Evaluation of a Recombinant Hc of *Clostridium botulinum* Neurotoxin Serotype F as an Effective Subunit Vaccine

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A new gene encoding the Hc domain of *Clostridium botulinum* neurotoxin serotype F (FHc) was designed and completely synthesized with oligonucleotides. A soluble recombinant Hc of *C. botulinum* neurotoxin serotype F was highly expressed in *Escherichia coli* with this synthetic FHc gene. Subsequently, the purified FHc was used to vaccinate mice and evaluate their survival against challenge with active botulinum neurotoxin serotype F (BoNT/F). After the administration of FHc protein mixed with Freund adjuvant via the subcutaneous route, a strong protective immune response was elicited in the vaccinated mice. Mice that were given two or three vaccinations with a dosage of 1 or 10 μg of FHc were completely protected against an intraperitoneal administration of 20,000 50% lethal doses (LD₅₀) of BoNT/F. The BoNT/F neutralization assay showed that the sera from these vaccinated mice contained high titers of protective antibodies. Furthermore, mice were vaccinated once, twice, or three times at four different dosages of FHc using Alhydrogel (Sigma) adjuvant via the intramuscular route and subsequently challenged with 20,000 LD₅₀ of neurotoxin serotype F. A dose response was observed in both the antibody titer and the protective efficacy with increasing dosage of FHc and number of vaccinations. Mice that received one injection of 5 μg or two injections of ≥0.04 μg of FHc were completely protected. These findings suggest that the recombinant FHc expressed in *E. coli* is efficacious in protecting mice against challenge with BoNT/F and that the recombinant FHc subunit vaccine may be useful in humans.

The *Clostridium botulinum* neurotoxins synthesized by *C. botulinum* are highly toxic. Botulinum neurotoxins can be divided into seven serotypes (A to G), each with similar structures but distinct antigenicities. Human botulism is commonly associated with neurotoxin serotypes A, B, E, and F. Each neurotoxin consists of a heavy chain (100 kDa) and a light chain (50 kDa) and contains three functional domains. The carboxyl-terminal portion of the heavy chain (Hc receptor domain, 50 kDa) mediates binding to the target neurons. The amino-terminal portion of the heavy chain (H₅ translocation domain, 50 kDa) mediates transmembrane internalization of the toxin (5). The light chain is identified as a zinc endopeptidase, which blocks neuromuscular transmission (17, 20).

Prevention of botulism can be efficiently achieved by vaccination which generates neutralizing antibodies against botulinum neurotoxin. Currently, the most widely available vaccine against botulinum neurotoxin for humans under IND (for investigational new drugs) status is a formalin-inactivated pentavalent vaccine against serotypes A through E (14). However, other than neurotoxin serotype G, serotype F still has not been included in this vaccine, so it cannot protect against serotype F neurotoxin. Thus, a vaccine specific against botulinum neurotoxin serotype F (BoNT/F) is urgently needed. To overcome drawbacks of toxoid vaccines, including high cost, time consumption in production, and hazardous detoxification, a new type of recombinant Hc subunit vaccine against serotype F neurotoxin and other serotypes was investigated (6, 14). Also, DNA vaccines encoding the Hc domains of serotype F have been described as next-generation botulinum vaccines (3, 11).

As shown by previous studies (22), a soluble and stable recombinant Hc domain of *C. botulinum* neurotoxin serotype A was highly expressed in *Escherichia coli*, and the efficacy of this AHc vaccine against biologically active BoNT/A was demonstrated. In the present study one synthetic gene encoding the Hc domain of BoNT/F (FHc) was constructed and was highly expressed in *E. coli*. In addition, the recombinant FHc protein provided a protective immune response against BoNT/F in mice.

**MATERIALS AND METHODS**

**Construction of the FHc gene.** Initially, construction of the synthetic FHc gene encoding the Hc domain of BoNT/F (FHc, amino acids 858 to 1278, ~50 kDa) was divided into three sections: A (N-terminal end of Hc) to C (C-terminal end of Hc). In brief, each section (about 435 bp in length) was constructed with 12 overlapping oligonucleotides that were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). These oligonucleotides were typically about 50 bases in length and contained complementary sequences (15 bases) at the 5’ ends. The 3’ ends were annealed by T4 DNA polymerase (TaKaRa) and further cloned into pMD18-T (TaKaRa) in *E. coli* strain DH5α, respectively. After sequencing, sections B and C were fused into BC by fusion PCR. Following the same methods, ABC, containing the complete FHc gene of 1,263 bp, was obtained and cloned into pMD18-T. The correct clone with the FHc gene, confirmed by sequencing, was named pMD18-T-FHc.

