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Original Article

Characterization of the Monoclonal Antibody Response to Botulinum Neurotoxin Type A in the Complexed and Uncomplexed Forms

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SUMMARY: *Clostridium botulinum* produces large complex toxins, which include botulinum neurotoxin (BoNT) and auxiliary non-toxic proteins. We prepared monoclonal antibodies (mAbs) from mice that were immunized several times with BoNT/A after basal immunization with toxoid. We then examined the reactivities of these mAbs to BoNT and toxoid and showed that some mAbs reacted to only BoNT. This result indicates that the antigenicity of BoNT/A partially disappeared with formalin treatment. Some mAbs that specifically recognized either BoNT/A1 or BoNT/A2 were considered useful as detection antibodies specific for the BoNT/A subtype. Results of a neutralizing test with mAbs against either BoNT/A1 or BoNT/A2 showed that neutralizing antibody recognition sites were present in the light chain, heavy chain (N-terminal half), and heavy chain (C-terminal half) domains. Investigation of the different binding capabilities of the mAbs to BoNT and the complex toxin by immunoprecipitation suggested that the light chain of BoNT is exposed at the molecular surface of the complex toxin since there was no difference in the binding of light chain-specific mAb to BoNT and the complex toxin. The heavy chain is related to BoNT binding to non-toxic components, because the reactivity of the heavy chain to some mAbs was influenced by non-toxic components.

INTRODUCTION

Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* are classified into 7 serotypes (A through G) on the basis of differences in antigen specificity. BoNTs are synthesized as single-chain peptides with a molecular mass of about 150 kDa, which are proteolytically activated into compounds with a light chain (L-chain, 50 kDa) and a heavy chain (H-chain, 100 kDa) linked by a disulfide bond (1). The L-chain acts as a zinc-dependent endopeptidase. The H-chain is composed of 2 functional domains; the N-terminal half (H_N) functions as the translocation domain, and the C-terminal half (H_C) as the receptor-binding domain (2). BoNTs are produced as complexes, which associate with non-toxic components. The sizes of complex toxins differ, from 900 kDa (LL toxin for type A) to 500 kDa (L toxin for types A, B, C, D, and G), down to 300 kDa (M toxin for types A, B, C, D, E, and F) (3,4). The M toxin consists of BoNT and non-toxic-non-hemagglutinin (NTNH), which shows no hemagglutination activity, whereas the L toxin consists of BoNT, NTNH, and hemagglutinin (HA). The LL toxin is a dimer of the L toxin and is probably linked via one of the HA subcomponents (5). The complex toxin dissociates to BoNT and the non-toxic components at a pH above 7.2 (6). Recently, the crystal structures of BoNT and 3 HA subcomponents (HA1, HA2, and HA3) were reported (7–11). Hasegawa et al. have modeled the structure of the botulinum toxin type D complex (10). However, the binding state of BoNT and the non-toxic components has not been clarified yet.

Type A organisms have been classified into 5 subtypes (A1 to A5) on the basis of the amino acid sequence variability of BoNT, and almost all strains belong to the A1 or A2 subtype (12–14). The toxin forms produced by the subtypes A1 and A2 are LL, L, and M toxins and M toxin alone, respectively (15). BoNT/A2 exhibits 89.9% amino acid sequence homology with BoNT/A1 (16). A1 and A2 toxins show no difference in lethal toxicity (15). However, it has been shown that BoNT/A2 is a more potent neuromuscular blocker than BoNT/A1 (17), suggesting that the subtypes possess different epitopes due to their amino acid sequence variability. Specific antibodies against A1 and A2 toxins have not been identified, so an immunological diagnostic method for discrimination has not been established.

In several previous studies, formalin-detoxified botulinum toxin (toxoid) was used as the antigen to prepare monoclonal antibodies (mAbs) against the toxin. However, the toxoid is altered in terms of its conformational structure, and it is not necessary to maintain the antigenicity of the toxin completely. In this study, we prepared mAbs using mice immunized several times with BoNT/A after basal immunization with toxoid, and investigated the binding patterns of each domain of BoNT and the non-toxic components in the complex toxin, based on the binding activity of mAbs to the complex toxin.

MATERIALS AND METHODS

Preparation of botulinum toxin: *C. botulinum* type A

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strains 62A (A1 subtype) and Chiba-H (A2 subtype) were used for the purification of toxins. The complex toxin was purified as previously reported (18). BoNT/A was isolated from the individual M toxin by anion exchange chromatography as previously described (19), and stored in 50 mM phosphate buffer, pH 7.5, at −80°C until use.

