Purification and Characterization of a Novel Subtype A3 Botulinum Neurotoxin

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Botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* are of considerable importance due to their being the cause of human and animal botulism, their potential as bioterrorism agents, and their utility as important pharmaceuticals. Type A is prominent due to its high toxicity and long duration of action. Five subtypes of type A BoNT are currently recognized; BoNT/A1, -/A2, and -/A5 have been purified, and their properties have been studied. BoNT/A3 is intriguing because it is not effectively neutralized by polyclonal anti-BoNT/A1 antibodies, and thus, it may potentially replace BoNT/A1 for patients who have become refractive to treatment with BoNT/A1 due to antibody formation or other modes of resistance. Purification of BoNT/A3 has been challenging because of its low levels of production in culture and the need for innovative purification procedures. In this study, modified Mueller-Miller medium was used in place of traditional toxin production medium (TPM) to culture *C. botulinum* A3 (CDC strain) and boost toxin production. BoNT/A3 titers were at least 10-fold higher than those produced in TPM. A purification method was developed to obtain greater than 95% pure BoNT/A3. The specific toxicity of BoNT/A3 as determined by mouse bioassay was 5.8×10^7 50% lethal doses (LD_{50})/mg. Neutralization of BoNT/A3 toxicity by a polyclonal anti-BoNT/A1 antibody was approximately 10-fold less than the neutralization of BoNT/A1 toxicity. In addition, differences in symptoms were observed between mice that were injected with BoNT/A3 and those that were injected with BoNT/A1. These results indicate that BoNT/A3 has novel biochemical and pharmacological properties compared to those of other subtype A toxins.

Clostridium botulinum produces a characteristic botulinum neurotoxin (BoNT), which is classified by the Centers for Disease Control and Prevention (CDC) as one of the six highest-risk threat agents for bioterrorism (“category A agents”) (1). BoNTs are produced by neurotoxicogenic clostridia as ~150-kDa single-chain proteins that can be cleaved into a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) by endogenous or exogenous proteases (4). The action of proteases on BoNTs is partially responsible for activation to the toxic form. The LC is a zinc metalloprotease which cleaves different SNARE proteins depending on the serotype, causing various symptoms in mice that include paralysis, labored breathing, and eventual death (6, 10, 25, 26). The HC consists of a C-terminal binding domain (H_C), which is responsible for binding to gangliosides and protein receptors on neuronal cells, and an N-terminal translocation domain (H_N), which is involved in the delivery of the catalytic light chain to the neuronal cytosol (17, 18). Based on neutralization of toxicity by type-specific antisera, BoNTs have traditionally been categorized into seven serotypes (BoNT/A to -/G), among which BoNTs A, B, E, and F are known to cause human botulism (11, 28, 29). Type A is of particular importance and interest since it causes the most severe human botulism and thus is considered to be a significant bioterrorism threat (1). Type A toxin is also the serotype most commonly used in the pharmaceutical industry (16) to treat a wide variety of neuronal disorders (27). In addition, type A toxin is used as a cosmetic treatment to smooth wrinkles by paralyzing facial muscles (2, 3).

Within serotype A, five different subtypes have been identified (A1, A2, A3, A4, and A5) (7, 9, 14). BoNT/A1 is the best studied and is currently used for clinical purposes. BoNT/A1 and BoNT/A5 are similar genetically, both containing a hemagglutinin (HA) neurotoxin gene cluster, while BoNT/A2, BoNT/A3, and BoNT/A4 contain an OrfX neurotoxin gene cluster (13, 14). Both

bont/A3 and *bont/A4* gene clusters are located on large plasmids instead of on the chromosome (21, 30).

BoNT/A3 was associated with a famous botulism outbreak in 1922 caused by potted duck meat at the rural Hotel Loch Maree in the Scottish Highlands (12). This botulism outbreak was extensively investigated and prompted the United Kingdom Ministry of Health to establish a program to make antitoxin available for potential botulism outbreaks (12). The *C. botulinum* strain A3 isolated from the duck meat paste is the only known A3 strain.

