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Synergistic capture of *Clostridium botulinum* Type A neurotoxin by scFv antibodies to novel epitopes

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Abstract

A non-immune library of human single chain fragment variable (scFv) antibodies displayed on *Saccharomyces cerevisiae* was screened for binding to the *Clostridium botulinum* neurotoxin serotype A binding domain [BoNT/A (H_C)] with the goal of identifying scFv to novel epitopes. To do this, an antibody-mediated labeling strategy was used in which antigen-binding yeast clones were selected after labeling with previously characterized monoclonal antibodies (MAbs) specific to the H_C. Twenty unique scFv clones were isolated that bound H_C. Of these, three also bound to full-length BoNT/A toxin complex with affinities ranging from 5 nM to 48 nM. Epitope binning showed that the three unique clones recognized at least two epitopes distinct from one another as well as from the detection MAbs. After production in *E. coli*, scFv were coupled to magnetic particles and tested for their ability to capture BoNT/A holotoxin using an Endopep-MS assay. In this assay, toxin captured by scFv coated magnetic particles was detected by incubation of the complex with a peptide containing a BoNT/A-specific cleavage sequence. Mass spectrometry was used to detect the ratio of intact peptide to cleavage products as evidence for toxin capture. When tested individually, each of the scFv showed a weak positive Endopep-MS result. However, when the particles were coated with all three scFv simultaneously, they exhibited significantly higher Endopep-MS activity, consistent with synergistic binding. These results demonstrate novel approaches toward the isolation and characterization of scFv antibodies specific to unlabeled antigens. They also provide evidence that distinct scFv antibodies can work synergistically to increase the efficiency of antigen capture onto a solid support.

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Keywords

Affinity reagents; molecular probes; scFv; antibodies; BoNT/A; yeast display

Introduction

The goal of this study was to isolate scFv antibodies from a non-immune yeast-displayed scFv library that bind to the *Clostridium botulinum* serotype A neurotoxin (BoNT/A). Specifically, we sought to 1) isolate pairs of BoNT/A (H_c)-specific antibodies from a non-immune library that can be used for holotoxin detection in an antibody sandwich assay; 2) validate an antibody-mediated antigen-labeling method as a means to screen yeast libraries with unmodified antigens; 3) test the hypothesis that distinct scFv antibodies can work synergistically to capture an antigen from solution; and 4) identify new antibodies to potentially novel epitopes.

Most existing BoNT/A antibodies have been isolated directly or indirectly from animals that were immunized with the fragments of the neurotoxin. As the immune response progresses *in vivo*, antibodies become more focused predominately on immunodominant epitopes. Our selections were designed to isolate single chain fragment variable (scFv) antibodies that bind to novel epitopes on the neurotoxin. The selections were performed with a human nonimmune library and most of the people whose nucleic acid was used to construct the library were unlikely to have been immunized with BoNT/A. Additionally, because selections were performed completely *in vitro*, we hypothesized a reduced likelihood of immunodominance and a greater probability of finding antibodies to novel epitopes (Cobey and Pascual, 2011).

The BoNT/A toxin consists of a 50 kDa light chain (LC) linked via disulfide bonds to a 100 kDa heavy chain (HC) (Lacy, et al., 1998). The HC is comprised of a ~50 kDa translocation domain (H_n) and a ~50 kDa receptor binding domain (H_c). Within the LC is the zinc endopeptidase which catalyzes the cleavage of specific peptide sequences within neuronal proteins (Lacy, et al., 1998, Singh, 2000). In the laboratory, the receptor binding domain of the toxin can be expressed alone. It is referred to as BoNT/A (H_c), and it is used as a nontoxic surrogate for the holotoxin (Byrne, et al., 1998). H_c has been shown to contain many of the immunogenic epitopes found on the holotoxin, as immunization of mice with H_c were significantly protected from subsequent challenge with holotoxin (Byrne, et al., 1998).

Typically, detection or neutralization of botulinum neurotoxin is mediated by antibodies directed against the H_c. These antibodies may inhibit binding of the toxin to its cellular receptor (Lacy, et al., 1998). Several studies have isolated scFv antibodies that bound to non-overlapping epitopes of BoNT/A (H_c) from both nonimmune and immune libraries of phage-displayed human or murine scFv (Amersdorfer and Marks, 2000, Amersdorfer, et al., 2002, Mullaney, et al., 2001). ScFv antibodies were isolated from mice immunized with BoNT/A (H_c) or humans immunized with pentavalent toxoid (Levy, et al., 2007, Razai, et al., 2005, Amersdorfer and Marks, 2000, Amersdorfer, et al., 2002). However, to date no

one has isolated functional scFv that bind BoNT toxins from a non-immune human yeast display library.

The present study isolated scFv from a non-immune yeast-display library of human-derived scFv antibodies. Selections conducted on yeast-displayed scFv libraries are typically performed using biotinylated antigens. Unfortunately, many antigens prove difficult to label efficiently or degrade during the labeling process. Additionally, labeling can alter or mask epitopes on an antigen. To obviate direct labeling of the H_c while selecting scFv that bind novel epitopes, we developed an antibody-mediated labeling strategy for screening the library. This strategy used three monoclonal IgG detection antibodies each of which bind to different epitopes on the BoNT/A (H_c) (Amersdorfer and Marks, 2000, Amersdorfer, et al., 2002, Mullaney, et al., 2001). We isolated a panel of scFv that bound H_c and then tested them for binding to native holotoxin. The toxin-binding scFv were then subjected to epitope binning (Siegel, et al., 2004, Gray, et al., 2010) to determine whether the scFv bind distinct epitopes on the H_c.

