

Protective Vaccination with a Recombinant Fragment of *Clostridium botulinum* Neurotoxin Serotype A Expressed from a Synthetic Gene in *Escherichia coli*

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A completely synthetic gene encoding fragment C, a ~50-kDa fragment, of botulinum neurotoxin serotype A was constructed from oligonucleotides. The gene was expressed in *Escherichia coli*, and full-sized product was produced as judged by Western blot (immunoblot) analysis. Crude extracts of *E. coli* expressing the gene were used to vaccinate mice and evaluate their survival against challenge with active toxin. Mice given three subcutaneous vaccinations were protected against an intraperitoneal administration of 10^6 50% lethal doses (LD_{50}) of serotype A toxin. The same mice survived when challenged with 3 LD_{50} of botulinum toxin serotype E but died when challenged with 10 LD_{50} of serotype E or 3 LD_{50} of serotype B. Purified fragment C was compared with the botulinum toxoid vaccine in a vaccination and challenge study. Fragment C was as efficacious in protecting against challenge with active botulinum neurotoxin serotype A as the toxoid vaccine. This recombinant protein product has many properties that make it a good candidate for human use to protect against botulinum toxin.

The clostridial neurotoxins are the most toxic substances known to science. The neurotoxin produced from *Clostridium tetani* (tetanus toxin) is encountered by humans as a result of wounds and remains a serious public health problem in developing countries around the world. Humans are usually exposed to the neurotoxins produced by *Clostridium botulinum* (botulinum toxins) through food poisoning, although there are rare incidents of wound botulism and colonizing infections in neonates known as infant botulism (17). Tetanus and botulinum toxins share a number of structural and functional features (4, 9, 16) in spite of radical differences in the clinical symptoms of poisoning. Each of the toxins is a protein with an approximate molecular mass of 150 kDa. The toxins may each be viewed as composed of three functional domains. The carboxyl-terminal 50 kDa of the toxins mediates binding to the target neurons. The 50-kDa middle one-third of the toxins assists or is responsible for internalization of the toxins. The amino-terminal 50 kDa is an enzyme, recently identified as a zinc protease (15). The protease activity is expressed intraneuronally, and the apparent molecular targets for the various serotypes are different and most have been identified (12).

Tetanus poisoning in industrial countries is not a public health problem as a result of the availability and widespread use of a safe, effective, and inexpensive vaccine. This vaccine is basically a formalin-inactivated culture supernatant from *C. tetani* grown in fermentors. A similar type of vaccine is available to protect against botulinum toxin poisoning. However, it suffers from several major problems, most notably its cost. Since botulinum toxin is found as seven serotypes, complete protection can be afforded only by making seven distinct vaccines and combining them for human administration. Presently, only five of the seven serotypes are represented in the botulinum vaccine. In addition, some of the serotypes are not

produced in high levels by any strain in culture, and obtaining sufficient toxin for purification and toxoiding is laborious.

We have undertaken through the use of molecular genetics the development of a new generation botulinum vaccine. We initially used the PCR and the native gene of the toxin to prepare a construct encoding a nontoxic 50-kDa carboxyl-terminal fragment (H_c) (11) of the toxin. Expressed as a fusion product, this fragment produced immunity in mice when used as an immunogen (6a). However, several technical difficulties were encountered with the construct and its product, so we elected to make a completely synthetic gene encoding the toxin fragment.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5 α was from Life Technologies, Gaithersburg, Md. The plasmids pUC18 and pTrc 99 A were from Pharmacia-LKB, Piscataway, N.J.; pUC19, pBR322, and pMal-P2 were obtained from New England Biolabs, Inc., Beverly, Mass. Construction of the assembly vector, pHL31, involved cloning two complementary oligonucleotides between the *Eco*RI and *Hind*III sites of pUC19, resulting in a polylinker containing sites for *Xho*I, *Bam*HI, *Ecl*XI, *Aat*II, and *Bgl*II.