BoNT/A3 has certain intriguing properties compared to other subtype A toxins. BoNT/A3 is not effectively neutralized by anti-BoNT/A1 antibodies (J. Marks, personal communication; 22), which indicates that certain epitopes responsible for neutralization are different between BoNT/A1 and BoNT/A3. This difference in immunogenicity leads to the possibility that BoNT/A3 could potentially replace BoNT/A1 for those patients who have developed neutralizing antibodies to BoNT/A1 due to repeated treatments or immunization with BoNT/A1. As discussed below, symptoms elicited in mice by BoNT/A3 are distinct from those seen with BoNT/A1, suggesting that it may target distinct neurons or have a unique mode of action.

Although BoNT/A3 is a novel and interesting toxin, purification of BoNT/A3 has been challenging, due primarily to its low levels of production. Representative cultures produce approximately 10^3 to 10^4 mouse 50% lethal doses (LD_{50}) per ml, while cultures of *C. botulinum* A1 typically produce greater than 10^6

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LD₅₀ per ml. There are no reports of purification and characterization of BoNT/A3. In this study, we established a production and purification method that results in BoNT/A3 of greater than 95% purity. The results obtained from this study indicate that BoNT/A3 is unique among the characterized type A BoNTs, may have utility as a pharmaceutical, and also will be important in the development of vaccines and antisera to treat botulism outbreaks and to counteract potential bioterrorist attacks.

MATERIALS AND METHODS

Medium, strain, and purification materials. CDC A3, a strain of *C. botulinum* A3, was kindly provided by Susan Maslanka and Brian Raphael at the CDC. The strain appeared to be identical by multilocus sequence typing to the Loch Maree strain obtained from other culture collections. After considerable evaluation of nutritional requirements as related to BoNT production, the CDC strain was grown in modified Mueller-Miller medium (23): NZ Case TT (20 g/liter) (Kerry Biosciences), Na₂HPO₄ (1 g/liter), KH₂PO₄ (0.15 g/liter), MgSO₄ · 7H₂O (0.15 g/liter), FeSO₄ · 7H₂O (1% solution, 0.04 g/liter), cysteine HCl (0.25 g/liter), beef heart infusion (0.5 ml/liter) (Difco), glucose (10 g/liter). The medium pH was adjusted to 7.3 using NaOH.

Acid precipitation and extraction of crude A3 neurotoxin from the culture with or without addition of RNA. Two bottles of 1.5 liters of sterile modified Mueller-Miller medium were inoculated with 1.5 ml of actively growing CDC A3 culture (24 h) and incubated statically for 5 days at 37°C. The 5-day culture was cooled down for 60 min on ice, and 0.3 g total RNA from *Torula* yeast (0.2 g/liter) (Sigma) was added to one of two bottles containing the 1.5-liter A3 culture to determine if RNA would help to precipitate the toxin more efficiently. The pH of the culture in both bottles was then adjusted to 3.5 using 3 N H₂SO₄ and the bottles were allowed to stand at room temperature for 1 h. The difference in the amount of settled precipitate between the two bottles with or without added RNA was visually observed and then confirmed by toxicity testing. The acid precipitate was collected by centrifugation and washed with a total of 200 ml distilled water. The pellet was extracted twice by gentle stirring for 2 h at room temperature in 200 ml 0.1 M sodium citrate buffer, pH 5.5, containing 200 μl aprotinin. The extracts were centrifuged, and the supernatants, which were 60% saturated with ammonium sulfate, were stored at 4°C. Sample aliquots from the whole culture, supernatant after acid precipitation, and extract supernatant were saved for toxicity testing.

Toxicity testing of cultures with and without RNA addition. Toxin recovery percentages were compared via toxicity testing. Samples which included A3 crude culture, supernatants after acid precipitation from the culture either with or without added RNA, and extract supernatants were collected. Toxicity was determined by intravenous (i.v.) injection of the nicked (trypsinized) or unnicked samples into groups of mice. Each mouse was injected with 100 μl sample diluted 1:1 in gelatin phosphate buffer (30 mM sodium phosphate, pH 6.3, plus 0.2% gelatin), and 4 mice were used per group. i.v. time to death was converted to intraperitoneal (i.p.) LD₅₀/ml by comparison to a standard type A1 curve developed in our laboratory (20).

