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Construction and functional evaluation of a single-chain antibody fragment that neutralizes toxin AahI from the venom of the scorpion *Androctonus australis hector*

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9C2 is a murine monoclonal IgG that participates in the neutralization of *Androctonus australis hector* scorpion venom. It recognizes AahI and AahIII, two of the three main neurotoxins responsible for almost all the toxicity of the venom when injected into mammals. Using PCR we cloned the antibody variable region coding genes from 9C2 hybridoma cells and constructed a gene encoding a single-chain antibody variable fragment molecule (scFv). This scFv was produced in the periplasm of *Escherichia coli* in a soluble and functional form and purified in a single step using protein L−agarose beads yielding 1–2 $mg \cdot L^{-1}$ of bacterial culture. scFv9C2 was predominantly monomeric but also tended to form dimeric and oligomeric structures, all capable of binding toxin AahI. The affinity of scFv and the parental mAb for toxin AahI and homologous toxin AahIII was of the same magnitude, in the nanomolar range. Similarly, purified forms of scFv9C2 completely inhibited the binding of toxin AahI to rat brain synaptosomes. Finally, scFv9C2 was efficient in protecting mice against the toxic effects of AahI after injection of the toxin and scFv to mice by the intracerebroventricular route in a molar ratio as low as 0.36 : 1. Thus, we produced a recombinant scFv that reproduces the recognition properties of the parent antibody and neutralizes the scorpion neurotoxin AahI, thereby opening new prospects for the treatment of envenomation.

Keywords: neurotoxins; neutralization; scFv; scorpion venom.

Poisoning by scorpion venom is a major health hazard in many tropical and subtropical regions. Scorpion venoms contain toxins that act on ion channels of excitable cells and that are likely responsible for noxious effects when people are stung [1]. The venom of the North African scorpion *Androctonus australis hector* contains several potent neurotoxins that act on potential-dependent sodium channels. Toxins I, II and III (AahI, AahII and AahIII) are the major components of the venom in terms of quantity and toxicity; they are responsible for almost all of the toxicity of the venom when injected into mammals [2,3]. They belong to two distinct structural and immunological groups. Specific rabbit antisera directed against the neurotoxins of the different groups do not cross-react. Toxins AahI and AahIII are members of the same group, and toxin AahII belongs to another group [4]. At present, the only specific treatment for scorpion envenomation is purified heterologous polyclonal IgG or $F(ab)'_2$ fractions prepared from hyperimmune animal sera [5]. These antivenom molecules are not readily produced in a reproducible manner and do not diffuse easily or rapidly from the vascular compartment when injected. Fab fragments diffuse more rapidly and are distributed more widely in tissues, but their preparation by enzyme digestion is tedious, they may still have adverse effects when injected, such as early anaphylactic and late serum sickness, and their renal tolerance remains to be explored. Therefore, improved antivenom production, tolerance and efficiency are all required [6]. Highly specific monoclonal antibodies (mAb) directed against toxins of each immunological group have been produced. Our group recently showed that the *Androctonus australis* venom can be neutralized efficiently by two of these mAbs (4C1, 9C2) directed against one toxin in each immunological group [7,8]. However, the replacement of polyclonal antibody fragments with mouse or human mAb for passive immunotherapy is probably not feasible for several reasons, including human anti-mouse antibody (HAMA) response, instability of the human hybridoma cell lines, and cost [9,10].

Recombinant single-chain antibody variable fragments (scFvs) prepared from antibody-secreting hybridoma by genetic engineering have properties that mAbs or their functional fragments prepared by proteolysis [Fab and $F(ab)'_2$] do not have, and are possibly more efficient at capturing highly diffusible molecules such as low molecular mass toxins [11]. scFvs consist of the variable regions of the antibody heavy (V_H) and light (V_L) chains fused together into a single polypeptide chain via a short flexible linker. They, thus incorporate the complete antigen-combining site in a single polypeptide chain of minimal size (29 kDa). They have a homogeneous structure that remains stable even at low concentration and physiological temperature. scFvs

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Abbreviations: Aah, *Androctonus australis hector*; i.c.v., intracerebroventricular; IPTG, isopropyl thio-β-D-galactoside; RIA, radioimmunoassay; scFv, single-chain antibody variable fragment.

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exhibit accelerated biodistribution, lower retention and faster blood clearance than intact IgG or Fabs [12]. They elicit little or no immune response after administration to patients because they last only a short time in the circulation and they lack the constant domains of the antibody molecules [13]. Finally, immunocomplexes made by associating a scFv and a low molecular mass soluble antigen are small and are therefore eliminated rapidly by the kidney. For all these reasons scFvs may be the most useful material for several clinical applications including detoxification and envenoming treatments [14,15].

In a previous study we prepared from hybridoma 4C1 a fully functional scFv that actively neutralizes toxin AahII, but the production yield was low and did not allow efficient purification of the scFv [15]. Here, we report the genetic engineering, production and characterization of a soluble scFv that replicates the affinity and specificity of an anti-AahI mAb combining site. Hybridoma 9C2 was selected as a source of V_H and V_L cDNA because it secretes a particularly interesting IgG that neutralizes *Androctonus australis hector* scorpion neurotoxin I and participates in the neutralization of the toxic effect of the whole venom in mice [8]. This neutralizing capability is likely due to the very high affinity of mAb9C2 for toxin I (AahI), but also to its ability to recognize homologous toxin III (AahIII). Here, scFv9C2 was produced in large quantities suitable for purification and functional characterization. ScFv9C2 binding site replicates the affinity, specificity and toxin neutralizing ability of the parental antibody.

