

Generation of a Recombinant Full-Length Human Antibody Binding to Botulinum Neurotoxin A

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Abstract In order to develop a recombinant full-length human anti-botulinum neurotoxin A (BoNT/A) antibody, human peripheral blood mononuclear cells (PBMC) were collected from three healthy volunteers and induced for BoNT/A-specific immune response by in vitro immunization. The genes encoding human Fd fragment, consisting of antibody heavy chain variable region and constant region 1 with the genes encoding antibody light chain, were cloned from the immunized PBMC. Afterwards, one combinatory human antigen-binding fragment (Fab) library was constructed using a lambda phage vector system. The size of the constructed library was approximately 10^5 *Escherichia coli* transformants. After screening the library by BoNT/A antigen using a plaque lifting with immunostaining approach, 55 clones were identified as positive. The Fab gene of the most reactive clone exhibiting particularly strong BoNT/A binding signal was further subcloned into a full-length human IgG1 antibody gene template in an adenoviral expression vector, in which the heavy and light chains were linked by a foot-and-mouth-disease virus-derived 2A self-cleavage peptide under a single promoter. After the full-length human IgG1 was expressed in mammalian cells and purified with protein L column, sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the heavy and light chains of the antibody were cleaved completely. The affinity expressed as the dissociation constant (K_d) for the recombinant human antibody to bind to BoNT/A was determined by indirect enzyme-linked immunosorbent assay and results confirmed that the recombinant full-length human antibody retained BoNT/A-binding specificity with K_d value of 10^{-7} M.

Keywords Recombinant full-length human antibody · Botulinum neurotoxin A · In vitro immunization · Fab library · 2A self-cleavage linker · Monocistronic expression vector · Mammalian expression

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Introduction

Botulinum neurotoxins (BoNTs) produced by the bacterium of *Clostridium botulinum* are the most poisonous naturally occurring substances affecting humans and other mammals [1, 2]. To date, seven serotypes of BoNT, classified A through G, are recognized. All seven subtypes share important structural and functional features, but only serotypes A, B, and E are clinically important agents of human botulism [2–4]. They bind to the presynaptic nerve terminal at the neuromuscular junction and cholinergic autonomic sites and thus disrupt cellular communication at the neuromuscular junction, resulting in muscular weakness and paralysis [5]. Since BoNTs are so lethal and easy to manufacture, deliver, and weaponize, these pose a threat to the public health as bioterrorist and biowarfare agents [6].

Monoclonal antibodies (mAbs) to BoNTs could provide an efficient passive immunity to endangered individuals [7]. Therefore, many murine anti-BoNTs antibodies have been developed [8–13]. However, the disadvantages of using murine mAbs as immunotherapeutic agents in humans are apparent [14, 15], the key one being the human anti-mouse antibody response [14]. Because rodent antibodies are foreign proteins, the human immune system mounts its own antibody response to these, leading to rapid clearance, reduced efficacy, and anaphylaxis, which can sometimes be fatal [16, 17]. Limitations in the use of murine mAbs in clinical applications have boosted the development of human antibodies [18–23].

Antigen-specific recombinant human antibody fragments can be developed by phage display libraries with antibody fragments on their surface [22]. Using this strategy, a diverse set of human antibody fragments such as antigen-binding fragment (Fab) or single chain variable fragment (scFv) from volunteers to a variety of infectious agents had been developed [24–26]. To further develop full-length human antibodies, antibody fragments have to be converted into full-length antibody versions by gene engineering.

Antibodies are large molecules composed of two chains, the heavy chain (HC) and light chain (LC), which need to be assembled into a four subunit structure to exert their function. At present, it is a challenge to produce HC and LC from a monocistronic construct. It has been found that the foot-and-mouth disease virus (FMDV)-derived 2A self-cleavage sequence could be introduced between HC and LC DNA sequences to express a full-length antibody from a single open reading frame driven by a single promoter in an adenoviral vector [27]. Also the 2A oligopeptide sequence can undergo self-cleavage to generate the antibody HC and LC after translation.

