Bivalent Recombinant Vaccine for Botulinum Neurotoxin Types A and B Based on a Polypeptide Comprising Their Effector and Translocation Domains That Is Protective against the Predominant A and B Subtypes

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The botulinum neurotoxins (BoNTs) are a large family of extremely potent, neuroparalytic, dichain proteins which act at the peripheral nervous system. The wide genetic diversity observed with this neurotoxin family poses a significant challenge for the development of an effective botulinum vaccine. The present study describes a vaccine development platform based on protein fragments representing the N-terminal two-thirds of each toxin molecule. These fragments, designated LH_A, comprise the light chain and translocation domains of each neurotoxin and are devoid of any neuron-binding activity. Using codon-optimized genes, LH_A fragments derived from BoNT serotypes A and B were expressed in Escherichia coli in high yield with >1 g of purified, soluble fragment recoverable from 4.5 liter-scale fermentations. The protective efficacy of LH_A/A was significantly enhanced by treatment with formaldehyde, which induced intramolecular cross-linking but virtually no aggregation of the fragment. A single immunization of the modified fragment protected mice from challenge with a 10^3 50% lethal dose (LD50) of BoNT/A1 with an 50% effective dose (ED50) of 50 ng of the vaccine. In similar experiments, the LH_A/B vaccine was shown to protect mice against challenge with BoNT/A subtypes A1, A2, and A3, which is the first demonstration of single-dose protection by a vaccine against the principal toxin subtypes of BoNT/A. The LH_A/B vaccine was also highly efficacious, giving an ED50 of ~140 ng to a challenge of 10^4 LD50 of BoNT/B1. In addition, LH_A/B provided single-dose protection in mice against BoNT/B4 (non-proteolytic toxin subtype).

The clostridial neurotoxins include tetanus toxin and the seven antigenically different botulinum neurotoxins (BoNTs), all of which exert their action by blocking the calcium-mediated release of neurotransmitters (24). The BoNTs act principally on the peripheral nervous system, where they inhibit the release of acetylcholine at the neuromuscular junction, an action that results in a widespread descending flaccid paralysis and ultimately the syndrome botulism. Because of the high potencies of the BoNTs, they are considered potential reagents for bioterrorist use and are currently designated by the Centers for Disease Control and Prevention as category A bioterror agents (1).

In their most active forms, the BoNTs consist of two subunits: a light chain (~50 kDa) linked by a disulfide bond to a heavy chain (~100 kDa). Structurally, these subunits are arranged into three distinct domains (17, 30): a 50-kDa HC domain that consists of two subdomains (of which the C-terminal subdomain is involved in neuronal acceptor binding), a translocation domain represented by the N-terminal half of the heavy chain (Hc domain), and a light-chain, effector domain (LC). Collectively, these domains enable the BoNTs to bind and translocate to within the presynaptic nerve terminal (6), where they act, via highly specific, zinc-dependent protease actions within the LC domain, to disable the process of calcium-mediated transmitter release (24).

While architecturally and mechanistically similar, the various serotypes of the BoNTs differ significantly in their primary structures (19) with the result that antibodies raised against one BoNT serotype offer no, or very little, protection against the biological action of another. Separate antigens are therefore required for each serotype to provide complete protection against the full spectrum of BoNTs. Vaccine development is further complicated by the occurrence of subtypes within most of the BoNT serotypes (13). For BoNT serotype A, for example, four subtypes have thus far been identified (designated BoNT/A1 to BoNT/A4) which display between 7 and 16% heterology in their primary nucleic acid sequences (2). These sequence variations occur primarily within surface-exposed regions on the molecule, thus maximizing their impact on antibody binding and neutralization and hence vaccine efficacy. Providing adequate cross-protection against the principal subtypes of each BoNT serotype must therefore be an important consideration in design of both vaccines and antibody-based therapeutics for the BoNTs.