MATERIALS AND METHODS

Toxins

Toxins AahI, AahII and AahIII were obtained from the venom of the scorpion *Androctonus australis hector* and carefully purified and characterized in the laboratory [16].

mAb 9C2

Production of anti-AahI mAb 9C2 was as described in Clot-Faybesse *et al*. [8]. IgG from hybridoma 9C2 (IgG_{2a}, κ) were obtained by purification on Protein A–Sepharose (Amersham Pharmacia Biotech, France) and then dialysed in borate buffer saline, pH 7.9.

Cloning of V_H and V_L 9C2 genes

mRNA from freshly subcloned hybridoma 9C2 cells was isolated and first-strand cDNA synthesized as in Billiald *et al*. [17]. cDNAs encoding the antibody variable domains (V_H and V_L) were amplified by PCR under standard conditions using *Taq* polymerase (Promega, Charbonnières, France) in a thermocycler (MiniCycler, MJ Research, Inc). V_H was amplified with the forward primer V_HFor [5′-CGGGATCCTCTAGACAGTGGATA(GA)AC(AC)GAT-GG-3′], which hybridizes to the γ-chain constant domain and with the reverse primer V_{H1}Rev [5′-GCAGCAGCTA-CAGGTGTCCA(C,T)TC-3′), which hybridizes to the signal sequence region of the antibody 9C2 γ-chain. The primers for amplifying V_L were MKCFor (5′-GGATACAGTTGG-TGCAGCATC-3′), which hybridizes to the κ-chain constant domain, and MKVRev (5′-GCTGTGGCTTACAGATGCC-3′), which hybridizes to the signal sequence region of the mouse antibody κ-chain. V_H1Rev and MKVRev sequences were deduced from signal sequences registered in the EMBL data bank (Accession nos M20835 and M91701). V_HFor and MKCFor sequences have been reported previously [15]. cDNA fragments were ligated into the pGEMT vector (Promega, Charbonnières, France) and the recombinant plasmids were purified by alkaline lysis. The sequences of the cloned DNA encoding the V_H and V_L inserts were determined by Taq-Dye-Terminator-Cycle-Sequencing (Applied Biosystems 377). The sequences of the V genes were determined on two independent batches of RNA to ensure accuracy. The cDNA sequences isolated were analysed using programs from the University of Wisconsin Genetics Computer Group (Molecular Biology Software, Wisconsin Package, GCG V10.1).

Construction, expression and purification of scFv9C2

scFv9C2 was created by joining the 9C2 V_H and V_L genes together by PCR splicing with overlap extension using oligonucleotides that encoded a 15 amino-acid linker $(Gly_4Ser)_3$ between the C-terminus of the V_H and the N-terminus of the V_L gene. The ends of the variable genes were first modified by PCR using primers V_{H2}Rev (5′-CAGGTCCA-ACTGCAGCAGCCTGG-3′), which encodes the N-terminal wild-type sequence of the V_H, and LinkFor (5′-ACCACCGGATCCGCCTCCGCCTGAGGAGACTGT-GAGAGT-3′) for V_H and LinkRev (5′-GGAGGCGGATC-CGGTGGTGGCGGATCTGGAGGTGGCGGAAGCGAC-GTCCAGATGACTCAG-3′) and scFv-flagFor (5′-TAGT-AGCTCGAGCTATGTCGCAGGATTAGTTATAGTGACAG-GAGGGTCACTGGCTACTTTGATYTCCAGCTTGGT-3′) for V_L. The modified genes were purified on agarose gels and 200 ng of each fragment was combined in a PCR reaction with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and outstream primers V_{H2}Rev and scFv-flagFor. The PCR program was as reported previously [17], and the resulting 0.7 kb PCR fragment was ligated into the pGEMT vector, cloned into *Escherichia coli* and sequenced.

The scFv gene was cloned in frame with the leader sequence pelB into the expression vector pSW1 as follows [18]. The vector pGEMT containing the scFv gene was digested with *Pst*I and *Xho*I. This gave two fragments, one of 624 bp and the other of 135 bp, because of the presence of two *Pst*I restriction sites in the scFv DNA sequence. The purified 135 bp fragment restricted with *Pst*I at its 5′-end and *Xho*I at its 3′-end was inserted into the pSW1 vector restricted in the same manner. The recombinant vector was then linearized with *Pst*I, dephosphorylated with calf intestinal alkaline phosphatase and ligated to the 624-bp fragment cut at both ends with *Pst*I. The resulting plasmid was used to transform *E. coli* TG1. Clones containing both inserts fused in the right orientation were identified by PCR screening and DNA sequencing with appropriate primers. The constructed vector named pSW1-9C2 was cloned in *E. coli* HB2151. All basic molecular biology procedures were carried out as in Sambrook *et al*. [19]. The scFv gene was expressed as in Mousli *et al*. [20] with the following modifications. The recombinant bacteria were grown at $D_{600} = 1$ and induced with 0.5 mM isopropyl thio-β-D-galactoside (IPTG) at 20 °C for 16 h. Cells were pelleted

