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Four Different Monoclonal Antibodies Against Type C_1 Toxin of *Clostridium botulinum*

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Monoclonal antibodies against type C_1 toxin produced by *Clostridium botulinum* type C strain Stockholm (C-ST) were prepared by fusion of BALB/c myeloma cells P3X63-Ag8, with spleen cells from the mice immunized by C-ST toxoid. About 5% of single-cell colonies in wells were found to produce antibodies against the toxin as determined by an enzyme-linked immunosorbent assay (ELISA). Four different hybridoma cell lines, no. 9, 12, 14, and 17, were established, cloned by limiting dilution, and intraperitoneally injected into mice to obtain the ascites fluids containing high-titered antibodies. The reactions of these antibodies to type C_1 and D toxins of strains C-ST, D-1873, and D-South African (D-SA) were observed by both neutralization and ELISA tests. Three monoclonal antibodies, no. 9, 14, and 17, reacted with C-ST toxin, but only no. 17 highly neutralized the toxin. These antibodies did not react with type D toxins. On the contrary, no. 12 reacted with toxins of both C-ST and D-SA (but not of D-1873) and commonly neutralized these two toxins. This indicates that there is a common antigenic part between C-ST and D-SA toxin molecules which participates in the toxin-neutralizing reaction. The neutralization profiles of C-ST toxin by no. 12 and 17 antibodies were different in a time-to-death test of mice. The mechanisms of neutralization by no. 12 and 17 may be different.

Type C and D strains of *Clostridium botulinum* have been reported to produce three different types of toxins, C_1, C_2, and D, in the proper medium. These conclusions were based on the cross-neutralization between each toxin type and antisera raised with detoxified culture fluids of type C and D. Recently, we purified C_1 toxin from strain C-Stockholm (C-ST) and D toxins from strains D-South African (D-SA) and D-1873 and prepared antisera against these three toxins in rabbits (3, 4). From the results of toxin neutralization and agar gel diffusion tests, the following conclusions were obtained. (i) C-ST, D-SA, and D-1873 toxins have common antigenic parts in their toxin molecules. (ii) C-ST and D-SA toxins have additional antigenic common parts. (iii) Antigenicities of D-SA and D-1873 toxins are not all identical. (iv) Cross-neutralization is due to the common parts mentioned above.

To verify the above conclusions, we prepared monoclonal antibodies against C-ST toxin. The antibodies reacting with only C-ST toxin and the antibody reacting with both C-ST and D-SA toxins have been obtained. Formation and some characteristics of these antibodies are described in this paper.

MATERIALS AND METHODS

Bacterial strains and cells. *C. botulinum* type C strain C-ST and type D strains D-1873 and D-SA were used. The origin of these cultures was reported previously (2). BALB/c myeloma cell line P3X63-Ag8 was obtained from T. Togashi, School of Medicine, Hokkaido University, Hokkaido, Japan, and had been originally supplied by the Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden.

Chemicals and media. Bacterial strains were incubated in the specific medium for toxin production, and type C_1 and D toxins were purified as reported previously (3, 8). Myeloma cells were maintained in Dulbecco-modified Eagle (DME) medium purchased from GIBCO Laboratories, Grand Island, N.Y. and supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 1% tylosin (GIBCO), and streptomycin. Before the fusion experiment, myeloma cells were incubated in DME medium with 20% FCS (DME-20). Hypoxanthine, aminopterin, thymidine, alkaline phosphatase, and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, Mo. Polyethylene glycol 1000 and *p*-nitrophenyl phosphate were from Wako Pure Chemical Co., Osaka, Japan. Ampholine was from LKB-Produkter AB, Bromma, Sweden, and CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Formation of hybridoma cell lines. Purified C-ST toxin (300 μg/ml) was made into toxoid by dialyzing