Current vaccines for the BoNTs consist of formaldehyde-inactivated toxin complexes which were first developed in the 1950s. Although these vaccines are effective, they require specialized high containment manufacturing facilities and are difficult and expensive to manufacture in large quantities (9). The
initial design of recombinant vaccines was undertaken with the rationale of inhibiting a key facet of the biological activity of the BoNTs, such as receptor binding. Thus, first-generation recombinant vaccines under development are based on the receptor-binding domains (HC fragments) of each BoNT. These fragments, produced in *Pichia pastoris*, have been shown to provide a protective immune response in mice and have recently entered clinical trials (3, 4, 27). The HC fragments derived from the various BoNTs, however, differ markedly in their isoelectric points (pI 5.7 to 9.1), which make formulation of a multivalent vaccine difficult. More recent studies indicate that antibodies directed against the light chain and the HN region of the BoNT molecule can also provide a neutralizing immune response (5, 6).

The LH N fragment of the BoNTs is a polypeptide of ~100 kDa consisting of the light-chain domain in close association with the translocation domain (Fig. 1). A polypeptide belt from the latter surrounds the light chain under nonreducing conditions. In initial studies, the LH N fragment of BoNT/A was produced by prolonged trypsin digestion of the neurotoxin and shown to be a soluble, immunoactive fragment (26). Subsequently, LH N fragments from several BoNT serotypes have been produced by recombinant DNA technology and demonstrated to be useful as the core of a range of potential novel therapeutics (10, 29). In the present study, LH N fragment-based vaccines for BoNT/A and BoNT/B are described. A derivative of the LH N/A vaccine is shown to have exceptional efficacy in animal studies providing single-dose protection against BoNT/A subtypes A1, A2, and A3. The LH N/B vaccine is shown to provide protection against BoNT/B subtypes B1 and B2 (nonproteolytic).

**MATERIALS AND METHODS**

BoNTs. BoNT/A1, BoNT/A2, BoNT/B1, and BoNT/B2 were purified from *Clostridium botulinum* strains ATCC 3502, NCTC 2012, Okra, and Eklund 17B strains, respectively, using the exchange chromatography methods described previously (25). BoNT/A1 was obtained from Metabiologics, Inc.

Expression and purification of LH N recombinant fragments. (i) Expression of LH N fragments. Synthetic genes coding for BoNT/A1 (amino acid residues 1 to 871; Swiss-Prot P10845) and BoNT/B1 (amino acid residues 1 to 858; Swiss-Prot P10844) were optimized for expression in *Escherichia coli* and synthesized (Geneart AG) with NdeI and Xhol restriction sites at the 5' and 3' ends, respectively, to allow insertion into a PET26b expression vector. Both LH N sequences contained two amino acid substitutions for LH N/A (E224Q and H227Y) and for LH N/B (E231Q and H234Y) to remove their endopeptidase activity. After transformation into *E. coli* ER25566, cells were inoculated into 50 ml of inoculum medium containing phytone yeast extract plus essential salts, followed by incubation with agitation for 16 to 20 h at 37°C. This mixture was then used to inoculate 500 ml of growth medium, containing phytone yeast extract plus carbon source and essential salts, which was similarly incubated and subsequently used to inoculate 4.5 liters of growth medium in a 5-liter fermenter. The ER25566 cells were grown in the fermenter at 37°C with agitation to maintain dissolved oxygen levels at 30% until the OD600 was between 15 to 20 (approximately 6 to 8 h after seed inoculation). The fermentor temperature was reduced to 16°C and dissolved oxygen was reduced below 10% by increasing agitation and aeration, and the culture was incubated for a further 16 to 29 h. The process of slowing down the agitation and decreasing the oxygen levels in the fermenter triggered expression of the LH N fragments in an “autoinduction” process similar to that described by Studier (28).

(ii) Purification of LH N/A. Soluble recombinant LH N/A was extracted from *E. coli* cell paste by resuspension with 20 mM Tris-HCl (pH 8.0)–25 mM EDTA on ice with stirring for 1 h. The suspension was passed twice through an APV1000 homogenizer (9,000 lb/in²), clarified by microfiltration using a hollow-fiber cartridge (500-kDa cutoff), concentrated by ultrafiltration (30-kDa cutoff), and dialyzed into 20 mM Tris-HCl (pH 8.0)–25 mM EDTA. All column chromatography steps for LH N/A occurred at room temperature. The extract was then made 1 M with (NH₄)₂SO₄ and applied to a Toyopearl Phenyl-650 M column equilibrated with 20 mM Tris-HCl (pH 8.0), 5 mM EDTA (buffer B) and loaded onto a Q-Sepharose column as described above for LH N fragment (buffer B) and loaded onto a Q-Sepharose column equilibrated with buffer B. After a wash with buffer B containing 50 mM NaCl, the LH N/A sample was eluted from the column with buffer A containing 0.5 M (NH₄)₂SO₄. The eluate was concentrated and dialyzed against 10 mM Tris-HCl–0.1 mM EDTA (buffer A) and loaded onto a Q-Sepharose column equilibrated with buffer A. After a wash with buffer B containing 50 mM NaCl, the LH N/A sample was eluted with buffer B containing 130 mM NaCl. The eluate from the Q-Sepharose column was concentrated and dialyzed against 10 mM sodium phosphate (pH 6.5). LH N/A was eluted with 105 mM sodium phosphate (pH 6.5). The eluate from the hydroxyapatite column was concentrated and dialyzed against 10 mM HEPES (pH 7.4)–100 mM NaCl, filtered (0.2-μm pore size), and stored at −70°C.

