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14. ABSTRACT

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Human monoclonal antibodies as a countermeasure against Botulinum toxins

ABSTRACT

In this report, we summarize our studies to find novel, neutralizing antibodies against BoNT/E, as well as the investigation into methods to generate cell lines expressing neutralizing antibodies or antibody-like molecules with specificities against BoNT/A, /B, and /E. Through a series of rat and rabbit immunizations, we generated twelve recombinant MAbs capable of neutralizing 100LD50s of BoNT/E in murine models. In parallel, multiple methods using both pro- and eukaryotic expression systems were tested for their ability to express neutralizing MAbs or MAb-like scaffolds with specificities against all three BoNTs. One system showing efficacy involved the generation of IgG-based expression constructs in which the full length light and heavy chains were expressed as a single amino acid sequence using a (glycine-serine4)6 linker. Co-transfection of previously generated humanized BoNT/A and /B clones using this linker system in conjunction with dual selection markers showed that it was possible to generate a stable 293F cell line secreting both MAbs, which retained specificity to both toxins. Additionally, this material could be purified using traditional methods and mass spectrometry (MS) revealed both homo- and heterodimer pairing of the single chains via the hinge region of IgG. Lastly, two of the twelve BoNT/E neutralizing MAbs were humanized and linked in a similar manner and tested for in vivo efficacy. One clone (199B13) was able to completely neutralize 100LD50s of BoNT/E. This confirms the concept of the light-heavy chain linked IgG system and will permit the generation of a single production cell line generating material capable of neutralizing BoNT/A, /B, and /E.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received   Paper

   TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received   Paper

   TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>   <u>Paper</u>

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>   <u>Paper</u>

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

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<u>Received</u>   <u>Paper</u>

TOTAL:

Number of Manuscripts:

Books

<u>Received</u>   <u>Paper</u>

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Morphotek	1.00	
FTE Equivalent:	**1.00**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

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The number of undergraduates funded by this agreement who graduated during this period: 0.00

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Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): 0.00

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The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

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Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Luigi Grasso	0.15
Brad Kline	0.30
Rina Kennedy	0.31
Qimin Chao	0.01
Bryon Martinez	0.14
Jackie Balogach	0.03
Matt Glatfelter	0.03
Matt Goserud	0.01
Shangong Wan	0.01
Marianne Henry	0.03
Christine DiGiovanni	0.02
David Chin	0.06
FTE Equivalent:	**1.10**
Total Number:	12

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

In this report, we describe the discovery, characterization, and humanization of anti-BoNT/E MAbs which are capable of neutralizing 100LD50s. Below are listed the specific aims of the project along with commentary on deliverables generated.

5.1 Aim 1: Generation of anti-BoNT/E MAbs

We described the generation and characterization of MAbs derived from both rat and rabbit origins. While a few rat clones were capable of neutralizing 10LD50s of BoNT/E, these were not deemed efficacious and were therefore discontinued. However, studies using rabbit anti-serum, followed by rabbit-human xiMAbs described the discovery of 12 MAbs which completely protected from challenge with 100LD50s BoNT/E.

5.2 Aim 2: Humanization of anti-BoNT/E MAbs

Of the 12 neutralizing xiMAbs generated, two were humanized and expressed as single chain constructs. One of these (zu199B13) was capable of protecting against 100LD50s of BoNT/E in vivo. It is also important to note that 10 other xiMAbs were neutralizers. However, due to time and manpower constraints we only focused on two MAbs. Future studies could be focused on the humanization and characterization of these remaining antibodies in the single chain format.

5.3 Aim 3&4: Engineering of a single cell line capable of producing three humanized IgGs against BoNT/A, /B, and /E

While the characterization of MAbs generated by the immunizations was underway, we examined a variety of pro- and eukaryotic scaffolds for production of trivalent neutralizing constructs. In both prokaryotic methods (in-line Fv and triabodies), reactivity against BoNTs could be observed, but either the affinities or percentage of correctly folded material negated their further study. In eukaryotic (293F) cells, linked full length IgMs and IgGs were tested for expression. While the IgM forms did not express, the IgG forms did. We therefore tested the feasibility of this platform by stably co-transfecting single chain forms of our previously generated BoNT/A and /B clones. Supernatants from a subclone (G-10) showed reactivity against both BoNT/A and BoNT/B by ELISA. Based on these observations, we believe this platform can be extended in future studies to a third anti-BoNT/E single chain MAb.

5.4 Aim 5: Development of purification method for MAbs generated in Aims 3 and 4.

Antibodies from the G-10 clone were secreted into the supernatant and could be purified using standard protein A based methods. Molecular characterization by ESI-MS showed the presence of both homo- and heterodimers as expected. On Coomassie gels, the linked antibodies ran at the expected apparent molecular masses, suggesting aberrant multimerization was not occurring, and that no proteolytic degradation was observed.

5.5 Aim 6: Characterization of linked IgGs for in vivo protection against BoNT/A, /B, and E

Two of the neutralizing xiMAbs generated from rabbits were humanized and expressed as linked single chain IgGs. One of these (zu199B13) was able to completely protect mice against challenge with 100LD50s of BoNT/E. Based on these results, we believe this construct can be transfected into the G-10 cell line, making it express three independent IgGs capable of neutralizing BoNT/A, /B, and /E.

5.6 Aim 7: Generation of a master cell bank of the production line

Because of the lag of timelines for the project to complete immunizations, this aim could not be completed. However, with the materials generated in this report, this could be completed in about six months' time.

Technology Transfer

DTRA Final Report: Human monoclonal antibodies as a countermeasure against *Botulinum* toxins

Author: Brad Kline

Contributors: Luigi Grasso, Rina Kennedy, Bryon Martinez, Earl Albone, Qimin Chao.

Report Date: November 30, 2012

Abstract

In this report, we summarize our studies to find novel, neutralizing antibodies against BoNT/E, as well as the investigation into methods to generate cell lines expressing neutralizing antibodies or antibody-like molecules with specificities against BoNT/A, /B, and /E. Through a series of rat and rabbit immunizations, we generated twelve recombinant MAbs capable of neutralizing $100LD_{50}$s of BoNT/E in murine models. In parallel, multiple methods using both pro- and eukaryotic expression systems were tested for their ability to express neutralizing MAbs or MAb-like scaffolds with specificities against all three BoNTs. One system showing efficacy involved the generation of IgG-based expression constructs in which the full length light and heavy chains were expressed as a single amino acid sequence using a $(glycine-serine_4)_6$ linker. Co-transfection of previously generated humanized BoNT/A and /B clones using this linker system in conjunction with dual selection markers showed that it was possible to generate a stable 293F cell line secreting both MAbs, which retained specificity to both toxins. Additionally, this material could be purified using traditional methods and mass spectrometry (MS) revealed both homo- and heterodimer pairing of the single chains via the hinge region of IgG. Lastly, two of the twelve BoNT/E neutralizing MAbs were humanized and linked in a similar manner and tested for in vivo efficacy. One clone (199B13) was able to completely neutralize $100LD_{50}$s of BoNT/E. This confirms the concept of the light-heavy chain linked IgG system and will permit the generation of a single production cell line generating material capable of neutralizing BoNT/A, /B, and /E.

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Figures and Tables

1 - Generation of anti-BoNT/E monoclonal antibodies (MAbs)

This section describes the rodent immunization campaigns and the analysis of antibodies generated from them for their ability to bind and neutralize BoNT/E.

1.1 Generation and analysis of rat MAbs at Covance

Historically, the generation of BoNT/E neutralizing MAbs by murine immunization was never successful for our lab (data not shown). In recent years, we have focused on both rat and rabbit immunizations because of the robust immune responses we have observed with human and non-human protein antigens. Because of the inherent toxicity of native BoNT/E, we proceeded with immunizations utilizing a toxoid form of the toxin. A recent study by Keller et al (Keller 1374-79) using *in vitro* and *in vivo* methods demonstrate that when a toxoid resembles the native toxin, it elicits very high protective antibody titers that effectively cross-recognize the native neurotoxin. On the other hand, if a toxoid does not resemble the native toxin by *in vitro* analysis, such as when formalin conditions are too harsh, then the toxoid elicits little, if any, protection *in vivo*. Toxin-specific immunogenicity, in fact, is quite poor in such instances. We therefore generated BoNT/E toxoid using Keller's method (see Table 1 legend) for immunization of both rats and rabbits. Five rat immunizations were performed at Covance, Inc. (Denver, PA). From these, rat #46 was used for the generation of hybridomas performed by Covance. Supernatants were screened at Covance for reactivity against

BoNT/E coated ELISA plates (Table 1). Most of the hybridomas showed intermediate reactivity to BoNT/E, but clone 881 exhibited reactivity greater than 50-fold above background. It was also the only clone producing amounts of antibody capable of performing *in vivo* protection assays. Therefore, the hybridoma was scaled up in culture and antibody purified to test its neutralizing abilities in mice. As shown in Table 2, 10 to 75 LD_{50}s of BoNT/E were co-administered with the antibody and in all cases, no neutralization was observed.

