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SPECIFICITY AND BINDING AFFINITY OF AN ANTI-CROTOXIN COMBINATORIAL ANTIBODY SELECTED FROM A PHAGE-DISPLAYED LIBRARY

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Abstract—A crotoxin-specific, monoclonal, high-affinity, single-chain antibody variable region (scFv) was generated by combinatorial methods using Pharmacia's Recombinant Phage Antibody System. A high-affinity clone, designated A10G, was selected, and its DNA sequence was determined. Protein A10G showed high reaction specificity, with only the closely related rattlesnake neurotoxins, concolor toxin and Mojave toxin, showing cross-reactivity out of eleven group II phospholipase A_2s (PLA_2s) screened. No group I PLA_2s cross-reacted in enzyme-linked immunosorbent assays. The gene coding for A10G was subcloned into an expression vector, and the resulting expressed nonfusion protein, designated A10GPE, was renatured and purified to apparent homogeneity. Dissociation constants of A10G with intact crotoxin and crotoxin basic subunit were determined to be 7×10^{-10} and 6.8×10^{-9} M, respectively. When A10GPE was preincubated with either the basic subunit or intact crotoxin at molar ratios of up to 5:1, no inhibition of phospholipase activity was observed. Expressed protein, however, could partially neutralize the lethality of Mojave toxin, a crotoxin homolog, in mice.

Key words: combinatorial antibody; antibody DNA sequence; phage panning; protein expression; crotoxin; snake venom; neurotoxicity

Immune passive therapy is the treatment of choice for snakebite victims. This practice requires production of high-titer antisera from animals, which may be difficult to generate with some snake venoms containing high concentrations of potent neurotoxins. In these circumstances, antisera spiked with neutralizing mAbs† can be used to enhance the neutralizing ability of antivenoms [1]. While effective under laboratory conditions, production of large amounts of mAbs for therapeutic use is not presently economically feasible. Combinatorial antibody generation using molecular cloning techniques is an alternative to the production of mAbs using traditional hybridoma technology. We describe here the utility of this approach in the preparation of a crotoxin-specific mouse scFv antibody.

Crotoxin is a heterodimeric, protein neurotoxin [2, 3] from the venom of the South American rattlesnake *Crotalus durissus terrificus* [4, 5] and is representative of all rattlesnake neurotoxins. Its basic subunit is an active, group II PLA_2 and has an i.v. LD_{50} of ≈0.5 μg/g in mice, which is about one-tenth as toxic as the intact toxin.

Using the combinatorial approach to generate antibody libraries [6, 7] provides a means of capturing the diversity of the antibody repertoire. Expressed scFv proteins may be displayed on the surface of filamentous phage as functional, fusion proteins with coat protein III (pIII) [8, 9], which provides a useful handle of selection [10–12] using the appropriate binding antigen. Because of the physical link between the Ab gene and the expressed fusion protein, coding regions for scFv proteins can be sequenced easily, facilitating studies on antibody variable domain-related gene structure and antibody function relationships, as well as antigen–antibody interactions and antibody mutagenesis. Once selected, subcloning and expression of nonfusion scFv proteins by *Escherichia coli* permit large-scale production of selected antibodies, enabling their use in research and clinical applications [13, 14].

This paper describes the construction of a combinatorial antibody library from crotoxin-binding enriched mouse spleen B cells. From this library a single-chain V_H and V_L fragment combinatorial antibody was selected, which displays high specificity and affinity for crotoxin.

MATERIALS AND METHODS

Materials

Crotoxin was purified [15] from lyophilized crude venom of *C. d. terrificus* supplied by the Butantan Institute, Sao Paulo, Brazil, unless noted otherwise. TiterMax adjuvant, transglutaminase, and β-bungarotoxin were purchased from the Sigma Chemical Co. (St. Louis, MO). Air-dried crude venom of *C. d. collilineatus* was obtained as previously described [16]. Porcine pancreatic phospholipase A_2, transferrin, creatinase, and neuraminidase were purchased from Boehring-Mannheim (Indianapolis, IN). Other toxins used were purified as described [17], except for myotoxin II from *Bothrops asper* [18]; myotoxin III from *B. asper* [19]; CbIα, CbIβ, and CbII (all are PLA_2 active neurotoxins) from *Pseudo-*

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† Abbreviations: CDR, complementarity-determining region; Fv, antibody variable region; mAb, monoclonal antibody; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; pfu, plaque forming unit(s); PLA_2, phospholipase A_2; scFv, single-chain Fv and V_L and V_H, light and heavy chain variable regions.

cerastes fieldi [20]; and caudoxin from *Bitis caudalis**. Restriction and molecular cloning enzymes were purchased from Promega (Madison, WI). All other chemicals unless otherwise specified were purchased from Sigma and were the highest purity available.

Protein concentration determinations

Most toxin concentrations were determined by weighing freeze-dried material recovered after extensive dialysis against deionized water. Crotoxin and related rattlesnake toxin concentrations were determined by absorbance at 280 nm using reported molar extinction coefficients [21]. Non-toxin protein concentrations were determined by BCA assays according to instructions provided by Pierce (Rockford, IL).

