Binding and Cytotoxic Effects of *Clostridium botulinum* Type A, C₁ and E Toxins in Primary Neuron Cultures from Foetal Mouse Brains

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Binding of purified *Clostridium botulinum* type A, C₁ and E toxins to cultured cells was studied by an immunocytochemical method. Type A and C₁ toxins bound strongly to neuron cultures prepared from brains of foetal mice, but binding of type E toxin was weak. None of the toxin types bound to the feeder layer, composed of non-neuronal cells. The heavy-chain component of the type C₁ toxin bound to neurons, but the light chain component did not. Type C₁ toxin also bound only to cell lines of neuronal origin. When type C₁ toxin [final concentration 4×10^2 LD₅₀ (10 ng) per well] was added to primary neuron cultures in 96-well plates, degeneration of neuronal processes and rounding of neuronal somas were observed, but type A and E toxins did not produce such changes. The binding and cytotoxic activities of type C₁ toxin were blocked by heat treatment (80 °C for 30 min) or by preincubation of the toxin with polyclonal anti-C₁ IgG and some of the monoclonal antibodies which neutralized the toxin activity in mice. In the neuronal processes treated with C₁ toxin, many degenerated mitochondria, membranous dense bodies and vesicles were observed by electron microscopy; these ultrastructural changes were similar to those of Wallerian degeneration *in vivo*.

INTRODUCTION

Clostridium botulinum strains are classified into seven types (A through G) based on the antigenicity of toxins produced. Although the toxins differ antigenically, their pharmacological action seems to be the same: they inhibit the release of acetylcholine from cholinergic nerve endings (Dickson & Shevky, 1923; Burgen *et al.*, 1949; Thesleff, 1960). However, the mechanism of action of the toxin is still not clear. In order to analyse it, synaptosomes and primary neuron culture have been used (Habermann, 1974; Habermann & Heller, 1975; Kitamura, 1976; Bigalke *et al.*, 1978; Agui *et al.*, 1983, 1985). Wonnacot & Marchbanks (1976) studied the activity of crude type A toxin in synaptosomes prepared from brains of guinea-pigs. They found that toxin did not reduce the formation of acetylcholine, but depressed its release. Bigalke *et al.* (1978) first examined the effect of the toxin (crystalline type A toxin, *M_r* 900 000) on primary nerve cell cultures derived from the central nervous system of rat embryo. The toxin inhibited both formation and release of acetylcholine, but no cell damage was observed by phase-contrast microscopy.

All toxin types except G have been purified (Sugiyama, 1980). In culture fluids (or in an acid solution), the neurotoxins are associated with nontoxic proteins which possess or lack haemagglutinating activity and exist as large-sized units of *M_r* 300 000 to 900 000. These

Abbreviations: FCS, foetal calf serum; Hc heavy-chain component; Lc, light-chain component.

complexes are dissociated into neurotoxins and nontoxic proteins in an alkaline solution (or in the small intestine), and only the neurotoxins attach to the cholinergic nerve terminals *in vivo*. All neurotoxin types have a similar size (M_r about 150000), and are composed of two subunits designated as heavy-chain component (Hc or fragment I) and light-chain component (Lc or fragment II). Evidence indicating that the Hc component is responsible for binding of the toxin to receptors have been obtained (Kozaki, 1979; Agui *et al.*, 1983).

We previously purified type C₁ neurotoxin and its Hc and Lc components, and prepared polyclonal and monoclonal antibodies against them (Syuto & Kubo, 1977, 1981; Oguma *et al.*, 1981, 1982, 1984). This report describes the binding of purified type C₁ neurotoxin and its subcomponents to primary neuron cultures prepared from the central nervous system of mouse embryos, and its cytotoxic effects in this system. Effects of purified type E neurotoxin and type A progenitor toxin (M_r 300000) on neurons were also studied.

METHODS

Toxin and its subcomponents. The type C₁ neurotoxin (M_r 150000) and its Hc and Lc components were prepared from the culture fluid of *Clostridium botulinum* type C strain Stockholm (C-ST) by the procedure reported previously (Syuto & Kubo, 1977, 1981). The type E neurotoxin (M_r 150000) was also prepared in our laboratory (Yokosawa *et al.*, 1986). Type A toxin was given by I. Ohishi (Veterinary Public Health, College of Agriculture, University of Osaka Prefecture). The M_r of type A toxin is 300000 and it was designated as M-progenitor toxin by Sugii & Sakaguchi (1975). The specific activity of the type C and E toxins employed was 4×10^7 50% lethal dose (LD_{50}) mg^{-1} and 4×10^6 LD_{50} mg^{-1} , respectively, and that of type A toxin was 1×10^8 LD_{50} mg^{-1} . These toxin preparations were diluted with 0.1 M-phosphate-buffered saline (PBS, pH 7.2; containing, per litre, 5.2 g NaCl, 0.13 g KCl, 2.784 g $Na_2HPO_4 \cdot 12H_2O$ and 0.2 g KH_2PO_4) and then used for binding and degeneration tests.

