The receptor binding domain of botulinum neurotoxin (BoNT), also designated the C terminus of the heavy chain (H_c), is a promising vaccine candidate against botulism. In this study, a highly efficient expression system for the protein was developed in *Escherichia coli*, which provided yields that were 1 order of magnitude higher than those reported to date (350 mg H_c per liter). The product was highly immunogenic, protecting mice from a challenge with 10^5 50% lethal dose (LD_50) after a single vaccination and generating a neutralizing titer of 49.98 IU/ml after three immunizations. In addition, a single boost with H_c increased neutralizing titers by up to 1 order of magnitude in rabbits hyperimmunized against toxoid. Moreover, we demonstrate here for the first time *in vivo* inhibition of BoNT/A intoxication by H_c/A, presumably due to a blockade of the neurotoxin protein receptor SV2. Administration of H_c/A delayed the time to death from 10.4 to 27.3 h in mice exposed to a lethal dose of BoNT/A (P = 0.0005). Since BoNT/A and BoNT/E partially share SV2 isoforms as their protein receptors, the ability of H_c/A to cross-inhibit BoNT/E intoxication was evaluated. The administration of H_c/A together with BoNT/E led to 50% survival and significantly delayed the time to death for the nonsurviving mice (P = 0.003). Furthermore, a combination of H_c/A and a subprotective dose of antitoxin E fully protected mice against 850 mouse LD_50 of BoNT/E, suggesting complementary mechanisms of protection consisting of toxin neutralization by antibodies and receptor blocking by H_c/A.

Botulinum neurotoxins (BoNTs) are the most poisonous substances known, with estimated 50% lethal dose (LD_50) values of 1 ng/kg body weight (1). There are seven serologically distinct serotypes of neurotoxins (designated A to G), which are mainly produced by the anaerobic, spore-forming bacterium *Clostridium botulinum*. The neurotoxins are 150-kDa proteins, consisting of a 100-kDa heavy chain joined to a 50-kDa light chain via a disulfide bond. The molecular mechanism of BoNT intoxication includes three steps, mediated by its three structural domains. The first step involves attachment of the receptor binding domain, located on the C terminus of the heavy chain (H_c), to receptors and subsequent internalization by endocytosis. The next step involves translocation and release of the light chain into the cytosol, a step consi- dered to be facilitated by the translocation domain found on the N terminus of the heavy chain (H_cN). The third active site is cleaved by the translocation domain found on the N terminus of the heavy chain (H_cN). The final step is the cleavage of one of three soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins by the light chain, which possesses endopeptidase activity, thereby preventing release of the neurotransmitter acetylcholine from nerve cells into the synapses (2–4).

Among the three structural domains of the neurotoxins, the H_c fragment is the domain most often investigated for therapeutic purposes, primarily as an antigen for active immunization but also as part of a vehicle for intracellular transport of light chain inhibitors (5, 6). The first demonstration of its use as a vaccine was published by Clayton et al., who reported that vaccination of mice with a recombinant H_c fragment of neurotoxin serotype A elicited protective immunity against challenge with the homologous toxin (7). This finding prompted extensive research efforts in which the receptor binding domains of all seven serotypes were produced and shown to induce protective immune responses (6, 8). Moreover, a recombinant botulinum vaccine composed of the H_c fragment of botulinum neurotoxins A and B and produced by the DynPort Vaccine Company for the U.S. Department of Defense is currently under clinical investigation (9).

Another potential therapeutic use of the H_c fragment is as a part of a protein vehicle to deliver cargo molecules specifically to neurons (5, 10). The cargo molecules can be botulinum neurotoxin light chain inhibitors or drugs related to other neuronal disorders. This approach might create opportunities to treat botulinum neurotoxin intoxication at a stage when the toxin already has been internalized into cells and no longer is available in the blood for antidote treatment. In addition to the two therapeutic uses described above, the inhibition of botulism may occur by direct competition of the H_c fragment with the neurotoxin for receptor binding. Indeed, studies that aimed to elucidate the protein receptor of several BoNT serotypes demonstrated reduced toxin activity in the presence of H_c both in hippocampal neuron cultures and in mouse phrenic nerve (MPN) hemidiaphragm (11–13). However, this phenomenon has never been demonstrated in vivo.

The first attempts to produce recombinant H_c fragments of neurotoxin A were performed using *Escherichia coli* as a host (7, 14). However, as most of the expressed protein was insoluble in this system, subsequent studies have used the alternative host *Pichia pastoris*. Using this system, H_c fragments of botulinum A, B, C, D, E, and F have been produced with satisfactory yields (15–20). It is important to note, however, that *P. pastoris* is considered to be a less attractive host than *E. coli* for recombinant gene expression, from the perspectives of both genetic manipulation and production processes, and there is still an interest in improv-
ing the expression yields of H₂ in *E. coli* (8). In this work, we present an efficient expression system for the BoNT/A H₂ fragment in *E. coli* and demonstrate, for the first time, in vivo inhibition and cross-inhibition of BoNT/A and BoNT/E by the recombinant product.

**MATERIALS AND METHODS**

**Ethics statement.** 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.

**Materials.** All chemicals were purchased from Sigma-Aldrich unless otherwise stated. The yeast extract and tryptone were from Becton, Dickenson and Company (Franklin Lakes, NJ). Mouse anti-H₂/A monoclonal antibody was prepared as described previously (21). Rabbit anti-H₂/A polyclonal antibodies were purified from sera of hyperimmune rabbits that had been immunized with H₂/A, as described previously (22). Rabbit antibody against peptide amino acids 1279 to 1295 of botulinum A was obtained from hyperimmune rabbits that had been immunized with the peptide, with keyhole limpet hemocyanin (KLH) as a carrier.

