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Development of human-like scFv-Fc antibodies neutralizing Botulinum toxin serotype B

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Botulinum neurotoxins (BoNTs) are responsible for human botulism, a life-threatening disease characterized by flaccid muscle paralysis that occurs naturally by food poisoning or colonization of the gastrointestinal tract by BoNT-producing clostridia. BoNTs have been classified as category A agents by the Centers for Disease Control and Prevention. To date, 7 subtypes of BoNT/B were identified showing that subtypes B1 (16 strains) and B2 (32 strains) constitute the vast majority of BoNT/B strains. Neutralizing antibodies are required for the development of anti-botulism drugs to deal with the potential risk. In this study, macaques (*Macaca fascicularis*) were immunized with recombinant light chain (LC) or heavy chain (HC) of BoNT/B2, followed by the construction of 2 hyper-immune phage display libraries. The best single-chain variable fragments (scFvs) isolated from each library were selected according to their affinities and cross reactivity with BoNT/B1 toxin subtype. These scFvs against LC and HC were further analyzed by assessing the inhibition of in vitro endopeptidase activity of BoNT/B1 and B2 and neutralization of BoNT/B1 and B2 toxin-induced paralysis in the mouse ex vivo phrenic nerve assay. The antibodies B2–7 (against HC) and BLC3 (against LC) were produced as scFv-Fc, and, when tested individually, neutralized BoNT/B1 and BoNT/B2 in a mouse ex vivo phrenic nerve assay. Whereas only scFv-Fc BLC3 alone protected mice against BoNT/B2-induced paralysis in vivo, when B2–7 and BLC3 were combined they exhibited potent synergistic protection. The present study provided an opportunity to assess the extent of antibody-mediated neutralization of BoNT/B1 and BoNT/B2 subtypes in ex vivo and in vitro assays, and to confirm the benefit of the synergistic effect of antibodies targeting the 2 distinct functional domains of the toxin in vivo. Notably, the framework regions of the most promising antibodies (B2–7 and BLC3) are close to the human germline sequences, which suggest that they may be well tolerated in potential clinical development.

Introduction

Botulinum neurotoxins (BoNT/s) are proteins secreted by the anaerobic spore-forming bacteria, *Clostridium botulinum*, which is responsible for the naturally-occurring lethal disease, botulism. BoNTs are classified in 7 serotypes (A to G) where BoNT/A, B and E are mainly implicated in natural human intoxication, with A and B serotypes being responsible for more than 90% of the cases.[1,2] Due to their high toxicity and ease of production and dissemination, BoNTs are also considered by the Centers for Disease Control and Prevention as one of the major threats for bioterrorism as a "Category A" agent.[3] In fact, BoNT/A and BoNT/B are considered to be the most toxic substances known, with a human LD_{50} estimated between 1 and 10 $ng.kg^{-1}$ by intravenous, subcutaneous, intra-peritoneal and intramuscular routes; between 10 and 21 $ng.kg^{-1}$ by pulmonary route and at 1 $\mu g.kg^{-1}$ by oral route.[3-9] To date, 7 subtypes of BoNT/B (BoNT/B1 to B7) have been identified.[10,11] The analysis of 53

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Clostridium botulinum B strains showed that subtypes B1 (16 strains) and B2 (32 strains) constitute the vast majority of BoNT/B strains. At the amino acid sequence level, BoNT/B1 and BoNT/B2 holotoxins present an overall identity of 96%, and their heavy chains present an identity of 91%.[12] BoNTs are composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC). The toxin binds to the neuronal cell surface due to the interaction between the C-terminus of the HC and high affinity receptors on the neural cell surface, and then penetrates into the cell by endocytosis.[13] The endosome acidification triggers the translocation of the LC from the lumen of the vesicles into the cytoplasm. The LC acts as a zinc-metalloprotease that cleaves components of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, implicated in the fusion of the exocytosis vesicle with the cell plasma membrane. BoNT/B selectively cleaves synaptobrevin 2 (also known as VAMP2),[14] resulting in the inhibition of the acetylcholine release at the neuromuscular junction, causing a life-threatening progressive descending flaccid paralysis that requires long term treatment in intensive care unit.[15,16]

Botulinum neurotoxin serotypes A and B are increasingly being used therapeutically to locally paralyse muscles for clinical or cosmetic benefit. Initially used to treat strabismus in the 1970s, BoNTs have more than a hundred possible medical applications, including treatment of movement disorders, vascular cerebral stroke, chronic pain, hyperhidrosis and inflammation.[17,18] Vaccination against botulism is ethically disputable since it could potentially prevent many patients from the benefit of these wide therapeutic applications.[19]

The current treatment against botulism is long, expensive and difficult to apply on a large scale. It requires respiratory assistance and the injection of anti-toxin antibodies.[20] A human-immunoglobulin preparation that neutralizes BoNTs (Big-IV, traded as BabyBIG®, supplied by the California Department of Public Health) was developed from pooled plasma of subjects immunized with a pentavalent vaccine (composed of BoNT/A, B, C, D and E toxoids) who were selected for their high titres of neutralizing antibodies against BoNT/A and B.[21] Because of its human origin, this immunoglobulin preparation is well tolerated, but expensive and available in very limited quantity, and thus it is only used for the treatment of botulism in patients below one year of age. Considering these limitations, botulism is treated by injections of horse-derived trivalent (A, B, E) antitoxin (Botulismus–Antitoxin Behring, Novartis, Basel, Switzerland), which is available in a larger quantity. In March 2013, the Food and Drug Administration (FDA) approved an Equine heptavalent botulism antitoxin (HBAT), manufactured by Cangene Corporation, for the treatment or suspected exposure to Botulinum neurotoxin serotypes A, B, C, D, E, F and G. The animal origin of these antitoxins increases their immunogenicity and thus the risk of serum sickness or anaphylaxis.[22] To overcome these limitations, recombinant human antibodies that could improve the safety and tolerance of antitoxin treatment are of particular interest.

Several studies to date isolated neutralizing antibodies directed against the HC or LC of BoNTs, starting from an immune or non-immune human library.[23-25] Kalb et al. have isolated several antibodies targeting the BoNT/B-LC and cross-interacting with BoNT/B1 and B2 with picomolar affinities (flow fluorometry evaluation).[23] Two of these antibodies, 2B27 and 1B10.1, seemed to inhibit the catalytic activity of some BoNT/B subtypes by interacting with the LC in vitro, determined by endopeptidase-MS.[23] A human IgG (30B) recognizing BoNT/B-HC with a high affinity (1.12 10^{-12} M) was previously isolated by hybridoma technology starting from the lymphocytes of human volunteers vaccinated with the pentavalent botulinum toxoid vaccine. This antibody only showed partial inhibition of BoNT/B1 in vivo as it delayed paralysis, but did not prevent mice mortality.[24] Studies by Kalb et al. reported in vitro inhibition of the enzymatic activity of different BoNT/B subtypes using a panel composed of 24 fully human antibodies directed against BoNT/B1, B2, B3, B4 and B5 toxins in complex form.[23] These IgGs were initially isolated as antibody fragments from an immune library and were engineered to improve their affinity or cross-reactivity, but were not tested for toxin neutralization properties using in vivo or ex vivo models, which are required to confirm neutralization.[26,27] The first report of the potentially neutralizing human monoclonal antibody against BoNT/B, derived from naïve single-chain variable fragment (scFv) phage display library against BoNT/B HC domain was reported by Zhou et al.[28] These studies also did not include in vivo or ex vivo neutralization studies, but relied exclusively on in vitro cell-based assay. More recent studies by Garcia-Rodriguez et al. confirmed the neutralizing property of one human monoclonal antibody (previously reported by Kalb et al.), 2B18, which recognizes a conserved epitope within Hn domain on multiple serotypes.[29] This antibody, at 25 μg, fully protected mice exposed to 200 LD_{50} of BoNT/B1 toxin. To our knowledge human-like neutralizing antibodies against BoNT/B toxin targeting its LC have not been published to date.

This study developed human-like neutralizing antibodies against BoNT/B by targeting the toxin HC and LC domains. The recombinant form of the HC and LC of BoNT/B2 were used to immunize macaques, from which the immune antibody phage-display library was built. The best scFvs isolated from each library were selected according to their affinities and cross reactivity with BoNT/B1. Screening for the best candidates was performed with holotoxins BoNT/B1 and B2 by assessing inhibition of in vitro endopeptidase activity and neutralization in the ex vivo mouse phrenic nerve-hemidiaphragm (MPNH) assay for scFvs targeting LC and HC, respectively. The more stable form of selected antibodies, expressed as a fusion protein (scFv-Fc), were further studied for their neutralization properties in the mouse ex vivo MPNH assay and in vivo mouse paralysis model.

The study reported here provided an excellent opportunity to assess the extent of antibody-mediated neutralization of BoNT/B1 and BoNT/B2 subtypes in ex vivo and in vitro assays and to confirm in vivo the benefit of synergistic effect of selected antibodies targeting the HC and LC domains of BoNT/B.

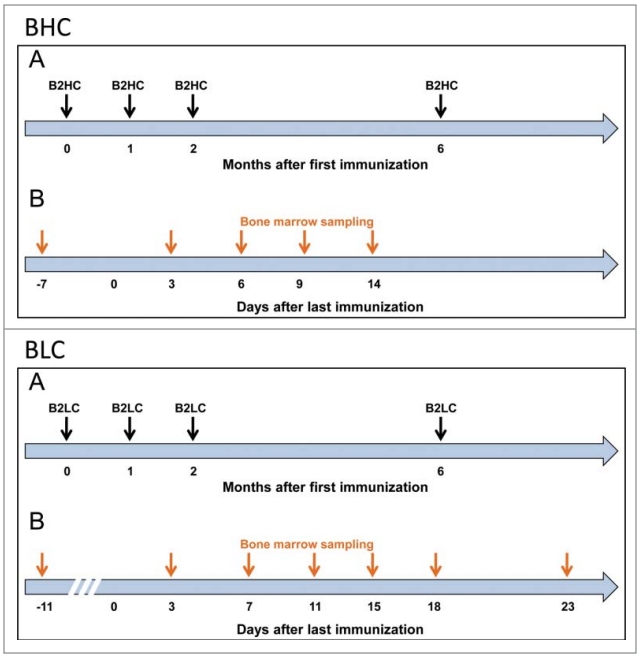

Figure 1. Immunization scheme and bone marrow sampling times. Protocol for hyperimmunization of cynomolgus macaques (*Macaca fascicularis*) with BoNT/B2-HC (BHC) and BoNT/B2-LC (BLC). (**A**): The first 3 injections were administered at one month intervals and the fourth injection was administered 4 months after the third. Animals were bled for donation of serum before the first immunization (day 0) and 3 to 5 d prior to the first booster injection. For BoNT/B2-LC, serum sampling was also performed 8 days after the third injection and after the last immunization of each animal. (**B**): Bone marrow sampling was performed 7 to 11 d prior to the first immunization and at regular intervals after the last immunization: on days 3, 6, 9, and 14 for BoNT/B2-HC; on days 3, 7, 11, 16, 18 and 23 for BoNT/B2-LC.