(iii) Purification of LH N/B. The purification process for LH N/B was very similar to that of LH N/A, except that it was performed at temperatures between 2 and 8°C. After homogenization, the clarified material was concentrated and dialyzed before loading onto a Toyopearl Phenyl-650 M column equilibrated with chilled buffer A and 1 M (NH₄)₂SO₄. After the column was washed with equilibration buffer, LH N/B was eluted with buffer A containing 0.7 M (NH₄)₂SO₄ and 0.2% formaldehyde. The eluate was concentrated and dialyzed against chilled buffer B and then loaded and eluted from a Q-Sepharose column as described above for LH N/A. The eluate from this column was concentrated and then dialyzed against buffer C using a 30-kDa cutoff polysulfone membrane, and then the filtrate loaded onto a Toyopearl Phenyl-650 M column equilibrated with chilled buffer C. The column was washed with 40 mM sodium phosphate (pH 6.5). LH N/B was eluted with 105 mM sodium phosphate (pH 6.5). The eluate from the hydroxyapatite column was concentrated and stored at 70°C as described above for LH N/A. Prior to the efficacy tests, purified LH N/A and LH N/B proteins were filtered through Mustang E filters to reduce protein-associated endotoxin levels to <1 endotoxin unit/mg of protein as assessed by chromogenic Limulus amebocyte lysate assay.

**Formulation and formaldehyde treatment.** LH N fragments were adsorbed onto Alhydrogel (Biosector 1.3) such that the final formulation contained 10 mM HEPES (pH 7.4), 100 mM NaCl. Mixtures were then incubated at 35°C for 24 h and adsorbed onto Alhydrogel. Formaldehyde was removed by dialysis against 10 mM HEPES (pH 7.4)–100 mM NaCl. The concentration of vaccine efficacy. The efficacy of the recombinant vaccine candidates was determined by using the mouse challenge assay in which MF1 mice, separated into groups of 10 animals, were immunized with various doses of formulated vaccine (doses in 0.2 ml were administered subcutaneously). For one-dose studies, mice were immunized on day 0 and then...
challenged on day 28 with a 10^3 mouse intraperitoneal 50% lethal dose (LD_{50}) of BoNT (in 0.5 ml administered intraperitoneally). Survival of mice was monitored over 4 days postchallenge. For two-dose studies, animals were immunized on days 0 and 14 and challenged on day 28. ED_{50} values, i.e., the vaccine dose required to protect half the animals in a group from the challenge dose, were calculated by using four-parameter logistic curve analysis (SigmaPlot).

For efficacy studies in guinea pigs, groups of three animals were immunized with a single dose of formulated LHN, fragment and bled 28 days postimmunization. Serum pools, combining an equal volume from each of three animals, were assessed for toxin neutralization in mice. For these studies, various dilutions of the guinea pig serum were made with phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin/ml. These dilutions were mixed with a fixed concentration of BoNT/A toxin at 200 LD_{50} per ml. After incubation for 2 h at 22°C, 0.5 ml from each serum dilution-toxin mixture was injected into groups of four mice. The endpoint of titration was based on the death or survival of the mice after 96 ± 2 h. The dilution of test serum that protected 50% of mice (i.e., the 50% effective dose [ED_{50}]) was calculated by probit analysis for the test serum and reference antisera, and the results are reported in international units/ml and represent the neutralizing antibody concentrations.