and soluble periplasmic proteins were extracted from cells by osmotic shock. The proteins were then dialysed extensively against Tris/HCl 50 mM, EDTA 5 mM, pH 7.5, clarified by centrifugation (15 000 ***g***, 4 °C, 20 min), and filtered through a 0.2-μM pore size membrane (Sartorius, Göttingen, Germany). Preparations containing the scFv protein were pooled and stored at −20 °C until use. The scFv9C2 protein was purified by loading periplasmic preparations extracted from 500 mL bacterial culture onto a column of protein L−agarose (0.5 mL; Actigen, Cambridge, UK). The column was washed with 20 mL Tris/HCl 50 mM, EDTA 5 mM, pH 7.5 and the recombinant protein was eluted in 0.5 mL fractions with glycine 0.1 M, pH 2 and immediately neutralized with Tris 1 M, pH 8.9 (70 μL). Fractions containing the recombinant protein were pooled, dialysed against Hepes 25 mM, pH 7.4 and then filtered through a 0.2-μm pore membrane (Sartorius). The integrity of the recombinant fusion protein and its purification were checked using SDS/PAGE on homogeneous 12.5% gel and Coomassie Brilliant Blue or silver-staining. Western blotting with anti-flag mAb MRC-OX74 was also carried out for the specific detection of the recombinant scFv [15,21]. The dot-immunoblotting protocol was adapted from Mousli *et al*. [20]. Samples were applied directly to a nitrocellulose membrane for 1 h without any treatment with SDS or 2-mercaptoethanol. The membrane was blocked with BSA 4% and then incubated for 30 min with protein L−peroxidase (Actigen, Cambridge, UK) and developed with Opti-4CN™ substrate as recommended by the supplier (Bio-Rad, Ivry, F).

The protein L-purified scFv preparations were resolved by size-exclusion FPLC on a Superdex™ 75 HR10/30 column (resolution range: 3000–70 000; Amersham Pharmacia Biotech, France). The column was calibrated with the following standards from Boehringer: IgG (150 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.5 kDa). Sample volumes were 200 μL. Elution was performed at 0.5 mL·min^{-1} with borate buffer saline, pH 7.9. The protein content of IgG or mAb fragment solutions was measured by UV spectrophotometer at $A_{278} = 1.5$ cm^2·mg^{-1}. Concentration procedures were performed by ultrafiltration with a 10K (IgG) or 3K (scFv) Microsep device (Filtron).

Immunoassays

The binding of scFv preparations to ^{125}I-labelled AahI was assessed by radioimmunoassay (RIA). Toxin AahI was labelled as described in Rochat *et al*. [22]. Twenty-five microlitres of each dilution of different concentrations of IgG or scFv was mixed with 25 μL of ^{125}I-labelled AahI (0.5×10^{-10} M) in NaCl/P$_i$/0.1% BSA (final volume: 150 μL). The mixtures were incubated for 90 min at 37 °C and then overnight at 4 °C. Bound antigen was separated from free antigen by adsorption of the free antigen onto activated charcoal. A suspension (0.5 mL) containing 0.8% charcoal (Sigma) and 0.08% dextran T-70 (Sigma) in NaCl/P$_i$−0.2% BSA was added to each tube, and the mixtures were incubated for 10 min at 4 °C and then centrifuged at 9000 ***g*** for 10 min. The radioactivity of the supernatants was measured with a gamma counter (RIASTAR, Packard). All assays were performed in duplicate. For competitive experiments, 25 μL of IgG at 0.2×10^{-9} M or scFv9C2 at 1×10^{-9} M was mixed with 25 μL of ^{125}I-labelled AahI (0.4×10^{-10} M) in the presence of a series of concentrations of unlabelled AahI, AahII and AahIII. Results are expressed as B/B_o, where B and B_o are the radioactivity bound to antibody in the presence (B) or absence (B_o) of unlabelled ligand.

Neutralization assays

Receptor binding assay. The rat brain synaptosomal fraction was prepared as described previously [23]. 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. Thirty microlitres of ^{125}I-labelled AahI (1.2×10^{-10} M) was incubated with 30 μL of each of a series of dilutions of IgG or scFv preparations for 90 min at 37 °C. The synaptosomal preparation was added (1 mg·mL^{-1}, 60 μL) and the incubation was continued for an additional 30 min at 37 °C. The mixtures were centrifuged at 9000 ***g*** and washed three times. The radioactivity of the pellets was measured. Assays were performed in duplicate.

Assays in vivo. The neutralizing capacity of scFv was tested via the intracerebroventricular (i.c.v.) route. Amounts of AahI equal to or higher than the value of the lethal dose (LD_{50} = 12 ng for 20 g C57BL/6 mice) were pre-incubated for 90 min at 37 °C with an equal volume of scFv preparations. Female C57BL/6 mice (groups of six) were injected via the i.c.v. route. Surviving mice were recorded after 24 h. Mice were raised and housed in the conventional facilities of the laboratory. Rooms and experiments were in accordance to the European guideline (no. 86/609/CEE) on animal welfare.

RESULTS

Construction and purification of scFv9C2

Hybridoma 9C2 secretes an IgG$_{2a}$ (kappa chain) that has a high affinity for an epitope common to two (AahI and AahIII) of the three most potent neurotoxins of the scorpion venom. To generate a scFv fragment, the V_H and V_L genes were cloned from cDNA and joined by a sequence encoding the 15 amino-acid residue flexible (Gly$_4$Ser)$_3$ linker in a PCR amplification. The resulting DNA was inserted into the bacterial expression vector pSW1. The sequences of the V_H and V_L domains assembled in the scFv gene were the same as those obtained after cloning V_H and V_L in pGEMT. Primers VH2Rev, LinkFor, LinkRev and scFv-flagFor used to construct the scFv gene were designed so that they matched the ends of the variable domain cDNA and did not introduce any mutation in the deduced amino-acid sequence of the antibody variable domains. This procedure is to be emphasized because the introduction of mutations into the amino-acid sequence of V_H or V_L FR1 domains could influence both the activity and production yield of the recombinant antibody fragment. No mutation was introduced by *Pfu* polymerase, which was selected for the assembling PCR amplification because it has one of the lowest error rates of any of the thermostable DNA polymerase tested to date. Figure 1 shows the nucleotide and deduced amino-acid sequences of the scFv9C2 gene.