Immunization

Sixteen 18–22 g BALB/C male mice were immunized by injecting 100 μL of the emulsion (equal volumes of crotoxin basic subunit in H_2O and TiterMax adjuvant) containing 50 μg of antigen subcutaneously in four sites. Mice were boosted in 2 weeks with the same amount of basic subunit-TiterMax emulsion using the same procedure. Sera were collected weekly and assayed for antibody titer by ELISAs. Twelve days after the boost, eight animals with the highest anti-intact crotoxin antibody titer were injected intraperitoneally with 50 μg of the same antigen in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 140 mM NaCl at pH 7.4). Twenty micrograms of antigen in PBS was injected i.v. the next day via the tail vein. Spleens were harvested 3 days later.

Preparation of spleen cells

Spleens from immunized mice were aseptically removed, and residual fat and connective tissue were trimmed away and placed in petri dishes containing 25 mL Dulbecco's modified Eagle's medium with 3% fetal bovine serum (Gibco-BRL Life Technologies, Grand Island, NY). Spleen contents were expelled through one end of the organ by compression of the spleen and dispersed by gentle pipetting. Cells were washed twice by centrifugation at 400 *g* for 5 min and resuspended in the same medium at a final cell concentration of about 10^6 cells/mL.

Panning of crotoxin-reactive B cells using streptavidin-coated paramagnetic particles

Panning was carried out to select B cells expressing crotoxin-reactive antibody on their surface. Suspended spleen cells from six animals were incubated with 0.1 μM biotinylated intact crotoxin [1] in a 5–7% CO_2 incubator at 37° for 2 hr with occasional gentle swirling. Streptavidin-paramagnetic particles (1 mL) (Promega; binding capacity: 107 μg of biotinylated rabbit IgG/mL of particles) prewashed three times with the cell suspension medium were transferred to the cell suspension in a sterile test tube and incubated at room temperature for 1 hr with shaking. The B cell-biotinylated crotoxin–streptavidin-coated magnetic particle complexes formed were captured with a magnetic stand and washed three times with cell suspension medium.

* Ritchey D and Kaiser II, unpublished results.

Total RNA extraction and mRNA purification

Total RNA was prepared by directly extracting the captured B cell-crotoxin–streptavidin-coated magnetic particle complexes [22]. Messenger mRNA was purified from total RNA using the Poly A-Tract mRNA Isolation System from Promega.

Construction of phage antibody library and panning to select crotoxin binding scFv

The Recombinant Phage Antibody System from Pharmacia (Piscataway, NJ) was used to prepare a phage antibody library from the purified mRNA according to the manufacturer's instructions. *E. coli* XL1-Blue MRF′ electroporation competent cells (Stratagene, La Jolla, CA) were electroporated with antibody cDNA ligated to phagemid pCANTAB (Pharmacia) by a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA) at settings of 25 μF, 1.8 kV, and 200 ohms using cuvettes with a 0.1 cm electrode gap. Electroporation efficiency was 2×10^7 clones/μg phagemid DNA. Phage were rescued by adding M13KO7 helper phage to a mid-log-phase *E. coli* culture ($A^{1\ cm}_{600\ nm} = 0.5$, Gilford 240 spectrophotometer) at a concentration of 2.5×10^9 pfu/mL. Expressed antibodies (as fusion proteins to M13 phage coat protein III) were selected by incubating phage (concentration ~10^{10} pfu/mL) with 0.1 μM biotinylated intact crotoxin at room temperature for 30 min in a 5% nonfat milk (w/v)–PBS solution. Streptavidin paramagnetic particles (250 μL) prewashed three times with the same buffer were added, incubation was continued for 30 min, and the phage-biotinylated crotoxin–streptavidin paramagnetic particles were captured with a magnetic stand and washed five times with the same buffer. Bound phage were eluted by adding 0.5 mL of 100 mM triethylamine (pH 11.6) at room temperature and incubating for 5 min. Elution buffer containing the phage was neutralized with 50 μL of 2 M Tris–HCl (pH 8.0) and used to infect *E. coli* cells. Panning was repeated several times until positive clones were sufficiently enriched to give significant ELISA readings. After the final round of panning, phage samples from 94 single randomly picked colonies of *E. coli* cells were used for ELISAs. Six clones out of these 94 showed specific binding to intact crotoxin. One of them, designated A10G, was chosen for further analysis.

ELISA

ELISAs, using A10GPE protein, essentially followed the procedure of Francis *et al.* [23], which used intact crotoxin to coat the plates and goat anti-mouse IgG Fab Specific Peroxidase conjugate as the second antibody (Sigma, Catalog No. A-9917). ELISAs, using phage-displayed antibodies, were performed following Pharmacia's instructions. Briefly, ELISA plates were coated with intact crotoxin or other snake toxins, washed [23], blocked with 5% nonfat milk (w/v) in PBS at room temperature for 2 hr, and rinsed with wash buffer (0.5% Tween-20 in PBS). Phage dilutions were made in antibody dilution buffer (2% nonfat milk in PBS) and incubated in the wells for 30 min at room temperature, washed, and incubated for 2 hr with Pharmacia's Anti-M13 peroxidase conjugate, diluted 1:10,000 in antibody buffer. Color was developed as described [23].

