), with the exception that polyethylene glycol 1540 was used instead of polyethylene glycol 1000 as a fusion agent. BALB/c mice were immunized with a total of 100 μg of toxoids (prepared by dialyzing purified toxins against 0.01 M phosphate-buffered saline [pH 8.0] containing 0.4% Formalin for 7 days at 30°C). To obtain antibodies that would recognize the antigenic determinants common to D-1873 and D-SA toxins, mice were basally immunized by an intraperitoneal injection of D-1873 toxoid (50 μg), followed by a booster of D-SA toxoid (50 μg) intravenously. To procure the antibodies that would recognize the determinants common to the three toxins, mice were given a booster injection with the mixture of D-SA and C-ST toxoids (25 μg each). Myeloma cells of secreting type were first used as fusion cells because this cell line yielded a much higher fusion rate than did the nonsecreting cell line. However, after the latter cells were cloned by limiting dilution and an actively growing clone was selected, this cell line yielded a fusion rate similar to the former one; hybrid cells appeared in 5 to 40% of the wells of 96-well microplates. The hybridomas formed with both cell types are listed here. The cells producing antibody were expanded, recloned by the limiting dilution method, and injected into mice intraperitoneally to obtain ascites fluid containing high-titer antibodies. The fusion experiment was carried out 10 times, and a total of 28 cell lines were established.

The established cell lines were designated with the capital letters C, D, and/or A before their cell line numbers, indicating a positive reaction in the ELISA to C-ST, D-1873, and/or D-SA toxins, respectively.

Cell lines formed with mouse myeloma cells of nonsecreting type were marked with the letter N.

ELISA. Antibody-producing hybridomas were screened by ELISA. Reactions between the ascites fluids and the three purified toxins and the Hc and Lc components of C-ST and D-1873 toxins were also observed. The ELISA procedure used has been described previously (5). Toxin or toxin component (600 ng) was bound to each well of the microplates (Nunc; model 239454) and then sequentially mixed with 0.1 ml of diluted samples containing antibodies, alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin G conjugate, and 2.5 mM *p*-nitrophenyl phosphate. The amount of *p*-nitrophenyl phosphate was changed from 0.2 to 0.1 ml. After the reaction was terminated (by the addition of 25 μl of 3 N NaOH), supernatant from each well was diluted 20 times with water, and the absorbance at 420 nm was determined.

Toxin neutralization. Neutralization of C_1 and D toxins from different strains by ascites fluid was studied. The ascites fluid, diluted 1:50 with 0.01 M phosphate-buffered saline (pH 7.2), was mixed with an equal volume of toxin containing 10 times the 50% lethal dose (LD_{50}). Except for the three purified preparations, toxins were supernatants of cooked meat cultures. Cultures diluted with 0.02 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.0)–0.2% gelatin containing 100 LD_{50}/ml were divided into small portions and stored at −80°C. Before use, they were diluted 10 times with the same buffer, mixed with antitoxin, and incubated at 37°C for 2 h. Two white mice (DDY; 25 to 30 g) were injected intraperitoneally with 0.5 ml of the incubated mixture and observed for 6 days.

Protein concentration. The amount of protein in the immunoglobulin preparations, purified toxins, Hc and Lc components, and toxoids was determined by the method of Lowry et al. (3). Concentrations of purified C-ST toxin were also estimated spectrophotometrically by absorbance at 278 nm. The $E^{1\%}_{1\,cm}$ for C-ST was 14.18 (11).

RESULTS

Toxin neutralization by polyclonal antibodies. The minimum amount of immunoglobulin preparation against C-ST and Hc and Lc components derived from it needed to neutralize 10 LD_{50} of C-ST toxin per ml was about 0.5 μg/ml. The neutralization of D-1873 toxin by anti-D-1873-Hc and anti-D-1873-Lc sera required a similar amount. Therefore, the neutralization test was performed by mixing 10 μg of immunoglobulin preparation per ml with an equal volume of 10 LD_{50} of toxin solution per ml. Since immunoglobulin was not prepared from anti-D-SA serum, it was used after a $1:10^3$ dilution since the titer of this serum to neutralize D-SA toxin was about $1:10^4$. Toxins from strains C-6812 and C-6813 were neutralized by both anti-C-ST and anti-D-1873 sera, as well as by the antisera to C-ST-Lc and D-1873-Hc components (Table 1). Anti-C-ST-Hc and anti-D-1873-Lc sera did not neutralize these toxins, although 100 μg of immunoglobulin preparation per ml was used.

