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Selection and characterization of a human monoclonal neutralizing antibody for *Clostridium Botulinum* neurotoxin serotype B

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ABSTRACT

Botulinum neurotoxins (BoNTs) are causative agents for botulism and are identified as a category A bioterror agents by the Centers for Disease Control and Prevention (CDC). Current antitoxins against BoNTs intoxication have some limitations including side effects or limited supply. As an alternative, neutralizing monoclonal antibodies will play an increasing role as BoNTs therapeutics. To date, no human anti-BoNT/B neutralizing monoclonal antibodies have yet to be reported. Herein, we describe an improved selection approach and characterization of a human monoclonal antibody, F2, which is capable of binding BoNT/B with high specificity and displays neutralizing activity in an in vitro cell-based assay. Through surface plasmon resonance studies, we have determined its association and dissociation rate constants. In sum, our data demonstrate that monoclonal antibody F2 is a promising BoNT/B therapeutic lead for further development.

Botulinum neurotoxins (BoNTs) are produced by various strains of the anaerobic bacterium, *Clostridium Botulinum,* and are classified into seven serotypes based on their distinct antigenicity (designated as serotype A-G).[1] They are causative agents of botulism, which is characterized by flaccid paralysis, and are the most potent toxins known to humans. Botulinum neurotoxins/A, /B, and /E account for nearly all recorded cases of human botulism, and almost all infant botulism in the United States results from either BoNT/A or BoNT/B.[2] These neurotoxins have a similar structure, consisting of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) linked by a disulfide bond.[1] The HC is mainly involved in the cell-binding, internalization and translocation. More specifically, the 50 kDa C-terminal portion of the heavy chain (H_C) is believed to preferentially target the BoNTs to the peripheral presynaptic termini at the neuromuscular junction.[3] Once engulfed inside a neuronal cell, the N-terminal half of the HC (H_N) facilitates translocation of the LC into the cytosol.[4,5] The LC domain is a group of Zinc-dependent endoproteases[6] that specifically cleave SNARE proteins (SNAP-25, VAMP, and syntaxin) that are essential for release of the neurotransmitter acetylcholine.

Due to their extreme potency and high lethality, BoNTs are classified as one of the six highest-risk threat agents for bioterrorism by the US Centers for Disease Control and Prevention (CDC). Currently, human or equine antisera is considered the most effective immunotherapeutic for BoNT exposure,[7,8] however either supply is a limiting factor or severe side effects[9] (e.g., allergic reactions, serum sickness, and anaphylaxis) are problematic. Advancements in monoclonal antibody (mAbs) generation/engineering has overcome these barriers by providing highly specific human antibodies with unlimited supply, reduced allergic effects, and improved pharmacological properties.[10]

Phage display is a powerful technique in which peptides or proteins can be expressed on the surface of bacteriophage and selected against a target antigen. In general, phage display method has been proven to be a fast, cost-effective alternative for mAb generation. Attractively, these selected mAbs can be easily manipulated to improve their affinity or converted into various antibody formats based on clinical utility. Additionally, if human antibodies are desired, a human antibody gene repertoire as the source for phage display libraries, that is, antibodies with human origin can be directly isolated and applied to downstream clinical trials, bypassing tedious humanization procedures. Here, we report the use of a human naïve scFv-phage display library for the production of human neutralizing mAbs against BoNT/B.

BoNT H_C domain contains regions thought to bind to presynaptic neuronal receptors, the first requisite step for intoxication, and results in protective immunity when used as an immunogen.[11,12]

Abbreviations: scFv, single-chain variable fragment; Fab, antigen binding fragment; mAb, monoclonal antibody; VMP2, vesicle-associated membrane protein 2; IPTG, isopropyl-β-D-thiogalactopyranoside.