Purification of BoNT/A3. One hundred milliliters of ammonium sulfate precipitate from the first extraction was collected by centrifugation and resuspended in 20 ml 50 mM NaPO₄ buffer, pH 6.0. RNase A (Sigma) was added at the final concentration of 100 μg/ml and incubated for 3 h at 37°C. The solution after digestion was centrifuged to remove insoluble material. Trypsin (Worthington) was then added to the solution to a final concentration of 100 μg/ml, and the solution was incubated for 30 min at 37°C to nick the remaining single-chain toxin. Soybean trypsin inhibitor (SBTI) (Sigma) was added to the solution to a final concentration of 200 μg/ml, and the solution was incubated for 10 min at room temperature to inactivate trypsin. The treated solution was precipitated with solid ammonium sulfate (39 g/100 ml) and stored at 4°C. To obtain purified BoNT/A3, the following chromatography steps were used.

DEAE chromatography, pH 5.5. The RNase A- and trypsin-treated ammonium sulfate-precipitated pellet was collected by centrifugation; resuspended in 1 ml of 0.05 M sodium citrate, pH 5.5; and dialyzed for 4 h at room temperature with 3 dialysis changes at 1-h intervals. The dialyzed solution was centrifuged to remove insoluble material, and the supernatant was loaded on an 8-ml (0.9-cm by 14-cm) DEAE Sephadex A-50 column (Sigma) equilibrated with 50 mM sodium citrate buffer, pH 5.5, at room temperature. The column was washed with 50 mM sodium citrate buffer, pH 5.5. The unbound fractions were collected directly, and the bound proteins were eluted with 0.05 M sodium citrate, pH 5.5, buffer containing 0.8 M NaCl. Fractions were monitored at an optical density at 278 nm (OD₂₇₈) and analyzed by SDS-PAGE. The unbound fractions containing the crude toxin complex were pooled as unbound pool 1 (*A*₂₆₀/*A*₂₇₈ ratio less than 0.6) and unbound pool 2 (*A*₂₆₀/*A*₂₇₈ ratio greater than 0.6), precipitated with solid ammonium sulfate (39 g/100 ml), and stored at 4°C.

CM Sepharose chromatography. The ammonium sulfate-precipitated crude toxin complex from the DEAE (pH 5.5) column was collected by centrifugation and resuspended in 1 ml 0.025 M sodium citrate buffer, pH 6.0. The solution was dialyzed at room temperature with 3 dialysis changes at 1-h intervals and loaded onto an 0.9-cm by 12-cm CM Sepharose column (Sigma). The column was washed with 25 mM sodium citrate buffer, pH 6.0. The unbound fractions were collected directly, and the bound proteins were eluted with 0.025 M sodium citrate buffer, pH 6.0, containing 0.5 M NaCl. The fractions were monitored at OD₂₇₈ and analyzed by SDS-PAGE. The fractions containing ~95% pure toxin complex (TC) were pooled as unbound pool 1 (*A*₂₆₀/*A*₂₇₈ ratio less than 0.6) and unbound pool 2 (*A*₂₆₀/*A*₂₇₈ ratio greater than 0.6) and precipitated by addition of ammonium sulfate (39 g/100 ml).

Mono-Q FPLC. The precipitated toxin complex from the CM Sepharose chromatography was collected by centrifugation; resuspended in 4 ml 20 mM sodium phosphate buffer, pH 8.0; and dialyzed at room temperature with 3 dialysis changes at 1-h intervals. The dialyzed solution was loaded on a fast protein liquid chromatography (FPLC) Mono-Q column (1 ml/min) (Pfizer-Pharmacia, Inc.) for separation of the toxin from non-toxic nonhemagglutinin (NTNH). A NaCl gradient from 0 to 0.35 M was applied to the column to elute the bound material. The fractions were monitored at OD₂₇₈ and analyzed by SDS-PAGE. Data showed that the A3 toxin, along with several minor contaminating proteins, was recovered in the first peak at 147 mM NaCl. Those fractions were pooled and precipitated with ammonium sulfate. Purity was determined by densitometry of a 4 to 12% SDS-PAGE gel (Invitrogen).

Specific toxicity determination. The specific toxicity of the purified 150-kDa BoNT/A3 was determined by i.p. injection of the following 5 different toxin amounts into groups of mice (4 mice/group): 25 pg, 20 pg, 15 pg, 10 pg, and 5 pg/mouse. The toxin was diluted in 0.5 ml gelatin phosphate buffer and injected i.p. The injected mice were observed for 4 days. The LD₅₀/mg of toxin was calculated using the method of Reed and Muench (24).