Table 1- Immunization and screening of rat hybridomas for BoNT/E reactivity

Clone Number	ELISA Signal	Fold Above Background
881	1.797	51.2
531	0.579	15.8
697	0.573	15.7
36	0.418	11.1
724	0.415	11.1
902	0.396	10.5
499	0.374	9.9
694	0.363	9.5
750	0.35	9.2
508	0.347	9.1
630	0.344	9
410	0.295	7.6
666	0.274	7
939	0.154	3.5
664	0.135	2.9
926	0.117	2.4
126	0.116	2.4
922	0.113	2.3
925	0.105	2.1
940	0.105	2.1
930	0.093	1.7
909	0.076	1.2
345	0.075	1.2
368	0.069	1
124	0.066	0.9
5	0.066	0.9
45	0.064	0.9
646	0.051	0.5
233	0.05	0.5
294	0.036	0

Legend: Five rats (#43-47) were immunized with toxoid version of BoNT/E at Covance. For toxoid generation in all immunizations, research-grade BoNT/E was purchased from Metabiologics, Inc. Briefly, 30 mg BoNT/E was diluted in PBS to a protein concentration of 0.16 mg/mL. Buffered formalin was prepared by dissolving 20 mg of p-formaldehyde/mL PBS and heating to 60°C for 45 min and diluted into toxin-PBS solutions to produce a final formalin concentration of 15mM. Reaction mixtures proceeded at 37°C without agitation for two weeks. The reaction was subsequently moved to 4°C and the residual toxicity was determined: two mice were injected with 50 μg toxoid i.p. and both mice survived challenge, indicating the lack of toxicity in the toxoid preperation. This material was used for all subsequent immunizations in the report. Based on serum reactivity ELISAs, Rat #46 received a final i.v. boost and fusion done at Covance. Thirty positive hybridoma fusions were generated (at Covance) by supernatant ELISAs conducted against BoNT/E coated plates. Clone 881 (yellow) expressed sufficient quantities to be tested with in vivo BoNT/E challenge.

Table 2- *In vivo* efficacy study with rat anti-BoNT/E clone 881

BoNT/E Administered	150 µg dose of pure 881 Mab	Dead/Total mice
0 LD_{50}	+	0/2 mice
10 LD_{50}	-	2/2 mice
10 LD_{50}	+	4/4 mice
25 LD_{50}	+	4/4 mice
50 LD_{50}	+	4/4 mice
75 LD_{50}	+	4/4 mice

Legend: CD-1 mice were challenged by intraperitoneal (i.p.) injection of 10 to 75 mouse LD50s of BoNT/E (MetaBiologics Inc.), while administering a single dose of 150 µg of anti-BoNT/E MAb 881, corresponding to approximately 7 mg/kg (or a hypothetical 500 mg dose for a 70 Kg person). Individual MAbs were incubated with various doses of a BoNT/E for 1 hour at room temperature. The toxin and antibody mixture were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored for survivors twice a day after challenge for 4 days. Two to four mice per cohort were employed and survival of all treated animals within a cohort signify full protection by the antitoxin.

1.2 In-house generation and analysis of rat MAbs using rat organs from Covance immunizations

The poor fusion efficiency of rat hybridomas generated at Covance prompted us to request harvested spleen and lymph nodes from the remaining immunized Covance rats. Our expertise in high rat fusion efficiencies led us to believe that more MAb leads could be generated and subsequently tested *in vivo*. Two fusion campaigns using splenic B cells (1704) and peripheral B cells (1705) were performed in parallel (Table 3). From these fusions, a total of 52 confirmed clones reacted by ELISA. Clones were scaled up for IgG purification and subsequent use in *in vivo* challenge models. An example assay is shown in Figure 1. Of the MAbs tested, two clones (64 and 74) were capable of neutralizing $10LD_{50}$s of BoNT/E *in vivo*. Based on these results, the antibodies were then tested for their ability to neutralize the required $100LD_{50}$s of toxin. In this case, no protection was observed and all mice died (data not shown).

Table 3- Summary of BoNT/E reactivity screening for in-house fusion of rat hybridoma spleen and lymph nodes

Campaign 1704	#	%	Campaign 1705	#	%
Week 3 plating efficiency	ND	69.2	Week 3 plating efficiency	ND	38.1
Hybridomas producing IgG	622	3.2	Hybridomas producing IgG	8047	48.5
BoNT/E reactive IgG+ wells	44	7.1	BoNT/E reactive IgG+ wells	114	1.4
Repeat confirmed BoNT/E reactivity	33	75.0	Repeat confirmed BoNT/E reactivity	19	16.7

Legend: Covance Rat 45 was subcutaneously boosted with BoNT/E toxoid and delivered to Morphotek. Spleen and Lymph Node B cells isolated at Morphotek. Fusions were performed at Morphotek. Campaign 1704, Rat 45 Spleen hybridoma fusions; Campaign 1705, Rat 45 Lymph Node fusion. 200 96 well plates (19,200 wells) were screened in 1704, 173 96 well plates (16,608 wells) in 1705. IgG -producing wells were determined by FRET analysis. Those wells were consolidated and supernatants tested for BoNT/E first by FRET, and reconfirmed by ELISA.

Figure 1- In-house generated rat MAbs *in vivo* efficacy challenge at 10LD$_{50}$s BoNT/E
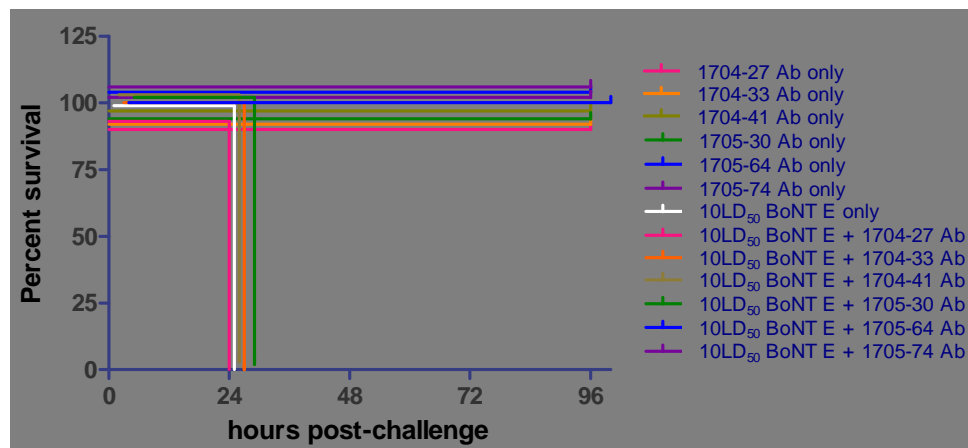
Legend: To determine the in vivo efficacy of anti-BoNT/E MAbs, a standard mouse model was employed. CD-1 mice were challenged by intraperitoneal (i.p.) injection of 10 mouse LD50 of BoNT/E (MetaBiologics Inc., WI), while administering a single dose of 150 µg of anti-BoNT/E MAb, corresponding to ~7 mg/Kg (or a hypothetical 500 mg/dose for a 70 Kg person). Individual MAbs were incubated with BoNT/E for 1 hour at room temperature. The toxin and antibody mixtures were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored for survivors twice a day after challenge for 4 days. Two (Ab only) or four (Ab+toxin) mice per cohort were employed and survival of all treated animals within a cohort signify full protection by the antitoxin.

## 1.3 In-house generation and analysis of recombinant chimeric rabbit-human MAbs (xiMAbs) using rabbit organs from Covance immunizations

1.3.1 Analysis of rabbit antisera for BoNT/E neutralization

At the same time rat immunizations were underway, parallel rabbit immunizations using BoNT/E toxoid were being performed (Covance). Ten rabbits were immunized and production bleeds sent regularly for testing at Morphotek. Using BoNT/E coated ELISA plates, the sera were serially diluted and tested for toxin reactivity. While pre-bleed sera before immunization showed no toxin reactivity (data not shown), the immunized rabbits exhibited a robust reaction against BoNT/E *in vitro* (Figure 2). The PBMCs and splenic B cells from the rabbits were harvested and frozen for subsequent cloning as described in the below paragraphs and figures. This promising reactivity led us to postulate that the antitoxin sera might confer resistance against BoNT/E challenge *in vivo*. To test this, dilutions of sera from two of the rabbits (74 and 81) were pre-mixed with 100LD50s of BoNT/E and injected into mice (Table 4). Even at a 1:1000 dilution, the post-immunization sera protected all of the mice tested. In contrast, the pre-immune sera did not protect, even when not diluted. These promising results suggested the presence of BoNT/E neutralizing MAbs could be isolated from rabbit B cells.

Figure 2- Serum BoNT/E reactivity of rabbits immunized with BoNT/E toxoid
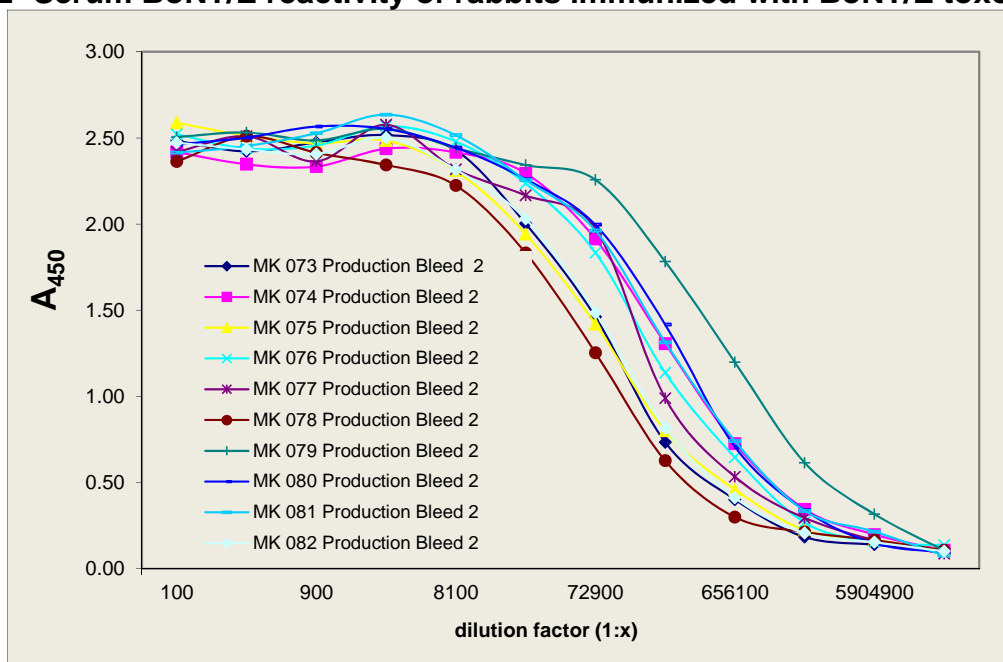
Legend: Ten rabbits (#73-82) were immunized with Toxoid version of BoNT/E (Covance). Serum dilutions were analyzed for BoNT/E reactivity by ELISA. Based on serum reactivity ELISAs, PBMCs and spleens from #74 and 81 were harvested and B cells isolated. Single B cells were cloned and those secreting IgG were tested for BoNT/E reactivity by ELISA. Variable domains for heavy and light chains were amplified from reactive cells by RT-PCR for subsequent recombinant expression and sequence analysis.

