Title
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Permalink
https://escholarship.org/uc/item/75n6n0qs

Journal
PloS one, 12(3)

ISSN
1932-6203

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Publication Date
2017

DOI
10.1371/journal.pone.0174187

Peer reviewed
A three monoclonal antibody combination potently neutralizes multiple botulinum neurotoxin serotype F subtypes

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Abstract

Human botulism is primarily caused by botulinum neurotoxin (BoNT) serotypes A, B and E, with around 1% caused by serotype F (BoNT/F). BoNT/F comprises at least seven different subtypes with the amino acid sequence difference between subtypes as high as 36%. The sequence differences present a significant challenge for generating monoclonal antibodies (mAbs) that can bind, detect and neutralize all BoNT/F subtypes. We used repertoire cloning of immune mouse antibody variable (V) regions and yeast display to generate a panel of 33 lead single chain Fv (scFv) mAbs that bound one or more BoNT/F subtypes with a median equilibrium dissociation constant ($K_D$) of $4.06 \times 10^{-9} \text{M}$. By diversifying the V-regions of the lead mAbs and selecting for cross reactivity we generated five mAbs that bound each of the seven subtypes. Three scFv binding non-overlapping epitopes were converted to IgG that had $K_D$ for the different BoNT/F subtypes ranging from $2.2 \times 10^{-8} \text{M}$ to $1.47 \times 10^{-12} \text{pM}$. An equimolar combination of the mAbs was able to potently neutralize BoNT/F1, F2, F4 and F7 in the mouse neutralization assay. The mAbs have potential utility as diagnostics capable of recognizing the known BoNT/F subtypes and could be developed as antitoxins to prevent and treat type F botulism.

Introduction

Botulism occurs in infants and adults and is caused by botulinum neurotoxin (BoNT), the most poisonous substance known. [1,2]. Botulism is characterized by flaccid paralysis, which if not rapidly fatal requires prolonged hospitalization in an intensive care unit and mechanical ventilation. The catalytic domain of BoNT is the light chain (LC), a heavy chain (HC) is comprised of a translocation domain (H$_T$) and a receptor-binding domain (H$_R$). There are at least seven distinct BoNT serotypes (A-H) [3–5], defined by their neutralization by serotype specific
antitoxin; an antitoxin against one serotype will not neutralize another serotype [6,7]. For six serotypes (A–F), there also exist multiple subtypes, which can vary at the amino acid level by a few percent to up to 36% for BoNT/F [8–10]. Subtype sequence differences may result in changes in surface epitopes that can cause a reduction in antitoxin potency [11].

Four BoNT serotypes (A, B, E, and F) cause virtually all human botulism. BoNTs are also classified as Category A bioterror threat agents, HHS Tier 1 select agents/toxins, one of the seven highest-risk threat agents for bioterrorism [12]. The only treatment for botulism is antitoxin [13]. Equine antitoxin [14,15] and human botulism immune globulin [16,17] are currently licensed to treat adult and infant botulism respectively. Human botulism immune globulin (BabyBIG) is produced by plasmaphersing immunized laboratory personnel who are at risk of exposure to BoNT [18]. Although human botulism immune globulin (licensed for serotypes A and B only) has been shown to be both safe and effective for treating infant botulism scaling of this product to treat adult botulism or for the bioterror drug repository is not feasible [18]. A heptavalent (serotypes A–G) equine botulism antitoxin (HBAT) produced from immunized horses is also licensed in the United States for the treatment of botulism [19]. As a foreign protein, HBAT is immunogenic, and hypersensitivity reactions, including serum sickness and asystole, have been reported [19]. HBAT is an immunoglobulin fragment antigen-binding (Fab')2 whose seven serotype-specific components have short serum half-lives (7.5–34.2 hours), which preclude its effective use for prophylaxis and may predispose to relapse of botulism after treatment [20]. As an alternative, highly potent human monoclonal antibody (mAb)-based antitoxins composed of three mAbs [21] are being developed for serotypes A, B, C, D and E, with the most advanced (serotype A) having completed Phase 1 human testing [22]. These mAbs bind non-overlapping epitopes on the BoNT molecules of the different BoNT subtypes [23,24].

For this work, we sought to generate a panel of mAbs that bound most, or all, of the highly disparate (up to 36% different at the amino acid level) BoNT/F sub-serotypes for the purpose of generating a therapeutic antitoxin.