To demonstrate the functional activity of these scFv, we used a previously described assay for detection of BoNT termed the Endopep-MS (Kalb, et al., 2010, Kalb, et al., 2009, Barr, et al., 2005, Boyer, et al., 2005, Kalb, et al., 2005). In simple matrices, this method was reported to detect all seven BoNT toxin subtypes. Endopep-MS involves incubation of BoNT with a peptide substrate that mimics the natural target of the toxin, either synaptosome-associated protein (SNAP-25), vesicle-associated membrane protein 2 (VAMP-2), or synaptobrevin 2 (Ekong, et al., 1997, Hallis, et al., 1996, Schiavo, et al., 2000, Schmidt and Stafford, 2003, Schmidt, et al., 2001). BoNT cleaves the peptide substrate in a specific location and amino acid sequence which varies by toxin-type (Barr, et al., 2005, Boyer, et al., 2005, Kalb, et al., 2005, Schiavo, et al., 2000). Peptide cleavage products are detected by mass spectrometry and sizes of the signal peaks for each of the cleaved peptide fragments is proportional to the amount of BoNT toxin captured on the particles. If the peptide substrate remains intact or is cleaved in an aberrant fashion, then it can be inferred that BoNT toxin is not present. Previous reports (Barr, et al., 2005, Boyer, et al., 2005, Kalb, et al., 2005) have shown that this method can detect BoNT at levels comparable to or lower than levels detected with mouse bioassays (Kautter and Solomon, 1977). The Endopep-MS method alone was not sufficient to detect low levels (100 mouse LD₅₀) of BoNT in dilute samples. However, the use of magnetic particles coated with anti-BoNT/A scFv, as described herein, allows for immuno-concentration of the toxin prior to the Endopep-MS assay.

Here we report the screening and selection of scFv from a non-immune yeast display library using an antibody-mediated selection methodology and unlabeled BoNT/A (H_c). The scFv were characterized with respect to affinity, holotoxin binding, and binding to novel epitopes. Finally, after producing soluble forms of the scFv, the Endopep-MS method was used to detect synergistic antigen binding by multiple scFv.

Materials and Methods

Reagents

Botulinum neurotoxin is very toxic and must be handled using care and appropriate safety measures. All neurotoxins were handled in a level 2 biosafety cabinet equipped with HEPA filters. The BoNT/A holotoxin complex was obtained from Metabio (Madison, WI) at 1 mg/mL total protein in 0.02 M sodium phosphate buffer, pH 7.0.

BoNT/A (H_c) was produced and provided by Dr. Leonard A. Smith (Division of Toxinology, United States Army Research Institute for Infectious Diseases, Frederick, MD).

All secondary reagents for immuno-cytometry were acquired through Molecular Probes (Invitrogen, Carlsbad, CA). Nickel-NTA sepharose for purification of secreted scFv was obtained from Qiagen.

The polyclonal rabbit anti-BoNT/A specific IgGs were provided in 150 mM potassium phosphate (pH 7.4) at a concentration of 4.61 mg/mL. Talon and Dynabeads[®] Protein G were purchased from Dynal (Lake Success, NY) at 1.3 g/cm³ in phosphate buffered saline (PBS) (pH 7.4) containing 0.1% Tween[®]-20 and 0.02% sodium azide. The immobilization of IgG to the Protein G beads was performed as described in the manufacturer's protocol.

BoNT/A-specific MAbs 3D12 (Amersdorfer, et al., 2002), B4 (Levy, et al., 2007), and AR1 (Razai, et al., 2005) were described previously. Each antibody was biotinylated using the Pierce EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific, Rockford, IL) and degree of biotinylation was quantified by using the Pierce Biotin Quantitation (HABA) Assay (Thermo Scientific, Rockford, IL).

Antibodies 3D12, B4, and AR1 were additionally labeled with the fluorescent dye Alexa-633 (A633) using the Alexa Fluor[®] 633 carboxylic acid, succinimidyl ester, labeling kit (Invitrogen) per manufacturer's instructions.

Except where indicated, all chemicals were from Sigma-Aldrich (St. Louis, MO). Peptides were synthesized by Los Alamos National Laboratory (Los Alamos, NM,) and are identical to those reported previously (Kalb, et al., 2010, Kalb, et al., 2009, Barr, et al., 2005, Boyer, et al., 2005, Kalb, et al., 2005). Specifically, the peptide substrate has the sequence biotin-KGSNRTRIDEANQRATRMLGK-biotin.

ScFv selections from the yeast-displayed human non-immune library

Selections, including growth and induction, were performed as described previously (Feldhaus and Siegel, 2004, Feldhaus, et al., 2003, Siegel, et al., 2004, Chao, et al., 2006). For round 1 (R1) and round 2 (R2) of selection, 10^{10} yeast were incubated in 10 mL wash buffer (PBS 0.5% BSA) containing 100 nM BoNT/A (H_c). H_c -bound yeast were then labeled by incubation with a cocktail of the detection MAbs 3D12-biotin and B4-biotin (1 μ g each anti-BoNT/A IgG). Following the second magnetic sort, the eluted yeast [R2 output] were split in half. Half was expanded in 500 mL synthetic dextrose casamino acid (SDCAA) media, while the remainder was incubated with 1 μ g mL⁻¹ streptavidin-phycoerythrin (SA-

PE). Yeast were then sorted by FACS for the top 10% [round 3a (R3a) output] and top 0.1% [round 3b (R3b) output] of PE positive cells and expanded into 5 mL of SDCAA media. For round 4 (R4) of the selection, the R3a output again was incubated with 100 nM H_c and then half was incubated with the detection antibody 3D12-A633 and the second half incubated with B4-A633. In both cases, a negative control containing no H_c, but containing 1 µg mL⁻¹ of secondary antibody, was performed. In the case of B4-A633, little background was observed in the no antigen control. We sorted the top 1% of PE positive (R4b-B4 output) yeast following incubation with H_c and grew them on SDCAA–HUT plates. In the case of 3D12-A633, the no antigen control revealed a high degree of background binding, and was unable to be immediately sorted.

