A VH,H That Neutralizes the Zinc Metalloproteinase Activity of Botulinum Neurotoxin Type A*

The current treatment of botulism is to administer animal-derived antitoxin, which frequently causes severe adverse reactions in the recipients. In this study, a heavy chain antibody fragment (VH/VHH) phage display library was constructed by amplification of the immunoglobulin genes of a nonimmune camel, Camelus dromedarius, using primers specific to human VH gene segments. A recombinant light chain of type A botulinum toxin, BoTxA/LC, with zinc endopeptidase activity was used in phage bio-panning to select phage clones displaying BoTxA/LC-bound VH/VHH. Soluble VH/VHH were produced and purified from 10 VH/VHH phagemid-transformed E. coli clones. Complementary determining regions (CDRs) and immunoglobulin frameworks (FRs) of the 10 camel VH/VHH-deduced amino acid sequences were determined. FR2 sequences of two clones showed a hallmark of camel VH,H, i.e. (F/Y)4E49R50(G/F)52. The remaining eight clones had an FR2 amino acid tetrad of conventional VH, i.e. V52G49L50W52. VH,H of one clone (VHH17) neutralized the SNAP25 hydrolytic activity of BoTxA/LC, whereas mouse polyclonal anti-BoTxA/LC did not have such activity. Mimotope sequences of VHH17 matched with the 194–206 amino acid residues of BoTxA/LC, which are located near the S1 subsite of the catalytic cleft of the enzyme. Molecular docking revealed that CDR3 of the VH,H bound to epitope in the toxin enzymatic cleft. Therefore, the BoTxA/LC neutralization by the VH,H should be due to the VH,H insertion into the enzymatic cleft of the toxin, which is usually inaccessible to a conventional antibody molecule. This antibody fragment warrants further development as a therapeutic agent for botulism.

Botulism is a clinical manifestation characterized by generalized flaccid paralysis and respiratory insufficiency, which may be fatal if not treated properly. It is caused mainly by consumption of food contaminated with neurotoxins of Clostridium botulinum (BoTx). The BoTx are zinc-dependent endopeptidases that cleave SNARE proteins used for the exocytosis of the neurotransmitter at the motor nerve end plate (1, 2). BoTx are recognized as the most potent toxic substance of humans with a lethal dose as low as 1 ng/kg body weight (3–5) and are classified as a category A bio-weapon by the Centers for Disease Control and Prevention (6–7). Presently, there are seven antigenic types of BoTx, serotypes A–G (3–5). Among these, serotype A causes the most serious clinical manifestations in humans due to its prolonged localization within the cytoplasm of the affected neuron (8).

The molecular structure of BoTx has been revealed by crystallography as an A-B toxin (9, 10). The two peptides are synthesized as a single polypeptide, which is modified post-translationally to a 150-kDa, di-chain active holotoxin. Each molecule of the holotoxin is composed of an A subunit or light chain (~50 kDa), which is linked to a B subunit or heavy chain (~100 kDa) by a single disulfide bond. The heavy chain is responsible for receptor binding, internalization, and translocation of the holotoxin into the endosome of cholineric neurons (11). After an early endosomal exit, the light chain hydrolyzes SNARE proteins such as SNAP25 (for types A, C, and E BoTx), synaptobrevin (for types B, D, F, and G BoTx), and syntaxin (type C BoTx) resulting in the disruption of the neurotransmission process (12, 13).

A licensed BoTx antagonist is not available. Patients with botulism are treated with animal-derived anti-BoTx antibodies together with supportive measures, such as artificial respiration. There are several drawbacks of using the anti-toxin of heterologous species. The animal antibodies often elicit allergic reactions, which may be as serious as fatal anaphylaxis, as well as an anti-isotype/idiotype response that causes serum sickness (6). Besides, a prolonged immunization process of the donor animals is required before a satisfactory level of the antitoxin is reached. Because of their small size (~15–20 kDa), high tissue-penetrating efficacy, and relative stability, single domain heavy

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chains ($V_{\text{i}}H$) from a dromedary ($Camelus dromedarius$), which are devoid of a variable light chain domain have attractive molecular structures for a potent enzyme/toxin inhibitor (14–20). $V_{\text{i}}H$ could directly recognize the conformational structure within the pocket of an enzyme active site, which can never be reached by the large sized conventional heavy light chain antibody (21–23). In this study, $V_{\text{i}}H$ produced from a phage clone derived from a VH/$V_{\text{i}}H$ phage display library constructed from immunoglobulin genes of B cells of a nonimmune Arabian camel, $C. dromedarius$, are used to bind specifically to the catalytic light chain of the type A botulinum neurotoxin and to inhibit the toxin endopeptidase activity. Experimental details and results are reported herein.