ELISA with monoclonal antibodies. Ascites fluids were diluted 1:100 and used in an ELISA against the three purified toxins and the Hc and Lc components of C-ST and D-1873 toxins. Antibodies could be grouped by their ELISA spectra into those reacting (i) with C-ST and D-SA but not with D-1873, (ii) with D-1873 and D-SA but not with C-ST, (iii) with all three toxins, or (iv) only with one of the three toxins. The components recognized by antibodies were easily deciphered in all cases except N-C-45, N-C-109, and N-DA-20, where weak reactions made determinations difficult (Table 2 and Fig. 1a and b). The part of D-SA toxin reacting with N-A-70 and N-A-145 was not determined since the necessary Hc and Lc components were not available (Table 2).

Six antibodies, CDA-69, N-CDA-14, N-CDA-175, CDA-30, N-CDA-5, and N-CDA-135, reacted with the three toxins. The first three gave similar titration curves with the three toxins and reacted with the Hc component. The last three antibodies reacted with the Lc component but demonstrated different reaction curves in the ELISA. The reaction of CDA-30 with C-ST toxin and the reactions of N-CDA-5 and N-CDA-135 with D-SA toxin were weak relative to their reactions with other toxins (Fig. 1c and d and Table 2).

Toxin neutralization by monoclonal antibodies. Of the 28 antibodies, 17 neutralized toxin (Table 2). Two of the five antibodies reacting with only C-ST toxin in the ELISA neutralized type C_1 toxin from many strains, with the exception of C-6812 and C-6813. Two antibodies reacting

TABLE 1. Neutralization test with polyclonal antibodies[a]

Antibody	Toxin neutralization										
	Type C							Type D			
	ST	468	203	D6F	CB19	6812	6813	1873	CB16	SA	4947
Anti-C-ST	+	+	+	+	+	+	+	−	−	−	−
Anti-C-ST-Hc	+	+	+	+	+	−	−	−	−	−	−
Anti-C-ST-Lc	+	+	+	+	+	+	+	−	−	−	−
Anti-D-1873	−	−	−	−	−	+	+	+	+	+	+
Anti-D-1873-Hc	−	−	−	−	−	+	+	+	+	+	+
Anti-D-1873-Lc	−	−	−	−	−	−	−	+	+	+	+
Anti-D-SA	−	−	−	−	−	−	−	+	+	+	+

[a] 10 μg of immunoglobulin G preparation and anti-D-SA toxin serum diluted 10^3 were mixed with an equal volume of 10 LD_{50} of toxin per ml, incubated at 37°C for 2 h, and then injected into mice intraperitoneally.

TABLE 2. Characterization of monoclonal antibodies by ELISA and neutralization test[a]

Antibody	ELISA				Neutralization										
	Toxin			Reactive component	Type C							Type D			
	C-ST	D-1873	D-SA		ST	468	203	D6F	CB19	6812	6813	1873	CB16	SA	4947
C-9	+	−	−	Hc	−	−	−	−	−	−	−	−	−	−	−
C-14	+	−	−	Hc	−	−	−	−	−	−	−	−	−	−	−
C-17	+	−	−	Hc	+	+	+	+	+	−	−	−	−	−	−
N-C-45	+	−	−	?[b]	−	−	−	−	−	−	−	−	−	−	−
N-C-109	+	−	−	?	+	+	+	+	+	−	−	−	−	−	−
D-26	−	+	−	Hc	−	−	−	−	−	−	−	−	−	−	−
D-34	−	+	−	Hc	−	−	−	−	−	−	−	−	−	−	−
N-D-16	−	+	−	Hc	−	−	−	−	−	−	−	−	−	−	−
D-162	−	+	−	Hc	−	−	−	−	−	−	−	+	−	−	−
D-210	−	+	−	Hc	−	−	−	−	−	−	−	+	−	−	−
D-28	−	+	−	Hc	−	−	−	−	−	+	+	+	+	−	−
N-D-1	−	+	−	Hc	−	−	−	−	−	+	+	+	+	−	−
N-D-2	−	+	−	Hc	−	−	−	−	−	+	+	+	+	−	−
N-A-70	−	−	+	NT[c]	−	−	−	−	−	−	−	−	−	+	+
N-A-145	−	−	+	NT	−	−	−	−	−	−	−	−	−	+	+
CA-12	+	−	+	Hc	+	+	+	+	+	−	−	−	−	+	+
N-CA-137	+	−	+	Hc	+	+	+	+	+	−	−	−	−	+	+
N-CA-11	+	−	+	Hc	±	±	±	±	±	−	−	−	−	+	+
N-CA-27	+	−	+	Hc	±	±	±	±	±	−	−	−	−	+	+
N-DA-20	−	+	+	?	−	−	−	−	−	−	−	−	−	−	−
N-DA-75	−	+	+	Lc	−	−	−	−	−	−	−	+	+	+	+
N-DA-122	−	+	+	Lc	−	−	−	−	−	−	−	+	+	+	+
CDA-69	+	+	+	Hc	±[d]	±	±	±	±	±	±	±	±	±	±
N-CDA-14	+	+	+	Hc	±	±	±	±	±	±	±	±	±	±	±
N-CDA-175	+	+	+	Hc	−	−	−	−	−	−	−	−	−	−	−
CDA-30	(+)[e]	+	+	Lc	−	−	−	−	−	−	−	−	−	−	−
N-CDA-5	+	+	(+)	Lc	−	−	−	−	−	−	−	−	−	−	−
N-CDA-135	+	+	(+)	Lc	−	−	−	−	−	−	−	−	−	−	−

