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INTERACTION BETWEEN *CLOSTRIDIUM BOTULINUM* NEUROTOXIN AND GANGLIOSIDES

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Summary

The effect of gangliosides on *Clostridium botulinum* type A neurotoxin was examined in terms of detoxification. The molar concentrations of gangliosides necessary to detoxify 50% of 1 M *Cl. botulinum* neurotoxin were as follows: G_{M1}, 2073; G_{M2}, 2439; G_{M3}, 6098; G_{D1a}, 610; G_{D1b}, 488; G_{T1a}, 829; G_{T1b}, 6 and G_{Q1b}, 27. Inhibition by gangliosides of the neurotoxin binding to synaptosomes showed that G_{T1b} was highly effective, but the others were not. Low-temperature treatment inhibited the detoxification of neurotoxin by G_{T1b} and the binding of ^{125}I-labelled neurotoxin to the synaptosome fraction. ^{125}I-labelled neurotoxin was mixed with G_{M1} or G_{T1b} and their molecular size was estimated by sucrose-density-gradient centrifugation. When ^{125}I-labelled neurotoxin was incubated with G_{M1}, a single radioactive peak having a sedimentation coefficient of 7.3 S appeared. When incubated with G_{T1b}, however, ^{125}I-labelled neurotoxin gave three peaks having sedimentation coefficients 14, 10 and 7.3 S, respectively. The present results indicated that the location and the number of sialic acids in ganglioside molecules are of significance in the detoxification and the binding of *Cl. botulinum* neurotoxin with ganglioside molecules.

Introduction

It is well known that the neurotoxin produced by *Clostridium botulinum*, when orally administered, leads to death of the animal by eventually paralyzing

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the diaphragm. Physiological and pharmacological studies indicate that *Cl. botulinum* toxin blocks the release of acetylcholine from the presynapse at the neuromuscular junction [1—9]. The mechanism of this blockade has not yet been elucidated on a molecular level.

Morphological studies have indicated that *Cl. botulinum* neurotoxin is accumulated on the neuromuscular junction in vivo [10] and in vitro [11,12]. *Cl. botulinum* neurotoxin not only binds to peripheral cholinergic synapses but also, when *Cl. botulinum* type A [13—15] or type E (proto-Eα [16] and act.-Eα [16], Kitamura, M., unpublished data) toxins are added in vitro to the homogenate of the central nervous system, the toxin binds to synaptosomal fractions [13—15] and especially to synaptosomal plasma membranes [15]. A recent interesting study elucidated that the *Cl. botulinum* neurotoxin is preferentially bound to the presynaptic membranes in synaptosomal fractions in vitro [17]. This result strongly indicates that the *Cl. botulinum* neurotoxin recognizes some specific substance(s) in the presynaptic membrane having affinity for the neurotoxin.

On the other hand, it has been reported that various kinds of gangliosides detoxify *Cl. botulinum* neurotoxin, especially with G_{T1} fractions [18,19]. The gangliosides are highly concentrated in nervous tissue, where major molecular species have been purified and characterized [20,21]. Recent studies, however, revealed that in addition to those gangliosides, there exist numerous new or unknown molecular species, most of which belong to minor components [22,23]. An introduction of various new techniques has enabled their isolation in high purity [22,23] and the determination of their chemical structures [24—26]. At present, however, the functional roles of gangliosides in the presynaptic membranes are still not clear.

We hope to elucidate the molecular mechanism of acetylcholine blockade by *Cl. botulinum* neurotoxin at a presynaptic membrane level with special emphasis on the relationship between the toxin and gangliosides. The present paper deals with the relationship between the neurotoxin isolated from *Cl. botulinum* type A crystalline toxin and various molecular species of gangliosides from the human brain.

Materials and Methods

Preparation of neurotoxin from Cl. botulinum type A crystalline toxin. The *Cl. botulinum* type A crystalline toxin was supplied by Dr. E.J. Schantz (University of Wisconsin, U.S.A.). The neurotoxin, a toxic component (α-fraction) of the type A crystalline toxin of molecular weight 150 000 and $s_{20,w}$ 7.2 [27], was isolated from the crystalline toxin by DEAE-Sephadex column chromatography [28]. The isolated neurotoxin was homogeneous in disc electrophoresis and immuno-agar-diffusion tests.

