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Molecular Characterization of Murine Humoral Immune Response to Botulinum Neurotoxin Type A Binding Domain as Assessed by Using Phage Antibody Libraries

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To produce antibodies capable of neutralizing botulinum neurotoxin type A (BoNT/A), the murine humoral immune response to BoNT/A binding domain (H_C) was characterized at the molecular level by using phage antibody libraries. Mice were immunized with BoNT/A H_C, the spleens were harvested, and single-chain Fv (scFv) phage antibody libraries were constructed from the immunoglobulin heavy and light chain variable region genes. Phage expressing BoNT/A binding scFv were isolated by selection on immobilized BoNT/A and BoNT/A H_C. Twenty-eight unique BoNT/A H_C binding scFv were identified by enzyme-linked immunosorbent assay and DNA sequencing. Epitope mapping using surface plasmon resonance in a BIAcore revealed that the 28 scFv bound to only 4 nonoverlapping epitopes with equilibrium constants (K_d) ranging from 7.3×10^{-8} to 1.1×10^{-9} M. In a mouse hemidiaphragm assay, scFv binding epitopes 1 and 2 significantly prolonged the time to neuroparalysis, 1.5- and 2.7-fold, respectively, compared to toxin control. scFv binding to epitopes 3 and 4 showed no protection against neuroparalysis. A combination of scFv binding epitopes 1 and 2 had an additive effect on time to neuroparalysis, which increased to 3.9-fold compared to the control. The results suggest that there are two "productive" receptor binding sites on H_C which lead to toxin internalization and toxicity. Blockade of these two epitopes with monoclonal antibodies may provide effective immunoprophylaxis or therapy against BoNT/A intoxication.

Botulism is a life-threatening flaccid paralysis caused by a neurotoxin produced by the anaerobic bacterium *Clostridium botulinum*. The disease typically results from ingestion of preformed toxin present in contaminated food (15) or from toxin produced in vivo from infected wounds (50) or in the intestines of infants (2) (or occasionally adults). In severe cases, patients require prolonged hospitalization in an intensive-care unit and mechanical ventilation. Specific therapy consists of administration of botulism antitoxin trivalent (equine) (48); however, this product has a high incidence of side effects, including serum sickness and anaphylaxis (5). To avoid these side effects, human BIG has been produced from immunized volunteers and its efficacy is being determined in a prospective randomized trial in infants with botulism (1). While theoretically nontoxic, human BIG also has limitations, largely related to production issues. These include potential transmission of blood-borne infectious diseases, variability in potency and specificity between lots, and the need to immunize humans. The latter issue has taken on increased importance with the use of BoNTs for the treatment of a range of neuromuscular diseases (28, 41). Immunization of volunteers for production of BIG would deprive them of subsequent botulinum therapy.

As an alternative to immune globulin, neutralizing monoclonal antibodies with defined potency and specificity could be produced in unlimited quantities. To date, however, no efficacious neutralizing antibotulinum monoclonal antibodies have been produced (38). Potential explanations for this failure include the following: (i) a neutralizing epitope(s) is less immunogenic than other epitopes; (ii) too few unique monoclonal antibodies have been studied; (iii) a toxoid immunogen (formaldehyde-inactivated crude toxin) which poorly mimics the conformation of the neutralizing epitope(s) has been used; and (iv) multiple epitopes must be blocked in order to achieve efficient neutralization (32). To address these issues, and to generate neutralizing antibodies to BoNT/A, we have produced and characterized a large panel of monoclonal antibodies from immunized mice. To generate antibodies capable of preventing the binding of toxin to its cellular receptor(s), mice were immunized with BoNT/A H_C (33). This domain contains the region(s) thought to bind to presynaptic neuronal receptors, the first requisite step for intoxication, and results in protective immunity when used as an immunogen (11, 33). To produce and characterize the greatest number of monoclonal antibodies possible, we used phage display (10, 37; reviewed in reference 34). Murine V_H and V_κ genes were used to construct libraries of millions of recombinant scFv, which were displayed on the surface of filamentous bacteriophage (Fig. 1). Phage displaying antibodies binding BoNT/A were isolated by affinity chromatography. Here we report the molecular and biophysical characterization of these antibodies, including specificity, affinity, epitopes recognized, and in vitro neutralization capacity.

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MATERIALS AND METHODS

Abbreviations used. AMP, ampicillin; BIG, botulinum immune globulin; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; CDR, complementarity-

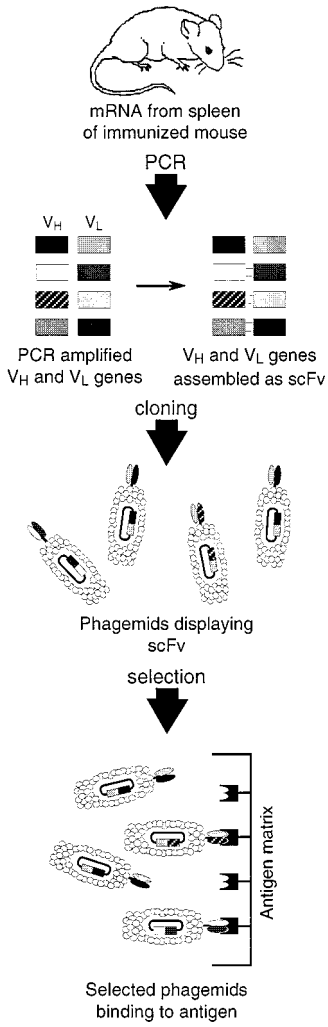

FIG. 1. Strategy for in vitro antibody production using phage display libraries. mRNA is prepared from splenocytes, first-strand cDNA is prepared, and antibody V_H and V_L genes are amplified by PCR. V_H and V_L genes are spliced together randomly by using PCR to create a repertoire of scFv genes. The scFv gene repertoire is cloned into a phagemid vector in frame with a gene (gIII) encoding a phagemid minor coat protein (pIII). Each phage in the resulting phage antibody library expresses an scFv-pIII fusion protein on its surface and contains the gene encoding the scFv inside. Phage antibodies binding a specific antigen can be separated from nonbinding phage antibodies by affinity chromatography on immobilized antigen. A single round of selection increases the number of antigen-binding phage antibodies by a factor ranging from 20 to 10,000, depending on the affinity of the antibody. Eluted phage antibodies are used to infect *E. coli*, which then produces more phage antibodies for the next round of selection. Repeated rounds of selection make it possible to isolate antigen-binding phage antibodies that were originally present at frequencies of less than one in a billion.

determining region; ELISA, enzyme-linked immunosorbent assay; GLU, glucose; HBS, HEPES-buffered saline (10 mM HEPES, 150 mM NaCl [pH 7.4]); H_C, C-terminal domain of BoNT heavy chain (binding domain); H_N, N-terminal domain of BoNT heavy chain (translocation domain); IgG, immunoglobulin G; IMAC, immobilized-metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; KAN, kanamycin; K_d, equilibrium constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; MPBS, skim milk powder in PBS; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline (25 mM NaH_2PO_4, 125 mM NaCl [pH 7.0]); RU, resonance units; scFv, single-chain Fv antibody fragments; TPBS, 0.05% (vol/vol) Tween 20 in PBS; TMPBS, 0.05% (vol/vol) Tween 20 in MPBS; TU, transducing units; V_H, immunoglobulin heavy-chain variable region; V_κ, immunoglobulin kappa light-chain variable region; V_L, immunoglobulin light-chain variable region.