Materials and methods

Ethics

The USAMRIID Institutional Animal Care and Use Committee approved the animal care and use protocol to conduct the animal studies reported here. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC/I) and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. The specific national regulations and guidelines to which this animal care and use protocol adheres are the following: (1) 7 USC, Sections 2131–2159, Chapter 54 “Animal Welfare Act”, and (CFR, Chapter 1, Subchapter A, Parts 1–4 “Animal Welfare Regulations”; (2) Health Research Extension Act of 1985, Public Law 99–158 “Animals in Research” and the Public Health Service Policy in Humane Care and Use of Laboratory Animals; (3) Biosafety in Microbiological and Biomedical Laboratories, 5th Edition, NIH, Human and Health Services Publication (CDC) 21–112; (4) Army Regulation 40–33 “The Care and Use of Animals in DOD Research, Development, Test and Evaluation or Training Programs” and (5) DOD Instruction 3216.01 “Use of Animals in DOD Programs”. DOD uses the “The Guide for the Care and Use of Laboratory Animals”, 8th Edition, Institute for Laboratory Animal Research, National Research Council, as a guideline for evaluation and accreditation of program and it is
based on the actual national regulations and guidelines for animal care and use programs. The animals used in this study were euthanized using carbon dioxide gas following the AVMA Guidelines on Euthanasia prior to spleen removal.

The University of California, San Francisco (UCSF) Institutional Review Board approved the human use protocol used for the studies described here. Human donors were laboratory workers being immunized to work with BoNT who were recruited via an informational letter and who signed informed consent.

Materials

_Saccharomyces cerevisiae_ strain EBY100 was used for library construction and BoNT/F domain display. _Escherichia coli_ DH5α was used for subcloning and preparation of plasmid DNA, while the strain BL21 was used for BoNT/F fragment expression. Chinese hamster ovary (CHO) cells were used for immunoglobulin G (IgG) expression. Yeast Peptone dextrose (YPD) medium was used for growth of EBY100 cells. Selective growth dextrose casamino acids media (SD-CAA) was used for selection of pYD4-transformed EBY100 as previously described [25] and selective growth galactose casamino acids media (SG-CAA), for induction of scFv expression on the surface of EBY100. 2 x YT was used for BL21 and DH5α growth. Pure holo-toxin BoNT/F1 and polyclonal rabbit anti-BoNT/F antibody were purchased from Metabiologics Inc. (Madison, WI, USA). Clostridial culture supernatants containing unpurified BoNT holotoxins BoNT/B, A, F, and F7 were expressed from strains An436, SU1904, Eklund 202F, and Sullivan, respectively. BoNT/F5 culture supernatant was a kind gift of Susan Maslanka and Brain Raphael at the Centers for Disease Control and Prevention. Mouse anti-SV5 antibody was purified from a hybridoma cell line and labeled with AlexaFluo-488 or AlexaFluo-647 labeling kit (Invitrogen, Carlsbad, CA, USA). All the secondary antibodies including PE (APC)-conjugated goat anti-human-Fc, goat anti-mouse Fc and goat anti-human F(ab') were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Protein expression and purification

The cDNA of 14 BoNT/F fragments including F1-7 N-terminal portion of the light chain (LC-H$_N$) and F1-7 C-terminal portion of the heavy chain (H$_C$) were amplified from synthetic BoNT/F1-7 genes. All the cDNAs were subcloned into the plasmid pET28b with a hexahistidine (His) tag (for the FLC-H$_N$ fragments, F1H$_C$ and F7H$_C$) or a maltose binding protein (MBP) tag (F2-6H$_C$). The fragments were expressed in the _E. coli_ strain BL21 and purified as described [26]. The MBP-tagged BoNT/F H$_C$ fragments were purified using the agarose Dextrin Sepharose High Performance (GE Healthcare, Pittsburg, PA, USA), eluted into PBS with 10mM maltose. The His-tagged fragments were purified with a Ni-NTA agarose (Qiagen, Valencia, CA). The recombinant proteins were dialyzed with PBS buffer overnight to remove imidazole or maltose prior to use. IgG were generated by subcloning V$_H$ and V$_k$ genes into a mammalian expression vector with human heavy and kappa light chain constant regions, establishment of stable CHO cell lines by transfection and purification of IgG by Protein G chromatography as previously reported [21].

Mouse immunization and spleen harvest

For the BoNT/F toxin immunization, mice were vaccinated three times at two week intervals (Days 1, 14 and 28) with 10 μg/mouse of catalytically inactive BoNT/ACEF HC. Mice boosted with BoNT/F5 were also vaccinated with BoNT/F5 LC. Mice were boosted twice with 1 μg active toxin (1.0–1.8 x 10$^4$ lethal dose 50% (LD$_{50}$) BoNT/F1, BoNT/B2, and BoNT/F6; 2.5 x 10$^3$ LD$_{50}$ BoNT/Af4; 0.5 x 10$^3$ LD$_{50}$ BoNT/F7). BoNT/F5 boosts were done with nontoxic...
BoNT/F5 LC-H₂N. For BoNT/F LC-H₂N immunization, 0.25–2.5 μg/mouse of antigen were subcutaneously injected with adjuvant on Days 1, 14 and 28, 0.25–2.5 μg/mouse and 0.5–5.0 μg/mouse of antigen was intramuscularly injected with adjuvant; at day 42, proteins were intravenously injected in PBS. Antibody titers after the third vaccination ranged from 0.2–10 x 10⁻⁵. Mice were euthanized and spleens were removed 3 to 5 days after their final vaccinations and processed to extract mRNA for scFv library construction.

