INFECTION AND IMMUNITY, Sept. 2005, p. 5450–5457 Vol. 73, No. 9
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.9.5450–5457.2005
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Sequence Variation within Botulinum Neurotoxin Serotypes Impacts Antibody Binding and Neutralization

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Received 22 October 2004/Returned for modification 25 January 2005/Accepted 26 April 2005

The botulinum neurotoxins (BoNTs) are category A biothreat agents which have been the focus of intensive efforts to develop vaccines and antibody-based prophylaxis and treatment. Such approaches must take into account the extensive BoNT sequence variability; the seven BoNT serotypes differ by up to 70% at the amino acid level. Here, we have analyzed 49 complete published sequences of BoNTs and show that all toxins also exhibit variability within serotypes ranging between 2.6 and 31.6%. To determine the impact of such sequence differences on immune recognition, we studied the binding and neutralization capacity of six BoNT serotype A (BoNT/A) monoclonal antibodies (MAbs) to BoNT/A1 and BoNT/A2, which differ by 10% at the amino acid level. While all six MAbs bound BoNT/A1 with high affinity, three of the six MAbs showed a marked reduction in binding affinity of 500- to more than 1,000-fold to BoNT/A2 toxin. Binding results predicted in vivo toxin neutralization; MAbs or MAb combinations that potently neutralized A1 toxin but did not bind A2 toxin had minimal neutralizing capacity for A2 toxin. This was most striking for a combination of three binding domain MAbs which together neutralized >40,000 mouse 50% lethal doses (LD_{50}s) of A1 toxin but less than 500 LD_{50}s of A2 toxin. Combining three MAbs which bound both A1 and A2 toxins potently neutralized both toxins. We conclude that sequence variability exists within all toxin serotypes, and this impacts monoclonal antibody binding and neutralization. Such subtype sequence variability must be accounted for when generating and evaluating diagnostic and therapeutic antibodies.

Botulism is caused by botulinum neurotoxin (BoNT) produced by members of the genus *Clostridium* and is characterized by flaccid paralysis, which, if not rapidly fatal, requires prolonged hospitalization in an intensive care unit and mechanical ventilation. Naturally occurring botulism is found in infants or adults whose gastrointestinal tracts become colonized by neurotoxigenic clostridia (infant or intestinal botulism), after ingestion of contaminated food products (food botulism), or in anaerobic wound infections (wound botulism) (10). BoNTs are also classified by the Centers for Disease Control and Prevention as one of the six highest-risk threat agents for bioterrorism (the "category A agents") due to their extreme potency and lethality, ease of production and transport, and need for prolonged intensive care (3). Both Iraq and the former Soviet Union produced BoNT for use as weapons (8, 53), and the Japanese cult Aum Shinrikyo attempted to use BoNT for bioterrorism (3). As a result of these threats, specific pharmaceutical agents are needed for prevention and treatment of intoxication.

No specific small-molecule drugs for prevention or treatment of botulism exist, but an investigational pentavalent toxoid vaccine is available from the Centers for Disease Control and Prevention (45), and a recombinant vaccine is under development (46). Regardless, mass civilian or military vaccination is unlikely, due to the rarity of disease or exposure and the fact that vaccination would prevent subsequent medicinal use of BoNT. Postexposure vaccination is useless, due to the rapid onset of disease. Toxin-neutralizing antibody (Ab) can be used for pre- or postexposure prophylaxis or for treatment (14). Small quantities of both equine antitoxin and human botulinum immunoglobulin (Ig) exist and are currently used to treat adult (7, 19) and infant (4) botulism, respectively. Recombinant monoclonal antibody (MAb) could provide an unlimited supply of antitoxin free of infectious disease risk and not requiring human donors for plasmapheresis. Given the extreme lethality of the BoNTs, MAbs must be of high potency in order to provide an adequate number of doses at reasonable cost. The development of such MAbs has become a high-priority research aim of the National Institute of Allergy and Infectious Diseases (http://www2.niaid.nih.gov/Biodefense/Research/high_priority.htm). While no single highly potent MAbs have been described to date, we recently reported that combining two to three MAbs could yield highly potent BoNT neutralization (39).

The development of MAb therapy for botulism is complicated by the fact that there are seven BoNT serotypes (A to G) (16) that show little, if any, antibody cross-reactivity. While only four of the BoNT serotypes generally cause human disease (A, B, E, and F), there has been one reported case of infant botulism caused by BoNT serotype C (BoNT/C) (40), one outbreak of food-borne botulism linked to BoNT/D (11), and several cases of suspicious deaths where BoNT/G was isolated (47). Aerosolized BoNT/C, BoNT/D, and BoNT/G

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TABLE 1. Clostridial strains used in sequence analyses

Serotype	Subtype	Strain	Accession no.[b]	Reference or source
A	A1	NCTC 2916	X52066	50
		62A	M30196	6
		ATCC 3502		M. Peck, unpublished
		Hall hyper	AF461540	12
		Hall Allergan[a]	AF488749	58
	A2	Kyoto-F	X73423	57
		FRI-H1A2	AY953275	M. Bradshaw et al., unpublished
B	B1	Danish	M81186	56
		BGB		25
		Okra		21
	B2	Strain 111	AB084152	21
	Nonproteolytic B	Eklund 17B	X71343	20
	Bivalent B	CDC 588	AF300465	23
		CDC 593	AF300466	23
		CDC 1436	AF295926	23
		CDC 3281	Y13630	44
C	C1	Stockholm	X66433	18
			X62389	22
		C 468	X72793	17
		Yoichi	AB061780	42
	C/D	6813	D49440	36
		6814	AB037166	
		TW/2003	AY251553	
D	D	BVD/−3	X54254	5
		CB-16	S49407	48
		1873	AB012112	38
	D/C	South Africa	D38442	36
		D 4947	AB037920	26
E	E botulinum	NCTC 11219	X62683	55
		Beluga	X62089	41
		35396	AB082519	48 52
	E butyricum	BL5262, BL6340	X62088	41
			X62088	
		BL5520	Q9FAR6	54
		KZ 1886	AB037708	54
		KZ 1887	AB037709	54
		KZ 1889	AB037710	54
		KZ 1890	AB037711	54
		KZ 1891	AB037712	54
		KZ 1897	AB037706	54
		KZ 1898	AB037707	54
		KZ 1899	AB037705	54
		LCL 063	AB037713	54
		LCL 095	AB037714	54
		LCL 155	AB037704	54
F	Proteolytic F	Langeland	X81714	20
			L35496	M. J. Elmore, unpublished
	Nonproteolytic F	Eklund 202F	M92906	13
	F baratii	ATCC 43756	X68262	51
	Bivalent F	CDC 3281 (Bf)	Y13631	44
G	G	1113/30	X74162	9

[a] Hall Allergan (GenBank accession no. AF488749) was obtained from Ed Schantz' lab at the University of Wisconsin—Madison and is likely the same strain as Hall hyper (accession no. AF61540).
[b] Accession numbers are from the NCBI databases.

have also been shown to produce botulism in primates by the inhalation route (34) and would most likely also affect humans. Thus, it is likely that any one of the seven BoNT serotypes can be used as a biothreat agent.

