INFECTION AND IMMUNITY, May 2003, p. 2941–2944
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.5.2941–2944.2003

Expression of H_C Subunits from *Clostridium botulinum* Types C and D and Their Evaluation as Candidate Vaccine Antigens in Mice

L. A. Woodward,[1,2]* H. Arimitsu,[2] R. Hirst,[1] and K. Oguma[2]

Department of Microbiology and Immunology, James Cook University, Townsville 4811, Australia,[1] and Department of Bacteriology, Graduate School of Medicine and Dentistry, Okayama University, Okayama 700-8558, Japan[2]

Received 24 October 2002/Returned for modification 22 November 2002/Accepted 29 January 2003

Two proteins representing the heavy-chain subunits of botulinum neurotoxin types C and D were expressed in *Escherichia coli*, and their vaccine potential was evaluated. Mice were vaccinated with doses ranging from 0.5 to 10 μg and were challenged with 10 to 10^5 50% lethal doses of toxin. For the type C subunit protein, C50, two doses of 2 μg were required for full protection, while, for type D subunit protein, D50, two 1-μg doses were required. A bivalent vaccine consisting of a mixture of these two proteins also provided protection against both botulinum neurotoxin type C and type D challenge. Antibody levels in serum were determined by both enzyme-linked immunosorbent assays and serum neutralization assays

Botulism is an intoxication caused by neurotoxins produced by *Clostridium botulinum*. Seven antigenically distinct botulinum neurotoxins (BoNTs), designated A through G, are produced with *C. botulinum* strains identified according to the major neurotoxin type produced. The different toxin types are pharmacologically and structurally similar, consisting of a single polypeptide chain that is posttranslationally nicked to yield a dichain molecule with a light chain (50 kDa), joined by a disulfide bond to a 100-kDa heavy chain (H_C) (4, 5). The 50-kDa carboxy-terminal end of the H_C contains the major determinants responsible for specific toxin binding at the neuromuscular junction (1, 11, 13). BoNT types C and D have both been isolated from cattle suffering paralytic disease, and only these strains are associated with botulism in cattle in Australia (3, 14). To combat the disease, a bivalent vaccine consisting of formalin-inactivated type C and D toxins is presently available, and although it is efficacious, various concerns regarding reliable vaccine availability have been expressed by members of the Australian cattle industry. Previous studies have shown that the H_C subunits of the tetanus toxin and various BoNTs are capable of evoking a protective immune response in mice (2, 6, 7, 9, 12). Such a subunit vaccine for BoNT types C and D would offer several advantages over the present vaccine, potentially eliminating or minimizing production problems that can sometimes be experienced.

Primers were designed to amplify the H_C-encoding region of the BoNT type C and type D genes. For ease of subsequent manipulations, appropriate restriction enzyme sites were incorporated into the primers. The templates for the PCRs were DNA fragments CH3, derived from the BoNT type C gene (8) (GenBank accession no. D90210), and H1, derived from BoNT type D (15) (GenBank accession no. S49407), inserted into the cloning plasmid pUC19 (New England BioLabs, Beverly, Mass.). Table 1 shows the primers, sequences, and enzymes.

Expression plasmids were used to transform *Escherichia coli* DH5α cells (Life Technologies), which were then grown overnight in Luria-Bertani broth supplemented with ampicillin and were then subcultured into Terrific broth containing ampicillin. Expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (final concentration) when cultures reached an optical density (OD) at 600 nm of approximately 0.8 to 1.5, and the cultures were transferred to a 25°C shaking incubator for growth overnight. Protein purifications were carried out by using a 50% nickel-nitrilotriacetic acid agarose (Ni-NTA) slurry (Qiagen, Valencia, Calif.) following the manufacturer's protocol for insoluble proteins. Expressed proteins were eluted from the Ni-NTA column with buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting and were probed with antihistidine antibody or anti-BoNT antisera. Protein visualization for Western blot analysis incorporated an enhanced chemiluminescence method (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Eluted fractions containing expressed proteins were pooled and dialyzed overnight, at 4°C, against phosphate-buffered saline (PBS). Protein concentrations were estimated by using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's protocol.

Vaccine antigens were diluted in sterile PBS and were emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.). An equal volume of sterile 2% Tween 20–140 mM NaCl was added and was further emulsified. Six-week-old female ddY mice from Shimizu Laboratory Supplies, Kyoto, Japan, were injected via the intraperitoneal (i.p.) route with 200 μl of each vaccine antigen, covering a dose range of 0.5 to 10 μg. Booster injections were prepared in the same manner, but with incomplete Freund's adjuvant, and were given 2 weeks following initial vaccination. Bivalent vaccines were prepared by combining 10 μg each of C50 and D50 subunit proteins, and adjuvant was incorporated as described above. In some cases vaccines were prepared without adjuvant; the protein antigens (10 μg) were diluted in sterile PBS and 200 μl was injected i.p. Five days prior to toxin challenge, blood was obtained from test animals and the serum was stored at −20°C. To establish the 50% lethal dose (LD_{50}),

* Corresponding author. Present address: Advanced Analytical Centre, James Cook University, Townsville 4811, Australia. Phone: (61) 7 4781 6138. Fax: (61) 7 4781 5550. E-mail: lynn.woodward@jcu.edu.au.

TABLE 1. Primers and sequences[a]

Primer	Sequence	Restriction enzyme
C5′	5′-AATACCATGGAAAATACAATACCCTTTAAT-3′	*Nco*I
C3′	5′-TCTTCTGAATTCACTCTGGAAGATTATAGACAG-3′	*Eco*RI
D5′	5′-GAAACCATGGTCCCTTTTAATATTTTTTCA-3′	*Nco*I
FUP (D3′)	5′-GTTTTCCCAGTCACGAC-3′	

[a] Incorporated restriction enzyme sites are underlined. PCRs contained 1× PCR buffer; dATP, dCTP, dGTP, and dTTP (200 μM each); 5′ and 3′ primers (0.5 μM each); DNA template (1 or 0.5 ng); $MgCl_2$ (1.25 mM); and *Taq* DNA polymerase (2.5 U) (Applied Biosystems, Foster City, Calif.). Typical PCR protocol was as follows: initial heating to 94°C for 1 min and then cycles of 94°C, 45 s; 54°C, 30 s; and 72°C, 45 s, for a total of 30 cycles. Products were initially cloned into pGEM-T (Promega Corp., Madison, Wis.) prior to subcloning into ProEXHT-A (Life Technologies, Mt. Waverley, Australia) expression plasmid. All constructs were confirmed by DNA sequencing.

toxin was diluted in sterile 0.01 M PBS (pH 6.0)–0.2% gelatin and each mouse was injected with 500 μl i.p. Two mice were challenged with each dilution of toxin and were observed for 4 days. In vaccine trials, mice were challenged with toxin doses ranging from 10 to 10^5 LD_{50}s 2 weeks after the final booster dose and were observed for a further 4 days. All animal work was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health & Medical Research Council).