Oligonucleotide design. Family-specific murine V_H and V_κ primers were designed as previously described for human V-gene primers (35, 36) to amplify full-length rearranged V genes. Briefly, murine V_H and V_κ DNA sequences were collected from the Kabat et al. (30) and GenBank databases, aligned, and classified by family, and family-specific primers were designed to anneal to the first 23 nucleotides comprising framework 1. Similarly, J_H and J_κ gene-segment-specific primers were designed to anneal to the final 24 nucleotides comprising each of the 4 J_H and 5 J_κ gene segments (30).

Vector construction. To construct the vector pSYN3, a 1.5-kb stuffer fragment was amplified from pCANTAB5E (Pharmacia Biotech, Milwaukee, Wis.) by using PCR and the primers LMB3 (35) and E-tagback (5′-ACCACCGAATTCT TATTAATGGTGATGATGGTGGATGACCAGCCGGTTCCAGCGG-3′). The DNA fragment was digested with *Sfi*I and *Not*I, gel purified, and ligated into pCANTAB5E digested with *Sfi*I and *Not*I. Ligated DNA was used to transform *Escherichia coli* TG1 (19), and clones containing the correct insert were identified by DNA sequencing. The resulting vector permits subcloning of phage-displayed scFv as *Sfi*I-*Not*I or *Nco*I-*Not*I fragments for secretion into the periplasm of *E. coli* as native scFv with a C-terminal E epitope tag followed by a hexahistidine tag.

Immunizations. For construction of library 1, BALB/c mice (16 to 22 g) were immunized at 0, 2, and 4 weeks with pure BoNT/A H_C (Ophidian Pharmaceuticals, Madison, Wis.). Each animal was given subcutaneously 1 μg of material adsorbed onto alum (Pierce Chemical Co., Rockford, Ill.) in a volume of 0.5 ml. Mice were challenged 2 weeks after the second immunization with 100,000 50% lethal doses of pure BoNT/A and were sacrificed 1 week later. For construction of library 2, CD-1 mice (16 to 22 g) were immunized at 0, 2, and 4 weeks with pure BoNT/A H_C and were sacrificed 2 weeks after the third immunization. For both libraries, the spleens were removed immediately after sacrifice and total RNA was extracted by the method of Cathala et al. (7).

Library construction. First-strand cDNA was synthesized from approximately 10 μg of total RNA exactly as previously described (35), except that immunoglobulin mRNA was specifically primed with 10 pmol each of MIgG1 For, MIgG3 For, and MC_κ For (Table 1). For construction of library 1, rearranged V_H and V_κ genes were amplified from first-strand cDNA by using commercially available V_H and V_κ back primers and J_H and J_κ forward primers (Recombinant Phage Antibody System; Pharmacia Biotech). For library 2, equimolar mixtures of family-specific V_H and V_κ back primers were used in conjunction with equimolar mixtures of J_H or J_κ gene-segment-specific forward primers in an attempt to increase library diversity (see "Oligonucleotide design" above). Rearranged V_H and V_κ genes were amplified separately in 50-μl reaction mixtures containing 5 μl of the first-strand cDNA reaction mixture, 20 pmol of an equimolar mixture of the appropriate back primers, 20 pmol of an equimolar mixture of the appropriate forward primers, 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μg of bovine serum albumin/ml, and 1 μl (5 U) of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) in the buffer supplied by the manufacturer. The reaction mixture was overlaid with paraffin oil (Sigma) and cycled 30 times (at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min). Reaction products were gel purified, isolated from the gel by using DEAE membranes, eluted from the membranes with high-salt buffer, ethanol precipitated, and resuspended in 20 μl of water (43).

scFv gene repertoires were assembled from purified V_H and V_κ gene repertoires and linker DNA by using splicing by overlap extension. Linker DNA encoded the peptide sequence $(G_4S)_3$ (27) and was complementary to the 3′ ends of the rearranged V_H genes and the 5′ ends of the rearranged V_κ genes. The V_H and V_κ DNAs (1.5 μg of each) were combined with 500 ng of linker DNA (Recombinant Phage Antibody System; Pharmacia Biotech) in a 25-μl PCR mixture containing 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 μg of bovine serum albumin/ml, and 1 μl (5 U) of *Taq* DNA polymerase (Promega) in the buffer supplied by the manufacturer, and the mixture was cycled 10 times (at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min) to join the fragments. Flanking oligonucleotide primers (RS, provided in the Recombinant Phage Antibody System kit, for library 1 and an equimolar mixture of V_HSfi and J_κNot primers [Table 1] for library 2) were added, and the reaction mixture was cycled for 33 cycles (at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) to append restriction sites. scFv gene repertoires were gel purified as described above, digested with *Sfi*I and *Not*I, and purified by electroelution, and 1 μg of each repertoire was ligated into either 1 μg of pCANTAB5E vector (Pharmacia Biotech) (library 1) or 1 μg of pHEN-1 (25) (library 2) digested with *Sfi*I and *Not*I. The ligation mix was purified by extraction with phenol-chloroform, ethanol precipitated, and resuspended in 20 μl of water, and 2.5-μl samples were electroporated (16) into 50 μl of *E. coli* TG1 (19). Cells were grown in 1 ml of SOC (43) for 30 min and then plated on TYE (39) medium containing 100 μg of AMP/ml and 1% (wt/vol) GLU (TYE-AMP-GLU). Colonies were scraped off the plates into 5 ml of 2× TY broth (39) containing 100 μg of AMP/ml, 1% GLU (2× TY-AMP-GLU), and 15% (vol/vol) glycerol for storage at −70°C. The cloning efficiency and diversity of the libraries were determined by PCR screening (20) exactly as described in reference 36.

Preparation of phage. To rescue phagemid particles from the libraries, 10 ml of 2× TY-AMP-GLU was inoculated with an appropriate volume of bacteria (approximately 50 to 100 μl) from the library stocks to give an A_{600} of 0.3 to 0.5 and bacteria were grown for 30 min with shaking at 37°C. About 10^{12} PFU of VCS-M13 (Stratagene) particles were added, and the mixture was incubated at