Variability of the BoNT gene and protein sequence within serotypes has also been reported, and there is evidence that such variability can affect the binding of monoclonal antibodies to BoNT/A (15, 29). The extent of such toxin variability within the different serotypes, or its impact on the binding and neutralization capacity of monoclonal antibody panels, is currently

TABLE 2. Classification of *Clostridium botulinum* neurotoxin gene sequences[a]

Serotype	No. of complete sequences	No. of partial sequences	No. of subtypes	Minimum and maximum amino acid differences within serotype (%)
A	7		2	10.1
B	9	3	4	3.6–7.7
C	6		2	24.0–24.2
D	5		2	23.7–23.9
E	17	6	3	2.6–5.1
F	4	2	4	10.7–31.6
G	1		1	
Total	49	11	18	

[a] Subtypes were defined as differing by at least 2.6% at the amino acid level.

not clear. For this work, we determined the extent of BoNT toxin variability within serotypes by compiling and analyzing all available published BoNT sequences. We then determined the ability of a panel of six BoNT/A MAbs to bind to, and neutralize, the two different types of BoNT/A, A1 and A2. The results have important implications for the generation of diagnostic and therapeutic BoNT antibodies as well as vaccines.

MATERIALS AND METHODS

Toxin gene sequences. The NCBI databases and Medline were searched to identify published or archived sequences of botulinum neurotoxin genes or proteins. The neurotoxin gene of clostridial strain FRI-H1A2 was sequenced for this work (unpublished data) (GenBank accession number AY953275). The neurotoxin gene sequence of clostridial strain NCTC 3502 was a gift of Michael Peck. Gene sequences were entered into Vector NTI (Invitrogen, San Diego, CA), translated, classified by serotype, and aligned. Phylogenetic trees were constructed using ClustalW.

Toxins and antibodies. Purified pure and complexed botulinum neurotoxins A1 (Hall hyper) and A2 (FRI-H1A2) were purchased from Metabiologics Inc. (Madison, WI). Antibodies S25 and C25 were derived from a single-chain Fv phage display library constructed from the V genes of a mouse immunized with recombinant BoNT/A1 C-terminal heavy chain (H_C) and boosted with BoNT/A1 toxin (1, 39). Antibody 3D12 was derived from a single-chain Fv phage display library constructed from the V genes of a human volunteer donor immunized with investigational pentavalent toxoid (2, 39). The subtype of BoNT/A in the toxoid is known to be BoNT/A1. Antibody B4 was derived from a single-chain Fv phage display library constructed from the V genes of a mouse transgenic for the human immunoglobulin locus (Xenomouse) immunized with recombinant BoNT/A1 H_C (I. Geren and J. D. Marks, unpublished data). The V genes of each of these four antibodies were cloned into a mammalian expression vector containing human IgG1 and kappa constant regions as previously described (39). Stable CHO DG44 cell lines were established, and IgG was purified using protein G as previously described (39). Antibody purity and concentration were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and absorbance at 280 nm. Antibodies 9D8 (murine IgG1/kappa) and 7C1 (murine IgG1/kappa) were derived from hybridomas generated from mice immunized with recombinant BoNT/A H_C and boosted with BoNT/A toxin. IgG was purified from hybridoma supernatants using protein G, and purity and concentration were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and bicinchoninic acid assay (Pierce Chemical Co.). For subsequent studies, IgG antibodies were stored in phosphate-buffered saline, pH 7.4, at approximately 1 to 3 mg/ml.

Toxin capture ELISA. For toxin capture enzyme-linked immunosorbent assay (ELISA), 96-well microtiter plates (Immunolon 2; Dynatech) were coated with antibody at 2 μg/ml overnight at 4°C. After blocking for 30 min in 5% skim milk–phosphate-buffered saline, toxins were applied in half-log dilutions from 100 nM to 1 pM in duplicate and incubated for 90 min at 37°C. Plates were washed and incubated with equine anti-BoNT/A antibody (PerImmune), diluted to 0.2 IU/ml, for 60 min followed by washing and incubation with a 1:1,000 dilution of goat anti-horse antibody conjugated to horseradish peroxidase (KPL) for 60 min. Plates were developed with ABTS [2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid)] (KPL). Average absorbance at 405 nm after subtraction of background control was plotted against toxin concentration.

Measurement of antibody affinity for toxin. IgG association and dissociation rate constants for purified BoNT/A1 and BoNT/A2 toxins were measured using surface plasmon resonance with a BIAcore 1000 (Pharmacia Biosensor) and used to calculate the K_D as previously described (39). Briefly, approximately 100 to 400 response units of purified IgG (10 to 20 μg/ml in 10 mM acetate, pH 3.5 to 4.5) was coupled to a CM5 sensor chip using NHS-EDC [*N*-hydroxysuccinate and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide] chemistry. The association rate constant for purified BoNT/A1 and BoNT/A2 neurotoxins was measured under continuous flow of 15 μl/min using a concentration range of 50 nM to 800 nM toxin. Association rate constant (k_{on}) was determined from a plot of [ln(dR/dt)]/t versus concentration, where dR is the derivative of response and dt is the derivative of time. The dissociation rate constant (k_{off}) was determined from the dissociation part of the sensorgram at the highest concentration of toxin analyzed using a flow rate of 30 μl/min to prevent rebinding. K_D was calculated as k_{off}/k_{on}.