Yeast-displayed, scFv antibody library construction and sorting

Total RNA was isolated from the blood of healthy human donors immunized with investigational pentavalent BoNT ABCDE toxoid (formalin-inactivated toxins) or from spleens of immunized mice. cDNA synthesis, V₃ and V₅ gene repertoires preparation and library construction were completed as previously described [26]. Library size of resultant libraries is given in S1 Table. Finally, the libraries were cultured in SD-CAA media and were induced with SG-CAA media at 18˚C over night.

Monoclonal BoNT/F antibodies were isolated as described [25,26] by using 100nM of BoNT/F1 with 1 hour of incubation at room temperature. Then yeasts were incubated with 2 μg/mL of rabbit anti-BoNT/F polyclonal antibody (Metabiologics Inc.) for 60 min. at 4˚C, washed, and then incubated with 1 μg/mL of Alexa-488-labeled goat anti-rabbit Fc antibody (Jackson ImmunoResearch Laboratories) and 1 μg/mL Alexa-647-labeled anti-SV5 mAb. After washing, yeasts were sorted by flow cytometry and identified as previously described [25,26]. For the initial library sorting, positive binding population were gated as many as possible to obtain all the potential binders; but for the libraries for affinity maturation, less than 1% of positive binder were gated to obtain the colonies with improved affinity.

Measurement of yeast-displayed scFv Kᵦ

The equilibrium dissociation constant (Kᵦ) of yeast-displayed scFv was measured by flow cytometry, as previously described [26–29], except that mAbs used were 6F5.1 and 6F11 (for LC-H₂N binders) or mAbs 6F8 and 6F3 (for H₂ binders) as secondary antibodies. Briefly, 2x10⁶ yeast were incubated in serially diluted BoNT/F toxin or fragments for 1 hour at room temperature, then with a detection antibody and a R-phycoerythrin (PE)-conjugated goat anti-human or anti-mouse antibody together with Alexa Fluor™ 647-labeled SV5 tag antibody. Mean fluorescence intensity (MFI) was measured by flow cytometry as previously described [26,27].

Ability of mAbs to bind other BoNT/F subtypes

The yeast-displayed scFv were incubated with 100nM BoNT/F1, F2, F3, F5, F6, F7 LC-H₂N or BoNT/F4 holotoxin (for LC-H₂N binders) or BoNT/F1, F2, F3, F4, F5, F6, F7 H₂ individually at room temperature for 1 hour. All subsequent washing and staining steps were performed at 4˚C using ice-cold FACS buffer as above. Yeast-displayed scFvs were incubated for 30 minutes with 2 μg/mL of a mixture of Alexa-647 conjugated IgGs 6F5.1 and 6F11 (for LC-H₂N binders) or mAbs 6F3 and 6F8 (for H₂ binders) as the secondary antibody for binding detection, together with an Alexa-488 labeled anti-SV5 IgG for detection of scFv expression. Finally, the yeasts were washed using cold FACS buffer and BoNT/F subtype binding was measured by flow cytometry.

Affinity maturation

The antibodies that potentially bound all seven subtypes of BoNT/F were affinity matured by chain shuffling, followed by error prone PCR mediated random mutagenesis. For the chain
shuffling libraries, the $V_H$ gene of the scFv was PCR-amplified with primers with gap tails and gel purified. The plasmids containing a pYD4-$V_K$ repertoire [25] were linearized by digestion with NcoI/Sall and were purified from an agarose gel by using GeneClean Turbo Kit (MP Biomedicals, Santa Ana, CA, USA). The gap-tailed $V_H$ gene was transformed into EBY100 together with the linearized pYD4-$V_K$ plasmids using LiAC as described previously [28]. For the random mutagenesis libraries, the cDNA encoding scFvs were PCR-amplified with gap-tailed primers by using Paq5000™ DNA polymerase (Agilent, Santa Clara, CA, USA), in which 0.05 mM MnCl$_2$ was applied to introduce random mutation. The cDNA with mutations was gel purified and transformed into EBY100 together with NcoI/NotI digested pYD4 plasmid by LiAC method as described previously [28]. The library size was determined by plating serially diluted transformation mixture on SD-CAA plates. The scFv libraries were induced by culturing in SG-CAA media with 10% SD-CAA for at least 24 hours.

In order to isolate the colonies that had improved affinity to all of the BoNT/F subtypes, each library was sorted at least five times; each subsequent round of sorting used a different BoNT/F subtype, with the antigen concentration decreased from 100nM to 100pM from the first to last round of sorting. After the final round of sorting, the collected yeast clones were grown on SD-CAA plates followed by plasmid isolation and DNA sequencing. $K_D$ values were measured and the colonies with highest affinity to each BoNT/F subtype were selected.

