Affinity maturation of human botulinum neurotoxin antibodies by light chain shuffling via yeast mating

J. Lou1, L. Geren1, C. Garcia-Rodriguez1, C. M. Forsyth1, W. Wen1, K. Knopp1, J. Brown2, T. Smith2, L. A. Smith2 and J. D. Marks3

1Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco Rm 3C-38, NH, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110 and 2Toxinology Division, USA/RID, Frederick, MD 21702, USA

3To whom correspondence should be addressed.
E-mail: marksj@anesthesia.ucsf.edu

Received December 22, 2009; revised December 22, 2009; accepted December 26, 2009

Edited by Andrew Bradbury

Botulism is caused by the botulinum neurotoxins (BoNTs), the most poisonous substance known. Because of the high potency of BoNT, development of diagnostic and therapeutic antibodies for botulism requires antibodies of very high affinity. Here we report the use of yeast mating to affinity mature BoNT antibodies by light chain shuffling. A library of immunoglobulin light chains was generated in a yeast vector where the light chain is secreted. The heavy chain variable region and the first domain of the constant region (VH–CH1) from a monoclonal antibody was cloned into a different yeast vector for surface display as a fusion to the Aga2 protein. Through yeast mating of the two haploid yeasts, a library of light chain-shuffled Fab was created. Using this approach, the affinities of one BoNT/A and two BoNT/B scFv antibody fragments were increased from 9- to more than 77-fold. Subcloning the V-genes from the affinity-matured Fab yielded fully human IgG1 with equilibrium binding constants for BoNT/A and BoNT/B of 2.51 × 10^−11 M or lower for all three monoclonal antibodies. This technique provides a rapid route to antibody affinity maturation.

Keywords: antibody engineering/botulinum neurotoxin/FACS/molecular evolution/yeast mating

Introduction

Botulism is caused by botulinum neurotoxin (BoNT), the most poisonous substance known (Gill, 1982). Human botulism is caused by BoNT serotypes A, B, E and F and is characterized by flaccid paralysis which, if not fatal, requires prolonged hospitalization in an intensive care unit and mechanical ventilation. Besides causing naturally occurring botulism, BoNTs are also classified by the Centers for Disease Control and Prevention as one of the six highest risk threat agents for bioterrorism (Arnon et al., 2001). Both Iraq and the former Soviet Union produced BoNT for use as weapons (Bozheeva et al., 1999) and the Japanese cult Aum Shinrikyo attempted to use BoNT for bioterrorism (Arnon et al., 2001).

As a result of these threats, there is an urgent need for rapid and very sensitive diagnostic assays that can detect BoNTs, as well as therapies that are safe, effective and can be produced in large quantities for stockpiling. There are a number of assays under development, and many of these rely on high-affinity polyclonal or mAbs (Varnum et al., 2006; Kalb et al., 2008, 2009). The mainstay of treatment for botulism is poly- or oligoclonal antitoxin (Franz et al., 1993; Nowakowski et al., 2002). Given the extraordinary toxicity of BoNTs, antitoxin must be of high potency. Thus, mAbs are an important resource for the detection, diagnosis and treatment of botulism (Amsdersorfer et al., 1997, 2002). Such mAbs should ideally be of high affinity for more sensitive BoNT detection and more potent BoNT treatment (Nowakowski et al., 2002; Razai et al., 2005; Garcia-Rodriguez et al., 2007).

In vitro antibody display technologies, such as phage and yeast display, have been widely utilized to increase antibody affinity (reviewed in Marks and Marks, 1996; Bradbury and Marks, 2004). Although initial reports utilized phage display (Clackson et al., 1991; Marks et al., 1992), yeast display has become widely utilized due to its ability to finely discriminate between mutants of close affinity (Boder and Wittrup, 1997; Boder et al., 2000). Antibody variable region genes (V-genes) are diversified either randomly (Hawkins et al., 1992) or using spiked oligonucleotides (Yang et al., 1995; Schier et al., 1996a, b), and higher affinity mutants are selected using various types of affinity chromatography or flow cytometry. The introduction of mutations by shuffling the wild-type light chain variable region gene (V_L, light chain shuffling) has a number of attractive advantages (Marks et al., 1992; Figini et al., 1994; Schier et al., 1996a, b). First, when the wild-type antibody is isolated via combinatorial libraries constructed from immune sources, the probability that the original VH–V_L pairing is recapitulated is virtually zero. Second, naturally occurring V-genes are utilized, which might reduce immunogenicity or improve stability.