Expression of A10GPE

Strategies for expression of A10G as a nonfusion protein, designated A10GPE, and sequences of oligonucle-

otide primers used for amplification are represented in Fig. 1. PCR methodology was used for subcloning of DNA fragment A10G. Oligonucleotide primers used in the PCR reactions were synthesized by Macromolecular Resources (Fort Collins, CO). Reaction conditions for amplification of DNA fragments with Taq polymerase (Promega) were amplification buffer containing 1.5 mM $MgCl_2$ (from Promega), a 400 μM concentration of each dNTP, 5.0 units of polymerase, 10 ng of template DNA, and 30 pmol of primer DNA. Reactions were incubated in a thermal cycler using the following PCR program: 30 cycles; 94° for 1 min; 55° for 2 min; 72° for 2 min. Following amplification, PCR products were purified on 1% low melting temperature agarose gel (Seaplaque, FMC), digested with restriction enzymes *BamH* I and *Nde* I, and cloned into *BamH* I–*Nde* I digested pET 11b expression plasmid (Novagen). Correct clones were identified by DNA sequencing. A10GPE protein was expressed in *E. coli* strain BL21 (DE3) by adding IPTG (2 mM) to a mid-log phase ($A^{1\,cm}_{600\,nm}$ = 0.5) culture (250 mL) in LB medium at 37°. *E. coli* cells were harvested after 3 hr by centrifugation, and proteins were extracted and separated into soluble and insoluble fractions according to Novagen's instructions. The pellet (containing the expressed protein) was washed two times by resuspension in 25 mL of 50 mM Tris–HCl (pH 8.0), 2 mM EDTA and centrifugation at 12,000 *g* for 10 min.

DNA and protein sequencing

DNA was sequenced by the dideoxy method [24]. Primers used for phagemid DNA sequencing were purchased from Pharmacia. Novagen's T7 promoter and T7 terminator primers were used to sequence the subcloned A10GPE sequence in plasmid pET 11b. Seven amino acid residues of the N-terminus of the expressed A10GPE protein were sequenced as described [19].

Preparation of crotoxin affinity column

A crotoxin affinity column was prepared by coupling intact crotoxin to cyanogen bromide-activated Sepharose 4B (Sigma) at a concentration of 5 mg intact crotoxin/mL gel as described [25]. The packed column (1 × 20 cm) was washed alternately with 10× column volume of washing buffer [20 mM sodium phosphate (pH 7.3), 0.5 M NaCl, 0.02% sodium azide] and elution buffer [0.1 M glycine–HCl (pH 2.5)] four times before use.

Refolding and purification of A10GPE

Washed pellet recovered from the previous step was dissolved in 5 mL of 50 mM Tris–HCl (pH 8.0), 7.5 M guanidine hydrochloride, and 3 mM EDTA. Refolding of A10GPE was performed by dilution into 1 L of PBS. After standing at room temperature for about 48 hr, the protein solution was concentrated to 2.5 mL by ultrafiltration through a YM-10 membrane (Amicon). This solution was loaded onto a crotoxin affinity column and washed with the washing buffer until absorbance at 280 nm returned to baseline. Bound protein was eluted with elution buffer, and the pH of each fraction (4 mL) was raised immediately by the addition of 0.5 mL of 2 M Tris–HCl (pH 8.0). Recovered, bound protein fractions were pooled, dialyzed against PBS at 4°, concentrated by ultrafiltration, and stored at 4°.

Comparing epitopes of different scFv antibodies

Crotoxin-specific scFv antibodies were compared to determine whether they interacted with the same or different epitopes on crotoxin, using the method of Friguet *et al.* [26]. ELISA procedures were performed as above, with plates coated with 0.25 μg/mL of intact crotoxin. Two scFvs were mixed and tested in each assay. Each scFv concentration was the lowest that still generated

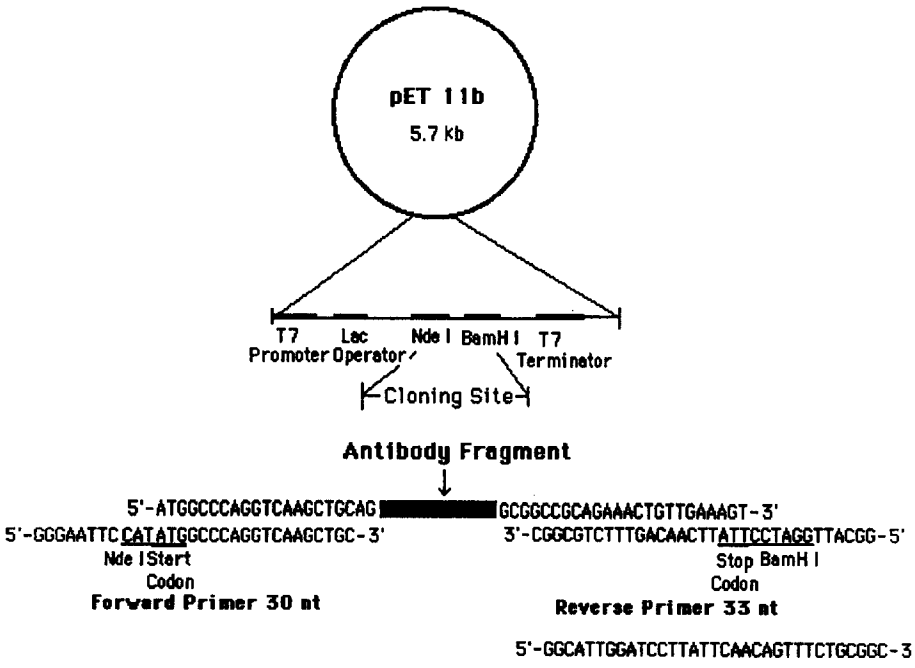

Fig. 1. Schematic representation of the expression plasmid, pET 11b, sequences of primers used for PCR-amplification of DNA fragment A10G, and the restriction sites used for insertion of the fragment. *Nde* I and *Bam*H I restriction sites on the antibody fragment are introduced by the forward and the reverse primers.

maximal ELISA response. ELISA signals obtained with a mixture of two scFvs were compared with the sum of individual responses for each scFv assayed alone. Whether two scFvs have the same or distinct epitopes was determined using an additivity index as described [26].