EXPERIMENTAL PROCEDURES

Production of a Full-length Recombinant Light Chain of Type A Botulinum Neurotoxin (BoTxA/LC)—Chromosomal DNA of serotype A $C. botulinum$ was used as a template for amplifying a gene sequence encoding the full-length BoTxA/LC. The 1.4-kb DNA amplicon of the toxin gene segment was cloned into pQE30 expression vectors (Qiagen), and the recombinant expression vectors were introduced into competent SG13009 (pREP4) Escherichia coli cells by a heat-shock method. The transformed SG13009 (pREP4) E. coli cells were selected from an overnight Luria-Bertani (LB) agar plate containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (LB-AK) and screened by PCR for the presence of the BoTxA/LC plasmid vectors. Selected transformed E. coli clones were individually grown in LB-AK broth at 25 °C with shaking until the absorbance at 600 nm ($A_{600\text{nm}}$) was 0.2. Isopropyl-β-D-thiogalactopyranoside (USB) was added to 0.5 mm, and the culture was incubated further at 25 °C for 16 h. Bacterial cells were collected by centrifugation and sonicated in a lysis buffer (5 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.3). The homogenate was treated further at 25 °C for 10 min. The recombinant BoTxA/LC in the bacterial lysate was purified by nickel-nitrilotriacetic acid-agarose (Invitrogen) according to the manufacturer’s instruction.

Determination of the Enzymatic Activity of the Recombinant BoTxA/LC—The endopeptidase activity of the recombinant BoTxA/LC was determined by Western blot analysis and fluorogenic assay. For Western blotting (24, 25), 20 µl of 10 µM recombinant BoTxA/LC were added to 200 µg of a SK-N-MC human neuroblastoma cell lysate in a working buffer (40 mM HEPES, pH 7.4, and 0.5 mM ZnCl$_2$), and the mixture was incubated at 37 °C for 24 h. The preparation was subjected to SDS-PAGE, transblotted onto a nitrocellulose membrane (NC), and probed with rabbit polyclonal anti-SNAP25 antibodies (Zymed Laboratories Inc.), which recognized only intact SNAP25. Goat anti-rabbit immunoglobulin-alkaline phosphatase (AP) conjugate (Southern Biotech) served as secondary anti-isotype antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium $^{\text{TM}}$ (KPL) was used as AP substrate. For the fluorogenic assay, the methods described by Schmidt (26) and Palmer (27) were followed with modifications. Briefly, a fluorogenic oligopeptide substrate representing amino acid residues 187–203 of SNAP25, which is a cleavage motif of BoTxA, was synthesized (Anspec, Inc.). The sequence of the synthetic peptide was SNRTRIDEAN(N-2,4-dinitrophenyl-K)RA(3-iodoacetamido-4-methyl-7-dimethylaminocoumarin-C-RML, which would be cleaved by BoTxA/LC between the Lys197 and Arg198 residues. The fluorescence signal from 3-iodoacetamido-4-methyl-7-dimethylaminocoumarin was quenched by N-2,4-dinitrophenyl in the intact fluorogenic substrate but became detectable upon the substrate cleavage when exposed to active BoTxA/LC. Hydrolytic rates of the fluorogenic substrate by BoTxA/LC were measured as the following: mixtures of various amounts of fluorogenic substrate (10, 12.5, 15, 17.5, 20, 25, and 50 µM) and a fixed amount (0.25 µM) of BoTxA/LC were separately made in 60 µl of working buffer contained in wells of a clear bottom 96-well assay black plate (Costar, Corning) at 25 °C. The excitation and emission maxima were 398 and 485 nm, respectively. The fluorescence signal of each reaction mixture was monitored at 2-s intervals using the VarioSkan Flash $^\text{®}$ microplate reader (Thermo Fisher Scientific). Initial hydrolysis rates or velocity ($V_i$) were derived from 5 to 8 min of the reactions and expressed as fluorescence units/sec. The $V_i$ values were fitted with the Michaelis-Menten equation by using GraphPad Prism$^5$ software to calculate maximum velocity ($V_{\text{max}}$) and $K_m$. The $V_i$ units were from conversion of fluorescence units/min into pmol/min by using an equation derived from a standard curve. The standard curve was constructed by plotting varying amounts of fluorescent products, (RA-DIA-CIA)CRML, from 5 pmol to 1 nmol against the fluorescence intensities. The $K_{\text{cat}}$ was then calculated by dividing the $V_{\text{max}}$ with molarity of the enzyme concentration.

Preparation of Mouse Anti-BoTxA/LC—Three ICR mice (from the National Laboratory Animal Center, Mahidol University, Nakhon-Pathom, Thailand) were housed for 1 week before commencing immunization. Each mouse received three injections of 10 µg of purified BoTxA/LC at weekly intervals. The immunogen was mixed with an equal volume of Alum’s adjuvant (Pierce, Thermo Fisher Scientific) and administered intraperitoneally. One week after the last booster, the mice were bled, and serum samples were collected separately. The titers of the antibodies to BoTxA/LC in the immune sera were measured by indirect ELISA using goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (Southern Biotech), and a chromogenic substrate, i.e. 2,2’-azino-di(3-ethylbenz-thiazoline-6-sulfonate) (Kirkegaard and Perry Laboratories, Inc.), as detection reagents. The optical density (OD) of the content of each well was determined at $A_{505\text{nm}}$ against the background (wells with normal mouse serum). The immune sera were kept at −20 °C until use.