Preparation of recombinant H_Cs: Recombinant H_Cs (H_C/A1, aa: 860–1296; H_C/A2, aa: 860–1296) were expressed according to the Gateway technology protocol (Invitrogen, Carlsbad, Calif., USA). DNA fragments encoding the H_C of BoNT were amplified by polymerase chain reaction (PCR) from genomic DNA using the following primers: forward, 5′-CACCAATATTA TTAATACTTCTATA-3′ (for 62A), 5′-CACCAATA TTGTTAATACCTCTATA-3′ (for Chiba-H); reverse, 5′-CCAATGCATTGGTTCTGCAGTTACAGTGGCC TTTC-3′ (for 62A), 5′-TGAAAGCTTTTACAGTGAA CTTTC-3′ (for Chiba-H).

The PCR product was purified using the Wizard® SV Gel and PCR Clean Up System (Promega, Fitchburg, Wis., USA). The purified H_C DNA fragment was inserted into pENTR TOPO vector (Invitrogen) by the TOPO cloning reaction to create the entry clone (H_C/TOPO), and transformed into *Escherichia coli* JM109 competent cells. The transformants were selected on Luria-Bertani (LB) plates containing 50 μg/ml kanamycin, and positive clones were confirmed by PCR. One of the positive clones was then cultured overnight in 3 ml of LB medium containing 50 μg/ml kanamycin, and the plasmid was purified using the GenElute plasmid Midiprep Kit (Sigma, St. Louis, Mo., USA). The purified H_C/TOPO vector was recombined with the destination vector pDEST 17 (Invitrogen) via an LR reaction to create the expression clone (H_C/pDEST 17), which was then transformed into *E. coli* DH5α competent cells. Positive expression clones were selected on an LB plate containing 100 μg/ml ampicillin, and confirmed by PCR. The plasmid H_C/pDEST 17 was then purified from a positive clone. The H_C DNA was amplified by PCR from the expression clone, and the purified PCR product was sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit, an ABI 3130 Genetic Analyzer, and the Sequencing Analysis software (all from Applied Biosystems, Foster City, Calif., USA).

Recombinant H_C was expressed in *E. coli* BL21 CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, Calif., USA). The expression clone H_C/pDEST 17 was used to transform *E. coli* BL21 CodonPlus (DE3)-RIL cells. A single transformed colony was selected on an LB plate containing 100 μg/ml ampicillin and cultured overnight in 3 ml LB medium containing 100 μg/ml ampicillin. One milliliter of culture was transferred to 100 ml of LB medium containing 100 μg/ml ampicillin, and shaken at 37°C until an OD_{600} of 0.4–0.7 was reached. Protein expression was then induced by addition of isopropyl-β-D(−)thiogalactopyranoside (IPTG; Wako Pure Chemicals, Osaka, Japan) at a final concentration of 12.5 μM. Cells were allowed to grow further at 20°C for 24 h and then harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The cell pellet was suspended in 8 ml of Bacterial Protein Extraction Reagent (B-PER; Pierce, Rockford, Ill., USA) containing 80 μl of protease inhibitor cocktail (100 mg/ml; Sigma) and 80 μl of lysozyme (10 mg/ml; Sigma) and placed on ice for 1 h. The suspension was then sonicated 6 times for 10 s each and centrifuged at 27,000 × *g* for 15 min at 4°C. The supernatant was loaded onto a Ni Sepharose 6 Fast Flow column (0.6 ml; GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM phosphate buffer, pH 8.0, containing 0.5 M NaCl and 20 mM imidazole. The column was washed with 10 column volumes of the equilibration buffer, and recombinant H_C was eluted with a linear gradient from 20 to 500 mM imidazole in 20 mM phosphate buffer, pH 8.0, containing 0.5 M NaCl. After dialysis against 50 mM phosphate buffer, pH 7.5, the pure recombinant H_C was stored at −30°C until use.

Preparation of mAb against BoNT/A: Purified BoNTs (0.2 mg/ml) were detoxified by dialyzing against 0.1 M sodium phosphate buffer, pH 7.0, containing 0.4% formalin at 30°C for 7 days. BALB/c mice were immunized 3 times with toxoid (25 μg, 0.5 ml) every 2 weeks by intraperitoneal (i.p.) injection, the first time with Freund's complete adjuvant, and the other times with Freund's incomplete adjuvant. After examining the toxin-neutralizing activity of the immunized sera, the mice were further immunized 5 times with gradually increasing amounts of BoNT/A (1–16 μg), with 1-week intervals. Three days prior to fusion, the mouse was boosted with an intravenous (i.v.) injection of BoNT/A (10 μg, 0.1 ml). Spleen cells were fused with myeloma cells (P3X63Ag8U1, P3U1) in 50% polyethylene glycol 4000 (Merck, Darmstadt, Germany) containing 10% dimethyl sulfoxide (DMSO) as previously described (20). The resulting hybridomas were selected in HAT medium and cloned twice by limiting dilution as previously described (20).

