Neutralizing Human Anti Crotoxin scFv Isolated from a Nonimmunized Phage Library*

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Combinatorial phage display technology offers a new possibility for making human antibodies which could be used in immune therapy. We explored the use of this technology to make human scFvs specific for crotoxin, the main toxic component of the venom of the South-American rattlesnake *Crotalus durissus terrificus*. Crotoxin, a phospholipase A_2 neurotoxin constituted by the association of two subunits, exerts its lethal action by blocking neuromuscular transmission. This is the first report of human anticrotoxin scFvs (scFv 1, scFv 6 and scFv 8) isolated from a naive library of more than 10^{10} scFv clones with *in vivo* neutralizing activity. Nevertheless, differences are observed at the level of biological and immunological effects. Only scFv 8 is able to reduce the myotoxicity induced by crotoxin and scFv 1 is capable of altering the *in vitro* enzymatic activity of this toxin. All three scFvs recognize a region of one subunit located at the junction with the other one. Moreover these scFvs share strong amino acid homologies at the level of either the heavy or the light chain. Taken together, our results suggest that the use of human anticrotoxin scFvs may lead to a new and less aggressive passive immune therapy against poisoning by the venom of *Crotalus durissus terrificus*.

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INTRODUCTION

Up to now, the heterologous antibody administration has been the treatment of choice for snakebite victims. This practice requires the production, normally by horse immunization, of a high-titer anti-venom serum, which takes a long time to be obtained and is difficult to generate as some venoms contain either potent neurotoxins and/or components associated with the down modulation of the immune response [1]. In addition, owing to the large amount of heterologous proteins administered, the serum therapy may induce adverse reactions. In these circumstances human antibodies would be ideal for immunotherapeutic applications.

The development of recombinant techniques for the cloning and expression of cDNAs encoding the variable regions of the H and L chains of antibodies allows the *in vitro* generation of large antibody repertoires revolutionizing the field of antibody engineering [2–4]. The process of a polymerase chain reaction (PCR) amplification and the selection used in the phage display of combinatorial libraries constitutes a selection step analogous to that occurring during the primary immune response [5–7] allowing the *in vitro* construction of fragments of antibodies of diverse specificities. Moreover it circumvents the ethical problem regarding the source of V-genes from immunized donors. Thus, the display of repertoires of antibody fragments on the surface of filamentous bacteriophages offers a new way of making human antibodies. Furthermore, scFvs are particularly suitable for therapeutic use as their small size increases their biodistribution and clearance properties [8–10].

Vaughan *et al.* [11] have constructed a phage scFv library consisting of more than 10^{10} clones, using V-genes isolated from 43 nonimmunized donors. To construct this library, the V-genes were amplified by PCR, using primers corresponding

* This article is dedicated to the memory of Dr Diva Cardoso.

to all known VH, Vκ and Vλ gene families. ScFv fragments were cloned in a phagemid vector, enabling, alternatively, scFv-phage or soluble scFv to be produced without subcloning and using the same infected *E. coli* cells.

Crotoxin (CTX) a highly toxic β-neurotoxin with phospholipase (PLA_2) activity is the main neurotoxic component of the venom of the rattlesnake *Crotalus durissus terrificus* (CDT). CTX acts primarily at the presynaptic level by altering the neurotransmitter release. It also induces the degeneration of muscular fibers [12]. CTX is composed of two noncovalently associated subunits: an acidic, nonenzymatic and nontoxic subunit (CA) and a basic subunit displaying high enzymatic activity and moderate toxicity (CB) [13]. CB is a presynaptic toxin blocking the neuromuscular transmission and is responsible for the myonecrosis which contributes to the poisoning of envenomed victims [14]. In the intact CTX, CA behaves as a chaperon, preventing the nonspecific adsorption of CB on membranes, thus enhancing the capacity of CB to reach its target at neuromuscular junctions [13]. The toxicity induced by CTX or CB can be neutralized by polyclonal or monoclonal antibodies (MoAb) directed against CB [15, 16]. The mechanism of protection associated with these antibodies has been attributed to their capacity: (i) to block the CTX phospholipase A_2 (PLA_2) activity; (ii) to compete with CTX target binding or (iii) to dissociate the CA-CB subunits [15]. A murine anti-CTX scFv with a limited neutralization power has been obtained from a combinatorial antibody library made from CTX immunized mice spleen cells [17]. Using a semisynthetic combinatorial phage-scFv library, Lafaye *et al*. [18] obtained the first human anti-CTX scFvs showing *in vitro* biological properties.