Construction of the Phage Display C. dromedarius VH/$V_{\text{i}}H$ Library—Peripheral blood mononuclear cells were isolated from 50 ml of venous blood collected from an eight-month-old naïve male dromedary ($C. dromedarius$) from the Chockchai Farm, Nakhon Ratchasema province, Thailand, using Ficoll-Paque $^\text{TM}$ (Amersham Biosciences). The total RNA was extracted from the cells by TRIzol$^\text{TM}$ reagent (Invitrogen), and the mRNA was reverse-transcribed to cDNA using Revert Aid$^\text{TM}$ (Fermentas Life Sciences). The gene fragments encoding variable domains of the dromedary VH/$V_{\text{i}}H$ were PCR-amplified using the cDNA as template, as well as 14 forward and three reverse human immunoglobulin-specific primers.

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The antibody was checked by SDS-PAGE and stained with Coomassie Brilliant Blue G-250 dye (USB). The preparation was dialyzed against phosphate-buffered saline. High purity VH/VH was prepared by subjecting the preparation in phosphate-buffered saline to an affinity anti-E-tag column (GE Healthcare Bio-Sciences AB).

\[ \text{Detection of the Binding of the Phage-derived VH/VH to the BoTxA/LC} \]

- Indirect and dot-ELISAs were used to detect the binding of the VH/VH derived from different recombinant phagemid-transformed HB2151 E. coli clones. For indirect ELISA, 1 μg of purified recombinant full-length BoTxA/LC or BSA, which served as an antigen control, in 100 μl of carbonate-bicarbonate buffer, pH 9.6, was added to each well of an ELISA plate. The antigen-coated wells were blocked with 1% BSA in phosphate-buffered saline and were then incubated with individual HB2151 E. coli lysates. After washing, the amount of bound VH/VH in each well was detected using mouse anti-E Tag antibody, goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (Southern Biotech), and ABTS substrate. The OD of the content of each well was determined at 405 nm against a blank (wells filled with phosphate-buffered saline instead of VH/VH-containing E. coli lysate). A well filled with a lystate of normal HB2151 E. coli instead of a VH/VH-containing E. coli lystate served as a background control. The VH/VH in the E. coli lystate that yielded an OD two times higher than that of the BSA control was selected. For the dot-ELISA, 3-μl aliquots of the BoTxA/LC (~1 μg) were dotted on separated nitrocellulose membranes (NCS; Hybond ECL, Amersham Biosciences). The antigen-dotted membrane was immersed into a solution of 3% BSA in washing buffer for 1 h, washed with washing buffer, and cut into squares with one antigen dot on each square. The NC pieces were incubated with individual VH/VH-containing HB2151 E. coli lysates. A lystate of normal HB2151 E. coli was used as a negative VH/VH control. After washing, the bound VH/VH on each NC piece was detected by mouse anti-E Tag antibody, goat anti-mouse immunoglobulin-AP conjugate, and a chromogenic substrate. E. coli lysates that contained VH/VH (positive) turned purplish-blue and thus could be differentiated from the negative VH/VH control.

\[ \text{Characterization of the VH/VH} \]

- The restriction fragment length polymorphism (RFLP) of the VH/VH sequences amplified from the selected HB2151 E. coli clones was determined using MvaI restriction endonuclease (29). The nucleotide sequences were also determined by DNA sequencing. The deduced amino acid sequences of all clones were then aligned with the cameld VH/VH and human VH sequences of the International Immunogenetics Information System database. The immunoglobulin frameworks and the CDRs of the individual VH/VH sequences were predicted using the International Immunogenetics Information System server.

\[ \text{Neutralization of BoTxA/LC by VH/VH} \]

- To screen for the transformed HB2151 E. coli clones that could express VH/VH with BoTxA/LC-neutralizing activity, the VH/VH of each selected E. coli clone was incubated with BoTxA/LC at a molar VH/VH:BoTxA ratio of 100:1 at 37°C for 1 h. A lystate of neuroblastoma cells was added to the toxin-antibody mixture and was further incubated at 37°C for 24 h. The mixture was

(28) Each primer sequence was flanked with SfiI and NotI endonuclease restriction sites at the 5’ and 3’ ends, respectively. During PCR, the immunoglobulin forward primers annealed to the 5’ ends of the VH/VH exons and the reverse primers to the 3’ ends of the JH exons of all immunoglobulin gene families. The amplified products were verified by agarose gel electrophoresis, and the VH/VH DNA amplicons of ~400 bp were extracted from the agarose gel slabs and purified using GeneClean™ II kit (MP Medicals). The purified DNA was digested with SfiI and NotI endonucleases and ligated into a pCANTAB5E phagemid vector™ (Amersham Biosciences) precut with the same enzymes, and the ligation mixture was introduced into competent TG1 E. coli cells by the electroporation method. An aliquot of the transformation mixture was plated onto a sodium, bacto tryptone, ampicillin, and glucose agar plate, and the plate was incubated at 37°C overnight to estimate the transformation efficiency. The VH/VH-displaying phage particles were rescued from the remaining portion of the transformation mixture by co-infecting the phagemid-transformed E. coli with M13K07 helper phages, and the titer of the rescued phage repertoire was determined (28).