Antibody levels in serum were determined by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed, 96-well microtiter plates (Costar, Laguna Niguel, Calif.) were coated with 50 μl of either purified C50 or D50 recombinant protein (1 μg/ml in 70 mM Na_2HPO_4) or BoNT type C or type D 16S toxin (20 μg/ml in PBS, pH 6.0). Plates were washed with 0.5% Tween–PBS, and nonspecific binding sites were blocked with 10% skim milk–PBS prior to the addition of 50 μl per well of mouse serum, serially diluted in skim milk–PBS. Plate contents were incubated for 1 h at room temperature before horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako Japan Co. Ltd., Kyoto, Japan), diluted 1:1,000 in skim milk–PBS, was added. After a further hour's incubation, the chromogen, *o*-phenylenediamine, was added. The color reaction was terminated by the addition of 2 M sulfuric acid, and the OD was measured at 490 nm on a Bio-Rad Novapath Microplate Reader (Bio-Rad Laboratories). The ELISA titer was taken as the reciprocal value of the last dilution giving an OD reading greater than the average plus 3 standard deviations of the OD obtained for eight replicates of pooled sera from 20 unvaccinated mice, run in parallel on each plate.

For some animals, antibody levels in serum were also measured by a serum neutralization assay. Mouse serum, diluted 1 in 10 in PBS, was mixed with an equal volume of toxin containing 10 LD_{50}s of BoNT type C or D diluted in 0.01 M PBS (pH 6.0)–0.2% gelatin. Samples were incubated at 37°C for 1 h, and 200 μl was injected i.p. into each of two mice per sample. Mice were observed for 4 days, and all deaths were recorded.

ELISA titers were log transformed to normalize the data. A one-way analysis of variance ($P = 0.05$) was used to compare the effects of different vaccination regimes. Spearman's correlation was performed to identify significant relationships between different data sets. Correlation coefficient (r), P, and level of significance (α) are reported.

Optimization of expression conditions, specifically the reduction of expression temperature to 25°C, resulted in a significant reduction in the presence of lower-molecular-weight proteins observed following expression at 37°C. Products of approximately 90% purity, as assessed by SDS-PAGE and Western blot analysis, were obtained following purification with Ni-NTA agarose (Fig. 1). Occasional batches contained a minor amount of lower-molecular-weight proteins, presumably degradation products of the full-length protein. These bands reacted with both Penta-His and anti-BoNT antibodies. Initial experiments resulted in yields of purified proteins of approximately 0.5 mg per liter of culture. When the pH of the elution buffer was reduced from 5.9 to 4.5, significantly higher yields in the region of 2.0 to 3.0 mg per liter of culture were obtained. Under these conditions, proteins precipitated upon dialysis against PBS but were still recognized by Penta-His antibodies,

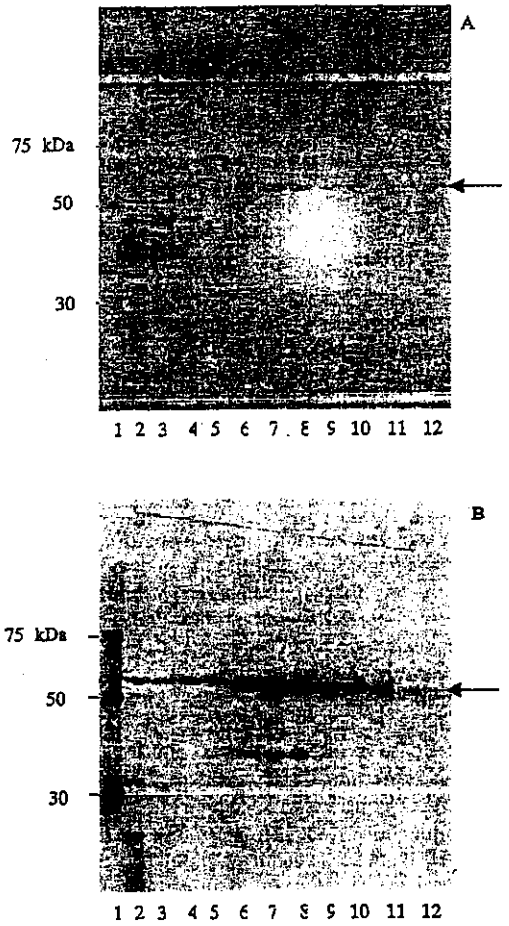

FIG. 1. SDS-PAGE (A) and a Western blot, stained with Penta-His antibody (B), of purification of D50 protein (arrowed). Proteins eluted with buffer E, pH 4.8. Lanes: 1, Qiagen His_6 protein ladder; 2, broth culture; 3, column flowthrough; 4, column wash 1; 5, wash 2; 6, column elution 1; 7, elution 2; 8, elution 3; 9, elution 4; 10, elution 5; 11, elution 6; and 12, elution 7. Lower-molecular-weight proteins were present in occasional batches for both C50 and D50 expression. These proteins reacted with both anti-His and anti-BoNT antisera and appear to represent degradation products of the full-length products.

TABLE 2. Effect of vaccination with subunit proteins on animal survival following toxin challenge[a]

Immunogen(s) and dose (μg)	No. of survivors/total challenged at challenge dose (no. of LD_{50}s)				
	10	10^2	10^3	10^4	10^5
C50					
0.5	2/5				
1	2/5				
2	5/5				
5	5/5	5/5			
10	5/5	5/5	4/5	2/5	2/5
10, no adjuvant	4/5	4/5	4/5		
10*	0/5 (type D)				
Adjuvant only	0/5				
D50					
0.6	2/5				
1	5/5	3/5			
10	5/5	5/5	5/5	3/5	3/5
10, no adjuvant	4/5	4/5			
10*	1/5 (type C)				
Adjuvant only	0/5				
C50 + D50					
10 (C challenge)	5/5		5/5**		
10 (D challenge)	4/5		2/5		

[a] All vaccines were given i.p. as two doses, 2 weeks apart. Animals were challenged with homologous toxin type corresponding to the vaccinating protein, except in cross-protection studies, indicated by a single asterisk. For bivalent-vaccine studies, equal amounts of C50 and D50 proteins were incorporated into the vaccine and animals were challenged with either BoNT type C or D as indicated. **, animals given a second challenge of BoNT type D (10^3 LD_{50}S). Five of five survived.