We recently reported the use of yeast display to generate very large combinatorial Fab libraries by first creating separate light chain and VH–CH1 libraries and then combining the libraries by yeast mating (Weaver-Feldhaus et al., 2004). This approach was used to isolate lead BoNT antibodies from the RNA of immunized donors. Here we report the modification and use of this approach to create light chain-shuffled libraries and their use to rapidly affinity mature three different BoNT antibodies to the low picomolar range.

Abbreviations: BoNT/A, botulinum neurotoxin serotype A; BoNT/B, botulinum neurotoxin serotype B; BoNT/A/HN, botulinum neurotoxin serotype A translocation domain, BoNT/A/LC, botulinum neurotoxin serotype A catalytic domain; CDR, complementary determining region; Fab, antigen-binding fragment of immunoglobulin with variable domain and first constant domain; FACS, fluorescent-activated cell sorting; IgG, immunoglobulin G; K_D, dissociation equilibrium constant; k_on, association rate constant; k_off, dissociation rate constant; mAb, monoclonal antibody; PBS, phosphate buffered saline; PCR, polymerase chain reaction; scFv, single-chain format of antibody variable regions; VH, heavy chain variable region; VL, light chain variable region.

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Table 1. Equilibrium dissociation constants ($K_D$) for wild-type and affinity-matured yeast displayed BoNT antibodies in both scFv and Fab formats were measured by flow cytometry.

<table>
<thead>
<tr>
<th>Clone</th>
<th>BoNT $K_D$ by FACS ($\times 10^{-12}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>scFv</td>
</tr>
<tr>
<td>BoNT/A1</td>
<td></td>
</tr>
<tr>
<td>BoNT/A2</td>
<td></td>
</tr>
<tr>
<td>ING1</td>
<td>2G11</td>
</tr>
<tr>
<td>B6</td>
<td>B6.1</td>
</tr>
<tr>
<td>B11</td>
<td>B11E8</td>
</tr>
<tr>
<td></td>
<td>2714</td>
</tr>
<tr>
<td></td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>2620</td>
</tr>
<tr>
<td></td>
<td>193</td>
</tr>
</tbody>
</table>

NM, not measurable.

Results

Antibodies selected for affinity maturation by light chain shuffling

One BoNT/A and two BoNT/B scFv antibodies were chosen for affinity maturation. The BoNT/A scFv antibody ING1 was selected from an immune scFv yeast display library constructed from a human donor immunized with pentavalent botulinum toxoid. ING1 binds the BoNT/A1 translocation domain (H$_N$) with an equilibrium dissociation constant ($K_D$) of $5.28 \times 10^{-9}$ M as a yeast-displayed scFv (Table I) and binds the BoNT/A2 subtype with comparable affinity. Two BoNT/B scFv antibodies (B6 and B11) were also selected from an immune scFv yeast display library constructed from a human donor immunized with pentavalent botulinum toxoid. B6 binds the BoNT/B1 light chain (L$_C$) with a $K_D$ of $2.7 \times 10^{-9}$ M and B11 binds the BoNT/B2 H$_N$ with a $K_D$ of $2.6 \times 10^{-9}$ M as yeast-displayed scFv (Table I). Both scFv bind the BoNT/B1 subtype with comparable affinities to the binding of the BoNT/B1 subtype.

