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Research Article

Using a recombinant bispecific antibody to block Na^+-channel toxins protects against experimental scorpion envenoming

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Abstract. In recent years, several molecular engineering methods of designing bispecific antibodies in various formats have been developed. Tandem-scFvs comprising two scFvs fused together via a peptide are 55-kDa molecules, and are one of the most promising and most straightforward approaches to bispecific antibody production. We report an attempt to design more effective antivenoms to the *Androctonus australis* scorpion using murine scFvs as building blocks to create a unique bispecific molecule that neutralizes the potent neurotoxins *Aah*I and *Aah*II. The tandem-scFv was produced in recombinant bacteria, purified by immobilized metal ion affinity chromatography, and analyzed by polyacrylamide gel electrophoresis, Western blot, gel filtration, mass spectrometry, and direct and competitive radioimmunoassay. *In vivo*, it neutralized the binding of the *Aah*I and *Aah*II toxins to their receptor, and protected mice against experimental envenomation. The findings reported here highlight the potential of recombinant antibody fragments for protecting against scorpion venom toxicity.

Keywords. Scorpion, toxin, immunotherapy, antibody engineering, bispecific antibody, tandem-scFv.

Introduction

Scorpion stings are a common hazard facing human beings in tropical and subtropical regions, especially in North Africa, Central and South America, India and the Middle East, where they constitute a serious public-health problem [1]. Accurate epidemiological data are not available, but approximately 200 000 scorpion stings are known to occur each year in Mexico [2], 45 000 in Tunisia [3] and 18 000 in Saudi Arabia [4].

Scorpion venoms are complex mixtures containing mucopolysaccharides, phospholipases, proteases inhibitors, bioamines but relatively low levels of toxins. Scorpion toxins target the sodium, potassium, calcium and chloride channels, causing direct effects and triggering the release of neurotransmitters such as catecholamines, and leading to signs of autonomic system overactivity. Immediate local signs of moderate envenoming, characterized by intense local pain, appear a few minutes after the sting and are usually treated with local anesthetics. Systemic clinical signs (fever, sweating, hypertension, vomiting and priapism) are observed 2–4 h after the sting and supportive treatment remains mostly symptomatic [5]. Patients with severe envenomation are usually kept under close surveillance in an intensive care unit, the main causes of death being cardiovascular shock, respiratory failure or pulmonary edema.

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The only specific treatment for severe scorpion envenomations is serum therapy, which is widely used in many areas, such as North Africa and Mexico. Significant reduction in mortality rates, particularly in children and elderly people, has been attributed to the intensive serum therapy campaigns conducted over the last few years particularly in Mexico, where there were 70 deaths in 2002 as compared to 700 in the 1970s [2]. However, the usefulness and efficacy of antivenoms have also been questioned for several reasons [6]. First, the low molecular weight of scorpion neurotoxins means that they diffuse extremely rapidly into tissues and bind to their target almost irreversibly, and some clinicians consider that the time between the scorpion sting and the administration of the antivenom is often too long to enable IgGs or $F(ab')_2$ to bind to the free toxins [7]. The low specific activity of the conventional antivenoms that are still made from equine polyclonal antibody fragments raised against the whole venom is another potential cause of ineffectiveness. This low activity means that patients are injected with large quantities of heterologous proteins, even though the quantity of lethal toxins in the venom is low, thus increasing the potential risk of major immune adverse reactions, such as serum sickness and anaphylaxis [8].

Over the last decade, antibody engineering has provided vastly enhanced opportunities for designing novel antigen-binding molecules with tailor-made properties for diagnostic, imaging and therapeutic applications [9, 10]. Novel antibody formats with improved pharmacokinetic properties, and greater affinity or specificity for their target have emerged as credible alternatives to conventional drugs and immunochemical reagents [11–13]. The rapid increase in the availability of these potentially therapeutic molecules, plus the limitations of conventional antivenoms have led to a search for anti-scorpion toxin antibodies and the design of new antibody formats with promising specific activity, stability, tissue penetration and neutralizing properties. Pioneering studies have been carried out by designing murine recombinant single-chain antibody fragments (scFvs) or Fabs that neutralize individual neurotoxins [2, 14, 15]. More recently, human scFvs directed against the scorpion neurotoxin *Cn*II were selected using the phage-display technology [16]. In addition, camelids antibodies with particular features conferring therapeutic effects have been produced against *Androctonus australis* scorpion toxins [17].

A. australis is one of the most dangerous scorpions in the world, which is responsible for serious envenomations in North Africa. When tested in mice, almost all the toxicity of the venom is accounted for by three small basic neurotoxins of about 7 kDa that act on the voltage-gated sodium channels of excitable cells. These toxins correspond to less than 5% of the total protein fraction of the venom, and belong to two distinct structural and immunological groups: group I (the *Aah*I and *Aah*III toxins) and group II (*Aah*II) [18]. Recombinant scFvs, which are the smallest antigen-binding entities consisting of the variable domains of an antibody linked together via a 15-residue peptide, have been produced for both these groups of toxins [19, 20]. They do indeed neutralize the activity of the toxins, but their short *in vivo* half-lives make them unsuitable for clinical use. A bivalent diabody (a dimer of an scFv with a linker reduced to 5 residues), which is similar in size to Fabs (50 kDa), has proved to be more effective than the homologous monovalent scFv in terms of its structural stability and time of residence in the body. It is able to protect mice experimentally challenged with a single toxin (*Aah*I). However, due to structural polymorphism between *Aah*I and *Aah*II, it fails to protect mice challenged with the whole venom, which makes it of limited therapeutic potential [14].

Protection against the toxicity of the whole venom will certainly involve designing a bispecific small molecule (50 kDa) able to neutralize toxins from groups I (*Aah*I and *Aah*III) and II (*Aah*II). There are several possible basic strategies, since single-chain diabodies (scDb) and tandem-scFvs are both single polypeptide chains that are more stable than heterodimeric bispecific diabodies (Fig. 1) [21].