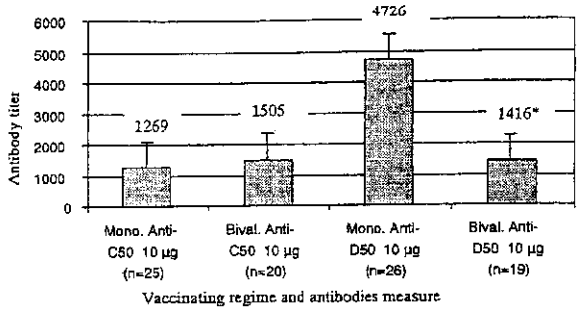

FIG. 2. Effect of combining vaccinating proteins on immunogenicity of components. Anti-C50 and anti-D50 antibody levels in serum were measured by ELISA for mice receiving either monovalent (Mono.) or bivalent (Bival.) vaccines. The geometric mean titer of each group and the standard error are presented. *, the mean anti-D50 titer of the group receiving D50 as part of a bivalent vaccine was significantly less than that of the group receiving D50 as a monovalent vaccine.

while type C derivatives were recognized by anti-BoNT type C serum. The precipitated proteins were stable, with storage for 1 month at −20°C, 4°C, room temperature, and up to 37°C resulting in very little degradation of product as assessed by SDS-PAGE and Western blot analysis.

Table 2 summarizes the results of the vaccine efficacy studies for the toxins. None of the control animals that received only adjuvant survived toxin challenge. For full protection against a toxin challenge of 10 LD_{50}s of BoNT type C, two 2-μg doses of the C50 and two doses of 1 μg of the D50 protein were required (for BoNT type C the LD_{50} was 5×10^{-7}, and for BoNT type D, 1×10^{-7} when 500 μl was injected i.p.). Little or no cross-protection was observed with the monovalent vaccines. A combination of 10 μg each of C50 and D50 proteins in a bivalent vaccine provided full protection against up to 1,000 LD_{50}s of BoNT type C but only partial protection against 10 and 1,000 LD_{50}s of BoNT type D. Anti-C50 antibody levels in serum did not differ significantly between animals receiving the monovalent or bivalent vaccines; however, D50 antibody levels were significantly ($P = 0.05$) lower in those mice receiving the bivalent vaccine (Fig. 2).

When sera from mice vaccinated with either C50 or D50 vaccine antigens were tested by ELISA, there was a positive correlation ($r = 0.784$ or $r = 0.866$, respectively; $P = 0.000$; $\alpha = 0.01$) between titers obtained when H_C subunit proteins and 16S toxins were used as the ELISA capture antigen. Following vaccination with C50 and D50 protein, 100 and 78% of animals with ELISA titers that were >160 of anti-C H_C and anti-D H_C, respectively, survived challenge with up to 1,000 LD_{50}s of homologous toxin. A number of sera were also tested for their ability to neutralize toxin. Serum from animals vaccinated with the C50 protein and surviving a challenge of up to 1,000 LD_{50}s did not consistently neutralize 10 LD_{50}s of homologous toxin. Similar results were observed for animals vaccinated with the D50 protein.

A relatively low yield of around 2.0 to 2.5 mg of purified proteins per liter of culture was obtained in this study. The low level of expression may be explained by factors including protein degradation, mRNA instability, plasmid instability, and differences in codon bias between the heterologous gene and *E. coli* (10). Considerable protein degradation occurred when expression was carried out at 37°C. In this study, a reduction in expression temperature to 25°C appeared to minimize the amount of protein degradation occurring.

The BoNT type C and type D H_C subunit proteins, C50 and D50, respectively, were successful in evoking a protective immune response when used as vaccinating antigens in mice. Furthermore, animals showed a dose-dependent response to vaccination, as there was a direct relationship between survival following toxin challenge and antibody titer. A direct comparison of the vaccine efficacy demonstrated in this study with other studies is difficult to make due to the use of different mouse strains and toxin forms. In this study, animals were challenged with 16S toxin. As this form consists of toxin and associated nontoxic components, the challenge via the i.p. route with 16S toxin may result in a lower level of protection due to blocking of protective antibodies by the nontoxic components.

A bivalent-vaccine formulation combining the two H_C proteins, C50 and D50, did not appear to affect the immunogenicity of the C50 protein. However, the mean anti-D50 ELISA titer for the bivalent vaccinated group was significantly less than for the corresponding monovalent vaccinated group, as was the level of protection following toxin challenge. Although the type D component in the bivalent vaccine appeared to be less immunogenic, it is interesting that animals receiving the

bivalent vaccine and surviving challenge with 1,000 LD_{50}s of type C toxin survived a subsequent challenge with 1,000 LD_{50}s of type D toxin. As there is reported to be little or no cross-protection afforded by the monovalent vaccines, then the protection provided must be attributed to anti-D50 antibodies. Although these results are encouraging, further development of the bivalent-vaccine formulation is required.

Anti-H_C ELISA antibody titers appear to correlate closely with survival following toxin challenge. It would be expected that the serum neutralization assays should give the best indication of the ability of an animal to survive toxin challenge, as only neutralizing antibodies are measured. However, in this study, the toxin neutralization assay did not correlate well with protection. This may be attributed to the presence of the nontoxic components in the 16S toxin, as discussed previously. ELISA offers several advantages over the serum neutralization assay, most notably the move away from use of animals, allowing a reduction in the time required for an assay to be performed and in the costs associated with it. With the move away from toxoid vaccines to subunit alternatives, the serum neutralization assay may prove to be less reliable at predicting protection.