**Bacteria and toxins.** *E. coli* strains and plasmids were purchased from Novagen (Madison, WI). *Clostridium botulinum* A, B, and E strains were obtained from the Israel Institute for Biological Research collection (strains A198, B392, and E450, respectively). Sequence analysis revealed conformity of the neurotoxin genes with serotypes 62A (GenBank accession number M30196), Danish (GenBank accession number M81186), and NCTC11219 (GenBank accession number X62683) for *Clostridium botulinum* types A, B, and E, respectively (23–25). Toxins were prepared from concentrated supernatants of cultures grown for 6 days in anaerobic culture tubes. BoNT/E was activated with trypsin (0.1% at 37°C for 45 min). The activity of all toxin preparations was at least 3 × 10⁶ mouse 50% lethal dose (MLD₅₀)/ml. BoNT/E toxoid was prepared by incubation of the toxin in the presence of 0.2% formalin at 30°C for 28 days, followed by extensive dialysis against 50 mM citrate buffer (pH 5.5).

**Construction of H₂ fragment expression plasmids.** A synthetic gene encoding the H₂ fragment of BoNT/A (strain 62A; GenBank accession number BAH79821.1) with optimized codon usage for expression in *E. coli* and a C-terminal His tag was synthesized by GenScript (Piscataway, NJ). The H₂ fragment gene was cloned with trxA by overlap extension PCR. First, the *trxa* gene was amplified by PCR from an *E. coli* colony using the following primers: *trxa* N-terminus (primer 1), 5′-AGTCTGGTAC ATATGAGCGATAAAATTATTCACCTG (bold type indicates the NdeI site); *trxa* C-terminus (primer 2), 5′-AAGTTCATTGTTATTTTGCGGATTAGCAGGTTTACCAGTCTGAGG. The H₂ fragment gene was amplified using the following primers: H₂ fragment N-terminus (primer 3), 5′-TCAAATGGCGTAAAGATATCCAGTACATTAGAATGACATTACATTACCTGCCTCG (bold type indicates the BamHI site). Primers 2 and 3 were designed to anneal at their 5′ termini. The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega; Madison, WI) and were mixed together with primers 1 and 4 to fuse the genes by overlap extension PCR. The product of the reaction was digested with NdeI and BamHI and ligated to the vectors PET-9a and PET-22b (+), digested similarly. A similar procedure was used to obtain a construct that possessed a ribosome, binding site (RBS) upstream of the H₂ fragment gene, but in this case, primers 2 and 3 were replaced by primers 5 and 6, as follows: *trxa* C-terminus with *rbs* (primer 5), GTATATCCTCCTCGAATTTCTGACCAGGTAGTCTGCG; H₂ fragment N-terminus with *rbs* (primer 6), CTAACCTGGCATAAGAATTATTCACCTG (the rbs site is underlined). The rbs site sequence is from the T7 major capsid protein.

**Growth of cultures for optimization studies.** During optimization, the cells were grown in 250-ml polycarbonate, baffled shake flasks (Nalgene; Nalge Nunc, Rochester, NY) containing 40 ml terrific broth (TB) medium (tryptone, 12 g/liter; yeast extract, 24 g/liter; glycerol, 0.4% [vol/vol]; potassium phosphate, 89 mM). Cultures expressing the H₂ fragment from the vector PET-9a (T7 promoter) were grown overnight at 37°C without induction. For cultures expressing the H₂ fragment from the vector PET-22b (+) (T7lac promoter), the optical density was monitored and, when the cells reached an A₆₀₀ value of 0.6, the cultures were induced for 3 to 4 h with IPTG (isopropyl-β-D-thiogalactoside) (0.2 mM).

**Soluble H₂/A quantification assay.** The assay was used to estimate the soluble H₂/A fragment yield in cultures grown under various conditions during expression optimization. The assay included two steps. First, samples (0.5 ml) withdrawn from the cultures were chemically disrupted with a Celllytic B Plus kit (Sigma-Aldrich), according to the manufacturer’s instructions, and the soluble proteins were separated from the insoluble cell fraction by centrifugation. The H₂ fragment concentrations in the supernatants were then estimated by sandwich enzyme-linked immunoassorbent assay (ELISA), as follows. Plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 μl of a mouse anti-H₂/A monoclonal antibody (21) diluted to a final concentration of 4 μg/ml in coating buffer (50 mM Na₂CO₃, pH 9.6) and were incubated overnight at 4°C. The plates were then washed with wash solution (0.09% NaCl, 0.05% Tween 20) and blocked for 1 h at 37°C with TSTA buffer (50 mM Tris, 0.9% NaCl, 0.05% Tween 20, 2% bovine serum albumin; 200 μl per well). After washing, the plates were incubated for 1 h at 37°C with serial dilutions (50 μl per well, in duplicate) of the tested supernatant and pure H₂/A standard in TSTA buffer. The plates were then washed with wash solution and incubated for 1 h with rabbit anti-H₂/A fragment polyclonal antibody diluted in TSTA buffer to a final concentration of 0.5 μg/ml. After additional washing, the plates were incubated for 1 h at 37°C with 50 μl of alkaline phosphatase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:1,500. Finally, the plates were washed with wash solution, and the colorimetric reaction proceeded using the substrate p-nitrophenyl phosphate (1 mg/ml in 0.2 M Tris buffer). The absorbance at 405 nm was continuously measured for 15 min in 30-s intervals, and the H₂ fragment concentration was determined by interpolation from a standard curve prepared with pure H₂/A.