Measurement of in vivo toxin neutralization. Fifty micrograms of the appropriate IgG was added to the indicated number of mouse 50% lethal doses (LD_{50}s) of BoNT/A1 neurotoxin complex (Hall strain) or BoNT/A2 neurotoxin complex (FRI-H1A2 strain) in a total volume of 0.5 ml of gelatin phosphate buffer and incubated at room temperature for 30 min. Both BoNT/A1 and BoNT/A2 complex toxins were titrated to determine the LD_{50}. Average specific activities of the two toxins were as follows: 3.6×10^7 LD_{50}s/mg ($n = 2$) for the Hall complex and 7.5×10^7 LD_{50}s/mg ($n = 2$) for the FRI-H1A2 complex. Since the molecular weight of the BoNT/A1 complex is ~500,000 Da and that of the A2 complex is ~300,000 Da, that would be 1.8×10^{16} and 2.25×10^{16} LD_{50}s per mole for the two toxins, respectively. Thus, BoNT/A1 and BoNT/A2 toxins are approximately equally potent.

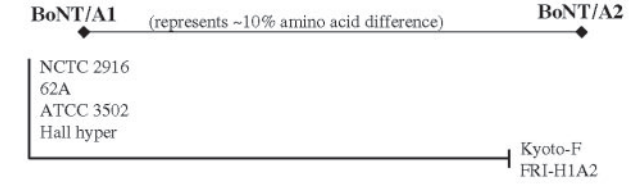
For pairs of MAbs, 25 μg of each MAb was added, and for the combination of three MAbs, 16.7 μg of each MAb was added. Stoichiometric ratios of total antibody to toxin were 500:1 at 10,000 LD_{50}s of toxin. The mixture was then injected intraperitoneally into female CD-1 mice (16 to 22 g on receipt). Mice were studied in groups of 10 and were observed at least daily. The final death tally was determined 5 days after injection. Studies using mice were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals of the National Research Council (38a). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

RESULTS

Sequence variation within botulinum neurotoxin serotypes. To determine the extent of sequence variability within toxin serotypes, the literature was searched, revealing 60 published neurotoxin sequences. These data included 49 complete toxin gene sequences and 11 partial toxin gene sequences (Table 1). The 49 complete sequences were classified by serotype and aligned, and the extent of sequence identity was determined (Table 2). Of the 49 sequences analyzed, there were 7 BoNT/A, 9 BoNT/B, 6 BoNT/C, 5 BoNT/D, 17 BoNT/E, 4 BoNT/F, and 1 BoNT/G sequences. Within serotypes, two types of toxin gene sequences were observed, those that were virtually identical to each other (see below) and those that differed by at least 2.6% at the amino acid level. Such sequence variability was observed within all six serotypes where more than one toxin gene had been sequenced (BoNT serotypes A, B, C, D, E, and F). Within serotypes, variability ranged from a high of 32% for BoNT/F to a low of 2.6% for BoNT/E (Table 2). Three BoNT/C/D and two BoNT/D/C mosaic strains were sequenced. These strains typically contained light chains and N-terminal heavy chains that matched their parental serotype, with the terminal third of the neurotoxin sequence having strong, but not absolute, identity with the alternative serotype of the mosaics (Table 1).

The two toxin serotypes causing more than 90% of human

A



B Percent amino acid identity between BoNT/A1 and BoNT/A2

	Holotoxin	Light chain	Heavy chain	H_N	H_C
BoNT/A1 vs BoNT/A2	89.9	95.1	87.1	87.1	87.2

C

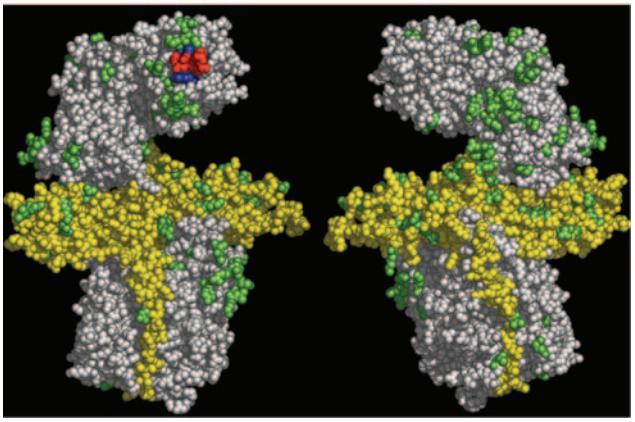
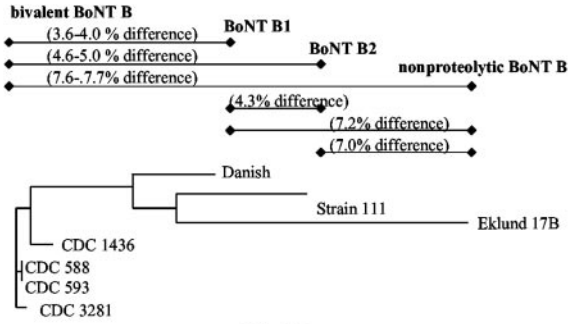


FIG. 1. Analysis of BoNT/A gene sequences. (A) Phylogenetic tree of BoNT/A genes reveals two clusters, A1 and A2. (B) Homology of the different domains of BoNT/A1 and BoNT/A2. (C) Model of the amino acid side chain differences between BoNT/A1 and BoNT/A2. Side-chain differences between BoNT/A2 and BoNT/A1 were modeled into the X-ray crystal structure of BoNT/A1 (3BTA [31]) using Modeler (43) in the Insight suite (Accelrys, San Diego, CA). Two different views of the toxin are shown rotated 180° about the *y* axis (Pymol; Delano Scientific, South San Francisco, CA). The BoNT/A heavy-chain binding domain is in white at the top of the figure, with the putative ganglioside binding residues (3BTA E1202, H1252, and W1265) in blue with the ganglioside (red) modeled by superposition using the O suite program LSQMAN (24) from the BoNT/B crystal structure (1F31 [49]). The root mean square deviation between the three ganglioside binding residue C-α positions from BotNT/A and BotNT/B is 0.22 Å. These residues are in the C-terminal subdomain, for which the root mean square deviation between the binding domain structures (3BTA residues 1091 to 1295 and 1F31 residues 1092 to 1290) is 1.39 Å. The heavy-chain translocation domain is in orange and the light-chain catalytic domain is in white at the bottom of the figure. Side-chain differences between BoNT/A1 and BoNT/A2 toxins are shown in green.