**Endopeptidase and ELISAs.** (i) **Endopeptidase assay.** Endopeptidase activities of LHN, fragments were measured essentially by the assay procedure described by Hallis et al. (12), in which the formation of the BoNT substrate was measured using specific antibodies in an enzyme-linked assay system.

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(ii) **ELISA.** The ELISA described here was used to determine the purified protein yields of LHN/A and LHN/B, as well as to quantify purified BoNTs and LHN/A and LHN/B proteins for the endopeptidase assay described above. For these assays, antibodies raised in goats to toxins of the purified BoNTs were used. Purified immunoglobulin G was coated onto microtiter plates (at 5 μg ml^-1; 100 μl/well), followed by incubation for 1 h at 37°C. After a wash with PBS containing 0.1% Tween 20 (PBST), the plates were incubated for 1 h at 37°C (150 μl/well) with blocking buffer (PBST containing 5% fetal bovine serum). After being washed with PBST, the test samples (BoNTs or LHN, fragments) were diluted in blocking buffer and incubated for 1 h at 37°C (100 μl/well). Plates were washed with PBST, incubated with 1 μg of biotinylated goat antibody ml^-1 raised against toxin in blocking buffer for 1 h at 37°C (100 μl/well), and washed with PBST, and then a 1/1,000 dilution of a streptavidin-horseradish peroxidase conjugate (Sigma) was added for 10 min at 37°C. After a washing step with PBST, the substrates 3,3',5,5'-tetramethybenzidine and H_2O_2 were added for 10 to 15 min before the reaction stopped with TMB stop solution. The plates were read at 450 nm.

**RESULTS**

**Design of vaccine candidates.** Previous studies have shown that a trypsin-resistant LHN, fragment can be derived from BoNT/A, which represents the N-terminal two-thirds (residues 1 to 871) of the neurotoxin (Fig. 1) (26). Lacking the receptor-binding domain, the toxicity of the LHN, fragment is greatly reduced (2 × 10^{12}-fold) compared to BoNT/A but is still measurable with a mouse LD_{50} dose of ~150 μg. For vaccine development, a recombinant, nontoxic derivative of the LHN, fragment was designed in which two mutations were introduced (E_{224}Q and H_{227}Y) to abolish the endopeptidase activity within the light-chain domain. A synthetic gene encoding the derivative LHN, was synthesized by PCR. The gene, optimized for expression in E. coli, contained NdeI and XhoI restriction sites at the 5' and 3' ends, respectively, to allow subcloning into expression vectors. An analogous, mutated gene for LHN, (residues 1 to 858 of BoNT/B) was also synthesized.

**Expression and purification.** Both LHN/A and LHN/B were expressed as soluble recombinant fragments and purified by a combination of hydrophobic interaction, ion exchange, and hydroxyapatite chromatography. Typical yields of purified LHN,A from three 4.5-liter fermentation runs were 1.2, 1.3, and 1.4 g, with overall recoveries of 62, 67, and 62%, respectively. The purity was >95% as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Comparable purity and yields were obtained with LHN,B at the same scale with an average yield of 1.47 g of purified fragment from three fermentation runs.

The endopeptidase activity of purified LHN,A was assessed using an antibody-based assay system and compared to that of native, recombinant LHN, A. The endopeptidase assay is based on a modified ELISA format that uses antibodies which specifically recognize the cleavage products of the BoNTs. Using these antibodies, the cleavage of the human synaptosomal-associated protein SNAP-25 by BoNT/A and BoNT/E and of synaptobrevin by BoNT/B can be measured at concentrations of <1 ng/ml. At the highest concentration of LHN,A used (1.8 mg ml^{-1}), the fragment displayed no measurable endopeptidase activity, and this was reduced >10^{7}-fold compared to the native fragment. In contrast, LHN,B showed virtually identical reactivity in an antigen ELISA compared to the holotoxin (Fig. 3). Virtually identical data were obtained for LHN,B (data not shown).