Results

Animal immunization and library construction

Non-toxic recombinant BoNT/B2-HC or BoNT/B2-LC were used to immunize 2 macaques by the immunization schedule depicted in **Figure 1**. The same proteins were also used as coating antigens for ELISA to determine antibody titre in animals, which increased by 130-fold for B2-HC and 250-fold for B2-LC, between samples taken just before the first immunization and just before second immunization, one month later. After the fourth and last immunization of each animal, a titre of 1/100,000 (BoNT/B2-HC immunization) or 1/300,000 (BoNT/B2-LC immunization) was observed against the immunogen. Both titres were identical to those observed after the third injection (typically between 3 to 11 d after the third immunization) with the same antigen, confirming that a hyper-immune response had been reached. **Figure 1** also indicates the scheme for bone marrow sampling times post immunization.

For construction of the BoNT/B2-HC library, the RNA from the macaque bone marrow was transcribed into cDNA and, with primer sets specific for macaque Fd and VLκ DNA regions, the V-genes were amplified. The amplification increased and reached an optimum 3 d after the fourth and last immunization when all primer pairs allowed a stronger amplification compared to other days. Considering the absence of amplification before the final immunization, the PCR products amplified at the third day were considered as specifically encoding variable regions of antibodies directed against BoNT/B2-HC, thus a hyper-immune response was raised against the immunogen.[30] Two sub-libraries, with a diversity of $2.6 \times 10^5$and 2.3×10^5clones coding for Fd and VLκ regions, respectively, were constructed in the pGEM®-T vector. These two sub-libraries were then combined together to generate a final phage-displayed scFv hyper-immune library composed of 1.1×10^8 clones in pHAL32 vector, with 95% full size scFv inserts.

For construction of BoNT/B2-LC library, the V-Gene encoding DNA fragments was similarly amplified starting from the bone marrow of a second macaque, with the same specific primer sets.[31] The amplification was optimal 7 d after the fourth and last immunization. All Fd PCR products and all VLκ PCR products were separately pooled and inserted into pGEM®-T vector. Two sub-libraries of 5×10^5 clones for the DNA encoding the Fd fragment, and 4.65×10^5 clones for the DNA encoding the VLκ chain, were generated. Starting from these 2 sub-libraries, a final phage-displayed scFv library, with a diversity of 1.2×10^8 clones, was constructed in pHAL35 vector, with 93% full-size scFv inserts.

Panning of the library for the isolation of cross-reacting scFvs

To isolate scFvs cross-reacting with BoNT/B1 and BoNT/B2 subtypes, the 2 phage-displayed scFv hyper-immune libraries were subjected to an innovative 2-time panning. First, a 3 round panning was performed on the BoNT/B1 holotoxin with an increasing number of washes, then a fourth and last round of panning was performed against the recombinant chain of the different BoNT/B subtype directly with a high number of washes to select only the cross-reactive phage at the end of the panning.

For the panning of the hyper-immune library directed against BoNT/B2-HC (**Fig. S1A**), the count of phage eluted after the first round was 4.6×10^5, decreasing 16-fold after the second round to 2.84×10^4, and then increasing 4-fold after the third round to 1.32×10^5. After the fourth and last round, the number of eluted phage increased dramatically by more than a hundred-fold to 1.54×10^7. To evaluate the cross-reactivity of the eluted phage, a phage-ELISA was performed with BoNT/B1 and BoNT/B2-HC as coating antigens. Signals for both antigens increased progressively and specifically after each round, which confirms enrichment of scFvs specific for both BoNT/B1 and BoNT/B2-HC during the course of the panning.

For the panning of the library directed against BoNT/B2-LC, (**Fig. S1B**) the number of eluted phage increased from 4.6×10^5 (first round) to 1.6×10^8 (third round), which corresponded to a successful enrichment of phage specific to BoNT/B1 holotoxin. After the fourth round of panning on BoNT/B2-LC, 1.3×10^8 phage were eluted which corresponded to the specific enrichment of phage specific to both BoNT/B1 and B2 subtypes. The cross-reactivity of the phage eluted after each of the 4 rounds of panning against BoNT/B1 and BoNT/B2-LC was assessed in

phage-ELISA against both antigens. The reactivity increased after each round, leading to an optimum reactivity after the fourth and last round (10- to 13-fold increase of reactivity against BoNT/B2-LC and BoNT/B1 between the first and last round, respectively). As expected, after round 2, eluted phage were as reactive against BoNT/B1 as against BoNT/B2-LC, highlighting their cross-reacting properties. No reactivity was observed against keyhole limpet hemocyanin (KLH) or lethal factor (LF), confirming their specificity.

Expression and purification of soluble cross-reacting scFvs targeting BoNT/B-HC or BoNT/B-LC

One hundred clones were randomly handpicked after the fourth round of panning against BoNT/B2-HC, and then sequenced to express the non-redundant and non-recombined scFvs cross-reacting with BoNT/B1 and BoNT/B2. Seventeen sequences were recombined and thus discarded, and 83 non-recombined sequences were identified. Among these 83 non-recombined sequences, only 10 different scFv sequences were identified: 7 scFvs (B2–1, B2–6, B2–7, B2–11, B2–29, B2–63 and B2–75) were represented once and 3 scFvs (B2–48, B2–86 and B2–119) were represented several times. It is interesting to note that among the 3 scFvs represented several times, scFv B2–48 and B2–119 were over-represented, with 43 and 31 occurrences, respectively. The 10 different scFvs were expressed in *E. coli* strain HB2151 as soluble scFvs and their affinities were measured by surface plasmon resonance (SPR). The affinities ranged from 2.29 nM (B2–6) to 15 nM (B2–63) for the holotoxin BoNT/B1 (**Table 1**), and from 0.2 nM (B2–119) to 11 nM (B2–29) for BoNT/B2-HC, with affinities against BoNT/B2-HC (the immunogen) always being more favorable than against BoNT/B1. The affinity of scFv B2–7 was 5 nM and 0.6 nM for the holotoxin BoNT/B1 and BoNT/B-HC, respectively. The $\mathbf{K}_{on}$ ($M^{-1} \times s^{-1}$) and $\mathbf{K}_{off}$ (s^{-1}) values were also calculated for scFv targeting the LC (**Table 1**), and were most favorable for BLC3 and BLC42, which were confirmed to bind to the same or very close epitope on the BoNT/B1 toxin.

Table 1. Affinities of the scFvs for BoNT/B1 isolated after panning

Sequence	Affinity for BoNT/B1 (K_D, nM)	K_{on} (1/Ms)	K_{off} (1/s)
BLC3	0.4	7.21×10^5	2.91×10^{-4}
BLC4	2.8	4.01×10^4	1.12×10^{-4}
BLC6	1.29	4.68×10^4	6.06×10^{-5}
BLC7	3	7.96×10^4	2.45×10^{-4}
BLC13	9.3	1.4×10^4	1.3×10^{-4}
BLC14	4.15	4.45×10^4	1.84×10^{-4}
BLC16	1.68	5.93×10^4	9.98×10^{-5}
BLC17	2.3	4.26×10^4	9.81×10^{-5}
BLC18	17	7.46×10^3	1.27×10^{-4}
BLC19	1.64	6.22×10^4	1.02×10^{-4}
BLC28	0.74	1.05×10^5	8.13×10^{-5}
BLC29	0.66	1.28×10^5	8.58×10^{-5}
BLC30	12.2	—	—
BLC33	1.46	1.46×10^5	2.14×10^{-4}
BLC42	0.73	2.46×10^5	1.79×10^{-4}
BLC43	3.84	4.57×10^4	1.76×10^{-4}
BLC44	2.12	8.03×10^4	1.7×10^{-4}
BLC49	0.648	1.75×10^5	1.13×10^{-4}
BLC51	4.9	3.54×10^4	1.75×10^{-4}
BLC59	0.524	2.32×10^5	1.22×10^{-4}
BLC61	2.39	6.42×10^4	1.54×10^{-4}
BLC63	5.98	4.03×10^4	2.37×10^{-4}
BLC66	4.6	2.43×10^4	1.12×10^{-4}
BLC77	5.3	2.65×10^4	1.41×10^{-4}
BLC83	6	4.5×10^4	2.71×10^{-4}
BLC119	16.5	1.61×10^4	2.67×10^{-4}

Sequence	Affinity for BoNT/B1 (K_D, nM)	K_{on} (1/Ms)	K_{off} (1/s)
B2–1	8.68	5.92×10^4	5.14×10^{-4}
B2–6	2.29	—	—
B2–7	4.83	$2.07 \times 10^{8*}$	—
B2–11	8	—	—
B2–29	12	—	—
B2–48	8.3	—	—
B2–63	14.1	$7.11 \times 10^{7*}$	—
B2–75	Non-specific	—	—
B2–86	6	—	—
B2–119	13	—	—

*KA (1/M).

— = not determined.

Affinities of the 36 scFvs, 10 isolated after the panning of the libraries directed against BoNT/B2-HC and 26 against BoNT/B2-LC were measured by surface Plasmon resonance (Biacore® technology) for holotoxin BoNT/B1.

After the fourth round of panning of the library directed against BoNT/B2-LC, 130 clones were randomly picked from the very high number of eluted phage, and their DNA was extracted and sequenced. Thirty-one non-recombined and non-redundant scFv sequences were identified. Twenty-six sequences (83.87% of the non-recombined, non-redundant sequences) were found in a single copy, whereas the other 5 sequences (16.13%) were found in at least 2 copies. The 26 single copy sequences were expressed as soluble scFvs, purified and their affinities for BoNT/B1 and BoNT/B2-LC were measured. Affinities for BoNT/B1 ranged from 0.4 nM (BLC3) to 17 nM (BLC18) (**Table 1**). Affinities for BoNT/B2-LC were performed only on a sub-set (50%) of scFvs and they were generally less favorable than for the holotoxin BoNT/B1, with an affinity of 50 nM determined for BLC3.