Epitope mapping
cDNA encoding each subtype of the BoNT/F1 LC, H$_N$ and H$_C$ domains were PCR amplified from the seven synthesized BoNT/F genes. Gel purified cDNA fragments were digested with NcoI/NotI and cloned into the plasmid pYD4. The recombinant plasmids were then used to transform EBY100 by LiAC, which were grown on SD-CAA plate for 72 hours. Individual colonies were picked, grown in SD-CAA medium and were induced with SG-CAA medium at 18˚C for 48 hours to express the BoNT/F domain on the yeast surface. Yeast colonies were incubated with 2 μg/ml one of the BoNT/F IgGs at room temperature for 1 hour, followed by PE-conjugated goat anti-mouse IgG together with Alexa-488 labeled anti-SV5 IgG at 4˚C for 30 minutes. Yeast clones were washed and assayed by flow cytometry.

BoNT/F modeling and comparison
BoNT/F1 was modeled on BoNT/A crystal structure (pdb ID: 2NZ9) using the software UCSF-Chimera [30]. The modeled LC was replaced by the crystal structure of BoNT/F LC (pdb ID: 2A97), leading to a BoNT/F1 model containing an experimentally determined structure of LC and a modeled F$_N$ and F$_C$. In order to compare the structure of BoNT/F subtypes, the amino acid sequence of BoNT/F1 was aligned with the other subtypes using the online tool “MultAlin”, (http://multalin.toulouse.inra.fr/multalin), [31]. The alignment was overlaid on the model structure into Esprit 3 online (http://esprit.ibcp.fr) [32]. which then calculated the similarity score based on the physiochemical property of residues, and generated a file with the structure comparison that was edited with Pymol [32]. The difference of the amino acids was coded between red and white; the more different, the more red.

Mouse neutralization assay
The mouse neutralization assay was performed as described previously [11]. Briefly, mAbs or HBAT were mixed with 1 μg of the indicated BoNT/F subtype and injected into mice. The toxin-exposed mice were observed at least twice daily. Most animals receiving lethal doses of BoNT become moribund within 12 hours, frequently within 4 hours.
Results and discussion

Comparison of the surface amino acid differences between BoNT/F subtypes

The BoNT/F subtypes differ from each other by 1.1% to 36.1% at the amino acid level [10]. BoNT/F1 differs from the other BoNT/F subtypes by 7.7% to 30.1% at the amino acid level. To visualize the impact of these differences on potential mAb binding, we modeled the differences on a model of BoNT/F. The BoNT/F1 model was generated by using a combination of the BoNT/F1 LC crystal structure (pdb ID: 2A97) and a model of the BoNT/F1 HC based on the crystal structure of BoNT/A1 complexed to mAb CR1 (pdb ID: 2NZ9). Differences in the amino acid sequence between BoNT/F1 and the other BoNT/F subtypes result in differences on the BoNT/F surface (Fig 1). These differences are greatest for BoNT/F5 (31.1% different).
and BoNT/F7 (26.3% different). The difference between BoNT/F5 and BoNT/F7 are as high as 36.3%. Differences are greatest between the BoNT/F1 LC and the LC of BoNT/F5 (52.4% different) and the LC of BoNT/F7 (36.7% different). In contrast, BoNT/F2 and BoNT/F3 surfaces are highly homologous. Diversity between subtypes makes it challenging to generate high affinity mAbs to all the subtypes.

**Yeast-displayed scFv antibody library**

scFv yeast-display libraries were constructed from spleens of immunized mice. Since BoNT/F1 was the only holotoxin commercially available, we produced recombinant BoNT/F domains for immunization of the other subtypes. Synthetic genes encoding the BoNT/F binding domain (H$_C$) or translocation domain (H$_N$) fused to the light chain (LC) (LC-H$_N$) of sub-serotypes F1, F2, F3, F4, F5, F6 and F7 were designed and cloned into plasmid pET28b to contain His tags or MBP tags. His-tagged BoNT/F1 and BoNT/F7 H$_C$ and MBP-tagged BoNT/F2, 3, 4, 5, 6 H$_C$ were expressed in the cytoplasm and could be purified (S1 Fig). His-tagged BoNT/F1, F2, F3, F5, F6 and F7 LC-H$_N$ could be expressed and purified to varying levels of purity (S1 Fig), while BoNT/F4 LC-H$_N$ did not express as soluble protein in the cytoplasm. Mice were initially immunized with one of the non-toxic domains and then boosted with either BoNT/F1 holotoxin or the domain used for the initial immunization (S1 Table). BoNT/F2 LC-H$_N$ was not used for immunizations due to its high level of identity to BoNT/F1.

Total RNA was isolated from mouse spleens and used to construct scFv libraries in the yeast display vector pYD4 exactly as described [25,26]. Libraries varied in size from 1.6x 10$^7$ to 9.0 x 10$^7$, with a 3-human donor library mixture of 2.0 x 10$^8$ (S1 Table). Total RNA was also isolated from white blood cells from twelve human donors and was used for scFv library construction as previously described [26].