Cell lines. The cell lines used are shown in Table 2. NS20Y, N1E-115 and PC12D were of neuronal origin, whereas C6 was derived from a glial cell. They were provided by I. Matsuoka (Faculty of Pharmaceutical Sciences, Hokkaido University, Hokkaido, Japan). HeLa, Vero, L929 and FL were maintained in our laboratory. Li-7, which had been established from a human hepatoma, was from Y. Tsukada (Department of Biochemistry, School of Medicine, Hokkaido University). These cell lines were maintained in Dulbecco's modified Eagle's medium (DME) purchased from Flow Laboratories, which was supplemented with 50 ml foetal calf serum (FCS) from the same supplier, 0.29 g L-glutamine, 3.7 g sodium bicarbonate, and 100 mg each of penicillin G and streptomycin sulphate per litre.

Preparation of primary neurons. The procedure was slightly modified from that reported by Sotelo *et al.* (1980). The primary neuron culture was established in DME-DI, which is DME modified to contain, per litre, 1.5 g $NaHCO_3$, 10 g glucose and 80 units insulin (Sigma, I 5500). Foetuses were obtained from a mouse (ICR-JCL strain) on the 11th day of gestation, and placed in DME-DI without FCS. The cephalic regions of the foetuses were dissected out into 10 ml DME-DI containing 20% FCS, minced with scissors, and then gently dissociated by three passages through an 18-gauge needle fitted to a 20 ml syringe. Thereafter, the needle was replaced successively by gauges 19, 20, 21 and 22, and the syringe was filled and emptied three times through each needle size. The resulting suspension was centrifuged at 300 r.p.m. (18 g) for 3 min to remove larger tissue particles. For the binding experiment, ten drops of the cell suspension thus obtained were added with a Pasteur pipette to each 35 mm well of a six-well plate (Falcon 3001), already containing a sterile 22 mm coverslip (Matsunami, Osaka) and 2.5 ml DME-DI with 20% FCS. The cell population density in each well was approximately 5×10^4 ml^{-1} . To study the effect of toxins on primary neurons (cytotoxicity test), each well of a 96-well microplate (Costar 3596) was filled with 120 μ l of suspension containing 8×10^5 cells ml^{-1} . The time from removal of foetuses to plating was within 30 min. The cells were incubated with 7.5% (v/v) CO_2 . On the 4th day the medium was replaced by DME-DI containing 20% FCS and 10 μ g cytosine arabinoside (Sigma, C-1768) ml^{-1} , and incubation was continued for 48 h. After that, DME-DI with 10% FCS was used, and exchanged every other day. Neurons were observed every day by differential interference contrast microscopy and by haematoxylin-eosin and Bodian's stain.

Antibodies against toxins. Polyclonal rabbit serum against the purified C-ST toxin previously prepared was applied to an affinity column of the C-ST toxin, then immunoglobulin fractions were obtained (Oguma *et al.*, 1981). Polyclonal rabbit anti-E IgG was also prepared in our laboratory (Yokosawa *et al.*, 1986). Polyclonal rabbit anti-A IgG was given by I. Ohishi. In addition, four monoclonal mouse (BALB/c) antibodies (C-9, CA-12, C-14 and C-17) against C-ST toxin were employed (Oguma *et al.*, 1982, 1984). The activity of type C toxin antibodies had been checked by toxin neutralization, enzyme-linked immunosorbent assay (ELISA) and Western dot-blotting tests.

Toxin neutralization, ELISA and Western dot-blotting tests. Toxin neutralization and ELISA tests were done as reported previously (Oguma *et al.*, 1982, 1984). Polyclonal anti-C-ST IgG (10μ g ml^{-1}) and 1 in 100 dilutions of monoclonal ascites fluids were mixed with an equal volume of toxin. After incubation at 37 °C for 60 min, each

mixture was injected into three white mice (DDY, 25–27 g) by both intravenous and intraperitoneal routes. For intraperitoneal injection, $10 \text{ LD}_{50} \text{ ml}^{-1}$ (250 pg ml^{-1}) of toxin was used; 0.5 ml of the mixtures was injected and the mice were observed for 6 d. For intravenous injection, $2 \times 10^5 \text{ LD}_{50} \text{ ml}^{-1}$ ($5 \text{ } \mu\text{g ml}^{-1}$) was used, and 0.1 ml of the mixtures was injected. The average time to death and the percentage of toxin neutralized were calculated.

ELISA was done in 96-well microplates (Nunc; model 239454). Toxin or each component (600 ng) was bound to a well, and then the wells were sequentially exposed to 0.1 ml polyclonal IgG ($10 \text{ } \mu\text{g ml}^{-1}$) or 1 in 100 dilutions of monoclonal ascites fluids, alkaline phosphatase-labelled rabbit anti-mouse IgG conjugate, and 2.5 mM-*p*-nitrophenyl phosphate.