The second half of the R2 output, which had been expanded as described above, was grown to 10⁹ yeast and induced for scFv expression. The yeast were incubated with 100 nM H_c and followed by detection with a third detection MAb, AR1-biotin, followed by SA-PE. AR1-biotin was chosen as it binds to a third unique epitope on H_c distinct from MAbs 3D12 and B4 (Razai, et al., 2005). The top 1% of PE positive yeast (R3c output) were sorted and grown on SDCAA –HUT plates. Isolated yeast colonies from both the R3a-B4 and R3c-AR1 outputs, each clonal for a single scFv antibody, were then screened further as described below.

Negative sorting of R3a output with MAb 3D12-A633

As described above, the R3a output showed a high degree of background binding to MAb 3D12-A633 in the absence of BoNT/A (H_c). We applied a technique, which we termed negative sorting, in which the output was incubated with 1 µg MAb 3D12-A633 and the nonspecific binding was allowed to occur. Despite the presence of A633 positive yeast, a sort gate was created encompassing the *c-myc*-FITC positive and A633 negative yeast. From this gate, 1.8×10^6 yeast were sorted and, without any expansion, were split into two aliquots. Half of the yeast was again incubated with 100 nM H_c, and then both aliquots were incubated with 0.25 µg mL⁻¹ MAb 3D12-A633.

ScFv clone analysis

Yeast clones derived from the R4 outputs were screened for binding to 100 nM H_c using MAbs B4-A633 and AR1-A633 separately for detection. All clones were tested for specificity using at least three A633-labeled and three biotinylated proteins (data not shown). Clones shown to bind any irrelevant protein or secondary detection MAbs were discarded. All H_c-binding clones were subjected to further scrutiny by amplifying the scFv gene from the plasmids and performing *Bst*N1 fingerprint analysis to identify unique clones. Unique clones were then sequenced to determine variable heavy (V_H) and variable light (V_L/V_k) gene family usage.

Twenty clones, chosen for their high degree of H_c binding, were further tested by flow cytometry for binding to 25 nM of native BoNT/A holotoxin using MAbs B4-A633 and AR1-A633 for detection. Five scFv bound to the holotoxin, of which three were subsequently produced in *E. coli* as described previously (Miller, et al., 2005).

Affinity of scFv by flow cytometry and Biacore

A flow cytometry assay to determine the affinity of scFv displayed on the surface of yeast has been described previously (Van Antwerp and Wittrup, 2000, Siegel, et al., 2004, Feldhaus and Siegel, 2004, Chao, et al., 2006). In this assay, yeast-displaying scFv were incubated with twofold serial dilutions of BoNT/A (H_c) spanning 3.125–250 nM in concentration and binding was detected with AR1-biotin. Samples were analyzed by flow cytometry, results graphed as a function of [H_c] versus mean PE fluorescence, and affinity determined by a nonlinear least squares fit of the curves as previously described (Feldhaus, et al., 2003, Van Antwerp and Wittrup, 2000, Kemmer and Keller, 2010).

Biacore assays were performed with the purified scFv using a Biacore 3000 instrument, and data were fit using Scrubber-2 [Developed at CBIA, University of Utah (www.cores.utah.edu/interaction)]. Approximately 12,000 response units (RU) of mouse anti-*c-myc* MAb clone 9e10 (Santa Cruz Biochemicals) was covalently linked to a Biacore CM5 chip using EDC/NHS amine coupling chemistry. Approximately 100 RU of scFv were captured onto the chip for each binding cycle. BoNT/A (H_c) spanning 0.6–75 nM in concentration was injected in triplicate and in random order over the captured scFv and reference (anti-*c-myc* only) flow cells at a flow rate of 100 $\mu\text{L min}^{-1}$. Buffer injections (identical to the H_c buffer) were performed every fourth injection for the purpose of double referencing. Between cycles, the chip surface was regenerated down to the anti-*c-myc* MAb by injecting 0.2 M glycine pH 1.5 buffer for 6 seconds at a flow rate of 100 $\mu\text{L min}^{-1}$. To determine the kinetic parameters of the interactions (the association and dissociation rate constants), each data set was double-referenced and globally fit to a simple 1:1 binding isotherm.

