A recombinant single-chain antibody fragment that neutralizes toxin II from the venom of the scorpion *Androctonus australis hector*

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Abstract Monoclonal antibody 4C1 specifically binds to and neutralizes the most potent neurotoxin (AahII) of the scorpion *Androctonus australis*. The cDNAs encoding the variable regions of this antibody were isolated by PCR-mediated cloning. A single-chain Fv gene was engineered and expressed in *Escherichia coli*. The recombinant protein had neutralizing activity similar to that of the intact antibody in vitro and in vivo. We have thus neutralized the pharmacological and biological properties of a scorpion neurotoxin with a single-chain Fv, which opens new perspectives for the treatment of envenomizations.

Key words: Neurotoxin; Single-chain antibody fragment; Single-chain antibody; Neutralization; Scorpion; *Androctonus australis hector*

1. Introduction

Antivenom serotherapy was first reported in 1894 and antivenoms are still the only specific treatment for scorpion stings [1]. However, such treatment has several limitations. Antivenoms are usually heterologous polyclonal immunoglobulins or $F(ab')_2$ fractions from immunized animals [2]. They are difficult to prepare reproducibly, even under standardized conditions, and their neutralizing properties may vary from one batch to another. Heterologous antibodies and $F(ab')_2$ molecules have much shorter half-lives in human plasma than human IgG and do not diffuse easily from the vascular compartment [3]. This could probably be slightly improved by using Fab fragments, but they can still produce adverse effects involving allergic hypersensitivity [4]. Monoclonal antibodies may offer substantial advantages in terms of potency, reproducibility and freedom from contaminants but they are not easy to use. The production of human monoclonal antibodies often has problems, mostly related to the instability of the hybridoma cell line, and the therapeutic use of murine monoclonal antibodies which is sometimes effective under laboratory conditions is at present not economical [5,6]. One way of avoiding most of these problems is to engineer novel recombinant antigen binding proteins that have increased efficiency and new properties. Fv fragments, which are the minimal structure likely to retain the binding characteristics of an antibody, can now be produced in heterologous systems [7,8]. The recombinant variable domains (V_H and V_L) are genetically linked in a single-chain (scFv) construct via a short flexible peptide that connects the C-terminus of one domain (V_H) to the N-terminus of the other (V_L) [9]. This stabilizes their association. scFvs can be derived from hybridoma or combinatorial antibody libraries by phage display technology [10,11] and may be very useful in many fields, such as research, immunodiagnosis and immunotherapy [12–14]. scFvs are cleared more rapidly from the blood to extravascular spaces than the much larger IgGs, $F(ab')_2$ and Fab molecules, resulting in better tissue penetration [15–17]. Thus, scFvs are potentially very useful molecules for treating cancer and detoxication [18–20]. The small size of the scFv permits their rapid diffusion in the body for detoxication and the fast in vivo clearance of immunocomplexes via the urine. This minimizes the chance of late release of bound toxin and the reemergence of toxicity. Lastly, the absence of the immunoglobulin constant domains decreases the risk of an immunological response from the patient.

Hybridoma 4C1 secretes a high affinity murine monoclonal antibody directed against the main toxic component (AahII) of the venom from the North African scorpion *Androctonus australis hector*. AahII is a 64 amino acid residue polypeptide that is highly toxic to mammals because it interacts with potential-dependent sodium channels [21,22]. Antibody 4C1 can competitively neutralize the toxin binding to its receptor because the epitope overlaps or is close to the receptor binding region of the toxin [23]. This report describes the engineering of an scFv derived from antibody 4C1 which is efficiently produced by recombinant bacteria in a soluble, active form. The scFv 4C1 can neutralize the pharmacological and biological effects of the toxin.