Table 4- Rabbit antisera generated by BoNT/E toxoid immunization protect against *in vivo* challenge in mice

| LD$_{50}$ BoNT E	Serum	# mice	0/2	0/2	0/2	0/2
0 LD$_{50}$ BoNT E	Neat prebleed 81	2	0/2	0/2	0/2	0/2
0 LD$_{50}$ BoNT E	Neat 74	2	0/2	0/2	0/2	0/2
0 LD$_{50}$ BoNT E	Neat 81	2	0/2	0/2	0/2	0/2
100 LD$_{50}$ BoNT E	none	2	2/2			
100 LD$_{50}$ BoNT E	Neat prebleed 74	2	2/2			
100 LD$_{50}$ BoNT E	Neat prebleed 81	2	2/2			
100 LD$_{50}$ BoNT E	Neat 74	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	Neat 81	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:10 of 74	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:10 of 81	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:100 of 74	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:100 of 81	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:1000 of 74	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:1000 of 81	4	0/4	0/4	0/4	0/4
100 LD$_{50}$ BoNT E	1:10000 of 74	4	3/4	3/4	3/4	3/4
100 LD$_{50}$ BoNT E	1:10000 of 81	4	3/4	3/4	3/4	3/4

Legend: To determine the in vivo efficacy of anti-BoNT/E antisera, a standard mouse model was employed. CD-1 mice were challenged by intraperitoneal (i.p.) injection of 100 mouse LD50 of BoNT/E (MetaBiologics Inc., WI),

while administering a single dose of anti-BoNT/E anti-sera (Neat up to a 1:10,000 dilution in PBS). Individual MAbs were incubated with BoNT/E for 1 hour at room temperature. The antiserum-toxin mixtures were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored for survivors twice a day after challenge for 4 days. Two (antisera only or BoNT/E only) or four (sera+toxin) mice per cohort were employed and survival of all treated animals within a cohort signify full protection by the antitoxin.

1.3.2 Cloning and expression of recombinant rabbit-human xiMAbs

Based on the neutralizing properties of sera from rabbits 74 and 81, single B cells from both animals were seeded into 384 well plates from both PBMCs (campaign 1710) and spleens (campaign 1714) and their IgGs analyzed for BoNT/E reactivity by ELISA. All B cell clones which exhibited toxin reactivity were harvested for RNA isolation, and RT-PCR performed to rescue the variable light (VL) and variable heavy (VH) domains of their antibodies. DNA sequencing was performed and the variable domains translated *in silico* and aligned (Figure 3). To eliminate duplicate clones and lower the number that would need to be expressed, they were grouped based on the uniqueness of their VH CDR3 sequences. For example, 3M18 and 103D5 were the same clone, so only 3M18 was subcloned into an expression vector for further analysis. Even by eliminating duplicates, we were able to clone and express 29 unique sequences for *in vivo* characterization, suggesting that perhaps some of the sequences encoded against neutralizing epitopes on BoNT/E.

Figure 3- Amino acid sequence homology of rabbit lead VH CDR3s

```
                                (1)  1         10              29
1710_101N21_2_P_HC_Rb_HC_1 (1)  YFCARWGYNTALTTY--TNNLWG------
1714_229M15_2_P_HC_RB_HC_1 (1)  YFCARGTYPS----YIADFNLWG------
 1714_232L9_2_P_HC_RB_HC_1 (1)  YFCARGTYPS----YIADFNLWG------
 1714_291L4_2_P_HC_RB_HC_1 (1)  YFCARGTYPS----YIADFNLWG------
1714_349I24_2_P_HC_RB_HC_1 (1)  YFCARGTYPS----YIADFNLWG------
 1714_353D5_2_P_HC_RB_HC_1 (1)  YFCARGTYPS----YIADFNLWG------
 1710_105A7_2_P_HC_Rb_HC_1 (1)  YFCARNPG------W--SSNMWG------
1710_108L17_2_P_HC_Rb_HC_1 (1)  YFCARNPG------W--SSNMWG------
1714_304H14_2_P_HC_RB_HC_1 (1)  YFCARNPG------W--SSNMWG------
  1714_76F3_2_P_HC_RB_HC_1 (1)  YFCARKLGGDSDD-Y--VWSMWG------
1714_322O21_2_P_HC_RB_HC_1 (1)  YFCARNHGGYDAY-Y--TCGLWG------
1714_362C21_2_P_HC_RB_HC_1 (1)  YFCARDAG------Y--RSNLWG------
1710_105C13_2_P_HC_U_Rb_HC_1 (1) YFCARDYGV-----S--DFNLWG------
1714_103D5_2_P_HC_RB_HC_1 (1)  YFCARRYGSS----GAWYFDIWG------
 1714_3M18_2_P_HC_RB_HC_1 (1)  YFCARRYGSS----GAWYFDIWG------
1714_182C9_2_P_HC_RB_HC_1 (1)  YFCARDYGG-----G--GFNIWG------
1714_170N6_2_P_HC_RB_HC_1 (1)  YFCAREYKYADHGHYGDAFDPWG------
1714_61D22_2_P_HC_RB_HC_1 (1)  YFCAREDIN-----Y--AFNIWG------
1714_223A8_2_P_HC_RB_HC_1 (1)  YFCARVGTGAAGYGF--AFNIWG------
1710_104A7_2_P_HC_Rb_HC_1 (1)  YFCAR-YSSGT----DSYFNIWG------
1714_231K18_2_P_HC_RB_HC_1 (1)  YFCAR-YSSGS----DSYFNIWG------
1714_69E18_2_P_HC_RB_HC_1 (1)  YFCARGGSSSSYS-Y--AFNIWG------
1714_133H15_2_P_HC_RB_HC_1 (1)  YFCTAGSSS----------DIWG------
 1714_56N2_2_P_HC_RB_HC_1 (1)  YFCVRGVSSE----NYSWFTLWG------
 1710_148J8_2_P_HC_Rb_HC_1 (1)  YFCARGYDSDVHA-YSIIHDLWG------
1714_255A5_2_P_HC_RB_HC_1 (1)  YFCARGYSGDWRG-Y--YFNLWG------
1714_321E4_2_P_HC_RB_HC_1 (1)  YFCARGGHGDS------YFQLWG------
1710_123B22_2_P_HC_Rb_HC_1 (1)  YFCTKSGSLW----G--------------
1714_395H17_2_P_HCURbHC_1 (1)  YFCTVALSRW----G--------------
1714_204D5_2_P_HC_RB_HC__1 (1)  YFCAGGWNLW----G--------------
1710_129G19_2_P_HC_Rb_HC_1 (1)  YFCARGDYDS--------SDWVDYFDLWG
1714_185B18_2_P_HC_RB_HC_1 (1)  YFCARGYVASS------VTTMPDYLDLWG
1714_202G3_2_P_HC_RB_HC_1 (1)  YFCARGMFNLW------G-----------
 1714_5J16_2_P_HC_RB_HC_1 (1)  YFCAR-DPNGG----SPYWGLWG------
1714_80O15_2_P_HC_RB_HC_1 (1)  YFCARGDANLV------PWG---------
1710_145I13_2_P_HC_Rb_HC_1 (1)  YFCARGGADSSTG-S--HFNLWG------
1714_315A23_2_P_HC_RB_HC_1 (1)  YFCVRGGFD-----S--GFNIWG------
 1714_87A5_2_P_HC_RB_HC_1 (1)  YFCVGGGFD-----S--GFSVWG------
1714_263B10_2_P_HC_RB_HC_1 (1)  YFCAGGGYWS----SGYEFDPWG------
1714_102F19_2_P_HC_RB_HC_1 (1)  YFCVRGGLS-----V--GTGIWG------
1714_61I17_2_P_HC_RB_HC_1 (1)  YFCARGGYSNLWG----------------
1714_331O20_2_P_HC_RB_HC_1 (1)  YFCAR-GYSDY----NTFMGLWG------
1710_114L14_2_P_HC_Rb_HC_1 (1)  YFCARVGASNIDWLD--VGDAWG------
 1714_101E4_2_P_HC_RB_HC_1 (1)  YCAR-TNSGV----GGYGNIWG------
1710_199B13_2_P_HC_Rb_HC_1 (1)  YFCARVPVTGSGA-W--TADLWG------
1714_244P5_2_P_HC_RB_HC_1 (1)  YFCAR-VASGS----GDAADPWG------
1714_371C11_2_P_HC_RB_HC_1 (1)  YFCARAAYAGSND-YIDTFDPWG------
1714_167D10_2_P_HC_RB_HC_1 (1)  YFCARDGNNGLYL-HYYGMDLWG------
1714_250L5_2_P_HC_RB_HC_1 (1)  YFCARDLDTG----GEYYFDLWG------
1714_348F6_2_P_HC_RB_HC_1 (1)  YFCARENDSGGYGSP--AIDLWG------
1714_99F17_2_P_HC_RB_HC_1 (1)  YFCAR-ARYGG----GGYADLWG------
1710_118O8_2_P_HC_Rb_HC_1 (1)  YFCARESYISWSS-GSEGYNLWG------
1714_79G17_2_P_HC_RB_HC_1 (1)  YFCARDTLG-----S--GYHLWG------
1710_122P6_2_P_HC_Rb_HC_1 (1)  YFCGRGAAGS----WWNGLELWG------
1710_124N20_2_P_HC_Rb_HC_1 (1)  YFCGRSIGRG----LWG-----------
1714_67B14_2_P_HC_RB_HC_1 (1)  YFCGR-IENGY----LDRLDLWG------
 1714_9L24_2_P_HC_RB_HC_1 (1)  YFCARSSVSGY----SDRLDLWG------
1710_137O11_2_P_HC_Rb_HC_1 (1)  YFCAREDYDTYDN-A--YLKLWG------
1714_129J1_2_P_HC_RB_HC_1 (1)  YFCARVFPGTI------ISDIWG------
1714_341O2_2_P_HC_RB_HC_1 (1)  YFCARGFPGWT----SSDIWG------
 1714_12G4_2_P_HC_RB_HC_1 (1)  YFCARGVTG-----S--YYDIWG------
1714_89M22_2_P_HC_RB_HC_1 (1)  YFCARGASGGGDN-SVDVFDLWG------
                  Consensus (1)  YFCAR            F LWG
```