Epitope binning assays

Yeast-displaying the three holotoxin-binding scFv were incubated with a 0.01 $\mu\text{g mL}^{-1}$ of mouse anti-*c-myc* MAb for one hour followed by a 0.005 $\mu\text{g mL}^{-1}$ goat-anti-mouse-FITC to detect scFv expression. Yeast were then washed three times with 500 μL PBS, and then incubated with 100 nM unlabeled H_c for one hour. Unbound antigen was removed by three washes with PBS, and the yeast were resuspended and split evenly into three tubes. Bound H_c was detected by separately incubating the yeast with 0.01 μg of the three biotinylated detection MAbs (AR1, B4, and 3D12) separately for 30 minutes on ice. After three washes to remove unbound MAbs, the bound H_c -MAb complexes were detected by adding SA-PE at a 1:800 dilution. Yeast were analyzed for their FITC and PE fluorescence by flow cytometry. A positive PE signal suggested that the yeast-bound scFv was capturing the H_c by binding to an epitope that is non-overlapping and non-competing with the biotinylated detection MAbs.

Endopep-MS assay

The entire Endopep-MS experiment was performed in triplicate with each scFv or combination of scFv. The BoNT/A-specific peptide substrate was resuspended in dH_2O at a concentration of 1 nmol/ μL . For the Endopep-MS assay, 50 μg of the IgG was coupled to 100 μL of Dynabeads[®] Protein G beads. For the coupling of scFv to the magnetic beads, 50 μg of scFv 14–2 and scFv 19–3, or 20 μg of scFv 4–2 were added to 100 μL or 40 μL of

Talon beads, respectively. A fourth coupling was performed by adding a cocktail of 17 μg of each of the 3 scFv to 100 μL Talon beads. The Talon beads were washed 2X with 500 μL PBS prior to use. Beads were washed 3X with PBS and then reconstituted in 100 μL of PBS before use in the Endopep-MS reaction.

A total of seven reactions were performed as described below: The positive control was 50U of BoNT/A toxin (2 μL of 25U μL^{-1}) spiked into a solution of 17 μL reaction buffer [0.05 M HEPES (pH 7.3), 25 mM dithiothreitol (DTT), 20 mM ZnCl_2 , and 1 mg mL^{-1} bovine serum albumin (BSA)] with 1 μL peptide substrate for a total volume of 20 μL . A second positive control consisted of Protein G-purified rabbit anti-BoNT/A IgG antibodies. The negative control was 19 μL reaction buffer and 1 μL peptide substrate. For testing the scFv and IgG antibodies, a solution of BoNT/A in PBS was made up at a concentration of 0.5 U μL^{-1} . One hundred μL (50 U) of the BoNT/A solution was added to 20 μL of each of the three scFv separately, to 20 μL of the three scFv cocktail, and to 20 μL of the IgG coupled to the Dynabeads[®] Protein G beads. The samples were incubated in a water bath at 37°C for 2 hours with no agitation. The beads were washed 3X with 500 μL PBST [10 mM sodium phosphate (pH 7.4), 0.9% NaCl, and 0.05% Tween-20] followed by two 500 μL washes with dH_2O . Following incubation with the BoNT/A toxin, the bead pellets were reconstituted in 19 μL of reaction buffer and 1 μL of peptide substrate was added. All samples, including the positive and negative controls, were incubated for 4 hours in a water bath at 37°C without agitation.

MALDI-MS analysis of Endopep reactions

Following incubation of the reactions, 2 μL of each sample was added to 18 μL of alpha-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg mL^{-1} in 50% acetonitrile, 0.1% TFA, and 1 mM ammonium citrate (CHCA matrix). This mixture was then applied in duplicate at 0.5 μL per sample well to a 192-spot stainless steel matrix-assisted laser desorption/ionization (MALDI) plate (Applied Biosystems, Framingham, MA).

Mass spectra of each sample well were obtained by scanning from 650 to 4500 m/z in MS positive-ion reflector mode on the Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA). The instrument uses a nitrogen laser at 337 nm, and each spectrum is an average of 2400 laser shots.

Results

Selection of BoNT/A (H_c)-binding clones from the yeast-displayed human non-immune scFv library

Selection proceeded by using a novel antibody-mediate strategy, as follows. During early rounds of selection, the library (1×10^{10} yeast) was incubated with excess unlabeled H_c antigen (100 nM) for 45 minutes, which is enough time for binding to proceed to saturation. Yeast cells that bound H_c were detected by incubation with MAbs B4-biotin and 3D12-biotin simultaneously. In R1 we isolated 1.5×10^7 H_c -binding yeast by incubation with streptavidin-magnetic particles followed by magnetic cell sorting (MACS). The R1 output was then expanded to 1×10^{10} yeast, and for R2 the incubations were performed identically

to R1 except that the R1 output was incubated with anti-biotin magnetic particles rather than streptavidin particles to reduce the likelihood of isolation of streptavidin-specific yeast. The R2 output, which consisted of 5×10^7 yeast from the second magnetic column, was incubated with SA-PE and then sorted using two overlapping gates for PE positive fluorescence as shown in Figure 1A. Two populations were sorted from R3: the R3a output (approximately 1.7×10^6 yeast) included all of the *c-myc*-positive, PE-positive yeast (3.42% of the total R2 output) and was sorted from the large gate shown in Figure 1A. A second sort was performed using the smaller gate in Figure 1A which included only the highest 1% of PE positive yeast (0.07% of the total R2 output). We sorted 25,000 yeast from this gate to generate the R3b output.