TABLE 1. Oligonucleotide primers used for PCR of mouse immunoglobulin genes

Procedure	Primer	Sequence[a]
1st-strand cDNA synthesis	Heavy-chain constant region	
	MIgG1/2 For	5′ CTG GAC AGG GAT CCA GAG TTC CA 3′
	MIgG3 For	5′ CTG GAC AGG GCT CCA TAG TTC CA 3′
	κ constant region, $MC_κ$ For	5′ CTC ATT CCT GTT GAA GCT CTT GAC 3′
Primary PCR	V_H back	
	V_H1 Back	5′ GAG GTG CAG CTT CAG GAG TCA GG 3′
	V_H2 Back	5′ GAT GTG CAG CTT CAG GAG TCR GG 3′
	V_H3 Back	5′ CAG GTG CAG CTG AAG SAG TCA GG 3′
	V_H4/6 Back	5′ GAG GTY CAG CTG CAR CAR TCT GG 3′
	V_H5/9 Back	5′ CAG GTY CAR CTG CAG CAG YCT GG 3′
	V_H7 Back	5′ GAR GTG AAG CTG GTG GAR TCT GG 3′
	V_H8 Back	5′ GAG GTT CAG CTT CAG CAG TCT GG 3′
	V_H10 Back	5′ GAA GTG CAG CTG KTG GAG WCT GG 3′
	V_H11 Back	5′ CAG ATC CAG TTG CTG CAG TCT GG 3′
	$V_κ$ back	
	$V_κ$1 Back	5′ GAC ATT GTG ATG WCA CAG TCT CC 3′
	$V_κ$2 Back	5′ GAT GTT KTG ATG ACC CAA ACT CC 3′
	$V_κ$3 Back	5′ GAT ATT GTG ATR ACB CAG GCW GC 3′
	$V_κ$4 Back	5′ GAC ATT GTG CTG ACM CAR TCT CC 3′
	$V_κ$5 Back	5′ SAA AWT GTK CTC ACC CAG TCT CC 3′
	$V_κ$6 Back	5′ GAY ATY VWG ATG ACM CAG WCT CC 3′
	$V_κ$7 Back	5′ CAA ATT GTT CTC ACC CAG TCT CC 3′
	$V_κ$8 Back	5′ TCA TTA TTG CAG GTG CTT GTG GG 3′
	J_H forward	
	J_H1 For	5′ TGA GGA GAC GGT GAC CGT GGT CCC 3′
	J_H2 For	5′ TGA GGA GAC TGT GAG AGT GGT GCC 3′
	J_H3 For	5′ TGC AGA GAC AGT GAC CAG AGT CCC 3′
	J_H4 For	5′ TGA GGA GAC GGT GAC TGA GGT TCC 3′
	$J_κ$ forward	
	$J_κ$1 For	5′ TTT GAT TTC CAG CTT GGT GCC TCC 3′
	$J_κ$2 For	5′ TTT TAT TTC CAG CTT GGT CCC CCC 3′
	$J_κ$3 For	5′ TTT TAT TTC CAG TCT GGT CCC ATC 3′
	$J_κ$4 For	5′ TTT TAT TTC CAA CTT TGT CCC CGA 3′
	$J_κ$5 For	5′ TTT CAG CTC CAG CTT GGT CCC AGC 3′
Reamplification	Restriction site containing	
	V_H *Sfi* back	
	V_H1 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTT CAG GAG TCA GG 3′
	V_H2 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAT GTG CAG CTT CAG GAG TCR GG 3′
	V_H3 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG AAG SAG TCA GG 3′
	V_H4/6 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTY CAG CTG CAR CAR TCT GG 3′
	V_H5/9 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTY CAR CTG CAG CAG YCT GG 3′
	V_H7 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAR GTG AAG CTG GTG GAR TCT GG 3′
	V_H8 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTT CAG CTT CAG CAG TCT GG 3′
	V_H10 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAA GTG CAG CTG KTG GAG WCT GG 3′
	V_H11 Sfi	5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG ATC CAG TTG CTG CAG TCT GG 3′
	$J_κ$ *Not* forward	
	$J_κ$1 Not	5′ GAG TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAG CTT GGT GCC TCC 3′
	$J_κ$2 Not	5′ GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAG CTT GGT CCC CCC 3′
	$J_κ$3 Not	5′ GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAG TCT GGT CCC ATC 3′
	$J_κ$4 Not	5′ GAG TCA TTC TCG ACT TGC GGC CGC TTT TAT TTC CAA CTT TGT CCC CGA 3′
	$J_κ$5 Not	5′ GAG TCA TTC TCG ACT TGC GGC CGC TTT CAG CTC CAG CTT GGT CCC AGC 3′

[a] R, A/G; Y, C/T, S, G/C; K, G/T; W, A/T; M, A/C; V, C/G/A; B, G/C/T; H, C/A/T.

37°C for 30 min without shaking, followed by incubation at 37°C for 30 min with shaking. Cells were spun down, resuspended in 50 ml of 2× TY broth containing 100 μg of AMP/ml and 50 μg of KAN/ml (2× TY-AMP-KAN), and grown overnight with shaking at 25°C. Phage particles were purified and concentrated by two polyethylene glycol precipitations (43), resuspended in 5 ml of PBS, and filtered through a 0.45-μm-pore-size filter. The phage preparation consistently resulted in a titer of approximately 10^{13} TU of AMP-resistant clones/ml.

Selection of phage antibody libraries. Both libraries were selected by using 75- by 12-mm immunotubes (Maxisorp; Nunc) coated with 1 ml of BoNT/A (50 μg/ml; kindly provided by Ray Stevens) or BoNT/A H_C (10 μg/ml) in PBS

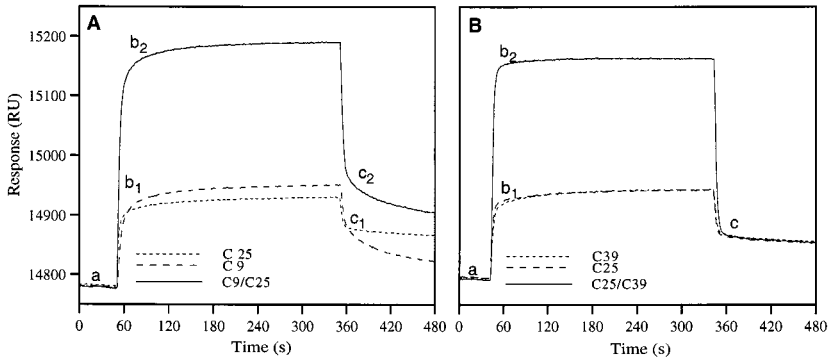

FIG. 2. Sensorgrams illustrating technique used to epitope map scFv binding to BoNT/A H_C. Epitope mapping was performed by using surface plasmon resonance in a BIAcore, with scFv studied in pairs. Each scFv was injected into the BIAcore and allowed to bind to BoNT/A H_C coupled to the sensor chip surface until saturation was achieved. The amount (in RU) bound for each scFv alone was compared to the amount bound when the two scFv were mixed and injected together. Point a, baseline, followed by the beginning of injection; points b_1 and b_2, initial association phase; points c_1 and c_2, beginning of dissociation. The differences in RU between points a and c equal the amount of scFv bound to BoNT/A H_C. (A) Two scFv recognizing different epitopes are studied (C25 and C9). The amount bound for the two scFv injected together (C9/C25; point c_2) is the sum of the two scFv injected alone (c_1). (B) Two scFv recognizing the same epitope are studied (C39 and C25). The amount bound for the two scFv injected together (C25/C39; point c) is the same as that for the two scFv injected alone (c). The large differences in RU between points b_1 and c_1, b_2 and c_2, and b_1 and c are due to differences in refractive index between scFv and running buffer.