Computational analysis

The IMGT/V-QUEST tool was used to analyze the DNA sequence of the isolated scFv to identify the V, D and J genes from which they would have derived if they had been of human origin. For the scFv isolated after the panning of the library directed against BoNT/B2-HC, 7 occurrences of IGHV4-b*01 were observed, 2 of IGHV3–66*02 and one of IGHV4–4*02, were observed. These IGHVs were combined with IGHD1–1*01 (7 occurrences), IGHD3–16*01, IGHD3–3*01 or IGHD5–18*0 (one occurrence each), and with IGHJ5*01, IGHJ5*02, IGHJ4*02 or IGHJ3*01 (1, 7, 1 and 1 occurrences, respectively). The genic diversity of the VLκ region was slightly more important as 6 V genes were identified: IGKV1–12*01, IGKV1–27*01, IGKV1–37*01, IGKV1–17*01, IGKV1–8*01 and IGKV1–39*01 (1, 1, 4, 1, 2 and 1 occurrences, respectively). Nevertheless, these 6 genes were combined only with IGKJ1*01 or IGKJ4*01 (9 and 1 occurrences, respectively). The percentage

Table 2. Human germlines gene and Germinality Index (GI) Human germline genes most similar to the genes coding for the 36 selected scFv were retrieved by IMGT/V-QUEST. The percentage identity between the scFv framework regions and those of the most similar human germline gene sequences (referred to as Germinality Index, GI) was calculated to indirectly predict their immunogenicity

	Heavy chain			Light chain		GI		
scFv	V	D	J	V	J	Heavy chain	Light chain	Mean
BLC3	IGHV4–28*06	IGHD5–12*01	IGHJ4*02	IGKV1–12*01	IGKJ2*01	85.71	85.71	85.71
BLC4	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	87.91	84.26	86.08
BLC6	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	85.71	83.14	84.42
BLC7	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	85.71	81.81	83.76
BLC13	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	82.41	83.14	82.77
BLC14	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	84.44	82.02	83.23
BLC16	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	85.71	82.02	83.86
BLC17	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	84.61	83.14	83.87
BLC18	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	85.71	85.22	85.46
BLC19	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	86.81	82.02	84.41
BLC28	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	86.81	82.02	84.41
BLC29	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	84.61	82.02	83.31
BLC30	IGHV3–66*02	IGHD1–1*01	IGHJ5*02	IGKV3–15*01	IGKJ3*01	87.91	66.29	77.1
BLC33	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	87.91	79.77	83.84
BLC39	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	86.81	72.14	79.47
BLC42	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	84.61	82.02	83.31
BLC43	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	86.81	82.02	84.41
BLC44	IGHV4-b*01	IGHD5–12*01	IGHJ4*02	IGKV1–5*03	IGKJ2*01	84.61	86.51	85.56
BLC49	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	85.71	82.15	83.93
BLC51	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	82.41	83.14	82.77
BLC59	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	84.61	80.89	82.75
BLC61	IGHV4-b*02	IGHD2–8*02	IGHJ5*02	IGKV1–8*01	IGKJ1*01	82.41	82.55	82.48
BLC63	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	75.82	79.77	77.79
BLC66	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	87.91	84.09	86
BLC77	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ1*01	86.81	85.39	86.1
BLC80	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*03	85.71	80.68	83.19
BLC83	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	84.61	82.02	83.31
BLC88	IGHV4–4*02	IGHD4–17*01	IGHJ4*02	IGKV1–5*03	IGKJ2*01	85.71	84.26	84.98
BLC90	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	86.81	82.02	84.41
BLC99	IGHV3–66*02	IGHD1–1*01	IGHJ5*02	IGKV1–17*01	IGKJ3*01	84.61	87.05	85.83
BLC119	IGHV3–15*01	IGHD5–12*01	IGHJ4*02	IGKV2–30*02	IGKJ2*01	85.71	82.02	83.86
B2–1	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–37*01	IGKJ4*01	85.71	78.4	82.06
B2–6	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–37*01	IGKJ4*01	85.71	76.13	80.92
B2–7	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–37*01	IGKJ4*01	85.71	77.27	81.49
B2–11	IGHV3–66*02	IGHD3–16*01	IGHJ5*01	IGKV1–12*01	IGKJ1*01	86.81	90.9	88.85
B2–29	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–27*01	IGKJ4*01	89.01	86.36	87.68
B2–48	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–37*01	IGKJ4*01	85.71	77.27	81.49
B2–63	IGHV4–4*02	IGHD3–3*01	IGHJ4*02	IGKV1–17*01	IGKJ4*01	91.2	87.64	89.42
B2–75	IGHV3–66*02	IGHD5–18*01	IGHJ3*01	IGKV1–39*01	IGKJ4*01	81.31	82.95	82.13
B2–86	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–8*01	IGKJ4*01	85.71	77.27	81.49
B2–119	IGHV4-b*01	IGHD1–1*01	IGHJ5*02	IGKV1–8*01	IGKJ4*01	85.71	77.27	81.49

of similarity between the framework regions of each scFv peptide sequence and the most similar human germline peptide sequences (percentage referred to as Germinality Index, GI) was calculated for each isolated scFv and averaged (average of the antibody heavy and light chain of the framework regions of each scFv). For the 10 isolated scFvs, GIs ranged from 80 to 89%, and the mean GI was 82.7%, which highlights their human-like character. The G-score, which evaluates the "humanness," and also predicts scFv potential immunogenicity by comparison with human expressed sequences, was calculated for each VH and VL region and averaged for each scFv. The scFv mean G-scores for the 10 selected scFvs ranged from −0.60 to −1.87, with a mean value equal to −1.45 (**Table S1**).

Similarly, for the library directed against BoNT/B2-LC, scFv sequences identified after clones-sequencing were subjected to a computational analysis. As shown in **Table 2**, low gene diversity was observed. Indeed, for IGHV genes, only 5 genes from IGVH3 and IGVH4 were observed with 25 occurrences of IGHV3–15*01, 2 occurrences of both IGHV3–66*02 and IGHV4-b*02, and finally one occurrence of each of IGHV4–28*06 and IGHV4-b*01. These 5 VH genes were combined with one of the 28 occurrences of IGHJ4*02 or with one of the 3 of IGHJ5*02. For antibody light chains, the use of 3 different IGKV genes (IGKV1, 2 and 3) was observed: IGKV2–30*02 (25 occurrences), IGKV1–5*03 (2 occurrences) and IGKV1–12*01, IGKV1–8*01, IGKV3–15*01

and IGKV1–17*01 (1 occurrence each). These IGKV genes are also combined to 4 different IGKJ (of IGK families 1, 2 and 3). For the antibody heavy chain, 25 associations "IGHV3–15*01/IGHD5–12*01/IGHJ4*02"were observed and were principally associated with one of the 17 "IGKV2–30*02/IGKJ2*01" combinations. Even with apparent low diversity, the GI score assessments revealed the presence of several point-mutations, thus increasing the sequence diversity. The GIs of the 26 isolated scFvs are presented in **Table 2**, with values ranging from 75.82% to 87.91% for the antibody heavy chains and from 79.77% to 84.09% for antibody light chains, resulting in an average value for the whole scFv molecule of between 77.79% and 86% (**Table 1**). The mean G-scores for 26 selected scFvs ranged between −1.0035 and −2.746, underlining that some scFvs are close to human antibody sequences (**Table S1**).

Differential dose response curves of BoNT/B1 and BoNT/B2 subtypes in ex vivo and in vitro assays

In order to select potentially cross-neutralizing scFvs directed against the BoNT/B2-LC or HC, during screening studies we applied the in vitro endopeptidase assay or the ex vivo MPNH assay using both BoNT/B1 and BoNT/B2 subtypes.

For the in vitro endopeptidase assay, dose response curves were compared for complex forms of BoNT/B1 and BoNT/B2 and for pure holotoxin BoNT/B1. Interestingly, complex BoNT/B2 subtype exhibited at least 2,000-fold higher enzyme activity in this model compared to complex BoNT/B1 (**Fig. S2**), despite comparable activities in the mouse LD_{50} and paralysis in vivo assays. Additional studies with purified LC of BoNT/B1 and BoNT/B2 did not show a difference in enzyme activity, suggesting that complex BoNT/B2 toxin may be more stable in this assay compared to BoNT/B1 toxin.

The complex form of BoNT/B1 had ~4 times higher endopeptidase activity relative to pure BoNT/B1 toxin (**Fig. S2**), which was also confirmed in vivo when the 2 preparations were tested together in the paralysis assay. Such differences are often observed for preparations calibrated in different laboratories and are not unexpected.

A full dose range for pure BoNT/B1 subtype (60 $LD_{50}.mL^{-1}$ to 240 $LD_{50}.mL^{-1}$) has been previously assessed using in-bred Balb/c mice in the ex vivo MPNH assay.[32] A similar dose response curve could not be established with complex BoNT/B2 because there was no significant dose effect above 50 $LD_{50}.mL^{-1}$, and a very steep dose-effect at lower concentrations, which prevented similar fitting. However, complex BoNT/B2 was found to be 500-fold more potent relative to pure BoNT/B1 in the MPNH assay since 0.2 $LD_{50}.mL^{-1}$ of complex BoNT/B2 induced similar paralysis to 100 LD_{50} of pure BoNT/B1. The much greater paralytic effect of complex BoNT/B2 was initially evidenced from a 50% paralysis time that was 3-times shorter with 200 $LD_{50}.mL^{-1}$ of BoNT/B2 complex than with 240 $LD_{50}.mL^{-1}$ of pure BoNT/B1 (**Fig. 2**). To further study the exacerbated activity of BoNT/B2 subtype, human neuronal cells (SH-SY5Y), differentiated as previously described,[33] were exposed to different doses of subtypes and noradrenaline release levels assessed after 48 h. Complex BoNT/B2 was found to be at least 30 times more potent than pure BoNT/B1 in this neuronal cell model (data not shown).

Since BoNT/B2 toxin was confirmed to be relatively more potent in several in vitro and ex vivo models, these studies highlight the assay specific differences between serotypes and subtypes of toxin and the need for determination of optimum doses in each assay system.

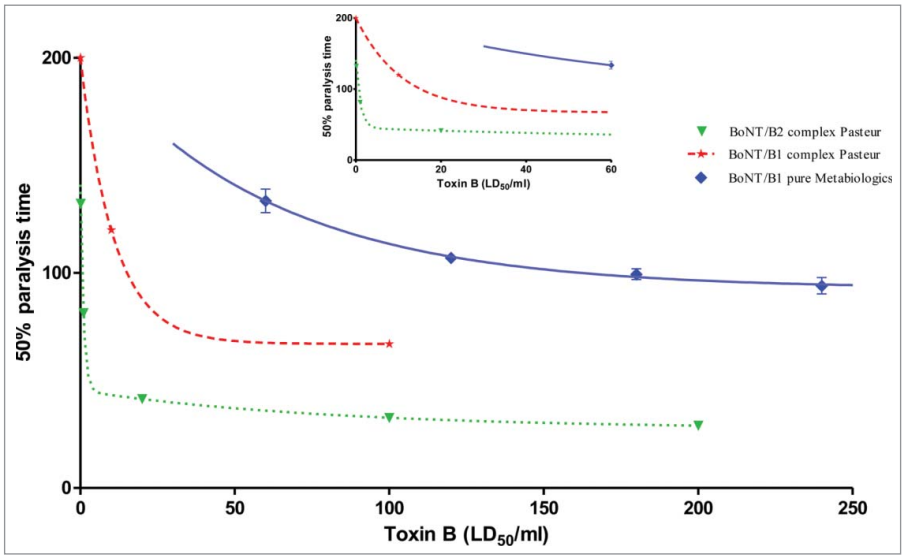

Figure 2. Dose response curves of BoNT/B1 and BoNT/B2 subtypes in ex vivo MPNH. Dose response curves for complex BoNT/B1, complex BoNT/B2, and pure BoNT/B1 were generated in MPNH on tissues taken from in-bred Balb/c mice. Time to 50% paralysis was calculated by linear regression of each individual twitch response curve. Each point is the mean ± SEM of 3 to 6 hemidiaphragm preparations except for complex BoNT/B1, which was from a single hemidiaphragm preparation. Where no error bar is visible, it is concealed by the symbol.