**Expression and purification of H₂/A.** *E. coli* BL21(DE3) carrying the plasmid pPET-9a-trxa-rbs-H₂/A was grown overnight without induction in 2-liter, polycarbonate, baffled shake flasks (Nalgene) containing 0.5 liter of TB medium supplemented with kanamycin (30 μg/ml). The flasks were incubated at 37°C with shaking (250 rpm). The cells from 1 liter of overnight culture (A₆₀₀ value of approximately 20) were harvested, resuspended in 100 ml binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), and disrupted by sonication. The cell extract was clarified by centrifugation (14,000 × g, 30 min) and loaded onto a HisTrap FF 5-m column (GE Healthcare) mounted on an AKTA Explorer fast protein liquid chromatography system (GE Healthcare). The column was washed with 10 column volumes of binding buffer and 10 column volumes of binding buffer containing 40 mM imidazole. The protein was eluted from the column with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The pure protein was dialyzed against 50 mM sodium phosphate, 50 mM NaCl (pH 6.5), and stored at −70°C. The concentration of pure H₂/A fragment was determined at 280 nm using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA), with the following parameters: molecular weight, 50,519 g/mol; extinction coefficient, 86,250 cm⁻¹ M⁻¹ (calculated using the Peptide Properties Calculator [www.basic.northwestern.edu/biotools/proteincalc.html]). The fraction of H₂/A in the total soluble cell protein preparation was determined with an Experion automated electrophoresis system (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions. The purity of the H₂/A fragment was determined with a Waters Acquity ultra-performance liquid chromatography (UPLC) system equipped with a BEH C₁₈ column, using a water/acetonitrile gradient of 70:30 to 20:80 over 5 min of run time.

**Vaccination of mice with the H₂ fragment.** Pure H₂/A fragment and formalin-inactivated toxoid (toxoid) were diluted with phosphate-buffered saline and adsorbed to aluminum hydroxide [final concentration of Al(OH)₃ 0.6% (wt/vol)] to obtain 2 or 5 μg protein per injection dose (0.5 ml). The vaccine preparations were injected subcutaneously into 10
mice (CD-1; Charles River UK) per group. For the challenge protection assay, the mice were immunized once (2 μg of antigen) and challenged with 10^5 MsLD₅₀ after 21 days. Survival was monitored for 7 days. For the determination of neutralizing antibodies, the mice were immunized three times, at 21-day intervals, with vaccine preparations containing 5 μg protein per injection. Two weeks after the last injection, the mice were bled and sera were pooled for each group. The neutralizing antibody concentration was determined according to the European Pharmacopoeia (26).

Booster vaccination with the H₉ fragment of rabbits hyperimmune to botulinum toxoid. A hyperimmune status of rabbits (NZW; Charles River UK) was achieved with the following immunization regimen: at 0, 1, and 2 months, 20 μg of toxoid adsorbed to aluminum hydroxide [final concentration of Al(OH)₃, 0.5% (wt/vol)]; at 5, 8, 11, and 14 months, 250 μg of soluble toxoid. The injections were given subcutaneously at 3 or 4 sites. The indicated amount of toxoid refers to the estimated amount of neurotoxin. At the fifth vaccination, the serum neutralizing titers reached a plateau, with values ranging from 70 to 150 IU/ml. No further increase in neutralization titers was observed with administration of the sixth and seventh boosters (average of 98.6 ± 30 IU/ml for the whole group of six rabbits), indicating that hyperimmunity had been attained. Seventeen months after the first vaccination, the rabbits were divided into three groups (n = 2), which received boosts as follows (soluble antigens): (i) 250 μg of toxoid (control group), (ii) 250 μg of H₉/A, or (iii) 2.5 mg of H₉/A.

In vivo inhibition test. Mice (3 or 6 per group) were injected intraperitoneally (i.p.) with 1 ml of 5 MsLD₅₀ of BoNT (A, B, or E), in the presence (test group) or absence (control group) of H₉/A at different doses. Following injection, survival was monitored for 1 week.

Combined treatment with antitoxin E and H₉/A against BoNT/E intoxication. The combined effect of H₉/A and antitoxin E was tested according to the European Pharmacopoeia neutralizing antibody assay (26). Serial dilutions of World Health Organization standard antitoxin E (from 0.08 to 0.12 IU/ml, at 0.02-IU/ml intervals) were incubated for 1 h at 25°C with BoNT/E (850 MsLD₅₀/ml), in the presence or absence of H₉/A (2.5 mg/ml). The mixtures (1 ml) were injected i.p. into mice (n = 8), and survival was monitored for 1 week.

Statistical analysis. A 4-parameter logistic regression model was used to construct a standard curve for pure H₉/A, using SoftMax Pro 5.4 (Molecular Devices) for estimation of expression levels. Comparisons of survival curves were performed with the log-rank (Mantel-Cox) test, using GraphPad Prism 5 software. Differences were considered significant when P was <0.05.

RESULTS

Rationale for expression construct design. A synthetic gene encoding the receptor binding domain of botulinum neurotoxin A was prepared. The gene was designed to contain high-frequency E. coli codons, and the overall GC content was increased from 24% (in the native clostridial gene) to 42%. To facilitate purification, a 6×His tag was added to the C terminus of the protein.

For optimal solubility of the recombinant protein, the expression constructs were designed to promote coexpression of thioredoxin A (TrxA) (27–29). Two configurations of the trxA and H₉ fragment genes were tested (Fig. 1). The first configuration included the H₉ fragment gene immediately downstream of the stop codon of trxA (trxA-H₉). In an attempt to increase expression levels further by enhancing gene translation, a second configuration was prepared, in which an additional ribosome binding site (rbs) sequence was inserted upstream of the H₉ fragment gene (trxA-rbs-H₉).

The expression of the recombinant protein for both gene configurations was tested by using two strategies for transcription regulation. The first used the T7lac promoter, representing tight transcription regulation [vector pET-22b(+)]. In this system, protein expression depends predominantly on induction by IPTG. This strategy has been used in most of the studies expressing the H₉ fragment in E. coli reported to date (8, 30, 31). Because expression under less-stringent transcription regulation has been demonstrated to yield high expression levels for various recombinant proteins (32–34), this second strategy was tested using a T7 promoter (vector pET-9a).

All tested plasmids were transformed into E. coli BL21(DE3). In addition, since the native receptor binding domain of the neurotoxin contains a single disulfide bond (between Cys1235 and Cys1280), the use of E. coli Origami B also was examined. This strain is a trxB-gor mutant and thus has the potential to enhance cytoplasmic disulfide bond formation (35).