2. Materials and methods

2.1. Cell lines, Escherichia coli strain and vectors

The mouse hybridoma cell line 4C1 secreting a monoclonal antibody directed against the scorpion toxin AahII was originally produced by Bahraoui et al. [23]. Here, hybridoma cells were cloned once by limiting dilution. Control tests were done with clone 4C1(BC5) expanded in BALB/c mice. The secreted immunoglobulins were purified on protein-A Sepharose (Pharmacia) [24]. Antigen-mediated ELISA was used to determine the heavy chain isotype (Sigma ISO-2 kit), and the light chain isotype (rat monoclonal antibodies anti-mouse kappa or lambda chain from Sigma).

E. coli HB21 51 transformed with plasmid pHEN1A58(Ile)scFv was used to produce the irrelevant scFvA58 protein directed against an epitope of scorpion hemocyanin [10].

The MRC-OX74 antibody directed against the peptide flag VASDPPVTITNPAT was a gift from Dr. N. Barclay [25]. *E. coli* strain JM109 (Promega, Lyon, France) was used as the cloning host

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Abbreviations: BSA, bovine serum albumin; Fv, antibody variable fragment; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; i.c.v., intracerebroventricular; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RIA, radioimmunoassay; scFv, single-chain antibody fragment

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and strain HB2151 as the gene expression host. pGEMT (Promega, Lyon, France) was used for cloning and sequencing hybridoma-derived immunoglobulin variable regions. The scFv gene was ligated and expressed in the pHEN1 phagemid kindly provided by Dr. G. Winter [26].

2.2. Cloning of cDNAs encoding the variable regions of antibody 4C1

Total RNA was prepared from 10^7 freshly subcloned hybridoma 4C1(BC5) cells using the RNAnow kit (Biogentex, USA) and first strand cDNA was synthesized using an oligodT_{12-18} primer [10]. The V_H and V_L chain domains were amplified by PCR with primers designed after analysis of the Kabat data base [27]. V_H was amplified with the forward primer MHFor (5′-CGG GAT CCT CTA GAC AGT GGA TA(GA) AC(AC) GAT GG-3′) complementary to the CH1 domain of the γ chain and the reverse primer MHRev (5′-ATG AAC TTC GGG CTC AGC TTG ATT TT-3′) complementary to the γ chain sequence leader. The primers to amplify V_L were MKCFor (5′-GGA TAC AGT TGG TGC AGC ATC-3′) which hybridizes to the κ light chain constant domain and MKRev (5′-ATG AAG TTG CCT GTT AGG CTG TTG GTG CT-3′), which hybridizes to the light chain sequence leader. The 50 μl PCR mixtures contained 50 ng hybridoma cDNA, 20 pmol of each appropriate primer, 250 μM of each dNTP, 1×Taq buffer (Promega, Lyon, France) and 1 U *Thermus aquaticus* (*Taq*) polymerase. Amplification included 25 cycles of 1.5 min at 94°C, 2.5 min at 55°C and 3 min at 72°C in a thermocycler (PTC-150, MJ Research). The amplified DNAs were ligated into the pGEMT vector and the recombinant plasmids purified by alkaline lysis. The DNA sequences of the cloned V_H and V_L inserts were determined using the PRISM Cycle Sequencing kit (ABI) and M13 Forward and Reverse primers. The sequences of the V genes were determined on two independent batches of RNA preparations to ensure accuracy. The sequences were analyzed with the programs from the University of Wisconsin Genetics Computer Group.