Generation of BoNT Fab from the V-genes of scFv by yeast mating

To determine the feasibility of affinity maturing ING1, B6 and B11 by yeast mating, yeast-displayed Fab was constructed, and the display level and $K_D$ for BoNT were determined. The strategy used to generate the Fab is shown in Fig. 1. The $V_L$ gene from each of the three scFv was PCR amplified and cloned directly into Saccharomyces cerevisiae strain JAR300 by gap repair into BamHI- and NheI-digested pPNL20s. This fuses the $V_H$ gene to the $C_L$ gene and results in secretion of the light chain. The $V_L$ gene from each of the three scFv was PCR amplified and cloned directly into S.cerevisiae strain YVH10 by gap repair into XhoI- and BsiWI-digested pPNL30s. This fuses the $V_L$ gene to the $C_L$ and results in secretion of the light chain. To create the Fab, S.cerevisiae JAR300 (a-mating type) containing the relevant $V_{H_1} - C_{H_1}$ in pPNL20s was mixed with S.cerevisiae YVH10 (a-mating type) containing the relevant light chain in pPNL30s and the resulting diploid yeast was selected on uracil, tryptophan plates (Fig. 1).

Fab display was induced and the display level was measured by flow cytometry using anti-SV5 antibody which bound a C-terminal epitope tag on the kappa constant region. Binding to BoNT was also quantitated by flow cytometry. All three scFv were well displayed on yeast and bound BoNT (Table I and Fig. 2). In contrast, the three Fabs were poorly displayed: B6 Fab showed minimal surface display, B11 Fab showed better display than B6, but of only a minority of the yeast population, and ING1 showed no detectable surface display (Fig. 2). For all three Fabs, levels of $V_{H_1} - C_{H_1}$ display [as quantitated using an antibody (anti-myc) binding a C-terminal epitope tag] were comparable to those of the Fab quantitated by measuring the presence of the kappa light chain (data not shown). With respect to BoNT binding, for B11 the population of yeast that showed surface display bound BoNT, whereas no detectable binding could be detected for the poorly displayed B6 Fab. For these two Fabs, the poor BoNT binding and display levels precluded the ability to measure a binding constant. ING1 Fab, despite showing no quantifiable Fab surface display, bound BoNT. The reasons for this are unclear, but could represent greater sensitivity of BoNT binding detection, or perhaps inaccessibility of the SV5 epitope tag [although display was also not detected using anti-kappa constant region antibody (data not shown)]. To determine whether this could be explained by the fact that the $V_{H_1} - C_{H_1}$ alone bound BoNT and there was no light chain present, the $V_{H_1} - C_{H_1}$ only was displayed on the yeast surface; no BoNT binding was detected (data not shown). ING1 Fab bound BoNT/A1 and BoNT/A2 with affinities ~4-fold lower than the parental scFv (Table I). This difference may partly reflect the inability to only gate the Fab displaying yeast population, as can be done for the scFv. Generally, only 50% of the yeast will show surface display ( Razai et al., 2005).

Generation and sorting of Fab chain-shuffled libraries

We hypothesized that the poor surface display and antigen binding of the three Fabs could be due to light chain sequences that resulted in poor light chain folding and Fab secretion and subsequent display. Such effects could be solved by replacing the wild-type light chain with alternatives, as occurs during light chain shuffling. We therefore proceeded to light chain shuffling of the three Fabs. The strategy shown in Fig. 1 was used to create ING1, B6 and B11 light chain-shuffled libraries. A library of human kappa and lambda light chains was cloned directly into the vector pPNL30s in S.cerevisiae strain YVH10 by gap repair. The resulting light chain library contained $5 \times 10^7$ transformants containing a light chain insert and was diverse as determined by DNA sequencing. To create Fab light chain-shuffled libraries, S.cerevisiae JAR300 (a-mating type) containing the relevant $V_{H_1} - C_{H_1}$ (ING1, B6 or B11) in pPNL20s was mixed with S.cerevisiae YVH10 (a-mating type) containing the light chain library in pPNL30s and the resulting diploid yeast selected on uracil, tryptophan plates. The number of diploid yeast colonies was at least 100 times greater than the size of the light chain-shuffled library, suggesting that the light chain library diversity was captured in the chain-shuffled library. Analysis of 12–48 colonies from each mating indicated that each had the expected wild-type $V_{H_1}$ gene and a different $V_L$ gene. Approximately 30% of the
clones expressed an Fab on the yeast surface, as determined by staining with anti-SV5 antibody. Higher affinity chain-shuffled Fabs were isolated by FACS. For each of the three chain-shuffled libraries, Fab expression was induced and yeast stained with 5·0 × 10^−27 M (50 nM) BoNT/A1 or BoNT/B1 for the first two rounds of sorting with the majority of BoNT binding yeast collected (Fig. 3). Subsequent rounds of sorting were increasingly stringent with the antigen concentration decreased and < 1% of the yeast collected (B11 sorting shown as an example in Fig. 3). A total of six rounds of sorting were performed for each Fab library, after which the sort output was plated for characterization of individual yeast displayed Fab. Ninety-six clones were randomly picked into 96-well microtiter plates from each of the three Fab sortings, and Fab expression and BoNT binding quantitated using a single antigen concentration (5·0 × 10^-10 M, 500 pM). Although almost 100% of the picked clones showed a positive BoNT binding signal, the mean fluorescence intensity (MFI) varied significantly among them.
Twelve individual clones with the highest MFI were chosen for further analysis.

