Efficacy of a Potential Trivalent Vaccine Based on Hc Fragments of Botulinum Toxins A, B, and E Produced in a Cell-Free Expression System

R. Zichel, A. Mimran, A. Keren, A. Barnea, I. Steinberger-Levy, D. Marcus, A. Turgeman, and S. Reuveny

Department of Biotechnology and Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, P.B. 19, Ness Ziona, 76100 Israel

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Botulinum neurotoxins produced by the anaerobic bacterium Clostridium botulinum are the most potent toxins in nature. Traditionally, people at risk are immunized with a formaldehyde-inactivated toxin complex. Second generation vaccines are based on the recombinant carboxy-terminal heavy-chain (Hc) fragment of the neurotoxin. However, the materialization of this approach is challenging, mainly due to the high AT content of clostridial genes. Herein, we present an alternative strategy in which the native genes encoding Hc proteins of botulinum toxins A, B, and E were used to express the recombinant Hc fragments in a cell-free expression system. We used the unique property of this open system to introduce different combinations of chaperone systems, protein disulfide isomerase (PDI), and reducing/oxidizing environments directly to the expression reaction. Optimized expression conditions led to increased production of soluble Hc protein, which was successfully scaled up using a continuous exchange (CE) cell-free system. Hc proteins were produced at a concentration of more than 1 mg/ml and purified by one-step Ni²⁺ affinity chromatography. Mice immunized with three injections containing 5 μg of any of the in vitro-expressed, alum-absorbed, Hc vaccines generated a serum enzyme-linked immunosorbent assay (ELISA) titer of 10⁵ against the native toxin complex, which enabled protection against a high-dose toxin challenge (10⁵ to 10⁶ mouse 50% lethal dose [MsLD₅₀]). Finally, immunization with a trivalent HcA, HcB, and HcE vaccine protected mice against the corresponding trivalent 10⁵ MsLD₅₀ toxin challenge. Our results together with the latest developments in scalability of the in vitro protein expression systems offer alternative routes for the preparation of botulinum vaccine.

Botulinum neurotoxins (BoNT) are the most potent toxins known in nature, having an estimated human 50% lethal dose (HLD₅₀) of 1 ng/kg body weight (2). There are seven different serotypes of the toxin, designated A to G, of which types A, B, E, and F are toxic for humans. The neurotoxins are produced by several species of the genus Clostridium, in particular, Clostridium botulinum (several subtypes of BoNT A to F), C. butyricum (2 known E subtypes), C. baratii (one F subtype), and C. argentinense (BoNT G). Each BoNT serotype is defined by the specificity of antibody neutralization.

Historically, vaccines against botulinum were based on formaldehyde-inactivated toxoids adsorbed on aluminum hydroxide. The licensed pentavalent (ABCDE) vaccine was manufactured in the early 1960s and is currently available only in limited supply (15, 42). In the late 1980s, it was shown that the 50-kDa carboxy-terminal fragment of the botulinum toxin heavy chain (Hc) could serve as a nontoxic vaccine candidate (13, 32). However, early attempts to express these fragments as recombinant proteins in cellular systems showed poor results, mainly because of a codon bias between native, AT-rich, clostridial genes and host codon usage (29, 39). This problem was addressed both in Escherichia coli and in the methylotrophic yeast Pichia pastoris by the generation of synthetic genes in which all rare codons were replaced by the more abundant codons of the host (6, 9, 35).

Hc proteins expressed in E. coli were mostly extracted as intracellular proteins and could hardly be collected as soluble secreted products (9, 19, 38). Similarly, secreted Pichia Hc was shown to be glycosylated in a manner that abolished its protective activity (41). Therefore, the nonglycosylated protein had to be purified from intracellular compartments. These observations have led to a state in which optimal production of Hc proteins is dependent on the generation of synthetic genes and subsequent product purification from intracellular compartments. Indeed, a new generation botulinum vaccine based on synthetic Hc genes expressed in P. pastoris is being developed by the U.S. army for humans at risk (5).

An alternative strategy for the expression of such unique recombinant proteins relies on the use of cell-free expression systems (CFES). These systems include all the regulatory elements required for in vitro transcription and translation of a protein from a template DNA (12, 18, 21). As the cell wall barrier is not present in cell-free systems, the reaction environment can be readily controlled, and different components can be directly added to promote protein folding and solubility (36). Moreover, the open nature of the cell-free system also allows rare tRNAs to be added directly into the reaction mix, enabling efficient expression of AT-rich and codon-biased genes (49).