In vitro endopeptidase inhibition properties of scFvs and scFv-Fcs directed against BoNT/B-LC

Initial screening of the 26 isolated scFvs was performed with the BoNT/B2 subtype in the endopeptidase assay because BoNT/B2-LC was used for the immunization. Six scFvs were identified as having some inhibitory properties against this subtype. ScFv BLC3 and BLC42 were selected for further studies because a quantity of ~2 μg inhibited 50% of the enzyme activity induced by 0.2 LD_{50} of complex BoNT/B2 (**Fig. 3A**). When a more stable version of this clone (scFv-Fc) was used in the same assay, 50% inhibition of the same dose of toxin was observed at 0.001 μg, representing some 2000-fold increase in the inhibition ratio. However, as in previous studies with scFv-Fcs against BoNT/A-LC,[31] high background

prevented analysis of the full dose response (data not shown).

All 26 clones were also tested for their ability to inhibit pure BoNT/B1 endopeptidase activity, initially with 70 LD_{50} and subsequently with 30 LD_{50}, using the same initial protein concentration of 10 $\mu g.mL^{-1}$ for each clone. Only two scFvs (BLC3 and BLC42) also inhibited BoNT/B1 subtype, with scFv BLC3 showing 50% inhibition of 30 $LD_{50}.mL^{-1}$ (150 $pg.mL^{-1}$) at ~0.5–1.0 $\mu g.mL^{-1}$ (molar ratio ~20,000:1 for scFv:toxin). The scFv BLC3 was selected for further inhibition studies because BLC3 and BLC42 were confirmed to compete for the same epitope on BoNT/B1 and the affinity of BLC3 for BoNT/B1 was slightly higher compared to BLC42. The inhibition properties of scFv BLC3 was further confirmed using complex form of BoNT/B1 at 10 $LD_{50}.mL^{-1}$ where 50% inhibition was seen at ~2–3 $\mu g.mL^{-1}$ (**Fig. 3B**), confirming cross-inhibition property of BLC3. At an equivalent range of concentrations, scFvs to BoNT/A-LC did not inhibit endopeptidase activity of either BoNT/B1 or B2 subtype. Assay specificity was confirmed with polyclonal antibody to BoNT/B serotype.

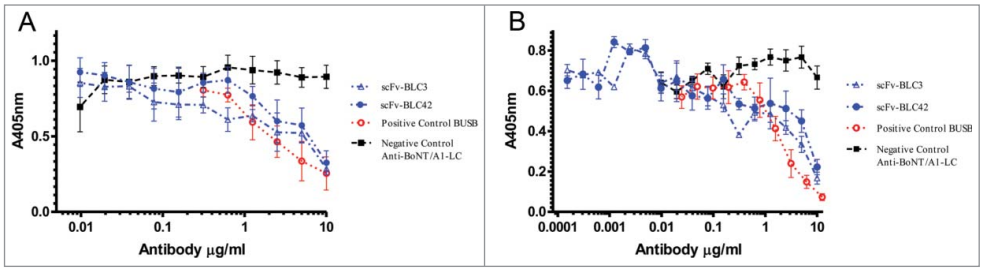

Figure 3. Endopeptidase inhibition activity of scFvs targeting BoNT/B-LC. Endopeptidase activity on 35 amino acid VAMP2 peptide substrate (VAMP2 60–94) by complex BoNT/B2 (0.2 $LD_{50}mL^{-1}$). (**A**) and complex BoNT/B1 (10 $LD_{50}.mL^{-1}$) (**B**), was inhibited dose dependently by selected scFvs targeting BoNT/B-LC. Doubling dilutions of scFv BLC3 and BLC42 were used from 10 to 0.01 $\mu g.mL^{-1}$. scFvs against BoNT/A1-LC[49] were included as negative controls. Polyclonal antibody to BoNT/B was used as a positive control. Each point is a mean of triplicate determinants within the same experiment with ± SEM for error bars. Data is representative of at least 3 independent experiments. Where no error bar is visible, it is concealed by the symbol.

Ex vivo neutralization properties of scFvs and scFv-Fc directed against BoNT/B-LC and -HC

The scFvs BLC3 and BLC42 were also tested in the ex vivo MPNH assay, but their neutralization capacities were found to be weak (data not shown). The use of a potentially more stable version of the BLC3 (scFv-Fc) resulted in much stronger neutralization of pure BoNT/B1 (100 $LD_{50}.mL^{-1}$) and of complex BoNT/B2 (0.2 $LD_{50}.mL^{-1}$) (**Fig. 4A and 4B**). At 2$\mu g.mL^{-1}$, BLC3 increased 50% paralysis time for BoNT/B2 by 147 minutes and for BoNT/B1 by 56 minutes, resulting in 38% and 26% inhibition of toxin-induced paralysis, respectively (**Table 3a and 3b**). Neutralization of BoNT/B2 subtype, but not of BoNT/B1, by scFv BLC3 was better than that for the commercial polyvalent F(ab)2 antitoxin, at 20 $mIU.mL^{-1}$, which increased 50% paralysis time by about 100 min for both subtypes (**Fig. 4A and 4B**).

Initial screening of all the anti-BHC scFvs in the MPNH assay identified 6 scFvs (>50%) with some inhibitory properties against BoNT/B1 (100 $LD_{50}.mL^{-1}$), with scFv B2–7 showing the strongest neutralization at 29 $\mu g.mL^{-1}$, which was comparable to that observed with commercial polyvalent $F(ab)_2$ antitoxin at 20 $mIU.mL^{-1}$. B2–7 was thus selected for further characterization against 0.2 $LD_{50}.mL^{-1}$ of complex BoNT/B2 (equipotent paralytic dose to 100 $LD_{50}.mL^{-1}$ of pure BoNT/B1). Dose response for neutralization of complex BoNT/B2 by scFv B2–7 (dose range between 0.1 and 9.85 $\mu g\ mL^{-1}$) showed a 52 min delay in the 50% paralysis time, representing 23% inhibition at 5.0 μg, which increased to a 374 min delay and 73% inhibition at 10 μg of scFv B2–7 (supplementary data **Fig. 3** and supplementary data **Table 2**).

Further characterization studies of B2–7 against 100 $LD_{50}.mL^{-1}$ of pure BoNT/B1 were performed using the

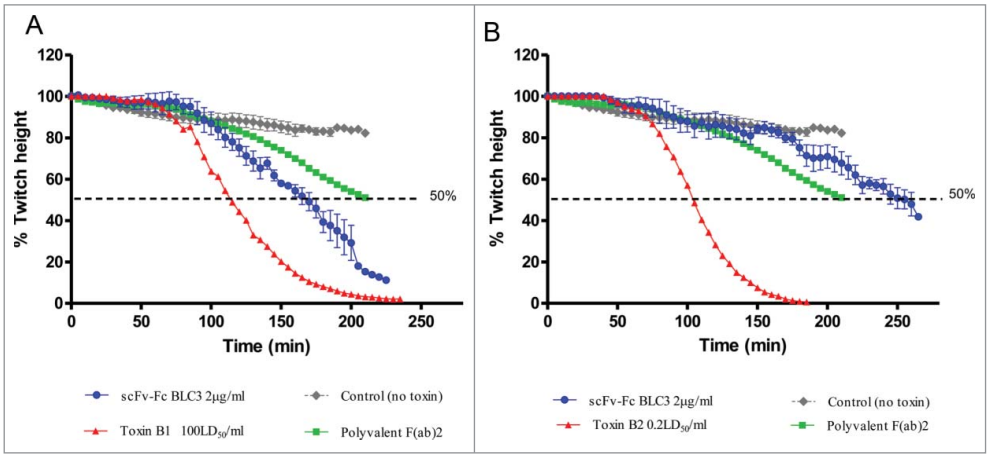

Figure 4. Neutralization activity in MPNH of scFv-Fc BLC3 targeting BoNT/B-LC. Neutralization of 100 $LD_{50}mL^{-1}$ of pure BoNT/B1 (**A**) and 0.2 $LD_{50}.mL^{-1}$ of complex BoNT/B2 (**B**) by scFv-Fc BLC3 at 2 $\mu g.mL^{-1}$, was studied in the MPNH assay. Paralysis of the tissue induced by the toxin alone is indicated in red. Dotted line indicates 50% paralysis. Antibody neutralization results in prolongation of the 50% paralysis for the same dose of toxin. In neutralization experiments (blue), each data point is a mean of 3 separate hemidiaphragm preparations with error bar ± SEM. Commercial equine F(ab')2 polyclonal serum was used as a positive control at 20 $mIU.mL^{-1}$ (green). Control tissues were not exposed to the toxin (gray).

Table 3a. Neutralization of pure BoNT/ B1 (100 $LD_{50}.mL^{-1}$) by scFv-Fc BLC3 in the mouse phrenic nerve-hemidiaphragm assay

scFv-Fc-BLC3 ($\mu g.mL^{-1}$)	50% Paralysis time (min) (mean, n = 3)	Prolongation time (min) (mean, n = 3)	% Inhibition (mean, n = 3)
2.0	169	56	26.0
Control (no toxin)	647		100

Table 3b. Neutraliszation of complex BoNT/B2 (0.2 $LD_{50}.mL^{-1}$) by scFv-Fc BLC3 in the mouse phrenic nerve-hemidiaphragm assay

scFv-Fc BLC3 ($\mu g.mL^{-1}$)	50% Paralysis time (min)(mean, n = 3)	Prolongation time (min)(mean, n = 3)	% Inhibition (mean, n = 3)
2.0	247	147	38.1
Control (no toxin)	647		100

scFv-Fc form of B2–7. These neutralization studies in MPNH using lower dilutions of B2–7 showed significant neutralization of pure BoNT/B1 toxin between 1.0 to 5.0 $\mu g.mL^{-1}$, with the minimum neutralizing capacity at ~0.1 $\mu g.mL^{-1}$. The 50% paralysis time was delayed by 27 min at 0.1 $\mu g.mL^{-1}$ and 67 min at 1.0 $\mu g.mL^{-1}$ respectively, representing 21% to 28% neutralizing capacity (**Fig. 5A**, **Table 4a**). Due to the non-specific effect of this antibody at doses exceeding 5 $\mu g.mL^{-1}$, it was not possible to assess neutralization at higher doses of B2–7 in the scFv-Fc form.

Final neutralization studies were performed using the scFv-Fc form of B2–7 antibody (dose range 1.0 to 30 $\mu g.mL^{-1}$) against the BoNT/B2 subtype. Significant neutralization of complex BoNT/B2 was obtained at 10–30 $\mu g.mL^{-1}$, with the minimum neutralizing capacity below 1.0 $\mu g.mL^{-1}$. The prolongation of paralysis time ranged from 30 to 80 min, with 28% neutralization obtained at 30 $\mu g.mL^{-1}$ (**Fig. 5B**, **Table 4b**).

The neutralization dose response for scFv and scFv-Fc B2–7 was not different, and both preparations showed similar inhibitions of ~26–28% over a 5- to 10-fold change in antibody dose (**Table 4a and 4b**).