Gangliosides. The various molecular species of gangliosides: G_{M1}, G_{M2}, G_{M3}, G_{D1a}, G_{D1b}, G_{T1a}, G_{T1b} and G_{Q1b} were isolated in high purity from the human brain by the previous method [22,23]. The isolated gangliosides were prepared in the form of an ammonium salt. The gangliosides were dissolved in distilled water to a concentration of 200 nmol per ml and were stored at —20°C until use. The homogeneity of gangliosides was checked by thin-layer chromatography.

Determination of neurotoxicity. The intravenous injection methods described previously were used for the determination of toxicity in terms of LD_{50} per ml [29]. Six mice were injected with 0.1 ml of the sample chilled in an ice bath.

Synaptosome preparation. The large synaptosomes of savanna monkey (*Cercopithecus aethiops*) brain were prepared according to the slight modification of the procedure of Kelly et al. [30].

Conjugation labelling methods for iodination of the neurotoxin. Iodination of the neurotoxin was performed either by use of *N*-succinimidyl 3-(4-hydroxyl 5-[^{125}I]iodophenyl) propionate (Bolton and Hunter reagent purchased from New England Nuclear, MA) according to the method of Bolton et al. [31], or by the chloramine T method [32].

Sucrose-density-gradient centrifugation. A linear density-gradient of 5—15% sucrose (w/w) was prepared at 0°C by mixing 2.4 ml each of 5 and 15% sucrose solution in 10 mM phosphate buffered saline (pH 7.2). 0.2 ml portions of the samples were layered on top of the sucrose gradient and centrifuged in an SW65 rotor in a Beckman L_2-65B ultracentrifuge at 64 000 rev./min for 3 h at 5°C. The value of $s_{20,w}$ was estimated by the method of Martin and Ames [33].

Inhibition by different gangliosides of binding of [^{125}I]neurotoxin to the synaptosome. 10 μl of gangliosides (200 nmol per ml) were mixed with the large synaptosome suspended in 0.32 M sucrose in 1 mM Tris · HCl buffer (pH 7.2) and 100 μl of [^{125}I]neurotoxin conjugated by the Bolton and Hunter method. After incubation at 37°C for 20 min, the suspension was centrifuged at 10 000 × *g* for 5 min. The radioactivity of the suspension and of the resulting supernatant of each sample were determined, respectively, from which the binding ratio,

$$\frac{\text{c.p.m., suspension} - \text{c.p.m., supernatant}}{\text{c.p.m., suspension}}$$

was calculated.

Results

The effect of gangliosides on the neurotoxin were examined in terms of detoxification. As shown in Table I, the ability of various gangliosides to detoxify the *Cl. botulinum* neurotoxin varies with their molecular structures. The monosialo gangliosides (G_{M1}, G_{M2} and G_{M3}) were not effective, and disialo gangliosides (G_{D1a}, G_{D1b}) and G_{T1a} were weakly effective. However, G_{T1b} and G_{Q1b} were found to be strongly effective.

The detoxification of the neurotoxin was examined at different concentrations of gangliosides. A curve relating the survival of toxicity vs. concentration of gangliosides showed a linearity with G_{T1b} and G_{Q1b}, as shown in Fig. 1. The linear curve of G_{Q1b} was shifted in parallel to the right compared with that of G_{T1b}. The similar linearity and parallel shift also were observed in other gangliosides examined. From these results, the molar concentrations of gangliosides which give 50% detoxification of 1 M *Cl. botulinum* neurotoxin were estimated (Table II). A 2073 to 6098 M concentration of monosialo gangliosides was required for G_{M1}, G_{M2} and G_{M3}, and a 488 to 610 M concentration of disialo