Media and cultural conditions. *E. coli* strains were grown in Luria broth (Difco Laboratories, Detroit, Mich.) or on Luria broth agar (Difco) (10). Media were supplemented with ampicillin (100 μ g/ml) or carbenicillin (100 μ g/ml) when appropriate. *E. coli* isolates were stored at -70°C in 20% glycerol in phosphate-buffered saline.

Enzymes and chemicals. Restriction endonucleases were from New England Biolabs (*Aat*II, *Msc*I, *Bsm*I, and *Alw*NI) or Boehringer-Mannheim, Indianapolis, Ind. (*Ecl*XI). All other restriction endonucleases were from Life Technologies. T4 DNA ligase was from Life Technologies or Takara Biochemical Corp., Berkeley, Calif. Molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad Laboratories, Melville, N.Y. All other chemicals were from Sigma Chemical Co., St. Louis, Mo., unless stated otherwise. All oligonucleotides were synthesized by Macromolecular Resources, Ft. Collins, Colo. Plasmids were prepared by the method of Birnboim and Doly (1). Sequencing was performed under good laboratory practice procedures by Lark Sequencing Technologies, Inc., Houston, Tex. Antiserum used for Western blots (immunoblots) was obtained from a horse that had been hyperimmunized to serotype A toxin with toxoid and boosted with native toxin.

Gene construction. The gene for fragment C was assembled from five subunits. Subunit 1 was constructed by cloning three large oligonucleotide pairs (100 to 120 bases) into pUC18, followed by excision and splicing. Subunits 2 to 5 were

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constructed by annealing small complementary oligonucleotides of ~60 bases, followed by ligation and cloning.

(i) **Construction of subunit 1.** Oligonucleotide pairs were annealed in 50 mM Tris-HCl (pH 7.2)–10 mM MgCl₂–100 μM dithiothreitol by incubation at 95°C for 5 min and cooling to room temperature. The final double-stranded DNA concentrations of oligonucleotides were 13 to 24 μg/ml. Ligations into digested pUC18 were conducted at concentrations of 13 ng of oligonucleotide per ml and 256 ng of vector DNA per ml. Agarose gel-purified plasmid fragments, from three original clones, were ligated at a concentration of 37 ng/ml. *E. coli* was transformed by a CaCl₂ procedure (14).

(ii) **Construction of subunits 2 to 5.** Oligomers were resuspended in water to a final concentration of 1 mg/ml. A typical annealing reaction consisted of 30 μg of two complementary oligonucleotides in a final volume of 1 ml containing 20 mM Tris-HCl, (pH 7.5), 10 mM MgCl₂, and 50 mM NaCl. The reactants were heated to 70°C for 5 min and then allowed to cool to 25°C over a 20-min period. The sets of annealed oligomers were then ligated, forming a subunit. Typically, 1 μg each of four annealed oligomer pairs constituting one subunit were mixed with 80 μl of 5× ligase buffer and 20 U of bacteriophage T4 ligase (Life Technologies) in a final volume of 400 μl. After overnight ligation at 15°C, the products were separated on a 6% nondenaturing polyacrylamide gel. The full-length subunit was cut out and eluted from the gel by the crush-and-soak method (14).

(iii) **Assembly of subunits.** Subunits 1, 3, and 4 were cloned directly into the assembly vector (pHL31 or derivatives), while subunits 2 and 5 were cloned into an intermediate cloning vector, amplified, and then cloned into the assembly vector. Subunit 2 was cloned initially into pBR322 at the *Bam*HI and *Eco*XI sites, whereas subunit 5 was cloned initially into pMal-P2 at the *Bgl*II and *Hind*III sites. The initial cloning vectors were digested with restriction enzymes as directed by the manufacturers (Life Technologies and Boehringer-Mannheim) and then electrophoresed on a 1.0% low-melting-point agarose gel (SeaPlaque; FMC, Rockland, Maine). The vector band was excised and recovered from the gel (18). A typical ligation reaction mixture containing 100 ng of vector DNA and 25 ng of subunit DNA in a volume of 7 μl of water was heated at 45°C for 5 min and put on ice. Subsequently, 2 μl of 5× ligase buffer and 1 U of T4 DNA ligase were added. After overnight incubation at 15°C, the ligation product was transformed into competent *E. coli* DH5α, and recombinant clones were selected on Luria broth agar containing 100 μg of ampicillin per ml. Clones were screened by digestion of Magic miniprep (Promega Corp., Madison, Wis.) plasmid DNA with the appropriate restriction enzymes.