SDS–PAGE and western blots

SDS–PAGE on 12 and 15% gels were as described [5]. For western blots, proteins from SDS–PAGE were transferred to nitrocellulose filters using the MilliBlot-SDE Transfer System from Millipore. Visualization of transferred proteins was carried out as described by John and Kaiser [27].

Phospholipase and lethality assays

Phospholipase A_2 activity against egg yolk L-α-phosphatidylcholine followed the methods of Aird and Kaiser [5]. Lethality neutralization studies were as previously described [1, 28].

Measurement of binding dissociation constants

Dissociation constants (K_d) of antigen–antibody interactions were determined for both A10G and A10GPE under equilibrium conditions, in two or more independent experiments as described by Friguet *et al.* [29]. Under ELISA conditions, the A_{414} for binding of either A10G or A10GPE to intact crotoxin was within the linear portion of A_{414} versus A10G phage concentration curve (2×10^9–10^{10} pfu/mL), and A10GPE protein concentration curve (1 to 8.5×10^{-10} M). Dissociation constants were deduced from Scatchard plots for protein A10GPE. When A10G phage was used for dissociation constant determinations, crotoxin concentrations were at least ten times that of phage scFv concentrations. In such a situation, the simplified Klotz plot can be used, which is independent of phage scFv protein concentration [29]. For both A10G and A10GPE, the equilibrium in solution was reached within 15 min. It should be noted that pfu cannot be converted directly to scFv protein concentration because phagemid vector requires the use of helper phage to make phage. The pIII coat protein from the helper phage competes with pIII fusion protein for incorporation into the phage particle. However, an estimation can be made, since on average there is slightly less than one fusion protein per phage [30, 31]. Therefore, estimations of phage scFv protein concentrations were conducted as follows: the pfu of phage solutions were determined by plating infected *E. coli* cells on ampicillin-agarose plates. Counted pfu values were multiplied by two to determine the actual number of physical phage particles [32]. This value was divided by Avogadro's number to give an estimate of the fusion protein molar concentration.

Carboxymethylation of crotoxin

Crotoxin was reduced, carboxymethylated [33], dialyzed against deionized water, lyophilized, and stored at −20°.

Phosphatidylcholine binding assay

A10GPE was coated on an ELISA plate (1 μg/mL in PBS at 4° overnight), and its ability to bind radioactive 1-palmitoyl-2-[1-^{14}C]palmitoyl-L-α-phosphatidylcholine (sp. act. 550 μCi/mmol) in the presence and absence of a 100-fold excess of unlabeled phosphatidylcholine was determined. After 30 min at room temperature, the plates were washed, the individual well was cut from the plate, and the radioactivity of each well was determined by scintillation counting.

RESULTS

Immunization of mice

Sera from immunized animals were collected weekly and assayed for antibody titer by ELISA. Positive, significant responses were seen in all sixteen animals. All eight animals chosen for B cell preparation had ELISA absorbance readings above 0.5 after serum dilutions of 1:200,000.

Construction of scFv fusion library

The mouse scFv library was constructed using primers and phagemid vector from Pharmacia's Recombinant Phage Antibody System by both PCR and conventional cloning techniques. The first recombinant antibody library (size 10^6 independent clones) using mRNA from unselected B cells and expressed as a fusion protein to M13 phage pIII was unsuccessful. Of 785 independent colonies randomly selected from the unamplified library, none reacted specifically with intact crotoxin by ELISA. Five rounds of panning of the full-sized library yielded a positive clone that bound non-specifically to a variety of different proteins (data not shown). To improve the proportion of crotoxin positive clones, a procedure was devised to enrich crotoxin binding B cells by panning (see Materials and Methods), which permitted the selection of B lymphocyte cells bearing surface antibody specific for crotoxin. After crotoxin specific B cell selection, a second library containing 1.5×10^6 independent clones was constructed, which had a crotoxin specific positive clone ratio of 6/288. Some clones in the library still showed non-specific binding.

Phage panning and selection of positive clones

After three rounds of panning of the second scFv library, six crotoxin specific positive clones that gave the strongest ELISA signals were selected. Epitope comparison indicated that all six shared the same or a similar antigen epitope recognition site (data not shown). All of these clones may be siblings derived from one original clone. Clone A10G was chosen for further study.

DNA sequencing

Both strands of clone A10G DNA were sequenced. Codons were numbered according to Kabat *et al.* [34] and aligned against mouse germ-line DNA sequences. CDR regions were designated after alignment to other antibody sequences according to Ref. 34. The V_H sequence (Fig. 2A) indicated that germ-line DNA lg V2b-3 [35] gene fragment was used to code for its V segment. It belongs to mouse heavy chain subgroup II(c). Some antibodies recognizing Glu-Tyr-containing epitopes share the same heavy chain V segment [35]. Sequences for heavy chain D and J minigenes are indicated in Fig. 2A. Segment D of the heavy chain variable region showed considerable variation to the known mouse sequences. However, it closely resembled D-minigene DFL16.1 [34]. J_{H2} has high homology to A10G J segment [34]. V_L DNA sequence (Fig. 2B) was almost identical to a phosphatidylcholine binding antibody, CH32 (>99% homology, Ref. 36). It is known that CH32 V_L fragment is unmutated compared to germ-line DNA sequence [37].