In our study, human peripheral blood mononuclear cells (PBMC) were collected from three healthy volunteers and then immunized with BoNT A (BoNT/A) with recombinant interleukin (rIL)-1 and rIL-2 in vitro. Next, the genes encoding human Fd consisting of HC variable region (VH) and constant region 1 (CH1) with the genes encoding human LC were cloned from the RNA extracted from the immunized PBMC. One combinatory human Fab library was then constructed using a lambda phage vector system. After screening the library using BoNT/A antigen by a plaque lifting with immunoscreening approach, 55 clones were identified as positive. The Fab gene, from the most reactive clone exhibiting particularly strong BoNT/A binding signal, was subcloned into a full-length human IgG1 antibody gene template. The HC and LC, linked by 2A self-cleavage peptide, were in a single open reading frame driven by a single promoter. After expression in mammalian cells and purification, the antibody was demonstrated to be properly dimerized and to retain BoNT/A-binding specificity with K_d of 10^{-7} M.

Materials and Methods

Isolation of Human PBMC

Human PBMC were isolated from three healthy volunteers. Blood (45 ml) was collected in Becton-Dickinson vacutainers (BD Biosciences, Mississauga, ON, Canada) containing sodium heparin and then diluted in two blood volumes of Dulbecco Modified Eagle Medium (DMEM) (GIBCO Laboratories, Burlington, Ontario, Canada) and layered over Nycomed 1.077 density gradient centrifugation media (Nycomed Pharma, Oslo, Norway). Tubes were centrifuged at $1,000\times g$ for 15 min with no brake. Buffy coats at the medium interface, containing mononuclear cells, were collected and washed once in DMEM.

In Vitro Immunization

Isolated PBMC were immunized in vitro by culturing for 3–4 days in completed DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS) (GIBCO Laboratories), 15 ng/ml rIL-1 (Roche Diagnostics, QC, Canada), 250 ng/ml rIL-2 (Roche Diagnostics) and 0.05–0.5 $\mu\text{g/ml}$ highly purified BoNT/A obtained from Food Research Institute, University of Wisconsin, Madison, WI.

Isolation of RNA from In Vitro Immunized Human PBMC

After in vitro immunization, the PBMC were collected by centrifugation at $800\times g$ for 15 min. Cells were lysed in guanidine isothiocyanate (Sigma-Aldrich, Oakville, ON, Canada) by four to five passages through a 26 gauge syringe needle, RNA was purified on CsCL gradients (Sigma) and a yield of about 50–75 μg of total RNA was successfully isolated.

Construction of Human Fab Lambda Phage Library

As illustrated in Fig. 1, by following the Stratagene ImmunoZap Manual (Stratagene, La Jolla, CA), human RNA was converted into complementary DNA (cDNA) using reverse transcriptase with oligo(dT) as a primer. The product was used as a template for amplification of human IgG Fd and LC DNA fragments by PCR using human Fd- and LC-specific PCR primers. The PCR amplified LC DNA fragments were digested with *Sac I* and *Xba I* restriction endonucleases and ligated into lambda phage ImmunoZap L vector arms. Similarly, the amplified Fd DNA fragments were digested with *Xho I* and *Spe I* restriction endonucleases and ligated into lambda phage ImmunoZap H vector arms. The ligated recombinant lambda phage ImmunoZap L and H DNAs were then packaged and plated. Next, lambda phage libraries of Fd and LC were cloned and amplified. Lambda phage DNA prepared from various LC and Fd libraries was separated on 0.8% agarose gel. The gel-purified LC lambda phage DNA was digested with *EcoR I* and *Mlu I* restriction endonucleases and the gel-purified Fd lambda phage DNA was digested with *EcoR I* and *Hind III* restriction endonucleases. Both products were ligated to create a combinatory lambda phage vector which expressed both Fd and LC gene fragments on a bicistronic messenger RNA (mRNA). The resultant phage was packaged and plated as a Fab (combination of Fd and LC) lambda phage library.