We believe this to be the first report on the production of BoNT type C and type D H_C subunit proteins and an evaluation of their use as immunogens in monovalent and bivalent formulations. A bivalent-vaccine formulation is an important consideration, as botulinum vaccines for both humans and animals must generally, protect against more than one of the botulinum neurotoxins. The subunit mono- and bivalent vaccines described here were able to evoke protective immune responses, and they could readily be manufactured on a commercial scale. The production of such vaccines has the potential to decrease the manufacturing process for the present toxoid vaccines from >30 weeks, with the substantial use of animals required to determine vaccine efficacy, to approximately 12 weeks with minimal use of animals.

We acknowledge Kenji Yokota and Nazira Mahmut of Okayama University and James Burnell of the Department of Biochemistry and Molecular Biology, James Cook University, for their help with this study.

Editor: J. T. Barbieri

REFERENCES

1. **Black, J. D., and J. O. Dolly.** 1986. Interaction of ^{125}I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell Biol. 103:521–534.
2. **Clayton, M. A., J. M. Clayton, D. R. Brown, and J. L. Middlebrook.** 1995. Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*. Infect. Immun. 63:2738–2742.
3. **Craven, C.** 1964. Control of a botulinum-like disease in north Queensland. Aust. Vet. J. 40:127–130.
4. **DasGupta, B. R.** 1989. The structure of botulinum neurotoxins, p. 53–67. *In* L. L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, New York, N.Y.
5. **DasGupta, B. R., and H. Sugiyama.** 1972. A common subunit structure in *Clostridium botulinum* type A, B and E toxins. Biochem. Biophys. Res. Commun. 48:108–112.
6. **Fairweather, N. F., V. A. Lyness, and D. J. Maskell.** 1987. Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli*. Infect. Immun. 55:2541–2545.
7. **Holley, J. L., M. Elmore, M. Manchline, N. Minton, and R. W. Titball.** 2001. Cloning, expression and evaluation of a recombinant sub-unit vaccine against *Clostridium botulinum* type F toxin. Vaccine 19:288–297.
8. **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.
9. **Makoff, A. J., S. P. Ballantine, A. E. Smallwood, and N. F. Fairweather.** 1989. Expression of tetanus toxin fragment C in *E. coli*: its purification and potential use as a vaccine. Bio/Technology 7:1043–1046.
10. **Makrides, S. C.** 1996. Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol. Rev. 60:512–538.
11. **Nishika, T.-I., Y. Kamata, Y. Nenoto, A. Omori, T. Ito, M. Takahashi, and S. Kozaki.** 1994. Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. J. Biol. Chem. 269:10498–10503.
12. **Potter, K. J., M. A. Bevins, E. V. Vassilieva, V. R. Chiruvolu, T. Smith, L. A. Smith, and M. M. Meagher.** 1998. Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 13:357–365.
13. **Shone, C. C., P. Hambleton, and J. Melling.** 1985. Inactivation of *Clostridium botulinum* type A neurotoxin by trypsin and purification of two tryptic fragments. Eur. J. Biochem. 151:75–82.
14. **Simmons, G. C., and L. Tammemagi.** 1964. *Clostridium botulinum* type D as a cause of bovine botulism in Queensland. Aust. Vet. J. 40:123–127.
15. **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-16f genome. J. Vet. Med. Sci. 54:905–913.

INFECTION AND IMMUNITY, Mar. 2003, p. 1599–1603 Vol. 71, No. 3
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.3.1599–1603.2003

Purification of Fully Activated *Clostridium botulinum* Serotype B Toxin for Treatment of Patients with Dystonia

Hideyuki Arimitsu,[1] Kaoru Inoue,[1] Yoshihiko Sakaguchi,[1] Jaechul Lee,[1] Yukako Fujinaga,[1] Toshihiro Watanabe,[2] Tohru Ohyama,[2] Robert Hirst,[3] and Keiji Oguma[1]*

Department of Bacteriology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558,[1] and Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493,[2] Japan, and Department of Microbiology and Immunology, James Cook University, Townsville 4811, Australia[3]

Received 9 September 2002/Returned for modification 1 November 2002/Accepted 2 December 2002

***Clostridium botulinum* serotype B toxins 12S and 16S were separated by using a beta-lactose gel column at pH 6.0; toxin 12S passed through the column, whereas toxin 16S bound to the column and eluted with lactose. The fully activated neurotoxin was obtained by applying the trypsin-treated 16S toxin on the same column at pH 8.0; the neurotoxin passed through the column, whereas remaining nontoxic components bound to the column. The toxicity of this purified fully activated neurotoxin was retained for a long period by addition of albumin in the preparation.**

Clostridium botulinum strains produce immunologically distinct neurotoxins (serotypes A to G). The molecular masses of neurotoxin types A to G are approximately 150 kDa. The neurotoxins are produced as a single form and become dichain-form light (50-kDa) and heavy (100-kDa) chains by cleavage with proteases such as trypsin at about one-third of the distance from the amino terminus, and the toxic activity of the dichain form becomes fully activated (1). In culture fluid and food with acidic conditions, the neurotoxins associate with nontoxic components and form large complexes designated progenitor toxins. Under alkaline conditions, the progenitor toxins dissociate into neurotoxin and nontoxic components (10, 18). The progenitor toxins are found in three forms with molecular masses of 900 kDa (19S), 500 kDa (16S), and 300 kDa (12S) (14). The 12S toxin is composed of a neurotoxin and a nontoxic component having no hemagglutinin (HA) activity (designated nontoxic non-HA [NTNH]), whereas the 16S and 19S toxins are composed of a neurotoxin, NTNH, and HA. The serotype A strain produces three forms of toxins (19S, 16S, and 12S). Type B, C, and D strains produce the 16S and the 12S toxins. We purified different-sized progenitor toxins from serotype A, C, and D cultures (2, 4, 6, 12, 13) and demonstrated that (i) the 19S toxin is a dimer of the 16S toxin; (ii) HA consists of four subcomponents with molecular masses of 52 to 53, 33 to 35, 19 to 23, and 15 to 17 kDa, designated here as HA3b, HA1, HA3a, and HA2, respectively; and (iii) NTNH of the 12S toxin has a cleavage site(s) at the N-terminal region.