overnight at 4°C. Tubes were blocked for 1 h at 37°C with 2% MPBS, and selection, washing, and elution were performed exactly as described in reference 35 by using phage at a concentration of 5.0×10^{12} TU/ml. One-third of the eluted phage was used to infect 10 ml of log-phase *E. coli* TG1, which was plated on TYE-AMP-GLU plates as described above. The rescue-selection-plating cycle was repeated three times, after which clones were analyzed for binding by ELISA. Libraries were also selected on soluble BoNT/A H_C. For library 1, 1.0 mg of BoNT/A H_C (700 μg/ml) was biotinylated (Recombinant Phage Selection Module; Pharmacia) and purified as recommended by the manufacturer. For each round of selection, 1 ml of phage (approximately 10^{13} TU) were mixed with 1 ml of PBS containing 4% skim milk powder, 0.05% Tween 20, and 10 μg of biotinylated BoNT/A H_C/ml. After 1 h at room temperature, antigen-bound phage were captured on blocked streptavidin-coated M280 magnetic beads (Dynabeads; Dynal) exactly as described in reference 45. Dynabeads were washed a total of 10 times (three times in TPBS, twice in TMPBS, twice in PBS, once in MPBS, and two more times in PBS). Bound phage were eluted from the Dynabeads by incubation with 100 μl of 100 mM triethylamine for 5 min and were neutralized with 1 M Tris-HCl, pH 7.5, and one-third of the eluate was used to infect log-phase *E. coli* TG1. For library 2, affinity-driven selections (22, 45) were performed by decreasing the concentration of soluble BoNT/A H_C used for selection (10 μg/ml for round 1, 1 μg/ml for round 2, and 10 ng/ml for round 3). Soluble BoNT/A H_C was captured on 200 μl of Ni^{2+}-NTA (Qiagen) via a C-terminal hexahistidine tag. After capture, the Ni^{2+}-NTA resin was washed a total of 10 times (5 times in TPBS and 5 times in PBS), bound phage were eluted as described above, and the eluate was used to infect log-phase *E. coli* TG1.

Initial characterization of binders. Initial analysis for binding to BoNT/A, BoNT/A H_C, and BoNT/A H_N (kindly provided by Ray Stevens) (8) was performed by ELISA using bacterial supernatant containing expressed scFv. Expression of scFv (13) was performed in 96-well microtiter plates exactly as described in reference 35. For ELISA, microtiter plates (Falcon 3912) were coated overnight at 4°C with either BoNT/A, BoNT/A H_C, or BoNT/A H_N (10 μg/ml) in PBS and then were blocked with 2% MPBS for 1 h at room temperature. Bacterial supernatants containing expressed scFv were added to wells and incubated at room temperature for 1.5 h. Plates were washed six times (3 times with TPBS and 3 times with PBS), and binding of scFv was detected via their C-terminal peptide tags (E epitope tag for library 1 in pCANTAB5E and myc epitope tag [42] for library 2 in pHEN-1) by using either anti-myc tag antibody (9E10; Santa Cruz Biotechnology) or anti-E antibody (Pharmacia Biotech) and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described in references 35 and 44. The number of unique binding scFv was determined by *Bst*N1 fingerprinting and DNA sequencing.

Subcloning, expression, and purification of scFv. To facilitate purification, scFv genes were subcloned into the expression vector pUC119mycHis (46) or pSYN3, resulting in the addition of a hexahistidine tag at the C-terminal end of the scFv. Two-hundred-milliliter cultures of *E. coli* TG1 harboring one of the appropriate phagemids were grown, expression of scFv was induced with IPTG (13), and the cultures were grown at 25°C overnight. scFv was harvested from the periplasm (6), dialyzed overnight at 4°C against IMAC loading buffer (50 mM sodium phosphate [pH 7.5], 500 mM NaCl, 20 mM imidazole), and then filtered through a 0.2-μm-pore-size filter. scFv was purified by IMAC (24) exactly as described in reference 46. To separate monomeric scFv from dimeric and aggregated scFv, samples were concentrated to a volume of <1 ml in a centrifugal concentrator (Centricon 10; Amicon) and fractionated on a Superdex 75 column (Pharmacia) by using HBS. The purity of the final preparation was evaluated by assaying an aliquot by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein bands were detected by Coomassie blue staining. The concentration was determined spectrophotometrically, on the assumption that an A_{280} of 1.0 corresponds to an scFv concentration of 0.7 mg/ml.

Measurement of affinity and binding kinetics. The K_ds of purified scFv were determined by using surface plasmon resonance in a BIAcore (Pharmacia Biosensor AB). In a BIAcore flow cell, approximately 600 RU of BoNT/A H_C (15 μg/ml in 10 mM sodium acetate [pH 4.5]) was coupled to a CM5 sensor chip by using *N*-hydroxysuccinimide–*N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide chemistry (29). This amount of coupled BoNT/A H_C resulted in a maximum RU of 100 to 175 of scFv bound. For regeneration of the surface after binding of scFv, 5 μl of 4 M $MgCl_2$ was injected, resulting in a return to baseline. The surface was reused 20 to 30 times under these regeneration conditions. Association was measured under a continuous flow of 5 μl/min with a concentration range from 50 to 1,000 nM. k_{on} was determined from a plot of ln $(dR/dt)/t$ versus concentration, where R is response and t is time (31). k_{off} was determined from the dissociation part of the sensorgram at the highest concentration of scFv analyzed (31) by using a flow rate of 30 μl/min. K_d was calculated as k_{off}/k_{on}.

Epitope mapping. Epitope mapping was performed by using surface plasmon resonance in a BIAcore. In a BIAcore flow cell, approximately 1,200 RU of BoNT/A H_C was coupled to a CM5 sensor chip as described above. With a flow rate of 5 μl/min, a titration of 100 nM to 1 μM scFv was injected over the flow cell surface for 5 min to determine an scFv concentration which resulted in near saturation of the surface. Epitope mapping was performed with pairs of scFv at concentrations resulting in near saturation and at least 100 RU of scFv bound. The amount of scFv bound was determined for each member of a pair, and then the two scFv were mixed together to give a final concentration equal to the concentration used for measurements of the individual scFv. scFv recognizing different epitopes showed an additive increase in the RU bound when injected together (Fig. 2A), while scFv recognizing identical epitopes showed only a minimal increase in RU (Fig. 2B).

In vitro neutralization studies. In vitro neutralization studies were performed by using a mouse hemidiaphragm preparation, as described by Deshpande et al. (14). Briefly, left and right phrenic nerve hemidiaphragm preparations were excised from male CD/1 mice (25 to 33 g) and suspended in physiological solution (135 mM NaCl, 5 mM KCl, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 11 mM GLU). The incubation bath was bubbled with 95% O_2–5% CO_2 and maintained at a constant temperature of 36°C. Phrenic nerves were stimulated supramaximally at 0.05 Hz with square waves of 0.2 ms duration. Isometric twitch tension was measured with a force displacement transducer (Model FT03; Grass) connected to a chart recorder. Purified scFv were incubated with purified BoNT/A for 30 min at room temperature and then added to the tissue bath, resulting in a final scFv concentration of 2.0×10^{-8} M and a final BoNT/A concentration of 2.0×10^{-11} M. For each scFv studied, time to 50% twitch tension reduction was determined three times for BoNT/A alone and three times for scFv plus BoNT/A. The combination of S25 and C25 was studied at a final concentration of 2.0×10^{-8} M each. Differences between times to 50% twitch reduction were determined by a two-tailed t test, with a P value of <0.05 considered significant.