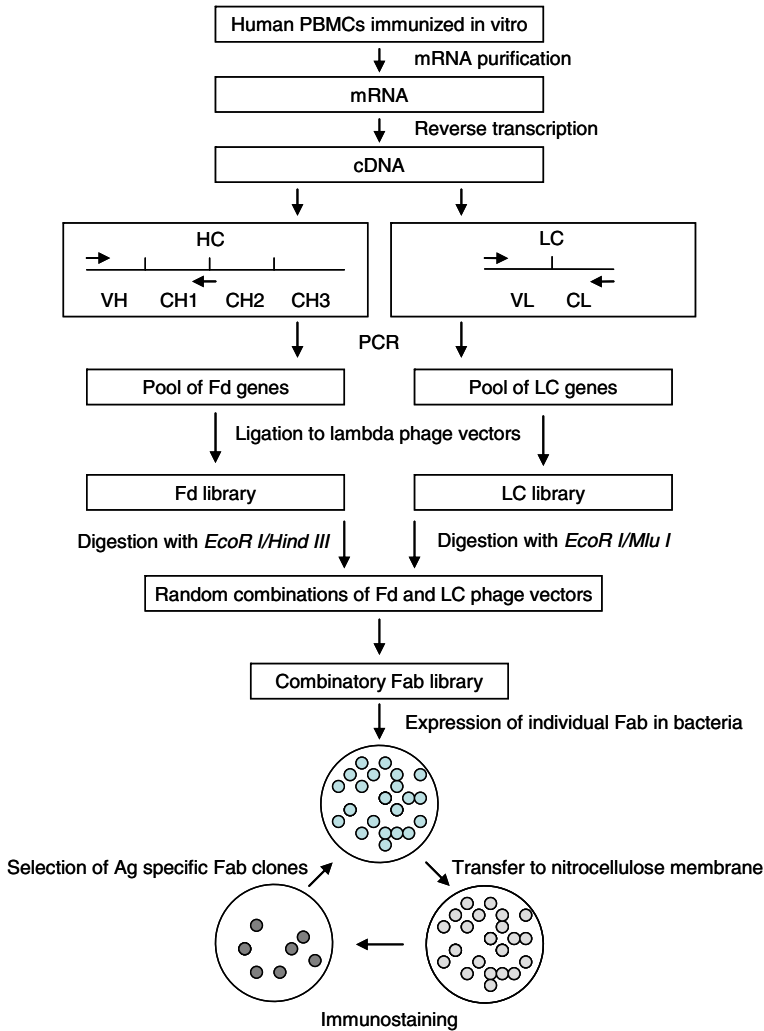


Fig. 1 Combinatory Fab library approach for selection of anti-BoNT/A Fab

Selection of Recombinant Human Anti-BoNT/A Fab

Screening of the Fab library for anti-BoNT/A Fab was performed by a plaque lifting with immunostaining approach. Plaque lifts were taken onto nitrocellulose membranes. Briefly, *Escherichia coli* was infected with recombinant phages and plated, nitrocellulose filters (Sigma-Aldrich) soaked in 10 mM isopropyl β -D-thiogalactopyranoside (Sigma-Aldrich) were overlaid onto the agar plates and incubated at room temperature overnight to induce expression of Fab antibodies. For selection of anti-BoNT/A Fab positive clones, the filters were then stained against BoNT/A. Briefly, the filters were blocked with 5% nonfat dry milk–tris buffered saline (TBS) (Sigma-Aldrich) for 1 h and probed with 100 ng/ml of BoNT/A for 1 h at room temperature. The filters were washed in TBS containing 0.1% tween-20 (TBST) for three times and then incubated with a 1:500 dilution of horse anti-BoNT/A antisera for 1 h at room temperature. After washing three times with TBST, the

filters were incubated with 1:1,000 dilution of a goat anti-horse-alkaline phosphatase conjugate (Stratagen, La Jolla, CA) for 1 h at room temperature. After washing three times with TBST, the filters were developed by adding CSPD chemiluminescent substrate (Tropix, Bedford, MA) according to the instructions of the manufacturer.