Recently, serotype A and B progenitor toxins have been used for treating patients with strabismus, blepharospasm, nystagmus, facial spasm, spastic aphonia, and many other forms of dystonia (9, 11). In both toxin types, progenitor toxins are used because they are easily obtained and are more stable than neurotoxin. The treatment is very effective but has a serious side effect for some patients in whom antiprogenitor toxins, including antineurotoxin antibodies, are produced after several injections. It seems that using neurotoxin alone is better than using the progenitor toxin (a complex of the neurotoxin and a nontoxic component). Furthermore, it has been reported that serotype B toxin, which is used therapeutically at present, is partially cleaved (16) and therefore the toxin is not fully activated. In this paper, we report a simple procedure for large-scale purification of botulinum serotype B progenitor toxin and neurotoxin, which have fully activated toxicity.

C. botulinum serotype B proteolytic strain Lamanna was cultured by the dialysis tubing method (17). The toxins were precipitated with 60% saturated ammonium sulfate, treated with protamine, and then applied to an SP-Toyopearl 650 M column (1.4 by 26 cm; Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium acetate buffer (pH 4.2) in the same manner as for the purification of serotype A toxin (6). All the chromatography steps discussed in this paper were performed at room temperature. The proteins were eluted with an NaCl gradient (0 to 0.5 M), and 2.5-ml fractions were collected. Four protein peaks were eluted (Fig. 1). Peak 1 was eluted as a shoulder of peak 2. Peak 2 possessed HA activity, but peak 1 did not, and both had very low toxicity. The toxin titer (minimum lethal dose [MLD]/ml) was obtained by injecting the diluted preparation into three mice intraperitoneally, each receiving 0.5 ml. The HA titer was obtained by reacting twofold-diluted preparations with an equal volume of 1% neuraminidase-treated human erythrocytes in a microtiter plate (7, 8). Peak 3 possessed both toxicity (8×10^7 MLD/ml) and HA activity. Peak 4, which eluted as a shoulder of peak 3, possessed high toxicity (2×10^8 MLD/ml) but a low HA activity. Therefore, it was speculated that peak 3 and peak 4 were 16S and 12S toxins, respectively. However, the two toxins could not be clearly separated either by the cation-exchange column or by gel filtration, especially when the amount of preparation was large. Therefore, we decided to establish a new purification procedure.

Recently, it was found that serotype A and B HA-positive

* Corresponding author. Mailing address: Department of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Phone: 81-86-235-7162. Fax: 81-86-235-7162. E-mail: kuma@md.okayama-u.ac.jp.

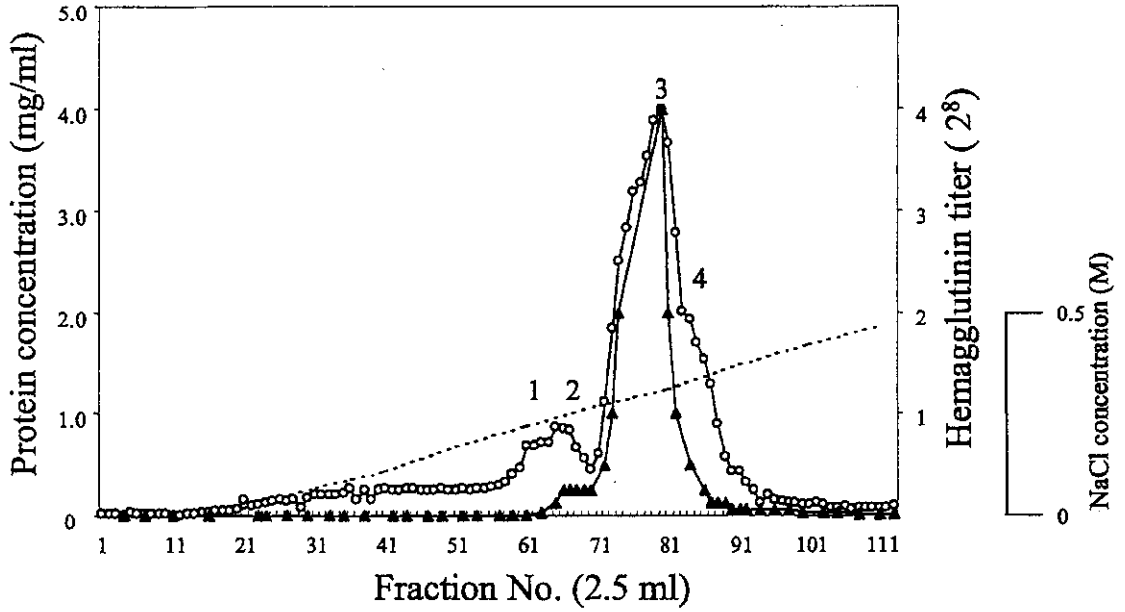

FIG. 1. Separation of progenitor toxins by SP-Toyopearl 650 M cation-exchange column chromatography. An ammonium sulfate-precipitated preparation treated with protamine was applied to the column and eluted with an NaCl gradient. The HA activities of some fractions were determined. O, protein; ▲, HA titer.

toxins could bind to both erythrocytes and the epithelial cells of the small intestine mainly via HA1 and that these bindings were effectively inhibited by lactose (3, 8) (data concerning serotype B have not been published yet), indicating that HA-positive toxins can bind to lactose via HA1. Based on these data, we planned to separate HA-positive 16S toxin and HA-negative 12S toxin by use of affinity gel column-linked lactose; it was speculated that the 12S toxin would pass through the

