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Development of neutralizing scFv-Fc against botulinum neurotoxin A light chain from a macaque immune library

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Botulinum toxins (BoNTs) are among the most toxic substances on earth, with serotype A toxin being the most toxic substance known. They are responsible for human botulism, a disease characterized by flaccid muscle paralysis that occurs naturally through food poisoning or the colonization of the gastrointestinal tract by BoNT-producing clostridia. BoNT has been classified as a category A agent by the Centers for Disease Control, and it is one of six agents with the highest potential risk of use as bioweapons. Human or human-like neutralizing antibodies are thus required for the development of anti-botulinum toxin drugs to deal with this possibility. In this study, *Macaca fascicularis* was hyperimmunized with a recombinant light chain of BoNT/A. An immune phage display library was constructed and, after multistep panning, several scFv with nanomolar affinities that inhibited the endopeptidase activity of BoNT/A1 in vitro as scFv-Fc, with a molar ratio (ab binding site:toxin) of up to 1:1, were isolated. The neutralization of BoNT/A-induced paralysis by the SEM120-IID5, SEM120-IIIC1 and SEM120-IIIC4 antibodies was demonstrated in mouse phrenic nerve-hemidiaphragm preparations with the holotoxin. The neutralization observed is the strongest ever measured in the phrenic nerve-hemidiaphragm assay for BoNT/A1 for a monoclonal antibody. Several scFv-Fc inhibiting the endopeptidase activity of botulinum neurotoxin A were isolated. For SEM120-IID5, SEM120-IIIC1, and SEM120-IIIC4, inhibitory effects in vitro and protection against the toxin ex vivo were observed. The human-like nature of these antibodies makes them promising lead candidates for further development of immunotherapeutics for this disease.

Introduction

The Gram-positive, anaerobic spore-forming bacterium *Clostridium botulinum* and other *Clostridium* spp. secrete seven serotypes (A–G) of botulinum neurotoxin (BoNT). Four of these neurotoxins (A, B, E, and rarely F) cause human botulism,[1] a disease characterized by flaccid muscle paralysis that occurs following food poisoning or the colonization of the gastrointestinal tract by BoNT-producing clostridia.[2] In recent years, several cases of botulism caused by BoNT/A, B and E have been reported worldwide.[3-5] BoNT/F causes only 1% of food poisoning-related cases of botulism.[6] A new botulinum toxin, BoNT/H, has also recently been discovered.[7,8]

BoNTs are classified as category A agents by the Centers for Disease Control and are among the six agents with the highest risk of potential use as bioweapons.[9,10] Botulinum neurotoxin A (BoNT/A) is the most toxic substance on earth with LD_{50} values (lethal dose) of 1 ng/kg by the intravenous and subcutaneous routes and 3 ng/kg by the pulmonary route. These values are estimated doses for humans, extrapolated from experiments with non-human primates.[11] BoNTs are produced as 150 kDa single-chain protoxins, which are subsequently activated by proteolytic cleavage to generate a disulfide bond-linked structure containing a 50 kDa light chain and a 100 kDa heavy chain. The heavy chain contains two functional domains (Hc and Hn). These domains are required for toxin uptake into nerve cells by receptor-mediated endocytosis and for the translocation of the light chain across the membrane into the neuronal cytosol.[12] The toxicity of BoNTs is due to the catalytic domain of the light chain (a zinc endopeptidase), which cleaves the SNARE complex (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) involved in the fusion of the synaptic vesicle with the

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presynaptic membrane. The light chain of BoNT/A cleaves SNAP-25 (synaptosomal-associated protein 25 kDa) through its zinc metalloprotease activity and inhibits neurotransmitter release, thereby causing flaccid paralysis, which requires intensive care in hospital. Vaccination is an effective strategy for protection against exotoxins, such as BoNTs, but universal vaccination would impede the increasing use of BoNTs for medical purposes, including the treatment of glandular hypersecretion, skeletal or smooth muscle hyperactivity and chronic pain-associated conditions.[13,14]

The current approach to the treatment of botulism consists of medical care and passive immunization with human anti-botulism immunoglobulins, such as BabyBIG,[15] or with the new heptavalent equine anti-toxin serum consisting of Fab and $F(ab')_2$ (HBAT) (http://www.cdc.gov). The human serum stock of BabyBIG is limited and the equine serum may cause hypersensitivity and serum sickness.[15] The rationale behind this antitoxin treatment is the removal of the toxin from the bloodstream before it can be taken up by neurons or the inhibition of its translocation by binding to the Hn domain of BoNT. Immunotherapeutic treatments are no longer effective once the toxin has bound and is taken up into the neuron, and such treatments cannot be used to reverse paralysis. Antibodies, however, may be useful for the direct neutralization of the proteolytic activity of the light chain, to prevent cleavage of the SNARE complex. The human IgG 4LCA directed against the light chain of BoNT/A, isolated by hybridoma technology, has been shown to neutralize the proteolytic activity of the light chain in vitro and to display protective activity in vivo.[16] A llama antibody inhibiting the cleavage of SNAP-25 by the light chain of BoNT/A has also been reported.[17]

In this study, we describe the isolation of several recombinant scFv with nanomolar affinities inhibiting the endopeptidase activity of BoNT/A in vitro and with neutralizing properties ex vivo, from an immune phage-display library for macaque (*Macaca fascicularis*).

The immunization of macaques has been used for the isolation of several antibody fragments with nanomolar and picomolar affinities against Crf2, a surface antigen of *Aspergillus fumigatus*[18] and several toxins such as tetanus toxin,[19] ricin,[20] and the two units of anthrax lethal toxin,[21,22] Immune libraries originating from macaques have also been used to isolate antibody fragments directed against several viruses, such as Venezuelan equine encephalitis virus (VEEV)[23] and simian immunodeficiency virus (SIV).[24] Based on the phylogenetic proximity between nonhuman primates (NHPs) and humans, the use of NHPs makes it possible to isolate human-like antibody fragments for therapeutic applications. Our immunization strategy was based on the use of a hyperimmune library, potentially spanning most of the epitopes of BoNT/A light chain (BoNT/A-L) with high-affinity antibodies rather than being directed against non-relevant antigens. The diversity of a previous anti-BoNT/A1-L library was limited by the frequent presence of *Nco*I and *Hind*III restriction sites within the genes encoding the scFv. Nevertheless, one scFv (2H8) inhibiting the endopeptidase activity of BoNT/A in vitro was isolated.[25] In this study, we designed a new vector for macaque antibody-phage display and constructed a new immune library, using *Sfi*I rather than *Nco*I restriction sites. This strategy yielded a higher proportion of full-sized inserts during library construction. We describe the isolation and characterization of several antibodies from an NHP antibody gene library by phage display, and their inhibition of the proteolytic activity of BoNT/A and neutralization of the toxin ex vivo.

Results

Animal immunization and antibody phage-display library construction

The light chain of botulinum neurotoxin A1 (BoNT/A1-L) was used for immunization. The immune response was evaluated by enzyme-linked immunosorbent assay (ELISA) with the whole toxin complex (BoNT/A1), to verify binding. Antibody titer was measured 10 d after the third boost, by ELISA, and was found to be 1 per 100 000. Eight months after the third immunization, a fourth boost was administered. Six and 10 d after the last immunization, PCR products were obtained with all the oligonucleotides used to amplify the genes encoding VH and VL. The measured titer on day 10 was again 1 per 100 000, indicating that a stable, hyperimmune response had been achieved. The DNA fragments corresponding to the genes encoding VH and VL, which was amplified on days 6 and 10 after the fourth injection, were pooled and inserted into pGemT for the construction of κ light chain and Fd sublibraries, containing 10^4 and 5×10^4 independent clones. For final construction of the immune library, the phage-display vector pHAL32 was used. This vector is a modified form of pHAL14, with an *Sfi*I restriction site for VH cloning. It was constructed specifically for this project, to allow the cloning of anti-BoNT/A1-L V genes containing *Nco*I sites. The scFv library was constructed by two consecutive cloning steps. First, VL gene fragments were cloned, using *Mlu*I and *Not*I, and then VH fragments, using *Sfi*I and *Hind*III. The final scFv antibody gene library consisted of 3.2×10^8 independent clones with a full-size insert rate of 80%. Finally, the immune library was packaged with M13K07 or Hyperphage, resulting in phage titers of 9×10^{13} and 6×10^{11} cfu/mL, respectively.