**mAb isolation and initial characterization**

After three rounds of sorting, individual colonies were picked and characterized for binding, yielding 33 unique mAbs as determined by DNA sequencing of the scFv genes; 26 of the mAbs were from the murine libraries and seven from the human libraries (Table 1). Four of these mAbs (46A5, 46F8, 47A12, and 54D7) did not bind BoNT/F1 holotoxin. The affinity of the yeast-displayed scFv was measured by flow cytometry, demonstrating a K$_D$ to BoNT/F1 ranging from 4.0 x 10$^{-11}$ M to 1 x 10$^{-7}$ M, with a median K$_D$ of 4.06 x 10$^{-9}$ M. (Table 1).

BoNT/F subtype cross reactivity of the mAbs was tested by flow cytometry. The scFv 4E17 bound all seven BoNT/F subtypes (Table 1 and S2 Fig). BoNT scFv 6F8 and 37B6 bound six of the seven subtypes but did not show binding to BoNT/F7 (Table 1). scFv 38B8, 38C1, 54D7, 57A8 and 57C3 bound five of the seven subtypes of BoNT/F. The other mAbs showed varying levels of cross reactivity to the other subtypes (Table 1 and S2 Fig).

**Broadening sub-serotype specificity and increasing affinity**

To broaden subtype specificity and increase affinity, the V-genes of selected scFv were mutated, displayed on yeast and sorted for higher affinity for multiple subtypes [28,29,33,34]. The V$_H$ gene of 4E17 was shuffled into a V$_L$ library, leading to 4E17.2 (6F5), which showed improved affinity to BoNT/F1 but low affinity to other subtypes. Then V gene of 4E17.2 (6F5) was randomly mutated by error prone PCR and sorted using extremely low BoNT/F1 holotoxin concentrations (100pM), which yielded the scFv 6F5.1 which has a 3.75 to >700 fold increase in affinity for the BoNT/F subtypes with the K$_D$ ranging from 0.32 nM for BoNT/F4 to 2.97nM for BoNT/F7 (Table 2, Fig 2). IgG produced from the 6F5.1 scFv showed high affinity binding to all seven BoNT/F subtypes, with K$_D$s ranging from 26 pM against BoNT/F4...
Table 1. Binding characteristics of selected mAbs against BoNT/F.

| mAb name | Origin | Yeast \(K_D\) (BoNT/F1 Holotoxin) | Domain | Subtype specificity |
|----------|--------|----------------------------------|--------|--------------------|
| 6F3      | Mouse  | \(2.87 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F7             |
| 6F4      | Mouse  | \(1.50 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F2, F3, F4, F5, F6, F7 |
| 6E17.2   | Human  | \(0.55 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F5, F7     |
| 6E6      | Mouse  | \(1.55 \times 10^{-9} \text{M}\) | \(LC\)  | F1, F3, F4         |
| 6E8      | Mouse  | \(0.30 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F2, F3, F4, F5, F6 |
| 6F9      | Mouse  | \(0.04 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F5     |
| 6F10     | Mouse  | \(1.20 \times 10^{-9} \text{M}\) | \(LC-H_N\) | F1, F6             |
| 28H4     | Mouse  | \(20.0 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F6     |
| 30C8     | Mouse  | \(0.20 \times 10^{-9} \text{M}\) | \(LC-H_N\) | F1, F3, F4, F5     |
| 29A2     | Mouse  | \(0.99 \times 10^{-9} \text{M}\) | \(LC-H_N\) | F1               |
| 28C9     | Mouse  | \(15.91 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 32G2     | Mouse  | \(5.18 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 37B4     | Human  | \(10.14 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F6     |
| 37B6     | Human  | \(23.75 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F2, F3, F4, F5, F6 |
| 38B8     | Human  | \(18.32 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F5, F6 |
| 38C1     | Human  | \(30.26 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F5, F6 |
| 38D11    | Human  | \(29.13 \times 10^{-9} \text{M}\) | \(H_N\) | F1               |
| 38F8     | Human  | \(29.36 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F4, F6     |
| 44A12    | Mouse  | \(1.90 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 44C2     | Mouse  | \(26.93 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 44C4     | Mouse  | \(29.32 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 44F4     | Mouse  | \(16.85 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 46.25    | Mouse  | \(13.84 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F6         |
| 46A5     | Mouse  | No binding                       | \(LC-H_N\) | F3               |
| 46F8     | Mouse  | No binding                       | \(LC-H_N\) | F3               |
| 46E12    | Mouse  | \(4.06 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F3, F5, F6, F7 |
| 47A12    | Mouse  | No binding                       | \(H_N\) | F3, F5, F6         |
| 54D7     | Mouse  | No binding                       | \(LC-H_N\) | F3, F4, F5, F6, F7 |
| 55A9     | Mouse  | \(5.25 \times 10^{-9} \text{M}\) | \(H_N\) | F1, F4             |
| 56E11    | Mouse  | No binding                       | \(LC-H_N\) | F3               |
| 57A6     | Mouse  | \(28.67 \times 10^{-9} \text{M}\) | \(LC-H_N\) | F1, F3, F5, F6, F7 |
| 57C3     | Mouse  | \(>100 \times 10^{-9} \text{M}\) | \(LC-H_N\) | F1, F3, F5, F6, F7 |