Expression optimization studies. To evaluate soluble H₉/A levels obtained with the various expression constructs, small-scale cultures (40 ml) were grown, and the soluble cell extracts were analyzed by ELISA to determine H₉/A concentrations. Two genetic elements had major contributions to enhanced H₉/A expression (Table 1). Use of the T7 promoter provided levels of protein expression 2 orders of magnitude higher than the levels obtained using the more commonly used T7lac promoter. For example, the construct pET-22b-trxA-H₉ (T7lac promoter) in BL21(DE3) yielded 0.49 mg/liter, while the same host carrying the plasmid pET-9a-trxA-H₉ (T7 promoter) yielded 93 mg/liter. The second genetic element that contributed to improved expression was rbs added upstream of the H₉ gene (configuration of trxA-rbs-H₉). This addition resulted in 3- to 8-fold increases in expression levels in all tested hosts and vectors. No significant expression advantage was observed when the Origami B host strain was used instead of BL21(DE3). The combination of the T7 promoter and rbs resulted in a yield of 293 mg/liter [pET-9a-trxA-rbs-H₉ in BL21(DE3)], 1 order of magnitude higher than reported previously for H₉/A expression in E. coli (8, 29, 31). The high yield of this construct was due to both the higher specific yield and the greater cell mass at culture harvest (Table 1). When cells were grown at 18°C, an even higher specific yield was obtained with this system (22.2 mg/liter per A₅₆₂₅ unit), and the expression levels reached 0.5 g of H₉/A per
liter of culture (data not shown). This construct was chosen for further expression and purification of recombinant Hc/A.

Recombinant Hc/A was purified from 1 liter of culture using immobilized metal chelate affinity chromatography (IMAC). SDS-PAGE and Western blot analyses (Fig. 2A and B) indicated that Hc/A was the major protein in the soluble cell fraction (45% of total soluble protein, based on automated electrophoretic analysis). The protein was eluted from the Ni2⁺ column as a single band, with a purity of >99% as determined by UPLC (Fig. 2C). A total of 350 mg of pure Hc fragment was obtained from 1 liter of culture, with a purification process yield of 93%.

**Immunogenicity of recombinant Hc.** Single vaccinations of mice (n = 10) with 2 μg aluminum hydroxide-adsorbed Hc fragment resulted in full protection (10/10 mice) against challenge with 10⁸ MsLD₅₀ (Table 2), while similar vaccinations with formalin-inactivated toxin resulted in only 50% survival (5/10 mice). The neutralizing antibody concentrations in pooled sera of mice that had been vaccinated thrice with 5 μg of Hc fragment or toxoid were 50.0 and 12.5 IU/ml, respectively (Table 2).

The immunogenicity of the Hc fragment produced was further examined by administration of the recombinant protein as a booster vaccine to rabbits that had been vaccinated previously to hyperimmune status with formalin-inactivated toxin (Table 3). The hyperimmune status of the rabbits (n = 6) was established by three immunizations with 20 μg of aluminum hydroxide-adsorbed detoxified toxin followed by four immunizations with 250 μg of soluble toxoid. As of the fifth vaccination, serum neutralizing titers stabilized in the range of 70 to 150 IU/ml, with no further increase in titers with the sixth and seventh boosters (average of 98.6 ± 30 IU/ml for the whole group of six rabbits), indicating the attainment of hyperimmunity. Rabbits were then divided into three groups (two rabbits per group); one group received an eighth booster vaccination with 250 μg formalin-inactivated toxin (control group) and the other two groups were vaccinated with either 250 μg or 2.5 mg Hc/A. No increase in neutralizing antibody concentrations was measured in the control group upon vaccination with 250 μg toxoid (83 IU/ml before and after the booster). However, a single booster vaccination with 250 μg of the recombinant Hc increased serum neutralizing titers by 2.9- to 5.8-fold (from an average titer of 103 IU/ml before the booster to 425 IU/ml after the booster). Moreover, increasing the Hc/A dose to 2.5 mg increased neutralizing antibody concentrations by 1 order of magnitude (from an average titer of 110 IU/ml before the booster to 1,042 IU/ml after the booster).

**In vivo inhibition of botulinum neurotoxin A by the Hc fragment.** The only currently approved treatment against botulinum toxin intoxication is the administration of an antitoxin prepara-

### Table 1 Comparison of soluble Hc/A expression levels obtained with different constructs

| E. coli strain          | Plasmid                | Promoter       | A₅₅₀ at harvest | Hc fragment yield (mg/liter)ᵃ | Specific yield (mg/liter Hc/A₅₅₀ unit) |
|-------------------------|------------------------|----------------|-----------------|------------------------------|---------------------------------------|
| BL21(DE3)               | pET-22b(+)–trxA–Hc     | T7lac          | 2.2             | 0.5                          | 0.2                                   |
| BL21(DE3)               | pET-22b(+)–trxA–rbs–Hc | T7lac          | 2.7             | 2.2                          | 0.8                                   |
| Origami B               | pET-22b(+)–trxA–Hc     | T7lac          | 2.7             | 0.6                          | 0.2                                   |
| Origami B               | pET-22b(+)–trxA–rbs–Hc | T7lac          | 2.61            | 4.9                          | 1.9                                   |
| BL21(DE3)               | pET-9a-trxA–Hc         | T7             | 18.9            | 93                           | 4.9                                   |
| BL21(DE3)               | pET-9a-trxA–rbs–Hc     | T7             | 21.9            | 293                          | 13.4                                  |

ᵃHc fragment yield values are expressed as mg protein per liter of culture.

### Table 2 Immunogenicity of recombinant Hc in mice

| Antigen        | No. of surviving mice/no. of treated miceᵃ | Neutralizing antibody titer (IU/ml)ᵇ |
|----------------|--------------------------------------------|--------------------------------------|
| Hc fragment    | 10/10                                      | 50.0                                 |
| Toxoid         | 5/10                                       | 12.5                                 |

ᵃMice were vaccinated once with 2 μg antigen and challenged with 10⁸ MsLD₅₀ after 21 days.
ᵇMice (n = 10) were vaccinated thrice with 5 μg antigen at 21-day intervals. Neutralizing antibody concentrations were determined in pooled sera according to the European Pharmacopoeia (26).