Here, we report the production of a bispecific tandem-scFv directed against the *Aah*I and *Aah*II toxins. This molecule consists in two scFvs joined together at the genetic level by a cDNA sequence encoding a peptide linker. Each scFv moiety comprises variable domains (VH and VL), which both have the same specificity. Using detailed biochemical analysis we demonstrate that significant yields of fully functional tandem-scFv can be obtained from recombinant bacteria. In addition, immobilized metal ion affinity chromatography (IMAC)-purified tandem-scFv neutralizes the most potent toxins in *A. australis* venom, and protects experimentally envenomed mice against the overall toxicity of the venom. These findings confirm the potential ability of recombinant antibodies to neutralize scorpion toxins, and constitute another step towards a new generation of antivenoms.

Materials and methods

Venom, toxins and anti-toxin antibodies

Venom and toxins were prepared as in [22]. IgG and recombinant anti-*Aah*I antibody fragments derived from hybridoma 9C2 [Fab, $scFv_{15}9C2$, and $scFv_5 9C2$ (also known as diabody 9C2)] were produced and purified as previously described [14, 23]. Murine polyclonal sera specific for the *Aah*I or *Aah*II toxins have been characterized previously [24].

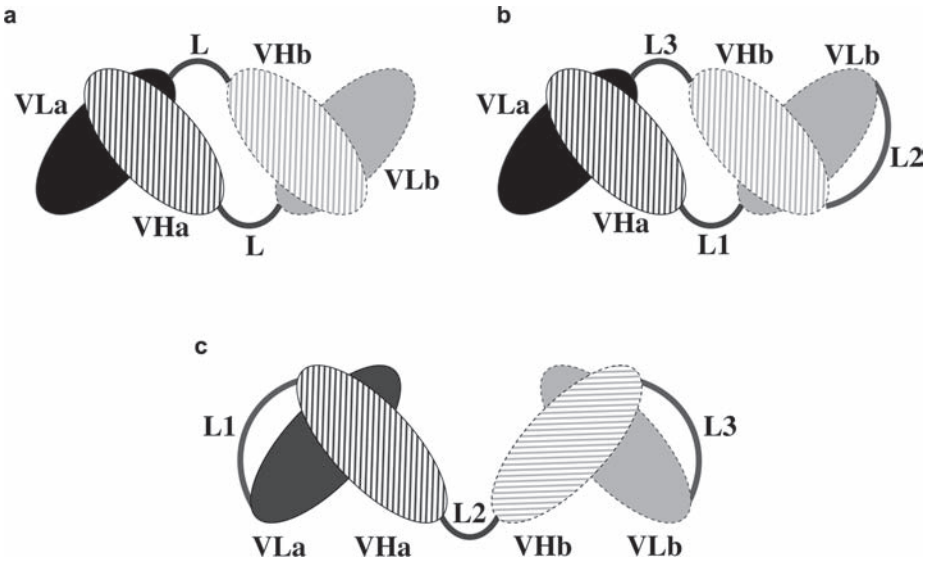

Figure 1. Schematic representation of small, bispecific antibody fragments restricted to antibody V domains. (*a*) Heterodimeric diabody; (*b*) single-chain diabody; (*c*) tandem-scFv. L1-3: flexible peptide linkers; VH and VL: heavy and light variable domains of antibodies (a) and (b).

Oligonucleotides

All oligonucleotides were synthesized by Sigma-Proligo (Paris, France). The sequences of the oligonucleotides are shown below. The restriction sites of interest [*Hin*dIII (AAGCTT), *Bss*HII (GCGCGC), *Bam*HI (GGATCC), *Nhe*I (GCTAGC), *Xho*I (CTCGAG)] are underlined, and the cDNA sequences encoding either part of the linkers or the His_6 flag are shown in italics. pNA1Rev: 5′-AC GCC AAG CT T GCC AAA TTC TAT TTC AAG GAG ACA GTC ATA ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC; pNA1For: 5′-GTG ACC CTC GAG TTA GGC ATG CGC GCC GCT TGC TGC GAG TAA TAA CAA TCC AGC GGC TGC CG; L9E2Mrev: 5′-AAG TGC GCG CAT GCC GAC GTC CAG ATG ACT; L9E2MFor: 5′-*CT ACC GGA TCC TTC GCT ACT CTT GCC GCT ACC GGA AGT AGA GCC* TTT GAT CTC CAG CTT GGT; H9E2Mrev: 5′-*GC GAA GGA TCC GGT AGC ACT AAA GGT* CAG GTC CAA CTC CAG CAG CC; H9TandFor: 5′-*CTC GAG CTC GAG TTA GCT AGC ACC GCC TCC* GGA GAC TGT GAG AGT GGT; H4pNA1Rev: 5′-*GGC GGT GCT AGC* GAA GTG CAT CTG GTG GAG; HisFlagFor: 5′-*G CAA TTC CTC GAG TTA GTG ATG GTG ATG GTG ATG* TTT GAT CTC CAG CTT GGT GCC.

Plasmid construction

To design the pNA1 plasmid (scFv display vector) a cloning sequence was created by overlap PCR of two long oligonucleotides (pNA1Rev and pNA1For), and introduced into vector pSW1 after restriction with *Hin*dIII and *Xho*I. This created a bacterial leader sequence and a cloning cassette (*Bss*HII, *Xho*I).

The cDNA encoding the anti-*Aah*I scFv-9C2 assembled in the order VL-to-VH was constructed by overlapping PCR, using L9E2Mrev and H9TandFor as the primers. To do this, cDNAs encoding the 9C2 VL and VH domains (accession no. AJ278442 and AJ278443) were first PCR-amplified from pSW1-$scFv_{15}$9C2 [14] using the following pairs of primers (L9E2MRev and L9E2MFor) and (H9E2MRev and H9TandFor), respectively. The resulting 774-bp cDNA was gel purified, digested with *Bss*HII and *Xho*I, and then inserted into pNA1 restricted in the same manner to create pNA1-scFv9C2. This intermediate vector is suitable for expressing scFv9C2 with the VL-to-VH arrangement. The cDNA encoding the anti-*Aah*II scFv 4C1 cDNA in the VH-to-VL orientation was then PCR-amplified from pHEN1-scFv4C1 with the primers H4pNA1Rev and HisFlagFor [19]. These primers can be used to generate restriction sites suitable for cloning into pNA1-scFv9C2, and introduce a sequence encoding an His_6 flag at the 3′ end. The resulting 787-bp cDNA was gel purified, restricted at both ends with *Nhe*I and *Xho*I, and cloned into vector pNA1-scFv9C2 restricted in the same manner. A recombinant plasmid with inserts in the correct orientation (also known as pNAT94H6) was selected by PCR screening using appropriate primers and sequencing.