Now, we report the isolation of scFvs specific for CTX from the large naive phage display library described above [11]. After three rounds of panning, 14 scFv-phage clones displaying the desired specificity were obtained. Among them, three different clones expressing soluble scFv molecules have been extensively studied. This is the first report of antivenom recombinant antibody fragments showing both *in vitro* and *in vivo* biological properties, since the scFvs are able to neutralize the lethality induced either by CTX alone or the whole venom of CDT.

MATERIALS AND METHODS

Reagents. CTX was isolated from CDT venom [19] and its subunits prepared by chromatography in 6 M urea, as described by Hendon and Fraenkel-Conrat [20].

Phage-scFv library-Production of phage particles and biopanning. The phage library, consisting of more than 10^{10} scFv clones expressed as fusions with the gene 3 protein was constructed from V-genes isolated from 43 nonimmunized donors using polymerase chain reaction (PCR) primers corresponding to all known VH and VL (κ and λ) gene families [11]. The panning procedure was adapted from the protocol described by Lafaye *et al*. [18] using nonporous oxirane acrylic beads coated with crotoxin. Briefly, 500 mg of beads were coated with crotoxin by 48 h incubation in phosphate-buffered saline (PBS) containing 50% (w/v) beads and 20 μM protein with gentle agitation at room temperature. The beads were then washed three times with PBS-Tween 0.1% and stored in PBS at 4 °C. An aliquot of 10 mg beads corresponding to 400 nM of protein was used for each panning experiment. TU input phages, $1–5 \times 10^{11}$, were panned by incubation with the coated beads for 2 h at 37 °C with gentle agitation. The samples were then washed eight times with TBS-Tween 0.5% for 60 mn. Bound phages were recovered by adding 200 μl of elution buffer (0.1 M HCl, pH 2.2 adjusted with glycine, 1 mg/ml BSA) and gently mixing for 10 mn at room temperature. Eluates were then neutralized by addition of 11 μl of 2 M Tris and titered by infecting exponentially growing *E. coli* TG1 and plating on LB agar plates containing 100 μg/ml ampicillin. Phage particles were isolated by PEG precipitation and then purified on CsCl gradient, as described by Parmley and Smith [21]. They were stored at 4 °C in PBS without calcium and magnesium salts (Eurobio).

DNA sequencing. Phagemid encoding DNA was used to transfect *E. coli* XL1-Blue cells. DNA was extracted using Nucleobond AX kit (Machery-Nagel, Düren, Germany) and both strands sequenced by Genome Express (Grenoble, France). Primers used are the following: pUC 19 reverse (anneals 5′ to scFv), fdtetseq (anneals 3′ to scFv), PCR-H link (anneals to the scFv linker) and myc seq 10 (anneals to the scFv myc tag) [11]. The germline genes were determined using the DNAPLOT analysis software (Cologne, Germany), which was modified for alignment to V BASE in collaboration with Ian Tomlinson (MRC centre for Protein Engineering, Cambridge, UK). Similarities between the amino acid sequences of the variable domains were determined by Bestfit analysis (GCG, Madison, WI, USA). The DNA sequences of the scFvs are accessible in Genbank under the following references: scFv1, AJ132607; scFv6, AJ132608; scFv8, AJ132609.

Expression, purification and characterization of soluble scFv fragments. Soluble scFv clones were obtained according to the method of Vaughan *et al*. [11]. Briefly, the production of scFv was induced during 3–4 h at 30 °C by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 2 mM and directed to the bacterial periplasm under the control of pelB signal sequence. In order to obtain a soluble sonicated extract, the cultures were pelleted by centrifugation, resuspended in Tris buffer (20 mM Tris-HCl pH 8.0 with 200 mM of PMSF), and sonicated at 60 Hz during 2 min, after which the supernatant was harvested by centrifugation. The soluble periplasmic extract was obtained by resuspending the pelleted cells in Tris buffer containing 500 mM of sucrose, leaving them on ice for 30 min and then centrifugating them. Purified scFvs were obtained by IMAC using a chelating agarose charged with Ni^{2+} (Superflow Ni-NTA, Qiagen Ltd, UK) and equilibrated with washing buffer (20 mM Tris-HCl, 500 mM NaCl and 30 mM imidazole, pH 8.0). Absorbed material was eluted with buffer containing 1 M imidazole. The enriched scFv fractions were pooled, desalted, and concentrated to 2 ml using ultra filtration units (Amicon). The protein content was measured using Bradford reagent. The purity of the final preparation was evaluated by SDS-PAGE and Coomassie staining and by Western blot. Monomeric and multimeric species of the purified scFvs were separated by gel filtration on a Superdex 75 HR 10/30 column in 50 mM Tris-HCl pH = 7.5 containing 150 mM NaCl, at a flow rate of 0.5 ml/min. The column was calibrated with chromatography standards (Bio-Rad). Dimeric/monomeric fraction concentration ratio was determined by calculating the area of corresponding peaks.