Isolation of scFv directed against BoNT/A1

A multi-step panning against holotoxin BoNT/A1 or recombinant BoNT/A-L was performed with Hyperphage and M13K07 packaged antibody-phage libraries. For the Hyperphage packaged library, 6.2×10^3 antibody-phage were isolated against BoNT/A1 holotoxin after the third panning round. Using the M13K07 packaged library, 1.3×10^5 antibody-phage were isolated against the BoNT/A1 holotoxin. For the panning against BoNT/A-L, only the M13K07 packaged library was used and resulted in 5.5×10^8 antibody-phage after the third round of panning. A total of 92 individual clones from each panning were produced as soluble scFv for screening by ELISA. Antigen binding was tested to BoNT/A1 holotoxin and BoNT/A1-L by ELISA (data not shown). In total, 69 ELISA positive scFv clones binding to BoNT/A1 were identified. The diversity was analyzed by *Bst*NI digest and the corresponding DNA was sequenced. The

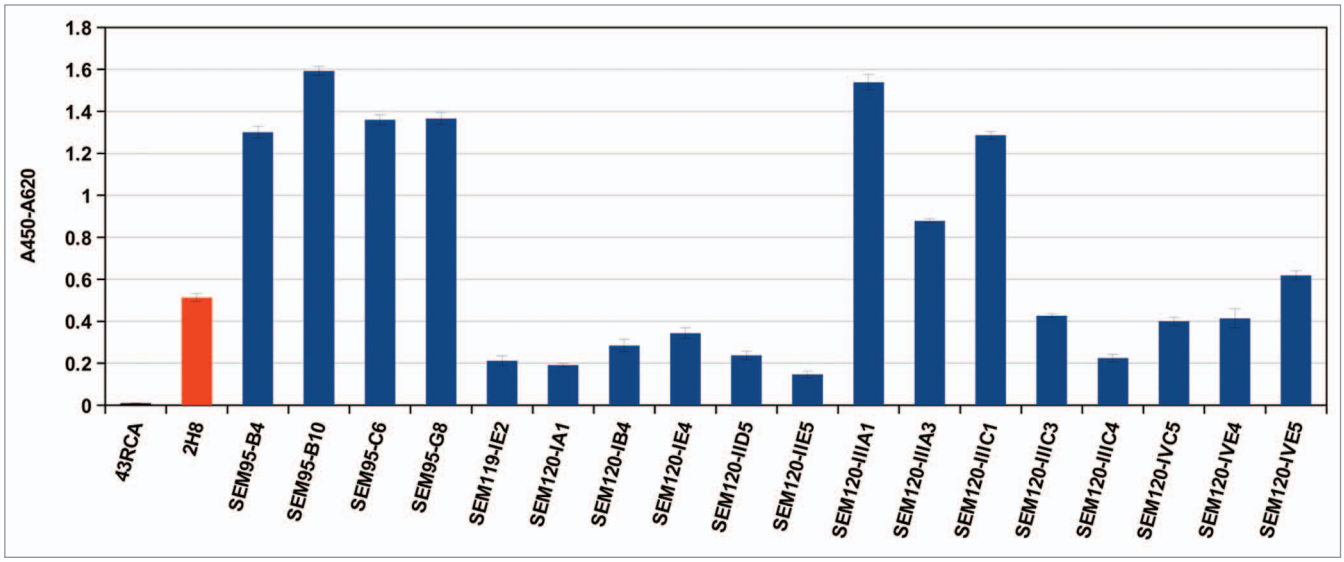

Figure 1. Validation of BoNT/A light chain binding by antigen ELISA. One µg of the 19 selected binders against BoNT/A1 (including 2H8 as a positive control[25]) and the anti-ricin antibody 43RCA (negative control)[20] were tested as scFv-Fc fusion with a murine IgG2c Fc part on 100 ng directly immobilized BoNT/A light chain. The bound scFv-Fc was detected with goat anti-mouse (Fc specific) HRP-conjugated antibodies (1:20 000).

sequences obtained revealed 22 unique full-size scFv among the 69 clones analyzed. Five clones were obtained by panning against the light chain with the M13K07-packaged library, two were obtained by panning against the holotoxin with the Hyperphage-packaged library, and 15 from the panning against holotoxin with the M13K07-packaged library.

Nineteen of the 23 anti-BoNT/A1 binders, including scFv from a previously produced anti-BoNT/A1 library,[25] were recloned and produced as scFv-Fc with a murine IgG2c-Fc part. The yield of the remaining four antibodies was too low for further analysis. The antigen binding of the 19 scFv-Fc was compared and validated by ELISA on immobilized BoNT/A light chain (**Fig. 1**).

Analysis of germline genes

An analysis of the human germline genes most similar to the genes encoding the 22 selected scFv in addition to 2H8 showed that VH and VL sequences were highly diverse. Almost all the IGHV genes were represented, with the exception of IGHV2 and IGHV5, but IGHV4 predominated (**Table 1**). These genes were combined with representatives of all IGDH genes, mostly in combination with IGJH4. IGJH4 was the predominant J gene, but there was one occurrence of IGJH1 and two occurrences of IGJH5. Nevertheless, we did not expect this limitation to have a severe effect on the diversity of the VH paratopes, because these genes encode only the last part of CDR3 and FR4. The diversity of VL was more limited than that of VH, due to the presence mostly of IGKV1, except for two occurrences of IGKV2 and two occurrences of IGKV3. These genes were combined only with representatives of IGKJ1, IGKJ2, and IGKJ3, with IGKJ1 predominating. The germinality index (GI) for each VH and VL was calculated and provided an indication of the identity between framework regions of the selected scFv and those encoded by the most similar human germline genes, as a percentage (**Table 1**).

In vitro endopeptidase immuno-assay

Nineteen antibodies were produced as scFv-Fc in mammalian cells. This type of antibody consists of a scFv fused to an IgG Fc part (~100 kDa as dimer). The resulting bivalent molecule is comparable to an IgG.[26] Here, a murine IgG2c Fc part was used, resulting in a chimeric macaque-murine antibody. Afterwards, the antibodies were tested in a single experiment against BoNT/A1 in vitro (**Fig. 2**). Several experiments were performed on each antibody to select the best data covering the dose range at which inhibition was observed. Where duplicates were performed in re-tests for some antibodies, the range between the duplicate values did not, on average, exceed 15% (data not shown). Eight antibodies gave higher levels of endopeptidase inhibition than the 2H8 antibody. Notably, many of the antibodies tested yielded a background (the titration data for these clones are not shown). The scFv-Fc concentration inhibiting BoNT/A1 activity by 50% in vitro was determined, the molar ratio for 50% inhibition is shown in **Table 2**. SEM120-IIIC4 gave the highest level of inhibition, and the molar ratio of antibody to toxin at which 50% inhibition of endopeptidase activity was obtained was found to be almost 0.5:1 for this antibody. This corresponds to a molar ratio of 1:1 per binding site. All antibodies inhibiting endopeptidase more strongly than 2H8 and giving little or no background signal were selected for further ex vivo studies, on the basis of clear dose-dependent inhibition.

Ex vivo assessment of scFv-Fc in phrenic nerve-hemidiaphragm assays

To confirm that the antibodies with inhibitory properties in the endopeptidase assay had toxin neutralization properties, the ex vivo neutralization capacity of eight antibodies in the scFv-Fc format was tested by the phrenic nerve-hemidiaphragm assay using 30 µg/mL of each antibody (**Fig. 3**; **Table 2**). Neutralization tests using the hemidiaphragm were initially performed at the highest possible ex vivo concentration for the selection step (data

Table 1. Human germline genes most similar to the genes encoding the 23 selected scFv

scFv	library (antigen for panning, helperphage)	VH			VL		GI [%]	
		V	D	J	V	J	VH	VL
2H8	[25]	IGHV1–2*02	IGHD2–21*01	IGHJ5*02	IGKV3–11*01	IGKJ2*04	85.7	92.1
SEM95-A9	BoNT/A-L, M13-K07	IGHV3 h*01	IGHD6–13*01	IGHJ4*02	IGKV3–7*02	IGKJ1*01	79.1	77.5
SEM95-B4		IGHV3–66*01	IGHD2–15*01	IGHJ4*02	IGKV1–12*01	IGKJ1*01	89.0	92.1
SEM95-B10		IGHV3–66*01	IGHD2–21*01	IGHJ4*02	IGKV2–30*02	IGKJ1*01	84.6	86.5
SEM95-C6		IGHV3 h*01	IGHD6–13*01	IGHJ4*02	IGKV2–30*02	IGKJ1*01	79.1	86.5
SEM95-G8		IGHV3 h*01	IGHD6–13*01	IGHJ1*01	IGKV2–30*02	IGKJ1*01	87.9	86.5
SEM119-IE2	holotoxin, Hyperphage	IGHV4–59*04	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	90.1	88.7
SEM119-IE3		IGHV3–66*02	IGHD3–9*01	IGHJ4*02	IGKV1–9*01	IGKJ1*01	87.9	89.9
SEM120-IA1	holotoxin, M13-K07	IGHV4–39*07	IGHD1–26*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	87.9	85.4
SEM120-IB4		IGHV4–59*04	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	91.2	89.9
SEM120-IE4		IGHV4–59*04	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	91.2	87.6
SEM120-IID5		IGHV4–59*04	IGHD1–26*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	89.0	89.9
SEM120-IIE5		IGHV4–59*04	IGHD4–17*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	93.4	86.5
SEM120-IIIA1		IGHV4–39*07	IGHD1–26*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	89.0	85.4
SEM120-IIIA3		IGHV4–59*04	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	91.2	88.7
SEM120-IIIB3		IGHV4–59*04	IGHD1–26*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	89.0	85.4
SEM120-IIIC1		IGHV1–8*02	IGHD3–22*01	IGHJ4*02	IGKV1–39*01	IGKJ3*01	86.8	87.6
SEM120-IIIC3		IGHV4–28*01	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ3*01	92.3	85.4
SEM120-IIIC4		IGHV4–59*04	IGHD1–26*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	90.1	89.9
SEM120-IVC5		IGHV1–2*02	IGHD2–21*01	IGHJ5*02	IGKV3–20*01	IGKJ2*03	86.8	88.7
SEM120-IVD3		IGHV4–28*01	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	89.0	89.9
SEM120-IVE4		IGHV4–28*01	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	86.8	88.7
SEM120-IVE5		IGHV4–59*04	IGHD3–22*01	IGHJ4*02	IGKV1–13*02	IGKJ1*01	90.1	88.7