The subclass of each mAb was determined using a Mouse MonoAb ID Kit (ZYMED, Carlsbad, Calif., USA). Each hybridoma was cultured in serum-free medium (ASF104; Ajinomoto, Tokyo, Japan). The supernatant was collected by centrifugation at 180 × *g* for 10 min, and mixed with an equal volume of saturated ammonium sulfate (pH 7.0). The mixture was allowed to stand at 4°C overnight, and centrifuged at 27,000 × *g* for 15 min at 4°C. The pellet was dissolved in 20 mM phosphate buffer, pH 7.0, and dialyzed with the same buffer. Each mAb was purified using a HiTrap™ Protein G HP column (GE Healthcare), and dialyzed with 75 mM phosphate buffer, pH 7.4, containing 75 mM NaCl. The mAbs were stored at −30°C until use.

Amino acid sequence of the mAb variable region: Total RNA was prepared from hybridoma cells using ISOGEN (Wako) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA as previously reported (21). The cDNA was then used as the template for PCR using the following set of primers, designed as previously described (21): forward, 5′-GTTAGATCTCCAGCTTGGTCCC-3′ [for the variable region of the light chain (VL)], 5′-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3′ [for the variable region of the heavy chain (VH)]; reverse, 5′-GACATTCAGCTGACCCAGTCTCCA-3′ (for VL), 5′-AGGTSMARCTGCAGSAGTCWGG-3′ (for VH) (S, C or G; M, A or C; R, A or G; and W, A or T).

The PCR product was purified using the Wizard® SV Gel and PCR Clean Up System (Promega), and se-

quenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The amino acid sequences were determined from the nucleotide sequence using BLAST (NCBI).

Enzyme-linked immunosorbent assay (ELISA): ELISA was carried out according to a previously described method with some modifications (20). Briefly, each well was coated with 0.1 ml of BoNT or toxoid (5 μg/ml) in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS for ELISA) at 37°C for 2 h, and blocked with 0.2 ml of 1% skim milk (Sigma) at 4°C overnight. After washing, 0.1 ml of purified mAb (1 μg/ml) was added to each well and incubated for 2 h at 37°C. After washing again, 0.1 ml of goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, Calif., USA) diluted 2,500-fold was added to each well for 2 h at 37°C. After further washing, 0.18 ml of a substrate solution (0.8 mg/ml 5-aminosalicylic acid and 0.05% H_2O_2, 9:1) was added to each well. After 45 min at 37°C, the reaction was terminated by adding 0.02 ml of 0.1 N NaOH. The reaction products were measured at 450 nm using a microplate reader (Model 680; Bio-Rad).

Competitive ELISA: The purified mAbs were biotinylated using EZ-LinkTM Sulfo-NHS-Biotin (Pierce) according to the manufacturer's instructions. Competitive ELISA was carried out according to a previously described method with some modifications (15). In order to examine the working concentration of biotinylated mAbs, serially diluted biotinylated mAbs were incubated in the ELISA plate coated with BoNT (1 μg/ml, 0.1 ml/well) for 2 h at 37°C. After washing, diluted peroxidase-labeled avidin (1:10,000; ZYMED) was added and the plate incubated at 37°C for 30 min. After washing, 150 μl of 0.4 mg/ml *o*-phenylenediamine (Nacalai Tesque, Kyoto, Japan) containing 0.05% H_2O_2 was added and reacted at 37°C for 30 min; 50 μl of 2.5 M H_2SO_4 was then added to stop the reaction, and absorbance was measured at 490 nm. When the absorbance was about 1.0, the concentration of biotinylated mAb was at the working concentration.

For competitive ELISA, the diluted biotinylated mAb (2 times the working concentration) was mixed with an equal volume of each diluted homologous or heterologous mAb (200 μg/ml, 20 μg/ml, 2 μg/ml), and ELISA was carried out as described above. The data are expressed as percent inhibition calculated by the following equation: [1-(OD_{490} in 200 μg/ml of mAb/OD_{490} in the absence of mAb)] $\times$ 100 (%).

Immunoblotting: SDS-PAGE was performed using a 10% gel under reducing conditions by the method of Laemmli (22), and the separated bands were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Mass., USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (25 mM Tris–HCl, pH 7.5, 137 mM NaCl), containing 0.05% Tween 20 (TBST). The separated bands were probed with mAbs diluted to 1 μg/ml with TBST for 1 h at room temperature. After washing, the membrane was incubated with the goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Bio-Rad), diluted 3,000-fold, for 30 min at room temperature, and then specific bands were visualized with a mixture of 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (Promega).