ELISA. scFv-phages were assayed by a modification of the method of Lafaye *et al*. [22]. Microtiter plates (Nunc, Denmark) were incubated with 1 μg/ml of various antigens diluted in PBS overnight at 4 °C. Plates were washed four times with PBS containing 0.1% Tween 20 (buffer A) and saturated with buffer A containing 0.5% gelatin (buffer B) for 30 min. Phages were then added for 2 h at 37 °C. Binding of scFv-phages was detected using peroxidase-conjugated rabbit anti-M13 phage

antibodies (Pharmacia Biotech, Uppsala, Sweden). After washes with buffer A, freshly prepared 0.2% orthophenylenediamine (Dakopatts A/S, Glostrup, Denmark) containing 0.03% H_2O_2 in 0.1 M citrate buffer, pH 5.2, was added to each well. The peroxidase reaction was stopped by the addition of 3 M HCl, and the optical density was measured at 490 nm. The reactivity of soluble scFvs against antigens (CTX or CB) was also determined by ELISA. The soluble scFv bound to antigen was detected using an anti *c-myc* tag MoAb (9E10 antibody, Pharmigen) followed by peroxidase-conjugated goat antimouse IgG (Diagnostic Pasteur, France).

Avidity determination. Phage-scFv or soluble corresponding scFvs were incubated overnight at 4 °C with increasing amounts of CTX (10^{-9}–10^{-6} M). The remaining free antigen binding sites were then quantified by ELISA as described above.

Competition assay to determine the antibody-binding region. This assay was used to measure the ability of the different scFvs to compete with mouse anti-CB MoAb for binding to the antigen. MoAb B142–7, B65–5, B69–10, B103–7 and B32–13 recognize specific epitopic regions on CB subunit as previously described [15]. The CB subunit was coated onto a 96 well microtiter plate, and saturated with buffer B. Various concentrations of the mouse MoAb were added. After a 2 h incubation at 37 °C, plates were washed with buffer A and the scFvs were added. Plates were incubated for 30 min at room temperature, washed and rabbit polyclonal anti-*c-myc* tag antibodies were added for 1 h at 37 °C. After washes, plates were incubated with peroxidase conjugated goat antirabbit antibodies for a further hour, washed and revealed as previously described. For each inhibitor concentration, the percent inhibition was calculated as follows:

$$\% = \frac{\mathrm{Io} - \mathrm{Ic}}{\mathrm{Io}} \times 100$$

Io where Io and Ic are, respectively, the absorbances at 490 nm without or with the inhibitor.

Phospholipase A_2 assay. PLA_2 activity of CTX was evaluated by a fluorimetric method using a fluorescent labelled phospholipid as described by Radvanyi *et al*. [23]. The effect of soluble scFv molecules on the enzymatic activity of CTX was measured by analyzing the residual activity displayed by CTX preincubated with variable amounts of scFv. The results are expressed as percent of the enzymatic activity of CTX in the absence of scFvs.

Neutralization of the toxic effect of CTX and CDT venom by the soluble scFvs. To evaluate the effect of scFvs on the lethality induced by CTX, two i.v. LD_{50} of CTX (2 μg) were preincubated with various concentrations of each scFv and injected into 20 g BALB/*c* mice. The mortality was followed every 24 h during 3 days and the results are expressed as the rate of survival (%) of mice 72 h after the CTX/scFv injection. The effect of scFvs on the lethality induced by the whole venom was assayed as described for CTX except that BALB/*c* mice were given 2 i.p. LD_{50} of CDT venom preincubated with 300 μg of scFvs.

Effect of soluble scFvs on the in vivo mycotoxicity of CTX. The effect of scFvs on the myotoxicity induced by CTX was evaluated by measuring the increase of the serum creatine kinase (CK) level in animals injected with the CTX/scFv mixture. BALB/*c* mice were given CTX (1.5 μg) preincubated with 100 μg of each anti-CTX-scFv by injection in the gastrocnemius muscle. Blood was collected with capillary tubes from the retroorbital plexus 2 h after injection as the maximal CK level was observed at this time in mice that did not receive scFvs. The CK level was determined following instructions of a commercial kit (CK (NAC), Merck, Germany) and enzyme activity was expressed in units/ml (U/ml), one unit resulting in the phosphorylation of one μmole of creatine per min at 25 °C.

Statistical analysis. Differences between values were examined by the Student's *t*-test. Differences yielding *P*-values of <0.05 were regarded as significant.