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            PstI
CAG GTC CAA CTG CAG CAG CCT GGT GCT GAG GTT GTG AAG CCT GGG GCC TCA    51
 Q   V   Q   L   Q   Q   P   G   A   E   V   V   K   P   G   A   S    17

GTG AGG CTG TCC TGC AAG GCT TCT GGC TAC TCT TTC ACC AGC AAC TGG ATA   102
 V   R   L   S   C   K   A   S   G   Y   S   F   T   S   N   W   I    34
                                                      CDR H1

AAC TGG GTG AAA CAG AGG CCT GGA CAA GGC CTT GAG TGG ATT GGA AAT ATT   153
 N   W   V   K   Q   R   P   G   Q   G   L   E   W   I   G   N   I    51

TCT CCT GGT GGT AGT AAC ACT AAC CAC AAT GAG AAG TTC AAG AGT AAG GCC   204
 S   P   G   G   S   N   T   N   H   N   E   K   F   K   S   K   A    68
                        CDR H2

ACA CTG ACT GCG GAC ACA TCC TCC AGC ACA GCC TAC ATG CAG CTC AGC AGC   255
 T   L   T   A   D   T   S   S   S   T   A   Y   M   Q   L   S   S    85

CTG ACA TCT GAC GAC TCT GCG GTC TAT TAT TGT GCA AGA TAC GAT GGT GAC   306
 L   T   S   D   D   S   A   V   Y   Y   C   A   R   Y   D   G   D   102

TAC GGC TCC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA   357
 Y   G   S   F   D   Y   W   G   Q   G   T   T   L   T   V   S   S   119
    CDR H3

GGC GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC GTC   408
 G   G   G   G   S   G   G   G   G   S   G   G   G   G   S   D   V   136

CAG ATG ACT CAG TCT CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC   459
 Q   M   T   Q   S   P   A   S   L   S   V   S   V   G   E   T   V   153

ACC ATC ACA TGT CGA GCA AGT GAG AAT ATT TAT CGT AAT TTA GCA TGG TAT   510
 T   I   T   C   R   A   S   E   N   I   Y   R   N   L   A   W   Y   170
                             CDR L1

CAG CAG AAA CAG GGA AAA TCT CCT CAG CTC CTG GTC TAT GCT GCA ACA AAT   561
 Q   Q   K   Q   G   K   S   P   Q   L   L   V   Y   A   A   T   N   187
                                                        CDR L2

TTA GCA GCT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG   612
 L   A   A   G   V   P   S   R   F   S   G   S   G   S   G   T   Q   204

                            PstI
TAT TCC CTC AAG ATC AAC AGC CTG CAG TCT GAA GAT TTT GGG AGT TAT TAC   663
 Y   S   L   K   I   N   S   L   Q   S   E   D   F   G   S   Y   Y   221

TGT CAA CAT TTT TGG AAT ATT CCA TTC ACG TTC GGC TCG GGG ACC AAG CTG   714
 C   Q   H   F   W   N   I   P   F   T   F   G   S   G   T   K   L   238
        CDR L3

GAG ATC AAA GTA GCC AGT GAC CCT CCT GTC ACT ATA ACT AAT CCT GCG ACA   772
 E   I   K   V   A   S   D   P   P   V   T   I   T   N   P   A   T   253

   Xho I
TAG CTC GAG
 *   L   E
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Fig. 1. Nucleotide and deduced amino-acid sequences of AahI-specific scFv9C2. The nucleotide sequences corresponding to the restriction sites *Pst* 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 (CDR) of V_H and V_L are underlined.

The deduced amino-acid sequences were compared with those of variable regions of immunoglobulins stored in the SWISSPROT database. We identified the three loops corresponding to the complementary determining regions of the V_H and V_L regions, and the amino-acid residues involved in the canonical structures. The heavy chain of 9C2 showed 85.34% similarity with the V_H of an antibody directed against 4-hydroxy-3-nitrophenyl acetyl (Accession no. B22769). Likewise, 86% similarity was found between 9C2 light (kappa) chain and the closest antibody V_L sequence belonging to an antibody directed against cytochrome *c* (Accession no. S19112). The cDNA sequences of antibody 9C2 V_H and V_L are now registered in the EMBL data bank (Accession nos AJ278443 and AJ278442). The scFv gene was expressed by growing the recombinant bacteria induced with IPTG. Intact recombinant scFv was found in the periplasm as indicated by Western blot using the antitag serum MRC-OX74 and dot-immunoblotting using protein L–peroxidase (Fig. 2). Affinity purification of scFv9C2 was carried out using agarose beads conjugated to recombinant protein L that bind specifically to the κ light chain variable domain of many immunoglobulins [24]. The periplasmic extracts from bacteria induced with IPTG for the production of the scFv were loaded onto the column and eluted fractions were collected and checked for the presence of the recombinant scFv. Appropriate fractions were pooled and a typical 1 L recombinant bacteria culture led to the production of 1–2 mg of recombinant soluble scFv9C2 (Fig. 2, lane 4). When periplasmic extracts from non-induced bacteria were loaded onto the column under similar conditions, no adsorbed proteins were detected.