For all further incubations, we used 1×10^7 yeast per stain regardless of the diversity of the output. Figure 1, Panels B-E show the binding of the R3a and R3b outputs to H_c as detected with MAb B4-A633. When we compared the R3a and R3b yeast stained with 100 nM H_c and B4-A633 (Panels C and E, respectively), the apparent affinities of the R3b-derived yeast were not much better than the R3a-derived yeast as judged by the intensity of the A633 signal. However, we did observe approximately three fold greater number of H_c -binding yeast in the R3b population (Figure 1E). The R3a output showed approximately 7 fold increased binding in the presence of H_c [0.02% A633-positive yeast the absence of H_c (Figure 1B) versus 0.14% binding to 100 nM H_c (Figure 1C)]. The R3b output showed much greater specificity for antigen resulting in approximately 39 fold over background [0.01% A633-positive yeast in the absence of H_c (Figure 1D) versus 0.39% binding in the presence of H_c (Figure 1E)]. A total of 13,500 FITC+/PE+ yeast were sorted from the R3a output (Figure 1C) and 776 yeast were sorted from the R3b output (Figure 1E).

A second sort of the R2 output was performed following incubation with 100 nM H_c followed by a third detection MAb, AR1-biotin. Figure 1F and 1G show the binding of the R3A output in the absence or presence of H_c , respectively. Despite the presence of ~0.03% of PE-positive yeast in the absence of H_c (Panel 1F), results showed approximately 4 fold greater PE-positive yeast in the presence of H_c (0.13%) (Panel 1G). We sorted 11,300 additional FITC+/PE-positive yeast from these incubations.

Negative sorting of round 3b output with MAb 3D12-A633

Incubation of the R2 output with MAb 3D12-A633 resulted in a high level of nonspecific binding in the absence of antigen (Figure 2A). Although the population of scFv expressing yeast had dropped dramatically as determined by the low *c-myc*-FITC signal (15.6% of the total yeast expressed scFv), approximately 0.22% of the population (both *c-myc* positive and *c-myc* negative yeast) bound nonspecifically to either MAb 3D12-A633 or to the A633 dye itself (Figure 2A). To reduce secondary reagent binding, and increase *c-myc* expression, a negative sorting approach was employed. The R2 output was labeled with anti-*c-myc*, GaM-A488, and 3D12-A633 in the absence of H_c . Two million *c-myc*-positive, A633-negative yeast were sorted from the dashed sort gate shown in the lower right quadrant of the bivariate plot in Figure 2A. A sample of the sorted yeast (50000 events) was immediately reanalyzed (Figure 2B), revealing that the population was now enriched for *c-myc* positive yeast (78.2% of the total). Incubation of another sample (20000 events) with MAb 3D12-

A633 in the absence of H_c confirmed a reduction in the nonspecific binding population (A633-positive yeast) from 0.22% down to 0.07% of the total sorted population (Figure 2C).

The remaining sorted yeast were incubated with 100 nM H_c followed by 3D12-A633 (Figure 2D). Two populations of H_c-binding yeast were observed, one which was *c-myc*-negative (upper left quadrant of Figure 2D) and another that was *c-myc*-positive (upper right quadrant of Figure 2D). The *c-myc* negative population showed 1.03% binding to H_c while the *c-myc* positive population showed 0.30% binding to H_c. Five hundred yeast were sorted from each of the *c-myc* negative and *c-myc* positive, H_c-binding populations.

Clone analysis

In total, 204 clones were analyzed for binding to BoNT/A (H_c) and secondary reagents. Clones were also screened for non-binding to 100 nM of various irrelevant proteins including human EGF-biotin, Bcl2-biotin (from *Bacillus anthracis*), and three biotinylated peptides (data not shown). From the 204 yeast analyzed, we isolated 18 unique H_c-specific clones. Two additional clones were isolated using the negative sorting technique. The 20 unique clones, including the MAb first used to detect them, the V_H and V_L/V_K gene family, their binding to holotoxin, and the number of times the clone was isolated are shown in Supplemental Table 1.

All twenty clones were tested for binding to 25 nM BoNT/A holotoxin of which five bound holotoxin (Supplemental Table 1). Two of the five (scFv F2B2 and scFv F8B6) bound holotoxin poorly, and sequence analysis of both could not identify a functional variable light chain gene. These two clones were removed from further analysis. ScFv genes from the remaining three BoNT/A-binding clones were cloned into pET27b and expressed in *E. coli* as previously described (Miller, et al., 2005). Secreted and purified scFv from all three clones exhibited H_c binding activity as determined by Biacore analysis.

Analysis of 24 clones from the negatively sorted population revealed two unique H_c-binding clones, one which was *c-myc* positive and the other *c-myc* negative. *Bst*N1 fingerprint analysis and DNA sequencing revealed that both clones isolated from the negative sorts with 3D12-A633 were unique to that sort with that antibody, and were not identified in any other sorts. The *c-myc*-positive clone, named scFv 4–2 (G8C6), is one of the three clones that bound holotoxin and is characterized throughout the study. The *c-myc* negative clone, named F11C4, did not bind holotoxin and was not analyzed further.

Between rounds of selection, yeast were typically expanded 100–1000 fold. As a result, many H_c binding yeast were propagated repeatedly, resulting in clone redundancy. In summation, eleven of the twenty clones were isolated only one time, five were isolated two times, and four were isolated between three and fourteen times. A clone isolated fourteen times, designated scFv 14–2, was isolated at least once by all three MAbs, was the highest affinity clone (27 nM by flow cytometry; 4.67 nM by Biacore), and bound to BoNT/A holotoxin. In contrast, scFv 19–3 was isolated twice with two MAbs while scFv 4–2 was isolated only through the negative sort experiment.