Selection and Production of BoTxA/LC-specific VH/VH—The recombinant BoTxA/LC was used as an antigen in a phage-antibody-panning process to select phage clones displaying VH/VH that bound to the protein (28). The protein (5 μg) was immobilized on the surface of each well of a microtiter ELISA plate (Costar, Corning). The so-constructed phage display VH/VH library (~5 × 1010 phage particles) was added into the antigen-coated wells and kept at 25°C for 1 h. Unbound phage particles were removed by successive washing with a washing buffer (0.15 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20; washing buffer). Bound phage particles were directly supplemented with exponential phase grown HB2151 E. coli cells. The phage transformed HB2151 E. coli cells were grown on LB-AG (LB-A containing 2% of glucose) selective agar plates. For indirect ELISA, 1 g of purified recombinant full-length BoTxA/LC or BSA, which served as an antigen control, in 100 μl of carbonate-bicarbonate buffer, pH 9.6, was added to each well of an ELISA plate. The antigen-coated wells were blocked with 1% BSA in phosphate-buffered saline and were then incubated with individual HB2151 E. coli lysates. After washing, the amount of bound VH/VH in each well was detected using mouse anti-E Tag antibody, goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (Southern Biotech), and ABTS substrate. The OD of the content of each well was determined at 405 nm against a blank (wells filled with phosphate-buffered saline instead of VH/VH-containing E. coli lysate). A well filled with a lystate of normal HB2151 E. coli instead of a VH/VH-containing E. coli lystate served as a background control. The VH/VH in the E. coli lystate that yielded an OD two times higher than that of the BSA control was selected. For the dot-ELISA, 3-μl aliquots of the BoTxA/LC (~1 μg) were dotted on separated nitrocellulose membranes (NCS; Hybond ECL, Amersham Biosciences). The antigen-dotted membrane was immersed into a solution of 3% BSA in washing buffer for 1 h, washed with washing buffer, and cut into squares with one antigen dot on each square. The NC pieces were incubated with individual VH/VH-containing HB2151 E. coli lysates. A lystate of normal HB2151 E. coli was used as a negative VH/VH control. After washing, the bound VH/VH on each NC piece was detected by mouse anti-E Tag antibody, goat anti-mouse immunoglobulin-AP conjugate, and a chromogenic substrate. E. coli lysates that contained VH/VH (positive) turned purplish-blue and thus could be differentiated from the negative VH/VH control.

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subjected to SDS-PAGE and probed with rabbit polyclonal antibody to SNAP25 followed by goat anti-rabbit immunoglobulin-AP conjugate and substrate. The intensity of the SNAP25 protein band was quantified by densitometric analysis with AlphaDigiDocTM 1201 software (version 3.3.0, Alpha Innotech). Percent cleavage inhibition of SNAP25 by VH/VH was calculated: % inhibition = (intensity value of sample ÷ intensity value of 100% inhibition control) × 100. The neutralizing activity of the selected clone was confirmed by mixing the antibody with the toxin either at a molar ratio of 3:1 or as otherwise indicated and incubated at 37 °C for 1 h before subjecting to the fluorometric assay. A zinc-chelating agent, i.e. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, Sigma) was used for positive toxin inhibition control.

Affinity Measurement of the VH/VH—The affinity (dissociation constant, \( K_d \)) of the VH/VH was determined by using ELISA as described previously (30). Briefly, 10 nM of the VH/VH was mixed with various amounts of soluble BoTxA/LC, and the tubes containing the reaction mixtures were kept at 20 °C for 16 h. Free VH/VHH was detected by ELISA. The contents of each tube (100 μl) were transferred to a well of a microtiter plate previously coated with 1 μg of BoTxA/LC. After incubation, VH/VH captured by the immobilized toxin was detected by using mouse monoclonal anti-E Tag, goat anti-mouse IgG-horseradish peroxidase conjugate, and 3,3',5,5'-tetramethylbenzidine substrate (Zymed Laboratories Inc.), respectively. OD of the content of each well was determined at \( A_{405\text{ nm}} \) against a blank (well filled with diluent instead of VH/VH). \( K_d \) was then calculated from a Klotz plot by linear regression analysis.