**Expression of FHc in *E. coli* and its purification.** The oligonucleotide primers for PCR amplification of the synthetic FHc gene with pMD18-T-FHc as a template were designed as follows (the underlined sequences indicate the enzyme recognition sites): F-HcE (EcoRI), 5’_GCCGGAATTCTAATGTACTTC_; and R-HcX (XhoI), 5’_CTAGCTCGAGGTTTTCCTGAAA; and R-HcX (XhoI), 5’_CTAGCTCGAGGTTTTCCTGAAA. The PCR products were digested with EcoRI and XhoI to excise the FHc DNA fragment, which was then cloned into an expression vector pTIG-Trx plasmid digested by the same enzymes to create recombinant...
plasmid pTIG-Trx-FHc as described previously (22). The nucleotide of the cloned gene FHC was confirmed by sequencing to ensure authenticity. The correct clone pTIG-Trx-FHc was transformed into E. coli strain BL21(DE3) cells (Stratagene), and cultures of the recombinant BL21 were grown in L broth containing 100 μg of ampicillin/ml at 37°C until the optical density at 600 nm was 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside; 1 M; Sigma) was added to the culture at a final concentration of 0.4 mM, and growth was continued at 25 rpm for 3 h at 30°C. The cells were grown in 1000 ml of L broth, and harvested cells were resuspended in buffer A (20 mM NaH₂PO₄ [pH 7.4], 300 mM NaCl) and lysed by sonication. The resulting lysates were centrifuged at 15,000 × g for 30 min at 4°C, and the His₆ tag fusion proteins were purified by nickel affinity column chromatography (Amersham Biosciences) according to the recommendation of the manufacturer. In brief, the lysates were loaded onto a column of nickel-nitritotriacetic acid resin (5-ml bed volume) that had been equilibrated with 25 ml of buffer A. The column was washed with 40 ml of buffer A, followed by 20 ml of buffer B (buffer A containing 20 mM imidazole) and then eluted with 10 quantities of 1 ml of buffer C (buffer A containing 500 mM imidazole). The soluble fraction and the purified FHc were verified by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and Western blotting with hyperimmune horse toxin F antiserum (obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond-C; Amersham) with a semidry blot apparatus (semidy transfer cell; Bio-Rad).

Vaccination of mice and challenge with BoNT/F. Female BALB/c mice (specific pathogen free), 6 weeks of age, were randomly assigned to different groups. In the first vaccination study, groups of six mice were vaccinated with 1 or 10 μg of FHC. The FHC protein in phosphate-buffered saline (PBS) was mixed 50:50 with complete Freund adjuvant (Sigma) for the first vaccination and with incomplete Freund adjuvant (Sigma) for the second or third vaccinations. Each mouse was treated with 0.4 ml of the material in either two (days 0 and 14) or three vaccinations (days 0, 14, and 28) via the subcutaneous route. Each vaccination group was repeated once. In the second vaccination study, groups of six mice were vaccinated intramuscularly (i.e., in each thigh quadriceps bilaterally) with either two or three doses of 0.04, 0.2, 1, or 5 μg of FHC. Vaccine was diluted in 25% (vol/vol) Alhydrogel (Sigma) and then clutered with 10 quantities of 1 ml of FHC (buffer A containing 500 mM imidazole). The soluble fraction and the purified FHc were verified by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and Western blotting with hyperimmune horse toxin F antiserum (obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond-C; Amersham) with a semidry blot apparatus (semidy transfer cell; Bio-Rad).

FIG. 1. Construction of the FHc gene. (a) The synthetic FHc gene was constructed from three sections (A, B, and C), each of which was constructed from 12 overlapping oligonucleotides. (b) These sections (A to C) were annealed, ligated, and made double stranded by using PCR. The three sections were cloned into plasmid pMD18-T (T vector), and the nucleotide sequence of the three sections was sequenced. (c) After the 855-bp BC section was constructed by fusion PCR, the synthetic FHc gene was constructed by fusion PCR and cloned into pMD18-T, the nucleotide sequence of complete FHc was sequenced.

Statistical analysis. The ELISA antibody titer was statistically analyzed by using the Student t test between group differences or with the paired t test between two- and three-dose groups. Two-factor analysis of variance was also used to determine whether the data were statistically significant.