The Western dot-blotting test was done as follows. Toxin or each component ($2 \text{ } \mu\text{g}$) was spotted on a nitrocellulose membrane. The membrane was blocked with 10% (w/v) skim milk and then reacted with 2 ml monoclonal antibodies (1 in 1000 dilutions of ascites fluids in 0.01 M-PBS pH 7.2 containing 3% bovine serum albumin) at 4 °C overnight by gently mixing in a tray. The membrane was washed with 50 mM-Tris/HCl buffer, pH 7.4, containing 0.05% Tween 20 and 0.5 M-NaCl, and then reacted with 2 ml peroxidase-conjugated rabbit anti-mouse IgG in a tray (DAKO) and diluted 1 in 1000 with PBS pH 7.2 containing 10% skim milk. After washing, diaminobenzidine (0.2 mg ml^{-1} in 50 mM-Tris/HCl, pH 7.6) and 0.01% H_2O_2 were added as a substrate.

Characterization of primary neurons. Primary neurons (day 10) grown on coverglasses in 35 mm dishes were fixed with 100% cold acetone ($-20 \text{ }^\circ\text{C}$) for 10 min. The fixed preparations were transferred to a 100 mm plastic dish, in which a wet filter paper had been set. The cells were then treated for 40 min at room temperature with 100 μl 1 in 1000 diluted rabbit anti-68K-neurofilament IgG (Monosan), anti-glial fibrillary acidic protein IgG (DAKO), anti-S-100 ($\beta\beta$) IgG or anti- γ -enolase IgG (Liem *et al.*, 1978; Dahl & Bignami, 1979; Isobe & Okuyama, 1978; Kato *et al.*, 1981). Antibodies against S-100 and γ -enolase were provided by K. Kato, Department of Biochemistry, Institute for Developmental Research, Aichi Prefectural Colony, Japan. After the reaction, the coverglasses were washed three times with PBS, and 100 μl biotinylated goat anti-rabbit IgG ($5 \text{ } \mu\text{g ml}^{-1}$; provided by T. Takami, Department of Pathology, Sapporo Medical College) was added, followed by incubation for 40 min at room temperature. The cells were washed three times with PBS, then reacted with 100 μl FITC-avidin D ($25 \text{ } \mu\text{g ml}^{-1}$) for 40 min at room temperature (Heitzmann & Richards, 1974). After three washes with water, the cells were mounted with 10% (v/v) glycerol and observed by fluorescence microscopy.

Binding of the toxins. Primary neurons and cell lines cultured on coverglasses in 35 mm dishes were separately fixed with freshly prepared 4% (v/v) paraformaldehyde, 100% acetone, 100% methanol, 100% ethanol, or periodate/lysine/paraformaldehyde at 4 °C for 30 min, and washed with PBS. A 100 μl volume of each tenfold diluted C-ST toxin preparation (1×10^3 to $1 \times 10^6 \text{ LD}_{50} \text{ ml}^{-1}$) was reacted with neurons for 20 min at room temperature. The neurons were then washed three times with PBS, and 100 μl antitoxin serum ($10 \text{ } \mu\text{g ml}^{-1}$) was added. After incubation at room temperature for 40 min, the neurons were washed, and then successively reacted with biotinylated goat anti-rabbit IgG and FITC-avidin D as mentioned above. Control experiments were performed in the absence of toxin. The Hc and Lc components (5 and $50 \text{ } \mu\text{g ml}^{-1}$) of C-ST toxin were also employed instead of the toxin.

The extent of binding was evaluated by using an epi-fluorescence microscope (Nikon Fluophoto) with blue excitation (IF 420–490 nm, DM 505, BF 515), and HFM 35 A. Each preparation was photographed with a magnification of 25 on Ektachrome 400 (Kodak). When the fluorescence was clearly observed by an exposure of 15 s, it was evaluated as strongly positive (+ +), and by an exposure of 60 s, positive (+). When an exposure of 240 s showed little fluorescence, it was evaluated as negative (–).

Cytotoxic effect of toxin on primary neurons. The primary neurons in 96-well microplates were used at day 10. To each well was added 4 μl tenfold diluted toxin preparations (1×10^3 to $1 \times 10^6 \text{ LD}_{50} \text{ ml}^{-1}$, corresponding to 100 pg to 100 ng protein per well) and incubated for 5 d with 7.5% CO_2 . The culture was observed every day by differential interference contrast microscopy. Buffer solution (0.1 M-PBS, pH 7.2) only, or heat-treated (80 °C for 30 min) toxin, was used as the control.

Inhibition of binding and cytotoxic activity by polyclonal and monoclonal antibodies. An anti C-ST IgG preparation containing $20 \text{ } \mu\text{g protein ml}^{-1}$, and 1 in 100 dilutions of monoclonal ascites fluids of C-9, CA-12, C-14 and C-17 were used, with a C-ST toxin preparation of $2 \times 10^5 \text{ LD}_{50} \text{ ml}^{-1}$ ($5 \text{ } \mu\text{g ml}^{-1}$). The toxin was mixed with an equal volume of antibodies, incubated at 37 °C for 60 min, and then reacted with neurons. For the binding inhibition test, 50 μl toxin (total 250 ng) was mixed with different antibodies, and 100 μl of each mixture was reacted with paraformaldehyde-fixed neurons on the coverglass, and then observed by the indirect biotin-avidin (FITC) system. For the degeneration inhibition test, 4 μl toxin (total 20 ng) was mixed with antibody, and then 4 μl of each mixture was added to neurons cultured in 96-well microplates.