Observation of botulism symptoms in the mice i.v. injected with BoNT/A3. Two groups of mice (4 mice/group) were i.v. injected with BoNT/A3 at a dose of ~5 × 10⁵ LD₅₀/ml or 1 × 10⁵ LD₅₀/ml, respectively. Mice were observed for symptoms of botulism from the time of injection until the time of death.

Neutralization of BoNT/A3 using anti-BoNT/A1 antibody. Serum from rabbits immunized with BoNT/A1 toxoid was fractionated by protein A chromatography to obtain polyclonal anti-BoNT/A1 IgG. One microliter of the purified antibody was able to neutralize ~ 5,000 LD₅₀ of BoNT/A1. In this study, 2 μl of antibody was mixed with 15,000 LD₅₀, 10,000 LD₅₀, and 5,000 LD₅₀ of BoNT/A1 or 15,000 LD₅₀, 10,000 LD₅₀, 5,000 LD₅₀, 2,500 LD₅₀, 1,000 LD₅₀, and 100 LD₅₀ of BoNT/A3, respectively, to compare the abilities of the anti-BoNT/A1 antibody to neutralize either BoNT/A1 or BoNT/A3. Toxin was diluted with gelatin phosphate to achieve the appropriate LD₅₀ concentrations. The different mixtures of toxin and antibody were incubated at 37°C for 90 min prior to injection.

FIG 1 Photograph showing the acid precipitation difference between cultures with (right bottle) and without (left bottle) addition of RNA. The arrow indicates the visible precipitation.

Each mouse within the group (4 mice) was injected with 0.5 ml of the toxin-antibody mixture and observed for 4 days for symptoms.

RESULTS

Effect of modified Mueller-Miller medium on production of BoNT/A3. In this study, a modified Mueller-Miller medium was used instead of toxin production medium (TPM) for culture, which resulted in greater than a 10-fold increase in BoNT/A3 from 10^3 to 10^4 to 10^4 to 10^5 LD₅₀/ml. Modified Mueller-Miller medium was used following the examination of effects of various media and conditions on BoNT/A3 production.

Comparison of toxin recoveries between the culture with and that without RNA added before acid precipitation. Precipitation differences were visible after adding RNA to the culture (Fig. 1). The pellet collected from the culture with the RNA added was larger than that from the culture without the RNA added. i.v. injection was performed as described in Materials and Methods. Specific toxicity (LD₅₀/ml) and total toxicity (LD₅₀/ml × total volume) were determined (Table 1). Data showed that only 2.9% of toxin was left in the supernatant after addition of RNA, while 17.8% of toxin was still left in the supernatant without addition of RNA to the culture before acid precipitation. Data indicated that RNA does play an important role in increasing the percentage of toxin precipitation. Data also indicated that approximately 100%

of toxin was recovered from the first extraction and that trypsinized (nicked) toxin had an ~2-fold-higher specific toxicity than did nontrypsinized toxin.

Purification of BoNT/A3. After crude BoNT/A3 extract was obtained from the culture (see Materials and Methods for details), three chromatography steps were used to purify BoNT/A3. First, the crude toxin extract was chromatographed on a DEAE-Sephadex A-50 column. Nucleic acid and a major protein band of ~38 kDa were removed from the crude toxin extract (Fig. 2 A). The majority of BoNT/A3 complex remains in the fractions from the first unbound peak. Pool 1 containing the toxin complex was further purified by chromatography on a CM Sepharose column. The unbound fractions in pool 1 from the CM chromatographic step yielded 95% pure toxin complex (TC) containing BoNT/A3 and NTNH with an A_{260}/A_{278} ratio of less than 0.6 (Fig. 2B). BoNT/A3 was then separated from NTNH using Mono-Q FPLC. A 95% pure BoNT/A3 was eluted at a salt concentration of 147 mM (Fig. 2C). The purified 150-kDa toxin was confirmed by SDS-PAGE under both unreduced and reduced conditions and by mouse bioassay. Approximately 210 μg of pure BoNT/A3 was obtained at this final step; therefore, a total of 420 μg of pure BoNT/A3 was expected to be obtained from the 1.5-liter starting culture because only half of the extract was used in the toxin purification.

BoNT/A3 toxicity testing. The toxicity of BoNT/A3 was determined by i.p. injection as described in Materials and Methods. The specific toxicity of the 150-kDa protein was determined to be $\sim 5.8 \times 10^7$ LD₅₀/mg. This specific toxicity is close to that of BoNT/A1 purified in our laboratory, which routinely has $\sim 1 \times 10^8$ to 2×10^8 LD₅₀/mg.