Percent Amino Acid Identity Among BoNT B Strains

	holotoxin	light chain	heavy chain	H_N	H_C
BoNT B1 vs:					
BoNT B2	95.7	99.5	93.6	95.8	91.8
BoNT B np	92.8	97.7	90.2	92.5	88.2
BoNT B bivalent	96.0-96.4	98.9-99.1	94.6-94.9	94.3-95.0	94.7-94.9

FIG. 2. Analysis of BoNT/B gene sequences. Phylogenetic tree of BoNT/B genes reveals four clusters: BoNT/B1, BoNT/B2, nonproteolytic BoNT/B, and bivalent BoNT/B. Percent differences between clusters range from 3.6 to 7.7%. As with BoNT/A, the greatest differences are seen in the heavy chain.

botulism (BoNT/A and BoNT/B [10]) were analyzed in more detail. Of the seven published BoNT/A toxin sequences, five (62A, NCTC 2916, ATCC 3502, and Hall hyper [Hall Allergan]) were virtually identical (99.9 to 100% identity) and have been classified as subtype A1 (Fig. 1A). The other two BoNT/A sequences (Kyoto-F and FRI-H1A2) were 100% identical and have been classified as subtype A2 (Fig. 1A). The A1 toxins differed from the A2 toxins by 10.1%, with the greatest difference in sequence in the receptor binding domain (H_C). (Fig. 1B). Of the 126 amino acid differences between BoNT/A1 and BoNT/A2, 85% are solvent exposed, 12% are partially exposed, and 2.4% are completely buried based on molecular surface calculations per residue with Pymol (DeLano Scientific, San Carlos, CA). Besides being greater in number, the H_C amino acid differences tended to be located in solvent-accessible amino acids exposed on the toxin surface (Fig. 1C). A number of these differences clustered around the putative ganglioside binding site (Fig. 1C). The sequence of the catalytic domain (light chain) was more conserved (Fig. 1B), with the differences more likely to be buried (Fig. 1C). The nine published BoNT/B sequences could be grouped into four subtypes based on DNA and protein homology (Fig. 2). These groups included the bivalent BoNT/B (BoNT Ab 1436, BoNT Ab 588, BoNT Ab 593, and BoNT Bf 3281), BoNT/B1 (BoNT/B Danish), BoNT/B2 (BoNT/B strain 111), and the nonproteolytic BoNT/B (BoNT/B Eklund 17B). These toxins differed from each other by 3.6% to 7.7% at the amino acid level, with greater differences in the heavy chain than in the light chain (Fig. 2).

Impact of BoNT/A toxin sequence variation and antibody binding. To determine the impact of BoNT/A toxin sequence variability on immune recognition, we measured the ability of six monoclonal antibodies raised against BoNT/A1 to bind to BoNT/A1 and BoNT/A2 by capture ELISA. Binding to both pure neurotoxin and neurotoxin complex was determined. Four MAbs (3D12, C25, B4, and S25) bound to nonoverlapping epitopes on the BoNT/A H_C, as determined by ELISA on recombinant H_C. 3D12 and S25 have been previously epitope mapped to the C-terminal subdomain of BoNT/A H_C, while C25 recognizes a complex epitope formed by the two H_C subdomains (37). One MAb (9D8) bound the BoNT/A translocation domain (H_N) as determined by ELISA on recombinant H_N (data not shown). One MAb (7C1) bound the BoNT/A light chain, as determined by ELISA on recombinant light chain.

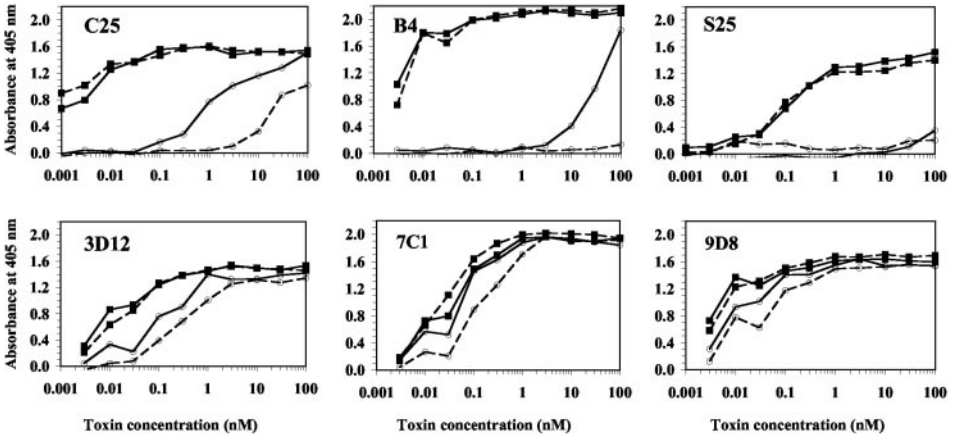


FIG. 3. Binding of BoNT/A monoclonal antibodies to BoNT/A1 and BoNT/A2 toxins as determined by capture ELISA. Wells were coated with the indicated MAb followed by various concentrations of pure or complex BoNT/A1 or BoNT/A2. Toxin binding was detected using polyclonal equine BoNT/A antisera. A1 toxins are indicated by solid squares; A2 toxins are indicated by open circles. Pure toxins are solid lines; toxin complexes are dashed lines. Differences in binding signals for MAbs C25 and B4 between A1 and A2 pure and complex toxins may reflect the presence of different sets of accessory proteins (reference 30 and see the text).

Three of the four antibodies which bound the BoNT/A H_C showed a marked reduction in binding to BoNT/A2 toxin compared to BoNT/A1 toxin (Fig. 3). In contrast, the two non-H_C-binding MAbs showed comparable ELISA signals on both A1 and A2 toxins (Fig. 3). Binding signals were generally comparable for binding to pure versus complex neurotoxin, except for the binding of MAbs C25 and B4 to pure BoNT/A2 compared to complex A2. These binding differences may reflect a blockade of toxin epitopes in the A2 complex toxin by the accessory proteins. Since A1 toxin complex does not share an identical set of accessory proteins, it is possible that the same epitopes are not blocked on the A1 complex toxin, thus explaining the binding data. While it is likely that observed differences in binding to BoNT/A1 compared to that of BoNT/A2 reflected differences in the relative affinities of the capture MAb for the two toxins subtypes, we cannot exclude differential binding of the detection polyclonal antibody. To more accurately quantitate the difference in binding to A1 and A2 toxins, the equilibrium dissociation constant and binding rate kinetics were measured for the binding of each MAb to purified A1 and A2 toxins (Table 3). All MAbs bound A1 toxin with high affinity (K_D ranging between 6 and 0.17 nM). The three MAbs which demonstrated decreased binding to A2 toxin by capture ELISA (C25, S25, and B4) showed a 553- to more than 1,200-fold reduction in affinity for A2 toxin compared to A1 toxin. It was not possible to measure a K_D for the B4 MAb binding to BoNT/A2 due to very-low-affinity binding. The majority of the reduction in affinity was due to a large decrease in the association rate constant (Table 3). In contrast, three MAbs (3D12, 9D8, and 7C1) showed comparable high affinity for both A1 and A2 toxins.