Construction and Expression of a Full-Length Human Antibody Derived from Anti-BoNT/A Fab

The most reactive one, 510-37 which exhibited particularly strong BoNT/A binding signal was selected to be converted into a full-length human antibody version. The recombinant adenoviral construct, pAd-hu1A4A1IgG1 was previously constructed in our lab (manuscript in preparation). It encoded a full-length human IgG1 antibody against Venezuelan equine encephalitis virus from a single open reading frame driven by a single promoter in an adenoviral vector. In the single open reading frame, the LC sequence was followed by a 2A linker before the HC so as to produce HC and LC in one monocistronic vector shown in Fig. 2. In order to clone 510-37 VH and light chain variable region (VL) genes into this full-length human antibody template, the insert in 510-37 clone was sequenced and then its VH and VL genes were synthesized by Genscript Corporation, Piscataway, NJ (www.genscript.com). When 510-37 VH and VL genes replaced the corresponding genes in pAdhu1A4A1IgG1, the resultant pAdhu510-37IgG1 was co-transformed with the

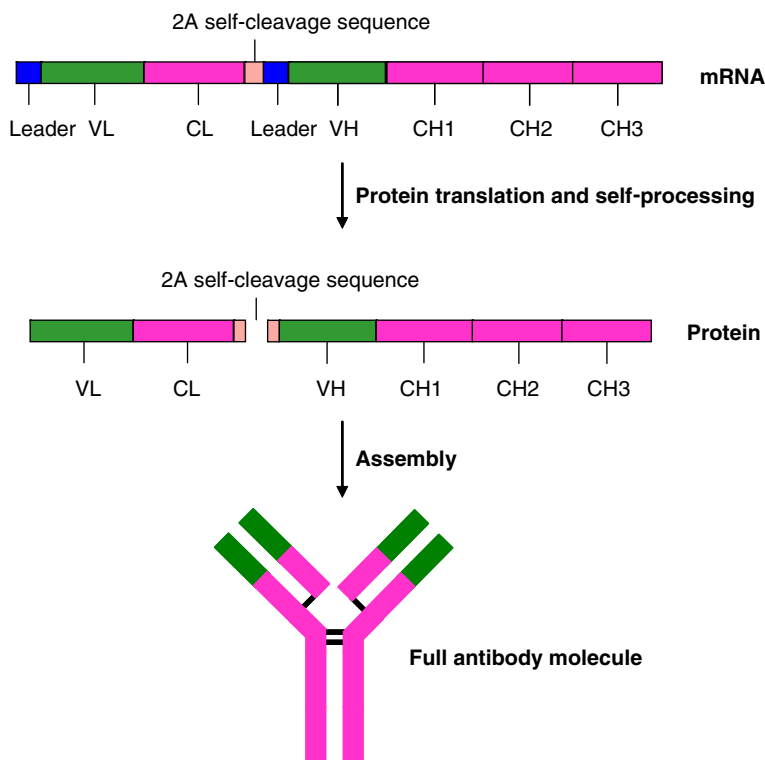


Fig. 2 Schematic diagram showing full-length antibody expression strategy using a monocistronic vector with 2A self-cleavage sequence as a linker between HC and LC

pAdEasy-1 vector (Qbiogene, Carlsbad, CA) into *E. coli* cells to produce recombinant adenoviral genomic construct for the full-length human anti-BoNT/A antibody, hu510-37IgG1. The pAd-hu510-37IgG1 was linearized with *Pac I* restriction endonuclease and transfected into HEK 293 cells (American Type Culture Collection, Manassas, VA) cultured in DMEM supplemented with 5% FBS for amplification and then the amplified adenoviruses were purified by a chromatographic method. To express hu510-37IgG1, HEK 293 cells were infected with the recombinant adenovirus at a multiplicity of infection of 1. The infected cells were cultured for 1 week and culture supernatant was harvested. The expressed hu510-37IgG1 was purified using protein L agarose gel from Pierce (Brockville, ON). Briefly, culture supernatant was dialyzed against phosphate buffer saline (PBS) (Sigma-Aldrich) for 12 h and then concentrated using polyethylene glycol to less than 50 ml. The concentrated sample was incubated with 2 ml protein L agarose gel at 4°C for 1 h. The gel and supernatant mixture was then loaded onto an empty column, which was subsequently washed with binding buffer. Bound hu510-37IgG1 was eluted with elution buffer. The eluted antibody was further desalted using an excellulose column (Pierce) and then concentrated by Centricon YM-30 (Millipore Corp., Bedford, MA).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Antibodies were separated by 10% SDS-PAGE gels using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Mississauga, ON). The bands were visualized by SimplyBlue SafeStain staining (Invitrogen, Burlington, ON). The molecular weights of the samples were estimated by comparison to the relative mobility values of standards of known molecular weights.