---

**FIG 2** Purification of the receptor binding domain from E. coli BL21(DE3) carrying the plasmid pET-9a-trxA-rbs–Hc. SDS-PAGE (A) and Western blot (B) analyses were performed with samples withdrawn during the purification process. Lane 1, molecular mass markers (in kDa); lane 2, soluble cell fraction; lane 3, insoluble cell fraction; lane 4, flowthrough from loading of the soluble cell fraction on the IMAC column (unbound proteins); lane 5, eluted protein. The Hc fragment was detected using rabbit antibodies directed against amino acids 1279 to 1295 of BoNT/A. (C) The purity of the final product was analyzed using a UPLC system equipped with a C18 column. The protein was found to be >99% pure. AU, absorbance units.
tion, which neutralizes free toxin molecules by direct binding. As the Hc fragment possesses receptor-binding capacity (36), we hypothesized that recombinant Hc/A could have a therapeutic effect in vivo by competing with the toxin for available receptors. Preliminary examination of this hypothesis was conducted by administration of 5 MsLD50 BoNT/A to mice (n = 3) in the presence of different doses of the Hc fragment (Table 4). For all Hc/A-treated groups, a delay in the median time to death (TTD) was observed, in comparison with the control group. The delay was dose-dependent, ranging from 2.4 h for 25 μg Hc/A to 22.2 h for 2.5 mg Hc/A (P = 0.02). To validate this observation, mice (n = 6) were exposed to 5 MsLD50 BoNT/A in the presence or absence of 2.5 mg Hc/A (Fig. 3A). In the absence of Hc/A, the TTD ranged from 8 to 15 h postexposure, with a median survival time of 10.4 h; for mice treated with the Hc fragment, the TTD ranged from 22 h post intoxication to as long as 38 h (median TTD, 27.25 h; P = 0.0005).

**Hc/A delays the time to death for botulinum neurotoxin E intoxicated mice and enhances the potency of an antitoxin E preparation.** Both BoNT/A and BoNT/E bind the protein receptor SV2. However, the two toxin serotypes differ in their specificities for different isoforms of the receptor. While BoNT/A binds all three isoforms of SV2 (SV2A, SV2B, and SV2C), BoNT/E binds almost exclusively glycosylated SV2A and SV2B (13, 37–39). As the receptors for BoNT/E are included in the BoNT/A receptor repertoire, we examined whether Hc/A possessed a therapeutic effect against BoNT/E intoxication. Mice (n = 6) were injected with 5 MsLD50 of BoNT/E in the presence or absence of Hc/A (2.5 mg). Fifty percent of treated mice with Hc/A completely survived the BoNT/E challenge (Fig. 3B). For Hc/A-treated animals that did not survive, the TTD ranged from 11 to 15 h and was significantly longer than the TTD of control mice (range, 6.2 to 11 h; P = 0.003). As expected, Hc/A had no effect on the TTD of mice intoxicated with BoNT/B (Fig. 3C), consistent with the fact that BoNT/B utilizes a different protein receptor than do BoNT/A and BoNT/E, the transmembrane proteins synaptotagmin I and II (40).

Next we tested whether Hc/A would have a therapeutic effect over that of the antitoxin preparation, the only currently approved treatment for botulism. We hypothesized that such a combined treatment might benefit from two complementary mechanisms, i.e., direct toxin neutralization by antibodies and receptor blocking by the Hc fragment. To test this approach, the therapeutic effect of Hc/A combined with serotype E antitoxin was evaluated in mice intoxicated with BoNT/E.

A high dose of 850 MsLD50 BoNT/E was incubated with serial dilutions (0.08 to 0.14 IU) of serotype E standard antitoxin (World Health Organization), in the presence or absence of Hc/A. The mixtures were injected into mice, and survival was monitored for 96 h (Table 5). For mice treated with antitoxin alone, 100% survival was obtained at antitoxin doses higher than 0.12 IU, while no survival was observed for doses of 0.1 IU and below. The addition of Hc/A to the toxin/antitoxin mixture fully protected mice treated with the subprotective antitoxin dose of 0.1 IU. For a dose of 0.8 IU, Hc/A only delayed the TTD. Hence, the protective contribution of Hc/A was equivalent to increasing the antitoxin dose by 20%.

**DISCUSSION**

The receptor binding domain of botulinum neurotoxins is of major interest for several therapeutic applications. Early attempts to express the Hc fragment in *E. coli* resulted in poor yields, mainly due to the low solubility of the recombinant product (7, 14). As a result, expression efforts were shifted to the methylotrophic yeast *P. pastoris*, in which the Hc fragments of serotypes A, B, C, D, E, and F were successfully expressed in sufficient amounts for vaccine production (3, 6). Recently, the expression of the Hc fragment in *E. coli*, which is considered to be a more attractive host than the yeast, is again being examined, with significantly higher yields now being achieved (8, 29, 31). A major difference between the early and recent expression studies in *E. coli* is the starting point of the recombinant protein. Before the crystal structure of BoNT/A was available, the receptor binding domain was predicted to begin at amino acid 861, based on sequence alignment and theoretical secondary structure analysis (41). In 1998, the crystal structure of BoNT/A was elucidated, and the receptor binding domain was shown to consist of amino acids 861 to 1296 (42). According to this structure analysis, amino acids 861 to 870 form a hydrophobic α-helix that is part of the translocation domain (Hn). Expression of the Hc fragment with this extra helical element might result in an unstable product due to the exposure of hydrophobic residues to the solvent. Such instability may explain the low yields of soluble protein in early studies. In the current study, several parameters were screened to optimize Hc expression in *E. coli* beyond the previously reported levels.