Observation of botulism symptoms in the mice i.v. injected with BoNT/A3. The mice injected i.v. with a dose of 5×10^5 LD₅₀ of BoNT/A3 started to show initial botulism symptoms approximately 35 to 40 min after the injection. Paralysis started with the front legs and spread to the whole body, and then mice died around 68 min after injection, on average. Symptoms observed with toxin A1 such as ruffled fur or wasp-like abdomen, labored breathing, and spasticity immediately prior to death were not observed (Table 2).

Neutralization of BoNT/A3 and BoNT/A1 using an anti-BoNT/A1 antibody. The neutralization results showed that 2 μl of anti-BoNT/A1 antibody was able to fully neutralize 10,000 LD₅₀ but not 15,000 LD₅₀ of BoNT/A1. However, the same quantity of anti-BoNT/A1 antibody neutralized only ca. 1,000 LD₅₀ but not 2,500 LD₅₀ of BoNT/A3. It was determined that 2 μl of anti-BoNT/A1 can neutralize 12,245 LD₅₀ of BoNT/A1 versus only 1,582 LD₅₀ of BoNT/A3, which was an approximately 8-fold difference. As much as a 50-fold difference in neutralization of A1 versus A3 toxin by anti-A1 specific monoclonal antibodies has

TABLE 1 Comparison of percentages of recovery between the culture with addition of RNA and the culture without addition of RNA before acid precipitation

Culture	Avg time to death (min)	Specific toxicity (LD ₅₀ /ml)	Total toxicity (LD ₅₀ /ml × total vol)	% recovery
A3 culture	118.31 ± 14.59	9.19×10^3	5.51×10^7	
Acid supernatant without addition of RNA	124.13 ± 27.04	6.57×10^3	9.85×10^6	17.8 (of starting toxicity)
Acid supernatant with addition of RNA	155.88 ± 20.13	1.06×10^3	1.58×10^6	2.9 (of starting toxicity)
A3 extract 1, unnicked	80.50 ± 3.85	8.10×10^4	6.32×10^7	100
A3 extract 1, nicked	68.31 ± 6.02	1.63×10^5	1.27×10^8	100

FIG 2 Coomassie blue-stained SDS-PAGE gels under both nonreduced and reduced conditions show the BoNT/A3 purification process. (A) Coomassie blue-stained SDS-PAGE gel analysis of fractions after DEAE-Sephadex A-50 chromatography. Data showed that a BoNT/A3 complex with an A_{260}/A_{278} ratio of less than 0.6 was obtained in pool 1 (unbound fractions). (B) Coomassie blue-stained SDS-PAGE gel analysis of fractions after CM-Sephadrose chromatography. Data showed that a 95% pure BoNT/A3 complex was obtained in pool 1 (unbound fractions). (C) Coomassie blue-stained SDS-PAGE gel analysis of fractions after Mono-Q chromatography. Data showed that 95% pure BoNT/A3 was obtained in the fraction at a 147 mM salt concentration. UB, unbound; NR, nonreduced; R, reduced; HC, heavy chain; LC, light chain; M, marker.

been reported by other laboratories (J. Marks, personal communication). The data indicate that there may be important differences in epitopes between BoNT/A1 and BoNT/A3, leading to differences in neutralizing antibody formation in immunized animals.

DISCUSSION

C. botulinum type A subtype A3 toxin has drawn attention recently because of its unique characteristics. For instance, unlike BoNT/

A1, BoNT/A3 contains an OrfX neurotoxin gene cluster instead of an HA gene cluster (13, 19). The gene for BoNT/A3 was found to be located on a conjugative plasmid instead of on the chromosome (21). In addition, multilocus sequence type (MLST) profiling analysis showed that the A3 strain is clearly distinguished from other subtype A strains (15). Of considerable interest is that BoNT/A3 was found to be less effectively neutralized by anti-BoNT/A1 antibody (22).

However, the purification of BoNT/A3 presented considerable challenges. The A3 strain produced very low levels of BoNT/3, approximately 100 times lower than that of BoNT/A1 in toxin production medium, which is used for production of other subtypes of BoNT/A. The low level of production presented difficulties in the purification and characterization of BoNT/A3.