against 0.01 M phosphate-buffered saline (PBS), pH 8.0, containing Formalin at 0.4% for 1 week at room temperature. Unless otherwise stated, 0.01 M PBS at pH 7.2 was used throughout the experiments. This preparation was then filtered through a membrane filter (pore size, 450 nm), diluted twice with sterilized PBS, and kept at 4°C until use. BALB/c mice were immunized by the following schedule. Three basal immunizations of 0.1, 0.2, and 0.2 ml were done through the intraperitoneal route at 2-week intervals. After more than 2 weeks, a booster injection of 0.1 ml was given through the intravenous route. The mice were sacrificed on day 3, and the spleen cells were obtained. Erythrocytes were lysed by incubation for 10 min at 4°C in 0.83% NH_4Cl. The cells were washed three times with DME medium without FCS (DME-0) and resuspended in 10 ml of the same medium (total of 10^8 cells). Actively growing myeloma cells were also washed three times with DME-0 medium and resuspended in 10 ml of DME-0 (total of 10^7 cells). These two cells were mixed in a plastic tube (Falcon, model 2070F) and sedimented together into a pellet by centrifugation. The pellet was gently overlaid with 0.5 ml of 50% polyethylene glycol 1000 in DME-0 and centrifuged (9). Hereafter, polyethylene glycol was diluted by dropwise addition of 25 ml of DME-0 for 15 min, centrifuged, suspended in 25 ml of DME-20, and incubated in a tissue culture flask (Nunc, model 163371) at 37°C overnight. The cells were collected by centrifugation and suspended in 30 ml of DME-20 containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT-20). All of the centrifugations were done at $1,000 \times g$ for 5 min at 25°C. Three drops (total of 3.0×10^5 spleen cells) were distributed by Pasteur pipette to individual wells of 96-well microplates (Linbro, model 76-003-05). One drop of HAT-20 medium was added to each well every 2 days. When the well became full, one-third of the medium was aspirated off. At between 10 and 20 days, colonies were visible macroscopically. Antibody production was checked by an enzyme-linked immunosorbent assay (ELISA), and the selected cells were expanded into 24-well tissue culture plates (Linbro, model 76-033-05) and then into 15-ml flasks (Nunc, model 153732).

Cloning by limiting dilution. The hybridoma cell lines producing antibodies were cloned by the method of limiting dilution. The cells were diluted and plated as to 36 wells of a 96-well plate with an average of 5 cells per well, 36 wells with 1 cell per well, and the remaining 24 wells with 0.5 cell per well. The spleen cells of BALB/c mice were used as carrier cells in diluting the hybrid cells and also as feeder cells in culture. Six antibody-producing clones were selected, expanded, and assayed again for their antibody production by ELISA. The best one was stored frozen at −80°C as a stock culture. Cells (more than 10^7) were also collected by low-speed centrifugation and washed once with PBS, and each 0.3 ml of cell suspension in the same buffer was intraperitoneally injected into BALB/c mice to obtain ascites fluids.

ELISA. Hybridomas producing antibody to C-ST toxin were screened by ELISA. Enzyme-linked rabbit antibody against mouse immunoglobulin G (IgG) was prepared as follows. Mouse IgG (1 mg/ml), which had been purified by affinity chromatography with a protein A column, was mixed with an equal volume of Freund incomplete adjuvant. Two 2-ml subcutaneous injections of IgG-adjuvant emulsion and one 2-ml intravenous injection of IgG were done in rabbits at 3-week intervals. The serum was harvested 5 days after the last injection, and the immunoglobulin fraction reacting with mouse IgG was obtained by application on a column of Sepharose 4B conjugated with mouse IgG. The rabbit immunoglobulin thus obtained (0.5 mg) and alkaline phosphatase (ALPase, 800 U) were resolved in 0.5 ml of PBS. A 4-μl amount of 25% glutaraldehyde was added and mixed gently at room temperature for 2 h and then overnight at 4°C. The enzyme-conjugated immunoglobulin was dialyzed against 0.1 M Tris-hydrochloride buffer (pH 7.2), diluted 10 times with PBS containing 50% glycerol, 1% BSA, and 1 mM $MgCl_2$, and stored at −20°C. Before use, the immunoglobulin was diluted 1:100 by PBS with BSA (1%) and Tween 20 (0.01%). ELISA was done with microtiter plates (Nunc, model 239454) as described by Voller et al. (10). A 30-μl amount of C-ST toxin (20 μg/ml) and 60 μl of 0.01 M carbonate-bicarbonate buffer (coating buffer), pH 9.6, were successively poured in each well and kept in a refrigerator overnight. After being washed four times with PBS-BSA-Tween 20, each 100-μl amount of culture supernatants of hybridomas was transferred, incubated at 37°C for 2 h, and washed again four times; then 100 μl of enzyme-labeled rabbit anti-mouse IgG conjugate was added. The plate was allowed to incubate at 37°C for 2 h, washed, and mixed with 200 μl of 2.5 mM *p*-nitrophenyl phosphate dissolved in 0.01 M carbonate-bicarbonate buffer (pH 9.8) with 1 mM $MgCl_2$. After incubation at 37°C for 1 h, the reaction was terminated by addition of 25 μl of 3 N NaOH. The supernatant of each well was diluted 10 times with distilled water, and the absorbance at 420 nm was determined spectrophotometrically.