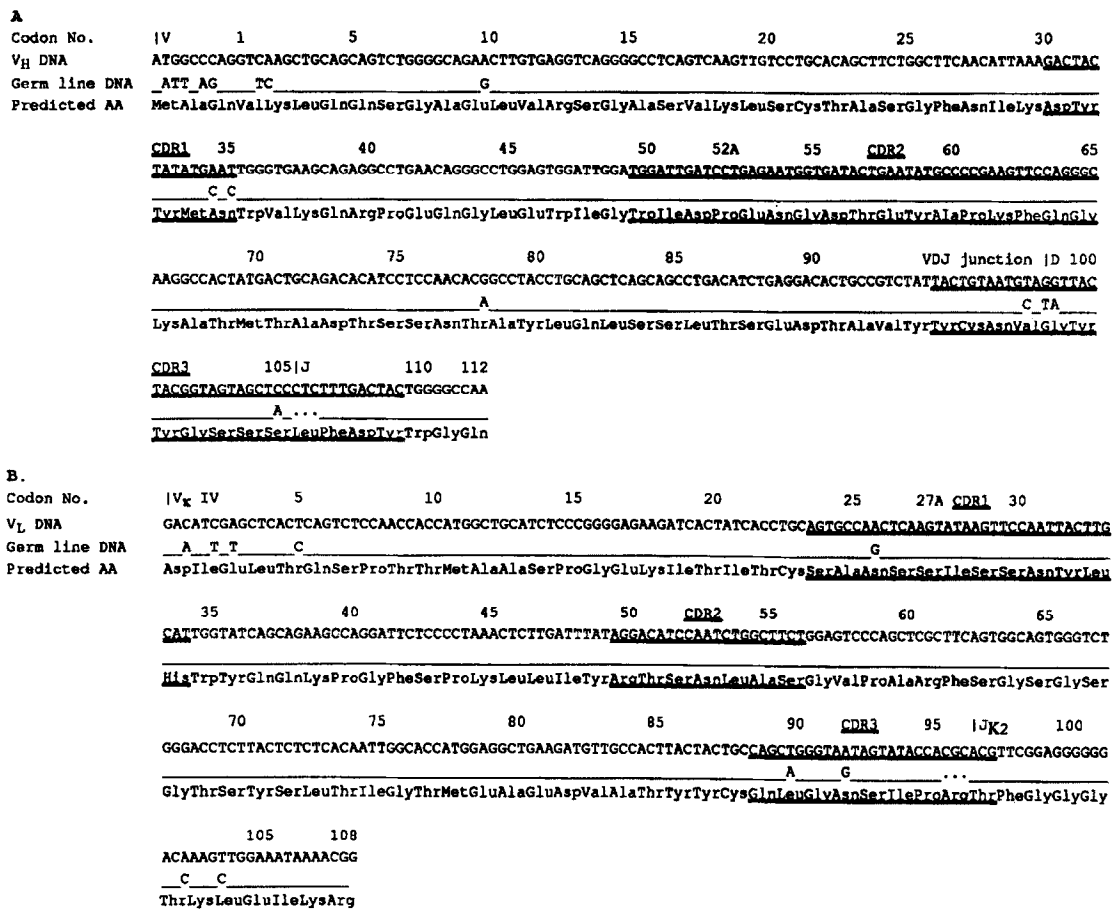

Fig. 2. Nucleotide and predicted amino acid sequences of the anti-crotoxin A10G heavy and light chain variable fragments. Nucleotide sequences are aligned against germ-line DNA sequences for comparison. Germ-line DNA positions that show identity positions with V_H and V_L sequences are indicated by a light line. Antibody CDR positions are indicated below the sequences by a heavy, solid line. Codons that were not assigned in the original germ-line DNA sequence are indicated by (•). (A) V_H; and (B) V_L. The nucleotide sequences reported in this paper have been submitted to the GenBank with accession numbers U14531 and U14532.

Expression, refolding, and purification of A10GPE protein

Proposed characterization studies of the antibody required us to clone, express, and purify A10G as a nonfusion protein. A10G gene was therefore subcloned into plasmid pET 11b, inserted into *E. coli* host strain BL21 (DE3), and expressed. DNA sequencing determined that the cloned DNA fragment was in the correct reading-frame and that it had the same sequence as A10G. Preliminary experiments with inclusion body pellets derived from small-scale cultures of the A10GPE scFv plasmid indicated that a 31-kDa protein was the main protein band present on SDS–PAGE gel and that the diluted and refolded material exhibited crotoxin specific binding (data not shown). Two other bacterial expression host strains, BL21(DE3)pLysS and BL21(DE3)pLysE, were also tested, but the expression levels were much lower (data not shown). In *E. coli* host strain BL21(DE3), we estimated that expressed protein accumulated to a level of at least 30% of the total bacterial protein 3 hr after induction (lane 2 in Fig. 3A).