Characterization of scFv9C2 preparations

Protein L-purified scFv showed a single band at ≈ 29 kDa when analysed by SDS/PAGE without mercaptan (Fig. 3B, lane 2). However, gel filtration of affinity-purified scFv on a Superdex 75 column revealed the presence of three peaks (Fig. 3A). Peak 1 (90–100 kDa), peak 2 (57 kDa) and peak 3 (30 kDa) represented, respectively, 21, 32 and 47% protein recovery. The elution profile was similar when a same volume of unconcentrated (0.185 mg·mL^{-1}) or concentrated (0.9 mg·mL^{-1}) preparations were analysed. All fractions (peaks 1–3) showed a single band at 29 kDa in SDS/PAGE (Fig. 3B, lane 4–6) and were capable of binding to AahI as determined by RIA using ^{125}I-labelled AahI (data not shown). Taken together, these observations allowed us to determine that the three peaks

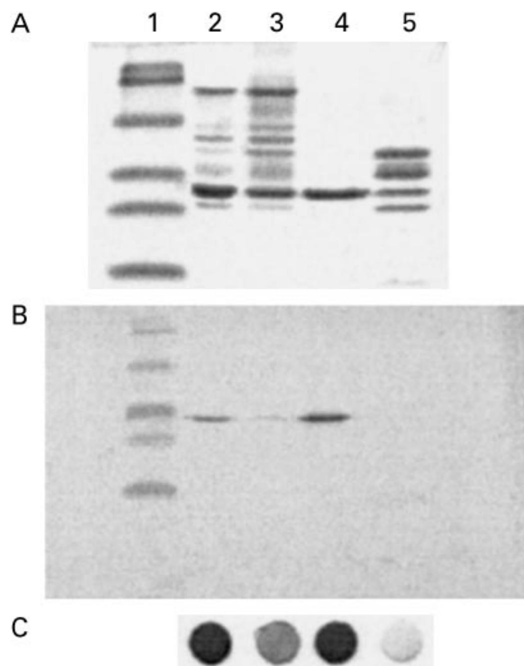

Fig. 2. Periplasmic production and protein L purification of a recombinant protein from pSW1-9C2 cultures in *Escherichia coli* HB2151. (A) SDS/PAGE stained with Coomassie Brilliant Blue. (B) Western blot analysis with antiflag MRC-OX74. (C) Dot immunoblotting with protein L−peroxidase. Lane 1, Molecular mass standards (102, 78, 49.5, 34.2, 28.3, 19.9 kDa); lane 2, periplasmic fraction of induced bacteria loaded onto a protein L−agarose column; lane 3, protein L−agarose column flow through fraction; lane 4, protein L–agarose column eluted fraction; lane 5, periplasmic fraction of uninduced bacteria.

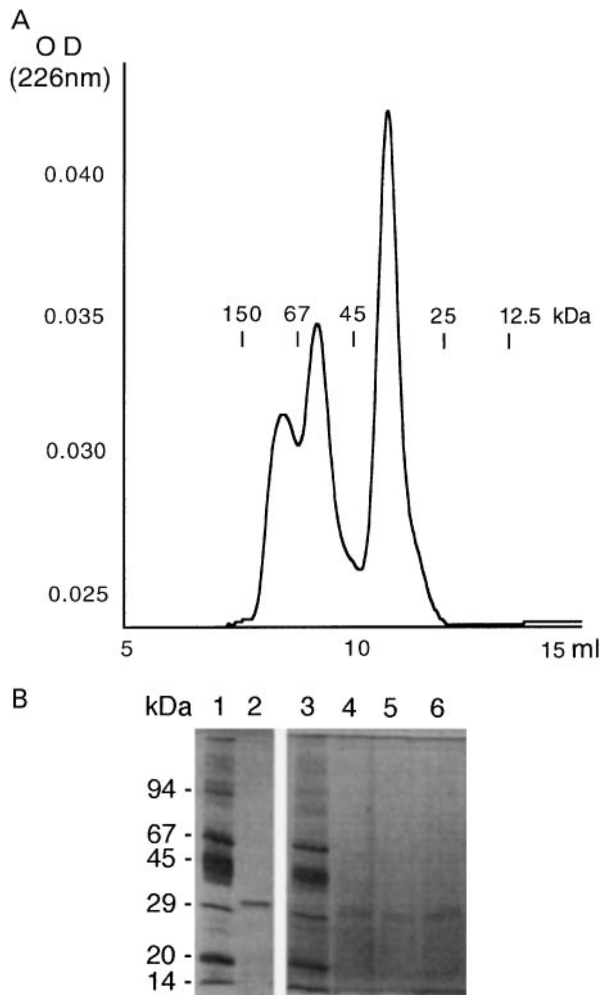

Fig. 3. ScFv characterization using Superdex S75 column. (A) The elution profile shows the different oligomeric forms present in protein L-purified scFv9C2. The elution volumes of marker proteins are indicated. (B) SDS/PAGE on silver-stained homogeneous 12.5% gel. Lanes 1 and 3, molecular mass standards (kDa); lane 2, protein L-purified scFv; lane 4, peak 1; lane 5, peak 2; lane 6, peak 3.

corresponded to different oligomeric states or a mixture of higher multimeric forms.