Electron microscopy. Neurons in the 96-well microplates were fixed with a mixture of 1% (v/v) paraformaldehyde and 3% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer pH 7.4 for 30 min at 4 °C, then they were washed three times with 0.1 M-cacodylate buffer. After postfixation with 1% (w/v) OsO_4 in 0.1 M-phosphate buffer for 30 min, the specimens were dehydrated in an ascending series of ethanol, and finally without the use of acetone, they were directly embedded in Epon 812 and treated at 60 °C for 72 h. Ultrathin sections prepared on an LKB 8800 microtome were stained with uranyl acetate and lead nitrate, then observed in a Hitachi H-300 electron microscope.

RESULTS

Characterization of toxin and monoclonal antibodies

The purity of the C-ST neurotoxin preparation employed was checked by SDS-PAGE (Fig. 1*a*). The reaction of four monoclonal antibodies (C-9, CA-12, C-14 and C-17) with C-ST toxin and its subcomponents was observed by toxin neutralization, ELISA and Western blotting tests. The results of the neutralization and ELISA tests were the same as those reported previously (Oguma *et al.*, 1982); all four antibodies reacted with the Hc component of the toxin, and CA-12 and C-17 possessed neutralizing activity (see Table 3). Western blotting was first performed with preparations treated with SDS and 2-mercaptoethanol. Both CA-12 and C-14 demonstrated one band with the Hc component (Fig. 1*b*), but no band appeared in the case of C-9 and C-17. These results indicated that the epitopes recognized by C-9 and C-17 were destroyed by SDS or heat (100 °C for 2 min) treatment. This was confirmed by both ELISA and dot-blotting tests using preparations with or without heat treatment. Hc and Lc components (100 µg ml⁻¹) were heated at 100 °C for 2 min, and then ELISA was performed as described in Methods. CA-12 and C-14 showed a positive reaction with the heat-treated Hc preparation, but C-9 and C-17 were negative. In dot-blotting tests with non-heat-treated preparations, all four monoclonal antibodies reacted with both whole toxin and its Hc component (Fig. 1*c*).

Establishment and characterization of primary neuron culture

The cephalic regions were obtained from embryonic mice, and dispersed in the medium (see Methods). Two hours after plates had been inoculated with the suspension of mouse brain, neurons began to come together, and form aggregations in many places. Some neuronal processes were found the next day (day 2). By day 4, the coverglass in the plate was fully covered with monolayered cells, on which aggregates of neurons were laid. The cells became morphologically stable on day 8. Some neurons extended processes to other neurons (Fig. 2*a*).

Fig. 1. SDS-PAGE and Western blotting. Purified C-ST toxin was treated with 1% SDS in 0.1 M-sodium phosphate buffer, pH 7.2, at 100 °C for 2 min in the presence (a-ii) or absence (a-i) of 1% 2-mercaptoethanol, and 20 µg of each protein sample was then loaded on an 8% polyacrylamide slab gel containing 0.1% SDS. Electrophoresis was performed at 40 mA. Preparation (a-ii) was transferred to a nitrocellulose membrane, and then sequentially reacted with monoclonal antibodies, peroxidase-conjugated rabbit anti-mouse IgG, and diaminobenzidine in 0.01% H₂O₂. Monoclonal antibodies CA-12 (b-i) and C-14 (b-ii) demonstrated one band with the Hc component. In a dot-blotting test with non-heat-treated preparations (each 2 µg), four monoclonal antibodies, C-9 (c-i), CA-12 (c-ii), C-14 (c-iii) and C-17 (c-iv), reacted with both whole toxin and the Hc component. Monoclonal antibody N-CDA-5 (b-iii, c-v), which reacted with the Lc component, was used as a control (Oguma *et al.*, 1984).

Many of the neuronal somas in the aggregates fluoresced when treated with anti- $\gamma\gamma$ -enolase IgG. However, some of the cells in the aggregates reacted with anti-glial fibrillary protein IgG and anti-S-100 ($\beta\beta$) IgG, indicating that the aggregates contained glial cells as well as neurons. Anti-68K-neurofilament IgG bound only to the processes, indicating that they were components of neurons.

Binding of the toxin and its components

The conditions necessary for binding of C-ST toxin to primary neuron culture were studied. The binding was strongly positive (see Methods) in neuron preparations which were not fixed or fixed with paraformaldehyde, positive in preparations fixed with acetone, methanol or ethanol, but negative in those fixed with periodate/lysine/paraformaldehyde. Since nonfixed neurons easily came off the coverglass during the experiment, paraformaldehyde-fixed neurons were used in the following experiment. A C-ST toxin preparation containing 1×10^4 LD₅₀ ml⁻¹ was needed for the detection of binding on neurons. Immunofluorescence was pale when the cells were incubated with the toxin for 5 min, but bright after 10 min or more of incubation. There was little difference in binding at 4 °C and 25 °C. Therefore binding tests with three types of toxins were performed by incubating the diluted toxin preparations with paraformaldehyde-fixed cells at 25 °C for 20 min. All the toxins bound diffusely to both neuronal aggregates and their processes (Fig. 2*b*), but they did not bind to the background feeder layer. The amount of type E toxin required for detection by fluorescence microscopy was 100 times more than those of type A and C₁ toxin (Table 1). An Hc preparation of C-ST toxin showed positive binding at 5 μ g ml⁻¹, while binding of the Lc component was negative even at 50 μ g ml⁻¹.