2.3. Construction and cloning of scFv-4C1 for expression in bacteria

A synthetic gene encoding scFv-4C1 was amplified by overlap extension using *Pyrococcus furiosus* (*Pfu*) polymerase (Stratagene, La Jolla, CA, USA) [10]. The genes encoding the variable domains were independently modified in an initial PCR amplification. The modifications at their 5′ and 3′ extremities were with primers MH2Rev (5′-CCG GCC ATG GCC GAA GTG CAT CTG GTG GAG-3′) and LinkFor (5′-ACC ACC GGA TCC GCC TCC GCC TGA AGA GAC AGT GAC CAG AGT CC-3′) for the V_H and LinkRev (5′-GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAT GTT (C,T)TG ATG ACC CA-3′) and FlagFor (5′-AGT AGC TCG AGC TAT GTC GCA GGA TTA GTT ATA GTG ACA GGA GGG TCA CTG GCT ACT TTG ATT TCC AGC TTG GT-3′) for the V_L domain. The four primers were designed to not introduce any mutation into the deduced peptide sequences of antibody 4C1 variable domains. Primers LinkFor and LinkRev carried overlapping sequences encoding the linker peptide $(Gly_4Ser)_3$. Primer MH2Rev carries an *Nco*I site suitable for cloning in the plasmid pHEN1. Primer FlagFor encoded the C-terminal Flag peptide tail recognized by antibody MRC-OX74. It carries a stop codon followed by a restriction sites (*Xho*I) suitable for cloning. The modified V_H and V_L genes were mixed together and reamplified in a second round of PCR with the external primers MH2Rev and FlagFor. The resulting PCR product was purified, cleaved successively with *Nco*I and *Xho*I and inserted in frame with the *Pel*B leader sequence into the pHEN1 vector linearized with the same restriction enzymes. The recombinant vector (pHEN1-4C1) was electroporated into competent HB2151 cells and positive clones were selected upon PCR amplification with appropriate primers. The entire DNA sequence of the cloned scFv insert was verified on both strands using sequencing primers adjacent to the *Nco*I and *Xho*I sites of pHEN1.

2.4. Bacterial expression of scFv-4C1

Non-suppressive HB2151 *E. coli* cells transformed with the scFv expression vector were grown as in [13]. Bacteria were induced for production of the scFv-4C1 protein with 0.5 mM isopropyl β-D-thiogalactoside for up to 72 h at temperatures of 16–30°C. The cells were harvested by centrifugation at 4000×*g* for 10 min at 4°C. Proteins released into the culture supernatant were precipitated with ammonium sulfate (60%) and resuspended in 25 mM HEPES pH 7.4 (5% of the initial volume). Soluble periplasmic proteins were extracted as in [13]. These preparations were then extensively dialyzed against 25 mM HEPES pH 7.4 buffer and clarified by centrifugation (15 000×*g*, 4°C, 45 min). The solutions were concentrated 5-fold with a Centricon 30 (Millipore) and then passed through an 0.2 μm pore size membrane (Sartorius, Göttingen, Germany).

The production of the recombinant protein was monitored by Western blot [28,29] which was detected by the peptide flag with monoclonal antibody MRC-OX74 [10].

2.5. Detection of scFv 4C1 activity

2.5.1. Protein concentration. The protein contents of synaptosome or scFv preparations were determined with the Micro BCA protein assay reagent (Pierce).

2.5.2. Radioimmunoassays (RIA). The binding of scFv preparations to [^{125}I]AahII was assessed. [^{125}I]AahII was prepared and purified [30]. Successive dilutions of IgG or scFv preparations were mixed with [^{125}I]AahII (1×10^{-10} M final concentration) in PBS-0.1% BSA (final volume: 150 μl) and incubated for 90 min at 37°C and then overnight at 4°C. Bound was separated from free antigen by adsorbing the free antigen onto activated charcoal. A suspension of 1.6% charcoal (Sigma) and 0.16% dextran T-70 (Sigma) in PBS-0.2% BSA was added to each tube (0.5 ml) and the mixtures were incubated for 30 min at 4°C, then centrifuged at 9000×*g* for 10 min. The radioactivity in the supernatants was measured with a gamma counter (Packard Crystal II). All assays were conducted in duplicate. IgG 4C1 (1×10^{-10} M) or scFv 4C1 preparation (1/18 final dilution) were mixed with [^{125}I]AahII (1×10^{-10} M) plus various concentrations of unlabeled AahII for competitive experiments.