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Hence, we used BoNT/B H_C domain as antigen to screen BoNT/B neutralizing antibodies. BoNT/B H_C domain (1088–1295) was prepared by PCR and subsequently inserted into the region between the NdeI and NotI sites on pET28b vector (Novagen). Rosetta2 (DE3) *Escherichia coli* (Novagen) were transformed with the recombinant plasmid and amplified in SB medium to an OD600 of approximately 0.6–0.8. The culture was induced for expression with 0.1 mM IPTG and was incubated at 25 °C overnight. The overnight culture was centrifuged at 5000*g*, 4 °C for 10 min; while cell pellets were resuspended in 40 mL PBS buffer supplemented with protease inhibitor (phenylmethylsulfonyl fluoride) and were lysed with a sonicator. The lysate was centrifuged at 35,000*g*, 4 °C for 20 min and the BoNT/B H_C domain protein within the supernatant was purified with a Ni–NTA resin column by immobilized metal affinity chromatography.

The construction of a human naïve scFv-phage display library has been described previously.[13] To reduce the susceptibility of BoNT denaturation on a solid phase surface,[14] we developed a solution phase selection approach for panning. In brief, phage library panning was performed on Ni–NTA resin coated with purified N-terminal hexahistidine tag BoNT/B H_C domain and blocked with Blocker™ Casein in PBS (Pierce). Upon incubation with the scFv-phage library, the Ni–NTA resin was washed 5 times with PBS and bound phage was eluted with 1 mL elution buffer (300 mM imidazole in PBS). The eluted phage was used to infect log-phase *E. coli* TG1. Titration of the phage libraries, phage rescue and preparation were performed as described previouly.[15]

Upon completion of the fourth round of panning, 96 single colonies were randomly picked for scFv-phage amplification and ELISA analysis. BoNT/B H_C was diluted to 5 μg/mL in PBS and immobilized on Costar ELISA plates (Corning) at 4 °C overnight. After blocking for 1 h at room temperature, the plates were incubated with diluted phage, which were at a concentration of 1:1 in Blocker™ Casein. Upon washing with PBS, the plates were incubated with 1:1000 diluted horseradish peroxidase (HRP)-conjugated mouse anti-M13 antibody (Amersham) and detected with TMB substrate (Pierce). The reaction was quenched with an equal volume of 2 M H_2SO_4 and the optical density was determined at 450 nm. A total of 36 clones were identified positive, of which five clones were unique in sequence. The binding ability and the specificity to the holotoxins of these five scFvs were further determined by ELISA. The results indicated scFv C4 and F2 can bind to the BoNT/B holotoxin with good specificity (Fig. 1).

Because scFv molecules are typically unstable, in addition to being prone to aggregation, we converted the selected scFv's into a more stable Fab format for further assay analysis. The construction and the expression of Fabs were conducted as previously described,[16] while the Fabs were purified by two-step affinity chromatography (Ni–NTA, Qiagen and GammaBind™ G Sepharose™, GE). The ELISA data clearly demonstrated that purified Fab C4 and F2 retained their binding activity and specificity (Fig. 2). The other Fabs bound to BoNT/B H_C, but unfortunately did not bind to the holotoxin (data not shown).

Recently, we have shown that BoNTs are susceptible to polystyrene induced denaturation,[14] thus, to confirm whether these selected antibodies recognize native toxin, a dot blot assay was performed with purified Fab samples. A series of 0.1 mg/mL BoNTs, BoNT/B H_C and BSA was blotted onto nitrocellulose membrane strips in a volume of 1 μL per spot. The membrane strips were air-dried and blocked in 4% skimmed milk in PBS. Fabs were diluted with 0.05% Tween 20 in PBS (PBS-T) and incubated with the membrane strips for 1 h at room temperature. Upon washing with PBS-T, the membrane strips were incubated with 1:1000 HRP-conjugated goat anti-human Fab antibody (Sigma). The binding was detected using WestDura substrate (Pierce). In this format Fab C4 and F2 bound to the native BoNT/B (Fig. 2). For the other Fabs, no significant signal could be seen on holotoxin, yet, a strong signal was seen with the H_C domain. This may imply that epitopes recognized by these Fabs are located at the interface between translocation domain and the neuron-specific binding domain, which is not exposed in the native holotoxin.