Impact of antibody binding on neutralization of A1 and A2 neurotoxins. We previously studied the in vivo neutralization capacity of three MAbs described here, 3D12, S25, and C25, for BoNT/A1 toxin. Despite showing significant in vitro neutralization of BoNT/A1, none of these three MAbs showed significant in vivo protection of mice receiving 50 μg of antibody and challenged with 20 mouse LD_{50}s of BoNT/A1 (only 10 to 20% survival [39]). Similarly, none of the remaining three MAbs reported here showed significant in vivo protection when mice were challenged with 20 mouse LD_{50}s of BoNT/A1 (only 10 to 20% survival [data not shown]). Since we previously reported significant synergy in in vivo protection when MAbs were combined, we studied the ability of MAb pairs and triplets to neutralize toxin in vivo. As previously observed, antibody pairs showed significantly greater BoNT/A1 neutralization than single MAbs, with even greater potency observed for combinations of three MAbs (Fig. 4 and 5A). Synergy was

TABLE 3. Association (k_{on}) and dissociation (k_{off}) rate constants and equilibrium dissociation constants (K_D) for BoNT/A IgG binding to BoNT/A1 and BoNT/A2[a]

Antibody	BoNT/A1			BoNT/A2		
	K_D (M^{-1})	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M^{-1})	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
C25	2.98×10^{-10}	1.5×10^{6}	4.47×10^{-4}	1.65×10^{-7}	2.09×10^{4}	3.63×10^{-3}
S25	1.69×10^{-9}	4.82×10^{5}	8.15×10^{-4}	2.14×10^{-6}	1.34×10^{3}	2.87×10^{-3}
3D12	1.68×10^{-10}	1.45×10^{6}	2.44×10^{-4}	1.04×10^{-9}	3.48×10^{5}	3.62×10^{-4}
B4	1.8×10^{-9}	7.2×10^{5}	1.31×10^{-3}	NM	NM	NM
7C1	5.9×10^{-9}	2.89×10^{5}	1.71×10^{-3}	5.1×10^{-9}	3.38×10^{5}	1.73×10^{-3}
9D8	1.21×10^{-9}	1.73×10^{5}	2.11×10^{-4}	1.3×10^{-9}	2.08×10^{5}	2.73×10^{-4}

[a] Association and dissociation rate constants were determined by surface plasmon resonance in a BIAcore, and K_D was calculated as k_{off}/k_{on}. NM, not measurable.

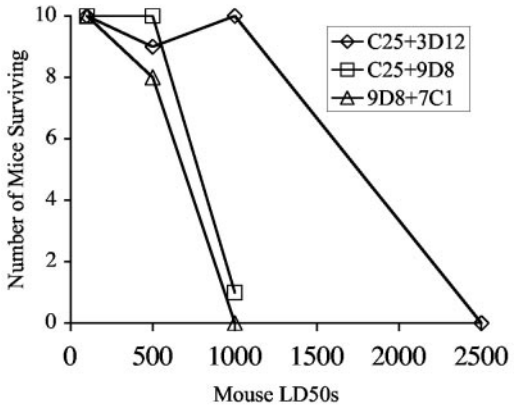


FIG. 4. Ability of MAb pairs to protect mice challenged with BoNT/A1 toxin. A range of mouse LD_{50}s of BoNT/A1 toxin complex was mixed with 50 μg of an equimolar ratio of the indicated MAbs, and the mixture was injected intraperitoneally. The number of mice surviving versus challenge dose is indicated.

observed for MAb pairs that included only one binding domain antibody (C25 + 9D8) or no binding domain antibodies (9D8 + 7C1) (Fig. 4) or combinations of three MAbs that included only one binding domain antibody (C25 + 9D8 + 7C1) (Fig. 5A). With respect to neutralization of BoNT/A2 toxin, only MAb pairs or triplets containing MAbs which bound BoNT/A2 with high affinity showed significant synergy for neutralization (Fig. 5B). The most potent MAb triplet (3D12 + 9D8 + 7C1) was able to completely protect mice from a challenge of 10,000 mouse LD_{50}s of A1 or A2 toxin. While this combination (3D12 + 9D8 + 7C1) was not as potent for neutralization of A1 toxin as a combination of three binding domain MAbs (C25 + 3D12 + B4), only one binding domain MAb bound A2 toxin with any affinity, and as a result, the C25 + 3D12 + B4 triplet neutralized less than 200 mouse LD_{50}s of A2 toxin.

DISCUSSION

Analysis of 49 complete published botulinum neurotoxin sequences revealed that within serotypes, toxin gene sequences were either virtually identical or differed from each other by at least 2.6% at the amino acid level. We have termed those toxins with this minimum difference (2.6%) to be subtypes of a given serotype. Such analysis revealed an average of 2.8 subtypes for the six serotypes where more than one toxin gene has been sequenced (range, 2 to 4 subtypes/serotype). While this analysis probably reveals the most frequent toxin subtypes, it is likely that additional toxin subtypes remain to be identified, given the relatively small number of toxin genes sequenced (on average, 8 toxin genes/serotype).

The importance of toxin subtypes is their impact on diagnostic tests and the development of toxin therapeutics. Clearly, this level of nucleotide polymorphism can affect DNA probe-based assays such as PCR. Importantly, the extent of amino acid substitution can affect the binding of monoclonal antibodies used for ELISA and other immunologically based diagnostic tests. We have clearly shown that the 10% amino acid difference between BoNT/A1 and BoNT/A2 subtypes has a dramatic effect on the binding affinity and ELISA signals of three of six monoclonal antibodies analyzed. Interestingly, the kinetic basis for the reduced MAb affinity is largely due to a decrease in the association rate constant rather than an increase in the dissociation rate constant. The impact of the difference in toxin amino acid sequence on the binding of polyclonal antibody is unknown. Clearly, toxin assays based on immunologic recognition will need to be validated using the different toxin subtypes.

The differences in binding affinity translate into significant differences in the potency of in vivo toxin neutralization. Since we have not observed potent in vivo toxin neutralization by single MAbs, we studied the impact of toxin sequence variation on the potency of MAb combinations. As with the binding studies, only MAb combinations binding tightly to both A1 and A2 subtypes potently neutralized toxin in vivo. Thus, the impact of subtype variability on potency must be evaluated in the development of antibody-based toxin therapy, whether such therapy is oligoclonal or polyclonal. Similarly, toxin vaccines based on a single subtype may need to be evaluated for their ability to protect against related subtypes.