2.5.3. Neutralization assays

2.5.3.1. Receptor binding assay. Rat brain synaptosomal fraction was prepared [31,32]. Binding assays were performed in 140 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES and 0.1% BSA at pH 7.4. [^{125}I]AahII (1×10^{-10} M) was incubated with dilutions of IgG or scFv preparations for 90 min at 37°C. Synaptosomal preparation was added (0.4 mg/ml) and the incubation was continued for 30 min at 37°C. The mixtures were centrifuged at 9000×*g* and washed three times. The radioactivity in the pellets was measured. All assays were conducted in duplicate.

2.5.3.2. Assays in vivo. The neutralizing capacity of scFv-4C1 preparations was tested by the intracerebroventricular (i.c.v.) route. Amounts of AahII equal to or greater than the lethal dose were incubated for 90 min at 37°C and 30 min at 4°C with an equal volume of scFv preparation. The mixture was then injected into female C57BL/6 mice of 20 g weight (groups of six) by the i.c.v. route. Surviving mice were recorded after 24 h.

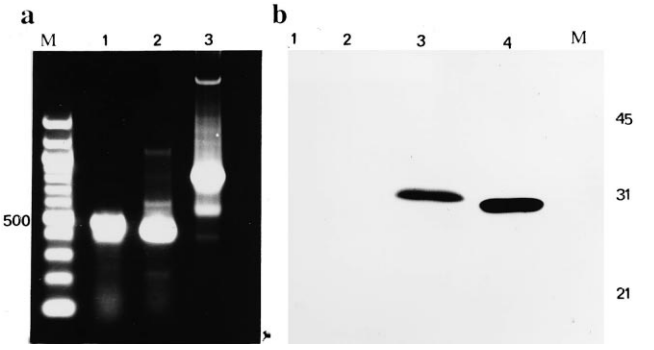

Fig. 1. Construction and expression of the scFv-4C1 chimeric gene. a: Molecular cloning of scFv-4C1. V_H (1) and V_L (2) domains were amplified by RT-PCR from hybridoma 4C1 RNA using external primers complementary to the leader sequence and constant domains. scFv gene (3) was assembled by splicing by overlap extension PCR with specific primers deduced from V_H and V_L sequences. Products were visualized on an agrose gel (1%). M, 100 bp DNA ladder. b: Production of the scFv-4C1 protein. Periplasmic (1, 3) and culture supernatant (2, 4) fractions (25 μg) of bacteria not induced (1, 2) or induced at 16°C for 48 h (3, 4) were analyzed by Western blot using the anti-flag MRC-OX74 antibody. M, molecular size marker in kDa.