Enzyme-Linked Immunosorbent Assay

The reactivity of purified hu510-37IgG1 to BoNT/A antigen was evaluated by enzyme-linked immunosorbent assay (ELISA). Nunc maxisorp™ flat bottomed 96 well plates (Canadian Life Technologies, Burlington, ON, Canada) were coated overnight at 4°C with BoNT/A antigen at a concentration of 10 µg/ml in carbonate bicarbonate buffer, pH9.6. The plates were washed five times with PBS containing 0.1% tween-20 (PBST) and then blocked in 2% BSA for 2 h at room temperature. After five washes with PBST, the plates were incubated for 2 h at room temperature with various concentrations of antibody diluted in PBST. Following five washes with PBST, the plates were incubated for 2 h at room temperature with horseradish peroxidase-conjugated rabbit anti-human IgG fragment crystallizable portion (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:5,000 in PBST. Finally, the plates were washed five times with PBST and developed for 10 min at room temperature with a 3,3',5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reactions were read at an absorbance of 615 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA).

Determination of the Affinity of hu510-37IgG1 Binding to BoNT/A

The affinity of hu510-37IgG1 binding to BoNT/A expressed as the dissociation constant (K_d) was measured by indirect ELISA [28]. Briefly, Nunc maxisorp flat-bottomed 96-well plate was coated overnight at 4°C with BoNT/A antigen at a concentration of 10 nM in carbonate bicarbonate buffer, pH9.6. Meanwhile, BoNT/A antigen at various concentrations (320, 160, 80, 40, and 20 nM) was incubated with 2.5 nM of hu510-37IgG1

antibody overnight at room temperature to reach equilibrium. To determine the concentrations of free antibody in the equilibrium mixture, 100 μ l of the equilibrium mixture was transferred to the BoNT/A-coated plate after the plate was blocking in 2% BSA for 2 h at room temperature. After incubation for 1 h at room temperature, the plate was washed with PBST and then incubated with horseradish peroxidase-conjugated rabbit anti-human IgG fragment crystallizable portion diluted 1:5,000 in PBST for 1.5 h at room temperature. Finally, the plate was washed five times with PBST and developed for 20 min at room temperature with a 3,3',5,5'-tetramethylbenzidine substrate. The reactions were read at an absorbance of 615 nm by the microplate autoreader. The K_d was deduced from the Klotz slope, by plotting the inverse value of the fraction of bound antibody against the inverse value of total antigen. The slope was calculated in EXCEL using the statistical functional CORREL.

Results

In order to induce BoNT/A-specific immune responses in human PBMC, the PBMC were immunized with BoNT/A in the presence of rIL-1 and rIL-2 for 3 days. An ELISPOT assay was performed to confirm the induction of antigen-specific B-cell responses (data not shown).

As illustrated in Fig. 1, to amplify the human Fd and LC genes of the antibody from the in vitro immunized PBMC, total RNA was extracted from the PBMC and then reverse-transcribed into cDNA using oligo (dT) as a primer. Human Fd and LC genes were amplified by PCR and cloned into lambda vectors, and packaged in vitro respectively. The two libraries were then combined by ligation. After packaging, the combinatory Fab library was titered. The library contained 10^5 independent clones.