A10GPE was solubilized and refolded as described in Materials and Methods. After 2 days of oxidation and subsequent concentration to 2 mL, the final purification by crotoxin affinity chromatography yielded 2 mg of active A10GPE in 2 mL PBS. Based on A_{280} profiles from affinity columns, we estimated recoveries to be about 50%. Recovered, bound protein fraction appeared to be more than 95% pure (lane 3 in Fig. 3A). SDS–PAGE showed the protein band under denaturing conditions to have an apparent molecular mass of 31 kDa (Fig. 3A). This apparent size was observed under both reducing (data not shown) and nonreducing (Fig. 3A) conditions. Western blots of similar SDS–PAGE gels gave a single band detected by goat anti-mouse antibody-alkaline phosphatase conjugate (Fig. 3B). Sequencing of the first seven amino acid N-terminal residues of this protein yielded Ala-Gln-Val-Lys-Leu-Gln-Gln, which compared with the predicted amino acid sequence from DNA of Met-Ala-Gln-Val-Lys-Leu-Gln-Gln. N-terminal Met was probably modified and hydrolyzed by *E. coli*. The calculated mass of the protein from the DNA sequence was 26,020 Da, compared with an M_r = 31,000 as determined by SDS–PAGE, indicating that the protein migrates more slowly than expected.

Cross-reactivity to various proteins

The cross-reactivity of scFv A10G to other structurally related or unrelated proteins was tested at phage concentrations of 1000× the minimum concentration for

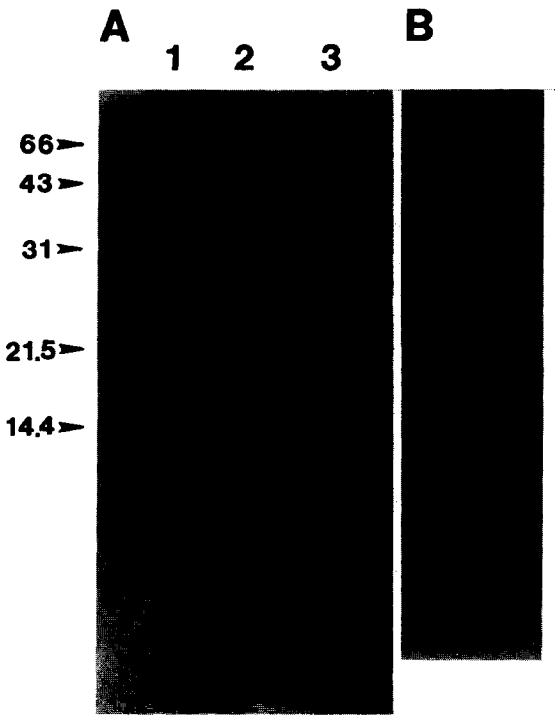

Fig. 3. SDS–PAGE and western blot analyses of expressed and purified A10GPE protein. (A) SDS–PAGE stained by Coomassie blue. Lane 1, molecular mass standards with their relative molecular masses shown at the left of the figure (kDa); lane 2, total protein from 30 μL *E. coli* BL21(DE3) culture, 3 hr after IPTG induction; and lane 3, protein A10GPE purified by affinity chromatography (10 μg). (B) Western blot of purified A10GPE (10 μg) detected by goat anti-mouse antibody conjugated to alkaline phosphatase.

intact crotoxin detection and to serial 5-fold dilutions. Of five structurally unrelated proteins tested (bovine serum albumin, transglutaminase, transferrin, creatinase, and neuraminidase), none cross-reacted with A10G. A10G did not bind to any of the eight toxic or nontoxic group I purified PLA_2s (porcine pancreatic PLA_2, taipoxin from *Oxyuranus scutellatus scutellatus* venom, textilotoxin from *Pseudonaja textilis* venom, β-bungarotoxin from *Bungarus multicinctus* venom, notexin, Notechis II-5, and scutoxin all from *Notechis scutatus scutatus* venom, and pseudexin (mixed isoforms) from *Pseudechis porphyriacus* venom). It also did not cross-react with a number of group II PLA_2s, including myotoxin II and III from *B. asper* venom; ammodytoxin A from *Vipera ammodytes ammodytes* venom; PLA_2 from *Crotalus atrox* venom; CbIα, CbIβ, and CbII, all from *Pseudocerastes fieldi* venom; and caudoxin from *Bitis caudalis* venom. A10G did not bind to crotoxin acidic subunit, even though the acidic subunit shares more than 50% protein sequence homology to the basic subunit. It binds strongly to intact crotoxin and its basic subunit, as well as other rattlesnake neurotoxins, such as Mojave toxin from *Crotalus scrutulatus scutulatus* and concolor toxin from *Crotalus viridus concolor,* which are known crotoxin homologs. Crotoxin and related rattlesnake neurotoxin basic subunits, as they are normally isolated, consist of multiple isoforms [16, 38, 39]. SDS–PAGE of either isolated basic subunits or crude venoms from these snakes give broad, strongly Coomassie blue stained bands with apparent molecular masses of about 14 kDA. With *C. d. collilineatus* venom, two distinct bands are observed on SDS–PAGE [16]. In results not shown, western blots of these gels generated staining intensities (using protein A10GPE as the primary detecting molecule) across the basic subunit band that correlated well with the intensity of protein staining. This suggests that the epitope recognized by protein A10GPE is conserved and found in all isoforms. Reduced and carboxymethylated crotoxin basic subunit did not bind to A10G, indicating that the synthetic antibody recognizes a conformational epitope.