**LHN,A formulation and efficacy evaluation.** With a theoretical pl value of 5.25, LHN,A was predicted to bind strongly to aluminum hydroxide (Alhydrogel) at physiological pH. In the presence of 10 mM HEPES (pH 7.4) and 100 mM NaCl, LHN,A (500 μg ml^{-1}) showed near-complete binding (>99%) to Alhydrogel solution (containing 3.1 mg of aluminum ml^{-1}) as assessed by protein assay of supernatant solutions after
centrifugation of the protein-Alhydrogel mixture. This formulation allowed administration of doses up to 100 μg of protein in mouse efficacy studies in which 0.2 ml was injected subcutaneously. In addition, it was determined that non-Alhydrogel-bound LH/N/A and LH/N/B vaccines were nontoxic to mice at doses of up to 750 μg/0.5 ml of PBS solutions (data not shown).

The protective efficacy was assessed as an ED50; the vaccine dose required to protect 50% of the mice in groups challenged with a BoNT dose of 103 mouse LD50. In initial studies, ED50 values were estimated after challenge with BoNT/A1 28 days after the administration of a single dose of vaccine (Table 1). Protection against BoNT/A1 challenge provided by the LH/N/A vaccine, several modifications to the protein were assessed against challenge with the BoNT/A 2 subtype at the highest vaccine dose tested.

Protection against BoNT/A1 challenge test provided by the LHN/A vaccine, several modifications to the protein were assessed against challenge with the BoNT/A 2 subtype at the highest vaccine dose tested. ED50 values were difficult to calculate accurately and were between 2 and 3 μg. No protection was observed against challenge with the BoNT/A2 subtype at the highest vaccine dose tested.

With relatively poor protection provided by the recombinant LH/N/A vaccine, several modifications to the protein were assessed. Conversion of the single-chain LH/N/A to the dimer form by trypsin treatment did not significantly improve the vaccine’s efficacy, and an ED50 value of 1.7 ± 0.39 μg was obtained. However, treatment with formaldehyde significantly enhanced the efficacy of LH/N/A as a vaccine. Incubation of LH/N/A (1 mg ml−1) with formaldehyde (0.2% at 35°C) for up to 96 h led to a broadening of the protein band on SDS-PAGE and also the appearance of a small proportion of a higher-molecular-mass band consistent in size with that of a dimer of the LH/N fragment (Fig. 2, lane 4). However, the appearance of HCHO-treated LH/N/A did not change significantly on SDS-PAGE after 24 h of incubation, so efficacy assessments were made. Initial ED50 tests on two different batches of LH/N/A suggested that the ED50 value was below 78 ng, and a third test gave an ED50 of 49 ± 7 ng against challenge with BoNT/A1. As well as providing >30-fold reduction in the ED50 value compared to the original untreated molecule, the HCHO-treated LH/N/A also afforded significantly better protection of animals at higher doses (Table 1). An even more marked enhancement in protection against challenge with BoNT/A2 was observed with almost complete protection of animals immunized with vaccine doses of >0.5 μg. In guinea pigs, protective efficacy was also significantly enhanced, although not to the same extent as in mice. For guinea pigs immunized with a single dose (10 μg) of untreated LH/N/A, the equivalent of 800 μl of pooled serum protected 50% of mice from challenge with 105 LD50 of BoNT/A1 in neutralization tests compared to the equivalent of 200 μl of serum from guinea pigs immunized with HCHO-treated LH/N/A, which represents a fourfold enhancement of efficacy.

To investigate possible mechanisms of the HCHO-mediated enhancement of efficacy, LH/N/A was treated with HCHO at lower protein concentrations (0.1 mg ml−1), which minimized the production of higher-molecular-mass forms of the fragment (Fig. 4, lane 2). Under these conditions, which generated only traces of the dimer LH/N/A form, an ED50 of 147 ± 13 ng was obtained that represents a statistically significant enhancement of efficacy compared to untreated controls. The efficacy enhancement would therefore not appear to be mediated by aggregated forms of the LH/N fragment. Native LH/N/A cleaved by trypsin appeared as two closely running bands of ~50 kDa on SDS-PAGE under reducing conditions which represent the light-chain and H/N fragments (Fig. 4). Under similar conditions, no such dissociation of the subunits was observed after HCHO treatment, which suggests a degree of intramolecular cross-linking had occurred.