[a] The ELISA test was performed with 1:100 diluted ascites fluid and 600 ng each of three purified toxins and Hc and Lc components of C-ST and D-1873 toxins. The neutralization test was done by mixing 1:50 diluted fluid with toxin.
[b] ?, Not decided.
[c] NT, Not tested.
[d] ±, Neutralized by ascites fluids diluted less than 20 times.
[e] (+), Weak reaction on ELISA.

with D-SA toxin in the ELISA neutralized D-SA and D-4947 toxins. The antibodies reacting with D-1873 toxin in the ELISA were divided into two groups, those neutralizing only D-1873 toxin and others neutralizing D-1873, D-CB16, C-6812, and C-6813 toxins.

The antibodies CA-12, N-CA-137, N-CA-11, and N-CA-27, which gave similar curves with C-ST and D-SA toxins in the ELISA, neutralized type C_1 toxin preparations, with the exception of C-6812 and C-6813, and type D toxin of D-SA and D-4947. However, the neutralization titer of these antibodies was different. CA-12 and N-CA-137 neutralized type C_1 and D toxins to a similar extent, but N-CA-11 and N-CA-27 neutralized type D toxin more than C_1 toxin (Fig. 1e and f and Tables 2 and 3). There may exist more than one common antigenic determinant between these toxin molecules.

Of six antibody preparations reacting with the three toxins in the ELISA, CDA-69 and N-CDA-14 neutralized all of the toxins examined, although their titers were low. They required the use of 1:20 dilutions of ascites fluid (Table 2).

The above-mentioned antibodies, with the exception of N-A-70 and N-A-145, recognized some antigenic parts on the Hc portion. The antibodies reacting with the Lc portion and possessing neutralizing activity were N-DA-75 and N-DA-122. These antibodies neutralized type D toxin from four different cultures (Tables 2 and 3). Antibody N-DA-20 also reacted with both D-1873 and D-SA toxins in the ELISA. This antibody, however, demonstrated a weak reaction to both components of D-1873 toxin and neutralized none of the toxins (Tables 2 and 3 and Fig. 1b).

DISCUSSION

Monoclonal antibodies were used in toxin neutralization tests and an ELISA to investigate the antigenicity and structure of type C_1 and D toxins. Neutralization tests were also performed with polyclonal antibodies.

Type C_1 toxin from all cultures except C-6812 and C-6813 demonstrated the same neutralization patterns to the antibodies. The molecular construction or the antigenic sites of these toxins might be identical. In contrast, reactions of the four type D toxin preparations were different. D-1873 and D-CB16 toxins belong to one group, whereas D-SA and D-4947 toxins belong to a second group. Even the antigenicity of D-1873 and D-CB16 toxins was not identical because two monoclonal antibodies neutralized only D-1873 toxin. D-SA and D-4947 toxins also possessed their particular antigenic