Table 2. The \(K_D\) values (x 10^{-9}M) of affinity and specificity-matured yeast-displayed scFv for the seven BoNT/F subtypes.

| Antibodies (epitopes) | Toxin* | 4E17.2 (6F5) --6F5.1(H_N) | 4E12-6F11(H_N) | 46.25-6F1 (H_N) | 47A12-6F13 (H_N) | 6F8 (H_C) | 28H4-6F15(H_C) |
|----------------------|--------|--------------------------|----------------|----------------|----------------|-----------|----------------|
| **F1**               | 1.5    | 0.4                      | 23.4           | 3.81           | 50.0           | 6.93      | 3.63           | 0.3            | 12.7           | 1.77           |
| **F2**               | 42.3   | 2.23                     | NB*            | 93.5           | NB             | 141       | NB             | 97.8           | 8.8            | NB             | 41.3           |
| **F3**               | 40.3   | 2.09                     | NB*            | >500           | 19.8           | >500      | 56.9           | >500           | 13.2           | 0.44           | >500           | 19.5           |
| **F4**               | >250   | 0.32                     | NB             | 3.61           | >500           | 38.9      | >500           | 24.1           | 5.32           | >500           | 53.4           |
| **F5**               | 14.1   | 0.68                     | NB             | >500           | NB             | 49.1      | >500           | 12.8           | 52.7           | >500           | 196            |
| **F6**               | 4.88   | 1.24                     | >500           | 8.42           | >500           | 70.3      | >500           | 6.23           | 4.95           | >500           | 60.3           |
| **F7**               | 101    | 2.97                     | >500           | 200            | >500           | 39.2      | >500           | 39.2           | NB             | >500           | 10.5           |

*NB: No binding detected

For \(H_N\) binders, BoNT/F1, F2, F3, F5, F6, F7 LC-H_N or BoNT/F4 holotoxin were used; For \(H_C\) binders, BoNT/F1, F2, F3, F4, F5, F6, F7H_C were used for \(K_D\) measurement.

https://doi.org/10.1371/journal.pone.0174187.t002

https://doi.org/10.1371/journal.pone.0174187.t001
holotoxin to 204 pM against BoNT/F7 LC-HN (Table 3). In addition, 4E17.2 is a highly cross reactive mAb that also binds HN of BoNT/A, BoNT/B, BoNT/E and BoNT/H [29,33].

A similar strategy was used for scFv 6F8, 37B6, 38B8, 38C1, 54D7, 57A8, 57C3, 46E12, 46.25, 47A12, and 28H4 with the first mutagenesis strategy being light chain shuffling followed by error prone mutagenesis. Selections and sorting were performed by simultaneous incubation of yeast-displayed scFv with two to up to five of the BoNT/F subtype fragments labeled with different fluorescent labels [34]. Using this strategy, the affinity and specificity of scFv
46E12, 46.25, 47A12, and 28H4, were improved to generate scFv 6F11, 6F12, 6F13 and 6F15, respectively. While the starting scFv bound 3–4 subtypes, the resultant scFv bound all seven subtypes (Fig 2), with the $K_D$ on yeast ranging from 3.81 nM to 200 nM for 6F11, 6.93 nM to >500 nM for 6F12, 3.63 nM to 97.8 nM for 6F13 and 1.77 nM to 60.3 nM for 6F15 (Table 2). Thus for each of these four scFv, it proved possible to impart a measurable $K_D$ where previously no detectable binding could be observed (Table 2 and Fig 2 versus S2 Fig). IgG constructed from the engineered 6F11 and 6F13 bound to each of the seven BoNT/F sub-serotypes with $K_D$ ranging from 1.47 pM to 12.8 nM for 6F11 and from 112 pM to 32 nM for 6F13 (Table 3). Interestingly, it was not possible to broaden the specificity of scFv 6F8, 37B6, 38B8, 38C1, 54D7, 57A8, or 57C3 to all seven subtypes.