RESULTS

Construction and cloning of the synthetic FHc gene. The sequence of native C. botulinum gene contains 76% A + T and includes numerous codons that are rarely used by E. coli genes, which may lead to the poor expression of recombinant C. botulinum genes in E. coli (16). To improve the expression level of gene, a new FHc gene was designed with reduced content of A + T (52.4%) that contains synonymous codons that are frequently used in E. coli. The complete FHc gene of 1,263 bp, which was constructed from three sections with synthetic oligonucleotides by fusion PCR, encodes 421 amino acids (amino acids 858 to 1278, ~50 kDa) at the C terminus of the heavy chain of BoNT/F (Fig. 1). To express recombinant FHc product in E. coli, the gene was cloned into plasmids pTIG-Trx to obtain the recombinant plasmid of pTIG-Trx-FHc. The synthetic FHc nucleotide sequence has been submitted to GenBank under accession no. EU386771.

Soluble expression of FHc in E. coli and its purification. Recombinant FHc was expressed in E. coli BL21 and identified by both its molecular mass and reaction with specific antibodies to BoNT/F in Western blots (Fig. 2). The results showed that the product of FHc, ~50 kDa, was soluble and had a high level of expression (ca. 15% of the cell protein). Small-scale purification of FHc was carried out by nickel-nitritotriacetic acid affinity chromatography for the soluble protein. Purified proteins were analyzed by SDS-PAGE (Fig. 2A) and Western blot (probed with anti-BoNT/F antisera) (Fig. 2B). Eluted fractions containing expressed proteins were pooled and dialyzed overnight at 4°C against PBS. Protein concentrations were estimated by using a BCA protein assay (Merck) according to

![Diagram](https://via.placeholder.com/150)
the manufacturer's protocol. The approximate yield by one-step purification was 20 mg of purified protein per liter of culture. The product was judged to be >95% pure by SDS-PAGE.

Serum antibody titers and BoNT/F neutralization assay. FHc-specific total IgG antibodies of sera from FHc-vaccinated mice were analyzed by the standard ELISA. Total IgG titers of serum samples from mice of the first vaccination study 2 weeks after the last vaccination (two or three vaccinations) are shown in Fig. 3. A dose response was observed in the antibody titer with increasing doses of FHc and number of vaccinations. The group titer of mice vaccinated with FHc of 10 \( \mu \)g was higher than that of mice vaccinated with FHc of 1 \( \mu \)g (\( P < 0.05 \)). The group titers of mice vaccinated with 2 doses of 1 \( \mu \)g of FHc were 25, 600 and the group titers were increased, reaching as high as 204, 800 for the mice that were vaccinated with three doses. Furthermore, the group titers of mice vaccinated with two doses of 10 \( \mu \)g of FHc exceeded 409,600 for mice receiving three vaccinations (\( P < 0.05 \)); IgG isotype titers were analyzed by ELISA. The data are expressed as the geometric mean titers ± the standard errors of the mean. Similar results were obtained in two additional experiments. Significant differences: *, \( P < 0.05 \); **, \( P < 0.01 \).

![Image](image-url)

FIG. 1. SDS-PAGE (A) and Western blot (B) analysis of recombinant soluble FHc expressed in E. coli. Lane 1, low-molecular-weight protein markers; lanes 2 and 7, supernatant from cells transformed with pTIG-Trx vector; lanes 3 and 5, supernatant from cells transformed with pTIG-Trx-FHc vector; lanes 4 and 6, purified FHc. The molecular masses of the protein standards are indicated on the left in kilodaltons. An arrow indicates the position of the FHc.

![Image](image-url)

FIG. 2. SDS-PAGE (A) and Western blot (B) analysis of recombinant soluble FHc expressed in E. coli. Lane 1, low-molecular-weight protein markers; lanes 2 and 7, supernatant from cells transformed with pTIG-Trx vector; lanes 3 and 5, supernatant from cells transformed with pTIG-Trx-FHc vector; lanes 4 and 6, purified FHc. The molecular masses of the protein standards are indicated on the left in kilodaltons. An arrow indicates the position of the FHc.

![Image](image-url)

FIG. 3. Total IgG titers and IgG1 and IgG2a isotype profiles of anti-FHc from mice vaccinated with recombinant FHc. Mice were vaccinated with two or three doses of 1 or 10 \( \mu \)g of FHc. Serum samples from mice vaccinated with FHc were collected 2 weeks after the last injections, and the specific anti-FHc total IgG and individual IgG isotype titers were analyzed by ELISA. The data are expressed as the geometric mean titers ± the standard errors of the mean. Similar results were obtained in two additional experiments. Significant differences: *, \( P < 0.05 \); **, \( P < 0.01 \).