Prior to direct cloning of subunits 1, 3, and 4, pHL vectors were digested with the appropriate restriction enzymes and then separated on a 1% low-melting-point agarose gel. The vector DNA was excised and recovered from the gel (18) and then dephosphorylated with bacterial alkaline phosphatase (Life Technologies) by adding 30 U of the enzyme to 500 ng of vector DNA in a final volume of 100 μl of 50 mM Tris (pH 8.0). Subunits cloned directly were first phosphorylated with bacteriophage T4 polynucleotide kinase (Life Technologies). Typically, H₂O was added to 100 ng of subunit DNA to a volume of 44 μl; 5 μl of 10× kinase buffer (100 mM MgCl₂, 0.15 mM β-mercaptoethanol, 0.1 mM ATP, 600 mM Tris-HCl [pH 7.8]) and 1 μl of polynucleotide kinase (10 U) were added subsequently. The 50-μl reaction mixture was incubated for 30 min at 37°C and subjected to phenol-chloroform extraction and ethanol precipitation.

(iv) **Mutagenesis of H_c synthetic gene to repair deletion errors.** Corrections were made with the Altered Sites in vitro mutagenesis system (Promega Corp.). The completed gene was cut out of pHL36 with *Xho*I and *Hind*III and cloned into pAlter (Promega Corp.) at sites *Sal*I and *Hind*III. Oligonucleotides having the corrected sequence were synthesized and annealed simultaneously as described by the manufacturer. Putative corrected clones were screened by sequence analysis of plasmid DNA. Sequencing of the corrected regions was performed at the U.S. Army Medical Research Institute of Infectious Diseases (Frederick, Md.) with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) prior to good laboratory practice sequencing. The plasmid with the corrected gene was designated pMutAC-1.

Nucleotide sequence accession number. The nucleotide sequence for the synthetic gene presented in this paper, *bntAC-1*, has been deposited in GenBank under the accession number U22962.

Expression of H_c. The H_c gene was excised from pMutAC-1 by use of *Nco*I and *Hind*III and cloned into the vector pTrc 99 A. Both the control and the vector with insert were used to transform *E. coli*, which was grown at 37°C to an optical density at 600 nm of 0.4, induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 0.3 mM), and harvested after 2 h by centrifugation. The cells were washed and sonicated, and samples were subjected to SDS-PAGE.

Ophidian Pharmaceuticals (Madison, Wis.) was supplied with plasmid pMutAC-1. Ophidian produced H_c in *E. coli* and purified H_c by proprietary techniques. The gene product was expressed with a carboxyl-terminal hexahistidine tag and was provided as a sterile solution at 0.5 mg/ml in saline with 10% glycerol.

Vaccination of animals. Mice were vaccinated with whole-cell lysates from induced cultures which had been transformed with vector alone or vector containing the H_c gene. Lysates were mixed 50:50 with complete Freund's adjuvant and used for all vaccinations. Animals received 0.5 ml of material subcutaneously at 0, 2, and 4 weeks.