Determination of the VH/VH Mimotope Sequence—The mimotopes of the VH/VH that gave the highest BoTxA/LC-neutralizing activity were determined by using a phage display 12-mer peptide library (Ph.D.-12™ Phage Display Peptide Library, New England Biolabs) as described previously (28). VH/VH (1 μg) was immobilized on the surface of each ELISA well at 4 °C overnight. After washing the well with Tris-buffered saline, pH 7.5, containing 0.1% Tween 20, the empty sites in the well were blocked by adding 200 μl of 0.5% BSA in Tris-buffered saline for 1 h. After washing to remove the blocking agent, 100 μl of the phage display 12-mer peptide library (diluted 1:10 with Tris-buffered saline, pH 7.5, containing 0.1% Tween 20), which contained \( \sim 1.5 \times 10^{11} \) plaque-forming units were added to the well, and the plate was kept at 25 °C for 1 h. Unbound phages were washed away with Tris-buffered saline, pH 7.5, containing 0.1% Tween 20, the bound phages were eluted with 0.2 M glycine-HCl solution, and the pH of the solution was immediately brought to neutral by adding 2 M Tris base. The eluted phages were allowed to amplify by infecting 20 ml of log phase-grown ER2738 E. coli (OD at \( A_{600\text{ nm}} \) ~ 0.3). After removing the bacterial cells by centrifugation at 12,000 × g, the phages in the supernatant were concentrated by adding polyethylene glycol/NaCl. The phages in the pellet were resuspended in phosphate-buffered saline and used in the next round of bio-panning. Three rounds of bio-panning were performed. The eluted phages from the third round of bio-panning were used to infect the ER2738 E. coli in top agarose overlaid on the LB agar containing isopropyl-\( \beta \)-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactopyranoside (X-gal). Well isolated blue plaques were randomly picked, inoculated individually in 1 ml of 1:100 overnight diluted ER2738 E. coli culture in LB broth, and incubated at 37 °C with shaking for 4 h. After removing the bacterial cells by centrifugation as above, the DNA of the individual phage clones was extracted from the culture supernatant using the phenol/chloroform method. The DNA of each phage clone was sequenced, and the mimotope peptides were...
deduced from the DNA sequences. The VH/V_{H}H mimotope sequences were subsequently aligned with the amino acid sequence of the type A botulinum neurotoxin of the GenBank database by ClustalW.

Homology Modeling and Molecular Docking—The interface binding between BoTXa/LC and the selected VH/V_{H}H was determined by using ZDOCK and RDOCK modules on Discovery Studio 2.1 (Accelrys Inc.). The BoTXa/LC model was derived from Protein Data Bank code 3BTA. The VH/V_{H}H model was built from Swiss-Model Web site using 2P49 (chain B) as a template. The VH/V_{H}H model was validated by using the Procheck program (31). BoTXa/LC and VH/V_{H}H proteins were added to a CHARMM polar heavy force field. The ZDOCK module was used to perform rigid body docking of the BoTXa/LC and VH/V_{H}H. The best hit cluster of pose from ZDOCK module was docked by a RDOCK module, which is a CHARMM-based energy minimization procedure. The visualized binding interface was analyzed by PyMOL (The PyMOL Molecular Graphics System, 2002).

**RESULTS**

**Recombinant BoTXa/LC**—A full-length recombinant light chain of type A botulinum neurotoxin (BoTXa/LC), ~50 kDa, was successfully produced and purified. The nucleotide sequence of the BoTXa/LC was verified by DNA sequencing, and the deduced amino acid sequence showed 100% homology to the sequence deposited in the GenBank database (accession number M30196). The produced recombinant BoTXa/LC had endopeptidase activity for SNAP25 as determined by Western blot analysis (Fig. 1), which digested SNAP25 in the neuroblastoma cell lysate treated with the BoTXa/LC, indicating that the SNARE protein was completely degraded by the toxin, whereas the untreated cell lysate (negative cleavage control) showed the SNAP25 band at ~27 kDa. The lysate treated with trypsin (positive cleavage control) showed no SNAP25 band.

From fluorescent assay, the kinetic parameters of the recombinant BoTXa/LC including $k_{cat}$ and $K_{m}$ were 2.03 s$^{-1}$ and 109.7 ± 32.38 μM, respectively.

**Mouse Polyclonal Anti-BoTXa/LC Antibody**—The highest reciprocal titer of immune serum against BoTXa/LC was 12,800. This serum was kept at 20 °C until use as antibody control in the toxin neutralization test below.

**VH/V_{H}H Library of the Nonimmune C. dromedaries**—By using the cDNA prepared from the total RNA extracted from ~3 × 10^7 peripheral blood mononuclear cells of the naïve C. dromedarius as templates, the VH/V_{H}H gene segments could
be amplified by nine combinations of the forward and reverse human immunoglobulin-specific primers: i.e. VH1a + JH1245, VH1a + JH3, VH1c + JH1245, VH1d + JH3, VH3a + JH1245, VH3a + JH3, VH3b + JH1245, and VH3b + JH3 (Fig. 2). The VH/VH\text{a} \text{H} ampiclons were pooled and used in the library construction. The size of the VH/VH\text{a} \text{H} library was 1.6 \times 10^5. A total of \sim 4 \times 10^4 VH/VH\text{a} \text{H}-displaying mature phage particles were obtained after co-infecting the phagemid transfected TG1 \textit{E. coli} with M13K07 helper phages in the phage rescuing process.