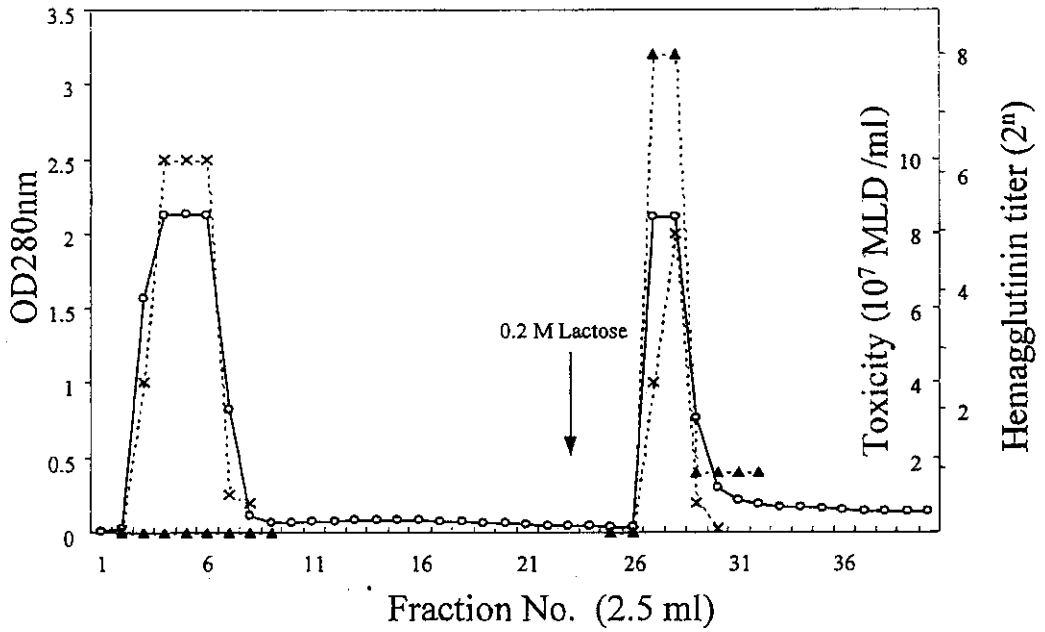

FIG. 2. Separation of 12S and 16S toxins by beta-lactose gel affinity column chromatography. Fractions 80 to 90 in Fig. 1 were pooled and concentrated, and 8.4 ml of the sample (52 mg) was applied to the column at pH 6.0. Similar results were obtained by employing fractions 70 to 79. O, protein; ▲, HA titer; ×, toxicity.

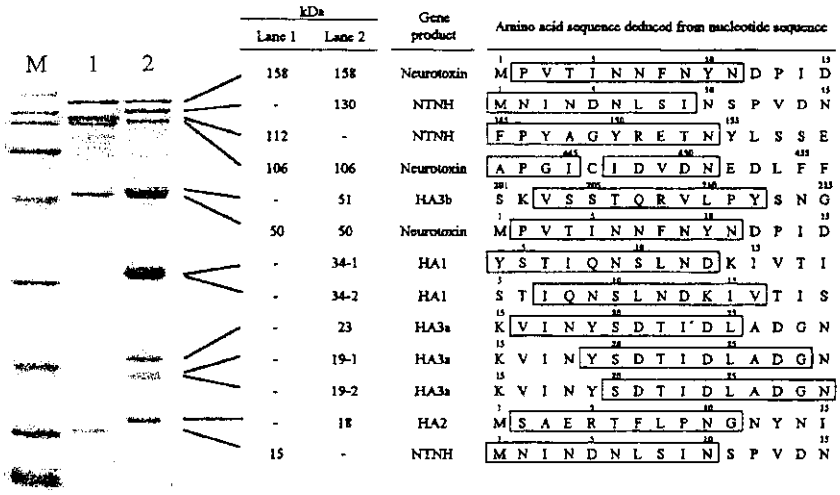

FIG. 3. SDS-PAGE profiles of the purified progenitor toxins. Each separated toxin (the same as in Fig. 2) and protein standards were heated at 100°C for 7 min in sample buffer with 2-mercaptoethanol. Electrophoresis was performed on a 12.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250. The N-terminal amino acid sequence of each band was also determined. The sequences corresponding to those deduced from the nucleotide sequences of genes are enclosed by open boxes. Lanes: M, molecular mass marker (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin. 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa); 1, unbound protein (12S toxin); 2, eluate (16S toxin).

column whereas the 16S toxin would bind to the column. Fractions 70 to 79 and 80 to 90 in Fig. 1 were separately pooled and concentrated with 80% saturated ammonium sulfate. After centrifugation at 15,000 × *g* for 30 min, each precipitate was suspended, dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and then applied to an aminophenyl beta-lactose gel column (1.0 by 6.0 cm; E-Y Laboratories Inc., San Mateo, Calif.) equilibrated with the same buffer. In both cases, the flowthrough fractions showed only high toxicity whereas the fractions eluted by the same buffer containing 0.2 M lactose

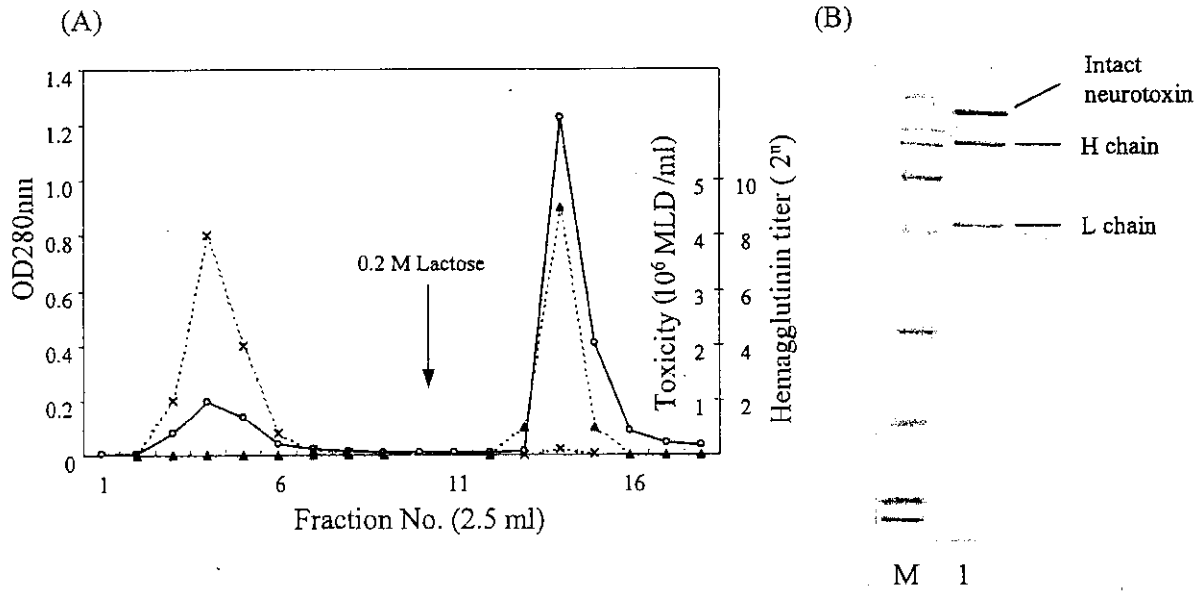

FIG. 4. (A) Separation of the neurotoxin by beta-lactose gel affinity column chromatography from the 16S toxin. The purified 16S toxin (3.5 mg) dialyzed against the buffer (pH 8.0) was applied to the column equilibrated with the same buffer. After the unbound protein was collected, the bound protein was eluted with the same buffer containing 0.2 M lactose. The HA and toxin titers of some fractions were determined. O, protein; ▲, HA titer; ×, toxicity. (B) SDS-PAGE profiles of the flowthrough protein (neurotoxin) shown in Fig. 4A. Electrophoresis was performed on a 12.5% polyacrylamide gel. Lanes: M, molecular weight marker; 1, unbound protein (neurotoxin).