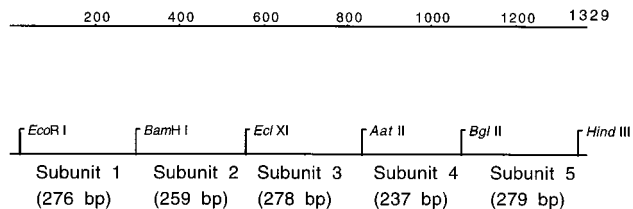


FIG. 1. Size and subunit map of synthetic gene for botulinum toxin serotype A H_c.

Mice vaccinated with purified H_c also received the material at 0, 2, and 4 weeks. Each animal was given subcutaneously 1 μg of material adsorbed onto alum (Pierce Chemical Co., Rockford, Ill.) in a volume of 0.5 ml. Final concentrations of H_c and alum were 2 μg/ml and 1.25 mg/ml, respectively.

In conducting the research described in this study, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, National Research Council.

RESULTS

Gene synthesis. When we attempted expression of the native gene sequence for H_c in *E. coli*, a number of technical difficulties arose, including the apparent instability of the gene and low levels of protein product (6a). One potential explanation for these problems is the high AT content of the native *C. botulinum* gene (13). We calculated the average AT content of the natural gene to be 76%. In addition, throughout the sequence there are a number of codons that are rarely employed in *E. coli* genes, which may also lead to poor expression (8). To improve expression levels, we designed a new H_c gene by reverse translating from the protein sequence with the single most frequently used codon of *E. coli* for each amino acid. This gene was examined manually for poly(A) runs, and when found, conservative codon substitutions were made to retain fidelity, with no poly(A) sequences longer than 5 bases. Additionally, internal restriction sites were designed to divide the gene into five fragments that were roughly equal in size (Fig. 1). The final design of the gene resulted in a marked reduction of the AT content to an average of 56%.

Initial, single-step attempts to assemble a synthetic gene for the H_c fragment of botulinum toxin serotype A from multiple overlapping oligonucleotides (60-mers) were unsuccessful. Consequently, an effort to assemble the first subunit of this gene from larger oligonucleotides was undertaken. Three pairs of oligonucleotides (104, 114, and 99 bp) were synthesized to provide terminal *Eco*RI- and *Aat*II-compatible ends for cloning into these sites in pUC18. The lengths of each were determined by the detection of sequences in the planned synthetic gene that could be used for blunt-end cloning, since the original small-fragment clones were assembled into the first subunit. Plasmids pAA1, pAA2, and pAA3 (Fig. 2) were constructed and verified by restriction endonuclease digestions. The 0.86-kbp *Alw*NI-*Ssp*I fragment of pAA1 and the 1.5-kbp *Alw*NI-*Msc*I fragment of pAA2 were ligated to construct pAA4 (Fig. 2). After restriction endonuclease mapping of this construction, the 0.97-kbp *Alw*NI-*Ssp*I fragment of pAA4 was ligated to the 1.49-kbp *Alw*NI-*Pvu*II fragment of pAA3 to yield pAA5, which completed the construction of the first subunit (Fig. 2).

While the work described above was under way, advances in synthetic nucleotide chemistry occurred such that oligomers could be efficiently phosphorylated on the synthesis columns after completion of the nucleotide chains. When oligomers were used in that form, the condensation and ligation reactions proved efficient. Thus, subunits 2 to 5 (Fig. 1) were made by