Clone family (brackets)
Clone family
Clone family

Legend: Clonal rabbit B cell culture supernatants were seeded onto BoNT/E coated ELISA plates and scored for toxin reactivity. B cells exhibiting reactivity were used as a source of RNA for RT-PCR reactions using primer pairs specific for rabbit VH and VL genes. Variable domain PCR fragments were gel purified and cloned into expression vector cassettes containing human kappa constant region (VL) and human IgG1 constant region (VH). Full length VH domain clones were DNA sequenced, translated in silico, and the CDR3 domains aligned (Vector NTI Advance ver. 11.5.1, Life Technologies, Carlsbad, CA). First number of clone name shows B cell origin. 1710 designates B cells of PBMC origin (plates 1-100 rabbit 74, 101-200 rabbit 81), 1714 splenic origin (plates 1-200 rabbit 74, 201-400 rabbit 81). Second number is clone plate and well origin. Brackets indicate examples of identical CDR3 clones.

1.3.1 *In vivo* efficacy challenge of rabbit-human xiMAbs with 100LD$_{50}$s of BoNT/E

Once cloned and stably expressed in human 293F cells, sufficient MAb was generated for testing *in vivo*. As shown in Table 5, 12 out of 29 (41%) of the clones completely protected against challenge with 100LD$_{50}$s of BoNT/E. To reconfirm how well they protected, two antibodies that expressed at high levels recombinantly (>50μg/mL) and that had completely divergent CDR3s were retested using both 100 and 200 LD$_{50}$s of BoNT/E (Figure 4). Because of the amounts of antibody required, clone 118O8 was only tested with 200 LD$_{50}$s of BoNT/E. Both 199B13 and 118O8 exhibited complete protection against 200 LD$_{50}$s of BoNT/E. This accomplished AIM 1 of the project, by generating novel BoNT/E MAbs that could neutralize greater than 100LD$_{50}$s of toxin.

Table 5- *In vivo* efficacy challenge using recombinant rabbit-human chimeric Mab leads in combination with 100LD$_{50}$ BoNT/E

150μg Mab + 100LD$_{50}$ BoNT/E	dead/total (24 hrs)	dead/total (48 hrs)	dead/total (72 hrs)	dead/total (96 hrs)
No Mab	2/2			
1710-137O11	2/2			
1710-145I13	2/2			
1710-148J8	1/2	1/2	1/2	1/2
➤ **1710-199B13**	0/2	0/2	0/2	0/2
1714-362C21	0/2	0/2	0/2	0/2
1714-102F19	1/2	1/2	1/2	1/2
1714-133H15	2/2			
1714-101E4	0/2	0/2	0/2	0/2
1714-89M22	2/2			
1714-87A5	2/2			
1714-202G3	2/2			
1714-371C11	0/2	0/2	0/2	0/2
1710-124N20	2/2			
1714-348F6	0/2	0/2	0/2	0/2
1714-322O21	0/2	0/2	0/2	0/2
1714-353D5	0/2	0/2	0/2	0/2
1714-9L24	1/2	1/2	1/2	1/2
1714-3M18	1/2	1/2	1/2	1/2
1714-129J1	1/2	2/2		
1714-99F17	0/2	0/2	0/2	0/2
1714-56N2	2/2			
1714-69E18	2/2			
1710-114L14	0/2	0/2	0/2	0/2
➤ **1710-118O8**	0/2	0/2	0/2	0/2
1714-104A7	2/2			
1710-122P6	0/2	0/2	0/2	0/2
1710-123B22	2/2			
1714-331O20	2/2			
1714-185B18	0/2	0/2	0/2	0/2

Legend: To determine the *in vivo* efficacy of anti-BoNT/E MAbs, a standard mouse model was employed. CD-1 mice were challenged by intraperitoneal (i.p.) injection of 100 mouse LD$_{50}$ of BoNT/E (MetaBiologics Inc., WI), while administering a single dose of 150 μg of anti-BoNT/E MAb, corresponding to ~7 mg/Kg (or a hypothetical 500 mg/dose for a 70 Kg person). Individual MAbs were incubated with BoNT/E for 1 hour at room temperature. The toxin and antibody mixtures were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored

for survivors twice a day after challenge for 4 days. Two mice per cohort were employed and survival of all animals treated within a cohort signify full protection by the antitoxin. Arrows indicate clones used in additional protection and humanization studies.

Figure 4- Clones 199B13 and 118O8 protect against 200LD$_{50}$ BoNT/E challenge *in vivo*
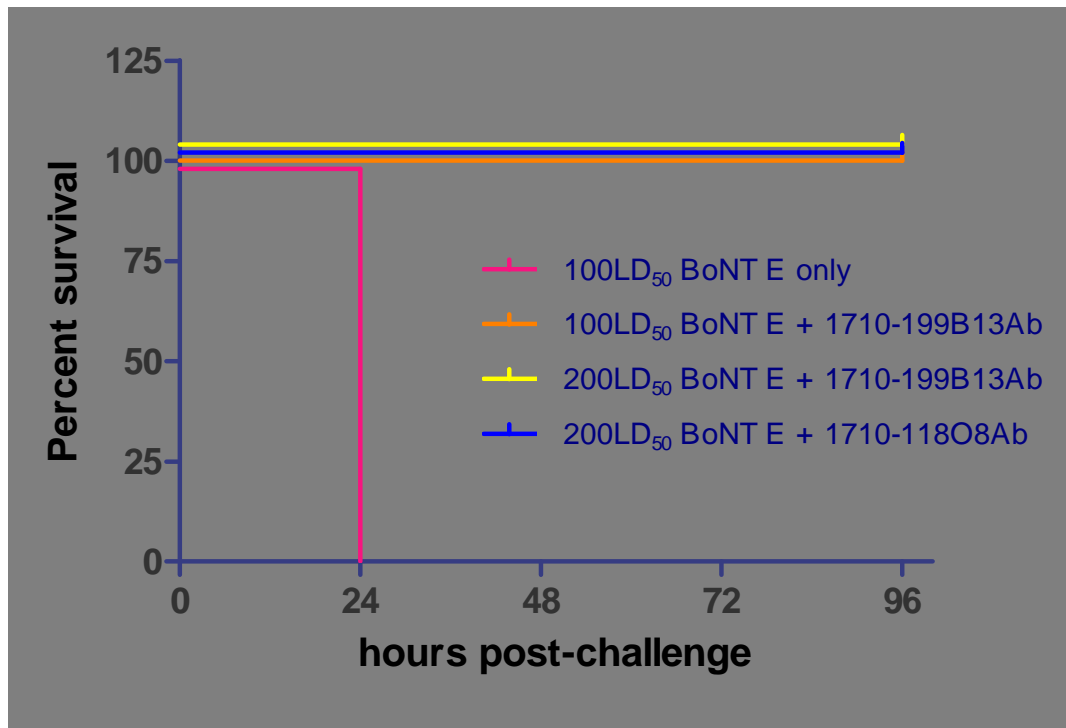
Legend: To determine the *in vivo* efficacy of anti-BoNT/E MAbs, a standard mouse model was employed. CD-1 mice were challenged by intraperitoneal (i.p.) injection of 100 and 200 mouse LD$_{50}$s of BoNT/E (MetaBiologics Inc., WI), while administering a single dose of 150 μg of anti-BoNT/E MAb, corresponding to ~7 mg/Kg (or a hypothetical 500 mg/dose for a 70 Kg person). Individual MAbs were incubated with various doses of BoNT/E for 1 hour at room temperature. The toxin and antibody mixtures were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored for survivors twice a day after challenge for 4 days. Two (118O8) or four (199B13) mice per cohort were employed (depending on MAb availability) and survival of all treated animals within a cohort signify full protection by the antitoxin.