Cell-free systems have the potential for higher productivity than current cellular production systems, since all of the cel-
lular resources can be efficiently directed toward production of the recombinant protein. Yet, until recently, only microgram quantities of recombinant protein could be expressed in CFES, mainly due to the rapid elimination of substrates and the accumulation of certain inhibiting by-products (18, 22, 25). This limitation was overcome by the development of continuous exchange-cell-free (CECF) systems. These systems enable the continuous supply of substrates and washing out of low-molecular-weight by-products known to inhibit the expression reaction, allowing for the continuous accumulation of the product in a membrane-separated reaction compartment (45). The use of CECF systems was shown to allow for the prolonged and efficient expression of proteins and to increase the product yield to the mg/ml range (23, 26, 50, 51). More recently, additional improvements were introduced to the field of cell-free biology that reduced costs and made this platform attractive for commercial production, especially for difficult-to-express proteins (8, 20, 37, 46). Moreover, the development of economical scale-up technologies allowed efficient cell-free expression of protein in a 1-liter reactor (50), and later this system was further scaled up to a 100-liter tank (10). The ability to produce large quantities of proteins, which are difficult to express in cellular systems, opens the possibility of using this system for botulinum vaccine production.

In this work, we tested the feasibility of designing a second generation multivalent botulinum vaccine based on recombinant Hc fragments of botulinum toxins A, B, and E expressed in a scalable, cell-free system using native gene templates. Indeed, DNAs encoding the Hc fragments of botulinum toxins A, B, and E were expressed using a commercial continuous-exchange cell-free system. The in vitro-expressed Hc proteins were immunorecognized specifically by anti-homolog toxin sera, and mice immunized with the in vitro-expressed recombinant Hc vaccine were protected from a high-dose homolog toxin challenge. Finally, mice immunized with a trivalent vaccine preparation of HcA, HcB, and HcE were protected against a high-dose botulinum A, B, and E multitoxin challenge.

**MATERIALS AND METHODS**

**Materials.** Chemicals and molecular reagents were purchased from Sigma-Aldrich and New England Biolabs, respectively, unless otherwise stated. **Bacteria and toxins.** *Clostridium botulinum* A, B, and E strains were obtained from the IBR collection (A198, B592, and E450, respectively). A sequence analysis revealed compliance of the neurotoxin genes with sequences 62A (accession no. M30196), Danish (accession no. M81168), and NCTC11219 (accession no. X62683) for *Clostridium botulinum* subtypes A1, B1, and E botulinum, respectively (4, 44, 54, 55).

*Clostridium botulinum* A, B, and E toxins were prepared from concentrated supernatant of cultures grown for 6 days in anaerobic culture tubes. Botulinum E was used in its activated form throughout the study. Activation was performed by treatment with 0.1% (wt/vol) trypsin (37°C, 1 h). Toxic activity was calibrated by the standard mouse bioassay (5).

**Antiser.** Rabbit and horse antitoxin (A, B, or E) sera were collected from hyperimmune rabbits immunized with the respective toxoid (50 by the standard mouse bioassay (5)). The primers used for cloning were 5'-TATGATGATGAGAACCCCCCCTGGGCGTTCCTCC-3' and 5'-CTTATGAAGAGGATTACAGATGATATTATGATACCATCAGAATTTACGAA-3' for HcA, 5'-GAACGCCGATCTTTACGTCACCCCTTCTAC-3' and 5'-CACAAGAATGGCGGCAGAATATATAAATTAGCGAAATTTAAA-3' for HcB, and 5'-GAACGC CGATCCTTTATTTTTCGCTTGATCTTCTTC-3' and 5'-CACAAGAATGCGGCGGCACTCATTACGATGATAAAATTTTA-3' for HcE. Cloning was verified using standard sequence analysis.

**In vitro Hc protein expression.** Proteins were expressed using the RTS-100 *E. coli* HY kit, RTS-500 *E. coli* HY kit, or RTS-9000 *E. coli* HY kit (Roche Diagnostics), according to the manufacturer's instructions. For expression optimization, different combinations of DNAK (70-kDa heat shock protein [HSP-70]) and GroE (HSP-60) supplements (Roche Diagnostics) and protein disulfide isomerase (PDI; Takara) were used according to the manufacturer's instructions, in reduced (1 mM glutathione [GSH]-0.1 mM glutathione disulfide [GSSG]) or oxidized (0.1 mM GSH–1 mM GSSG) environments. Product solubility was assessed by comparing fractions collected before and after centrifugation of the reaction mixture at 14,000 × g for 10 min.

**Polymerization and analysis.** At the end of protein synthesis, the mixture was diluted 1:25 in binding buffer (10 mM imidazole, 300 mM NaCl in 50 mM phosphate buffer, pH 7.5) and clarified through a 0.22-µm-pore filter. The filtered lysate was loaded onto a Ni²⁺-HiTrap chelating affinity chromatography column (1 ml; GE Healthcare). After washing with 60 mM imidazole, His-fused protein was eluted with 300 mM imidazole–300 mM NaCl–50 mM phosphate buffer, pH 7.5. Fractions containing Hc protein were pooled and dialyzed overnight against 50 mM NaCl–50 mM phosphate buffer, pH 6.5. Total protein concentrations were determined using the bicinchoninic acid (BCA) (Pierce Chemicals) standard assay, using BSA as a standard, and purity was determined by SDS-PAGE. A dynamic light scattering (DLS) analysis was conducted using the model 902 DLS instrument (Vicottek). Correlation functions were obtained via OmnI SIZE software.