RESULTS

Selection of CTX-binding clones

All the scFvs were cloned in the pCANTAB 6 vector [11] and incorporated at their C-terminus both a stretch of six histidines (His_6) for immobilized metal affinity chromatography (IMAC) and a sequence derived from the *c-myc* antigen enabling immunochemical detection. The presence of an amber codon between the gene coding for the scFv and gene 3 of phage M13 makes it possible to switch between the soluble scFv expression induced by IPTG and the phage display using M13K07 phage helper.

Phages displaying scFvs that specifically bound to CTX were selected by panning with antigen coated beads. After four rounds of selection, phage particles were prepared from 360 individual ampicillin resistant clones and analyzed for binding to CTX, CB or nonrelated Tetanus toxoid (TT) antigens. Fourteen positive clones specific to CTX were obtained. The four clones (clones 1, 3, 6 and 8) giving the best signal were amplified and quantified (data not shown). To further characterize the scFv fragments, soluble scFv from three clones (scFv 1, scFv 6 and scFv 8) were obtained from IPTG induced cultures. After IMAC purification (50–75% of recovery) of scFvs harvested in periplasmic extracts, their reactivity towards CTX was assayed by ELISA (Fig. 1). A SDS-PAGE gel was also performed and the presence of scFv molecules was revealed by a Western blot analysis using an anti-*c-myc* tag secondary antibody. A single positive band was present for each clone at 30 kDa consistent with the molecular weight of a scFv fused to *c-myc* and His_6 tags (data not shown). The binding characteristics of phage-scFvs and corresponding soluble scFvs were determined in competitive inhibition experiments involving CTX coated solid phase and soluble CTX. The concentrations of CTX giving 50% binding inhibition were estimated to be between 10^{-6} M and 5×10^{-7} M for phage-scFvs and corresponding soluble scFvs.

Equilibrium between the monomer and the dimer was studied by size-exclusion HPLC (data not shown). The dimerization constant of scFv 1 seems higher than those of scFvs 6 and 8. The monomer/dimer ratio was, respectively, 1 : 9, 1 : 1, and 9 : 1 for scFv 1, scFv 6 and scFv 8. Thus, scFv 1 is mostly in dimeric form while scFv 8 is mostly in monomeric form.

DNA sequencing

The nucleotide sequences encoding the variable domains of H and L chains (VH and VL) of clones 1, 3, 6 and 8 were determined. ScFv 3 and scFv 8 have the same nucleotide sequence for both VH and VL (data not shown). Therefore, only the properties of scFv 8 will be reported. Table 1 shows that the VH domains of scFv 1 and scFv 8 use VH segments from the VH3 family, respectively, the DP-47 and the DP-49 VH segments. DP-47 VH

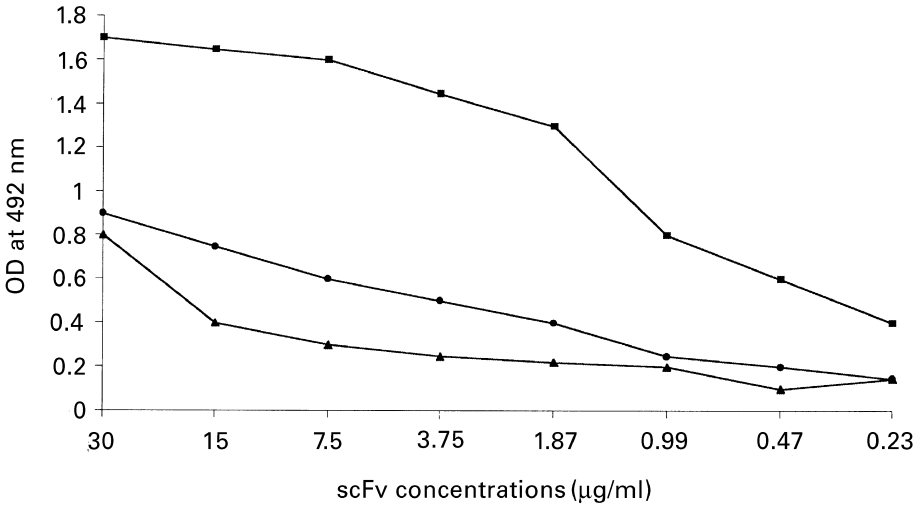

Fig. 1. Quantification of anticrotoxin scFvs by ELISA. Soluble scFv clones were produced in the bacterial periplasm after IPTG induction. Different concentrations of scFvs were assayed by ELISA for its crotoxin reactivity (■) scFv 1; (▲) scFv 6; (●) scFv 8.

segment had been found to be present in 12 clones out of 26 directed against various antigens by Vaughan *et al*. [11], but DP-49 VH segment had never been selected until now. However, they use the same D and J segments, consistent with the fact that their H3 hypervariable sequences are identical. In contrast, the amino acid sequence of VH6 is different from the two others since the DP-67 VH segment, from VH4 family is used. This segment had been found in 3 clones out of 26 in the original library screening [11]. On the other hand, concerning the VL domains, scFv6 and scFv1 presented strong similarities. They both use the DPL2 VL segment suggesting that these antibodies share homology in the L1 and L2 hypervariable loops. This segment had been found to be present in 9 clones out of 26 by Vaughan *et al*. [11]. Nevertheless, they use different J segments, consistent with the fact that their L3 hypervariable sequences are divergent. In contrast, VL8 is different since it uses the DPL 11 VL segment. This segment had been found to be present in 6 clones out of 26 by Vaughan *et al*. [11].