Neutralization test. Neutralizing activity of monoclonal antibodies was examined by injecting the mixture of toxin and antibody into three white mice (DDY, 25 to 27 g) through both intravenous and intraperitoneal routes. Antibody diluted in serial tenfold or twofold steps with PBS was mixed with an equal volume of toxin and incubated at 37°C for 2 h. For intraperitoneal injection, 10 times the 50% lethal dose (LD_{50}) of toxin per ml was used, and each 0.5 ml of the mixture was injected. The highest dilution of the antibody which neutralized the toxin was obtained. For intravenous injection, 8×10^4 or 2×10^4 LD_{50} of toxin per ml was mixed with an equal volume of diluted antibodies and incubated at 37°C for 2 h; then 0.1-ml amounts of the mixtures were injected into each of three mice, and the average time to death was calculated (1).

IF. Gel isoelectric focusing (IF) of monoclonal antibodies was performed by the method of Righetti and Drysdale with some modification (6). Each antibody (50 μg of protein) was mixed in the solution containing 5% acrylamide mixture (the volume ratio of acrylamide and bisacrylamide was 20:1), 2% ampholine (pH 3.5 to 10), 0.0005% riboflavin, 0.056% *N,N,N,N'*-tetramethylethylenediamine, and 0.01% ammonium persulfate. The mixture was poured in glass tubes (0.5 by 11 cm), and gels of 10-cm length were made by exposing them to UV light at room temperature. Solutions of 0.02 M H_3PO_4 and 1 M NaOH were used as electric fluids of anode and cathode, respectively. IF was performed in a cold room with constant voltage