**Western blot analysis.** Samples were separated by 10% SDS-PAGE using a Mini-Protein III electrophoresis cell (Bio-Rad Laboratories). Proteins were then transferred to a Hybond nitrocellulose membrane using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories) in Tris-glycine transfer buffer. Blotted membranes were rinsed with phosphate-buffered saline–0.05% Tween 20 (PBST) and blocked for 1 h at 37°C with 200 µl per well of 5% nonfat dry milk in PBST. The membranes were washed with PBST and then incubated at 4°C overnight with the appropriate antiserum diluted in 1% skim milk in PBST. The membranes were washed in PBST and incubated with alkaline phosphatase-conjugated secondary antibody in 1% skim milk in PBST for 1 h at room temperature. Membranes were washed in PBST, and the colorimetric reaction was developed with BCIP (5-bromo-4-chloro-3-indolylphosphate) liquid substrate (Sigma).

**Determination of Hc concentration by ELISA.** To evaluate the Hc expression level, enzyme-linked immunosorbent assay (ELISA) plates (Maxisorp; Nunc) were coated with 50 µl rabbit antitoxin diluted 1:4,000 in coating buffer (50 mM Na2CO3, pH 9.6) and incubated overnight at 4°C. Plates were then washed in PBST and blocked for 1 h at 37°C with 200 µl per well of 2% (wt/vol) bovine serum albumin (BSA) in Tris-NaCl, pH 7.6 (TSTA). After washing, plates were incubated with serial dilutions (50 µl, in duplicate) of the tested expression reaction samples in TSTA for 1 h at 37°C. Plates were then washed with PBST and incubated with the specific horse antitoxin (50 µl) diluted 1:500 in TSTA for 1 h at 37°C. After additional washing with PBST, the plates were incubated with 50 µl of conjugated goat anti-horse F(ab')2 diluted 1:700 in TSTA for 1 h at 37°C. Finally, plates were washed 4 times with PBST, and the color reaction was developed using p-nitrophenyl substrate (Sigma). Absorbance was measured at 405 nm, and the Hc concentration was determined by interpolation from a 1.56- to 12.5-ng/ml standard curve of pure Hc.

**Immunization of mice with Hc vaccine.** Pure HcA, HcB, or HcE was adsorbed to aluminum hydroxide [0.5% (wt/vol) final concentration of Al(OH)3] to gen-
erate monovalent (5 μg HcA, HcB, or HcE in a 0.2-ml dose) or trivalent (5 μg of each Hc in a 0.2-ml dose) vaccine preparations. ICR mice (Charles River) (10 per group) were immunized subcutaneously (s.c.) with the indicated preparation for three consecutive immunizations at 0, 4, and 8 weeks. Ten days after each immunization, serum samples were collected for titer analysis.

**Determination of antitoxin titer.** To evaluate the development of the immune response to the Hc vaccine, ELISA plates (Maxisorp; Nunc) were coated with 50 μl rabbit antitoxin diluted 1:4,000 in coating buffer (50 mM Na2CO3, pH 9.6) and incubated overnight at 4°C. Plates were then washed 4 times with 300 μl of PBST and blocked for 1 h at 37°C with 200 μl per well 2% (wt/vol) BSA in Tris-NaCl, pH 7.6 (TSTA). After washing, plates were incubated with botulinum toxin A, B, or E (300 ng/ml NT, 50 μl per well, in duplicates) in TSTA for 1 h at 37°C. Plates were then washed with PBST and incubated with serial dilutions of mouse sera (50 μl) in TSTA for 1 h at 37°C. After washing with PBST, the plates were incubated with 50 μl of horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (Jackson) diluted 1:700 in TSTA for 1 h at 37°C. Finally, plates were washed with PBST, and the colorimetric reaction was developed for 15 min using 50 μl tetramethylbenzidine (TMB) substrate (Sigma). The reaction was stopped with 50 μl 0.5 M H2SO4, and absorbance was measured at 450 nm. Titors were determined as the last dilution having a signal greater than 3 standard deviations above control naive sera.

**Mouse protection assay.** Ten days after the last vaccination, mice were challenged with a 107 to 109 mouse lethal dose (MLD50) of the homolog toxin (three mice/group), and survival was monitored for 7 days. Botulinum E challenge studies were conducted with trypically activated toxin E. All animal experiments were performed in accordance with Israeli law and were approved by the Ethics Committee for animal experiments at the Israel Institute for Biological Research.