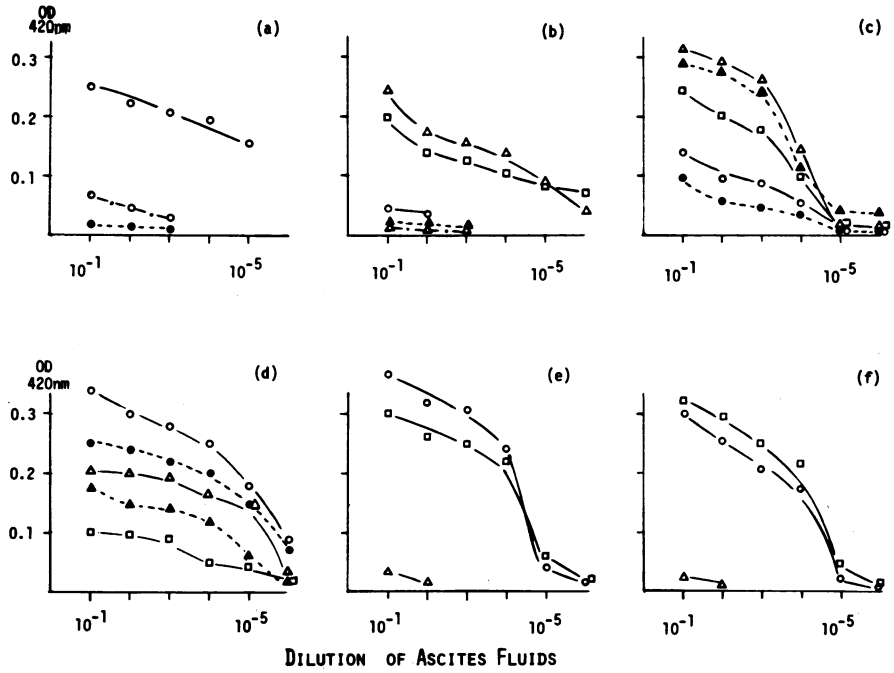

FIG. 1. Titration curves of ascites fluid to toxin and Hc and Lc components by ELISA. Serial 10-fold diluted ascites fluid, N-C-109 (a), N-DA-20 (b), CDA-30 (c), N-CDA-5 (d), N-CA-137 (e), and N-CA-11 (f), were reacted with each of three toxins and the Hc and Lc components of C-ST and D-1873 toxins. After alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G and the substrate were allowed to react, the yellow color of *p*-nitrophenol released was assayed. Symbols: mixed with ○——○, C-ST toxin; ○—·—○, C-ST-Hc component; ●-----●, C-ST-Lc component; △——△, D-1873 toxin; △—·—△, D-1873-Hc component; ▲-----▲, D-1873-Lc component; □——□, D-SA toxin. OD, Optical density.

determinant(s) recognized by two monoclonal antibodies. In previous studies with polyclonal antibodies, the serum fractions which neutralized both C-ST and D-SA toxins and formed a fused precipitin line with these toxins and the C-ST-Hc portion, and the fractions which neutralized type D toxins and formed a fused precipitin line with D-SA and D-1873 toxins were obtained (7). Here, we isolated four monoclonal antibodies which reacted with the C-ST-Hc portion in an ELISA and neutralized D toxin from D-SA and D-4947 and C_1 toxin from type C cultures except C-6812 and C-6813. Furthermore, two monoclonal antibodies which reacted with the D-1873-Lc portion and neutralized four type D toxin preparations were obtained. Therefore, we presumed that D-SA and D-4947 toxins contain on their Lc portion the majority of antigenic determinants which are common to type D toxin preparations and on the Hc portion the determinants found in the type C_1 toxin preparations except C-6812 and C-6813.

The antigenicity of C-6812 and C-6813 toxins was also complex. These toxins were neutralized by monoclonal antibodies which reacted with the D-1873-Hc portion in the ELISA and neutralized both D-1873 and D-CB16 toxins. Also from the results of the neutralization test with polyclonal antibodies, it was speculated that C-6812 and C-6813 toxins have Hc and Lc portions, part of which are similar to those of D-1873 and C-ST toxins, respectively. We presumed that such "mosaic" toxins may be formed by the recombination of phage genes controlling type C_1 and D toxin production (6).

Six monoclonal antibodies reacted with all three purified toxins in the ELISA. Three of six antibodies reacted with the Hc portion, whereas the others reacted with the Lc portion. Among the first three, two neutralized the toxicity of many type C_1 and D toxin preparations, although their titers were low. Among the latter three antibodies, one reacted better with D-1873 and D-SA toxins than with C-ST toxin, whereas two others reacted better with C-ST and D-1873 toxins than with D-SA toxin. These results suggest that more than one of the antigenic determinants common to these three toxins exist on each of the Hc and Lc portions of intact toxins. We are now trying the competitive ELISA analysis to determine whether these monoclonal antibodies are reacting with the same or different antigenic determinants.