In this study, we report the first method to purify 150-kDa BoNT/A3. The total yield of BoNT/A3 was approximately 1/10 of that of BoNT/A1, primarily due to the low production level. Different media were investigated, and modified Mueller-Miller medium was found to increase toxin production at least 10-fold compared to TPM (5, 8).

In addition to finding a suitable medium to increase toxin production, we also found that adding RNA to the 5-day A3 culture

TABLE 2 Differences in botulism symptoms between the mice i.v. injected with BoNT/A1 and those injected with BoNT/A3

Botulism symptom	Presence in mice injected with toxin:	
	A1	A3
Ruffled fur	Yes	No
Wasp-like abdomen	Yes	No
Jumping around before death	Yes	No
Front leg paralysis	No	Yes
Hind leg paralysis	No	Yes
Whole-body paralysis	No	Yes

before acid precipitation resulted in approximately 6-fold less toxicity remaining in the supernatant. Initial toxicity determinations indicated that the 150-kDa BoNT/A3 had a slightly lower specific toxicity than did BoNT/A1.

Surprisingly, differences in symptoms were observed between mice injected i.v. with relatively high doses of BoNT/A3 and those injected with BoNT/A1. These results suggest that BoNT/A1 and BoNT/A3 may have different receptors or trafficking pathways or that BoNT/A3 may target different neurons than those targeted by BoNT/A1. Previous studies have shown that the light chain (LC) of BoNT/A3 possesses the same cleavage site specificity for SNAP-25 as does the LC of BoNT/A1, although there is only 81% amino acid identity between them (31). These data indicate that the LC of BoNT/A3 likely does not play a role in the differences in symptoms observed. There is 86% amino acid identity between the heavy chains (HCs) of BoNT/A3 and BoNT/A1 (31). Among them, the receptor binding domain (C-terminal half of the heavy chain) has an 86.6% amino acid identity and the translocation domain (N-terminal half of the heavy chain) has an 85.4% amino acid identity, which are the lowest identity percentages among the type A subtypes. The amino acid differences in the HC of BoNT/A3 might cause structural changes influencing receptor binding, trafficking, or translocation of the LC into the neuronal cytosol.

Immunological neutralization experiments also showed that anti-BoNT/A1 polyclonal antibodies neutralized BoNT/A3 relatively poorly. We observed a 10-fold-decreased ability of anti-A1 polyclonal antibodies to neutralize BoNT/A3, while other laboratories have reported up to a 50-fold decrease in neutralization using crude culture supernatants (J. Marks, personal communication). Therefore, BoNT/A1 and BoNT/A3 must have different epitope structures that contribute to antibody neutralization.

Due to the differences observed in immunological neutralization, symptoms, and possibly cellular biology, BoNT/A3 may have novel applications as a pharmaceutical to treat neurological conditions. This leads to the possibility of BoNT/A3 replacing BoNT/A1 for those patients who have developed resistance to BoNT/A1 due to repeated treatments, antibody development, or immunization with BoNT/A1 in laboratory workers or soldiers. Moreover, since BoNT/A3 is not efficiently neutralized by anti-BoNT/A1 antibodies, anti-BoNT/A1 being the only subtype A serum available to treat botulism, BoNT/A3 may pose a potential bioterrorism threat. The ability to produce purified BoNT/A3 will be important in the development of additional specific vaccines and antiserum to combat potential bioterrorist attacks. Further studies are needed to evaluate the unique structure, cellular biology, and pharmacological and clinical properties of BoNT/A3.

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REFERENCES

1. Arnon SS, et al. 2001. Botulinum toxin as a biological weapon: medical and public health management. *JAMA* 285:1059–1070.
2. Ascher B, et al. 2010. International consensus recommendations on the