Immunoreactivity and specificity of scFv9C2 preparations for Aah toxins

Protein L-purified scFv preparation and pooled fractions from peaks 2 and 3 recognized ^{125}I-labelled AahI in a RIA and showed identical dose–response curves. The signal obtained when using complete IgG from 9C2 hybridoma (IgG9C2) was only slightly higher (Fig. 4A). The affinity and specificity of toxin recognition by scFv9C2 preparations were then assessed by competitive RIA with increasing concentrations of unlabelled AahI. The protein L-purified scFv9C2 preparations and the eluted fractions corresponding to peaks 2 and 3 showed competitive curves similar to that of IgG9C2 for AahI (Fig. 4B). The dissociation constants (K_d) for the interaction of AahI with protein L-purified scFv preparation or IgG9C2 were calculated using the Muller formula [25], K_d = 3/8 [I − T]

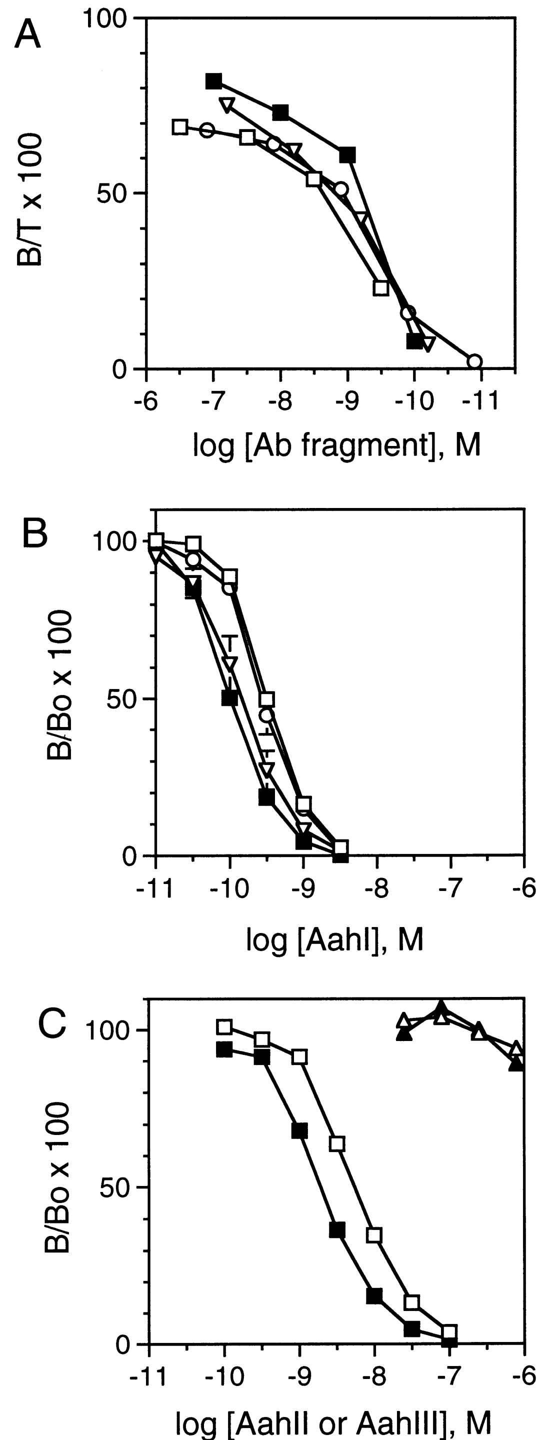

Fig. 4. Immunoreactivity of scFv9C2 preparations tested by radioimmunoassay. (A) Serial dilutions of different preparations of scFv9C2, protein L-purified scFv (□), Superdex S75 peak 2 (△), peak 3 (○) and IgG9C2 (■) were tested with ^{125}I-labelled AahI. (B) Competitive RIA were performed as described in Materials and methods through serial dilutions of unlabelled AahI. (C) Cross-reactivities of scFv9C2 (open symbols) and IgG9C2 (filled symbols) were tested using serial dilutions of unlabelled AahII (triangles) and AahIII (squares). B and B_o are the binding measured in the presence (B) or absence (B_o) of unlabelled ligand.

Table 1. ***In vivo*** **neutralizing capacity of scFv or IgG fraction.**

Antibody fragment	Molar ratio AahI : antibody	Injected AahI		Protected/ injected mice	Protective capacity [a]
		ng per mouse	LD_{50} per mouse		
scFv9C2	0.18 : 1	93	8	6/6	
	0.28 : 1	144	12	6/6	
	0.36 : 1	185	15	5/6	$< 6600\ LD_{50}\cdot mg^{-1}$ $< 200\ LD_{50}\cdot nmol^{-1}$
	0.45 : 1	185	15	1/6	
IgG9C2	1.5 : 1	272	22	5/6	$< 5500\ LD_{50}\cdot mg^{-1}$ $< 840\ LD_{50}\cdot nmol^{-1}$
IgG nonimmune	0.22 : 1	25	2	1/6	

[a] The protective capacity of the antibody fragment was expressed as the number of LD_{50} neutralized by mg (or nmol) of scFv or IgG9C2.

where I = IC_{50} and T = [^{125}I-labelled AahI]. They were 1.5×10^{-10} M (scFv9C2) and 0.2×10^{-10} M (IgG9C2).

The cross-reactivity of protein L-purified scFv preparation with the other main toxins of the Aah venom, AahII and AahIII, was determined in a similar competitive RIA with increasing concentrations of AahII or AahIII (Fig. 3C). IC_{50} values for AahIII were 6.3×10^{-9} M (scFv9C2) and 2.5×10^{-9} M (IgG9C2). As already demonstrated for IgG9C2 [8] no cross-reactivity was detected for scFv9C2 and AahII.