The human germline genes most similar to the genes encoding the 23 scFv were retrieved by IMGT/V-QUEST analysis. The germinality index (GI) of each scFv (VH+VL) is indicated in the column on the right. For comparison, the mean germinality index of 100 unpublished human scFv from a naive scFv library (HAL7/8) was 96.6 for VH and 94.8 for VL.

not shown), and then repeated at lower concentration (30 μg/mL) for potency comparison of the successful antibodies. A trivalent equine F(ab')2 polyclonal serum was used as a positive control and the anti-ricin antibody 43RCA as a negative control.[20] Five of the eight antibodies initially selected (SEM120-IID5, SEM120-IIIC1, SEM120-IIIC4, SEM95-G8 and SEM120-IE4) neutralized BoNT/A1 effectively, significantly delaying the action of the toxin during the test period. The time to 50% paralysis (50% PT) was doubled by each of the five antibodies, i.e., 202 min, 200 min, 188 min, 181 min and 173 min for SEM120-IID5, SEM120-IIIC1, SEM120-IIIC4, SEM95-G8 and SEM120-IE4, respectively. One antibody gave moderate levels of neutralization of the same toxin in the hemidiaphragm assay (SEM119-IE2, 50% PT: 164 min) and one antibody displayed weak neutralization ability (SEM120-IVC5, 50% PT: 116 min). One antibody was confirmed to be non-neutralizing (SEM95-C6, 50% PT: 90 min). SEM95-C6 and 43RCA (50%PT: 76 min) led to an increased toxicity, but the increased toxicity with SEM95-C6 is not significant.

Affinity measurement

Affinity was determined with Biacore and different antibody concentrations. Here, four antibodies identified as inhibitory in endopeptidase assays were analyzed (**Table 3**). Except for SEM120-IVC5, all monovalent affinities were in the subnanomolar range.

Epitope characterization

All antibodies with inhibitory activity in endopeptidase assays for which 50% inhibition was achieved were analyzed by immunoblotting to determine the nature of the epitopes involved. Here, the SDS-PAGE for BoNT/A light chains was performed under reducing (Laemmli buffer containing β-mercaptoethanol, 95 °C) or non-reducing conditions (Laemmli buffer, 56 °C) (**Fig. 4**). Most conformational epitopes should be preserved in non-reducing conditions, whereas the proteins are denatured in reducing conditions, with linear epitopes remaining. The BoNT/A light chain was detected with the antibodies in scFv-Fc format. One antibody, SEM120-IE4, displayed low levels of binding to BoNT/A-L under non-reducing conditions. Two antibodies, SEM95-C6 and SEM95-G8, bound to denatured antigen, indicating binding to a linear epitope. SEM95-C6 is particularly notable, because it has some SNAP25 inhibiting activity, but was not identified as neutralizing in the ex vivo assay. The SEM95-G8 antibody was neutralizing. We cannot provide further information about the neutralizing epitope because of European Community security concerns. We therefore focus here on the further analysis of the non-neutralizing epitope of SEM95-C6. Epitope mapping was performed on SEM95-C6 with an epitope mapping membrane with overlapping peptide fragments of 15 amino acids (aa) in length, with an offset of four aa for analysis of the complete BoNT/A light chain

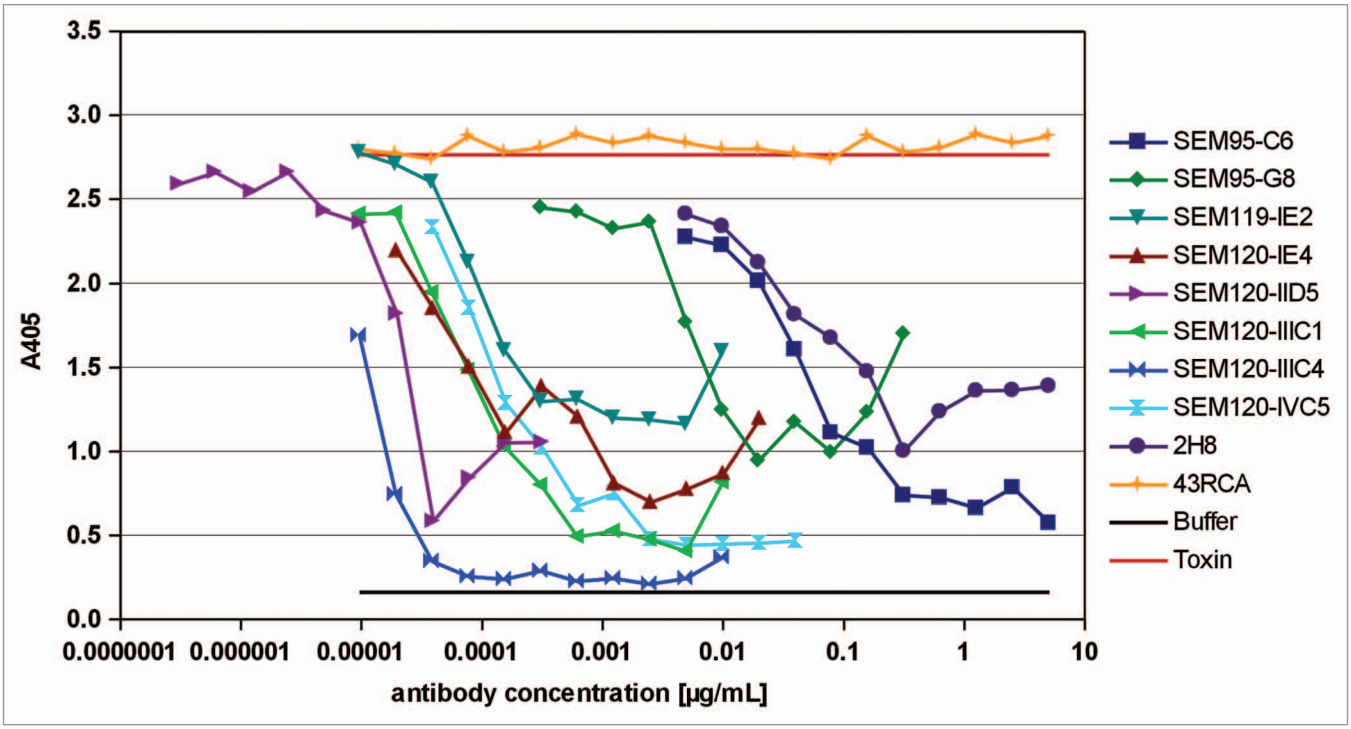

Figure 2. Analysis of endopeptidase inhibition. Dose-dependent inhibition of the endopeptidase activity of BoNT/A1 holotoxin (43.5 pg/mL; 10LD_{50}/mL) in vitro with various anti-BoNT/A light chain antibodies in scFv-Fc format. As negative control, an anti-ricin antibody (43RCA) was used.

(**Fig. 5**, membrane 1). Two possible regions (spot 7 and 17) were identified. Both sequences are not highly homologous (spot 7: VPVSYYDSTYLSTDN, spot 17: NGYGSTQYIRFSPDF). The tyrosine residues could be the reason why this antibody binds also to spot 17. Structure analysis with pdb structure 3BTA showed that only spot 7 corresponded to the toxin surface[41] (**Fig. 6A**). More detailed epitope mapping (**Fig. 5**, membrane 2) identified a 7 aa continuous epitope (YYDSTYL) at position 71–78 on the surface of the light chain. This analysis was performed with an epitope mapping membrane with nine aa peptides and an offset of one aa. The corresponding model is shown in **Figure 6B**. Binding to the continuous epitope YYDSTYL was confirmed by direct ELISA on a biotinylated peptide with a glycine-serine-linker (**Fig. 7A**). The specific binding to this peptide was validated by ELISA using BoNT/A-L and the peptide YYDSTYL for competition (**Fig. 7B**).