The parameter that had the most significant contribution to recombinant protein yields in our work was the use of the T7 promoter. Most other works in which the Hc fragment was expressed in *E. coli* used the more-stringent T7lac promoter (8, 29, 31). It is generally accepted that tight regulation of recombinant protein expression is desired, especially when the gene product may be toxic to the host and lead to construct instability. Such instability was reported by LaPenotiere and colleagues in attempts

---

**TABLE 3 Serum neutralizing titers of hyperimmune rabbits before and after booster vaccination with toxoid (control group) or Hc fragment**

| Rabbit no. | Booster | Before booster | After booster |
|------------|---------|----------------|--------------|
| 1          | 250 μg toxoid | 83             | 83           |
| 2          | 250 μg toxoid | 83             | 83           |
| 3          | 250 μg Hc fragment | 120           | 350          |
| 4          | 250 μg Hc fragment | 86             | 500          |
| 5          | 2.5 mg Hc fragment | 70             | 750          |
| 6          | 2.5 mg Hc fragment | 150            | 1,334         |

*Serum neutralizing antibodies after the last vaccination of the rabbits with 250 μg toxoid.

*Serum neutralizing antibodies 2 weeks after booster vaccination with the indicated immunogen (toxoid or Hc fragment).

---

**TABLE 4 Inhibition of BoNT/A intoxication by Hc/A**

| Hc dose | Median TTD (h) | Delay in TTD (h) | P<sup>b</sup> |
|---------|----------------|------------------|--------------|
| 0       | 12.1           |                  |              |
| 25 μg   | 14.5           | 2.4              | 0.65         |
| 250 μg  | 19.3           | 7.2              | 0.06         |
| 2.5 mg  | 34.3           | 22.2             | 0.02         |

<sup>a</sup> Mice (n = 3) were treated with 5 MsLD50 BoNT/A in the presence of the indicated dose of Hc/A.

<sup>b</sup> The individual significance of each treatment was calculated using the log-rank (Mantel-Cox) test, in comparison with the control group.
to express Hc/A in E. coli (14). However, in cases in which high levels of the recombinant protein are not detrimental to the host, it has been shown that higher protein yields can be obtained with less-stringently regulated protein expression systems (32–34, 43). In our study, use of the T7 promoter (vector pET-9a) provided yields that were 2 orders of magnitude higher than those obtained with the more-stringent T7lac promoter (Table 1).

The tested constructs in this work included a thioredoxin reductase gene (trxA) upstream of the Hc fragment gene, to enhance its solubility. This strategy was previously found to be essential for expression of the C-terminal quarter of botulinum neurotoxin A (44) and also has been successfully employed to express the Hc fragments of botulinum neurotoxins A and F (28, 29) and the Hc fragment of tetanus neurotoxin (27). We found here that the addition of a specific ribosome binding site upstream of the Hc fragment gene (configuration of trxA-rbs-Hc) further improved expression levels by 3- to 8-fold in all tested hosts and vectors.

The combination of the two genetic elements, i.e., a T7 promoter and a specific ribosome binding site, resulted in a highly efficient expression system that provided 350 mg of pure protein per liter of culture. This yield value is 1 order of magnitude higher than the values reported thus far for the expression of HC/A in E. coli (8, 29, 31). Furthermore, the yield of our expression system was even higher than that achieved with P. pastoris, which has been reported to reach 1.4 g of pure Hc/A per kg of cells (45). In the current work, 350 mg of pure protein was obtained from 23.3 g of E. coli wet mass. Therefore, a yield of 15.5 g of pure Hc/A per kg of cells is expected. Moreover, unlike expression in P. pastoris, which is extended, involves multiple steps, and requires a controlled fermentor (41), our protein expression procedure consists of a single step of overnight culture growth in shake flasks.

The Hc fragment produced exhibited good immunogenic properties. Mice immunized once with 2 µg of Hc fragment were fully protected against challenge with 10^8 MsLDs90 and, following three vaccinations with the protein, a neutralizing antibody concentration of 49.98 IU/ml was obtained. Similar results were reported for Hc fragments prepared by other groups with E. coli (30) and P. pastoris (20) as hosts.

The generation of high neutralizing antibody titers is desired in animals used for therapeutic antitoxin production, as they contribute to higher product specific activity and improve safety. Booster vaccination with Hc/A (250 µg) for rabbits hyperimmunized against toxoid led to 3- to 5-fold increases in neutralizing antibody titers, while the titers remained unchanged in rabbits boosted with an equal amount of toxoid. The lower potency of the toxoid can be attributed to chemical modification of the neutralizing epitopes by formaldehyde during the detoxification process (46). In another study, similar neutralizing antibody titers were generated in rabbits upon vaccination with formalin-inactivated BoNT/B and Hc/B (47). However, the authors suggested that Hc potency in that study was limited due to precipitation of the recombinant antigen. In our study, increasing the Hc booster dose to 2.5 mg increased the neutralizing antibody concentration by 1 order of magnitude over that obtained with 250 µg toxoid (from 110 IU/ml to 1,042 IU/ml). This result suggests that a 250-µg dose is suboptimal and that higher neutralizing antibody titers can be obtained by increasing the antigen dose. The equivalent of 2.5 mg of antigen in horses, animals commonly used for antiserum production, is approximately 100 mg (on a weight basis). Our protein expression system can easily support such an elevated dose, due to its high efficiency. However, vaccination with such a high antigen dose is not applicable with toxoid, due to poor yield and production safety issues (3, 48).