**Epitope determination and overlap analysis**

In order to confirm the binding domain of the cross-reactive antibodies, three domains of BoNT/F1, (LC, HN, and HC) and LC-HN were subcloned into the plasmid pYD4 and displayed on the surface of EBY100. The yeast-displayed domains were incubated with Alexa-647 conjugated IgG and Alexa-488 labeled antiSV5 IgG and detected by flow cytometry. The results demonstrated that 6F5.1, 6F11, 6F12, and 6F13 bound BoNT/F LC-HN and BoNT/F HN, indicating that they bound on HN domain, and that 6F8 and 6F15 bound BoNT/F HC (S3 Fig). Only 6F7 bound BoNT/F LC. A sandwich binding assay was designed to determine if the four BoNT/F HN binders shared overlapping or non-overlapping epitopes. BoNT/F1 was captured by a yeast displayed yeast-displayed scFv, and each Alexa labeled IgG was tested for the ability to simultaneously bind the scFv captured BoNT/F1 (Fig 3). 6F11 and 6F12 share an overlapping epitope, while 6F5.1 and 6F13 had epitopes that did not overlap with each other or with the epitopes of 6F11 and 6F12. Similarly, the HC binding 6F8 and 6F15 were able to bind BoNT/F1 simultaneously, indicating that their epitopes on the HC did not overlap (Fig 3).

**Mouse neutralization assay**

Our previous studies demonstrated that potency of in vivo BoNT neutralization by mAbs was greatest when three mAbs with high affinity and non-overlapping epitopes were combined [21]. A combination of mAbs 6F5.1, 6F11, and 6F13 were chosen for the mouse neutralization assay due to their ability to bind multiple BoNT/F subtypes, their non-overlapping epitopes, and their relatively high affinity when compared with the other mAbs reported here. Although 6F15 is also cross-reactive to all the seven subtypes, it had lower affinity to BoNT/F4, F5 and F6. HBAT was used as a comparator. Various doses of an equimolar combination of the three mAbs (0.5 µg – 100 µg) or the equine antitoxin (10 µg – 300 µg) were incubated with 1 µg of BoNT/F1, F2, F4, or F7; the mixtures were injected into mice, and the animals were monitored.

### Table 3. $K_D$ values of selected IgGs to BoNT/F toxin subtypes (x 10^{-12} M) as determined by flow fluorimetry in a KinExA [28].

| Toxin                  | 6F5.1 | 6F11 | 6F13 |
|------------------------|-------|------|------|
| BoNT/F1 holotoxin      | 90.93 | 1.47 | 327.31 |
| BoNT/F2 holotoxin      | 64.22 | 287.46 | 22150 |
| BoNT/F3 LC-HN          | 78.76 | 52.94 | 2860 |
| BoNT/F4 holotoxin      | 26.38 | 85.64 | 112.17 |
| BoNT/F5 holotoxin      | 152.01 | 12850 | 125.39 |
| BoNT/F6 holotoxin      | 26.67 | 142.56 | 822.63 |
| BoNT/F7 LC-HN          | 204.53 | 430.60 | 458.13 |

https://doi.org/10.1371/journal.pone.0174187.t003
for signs of botulism and mortality. As the antibody-toxin interactions are based on amounts, and not toxicities, of the toxins, an equimolar amount of BoNT/F subtypes (1 μg) was studied rather than an equipotent dose (LD₅₀). A broad range of specific activity of the different BoNT/F subtypes (> 40 fold difference in lethality was seen when comparing LD₅₀ of 1 μg of BoNT/F4 to 1 μg of BoNT/F1) (Table 4). Five micrograms of the mAb combination completely
protected mice that were challenged with 1 μg (20,000 LD₅₀) of BoNT/F1 and 1 μg of the mAb combination protected 8/10 mice, indicating a median effective dose (ED₅₀) of less than 1 μg (Table 4 and Fig 4). For BoNT/F7, the most frequently reported BoNT/F subtype causing human disease, complete protection of a 1 μg (11,000 LD₅₀) challenge dose of BoNT occurred at a mAb dose of 20 μg, with an ED₅₀ of less than 10 μg. The mAb combination was least potent for BoNT/F2, with an ED₅₀ of approximately 50 μg, likely reflecting the lower affinity of mAb 6F13 for this subtype (22 nM) compared to the other BoNT/F subtypes (112–458 pM, Table 3).

The mAb combination was approximately 150 times more potent in neutralizing BoNT/F1 than equine antitoxin on a weight basis and approximately 450 times more potent on a molar basis (IgG molecular weight = 150 kDa; Fab molecular weight = 50 kDa), since 1.0μg of mAbs neutralized 20,000 LD₅₀ of BoNT/F1. In contrast, 50μg of HBAT was needed to neutralize 6600 LD₅₀ of BoNT/F1. This difference would be expected given that only a fraction of polyclonal antitoxins bind the BoNT F1. For BoNT/F2, F4 and F7, the mAb combination also demonstrated much potent neutralizing capacity than HBAT, with the ED₅₀ of <50μg for BoNT/F2, <5.0μg for BoNT/F4 and <10μg for BoNT/F7. By comparison, the range of ED₅₀s of the equine antitoxin for the four BoNT subtypes were more than 100 fold lower than the mAb combination; the lowest ED₅₀ was for BoNT/F7, with 300μg of HBAT rescuing 9 of 10 mice with 344 LD₅₀ of BoNT/F7 challenge but without showing any protection if mice exposed to 11000 LD₅₀ of BoNT/F7 (Table 4 and Fig 4).