2 - Analysis of differently engineered antibody scaffolds for the production of multi-valent anti-BoNT therapeutics

This section describes the analysis of pro- and eukaryotic scaffolds for the production of therapeutics capable of neutralizing BoNT/A, /B, and /E.

2.1 Prokaryotic expression constructs examined as possible scaffolds for creating therapeutics

While we had shown in our study proposal that linking IgG/M heavy and light chains in eukaryotic cells would ensure correct pairing for BoNT reactivity, we wished to

explore other alternative strategies for producing therapeutics in an *E. coli* based system. Multiple forms of bi-and tri-specific constructs have been described (Todorovska et al. 47-66). We attempted to express and refold two different forms of multi-specific constructs in *E. coli*. One consisted of two chains of variable fragments encoding VH-VL-VH and VL-VH-VL specificities (In-line Fvs, Figure 5A), while the other consisted of single polypeptide chain of variable domains (Triabody, Figure 5B). In each case, the variable domains and linkers were arranged in such a manner as to promote the correct pair of VL/VH pairs encoding each anti-BoNT specificity. While the rat and rabbit immunization were still being characterized, and we did not have our final anti-BoNT/E lead MAbs yet, we used clone 74 (Figure 1) as a sample BoNT/E clone while assessing the scaffolds.

Figure 5- In-line Fv and triabody structures
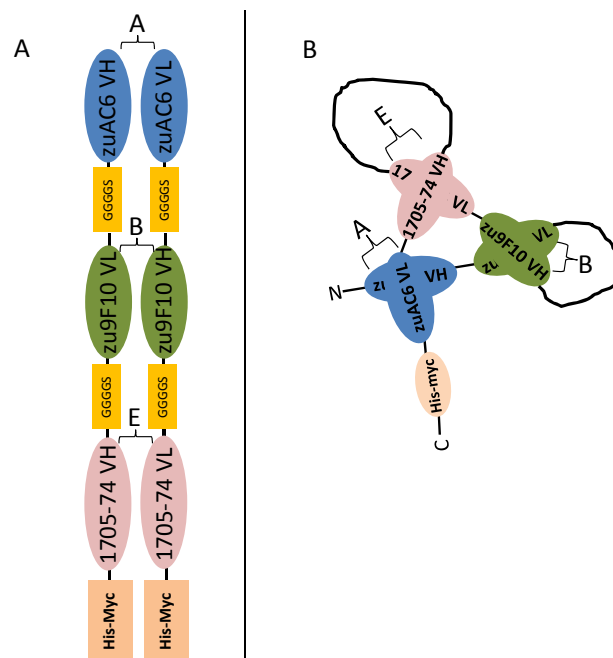
Legend: Schematic representation of in-line Fvs and Triabodies. A) In-line Fv structure. *E. coli* optimized constructs encoding anti-A VH-anti-B VL-anti-E VH and anti-A VL-anti-B VH-anti-E VL were expressed in parallel and combined as denatured inclusion bodies for refolding and analysis. B) Triabody structure. Single polypeptide chain in which VH and VL domains are arranged in such a way that small rigid (GG) and large flexible (G$_4$S)$_4$ linkers permit folding such that the correct VH/VL pairs generate toxin binding domains. In both structures, BoNT binding domains are designated as A, B, or E with a bracket.

2.1.1 Characterization of *E. coli* expressed constructs and their refolding for BoNT reactivity

The sequences from Figure 5 were codon optimized for *E. coli* expression (DNA 2.0) and expressed in BL21(DE3) cells to assess their expression and solubility. The triabody construct is shown in Figure 6. After IPTG induction, a distinct band of the correct molecular mass (82 KDa) was observed in the pellet, but not the supernatant, suggesting it was expressed in insoluble inclusion bodies. In parallel, the same

observation was made with in-line Fv expression, with bands in the insoluble fraction observed at their expected 42 KDa size (data not shown). Both scaffolds were scaled up, expressed, and their inclusion bodies isolated as per instructions using iFold kits (Millipore). The inclusion bodies from both expressed scaffolds were solubilized using guanidine and tested in matrices of refolding conditions using the iFold kit. The dialyzed samples were then tested for their ELISA reactivity against BoNT/A, /B, and /E coated plates, with the non-relevant protein coating mesothelin serving as a negative control. Figure 7 shows the results of the triabody preparation. Several wells showed conditions in which binding to all three toxins could be observed, while no binding to mesothelin was seen. For the triabody, condition D9 was used to refold a 200mL induced culture. For the in-line Fv, similar results were observed, but condition B12 was used to refold a 200mL culture.

After refolding and NTA resin purification via a C-terminal 6xHis tag, both constructs were tested for their ability to bind to a BoNT/A coated plate in a dose-dependent manner (Figure 8).   Both constructs exhibited binding to BoNT/A, with the in-line Fv showing greater binding. However, when compared to humanized standard IgG zuAC6, the binding was poor. This could be due to either: 1, the construct structure was decreasing the binding affinity to the target toxins. 2, the efficiency of refolding was poor, with only a fraction of material folded properly to bind to BoNT/A. In either case, we abandoned further prokaryotic expression and began to focus on eukaryotic expression.

**Figure 6- Test Induction of triabodies: soluble versus insoluble fractions**
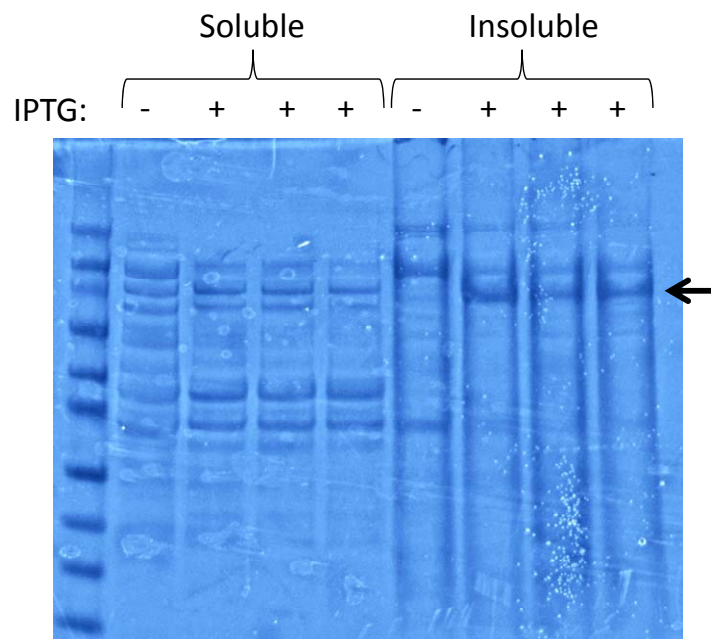
Legend: BL21(DE3) cells transformed with anti-BoNT/A,B,E triabody construct were grown overnight at 37°C with shaking. Four 25mL cultures of LB/Kan were inoculated with 0.25mL culture (1:100 dilution) and grown at 37°C with shaking until OD$_{600}\cong$0.9. At this point induced cultures were inoculated with IPTG at a final concentration of 1mM and shaken an additional 4 hours. Cells were pelleted and lysed in 1/3 volume B-PER reagent (Pierce) for 20 minutes on ice. Insoluble material was pelleted and saved, and supernatant mixed with protein loading buffer and heated for 5 minutes at 100°C . Insoluble pellets were resuspended directly in loading buffer, heated at 100°C for 5 minutes and sonicated on ice. Remaining insoluble material was pelleted

and supernatent harvested. 30 μL of each sample was electrophoresed on a 4-12% Bis-Tris acrylamide gel and Coomassie stained to visualize bands. Arrow indicates insoluble expression of triabody.