Discussion

Botulinum toxins (BoNTs) are among the most toxic substances on earth, with serotype A recognized as the most toxic substance known.[11] In this study, we focused on subtype A1, the most prevalent BoNT/A subtype.[27] Several antibodies targeting the heavy chain of botulinum toxins and inhibiting cell entry have already been isolated.[28-35] In contrast, antibodies inhibiting BoNT/A light chain endopeptidase activity have rarely been described. Only one human (4LCA), one llama (Aa1) and one macaque (2H8) antibody with such inhibitory properties have been described to date.[16,25,36] Such antibodies may also be protective through other mechanisms in vivo, such as toxin clearance via the recruitment of immune effectors.[37]

In this study, the antibody phage display library was constructed as previously described[18,20,22,38] for the generation of high-affinity scFv.[19] For immunization, only the non-toxic recombinant light chain of botulinum neurotoxin A1 (BoNT/A1-L) was used to induce a hyper-immune response. However, for validation of the immune response, we used antibody-phage display, affinity measurement and enzymatic assays based on the holotoxin to ensure that only binding fragments recognizing the complete toxin were selected. A previously constructed library in pHAL14, had a low proportion of full-sized inserts (37%). For BoNT/A1-L, VH1 and VH7 genes with *Nco*I restriction sites were particularly prevalent in the anti-BoNT/A immune response.[25] This had never before been reported for other macaque immune libraries.[18,20,22,38] It also contrasts with reports for human V genes, as *Nco*I sites have never been detected in VH genes (Hust, unpublished results). Therefore, a new vector (pHAL32), based on pHAL14, was designed for the construction of antibody-phage display libraries using *Sfi*I instead of *Nco*I for VH gene cloning. The use of pHAL32, increased the frequency of full-sized inserts in the library from 37% to 80%. This library was packaged with Hyperphage or M13K07 and screened by multistep panning, consisting of three rounds of screening, increasing in stringency due to additional washing steps. At the end of the third round of screening antibody clones were analyzed by ELISA to assess their reactivity with BoNT/A1. Sequencing identified 22 unique scFv and revealed a high level of diversity, suggesting that these scFv may target different epitopes

Table 2. BoNT/A1 endopeptidase inhibition and retardation of ex-vivo paralysis.

scFv-Fc antibody	Antibody concentration at 50% endopeptidase inhibition [µg/mL]	Molar ratio scFv-Fc: toxin	50% paralysis time [min]
SEM120-IIIC4	1.5×10^{-5}	0.5: 1	188
SEM120-IID5	4.0×10^{-5}	1: 1	202
SEM120-IIIC1	1.5×10^{-4}	5: 1	200
SEM120-IE4	6.0×10^{-4}	15: 1	173
SEM119-IE2	2.0×10^{-4}	5: 1	164
SEM120-IVC5	2.0×10^{-4}	5: 1	116
SEM95-C6	7.0×10^{-2}	2000: 1	90
SEM95-G8	1.2×10^{-2}	300: 1	181
2H8	2.0×10^{-1}	6500: 1	n.d.*
43RCA	-	-	76

The inhibition of endopeptidase activity was studied in vitro with BoNT/A1 toxin (43.5 µg/mL; 20 LD_{50}/mL). The antibody concentration yielding 50% inhibition is given, together with the molar ratio of scFv-Fc:toxin. The negative control (43RCA) gave no inhibition at a concentration of 5 µg/mL scFv-Fc. The ex-vivo paralysis time is given in minutes. * 2H8 did not show neutralization ex-vivo as scFv (not determined as scFv-Fc)

and perhaps inhibit the endopeptidase activity of the light chain. All scFv binding BoNT/A1 were converted into scFv-Fc antibodies for the assessment of their neutralizing properties. Here, the murine IgG2C Fc was used, resulting in a chimeric antibody. A cassette vector system with different Fc parts made it possible to select the most appropriate Fc part for the assay concerned.[26] Five of these scFv inhibited SNAP25 cleavage in the in vitro endopeptidase immunoassay, without interference or background. The best way to compare the inhibitory effects of several antibodies is to calculate the molar ratio of antibody to toxin at which an inhibition of endopeptidase activity is observed. The molar ratio at which 2H8, as an scFv, inhibits BoNT/A1 endopeptidase activity by 50% has been reported to be 64,000:1,[25] as an scFv-Fc, 50% inhibition was observed at a molar ratio of 6,500:1 for 2H8, presumably due to an avidity effect. The best molar ratios were those obtained for SEM120-IIIC4 (0.5:1). SEM120-IID5 (1:1) and SEM120-IIIC1 (5:1) as scFv-Fc antibodies. An antibody:toxin ratio of 0.5:1 corresponds to a 1:1 binding site:toxin ratio for SEM120-IIIC4. The human 4LCA IgG was tested in an in vitro endopeptidase assay with a single dose, yielding 63% inhibition of BoNT/A endopeptidase activity, with a corresponding molar ratio of 10 000:1.[16] In a previous study, the llama antibody Aa1 gave a 50% inhibition at a molar ratio of 200:1, but only against the light chain of BoNT/A. It was not tested against the complete toxin.[36] The affinities of the endopeptidase-inhibiting antibodies were determined. The monovalent affinities of these antibodies were in the low nanomolar to subnanomolar range.

Realistic evaluations of the potential of antibodies require tests of neutralization capacity. The in vitro inhibition assay was used to reduce the number of antibody clones tested in the next step. The mouse phrenic nerve-diaphragm assay is an ex vivo assay that closely mimics the in vivo respiratory paralysis caused by BoNT[39] and has been shown to be give results consistent with those of the mouse in vivo assay for assessments of the neutralizing activity of antibodies for the main toxin serotypes A, B and E, in tests of polyclonal antiserum standards.[40] However, the correlation between the results of ex vivo assays and in vivo assays for BoNT may depend on specific epitopes. In this ex vivo assay, strong inhibition was observed for SEM120-IID5, SEM120-IIIC1 and SEM120-IIIC4, identifying these antibodies as promising candidates for further development. For future immunotherapeutic purposes, the antibody will need to be produced with a human IgG1 Fc, as a complete IgG.

The SEM95-C6 antibody inhibited SNAP25 with low activity in the in vitro assay and had no neutralizing activity in the ex vivo assay. This antibody resulted in levels of toxicity slightly, but not significantly, higher than those for the pure toxin in the absence of antibody. An increase in toxicity was also observed for anti-BoNT/A antibodies, with a 1:1 molar ratio of antibody:toxin.[41] Therefore, this non-neutralizing epitope was further analyzed. The large, seven aa epitope identified for SEM95-C6 is part of four discrete regions within BoNT/A-L known to induce an immune response in rabbit.[42] This work showed that an antibody directed against this epitope is not neutralizing.

For optimal immune tolerance for human treatments, the framework regions of the macaque antibodies should be very similar to the corresponding human germline sequences. One way of estimating the potential immunogenicity of antibodies is to calculate the level of identity of the corresponding framework regions to the most similar human germline-encoding framework regions. This degree of identity is also known as the germinality index (GI).[21] GI indirectly predicts the tolerance of a VH or VL sequence, based on the assumption that germline encoding sequences are the best tolerated, as they are part of the IgM immunoglobulins. The GI values of the 23 scFv ranged from 79.1% to 92.3% for VH and from 77.5% to 92.1% for VL. For comparison, the mean GI values of 100 unpublished human scFv from a naïve scFv library (HAL7/8)[43] were 96.6% for VH and 94.8% for VL. The germline humanization of macaque antibodies is a potentially promising method for increasing immune tolerance for human treatment.[44] Another macaque antibody fragment ($35PA_{83}$) neutralizing the anthrax lethal toxin[21] has been subjected to germline humanization to increase