![Image](image-url)

TABLE 1. Protection of mice vaccinated via the subcutaneous route with recombinant FHc after neurotoxin challenge

<table>
<thead>
<tr>
<th>Vaccination dose (( \mu )g)</th>
<th>Challenge (LD(_{50}))^a</th>
<th>Two vaccinations</th>
<th>Three vaccinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( 2 \times 10^4 )</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>( 2 \times 10^3 )</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>( 2 \times 10^4 )</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>( 2 \times 10^3 )</td>
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<tr>
<td>PBS</td>
<td>( 2 \times 10^3 )</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

\( ^a \) Mice were challenged i.p. with different doses of biologically active BoNT/F 14 days after the last injection. Survival is expressed as the number of animals surviving of the six animals tested. Mice were vaccinated with two or three doses of 1 or 10 \( \mu \)g of FHc.

102,400, and the group titers were increased to a high level that exceeded 409,600 for mice receiving three vaccinations (\( P = 0.004 \); i.e., <0.01). The ELISA absorbance of the pooled sera from the negative control groups was low, showing serum titers of <200. In order to determine the nature of the immune response to FHc, isotypes of the IgG responses were carried out and are shown in Fig. 3. The titers of IgG1 were much higher than that of IgG2a in FHc-vaccinated mice, and the ratio of IgG1 to IgG2a was more than 15. These results suggest that FHc vaccination predominantly elicits Th2-type humoral immune responses.

To observe the protective antibodies against BoNT/F, sera from mice vaccinated with two or three doses of FHc in the first vaccination study were used in a BoNT/F neutralization assay. As shown in the assay, 4 \( \mu l \) of pooled sera from mice with two vaccinations fully protected mice against challenge with 100 LD\(_{50}\) of BoNT/F. The same amount of sera from mice with three vaccinations fully protected mice against challenge with 1,000 LD\(_{50}\) of BoNT/F. However, 64 \( \mu l \) of pooled sera from negative control groups did not protect the tested mice, which all succumbed within 12 h. Similar results were obtained in an additional experiment. According to the assay as described previously (4, 23), the calculated titers of protective antibodies neutralizing BoNT/F in the pooled sera were 25,000 LD\(_{50}\)/ml for the two-dose groups and 250,000 LD\(_{50}\)/ml for the three-dose groups.

**Efficacy of FHc subunit vaccine in mice.** In the preliminary protection study, mice were vaccinated with 1 or 10 \( \mu \)g of FHc via the subcutaneous route, and challenge was carried out i.p. with 2 \( \times 10^3 \) or 2 \( \times 10^4 \) LD\(_{50}\) of BoNT/F 2 weeks after the last vaccination. All of the mice vaccinated with two or three doses of FHc were fully protected against 2 \( \times 10^3 \) or 2 \( \times 10^4 \) LD\(_{50}\) doses of pure BoNT/F (i.p.) (Table 1). All of the vaccinated groups survived the challenge without adverse effects. In contrast, mice in the negative control groups succumbed to a typical botulism and died within 5 h. In addition, long-term protection was also tested. After 6 months, the vaccinated mice that received the three doses of FHc were rechallenged with 2 \( \times 10^3 \) LD\(_{50}\) doses of BoNT/F. One hundred percent protection was still maintained.

A more detailed study into the efficacy of the recombinant FHc in terms of the dose and the number of vaccinations that protected against neurotoxin challenge, using Alhydrogel adjuvant acceptable for human use, was performed in the mouse...
model. In the second vaccination study, mice were vaccinated intramuscularly either once, twice, or three times at four different dosages of FHc and subsequently challenged with 2 × 10^6 LD₅₀ of BoNT/F (Table 2). A dose response was observed in the antibody titer and protective efficacy with increasing FHc dose and number of vaccinations. Mice that received one injection of 5 µg and two or three injections of ≥0.04 µg of FHc were completely protected after challenge with 2 × 10^4 LD₅₀ of BoNT/F. Mice injected with one dose of ≤1 µg of FHc showed partial protection when challenged with biologically active BoNT/F. These results suggest that genetic engineering of the recombinant product FHc in a soluble form from E. coli BL21 is an effective immunogen and can protect against a challenge dose of biologically active BoNT/F.