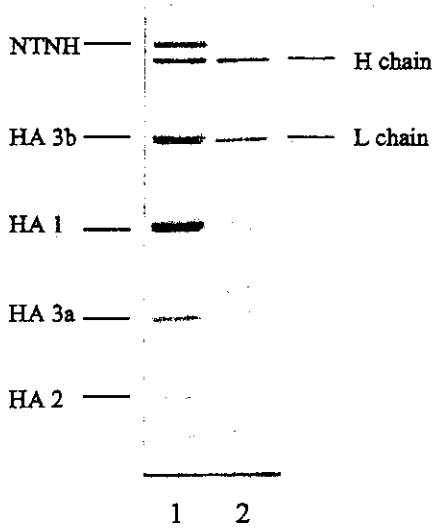

FIG. 5. SDS-PAGE profiles of fully activated 16S toxin and neurotoxin purified by beta-lactose gel affinity column chromatography. The 16S toxin was first incubated with trypsin for 1 h at 37°C and then purified by the lactose gel column at pH 6.0. By applying this fully activated 16S toxin on the same column at pH 8.0, the fully activated neurotoxin was obtained in the unbound fraction. Lanes: 1, fully activated 16S toxin; 2, fully activated neurotoxin.

showed both a high HA titer and toxicity, indicating that the former is 12S toxin and the latter is 16S toxin (Fig. 2). This was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by determining the N-terminal amino acid sequence of each band that appeared. The banding profiles of the flowthrough fraction (Fig. 3, lane 1) and the eluate (Fig. 3, lane 2) were similar to those of 12S and 16S toxins, respectively, of other serotypes (2, 4, 6, 12, 13), and the N-terminal sequences were identical to those deduced from the nucleotide sequences of the serotype B toxin genes previously published (Fig. 3) (15, 19, 20, 21). Thus, it was concluded that the molecular compositions of serotype B 12S and 16S toxins are similar to those of other serotypes.

The main fractions of peak 1 and peak 2 of Fig. 1 were also dialyzed and applied to the beta-lactose gel column. Both of them bound to the column and were eluted under the same conditions as those used for purification of the 16S toxin. By SDS-PAGE analysis, peak 1 proteins showed a single band with a molecular mass of 34 kDa, and peak 2 proteins showed four bands at 51, 34, 23, and 18 kDa (data not shown). Based on the N-terminal amino acid sequence, it was concluded that peak 1 and peak 2 proteins are HA1 and HA, respectively, as reported for serotype A toxins (6). Therefore, the beta-lactose gel was considered to be an affinity gel via at least HA1. The binding capacity of 1 ml of this matrix was estimated to be about 14 mg of 16S toxin. In one use of this purification method, 26 mg of the 12S toxin (3.2%) and 58 mg of the 16S toxin (7.2%) were obtained from the precipitates with 60% saturated ammonium sulfate of bacterial culture fluid (808.5 mg).

The progenitor toxins dissociate into a neurotoxin and a nontoxic component under alkaline conditions, 12S toxin dissociates into a neurotoxin and NTNH, and 16S and 19S toxins dissociate into a neurotoxin and a complex of NTNH and HA (10, 18). For purification of the neurotoxin, the purified 16S toxin thus obtained was first dialyzed against 10 mM sodium phosphate buffer (pH 8.0) to dissociate it to a neurotoxin and a nontoxic component, and then it was applied to the lactose gel column equilibrated with the same buffer (Fig. 4A). The neurotoxin passed through the column, whereas the nontoxic component bound to the column (the latter was eluted by the same buffer containing 0.2 M lactose). This was confirmed by SDS-PAGE; the flowthrough fraction demonstrated that the bands were consistent with the intact neurotoxin (158 kDa) and its heavy chain (106 kDa) and light chain (50 kDa) (Fig. 4B, lane 1). These results also indicated that the neurotoxin is not fully activated, even though this strain is proteolytic. Thus, we tried to purify the fully activated 16S and neurotoxin as follows. The partially activated 16S toxin preparation was incubated with bovine pancreatic trypsin (Sigma Chemical Co., St. Louis, Mo.) at pH 6.0 for 1 h at 37°C with a toxin-to-enzyme ratio of 100 to 1 in order to activate its toxicity. The preparation was then applied to the lactose gel column at the same pH. As expected, the fully activated 16S toxin (Fig. 5, lane 1) bound to the column, but the trypsin was washed out. The fact that trypsin does not bind to the column was confirmed independently (data not shown). This fully activated 16S toxin was dialyzed against the 10 mM sodium phosphate buffer (pH 8.0) and then layered on the column equilibrated with the same buffer. The fully activated neurotoxin (Fig. 5, lane 2) appeared in the unbound fractions. More simply, the fully activated neurotoxin could be obtained by applying the fully activated 16S toxin on the beta-lactose gel column at pH 6.0 and then changing the pH of the column with 0.1 M sodium phosphate buffer (pH 8.0) to dissociate the neurotoxin from the nontoxic component on the column.