Binding affinities of A10G and A10GPE to crotoxin

Dissociation constants for crotoxin and crotoxin-like proteins were determined using the indirect ELISA method [29]. Both A10G and A10GPE were found to be suitable for dissociation constant measurement because they satisfied two important criteria of the assay. (a) The ELISA response was linear in relation to the phage (2×10^9–10^{10} pfu/mL) or protein (1 to 8.5×10^{-10} M) concentrations. (b) Less than 15% of either phage or protein was bound to the plate over the range of concentrations used. Thus, the amount removed from solution would not affect significantly the equilibrium in the liquid phase. Scatchard analysis of protein A10GPE binding and a Klotz plot of A10G bindings at the indicated concentrations of intact crotoxin are shown in Fig. 4. K_d values of A10G and A10GPE with various crotoxin and crotoxin-like proteins are listed in Table 1. Both A10G and A10GPE bound strongly to intact crotoxin and A10G, to a lesser extent, to crotoxin basic subunit (Table 1), even though basic subunit was the original immunization antigen. Similar phenomena have been observed with monoclonal antibodies before [1]. This may be due to basic subunit aggregation. Both Mojave toxin and concolor toxin bound to A10G with similar affinities. These two rattlesnake neurotoxins are both heterodimeric proteins and are known to share high protein sequence identities to crotoxin [15, 40]. Dissociation constants of both A10G and A10GPE with the basic subunit of crotoxin and Mojave toxins were an order of magnitude lower than with intact crotoxin purified from venom of *C. d. terrificus* obtained from the Butantan Institute, Sao Paulo, Brazil. Intact crotoxin purified from venom of *C. d. terrificus* obtained from the Miami Serpentarium showed binding affinities similar to Mojave and concolor toxin.

Additional properties of A10G and A10GPE

Effects of scFv A10GPE on crotoxin PLA_2 activity were examined. A10GPE was preincubated with either intact or basic subunit of crotoxin at 37° for 30 min at molar ratios of 1:1 or 5:1 scFv:intact or basic subunit. No inhibition of phospholipase activity was observed. This indicates that the binding of this antibody to crotoxin was not directly on PLA_2 active or interfacial recognition sites; otherwise, the high affinity of A10GPE would presumably affect crotoxin PLA_2 activity.

Searching of the protein sequence database indicated that the V_L of A10G has high sequence identity with phosphatidylcholine-binding antibodies. When protein A10GPE was mixed with 1,2-dibutyryl-L-α-phosphatidylcholine (50–1000 μM), binding to intact crotoxin-coated ELISA plates was not affected (data not shown). Also, no substrate binding of ^{14}C-labeled phosphatidyl

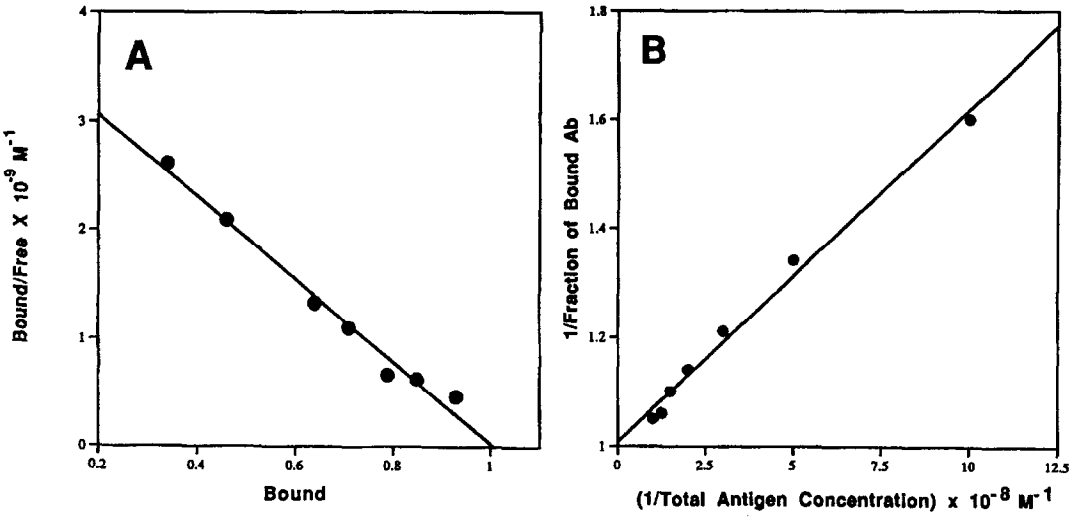

Fig. 4. (A) Scatchard plot of the binding of crotoxin to A10GPE. The free and bound antibody concentrations at equilibrium are related to the absorbance A measured in the ELISA. See Friguet *et al.* [29] for calculations. K_d was determined from the slope of the line. (B) Klotz plot of the binding of crotoxin to A10G measured by the ELISA. See Friguet *et al.* [29] for details on the calculation of the fraction of Ab bound. K_d was determined from the slope of the line.

choline was observed by intact crotoxin bound to ELISA plates at a micellular suspension equivalent of 10 μM. This was not too surprising considering that antigen binding entails contact with amino acid residues of both antibody subunits. The variable region of heavy chain of A10G, which is usually more important in epitope determination [41], does not show significant homology to phosphatidylcholine-binding antibodies.

Neutralization of lethality by A10GPE

Mojave toxin rather than crotoxin was used for lethality assays because it gave less variation in the experiment, and the entire sequence of Mojave toxin basic subunit is identical to one isoform of crotoxin basic subunit. Mice were injected using twice the i.v. LD_{50} dose of intact Mojave toxin in PBS containing 0.1% BSA. In sixteen animals, the average time-to-death was 2.2 ± 0.7 hr (standard deviation) after toxin injection alone. When the same amount of toxin was preincubated with A10GPE at an Ab:toxin molar ratio of 4:1, five out of sixteen mice survived beyond 24 hr. Of the eleven who expired, their time-to-death was 8.7 ± 1.9 hr. Compared with the 2.2 hr exhibited by the control group, this difference was significant ($P < 0.01$), as analyzed by a one-tailed Student's t-test with significance taken at $P < 0.05$. Therefore, binding of A10GPE to Mojave toxin caused in some cases delayed death and in others total neutralization of the toxicity.