**Statistical analysis.** Statistical analysis was carried out using repeated analysis of variance (ANOVA) performed with GraphPad Instat 3 software. Where indicated, the 25th percentile was calculated using GraphPad Prism version 5.00 for Windows. Differences were considered significant when P was <0.05.

**RESULTS**

**Expression of the clostridial AT-rich HcA gene in a cell-free system.** The compliance of AT-rich genes with cell-free expression was evaluated by testing the expression of the carboxy-terminal fragment of botulinum neurotoxin A heavy chain (HcA) using the native gene encoding amino acids 861 to 1296 as template content and is abundant with codons that are rare in AT-rich genes. To confirm efficient translation, the native gene encoding HcA was amplified at the NcoI/H9262 position (HcA-His6; 1,676 bp) and the second with a His6 tag terminal fragment of botulinum neurotoxin A heavy chain. The linear template system we used for efficient in vitro transcription and translation of the HcA gene encoding amino acids 861 to 1296 from botulinum toxin A. This gene has an approximately 70% AT content and is abundant with codons that are rare in E. coli codon usage. Expression was performed using the RTS-100 expression system for 6 h at 25°C. Total and soluble fractions of the reaction mixture were separated by SDS-PAGE (left panel) and analyzed by immunoblotting (right panel) using rabbit antipeptide specific to 17 aa at the C terminus of botulinum type A heavy chain. Lane 1, negative control (no DNA); lane 2, total HcA-His6; lane 3, soluble HcA-His6; lane 4, total His6-HcA; lane 5, soluble His6-HcA; lane M, molecular mass marker.

HcA expression was demonstrated at this stage by immunoblotting but not in SDS-PAGE, which is in agreement with the expected low yield of a batch mode cell-free system. The specific antipeptide sera positively reacted with the HcA that migrated, as expected, as a 47-kDa protein (Fig. 1B; right panel). Both HcA constructs (His6-HcA and HcA-His6) showed similar expression efficiencies and only partial solubility. Since the two constructs showed similar performances, we arbitrarily chose the HcA-His6 construct for further studies.

Expression of HcA using the linear template served as a proof of concept to demonstrate that the cell-free expression system contains the necessary elements for the expression of proteins encoded by native AT-rich genes. These results were the basis for further optimization studies of HcA expression and solubility, as well as for expanding the use of this expression system for HcB and HcE production.

**Optimization of expression level and solubility of Hc (A, B, and E) proteins.** The linear template system we used for feasibility studies was unable to produce the amount of DNA template required for scale-up studies. Hence, the HcA gene was cloned into a pIVEX2.3 plasmid (Roche Diagnostics) to generate HcA fused to a C'-terminal His6 tag (HcA-His6). The HcA construct was sequenced and found to be identical to the endogenous gene obtained from chromosomal Clostridium botulinum type A DNA.

To optimize the solubility and yield of the expressed protein, different combinations of chaperones, enzymes, and factors that promote native folding of proteins were added. Thus, expression experiments were conducted in the presence of the HSP-70 (DnaK/DnaJ/GrpE) or HSP-60 (GroE) chaperone systems. Since native botulinum toxins contain an intrachain disulfide bond at the Hc region (28), protein disulfide isomerase (PDI) was tested for its effect on expression and solubility under oxidizing (10:1 GSSG/GSH ratio) or reducing (10:1 GSH/GSSG ratio) environments. Reaction products (total and

**FIG. 1. In vitro expression of HcA using linear template DNA.** (A) Two-step PCR was used to add all the regulatory elements required for efficient in vitro transcription and translation of HcA. The first PCR generated universal sequences that complemented the 2nd PCR primers in which regulatory elements were added. The PCR products of the first (lanes 2 and 3) and second (lanes 4 and 5) PCRs for HcA-His6 (lanes 2 and 4) and His6-HcA (lanes 3 and 5) were separated on 1% agarose gel. (B) SDS-PAGE and immunoblotting of the in vitro-expressed proteins. The recombinant proteins were expressed using the RTS-100 expression system for 6 h at 25°C. Total and soluble fractions of the reaction mixture were separated by SDS-PAGE (left panel) and analyzed by immunoblotting (right panel) using rabbit antipeptide specific to 17 aa at the C terminus of botulinum type A heavy chain. Lane 1, negative control (no DNA); lane 2, total HcA-His6; lane 3, soluble HcA-His6; lane 4, total His6-HcA; lane 5, soluble His6-HcA; lane M, molecular mass marker.
soluble) were analyzed by ELISA and immunoblotting to evaluate HcA expression levels and solubility, respectively. As shown in Table 1 and Fig. 2, the addition of the HSP-70 chaperone system (but not HSP-60) increased HcA expression 4-fold, yielding a concentration of 38 μg/ml. Elevated HcA expression was not associated with reduced solubility (Fig. 2). All combinations of different redox environments, HSP-60, and PDI tested with or without HSP-70 did not increase HcA expression and solubility over the effect of HSP-70 alone. Therefore, all further expression experiments were conducted in the presence of HSP-70 alone.