**LH/N/A efficacy against BoNT/A subtypes.** An important consideration in vaccine design for the botulinum toxins is that they should offer protection against the principal subtypes of the relevant serotype. Both one- and two-dose ED50 tests were therefore conducted in which animals were challenged with either BoNT/A1, BoNT/A2, or BoNT/A3 toxin subtypes (Table 2). A single dose of formaldehyde-treated LH/N/A provided protection in mice against all three type A subtypes. In terms of the ED50, the protective efficacy against both BoNT/A1 and BoNT/A2 was reduced compared to that against BoNT/A3, approximately 6- and 45-fold, respectively. In studies evaluating the efficacy of two LH/N doses, substantial reduction in

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**TABLE 1. Single-dose efficacy studies of formaldehyde-treated LH/N/A vaccine**

<table>
<thead>
<tr>
<th>Vaccine dose (μg)</th>
<th>No. of surviving mice (of 10) at 4 days postchallenge with untreated LH/N/A</th>
<th>No. of surviving mice (of 10) at 4 days postchallenge with HCHO-treated LH/N/A</th>
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<tbody>
<tr>
<td></td>
<td>A1 challenge test 1</td>
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<tr>
<td>100</td>
<td>3</td>
<td>8</td>
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<tr>
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</table>

*a The ED50 values were as follows: A1 challenge test 1, 2.3 μg; A1 challenge test 2, 2.17 ± 0.5 μg; and A2 challenge test, >100 μg.

*b The ED50 values were as follows: A1 challenge test, 0.049 ± 0.007 μg; and A2 challenge test, 0.28 ± 0.02 μg.

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**FIG. 4. Intramolecular cross-linking of LH/N/A by formaldehyde.** Lane 1, LH/N/A control; lane 2, LH/N/A (0.1 mg ml−1) treated with 0.2% (vol/vol) formaldehyde for 24 h at 35°C; lanes 3 and 4, LH/N/A treated under nonreducing and reducing conditions (10 mM dithiothreitol), respectively; lanes 5 and 6, LH/N/A treated with 10 μg of trypsin ml−1 for 30 min at 37°C under nonreducing and reducing conditions (10 mM dithiothreitol), respectively; lanes 7 and 8, LH/N/A treated with trypsin and with 0.2% (vol/vol) formaldehyde for 24 h at 35°C under nonreducing and reducing conditions, respectively.
ED_{50} values were obtained for all three type A subtypes (Table 2).

**LH_{A/B} formulation and efficacy studies.** LH_{A/B} was formulated under conditions identical to those described for LH_{A} above. Under these conditions, nearly complete adsorption of LH_{A/B} was obtained in mixtures containing 500 μg of LH_{A/B} and Alhydrogel ml^{-1} (3.1 mg of Al ml^{-1}). The efficacy data for the LH_{A/B} vaccine are summarized in Table 3. In single-dose tests, LH_{A/B} displayed excellent protective efficacy, with ED_{50} values of <0.2 μg. In view of the significant enhancing effect of formaldehyde treatment on the efficacy LH_{A}, the effect of a similar modification of LH_{A/B} was assessed. Treatment of LH_{A/B} with 0.2% HCHO for 24 h at 35°C was found to induce band broadening and significantly more aggregation than observed with LH_{A} with prominent bands consistent with dimer and trimer formation evident on SDS-PAGE (Fig. 2, lane 6). In single-dose efficacy studies, ED_{50} values were obtained for untreated LH_{A/B} and HCHO-treated LH_{A/B} that were not significantly different (Table 2). The efficacy of LH_{A/B} vaccine was also assessed against challenge with BoNT/B4 subtype purified from the nonproteolytic C. botulinum strain, Ekland 17B. While protection was reduced two- to threefold compared to that against BoNT/B1, ED_{50} values of <1 μg were obtained after a single dose vaccine (Table 2).

**Bivalent efficacy assessment.** Formulations of LH_{A/B} and LH_{A/B} for bivalent efficacy studies contained final concentrations of 100 μg of each vaccine candidate ml^{-1}. For both LH_{A/B} and LH_{A/B}, single-dose ED_{50} values were obtained that were comparable to those obtained from monovalent tests (Table 4). No evidence of immunosuppression by either fragment was evident.