Apart from being a promising anti-botulinum vaccine candidate, the ability of the Hc fragment to bind BoNT receptors might be utilized to counteract botulism by receptor blockade. To date, inhibition of BoNTs by Hc fragments was demonstrated in hippocampal neuron cultures and in MPN hemidiaphragm throughout the elucidation of BoNT/D and BoNT/F protein receptors (11–13). To our knowledge, however, inhibition of botulism by Hc fragments was never demonstrated in vivo. Therefore, we wished to examine whether the receptor binding domain had a therapeutic effect as a receptor-blocking agent. Exposure of mice to BoNT/A in the presence of Hc/A resulted in a dose-dependently delayed median TTD. The most significant effect was obtained

### TABLE 5 Hc/A enhances the potency of an antitoxin E preparation

| Antitoxin E dose (IU) | No. of surviving mice<sup>a</sup> | Hc/A | +Hc/A |
|-----------------------|-------------------------------|------|-------|
| 0.08                  | 0                             | 0    | 0     |
| 0.10                  | 0                             | 0    | 0     |
| 0.12                  | 8                             | 8    | 8     |
| 0.14                  | 8                             | 8    | 8     |

<sup>a</sup>Values indicate the survival of mice injected with a mixture containing the indicated antitoxin dose and BoNT/E (850 MsLDs90), in the presence or absence of Hc/A (2.5 mg). Results are the summary of two independent experiments, each with four mice per group.

FIG 3 Hc/A delays the time to death for mice exposed to BoNT/A and BoNT/E but not BoNT/B. Mice (n = 6) were exposed to 5 MsLDs90 of BoNT/A (A), BoNT/E (B), or BoNT/B (C) in the presence (dashed line) or absence (solid line) of Hc/A, and survival was monitored. Time to death was significantly delayed as a result of coadministration of Hc/A for neurotoxins A and E (P = 0.0005 and P = 0.003, respectively, by the log-rank test) but not for neurotoxin B (P = 0.87).
with a dose of 2.5 mg, which delayed the median TTD from 12.1 h to 34.3 h. Yet, for all tested doses of H₂, survival was not observed. Additionally, a dose of 2.5 mg in mice weighing 25 g is equivalent to ~7 g of H₂/A in a 70-kg human (on a weight basis), which limits the therapeutic potential of the H₂ fragment as a receptor-blocking agent. The requirement for such a high dose of H₂/A can stem from the fact that, while a neurotoxin molecule requires only a single SV2 molecule to enter the cell, preventing its entrance requires blocking of most SV2 molecules. In addition, during synaptic vesicle recycling, the H₂ fragment is probably released from the receptor and, following receptor binding, dissociation from it will be reduced. Such improved affinity may be achieved using protein-engineering strategies such as rational design or directed evolution (49, 50).

Botulinum neurotoxins enter neural cells via two main protein receptors, SV2 and synaptotagmin. To date, it has been shown that BoNT/A, BoNT/D, BoNT/E, and BoNT/F bind different isoforms of SV2 (11–13, 37–39), while BoNT/B and BoNT/G use synaptotagmin I and II (51, 52). Consistent with this receptor specificity, administration of H₂/A to BoNT/E-intoxicated mice in our study led to 50% survival and significantly delayed the TTD for the nonsurviving mice. This observation is supported by data from Rummel et al., who demonstrated, using the MPN hemidiaphragm model, that the paralytic half time induced by BoNTs may be prolonged by H₂ fragments originating from neurotoxin serotypes that share the same protein receptors (13).

The protective effect of H₂/A against BoNT/E was stronger than that obtained for BoNT/A (50% survival and delayed TTD, respectively). This result is surprising and may be explained by differences in the affinities of the Hc fragments of BoNT/A and BoNT/E for their receptors. The two neurotoxins share the isoforms SV2A and SV2B as the protein receptors. However, while the affinities of the receptor binding domains of the neurotoxins toward SV2A were similar, SV2B showed substantially higher affinity for H₂/A than H₂/E (13). Therefore, H₂/A might displace BoNT/E from the receptor more effectively than it could displace BoNT/A, for which the affinity toward the receptor is identical. Similar behavior was obtained for BoNT/G in the MPN hemidiaphragm model. In this case, H₂/G extended the paralytic half time more than the homologous receptor binding domain (H₂/G). The authors suggested that the greater inhibition by H₂/G reflects the lower affinity of H₂/G toward the protein receptor synaptotagmin II (13).

The combination of H₂/A with a subprotective dose of antitoxin E fully protected mice from a high-dose BoNT/E challenge (850 MsLD₅₀). The improved protective effect presumably stems from both neutralization of BoNT/E by the antitoxin and receptor blocking by H₂/A. This approach utilizes the serotypic difference between BoNT/A and BoNT/E, as antitoxin E antibodies do not prevent H₂/A from binding SV2 and H₂/A does not occupy the neutralizing antibodies found in the antitoxin E preparation. Botulinum antitoxin is currently the treatment of choice for botulinum toxin intoxication. The efficacy of antitoxin treatment decreases with time postexposure and higher doses, which may induce adverse effects, are required to confer protection (53, 54).

Combined treatment consisting of an antitoxin and a receptor-blocking agent, such as the H₂ fragment or another antagonist analogue, is thus of interest and may be a promising approach to expand the time window for treatment and reduce the required antitoxin dose.

ACKNOWLEDGMENT
This work was funded by Israel Institute for Biological Research SB/5122-28.