Another problem in using the neurotoxin for treatment is the low stability of the neurotoxin in long-term storage. It was reported that the pH of the buffer affects the stability of toxicity

TABLE 1. Stability of fully activated neurotoxin under different storage conditions[a]

Buffer	Temp (°C)	pH	Toxin titer (MLD/0.5 ml) after storage for days indicated		
			40	90	180
20 mM sodium phosphate	4	6.0	<1		
		7.0	<1		
		8.0	<1		
	−80	6.0	1		
		7.0	1		
		8.0	1		
20 mM sodium phosphate + albumin	4	6.0	500	500	500
		7.0	500	500	500
		8.0	500	500	500
	−80	6.0	500	500	500
		7.0	500	500	500
		8.0	500	500	500

[a] The fully activated neurotoxin was diluted to 500 MLD/0.5 ml in buffer at different pHs with and without human serum albumin. After storage, the toxin titer remaining was determined for each sample.

of the serotype B progenitor toxin (16) and that albumin can be used to stabilize the serotype A toxin (5). Therefore, the fully activated neurotoxin was filtered with a 0.45-μm-pore-size membrane filter and then diluted to 1,000 MLD/ml in sterilized 20 mM sodium phosphate buffer with different pHs (6.0, 7.0, and 8.0), with or without human serum albumin (0.5 mg/ml). After storage of these preparations (0.5-ml volume each, in vials) at 4 or −80°C for 40, 90, or 180 days, the level of remaining toxicity was determined in an assay using mice. Regardless of temperature or pH, no reduction of toxicity was observed for the albumin-containing samples that had remained in storage for at least 6 months (Table 1). On the other hand, the samples without albumin dramatically lost their toxicity within 40 days.

These results may contribute to the development of new dosages of botulinum neurotoxin for the treatment of patients with dystonia. We are now investigating the effects of fully activated neurotoxin in animals electrophysiologically.

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and grants from the Japan Health Sciences Foundation and The Waksman Foundation of Japan.

REFERENCES

1. **DasGupta, B. R.** 1981. Structure and structure function relation of botulinum neurotoxins, p. 1–19. *In* George E. Lewis, Jr. (ed.), Biomedical aspects of botulism. Academic Press, London, United Kingdom.
2. **Fujinaga, Y., K. Inoue, S. Shimazaki, K. Tomochika, K. Tsuzuki, N. Fujii, T. Watanabe, T. Ohyama, K. Takeshi, K. Inoue, and K. Oguma.** 1994. Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. Biochem. Biophys. Res. Commun. **205**:1291–1298.
3. **Fujinaga, Y., K. Inoue, T. Nomura, J. Sasaki, J. C. Marvaud, M. R. Popoff, S. Kozaki, and K. Oguma.** 2000. Identification and characterization of functional subunits of *Clostridium botulinum* type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. FEBS Lett. **467**:179–183.
4. **Fujita, R., Y. Fujinaga, K. Inoue, H. Nakajima, H. Kumon, and K. Oguma.** 1995. Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. FEBS Lett. **376**:41–44.
5. **Goodnough, M. C., and E. A. Johnson.** 1992. Stabilization of botulinum toxin type A during lyophilization. Appl. Environ. Microbiol. **58**:3426–3428.
6. **Inoue, K., Y. Fujinaga, T. Watanabe, T. Ohyama, K. Takeshi, K. Moriishi, H. Nakajima, K. Inoue, and K. Oguma.** 1996. Molecular composition of *Clostridium botulinum* type A progenitor toxins. Infect. Immun. **64**:1589–1594.
7. **Inoue, K., Y. Fujinaga, K. Honke, K. Yokota, T. Ikeda, T. Ohyama, K. Takeshi, T. Watanabe, K. Inoue, and K. Oguma.** 1999. Characterization of haemagglutinin activity of *Clostridium botulinum* type C and D 16S toxins, and one subcomponent of haemagglutinin (HA1). Microbiology **145**:2533–2542.
8. **Inoue, K., Y. Fujinaga, K. Honke, H. Arimitsu, N. Mahmut, Y. Sakaguchi, T. Ohyama, T. Watanabe, K. Inoue, and K. Oguma.** 2001. *Clostridium botulinum* type A haemagglutinin-positive progenitor toxin (HA^+-PTX) binds to oligosaccharides containing Galβ1–4GlcNAc through one subcomponent of haemagglutinin (HA1). Microbiology **147**:811–819.
9. **Jankovic, J., and M. F. Brin.** 1991. Therapeutic uses of botulinum toxin. N. Engl. J. Med. **324**:1186–1194.
10. **Kozaki, S., S. Sakaguchi, and G. Sakaguchi.** 1974. Purification and some properties of progenitor toxins of *Clostridium botulinum* type B. Infect. Immun. **10**:750–756.
11. **Lew, M. F., A. Brashear, and S. Factor.** 2000. The safety and efficacy of botulinum toxin type B in the treatment of patients with cervical dystonia: summary of three controlled clinical trials. Neurology **55**(Suppl. 5):S29–S35.
12. **Oguma, K., K. Inoue, Y. Fujinaga, K. Yokota, T. Watanabe, T. Ohyama, K. Takeshi, and K. Inoue.** 1999. Structure and function of *Clostridium botulinum* progenitor toxin. J. Toxicol. **18**:17–34.
13. **Ohyama, T., T. Watanabe, Y. Fujinaga, K. Inoue, H. Sunagawa, N. Fujii, K. Inoue, and K. Oguma.** 1995. Characterization of nontoxic-nonhemagglutinin component of the two types of progenitor toxin (M and L) produced by *Clostridium botulinum* type D CB-16. Microbiol. Immunol. **39**:457–465.
14. **Sakaguchi, G., S. Kozaki, and I. Ohishi.** 1984. Structure and function of botulinum toxins, p. 435–443. *In* J. E. Alouf, F. J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, London, United Kingdom.
15. **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.
16. **Setler, P.** 2000. The biochemistry of botulinum toxin type B. Neurology **55**(Suppl. 5):S22–S28.
17. **Stern, M., and L. M. Wentzel.** 1950. A new method for the large-scale production of high-titre botulinum formol-toxoid type C and D. J. Immunol. **65**:175–183.
18. **Sugii, S., I. Ohishi, and G. Sakaguchi.** 1977. Correlation between oral toxicity and in vitro stability of *Clostridium botulinum* type A and B toxins of different molecular sizes. Infect. Immun. **16**:910–914.
19. **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.
20. **Yang, G. H., S. D. Rhee, H. H. Jung, and K. H. Yang.** 1996. Organization and nucleotide sequence of genes for hemagglutinin components of *Clostridium botulinum* type B progenitor toxin. Biochem. Mol. Biol. Int. **39**:1141–1146.
21. **Yang, G. H., S. D. Rhee, H. H. Jung, O. H. Jhee, and K. H. Yang.** 1998. Cloning and characterization of the upstream region of *Clostridium botulinum* type B neurotoxin gene. Biochem. Mol. Biol. Int. **45**:401–407.

Editor: J. T. Barbieri