**Characterization of chain-shuffled Fab clones**

The V_H and V_L genes were PCR amplified from the three sets of 12 yeast colonies and the V_H and V_L genes sequenced. For each of the three chain-shuffled libraries, sequence analysis revealed a single V_H gene (the same as the original V_H gene) paired with different V_L genes (Table II). Affinities (K_D) of the yeast displayed Fab were measured by flow cytometry for BoNT/A1 or BoNT/B1 and compared with the affinities of the parental scFv (Table II). Overall the affinities of the best ING1, B6 and B11 chain-shuffled Fab increased from 9- to 77-fold compared with their parental
scFv, with yeast displayed $K_D$ ranging from 34 to 1645 pM (Table II, Fig. 5). The level of surface Fab display for the affinity-matured Fab was also markedly improved compared with the wild-type parental Fab (Fig. 2).

In the case of the BoNT/A antibody ING1, seven unique Fabs were identified which had affinities 3- to 26-fold higher than the parental ING1 scFv (Table II). All seven had $V_L$ genes derived from the same $V_{k1}$ germline gene family and five of the seven were derived from the same IGKV1–39*01 germline gene. Compared with the parental $V_L$ gene, the $V_k$ genes from the affinity-matured Fab had 6–16 amino acid substitutions (Table II). In the case of the BoNT/B antibody B11, six unique Fabs were identified which had affinities 15- to 77-fold higher than the parental B11 scFv (Table II). Three of the six had $V_L$ genes derived from the same $V_k1$ germline gene family as the parental $V_k$ gene; however, three had $V_k$ genes derived from the $V_k3$ family. The $V_k$ genes from the affinity-matured Fab had 16–33 amino acid substitutions compared with the parental $V_k$ gene (Table II).

**Generation and characterization of IgG constructed from the $V$-genes of Fab**

To determine whether the affinities observed for the Fab were recapitulated in IgG, and to generate antibodies for the diagnosis and treatment of botulism, we converted 2G11, B6.1 and B11E8 to human IgG1/kappa antibodies. IgGs were expressed from stable CHO cell lines, purified using protein G and solution KD for BoNT/A or BoNT/B subtype measured by flow fluorimetry. The affinities of the three IgGs ranged from 25.1 to 6.59 pM for BoNT/A1 or BoNT/B1 and were increased from 4.85- to 42-fold compared with those measured for the yeast-displayed Fab (Table III). We have observed comparable increases in affinities for yeast-displayed scFv converted to IgG (Razai et al., 2005). 2G11 bound three BoNT/A subtypes (BoNT/A1, A2 and A3) with comparable, and high, affinity. B6.1 bound four BoNT/B subtypes (BoNT/B1, B2, B3 and B4) with comparable, and high, affinity. In contrast, B11E8 only bound BoNT/B1, B2 and B3 (Table III).