The PCR and overlap PCR program reactions were essentially as previously reported [25]. All basic molecular biology procedures were carried out as in [26]. Restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany). Taq and Pfu polymerases, T4DNA ligase and

calf intestinal phosphatase were from Promega (Charbonnières, France). *Escherichia coli* strain TG1 (Stratagene) was used for all the cloning steps. All chemicals were of standard grade from Sigma (St Quentin-Fallavier, France) or equivalent.

Bacterial expression and purification of tandem scFv

A 500-ml volume of 2xYT medium (DIFCO, Le Pont de Claix, France) containing 0.05 g/l ampicillin was inoculated with an overnight culture, grown at 37 °C from a single colony of *E. coli* HB2151 [K12, *ara*, Δ(*lac-pro*), *thi*/F' *pro*A^+B^+, lacl^q lacZΔM15], transformed with plasmid pNAT94H6. Various bacterial culture conditions were tested. Bacteria were grown at 30°–37 °C to A_{600nm} = 0.5–1.2. IPTG was then added at a final concentration of 0.84 mM and growth was continued at 15°–30 °C for another 8–16 h. Periplasmic extracts were prepared as previously reported [14].

The recombinant tandem-scFv (also referred to here as T94H6) extracted from 500 ml bacterial culture was first briefly incubated with 10 mM imidazol, and then purified by IMAC using 0.5 ml Ni^{2+}-NTA resin (Qiagen) packed in a disposable column. The matrix was washed thoroughly with a washing buffer (PBS, pH 7.4, containing 20 mM imidazol). Bound proteins were eluted in 0.5-ml fractions of elution buffer (PBS, pH 7.4 containing 150 mM imidazol), and immediately dialyzed against PBS. The elution fractions containing the functional recombinant protein were identified by direct ELISA using either *Aah*I or *Aah*II toxin, as previously reported [20]. Positive ELISA fractions were pooled and stored at –20 °C. Samples were also analyzed using reducing SDS-12% polyacrylamide gel electrophoresis with subsequent Western blotting. Immunostaining was performed with the penta-His monoclonal antibody from Qiagen. Dot-blots were performed using Protein L conjugated with peroxidase (Pierce), essentially as in [20]. IMAC-purified protein (200 µl, 100 µg/ml) was analyzed by gel filtration using a Superose 12HR prepacked 10/30 column (Amersham Bioscience, Les Ulis, France) calibrated using standards from Boehringer Mannheim (Meylan, France) and Protein L-purified diabody 9C2 prepared as reported previously [14]. Proteins were eluted with PBS at a rate of 0.5 ml/min and detected with a UV recorder at 280 nm. The integrity of the purified recombinant protein was analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Swiss Institute of Bioinformatics software (ProtParam tool) was used to determine the theoretical M_r of the recombinant tandem-scFv (M_r = 53 675) and its extinction coefficient [27]. The protein content of the solution was measured using UV spectrophotometry at A_{278nm} = 1.839 L g^{-1} cm^{-1}.

Toxin binding assays

Radioimmunoassays. The binding of antibody fragments to isolated *Aah*I or *Aah*II toxin was investigated by radioimmunoassay, essentially as in [19].

Toxins were radiolabeled with ^{125}I. Twenty-five microliters of each dilution of the various concentrations of antibody sample was mixed with 25 µl of ^{125}I-labeled *Aah*I or ^{125}I-labeled *Aah*II toxin (3.2×10^{-11} M and 5×10^{-11} M, respectively) in PBS-0.1% BSA (final volume: 150 µl), and the mixtures were incubated for 90 min at 37 °C, and then overnight at 4 °C. The bound antigen was separated from the free antigen by adsorbing the free antigen onto activated charcoal. A suspension (0.5 ml) containing 0.8% charcoal (Sigma) and 0.08% dextran T-70 (Sigma) in PBS-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 in the supernatants was measured with a gamma counter (RIAStar, Packard). All assays were carried out in duplicate. For competitive experiments IgG9C2 or antibody fragments (diluted to a concentration corresponding to 2.4×10^{-11} M and 8.1×10^{-9} M were mixed with radiolabeled toxin *Aah*I (3.2×10^{-11} M) or *Aah*II (5×10^{-11} M), respectively, plus various concentrations of unlabeled homologous toxin. Results are expressed as B/Bo, where B and Bo are the amounts of radioactivity bound to the antibody in the presence (B) or absence (Bo) of unlabeled ligand.

Two-site immunometric assay. Microtiter plates (96-well, Maxisorb, Nunc) were coated with 0.1 µg purified *Aah*II toxin diluted in PBS (pH 7.4) and saturated with PBS containing 3% BSA. Purified T94H6 (100 µl) preincubated with *Aah*I (0.1 µg) was added to each well. A polyclonal murine serum specific for toxin *Aah*I was then added, and immunocomplexes were revealed with an anti-mouse IgG (Fc specific)-alkaline phosphatase conjugate (Sigma), using *p*-nitrophenol phosphate as a substrate. The absorbance was read at 405 nm.

All incubations were carried out for 1 h at 37 °C. Three washings with PBS (pH 7.4) containing 0.1% Tween 20 were performed between each of the intermediate steps. Purified diabody 9C2 was used instead of T94H6 in control experiments. The assay was also carried out using *Aah*I as the immobilized toxin, *Aah*II as the free toxin, and a polyclonal murine serum specific for toxin *Aah*II but not for toxin *Aah*I.