From the results mentioned above, we concluded that there exist many common antigenic determinants between type C_1 and D toxins, and some of them participate in toxin neutralization. The distribution of these common antigenic determinants was different among toxin preparations. This

TABLE 3. Neutralization test of monoclonal antibodies[a]

Antibody	Titer[b]			
	C-ST	C-6812	D-1873	D-SA
CA-12	2,000	<20	<20	2,000
N-CA-11	20[c]	<20	<20	200
N-CA-27	20[c]	<20	<20	200
N-CA-137	200	<20	<20	200
N-DA-75	<20	<20	200	2,000
N-DA-20	<20	<20	<20	<20
N-DA-122	<20	<20	200	200
D-28	<20	2,000	2,000	<20
N-D-1	<20	200	200	<20

[a] Ascites fluid was diluted in serial 10-fold steps and then mixed with an equal volume of 10 LD_{50} of toxin per ml.

[b] Reciprocals of the final highest dilutions of ascites fluid which neutralized toxin.

[c] Ascites fluid diluted 40 times did not neutralize toxin.

causes the varying rate of (cross)neutralization between C_1 and D toxins and their antisera and causes confusion in the classification of cultures.

The reactions of some monoclonal antibodies to Hc and Lc components in the ELISA were very weak, although they showed strong positive reactions to intact toxins. This phenomenon can be explained by the hypothesis that these antibodies recognize either the three-dimensional configuration of intact toxins or the part(s) whose antigenicity is diminished during the process of separation of each component.

All monoclonal antibodies except N-DA-75 and N-DA-122 (and N-A-70 and N-A-145), which neutralized toxicity, reacted with the Hc portion in the ELISA. When polyclonal antibodies were prepared in rabbits by injecting purified C-ST toxoid and Hc and Lc components, the neutralizing activity of anti-Lc serum was very low compared with other sera (8). However, the anti-Lc serum obtained from whole anti-C-ST serum by affinity chromatography neutralized toxicity to the same degree as the original anti-C-ST or similarly prepared anti-Hc serum. The immunogenicity of the Lc component responsible for toxin-neutralizing activity might be low or might decrease when it is separated from the intact toxin.

The function of the Hc and Lc components is still not clear. We are presently investigating the matter, as well as studying the mechanism of neutralization by antibodies.

ACKNOWLEDGMENTS

We are grateful to R. Iwaki, Sapporo Medical College, Hokkaido, Japan, for critical reading of the manuscript. We also thank Toshi Abe and Tsuyako Hara for their excellent assistance.

This study was supported by Scientific Research Fund grants 58122001 and 58570192 from the Ministry of Education of Japan.

LITERATURE CITED

1. **Bulatova, T. I., K. I. Matveev, and V. S. Samsonova.** 1967. Biological characteristics of *Clostridium botulinum* type C strains isolated from minks in the USSR, p. 391–399. *In* M. Ingram and T. A. Roberts (ed.), Botulism 1966. Chapman and Hall, London.
2. **Jansen, B. C.** 1971. The toxic antigenic factors produced by *Clostridium botulinum* type C and D. Onderstepoort. J. Vet. Res. **38**:93–98.
3. **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.
4. **Miyasaki, S., M. Iwasaki, and G. Sakaguchi.** 1977. *Clostridium botulinum* type D toxin; purification, molecular structure and some immunological properties. Infect. Immun. **17**:395–401.
5. **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.
6. **Oguma, K., H. Iida, M. Shiozaki, and K. Inoue.** 1976. Antigenicity of converting phages obtained from *Clostridium botulinum* type C and D. Infect. Immun. **13**:855–860.
7. **Oguma, K., B. Syuto, T. Agui, H. Iida, and S. Kubo.** 1981. Homogeneity and heterogeneity of toxins produced by *Clostridium botulinum* type C and D strain. Infect. Immun. **34**:382–388.
8. **Oguma, K., B. Syuto, H. Iida, and S. Kubo.** 1980. Antigenic similarity of toxins produced by *Clostridium botulinum* type C and D strain. Infect. Immun. **30**:656–660.
9. **Ohishi, I.** 1983. Lethal and vascular permeability activities of botulinum C_2 toxin induced by separate injections of the two toxin components. Infect. Immun. **40**:336–339.
10. **Ohishi, I., M. Iwasaki, and G. Sakaguchi.** 1980. Purification and characterization of two components of botulinum C_2 toxin. Infect. Immun. **30**:668–673.
11. **Syuto, B., and S. Kubo.** 1977. Isolation and molecular size of *Clostridium botulinum* type C toxin. Appl. Environ. Microbiol. **33**:400–405.
12. **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. **266**:3712–3717.