Figure 7- Triabody iFold refolding condition matrix BoNT reactivity ELISA
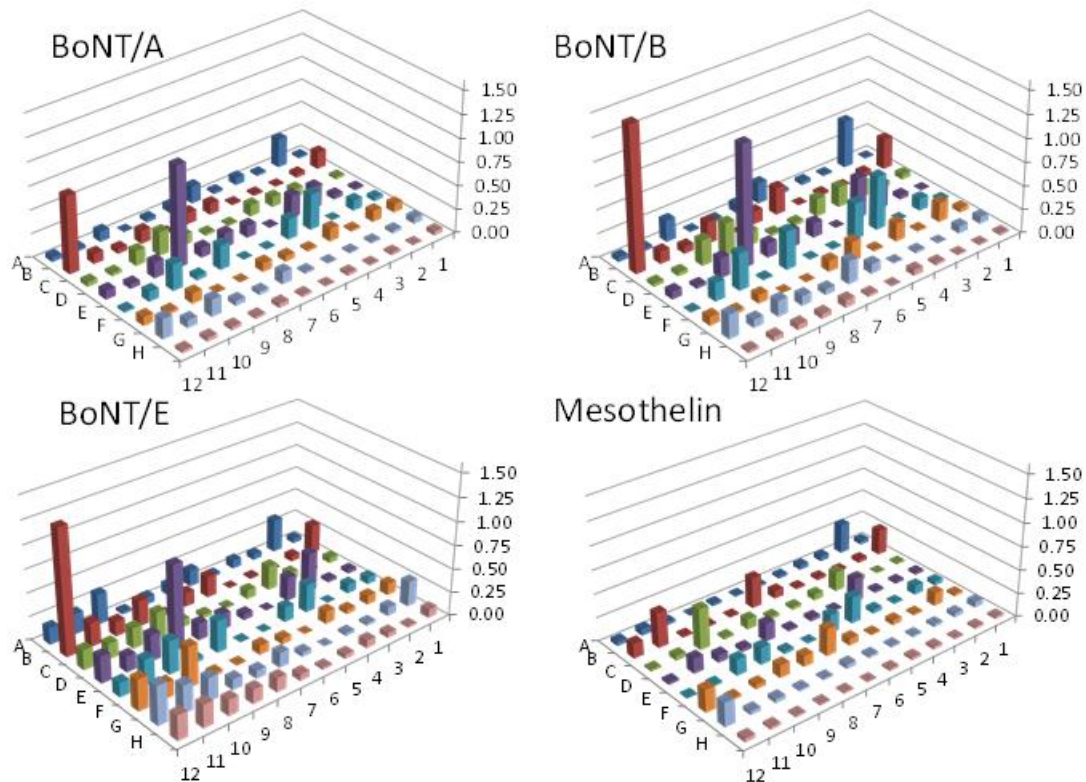
Legend: Triabody constructs were grown to log phase and induced overnight with 1mM IPTG. Inclusion bodies were isolated, resolubilized and refolded in 96 deep well format using the iFold kit (Millipore) as per manual. Samples were dialyzed in 96 well format overnight at 4°C using D-Tube96 Dialyzer (Millipore, MWCO 12-14kDa). 50 μL of material was added to ELISA plates coated with BoNT/A, B, E or mesothelin (neg control) shaking for 1 hour at room temperature. Plates were washed with TBST followed by incubation with mouse anti-myc mAb (Invitrogen). Plates were washed again and a 1:10,000 dilution of HRP-conjugated goat anti-mouse antibody (Jackson Immuno) was added with shaking. After a final wash, SureBlue substrate was added for 10 minutes, followed by H_2SO_4 and A_{450} was read to determine reactivity of refolded material.

Figure 8- BoNT/A reactivity of refolded in-line Fv and triabody constructs
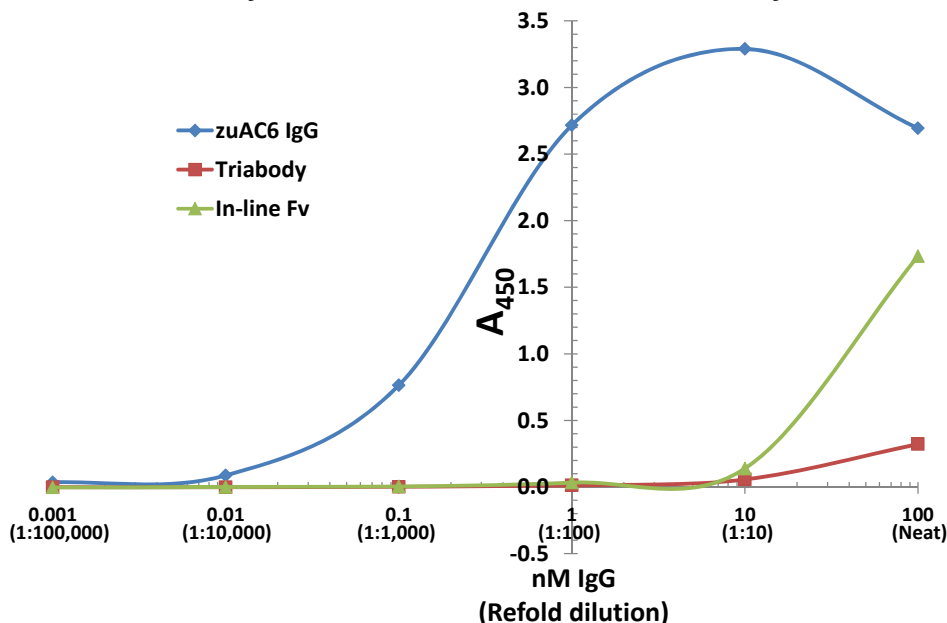
Legend: Reactivity of refolded in-line Fv and triabody structures for BoNT/A as compared to AC6 MAb.
Dilutions of refolded material from iFold condition D9 (triabody) or B12 (in-line Fv) were incubated on a BoNT/A
coated ELISA plate. For comparison, 0-100 nM (~15µg/mL) AC6 mAb were added on the same plate. Binding for
the refolded constructs was quantitated using anti-myc antibody as in Figure 7, while hAC6 IgG was quantitated
using HRP-conjugated goat anti-human secondary.

2.2   Characterization of eukaryotically expressed constructs and their BoNT reactivity

In parallel with the studies of Section 2.1, we investigated methods of eukarotic
expression to generate a therapeutic from a single cell line with neutralizing activity
against BoNT/A, /B, and /E. As described in the statement of work proposal, we
generated full length linked light/heavy chain IgG and IgM forms of our anti-BoNT/A
MAb (AC6) and anti-BoNT/B MAb (9F10). In each case, expression was driven by a
CMV promoter and downstream of the ORF was an internal ribosome entry site (IRES)
sequence, followed by a drug selection marker (Figure 9A and B). Once humanization
of a neutralizing BoNT/E MAb was complete, we could engineer it in a similar manner
using a third selection marker (Figure 9C). Using three individual selection markers, it
could be possible to generate a single cell line expressing linked IgG or IgM chains with
the trivalent neutralizing capabilities desired.

To see if eukaryotic cells could express and secrete such constructs, 293F cells
were co-transfected with linked AC6 and 9F10 IgG and IgM forms and selected with
zeocin and blasticidin. Resistant subclones were expanded and tested first for the
production of immunoglobulins and secondly for reactivity to BoNT/A and /B on toxin
coated ELISA plates. No significant amounts of IgMs could be detected from the
transfectants either in the medium or in cell lysates (data not shown), perhaps because
linked IgMs in the cell are unstable and sequentially degraded. However, subclones of
the IgG constructs were found to secret antibodies into the supernatant and exhibited

specificity against BoNT/A and BoNT/B. One such clone (G-10) is shown in Figure 10. Using 25 ng/mL of pure unlinked IgGs against BoNT/A (AC6) or BoNT/B (9F10), you can clearly see clone G-10 is producing antibodies with specificities against both toxins. In summary, this system could therefore be used to incorporated neutralizing BoNT/E IgGs by transfection of an additional construct using a unique drug selection marker.

Figure 9- Humanized single chain Ig structure construction
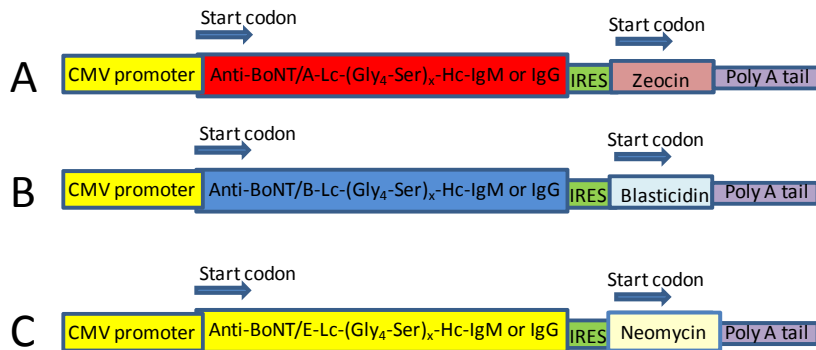
Legend: Single chain IgG and IgM constructs against BoNT/A (A, humanized clone AC6), BoNT/B (B, humanized clone 9F10), or BoNT/E (C, humanized clones 199B13 or 118O8) were synthesized with glycine-serine (G-S$_4$)$_6$ linkers connecting full length light and heavy chains, respectively (DNA 2.0, Menlo Park, CA). Each construct expresses an independent selection marker, permitting selection of all three constructs in a single cell line.

Figure 10- Dual BoNT/A and /B reactivity of linked antibodies secreted from stably transfected 293F cells
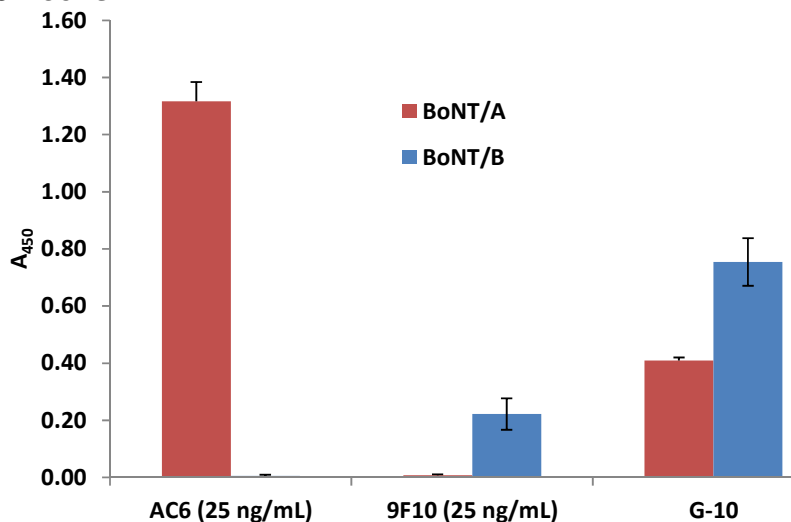
Legend: Supernatants from 293F cells stably co-transfected with linked humanized anti-BoNT/A (AC6) and -/B (9F10) constructs (clone G-10) were incubated on BoNT/A and BoNT/B coated ELISA plates (Metabiologics) in parallel with previously purified normal hAC6 and h9F10 IgGs in duplicate, shaking for 1 hour at room temperature. Plates were washed 3x with TBST and a 1:10,000 dilution of HRP-conjugated goat anti-human antibody (Jackson Immuno) was added with shaking. After a final wash, SureBlue substrate was added for 10 minutes, followed by H$_2$SO$_4$ and A$_{450}$ was read to determine dual reactivity of secreted product.