An unexpected finding in these studies was that MAbs bind-

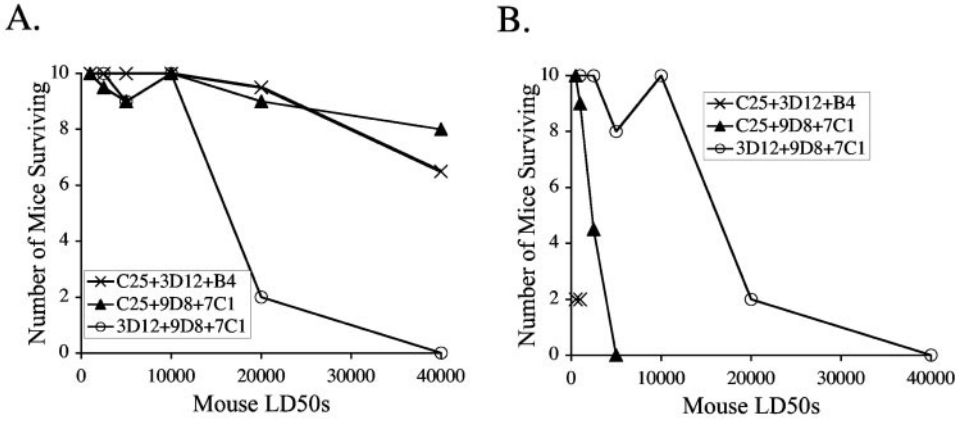


FIG. 5. Ability of MAb triplets to protect mice challenged with BoNT/A1 or BoNT/A2 toxins. A range of mouse LD_{50}s of BoNT/A1 toxin complex (A) or BoNT/A2 toxin complex (B) was mixed with 50 μg of an equimolar ratio of the indicated MAbs, and the mixture was injected intraperitoneally. The number of mice surviving versus challenge dose is indicated.

ing to the translocation domain and/or catalytic domains of BoNT had neutralizing activity, either when combined with each other or when combined with a MAb recognizing the BoNT receptor binding domain (H_C). Neutralizing activity has also been reported for MAbs binding the catalytic domain of tetanus toxin (32) and ricin (33). Since MAbs which do not bind to the BoNT receptor binding domain cannot strictly block the interaction of BoNT binding domain epitopes to cellular receptors and subsequent BoNT endocytosis, the mechanism by which they contribute to neutralization remains unknown. Possibilities include enhancement of BoNT clearance from the circulation upon binding of multiple MAbs (35), interference of receptor binding by a steric effect, interference with required intracellular toxin processes (endosomal escape or catalytic activity) (25), and/or altering intracellular BoNT trafficking. Regardless of the mechanism, the ability of non-binding-domain MAbs to neutralize toxin significantly increases the number of epitopes available for neutralizing MAb generation, increasing the likelihood of finding MAbs binding and neutralizing all BoNT subtypes.

While we only studied the impact of sequence variability on antibody binding and neutralization for a single serotype (BoNT/A), three serotypes (BoNT/C, BoNT/D, and BoNT/F) have subtypes which differ from each other by more than the 10% difference between BoNT/A1 and BoNT/A2 (10.7% to 31.6%). For these three serotypes, the impact of sequence variability on immune recognition is likely to be greater than that for BoNT/A. For two serotypes (BoNT/B and BoNT/E), sequence variability was less than that observed for BoNT/A (2.6% to 7.6%). The impact of this level of sequence variability will need to be evaluated but is clearly in a range that could affect MAb binding, as shown in previous evaluations of MAb binding to BoNT/B toxin (15, 28) and BoNT/E (27).

In conclusion, we report the existence of considerable sequence variability within six of the seven botulinum neurotoxin serotypes and show that this level of variability can significantly affect antibody binding and neutralization. Determining the full extent of such toxin diversity is an important first step in the development of immunological botulinum toxin assays, therapeutics, and vaccines. Once the sequence variability has been defined, it is likely that some number of these toxin variants will need to be produced for validation of detection assays, therapeutics, and vaccines.

ACKNOWLEDGMENTS

This work was partially supported by Department of Defense contract DAMD17-03-C-0076 and by National Institute of Allergy and Infectious Diseases cooperative agreement U01 AI056493. Funding for S.L.L. was through an American Cancer Society postdoctoral fellowship.