TABLE 2. Frequency of binding of clones from phage antibody libraries

Antigen used for selection	Frequency of ELISA-positive clones[a] in selection round:		
	1	2	3
Library 1[b]			
BoNT/A: immunotube[c]	20/184	124/184	ND
BoNT/A H_C: immunotube	7/92	86/92	88/92
BoNT/A H_C: biotinylated[d]	7/90	90/90	90/90
	14/48	48/48	ND
Library 2[e]			
BoNT/A: immunotube	ND	81/92	ND
BoNT/A H_C: immunotube	ND	ND	76/92
BoNT/A H_C: Ni^{2+}-NTA[f]	ND	ND	67/92

[a] Expressed as number of positive clones/total number of clones. For selections on BoNT/A and BoNT/A H_C, ELISA was done on immobilized BoNT/A and BoNT/A H_C, respectively. ND, data not determined from selection performed.

[b] Derived from a mouse immunized twice with BoNT/A H_C and once with BoNT/A.

[c] Immunotube selections were performed with the antigen absorbed onto immunotubes.

[d] Biotinylated selections were performed in solution with capture on streptavidin magnetic beads.

[e] Derived from a mouse immunized three times with BoNT/A H_C.

[f] Ni^{2+}-NTA selections were performed in solution with capture on Ni^{2+}-NTA agarose.

RESULTS

Phage antibody library construction and characterization. Two phage antibody libraries were constructed from the V_H and V_κ genes of immunized mice (Fig. 1). For library 1, a mouse was immunized twice with BoNT/A H_C and challenged 2 weeks after the second immunization with 100,000 50% lethal doses of BoNT/A. The mouse survived the BoNT/A challenge and was sacrificed 1 week later. The spleen was removed immediately after sacrifice, and total RNA was prepared. For library construction, IgG heavy-chain and kappa light-chain mRNA were specifically primed and first-strand cDNA was synthesized. V_H and V_κ gene repertoires were amplified by PCR, and V_H, J_H, V_κ, and J_κ primers were provided in the recombinant phage antibody system. The V_H and V_κ gene repertoires were randomly spliced together to create an scFv gene repertoire by using synthetic DNA encoding the 15-amino-acid peptide linker $(G_4S)_3$. Each scFv gene repertoire was separately cloned into the phage display vector pCANTAB5E (Pharmacia). After transformation, a library of 2.1×10^6 members was obtained. Ninety percent of the clones had an insert of the appropriate size for an scFv gene, as determined by PCR screening, and the cloned scFv genes were diverse, as determined by PCR fingerprinting (data not shown). DNA sequencing of 10 unselected clones from library 1 revealed that all V_H genes were derived from the murine V_H2 family and all V_κ genes were derived from the murine $V_\kappa4$ and $V_\kappa6$ families (30). Based on this observed V-gene bias, family-specific V_H and V_κ primers were designed along with J_H and J_κ gene-segment-specific primers (Table 1). These primers were then used to construct a second phage antibody library. For library 2, a mouse was immunized three times with BoNT/A H_C and sacrificed 2 weeks after the third immunization. The mouse was not challenged with BoNT/A prior to spleen harvest, as this led to the production of non-H_C-binding antibodies (see "Selection and initial characterization of phage antibodies" below). The spleen was harvested, and a phage antibody library was constructed as described above, except that V_H-, J_H-, V_κ-, and J_κ-specific primers were used. After transformation, a library of 1.0×10^6 members was obtained. Ninety-five percent of the clones had an insert of the appropriate size for an scFv gene, as determined by PCR screening, and the cloned scFv genes were diverse, as determined by PCR fingerprinting (data not shown). DNA sequencing of 10 unselected clones from library 2 revealed greater diversity than was observed in library 1; V_H genes were derived from the V_H1, V_H2, and V_H3 families, and V_κ genes were derived from the $V_\kappa2$, $V_\kappa3$, $V_\kappa4$, and $V_\kappa6$ families (30).

Selection and initial characterization of phage antibodies. To isolate BoNT/A binding phage antibodies, phage were rescued from the library and selected on either purified BoNT/A or BoNT/A H_C. Selections were performed on the holotoxin in addition to H_C, since it was unclear to what extent the recombinant toxin H_C would mimic the conformation of the H_C in the holotoxin. Selection for BoNT/A and BoNT/A H_C binders was performed on antigen adsorbed to polystyrene. In addition, H_C binding phage were selected in solution on biotinylated H_C, with capture on streptavidin magnetic beads (for library 1) or on hexahistidine tagged H_C, with capture on Ni^{2+}-NTA agarose (for library 2). Selections in solution were utilized based on our previous observation that selection on protein adsorbed to polystyrene could yield phage antibodies which did not recognize native protein (46). Selection in solution was not performed on the holotoxin due to our inability to successfully biotinylate the toxin without destroying immunoreactivity.

After two to three rounds of selection, at least 67% of scFv analyzed bound the antigen used for selection (Table 2). The number of unique scFv was determined by DNA fingerprinting followed by DNA sequencing, and the specificity of each scFv was determined by ELISA on pure BoNT/A and recombinant BoNT/A H_C and H_N. scFv binding BoNT/A but not binding H_C or H_N were presumed to bind the light chain (catalytic domain). A total of 33 unique scFv were isolated from mice immunized with H_C and challenged with BoNT/A (Table 3, library 1). When library 1 was selected on holotoxin, 25 unique scFv were identified. Only 2 of these scFv, however, bound H_C, with the majority (21) binding the light chain and 2 binding H_N. The two H_C binding scFv did not express as well as other scFv recognizing similar epitopes, and they were therefore not characterized with respect to affinity or neutralization capacity (see below). Selection of library 1 on H_C yielded an additional eight unique scFv (Tables 3 and 4). Overall, however, only 50% of scFv selected on H_C also bound holotoxin. This result suggests that a significant portion of the H_C surface may be inaccessible in the holotoxin. Alternatively, scFv could be binding H_C conformations that do not exist in the holotoxin. From mice immunized with H_C only (library 2), all scFv selected on holotoxin also bound H_C. As with library 1, however, only 50% of scFv selected on H_C bound holotoxin. In all, 18 unique H_C binding scFv were isolated from library 2, resulting in a total of 28 unique H_C binding scFv (Tables 3 and 4). scFv of identical

TABLE 3. Specificity of BoNT binding scFv selected from phage antibody libraries

scFv specificity	No. of unique scFv in:	
	Library 1	Library 2
BoNT/A H_C	10	18
BoNT/A H_N	2	0
BoNT/A light chain	21	0
Total	33	18

TABLE 4. Deduced protein sequences of V_H and V_L of BoNT/A H_C binding scFv, classified by epitope recognized