3 - Purification and molecular characterization of linked IgG molecules

This section describes the purification and analysis of bispecific IgG therapeutic secreted from the G-10 subclone described in Figure 10.

3.1 Purification of linked IgG material from clone G-10

An essential part in manufacturing any protein therapeutic is the ability to develop a purification procedure. To ensure linked IgGs could be purified using standard methods, the supernatant from clone G-10 was loaded onto a protein A column and eluted, then material was run under both non-reducing and reducing conditions (Figure 11). Under non-reducing conditions, a single band of material was observed with an apparent molecular mass of 160 KDa. This corresponds well to calculated mass of two interchain disulfide linked light chain- heavy chain constructs (~74.9 KDa each, plus glycosylation). Under reducing conditions, this interchain linkage is removed, yielding a single band of the correct molecular mass. In contrast, a normal IgG molecule is reduced into two distinct light and heavy chain bands. Taken together, this suggests the linked IgGs form the normal 2 LC/2 HC pairs observed with unlinked IgGs as no higher weight aggregates were observed. Secondly, the single chain is stable, as a single band of the correct molecular mass was observed under reducing conditions.

**Figure 11- Purification of G-10 cell line IgG, with reactivity to BoNT/A and /B**
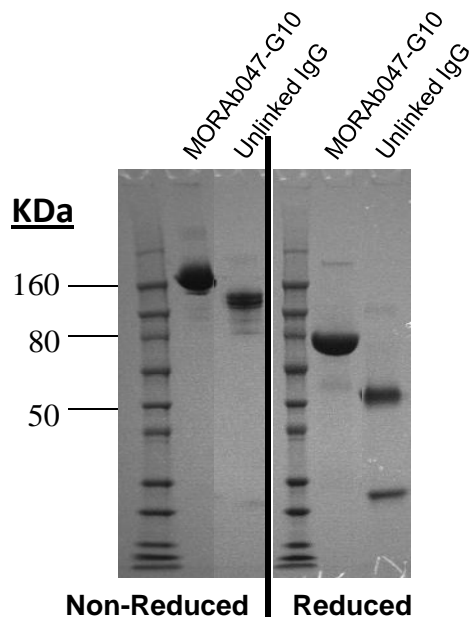
Legend: One liter of conditioned medium from a 293F cell line stably co-transfected with linked humanized AC6 and 9F10 constructs (Clone G-10) was purified by affinity chromatography on 1 mL rProteinA-4FF affinity resin (GE Healthcare) using an Xpress Twin (GE Healthcare). On-line desalting into 1X PBS was done using HiTrap desalting columns (coupled 2 x 5 mL). Purity was determined by SDS-PAGE and yield by BCA analysis. Normal unlinked IgG control was run to show differences in mass and separation of unlinked heavy and light chains upon reduction.

3.2 Mass spectrometry analysis of G-10 linked IgGs

Due to the structural nature of the linked IgGs, it is expected that the purified G-10 material should have heterogeneous intra chain disulfide pairs. For example, there should be a mix of AC6:AC6, AC6:9F10, and 9F10:9F10 molecules. To investigate the composition of the purified linked IgG material, electrospray ionization mass spectrometry (ESI-MS) was performed under non-reducing (Figure 12A) and reducing (Figure 12B) conditions. Under reducing conditions, two distinct peaks were observed corresponding to the molecular masses of the linked AC6 and 9F10 chains (Figure 12B). However, under non-reducing conditions, homo- and heterodimer molecules could be distinguished, corresponding to 9F10:9F10 IgGs, as well as AC6:9F10 IgGs. Interestingly, no AC6:AC6 homodimers were observed as would be expected. This may be perhaps there was too little to be detected, as the ratio of expression was estimated to be 3:1 favoring 9F10 over AC6.

Figure 12- ESI-MS characterization of purified G-10 IgGs
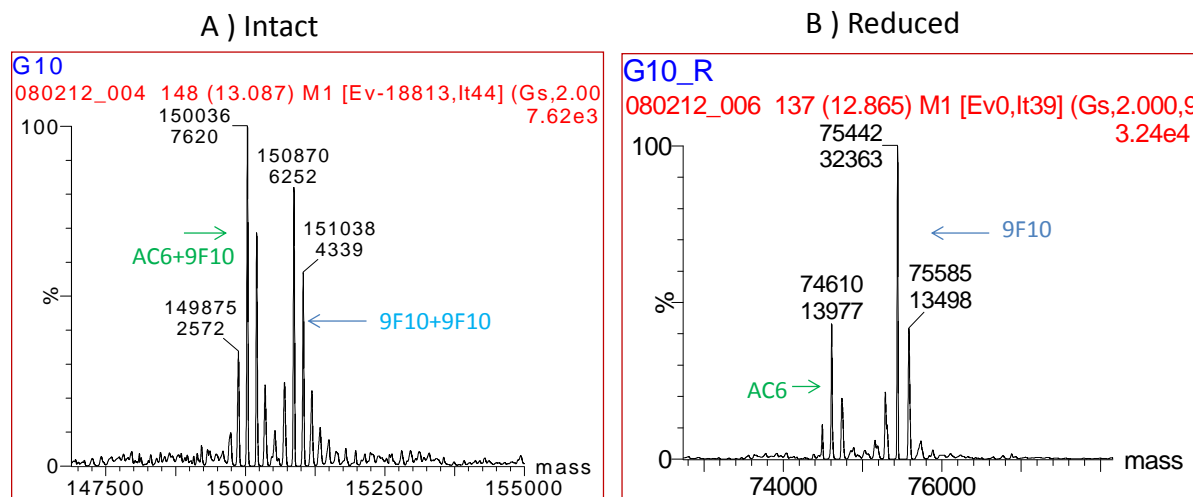
Notes: Purified G-10 (Figure 11) was deglycosylated using PNGaseF in 0.1M Tris buffer, pH 7.5, at 37°C overnight. G-10 was analyzed using Nano-ESI-MS, at 1mg/mL, injecting 1µL. A, intact material; B, Reduced. For reduction, G-10 was treated with 20mM DTT at 60 °C, 5min, prior to ESI-MS analysis. The results indicated G-10 was a mixture of homodimer of 9F10, and heterodimer of AC6-9F10. The ration was about 1:1 by intensity.

4 - Humanization and characterization of linked anti-BoNT/E MAbs *in vivo*

This section describes the humanization of two neutralizing rabbit MAbs from Table 5 *and their subsequent ability to neutralize 100LD$_{50}$s of BoNT/E in vivo.*

4.1 Humanization of 199B13 and 118O8 neutralizing rabbit-human xiMAbs

Of the 12 fully neutralizing MAbs generated from rabbits (Table 5), two (199B13 and 118O8) were chosen for humanization based on the criteria that they had completely independent CDR3 sequences and they expressed recombinantly at high levels as chimerics and were therefore believed to express well when humanized. The

amino acid sequences of the VH and VL pairs from each were used to search the online human germline database (NCBI) to find the their most homologous human counterparts (Table 6). Using these sequences, the CDRs of the VH and VL domains from each corresponding rabbit chain were grafted onto these backbones and the complete amino acid sequences of the linked light chain-heavy chain constructs codon optimized for 293F expression (DNA 2.0). Using the expression vector system described in Figure 9C, stable transfectants were expanded to generate humanized, linked forms of the two clones that were subsequently purified using the methods described in Figure 11.

Table 6- Amino acid alignment of rabbit 199B13 and 118O8 variable domains with closest human germline variable domains