Affinity of yeast-displayed and secreted scFv

Table 1 shows the affinities of the three BoNT/A binding clones, scFv 4–2 (G8C6), scFv 14–2 (G10C8), and scFv 19–3 (H3D1) as measured by Biacore analysis and flow cytometry. Flow cytometry measures the affinity of the scFv in its yeast-bound form (Chao, et al., 2006, Siegel, et al., 2004, Van Antwerp and Wittrup, 2000, Feldhaus and Siegel, 2004). Several investigators have demonstrated that the affinity constants measured with flow cytometry correlate closely with those measured by Biacore surface plasmon resonance (SPR) (Gai and Wittrup, 2007). ScFv 4–2, scFv 14–2, and scFv 19–3 had affinities to the H_c of 48 nM, 27 nM, and 45 nM, respectively, in this assay.

In order to determine kinetic rates of association (k_a) and dissociation (k_d), Biacore analysis was conducted on secreted scFv. By this method the affinities of scFv 14–2 and scFv 19–3 to the H_c were 4.67 nM, and 8.96 nM, respectively (Table 1). ScFv 4–2 was tested by Biacore, however due to a significant decay of the 4–2 scFv following capture by the *c-myc* MAb on the surface of the chip, we were unable to reliably measure its affinity by SPR. Supplemental Figure 1 shows the Biacore curves (seven H_c concentrations, each tested in triplicate) for scFv 14–2 and 19–3.

Epitope specificity

In epitope binning assays (Gray, et al., 2010, Siegel, et al., 2004), yeast-displaying scFv were incubated with H_c, split into three aliquots, and the bound H_c detected with one of the three biotinylated detection MAbs: 3D12-biotin, B4-biotin, or AR1-biotin. As seen in Figure 3, MAb B4 did not bind to H_c that had been captured by scFv 19–3. This is evidence that scFv 19–3 and MAb B4 share at least a portion of the same epitope. MAb B4 did bind H_c captured by either scFv 4–2 or scFv 14–2, so these scFv must bind different epitopes from both scFv 19–3 and B4. The other two scFv clones, scFv 4–2 and scFv 14–2 do not inhibit the binding of any of the three MAbs. Therefore, they may bind epitopes different from all three MAbs. The conclusion is that the three holotoxin-binding scFv bind at least two distinct epitopes on H_c.

Synergistic antigen capture

The Endopep-MS assay described by Barr *et al.* uses magnetic particles coated with scFv or IgG antibodies to capture BoNT toxins from environmental samples. The beads are then mixed with peptides which are specifically cleaved by BoNT (figure 4). Cleavage is detected by mass spectrometry to discern the peptide cleavage products (Figure 4B and 4C). Incubation of BoNT/A with its peptide substrate resulted in cleavage of the intact peptide [at m/z 1456.8 (doubly charged) and 2911.6 (singly charged)] into two fragments at m/z ~1215.7 and ~1714.9. Signals corresponding to the cleaved peptide fragments were absent in the negative control (Figure 5A) and were apparent in the positive controls containing pure toxin at a concentration of 2.9 U μ L⁻¹ (Figure 5B) or toxin captured by rabbit polyclonal anti-BoNT/A IgGs (Figure 5C). Thus, the presence of signals at m/z 1215.7 and 1714.9 indicates the presence of BoNT/A.

To assess BoNT/A binding by scFv antibodies, magnetic particles coated with scFv were incubated with buffer solution containing BoNT/A at 0.5 U μ L⁻¹. After washing, bound

toxin was detected by Endopep-MS. Particles coated with a BoNT/A-specific rabbit polyclonal antiserum exhibited capture and activity of at least some of the toxin (Figure 5C). When magnetic beads were coupled singly with scFv 4–2 (Figure 5D), scFv 14–2 (Figure 5E), or scFv 19–3 (Figure 5F), only modest binding activity was observed relative to the rabbit polyclonal antiserum control. When the BoNT/A spiked buffer solution was incubated with beads coated with a mixture of all three scFv antibodies, significantly greater binding activity was observed (Figure 5G). Signals at ~ 1215.7 and ~ 1714.9 m/z were consistently greater than signals seen with any individual scFv or with the rabbit polyclonal antiserum.

Discussion

This study introduced a new strategy for selecting scFv specific to an unlabeled antigen. Antibody-mediated labeling enables selections with antigens that are difficult to biotinylate. It may also enable the display of antigens in more native conformations. Moreover, detection MABs or scFv can be used to mask an epitope, thereby increasing the likelihood of isolating clones to different epitopes. The limitations of this method are that pre-existing antibodies (IgG, Fab, or scFv) are needed, and antibodies of low affinity may not bind to the antigen long enough to be detected.

A non-immune human scFv library was used for initial selections with the BoNT/A H_c, and scFv clones that bound H_c were detected by using labeled anti-BoNT/A IgG MABs identified previously (Razai, et al., 2005, Amersdorfer, et al., 2002, Levy, et al., 2007). Throughout selections, we incubated the yeast with excess antigen (100 nM H_c) for sufficient time (45 minutes) to allow antigen binding to proceed to saturation. This strategy assures the isolation of high-affinity as well as low-affinity clones.

In general, the scFv clones isolated with biotinylated MABs tended to be more H_c specific, and fewer bound secondary reagents, compared to clones isolated with the A633-labeled detection MABs. This may have been due to the intensity of the phycoerythrin portion of the SA-PE detection reagent. We also found that clones that were sorted from an apparently “higher affinity” gate, that is those clones with the highest mean fluorescence intensity for H_c binding, did not exhibit better affinity than clones isolated from a “lower affinity” gate and typically bound secondary reagents only. Additionally, selections performed at lower antigen concentrations also did not produce higher affinity clones, and similarly exhibited mostly secondary reagent binders.