Binding kinetics of Fab C4 and F2 was analyzed using surface plasmon resonance (SPR) on a BIAcore™ 3000 instrument (Biacore Ab, Uppsala, Sweden) at 25 °C. To accomplish this task Fab C4 and F2 were immobilized on CM5 sensor chip and targeted to a 300 RU setting using standard NHS/EDC coupling methodology. All measurements were conducted in HBS-EP buffer with a flow rate of 30 μL/min. BoNT/B holotoxin was diluted so as to obtain a series of concentrations between 15.82–250 nM. The concentration series were injected individually into the flow cell in triplicates, double referenced, and 4 M $MgCl_2$ was used to regenerate the chip for each round of analysis. The association and dissociation rate constants (k_a, $M^{-1}\,s^{-1}$ and k_d, s^{-1}), and affinity (K_D, M) were determined to be $2.33 \times 10^4\,M^{-1}\,s^{-1}$, $7.5 \times 10^{-3}\,s^{-1}$, and 3.2×10^{-7} M for Fab C4, and $3.3 \times 10^4\,M^{-1}\,s^{-1}$, $0.013\,s^{-1}$, and 4.0×10^{-7} M for Fab F2 using a 1:1 (Langmuir) binding fitted model. We note that the affinities found for Fab C4 and F2 are only moderate. However, such binding affinity is reasonable when panning a naïve library, which is constructed from "hosts" that were never exposed to a particular antigen.[17–19]

The neutralizing potency of Fab C4 and F2 against BoNT/B was evaluated by a sensitive in vitro cell-based assay with primary rat spinal cord (RSC) cells. Primary RSC cells were prepared[20] and seeded into the wells of collagen coated 96-well plates (BD Biosciences) at a density of 75,000 cells per well. The cells were allowed

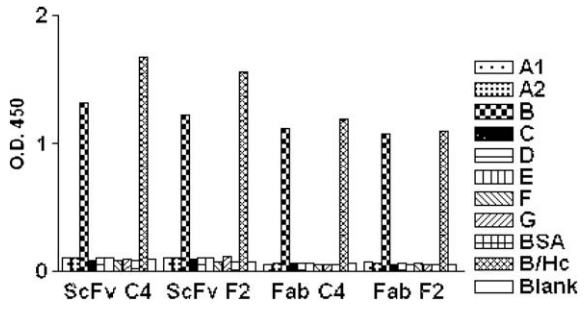

Figure 1. Cross-reaction ELISA. ScFvs or purified Fabs were added to an ELISA plate coated with BoNTs, BoNT/B H_C, BSA or a blank control. Data shown were from one representative experiment, which was repeated a total of 3 times. The figure demonstrates that scFv C4 and F2 have good specificity for BoNT/B and the purified Fabs retain their binding affinity and specificity.

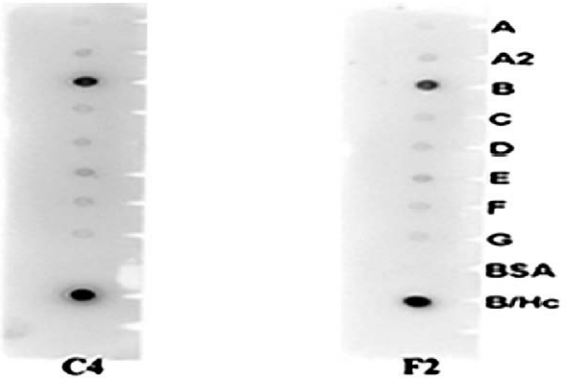

Figure 2. Dot blot assay. 0.1 μg BoNTs, BoNT/B H_C domain, together with BSA were spotted on nitrocellulose membrane strips. Fab F2 were incubated with the membrane strips and the bound Fabs were detected with an anti-Fab HRP conjugate.