**DISCUSSION**

As has been shown previously, these recombinant Hc subunit vaccines, synthesized in E. coli or yeast (7, 8, 12, 18), are less expensive and less hazardous to produce than the traditional toxin-inactivated vaccines. In addition, the pure and concentrated antigens may increase immunogenicity and protection efficacy in humans. Although recombinant Hc subunit vaccines from E. coli were reported in previous studies, low yields and/or poor solubility (the formation of inclusion bodies) have also limited their use for vaccine development (9, 10, 13, 21). Thus, it is desirable and practical to easily produce the soluble Hc in E. coli, but the yield of the recombinant protein needs to be increased to be economically feasible. Recently, our laboratory, among others, has expressed the soluble Hc domains in E. coli at levels sufficient for vaccine development (1, 2, 15, 19, 22). In addition, we have confirmed that a similar FHc domain can be produced in E. coli by using a completely synthetic gene encoding the Hc domain of C. botulinum BoNT/F and further exploited as a subunit vaccine against BoNT/F for human use.

In the present study a synthetic gene encoding the Hc domain of BoNT/F was designed, expressed, and purified from E. coli. The expression level of soluble recombinant protein FHc reach a level of 15% of cellular protein and demonstrated a yield of more than 20 mg of purified protein per liter of culture. This contrasts with 1 mg of protein per liter in a previous study for the FHc fused with glutathione S-transferase or maltose-binding protein carrier proteins (10). After the mice were vaccinated with recombinant FHc via the subcutaneous route, elevated titers of anti-FHc antibodies and BoNT/F neutralization antibodies in the pooled sera were observed. As a result, all of the mice vaccinated with two or three doses of 1 or 10 µg of FHc were fully protected against 2 × 10^6 LD₅₀ i.p. doses of pure BoNT/F. Long-term protection was also observed in FHc-vaccinated mice. In addition, a more detailed study of the efficacy of the recombinant FHc using Alhydrogel adjuvant acceptable for human use was performed in the mouse model. A dose response was observed in both the antibody titer and the protective efficacy with increasing FHc dosage and number of vaccinations. After mice were vaccinated with one dose of 5 µg and two or three doses of ≥0.04 µg of FHc, there was 100% protection when the animals were challenged with 2 × 10^4 LD₅₀ of pure BoNT/F. Based on these results, the recombinant FHc product shows strong immunogenicity and can protect mice against i.p. neurotoxin challenge, as well as previous subunit vaccines against BoNT/F and other BoNTs. The titers of IgG1 were much higher than those of IgG2a in FHc-vaccinated mice treated via the subcutaneous or intramuscular route (data not shown). This indicates that FHc vaccination predominantly elicits a Th2-type humoral immune response, which involves B-lymphocyte activation and antibody production. The hypothesis that protection against toxin is antibody mediated is reinforced by the high titers of the IgG1 subtype and neutralization antibodies observed in the present study.

We have made extensive efforts in the development of recombinant Hc vaccines against the four botulinum neurotoxin serotypes (A, B, E, and F) because they commonly cause human botulism. The approach to producing the recombinant Hc vaccines involves the cloning and expression of nontoxic Hc domains of botulinum neurotoxins, with an evaluation of immunogenicity and the protective efficacy of the Hc domains. It is encouraging that some problems existing in the expression of fragments derived from botulinum neurotoxins have been resolved in our laboratory, and the Hc domains of BoNT/B and BoNT/E have also been successfully expressed with this prokaryotic expression system. These recombinant Hc proteins possessing strong immunogenicity can further be used to prepare an antitoxin for treatment botulism by immunizing horses (data not shown). It is important to prepare an antitoxin for the treatment of patients with F serotype toxin botulism because these antitoxins currently used for humans tend to be bivalent (A/B) or trivalent (A/B/E) and do not offer protection against serotype F neurotoxin. It is suitable and practical to produce and purify the soluble recombinant protein FHc as a.

### Table 2. Survival and group antibody ELISA titers of mice after intramuscular vaccination with recombinant FHc

<table>
<thead>
<tr>
<th>Vaccination dose (µg)</th>
<th>Survival (no. of mice)</th>
<th>Group antibody ELISA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One vaccination</td>
<td>Two vaccinations</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>0.2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>0.04</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Mice were vaccinated either once, twice, or three times at four different dosages of FHc. Mice were challenged i.p. with 2 × 10^6 LD₅₀ of biologically active BoNT/F 14 days after the last injection.

b Survival is expressed as the number of animals surviving of the six animals tested.

c The antibody titers from pooled mouse serum were estimated as the reciprocal of the maximum dilution of serum giving an absorbance reading greater than 0.5 U.
subunit vaccine in a large scale using our prokaryotic expression system. Given all of these factors, the recombinant FHc vaccine produced in E. coli has promise to be developed as a safe and efficacious vaccine against BoNT/F for human use.

REFERENCES