Table 1. Deduced germline origin of the selected scFvs

	VH			VL	
Germline	Family (segment)	DH	JH	Family (segment)	JL
ScFv 1	VH3 (DP-47)	DK 1	JH 6	Vλ 1 (DPL 2)	JL 3
ScFv 6	VH4 (DP-67)	D21–10	JH 4	Vλ 1 (DPL 2)	JL 1
ScFv 8	VH3 (DP-49)	DK 1	JH 6	Vλ 2 (DPL 11)	JL 3

Human germline sequences were determined using the DNAPLOT software (Cologne, Germany) and V Base.

The deduced amino acid alignments of the H and L chain variable fragments (Fig. 2A, B, respectively) show that the H chains of scFv1 and scFv8 share a high degree of similarity (84%). The largest sequence differences were found in the H2 region. The similarity of the H chain of scFv6 with scFv1 or scFv8 is much lower (60%). However, the situation for the L chain is quite different. The similarity between scFv6 and scFv1 is higher (77%) than the similarity between scFv6 and scFv8 or scFv1 and scFv8 (65%). It should be noted that the largest sequence differences between scFv6 and scFv1 were found in L3.

Epitope mapping of anti-CTX scFvs

A preliminary experiment was performed by ELISA and showed that scFvs only react with CB (data not shown). In order to identify the epitopic regions recognized by anti-CTX scFvs, we examined their ability to bind to CTX, after preincubation with an anti-CB MoAb antibody at saturating concentrations. We used MoAbs directed against several different epitopes of CB. Murine B62–10, B142–7, B65–5 MoAbs recognize a CB epitopic region located at the interface between CA and CB subunits (BI-a region). B69–9 recognizes an epitopic region, located on CB, which is involved in its enzymatic activity (BII). B103–7 binds to another zone of the interface between CA and CB subunits (BIII) which is independent from BI-a. Finally, B32–13 recognizes a region not related to the others, named BIV [15]. As shown in Fig. 3, the binding of human scFvs to CB was inhibited in the presence of murine B142–7, B65–5 and B103–7 MoAbs. A weak competition was also observed between scFv8 and B62–10 MoAb. The other murine MoAbs did not significantly inhibit the binding of the three scFvs studied.

From these data we can conclude that scFvs recognize epitopes overlapping BI-a and BIII epitopic region. The closeness of the epitopes recognized could be explained by the fact that each scFv shared strong amino acid homologies either in the VH domain or the VL domain.

A

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                10                  20                  30   H1             40
scFv 1   G V Q L V Q S G G G L V Q P G G S L R L S C A A S G F T F S S Y A W S W V R Q A P G
scFv 6   Q - - - - - - - - - V - - - - R - - - - - - - - - - - - - S S Y G M H - - - - - - -
scFv 8   Q - - - - E - - - - V - - - - R - - - - - - - - - - - - - R N Y G V H - - - - - - -

                                 H2                                        80
scFv 1   R G L E W V S V I D G S G D N T F Y A D S V K G R F T I S R D N S K N T L Y L Q M N S
scFv 6   K - - - - I G S V H H S G . P T Y Y N P S L K S - V - M - V - T - - - Q F S - K L K -
scFv 8   K - - - -   A V I S Y D G S N K Y Y A D S V K G - - - - - - - - - - - - - - - - - -

                90                   H3                                110
scFv 1   L R V E D T A V Y Y C A R R W Y G G S G Y W G H F Y S . Y M D G W G K G T K V T V S S
scFv 6   V T A A D - - M - F - - - . E . G G S T W R S L Y K H Y Y M D V - - Q - - L - - - - -
scFv 8   - - - - - - - - - - - - - R W Y G G S G Y W G H F Y S . Y M D G - - - - - - - - - - -
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B

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                10                  20                    L1                    40
scFv 1   Q S V L T Q P P S A S G T P G Q R V T I S C S G S S S N I G . S N T V N W Y Q R L P G
scFv 6   S Y - - - - - - - V - - - - - - - - - - - - - S G S S S N I G . S N T V K - - - Q - - -
scFv 8   - P - - - - - A - V - - S   - - S I - - - - T G T S S D V G G Y N Y V S - - - Q H - -