In vivo neutralization of scFv-Fc against BoNT/B-HC and -LC

The scFv-Fc forms of BLC3 and B2–7, targeting BoNT/B-LC and HC, respectively, were tested in vivo both individually, and in combination for a synergistic neutralizing effect, in the mouse flaccid paralysis protection model against complex BoNT/B2 (0.2 LD_{50} per dose). Complete protection was achieved with 25 and 2.5 μg of BLC3, whereas B2–7 did not fully protect mice, at 25 μg per dose, but lowered the intensity of paralysis with no clear dose response. Results were comparable at 24 h and 48 h observations (**Fig. 6A and 6B**). Additional neutralization studies with higher concentrations (50 μg and 100 μg) of scFv B2–7 demonstrated a similar effect with no benefit of dose increase. However, the combination of BLC3 and B2–7 fully protected mice in vivo where protection was not observed by each antibody alone (0.25 μg of each antibody) (**Fig. 6A and 6B**), confirming effective synergistic effect.

Discussion

The aim of this project was to develop antibody fragments cross-neutralizing BoNT/B1 and BoNT/B2 subtypes based on the panning of a phage-displayed library using recombinant forms of HC and LC of BoNT/B2 toxin. The C-termini of BoNT/B1 and BoNT/B2 heavy chains (BoNT/B1-HC and BoNT/B2-HC) were targeted because this region is known to harbour neutralizing epitopes.[34,35] The recombinant form of BoNT/B2-LC was also used because previous studies have confirmed that antibodies specific to BoNT/A-LC can inhibit enzyme activity and fully protect mice from toxin challenge.[36] Macaques (*Macaca fascicularis*) were used for immunization with recombinant non-toxic antigens because antibodies derived from this species present a human-like character favorable for future therapeutic applications,[37,38] and for ethical concerns because immunization of human volunteers with potential bio-warfare agents such as BoNT is not conceivable. In addition, naïve

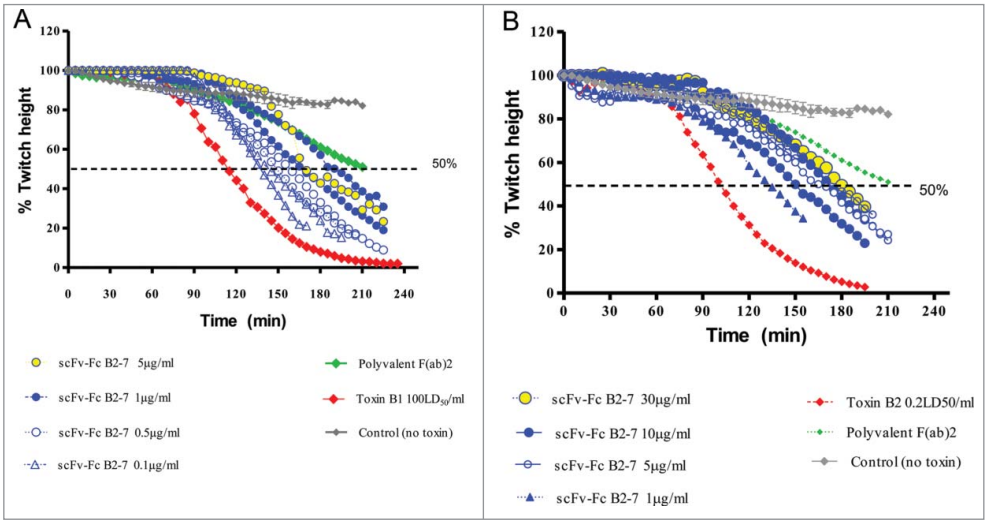

Figure 5. Neutralization activity in MPNH of scFv-Fc B2–7 targeting BoNT/B-HC. Dose dependent neutralization of pure BoNT/B1 (100 $LD_{50}.mL^{-1}$) (**A**) and complex BoNT/B2 toxin (0.2 $LD_{50}.mL^{-1}$) (**B**) by scFv-Fc B2–7, was studied in MPNH at concentrations between 30 and 0.1 $\mu g.mL^{-1}$. Paralysis of the tissue induced by the toxin alone is indicated in red. Dotted line indicates 50% paralysis. Antibody neutralization results in prolongation of the 50% paralysis for the same dose of toxin. Each neutralization set is from a single hemidiaphragm preparation per concentration. A commercial trivalent equine F(ab')2 polyclonal serum was used as positive control (green). Control tissues were not exposed to the toxin (gray).

Table 4a. Dose dependent neutralization of pure BoNT/B1 (100 $LD_{50}.mL^{-1}$) by scFv-Fc B2–7 in the mouse phrenic nerve-hemidiaphragm assay

scFv-Fc B2–7 ($\mu g.mL^{-1}$)	50% Paralysis time (min)	Prolongation time (min)	% Inhibition
0.1	140	27	21.6
0.5	165	52	25.5
1.0	180	67	27.8
5.0	170	57	26.3
Control(no toxin)	647		100

Table 4b. Dose dependent neutralization of complex BoNT/B2 (0.2 $LD_{50}.mL^{-1}$) by scFv-Fc B2–7 in the mouse phrenic nerve-hemidiaphragm assay

scFv-Fc B2–7 ($\mu g.mL^{-1}$)	50%Paralysis time (min)	Prolongation time (min)	% Inhibition
1.0	130	30	20.1
5.0	170	70	26.3
10	160	60	24.7
30	180	80	27.8
Control(no toxin)	647		100

libraries without affinity maturation have resulted in low neutralization capacities.[24] This animal model allows the injection of the antigen in the presence of a strong adjuvant that induces a hyper-immune response, and thus the isolation of high-affinity antibody fragments.[39-41]

To limit the number of recombinant antibodies for clinical development for the neutralization of the BoNT/B serotype, it is desirable to isolate antibodies that cross-react with BoNT/B1 and BoNT/B2, similar to the antibody C25 developed previously for the neutralization of BoNT/A1 and BoNT/A2 subtypes.[42] Murine antibody C25 directed against BoNT/A1-HC was later humanized (HuC25) to enable therapeutic use. This antibody was engineered to enhance its cross-reactivity with BoNT/A2, resulting in a mutant variant (CR2) with improved affinity to BoNT/A2 (from 109 nM to 87 pM), while retaining its affinity to BoNT/A1 (115 pM). It was later shown that CR2 had the same capacity to inhibit the enzymatic activity of BoNT/A1 and BoNT/A2.[43]

We hypothesized the possible isolation of a cross-neutralizing antibody from an immune NHP library without in vitro engineering, contrary to other publications.[42,43] The high percentage of identity (96%) between BoNT/B1 and BoNT/B2 subtypes also increases the probability of cross-neutralization. Because of BoNT/B toxicity, previous studies aimed at isolating neutralizing or detecting antibodies by targeting their non-toxic recombinant HC or LC domains. The production of antibodies that inhibit BoNT/B enzyme activity by targeting its LC have been reported and emphasize the presence of potentially protective epitopes in its sequence.[23,44] However, there is no published study to date describing antibodies targeting the BoNT/B-LC that also neutralize toxin activity in vivo. Antibodies simultaneously inhibiting in vitro catalytic activity of BoNT/B1 and B2 in the same range were noted by Kalb *et* al.[23] Nevertheless, as confirmed in the case of BoNT/A studies, to efficiently neutralise the toxin, scFvs need to be used in combination or undergo affinity maturation to improve their affinity.[24,45-47] Consequently, the same result should be expected for BoNT/B.

To isolate scFvs potentially neutralizing both BoNT/B1 and BoNT/B2, the 2 hyper-immune libraries were screened using a 2-step panning process. As BoNT/B1 holotoxin, BoNT/B2-HC and BoNT/B2-LC fragments were available for the study, our choice was to immunize with the recombinant BoNT/B2 non-toxic fragments (BoNT/B2-HC and BoNT/B2-LC) in order to focus the library on a region known to harbour neutralizing epitopes. The panning was then initiated with the holotoxin BoNT/B1 in order to select scFvs reacting with fully active toxin, which is also derived from a different subtype to the immunogen. This approach was validated because the number of eluted phage was comparable to that reported in previous studies,[39] and 26 (sub-) nanomolar scFvs targeting BoNT/B2-LC and 10 (sub-) nanomolar scFvs targeting BoNT/B2-HC were finally isolated.

After the third immunization with BoNT/B2-HC, the ELISA titre reached 1/100,000, with no further increase, indicating that the macaque had been hyper-immunized. Five days prior to the fourth injection, to control the evolution of the immune response, specific primer sets that specifically amplified the DNA coding for VH and VL antibody regions were used. No DNA coding for antibody fragments were amplified using primers specific for the amplification of VH or VLκ regions. After the last injection, bone marrow was iteratively sampled and optimal amplification of DNA coding VH and VL observed 3 d after the final boost. Because of the absence of amplification prior to the final immunization, the DNA amplified after the last immunization was regarded as specifically encoding variable regions reacting with the antigen. Due to the hyper-immune nature of the animal response, the timely PCR amplification, and according to our previous observations and definitions, the phage-displayed library built from this DNA was regarded as a hyper-immune library.[48] The phage-ELISA study confirmed the specificity of eluted phage for both BoNT/B1 and BoNT/B2-HC. The strong increase in the number of phage isolated after panning with BoNT/B2-HC was no surprise, as the panning was performed using the same molecule previously chosen for immunization. Nevertheless, among one hundred clones eluted from the panning randomly picked and sequenced, only 10 non-recombined and non-redundant scFvs were identified, which was lower than in some of our previous studies.[31,39] It may be noted that the 2 scFvs (B2–48 and B2–119) constituting the vast majority of the 100 sequenced scFvs (43 and 31 occurrences, respectively) presented the best affinities for BoNT/B2-HC among those observed, which may explain their high frequency. From these 10 different scFvs, 9 presented nanomolar or sub-nanomolar affinities for both BoNT/B1 and BoNT/B2-HC, with affinities for BoNT/B2-HC (from 0.2 nM to 11 nM) always being more favorable, probably as a consequence of its use as an immunogen.