Neutralizing potency of scFv9C2 preparations to the AahI binding on rat brain synaptosomes and to AahI toxicity in mice

The capacity of scFv9C2 to inhibit the biological effects of AahI was tested both *in vitro* and *in vivo*. The inhibition of the specific binding of ^{125}I-labelled AahI on rat brain synaptosomes was determined. The protein L-purified scFv9C2 and gel filtration-eluted fractions (peaks 2 and 3) of scFv showed dose-dependent inhibition (Fig. 5). No inhibition was detected when nonimmune IgG or irrelevant scFv were used as a control (not shown). Concentrations of 12×10^{-10} M protein L-purified scFv9C2 and 1.6×10^{-10} M IgG9C2 were able to inhibit half of the binding of ^{125}I-labelled AahI (0.4×10^{-10} M) on synaptosomal preparation.

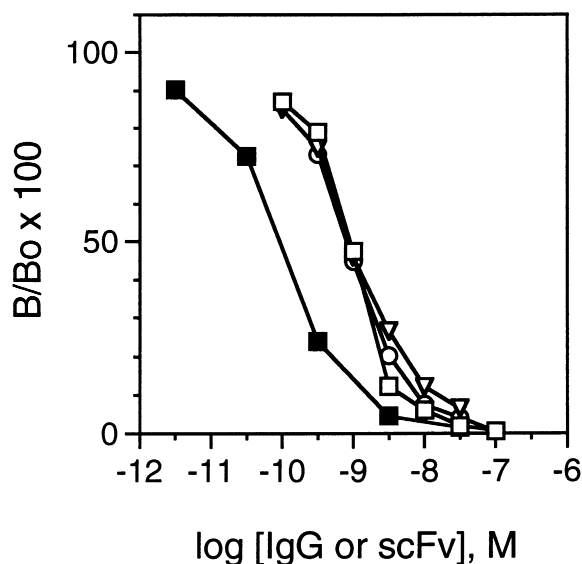

Fig. 5. AahI neutralizing capacity of scFv9C2 preparations tested by receptor assay. Binding of ^{125}I-labelled AahI to rat brain synaptosomes was inhibited by pre-incubation with various dilutions of protein L-purified scFv9C2 (□), Superdex S75 peak 2 (△), peak 3 (○) and IgG9C2 (■). B and B_o are the binding measured in the presence (B) or absence (B_o) of scFv or IgG.

The protective effect of scFv was evaluated by preincubating different amounts of AahI (LD_{50} = 12 ng) with an equal volume of purified scFv ($0.9\ mg\cdot mL^{-1}$) and then injecting the mixture via the i.c.v. route into C57BL/6 mice (six per AahI concentration). Controls were performed in the same way with IgG9C2 ($2\ mg\cdot mL^{-1}$) or mouse nonimmune IgG ($1.2\ mg\cdot mL^{-1}$). In the presence of scFv9C2, almost all the mice injected survived the injection of 15 LD_{50} when the ratio toxin/scFv ratio was 0.36 : 1, however, none survived when the ratio was 0.45 : 1 (Table 1). The protective capacity of scFv9C2 was therefore ≈ 6600 LD_{50} per mg of preparation (0.36 nmol of AahI per nmol of scFv). In the same way, the protective capacity of IgG9C2 was ≈ 5500 LD_{50} per mg of preparation (1.5 nmole of AahI per nmol of IgG). No significant protection was observed with the nonimmune IgG control.

DISCUSSION

scFvs are recombinant antigen-binding molecules with structural and functional properties that make them potentially more suitable than entire IgGs, or other antibody fragments, for medical applications in which the biological function of the antibody Fc domain is not required [26]. These applications include *in vivo* imaging with scFvs directed against tumour antigens [27], and *in vivo* detoxification with scFvs directed against highly diffusible drugs, such as digoxin, or low molecular mass animal toxins, such as scorpion neurotoxins [15,28]. In addition, scFvs genetically fused to an enzymatic tracer, such as alkaline phosphatase, luciferase or acetylcholinesterase, should provide suitable tools for developing rapid one-step immunoassays to monitor a patient's serum toxin

concentrations in order to assess envenomation severity [20,29–31]. Here, we produced and characterized a scFv that neutralizes AahI, one of the most potent neurotoxins from the venom of the scorpion *Androctonus australis hector* likely to be responsible for the lethal effect of stings. scFvs are usually prepared by assembling variable domain cDNA obtained either from a pre-existing hybridoma secreting a well-characterized IgG or from a combinatorial library, and they are then produced as soluble free protein or at the surface of phage. Here, scFv9C2 was designed from an existing neutralizing IgG molecule, with sub-nanomolar affinity for toxin AahI and homologous toxin AahIII, rather than by screening combinatorial libraries by the phage-display technology. The latter approach is often preferred because it allows the selection of human or mouse antibody fragments from naive libraries without the need for animal immunization, a tricky task, particularly when the immunogen is a low molecular mass, poorly immunogenic and highly toxic component [8]. However selection by the phage-display technology of antibody fragments against soluble toxins generally leads to the production of scFvs with low solubility and reduced binding affinity, in the range 10^{-5} to 10^{-7} M, making these scFvs unsuitable for therapeutic use [32,33].