Immunoprecipitation: Each mAb (6 μg) was added to 25 μl of a 20% slurry of protein G-Sepharose (GE Healthcare) in 20 mM phosphate buffer, pH 6.0, containing 0.15 M NaCl (PBS), and the mixture was mixed by rotating at 4°C for 1 h. mAb-bound Protein G-Sepharose beads were washed 3 times with PBS by centrifugation at 2,300 $\times$ *g* for 2 min. BoNT, M toxin, or L toxin (0.6 μg, 1.2 μg, or 2 μg, respectively, in 0.3 ml) was added to mAb-bound Protein G-Sepharose beads and the mixture was mixed by rotating at 4°C for 1 h. After washing 3 times with PBS, 10 μl of sample buffer (2$\times$ SDS sample buffer, 0.1 M DTT) was added to the Sepharose beads and boiled for 3 min. After centrifugation at 2,300 $\times$ *g* for 1 min, the supernatant was subjected to SDS-PAGE. The toxin was detected by immunoblotting using rabbit anti-BoNT/A IgG.

Surface plasmon resonance (SPR): Rabbit anti-mouse IgG antibody (anti-ligand antibody, 30 μg/ml; GE Healthcare) was immobilized on the surface of CM5 sensor chips (GE Healthcare) according to the manufacturer's instructions. Mouse mAbs were captured on the sensor chips using the capture method. The kinetics of mAbs binding to BoNT/A1 and BoNT/A2 were measured using a Biacore X100 instrument (GE Healthcare) according to the manufacturer's instructions, and the data were analyzed using the BIAevaluation Data Analysis Software (GE Healthcare).

Other methods: Protein concentrations were determined by the Bradford method (23) for immunoglobulin or the Lowry method (24) for the other proteins, using bovine gamma globulin or BSA as standard, respectively. The toxicity of BoNT/A was determined as previously reported (20). To examine the neutralization of mAbs, BoNT/A diluted to 40 LD_{50}/ml with gelatin-phosphate buffer was mixed with an equal volume of each mAb (40 μg/ml) and incubated for 30 min at room temperature. Mice (4 weeks, ddY; Japan SLC, Hamamatsu, Japan) were injected intraperitoneally with 0.5 ml of the mixture and observed for 4 days.

RESULTS

Properties of mAbs: We obtained 51 and 25 positive clones for BoNT/A1 and BoNT/A2, respectively. We first determined the recognition region of each mAb by immunoblotting; mAbs reacting with H-chains, but not with H_C, were considered to recognize H_N. In subsequent experiments, we examined the reactivities of mAbs to BoNT by immunoprecipitation, and selected positive clones that were applicable for the detection of BoNT. By competitive ELISA, we selected 8 and 7 mAbs against BoNT/A1 (mAb-A1) and BoNT/A2 (mAb-A2), respectively, each of which recognized different epitopes. The subclass of the mAbs was IgG1 or IgG2a (Tables 1 and 2). Of the 8 mAb-A1s, 2 reacted to the L-chain, 3 to H_N, and the others to H_C (Fig. 1A). Of the 7 mAb-A2s, 3 reacted to L-chain, 2 to H_N, and the others to H_C (Fig. 1B).

We determined the amino acid sequences of the VH and VL of each mAb. Their amino acid sequences in the VH region were significantly different, except in the case of 6D9 against BoNT/A1 and 5C7 against BoNT/A2, which showed differences in only 2 amino

Table 1. Properties of mAbs raised against BoNT/A1

mAb[1]	ELISA (OD_{450})[2]			Blotting	Neutralization[3]		Subclass
	BoNT/A1	BoNT/A2	Toxoid/A1		BoNT/A1	BoNT/A2	
1D4	0.879	0.795	0.787	L	−	−	IgG_1
1E2	0.741	0.820	0.725	L	++	+	IgG_1
1B12	0.748	0.372	0.505	H_N	++	−	IgG_1
1F11	0.953	0.404	0.853	H_N	++	−	IgG_1
10H3	0.725	0.090	0.453	H_N	−	−	IgG_1
4E4	0.967	0.005	0.002	H_C	++	−	IgG_{2a}
6D9	0.565	0.235	0.151	H_C	−	−	IgG_1
9A3	0.643	0.040	0.051	H_C	++	−	IgG_1

[1]: MAbs were raised against BoNT/A1.
[2]: Values were obtained with each mAb at 1 μg/ml.
[3]: Mix mAb (10 μg/ml) 300 μl with BoNT/A (40 LD_{50}/ml) 300 μl in GPB, and incubate at room temperature for 30 min. Inject intraperitoneally 0.5 ml of the mixture to a mouse and monitor the mouse for 4 days. −, time to death ≤24 h; +, 24 h< time to death ≤96 h; ++, time to death >96 h.