Biological activity of tandem-scFv

Neutralizing activity after intracerebroventricular injection. The neutralizing capacity of the recombinant antibody was tested via the intracerebroventricular (i.c.v.) route. Amounts of toxins (*Aah*I or *Aah*II) or puri-

fied venom equal to or greater than the lethal dose were incubated for 30 min at 37 °C with a constant volume of recombinant antibody preparation. The mixture was then injected into female C57BL/6 mice weighing 20 g by the i.c.v. route. The number of surviving mice was recorded after 24 h.

Protective activity after experimental envenomation. Female C57BL/6 mice weighing 20 g were used for *in vivo* protection assays. Purified *A. australis* venom was first calibrated to determine the murine LD_{50} by the subcutaneous route. The quantity of purified venom equivalent to 1 LD_{100} was then injected in a volume of 200 µl into mice by the subcutaneous route. Immediately after this, the IMAC-purified tandem-scFvT94H6 was intraperitoneally delivered to mice in a volume of 200 µl. The clinical signs and survival ratio were recorded for 24 h. In control experiments, tandem-scFv T94H6 was replaced either by an excess (20 µg) of Protein L-purified $scFv_5$9C2 (diabody) or by 0.1% BSA. The animal rooms and experiments complied with the Swiss regulation on animal welfare.

Results

Design and construction of bispecific tandem scFv

To generate the bispecific *Aah*I × *Aah*II tandem-scFv, the pNAT94H6 expression vector was designed after constructing a coding sequence and then cloning it into pSW1 as a *Hin*dIII/*Xho*I insert (Fig. 2). The cDNA cloned into pSW1 consisted of the pelB signal sequence, which was expected to direct the nascent recombinant protein to the periplasm of the bacteria, as previously described [28]. The pelB signal sequence was followed by the scFv 9C2 encoding sequence assembled in the VL-to-VH orientation. Here the 18 amino acid residue linker L_1 (GSTSGSGKSSEGSGSTKG) was introduced between the 9C2 variable domains in place of the usual $(G_4S)_3$ sequence to preserve the antigen-binding activity. In natural IgGs, the distance between the C terminus of VL and N terminus of VH is slightly greater than that between the C terminus of VH and N terminus of VL (39–43 Å *versus* 30–34 Å) [29]. The scFv 9C2 sequence was fused via a short linker, L_2 (GGGAS), to the scFv4C1 assembled in the VH-to-VL orientation with the $(G_4S)_3$ linker L_3, exactly as previously reported for the active,

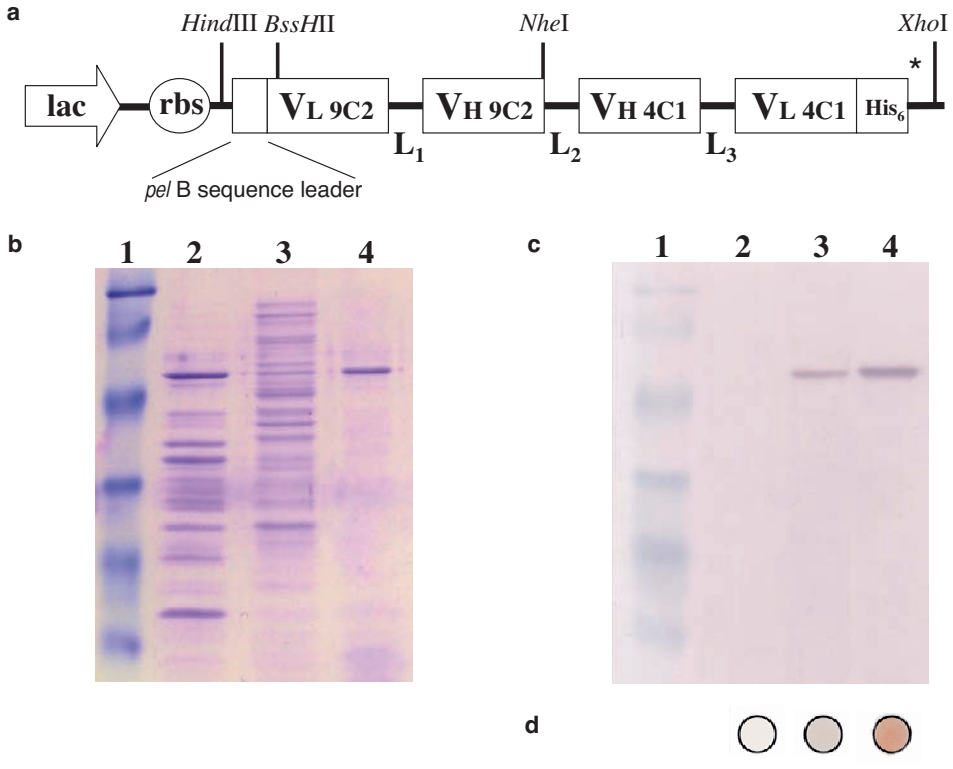

Figure 2. Construction of expression vector pNAT94H6 and purification of the tandem-scFv T94H6 protein from periplasmic extracts of recombinant *E. coli* HB2151. (*a*) Schematic representation of vector pNAT94H6. * indicates a stop codon. (*b*) SDS-PAGE stained with Coomassie brilliant blue. (*c*) Western blot carried out using an anti-$(His)_5$ flag monoclonal antibody. (*d*) Dot immunoblot carried out using protein L-peroxidase. Lane 1: molecular mass standards (118, 85, 47, 36, 26 and 20 kDa); lane 2: periplasmic fraction of uninduced bacteria; lane 3: periplasmic fraction of induced bacteria loaded on to Ni^{2+}-agarose gel column; lane 4: Ni^{2+}-agarose gel column eluted fraction.