- aesthetic usage of botulinum toxin type A (Speywood Unit)—part I: upper facial wrinkles. *J. Eur. Acad. Dermatol. Venereol.* 24:1278–1284.
3. Ascher B, et al. 2010. International consensus recommendations on the aesthetic usage of botulinum toxin type A (Speywood Unit)—part II: wrinkles on the middle and lower face, neck and chest. *J. Eur. Acad. Dermatol. Venereol.* 24:1285–1295.
4. Bandyopadhyay S, Clark AW, DasGupta BR, Sathyamoorthy V. 1987. Role of the heavy and light chains of botulinum neurotoxin in neuromuscular paralysis. *J. Biol. Chem.* 262:2660–2663.
5. Bellevue S. 2006. Master's thesis. University of Wisconsin, Madison, WI.
6. Binz T, et al. 1994. Proteolysis of SNAP-25 by types E and A botulinum neurotoxins. *J. Biol. Chem.* 269:1617–1620.
7. Carter AT, et al. 2009. Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. *BMC Genomics* 10:115.
8. DasGupta BR, Sathyamoorthy V. 1984. Purification and amino acid composition of type A botulinum neurotoxin. *Toxicon* 22:415–424.
9. Dover N, Barash JR, Arnon SS. 2009. Novel *Clostridium botulinum* toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. *J. Clin. Microbiol.* 47:2349–2350.
10. Foran P, Lawrence GW, Shone CC, Foster KA, Dolly JO. 1996. Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. *Biochemistry* 35:2630–2636.
11. Hatheway CL. 1988. Botulism, p 111–133. In Balows A (ed), *Laboratory diagnosis of infectious diseases: principles and practice*. Springer-Verlag, New York, NY.
12. Horowitz BZ. 2011. The ripe olive scare and Hotel Loch Maree tragedy: botulism under glass in the 1920's. *Clin. Toxicol.* 49:345–347.
13. Jacobson MJ, Lin G, Raphael B, Andreadis J, Johnson EA. 2008. Analysis of neurotoxin cluster genes in *Clostridium botulinum* strains producing botulinum neurotoxin serotype A subtypes. *Appl. Environ. Microbiol.* 74:2778–2786.
14. Jacobson MJ, et al. 2011. Purification, modeling, and analysis of neurotoxin BoNT/A5 from *Clostridium botulinum* strain A661222. *Appl. Environ. Microbiol.* 77:4217–4222.
15. Jacobson MJ, Lin G, Whittam TS, Johnson EA. 2008. Phylogenetic analysis of *Clostridium botulinum* type A by multi-locus sequence typing. *Microbiology* 154:2408–2415.
16. Johnson EA. 1999. Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. *Annu. Rev. Microbiol.* 53:551–575.
17. Lacy DB, Stevens RC. 1999. Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol.* 291:1091–1104.
18. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. 1998. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat. Struct. Biol.* 5:898–902.
19. Lin G, Tepp WH, Pier CL, Jacobson MJ, Johnson EA. 2010. Expression of the *Clostridium botulinum* A2 neurotoxin gene cluster proteins and characterization of the A2 complex. *Appl. Environ. Microbiol.* 76:40–47.
20. Malizio CJ, Goodnough MC, Johnson EA. 2000. Purification of *Clostridium botulinum* type A neurotoxin. *Methods Mol. Biol.* 145:27–39.
21. Marshall KM, Bradshaw M, Pellett S, Johnson EA. 2007. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. *Biochem. Biophys. Res. Commun.* 361:49–54.
22. Mazuet C, Dano J, Popoff MR, Creminon C, Volland H. 2010. Characterization of botulinum neurotoxin type A neutralizing monoclonal antibodies and influence of their half-lives on therapeutic activity. *PLoS One* 5:e12416.
23. Mueller JH, Schoenbach EB, Jezukawicz JJ, Miller PA. 1943. Production of tetanus toxin on peptone-free media. *J. Clin. Invest.* 22:315–318.
24. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27:493–497.
25. Schiavo G, Rossetto O, Santucci A, DasGupta BR, Montecucco C. 1992. Botulinum neurotoxins are zinc proteins. *J. Biol. Chem.* 267:23479–23483.
26. Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C. 1995. Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* 270:10566–10570.
27. Singh JA. 2010. Botulinum toxin therapy for osteoarticular pain: an evidence-based review. *Ther. Adv. Musculoskelet. Dis.* 2:105–118.

28. Smith LDS, Sugiyama H. 1988. Botulism. The organism, its toxins, the disease. Charles C Thomas, Springfield, IL.
29. Smith GR, Moryson CJ. 1977. A comparison of the distribution of *Clostridium botulinum* in soil and in lake mud. J. Hyg. (Lond.) 78: 39–41.
30. Smith TJ, et al. 2007. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One 2:e1271.
31. Webb RP, Smith TJ, Wright P, Brown J, Smith LA. 2009. Production of catalytically inactive BoNT/A1 holoprotein and comparison with BoNT/A1 subunit vaccines against toxin subtypes A1, A2, and A3. Vaccine 27:4490–4497.