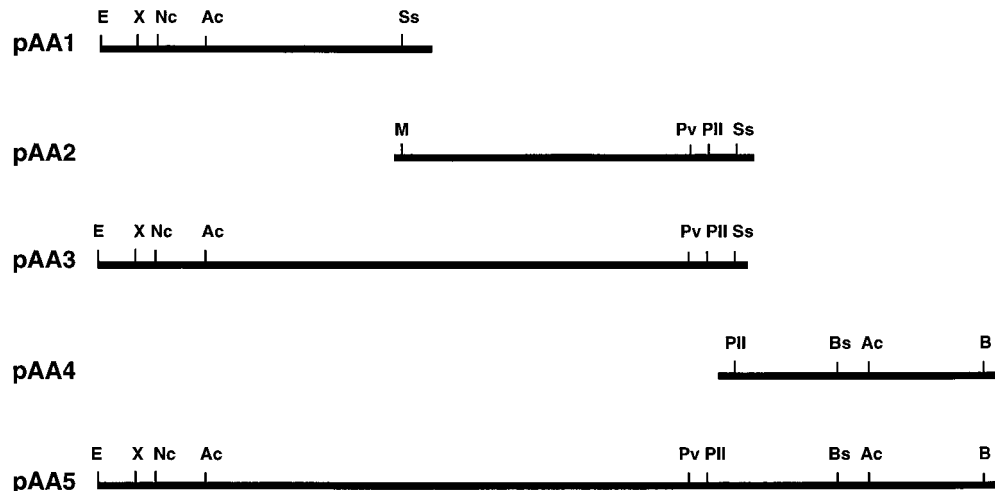


FIG. 2. Maps of the vectors used to construct subunit 1 of the H_c gene. Partial restriction maps for the inserts are shown. Restriction endonuclease site abbreviations: Ac, *AccI*; B, *Bam*HI; Bs, *Bsm*I; E, *Eco*RI; M, *Msc*I; Nc, *Nco*I; Pv, *Pvu*I; PII, *Pvu*II; Ss, *Ssp*I; X, *Xho*I.

ligations between oligomer pairs of 60 to 70 bases, having complementary homologous overhangs of 5 to 6 bases.

For assembly, subunit 1 was cloned into the pHL31 assembly vector as described in Materials and Methods; this construct was designated pHL32. Subunit 2 was excised from pBR322, cloned into pHL32, and designated pHL33. Next, subunit 3 was cloned directly into pHL33, producing pHL34. Subunit 5 was excised from pMal-P2, cloned into pHL34, and designated pHL35. Finally, subunit 4 was cloned directly into pHL35 and designated pHL36.

When the H_c gene was sequenced, several errors were found (Fig. 3). In every case, the errors were deletions of one or more bases. To correct the errors, the gene was cloned into the pAlter vector and *in vitro* mutagenesis was performed in one reaction containing five repair primers. Upon resequencing, the correct primary structure of the gene was confirmed. We designated this gene *bntAC-1*.

Gene expression. Expression of the *bntAC-1* gene was attempted after cloning into the vector pTrc 99 A. Whole-cell lysates were subjected to SDS-PAGE. Specific product could not be seen in Coomassie brilliant blue-stained gels. However, a Western blot analysis demonstrated immunoreactive material migrating at the expected molecular weight in lysates of the induced vector with insert (Fig. 4). The cell lysate from the noninduced vector with the H_c gene did not exhibit this band.

In addition, neither the induced nor noninduced lysates from pTrc 99 A without the H_c gene exhibited this band (Fig. 4).

Vaccination of animals. As an initial test of immunogenicity, mice were immunized with crude H_c from whole-cell lysates. Animals received material at 0, 2, and 4 weeks. Although none of the animals exhibited untoward symptoms on the first vaccination, several animals in both the control and insert groups died upon receiving the second and third vaccinations. All surviving animals were challenged with toxin at the first part of week 5. As demonstrated by the data in Table 1, vaccination with crude H_c led to protection against a dose of up to 3,000 50% lethal doses (LD_{50}) of serotype A botulinum toxin. To obtain some estimate of the upper level of protection achieved, we rechallenged the same animals 3 to 5 days later with various challenge doses of toxin of up to 10^6 LD_{50} and observed no animal deaths or symptoms of poisoning.

In a limited study, we determined whether there might be cross-protection against other serotypes of botulinum toxin conferred by immunization with serotype A H_c . Using the same animals listed in Table 1, we challenged three with serotype B (3 LD_{50}) with the result that none survived. Animals challenged with 3 LD_{50} of serotype E survived (three of three), but animals challenged with 10 LD_{50} of serotype E died (three of three).