The same optimization protocol was used to express recombinant HcB (aa 866 to 1292) and HcE (aa 855 to 1293), with a His₆ tag at the N'-terminal position. HcB and HcE were obtained at comparable levels to HcA and similarly presented optimal expression levels and solubilities solely in the presence of HSP-70 (data not shown).

**Scale up of Hc expression in a continuous-exchange cell-free system.** Extension of the cell-free expression batch reaction for longer than 6 h did not increase total HcA yield over the range of a few micrograms (data not shown). This limitation in yield is probably due to the accumulation of inhibiting by-products and depletion of reaction substrates in the batch mode (25).

To express HcA in quantities large enough for biochemical and immunological characterization and design of experimental vaccines, we adopted the optimal conditions of the batch system to a continuous-exchange cell-free system. This system was previously shown to allow the production of milligram quantities of recombinant protein per milliliter of reaction mixture (23, 26).

A DNA plasmid encoding HcA (15 μg) was added to a continuous-exchange cell-free reaction mixture (RTS-500; 1-ml volume) in the presence of an HSP-70 chaperone system. The reaction was conducted for 22 h at 25°C with constant shaking. The HcA concentration in this system reached 1,480 μg/ml, whereas the batch system generated only 38 μg/ml. The 30-fold increase in volumetric productivity as well as the increase in reaction volume (1 ml versus 50 μl in the batch system) resulted in the production of total HcA in quantities 3 orders of magnitude higher than those in the batch system (1.5 mg versus 2 to 3 μg, respectively). Further scale up from a 1-ml CECF chamber to a 10-ml CECF reaction chamber (RTS-9000) resulted in a proportional increase in total HcA levels (from 1.5 mg to 15 mg), confirming stable expression levels at different CECF scales and enabling the potential expression of more than 40 mg HcA in a single reaction of this specific system (3 × 10 ml). HcB and HcE expression was scaled up using essentially the same protocol, yielding 1 to 1.5 mg recombinant Hc per milliliter of reaction mixture.

**Purification and characterization of in vitro-expressed Hc proteins.** HcA was purified from the reaction lysate by one-step Ni⁺⁺-affinity chromatography. The purification process yield was 40%, and HcA was shown to be >95% pure, as demonstrated both by ELISA in which HcA concentration was shown to be 1 mg per mg protein and by SDS-PAGE (Fig. 3a, lane 2). HcB and HcE were purified in a similar manner, yielding similar quantities with over 95% purified soluble pro-

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**TABLE 1. Comparison of HcA expression levels under different expression conditions**

| Reaction mixture sample | HcA expression (mg/ml) |
|-------------------------|------------------------|
| No additives            | 10                     |
| GSSG, PDI               | 13                     |
| HSP-60                  | 10                     |
| HSP-70                  | 38                     |
| HSP-70, GSSG, PDI       | 40                     |
| HSP-70, HSP-60, GSSG, PDI | 35                   |

*Supernatant collected from the soluble fractions of the indicated reaction mixtures was analyzed for HcA concentration by ELISA. Results are average of two representative experiments.*

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**FIG. 2. Optimization of HcA expression in a cell-free system.** Plasmid (0.5 mg) encoding HcA was used as a template in RTS-100 cell-free reaction at 30°C for 5 h with constant shaking (220 rpm). Expression was conducted in the absence (control) or presence of different additives as indicated, including the HSP-60 and HSP-70 chaperone system, protein disulfide isomerase (PDI), and oxidized (GSSG) and reduced (GSH) glutathione. Total protein (T) and soluble fraction (S) were separated by SDS-PAGE and analyzed by immunoblotting using rabbit antipeptide aa 1279 to 1295 of botulinum A.

**FIG. 3. Characterization of in vitro-expressed Hc proteins.** (a) Purified preparations of HcA, HcB, and HcE were analyzed by SDS-PAGE. Lane 1, molecular mass marker (M); lane 2, HcA-His₆; lane 3, His₆-HcB; lane 4, HcE-His₆. (b) Purified HcA (lane 2) and botulinum A toxin complex (lane 3) were analyzed by immunoblotting using rabbit antipeptide aa 1279 to 1295 of botulinum A (left panel) and rabbit antitoxin A complex (right panel). The positions of HcA (lower arrow) and the botulinum A heavy chain (upper arrow) are marked.
tein (Fig. 3a). Due to structural considerations, HcB was expressed from a template 20 amino acids longer than that of HcA and HcE (28). This difference was demonstrated in the slightly higher molecular weight observed for HcB. A dynamic light scattering (DLS) analysis confirmed that cell-free-expressed Hc proteins appeared as a soluble homogeneous monomer. All three recombinant proteins remained soluble after being stored at $-100$ °C for at least 1 year.