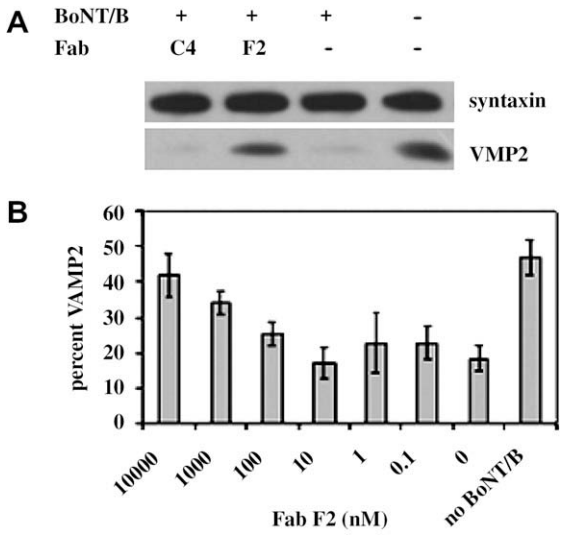

Figure 3. In vitro cell assay. Primary rat spinal cord cells were incubated with BoNT/B for 48 h in the presence or absence of Fab C4 or F2. (A) Protection of VAMP2 cleavage was detected when Fab C4 or F2 presence. Figure shown was from one representative experiment repeated a total of 3 times. (B) Dose and effect of Fab F2. Identical results were obtained for the 4 replicate sets of samples.

to mature for at least 19 days prior to use in the toxin assays. In order to determine whether these Fab fragments are able to neutralize BoNT/B activity in the RSC cell assay, 3 μM of Fab C4 or F2 were combined with 67 pM (500 pg) of BoNT/B in 50 μL of culture medium and incubated at 37 °C for 1 h. For the positive control, only BoNT/B was added, and for the negative control only culture medium was used. After the 1 h pre-incubation period, the Fab and/or BoNT/B mixtures were added to the 96-well plate containing RSC cells and incubated for 48 h at 37 °C, 5% CO_2, in a humidified atmosphere. Upon removal of the media, the cells were then lysed in 100 μL of 1 × LDS sample buffer (Invitrogen) and analyzed for VAMP cleavage by Western blot,[20] using an anti-VAMP antibody and an anti-syntaxin antibody (Synaptic Systems), where syntaxin was utilized as a control. Since Western blot analysis can only detect the disappearance of the VAMP2 band, and no cleavage product, the syntaxin control is essential to validate that the differences in VAMP band density were not caused by the variance of cell lysates. The VAMP2 bands were totally cleaved in the positive control (toxin only) as well as the sample incubated with Fab C4 (Fig. 3A). This clearly indicated that the Fab C4 did not protect VAMP2 from BoNT/B cleavage. We surmise this lack of protection is due to binding to a "non-neutralizing" epitope on BoNT/B. Gratifyingly, the difference in density of the VAMP2 band with Fab F2 versus the negative control (no toxin) was minimal. This, thus demonstrated that Fab F2 provided significant protection against BoNT/B toxicity. In order to determine the minimum inhibitory concentration of Fab F2, 10-fold serial dilutions of the Fab F2 were tested in the range of 10 μM to 0.1 nM with 100 pM BoNT/B (750 pg), all in replicates of four (Fig. 3B). These results indicate that a 1000-fold molar excess of the Fab F2 was required for inhibition of BoNT/B activity. This suggests that this Fab possesses weak neutralization activity, with possible causes due to: (1) a requirement of more B-type than A-toxin in the cell assay, and thus a lower sensitivity for evaluation of anti-BoNT/B antibodies. (2) A moderate affinity of the antibody for the holotoxin. Indeed, the latter scenario goes hand-in-glove with Georgiou's finding indicating that protection activity by an antibody correlates with its antigen affinity.[21] Affinity maturation of Fab F2 combined with phage display is in progress to obtain an improved neutralizing antibody with higher affinity.

In conclusion, we have demonstrated that human mAbs that specifically bind to BoNT/B can be discovered using a solution phase panning approach from naïve phage display libraries. The data we have presented suggest that mAb F2 may prove to be promising lead for the development of human therapeutics for BoNT/B intoxication.

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