Region	Epitope	Clone	Lib[a]	Sequence[b]						
				Framework 1	CDR 1	Framework 2	CDR 2	Framework 3	CDR 3	Framework 4
V_H	1	C15	1	QVKLQQSGAELVRPGASVKLSCKTSGYSFT	SYWMN	WVKQGPGQGLEWIG	MIHPSNSEIRFNQKFED	MATLTVDKSSSTAYMQLSSPTSEDSAVYYCAR	GIYYDYDGGNYYAMDY	WGQGTTVTASS
		C9	1	----------	-----	----------	----------N	----------	----V----------	--------V--
		1D5	2	E---VE----------N----A------	-----	----R---------	--------T-L----K-	K----------	-------E-Y--TL--	------L-V--
		C1	1	----------A------	-----	----R---------	-------DT--------	K------R-----IH----------	-L-GYGF WYFDV	--------V--
		S25	1	----------A----L-	-----	----R---------	-----D-DT--------	K------T----------	-L-NGF WYF-V	--------V--
		1B6	2	--Q----------V---I---A---T-I	D-A-H	----S-AKS-----	V-SSYYGDTDY--I-KG	K---------N----E-ARL--D---I-----	RGKG ----	--------V--
		1C9	2	--Q-K----------V---I---G---T-I	D-AVH	----SHAKS-----	V-STYYGDADY-PK-KG	K-----N---N----E-PRL------I-----	RGKG ----	-----S--V--
		1E8	2	E-Q--E--PG--K-SQ-LS-T-TVT---I-	D-AW-	-IR-F--KK---M-	Y-S YSGSTGYNPSLKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
		1G7	2	E-Q--E--PG -K-SQ-LS-T-TVT---I-	D-AWY	-IR-F--KK---M-	Y-S YSGSTGYNPSLKS	RISI-R-T-KNQFFL--N-V-T--TGT-----	-YD ----	-----S--V--
	2	1A1	2	EVKLVESGGGLVQPGGSRKLSCATSGFTFS	DYYMS	WIRQSPDKRLEWVA	TISDGGTYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCVR	HGYGNYPSH WYFDV	WGAGTTVTVSS
		1F1	2	----------L-----A------	N-G--	-V--T---------	M--S--S-N--S-----	-V--------S---------Q-------L-T-	--------Y -----	----------
		C39	1	Q-Q-Q-----S-K----L-----A------	-----	-V--T-E-------	------S----------	----------N----------I-----	YR-DEGL -Y	--Q--------
		C25	1	Q-Q-Q-------K----L-----A------	----Y	-V--T-E-------	------S----------	----------N----------S-	YR-DDAM -Y	--Q--------
		2G5	2	----------K----L-----A------	S-A--	-V--T-E-------	----------T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
		3C3	2	----K-------K----L-----A------	S-A--	-V--T-E-------	----------T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
		3F4	2	EG---------K----L-----A------	S-A--	-V--T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
		3H4	2	----------K---PL-----A------	S-A--	-V--T-EH------	-------F---T-N---	----------HN------H----------A-	NLPYDHV -Y	--Q--S-----
	3	1B3	2	EVQLQESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	DWSEGYYYYG MDV	WGQGTTVIVSS
		1C6	2	QI--LQ----------	-----	----------	----------	----------	---------- ---	----------
		2B6	2	VKLVESGP-L-KPSQSLSLTCTVTGYSIT-	D-AWN	-I--F--NK---MG	Y-N-----N-NP -L-N	-ISIT--T---QFF-KL--VTS----T-----	AGDGY-VD WYFDV	--T--------
		1G5	2	Q----Q--AEL----A-VKM--K---Y--T	--WTT	--K-R--Q----IG	D-YPGSGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
		1H6	2	-----Q--AEL-K--A-VKM--K---Y--T	--WTT	--K-R--Q----IG	D-YP-SGSTNYNEKF-S	KA-LTV-T-SS-A-M-LS--TS--S-------	ELGD A--Y	-----S-----
	4	1F3	2	EVQLQQSGAELVKPGASVKLSCKASGYTFT	SFWMH	WVKQRPGRGLEWIG	RLDPNSGETKYNEKFKS	KATLTVDKPSSTAYMELSSLTSEDSAVYYCAR	EAYGYWN FDV	WGTGTTVTVSS
		2E8	2	----------	-----	----------	----------K----	----------	------- ---	----------
V_L	1	C15	1	DIELTQSPAIMSASPGEKVIMTC	SASS SVSHMY	WYQQKPGSSPRLLIY	DTSNLAS	GVPIRFSGSGSGTSYSLTISRMEAEDSATYYC	QQWSSYPFT	FGSGTKLELKR
		C9	1	--D---------S-------I--	---- ---Y-H	-F-----T--KPW--	S------	---A----------SV----A-----	--Y-G--L-	--A-----I--
		1D5	2	----------A--------I--	----S I-S-NLH	-----SETSPKPW--	G------	---V----------S-----A-----	---G---L-	--G-----I--
		C1	1	----------	---- ---Y--	----------	-------	---V----------A-----	-------L-	--A--------
		S25	1	---------L-A--------I--	-V--S I-S-NLH	-----S-T--KPW--	G------	---V----------S-----A-----	-------L-	--A-----I--
		1B6	2	---------SLAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K----	RA---E-	-I-A-------R-DFT--INPV--D-V-----	--SNED-P-	--A--------
		1C9	2	---------SLAV-L-QRA-IS-	RA-ESVDSYGN-F-H	-------QP-K----	RA---E-	-I-A-------R-DFT---NPV--D-V-----	--SNED-Y-	--G-----I--
		1E8	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
		1G7	2	----------	---- ---Y-H	-----S-T--KRW--	---K---	---A----------S-----A-----	-----N-L-	--A--------
	2	1A1	2	DIELTQSPASLAVSLGQRATISC	RASESVDSYGNSFMH	WYQQKPGQPPKLLIY	LASNLES	GVPARFSGSGSRTDFTLTIDPVEADDAATYYC	QQNNEDPYT	FGGGTKLEIKR
		1F1	2	--------T----------	----------	----------	-------	----------	---------	----------
		C39	1	----------R------	----------H----	----------	-------	----------	---------	----------
		C25	1	----------	----------H---Q	----------	R-----P	-I---------G-------N------V-----	--S----F-	--S--------
		2G5	2	---------IMSA-P-EKVTTT-	S--S SV-Y--	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-------	--RSSY---	----DQAGN-S
		3C3	2	---------IMSA-P-EKVTTT-	----------H---Q	-F-----TS-K-W--	ST---A-	----------G-SYS---SRM--E-------	--RSSY---	----DQAGN--
		3F4	2	-T-------IMSA-P-EKVTMT-	S--S SV-Y-Y	-------SS-R----	DT---A-	---V-------G-SYS---SRM--E-------	--WSSY-P-	----------
		3H4	2	---------IMSA-P-EKVTMT-	---S- VSS-YL-	-------SS-R----	DT---A-	---V-------G-SYS---SRM--E-------	--WSSY-P-	----------
	3	1B3	2	DSELTQSPTTMAASPGEKITTTC	SASSS ISSNYLH	WYQQRPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPRT	FGGGTKLEIKR
		1C6	2	-I------ASL-V-L-RRA--S-	R--E-VEYYGTSLMQ	----K--QP------	AA--VE-	------------DF--N-HPV-E -I-M-F-	--SRKV-W-	----------
		2B6	2	YI------ASL-V-L-QRA--S-	R--E-VDSYGNSFM-	----K--QP------	LA---E-	----------R-DFT---DPV--D-A-----	--NNED-Y-	----------S
		1G5	2	-I------ASL-V-L-QRA--S-	R--E-VEYYGTSLMQ	----K--QP------	AA--VE-	-A----------DF--N-HPV-ED-I-M-F-	--SRKV-Y-	----------
		1H6	2	-I------AI-S------V----	-V--- ---SN--	----KS-T----W--	G------	---V----------SS-----A-----	--W--Y-L-	--A---V-LR-
	4	1F3	2	DIELTQSPASMSASPGEKVTMTC	RATSS VSSSYLH	WYQQKSGASPKLWIY	SASNLAS	GVPSRFSGSGSGTSYSLTISSVEAEDAATYYC	QQYIGYPYT	FGGGTKLEIKR
		2E8	2	--------TT-A------I-I--	S-S-- IG-N---	-----P-F----L--	RT-----	---A----------GAM----V-----	--GSSI---	----------

[a] Lib, library.
[b] Full-length sequences were not determined for clones C12, C13, C2, and S44 (all bind epitope 1). Accession can be made through GenBank with nos. AF003702 to AF003725.

or related sequences were isolated on both H_C immobilized on polystyrene and H_C in solution. Thus, in the case of H_C, the method of selection was not important.