**Discussion**

We report here the use of yeast mating to rapidly generate yeast surface display chain-shuffled Fab libraries from which affinity-matured Fab can be isolated by FACS. Three separate Fabs derived from scFv were affinity matured, with the average affinity increasing 37-fold. The mating system allows a simple way to rapidly construct large chain-shuffled Fab libraries without dependence on transformation efficiency. Poor surface display levels of the three Fabs constructed from the parental scFv genes did not prevent successful affinity maturation of each of the three Fabs. Interestingly, each of the matured Fab had markedly higher surface display levels than each of the parental Fab from which they were derived. The reason for the improved display is unclear; however, each matured Fab had many mutations in the framework regions as well as the CDRs. Two of the three affinity-matured Fabs used different $V$-genes or different $V$-gene families than the parental Fab. It is possible that these changes led to better $V_H$–$V_k$ packing and better surface display (Rothlisberger et al., 2005). The improved surface display was not due to mutations in the $C_k$ or $C_H1$ gene, which was the same for each of the affinity-matured Fabs.

There are many options for diversifying $V$-genes for affinity maturation, including the random introduction of mutations, for example, by error-prone PCR, CDR mutagenesis using spiked oligonucleotides and chain shuffling. However, affinity maturation by chain shuffling should be an optimal approach for immune repertoires since it is statistically improbable that the original immune cognate $V_H$–$V_L$ pair is recreated in the
primary immune library (Clackson et al., 1991). Combining chain shuffling with the stringent selection made possible by yeast display and flow cytometry should isolate the highest affinity antibodies present. Light chain shuffling has been previously used to increase the affinity of immune and non-immune antibody fragments using phage display, but not by using yeast display (Clackson et al., 1991; Kang et al., 1991; Marks et al., 1992; Figini et al., 1994). Yeast mating and display have been used, however, to combine libraries of randomly mutated V\textsubscript{H} and V\textsubscript{L} genes in the Fab format (Blaise et al., 2004). For protein-binding antibody fragments, there are few examples where the light chain shuffling has been used for antibodies from immune repertoires using any type of display technology. When applied to antibody fragments

Table II. Characteristics of affinity-matured BoNT antibodies

<table>
<thead>
<tr>
<th>Clone name</th>
<th>Amino acid sequence of V\textsubscript{L} CDRs</th>
<th>Number of mutations from wild-type V\textsubscript{L} gene</th>
<th>Germline family gene</th>
<th>BoNT K\textsubscript{D} by FACS (\times 10\textsuperscript{{12}} M\textsuperscript{\textdegree})</th>
<th>K\textsubscript{D} wild-type/BoNT/1 matured</th>
</tr>
</thead>
<tbody>
<tr>
<td>ING1</td>
<td>RASQ15IY1AN RASQ15IY1AN</td>
<td>0</td>
<td>IGKV1-39*01</td>
<td>5284</td>
<td></td>
</tr>
<tr>
<td>2G11</td>
<td>------------H --------------- ---------------</td>
<td>12</td>
<td>IGKV1-39*01</td>
<td>205</td>
<td>26</td>
</tr>
<tr>
<td>1D11</td>
<td>------------H --------------- ---------------</td>
<td>12</td>
<td>IGKV1-39*01</td>
<td>420</td>
<td>12</td>
</tr>
<tr>
<td>IC1</td>
<td>------------H --------------- ---------------</td>
<td>6</td>
<td>IGKV1-39*01</td>
<td>890</td>
<td>6</td>
</tr>
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<td>2B10</td>
<td>------------H --------------- ---------------</td>
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<tr>
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<td>------------H --------------- ---------------</td>
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<td>IGKV1-39*01</td>
<td>290</td>
<td>18</td>
</tr>
<tr>
<td>SC1</td>
<td>------------H --------------- ---------------</td>
<td>16</td>
<td>IGKV1-27*01</td>
<td>710</td>
<td>7</td>
</tr>
<tr>
<td>5G4</td>
<td>------------H --------------- ---------------</td>
<td>14</td>
<td>IGKV1-27*01</td>
<td>430</td>
<td>12</td>
</tr>
</tbody>
</table>

Clone name, location of mutations in the V\textsubscript{L} CDRs, number of mutations between the wild-type and affinity-matured V\textsubscript{L} and the germline gene family and germline gene of origin are indicated. Equilibrium dissociation constant (K\textsubscript{D}) was measured by flow cytometry.