Fig. 2. (a) Primary neurons observed by differential interference contrast microscopy on the 10th day of culture. Neuronal aggregates and processes were located on monolayered cells. (b) Immunofluorescence of primary neurons with bound C-ST toxin (10th day of culture). The toxin bound to neuronal processes and somas, but not to monolayered cells. (c) Partially degenerated neurons, 24 h after addition of C-ST toxin (10 ng per well). The neuronal processes had thinned and some swellings were observed. (d) Completely degenerated primary neurons, 48 h after addition of C-ST toxin (10 ng per well). Disappearance of processes and rounding of neuronal somas were observed. Bar, 100 μ m.

Table 1. *Binding to and degeneration of primary neurons by type A, C₁ and E toxins*

Binding and degeneration tests were done with diluted toxin preparations (1×10^3 to 1×10^6 LD₅₀ ml⁻¹). For the binding test, 100 µl of each diluted toxin preparation was reacted with neurons grown on coverglasses in 35 mm dishes; for the degeneration test, 4 µl of each toxin preparation was added to the neuron culture in 96-well microplates. The C₁ and E neurotoxin preparations of 1×10^4 LD₅₀ ml⁻¹ contained 0.25 µg and 2.5 µg protein ml⁻¹, respectively, and that of type A progenitor toxin preparation contained 0.1 µg protein ml⁻¹. The degree of binding was expressed as negative (-), positive (+) or strongly positive (++) (see Methods). The neurons treated with type C₁ toxin for 48 h were completely degenerated (++). Type A and E toxins had little effect on neurons (-).

Amount of toxin (LD ₅₀ ml ⁻¹)	Binding by toxins of type:			Degeneration by toxins of type:		
	A	C ₁	E	A	C ₁	E
1×10^3	-	-	-	-	-	-
1×10^4	+	+	-	-	-	-
1×10^5	+	++	-	-	++	-
1×10^6	++	++	+	-	++	-

Table 2. *Binding of C₁ toxin to neuronal and non-neuronal cell lines*

The degree of binding was assessed as described in Methods: ++, strongly positive; +, positive; -, negative.

Cell line	Binding of C-ST toxin
NS20Y (mouse neuroblastoma, cholinergic)	++
N1E-115 (mouse neuroblastoma, adrenergic)	++
PC12D (rat pheochromocytoma)	+
C6 (rat astrocytoma)	-
HeLa (human cervical cancer)	-
Vero (simian kidney)	-
L929 (mouse mesenchymal tissue)	-
Li-7 (human hepatoma)	-
FL (human amnion)	-

The binding of C-ST toxin to different cell lines was also studied (Table 2). The toxin bound to cell lines of neuronal origin, but not to those of non-neuronal origin.

Degeneration of primary neurons

The cytotoxic effect of the toxins on primary neurons cultured in 96-well microplates was studied. No change was found by light microscopy when type A and E toxins were added. In contrast, neuronal processes were degenerated after addition of 4 µl (10 ng) C-ST toxin preparation (Table 1). The neuronal soma became spherical, and processes were thinned and partially swollen at 24 h (Fig. 2c); this was evaluated as a positive response (+). At 48 h, all processes had disappeared (Fig. 2d); this was evaluated as a strongly positive response (++). These changes appeared only in neurons; the background feeder layer showed no change even after 5 d. The severity of the degeneration increased with increase in the amount of C-ST toxin inoculated. No morphological changes in the neurons were produced by PBS pH 7.2 or by 1×10^5 LD₅₀ ml⁻¹ (2.5 µg ml⁻¹) C-ST toxin heated at 80 °C for 30 min.

Inhibition of binding and degeneration by antibodies

Toxin neutralization, and inhibition of binding and degeneration, by antibodies were studied (Table 3). Anti-C-ST IgG completely inhibited toxin binding. The monoclonal antibody CA-12 inhibited binding, but C-14 and C-17 did not. Monoclonal antibody C-9 also inhibited toxin binding, but to a lesser extent than polyclonal antibody and CA-12.

Anti-C-ST IgG, CA-12 and C-17 inhibited the toxin-induced degeneration of neurons, whereas C-9 and C-14 did not (Table 3).

Fig. 3. Normal 'neuropil' of the neuron culture (10th day). Although many neuronal processes were observed, it was not possible to distinguish between axons and dendrites. The inset shows a primitive synaptic junction found in the 'neuropil'. Bar, 1 μ m.