The neutralization capacities of the scFvs, targeting the HC, against BoNT/B1 and BoNT/B2 subtypes were evaluated in the ex vivo hemidiaphragm assay, which has been shown to correlate well with the mouse in vivo assay for assessments of the neutralizing activity of polyclonal antibodies against toxin serotypes A, B

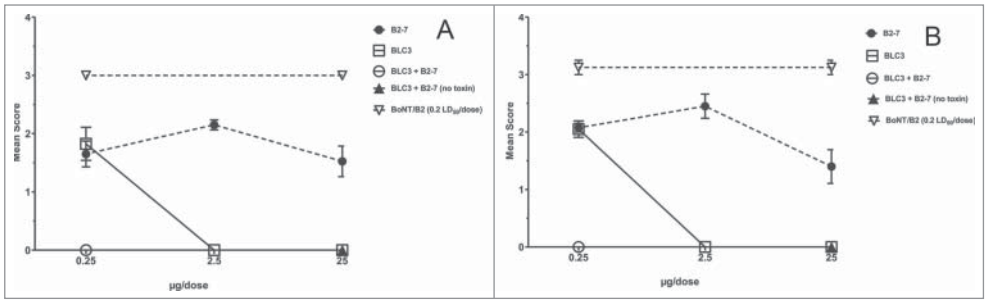

Figure 6. Neutralization activity of scFv-Fc BLC3 and B2–7 in vivo. The neutralization activity of scFv-Fc BLC3 and B2–7 was determined in mouse paralysis assay in vivo after 24 h (**A**) and 48 h (**B**). Complex BoNT/B2 toxin (0.2 LD_{50} per dose) was pre-mixed with each antibody at 0.25 µg, 2.5 µg and 25 µg per dose. When tested in combination, 0.25 µg of each of BLC3 and B2–7 were mixed with the same dose of complex BoNT/B2 toxin. Antibody:toxin mixtures were left for 30 min at room temperature before injecting subcutaneously 0.1 ml into left inguinocrural region of female MF1 strain of mice. Animals were scored at 24 and 48 h post injection. Results are expressed as mean score of 4 mice ± SEM. Positive control group of mice were injected with BoNT/B2 toxin alone, and negative control group were given the maximum concentration of antibody in the absence of toxin.

and E.[26] The best scFv candidate was B2–7, which significantly delayed the time to 50% paralysis induced by both BoNT/B1 and BoNT/B2 at 29 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$, respectively. Strong neutralization was also confirmed with the scFv-Fc fusion form of the B2–7 against the same concentrations of BoNT/B1 and BoNT/B2 subtypes, inducing a 67 min and 80 min prolongation of the 50% paralysis time at 1.0 and 30 $\mu g.mL^{-1}$, respectively. This antibody is the most promising candidate for further development from the panel studied, targeting the HC of BoNT/B. Although the affinity of B2–7 antibody for BoNT/B2-HC was higher (0.6 nM) in comparison to affinity for holotoxin BoNT/B1 (5 nM), neutralization in the ex vivo assay indicated 8% higher neutralization of BoNT/B1 at 1.0 µg. However, a narrow range (between 20–28%) of the inhibition profile was observed across the 50- to 30-fold change of concentration for both subtypes. Considering the mechanism of action of the toxin, the scFvs targeting BoNT/B-HC should inhibit the adhesion to the cell surface or the penetration into the cytosol by steric hindrance, but scFv or even scFv-Fc fragment of a single antibody may not protect from toxin challenge in vivo. This was confirmed in the mouse in vivo protection assay, where scFv-Fc B2–7 lowered the severity, but failed to fully protect the mice from BoNT/B2 toxin-induced paralysis, with no dose benefit. These results are comparable to those in the ex vivo MPNH neutralization assay, where prolongation time and 50% inhibition of paralysis of B2–7 was similar over a 10-fold range in dose. Biochemical characterization of B2–7 binding was not part of this study, but preliminary data suggests that this may not be an effective blocking antibody, thus explaining poor dose effect in neutralization studies. Neutralizing antibodies targeting only the BoNT/A-HC domains have been previously reported,[48] and their efficacy in vivo is considered to be increased by increasing the size of the fragment or including combinations.[47-49] Combining antibodies targeting both HC and LC to also achieve high protection in vivo was of interest.

In order to select scFvs targeting BoNT/B-LC that bind and neutralize BoNT/B1 and BoNT/B2, we applied the same strategy as for selection of antibodies targeting the BoNT/B-HC. The immunization of one monkey with 4 injections of the non-toxic BoNT/B2-LC led to a titre superior to 1/300,000 for BoNT/B2. Before the final immunization, no amplification of any VH or VL DNA was detected, thus it can be deduced that the amplification observed after the last boost corresponds to neo-synthesized antibodies specifically directed against the immunogen, BoNT/B2-LC. Starting from the amplicons obtained 11 d after the final boost, where the retro-amplification of VH and VL RNA was optimal, the hyper-immune library was generated in the pHAL35 vector. The increasing reactivity against both BoNT/B1 and BoNT/B2-LC observed after each round of panning emphasized the cross-binding properties of eluted phage. Considering these results, clones eluted after the fourth round of panning should present high affinity against both subtypes, and were thus used for further investigations. From the 26 different scFvs, 25 presented nanomolar affinities, of which 3 had sub-nanomolar affinities for BoNT/B1, with the highest affinity observed for BLC3 (0.4 nM) with favorable K_{on} and K_{off} values. In contrast to results with scFvs targeting BoNT/B-HC, affinities for BoNT/B2-LC, which was an immunogen, were less favorable, and were in the range of 38–280 nM, which could represent better binding of this antibody to the whole active toxin.

Because of their low nanomolar affinities, the 26 scFvs were assessed for their inhibition properties in the VAMP2 endopeptidase assay. Six (23%) scFvs inhibited the catalytic activity of 0.2 $LD_{50}.mL^{-1}$ complex BoNT/B2, and only 2 of these (7.6%) also inhibited 30 $LD_{50}.mL^{-1}$ of pure BoNT/B1 subtype. The inhibition of BoNT/B1 and BoNT/B2 catalytic activity observed for all scFvs was dose-dependent between 0.1 and 10 $\mu g.mL^{-1}$. The inhibition of endopeptidase activity was considerably improved when using potentially more stable scFv-Fc bivalent format of an antibody that is similar to IgG. This observation was consistent with our previously published studies with antibodies targeting BoNT/A-LC where a reduced inhibition ratio in comparison to scFv format was noted. In Chahboun et al., we reported in vitro inhibition property for scFv 2H8 targeting the BoNT/A-LC with a molar ratio of 64,000:1 (scFv:toxin).[31] In Miethe et al., inhibition was observed at a molar ratio of 6,500:1 for scFv-Fc 2H8 (scFv-Fc:toxin).[50] Furthermore, we observed similar results for antibody targeting the BoNT/E-LC (Miethe et al., submitted). The improved stability, and

apparent increase in affinity and steric hindrance improve IC_{50} values as well as the molar ratio after converting the scFv to either scFv-Fc or IgG. This is the reason why short chain scFv with in vitro inhibition properties will not necessarily protect in vivo. We observed this with all neutralizing antibodies targeting the LC of the toxin suggesting that the role of steric hindrance in neutralization is particularly relevant for antibodies targeting this domain. Use of scFv-Fc also increased the backgrounds at higher antibody concentration, which was also observed in the previous study.[50] It was confirmed that substrate size in the endopeptidase assay is not a contributing factor by using a larger 50 amino acid human VAMP2 peptide (VAMP2 45 to 93 sequences, including a C-terminal Cysteine residue) as a substrate, with comparable inhibition results. Furthermore, it was confirmed that BLC3 is of interest as a potentially neutralizing antibody for further development by performing the neutralization study in the MPNH as scFv-Fc to gain avidity. The scFv-Fc BLC3 neutralized paralysis and increased the 50% paralysis time by 56 min and by 147 min for BoNT/B1 and BoNT/B2, respectively. Neutralization of BoNT/B2 with 2 μg of scFv-Fc BLC3 was superior to neutralization with the commercial polyvalent $F(ab)_2$ antibody at 20 mIU. However, it should be emphasized that it is not possible to directly extrapolate this information to likely protection in vivo in comparison to current products, which are hyper immune horse antitoxin or human IgG. Here, scFvs appear to be more protective against BoNT/B2 than for the BoNT/B1 subtype, which was only used during the panning process.

Recombinant antibodies that inhibit the catalytic activity of BoNT/B by targeting its LC have been isolated after affinity maturation process, demonstrating comparable in vitro inhibition properties.[23] The inhibition of endopeptidase activity by antibodies directed against BoNT/B-LC is thought to mainly depend on their direct interaction with toxin, initially outside of the cells, but persisting within, and preventing the translocation of the toxin LC from the endosome to the cytosol by steric hindrance. Re-formatting the neutralizing antibody in order to facilitate its cell entry, without prerequisite interaction with the toxin, could allow neutralization after a longer delay post-intoxication and offer a longer window of opportunity in therapy. Consequently, the development of such monoclonal antibodies is of great interest due to their high specificity for toxin serotype. To our knowledge, neutralizing human-like monoclonal antibodies against BoNT/B -LC that also protect in vivo have not been published to date. Our studies demonstrated that scFv-Fc BLC3 at doses as low as 2.5 μg fully protected mice from paralysis induced by 0.2 LD_{50} of BoNT/B2 toxin. Whereas it is not possible to directly compare with other protection studies, which always use IgG, human monoclonal antibody targeting BoNT/A-LC at 100 μg dose fully protected mice from 25 LD_{50},[36] which is in a similar range as reported here. BLC3 demonstrated an affinity to the holotoxin BoNT/B1 that was over 100-fold higher than to the non-toxic immunogen, BoNT/B2-LC, which also suggests a preference to conformational epitopes on the whole toxin, and in turn may also contribute to its higher potency in vivo. That high affinity of short chain antibody fragments is important for in vivo efficacy has been reported for antibodies against BoNT/A-HC and LC.[49]

Several studies have confirmed synergistic effect when 2 or more antibodies are combined together to increase the neutralizing potency.[47-49] When the scFv-Fc B2–7 (targeting HC) and BLC3 (targeting LC) were combined, they fully protected mice in vivo at a dose where each antibody alone only decreased the symptoms of paralysis, without fully protecting, confirming effective synergistic effect.

The hyper-immune libraries generated during this study were obtained starting from a non-human primate. Even though they are close to their human counterpart, macaque IgGs are not identical and could induce a human-anti-macaque-antibody response.[31] This anti-antibody response leads to a decrease in efficacy of therapeutic antibodies due to their fast elimination from the bloodstream. A computational analysis was performed to predict the immunogenicity of isolated scFvs using 2 parameters: the GI and the G-score.[51] The GI allows the determination of the identity percentage between the scFv framework sequences and those of their closest germline human counterparts identified with the IMGT/DomainGapAlign tool. Because the germline sequences naturally code for human antibodies, they were considered as non-immunogenic. The closer a sequence is to germline, the more likely it is to be well tolerated.[52] The GI of B2–7, the most neutralizing scFv targeting the BoNT/B-HC, was found to be 80.5%, thus emphasizing the human-like quality of this antibody, but the value was lower than the average for human IgGs (about 95%[51]). The GI for BLC3, the most promising neutralizing antibody targeting BoNT/B-LC, presents an identity of 86.68% (87.64% for its heavy chain and 85.71% for its light chain), indicating that it should be well tolerated during therapeutic use.

To improve the tolerance of B2–7 and BLC3, these scFv can be subject to hyper-humanization process.[30,52,53] The process of humanization consists of the replacement of framework regions (humanization), or even complementarity-determining regions (hyper-humanization) of amino acids that are different between the scFvs and the closest human germline antibody sequence. Amino acids diverging from the human germline sequence are replaced with the amino acid present at the same position in the germline sequence. Introduction of such mutations could affect stability, and thus affinity. Such an approach was already successfully applied to the anti-anthrax toxin antibody, Fab35 PA_{83},[53] whereby germline humanization increased the GI index from 87% to 97%.

In conclusion, these results highlight the success of our strategy based on targeting the HC and LC domain of the toxin and the use of NHP hyper-immune libraries. Such a library was successfully screened here by panning using 2 antigens (BoNT/B1 and BoNT/B2-HC or BoNT/B2-LC). Furthermore, we applied relevant in vitro and ex vivo methods during the screening process to isolate scFvs B2–7 and BLC3 targeting BoNT/B HC and LC respectively. These two

scFvs cross-reacted and neutralized the 2 main subtypes of BoNT/B, BoNT/B1 and BoNT/B2, in ex vivo MPNH. Furthermore, when combined, these antibodies exhibited potent synergistic protection in vivo when studied in scFv-Fc format. The framework regions of B2–7 and BLC3 were close to the human germline sequences, predicting a good tolerance for clinical use, but their tolerance could be further increased by germline-humanization. The approach presented here is certainly of broad interest, in particular to isolate recombinant antibodies targeting infectious agents.