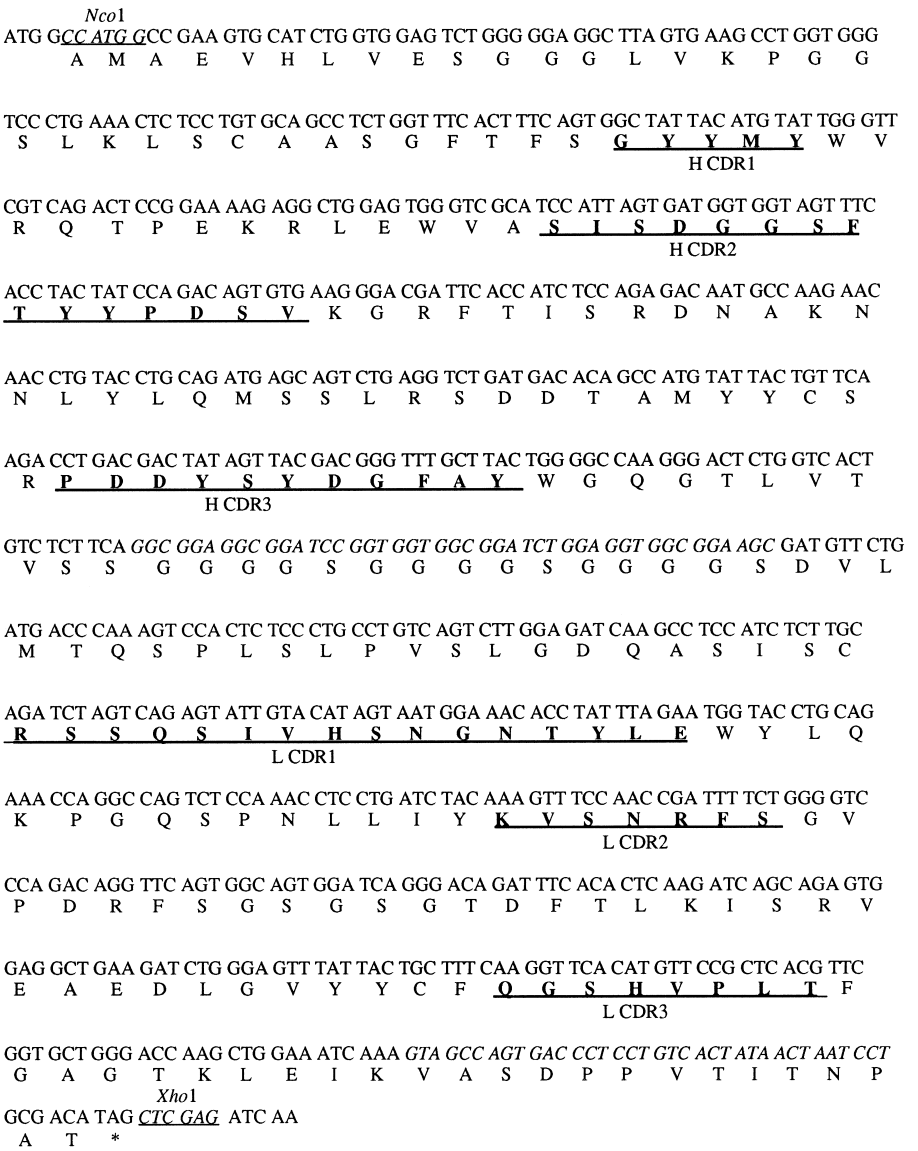

Fig. 2. The nucleotide and predicted amino acid sequences of the AahII-specific scFv-4C1. The nucleotide sequences corresponding to the restriction sites *Nco*I and *Xho*I used for cloning, the linker peptide $(Gly_4Ser)_3$ and the peptide flag are shown in italics. The deduced amino acid sequence of the complementary determining regions (CDRs) of V_H and V_L are underlined.

3. Results

3.1. Construction and sequence of the scFv-4C1 gene

Antibody 4C1 was shown to be an IgG1 (kappa chain). To generate an scFv fragment, the V_H and V_L genes were cloned from cDNA and joined by a sequence encoding the flexible $(Gly_4\text{-}Ser)_3$ linker in a PCR amplification (Fig. 1a). The resulting DNA was inserted into the bacterial expression vector pHEN1. The sequences of the V_H and V_L domains assembled in the scFv chimeric gene were the same as those obtained after cloning V_H and V_L in pGEMT (Fig. 2). The primers used to construct the scFv gene were designed so that they perfectly matched the ends of the variable region cDNAs. This is important because mutations induced by the V_H 5′ primer could influence the activity of the recombinant antibody [33,34]. No mutation was introduced by the *Pfu* DNA polymerase which was selected for the assembling PCR am-

Table 1
In vivo neutralization of AahII toxicity in C57BL/6 mice by scFv preparations

AahII injected (ng)	scFv preparation	
	4C1	A58
1.50		4/6
2.25		1/6
3.00	6/6	0/6
3.75	6/6	
4.50	4/6	
6.00	3/6	
7.50	0/6	

AahII solutions were incubated with AahII-specific scFv (4C1) or with irrelevant scFv (A58) before i.c.v. injection into mice. The LD_{50} of the toxic fraction in the absence of antibody was 1.5 ng per 20 g mouse. Results are expressed as the number of surviving mice/injected mice.

plification because it has the lowest error rate of any thermostable DNA polymerase tested to date [35].