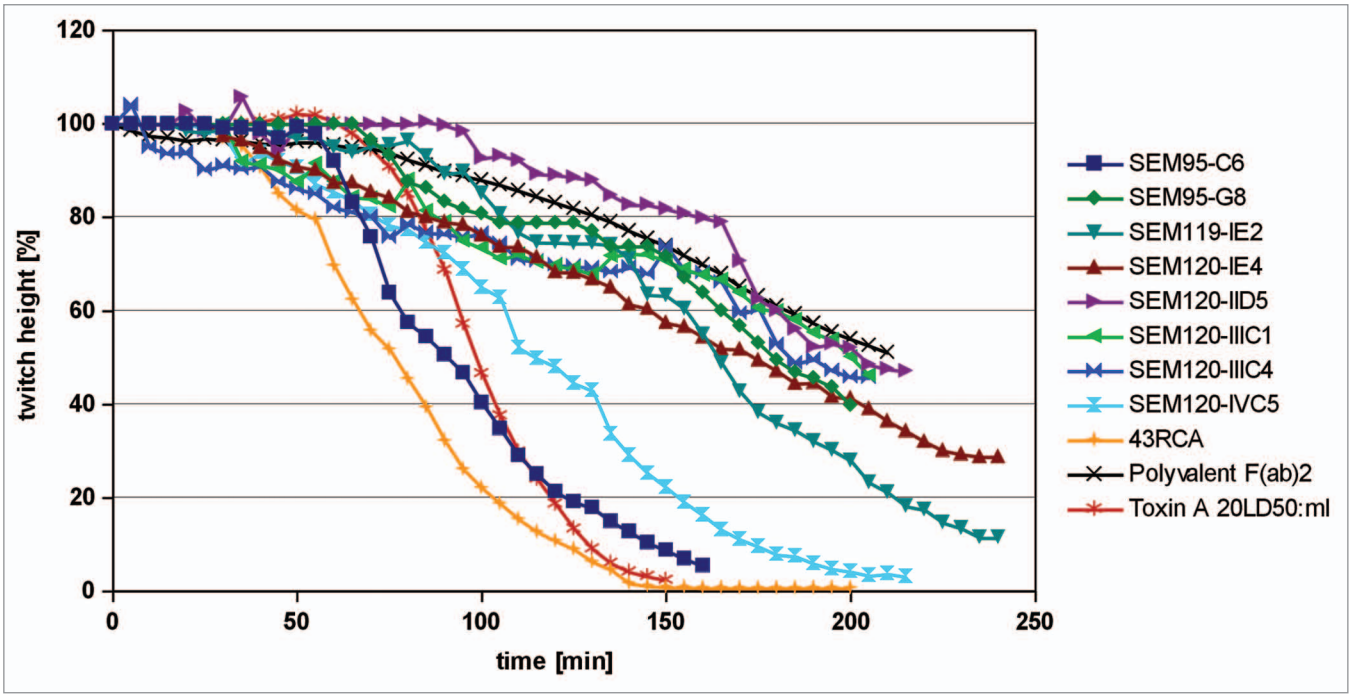

Figure 3. Analyzing the neutralization capacity. Neutralization of BoNT/A1 holotoxin (87 pg/mL; 20LD50/mL) by 30 µg/mL scFv-Fc in the mouse phrenic nerve-hemidiaphragm assay. More potent antibodies result in longer times to 50% paralysis of the hemidiaphragm for the same dose of toxin. As positive control, a trivalent equine F(ab')2 polyclonal serum was used and an anti-ricin antibody (43RCA) was used as negative control.

its GI value to 97.8%.[45,46] After the humanization of $35PA_{83}$, only four macaque residues needed to be changed to obtain the original structure. The GI values of the three best antibodies were slightly better (86.8–90% for each chain) than that of $35PA_{38}$ (87.6%), and successful germline humanization can therefore be reasonably anticipated.

The anti-BoNT/A1 antibodies SEM120-IID5, SEM120-IIIC1, and SEM120-IIIC4 are good candidates for the future development of immunotherapeutic treatments, particularly if combined with a neutralizing anti-BoNT/A heavy chain antibody.

Materials and Methods

Ethics statement and animal care

All animal studies presented were given specific approval from the Institut de Recherche Biomédicale des Armées ethics committee (Comité d'éthique de l'Institut de Recherche Biomédicale du Service de Santé des Armées) under authorization no. 2008/03.0 and were performed in accordance with all relevant French laws and ethical guidelines, including, in particular (1) "partie règlementaire 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," (2) "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."

Table 3. Affinities of four inhibiting antibodies (in scFv-Fc form)

Antibody	K_{off} [M]	K_{on} [M]	K_D [nM]
SEM95-C6	7.27×10^{-5}	8.44×10^{4}	0.86
SEM120-IIIC1	6.87×10^{-5}	8.34×10^{4}	0.82
SE120-IIIC4	1.18×10^{-4}	1.55×10^{5}	0.76
SEM120-IVC5	6.99×10^{-4}	4.90×10^{5}	1.43

Animal care procedures complied with the regulations detailed under the Animal Welfare Act[47] and in the Guide for the Care and Use of Laboratory Animals.[48] Animals were kept at a constant temperature (22 °C ± 2 °C) and relative humidity (50%), with 12 h of artificial light per day. They were housed in individual cages (6 per room), each of which contained a perch. Animals were fed twice daily, once with dried food and once with fresh fruits and vegetables, and water was provided at the same time. Food intake and general behavior were observed by animal technicians during feeding times, and veterinary surgeons were available for consultation if necessary. Veterinary surgeons also performed systematic visits to each NHP-room twice weekly. The environmental enrichment program for the nonhuman primates was limited to games with animal care staff and access to approved toys. The well-being of the animals was monitored by the attending veterinary surgeon. Animals were anesthetized before the collection of blood or bone marrow by an intramuscular injection of 10 mg/kg ketamine (Imalgene®, Merial). Analgesics were subsequently administered, through a single intramuscular injection of 5 mg/kg flunixine (Finadyne®, Schering Plough) in the days after interventions if the animal

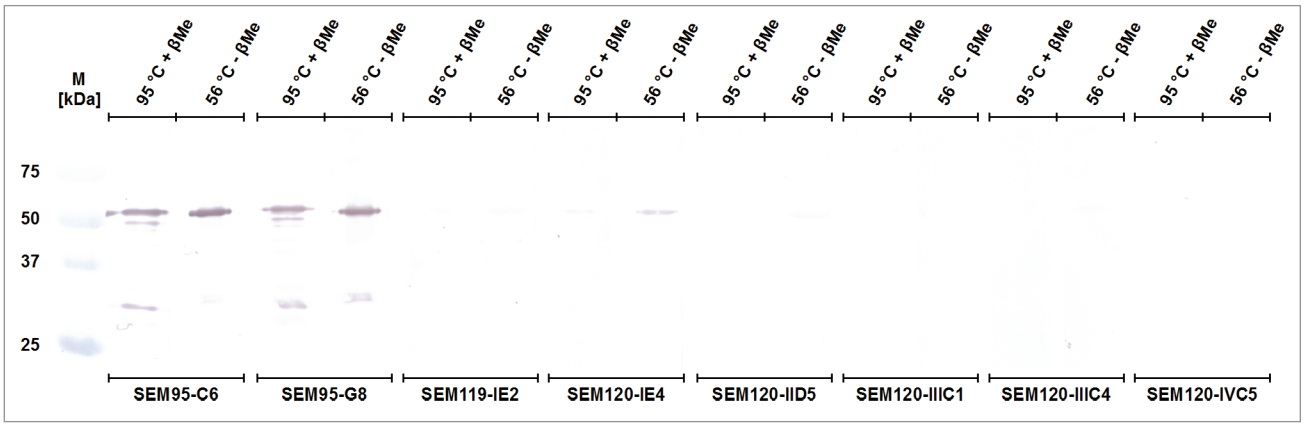

Figure 4. Determination of the epitope type of the BoNT/A endopeptidase-inhibiting antibodies by immunoblotting. SDS-PAGE of the BoNT/A light chain was performed under reducing conditions (Laemmli buffer containing β-mercaptoethanol, 95 °C) or non-reducing conditions (Laemmli buffer, 56 °C). Staining was performed with anti-BoNT/A antibodies in the scFv-Fc format, and binding was detected with goat anti-mouse (Fc-specific) secondary antibodies conjugated to alkaline phosphatase (1:10 000).

technicians suspected that the animal was in pain, on the basis of their observations of animal behavior. None of the nonhuman primates were killed during this study.