Fig. 5. Affinities of wild-type and affinity-matured yeast-displayed Fab and scFv. Equilibrium binding constants (K\textsubscript{D}) were determined for yeast-displayed Fab and scFv binding to purified BoNT/A1 or purified BoNT/B1 by flow cytometry.
from non-immune libraries, the increases in affinity have been relatively modest, in the range of 2- to 8-fold range (Osbourn et al., 1996; Schier et al., 1996a, b), with the largest reported increase in affinity 15-fold (Huls et al., 2001).

The magnitude of the increase in affinity from chain shuffling did not appear to be dependent on whether the source of the V\(_L\) genes was from the same donor as the initial V\(_H\)–V\(_L\) gene pairing or not. Presumably, light chain diversity of the V\(_L\) genes was from the same donor as the initial V\(_H\)–V\(_L\) gene pairing. Since it did not appear to be dependent on whether the source did not appear to be dependent on whether the source.

In conclusion, we describe a simple and rapid way to affinity mature scFv or Fab antibodies using light chain shuffling by yeast mating and surface display. This approach should be applicable to lead antibodies generated from both immune and non-immune display libraries. In the example provided here, three very high affinity antibodies were generated binding most or all subtypes of BoNT/A and BoNT/B. These specific antibodies should prove useful for both diagnostic and therapeutic applications.

### Materials and methods

#### Oligonucleotide primers

**Primers for V\(_L\) library construction.** For cloning human V\(_L\) genes into pPNL30s vector to make the V\(_L\) library: HuVK1aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTGCTC-3'; HuVK2aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'; HuVK3aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'; HuVK4aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'; HuVK5aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'; HuVK6aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'; HuVK7aBACKFabGap: 5'-ATCTCTCGAAAGAGAGGAAGTGCCGTGCTGAGTGCCGCTTTGAGCTGCTTGCTGCTC-3'.

**Other primers.** For subcloning the V\(_H\) gene of ING1, B6 or B11 into pPNL20s vector, the following specific primer pairs were used: ING1VHBACKFabGap (for ING1 and B6) 5'-AAGGCTTCTTGTGACAAGAACACCTGAGCTTTACCGAGGTCCAGCTGGTACGCTGGT-3'; ING1VHBACKFabGap (for B11) 5'-AAGGCTTCTTGTGACAAGAACACCTGAGCTTTACCGAGGTCCAGCTGGTACGCTGGT-3'; ING1VHBACKFabGap (for B11) 5'-AAGGCTTCTTGTGACAAGAACACCTGAGCTTTACCGAGGTCCAGCTGGTACGCTGGT-3'; B6VHFABFabGap (for B6 and B11) 5'-GGCCCTTTGGTCTGCTGCACTGGAGGACGGTACGGTGACTGGAGGAGACGCAGTTTGCACTGGGTATGCTGCTGCTC-3'; B6VHFABFabGap (for B6 and B11) 5'-GGCCCTTTGGTCTGCTGCACTGGAGGACGGTACGGTGACTGGAGGAGACGCAGTTTGCACTGGGTATGCTGCTGCTC-3'; B6VHFABFabGap (for B6 and B11) 5'-GGCCCTTTGGTCTGCTGCACTGGAGGACGGTACGGTGACTGGAGGAGACGCAGTTTGCACTGGGTATGCTGCTGCTC-3'; B6VHFABFabGap (for B6 and B11) 5'-GGCCCTTTGGTCTGCTGCACTGGAGGACGGTACGGTGACTGGAGGAGACGCAGTTTGCACTGGGTATGCTGCTGCTC-3'.