                     L2             60                  70                  80
scFv 1   A A P Q L L I Y N N D Q R P S G I P D R F S G S K S G T S G S L V I S R . L Q S E D E
scFv 6   T - - K - - - - G N D Q R P S G V - - - - - - - - - - - - - A - - T   T G   V R A - - -
scFv 8   K - - K - M - - E G S K R P S G V S N - - - - - - - - N T A - - T - - G A L - A - - -

                     L3             100
scFv 1   A D Y Y C A S W D D R L N G . R V F G G G T K L T V L G
scFv 6   - - - - - Q S Y D S S L R G S R V - - T - - - V - - - -
scFv 8   - - - - - S S Y . . T T R S T R V - - - - - R - - - - -
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Fig. 2. Alignment of deduced amino acid sequences of scFvs 1, 6 and 8. The deduced amino acid sequences are shown in one letter code, and the CDR regions are indicated. Amino acids identical to those of scFv 1 are indicated by dashes; dots indicate gaps between the alignment of the three sequences. A: H chain variable domain (VH) amino acid sequences; B: L chain variable domain (VL) amino acid sequences.

In vitro and in vivo assessment of the biological activities of anti-CTX scFvs

In vitro effect of scFvs on CTX enzymatic activity. CTX is composed of two subunits: a weakly toxic PLA_2 (CB), and a nontoxic, nonenzymatic subunit (CA). CA increases the toxicity and the specificity of the CB action while simultaneously reducing its enzymatic activity. The PLA_2 activity of CTX was measured *in vitro* by recording the changes of fluorescence accompanying the hydrolysis of a substrate analogue. CTX was incubated with different amounts of purified scFv1, scFv6 and scFv8. A dose dependent enhancement of CTX enzymatic activity was observed in the presence of scFv1 but not with scFv6 and scFv8 (Table 2).

In vivo effect of scFvs on the myotoxicity induced by CTX. CTX exerts a necrotic action on murine skeletal muscle cells, by causing damage to the plasma membrane. Consequently an increase in plasma creatine kinase (CK) levels is observed [24]. The myotoxic activity induced by purified CTX can therefore be assayed by measuring the raise of serum CK levels after venom injection. This raise in the level of serum CK was strongly reduced in mice to which CTX preincubated with scFv8 was injected (molar ratio scFv/CTX 50/1): 71% inhibition was observed (Table 3). The CK level was unchanged compared to the control in mice which were given CTX preincubated with scFv1 or scFv6.

Neutralization of the lethal potency of CTX. Different concentrations of scFv1, scFv6 and scFv8 were incubated with a fixed concentration of CTX, and the mixture was then injected to mice. All the scFvs studied show a neutralizing activity. However, the protection observed was different from one

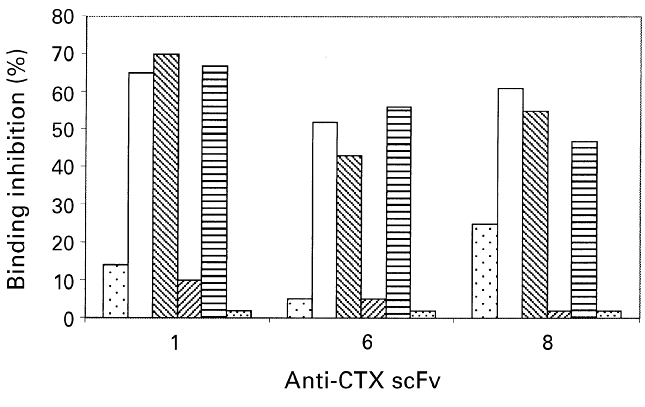

Fig. 3. Mapping of the epitopes of purified anti-CTX scFvs by ELISA. Inhibition assays were performed to measure the ability of different scFvs to compete with mouse anti-CB MoAbs for binding to crotoxin. Each MoAb recognizes a specific epitopic region on the CB subunit: ⊞ B 62–10; □ B 65–5; ▧ B 142–7; ▨ B 69–10; ▤ B 103–7; ▩ B32–13.