Table 1. Dissociation constants of A10G and A10GPE against crotoxin and crotoxin-like proteins

	K_d (×10^{-9} M)	
	Antibody	
Proteins	A10G	A10GPE
Intact crotoxin (Butantan Institute)	0.7 ± 0.2	0.2 ± 0.1
Intact crotoxin (Miami Serpentarium)	5.6 ± 0.4	6.4 ± 0.5
Crotoxin basic subunit (Butantan Institute)	6.5, 7.1	ND*
Mojave toxin	7.4, 6.9	2.5, 3.2
Concolor Toxin	6.1, 5.8	4.0, 3.7

Values are means ± SD of three independent data sets. Where only duplicate data sets were determined, both values are shown.

* Not determined.

DISCUSSION

Antibodies from both humans and mice normally have antigen-binding K_d values between 1×10^{-7} and 5×10^{-10} M [42]. Antibodies with higher protein binding affinities are rare [43]. Thus, protein A10G has a relatively high affinity for crotoxin. There are some affinity differences of scFv A10G (also A10GPE) towards crotoxin purified from different sources. Proteins A10G and A10GPE consistently showed about 8- and 32-fold differences, respectively, which is significant. This difference may be caused by basic subunit isoform compositions within each toxin preparation. These isoforms differ from each other by only one or a few amino acid residues [38, 39], but the protein sequences of all of these isoforms have not been determined. If replacement of only one residue contributes to this affinity change, this would suggest a direct contact of that residue to the antibody [44]. From our previous studies, residue 34 of one crotoxin basic subunit isoform (numbered according to Renetseder *et al.* [45]) changes from Gln in crotoxin from Butantan venom to Arg in crotoxin from Miami venom. This residue is a good candidate for direct contact with A10G antibody. It is a nonconserved residue that constitutes part of the calcium binding loop on the protein. Coincidently, other crotoxin-like proteins that cross-react with A10G, such as Mojave toxin and concolor toxin, have Arg at that position, at least in the isoform sequenced, and have binding affinities to A10G similar to crotoxin from Miami Serpentarium venom.

Our procedure of panning to select crotoxin positive B cells greatly improved the occurrence of crotoxin specific clones from none in 789 clones to six in 288, which makes direct selection of scFv phage clones from the library feasible without phage clone panning. Phage panning has proven to be a powerful selection process that is based primarily on affinity. However, the diversity of the library could be lost by this process as indicated by our second library panning. All specific clones selected from that library after three rounds of panning have the same or similar properties. The most common practice for panning is to attach target molecules to a solid phase and select specific molecules from a liquid phase. However, the binding to the immobilized target on a solid phase depends on a number of factors other than molecular affinity, such as the surface characteristics of the solid phase, number of attachment points (avidity effects), and the hydrodynamic conditions of washing and elution. All these factors will complicate the outcome of the panning process, and so far there is no satisfactory kinetic model to describe the behavior of molecules in two-phase systems. Our procedure of panning in liquid phase avoids these problems.

This description of preparing a crotoxin-specific combinatorial mAb is an alternative to the production of mAbs using traditional hybridoma technology. DNA sequence coding for A10G provides information on antibody structure/function relationships. Analysis of A10G DNA sequence indicated that at the 5′-end of both light and heavy chain fragments in framework I, there are base changes that were probably introduced by primers used in cDNA preparation from Pharmacia's antibody kit. Sequence comparisons (Fig. 2) indicate that most base differences between A10G and germ-line DNA are on CDR regions. Considering the low dissociation constant of A10G towards intact crotoxin, it is quite possible that these mutations from germ-line DNA contribute to the high affinity of A10G for crotoxin. Protein sequence at CDR regions could be useful to determine antibody–antigen interactions on the amino acid level. As the amount of data of high resolution Fab structure has grown in recent years, it has become increasingly possible to model the combining sites of other antibodies from their amino acid sequence based on these known structures [46]. This will greatly increase our understanding of specific antigen–antibody interactions.

There is an on-going debate of the role PLA_2 activity has on presynaptic neurotoxicity; the lack of PLA_2 inhibition by A10GPE indicates that it does not bind to either enzyme active site or to its interfacial binding site. Since A10GPE has some effect on neurotoxicity, this is a strong suggestion that the toxic site is distinct from the toxin enzyme active site or the interfacial binding site. We are also carrying out studies on identifying the epitope of A10GPE by protease digestion. If the epitope can be identified, it is likely to be on or in close proximity to the toxic site for A10GPE to exert its effect.

It has been demonstrated in this paper that A10GPE could at least partially neutralize rattlesnake neurotoxin *in vivo*. Even though revealing the mechanism of this partial lethality neutralization conferred by A10GPE requires detailed neurophysiological and pharmacokinetical studies, it is possible that binding of A10GPE to Mojave toxin interfered with its binding to the putative neuron binding sites(s), which caused in some cases delayed death and in others total neutralization of the toxicity. This demonstrates the potential of genetically engineered Abs for therapeutic treatment by supplementing toxin-specific antibodies to antivenom injections. This treatment could be made more effective by genetically engineering scFv antibody into a full size IgG molecule for engagement of cellular immune responses.

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