Table 3. Toxin neutralization, and inhibition of binding and degeneration, by various anti-C-ST antibodies

Anti-C-ST IgG (20 μ g ml⁻¹) and 1 in 100 dilutions of monoclonal ascites were separately mixed with an equal volume of C-ST toxin, incubated at 37 °C for 60 min, and then neutralization, binding and degeneration tests were performed. For the neutralization test with intravenous (i.v.) injection, and for the binding and degeneration tests, 2×10^5 LD₅₀ ml⁻¹ (5 μ g ml⁻¹) of toxin was used; for the neutralization test with intraperitoneal (i.p.) injection, 10 LD₅₀ ml⁻¹ was used.

Antibody	Toxin neutralization		Inhibition† of:	
	i.v.*	i.p.†	Binding	Degeneration
Polyclonal anti-C-ST IgG	90%	s	+	+
Monoclonal ascites C-9	48%	D	±	—
Monoclonal ascites CA-12	91%	s	+	+
Monoclonal ascites C-14	30%	D	—	—
Monoclonal ascites C-17	>99%	s	—	+

* Percentage of neutralized toxin.

† s, mouse survived; D, mouse died.

‡ +, Inhibited; ±, partially inhibited, —, not inhibited.

Electron microscopy

Normal primary neuron cultures showed many processes (Fig. 3), some of which formed primitive synaptic junctions (Fig. 3, inset). When the neurons were incubated with C-ST toxin (10 ng), some degenerative areas appeared in the middles and termini of the processes after 24 h. After 48 h, normal structures had disappeared, and degenerated mitochondria, membranous dense bodies and vesicles had accumulated in the swelled processes (Fig. 4). The neuronal somas became spherical and the nuclei were distorted, but intracytoplasmic organelles, except Nissl bodies, were preserved (Fig. 5). The feeder-layer cells were well preserved.

Fig. 4. Completely degenerated neuronal processes, 48 h after addition of C-ST toxin (10 ng). The processes were swollen and contained degenerated mitochondria, membranous dense bodies and vesicles. Bar, 1 μ m.

Fig. 5. Rounded neuronal soma, 48 h after addition of C-ST toxin (10 ng). The cell lacked processes, but its intracytoplasmic organelles, except Nissl bodies, were preserved. Bar, 1 μ m.

DISCUSSION

The primary neuron culture used in this study was prepared from the central nervous system of mouse embryos. It contained both neurons and non-neuronal cells. Neurons grew on the monolayered non-neuronal cells, and formed aggregates and a network of processes between them. However, the aggregates were composed of not only neurons but also a small number of glial cells. Synaptic junctions were found in this culture system, although they were very simple (Fig. 3, inset).

All toxin types tested A, C₁ and E, bound diffusely to both neuronal processes and aggregates but not to the background feeder layer. Furthermore, C-ST toxin bound only to cell lines of neuronal origin, but not to those of non-neuronal origin, including astrocytoma. Therefore, we presumed that the toxin bound to only neuronal processes and somas in aggregates of primary neuron culture. The toxin can be used as a neuron surface marker as has been shown for tetanus toxin (Mirsky *et al.*, 1978).

Binding of the botulinum toxin is specific to the presynaptic membranes of cholinergic nerve endings (Hirokawa & Kitamura, 1979; Dolly *et al.*, 1984; Black & Dolly, 1986). The present observations differ from those previously published in that each toxin bound to both processes and somas of primary neurons in addition to the nerve terminals. This diffuse binding indicates that the surface of neuronal soma and processes have toxin receptors. In cultured neurons the receptors are exposed to toxin because the neurons lack both the myelin sheath and the perineurium, which normally surround peripheral nerves except at their endings (presynaptic terminals of the end-plate), and thereby prevent toxin from reacting with the deeper-lying receptor. This may explain the diffuse binding. The chemical composition of the receptor for botulinum toxin is not yet clear. The toxin can bind to several kinds of gangliosides, especially G_{T1b} (Simpson & Rapport, 1971; Kitamura *et al.*, 1980). Agui *et al.* (1985) suggested that there may exist more than one receptor on synaptosomes, with different affinity constants. It is presumed that primary neurons also have several kinds of receptors in addition to the 'true' receptor (see below), some of which may exist on the surface of neuronal somas and processes. We may have observed toxin binding to all of these receptors; this would be an alternative explanation of the diffuse binding. Quantitative binding studies using isotope-labelled toxins and ELISA, are now in progress in our laboratory.

Kozaki (1979) pointed out that fragment I (Hc) of botulinum type B toxin competitively inhibited the binding of the type B toxin to synaptosomes of rat brain. This might suggest that the Hc portion contained the binding site. In our experiments, the Hc component bound to neurons but the Lc component did not.

The monoclonal antibody C-9 did not neutralize the lethal activity of C-ST toxin in mice, and did not inhibit the toxin binding to synaptosomes, but did inhibit its binding to G_{T1b} (Oguma *et al.*, 1984; Agui *et al.*, 1985). In contrast, monoclonal antibody CA-12 neutralized toxicity in mice and inhibited the binding to synaptosomes but not to G_{T1b}. Therefore, it was speculated that there may be receptor(s) other than G_{T1b} on synaptosomes. In our study, both C-9 and CA-12 inhibited the binding of toxin to neurons. The degree of inhibition by C-9, however, was weak as compared with CA-12. Furthermore, the degeneration of neurons was inhibited by CA-12 but not by C-9. These data also indicate that there may exist a receptor(s) other than G_{T1b} on neurons, and that G_{T1b} may not be a true receptor. Monoclonal antibody C-17 neutralized the toxicity in mice and completely inhibited the degeneration of neurons, but did not inhibit the binding to G_{T1b}, synaptosomes and neurons. This antibody may react with the part of the toxin which is necessary for expression of toxicity other than binding steps.