Epitope mapping. All 28 unique H_C binding scFv were epitope mapped by using surface plasmon resonance in a BIAcore. Epitope mapping was performed with pairs of scFv at concentrations resulting in near saturation of the chip surface and at least 100 RU of scFv bound. The amount of scFv bound was determined for each member of a pair, and then the two scFv were mixed together to give a final concentration equal to the concentration used for measurements of the individual scFv. scFv recognizing different epitopes showed an additive increase in the RU bound when injected together (Fig. 2A), while scFv recognizing identical epitopes showed only a minimal increase in RU (Fig. 2B). By this technique, mapping of the 28 scFv yielded 4 nonoverlapping epitopes recognized on H_C (Table 4). scFv recognizing only epitopes 1 and 2 were obtained from library 1, whereas scFv recognizing all 4 epitopes were obtained from library 2. Many of the scFv recognizing the same epitope (C1 and S25; C9 and C15; 1E8 and 1G7; 1B6 and 1C9; C25 and C39; 2G5, 3C3, 3F4, and 3H4; 1A1 and 1F1; 1B3 and 1C6; 1G5 and 1H6; 1F3 and 2E8) had V_H domains derived from the same V-D-J rearrangement, as evidenced by the high level of homology of the V_H CDR3 and V_H-gene segment (Table 4). These scFv differ only by substitutions introduced by somatic hypermutation or PCR error. For epitopes 1 and 2, most or all of the scFv recognizing the same epitope are derived from the same or very similar V_H-gene segments but differ significantly with respect to V_H CDR3 length and sequence (5 of 9 scFv for epitope 1; 8 of 8 scFv for epitope 2) (Table 4). These include scFv derived from different mice. Given the great degree of diversity in V_H CDR2 sequences in the primary repertoire (49), specific V_H-gene segments may have evolved for their ability to form binding sites capable of recognizing specific pathogenic antigenic shapes. In contrast, greater structural variation appears to occur in the rearranged V_κ genes. For example, three different germ line genes and CDR1 main-chain conformations (9) are observed for epitope 2, where all the V_H genes are derived from the same germ line gene. Such "promiscuity" in chain pairings has been reported previously (10).

Affinity, binding kinetics, and in vitro toxin neutralization. Affinity, binding kinetics, and in vitro toxin neutralization were determined for one representative scFv binding to each epitope. For each epitope, the scFv chosen for further study had the best combination of high expression level and slow k_{off}, as determined during epitope mapping studies. K_d for the four scFv studied ranged between 7.3×10^{-8} and 1.1×10^{-9} M (Table 5), values comparable to those reported for monoclonal IgG produced from hybridomas (18). C25 has the highest affinity ($K_d = 1.1 \times 10^{-9}$ M) reported for an anti-botulinum toxin antibody. k_{on} differed over 84-fold, and k_{off} differed over 33-fold, between scFv (Table 5). In vitro toxin neutralization was determined by using a mouse hemidiaphragm preparation and measuring the time to 50% twitch tension reduction for BoNT/A alone and in the presence of 2.0×10^{-8} M scFv. Values are reported in time to 50% twitch reduction. scFv binding to epitope 1 (S25) and epitope 2 (C25) significantly prolonged the time to neuroparalysis: 1.5-fold (152%) and 2.7-fold (270%), respectively (Table 5 and Fig. 3). In contrast, scFv binding to epitopes 3 and 4 had no significant effect on the time to neuroparalysis. A mixture of S25 and C25 had a significant additive effect on the time to neuroparalysis, with the time to 50% twitch reduction increasing 3.9-fold (390%).

TABLE 5. Affinities, binding kinetics, and in vitro toxin neutralization results of scFv selected from phage antibody libraries

scFv clone	Epitope	K_d[a] (M)	k_{on} (10^4 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	Paralysis time (min)[b]
S25	1	7.3×10^{-8}	1.1	0.82	85 ± 10[c]
C25	2	1.1×10^{-9}	30	0.33	151 ± 12[c]
C39	2	2.3×10^{-9}	14	0.32	139 ± 8.9[c]
1C6	3	2.0×10^{-8}	13	2.5	63 ± 3.3
1F3	4	1.2×10^{-8}	92	11	52 ± 1.4
C25 + S25					218 ± 22[c,d]
BoNT/A pure toxin (control)					56 ± 3.8

[a] k_{on} and k_{off} were measured by surface plasmon resonance, and K_d was calculated as k_{off}/k_{on}.

[b] Time to 50% twitch reduction in mouse hemidiaphragm assay using 20 nM scFv plus 20 pM BoNT/A compared to time for BoNT/A alone. For the C25 plus S25 combination, 20 nM each scFv was used. Each value is the mean ± standard error of the mean of at least three observations.

[c] $P < 0.01$ compared to BoNT/A.

[d] $P < 0.05$ compared to C25.

DISCUSSION

BoNTs consist of a heavy and a light chain linked by a single disulfide bond. The carboxy-terminal half of the toxin binds to a specific membrane receptor(s), resulting in internalization, while the amino-terminal half mediates translocation of the toxin from the endosome into the cytosol. The light chain is a zinc endopeptidase which cleaves an essential synaptosomal protein, leading to failure of synaptic transmission and paralysis. Effective immunotherapy must prevent binding of the toxin to the receptor, since the other two toxin functions occur intracellularly. Identification of epitopes on H_C which mediate binding is an essential first step, both to the design of better vaccines and to development of a high-titer neutralizing monoclonal antibody (or antibodies) for passive immunotherapy.

For this work, we attempted to direct the immune response to a neutralizing epitope(s) by immunization with recombinant BoNT/A H_C. This should lead to the production of antibodies which prevent binding of toxin to its cellular receptor(s). One limitation of this approach is the extent to which recombinant H_C mimics the conformation of H_C in the holotoxin. The fact that 50% of antibodies selected on H_C recognize holotoxin suggests significant structural homology for a large portion of the molecule. Although 50% of antibodies selected on H_C do not bind holotoxin, this could result from packing of a significant portion of the H_C surface against other toxin domains. Our results do not, however, exclude the possibility that some of these antibodies are binding H_C conformations that do not exist in the holotoxin or that conformational epitopes present in the holotoxin are absent from recombinant H_C. This could lead to failure to generate antibodies to certain conformational epitopes. Regardless, immunizing and selecting with H_C resulted in the isolation of a large panel of monoclonal antibodies which bind holotoxin. In contrast, monoclonal antibodies isolated after immunization with holotoxin or toxoid bind to other toxin domains (H_N or light chain) or to nontoxin proteins present in crude toxin preparations and toxoid (results from library 1, this work and reference 17).