Comparison of the current sequence with those in the immunological database of Kabat [27] indicated that the V_H region of antibody 4C1 belonged to the mouse heavy chain family XIV subgroup IIID, whereas the V_L regions belonged to the mouse κ light chain family IV subgroup I. Comparison of the deduced amino acid sequence with those of the immunoglobulin variable regions stored in the SwissProt data bank also permitted identification of the hypervariable regions or CDRs forming the antigen binding loops of the antibody. The cDNA sequences of antibody 4C1 V_H and V_L are now registered in the EMBL data bank (accession numbers Y17588 and Y17589).

3.2. Production of recombinant scFv protein

Recombinant bacteria in the exponential growth phase were induced at different temperatures. The scFv accumulated in the cytoplasm, mainly as inclusion bodies, whatever the induction time or temperature (data not shown). However, the presence of the *Pel*B signal peptide allowed production of small amounts of the scFv protein which was found in the periplasm of bacteria harboring the pHEN1-4C1 vector. It was detected in cells induced at 16°C for 48–72 h (Fig. 1b). The intact recombinant scFv was even released into the culture medium under these conditions and no degradation products were detected. This procedure provided enough material for us to evaluate the activity of scFv.

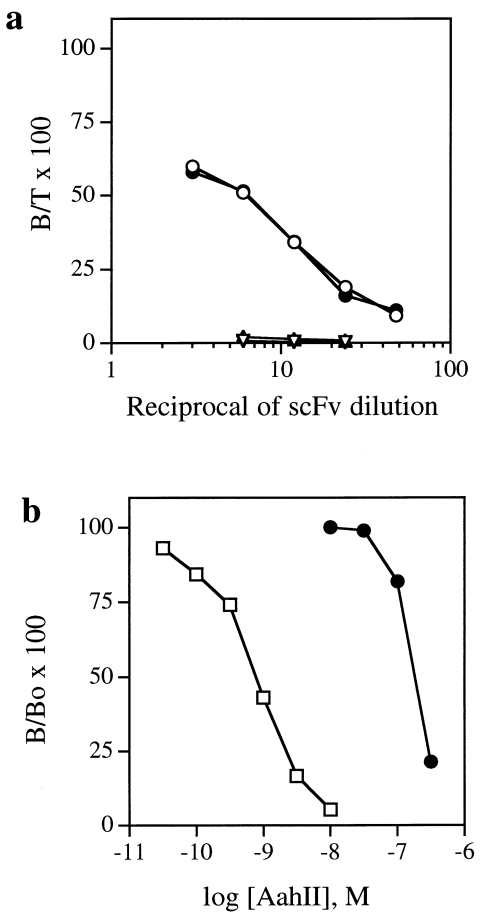

Fig. 3. Immunoreactivity of scFv-4C1 with AahII toxin tested by RIA. a: Serial dilutions of scFv 4C1 from culture supernatant (filled circle) or periplasmic (open circle) fractions or scFv A58 from culture supernatant (filled triangle) or periplasmic (open triangle) fractions were tested against [^{125}I]AahII. Results are expressed as B/T where B is the radioactivity bound to antibody and T is total radioactivity. b: Inhibition of [^{125}I]AahII binding to IgG (open square) or to scFv-4C1 from culture supernatant preparation (filled circle) was performed with unlabeled AahII. Results are expressed as B/Bo where B and Bo are the radioactivity bound to antibody in the presence (B) or absence (Bo) of unlabeled ligand.