Material and Methods

Toxins and antitoxins

Botulinum B1 and B2 complex toxins were prepared by Institut Pasteur (Paris, France) as previously described,[54,55] and activity determined as 2×10^7 $LD_{50}.mL^{-1}$, (3.6 $mg.mL^{-1}$ total protein from proteolytic strain *C. botulinum* B1 strain # 7273, batch 02) and 2×10^5 $LD_{50}.mL^{-1}$ (2.9 $mg.mL^{-1}$ total protein from proteolytic strain *C. botulinum* B2 strain # BL6 batch, 01), respectively. In both cases, the method yields crude complexes that are not trypsinized prior to use. We have not checked whether the preparations are M or L complex form, but usually there are only M complexes in *C. botulinum* B.[55] Pure hemagglutinin free BoNT/B1 was purchased un-nicked from Metabiologics Inc. (Madison, USA) with stated activity of 2×10^8 $LD_{50}.mg^{-1}$ (1 $mg.mL^{-1}$ from Okra strain) which was not confirmed. All toxin preparations were not further trypsinized and were diluted to 20,000 $LD_{50}.mL^{-1}$ in gelatin (0.2% w/v) phosphate (50 mM di-sodium hydrogen orthophosphate) (GPB) buffer, pH 6.5, and stored at −40°C until use.

The polyvalent antitoxin used as positive control in same neutralization assays is a $F(ab)^2$ immunoglobulin fraction from animal immune serum and currently licensed in the European Union by Novartis (Marburg, Germany). The activity of the batch used (lot #079012A) was determined by the manufacturer: the equine protein concentration was 27 $mg.mL^{-1}$ (85–90% purity) and the labeled neutralization potency was ∼1000 $IU.mL^{-1}$ against BoNT/A, ∼500 $IU.mL^{-1}$ against BoNT/B, and ∼50 $IU.mL^{-1}$ against BoNT/E.

Animal immunization and ethical statement

Two different male cynomolgus macaques *(Macaca fascicularis)* were immunized with 4 subcutaneous injections of 80 μg of the recombinant C-terminus of BoNT/B2-HC from strain F11 (amino acids 861 to 1291, with a N-terminal 6xHis-tag from pET28 vector) or with recombinant BoNT/B2-LC (amino acids 1 to 444, with a N-terminal 6xHis-tag from pET28 vector) in Freund's adjuvant, as previously described.[56] Complete Freund's adjuvant was used for the primary immunization (Sigma, Isle d'Abeau, France) and incomplete Freund's adjuvant for the following injections. The first 3 injections were administered at one-month intervals and the fourth injection was administered 4 months after the third (**Fig. 1**).

These animal experiments were approved and performed in compliance with all relevant French ethical guidelines and laws, in particular : 1) «Partie réglementaire du livre II du code rural (Titre I, chapitre IV, section 5, sous-section 3: expérimentation sur l'animal)» ; 2) «Décret 87–848 du 19–10/1987 relatif aux expériences pratiquées sur les animaux vertébrés modifié par le décret 2001/464 du 29/05/2001» ; 3) «Arrêté du 29 octobre 1990 relatif aux conditions de l'expérimentation animale pour le ministère de la défense» ; and 4) «Instruction 844/DEF/DCSSA/AST/VET du 9 avril 1991 relative aux conditions de réalisation de l'expérimentation animale."

The in vivo paralysis neutralization tests (Division of Bacteriology Project License 80/2634) were performed in compliance with the UK Home Office regulations for the use of animals in research and testing under the Animals (Scientific Procedures) Act 1986 (ASPA). ASPA was recently revised to transpose European Directive 2010/63/EU on the protection of animals used for scientific purposes. The revised EU legislation came into force on January 1, 2013. All experiments on animals at NIBSC (UK) are also approved by the local animal research oversight committee AWERB (Animal Welfare and Ethics Review Body).

Library construction

After the final immunization, bone marrow was sampled twice a week for 1 month, with sample volumes not exceeding 6 mL each (**Fig. 1**). Total RNA was extracted using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH USA). RNA coding Fd fragments of the γ chain and κ light chains were separately retro-amplified with 9 and 7 specific primer sets, respectively, and the best amplification was chosen to build the library.[52] The PCR products corresponding to the retro-amplification of the RNA coding Fd fragments and the γ chain and κ light chains were separately pooled to generate 2 sub-libraries in pGEM®-T vector (Promega, Madison, Wisconsin, USA): one for the antibody variable region of the light chain (VL) and one for the antibody variable region of the heavy chain (VH). The DNA encoding the VL and VH regions were amplified, with 2 oligonucleotide primer sets introducing restriction sites.[57] The library construction consisted of the cloning of the VL fragments into pHAL35 (BoNT/B2-LC library) or pHAL32 vector (BoNT/B2-HC library),[58] and then the cloning of the VH fragments into the same vector, containing the VL repertoire. For this, the pHAL35 or pHAL32 vector and the VL fragments were digested with MluI and NotI (New England Biolabs, Frankfurt, Germany), the enzymes were inactivated, pHAL35 or pHAL32 was dephosphorylated using calf intestinal phosphatase (MBI, Fermentas), and the DNA was purified. VL PCR products (270 ng) were inserted into 1 μg of the dephosphorylated pHAL35 or pHAL32 preparation in 4 separate ligation reactions. DNA was precipitated from the reaction mixes with ethanol and sodium acetate, the pellet was washed twice with 70% ethanol, and then 4 aliquots (25 μL) of XL1-Blue MRF' (Stratagene, Amsterdam, the Netherlands) were used for electroporation. Plasmids (VL library) were isolated using a Plasmid Midi Kit (QIAGEN, Hilden, Germany). The VL library and the VH fragments were digested with NcoI and HindIII (New England

Biolabs), and ligation and electroporation were then performed as described for VL. The library was packaged into M13K07 phage (Agilent, Waldbron, Germany).

Selection of BoNT/B1 and BoNT/B2–HC and –LC specific scFv using antibody phage display

Screening of scFvs against BoNT/B-HC and -LC was performed in 2 steps. Because pure BoNT/B2 holotoxin was not available for this study, the recombinant BoNT/B2-HC or BoNT/B2-LC were used as a surrogate for BoNT/B2. In the first step of panning, a plate (Nunc, Roskilde, Denmark) was coated overnight at 4°C with BoNT/B1 holotoxin (Metabiologics Inc., Madison, WI USA) at a concentration of 5 $\mu g.mL^{-1}$ in PBS. The next day, the plate was saturated with PBS-BSA (3%) for 2 h at 37°C and phage were incubated in the wells of plates for 2 h at 37°C. Three rounds of panning, composed of 5, then 10 followed by 20 washes with 5 min interval between each were performed in PBS-Tween (0.1%) as a washing buffer. An additional round of panning, composed of 20 washes, was performed using the same conditions, except that BoNT/B2-HC or BoNT/B2-LC (5 $\mu g.mL^{-1}$ in PBS) (Institute Pasteur, Paris) were used as the coating antigen. After each round of panning the plate was rinsed with sterile PBS, and the phage were eluted with trypsin (10 $mg.mL^{-1}$ in PBS, 30 min at 37°C). *E. coli* (SURE strain), previously cultured in SB medium supplemented with tetracycline (10 $\mu g.mL^{-1}$), were infected with the eluted phage.

ScFv sequencing

One hundred clones were randomly handpicked after the fourth round of panning and cultured in SB medium supplemented with carbenicillin (50 $\mu g.mL^{-1}$). DNAs were extracted by the Nucleobond AX100 kit (Macherey-Nagel, Duren, Germany) or the QIA cube apparatus (Qiagen, Hilden, Germany) and then sequenced by Beckman Coulter Genomics (Takeley, UK) using the primers MHLacZ-Pro_f (5′_GGCTCGTATGTTGTGTGG_3′) and MHgIII_r (5′_CTAAAGTTTTGTCGTCTTTCC_3′).[59] The DNA sequences were then translated to amino acid sequences (http://web.expasy.org/translate/) to identify and eliminate the redundant and the recombined sequences.

Production and purification of scFv and scFv-Fc

DNAs encoding the scFvs of interest were transformed into the non-suppressor *E.coli* strain HB2151 in order to express and purify soluble scFvs, as described previously.[60]

For scFv-Fc production, the mammalian expression vector pCSE2.5-mIgG2c-XP was used for the fusion of scFv to the gene fragment encoding the human IgG2 Fc part (hinge-CH2-CH3). The expression was performed as described previously.[61] In brief, HEK293–6E cells (National Research Council, Biotechnological Research Institute (BRI), Montreal, Canada) were cultured in the chemically defined medium F17 (Invitrogen, Life Technologies, Darmstadt, Germany), supplemented with $1g.L^{-1}$ pluronic F-68 BioChemica (Applichem, Darmstadt, Germany), 4 nM L-glutamine (PAA) and 25 $mg.L^{-1}$ G418 (PAA). DNA was transiently transfected into 25 mL HEK293–6E cells in Erlenmeyer shake flasks. After 48 hours cultivation at 110 rpm, 37°C and 5% CO_2 atmosphere in a Minitron orbital shaker (Infors, Bottmingen, Switzerland), one volume culture medium and a final concentration of 0.5% (w/v) of tryptone N1 (TN1, Organotechnie S.A.S., La Courneuve, France) was used for the purification on a UNOsphereSUPrA column (Biorad) with a Profinia apparatus (Biorad, Hercules), according to the manufacturer's instructions.

ELISA assays

During the course of immunization, the immune response in macaques was evaluated by an ELISA method as described previously,[60] except that in this study holotoxin BoNT/B1 (Metabiologics Inc., Madison, Wisconsin USA) or recombinant BoNT/B2-LC or BoNT/B2-HC (Institut Pasteur, Paris) were used instead of LF as coating antigen. Briefly, a Maxisorb plate (Nunc, Roskilde, Denmark) was coated overnight with 5 $\mu g.mL^{-1}$ of each antigen in PBS (Euromedex, Mundolsheim, France). After the coating, plates were saturated with PBS-BSA (3%) for 2 h at 37°C. The macaque serum was then incubated for 2 h at 37°C. The pre-immune serum was simultaneously incubated as a negative control. The reactions were developed with the anti-human-IgG (Fc specific)-HRP conjugated (Sigma Aldrich, Isle d'Abeau, France) substrate diluted 1/1,000 in the saturation buffer and incubated for 1 h at 37°C. Specific color was developed by addition of TMB (Sigma Aldrich, Isle d'Abeau, France) and read at 650 nm without stopping the reaction. The antibody titre was measured as the highest dilution of the immune serum giving a signal 3 times stronger than the negative control serum, at the same concentration.