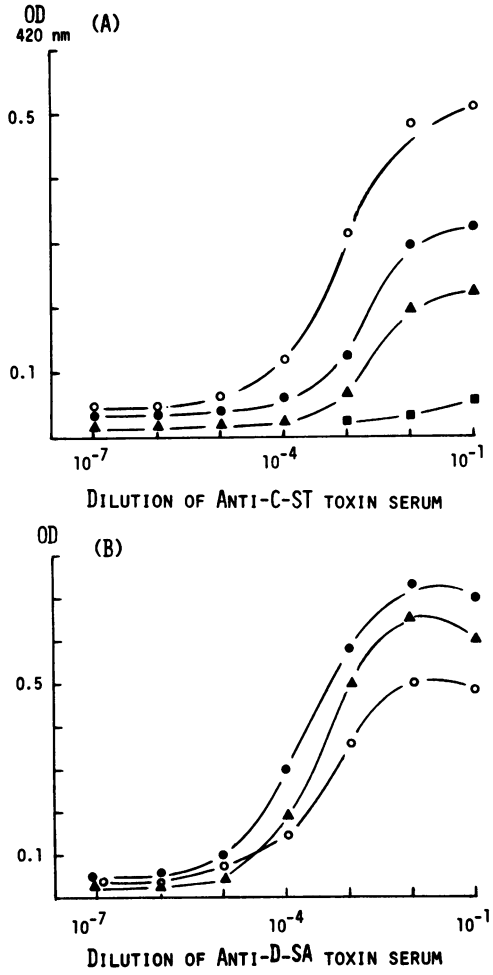

FIG. 1. Titration curves of mouse anti-toxin sera to different toxins by ELISA. Mouse anti-C-ST toxin (A) and anti-D-SA toxin (B) sera were diluted in serial tenfold steps and reacted with each of three toxins which had been prebound to the plastic plate. After ALPase-conjugated rabbit anti-mouse IgG and the substrate were allowed to react, the yellow color of *p*-nitrophenol released was assayed. Symbols: ○, mixed with C-ST toxin; ●, mixed with D-SA toxin; ▲, mixed with D-1873 toxin; ■, normal mouse serum mixed with C-ST toxin. OD, Optical density.

of 200 V for 12 h. For the first and the last 1-h periods, however, 100 and 300 V were used, respectively. The gels were stained with Coomassie brilliant blue G-250 in 3.5% perchloric acid by the procedure of Reisner et al. (5).

Other immunological methods. IgG which reacted with toxin specifically was purified from ascites fluids by using affinity chromatography of a column of C-ST toxin. An agar gel double-diffusion test was also done to characterize the antibodies. Details of these procedures were reported previously (3, 4).

RESULTS

Preparation of antitoxin sera. BALB/c mice were immunized by toxoid of C-ST and D-SA as described above. Antisera obtained were examined for their reactivity to C-ST, D-SA, and D-1873 toxins by both neutralization and ELISA tests (Fig. 1; see also Table 3). Anti-C-ST and anti-D-SA toxin sera cross-neutralized type D and C_1 toxins, respectively. Cross-reaction was also observed by ELISA. Data indicated that the parts common to C-ST and D-SA toxins are larger than the parts common to C-ST and D-1873 toxins.

Formation of hybridoma cell lines. The spleen cells were obtained from the mice immunized by C-ST toxoid and fused with myeloma cells (P3X63-Ag8). The fusion rate was 5 to 40%, and about 5% of single colonies in wells were found to produce antibody against toxin. Nonsecretor P3X63-Ag8-653 cells were also examined. However, only a few hybrid cells were obtained, and none of them produced antibody. Therefore, P3X63-Ag8 was used, although this cell line spontaneously produces mouse IgG1. The wells in which more than one colony had grown were discarded. During expansion, some of hybrid

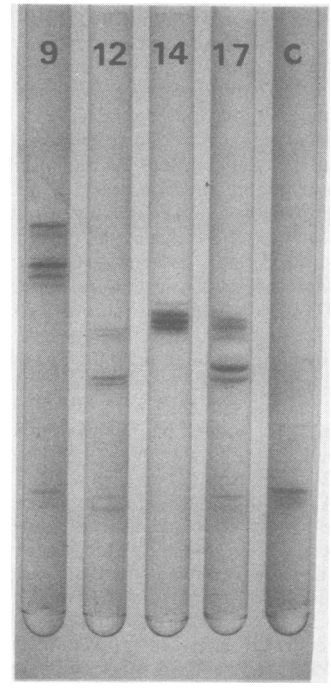

FIG. 2. IF of monoclonal antibodies. IgG was obtained from four ascites fluids (no. 9, 12, 14, and 17), and IF was performed as described in the text. As a control, the preparation that eluted from the same column which had been subjected to ascites fluid containing no antibody against C-ST toxin was migrated (C).