Thesleff (1960) observed no ultrastructural changes in the end-plates of frog and cat muscles paralysed by crude type A toxin. Zacks *et al.* (1962) observed the accumulation of ferritin-labelled type B toxin at end-plates by electron microscopy, but found no morphological abnormality in neurons. Duchen (1970, 1971) investigated the sprouting of nerve terminals when type A toxin was injected into muscles, but did not observe degenerative changes of neurons by electron microscopy. Also, Bigalke *et al.* (1978) observed no changes by light microscopy in a primary neuron culture treated with type A toxin. In our study, however, C-ST toxin induced severe degeneration of neuronal processes in which degenerated mitochondria,

membranous dense bodies, and vesicles were found. These degenerated products were similar to those found in Wallerian degeneration *in vivo* (Birks *et al.*, 1960; Lampert, 1967). In addition to the changes in the processes, the neuronal somas became spherical, with the nucleus located at one side; however, intracytoplasmic organelles except Nissl bodies were well preserved. These ultrastructural changes were similar to the axon reaction (retrograde degeneration) *in vivo* (Duchen, 1984). Therefore, it was suggested that C-ST toxin might disturb the axonal flow and cause the degeneration of neuron culture (Grafstein & Forman, 1980).

The reason why only C-ST toxin caused neuronal degeneration is not clear. The C-ST toxin might have some different activity compared with type A and E toxin. Further morphological, biochemical and electrophysiological examinations seem to be necessary with preparations of both neuromuscular junction and cultured neurons to clarify the mechanism of C-ST toxin activity.

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REFERENCES

- AGUI, T., SYUTO, B., OGUMA, K., IIDA, H. & KUBO, S. (1983). Binding of *Clostridium botulinum* type C neurotoxin to rat brain synaptosomes. *Journal of Biochemistry* **94**, 521–527.
- AGUI, T., SYUTO, B., OGUMA, K., IIDA, H. & KUBO, S. (1985). The structural relation between the antigenic determinants to monoclonal antibodies and binding sites to rat brain synaptosomes and G_{T1b} ganglioside in *Clostridium botulinum* type C neurotoxin. *Journal of Biochemistry* **97**, 213–218.
- BIGALKE, H., DIMPFEL, W. & HABERMANN, E. (1978). Suppression of ³H-acetylcholine release from primary nerve cell cultures by tetanus and botulinum-A toxin. *Naunyn-Schmiedeberg's Archives of Pharmacology* **303**, 133–138.
- BIRKS, R., KATZ, B. & MILEDI, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *Journal of Physiology* **150**, 145–168.
- BLACK, J. D. & DOLLY, J. O. (1986). Interaction of ¹²⁵I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. *Journal of Cell Biology* **103**, 521–534.
- BURGEN, A. S. V., DICKENS, F. & ZATMAN, L. J. (1949). The action of botulinum toxin on the neuromuscular junction. *Journal of Physiology* **109**, 10–24.
- DAHL, D. & BIGNAMI, A. (1979). Astroglial and axonal proteins in isolated brain filaments. I. Isolation of the glial fibrillary acidic protein and of an immunologically active cyanogen bromide peptide from brain filament preparations of bovine white matter. *Biochimica et biophysica acta* **578**, 305–316.
- DICKSON, E. C. & SHEVKY, E. (1923). Botulism. Studies on the manner in which the toxin of *Clostridium botulinum* acts upon the body. II. The effect upon the voluntary nervous system. *Journal of Experimental Medicine* **38**, 327–346.
- DOLLY, J. O., BLACK, J., WILLIAMS, R. S. & MELLING, J. (1984). Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. *Nature, London* **307**, 457–460.
- DUCHEN, L. W. (1970). Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: differences between fast and slow muscles. *Journal of Neurology, Neurosurgery and Psychiatry* **33**, 40–54.
- DUCHEN, L. W. (1971). An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast-skeletal muscle fibres of the mouse. *Journal of the Neurological Sciences* **14**, 47–60.
- DUCHEN, L. W. (1984). General pathology of neurons and neuroglia. In *Greenfield's Neuropathology*, 4th edn., pp. 1–52. Edited by J. Hume Adams, J. A. N. Corsellis & L. W. Duchen. London: Edward Arnold.
- GRAFSTEIN, B. & FORMAN, D. S. (1980). Intracellular transport in neurons. *Physiological Reviews* **60**, 1167–1283.
- HABERMANN, E. (1974). ¹²⁵I-labeled neurotoxin from *Clostridium botulinum* A: preparation, binding to synaptosomes and ascent to the spinal cord. *Naunyn-Schmiedeberg's Archives of Pharmacology* **281**, 47–56.
- HABERMANN, E. & HELLER, I. (1975). Direct evidence for the specific fixation of *Cl. botulinum* A neurotoxin to brain matter. *Naunyn-Schmiedeberg's Archives of Pharmacology* **287**, 97–106.
- HEITZMANN, H. & RICHARDS, F. M. (1974). Use of the avidin-biotin complex for specific staining of biological membranes in electron microscopy. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 3537–3541.
- HIROKAWA, N. & KITAMURA, M. (1979). Binding of *Clostridium botulinum* neurotoxin to the presynaptic membrane in the central nervous system. *Journal of Cell Biology* **81**, 43–49.
- ISOBE, T. & OKUYAMA, T. (1978). The amino-acid sequence of S-100 protein (PAP I-b protein) and its relation to the calcium-binding proteins. *European Journal of Biochemistry* **89**, 379–388.
- KATO, K., SUZUKI, F. & UMEDA, Y. (1981). Highly