TABLE I

DETOXIFICATION OF *CL. BOTULINUM* TYPE A NEUROTOXIN BY DIFFERENT GANGLIOSIDES

15 μl neurotoxin (82 μg toxin per ml) were mixed with 25 μl ganglioside (200 nmol/ml), 60 μl 0.5 M Tris · HCl buffer (pH 7.2) and 500 μl distilled water. After incubation at 37°C for 20 min, 50 μl albumin (10 mg/ml) in 0.05 M Tris · HCl buffer (pH 7.2) and 50 μl of a 10-fold concentration of 10 mM phosphate buffered saline (pH 7.2) was added to the reaction mixture. The preparation was injected into mice for determining toxicity. AcNeu, *N*-acetylneuraminic acid

Ganglioside	Structure of ganglioside	Toxicity survival	
		LD_{50}/ml	Percentage
Control		61 500	100.00
G_{M3}	AcNeu(α2-3)Gal(β1-4)Glc(β1-1)Cer	62 100	100.98
G_{M2}	GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 ↑ α2 AcNeu	51 400	83. 58
G_{M1}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 ↑ α2 AcNeu	57 850	94.07
G_{D1a}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 3 ↑ ↑ α2 α2 AcNeu AcNeu	33 800	54.96
G_{D1b}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 ↑ α2 AcNeu(8-2α)AcNeu	15 050	24.47
G_{T1a}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 3 ↑ ↑ α2 α2 AcNeu(8-2α)AcNeu AcNeu	35 850	58.29
G_{T1b}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 3 ↑ ↑ α2 α2 AcNeu AcNeu(8-2α)AcNeu	20	0.03
G_{Q1b}	Gal(β1-3)(GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer 3 3 ↑ ↑ α2 α2 AcNeu(8-2α)AcNeu AcNeu(8-2α)AcNeu	330	0.54

gangliosides for G_{D1a} and G_{D1b}. In trisialo gangliosides, concentrations 829 M of G_{T1a} and 6 M of G_{T1b} were necessary and in the tetrasialo ganglioside, 27 M of G_{Q1b} were required.

The inhibition experiment on the binding of ^{125}I-labelled neurotoxin to the synaptosome by gangliosides showed that G_{T1b} was most effective (70% binding), followed by G_{Q1b} (95% binding), while other gangliosides were not effective. The ratio in the control experiment without addition of ganglioside was

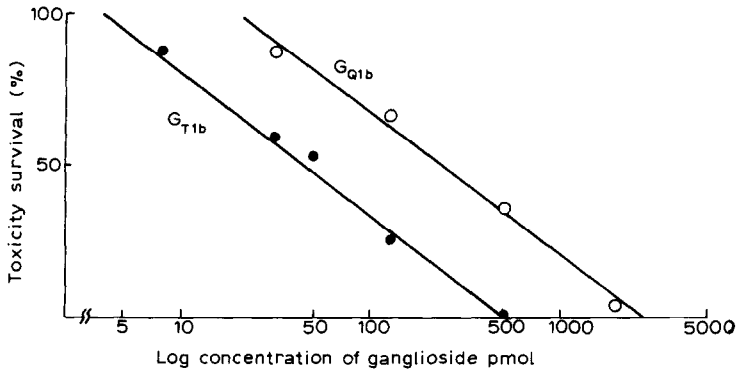

Fig. 1. Detoxification of the neurotoxin in different concentrations of gangliosides. The experimental procedure was the same as described in Table I except for the concentration of gangliosides.

TABLE II

THE MOLAR CONCENTRATION OF GANGLIOSIDES GIVING 50% DETOXIFICATION OF *CL. BOTULINUM* NEUROTOXIN

The values in the table were estimated from the results of detoxification of the neurotoxin in different concentrations of gangliosides (see Fig. 1).