TABLE 1. ELISA with monoclonal antibodies and toxins[a]

Dilution of ascites fluids	Absorbance of *p*-nitrophenol for cell line:											
	no. 9			no. 12			no. 14			no. 17		
	C-ST	D-SA	D-1873	C-ST	D-SA	D-1873	C-ST	D-SA	D-1873	C-ST	D-SA	D-1873
10^{-1}	0.52	0.03	0.03	0.54	0.50	0.04	0.48	0.03	0.02	0.50	0.04	0.02
10^{-2}	0.68	—[b]	—	0.66	0.61	—	0.63	—	—	0.71	—	—
10^{-3}	0.58	—	—	0.62	0.58	—	0.60	—	—	0.66	—	—
10^{-4}	0.23	—	—	0.21	0.18	—	0.29	—	—	0.25	—	—
10^{-5}	0.11	—	—	0.08	0.07	—	0.10	—	—	0.12	—	—

[a] ALPase-conjugated rabbit anti-mouse IgG and the substrate were made to react with the mixtures of each toxin and serial 10-fold-diluted ascites fluids, and the absorbance was determined.
[b] Less than 0.1.

cells died probably because we did not use the feeder layer at this time. Finally, four different cell lines, no. 9, 12, 14, and 17, were established. These cell lines were cloned by limiting dilution and were intraperitoneally injected into mice. Ascites fluids thus obtained were characterized as follows.

Characterization of monoclonal antibodies. (i) IF. IF was carried out with IgG purified from ascites fluids by affinity chromatography of a C-ST toxin-Sepharose 4B column. It was expected that each IgG would demonstrate one or few strong lines in the limited region. All of the cell lines except no. 14, however, showed several strong lines in a rather wide region (Fig. 2). Cells of no. 12 and 17 were again cloned by limiting dilution, and six single clones selected from each cell line were assayed for their antibody production by ELISA. All clones showed positive reactions. One out of six clones was expanded, ascites fluids were obtained, and the IF test was again performed. Both cell lines demonstrated patterns similar to those shown in Fig. 2. The lines that appeared near the bottom of the gels were not due to immunoglobulin because the preparation that eluted from a C-ST toxin column, which had been layered by ascites fluid containing no specific antibody against toxin, also demonstrated the lines in the same region. These lines may have been formed by a substance which nonspecifically bound to the column.

In the double-gel immunodiffusion test, none of the four antibodies formed a precipitin line with C-ST toxin.

(ii) ELISA. The reactions of ascites fluids to three toxins, C-ST, D-SA, and D-1873, and heavy-chain and light-chain components of C-ST toxin were observed (Tables 1 and 2). There was no antibody reacting with D-1873 toxin. Cell line no. 12, however, reacted with both C-ST and D-SA toxins to a similar extent. The other three antibodies showed positive reaction only to C-ST toxin. All four preparations reacted with heavy-chain component of C-ST toxin.

Then the subclass of the hybridoma antibodies was determined by Ouchterlony immunodiffusion test with rabbit IgG against different mouse immunoglobulin subclasses (IgG1, IgG2a, IgG2b, IgG3, and IgM) and κ and λ light chains which were purchased from Miles Laboratories, Inc., Elkhart, Ind. All four antibodies formed precipitin lines only with the sera against IgG1 and κ light chain.

(iii) Neutralization test. Four ascites fluids that had similar titers to C-ST toxin by ELISA were serially diluted in twofold steps and mixed with equal volumes of 10-LD_{50}/ml concentrations of

TABLE 2. ELISA with monoclonal antibodies and heavy-chain and light-chain components of C-ST toxin[a]

Antibody	Dilution of ascites fluids	Absorbance of *p*-nitrophenol	
		Heavy chain	Light chain
No. 9	10^{-1}	0.33	0.04
	10^{-2}	0.25	—[b]
	10^{-3}	0.18	—
No. 12	10^{-1}	0.41	0.06
	10^{-2}	0.38	—
	10^{-3}	0.35	—
No. 14	10^{-1}	0.39	0.05
	10^{-2}	0.28	—
	10^{-3}	0.22	—
No. 17	10^{-1}	0.33	—
	10^{-2}	0.28	—
	10^{-3}	0.22	—
Anti-C-ST[c]	10^{-1}	0.47	0.29
	10^{-2}	0.27	0.15
	10^{-3}	0.22	0.04

[a] The 20-μg/ml concentrations of heavy-chain and light-chain components previously obtained (7) were used.
[b] Less than 0.1.
[c] Mouse anti-C-ST toxin serum.