The scFv9C2 gene was assembled so that the primary structures of the parent antibody and the recombinant Fv were the same, even in the priming sites used for PCR of the variable antibody regions. Recent studies indicate that it is essential to preserve the N-terminal wild-type sequence of the variable domains for at least two reasons. First, N-terminal substitutions in V_H might affect bacterial expression and second, framework 1 of antibody variable domains (V_H and V_L) may provide the contact required for close association of antibody with antigen, while the CDRs dictate the fine specificity and strength of binding [34,35]. The expression system used here provided an efficient process for the continuous production of a high amount (1–2 $mg{\cdot}L^{-1}$ of bacterial culture) of specific and active soluble scFv9C2 in a short time and at low cost. Laborious procedures, such as sonication followed by detergent extraction, denaturation and re-folding, were not required. The possibility of isolating the soluble and functional scFv9C2 in a one-step affinity purification using protein L–agarose or of detecting it using protein L–peroxidase conjugate also means that the C-terminal peptide tag MRC-OX74 was not needed to monitor the purification procedure [24]. This is of interest in view of therapeutic applications of this scFv, because a peptide flag may contribute to side-effects. The purified scFv fraction showed a single band in SDS/PAGE migrating at ≈ 29 kDa, but as it is often observed for scFv constructs, it exhibited several multimeric forms [36], monomeric and dimeric structures representing 47 and 32%, respectively. Each of these forms retained the structural features that define the specificity of the parent antibody. They were capable of binding toxin AahI with a K_d of $\approx 1.5 \times 10^{-10}$ M, very close to that of the parent IgG (0.2×10^{-10} M). In addition, scFv9C2, like IgG9C2, recognized toxin AahIII. These two toxins belong to the same immunological group [4] and their sequences are 79% identical. They are well represented in *Androctonus australis hector* venom [37] and contribute to the high toxicity of this venom in mammals (LD_{50} of 380 and 480 ng per 20 g mouse, respectively, via the subcutaneous route), as does toxin AahII (subcutaneous LD_{50} of 180 ng per 20 g mouse), which belongs to another immunological group. As often reported, the affinity of scFvs was slightly lower than that of the parental IgG. This may be related to several factors, including not only the absence in the scFv structure of constant regions that contribute to stabilizing the IgG structure, but also the incorporation of the $(Gly_4Ser)_3$ linker sequence between the V_H and V_L domain, which may distort the antigen-binding pocket. The presence of a reporter flag sequence of 14 residues at the C-terminal extremity of the molecule may also participate in the decreased affinity of the scFv. Whatever the explanation, scFv9C2 preparations are all active and exhibit a high affinity in the nanomolar range as shown by the RIA data reported here. This high affinity for AahI is a promising step towards clinical applications of scFv9C2. Cano *et al.* [38] demonstrated that IgG with an affinity in the nanomolar range for digoxin can reduce the free toxin plasma level by 90% and reverse the drug normal distribution in rats when injected 30 min after digoxin infusion in a stoichiometric IgG/drug ratio.

Because the lethal effect of scorpion venom is due mainly to the binding of Aah toxins (AahII, AahI and AahIII) to voltage-dependent sodium channels [39], we measured the neutralizing potency of scFv9C2 in an inhibition test using rat brain synaptosomal preparation. All purified fractions of scFv9C2 completely inhibited the binding of ^{125}I-labelled AahI to its site with an IC_{50} of 12×10^{-10} M, slightly lower than that of IgG (1.6×10^{-10} M). In addition, as found for mAb9C2, scFv9C2 was efficient in protecting mice against the toxic effect of the AahI following injection into mice via the i.c.v. route in a molar toxin/scFv ratio as low as 0.36 : 1. The neutralizing capacity of scFv9C2 was ≈ 0.36 nmol of AahI per nmol of purified scFv (29 μg), whereas that of the parental bivalent IgG was ≈ 1.5 nmol per nmol of IgG (150 μg). This indicates that highly diffusible scFvs should be efficient in trapping the toxin in the body compartments before it binds to its receptor. Because scFvs do not accumulate in the tissues, the immunocomplexes made of a scFv and a scorpion neurotoxin should be eliminated quickly from the body via urine.

This report and a recent study [15] demonstrate that scFvs derived from antibody-secreting hybridoma are promising tools for the design of new antivenoms. They are among the smallest functional modules of antibodies required for high-affinity binding of antigen; they are therefore highly diffusible, potentially suitable for reversing the toxin's normal tissue distribution in the body, and only minimally, if at all, immunogenic. Thus, a cocktail of merely two scFvs, one neutralizing toxins AahI and AahIII (scFv9C2) and the other neutralizing toxin AahII (scFv4C1), might be efficient enough to neutralize the toxicity of the whole venom. This hypothesis is strongly supported by Clot-Faybesse *et al.* [8] who showed that a mixture of mAbs 9C2 and 4C1 can efficiently neutralize the Aah venom toxic fraction when injected into mice.

However, although scFvs penetrate deeper into tissues than $F(ab')_2$ and IgG, they might not be retained for long, and too rapid clearance would be a problem in clinical applications [40]. Recently, bivalent scFvs have emerged as more promising therapeutic agents in several circumstances,

revealing increased tissue retention, optimized clearance properties and improved *in vivo* stability [41,42]. Because scFv9C2 spontaneously forms bivalent and functional dimeric structures it would be of interest to analyse their proper *in vivo* biodistribution and pharmacological properties. In addition, covalently associated sc(Fv)$_2$ diabody Q2 made of the variable domains of antibodies 9C2 and 4C1 could be produced, thereby opening up the possibility of creating a novel generation of antivenom made of a single molecule with two antigen-binding sites with distinct specificity: one directed against the most potent neurotoxin of the venom (AahII) and the other capable of neutralizing the toxic effects of one of the other two toxins (AahI and AahIII).

In summary, scFv9C2 retained the biological properties of the parent IgG9C2. It recognized AahI in a nanomolar affinity range, cross-reacted with the toxin of the same immunological group (AahIII), and was able to neutralize the AahI toxin. This scFv construct is then a valuable tool to be further engineered for use in therapeutic and diagnostic applications.

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