### Table III. Solution equilibrium binding constants for IgG constructed from affinity-matured BoNT Fab

<table>
<thead>
<tr>
<th>Clone</th>
<th>IgG affinity by KinExA (K_D (\times 10^{-12} \text{M}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BoNT/A1</td>
</tr>
<tr>
<td>2G11</td>
<td>25.1</td>
</tr>
<tr>
<td>B6.1</td>
<td>6.82</td>
</tr>
<tr>
<td>B11E8</td>
<td>6.59</td>
</tr>
</tbody>
</table>

Equilibrium dissociation constants \(\left(K_D\right)\) for affinity-matured BoNT IgG were measured for the different BoNT subtypes by flow fluorimetry in a KinExA.
was expressed and purified from strains A254 and Ba207 (Hill et al., 2007). Mouse anti-SV5 antibody was purified from hybridoma supernatant using Protein G and directly labeled with Alexa-488 or Alexa-647 using a kit provided by Invitrogen/Molecular Probes. For flow cytometry (FACS), purified human or mouse IgGs specific to BoNT/A or BoNT/B were directly labeled with either Alexa-647 or Alexa-488 using a kit provided by Invitrogen/Molecular Probes.

Construction of a light chain repertoire in YVH10 for light chain shuffling

A single light chain repertoire in YVH10 was constructed containing kappa and lambda light chains (Fig. 1). First, the vector pPNL30 was modified to insert a 90 bp stuffer between the XhoI and the BsiWI sites. Primers 30S (5’-CCATCAGCAACTCGAGGCTGAAG-3’) and VKBACK (5’-TTTCAACTGCTCATCAGA-3’) were used to PCR amplify the stuffer from a VL insert into pNL30-EGF (Weaver-Feldhaus et al., 2004), and the stuffer subcloned back into the original pPNL30 vector digested with XhoI and BsiWI cut. This new vector pPNL30s makes the V\textsubscript{L} subcloning and screening easier and the SV5 tag out of reading frame without a V\textsubscript{H} gene. The VL repertoire from an immune scFv reper- toire and used to transform the yeast YVH10 with the primer list above and was then cloned into pPNL20s vector before yeast PCR. For Fab surface display, freshly saturated SD-CAA cultures of the diploid yeast libraries were induced in SG-CAA liquid media at 18°C for 24–48 h with shaking. Anti-SV5/Alexa-647 was used to quantitate the Fab expression after induction.

Selection of higher affinity clones from Fab libraries by FACS

Induced diploid yeast were flow sorted on a Becton-Dickinson FACS ARIA using procedures and protocols similar to those described previously (Weaver-Feldhaus et al., 2004) with some modification as detailed below. Briefly, an amount of diploid yeast at least 10 times larger than the library size or the sort output from the previous round were washed and re-suspended in FACS buffer and incubated with pure BoNT/A or B. Incubation times were chosen to ensure that the reaction had come to at least 90% of equilibrium, as determined as previously described (Razai et al., 2005). The volume for incubation of yeast with toxin was chosen to ensure that toxin was in at least a 5-fold excess over the number of expressed Fabs (assuming 5 x 10\(^5\) Fab/yeast) (Razai et al., 2005). For the first and second round of sorting of the ING1Fab library, 10 nM BoNT/A1 was used for staining yeast. To select for cross-reactivity with the BoNT/A2 subtype, decreasing concentrations of BoNT/A2 (10, 2, 1 and 0.5 nM) were used for the following four rounds of sorting. BoNT/B1 concentrations used for the six rounds of sorting of the B6Fab library were 100, 50, 10, 2, 1 and 0.5 nM, whereas those for the B11Fab library were 50, 25, 10, 1 and 0.5 nM. After incubation with toxin, yeasts were washed with ice-cold FACS buffer and re-suspended in a 1:40 dilution of Alexa-488-labeled anti-SV5 antibody and one of the following: Alexa-647-labeled 7C1 or 3D12 antibody (for the ING1Fab library), or Alexa-647-labeled anti-BoNT/B mAbs (A12 or 6A12) which bound the catalytic domain or receptor binding domain of the toxin. Cells were incubated for 30 min with secondary antibodies at 4–8°C, washed once with FACS buffer, re-suspended in 0.5–1 ml of FACS buffer and sorted. Typically 0.5–5% of the expressing and toxin binding population were gated for collection in the first two rounds sorting, and 0.1–0.5% of those population was gated in the following rounds of sorting. Collected cells were grown in SD-CAA media and used for the next round of sorting after induction in SG-CAA as described above. After the final round of sorting, 96 individual clones were picked, induced and stained with 500 pM of Alexa-647 BoNT/A or BoNT/B and Alexa-488 SV5 antibody to identify the best candidates in terms of expression and binding by FACS analysis. Using MFI as a single criterion after fixed concentration toxin staining, 12 clones were chosen for further characterization by DNA sequencing and affinity measurement by flow cytometry.
Quantitative equilibrium binding was determined using flow cytometry as described (Boder and Wittrup, 1997; Boder et al., 2000). In general, six different concentrations of one subtype of pure BoNT/A or BoNT/B was utilized spanning a range of concentrations from 10 times above to 10 times below the $K_D$. Incubation volumes and the number of yeasts stained were chosen to keep the number of antigen molecules in 10-fold excess above the number of expressed Fabs, assuming $5.0 \times 10^7$ Fab/yeast. Incubation times were chosen based on anticipated times to equilibrium calculated using approximations of the anticipated $K_{on}$ and $K_{off}$ (Razai et al., 2005). This was usually accomplished by overnight incubation with toxin. Binding of BoNT/A to yeast-displayed Fab was detected using a 1:400 dilution of 1 mg/ml monoclonal antibody binding a non-overlapping epitope labeled with Alexa-647. To quantify the affinity constant ($K_D$) within the surface display context, only the Fab displaying yeast (SV5 binding) was included in the analysis by co-staining with SV5-Alexa-488. Each $K_D$ was determined as the average of three separate inductions and measurements.