For phage-ELISA, a Maxisorb plate (Nunc, Roskilde, Denmark) was coated overnight with 5 $\mu g.mL^{-1}$ of either BoNT/B1 holotoxin, BoNT/B2-LC, BoNT/B2-HC, KLH or PBS-BSA (5%). Plates were then saturated in PBS-milk (3%) for 2 h at 37°C. Phage were then diluted in PBS-milk (1%) and incubated for 2 h at 37°C. The reactions were developed with the anti-M13-HRP-conjugated antibody (Amersham Biosciences, Little Chalfont, United Kingdom) diluted 1/1,000 in the saturation buffer. Plates were developed by addition of TMB substrate (Sigma Aldrich, Isle d'Abeau, France) and read at OD 650 nm, without stopping the reaction.

Affinity measurement and competition studies

Affinities of scFv and scFv-Fc antibodies were measured by SPR using a Biacore 3000 instrument (General Electric Healthcare-Biacore, Uppsala, Sweden). Each scFv was immobilised (600 RU) on a CM5 chip (Biacore) via amine coupling and at least 4 dilutions (in HBS-EP buffer, Biacore) of BoNT/B1 holotoxin (from 0.16 μM to 0.01 μM), BoNT/B2-HC (from 0.5 μM to 0.06 μM) or BoNT/B2-LC (from 0.5 μM to 0.06 μM) were tested for at least 1,000 seconds. For scFv-Fc measurements, BoNT/B1, BoNT/B2-HC or BoNT/B2-LC were immobilized (1,000 RU) on the chip, and at least 4 dilutions of the scFv-Fc in HBS-EP buffer (from 0.3 μM to 9 nM) were tested for at least 1,000 seconds. All the measurements were

performed under a flow rate of 30 $\mu l.min^{-1}$. Affinities were calculated using the BIAevaluation software (General Electric Healthcare-Biacore) according to Langmuir adsorption model and results were verified by internal consistency tests.[62]

Competition studies were performed by the SPR using Biacore 3000 instrument (General Electric Healthcare-Biacore, Uppsala, Sweden). The CM5 chip (Biacore) was first coated with BoNT/B1 holotoxin (0.16 μM) followed by either scFv BL3 or BLC42. This step was then followed by injection of either scFv BLC42 or BLC3 without regeneration between injections of the 2 different antibodies. Both combinations were studied to investigate if these 2 antibodies compete for the same epitope on the BoNT/B1 toxin.

Computational analysis

The sequences of interest were analyzed with IMGT/V-QUEST and IMGT/DomainGapAlign tools, available on-line from the International ImmunoGeneTics Information system (IMGT) (http://imgt.imgt.org),[53,63-65] to identify the human germline sequences that are the most similar to the isolated scFvs and to calculate the GI. The percentage of identity between the framework regions of the isolated scFv and those of the most similar human germline sequences, representing a first evaluation of immunogenicity, was also calculated.[52,53] A second evaluation of immunogenicity relied on the G-score (http://www.bioinf.org.uk). This parameter evaluates the "humanness" of a given variable region by comparison with expressed human variable regions belonging to the same family available from the Kabat database. Human variable regions have an average G-score equal to 0; a G-score higher than 0 indicates that a sequence has a similarity with human variable region sequences higher than human average, and conversely for G-scores lower than 0.[38]

In vitro inhibition of the BoNT/B endopeptidase activity

The principle of the endopeptidase assay for BoNT/B toxin is the same as previously described for BoNT/A and BoNT/E,[66] except that 35 amino acid VAMP2 peptide (amino acid VAMP2 60 to 94, purchased from Immune Systems Ltd, Paignton, UK), is used to coat Sulfhydryl 96-well ELISA plates (Costar plates, Corning Inc., NY USA) at 50 μL per well ($2mg.mL^{-1}$). The positive signal in the immunoassay is associated with BoNT/B cleavage of this substrate and is detected by an antibody to a newly exposed epitope on VAMP2. This antibody was made in house by immunization of rabbits with an octapeptide (VAMP2 amino acid sequence 77 to 84) conjugated to KLH through the N-terminal Cys residue, purified from serum and used in the assay at 60 $\mu g.mL^{-1}$.

Inhibition studies were performed to determine the concentrations at which scFvs could inhibit the endopeptidase activity conferred by a fixed dose of BoNT/B1 or BoNT/B2 subtype. The fixed toxin concentrations for inhibition studies were determined from dose response curves for each subtype (**Fig. S2**). Doses of toxin representing maximum activity were selected: for pure BoNT/B1 (Metabiologics, USA), a dose of 30 $LD_{50}.mL^{-1}$ was selected; for complex BoNT/B1 and BoNT/B2 (Institut Pasteur, Paris), 10 $LD_{50}.mL^{-1}$ and 0.2 $LD_{50}.mL^{-1}$ were selected, respectively. All toxins were diluted in reaction buffer (50 mM HEPES, 20 μM $ZnCl_2$, 5 mM DTT, pH 7.0) and mixed with an equal volume of a graded concentration of scFvs against the BoNT/B-LC. The toxin:scFv mixtures were briefly shaken on a plate shaker for 1 min at room temperature and incubated for 1 h at 37°C prior to transfer (50 μL per well) to 35 amino acid VAMP2 peptide coated sulfhydryl plate for estimation of endopeptidase activity. Plates were incubated for 18 h at room temperature and then washed 3 times with PBS supplemented with 0.05% (v/v) Tween 20. Reaction was detected by addition of 50 μL per well of a purified rabbit antibody to VAMP2 (77–84) at 60 $\mu g.mL^{-1}$ diluted in 2.5% (w/v) skimmed milk powder in PBS (MPBS). After 1 h incubation at room temperature, the plate was washed with PBS supplemented with 0.05% (v/v) Tween 20 and 100 μL per well of goat anti-rabbit (Sigma A0545) detection antibody (1/2000, diluted in 2.5% MPBS) was added and plates incubated again for 1.5 h at room temperature. After a final washing, ABTS substrate solution (2,2′-azino-bis(3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt, 0.05% (v/v) of 30% (w/v) hydrogen peroxide solution, 50 mM citric acid pH 4.0) was added at 100 μL per well. The color reaction was evaluated by determining absorbance at 405 nm with a plate reader (Multiskan MS, Labsystems).

To allow comparison between preparations, all antibody fragments were diluted to the same initial concentration of 10 $\mu g.mL^{-1}$ and serial doubling dilutions were made from this concentration. ScFv targeting BoNT/A-LC and inhibiting BoNT/A1 toxin[31] was included in the assays at the same concentration as a negative control.

***Ex vivo* mouse phrenic nerve neutralization studies**

Mouse phrenic nerve-hemidiaphragm (MPNH) preparation and neutralization studies were performed as described previously.[26,32] Briefly, left phrenic nerve-hemidiaphragm preparations were excised from male BALB/c strain of mice and installed in a 6 mL organ bath maintained at 37°C containing Krebs-gelatin buffer gassed with O_2 95% / CO_2 5%. Indirect stimulation via the phrenic nerve was achieved with a supramaximal voltage (~3 V, 1 Hz, 0.2 ms) and the resulting muscle contractions were measured with an isometric force transducer (FMI Gmbh, Seeheim, Germany) linked to a ML110 bridge amplifier and a Powerlab/4SP 4 channel recorder (AD Instruments, Chalgrove, UK). The hemidiaphragm resting tension was increased in a stepwise manner during the equilibration period until reproducible twitches were observed. Once the muscle twitch response to nerve stimulation had stabilized and remained at a constant magnitude for at least 30 min without further adjustment, the Krebs buffer was replaced with 6 mL of fresh Krebs buffer containing the toxin, and stimulation was resumed.

Toxin-induced paralysis was defined as a 50% decrease in the muscle twitch response to neurogenic stimulation, based on the magnitude of the contractions just before the addition of the toxin. The time to 50% paralysis after the addition of BoNT/B1 or BoNT/B2 was determined by fitting the linear part of the paralysis curve. The myotoxic effects of the toxin were also assessed by applying a short burst of direct (muscle) stimulation

(~30 V, 1 Hz, 1 ms) before adding the toxin at the end of the experiment.

Dose response curves for BoNT/B2 serotype were established to identify the optimum concentration of toxin suitable for the neutralization studies. The toxin dose selected as optimum for antibody inhibition studies typically induces ~80% of the maximum paralytic activity in the dose response curve (ensuring optimal precision) unless further scFv characterization requires weaker paralytic activity (**Fig. 2**).

A dose of 100 $LD_{50}.mL^{-1}$ pure BoNT/B1 (Metabiologics, USA) and a dose of 0.2 $LD_{50}.mL^{-1}$ complex BoNT/B2 (Institut Pasteur, Paris) were selected as inducing equivalent paralysis in the MPNH. Toxin neutralization was assessed by pre-mixing 500 $pg.mL^{-1}$ (100 $LD_{50}.mL^{-1}$) of pure BoNT/B1, or 2.9 $ng.mL^{-1}$ (0.2 $LD_{50}.mL^{-1}$) of complex BoNT/B2, with the various concentrations of either scFv or scFv-Fc preparations, and incubating for 30 min at 37°C before applying the mixture to the tissue.

Linear and non-linear regression analyses were performed with Prism 5.0 software (Graphpad, San Diego, CA, USA). The neutralization of toxin activity was proportional to the ability of the antibody fragments (scFv or scFv-Fc) to delay BoNT/B-induced paralysis. Greater antibody neutralizing potency was associated with the requirement of a longer period of time for the hemidiaphragm to reach 50% paralysis for the same dose of toxin. In some studies, commercial trivalent botulinum antitoxin was included as a positive control to provide an indication of protective activity.

In vivo neutralization studies in mouse flaccid paralysis assay

Botulinum toxin induces asymmetric flaccid muscular paralysis at the site of injection and this has formed the basis for development of an assay for toxin and serotype specific neutralization.[67,68] The method was further validated against the mouse LD_{50} test for neutralization of BoNT/A by specific IgG and its smaller fragments.[27]

Neutralization activity of selected scFv-Fcs (BLC3 and B2–7) was assessed in vivo, both individually and in combination, by pre-mixing complex BoNT/B2 (0.2 LD_{50} per dose) with the various concentrations of each antibody (0.25 μg, 2.5 μg and 25 μg per dose). For the combination protocol, 2 antibodies were used at 0.25 μg each and were mixed with the same dose of complex BoNT/B2 toxin. All dilutions were prepared in gelatin (0.2% w/v) phosphate (50 mM di-sodium hydrogen orthophosphate buffer, GPBS, pH 6.5), and toxin:antibody mixtures were left for 30 min at room temperature before injecting 0.1 mL subcutaneously into female MF1 strain of mice, each weighing between 16–20 g (n = 4 per dose). All injections were performed within 30 min and all the mice were injected in the left inguinocrural region. Scores were recorded at 24 and 48 h post injection with intensity of paralysis ranging from 0 (no sign of paralysis), to scores between 1 and 4, defined by an increasing extent of local flaccid paralysis. Positive control group of mice were injected with BoNT/B2 toxin alone, and negative control group of mice were injected with the maximum concentration of antibody used in the assay in the absence of toxin. In preliminary studies, the International Standard for type B antitoxin (NIBSC code 60/001, 2400 IU/ampoule) was used to confirm specific neutralization (data not shown).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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