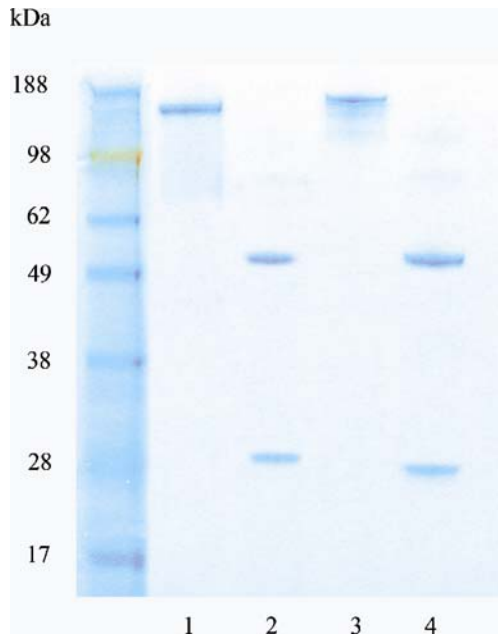
To select BoNT/A-specific phage clones, the library was screened with BoNT/A using a plaque lifting with immunostaining approach. Fifty-five clones were identified as positive. The most reactive one, 510-37, which exhibited particularly strong BoNT/A binding signal, was selected to construct a human full IgG antibody gene by cloning VH and VL from the Fab to a full human IgG template in the adenovirus vector with 2A linker between HC and LC as showed in Fig. 2 (manuscript in preparation).

In order to express the full human anti-BoNT/A IgG, HEK 293 mammalian cells were infected with the adenovirus pAd-hu510-37IgG1. The infected cells were cultured for 1 week and culture supernatant was harvested. The expressed hu510-37 IgG1 was purified using ImmunoPure Protein (L) agarose column. The purified product was subjected to 10% SDS-PAGE, and one obvious band of ~150 kDa in non-reducing condition and two clear bands of ~50 kDa and ~25 kDa without any extra bands in reducing condition (cleavage of disulfide bridges) were observed (Fig. 3). The purity estimated by SDS-PAGE was more than 90%. The yield was 1 mg/L after purification.

The specific binding reactivity of purified hu510-37 IgG1 to BoNT/A antigen was examined by an ELISA. When the plates were coated with 10 μ g/ml of BoNT/A, the hu510-37 IgG1 bound to BoNT/A in a dose-dependent manner (Fig. 4). The affinity of the purified hu510-37 IgG1 binding to BoNT/A antigen was determined by indirect ELISA. Klotz plot was constructed by plotting the inverse value of the fraction of bound antibody against the inverse value of total antigen as showed in Fig. 5. The K_d deduced from the slope was 10^{-7} M.

An anti-BoNT/A mAb (230F9) from abcam, which recognizes C-terminal of BoNT/A was used with hu510-37IgG1 in a competitive ELISA to see if hu510-37IgG1 bound to the

Fig. 3 SDS-PAGE analysis of the purified hu510-37IgG1. Samples were resolved by SDS-PAGE. Lanes 1 and 3 control human IgG1 and hu510-37IgG1 in non-reducing condition; lanes 2 and 4 control human IgG1 and hu510-37IgG1 in reducing condition



same epitope as 230F9 or not (data not shown). The result showed hu510-37IgG did not compete with 230F9, indicating hu510-37IgG binds to a different epitope from mAb 230F9.

Discussion

In this study, we constructed a combinatory lambda phage Fab antibody library using RNA from in vitro immunized human PBMC. The constructed phage library was screened by BoNT/A antigen for the isolation of phages displaying anti-BoNT/A human Fab antibody. Among selected clones, the most reactive clone revealing the highest activity against BoNT/A was chosen for conversion into a full human antibody version by cloning the VH