Gangliosides	Concentration of ganglioside (nmol) to 8.2 pmol toxin	Molar ratio ganglioside/toxin
G_{M3}	50.00	6097.6
G_{M2}	20.00	2439.0
G_{M1}	17.00	2073.2
G_{D1a}	5.00	609.8
G_{D1b}	4.00	487.8
G_{T1a}	6.80	829.3
G_{T1b}	0.05	6.1
G_{Q1b}	0.22	26.8

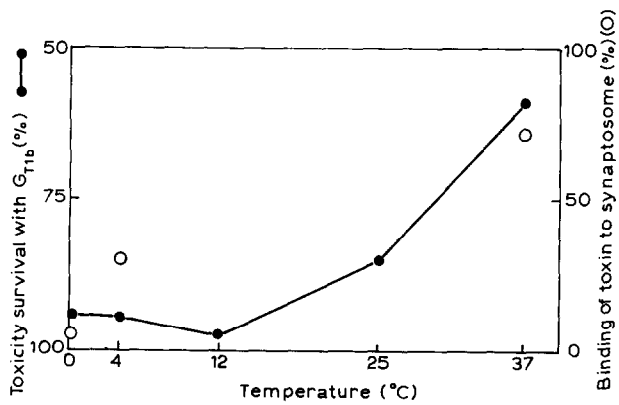

Fig. 2. The effect of detoxification of the neurotoxin by G_{T1b} and the binding of neurotoxin to synaptosome fraction at different temperatures. 20 μl neurotoxin (74 μg/ml) were mixed with 20 μl G_{T1b} (20 nmol/ml), 0.5 M Tris-HCl buffer (pH 7.2) and 500 μl distilled water. The mixture was kept at 0°C until incubation at different temperatures. After incubation at different temperatures for 20 min, 50 μl albumin (10 mg/ml) and 50 μl of 10-fold concentration of phosphate-buffered saline were added. 30 μl neurotoxin (74 μg/ml) were mixed with 100 μl of large synaptosome fraction suspended in 0.32 M sucrose in 1 mM Tris-HCl (pH 7.2) and 100 μl of phosphate-buffered saline. After incubation at each temperature for 20 min, the reaction mixture was centrifuged at 10 000 × *g* for 5 min. 200 μl of the supernatant was pipetted out and 50 μl albumin (10 mg/ml) and 450 μl phosphate buffered saline were added.

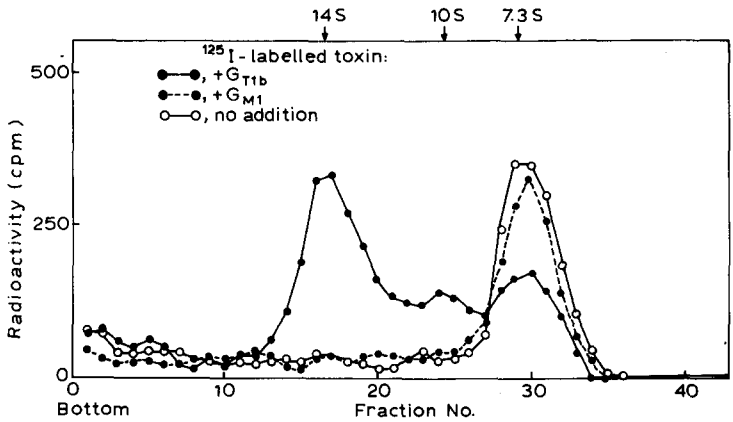

Fig. 3. Sucrose density-gradient centrifugation of the reaction mixture of ^{125}I-labelled neurotoxin and G_{M1} or G_{T1b}. 200 μl ^{125}I-labelled neurotoxin conjugated by the chloramine method was mixed with 5 μl gangliosides (200 nmol/ml) and incubated at 37°C for 20 min. The reaction mixture was then centrifuged.

taken as 100 per cent to calculate the binding percentage in each ganglioside sample.

The effect of G_{T1b} on detoxification of neurotoxin and the binding of neurotoxin to the synaptosome fraction were examined at different temperatures. The detoxification by G_{T1b} was not observed below 12°C (Fig. 2). The binding of the toxin to synaptosome was also weak at 0°C (Fig. 2).