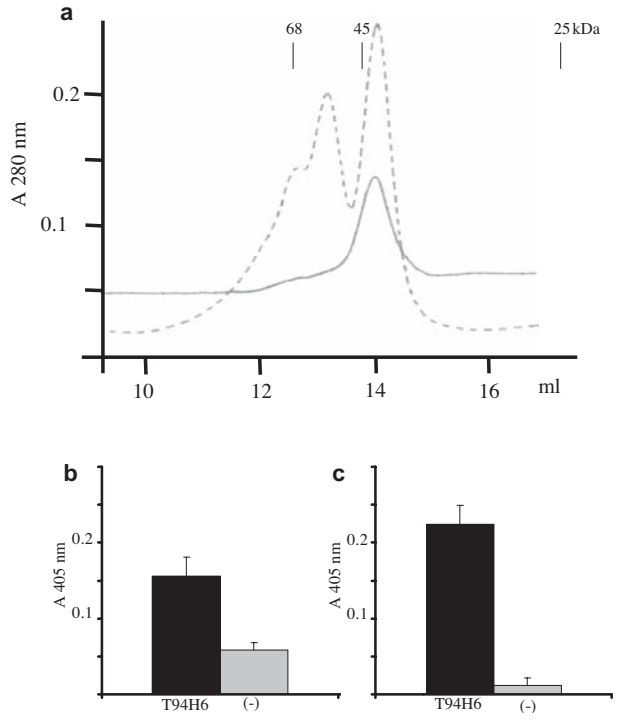

Figure 3. Characterization of the IMAC-purified tandem-scFv T94H6. (*a*) Elution profile of the IMAC-purified tandem-scFv T94H6 (continuous line) loaded onto Superose 12HR column calibrated with preparation of diabody 9C2 eluted as dimers or multimers (dotted line). (*b*, *c*) Binding activity of the recombinant protein eluted from the Superose 12 column tested in a two sites immunometric assay *versus* immobilized *Aah*I (*b*) or *Aah*II (*c*). (-) control using diabody 9C2 instead of the T94H6 preparation.

free scFv 4C1 [19]. The 5 amino acid residues sequence L_2 that joins the scFvs creates a steric constraint that is expected to prevent association between internal domains. At the 3′end of the cDNA there is a sequence encoding a $(His)_6$ tag to facilitate detection and purification, followed by a stop codon (TAA). Thus, the recombinant protein T94H6 encoded by the gene cloned into pSW1 was expected to be produced in the periplasm of recombinant bacteria after induction with IPTG. T94H6 is a single-chain polypeptide comprising two scFvs that have differing antigen-binding specificities and V domains with opposite orientations. We also constructed a vector (pNA1-scFv9C2) intended to express the scFv 9C2 alone and assembled in the VL-to-VH orientation to confirm that this arrangement did indeed preserve *Aah*I-binding properties (data not shown). We did not investigate the effect of different V-domain orders on the activity of the tandem-scFv, but the one selected (VL_{9C2}-VH_{9C2}-VH_{4C1}-VL_{4C1}) was expected to prevent the formation of non-functional scFv modules that can occur from adjacent internal domains in a tandem-scFv with VH_{9C2}-VL_{9C2}-VH_{4C1}-VL_{4C1} arrangement [30].

Bacterial expression, purification and structural characterization of tandem-scFv

The tandem-scFv T94H6 gene was expressed by growing the recombinant HB2151 bacteria induced with IPTG. Western blotting using the anti-His_5 antibody and rapid dot-immunoblotting using Protein L-peroxidase indicated that T94H6 was found in the periplasm of induced bacteria (Fig. 2). No degradation products were observed when the bacteria were grown at 37 °C to A_{600nm} = 1.2, and then induced with IPTG at 17 °C for 16 h while shaking (150 rpm/min), but under other conditions the yield and quality of the production may be significantly different (data not shown). Purification of tandem-scFv T94H6 was carried out by IMAC chromatography. The IMAC-purified T94H6 molecule migrated as a single main band, with electrophoretic mobility on SDS-PAGE under reducing conditions corresponding to an apparent molecular mass of 50 kDa (Fig. 2b). The IMAC-purified protein was recognized by an anti-His_5 monoclonal antibody with no detectable degradation products after Western blotting. After dot blotting under native conditions, IMAC-purified T94H6 also reacted with Protein L, which interacts with the 9C2 VL domain, but not with 4C1 VL (not shown here). In size exclusion HPLC, the IMAC-purified T94H6 molecule eluted as a single peak with a retention volume of 14 ml, corresponding to the monomeric T94H6 molecule of ~50 kDa (Fig. 3). Diabody 9C2, used here as a control, eluted as a dimer with a similar retention volume, but also as multimeric structures with lower retention volumes as previously reported [14]. T94H6 was very stable, and no further degradation products were observed when the eluted T94H6 peak was re-chromatographied after being stored for up to 7 days at 4 °C. The T94H6 molecule eluted from the Superose 12HR column was able to recognize toxins *Aah*I and *Aah*II simultaneously in a two-site immunometric assay (Fig. 3b, c). Mass spectrometric analysis of IMAC-purified T94H6 indicated an experimental relative molecular mass $(M+H)^+$ of 53 692 that closely matched the theoretical M_r of 53 675 calculated from the amino acid sequence. It confirmed that the IMAC-purified recombinant antibody, which was produced with a yield of 100 µg/l of culture, had been processed correctly.

Antigen-binding activity and stability

Tandem-scFv T94H6 recognized ^{125}I-labeled *Aah*I and ^{125}I-labeled *Aah*II in a direct RIA (Fig. 4a, b). The affinity of tandem-scFv T94H6 was also measured by competitive RIA (Fig. 4c, d). The K_D value deduced from the data in Figure 4c, as in [31, 32], was 10^{-10} M for all the antibody fragments derived from 9C2 variable domains, including T94H6. T94H6 also preserved a high affinity for toxin *Aah*II, with a K_D of 10^{-9} M, which matched the