Table 2. Properties of mAbs raised against BoNT/A2

mAb[1]	ELISA (OD_{450})[2]			Blotting	Neutralization[3]		Subclass
	BoNT/A1	BoNT/A2	Toxoid/A2		BoNT/A1	BoNT/A2	
3B10	0.063	0.824	0.305	L	−	++	IgG_1
4G12	0.745	0.805	0.432	L	−	+	IgG_1
9B3	0.614	0.894	0.466	L	−	−	IgG_1
2A12	1.185	0.969	0.404	H_N	−	−	IgG_1
5G2	0.025	0.539	0.222	H_N	−	++	IgG_1
5C7	0.601	0.591	0.085	H_C	−	−	IgG_1
6A5	0.015	0.865	0.338	H_C	−	+	IgG_{2a}

[1]: MAbs were raised against BoNT/A2.
[2],[3]: For footnotes, see Table 1, footnotes [2] and [3].

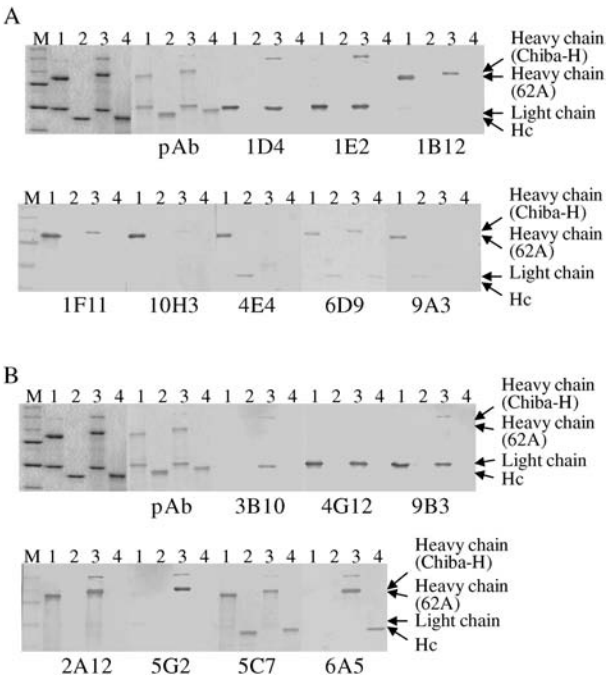

Fig. 1. Immunoblotting analyses of mAbs bound to different domains of BoNT/A. M, Marker; Lane 1, BoNT/A1, 0.6 μg, DTT+; Lane 2, Hc/A1, 0.2 μg, DTT+; Lane 3, BoNT/A2, 0.6 μg, DTT+; Lane 4, rHc/A2, 0.2 μg, DTT+. (A) mAbs were raised against BoNT/A1. (B) mAbs were raised against BoNT/A2.

acids. These differences suggest that each hybridoma was derived from a distinct B cell clone. Interestingly, the VL regions in all clones except 1D4, 1F11, and 5G2 were found to have identical sequences (Fig. 2).

Reactivity of mAbs with BoNT and toxoid: We determined the reactivity of each mAb with BoNT and toxoid using ELISA. Of the 8 mAb-A1s, 3 mAbs (10H3, 4E4, and 9A3) specifically reacted with the A1 subtype, and 2 mAbs (4E4 and 9A3) recognizing H_C reacted with BoNT/A1, but not with the toxoid. Five mAbs (1D4, 1E2, 1B12, 1F11, and 6D9) reacted with both the toxoid and BoNT/A2 (Table 1). Of the 7 mAb-A2s, 3 mAbs (3B10, 5G2, and 6A5) specifically reacted with BoNT/A2. 5C7, which recognized the H_C, reacted with both BoNT/A1 and BoNT/A2, but not with toxoid. Three mAbs (4G12, 9B3, and 2A12) reacted with both the toxoid and BoNT/A1 (Table 2).

We also investigated the neutralizing activity of each mAb against BoNT/A1 and BoNT/A2. Neutralizing antibody recognition sites were present in every domain (L-chain, H_N, and H_C) of both BoNTs. Neutralizing antibodies mostly neutralized only their immunogenic toxin, except 1E2, which neutralized both toxins (Tables 1 and 2).