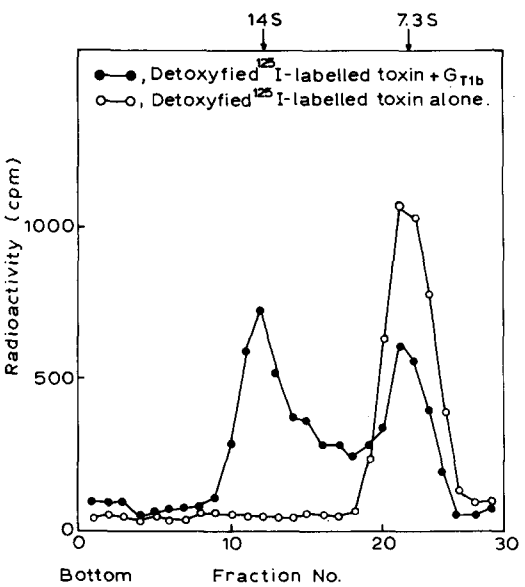

Fig. 4. Sucrose density-gradient centrifugation of the reaction mixture of detoxified ^{125}I-labelled neurotoxin and G_{T1b}. The detoxified ^{125}I-labelled neurotoxin was prepared by increasing the concentration of chloramine T when ^{125}I was conjugated to the neurotoxin.

^{125}I-labelled neurotoxin was mixed with G_{M1} or G_{T1b} and the molecular size of the complex was estimated by sucrose density-gradient centrifugation. When the toxin was incubated with G_{M1}, a single radioactive peak having $s_{20,w}$ 7.3 S was observed which is the same sedimentation coefficient as that of the toxin molecule itself. Incubation of the toxin with G_{T1b} gave three radioactive peaks having sedimentation coefficients of 14, 10 and 7.3 S, respectively (Fig. 3). When detoxified ^{125}I-labelled neurotoxin was mixed with G_{T1b}, two radioactive peaks having $s_{20,w}$ 14 and 7.3 S appeared (Fig. 4).

Discussion

The present paper concerns the interaction between *Cl. botulinum* neurotoxin that preferentially recognizes the presynaptic membrane [17] and various molecular species of gangliosides from the human brain that were more highly purified than before [22,23].

It was shown that the reactivities of the various molecular species of gangliosides were clearly different in terms of the detoxification of the neurotoxin. Both G_{T1b} and G_{Q1b} showed the strongest detoxification ability of any so far examined. Simpson and Rapport [18,19] previously reported that the number of sialic acid molecules per ganglioside molecule was important in detoxification. When G_{T1a} and G_{T1b} containing 3 mol residues of sialic acid per molecule of ganglioside were compared, however, G_{T1a} required about a 136-fold higher molar concentration than G_{T1b} for equal detoxification of the neurotoxin. This shows that the number of sialic acid molecules located at the internal site was more significant than that at the non-reducing terminal site of a ganglioside molecule. In addition, when G_{D1b}, G_{T1b} and G_{Q1b} which commonly contained 2 mol of sialic acid at the internal site were compared, G_{D1b} required an 80-fold higher molar concentration than G_{T1b} and an 18-fold higher concentration than G_{Q1b} for detoxification. These results indicate that 1 mol of sialic acid residue located at the non-reducing terminal site of the gangliosides is also essential for detoxification. The overall result shows that both the number and location of sialic acid residues in the carbohydrate backbone of a ganglioside molecule are important in detoxifying the neurotoxin. The binding of ^{125}I-labelled neurotoxin to synaptosome was inhibited most strongly by G_{T1b}. Low-temperature treatment inhibited the detoxification by G_{T1b} of neurotoxin and the binding of ^{125}I-labelled neurotoxin to the synaptosome fraction. These results indicate that G_{T1b} is probably involved in the binding of *Cl. botulinum* neurotoxin at presynaptic membranes as a receptor substance, or as a receptor structure. The possibility should not be ruled out that minor, unknown gangliosides other than those examined, also participate.

The interaction between neurotoxin and ganglioside(s) occurs by complex formation of the toxin with ganglioside(s). However, when detoxified ^{125}I-labelled neurotoxin was mixed with G_{T1b}, a complex of the toxin and G_{T1b} was also formed. These results may indicate that either the binding state of native and detoxified ^{125}I-labelled neurotoxin with G_{T1b} was different, or the binding sites in toxin molecules were different from the toxic sites.

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