**Generation of IgG from Fab**

IgGs were generated by cloning the Fab $V_H$ and $V_L$ genes into a vector for stable mammalian expression as previously described (Nowakowski et al., 2002; Razai et al., 2005). Clones containing the correct $V_H$ and $V_L$ genes were identified by DNA sequencing, and those plasmids were used to transfect CHO DG44 cells by electroporation. Stable cell lines were established by selection in G418 and expanded into 1 L spinner flasks. Supernatant containing IgG was collected and purified on Protein G (Pharmacia). IgG purity was assessed by native and denaturing SDS-PAGE and protein concentration determined by A280 nm.

**Measurement of IgG solution equilibrium binding constants by flow fluorimetry**

Equilibrium binding studies were conducted at room temperature using a KinExA 3000 flow fluorimeter to quantify the free BoNT/A or B at equilibrium using varying concentrations of antibody as previously reported (Blake et al., 1999; Razai et al., 2005). Studies of BoNT/A or B reaction mixtures were performed in PBS (pH 7.4), with 1 mg/ml BSA and 0.02% (w/v) sodium azide as a preservative. Antibody was serially diluted into a constant concentration of BoNT/A or B sufficient to produce a reasonable signal, where the antibody concentration was varied from <0.1 to >10-fold above the value of the apparent $K_D$. The BoNT/A or B concentrations were no more than 4-fold above the $K_D$ to ensure a $K_D$-controlled experiment. Samples were allowed to reach equilibrium for as long as 2 days, then each of the 12 dilutions was passed over a flow cell with a 4 mm column of Azlactone beads (Sapidyne Instruments) covalently coated with the corresponding antibody to capture the free BoNT/A or B. Passing an Alexa-647-labeled BoNT/A or B antibody binding a non-overlapping epitope over the beads produced a signal relative to the amount of free BoNT/A or B bound to the beads. All data points were run in duplicate and sample volume varied from 4 to 25 ml depending on antibody affinity. The equilibrium titration data were fit to a 1:1 reversible binding model using KinExA Pro Software (version 1.0.2; Sapidyne Instruments) to determine the $K_D$.

**Funding**

This work was partially supported by National Institutes of Health cooperative agreement U01 AI056493, Defense Threat Reduction Agency contract 1-07-C-0030 and Centers for Disease Control and Prevention contract 200-2006-16697.

**References**