To determine the affinity of the mAbs to A1 and A2 toxins, we measured the equilibrium dissociation constants (K_D) for the binding of each mAb to BoNT/A1 and BoNT/A2 using the Biacore X100. Five mAbs (1B12, 1F11, 4G12, 9B3, and 5C7), which cross-reacted with both toxins, showed different affinities between

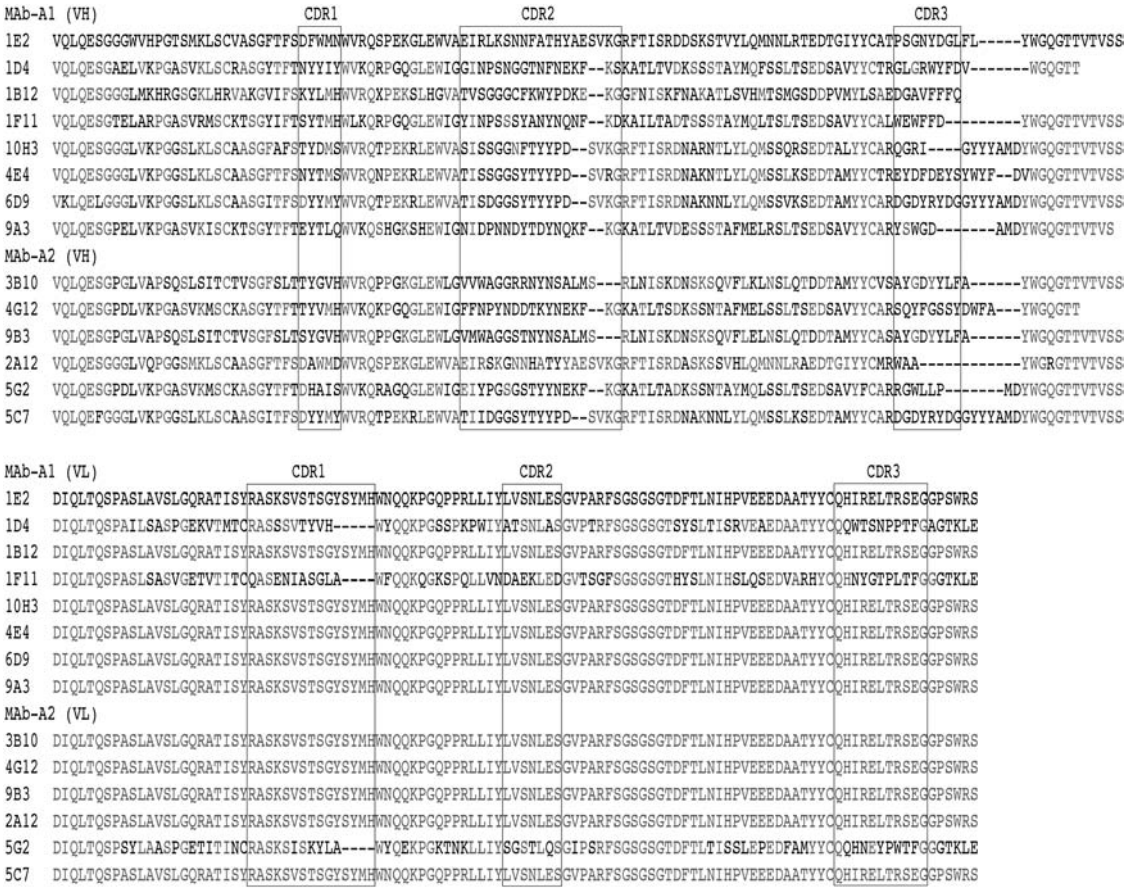

Fig. 2. Amino acid sequences of anti-BoNT mAb VH (upper) and VL (lower) regions. Homologous amino acid residuces are indicated gray letters. The complementarity determining regins (CDRs) are boxed. 6A5 was not determined.

Table 3. Equilibrium dissociation constants (K_D)[1] of BoNT/A1 and BoNT/A2 with mAbs against BoNT/A1

mAb[2]	K_D (M)[3] (BoNT/A1)	K_D (M)[4] (BoNT/A2)
1D4	3.8×10^{-9}	3.3×10^{-9}
1E2	9.2×10^{-10}	8.5×10^{-10}
1B12	2.5×10^{-9}	2.7×10^{-10}
1F11	6.2×10^{-10}	1.3×10^{-8}
10H3	1.3×10^{-9}	ND[5]
4E4	4.2×10^{-10}	ND
6D9	2.8×10^{-9}	3.2×10^{-9}
9A3	8.1×10^{-11}	ND

[1]: Association (k_{on}) and dissociation (k_{off}) rate constants were determined by surface plasmon resonance in biacore, and K_D was calculated as k_{off}/k_{on}.
[2]: MAbs were raised against BoNT/A1.
[3]: K_D for mAbs binding to BoNT/A1.
[4]: K_D for mAbs binding to BoNT/A2.
[5]: Not determinable.