**Conclusions**

Using repertoire cloning, a panel of murine scFv antibody fragments were generated that bound one or more of the BoNT/F subtypes. Only one of these lead scFv bound all seven BoNT/F subtypes, reflective of the fact that the subtypes differ by up to 36% at the amino acid level [10]. Using yeast display and the simultaneous staining and sorting of yeast-displayed scFv for binding to multiple subtypes, we significantly increased subtype cross-reactivity and

| BoNT/F subtype | mAbs 6F5.1+6F11+6F13 | Equine antitoxin (HBAT) |
|----------------|-----------------------|-------------------------|
| Experimentally-determined LD₅₀ of 1 μg of toxin | 20000 | 6600¹ |
| Antibody Dose (μg) | Mice surviving/ mice studied | |
| 0.5 | 1/10 | 20–50 |
| 1.0 | 8/10 | 2–5 |
| 2.0 | 8/10 | 5–10 |
| 5.0 | 10/10 | >300² |
| 10 | 8/10 | 10/10 |
| 20 | 10/10 | 0/10 |
| 50 | 7/10 | 0/10 |
| 100 | 7/10 | 2/10 |
| 150 | 10/10 | 0/10 |
| 300 | 10/10 | 0/10 |
| ED₅₀ (μg)³ | 0.5–1.0 | 100 |

¹ BoNT/F1 dose reduced as 50% survival not achieved at maximal HBAT dose with 20,000 LD₅₀ challenge.
² 300 μg of HBAT protected 9 of 10 mice from 1,000 LD₅₀ BoNT/F2 and 344 LD₅₀ of BoNT/F7 challenge.
³ The ED₅₀ is the mAb dose at which greater than 50% of the mice studied survived.

https://doi.org/10.1371/journal.pone.0174187.t004
affinity. As a result, four mAbs were generated that bound all seven BoNT/F subtypes with high affinity. Interestingly, three of the most cross reactive mAbs, 6F11, 6F12 and 6F13, were those that initially only showed measurable binding to three to four of the BoNT/F subtypes. Broadening specificity occurs through a combination of removing steric clashes, increasing complementarity determining region (CDR) flexibility and generating new contacts such as hydrogen bonds and van der Waals interactions while not changing key contact residues [34,35]. For whatever reason, in the examples here that only proved possible with the less cross-reactive lead mAbs.

The mAbs reported here have potential diagnostic and therapeutic utility due to their subtype cross reactivity. The ability to bind all seven BoNT/F subtypes allows sensitive detection...
of all known BoNT/F’s and increases the probability that yet-to-be discovered subtypes will also be detected [29,36]. Therapeutically, the mAbs reported could serve as leads for development of a neutralizing mAb combination, for example via humanization, as has been reported for BoNT/A [22]. While we did not evaluate the ability of the mAbs reported here to neutralize BoNT/F3, F5 and F6, due to lack of availability of the toxins, the mAb affinities for these subtypes would suggest that potent neutralization would be achieved. Compared to anti-toxins generated by animal immunizations, such mAb combinations are more potent, can be engineered to have broader subtype coverage as reported here and are expected to have a longer half-life in vivo reducing the risk of reintoxication [20].

Supporting information
S1 Fig. Analysis of the purity of recombinant BoNT/F domains. A. Cartoon of botulinum neurotoxin primary structure. B. SDS-PAGE analysis of BoNT/F fragments expressed from E. coli BL21 (DE3). The upper panel shows the expression of BoNT/F LC-HN fragments fused with a 6 x His tag (~110Kd). C. Expression of BoNT/F HC fragments fused with a 6 x His tag (BoNT/F1 HC and BoNT/F7 HC, (~50Kd) or fused to a maltose binding protein (MBP) tag (BoNT/F2, F3, F4, F5 and F6 HC, (~80Kd).

S2 Fig. Cross reactivity of selected mAbs against BoNT/F subtypes. Yeast displayed scFv were incubated with BoNT/F LC-HN fragments (scFv 4E17, 46E12, 46.25 and 47A120) or were incubated with BoNT/F HC fragments (6F8 and 28H4) and then an Alexa-647 labeled secondary IgG was used for binding detection. An Alexa-488 labeled anti-SV5 IgG was used to detect scFv mAb expression on yeasts. Binding to BoNT/F4 was measured using crude BoNT/F4 culture supernatant due to the absence of recombinant domains.

S3 Fig. Domain specificity of cross-reactive mAbs. Yeast displayed BoNT/F1 LC, HN, LC-HN or HC was incubated with Alexa-647 labeled IgG and Alexa-488 labeled anti-SV5 IgG.

S1 Table. Characteristics of yeast display libraries used for BoNT/F mAb generation.

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