REFERENCES
1. Gill DM. 1982. Bacterial toxins: a table of lethal amounts. Microbiol. Rev. 46:86–94.
2. Rusnak JM, Smith LA. 2009. Botulinum neurotoxin vaccines: past history and recent developments. Hum. Vaccines 5:794–805.
3. Smith LA, Rusnak JM. 2007. Botulinum neurotoxin vaccines: past, present, and future. Crit. Rev. Immunol. 27:303–318.
4. Montal M. 2010. Botulinum neurotoxin: a marvel of protein design. Annu. Rev. Biochem. 79:591–617.
5. Singh BR, Thirunavukkarasu N, Ghosal K, Ravichandran E, Kukreja R, Cai S, Zhang P, Ray R, Ray P. 2010. Clostridial neurotoxins as a drug delivery vehicle targeting nervous system. Biochimie 92:1252–1259.
6. Smith LA. 2009. Botulism and vaccines for its prevention. Vaccine 27: D33–D39.
7. Clayton MA, Clayton JM, Brown DR, Middleton J. 1995. Protective vaccination with a recombinant fragment of Closstridium botulinum neurotoxin type A expressed from a synthetic gene in Escherichia coli. Infect. Immun. 63:2738–2742.
8. Baldwin MR, Tepp WH, Przedpelski A, Pier CI, Bradshaw M, Johnson EA, Barbieri JT. 2008. Subunit vaccine against the seven serotypes of botulism. Infect. Immun. 76:1314–1318.
9. Shearer JD, Vassar ML, Swiderski W, Metcalfe K, Niemuth N, Henderson L. 2010. Botulinum neurotoxin neutralizing activity of immune globulin (IG) purified from clinical volunteers vaccinated with recombinant bostrulinum vaccine (rBV/A/B). Vaccine 28:7313–7318.
10. Foster KA. 2009. Engineered toxins: new therapeutics. Toxicon 54:587–592.
11. Fu Z, Chen C, Barbieri JT, Kim JJP, Baldwin MR. 2009. Glycosylated SV2 and gangliosides as dual receptors for botulinum serotype F. Biochemistry 48:5631–5641.
12. Peng L, Tepp W, Johnson EA, Dong M. 2011. Botulinum neurotoxin D uses synaptic vesicle protein SV2 and gangliosides as receptors. PLoS Pathog. 7:e1002008. doi:10.1371/journal.ppat.1002008.
13. Rummel A, Hafner K, Mehrhold S, Darashchonak N, Holt M, Jahn R, Beermann S, Karnath T, Bigalke H, Binz T. 2009. Botulinum neurotoxins C, E, and F bind gangliosides via a conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F utilising the three isoforms of SV2 as second receptor. J. Neurochem. 110:1942–1954.
14. LaPenotiere HF, Clayton MA, Middleton J. 1995. Expression of a large, nontoxic fragment of botulinum neurotoxin serotype A and its use as an immunogen. Toxicon 33:1383–1386.
15. Bole J, West M, Montgomery V, Tammariello R, Pitt MLM, Gibbs P, Smith I, LeClaire RD. 2006. Recombinant C fragment of botulinum neurotoxin B serotype (rBoNTB (H₁)) immune response and protection in the rhesus monkey. Toxicon 47:877–884.
16. Byrne MP, Smith TJ, Montgomery VA, Smith LA. 1998. Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from Pichia pastoris as a recombinant vaccine candidate. Infect. Immun. 66:4817–4822.
17. Byrne MP, Titball RW, Holley J, Smith LA. 2000. Fermentation, purification, and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from Pichia pastoris. Protein Expr. Purif. 18:327–337.
18. Dux MP, Barent R, Sinha J, Gouthro M, Swanson T, Barthali A, Inan M, Ross JT, Smith LA, Smith TJ, Webb R, Loveless B, Henderson I, Meagher MM. 2006. Purification and scale-up of a recombinant heavy chain fragment C of botulinum neurotoxin serotype E in Pichia pastoris GS115. Protein Expr. Purif. 45:359–367.
19. Dux MP, Huang J, Barent R, Inan M, Swanson ST, Sinha J, Ross JT, Smith LA, Smith TJ, Henderson I, Meagher MM. 2011. Purification of a recombinant heavy chain fragment C vaccine candidate against botulins.
num serotype C neurotoxin [rBoNTC(H\textsubscript{c})] expressed in *Pichia pastoris*. Protein Expr. Purif. 75:177–185.

20. Potter KJ, Zhang W, Smith LA, Meghner MM. 2000. Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 19:393–402.

21. Diamant E, Lahmi B, Keren A, Barnea A, Marcus H, Cohen S, Zichel R. 2013. Development of serotype-specific monoclonal antibodies against botulinum neurotoxins A, B, and E, based on a trivalent immunization protocol and simultaneous differential robotic screen. Toxicon 68:103.

22. Zichel R, Mimran A, Keren A, Barnea A, Steinberger-Levy I, Barnea A, Steinberger-Levy I, Nieman H. 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. 265:9153–9158.

23. Whelan SM, Elmorce MJ, Bodsworth NJ, Atkinson T, Minton NP. 1992. The complete amino acid sequence of the *Clostridium botulinum* type B neurotoxin derived by sequence analysis of the encoding gene. Eur. J. Biochem. 204:657–667.

24. Whelan SM, Elmorce MJ, Bodsworth NJ, Brehm JK, Atkinson Minton TP. 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. Appl. Environ. Microbiol. 58:2345–2354.

25. European Directorate for the Quality of Medicines and Healthcare. 2010. Development and preclinical evaluation of a new F(ab\textsubscript{2}) antitoxin against botulinum neurotoxin type A. Hum. Vaccin. 7:1090–1095.

26. Yu YZ, Zhang SM, Wang WB, Du Y, Zhu HQ, Wang RL, Zhou XW, Lin TJ. 2004. Roads from vaccines to therapies. Mov. Disord. 19(Suppl 8): S48–S52.

27. Bonsor DA, Sundberg EJ. 2011. Dissecting protein-protein interactions using directed evolution. Biochemistry 50:2394–3402.

28. Karanikolas J, Kuhlman B. 2009. Computational design of affinity and specificity at protein-protein interface. Curr. Opin. Struct. Biol. 19:458–463.

29. Jin R, Rummel A, Binz T, Brunger AT. 2006. Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. Nature 444:1092–1095.

30. Rummel A, Karnath T, Henke T, Bigalke H, Binz T. 2004. Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin E. J. Biol. Chem. 279:30865–30870.

31. Tacket CO, Shandera WX, Mann JM, Hargrett NT, Blake PA. 1984. Equine antitoxin use and other factors that predict outcome in type A foodborne botulism. Am. J. Med. 76:794–798.

32. Yu YZ, Zhang SM, Wang WB, Du Y, Zhu HQ, Wang RL, Zhou XW, Lin JB, Wang S, Yu WY, Huang PT, Sun ZW. 2010. Development and preclinical evaluation of a new F(ab\textsubscript{2})\textsuperscript{2} antitoxin against botulinum neurotoxin serotype A. Biochimie 92:1315–1320.