Animal immunization

A male cynomolgus macaque (*Macaca fascicularis*) was immunized with four subcutaneous injections of 80 μg recombinant botulinum neurotoxin light chain (BoNT/A1-L, Metabiologics Inc) at one-month intervals, except for the fourth injection, which was given eight months after the third.[25]

Construction of the anti-BoNT/A1-L scFv antibody library

RNA was isolated from the bone marrow of immunized animals with Tri Reagent® (Molecular Research Center Inc.) and was used for reverse transcription. Seven oligonucleotides were used for the amplification of DNA encoding the κ light chain and nine oligonucleotides were used to amplify the DNA encoding Fd fragments of the γ chain.[49] The corresponding PCR products were pooled and inserted into the pGemT vector (Promega). The corresponding PCR products in pGemT were re-amplified with two individual oligonucleotide sets introducing the required restriction sites for the cloning of the final library. The initial step for library construction was the insertion of VL fragments into pHAL32. This vector is based on pHAL14[23,43,50] and was specifically designed for this library. In the second cloning step, the VH fragments were inserted into pHAL32 containing the VL repertoire. For library construction, pHAL32 and the VL fragments were digested with *Mlu*I and *Not*I (New England Biolabs). The enzyme reaction was inactivated and pHAL32 was dephosphorylated with calf intestinal phosphatase (MBI Fermentas). The vector and VL repertoire were purified with NucleoSpinII Gel and a PCR Clean-up kit (Macherey-Nagel). 270 ng of VL was cloned into 1 μg of dephosphorylated vector separately in four ligation reactions, with T4 DNA-Ligase (Promega). The DNA was precipitated with ethanol and sodium acetate, the pellet was washed twice with 70% ethanol, resuspended in 30 μL H_2O and used for electroporation (1.7 kV) with 25 μL of XL1-Blue MRF' (Stratagene). The transformed bacteria were cultured on 2xYT agar plates (25 cm petri dishes) supplemented with 100 μg/mL ampicillin, 20 μg/mL tetracycline and 100 mM glucose. The colonies were harvested by resuspension in 40 mL of 2xYT medium with a Drigalsky spatula and plasmids were isolated with the Nucleobond Plasmid Midi Kit (Macherey-Nagel). The VL library and the VH fragments were digested with *Sfi*I and HindIII (New England Biolabs), ligated and electroporated as described for VL, but with the following modifications: 250 ng of the digested and purified VH repertoire was inserted into 1 μg of the VL library. The harvested bacteria of the final scFv antibody gene library were pooled, aliquoted and stored at -80 °C. The library was packaged with Hyperphage[51,52] and M13K07.

Selection of recombinant antibodies against BoNT/A1 and BoNT/A1-L by antibody phage display

For the isolation of recombinant scFv, a microtiter plate was coated overnight with holotoxin BoNT/A1 (Metabiologics) at a concentration of 20 μg/mL in PBS at 4 °C. The following day, the plate was blocked with 3% BSA in TBS (50 mM TRIS; 137 mM NaCl; 2.7 mM KCl; pH adjust to 8.0 with HCl) for 2 h at 37 °C, and the antibody phage display library was incubated for additional 2 h at 37 °C after a washing step. During the first round of panning, the plate was washed 10 times with TBS supplemented with 0.1% Tween 20, with an interval of five minutes between washes. The plate was finally rinsed with sterile TBS and phages were eluted with trypsin (10 mg/mL in TBS) for 30 min at 37 °C. The eluted phages were used to infect *E. coli* (SURE strain, Stratagene) cultured in SB (Super Broth) supplemented with tetracycline (10 μg/mL) and carbenicillin (50 μg/mL). For the production of new phage particles, infected *E. coli* were co-infected with helper phage and cultured overnight at 30 °C in SB supplemented with tetracycline (10 μg/mL), carbenicillin (50 μg/mL) and kanamycin (70 μg/mL). Phage particles were precipitated in PEG/NaCl [4% (w/v) PEG-8000, 3% (w/v) NaCl] and used for next round of panning. The second round of panning was performed as described above, but with the following modifications: the plate was washed 20 times during the second panning and then 30 times in the third panning,

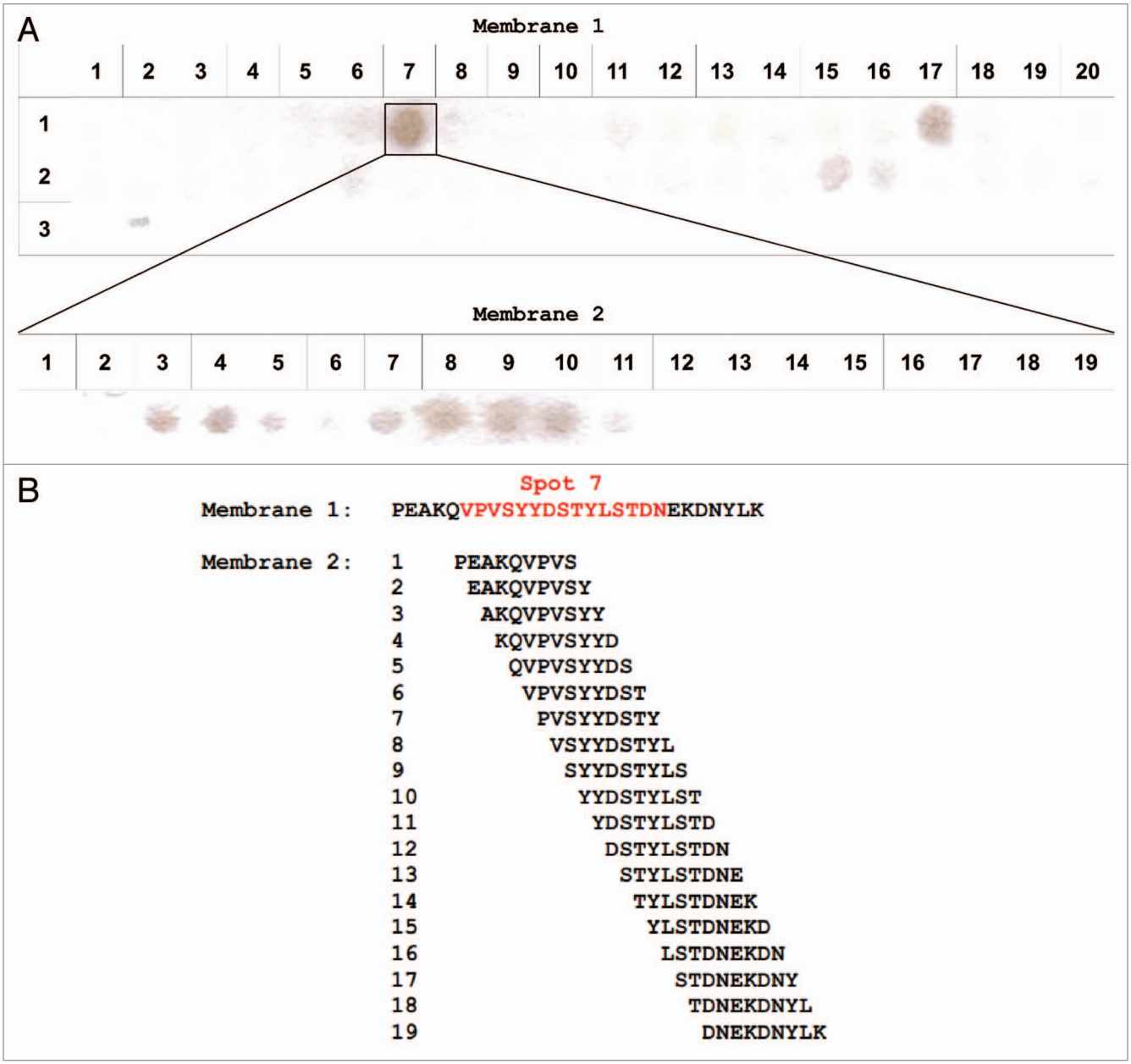

Figure 5. Epitope mapping analysis of the non-neutralizing antibody SEM95-C6. **(A)** Epitope mapping membrane 1 covers the complete BoNT/A light chain with peptides of 15 amino acids in length, with an offset of four amino acids. The membrane was stained with scFv-Fc (10 µg/mL). The bound scFv-Fc was detected with goat anti-mouse (Fc-specific) HRP-conjugated secondary antibody (1:20 000). For detailed analysis of the identified epitope, epitope mapping membrane 2 was used, which covered the light chain with peptides of nine amino acids in length, with an offset of one amino acid, as described above. (**B**) The amino acid sequences of the epitopes identified on membranes 1 and 2 are shown.

with TBS supplemented with 0.1% Tween 20 and a five-minute interval between washes. The infected *E. coli* from the third round of panning were grown on SB media in petri dishes and used for screening.

scFv and scFv-Fc antibody production and purification

All scFv fragments isolated by antibody-phage display were subcloned into pCSE2.5-HIS-XP and pCSE2.5-mIgG2c-Fc-XP and produced as scFv or scFv-Fc in HEK293–6E cells [National Research Council (NRC), Biotechnological Research Institute (BRI)] cultured in chemically defined medium F17 (Invitrogen, Life Technologies) supplemented with 1 g/L pluronic F68 (Applichem), 4 mM L-glutamine (PAA) and 25 mg/L G418 (PAA), as previously described.[26] The scFv-Fc produced were chimeric macaque-mouse antibodies. DNA was used for the transient transfection of 25 mL cultures of HEK293–6E cells in 125 mL Erlenmeyer shake flasks. After 48 h of culture with shaking at 110 rpm in a Minitron orbital shaker (Infors) at 37 °C, under an atmosphere containing 5% CO_2, one volume of culture medium, with a final concentration of 0.5% (w/v) tryptone N1 (TN1, Organotechnie S.A.S.) was used for the purification of