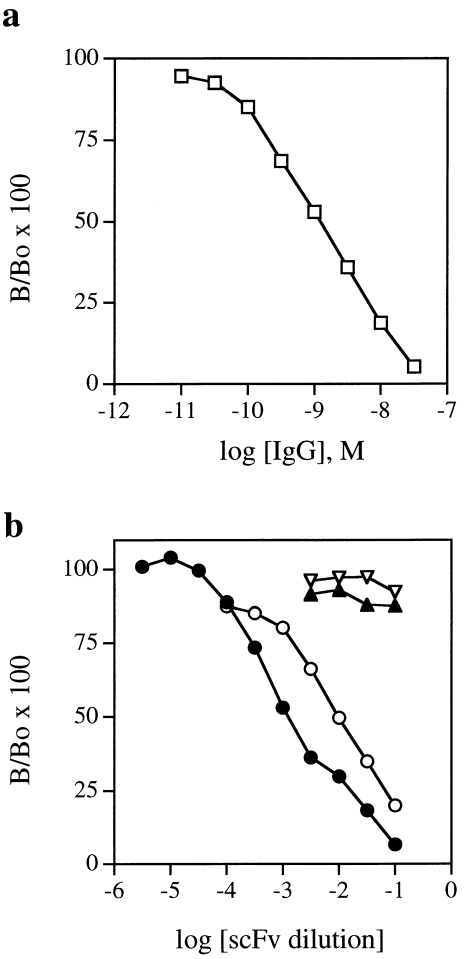

Fig. 4. AahII neutralizing capacities of IgG (a) and scFv-4C1 (b). Binding of [^{125}I]AahII (1×10^{-10} M) to rat brain synaptosomes was inhibited by incubation with IgG (open square) or periplasmic (open circle) or culture supernatant (filled circle) scFv-4C1 preparations. Periplasmic (open triangle) and culture supernatant (filled triangle) scFv A58 preparations were used as a control. B and Bo are the binding measured with (B) or without (Bo) IgG or scFv.

3.3. Immunoreactivity and specificity of scFv preparations for toxin AahII

scFv 4C1 recognized AahII in an RIA (Fig. 3a). The specificity of the recognition was assessed using the irrelevant preparation scFv A58 as control. The affinity of monoclonal antibody or scFv 4C1 for the native AahII toxin was measured by competitive RIA. The K_D values deduced from the data in Fig. 3b as in [23,36] were 0.4 nM (IgG 4C1) and 25 nM (scFv 4C1).

3.4. Neutralization of AahII binding to rat brain synaptosomes and AahII toxicity in mice by scFv preparations

Scorpion venom is lethal mainly because of AahII binding

to voltage-dependent sodium channels. The capacity of monoclonal antibody 4C1 or scFv 4C1 preparations to inhibit the effects of AahII was tested in vitro and in vivo. Both monoclonal antibody and scFv 4C1 showed dose-dependent inhibition of [^{125}I]AahII binding to its receptor on rat brain synaptosomes (Fig. 4). The irrelevant scFv A58 preparation did not inhibit binding. Half-maximal inhibition of AahII binding to synaptosomes was obtained with 1×10^{-9} M IgG 4C1 and 1/600 dilution scFv (10 mg of protein/ml).

The toxicity of AahII was measured in C57BL/6 mice by i.c.v. injection. The protective effect of scFv was evaluated by incubating AahII with an equal volume of scFv preparation and then injecting the mixture i.c.v. The controls contained the irrelevant scFv A58 preparation at the same protein concentration (5 mg/ml). scFv 4C1 ensured the survival of 50% of the mice injected after injection of 6 ng of toxin AahII (4 LD_{50}), whereas all the control mice died (Table 1). The protective capacity of scFv 4C1 was about 800 LD_{50} per ml preparation. The control scFv A58 gave no significant protection.