Previous selections on BoNT/A immune phage libraries by Amersdorfer *et al.* resulted in isolation of 28 scFv antibodies (Amersdorfer, et al., 1997). Clones analyzed for affinity to H_c using a flow cytometric assay ranged from 1 nM to greater than 100 nM. Two clones, S25 and C25 had affinities of 73 nM and 1 nM, respectively, and a third scFv isolated from a human immune library, 3D12, had an affinity of 37 nM (Amersdorfer, et al., 2002). Each of these scFv bound different epitopes of the BoNT/A holotoxin (Mullaney, et al., 2001).

Our selections using the non-immune human yeast-display scFv library identified 20 unique clones with affinities for the BoNT/A (H_c) that were similar to those seen with the immune library, ranging from 5 nM to 48 nM. Epitope binning revealed at least two non-overlapping

epitopes on the H_C were bound by the three scFv, although they have not been mapped to the exact epitope. These observations demonstrate the utility of the non-immune library for isolating affinity reagents to novel epitopes.

Clones were isolated that represented three of the six V_H families and five of the eleven V_L families described by Feldhaus *et al.* (Feldhaus, et al., 2003). Moreover, the percent usage of each family in clones that we identified was consistent with the usage described for the original library, suggesting that antibody-mediated selections do not introduce a selection bias with respect to V_H and V_L/V_k gene usage.

Five of twenty scFv tested bound to the BoNT/A holotoxin [scFv F2B2, scFv F8B6, scFv 4–2 (G8C6), scFv 14–2 (G10C8), and scFv 19–3 (H3D1)]. Two of these (scFv F2B2 and F8B6) bound very poorly and had no functional light chain as determined by sequence analysis. All the other clones bound only H_C (data not shown). Of the three that bound holotoxin, scFv 4–2 (G8C6) is one of two scFv isolated from the negative sorts, and was only isolated with the 3D12-A633 detection MAb. By contrast, clone scFv 14–2 (G10C8) was isolated 14 times, had the lowest affinity by flow cytometry and Biacore, and was isolated at least once with all three detection MAbs. All three scFv that bound holotoxin were active following secretion from *E. coli*.

The utility of negative sorting was illustrated by an experiment in which we sorted clones from the R2 output following incubation with 100 nM H_C and MAb 3D12-A633, but did not perform a negative sort. We then screened 24 sorted clones despite the strong presence of nonspecific binders present in the antigen binding region of the bivariate plots. Our hypothesis was that within this pool of nonspecific binders existed clones that bound specifically to H_C. However, analysis of 24 clones from this sort identified 22 secondary reagent binders, and none bound H_C. In contrast, negative sorting yielded two H_C-binding clones, both of which were not isolated in any other selections or with the other two detection MAbs.

The three MAbs used in the selections are known to bind different epitopes on BoNT/A-H_C (Mullaney, et al., 2001, Razai, et al., 2005, Levy, et al., 2007). If a MAb is blocked from binding to H_C following its capture by the yeast-displayed scFv, it could be inferred that the MAb and scFv share a common or overlapping epitope. ScFv 19–3 was observed to inhibit MAb B4 binding, but did not inhibit binding of MAbs 3D12 or AR1. Therefore, scFv 19–3 binds an epitope that overlaps the B4 epitope on BoNT/A. In contrast, scFv 4–2 and scFv 14–2 did not inhibit the binding of any of the three detection MAbs. Therefore, it can be predicted that scFv 19–3 could be used in an ELISA assay when paired with scFv 4–2 or scFv 14–2.

The fact that MAbs B4 and 3D12 were used in the first two rounds of the selection indicates that scFv 19–3 must have been isolated with MAb 3D12. This clone was eventually identified in the AR1-sorted round 3c output. This observation highlights the advantage of using multiple detection MAbs during early rounds of selections to optimize the likelihood of isolating clones that bind to many different epitopes. Taken together, the data indicate

that there are at least five non-overlapping epitopes on the BoNT/A H_c including: the AR1 epitope, B4 epitope, 3D12 epitope, scFv 19–3 epitope, and scFv 4–2/14–2 epitopes.

The Endopep-MS experiment demonstrated the function of soluble scFv following production and purification. This assay has been used many times to detect BoNT toxins in biological samples (Barr, et al., 2005, Kalb, et al., 2010, Kalb, et al., 2005, Kalb, et al., 2009, Kalb, et al., 2006). In our assay, the mass spectrometry signals observed with the polyclonal BoNT/A antiserum were consistently less intense than the signals observed when the three scFv were captured simultaneously. This may be due to the avidity of the three scFv working synergistically. However, the polyclonal BoNT/A antiserum has been shown to neutralize some activity of the toxin resulting in lowered peptide cleavage activity in the Endopep-MS assay (Kalb, et al., 2006). We cannot rule out that the larger signals observed with the three scFv may not be due to increase toxin capture but rather may be due to failure to neutralize the toxin. However, it is difficult to conceive of a mechanism by which three different antibodies neutralize the toxin individually, but not in combination. Given that the three scFv bound at least two different epitopes on the antigen, synergistic binding effects are more likely to account for our observations.