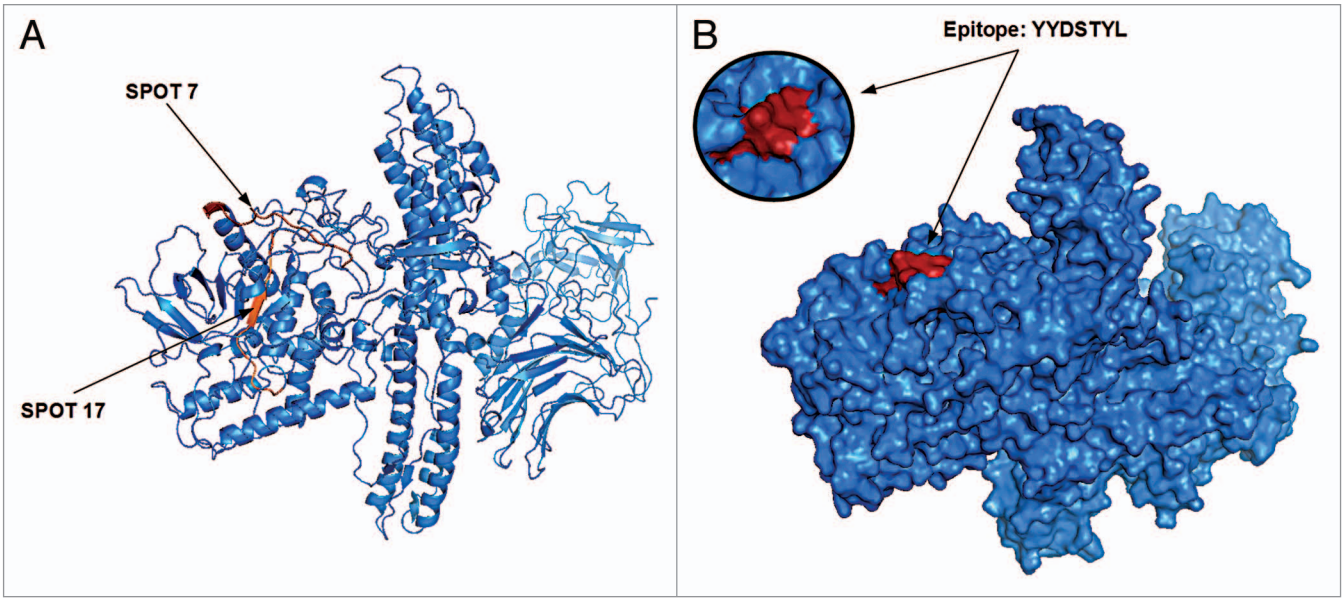

Figure 6. Structure of the botulinum neurotoxin (pdb structure 3BTA).[56] **(A)** Localization of spot 7 and 17 on the BoNT/A light chain. **(B)** Identified epitope of SEM95-C6 at positions 71–78 on the surface of the light chain of BoNT/A.

ScFv on a HIS Trap FF crude column (GE, Healthcare), whereas scFv-Fc were purified on a UNOsphere SUPrA column (Biorad) with a Profinia apparatus (Biorad, Hercules), according to the manufacturer's instructions.

ELISA

ELISA was performed in 96-well microtiter plates (MaxiSorp, Nunc). Each well was coated with BoNT/A1 holotoxin or recombinant BoNT/A-L (100 ng per well) (Metabiologics), in 100 μL of phosphate-buffered saline (PBS) by incubating overnight at 4 °C. The coated wells were washed three times with PBST (PBS + 0.05% Tween 20), with an ELISA washer (Tecan Columbus, Tecan). The wells were then blocked by incubation with 2% (w/v) skim milk powder in PBS supplemented with 0.1% Tween 20 (2% M-PBST) for 1.5 h at room temperature, and washed three times with PBST. For the antigen ELISA, soluble scFv or scFv-Fc was diluted in 100 μL of 2% M-PBST and incubated in the antigen-coated wells for 1.5 h at room temperature. The wells were then washed three times with PBST. Bound scFv from antibody-phage display were detected with the murine mAb 9E10 recognizing the c-myc tag and a goat anti-mouse antibody conjugated to horseradish peroxidase. Bound scFv-Fc were detected with a peroxidase-labeled goat anti-mouse antibody recognizing the murine part of the Fc fragment (A0168, Sigma-Aldrich), visualized in a detection reaction with TMB (3,3',5,5'-tetramethylbenzidine) as the substrate. The staining reaction was stopped by adding 100 μL of 1 N sulfuric acid. Absorbance at 450 nm (reference wavelength: 620 nm) was measured in a SUNRISE™ microtiter plate reader (Tecan).

Affinity measurement

The affinities of scFv-Fc antibodies were measured by surface plasmon resonance with a Biacore X instrument (General Electric-Biacore). Each scFv-Fc (1000 resonance units [RU]) was immobilized on a CM5 chip (General Electric-Biacore) via amine coupling, according to the manufacturer's instructions. During measurement, a flow rate of 30 μL/min was maintained and six dilutions of BoNT/A1 (0.16 μM to 5 nM) in HBS-EP buffer were tested for 1000 s each. After each dilution, the chip was regenerated with glycine (pH 1.5) and run for 30 s at 10 μL/min. Constants were calculated with Biaevaluation software[35] and checked in internal consistency tests.[53]

DNA sequencing and sequence analysis

Twenty-two scFv isolated by panning were sequenced by GATC Biotech (Konstanz, Germany) and compared with human germline sequences, with the IMGT/V-QUEST online tool from the International ImmunoGeneTics information system® (IMGT) (http://www.imgt.org). This tool identifies the human germline sequences most similar to any given variable region and calculates the germinality index (GI), defined as the percentage identity between a given framework region (FR) and the most similar human germline sequence.

Botulinum toxins and antitoxins

Purified type A1 hemagglutinin-free holotoxin was purchased from Metabiologics Inc at a concentration 2.3×10^8 LD_{50}/mg, (1 mg/mL from the Hall strain, lot. A062805–01) and diluted to 20000 LD_{50}/mL in gelatin (0.2% w/v) phosphate (50 mM disodium hydrogen orthophosphate) buffer pH 6.5 (GPB). The diluted preparation was dispensed into aliquots and stored frozen at -40 °C. Recombinant BoNT/A light chain was also purchased from Metabiologics. The polyvalent material used as a positive control in neutralization assays was a commercially available equine polyclonal F(ab')2 preparation (Novartis Vaccines).

Ex vivo neurotoxicity (mouse phrenic nerve-hemidiaphragm assay)

Left phrenic nerve-hemidiaphragm preparations were excised from male inbred mice (Balb/c) and installed in a 6 mL organ