- sensitive immunoassays for three forms of rat brain enolase. *Journal of Neurochemistry* **36**, 793–797.
- KITAMURA, M. (1976). Binding of botulinum neurotoxin to the synaptosome fraction of rat brain. *Naunyn-Schmiedeberg's Archives of Pharmacology* **295**, 171–175.
- KITAMURA, M., IWAMORI, M. & NAGAI, Y. (1980). Interaction between *Clostridium botulinum* neurotoxin and gangliosides. *Biochimica et biophysica acta* **628**, 328–335.
- KOZAKI, S. (1979). Interaction of botulinum type A, B and E derivative toxins with synaptosomes of rat brain. *Naunyn-Schmiedeberg's Archives of Pharmacology* **308**, 67–70.
- LAMPERT, P. W. (1967). A comparative electron microscopic study of reactive, degenerating, regenerating, and dystrophic axons. *Journal of Neuropathology and Experimental Neurology* **26**, 345–368.
- LIEM, R. K. H., SHU-HUI YEN, SALOMON, G. D. & SHELANSKI, M. L. (1978). Intermediate filaments in nervous tissues. *Journal of Cell Biology* **79**, 637–645.
- MIRSKY, R., WENDON, L. M. B., BLACK, P., STOLKIN, C. & BRAY, D. (1978). Tetanus toxin: a cell surface marker for neurones in culture. *Brain Research* **148**, 251–259.
- OGUMA, K., SYUTO, B., AGUI, T., IIDA, H. & KUBO, S. (1981). Homogeneity and heterogeneity of toxins produced by *Clostridium botulinum* type C and D strains. *Infection and Immunity* **34**, 382–388.
- OGUMA, K., AGUI, T., SYUTO, B., KIMURA, K., IIDA, H. & KUBO, S. (1982). Four different monoclonal antibodies against type C₁ toxin of *Clostridium botulinum*. *Infection and Immunity* **38**, 14–20.
- OGUMA, K., MURAYAMA, S., SYUTO, B., IIDA, H. & KUBO, S. (1984). Analysis of antigenicity of *Clostridium botulinum* type C₁ and D toxins by polyclonal and monoclonal antibodies. *Infection and Immunity* **43**, 584–588.
- SIMPSON, L. L. & RAPPORT, M. M. (1971). Ganglioside inactivation of botulinum toxin. *Journal of Neurochemistry* **18**, 1341–1343.
- SOTELO, J., GIBBS, C. J., JR, GAJDUSEK, D. C., TOH, B. H. & WURTH, M. (1980). Method for preparing cultures of central neurons: cytochemical and immunochemical studies. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 653–657.
- SUGII, S. & SAKAGUCHI, G. (1975). Molecular construction of *Clostridium botulinum* type A toxins. *Infection and Immunity* **12**, 1262–1270.
- SUGIYAMA, H. (1980). *Clostridium botulinum* neurotoxin. *Microbiological Reviews* **44**, 419–448.
- SYUTO, B. & KUBO, S. (1977). Isolation and molecular size of *Clostridium botulinum* type C toxin. *Applied and Environmental Microbiology* **33**, 400–405.
- SYUTO, B. & KUBO, S. (1981). Separation and characterization of heavy and light chains from *Clostridium botulinum* type C toxin and their reconstitution. *Journal of Biological Chemistry* **256**, 3712–3717.
- THESLEFF, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. *Journal of Physiology* **151**, 598–607.
- WONNACOTT, S. & MARCHBANKS, R. M. (1976). Inhibition by botulinum toxin of depolarization-evoked release by [¹⁴C]acetylcholine from synaptosomes *in vitro*. *Biochemical Journal* **156**, 710–712.
- YOKOSAWA, N., TSUZUKI, K., SYUTO, B. & OGUMA, K. (1986). Activation of *Clostridium botulinum* type E toxin purified by two different procedures. *Journal of General Microbiology* **132**, 1981–1988.
- ZACKS, S. I., METZGER, J. F., SMITH, C. W. & BLUMBERG, J. M. (1962). Localization of ferritin-labelled botulinus toxin in the neuromuscular junction of the mouse. *Journal of Neuropathology and Experimental Neurology* **21**, 610–633.