In summary, this report described a novel antibody-mediated labeling technique for screening yeast display libraries for scFv that bind multiple epitopes on an unlabeled antigen. It also demonstrated synergistic binding of scFv antibodies to an enzymatically active antigen. Synergistic antigen capture could be a broadly applicable strategy for improving the performance and utility of monovalent antibody-like molecules such as scFv.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in paper

scFv	single chain Fragment variable
MAb	monoclonal antibody
GaM	goat anti mouse IgG
A633	Alexa fluor-633
SA-PE	streptavidin conjugated to phycoerythrin
K_D	equilibrium affinity constant
BoNT/A	<i>C. botulinum</i> holotoxin

BoNT/A-H_C (or H_C alone)	<i>C. botulinum</i> neurotoxin serotype A receptor binding domain
V_H	variable heavy
V_L or V_k	variable light

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Figure 1.

Results of antibody-mediated BoNT/A H_c selections. Fluorescent-activated cell-sorting of antigen binding clones enriched on a magnetic column. Panel A shows the bivariate plot of the Round 2 output stained with 100 nM H_c and detected with MAbs B4-biotin and 3D12-biotin. Two overlapping sort gates are shown: the large gate was used to sort all of the H_c-binding yeast (R3a output) while the smaller gate was used to sort the top 10% of H_c-binding yeast (R3b output). Panels B and C shows binding of the R3a output to no antigen (Panel B) or 100 nM H_c (Panel C) followed by detection with MAb B4-A633. Panels D and E shows binding of the R3b output to no antigen (Panel D) or 100 nM H_c (Panel E) followed by detection with MAb B4-A633. Panels F and G shows binding of the R3c output to no antigen (Panel F) or 100 nM H_c (Panel G) followed by detection with MAb AR1-biotin and SA-PE.

Figure 2.

Negative sorting to enrich for scFv expressing yeast and reduce secondary reagent binding. Panel A shows binding of the R2 output to MAb 3D12-A633 in the absence of H_c . The dashed gate in the lower right quadrant of Panel A was used to sort the *c-myc* positive, 3D12-A633-negative yeast. Panels B shows 50,000 sorted, unstained yeast; Panel C shows binding of the 3D12-A633 antibody in the absence of H_c ; Panel D show the binding of sorted yeast to 100 nM H_c with detection by 3D12-A633. Gates used to sort the *c-myc*-negative/ H_c -binding yeast (upper left gate in Panel D) and the *c-myc*-positive/ H_c -binding yeast (upper right gate in Panel D) are shown.

Figure 3.

Epitope binning of BoNT/A-binding clones with IgG antibodies. The three BoNT/A-binding yeast isolates were incubated with 100 nM H_c , split into three aliquots, and bound H_c detected with each of the three biotinylated detection MAb separately. If the detection MAb is able to bind to H_c that has been captured by the yeast-displayed scFv, it will appear as a positive PE signal in the upper right quadrant of the bivariate plot. Weak PE fluorescence in this quadrant suggests that the biotinylated detection MAb is unable to bind to the H_c because it binds to an identical or overlapping epitope as the scFv. This inhibition was observed when H_c was captured by scFv 19–3 and the MAb B4-biotin was used for detection (lower right panel). This suggests that the scFv 19–3 and MAb B4 bind to a common or overlapping epitope.

Figure 4.

Pictorial illustration of the Endopep-MS assay for detection of botulinum neurotoxins. Panel A describes the four steps involved in the assay including 1) antibody-coated particles capture BoNT/A toxin; 2) toxin coated particles are incubated with a peptide containing the BoNT/A-specific cleavage site; 3&4) the peptide mixture is analyzed by MS for intact versus cleaved peptides. Panel B shows sizes of the intact peptide and the two cleavage products generated by toxin-specific cleavage. Panel C is a typical mass spectrometry plot of all of the possible signal peaks seen in the Endopep-MS assay. Peaks 1 and 3 are the cleaved peptide fragments at ~ 1215.7 and $\sim 1714.9 \text{ m/z}$, respectively. Peak 2 is the doubly charged intact peptide at $\sim 1455.8 \text{ m/z}$ and peak 4 is the singly charged intact peptide at $\sim 2911.6 \text{ m/z}$. A positive Endopep-MS would be a reduction in peaks 2 and 4 concurrent with an increase in peaks 1 and 3.

Figure 5.

Endopep-MS assay for detection of botulinum type A neurotoxin. The MALDI TOF-MS results of the Endopep-MS assay for each of the scFv antibodies as well as the control reactions are shown. Each panel is a MALDI TOF-MS spectrum where the x -axis shows the peptide mass-to-charge (m/z) ratio while the y -axis represents the percent relative intensity. Panel A) negative control (peptide substrate only); Panel B) positive control (50U purified BoNT/A toxin); Panel C) BoNT/A-specific rabbit polyclonal antiserum; Panel D) scFv 4-2; Panel E) scFv 14-2; Panel F) scFv 19-3; Panel G) mix of all three scFv.

Table 1

Biacore and flow cytometry affinity analysis of BoNT/A-specific scFv

Bot scFv Clone	On-rate (ka) M/s	Off-rate (kd) s ⁻¹	Affinity (nM)	Affinity by Flow cytometry
#4-2	<i>Not tested</i>	<i>Not tested</i>	<i>Not tested</i>	48 ± 9.2nM
#14-2	3.55 × 10 ⁵	1.66 × 10 ⁻³	4.67	27 ± 6.1nM
#19-3	1.28 × 10 ⁶	1.15 × 10 ⁻²	8.96	45 ± 5.5nM