### DISCUSSION

The development of an effective vaccine for the BoNTs represents a significant challenge due to the considerable antigenic diversity that exists with this neurotoxin family (13). The approach described here, based on the LH_{A} fragments of the BoNTs, represents an extremely promising vaccine design platform, offering some key advantages over an H_{A}-based vaccine with respect to the production of an efficacious vaccine product. Both LH_{A} and LH_{A/B} were expressed in E. coli as soluble, stable fragments in high yield and could be purified to >95% purity by a relatively simple three-column purification protocol. These protocols are fully compatible with current good manufacturing practices, and scaled-up processes yielding 600 to 800 mg of antigen/liter have now been achieved in pilot studies. Formulation of LH_{A} and LH_{A/B} onto Alhydrogel was achieved under identical buffer conditions, with both fragments displaying high-capacity binding. In addition, since the theoretical pI values for the LH_{A} fragments derived from all seven BoNT serotypes occupy a very narrow range (4.96 to 5.41), it is envisaged that formulation of a multivalent vaccine could be readily achieved. Indeed, preliminary formulation studies using a recombinant LH_{A/B} vaccine candidate suggest that a trivalent (LH_{A/B}/E) vaccine can be formulated under the conditions described here.

The first stage of the action of the BoNTs, once in the bloodstream, is binding to receptors on the presynaptic nerve surface at the neuromuscular junction, and this action is mediated by the C-terminal subdomain of the H_{C} binding region (23, 26). Initial approaches in the design of a recombinant vaccine were focused on the premise of blocking this stage of BoNT action and vaccine candidates were therefore based on the BoNT H_{C} fragments (27). More recently, a vaccine candidate based on enzymatically inactive holotoxin of BoNT/A has been described (21). Antibodies generated to this holotoxin vaccine were shown to block the binding of BoNT to gangliosides in solid-phase assays, and it was proposed that this was, at least in part, the mechanism of toxin neutralization. Previous studies with monoclonal antibodies have shown that the neutralization of in vivo BoNT activity is complex. To date, single monoclonal antibodies, even of high affinity, have not been completely effective at neutralizing toxin activity (20). Combinations of three or more antibodies to nonoverlapping epitopes may be required to give potent toxin neutralization. The studies described above, however, focused on antibodies directed at the H_{C} domain. The murine model efficacy data generated in the present study show that a polyclonal antibody response against the LC and H_{A} domains of BoNT efficiently

### Table 2. Summary of ED_{50} values for LH_{A} efficacy studies

<table>
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<th>Efficacy test</th>
<th>Mean ED_{50} (μg) ± SD</th>
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<tr>
<td></td>
<td>A_{1} challenge test</td>
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<tr>
<td>One dose</td>
<td>0.049 ± 0.007</td>
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<tr>
<td>Two dose</td>
<td>0.017 ± 0.014</td>
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### Table 3. Summary of ED_{50} values for LH_{A/B} efficacy studies

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<th>Efficacy test</th>
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<td>B_{1} challenge test</td>
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<td>One dose (untreated)</td>
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<tr>
<td>One dose (HCHO treated)</td>
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<td>Two dose</td>
<td>0.08 ± 0.001</td>
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### Table 4. Single-dose efficacy studies for a bivalent (A/B) vaccine formulation

<table>
<thead>
<tr>
<th>Vaccine dose (μg)</th>
<th>No. of surviving mice (of 10) at 4 days postchallenge</th>
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<td>3</td>
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a ND, not determined.

b BoNT/B_{subp}, subtype B_{4} purified from the nonproteolytic C. botulinum strain Ekland 17B.
neutralizes in vivo activity. These findings also demonstrate that antibodies directed at epitopes on the H$_C$-fragment of the BoNTs are not required to generate a potent toxin-neutralizing immune response. The mechanism by which antibodies to the LHN$_{a}$/A fragments neutralize the activity of the BoNTs is unclear. The BoNT receptor at the neuromuscular junction has been shown to consist of both ganglioside and protein components (7, 16), and the possibility that multiple antibody molecules binding to the LHN$_{a}$/portion of the BoNT molecule could interfere with its interaction with one or both acceptor components cannot be discounted. Inhibition of the later stages of BoNT action, such as translocation into the nerve ending, is also feasible. Other mechanisms, such as rapid antibody Fc-mediated clearing of BoNT from the bloodstream also need to be considered.