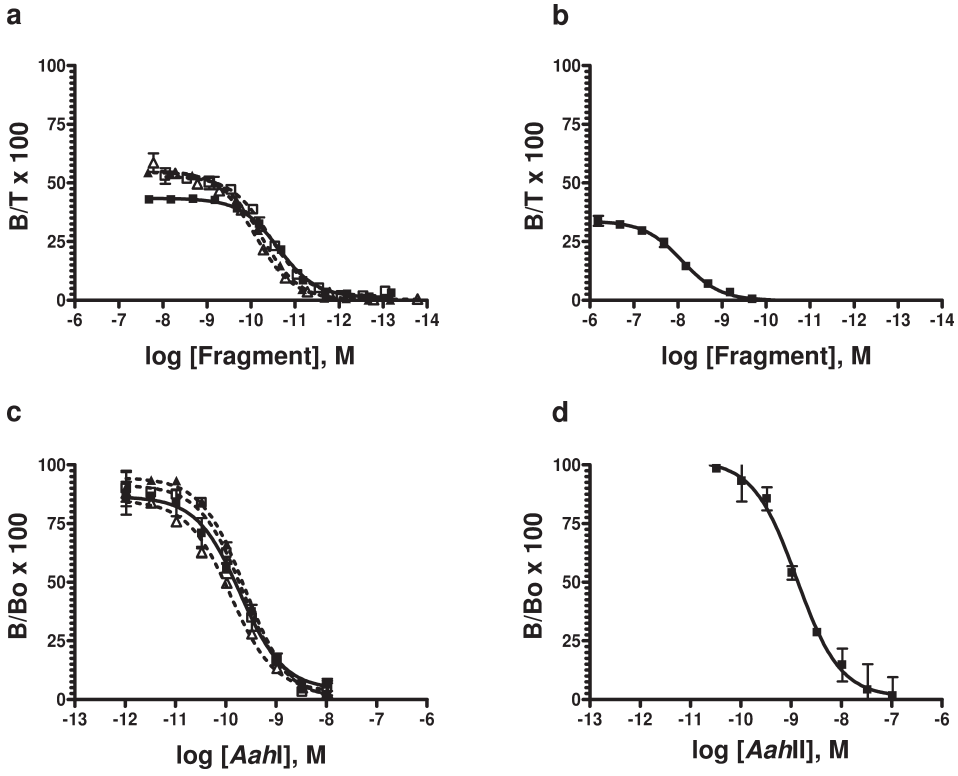

Figure 4. Immunoreactivity of tandem-scFv T94H6 with *Aah*I or *Aah*II toxins tested by RIA. (*a*, *b*) Serial dilutions of antibody preparations were tested against ^{125}I-labeled *Aah*I (*a*) or ^{125}I-labeled *Aah*II (*b*). Results are expressed as B/T, where B is the radioactivity bound to antibody, and T is the total radioactivity. (*c*, *d*) Inhibition of ^{125}I-labeled *Aah*I (*c*) or ^{125}I-labeled *Aah*II (*d*) binding to the antibody was performed with the homologous unlabeled toxin. Results are expressed as B/B_0, where B and B_0 are the radioactivity bound to antibody in the presence (B) or absence (B_0) of unlabeled ligand. Filled square: T94H6; filled triangle: $scFv_5$9C2; open square: $scFv_{15}$9C2; open triangle: Fab.

value previously determined under similar conditions for scFv 4C1 and the parental IgG [19].

The functional stability of IMAC-purified tandem-scFv T94H6 was also investigated in a direct ELISA after storage for up to 7 days either at 4 °C in PBS containing 0.1% BSA or at 37 °C after being diluted in bovine serum. Determination of the antigen-binding properties of samples taken at several time points showed no change in binding to individual toxins *Aah*I or *Aah*II for the T94H6 when stored at 4 °C. More than 90% of the toxin-binding activity was also preserved after incubation in bovine serum at 37 °C for up to 7 days.

In vivo biological activity of tandem scFv

Neutralizing capacity. Scorpion venom lethality is mainly due to *Aah*I and *Aah*II binding to site 3 of the voltage-dependent sodium channels. We first tested the neutralizing ability of tandem-scFv by injecting periplasmic extracts of T94H6 pre-incubated with isolated native toxins (*Aah*I or *Aah*II) or the Sephadex G-50-purified venom fraction to mice by the i.c.v. route. This experimental approach has previously been shown to be an effective way of testing various anti-toxin antibodies in a reliable manner without requiring large amounts of material. This is particularly useful in the case of isolated toxins, the purification of which is a lengthy and tedious task.

First, the toxicity of isolated *Aah*I, *Aah*II and of purified venom were measured after i.c.v. injection in C57BL/6 mice to provide an accurate determination of the LD_{50}. The neutralizing effect of T94H6 was then evaluated by incubating periplasmic extracts with an equal volume of toxin or purified venom, and injecting mice with the mixture. In the control experiments, BSA or a periplasmic extract containing scFv 9C2 was used in place of T94H6 preparation. The scFv9C2 periplasmic extract that replaced T94H6 in control experiments was first calibrated and then used at a concentration exhibiting the same anti-*Aah*I activity as the T94H6 preparation in RIA. When tested against isolated toxins, T94H6 ensured the survival of 100% of the mice injected with 3 LD_{50} of toxin *Aah*I (30 ng) (Table 1). It also neutralized *Aah*II, but to a lesser extent, as 50% of the mice injected with 3 LD_{50} of toxin *Aah*II (3 ng) survived. Finally, all the mice injected with 2 LD_{50} of the venom pre-incubated with T94H6 survived, *versus* only one of the six injected with venom pre-incubated with scFv9C2 (Table 2).

Table 1. *In vivo* neutralization of the toxicity of *Aah*I and *Aah*II pre-incubated with periplasmic extracts of tandem-scFv T94H6 and injected into mice by the intracerebroventricular (i.c.v.) route. x/y indicates the ratio of surviving/injected mice.

ng/mouse (LD_{50})	*Aah*I		ng/mouse (LD_{50})	*Aah*II	
	Surviving/injected (i.c.v.)			Surviving/injected (i.c.v.)	
	PBS-BSA	T94H6		PBS-BSA	T94H6
10 (1 LD_{50})	3/6	6/6	1 (1 LD_{50})	3/6	6/6
20 (2 LD_{50})	0/6	6/6	2 (2 LD_{50})	0/6	7/8
30 (3 LD_{50})		6/6	3 (3 LD_{50})		4/8
40 (4 LD_{50})		7/8	4 (4 LD_{50})		0/6
50 (5 LD_{50})		2/6			
60 (6 LD_{50})		0/6			