Table 2. Effect of the selected scFvs. on the enzymatic activity of crotoxin

Molar ratio: scFv/Toxin	Crotoxin (%)		
	ScFv 1	**ScFv 6**	**ScFv 8**
16/1	157 ± 15*	104 ± 4	ND
8/1	162 ± 14*	100 ± 4	107 ± 3
4/1	ND	94 ± 3	98 ± 3
1.6/1	100 ± 5	ND	95 ± 5
0.8/1	ND	93 ± 4	107 ± 3

CTX was incubated for 1 h in the presence of the indicated molar ratio of purified scFvs 1, 6 and 8. Aliquots were tested for their residual PLA_2 activity as described in Material and Methods. Results are expressed as the percentage of the specific activity measured in the absence of scFvs. The average and the S.D. correspond to two independent preparations, with three assays per preparation. (ND, not determined). * $P < 0.01$.

scFv to another. Indeed, scFvs 6 and 8 give a better protection than scFv1 (Table 4) as seen when using a scFv/CTX ratio of approximatively 45/1. Moreover more scFv1 than scFv6 or scFv8 was required in order to obtain the same level of 80% protection. It is important to note that mice were still alive after the periods indicated here, whereas mice which received 2 LD_{50} of CTX without scFv died between 12 and 18 h after injection.

The effect of scFvs 1, 6 and 8 on the lethality induced by whole CDT venom was also investigated. ScFv 6 protected the mice more efficiently than scFv1 and scFv8 (Table 5).

DISCUSSION

We report here the first human anti-CTX scFvs with *in vivo* neutralizing activity, obtained from a combinatorial library.

Table 3. Effect of scFvs on the myotoxicity induced by Crotoxin

CTX plus	CK level (U/ml)	Inhibition (%)
PBS	1516 ± 568	
ScFv 1	1540 ± 755	0
ScFv 6	1723 ± 766	0
ScFv 8	437 ± 166*	71

Each scFv was incubated for 1 h with CTX (molar ratio 50/1) and groups of mice ($n = 6$) were injected with scFv/crotoxin mixtures as described in Material and Methods. Mice were bled after 2 h and their serum CK level content was assayed. Results are expressed as means ± SD of CK concentration (U/ml). The inhibition percentages were compared with the CK level found in the serum of control mice. The average and the SD of two independent experiments are given. * $P < 0.01$.

Table 4. Effect of scFvs on the lethality induced by Crotoxin

Molar Ratio scFv/CTX	Number of live mice	Protection (%)
ScFv 1		
47	1/4	25
83	2/9	22
167	8/11	73
ScFv 6		
42	3/7	43
125	5/6	83
ScFv 8		
42	3/7	43
125	4/5	80
PBS	3/20	15

CTX (two i.v. LD 50) was incubated at different molar ratios with scFv 1, scFv 6 and scFv 8 as indicated in Material and Methods. The mixture was injected into BALB/c mice and the mortality was followed for 72 h. The results are expressed as the number of live mice 72 h. The results are expressed as the number of live mice 72 h after the injection of the mixture.

Immune passive therapy is the current treatment for snake bite poisoning, like that caused by the rattlesnake *Crotalus durissus terrificus*. Owing to its heterologous nature, the serum therapy can induce adverse reactions. Human monoclonal antibodies raised by molecular engineering could be used in order to avoid these problems. The use of phage display libraries to prepare antibodies circumvents the ethical problem regarding the impossibility of immunizing human donors for collecting specific V-genes [5–7]. Repertoires of V-genes from nonimmunized human donors, cloned into phagemid vectors, have been previously used to select and generate scFvs which bound specifically to various other antigens [11, 25].

Recently, we had already isolated biologically active anti-CTX phage-scFvs from a semisynthetic phage library [18]. The

Table 5. Effect of scFv on the lethality induced by *Crotalus durissus terrificus* whole venom

	Number of live mice	Protection (%)
PBS	2/20	10
ScFv 1	5/20	25
ScFv 6	14/25	56
ScFv 8	13/54	24

CDT venom (two i.p. LD 50) was incubated with a fixed amount of each scFv (300 μg) as indicated in Material and Methods. The mixture was injected into Balb/c mice and the mortality was followed for 72 h. The results are expressed as the number of live mice 72 h after the injection of the mixture.

phage-scFvs bound to different epitopes of CB subunit and one of them was able to increase the *in vitro* enzymatic activity of CTX. However soluble scFvs corresponding to these phage-scFvs could not be produced, making them unsuitable for therapeutic use. On the other hand, a murine anti-CTX scFv, obtained from a combinatorial antibody library from CTX immunized mice spleen cells, has been described [17] but it does not inhibit the *in vitro* phospholipase activity of CTX and has a limited neutralization power since mice injected with scFv-CTX mixture survived little beyond 24 h.