In its unmodified form, LHN$_{a}$/A was found to be a poorer antigen than LHN$_{a}$/B, the former failing to provide complete protection to a BoNT/A challenge even at relatively high protein doses. Neither biochemical nor functional analyses indicated that the fragment was misfolded or unstable. LHN$_{a}$/A showed the same reactivity in ELISA as native BoNT/A and was resistant to trypsin digestion, as has been observed previously with LHN$_{a}$/A derived by proteolytic digestion of the holotoxin (26). The ability of formaldehyde treatment of LH$_{a}$/A to significantly improve protective efficacy of LH$_{a}$/A was a surprising finding in the present study. Historically, formaldehyde-mediated inactivation of toxins has been used for the preparation of several vaccines, including anthrax, diphtheria, tetanus, and botulinum vaccines. A serious drawback of the use of formaldehyde in this context is that it often leads to a reduction in immunogenicity via the modification of key epitopes (31). Indeed, previous studies have shown that detoxification of BoNT/A with formaldehyde leads to a loss of epitopes within the H$_C$ region (11). It should be noted, however, that generation of a botulinum toxoid requires formaldehyde incubation over a significantly longer period (25 to 28 days) compared to the relatively short incubation period (24 h) required to enhance the immunogenicity of LH$_{a}$/A.

The modification of proteins by formaldehyde is complex and can be divided into three types: methylol groups, Schiff bases, and methylene bridges (18). Of these, the formation of methylene bridges between arginine, lysine, and histidine residues is the most stable. The mechanism by which formaldehyde treatment enhances the immunogenicity of LH$_{a}$/A can only be speculated upon. From the present study, potential immunostimulatory effects of higher-molecular-mass aggregates of the LH$_{a}$/A fragment can be discounted since, under formaldehyde treatment conditions that eliminated intermolecular cross-linking, a significant enhancement of protective efficacy was still obtained. Evidence for intramolecular cross-linking was obtained, and the broadening of the protein band on SDS-PAGE suggests that multiple protein conformations of monomeric LH$_{a}$/A are present in the mixture. One possibility is that formaldehyde treatment, via the formation of intramolecular methylene bridges, stabilizes a conformation(s) of LH$_{a}$/A that is more efficient at eliciting a protective immune response. The ability of formaldehyde to stabilize protein conformation and function via intramolecular cross-linking has been reported previously (8), and it is well established that peptides with a flexible structure elicit a weaker immune response than proteins that are more highly ordered in tertiary structure (14, 22). Regardless of the mechanism, it is of considerable interest to explore a similar strategy with other proteins and peptides to assess the general applicability of the approach. Such studies are now in progress with several antigens, including LH$_{a}$/E.

The extent of the antigenic variation with the BoNT family has only become apparent over the last 5 years with characterization of numerous toxin subtypes within most BoNT serotypes. The sequence heterology between subtypes is not insignificant (up to 16% for the BoNT/A subtypes), which can have a major impact on the antigenic profile of the molecule (2). Mice immunized with a single dose of formaldehyde-treated LH$_{a}$/A were protected against challenge with BoNT/A$_1$, BoNT/A$_2$, and BoNT/A$_2$, which is the first demonstration of single-dose protection against these subtypes by a recombinant type A vaccine. A significant difference in the protective efficacy between BoNT/A$_1$ and BoNT/A$_2$ was observed: protection against the latter was reduced ~42-fold compared to BoNT/A$_1$, and this reflects the greater degree of heterology within the LC region of A$_1$ compared to A$_1$ and A$_2$. However, because of its extraordinary potency against BoNT/A$_1$, a single dose of formaldehyde-treated LH$_{a}$/A gave ED$_{50}$ values in the low-microgram range against a BoNT/A$_1$ challenge. The LH$_{a}$/B vaccine also displayed cross-protection against BoNT/B subtypes in a murine model. In addition to BoNT/B$_1$, the protective efficacy against the BoNT/B$_2$ subtype, which is produced by nonproteolytic B strains, was clearly demonstrated in a murine model.

The LH$_{a}$/A fragment provides an excellent platform for design of a recombinant botulinum vaccine. Recombinant fragments derived from BoNT/A and BoNT/B can be produced in high yield, are stable and easy to formulate, and provide single-dose protective efficacy against a broad range of toxin subtypes in a murine model. LH$_{a}$/A and LH$_{a}$/B were formulated as a bivalent vaccine and fully retained the respective potencies of the monovalent components. Additional studies are under way that focus on the development of an LH$_{a}$/E component which may enable the generation of a trivalent botulinum vaccine.

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REFERENCES


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