Table 2. *In vivo* neutralization of the toxicity of purified *Androctonus australis* venom pre-incubated with periplasmic extracts of tandem-scFv T94H6 or anti-*Aah*I $scFv_5$9C2 and injected to mice by the i.c.v. route. x/y indicates the ratio of surviving/injected mice.

ng/mouse (LD_{50})	Surviving/injected (i.c.v.)		
	PBS-BSA 0.1%	$scFv_5$9C2	T94H6
30 (1 LD_{100})	1/6	2/6	6/6
46 (2 LD_{50})	0/6	1/6	6/6
69 (3 LD_{50})		1/6	2/6
92 (4 LD_{50})			1/6
115 (5 LD_{50})			1/6

Table 3. *In vivo* protection of mice experimentally envenomed by the subcutaneous injection of purified *A. australis* venom, and treated by intraperitoneal (i.p.) injection of antibody fragments. x/y indicates the ratio protected/injected mice. (*) indicates the molar ratio, taking into account the estimated amount of the *Aah*I and *Aah*II toxins in the venom according to [18].

Venom injected (s.c.)	Protected/injected mice (i.p.)						
µg/mouse	PBS-BSA 0.1%	$scFv_5$ 9C2 20 µg/mouse	Molar ratio* *Aah*I:antibody	T94H6 6 µg/mouse	Molar ratio* *Aah*II:antibody	T94H6 12 µg/mouse	Molar ratio* *Aah*II:antibody
3 µg (1 LD_{50})	4/8						
4 µg (1 LD_{100})	0/8	2/8	1 : 42	7/8	1 : 4	8/8	1 : 8
6 µg (2 LD_{50})				2/8	1 : 2.6	6/8	1 : 5.2

Protection against experimental envenomation. For the protection assays, C57BL/6 mice were injected with 1 LD_{100} of freshly calibrated purified venom by the subcutaneous route. Immediately afterwards, the mice were treated with IMAC-purified tandem-scFv T94H6 (6 µg in 200 µl) delivered by intraperitoneal injection. In control experiments, the T94H6 preparation was replaced either by an excess (20 µg) of Protein L-purified anti-*Aah*I diabody 9C2, or by 0.1% BSA in PBS. Control animals receiving only BSA treatment showed typical symptoms 10 min after envenomation, and all died within 4 h of being injected. When treated with the tandem-scFv T94H6 molecule, all the mice displayed some symptoms, such as convulsions and respiratory distress soon after envenomation, but only one out of the eight animals challenged with 1 LD_{100} died. All the others recovered, and were still alive 24 h later, demonstrating the effectiveness of the tandem-scFv T94H6. Increasing the amount of T94H6 molecule injected into the mice challenged with 1 LD_{100} or 2 LD_{50} of venom (12 µg in place of 6 µg) improved the protection (Table 3). Under the same experimental conditions, using anti-*Aah*I diabody 9C2 in place of the bispecific T94H6 molecule was not effective; six of eight mice died, and there was no significant delay in the onset of symptoms of poisoning.

Discussion

Conventional antivenoms commonly used for the treatment of serious envenomations are still produced from hyper-immunized animal sera with low specific activity

against the most potent toxins of the venom. No fundamental changes have been made since the discovery of serum therapy by Phisalix and Bertrand [33], and this approach still has many drawbacks [1, 34]. These include inter-batch variation, risks of contamination by pathogens, a low neutralizing antibody content making it necessary to inject a high, and potentially toxic dose, and finally life-threatening adverse immunological reactions. Consequently, improvements are required to make antivenoms safer and more effective [35].

Over the past decade, antibody engineering has led to a renaissance of passive therapy. Several antibody-derived molecules have been approved for medical applications in a wide range of disorders of major importance in developed countries (in the fields of oncology, hemostasis, immunology and, to a much lesser extent, anti-infectives) [36, 37]. Most of these molecules consist of antibody V domains derived from murine IgG-secreting hybridomas, in the same way as we have done here, even though other technologies (such as the antibody-phage display and library screening) can now be used to select human antibody V domains that are potentially less immunogenic. The advantages of the phage-display technique are not clear, and indeed remain controversial, particularly when libraries consist of a 'naïve' repertoire constructed by cloning the antibody V domain genes from non-immunized donors. Many experimental difficulties are experienced in constructing antibody-phage libraries and displaying large repertoires (10^{10}–10^{11}) essentially due to toxic effects on bacterial expression, improper folding or assembly and proteolysis of the displayed scFv [9]. Another major problem is maintaining the repertoire over time and delaying the inevitable drift of its content as far as possible. Thus, of the 18 recombinant antibodies already approved (2006) for medical purposes, 16 are derived from murine hybridomas, and only 2 have been selected from antibody-phage libraries (adalimumab and omalizumab). Recently, a human scFv with low affinity for the CnII toxin of the Mexican scorpion *Centruroïdes noxius* was isolated from a small size non-immune library. Directed evolution and phage display have made it possible to increase the affinity of scFv for its target 446-fold. Nevertheless, this affinity ($K_D = 4.1 \times 10^{-10}$ M) is no greater than that usually observed for conventional murine antibodies [16]. In addition, the neutralizing capacity of this 'evolved' scFv was weak (only investigated for 1 LD_{50}), and its protective capacity was not investigated. More innovative and efficient developments may emerge from the discovery of novel antibodies that are naturally devoid of the light chain and which constitute a significant part of the serum immunoglobulins in Camelids. These antibodies (HCAb) also lack the CH_1 domain, but harbor an intact variable domain (V_HH). V_HHs display unusually long surface loops, and are able to penetrate into cavities in target antigens or to bind with high affinity to low molecular weight haptens [38]. In addition, V_HHs are physically very stable, have low immunogenicity, and some of them have been produced at very high levels in recombinant bacteria (up to 10 mg/l). HCAbs (90 kDa), or their functional recombinant unit V_HH (15 kDa), have novel pharmacokinetic properties that may make them ideal for neutralizing the toxicity of low molecular weight scorpion toxins *in vivo*. Recently, the serum of camels immunized with the neurotoxins of *A. australis* scorpion was shown to contain HCAbs with high titer and venom neutralizing capacities [17]. Another recent pre-clinical study reported the neutralization of snake venom-induced hemorrhage by immune camelid sera [39]. However, these studies have not yet advanced to the stage of producing recombinant monoclonal V_HHs.