Using the phage-scFv library constructed by Vaughan *et al.* [11], we were able to isolate several clones of phage-scFv that react with CTX. Three clones [1, 6, 8] were selected since they express a high amount of specific and active soluble scFv molecules. These scFvs, which recognize epitopes carried by the CB subunit, were able to neutralize the lethality induced by CTX. Each scFv displayed a dose dependent protection. A more pronounced effect was observed with scFvs 6 and 8, as compared to scFv1. Mice injected with scFv/CTX mixtures survived beyond 72 h while mice injected with CTX alone died within 18 h. CTX is the main toxic component of the venom of CDT since it accounts for 90% of the toxicity of the venom. When experiments were performed using crude venom, scFv 6 appeared to be the most active, while scFv1 showed only a weak neutralizing activity, in agreement with the results observed when purified CTX is used. As for scFv8, different results were obtained when crude venom was used instead of CTX. This difference may be explained by the fact that the venom has components other than the CTX which may play an important role in the lethality. A similar result was observed by Kaiser *et al.* [26] with a neutralizing MoAb. A high molar ratio of scFv over CTX (more than 100/1) was necessary to attain a 80% neutralization threshold. The scFvs isolated from this naive library therefore had a low *in vivo* efficiency, which could be explained by their moderate affinity. These characteristics could be improved in the future by protein engineering.

To understand how the scFvs could neutralize CTX, it was important to define which regions of CB they recognize. Several epitopic regions of CB have been mapped by the use of murine MoAbs [15]. Three of them are involved in the stability of the interaction between CA and CB (BI-a, BI-b and BIII), one in the lethality of CTX (BIV) and the last one in the enzymatic activity of CB (BII): it could be composed of residues involved in the catalytic mechanism and/or in the binding of phospholipids. ScFvs 1, 6 and 8 recognized, with little differences from one scFv to another, an epitopic region located at the junction between the CB and CA subunits as deduced from competition experiments using anti-CB MoAbs, performed by ELISA. ScFvs competed with B103–7 and B142–7 but not with B62–10. As reported previously [15], B62–10 and B142–7 both recognize the BI-a epitopic region while B103–7 recognizes the BIII epitopic region. It could be possible that the BI-a and BIII regions significantly overlap or that the three scFvs recognize conformational epitopes spanning over the BI-a and BIII regions. It is important to emphasize that all the regions defined by murine MoAbs are based on phenomenological properties and not on topological structures. From these experimental competition data, we can conclude that all the anti-CTX scFvs recognized epitopes located in the zone of interaction between the CA and CB subunits.

The immunochemical and biological properties of these scFvs suggest that they recognize close but distinct epitopes. This result may be explained by the analysis of the sequence of the scFvs. The selected clones represent a reduced combination of only two types of VH and VL domains. However, although these scFvs share strong sequence homologies, we observed variations in the CDRs of the H and L chains, which could explain the different biological effect of these scFvs on the myotoxic and enzymatic activities of CTX. Only scFv 1 induced an enhancement of CTX enzymatic activity. Since CB has a higher enzymatic activity than CTX, the influence of scFv 1 on the pharmacological properties of CTX may be explained by a dissociation of the subunits of CTX. The predominant dimeric form of scFv1 may contribute to its efficiency in dissociating the complex between CA and CB.

Only scFv 8 was able to neutralize efficiently the myotoxic activity induced by CTX. This myotoxic effect displayed by CDT venom is ascribed to CTX or its CB subunit [12, 27]. Moreover polyclonal anti-CB antibodies are as efficient as serum raised against the whole venom to reduce the myotoxicity induced by CDT or CB subunit [16]. Although necrotic lesions occur mainly locally, at the site of venom injection, systemic effects are also common where muscles of the whole body are involved and consequently could play an important part in the lethality observed in humans [14]. Kini and Iwanaga [28] have examined the charge density distribution of PLA_2s in order to identify the involvement of charged amino acid residues in the determination of the pharmacological properties of these proteins. The PLA_2s which are also myotoxic have a characteristic cationic site region which is located on the outer surface in the three-dimensional structure of phospholipase. ScFv 8 could recognize this cationic site and interfere with charged amino acid residues.

Taken together our results suggest that the scFvs we have selected are able to neutralize important toxic effects induced by whole venom, CTX or its CB subunit. The neutralization effect of these scFvs may be owing to an ability to dissociate the CA and CB subunits. It has previously been shown that several anti-CB antibodies (monoclonal or polyclonal) neutralize the lethal potency of CTX [15, 16, 18]. One of the principal mechanisms of neutralization that was proposed was the dissociation of the CTX dimer, as the toxicity is the consequence of the synergy of the two subunits [15, 18]. More recently, other antivenom factors extracted from the plasma of different snakes have been shown to complex with PLA_2 subunits, separating them, and thus suppressing the synergistic activity between the two subunits [29].

In the present investigation we observed for the first time that scFvs isolated from a phage display library constructed from V-genes isolated from nonimmunized donors possess an *in vivo* neutralizing activity against a venom toxin. Thus the

generation of anti-CTX scFvs, selected from naive libraries, might become a new tool for the development of immunotherapies for poisoned victims.

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