To evaluate the immunologic properties of the Hc proteins expressed in the cell-free system, the in vitro-expressed HcA was tested and compared to native botulinum toxin A for reactivity with different antisera in an immunoblot analysis. Antiserum produced against the whole toxin complex reacted with HcA and produced a single band corresponding to the expected molecular weight of the recombinant protein (Fig. 3b, right panel). The same antiserum reacted with the toxin A complex that exhibited a multiband profile, in agreement with the presence of hemagglutinins (1, 27). Incubation with anti-peptide-specific antibody generated, as expected, a single band at 47 kDa representing in vitro-expressed HcA and a single band at 100 kDa for neurotoxin A heavy chain (Tox A). The same immunological properties were demonstrated for HcB and for HcE, using both activated and nonactivated forms of botulinum E toxin as antigen (data not shown), confirming that in vitro-expressed Hc proteins contain epitopes present on the native botulinum toxin homolog and are therefore suited to be tested as a candidate vaccine for botulinum.

**Evaluation of immunogenicity and protective efficacy of a monovalent Hc experimental vaccine.** To test whether in vitro-expressed recombinant Hc presents protective epitopes, alum-adsorbed Hc experimental vaccines were prepared. Three groups of ICR mice (10 each) were immunized by subcutaneous administration of 0.2 ml (5 μg) of each monovalent preparation (HcA, HcB, or HcE). Immunization included three consecutive injections, with a 4-week interval between each immunization. Ten days after each immunization, blood samples were collected and the antitoxin-specific antibody titer was measured by ELISA. Antitoxin titers were below 1,000 after the first immunization in all three experimental groups. However, there was a dramatic increase in antitoxin titers to values in the range of 50,000 after the second injection (Fig. 4). These further increased to values in the 100,000 range by the third injection. No significant difference in average antitoxin titer was observed between mice immunized with the different Hc serotypes ($P = 0.43$).

The development of the immune response to HcE was slightly delayed compared to that of anti-HcA and anti-HcB immune responses. The 25th percentile titer values after two injections were 4,750 in mice immunized with HcE compared to 45,000 and 52,500 in mice immunized with HcA or HcB, respectively, confirming the presence of low responders in this group.

The protective efficacy of the experimental vaccines was studied by challenging mice 10 days after the third immunization with $10^3$ to $10^6$ MsLD$_{50}$ of the toxin homolog. Mouse survival was observed for 7 days (Table 2). Mice immunized with HcA or HcB survived a challenge dose of up to $10^6$ MsLD$_{50}$ of the homolog toxin. However, mice immunized with HcE were protected only against $10^2$ MsLD$_{50}$ of botulinum toxin E and were only partially protected against the $10^4$ MsLD$_{50}$ challenge, as reflected by a mean time to death of 17 h compared to 1.5 h in control mice. A dramatic increase in protection was observed after a fourth injection with HcE vaccine (Table 2, right column). Mice became fully protected against $10^5$ MsLD$_{50}$ of botulinum toxin E. It should be noted that mice from all groups did not survive a low-dose challenge.

| Botulinum toxin challenge (MsLD$_{50}$) | No. of mice surviving/total when immunized with$^a$: | \(3\) injections | \(4\) injections |
|---------------------------------------|---------------------------------|-----------------|-----------------|
|                                       | HcA (3 injections) | HcB (3 injections) | HcE |
| $10^2$                                | ND               | ND               | 3/3  |
| $10^3$                                | ND               | ND               | 0/3  | 3/3  |
| $10^4$                                | 3/3              | 3/3              | 1/3  | 3/3  |
| $10^5$                                | 3/3              | 3/3              | 0/3  | 0/3  |

$^a$ Mice (12 mice per group) were immunized with three consecutive injections of monovalent HcA, HcB, or HcE vaccine or four consecutive injections of HcE vaccine. Ten days after the last immunization, mice were challenged with the homolog toxin as indicated. Survival was monitored for 7 days. ND, not determined.
of 10 MsLD50 of a heterologous toxin, demonstrating complete absence of cross-protection between different Hc serotypes. This observation is consistent with the botulinum toxin serotype classification that relies on the specificity of antibody neutralization.