A second vaccination study was performed in which purified

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1  GTCGAGCCAT  GGCTCGTCTG  CTGTCTACCT  TCACATGAATA  CATCAAGAAC  ATCATCAATA  CCTCCATCCT  GAACCTGCGC  TACGAATCCA  ATCACCTGAT
101  CGACCTGTCT  CGCTACGCTT  CCAAAATCAA  CATCGGTTCT  AAAGTTAACT  TCGATCOGAT  CGACAAGAAT  CAGATCCAGC  TGTTCATCT  GGAATCTTCC
201  AAAATCGAAG  TTTATCTGAA  GAAATGCTATC  GTATACAAC  CTATGTACGA  AAACCTTCTCC  ACCTCCTTCT  GGATCCGAT  CCGGAAATAC  TTcaactcca
301  TCTCTCTGAA  CAATGAATAC  ACCATCATCA  ACTGCATGGA  AAACAATTCT  GGTITGAAAG  TATCTCTGAA  CTACGGTGA  ATCATCTGGA  CTCTGCAGGA
401  CACTCAGGAA  ATCAAACAGC  GTGTGTATTT  CAAATACTCT  CAGATGATCA  ACATCTCTGA  CTACATCAAT  CGCTGGATCT  TCGTTACCAT  CACCAACAA
501  CGTCTGAATA  ACTCCAAAAT  CTACATCAAC  GGCCGCTTGA  TCGACCAGAA  ACCGATCTCC  AATCTGGGTA  ACATCCACGC  TTCTAATAAC  ATCATGTTCA
601  AACTGGACGG  TTGTCTGTAC  ACTCACCCGT  ACATCTGGAT  CAAATACTTTC  AATCTGTCTG  ACAAGAAGCT  GAACGAAAA  GAAaTCAAAG  ACCTGTACGA
701  CAACCACTCC  AATTTCTGGT  TCCTGAAAGA  CTTCTGGGgT  GACTACTCTG  AGTACGACAA  ACCGTACTAC  ATGCTGAATC  TGTACGATCC  GAACAAATAC
801  GTTGCAGTCA  ACAATGTAGT  TATTCGCGGT  TACATGTACC  TGAAAGGTCC  GCGTGGTCT  GTTATGACTA  CCAACATCTA  CCTGAACCT  TCCCTGTACC
901  GTGGTACCAA  ATTTCATCAT  AAGAAATACG  CGTCTGGTAA  CAAGACAAT  ATCGTTCGCA  ACAATGATCG  TGTATACATC  AATGTTGTAGT  TAAGAACAA
1001  AGAATACCGT  CTGGCTACCA  ATGCTTCTCA  GGCTGGTGA  GAAAAGATCT  TGCTCTCTCT  GGAATCCCG  GACGTTGGTA  ATCTGTCTCA  GGTAGTTGTA
1101  ATGAAATCCA  AGAACGACCA  GGGTATCACT  AACAAATGCA  AAaTGAATCT  GCAGGACAAC  AATGGTAAOG  ATATCGGTTT  CATCGGTTTC  CACCAGTTCA
1201  ACAATATCG  TAAACTGGIT  GCTTCCAAGT  GGTACAATCG  TCAGATCGAA  CGTTCCTCTC  GCACCTCTGG  TTGTCTCTGG  GAGTTCATCC  CGGTTGATGA
1301  CGGTTGGGGT  GAACGTCGCG  TGTAaCCCCG  GAAaGCTT

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FIG. 3. Nucleotide sequence of the synthetic gene for H_c . The sequence of the gene is shown with the deletion errors found after the initial sequencing (deletion errors in small letters).