	FWR1	CDR1	FWR2	CDR2	FWR3	CDR3	FWR4
Rabbit 199B13 VH	QSLEESGGRLVTPGTPLTLTCTVS	GFSLSRYAMN	WVRQAPGKGLEWIG	MITTDGSKYYASRAKG	RFTISK--TSTTVDLKMTSPTTGDTATYFCAR	VPVTGSGAWTADL	WGPGTLVTVSS
V 59.1% (55/93) IGHV3-30*15	.V....GV.Q..RS.R.S.AA.	..TF.S...H	...........VA	V.SY...N....DSV..	.....RDNSKN.LY.Q.S.LRAE...V.Y...		
Human Germline VH M77327	QVQLVESGGGVVQPGRSLRLSCAAS	GFTFSSYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCAR		
Humanized	QVQLVESGGGVVQPGRSLRLSCAAS	GFSLSRYAMN	WVRQAPGKGLEWVA	MITTDGSKYYASRAKG	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCAR	VPVTGSGAWTADL	WGSGTPVTVSS
199B13 LC	DGVMTQTPSSASEPVGGTVTIKC	QASQTIHSGLA	WYQQKPGQPPKLLIY	GASTLAS	GVPSRFKGSRSGTEYTLTISDLECADAATYYC	QTYYSISTYGNG	FGGGTEVVVK
70.0% (63/90) IGKV1-NL1*01	...S...L.AS..DR....T.	R....G.SNS..	.......KA....L.	A..R.E.	......S..G...D......S.QPE.F.....		
Human Germline VL Y14865	DIQMTQSPSSLSASVGDRVTITC	RASQGISNSLA	WYQQKPGKAPKLLLY	AASRLES	GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC		
Humanized	DIQMTQSPSSLSASVGDRVTITC	QASQTIHSGLA	WYQQKPGKAPKLLLY	GASTLAS	GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC	QTYYSISTYGNG	FGSGTKVEIK
118O8 HC	QSLEESGGRLVTPGGSLTLTCTAS	GFSISSHAMG	WVRQAPGKGLEYIG	IMDVSGVTYYAIWAKD	RFTIS-T-SSSTTVDLEMTSLTTEDTATYFCAR	ESYISWSSGSGEGYNL	WGPGTLVTVSS
V 60.6% (57/94) IGHV3-53*04	.V.....G..Q.....R.S.A..	..TV..NY.S	...........WVS	VIYSG.S....DSV.G	......-RHN.KN.LY.Q.N..RA....V.Y...		
Human Germline VH HM855453	EVQLVESGGGLVQPGGSLRLSCAAS	GFTVSSNYMS	WVRQAPGKGLEWVS	VIYSGGSTYYADSVKG	RFTISRHNSKNTLYLQMNSLRAEDTAVYYCAR		
Humanized	EVQLVESGGGLVQPGGSLRLSCAAS	GFSISSHAMG	WVRQAPGKGLEWVS	IMDVSGVTYYAIWAKD	RFTISRHNSKNTLYLQMNSLRAEDTAVYYCAR	ESYISWSSGSGEGYNL	WGSGTPVTVSS
118O8 LC	AIDMTQTPASVEVAVGGTVTIKC	QASQSIYNYLA	WYQQKPGQPPKRLIY	KASTLAS	GVPSRFKGSGSGAQYTLTISGVECADAATYYC	QQGYENDNVDNA	FGGGTEVVVK
67.4% (60/89) IGKV1-27*01	.Q...S.S.LSAS..DR....T.	R....G.S....	.......KV..L...	A....Q.	......S.....TDF.....SLQPE.V.....		
Human Germline VL X93622	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC		
Humanized	DIQMTQSPSSLSASVGDRVTITC	QASQSIYNYLA	WYQQKPGKVPKLLIY	KASTLAS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QQGYENDNVDNA	FGSGTKVEIK

Legend: Translated sequences of the VH and VL domains from rabbit clones 199B13 and 118O8 were used to determine their closest human homologues using IgBlast (National Center for Biotechnology Information, Bethesda, MD). Rabbit CDRs were grafted onto the human gerline homologues and full length light/heavy chain linked constructs synthesized with glycine-serine (G-S$_4$)$_6$ linkers connecting full length light and heavy chains, respectively (DNA 2.0, Menlo Park, CA).

4.2 *In vivo* efficacy challenge of humanized single chain 199B13 (zu199B13) and 118O8 (zu118O8) with 100LD$_{50}$s of BoNT/E

After purification of the newly designed single chain MAbs, we wished to determine if the humanized forms of these MAbs were still capable of neutralizing BoNT/E *in vivo*. In this particular experiment doses were increased to 17.5 (zu199B13) and 14.7 (zu118O8) mg/kg, as compared to 7 mg/kg used in previous experiments (Figure 13). Based on the post-purification concentrations, this was the maximum injectable dose for each. We reasoned if they could not protect against 100LD$_{50}$s at these doses, the MAbs were not worth pursuing as therapeutics. Under these conditions, clone zu118O8 did not elicit protection, with all mice dying within a 24 hour period. However, zu199B13 did protect completely, therefore fulfilling the aim of generating a single chain, humanized anti-BoNT/E MAb.

Figure 13- Linked humanized clone 199B13 protects against 100LD$_{50}$ BoNT/E challenge
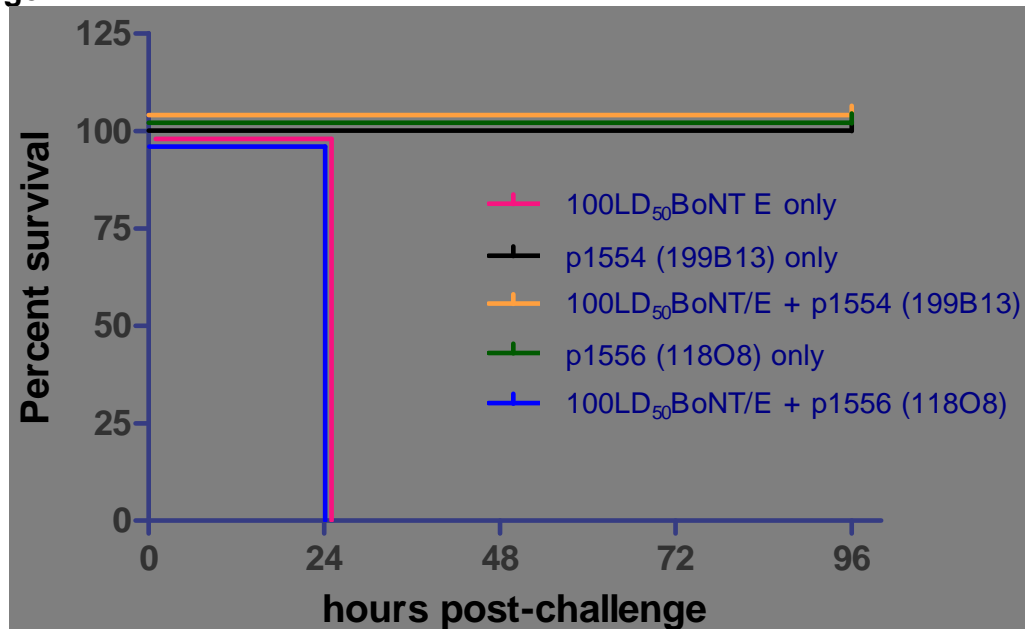
Legend: To determine the *in vivo* efficacy of linked, humanized anti-BoNT/E MAbs, a standard mouse model was employed. CD-1 mice were challenged by intraperitoneal (i.p.) injection of 100 mouse LD$_{50}$s of BoNT/Es (MetaBiologics Inc., WI), while administering a single dose of 375 μg (p1554, linked zu199B13) or 315 μg (p1556, linked zu118O8) anti-BoNT/E MAb. Individual MAbs were incubated with 100 mouse LD$_{50}$ doses of a BoNT/E for 1 hour at room temperature. The toxin and antibody mixtures were administered i.p. in a volume of 0.5 mL per mouse. The mice were scored for survivors twice a day after challenge for 4 days. Two mice per cohort were employed and survival of all treated animals within a cohort will signify full protection by the antitoxin.

5 - Conclusions

In this report, we describe the discovery, characterization, and humanization of anti-BoNT/E MAbs which are capable of neutralizing 100LD$_{50}$s. Below are listed the specific aims of the project along with commentary on deliverables generated.

5.1 Aim 1: Generation of anti-BoNT/E MAbs

We described the generation and characterization of MAbs derived from both rat and rabbit origins. While a few rat clones were capable of neutralizing 10LD50s of BoNT/E, these were not deemed efficacious and were therefore discontinued. However, studies using rabbit anti-serum, followed by rabbit-human xiMAbs described the discovery of 12 MAbs which completely protected from challenge with 100LD50s BoNT/E.

5.2 Aim 2: Humanization of anti-BoNT/E MAbs

Of the 12 neutralizing xiMAbs generated, two were humanized and expressed as single chain constructs. One of these (zu199B13) was capable of protecting against 100LD$_{50}$s of BoNT/E *in vivo.*   It is also important to note that 10 other xiMAbs were

neutralizers. However, due to time and manpower constraints we only focused on two MAbs. Future studies could be focused on the humanization and characterization of these remaining antibodies in the single chain format.

5.3 <u>Aim 3&4</u>: Engineering of a single cell line capable of producing three humanized IgGs against BoNT/A, /B, and /E

While the characterization of MAbs generated by the immunizations was underway, we examined a variety of pro- and eukaryotic scaffolds for production of trivalent neutralizing constructs. In both prokaryotic methods (in-line Fv and triabodies), reactivity against BoNTs could be observed, but either the affinities or percentage of correctly folded material negated their further study. In eukaryotic (293F) cells, linked full length IgMs and IgGs were tested for expression. While the IgM forms did not express, the IgG forms did. We therefore tested the feasibility of this platform by stably co-transfecting single chain forms of our previously generated BoNT/A and /B clones. Supernatants from a subclone (G-10) showed reactivity against both BoNT/A and BoNT/B by ELISA. Based on these observations, we believe this platform can be extended in future studies to a third anti-BoNT/E single chain MAb.

5.4 <u>Aim 5</u>: Development of purification method for MAbs generated in Aims 3 and 4.

Antibodies from the G-10 clone were secreted into the supernatant and could be purified using standard protein A based methods. Molecular characterization by ESI-MS showed the presence of both homo- and heterodimers as expected. On Coomassie gels, the linked antibodies ran at the expected apparent molecular masses, suggesting aberrant multimerization was not occurring, and that no proteolytic degradation was observed.

5.5 <u>Aim 6</u>: Characterization of linked IgGs for *in vivo* protection against BoNT/A, /B, and E

Two of the neutralizing xiMAbs generated from rabbits were humanized and expressed as linked single chain IgGs. One of these (zu199B13) was able to completely protect mice against challenge with $100LD_{50}$s of BoNT/E. Based on these results, we believe this construct can be transfected into the G-10 cell line, making it express three independent IgGs capable of neutralizing BoNT/A, /B, and /E.

5.6 <u>Aim 7</u>: Generation of a master cell bank of the production line

Because of the lag of timelines for the project to complete immunizations, this aim could not be completed. However, with the materials generated in this report, this could be completed in about six months' time.

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