TABLE 3. Neutralization test with monoclonal antibodies and toxins[a]

Antibody	Titer[b]		
	C-ST	D-SA	D-1873
Expt 1			
Anti-C-ST	1,600	80	16
Anti-D-SA	160	6,400	1,600
No. 9	8	<4	<4
No. 12	400	400	<4
No. 14	4	<4	<4
No. 17	1,600	<4	<4
Expt 2			
Anti-C-ST	160		
No. 12	20		
No. 17	40		

[a] Mixtures of 10-LD_{50}/ml concentrations of toxins with diluted ascites fluids (experiment 1) or with 130 μg of IgG per ml (experiment 2) were intraperitoneally injected into mice.

[b] Reciprocals of the final highest dilutions of sera to neutralize toxins.

toxins C-ST, D-SA, and D-1873. After incubation, 0.5-ml amounts of the mixtures were intraperitoneally injected into each of three mice (Table 3, experiment 1). Cell line no. 12 neutralized both C-ST and D-SA toxins to a similar extent. Cell line no. 17 neutralized only C-ST toxin, and no. 9 and 14 showed little neutralizing activity to any toxin; only less than 1:10-diluted sera could neutralize C-ST toxin. Neutralizing activity of no. 17 to C-ST toxin seemed to be greater than that of no. 12. Therefore, they were layered on a C-ST toxin column, and IgG was obtained. Neutralizing tests were performed with the same amount (130 μg/ml) of IgG (Table 3, experiment 2). Cell line no. 17 showed slightly higher activity than did no. 12. The difference of their neutralizing activity became clear by employing the time-to-death test of mice (1). The standard curve of time to death by C-ST toxin was first obtained (Fig. 3). Ascites fluids and IgG were mixed with final concentrations of 4×10^4 and 1×10^4 LD_{50} of toxin per ml, respectively. The neutralizing activity of no. 17 was much higher than no. 12 when a large amount of antibody was used. Cell line no. 17, however, showed an "all or none" effect on toxin neutralization; the activity rapidly decreased and became lower than that of no. 12 when the amount of antibody was below the critical point (Table 4). The effects of no. 9 and 14 were very little, but the survival time became slightly longer by mixing with each 1:10-diluted ascites fluid.

DISCUSSION

Monoclonal antibodies were prepared to investigate the structure and function of type C_1 and D toxins. Four different monoclonal anti-

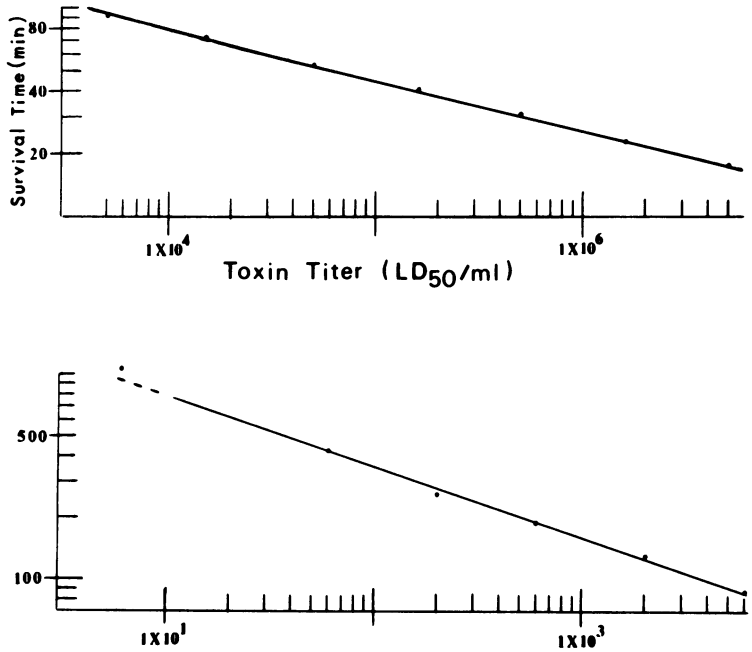

FIG. 3. Relation of log of concentration of C-ST toxin to mean survival time of mice. Each 0.1-ml amount of diluted C-ST toxin was intravenously injected into six mice, and mean survival time was calculated. Log of survival time and concentration of toxin were plotted.