Table 4. Equilibrium dissociation constants (K_D)[1] of BoNT/A1 and BoNT/A2 with mAbs against BoNT/A2

mAb[2]	K_D (M)[3] (BoNT/A1)	K_D (M)[4] (BoNT/A2)
3B10	ND[5]	8.9×10^{-10}
4G12	7.7×10^{-9}	3.7×10^{-8}
9B3	7.7×10^{-9}	1.5×10^{-8}
2A12	ND	ND
5G2	ND	8.7×10^{-8}
5C7	1.6×10^{-8}	2.7×10^{-9}
6A5	ND	2.7×10^{-9}

[1),3),4),5)]: For footnotes, see Table 3, footnotes 1), 3), 4), and 5).
[2]: MAbs were raised against BoNT/A2.

the 2 toxins (difference of approximately 2- to 21-fold). The K_D of 1B12 for BoNT/A1 was 9-fold higher than for BoNT/A2 (K_D for BoNT/A1: 2.5×10^{-9} M; K_D for BoNT/A2: 2.7×10^{-10} M). 1D4, 1E2, and 6D9 showed high affinity for both toxins (Tables 3 and 4).

The binding activity of the mAbs to complex toxins: To investigate the binding patterns of each domain of BoNT and non-toxic components in complex toxin, we examined the reactivity of mAbs and complex toxins (M toxin/A1, L toxin/A1, and M toxin/A2) by immunoprecipitation. In order to retain the structure of the complex toxins, all reactions were performed under a slightly acidic condition (pH 6.0) (Tables 5 and 6).

Of the 8 mAb-A1s, 2 mAbs (1D4 and 1E2), recognizing the L-chain, reacted with all of the complex toxins. Of the 3 mAbs recognizing H_N, 1F11 reacted with all of the complex toxins, but 1B12 and 10H3 did not react with any complex toxin. Of the 3 mAbs recognizing H_C, 4E4 and 9A3 did not react with any complex toxin, and 6D9 reacted with the M toxin, but not with the L toxin (Table 5).

Table 5. Binding of different forms of botulinum type A toxin with mAbs raised against BoNT/A1

mAb[1)]	Recognition domain	Immunoprecipitation[2)]				
		BoNT/A1	M toxin/A1	L toxin/A1	BoNT/A2	M toxin/A2
1D4	L	+	+	+	+	+
1E2	L	+	+	+	+	+
1B12	H_N	+	−	−	+	−
1F11	H_N	+	+	+	+	+
10H3	H_N	+	−	−	−	−
4E4	H_C	+	−	−	−	−
6D9	H_C	+	+	−	+	+
9A3	H_C	+	−	−	−	−

1): MAbs were raised against BoNT/A1.
2): Immunoprecipitation of botulinum toxin (4 nmol) with mAb (40 nmol) was done in 20 mM phosphate buffer, pH 6.0 containing 0.15 M NaCl.

Table 6. Binding of different forms of botulinum type A toxin with mAbs raised against BoNT/A2

mAb[1)]	Recognition domain	Immunoprecipitation[2)]				
		BoNT/A1	M toxin/A1	L toxin/A1	BoNT/A2	M toxin/A2
3B10	L	−	−	−	+	+
4G12	L	+	+	+	+	+
9B3	L	+	+	+	+	+
2A12	H_N	+	−	−	+	−
5G2	H_N	−	−	−	+	−
5C7	H_C	+	+	−	+	+
6A5	H_C	−	−	−	+	+

1): MAbs were raised against BoNT/A2.
2): For footnote, see Table 5, footnote 2).

Of the 7 mAb-A2s, 4G12 and 9B3, recognizing the L-chain, reacted with all of the complex toxins, and 3B10, which specifically recognized BoNT/A2, reacted with the M toxin of subtype A2. 2A12 and 5G2, recognizing H_N, did not react with any complex toxin. Of the 2 mAbs recognizing H_C, 5C7, which reacted with both BoNTs, bound to the M toxin, but not to the L toxin. Another mAb (6A5) specifically recognizing BoNT/A2 reacted, with the M toxin of the A2 subtype (Table 6).

DISCUSSION

In several previous studies, antitoxins were always prepared by immunizing animals with toxoid. However, the conformational structure of the toxoid is altered upon formalin treatment, and the antigenicity of the toxin is not necessarily maintained perfectly. In this study, we prepared mAbs using mice that were immunized several times with BoNT/A after basal immunization with toxoid. The reactivities of mAbs to BoNT/A and to the toxoid were investigated by ELISA. Some mAbs reacted with BoNT/A, but not with toxoid, and all of these mAbs recognized H_C. These mAbs specifically recognized native BoNT/A. This suggested that the antigenicity of H_C partially disappeared with formalin treatment (Tables 1 and 2).