Evaluation of immunogenicity and protective efficacy of a trivalent Hc experimental vaccine. Multivalent vaccines against botulinum toxins are beneficial since they are designed to protect against all toxins that are considered to be a threat for humans. To address this issue, we investigated the immunogenic and protective efficacy of a trivalent in vitro-expressed HcA, HcB, and HcE vaccine and compared it to the monovalent preparations. Mice were immunized with a trivalent vaccine containing 5 μg of each antigen (HcA, HcB, and HcE) adsorbed to alum (final volume, 0.2 ml/mouse), applying the same vaccination regime that was used for the monovalent vaccines. Ten days after each immunization, sera were collected and anti-botulinum toxin A, B, and E titers were measured. Figure 5 shows the antitoxin titers in mice immunized with the mono- or trivalent Hc experimental vaccine after the third injection. Antitoxin titers in mice immunized with the trivalent vaccine were lower than those obtained with the monovalent vaccine (2.2-, 1.9-, and 3.2-fold reductions compared to the monovalent vaccine for HcA, HcB, and HcE preparations, respectively). The reduced titers in mice immunized with trivalent vaccine were statistically insignificant, yet they were observed in all three serotypes, suggesting partial antigenic competition in the trivalent preparation. The delayed development of humoral immunity to botulinum toxin E observed in mice immunized with the monovalent preparation was even more pronounced in the trivalent platform (values for 25th percentile titer after two injections were 286 for toxin E and 27,500 for both toxins A and B).

Mice were challenged 10 days after three immunizations with the trivalent vaccine with 10^3 to 10^6 MsLD50 of botulinum toxin A, B, or E and observed for survival. The protection profile was essentially the same as with the monovalent vaccine. After three immunizations with the combined HcA, HcB, and HcE preparation, mice were protected from 10^6 MsLD50 of botulinum A and B and 10^3 MsLD50 of botulinum E. After an additional boost with the trivalent preparation, the anti-botulinum E titer dramatically increased, and mice were protected against a 10^5-MsLD50 botulinum toxin E challenge. These results suggest that the slightly reduced humoral immune response to the trivalent vaccine was not reflected in its protective properties.

Finally, the protective efficacy of the trivalent vaccine was evaluated against a trivalent challenge containing all three homologous toxins. Mice were immunized with four injections of the trivalent experimental vaccine and then challenged 10 days after the 4th injection with 10^5 MsLD50 botulinum toxins A, B, and E. The challenge was conducted with 2-day intervals between each serotype to distinguish between toxin serotypes in case of mortality. All mice survived the combined toxin challenge, confirming that the protective efficacy measured for the trivalent experimental vaccine in separate toxin serotype challenges is valid to a combined challenge of botulinum toxins A, B, and E.

![Figure 5](image-url)  
**FIG. 5.** Antitoxin antibody titer in mice immunized with monovalent and trivalent Hc vaccines. Mice were immunized with three consecutive injections containing 5 μg HcA, HcB, or HcE in alum (monovalent vaccine) or with a combined preparation containing 5 μg each HcA, HcB, and HcE (trivalent vaccine). Ten days after the third immunization, mice were bled and anti-toxin A, B, or E titers were measured by ELISA. The geometric mean and geometric standard deviation are presented for each group.

DISCUSSION

Botulinum is designated as a category A biothreat agent by the Centers for Disease Control (CDC) (http://emergency.cdc.gov/agent/agentlist-category.asp). The only licensed vaccine for botulism is the pentavalent (A to E) botulinum toxoid (PBT) that received Investigational New Drug (IND) status from the CDC in 1965 (43). A decline in PBT potency was first observed in the year 2001, which raised the demand for the renewal of botulinum countermeasures for prophylactic treatment. Extensive study was devoted in the past decade to develop second generation botulinum vaccines that are based on recombinant nontoxic fragments of the neurotoxin (47, 52). The 50-kDa carboxy terminal of the neurotoxin heavy chain (Hc) was found to carry most of the neutralizing epitopes (58) and is the leading candidate for recombinant botulinum vac-
The protective properties of Hc fragments expressed using synthetic genes in *E. coli* and *P. pastoris* have been demonstrated in various animal models, including nonhuman primates (5). However, expression of recombinant Hc proteins in these systems met some major challenges, including the demand for laborious synthetic gene generation and difficulties in purifying the recombinant protein as a soluble secreted product (38, 41).

Cell-free expression systems are particularly suited to meet the specific challenges raised through the expression of Hc proteins in cellular expression systems. Late advances in cell-free biology suggest production feasibility for pharmaceutical proteins that may economically compete with known commercialized cellular expression systems, especially for proteins that are difficult to express in cellular systems (46). In the present study, a cell-free expression system was used to express the native genes encoding the Hc fragments of botulinum toxins A, B, and E as soluble recombinant proteins for experimental vaccine evaluation.