To produce and characterize the greatest number of monoclonal antibodies possible, we used phage display. This approach makes it possible to create and screen millions of different antibodies for binding. The resulting antibody fragments are already cloned and can easily be sequenced to identify the number of unique antibodies. Expression levels in *E. coli* are

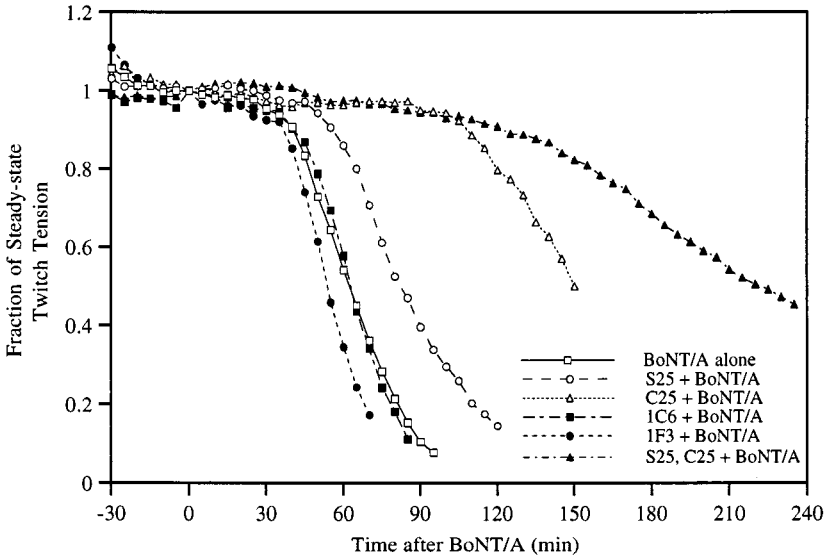

FIG. 3. Evaluation of scFv neutralization of BoNT/A in a mouse hemidiaphragm model. The twitch tension developed after electrical stimulation of a mouse hemidiaphragm was measured before (−30 to 0 min) and after the addition of 20 pM BoNT/A (control), 20 pM BoNT/A plus 20 nM scFv S25, C25, 1C6, or 1F3 (representing epitopes 1 to 4, respectively), or a combination of S25 and C25 at a final concentration of 20 nM each. Results are expressed as the fraction of steady-state twitch tension (at 0 min) versus time. scFv 1C6 and 1F3 do not alter the time to 50% twitch reduction, whereas scFv C25 and S25 significantly prolong it. The combination of S25 and C25 significantly prolonged the time to neuroparalysis compared to C25 or S25 alone.

typically adequate to produce milligram quantities of scFv, which can easily be purified by IMAC after subcloning into a vector which attaches a hexahistidine tag to the C terminus. Ultimately, the V_H and V_L genes can be subcloned to construct complete IgG molecules, grafted to construct humanized antibodies, or mutated to create ultrahigh-affinity antibodies. By this approach, 28 unique monoclonal anti-BoNT/A H_C antibodies were produced and characterized. The antibody sequences were diverse, consisting of 3 different V_H-gene families, at least 13 unique V-D-J rearrangements, and 3 V_κ-gene families. Generation of this large panel of BoNT/A H_C antibodies was a result of the choice of antigen used for immunization and selection (BoNT/A H_C). For example, a Fab phage antibody library constructed from the V genes of mice immunized with pentavalent toxoid yielded only two Fab which bound pure toxin (in this case, BoNT/B). The majority of the Fab bound nontoxin proteins present in the toxoid (17).

Despite the sequence diversity of the antibodies, epitope mapping revealed only four nonoverlapping epitopes. Epitopes 1 and 2 were immunodominant, being recognized by 21 of 28 (75%) of the antibodies. Interestingly, approximately the same number (three to five) of immunodominant BoNT/A H_C peptide (nonconformational) epitopes are recognized by mouse and human polyclonal antibodies after immunization with pentavalent toxoid and by horse polyclonal antibodies after immunization with formaldehyde-inactivated BoNT/A (3).

scFv binding epitopes 1 and 2 resulted in partial antagonism of toxin-induced neuroparalysis at the mouse neuromuscular junction. When administered together, the two scFv had an additive effect, with the time to neuroparalysis increasing significantly. These results are consistent with the presence of two unique receptor binding sites on BoNT/A H_C. While the BoNT/A receptor(s) has not been formally identified, the results are consistent with those of ligand binding studies, which also indicate two classes of receptor binding sites on toxin, high and low affinity, and have led to a "dual receptor" model for toxin binding (40). Whether both of these sites are on H_C, however, is controversial. In two studies, BoNT/A H_C partially inhibited binding and neuromuscular paralysis (4, 5), whereas Daniels-Holgate and Dolly (12) showed that BoNT/A H_C inhibited binding at motor nerve terminals but had no antagonistic effect on toxin-induced neuroparalysis at the mouse neuromuscular junction. Our results are consistent with the presence of two "productive" receptor binding sites on H_C which result in toxin internalization and toxicity. Differences in scFv potency may reflect differences in affinity of H_C for receptor binding sites or may reflect the greater than 10-fold difference in affinity of scFv for H_C. Finally, we have not formally shown that any of the scFv actually block binding of toxin to the cell surface. It is conceivable that the observed effect on time to neuroparalysis results from interference with a postbinding event.

scFv antagonism of toxin-induced neuroparalysis in the mouse hemidiaphragm assay was less than that (7.5-fold prolongation of time to neuroparalysis) observed for 2.0×10^{-9} M polyclonal equine antitoxin (PerImmune Inc.) (47a). This difference could be due to the necessity of blocking additional binding sites, differences in antibody affinity or avidity, or a cross-linking effect leading to aggregated toxin which cannot bind. Affinity of antibody binding is also likely to be an important factor, since the toxin binds with high affinity to its receptor (51) and can be concentrated inside the cell by internalization. Of note, the most potent scFv has the highest affinity for H_C. Availability of other scFv described here, which recognize the same neutralizing epitope but with different K_ds, should help define the importance of affinity. These scFv, however, differ by many amino acids and may also differ in fine specificity, making interpretation of results difficult. Alternatively, mutagenesis combined with phage display can lead to the production of scFv which differ by only a few amino acids in sequence but vary by several orders of magnitude in affinity (47). The same approach can be used to increase antibody affinity into the picomolar range (47).

The "gold standard" for neutralization is protection of mice against the lethal effects of toxin coinjected with antibody. While the relationship between in vitro and in vivo protection

has not been formally established, equine antitoxin potentially neutralizes toxin in both types of assays (see above and reference 21). Presumably, this relationship holds for the scFv reported here, but this will need to be verified experimentally. Such studies are not possible with small (25-kDa) scFv antibody fragments. The small size of scFv leads to rapid redistribution (the half-life at α phase is 2.4 to 12 min) and clearance (the half-life at β phase is 1.5 to 4 h) and antibody levels which rapidly become undetectable (26, 46), while toxin levels presumably remain high (23). Performance of in vivo studies will require the construction of complete IgG molecules from the V_H and V_L genes of scFv. Use of human constant regions will yield chimeric antibodies less immunogenic than murine monoclonals and much less immunogenic than currently used equine antitoxin. Immunogenicity could be further reduced by CDR grafting to yield humanized antibodies.

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