We determined the amino acid sequences of the VH and VL of each mAb. Their amino acid sequences in the VH region were significantly different. These differences suggest that each hybridoma was derived from distinct B cell clones. The VL regions in all clones except 1D4, 1F11, and 5G2 have been identified as perfectly identical sequences (Fig. 2). Since their mAbs recognized different epitopes, this data suggests that the VH plays an important role in antigen-binding specificity.

In amino acid sequences, BoNT/A1 and BoNT/A2 were found to be 89.9% identical in the holotoxin, 95.1% in the L-chain, 87.1% in the H_N, and 87.2% in the H_C. Of the 126 amino acid differences between BoNT/A1 and BoNT/A2, 85% are exposed to solvent and 12% are partially exposed (16). We prepared whole antibodies that reacted with the denatured toxin by immunoblotting, suggesting that they recognize linear epitopes. Some of them specifically reacted with either BoNT/A1 or BoNT/A2 (Tables 1, 2, 5, and 6). We suggest that these antibody-binding sites contain different amino acid residues exposed on the surface of the BoNT/A molecule. In addition, we first obtained a mAb (3B10) reacting with the L-chain, which specifically recognized BoNT/A2 (Table 2). This mAb also reacted with the M toxin/A2 by immunoprecipitation, so it was considered useful as a detection antibody specific for the A2 subtype toxin (Table 6).

Neutralizing antibodies against BoNT/A1 that recognized the L-chain, H_N, or H_C have been reported (20,25,26). We generated neutralizing antibodies against BoNT/A2 that recognized the L-chain, H_N, or H_C (Table 2). Therefore, it is suggested that the epitopes of neutralizing antibodies are present in every domain of both subtypes. Each neutralizing antibody mostly neu-

tralized only toxins of their own subtype. However, 1E2, which could neutralize both toxins, has a common epitope involved in toxin neutralization (Tables 1 and 2). Interestingly, the K_D of 1B12 for BoNT/A1 was 9-fold higher than that for BoNT/A2 (Tables 3 and 4). However, 1B12 neutralized only BoNT/A1, suggesting that the neutralization of mAb did not correspond to its affinity, and that the common epitope plays different roles in toxicity and is blocked by mAb binding between the 2 toxins. For the binding of mAbs to BoNT/A, the K_D values obtained by SPR did not necessarily correlate with OD values obtained by ELISA (Tables 1, 2, 3, and 4). It is possible that there is a change in the conformational structure of BoNT/A upon immobilization on ELISA plates.

Chen et al. (27) examined the interaction between the complex toxin and some mAbs against BoNT/A to analyze the structure of the complex toxin. In these experiments, they analyzed the direct binding of mAbs to the complex toxin that had been coated on the ELISA plate; however, they had treated the complex toxin under mildly alkaline conditions before coating. Since the complex toxin easily dissociates into BoNT and the non-toxic components (28), it is doubtful to evaluate the interaction of mAbs with the native state of the complex toxin. Therefore, we investigated the reactivities of each mAb with complex toxins by immunoprecipitation under slightly acidic conditions (pH 6.0) to retain the complex structure (Tables 5 and 6). Our results were consistent, in that each mAb cross-reacting with both BoNTs showed cross-reactivity to the M toxin of both subtypes. This was considered to be consistent with the binding pattern of NTNH and BoNT in the M toxin of both subtypes. All mAbs reacting with the L-chain (1D4, 1E2, 3B10, 4G12, and 9B3) always reacted with BoNT/A whether or not it was related to the complex form. This suggested that the epitopes of the mAbs reacting with the L-chain are exposed to the molecular surface even in complex toxins. Of the mAbs reacting with H_N, 1F11 reacted with all of the complex toxins, but the other mAbs (1B12, 10H3, 2A12, and 5G2) did not react with any of the complex toxins. This indicates that the non-toxic components structurally block some antibody binding sites, therby inhibiting the binding of these antibodies. Of the mAbs reacting with H_C, 4E4 and 9A3 did not react with any complex toxin. However, 6D9 and 5C7 reacted with the M toxin, but not with the L toxin. This indicates that the epitopes of some mAbs reacting with H_C are covered by a non-toxic component in the M toxin, and that all of them were covered by non-toxic components in L toxin. From these results, we expected that the L-chain of BoNT/A is exposed to the molecular surface of each complex toxin, that some regions of H_N and H_C are covered with a non-toxic component (NTNH) in the M toxin, and that some regions of H_N and the whole region of H_C are covered by non-toxic components (NTNH and HA) in the L toxin.

Conflict of interest None to declare.

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