Selection of Phage Clones Displaying VH/VH\text{a} \text{H} That Bound to BoTxA/LC—After a single round of phage bio-panning with immobilized recombinant BoTxA/LC, 60 transformed HB2151 \textit{E. coli} were randomly picked from the selective agar plate and screened for the presence of the VH/VH\text{a} \text{H}-coding sequences by PCR using the R1 and R2 primers. It was found that 39 of 60 clones (65%) were positive for VH/VH\text{a} \text{H} ampiclons. The lysates of these \textit{E. coli} clones grown under isopropyl-\beta-D-thiogalactopyranoside induction were subjected to Western blotting, and 28 clones (46.6%) could express the VH/VH\text{a} \text{H} proteins seen as bands at \sim 15–20 kDa (Fig. 3). The VH/VH\text{a} \text{H} produced by the 28 clones were tested for their antigenic specificity against homologous antigen, i.e. recombinant BoTxA/LC, by indirect ELISA and VH/VH\text{a} \text{H} from 10 clones (16.6%), namely clones 3, 5, 11, 15, 17, 20, 21, 22, 26, and 27 gave significant ELISA signals to the immobilized BoTxA/LC above the background BSA control (Fig. 4A). Binding of the VH/VH\text{a} \text{H} of the 10 clones to BoTxA/LC was confirmed by dot-ELISA (Fig. 4B). The VH/VH\text{a} \text{H} did not bind to recombinant light chain of type B botulinum neurotoxin, zinc-dependent metalloprotease lethal factor of anthrax toxin, neuraminidase of H5N1 virus, and lysate of the SK-N-MC neuroblastoma human cell line (data not shown).

Fig. 5 shows the RFLP patterns of the VH/VH\text{a} \text{H} nucleotide sequences of the 10 clones, which revealed completely different banding patterns (Fig. 5). Two clones were VH\text{a} \text{H} (designated as VH\text{a} \text{H}17 and VH\text{a} \text{H}21); the remaining eight clones were conventional VH (designated as VH3, VH5, VH11, VH15, VH20, VH22, VH26, and VH27). The hallmark tetrad amino acids of camelid VH\text{a} \text{H} located at FR2 are indicated by \textit{boldface} and \textit{type} \textit{face}. An asterisk identical amino acids; a colon indicates conserved amino acid substitution; and a period indicates a semiconserved amino acid substitution.
remaining eight clones lacked the tetrad but, instead, had the features of FR2 of conventional VH of mammals including human, mouse, and camelid, i.e. Val42-Gly49-Leu50-Trp52. Previous data have demonstrated that the highest similarity between camel VHH and human VH was 82.6% (32). In our data, the deduced amino acid sequences of the amplified camel VH using human primers showed 73.68–100% homology (average 91.26%) with the human VH, whereas the VHH using human primers showed 58.82–91.39% homology (average 76.13%) with the human VH (Table 1). A marked difference between the VHH and human VH was found at the tetrad amino acids of FR2, which determines the hydrophilicity of the former and hydrophobicity at the variable light chain-binding site of the latter.

### Neutralization Tests

-VH3, VH15, VH20, VH22, VH26, and VH27 and VHH17 and VHH21 were screened for their ability to neutralize the enzymatic activity of the recombinant BoTxA/LC. Fig. 7 shows the results of the Western blot assay testing the neutralizing activities of the VH/VHH of the eight clones. Unfortunately, clones 5 and 11 lost their ability to express VH. On an equal weight basis, VH15 and VH22 and VHH17 efficiently inhibited (83.33, 75.0, and 83.33%) the hydrolysis of SNAP25 by 10 nM BoTxA/LC, as shown by the presence of intensely stained SNAP25 bands in Fig. 7 (lanes 4, 8, and 5, respectively) and the densitometer readouts at the bottom of Fig. 7. VH20, VH26, and VH27 had less BoTxA/LC inhibitory activity (54.17, 41.67, and 50.0%) than the former three clones (lanes 6, 9, and 10). The VH of clone 3 showed trace toxin-inhibitory activity (29.17%; Fig. 7, lane 3), whereas