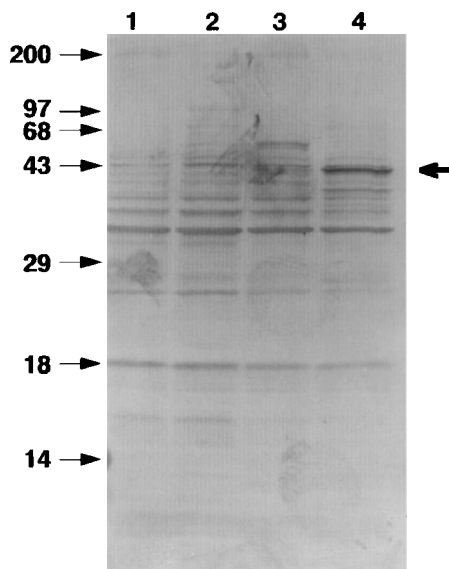


FIG. 4. Western blot analysis of *E. coli*-produced H_C. Bacteria were transformed with pTrc 99 A or pTrc 99 A with the *bntAC-1* insert. Cultures were induced, and after 2 h, whole-cell homogenates were prepared. Aliquots of the homogenates were subjected to SDS-PAGE, blotted, and developed with hyper-immune horse type A antiserum. Lanes 1, vector-transformed cells; 2, vector-transformed, induced cells; 3, *bntAC-1* vector-transformed cells; 4, *bntAC-1* vector-transformed, induced cells. Arrows on the left indicate the positions of the specified molecular weight standards (10³). The arrow on the right indicates the position of the H_C.

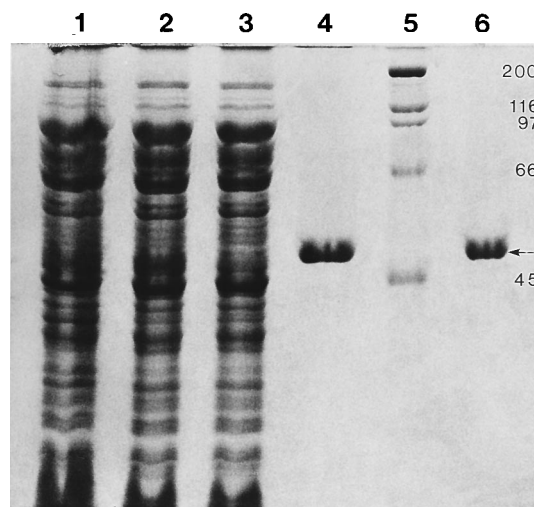


FIG. 5. SDS-PAGE of H_C prepared by Ophidian Pharmaceuticals. Lanes: 1, *E. coli* lysate; 2, soluble polypeptides from *E. coli* lysate; 3, nonretained polypeptides from affinity column; 4, eluted fragment C from affinity column; 5, Bio-Rad high-molecular-weight standards; 6, same as that described for lane 4 but with a freeze-thaw cycle. Molecular weights of the protein standards are indicated on the right (10³). An arrow indicates the position of the H_C.

H_C was used with an aluminum hydroxide adjuvant. The product from Ophidian Pharmaceuticals was judged to be >95% pure by SDS-PAGE (Fig. 5). A set of control animals was vaccinated with the currently available botulinum toxoid (pentavalent) adsorbed onto alum. The doses of toxoid were calculated on the basis of the serotype A component alone. Animals were vaccinated and challenged by the same schedule as that described above. At the high-dose range of both immunogens, there was complete protection of animals at all challenge doses except the highest (Table 2). At the lower-dose range of immunogen, little protection was observed with any of the toxin challenge doses (Table 2). These data indicate that the genetically engineered product is similar to the toxoid in its efficacy.