We used a linear template model to demonstrate efficient cell-free expression of native botulinum Hc genes and to select the preferred His6 tag orientation for each Hc serotype. The linear template platform is unique to cell-free systems and has been shown to enable rapid evaluation of expression by obtaining a correctly folded purified recombinant protein from a selected gene in only 1 day (34, 40). In this manner, our results confirmed the feasibility of Hc expression in a cell-free system. Tsuboi and his colleagues have recently used a wheat germ cell-free expression system to study the expression of AT-rich malaria proteins. The authors have demonstrated equal expression efficiencies for native and codon-optimized malaria genes, confirming that the use of a cell-free expression system can completely circumvent the codon bias barrier (49).

We studied the expression levels and solubilities of Hc proteins under the effect of two chaperone systems, different redox potentials, the enzyme disulfide isomerase (PDI), and combinations of these additives (18). Whereas control of expression level and solubility is quite limited in cellular expression systems (16), the cell-free system enables parallel screening of up to 96 different combinations of additives in a single experiment and eliminates the need to clone each of these additives at the gene level (14, 36). We have found that optimal expression and solubility of Hc were achieved in the presence of an HSP-70 system (DnaK/DnaJ/GrpE). Similar conditions were found to be optimal for all three Hc serotypes, probably due to the similarity in fragment length and high homology between the three recombinant proteins. This assumption is based on experiments in which expression of a shorter HcE fragment (aa R856 to K1252) showed optimal expression in the presence of HSP-60 and complete lack of expression with HSP-70 (data not shown). Other proteins required different combinations of chaperones and physical environmental conditions for optimal expression and solubility in cell-free systems (17, 24, 46). The immunological properties we determined for in vitro-expressed Hc confirmed that optimized expression yielded protein products that present binding and protective immune epitopes of the native botulinum toxins (7, 57).

To allow for immunological characterization and experimental vaccine preparation, we scaled up the optimal reaction obtained in batch mode to a continuous-exchange cell-free system (26, 30, 31). The total Hc level in the continuous exchange mode increased 3 orders of magnitude over that obtained in the batch mode, yielding more than 1 mg of recombinant Hc per ml of reaction mixture, in both 1-ml and 10-ml reaction chambers. Further scale up can be achieved using emerging techniques that were recently demonstrated in 1-liter and 100-liter cell-free reactors (10).

Mice immunized with monovalent in vitro-expressed HcA, HcB, or HcE preparations had comparable ELISA titers against each homologous toxin. Nevertheless, delayed development of immunity to HcE was demonstrated both by antitoxin ELISA titer and by the number of vaccinations required to induce full protection against a high-dose toxin challenge. Similar results were obtained after immunization with codon-optimized Hc fragments expressed in *E. coli* and *P. pastoris* (7, 11, 42, 53, 57).

After three consecutive immunizations with 5 μg in vitro-expressed HcA or HcB, mice were protected from a 106 MsLD50 challenge of the homolog toxin. This observation confirmed that in vitro-expressed Hc presents the essential protective epitopes of the native toxin in addition to the relevant immunorecognition epitopes and that its potency is comparable to that reported for recombinant, codon-optimized Hc proteins expressed in common cellular expression systems (42).

A trivalent experimental vaccine was prepared by combining in vitro-expressed HcA, HcB, and HcE. Antitoxin titers were reduced 2- to 3-fold in comparison to the monovalent preparations, although the differences were not statistically significant. Similar results have been presented by Ravichandran and coworkers for a trivalent mucosal HcA, HcB, and HcE vaccine (38). In both studies, the antitoxin E titer was reduced compared to that obtained against botulinum toxins A and B. In support of this observation, a detailed review of the immune response of humans to the pentavalent botulinum toxoid (ABCDE) demonstrated that it has consistently shown reduced immunity and neutralizing antitoxin E titers compared to antitoxin A and antitoxin B (43). Taken together, our results suggest that there is an absence of significant antigenic competition for Hc antigens in the multivalent form.

The protective potency of the trivalent vaccine was essentially equal to those of the monovalent preparations. The slightly reduced ELISA titers induced by the trivalent vaccine were not reflected by reduced protection. Equal or reduced potencies have been measured when other multivalent Hc vaccines have been compared to their monovalent counterparts, depending on the nature of vaccine and mode of administration (38, 48, 56). Our results suggest that the multivalent HcA, HcB, and HcE vaccine protected mice to the same extent as the monovalent preparations.

Finally, the combined preparation of in vitro-expressed HcA, HcB, and HcE protected mice from a multitoxin challenge containing 105 MsLD50 of toxins A, B, and E. To our knowledge, this is the highest dose of a multitoxin challenge that mice could withstand after a combined Hc vaccine (3).

Our study demonstrates the proof of concept for efficient in vitro expression of botulinum Hc native genes for experimental vaccine preparation. This commercially compliant, emerging strategy enables researchers to circumvent most of the obstacles that have been described in attempts to express these...
proteins in common cellular expression systems. Protective studies confirmed that in vitro-expressed Hc induces immunity that is comparable to that reported for recombinant Hc derived from cellular expression systems.

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