TABLE 4. Time-to-death test with monoclonal antibodies and C-ST toxin[a]

Dilution of antibody	Cell line no. 12			Cell line no. 17		
	Survival time (min)	Remaining toxin (LD_{50}/ml)	Neutralized toxin (%)	Survival time (min)	Remaining toxin (LD_{50}/ml)	Neutralized toxin (%)
Expt 1 (fold)						
100	112	3.0×10^3	93	310	1.5×10^2	>99
200	98	4.0×10^3	90	256	2.4×10^2	>99
400	85	7.0×10^3	83	78	9.0×10^3	77
800	75	1.1×10^4	73	70	1.5×10^4	64
Control[b]	55	4.0×10^4				
Expt 2 (μg)						
60	145	2.0×10^3	80	270	2.2×10^2	98
30	120	2.4×10^3	76	240	3.2×10^2	97
15	106	3.3×10^3	67	86	6.5×10^3	35
7.5	87	6.5×10^3	35	80	8.5×10^3	15
Control[b]	77	1.0×10^4				

[a] Diluted ascites fluids (experiment 1) and IgG (experiment 2) were mixed with equal volumes of 8×10^4 and 2×10^4 LD_{50} of toxins per ml, respectively. Each 0.1-ml amount was intravenously injected into three mice, and average survival time was calculated. The remaining toxin was estimated by the standard curve.

[b] Toxin alone.

bodies were obtained. Two of them, no. 9 and 14, were cloned once, and the other two, no. 12 and 17, which possessed the neutralizing activity of toxin, were cloned twice by the limiting dilution method. All of these cell lines except no. 14 showed several distinctly separated strong lines on IF. However, we are sure that these cell lines are "monoclones" because: (i) no. 12 and 17 were cloned twice as mentioned above, and their IF patterns were similar before and after the cloning; and (ii) all of single clones that appeared in the limiting dilution showed positive antibody production by ELISA.

The class of four hybridoma antibodies was IgG1 with κ light chain. Since the rabbit immunoglobulin used for screening the hybridoma was obtained by immunization with mouse IgG which had been purified by a protein A column, any hybridomas producing IgM or IgA antibodies would have been missed.

In the Ouchterlony test with C-ST toxin, whole mouse antitoxin serum formed a precipitin line, but four monoclonal antibodies did not. This indicates that there is no (little) repetition of these antigens in the C-ST toxin molecule or that all of these antibodies are univalent. A line was not formed by the mixture of four antibodies. Detailed experiments are necessary to clarify this result.

Cell line no. 12 neutralized both C-ST and D-SA toxins to a similar extent. Therefore, we concluded that C-ST and D-SA toxins have at least one common part in their molecules and that this part caused the cross-reaction observed between C-ST and D-SA toxins and their antisera.

Both no. 12 and 17 highly neutralized C-ST toxin, but their neutralizing behavior in the time-to-death test of mice was different. In our preliminary experiment observing the blocking activity of antibodies for toxin to bind to the synaptosomes, no. 12 inhibited the binding, but no. 17 did not. The different neutralizing profile may be due to the different mechanism of toxin neutralization. The IF patterns of no. 9 and 14 were different, but their reactions to toxin were similar. There is a possibility that they recognize the same part of the toxin.

Detailed characteristics of these antibodies are now being investigated. We are also trying to form monoclonal antibodies against D-1873 toxin and to get three different antibodies: one reacting with only D-1873 toxin, one reacting with both D-1873 and D-SA toxins, and one reacting with C-ST, D-1873, and D-SA toxins.

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