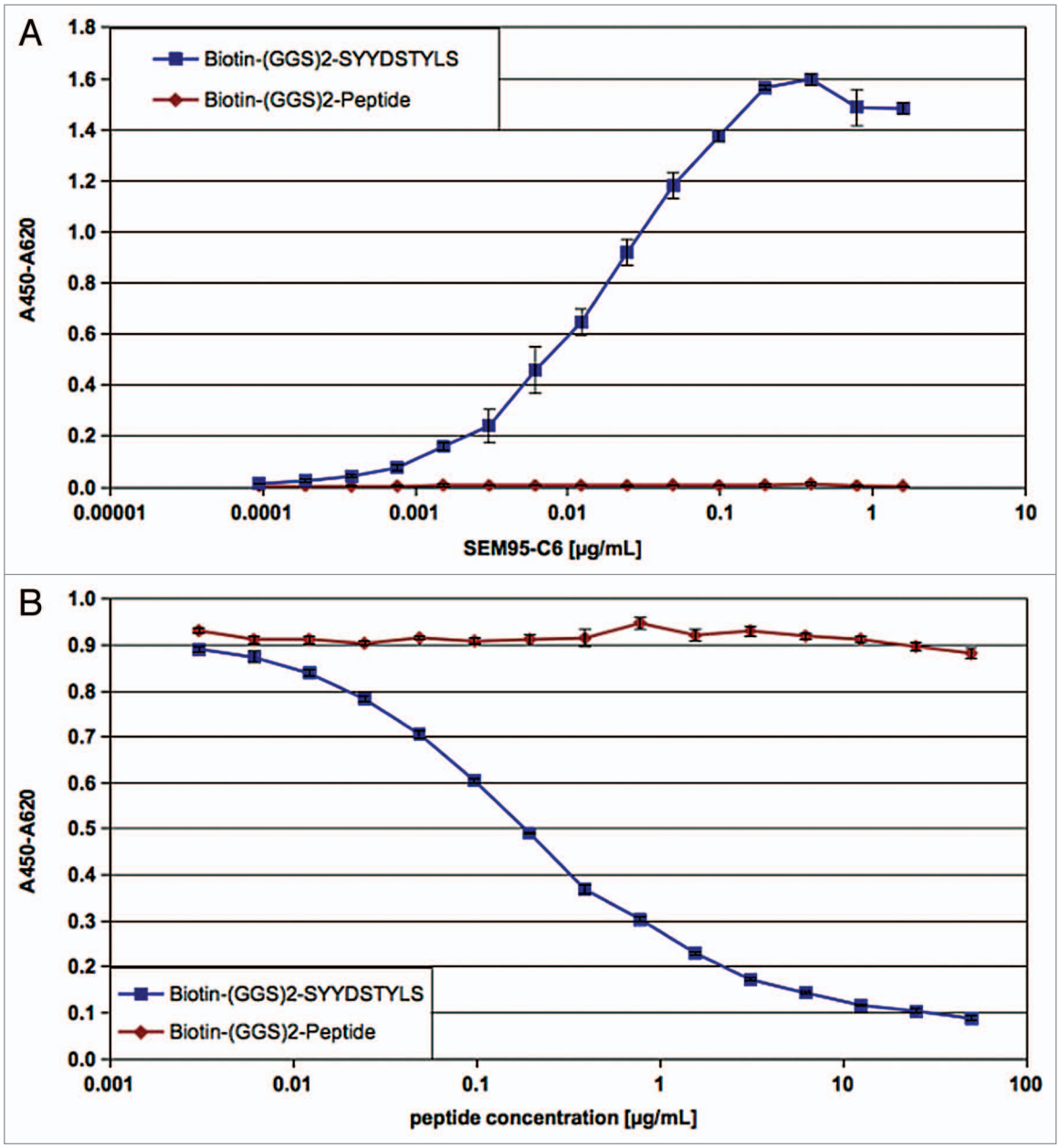

Figure 7. Validation of the epitope of the non-neutralizing antibody SEM95-C6. **(A)** Titration-ELISA of SEM95-C6 on biotinylated peptide. Plates were coated with 200 ng biotinylated peptide and incubated with serial dilutions of SEM95-C6. A non-specific peptide was used a control. The bound scFv-Fc was detected with goat anti-mouse (Fc-specific) HRP-conjugated secondary antibody (1:20 000). **(B)** Competitive ELISA for SEM95-C6. Plates were coated with 100 ng BoNT/A-L and incubated with 25 ng/mL SEM95-C6 in the presence of serial dilutions of biotin-(GGS)2-SYYDSTYLS or a non-specific peptide. The bound scFv-Fc was detected as described above.

bath maintained at 37 °C containing Krebs-gelatin buffer gassed with 95% O_2-5% CO_2 bubbled through the buffer. Continuous 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) linked to a ML110 bridge amplifier and a Powerlab/4SP 4 channel recorder (ADInstruments). The hemidiaphragm resting tension was increased in a stepwise manner during the equilibration period, until reproducible twitches were observed. The toxin was diluted in oxygenated Krebs buffer and incubated at 37 °C for 30 min before being applied to the tissue. Once the muscle twitch response to nerve stimulation had stabilized and remained of a constant magnitude for at least 30 min without further adjustment, the Krebs buffer was replaced with 6 mL of toxin in Krebs solution 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 botulinum

toxin type A1 was determined by fitting to 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 and at the end of the experiment.

Toxin neutralization by the scFv-Fc preparations was assessed by mixing 87 pg/mL (20 LD_{50}/mL) A1 holotoxin with 30 μg/mL of the scFv-Fc preparation and incubating for 30 min at 37 °C before applying the mixture to the tissue. The toxin dose considered optimal for antibody inhibition studies is typically that inducing about 80% maximum paralytic activity on the dose-response curve (ensuring optimal precision), unless further scFv-Fc characterization requires weaker paralytic activity. The organ bath was drained, the toxin/scFv-Fc mixture was added and twitch responses were recorded for up to 300 min (5 h) or until the twitch tension was no longer detectable. The time to 50% paralysis after the addition of the mixture of botulinum toxin/scFv-Fc was determined by fitting to the linear part of the paralysis curve.[39,40] Linear and nonlinear regression analyses were performed with Prism 5.0 software (Graphpad). The neutralization of toxin activity was proportional to the ability of the scFv-Fc to delay BoNT-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 vitro endopeptidase immuno-assay

The in vitro endopeptidase immunoassay for BoNT/A toxin described in a previous study[54] was modified and used to assess the inhibitory properties of scFv-Fc preparations. The curve obtained in this assay presents the ELISA signal as a function of BoNT/A endopeptidase activity and has a linear part, used in this study, in which a 50% decrease in signal strength corresponds to a 50% decrease in endopeptidase activity. BoNT/A1 holotoxin from Metabiologics was diluted to a stock concentration of 87 pg/mL (20 LD_{50}/mL) in reaction buffer (50 mM HEPES, 20 μM ZnCl, pH 7.0, 0.5% (v/v) Tween 20, 5 mM DTT). The scFv-Fc preparations were diluted in reaction buffer to a concentration of 10 μg/mL and 150 μL of the resulting dilution was added to all wells in column 1 of a 96-well low-cell binding polypropylene plate (Nunc). Afterwards 75 μL of reaction buffer were added to all wells in columns 2 to 11. 2-fold dilutions of purified scFv-Fc preparations were performed across the plate, initiated by the removal of 75 μL from the wells in column 1 and completed by the discarding of 75 μL from the wells in position 11. 75 μL of BoNT/A1 solution were added to all wells, to yield a final concentration of 43.5 pg/mL toxin, corresponding to 10 LD_{50}/mL. The toxin/antitoxin mixture was briefly shaken on a plate shaker for 1 min at room temperature, then incubated for 1 h at 37 °C, and was then transferred (50 μL per well) to another 96-well ELISA plate previously coated with custom-made SNAP-25 (aa 137–206) peptide. The plate was incubated for ~18 h at room temperature and washed three times in wash buffer (PBS + 0.05% Tween 20). SNAP-25 (aa 137–206) peptide cleaved by the BoNT/A toxin was detected with 100 μL per well of in-house rabbit anti-SNAP-25 (aa 190–197) antibody[54] in 2.5% M-PBST. The plate was incubated at room temperature for 90 min. It was then washed and 100 μL per well of goat anti-rabbit-HRP conjugate was added (Sigma A0545, diluted 1:2000 in 2.5% M-PBST). The plate was incubated for 90 min at room temperature, washed and 100 μL per well substrate solution (50 mM citric acid pH 4.0, 0.05% (w/v) ABTS (2,2'-azino-bis(3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt, and 0.05% (v/v) of a 30% (w/v) hydrogen peroxide solution) was added. The staining reaction was then evaluated by determining absorbance at 405 nm with a plate reader (Multiskan MS, Labsystems Oy).

SDS-PAGE and immunoblot analysis

All samples were analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE) in a 12% polyacrylamide gel under reducing or non-reducing conditions. The gel was stained with Coomassie blue or blotted onto a PVDF membrane. The membrane was blocked by incubation with 2% M-PBST for 1 h at room temperature. ScFv-Fc antibodies (10 μg/mL in 2% MPBST) were incubated with the membrane for 1.5 h at room temperature, and the membrane was then washed twice with PBST. Binding was detected by incubation with a goat anti-mouse (Fc-specific) antibody conjugated to alkaline phosphatase (Sigma, 1:10 000 in 2% MPBST) followed by the NBT/BCIP substrate.

Epitope mapping

The protein sequence of the BoNT/A1 light chain was divided into overlapping peptide fragments of 15 aa in length, with an offset of four aa for analysis of the complete BoNT/A1 or of nine aa in length with an offset of one amino acid. The peptides were synthesized by the SPOT technique,[55] N-terminally acetylated and covalently bound to a continuous cellulose membrane via their carboxy-termini (JPT Peptide Technologies GmbH). The membrane was first incubated for 5 min with methanol, to prevent the precipitation of hydrophobic peptides during subsequent washing with TBS (50 mM TRIS, 137 mM NaCl, 2.7 mM KCl, pH adjusted to 8.0 with HCl). The washed membrane was blocked by incubation for at least two hours with 2% skim milk powder in TBS (2% M-TBS), at room temperature, with shaking. The membrane was then incubated with 10 μg/mL scFv-Fc antibody in 2% M-TBS for 3 h with shaking and washed with TBS-T (TBS-buffer pH 8.0; 0.05% Tween 20) before incubation with an appropriate volume of peroxidase-labeled secondary antibody (goat anti-mouse (Fc-specific), A0168, Sigma-Aldrich) for 2 h, with shaking. The bound antibody was detected by incubation with DAB (3,3′-diaminobenzidine in PBS; 1:10 000 H_2O_2).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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