4. Discussion and conclusion

Recombinant scFvs are novel agents that may be more suitable than entire antibodies in certain medical applications, particularly when they are raised against soluble low molecular weight, diffusible toxins or tumor antigens [37,38]. This report describes the successful production of an scFv that binds to and neutralizes the toxin AahII from scorpion venom. We prepared an scFv from an existing well-characterized hybridoma rather than from Fv libraries by phage display technology because phage display often gives scFvs with poor affinity (10^{-5}–10^{-7} M) which are often not soluble [11,39]. The scFv-4C1 gene was assemble to ensure that the primary structures of the parent antibody and the recombinant variable regions were the same, even in the priming sites used for PCR of the variable antibody regions. Sequence differences may have an impact on antigen binding, especially for framework 1 as X-ray structures indicate that the N-terminal extremity of antibodies lies near the CDR loops. Such mutations may decrease affinity of scFvs compared to their parent antibody. The recombinant scFv-4C1 was soluble and retained the structural features that define the specificity of the parent antibody. The $(Gly_4Ser)_3$ linker sequence and reporter flag sequences incorporated had no significant effects on antigen binding. The apparent equilibrium dissociation constant of the scFv was 25×10^{-9} M, similar to the K_D of most antibodies [40]. Thus, scFv-4C1 has a high affinity for AahII. The recombinant protein was correctly produced without any detectable proteolysis, but the amounts of scFv secreted into the periplasm and released into the culture medium were quite low compared to the amount of recombinant protein trapped in the cytoplasm of the bacteria, mainly as insoluble inclusion bodies (data not shown). This might be due to such factors as the host strain, growth conditions, signal sequence and the structure of this particular scFv. Others have shown that acid residues at position 1 and 6 of the V_H framework 1, as is the case for scFv-4C1 (E1 and E6), may influence the processing of the exported protein after it has crossed the bacterial plasma membrane, and its folding efficiency [33,41]. However, the gene expression system used yielded material of a quality good enough for the direct, rapid evaluation of recombinant protein activity.

Bahraoui et al. [23] proposed two possible mechanisms of neutralization for antibody 4C1: steric hindrance due to the binding of the whole IgG to the toxin, or an overlap of the epitope 4C1 with the regions of the toxin that interact with its acceptor on the presynaptic membrane. This scFv, which is about 6 times smaller than the whole antibody 4C1, had neutralizing activity. One amino acid residue (K58) of the toxin is likely implicated in the pharmacological site and also in the epitope 4C1 [23], therefore we believe that neutralization is due to an overlap of the two sites. Modelling of the interactions between the toxin whose 3D structure is known and scFv 4C1 (whose variable domains have been identified here) might help to clarify this point. Many high resolution Fab structures are currently available, so that it should be possible to prepare a model of the combining site of scFv-4C1 from its sequence homologies with other antibodies.

In vitro and in vivo experiments indicate that scFv-4C1 has a high capacity to neutralize the pharmacological effects and lethal potency of toxin AahII (about 800 LD_{50} per ml of scFv preparation by the i.c.v. route). This promises well for the use of this molecule in detoxication. A scorpion sting must be treated with a highly diffusible antivenom, with relatively high affinity for its target toxin so as to trap the toxin, which is absorbed rapidly, becomes widely distributed and is slowly eliminated. scFv fits these requirements well and might be able to trap the toxin in the vascular and extravascular compartments before it binds to its acceptor. scFv-AahII immunocomplexes are also small, and can be easily eliminated in the urine. A production system that provides enough scFv for detailed neurophysiological and pharmacokinetic studies must now be developed. A baculovirus system using the same cloning sites as the pHEN1 vector, as recently reported, may be suitable for producing large quantities of recombinant scFv-4C1 in a medium devoid of contaminants toxic for man in contrast to *E. coli*, which can release endotoxins [20].

Thus, this report describes the first fully functional scFv that actively neutralizes the most potent toxin produced by the scorpion *A. australis* which is responsible for many serious envenomizations and some deaths in North Africa. scFvs are homogeneous, only slightly immunogenic and diffuse better than whole IgG or fragments prepared by proteolysis. We therefore believe that scFv-4C1 is a promising step towards designing new antivenoms with improved properties for immunotherapy.

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