DISCUSSION

The current vaccine used to protect humans against poisoning by botulinum toxin suffers from several shortcomings, the

TABLE 1. Protection of ICR mice immunized with H_C derived from the synthetic gene^a

Calculated challenge dose (LD ₅₀)	No. of survivors/total no.	
	Control (vector alone)	Experimental (vector with insert)
4	1/3	3/3
10		3/3
30	1/4	3/3
100		3/3
300		3/3
1,000		3/3
3,000		1/1

^a Animals received vaccinations of crude lysate at 0, 2, and 4 weeks. Challenges with serotype A were given intraperitoneally at 5 weeks.

majority of which stem from the difficulty and expense of culturing seven serotypes of *C. botulinum* and working with highly toxic preparations while processing them for inactivation by formalin. If an immunogenic nontoxic fragment of the toxin could be produced without the use of fully active toxin, cost should be reduced and safety should be increased. Such an advance has been made with tetanus toxin by Fairweather and colleagues (5, 6). Working initially with the natural gene (5, 7) and later with a synthetic gene (8) encoding fragment C of tetanus toxin, they achieved expression in several heterologous systems (2, 3, 13) and demonstrated that their product conferred protection in animal models. Much of the work reported here was patterned after their approach.

As expressed in *E. coli*, our synthetic gene produces a protein product with the expected molecular mass (~50 kDa) on SDS-PAGE that reacts in Western blots with antisera to sero-

TABLE 2. Comparative challenge of H_C-immunized and toxoid-immunized mice

Immunogen and dose (μg)	No. of survivors/total no. at challenge dose of ^a :				
	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶
Fragment C					
1.0	6/6	6/6	6/6	6/6	6/6
0.3	5/6	6/6	6/6	4/6	2/6
0.1	5/6	5/5 ^b	4/6	4/6	2/6
0.03	4/4 ^c	4/6	4/6	2/6	1/6
0.01	2/6	1/6	0/6	1/6	0/6
Toxoid					
1.0	6/6	6/6	6/6	6/6	3/6
0.3	5/5	6/6	6/6	5/6	3/6
0.1	6/6	5/6	5/6	4/6	0/6
0.03	6/6	6/6	4/6	3/6	0/6
0.01	1/6	1/6	0/6	1/6	0/6
Control					
0.0	1/15	0/12			

^a Neutralization results at 96 h.

^b One death occurred immediately following challenge injection.

^c One death occurred following immunization; one animal escaped.

type A botulinum toxin. While these data were encouraging, they gave no indication regarding the antigenicity or immunogenicity of the H_c product. Therefore, prior to undertaking purification of H_c, crude lysates were used to obtain an indication of its potential as an immunogen. In this initial evaluation, we did not anticipate the animals developing the extremely high levels of immunity that were ultimately determined. Thus, we initially challenged the mice with a dose range of 3 to 3,000 LD₅₀ and observed no toxic symptoms. We rechallenged them 3 to 5 days later with much higher toxin doses, up to 10⁶ LD₅₀, again with no observed toxic symptoms (data not shown). In addition, it did not appear that there was an appreciable degree of cross-protection against botulinum toxin serotypes B and E in animals immunized with the crude product.

After obtaining the encouraging results with crude H_c, we obtained purified *E. coli*-produced botulinum toxin serotype A H_c and vaccinated mice with that material. The results of this experiment allowed us to establish or clarify several important points. First, during the course of vaccination of the mice with pure H_c, none of the animals died exhibiting endotoxic-like symptoms, as did some of the mice in the experiment described in Table 1. However, there was one unexplainable death following vaccination with a low dose of H_c. We believe that other *E. coli*-derived materials were responsible for the deaths during vaccination in the experiment described in Table 1. This belief is further supported by a lack of deaths during the course of vaccination of animals with crude baculovirus-produced H_c (9a). A second point established was that the *E. coli*-derived H_c will elicit a response protective against toxin challenges at doses in the range of 10³ to 10⁶ LD₅₀. Finally, it is clear that the H_c will produce these high levels of protection when used with an adjuvant acceptable for human use (e.g., alum). If the results with mice translate to humans, this product is a new candidate vaccine for botulinum toxin poisoning. The material is nontoxic, can be produced without high-containment facilities, and is highly effective.

ACKNOWLEDGMENTS

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