### TABLE 1

Percent amino acid homology of the VH/VHH sequences from the 10 transformed E. coli clones with the closest human V region frameworks

| VH/VHH clone number | Closest human V region | Amino acid homology with human FRs |
|---------------------|------------------------|-----------------------------------|
|                     |                        | FR1 | FR2 | FR3 |
| VH3                 | AB879486 IGHV3–23’04  | 100.00 | 88.24 | 89.19 |
| VH5                 | Z17392 IGHV3–74’02    | 100.00 | 80.00 | 89.19 |
| VH11                | X56368 IGHV5–51’03    | 87.50 | 100.00 | 91.89 |
| VH15                | U29481 IGHV3–23’03    | 95.83 | 100.00 | 86.84 |
| VHH17               | Z27504 IGHV3–66’02    | 91.30 | 60.00 | 81.58 |
| VHH20               | Z27504 IGHV3–66’02    | 95.83 | 93.75 | 83.78 |
| VHH21               | AB879484 IGHV3–h’01(P)| 90.91 | 58.82 | 74.19 |
| VH22                | U29481 IGHV3–23’03    | 95.83 | 94.12 | 86.49 |
| VH26                | X92239 IGHV4–55’09(P)| 75.00 | 85.71 | 73.68 |
| VH27                | X92288 IGHV3–7’02     | 100.00 | 100.00 | 97.37 |

**FIGURE 7.** Results of the Western blot analysis for demonstrating the BoTxA/LC-neutralizing effect of camel VH/VHH. VH3, VH15, VH22 (lanes 4 and 8), and VHH17 (lane 5) efficiently inhibited the hydrolysis of SNAP25 by 10 nM BoTxA/LC, whereas VH20, VH26, and VH27 (lanes 6, 9, and 10) showed less toxin inhibitory activity than the former three clones. VH3 (lane 3) had trace toxin-inhibitory activity, whereas VHH21 (lane 7) had the lowest toxin inhibitory activity. Lane 1, reaction mixture of a neuroblastoma cell lysate with BoTxA/LC (negative cleavage inhibition control); lane 2, a cell lysate mixed with buffer without BoTxA/LC (100% cleavage inhibition control). M, precast protein molecular weight marker. The intensity of the SNAP25 protein band was quantified by using a densitometer. The % cleavage inhibition of SNAP25 by the VH/VHH was shown in a bar graph below. *, % cleavage inhibition of VH15, VH22, and VHH17.

**FIGURE 8.** Results of a fluorescence-based assay for detecting the BoTxA/LC inhibitory activity of the VH/VHH. VHH17 (bar 4), VH22 (bar 3), and VH15 (bar 2) exhibited 73, 64, and 58% inhibition of hydrolysis of SNAP25 fluorogenic substrate by the BoTxA/LC, respectively. Reaction mixtures of the fluorogenic substrate with 15 μM of BoTxA/LC (bar 1) and buffer without BoTxA/LC (Neg) served as positive and negative enzymatic hydrolysis controls, respectively. PTEN (5 μM), the zinc-chelating agent, served as positive inhibition control. Bar 5, BoTxA/LC treated with 1:1,000 mouse polyclonal antibodies. Percent inhibition of SNAP25 was calculated from % hydrolysis of SNAP25 = (Vtest / VBoTxA/LC control) × 100. Percent inhibition of hydrolysis of SNAP25 was % inhibition = 100 − % hydrolysis of SNAP25.
VHH21 showed the lowest toxin-inhibitory activity (8.33%) (Fig. 7, lane 7). VH15, VH22, and VHH17 were tested further for their toxin neutralization by using a fluorogenic substrate. The results of the fluorescent-based assay showed that VHH17 exhibited the highest % inhibition of hydrolysis of SNAP25 (73% inhibition), whereas VH15 and VH22 exerted 59 and 64% inhibition, respectively (Fig. 8). The mouse polyclonal antibody to BoTxA/LC at dilution 1:1,000 did not have any BoTxA/LC-neutralizing activity. VHH17 inhibited the endopeptidase activity of the BoTxA/LC in a dose-dependent manner. At 4.5 and 10 μM (contained 13.5 × 10^13 and 30.1 × 10^13 molecules, respectively), the VHH17 exhibited 73 and 92% inhibition of hydrolysis of SNAP25 by 1.5 μM of BoTxA/LC (contained 4.5 × 10^13 molecules), respectively.

**Affinity of VHH**—The dissociation constant (Kₐ) of the VHH17 derived from the Klotz plot was 11.6 nM.

**Mimotope of VHH**—10 randomly picked blue plaques were separately added to log phase-grown ER2738 E. coli. The DNA of each amplified 12-mer peptide display phage clone was extracted and sequenced, and the amino acids were deduced. The 10-amino acid mimotopes of the VHH17 (designated M1–M10) were aligned with the reference sequence of the BoTxA/LC of the GenBank™ database (accession number AAA23262.1). It was found that 7 of 10 sequences, i.e. M1 and M3–M8, matched with the amino acid residues 194–206 of BoTxA/LC (Fig. 9).

**Molecular Docking**—Interface binding of BoTxA/LC and VHH17 is shown in Fig. 10. The best docking result (the lowest docking energy of −20.39661 kcal/mol), which was obtained from using the ZDOCK and RDOCK modules, showed that the CDR3 of the VHH17 bound at the BoTxA/LC enzymatic groove.