Fig. 4 hu510-37IgG1 binding to BoNT/A by ELISA

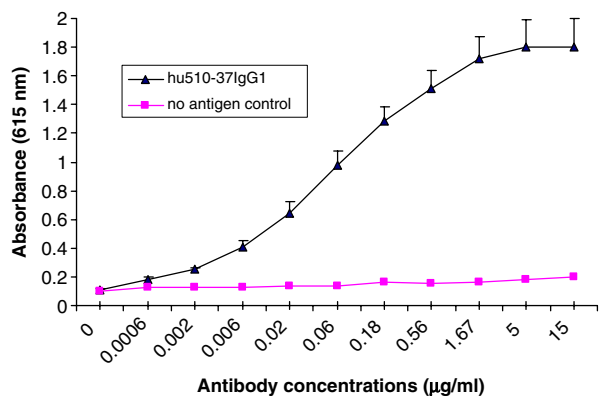
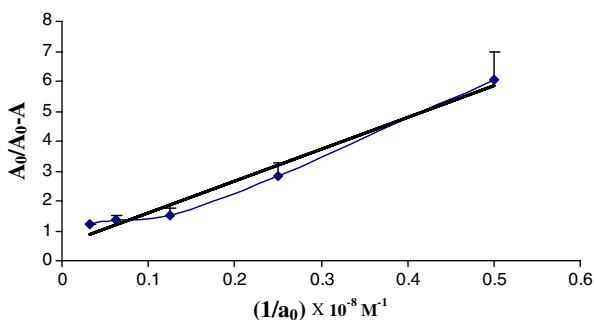


Fig. 5 Klotz plot of hu510-37IgG1 binding to BoNT/A by the competitive indirect ELISA. a_0 the total BoNT/A concentration, A_0 the absorbance measured for hu510-37IgG1 in the absence of BoNT/A, A the absorbance measured for bound hu510-37IgG1



and VL of the Fab into a full human IgG1 antibody gene template in an adenoviral expression vector.

Currently, there are approaches available to express two antibody chains in mammalian cells. However, most of these suffer from a drawback—the imbalance in HC and LC production in cells. Extra chain production has been found to be toxic to the antibody-expressing cells. That also explains the low yield of expressed antibodies from mammalian cells. The conventional approach is to use a two vector system [29]. Each vector carries HC or LC, respectively. It is difficult to control the amount of the two vectors transfecting each individual cell and frequently this results in the unequal molar production of HC and LC within cells. A second approach is to insert an internal ribosomal entry site (IRES) between HC and LC genes in a bicistronic vector under a single promoter [30]. Transcription from this bicistronic vector produces a single mRNA molecule encoding both HC and LC. IRES enables the ribosome to bind to the initiation site of LC. The HC and LC are translated separately from the same mRNA molecule and expression levels of both chains are assumed to be paired. However, the two gene products are not always expressed equally [31], possibly as a result of variability in ribosome recruitment of biologically active and reporter molecules. A third approach is that a bidirectional cassette, consisting of two promoters orientated in opposite directions, drives expression of both products, but this approach has not been widely tested [32]. Expression of two genes in a monocistronic vector with a linker between these has proved to be an effective approach to producing a balance of the two gene products. The linker can be either proteases, such as furin or a self-cleavage sequence, such as 2A. Although the furin linker has worked effectively in a human Fab construct with producing balanced Fd and LC [33], it has not worked well in our full antibody construct [34]. One possible reason is the furin linker sequence in a full antibody structure is probably inaccessible to the furin. Recently, an FMVD-derived 2A self-cleavage sequence was demonstrated to be able to cleave two chains efficiently enough to produce an equal mole of HC and LC when this sequence was introduced between the two chains in a single open reading frame driven by a single promoter in an adenoviral vector [27]. In this study, we used this approach to express the anti-BoNT/A full-length human antibody as shown in Fig. 2. After expression in mammalian cells, two chains were completely cleaved. The antibody was demonstrated to be properly dimerized without extra HC and LC and to retain BoNT/A-binding specificity with K_d of 10^{-7} M. An anti-BoNT/A mAb (230F9) from abcam, which recognizes C-terminal of BoNT/A was used in a competitive ELISA to see if hu510-37IgG1 bound to the same epitope as 230F9 or not. The result indicates hu510-37IgG binds to a different epitope from mAb 230F9. More works need to be done to map this antibody epitope.

Using this monocistronic vector with 2A linker system, not only can equal molar production of two chains be guaranteed, but also the methodology is simple dealing with one plasmid system, greatly reducing the time spent constructing and expressing a functional antibody when compared to the two vector systems.

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