REFERENCES

1. **Amersdorfer, P., C. Wong, S. Chen, T. Smith, S. Desphande, R. Sheridan, R. Finnern, and J. D. Marks.** 1997. Molecular characterization of murine humoral immune response to botulinum neurotoxin type A binding domain as assessed using phage antibody libraries. Infect. Immun. **65:**3743–3752.
2. **Amersdorfer, P., C. Wong, T. Smith, S. Chen, S. Deshpande, R. Sheridan, and J. D. Marks.** 2002. Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. Vaccine **20:**1640–1648.
3. **Arnon, S. A., R. Schecter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow, and K. Tonat.** 2001. Botulinum toxin as a biological weapon. JAMA **285:** 1059–1070.
4. **Arnon, S. S.** 1993. Clinical trial of human botulism immune globulin, p. 477–482. *In* B. R. DasGupta (ed.), Botulinum and tetanus neurotoxins: neurotransmission and biomedical aspects. Plenum Press, New York, N.Y.
5. **Binz, T., H. Kurazono, M. R. Popoff, M. W. Eklund, G. Sakaguchi, S. Kozaki, K. Krieglstein, A. Henschen, D. M. Gill, and H. Niemann.** 1990. Nucleotide sequence of the gene encoding Clostridium botulinum neurotoxin type D. Nucleic Acids Res. **18:**5556.
6. **Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
7. **Black, R. E., and R. A. Gunn.** 1980. Hypersensitivity reactions associated with botulinal antitoxin. Am. J. Med. **69:**567–570.
8. **Bozheyeva, G., Y. Kunakbayev, and D. Yeleukenov.** 1999. Former soviet biological weapons facilities in Kazakhstan: past, present, and future. Center for Nonproliferation Studies, Monterey Institute of International Studies, Monterey, Calif.
9. **Campbell, K., M. D. Collins, and A. K. East.** 1993. Nucleotide sequence of the gene coding for Clostridium botulinum (Clostridium argentinense) type G neurotoxin: genealogical comparison with other clostridial neurotoxins. Biochim. Biophys. Acta **1216:**487–491.
10. **Centers for Disease Control and Prevention.** 1998. Botulism in the United States, 1899–1998. Handbook for epidemiologists, clinicians, and laboratory workers. Public Health Service, U.S. Department of Health and Human Services, Atlanta, Ga. [Online.] http://www.bt.cdc.gov/agent/botulism/index.asp.
11. **Demarchi, J., C. Mourgues, J. Orio, and A. R. Prevot.** 1958. Existence du botulisme de type D. Bull. Acad. Nat. Med. **142:**580–582.
12. **Dineen, S. S., M. Bradshaw, and E. A. Johnson.** 2003. Neurotoxin gene clusters in Clostridium botulinum type A strains: sequence comparison and evolutionary implications. Curr. Microbiol. **46:**345–352.
13. **East, A. K., P. T. Richardson, D. Allaway, M. D. Collins, T. A. Roberts, and D. E. Thompson.** 1992. Sequence of the gene encoding type F neurotoxin of Clostridium botulinum. FEMS Microbiol. Lett. **75:**225–230.
14. **Franz, D. R., L. M. Pitt, M. A. Clayton, M. A. Hanes, and K. J. Rose.** 1993. Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism, p. 473–476. *In* B. R. DasGupta (ed.), Botulinum and tetanus neurotoxins: neurotransmission and biomedical aspects. Plenum Press, New York, N.Y.
15. **Gibson, A. M., N. K. Modi, T. A. Roberts, P. Hambleton, and J. Melling.** 1988. Evaluation of a monoclonal antibody-based immunoassay for detecting type B Clostridium botulinum toxin produced in pure culture and an inoculated model cured meat system. J. Appl. Bacteriol. **64:**285–291.
16. **Hatheway, C. L.** 1995. Botulism: the present status of the disease. Curr. Top. Microbiol. Immunol. **195:**55–75.
17. **Hauser, D., M. W. Eklund, P. Boquet, and M. R. Popoff.** 1994. Organization of the botulinum neurotoxin C1 gene and its associated non-toxic protein genes in Clostridium botulinum C 468. Mol. Gen. Genet. **243:**631–640.
18. **Hauser, D., M. W. Eklund, H. Kurazono, T. Binz, H. Niemann, D. M. Gill, P. Boquet, and M. R. Popoff.** 1990. Nucleotide sequence of Clostridium botulinum C1 neurotoxin. Nucleic Acids Res. **18:**4924.
19. **Hibbs, R. G., J. T. Weber, A. Corwin, B. M. Allos, M. S. Abd el Rehim, S. E. Sharkawy, J. E. Sarn, and K. T. J. McKee.** 1996. Experience with the use of an investigational F(ab′)2 heptavalent botulism immune globulin of equine origin during an outbreak of type E origin in Egypt. Clin. Infect. Dis. **23:**337–340.
20. **Hutson, R. A., M. D. Collins, A. K. East, and D. E. Thompson.** 1994. Nucleotide sequence of the gene coding for non-proteolytic Clostridium botulinum type B neurotoxin: comparison with other clostridial neurotoxins. Curr. Microbiol. **28:**101–110.
21. **Ihara, H., T. Kohda, F. Morimoto, K. Tsukamoto, T. Karasawa, S. Nakamura, M. Mukamoto, and S. Kozaki.** 2003. Sequence of the gene for Clostridium botulinum type B neurotoxin associated with infant botulism, expression of the C-terminal half of heavy chain and its binding activity. Biochim. Biophys. Acta **1625:**19–26.
22. **Kimura, K., N. Fujii, K. Tsuzuki, T. Murakami, T. Indoh, N. Yokosawa, K. Takeshi, B. Syuto, and K. Oguma.** 1990. The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. Biochem. Biophys. Res. Commun. **171:**1304–1311.
23. **Kirma, N., J. L. Ferreira, and B. R. Baumstark.** 2004. Characterization of six type A strains of Clostridium botulinum that contain type B toxin gene sequences. FEMS Microbiol. Lett. **231:**159–164.
24. **Kleywegt, G. T., and T. A. Jones.** 1994. A super position. CCP4/ESF-EACBM Newslett. Protein Crystallogr. **31:**9–14.
25. **Koriazova, L. K., and M. Montal.** 2003. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat. Struct. Biol. **10:**13–18.
26. **Kouguchi, H., T. Watanabe, Y. Sagane, H. Sunagawa, and T. Ohyama.** 2002. In vitro reconstitution of the Clostridium botulinum type D progenitor toxin. J. Biol. Chem. **277:**2650–2656.
27. **Kozaki, S., Y. Kamata, T. Nagai, J. Ogasawara, and G. Sakaguchi.** 1986. The