**DISCUSSION**

Because of their small size (~15–20 kDa) and long CDR3 sequences, which can be readily extended into the enzymatic cleft, the variable domains of camelid heavy chain antibodies (VHH domain) have been shown to be potent enzyme inhibitors (22, 23, 33, 34). VHH-conjugated with β-lactamase has been shown to exert anti-cancer activity (35). VHH-conjugated with human trypanolytic factor has been effective in the treatment of experimental African trypanosomiasis (36). Nevertheless, data in the literature are limited concerning the use of the VHH as a therapeutic agent for intoxications caused by bacterial toxic enzymes such as botulism. Current botulism immunotherapy based on animal-derived antitoxin is facing several obstacles. The polyclonal antibodies neutralize only circulating extracellular BoTx but are ineffective for the internalized toxin. Besides, the animal proteins induce anti-isotype/anti-idiotype responses in the antitoxin-treated human patients (6). Antibody that specifically interferes with the enzymatic activity of the botulinum toxin has never been available.

Recently, camel VHH (Nano-body®, Ablynx) specific to von
Willebrand factor was tested in a phase I clinical trial in humans and was found to be relatively safe without any untoward reactions in the recipients (37). Most of the antigen-specific V_{14}H antibodies so far reported in the literature were derived from immunized camels. In this study, however, the V_{14}H that bound to and neutralized the enzymatic activity of the type A botulinum neurotoxin was produced by using a phage clone selected from a display antibody library constructed from immunoglobulin genes of an 8-month-old naïve Arabian camel. Degenerate oligonucleotide primers specific to 14 VH (forward) and three JH (reverse) of human gene families were used. From the 42 forward and reverse primer combinations, only nine combinations yielded productive VH/V_{14}H PCR amplicons. These were combinations of degenerate forward VH1a, VH1c, and VH1d and VH3 with the reverse JH1245, and JH3 primers (Fig. 2). Previous data have shown that the genes of the VH3 family are shared among humans and camels (32). Our findings not only confirm the notion on VH3 but also add more information on the similarity of the VH1 gene family among the two species. Although the phage VH/V_{14}H library was constructed by using only nine PCR amplicons, phage clones displaying VH/V_{14}H specific to BoTxA/LC could nevertheless be appreciably selected from the library, implying a high diversity of the antibody gene repertoire in the constructed library. The diversity was confirmed by finding completely different DNA banding (RFLP) patterns after Mval digestion of the DNA segments of the 10 randomly selected phage clones (Fig. 5) and also by nucleotide sequencing (Fig. 6).

Recombinant BoTxA/LC with inherent zinc metalloproteinase was successfully produced. The recombinant protein cleaved SNAP25 in the presence of zinc, and the activity was abrogated in the reaction mixture containing a zinc-chelating agent, i.e. TPEN (Fig. 8). Among the 10 randomly selected phage clones derived from a single round of phage bio-panning using the recombinant BoTxA/LC as antigen, eight clones were conventional VH, and two clones (20%) were V_{14}H, according to the tetrad hallmarks of the FR2 sequences. Most reports in the literature performed multiple rounds of bio-panning for selecting antigen-specific phage clones. However, a single round of bio-panning was done in this study, as it has been found that multiple rounds of panning often yielded ineffective phage clones, i.e. positive binding to the antigen but a lack of antibody coding sequences or an inability to express the antibody (28). Although selected phage clones could produce VH/V_{14}H that exerted BoTxA/LC-neutralizing activity, the V_{14}H17 showed the best toxin inhibitory activity. The V_{14}H17 that was derived from the naïve library had affinity to the BoTxA/LC in the nanomolar range (11.6 nM), which is, more or less, similar to the antibodies used successfully in immunotherapeutic applications (affinities ranged from 0.08 to 32 nM). Thus, V_{14}H with high affinity to the targets could be isolated from the naïve library (39, 40).

The mouse polyclonal antibody raised against the recombinant BoTxA/LC did not have any BoTxA/LC-neutralizing activity. It was possible that either the BoTxA/LC enzyme crevices were inaccessible by the epitope-binding fragments of the conventional antibody (33) or the cleft molecular structures are not immunogenic (41).

Because the peptide substrate used in the fluorescent assay of this study was designed to clarify the cleavage motif of SNAP25, the inhibition of the BoTxA/LC-mediated substrate cleavage by the V_{14}H17 is likely to involve the toxin active site. A search of V_{14}H17 mimotopes using the 12-mer phage display peptide library revealed that the mimotopes were clustered at amino acids 194–206 of the botulinum type A light chain, which is located near the S1 subite of the catalytic cleft of the toxin (42). Molecular docking results showed that the CDR3 of the V_{14}H17 bound and inserted into the enzymatic groove of the toxin, which were confirmed to the mimotope results. The data of this study indicate that it should be worthwhile developing the V_{14}H17 further into a cell-penetrating version for specifically neutralizing the catalytic activity of the toxin that has gained cellular entry. This would be a novel in vivo immunotherapeutic strategy for botulism.

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