use of monoclonal antibodies to analyze the structure of *Clostridium botulinum* type E derivative toxin. Infect. Immun. **52:**786–791.
28. **Kozaki, S., Y. Kamata, T. Nishiki, H. Kakinuma, H. Maruyama, H. Takahashi, T. Karasawa, K. Yamakawa, and S. Nakamura.** 1998. Characterization of *Clostridium botulinum* type B neurotoxin associated with infant botulism in Japan. Infect. Immun. **66:**4811–4816.
29. **Kozaki, S., S. Nakaue, and Y. Kamata.** 1995. Immunological characterization of the neurotoxin produced by Clostridium botulinum type A associated with infant botulism in Japan. Microbiol. Immunol. **39:**767–774.
30. **Kubota, T., N. Yonekura, Y. Hariya, E. Isogai, H. Isogai, K. Amano, and N. Fujii.** 1998. Gene arrangement in the upstream region of Clostridium botulinum type E and Clostridium butyricum BL6340 progenitor toxin genes is different from that of other types. FEMS Microbiol. Lett. **158:**215–221.
31. **Lacy, D. B., W. Tepp, A. C. Cohen, B. R. DasGupta, and R. C. Stevens.** 1998. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. **5:**898–902.
32. **Lang, A. B., S. J. Cryz, U. Schurch, M. T. Ganss, and U. Bruderer.** 1993. Immunotherapy with human monoclonal antibodies: fragment A specificity of polyclonal and monoclonal antibodies is crucial for full protection against tetanus toxin. J. Immunol. **151:**466–472.
33. **Maddaloni, M., C. Cooke, R. Wilkinson, A. V. Stout, L. Eng, and S. H. Pincus.** 2004. Immunological characteristics associated with the protective efficacy of antibodies to ricin. J. Immunol. **172:**6221–6228.
34. **Middlebrook, J. L., and D. R. Franz.** 1997. Botulinum toxins, p. 643–654. *In* F. R. Sidell, E. T. Takafuji, and D. R. Franz (ed.), Medical aspects of chemical and biological warfare. TMM publications, Washington, D.C.
35. **Montero-Julian, F. A., B. Klein, E. Gautherot, and H. Brailly.** 1995. Pharmacokinetic study of anti-interleukin-6 (IL-6) therapy with monoclonal antibodies: enhancement of IL-6 clearance by cocktails of anti-IL-6 antibodies. Blood **85:**917–924.
36. **Moriishi, K., M. Koura, N. Abe, N. Fujii, Y. Fujinaga, K. Inoue, and K. Ogumad.** 1996. Mosaic structures of neurotoxins produced from Clostridium botulinum types C and D organisms. Biochim. Biophys. Acta **1307:**123–126.
37. **Mullaney, B. P., M. G. Pallavicini, and J. D. Marks.** 2001. Epitope mapping of neutralizing botulinum neurotoxin A antibodies by phage display. Infect. Immun. **69:**6511–6514.
38. **Nakajima, H., K. Inoue, T. Ikeda, Y. Fujinaga, H. Sunagawa, K. Takeshi, T. Ohyama, T. Watanabe, and K. Oguma.** 1998. Molecular composition of the 16S toxin produced by a Clostridium botulinum type D strain, 1873. Microbiol. Immunol. **42:**599–605.
38a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.
39. **Nowakowski, A., C. Wang, D. B. Powers, P. Amersdorfer, T. J. Smith, V. A. Montgomery, R. Sheridan, R. Blake, L. A. Smith, and J. D. Marks.** 2002. Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. Proc. Natl. Acad. Sci. USA **99:**11346–11350.
40. **Oguma, K., K. Yokota, S. Hayashi, K. Takeshi, M. Kumagai, N. Itoh, N. Tachi, and S. Chiba.** 1990. Infant botulism due to Clostridium botulinum type C toxin. Lancet **336:**1449–1450.
41. **Poulet, S., D. Hauser, M. Quanz, H. Niemann, and M. R. Popoff.** 1992. Sequences of the botulinal neurotoxin E derived from Clostridium botulinum type E (strain Beluga) and Clostridium butyricum (strains ATCC 43181 and ATCC 43755). Biochem. Biophys. Res. Commun. **183:**107–113.
42. **Sagane, Y., H. Kouguchi, T. Watanabe, H. Sunagawa, K. Inoue, Y. Fujinaga, K. Oguma, and T. Ohyama.** 2001. Role of C-terminal region of HA-33 component of botulinum toxin in hemagglutination. Biochem. Biophys. Res. Commun. **288:**650–657.
43. **Sali, A., and T. L. Blundell.** 1990. Definition of general topological equivalence in protein structures: a procedure involving comparison of properties and relationships through simulated annealing and dynamic programming. J. Mol. Biol. **212:**403–428.
44. **Santos-Buelga, J. A., M. D. Collins, and A. K. East.** 1998. Characterization of the genes encoding the botulinum neurotoxin complex in a strain of Clostridium botulinum producing type B and F neurotoxins. Curr. Microbiol. **37:**312–318.
45. **Siegel, L. S.** 1988. Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **26:**2351–2356.
46. **Smith, L. A.** 1998. Development of recombinant vaccines for botulinum neurotoxin. Toxicon **36:**1539–1548.
47. **Sonnabend, O., W. Sonnabend, R. Heinzle, T. Sigrist, R. Dirnhofer, and U. Krech.** 1981. Isolation of Clostridium botulinum type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. J. Infect. Dis. **143:**22–27.
48. **Sunagawa, H., T. Ohyama, T. Watanabe, and K. Inoue.** 1992. The complete amino acid sequence of the Clostridium botulinum type D neurotoxin, deduced by nucleotide sequence analysis of the encoding phage d-16 phi genome. J. Vet. Med. Sci. **54:**905–913.
49. **Swaminathan, S., and S. Eswaramoorthy.** 2000. Structural analysis of the catalytic and binding sites of Clostridium botulinum neurotoxin B. Nat. Struct. Biol. **7:**693–699.
50. **Thompson, D. E., J. K. Brehm, J. D. Oultram, T. J. Swinfield, C. C. Shone, T. Atkinson, J. Melling, and N. P. Minton.** 1990. The complete amino acid sequence of the Clostridium botulinum type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur. J. Biochem. **189:**73–81.
51. **Thompson, D. E., R. A. Hutson, A. K. East, D. Allaway, M. D. Collins, and P. T. Richardson.** 1993. Nucleotide sequence of the gene coding for Clostridium barati type F neurotoxin: comparison with other clostridial neurotoxins. FEMS Microbiol. Lett. **108:**175–182.
52. **Tsukamoto, K., M. Mukamoto, T. Kohda, H. Ihara, X. Wang, T. Maegawa, S. Nakamura, T. Karasawa, and S. Kozaki.** 2002. Characterization of Clostridium butyricum neurotoxin associated with food-borne botulism. Microb. Pathog. **33:**177–184.
53. **United Nations Security Council.** 1995. Tenth report of the executive committee of the special commission established by the secretary-general pursuant to paragraph 9(b)(I) of security council resolution 687 (1991), and paragraph 3 of resolution 699 (1991) on the activities of the Special Commission. United Nations Security Council, New York, N.Y.
54. **Wang, X., T. Maegawa, T. Karasawa, S. Kozaki, K. Tsukamoto, Y. Gyobu, K. Yamakawa, K. Oguma, Y. Sakaguchi, and S. Nakamura.** 2000. Genetic analysis of type E botulinum toxin-producing *Clostridium butyricum* strains. Appl. Environ. Microbiol. **66:**4992–4997.
55. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, T. Atkinson, and N. P. Minton.** 1992. The complete amino acid sequence of the Clostridium botulinum type-E neurotoxin, derived by nucleotide-sequence analysis of the encoding gene. Eur. J. Biochem. **204:**657–667.
56. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson, and N. P. Minton.** 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. Appl. Environ. Microbiol. **58:**2345–2354.
57. **Willems, A., A. K. East, P. A. Lawson, and M. D. Collins.** 1993. Sequence of the gene coding for the neurotoxin of Clostridium botulinum type A associated with infant botulism: comparison with other clostridial neurotoxins. Res. Microbiol. **144:**547–556.
58. **Zhang, L., W.-J. Lin, S. Li, and K. R. Aoki.** 2003. Complete DNA sequences of the botulinum neurotoxin complex of Clostridium botulinum type A-Hall (Allergan) strain. Gene **315:**21–32.

Editor: A. D. O'Brien