The smallest functional fragment derived from murine or human antibodies is the scFv molecule, which can subsequently be further engineered and rebuilt into multivalent and/or multispecific molecules with other unique and enhanced properties for various applications [40]. The monovalent scFvs that we had previously engineered from existing murine antibodies (9C2 and 4C1) were the smallest units able to bind the scorpion neurotoxins *Aah*I and *Aah*II [19, 20]. When rebuilt to form a bivalent diabody of 50 kDa, 9C2 performed better, and protected mice that had been challenged with isolated *Aah*I toxin under experimental conditions close to natural envenoming. However, diabody 9C2 did not protect mice against a venom challenge, mainly because the toxicity of the venom is not solely due to the *Aah*I (and *Aah*III) toxin but also to *Aah*II toxin.

Given this fact, and the importance of size for the pharmacokinetics and biodistribution of antibody molecules, we prepared a 50-kDa, bispecific antibody directed against the group-I toxins (*Aah*I and *Aah*III) on the one hand, and against group-II (*Aah*II) toxins on the other. The effectiveness of conventional antivenoms has previously been widely discussed in terms of the size and the valence of antibody fragments [1]. It is generally considered that antibody fragments similar in size to Fabs or $F(ab')_2$ are certainly the most effective molecules in terms of tissue penetration, bioavailability and detoxification [35]. The capacity of diabody 9C2 to protect mice challenged with *Aah*I toxin is consistent with this [14]. Conversely, IgGs (150 kDa) penetrate tissues much more slowly than highly diffusible scorpion toxins, are not uniformly distributed, and are associated with high levels of potential toxicity.

Pioneering studies have demonstrated the potential of small bispecific fragments limited to antibody V domains for applications in cancer therapy involving retargeting cytotoxic effector agents against tumor cells [41, 42]. Bispecific antibodies are designed in one of three ways: (i) by making heterodimeric diabodies in which the variable domains of an antibody are present

on different polypeptide chains [43], (ii) by making monomeric single-chain diabodies (scDb), which are significantly more stable than heterodimeric diabodies, since both subunits are now fused together via an appropriate linker overcoming the problem of non-functional homodimer formation [30], and finally (iii) by making a tandem-scFv consisting of two unmodified scFv molecules associated via a peptide of virtually any size [44] (Fig. 1). In our study we did not investigate heterodimeric diabodies, essentially because of the difficulty of expressing two different gene products in the same cell in similar amounts, and the possibility that each subunit may then form non-functional homodimeric structures, making the purification of bifunctional heterodimers a tedious process. We also preferred to investigate the tandem-scFv format rather than the scDb structure for several reasons. ScDbs are rigid molecules in which the V domains are tightly packed [45]. They are more compact in shape than tandem-scFvs, which could help to improve their diffusion into tissues. However, designing functional scDbs is not simple, and can call for detailed structural analysis and modeling to determine the most appropriate arrangement of the V domains and the length of the linkers required for correct V domain folding and the stable association into functional units [40]. Otherwise, mispairing of noncognate heavy and light chains could occur, resulting in the formation of inactive antigen-binding sites. We thought that joining the scFvs 9C2 and 4C1 in tandem with a central linker would be the simplest way of keeping two scFvs together as bispecific molecule. Unlike scDb, the two scFvs fold independently and the size of the central linker, which is critical for the structure of the whole scDb, does not have much influence on the folding of antigen-binding sites in tandem-scFv. In addition, to circumvent the unlikely possibility of intramolecular pairing, the first scFv (9C2) was in the VL-to-VH orientation, whereas the second one (4C1) had the opposite orientation (VH-to-VL).

Functional T94H6 was produced in recombinant bacteria with a yield of 100 µg protein /l culture, which was similar to other well-expressed tandem-scFvs [46]. IMAC-purified T94H6 was homogeneous, as shown by SDS-PAGE, Western-blot, mass spectrometry, and HPLC analyses. Unlike scDbs, which have a tendency to multimerize, T94H6 remained stable, free of higher molecular-mass species after storage at 4 °C, and did not loose its activity rapidly in serum at 37 °C, all of which is essential for homogeneous pharmacokinetic properties and effective detoxification *in vivo*. However, to ensure that it has these qualities, the T94H6 must be prepared scrupulously under the cell culture and induction conditions reported here. Otherwise it was difficult to express full-length soluble T94H6 due to its sequestration in the cytoplasm, and sensitivity to proteases, which leads to the formation of degradation products in the periplasm (data not shown). This means that other expression systems will have to be developed for the large-scale production of pure T94H6. The advantages and disadvantages of bacterial, yeast, plants, insect and mammalian expression systems for producing recombinant antibody fragments have been discussed in detail elsewhere as well as points to be considered for manufacturing [47, 48].

Tandem-scFv T94H6 is fully functional and possesses neutralizing capacity that usually correlates with antigen affinity [49]. This was confirmed here. T94H6 conserved the high affinity for *Aah*I ($K_D = 10^{-10}$ M) and *Aah*II ($K_D = 10^{-9}$ M) of the parental antibody, reflecting the stability of the immunocomplexes, which is essential for stable inactivation of the toxin, its clearance and to reverse its normal distribution in the body. We also noticed that T94H6 binding affinity was correlated to the neutralization of individual toxins, the neutralization of *Aah*I being more efficient than that of *Aah*II. This might restrict the therapeutic potential of T94H6 for several reasons. *Aah*II, which is the most abundant and the most potent of the three lethal toxins, may be responsible for up to 73% of the toxicity, even though there is considerable polymorphism between secretions from individual specimens of the same species [18]. Nevertheless, significant neutralization of the whole venom was also observed in this assay, which was particularly sensitive, the LD_{50} of individual toxins and whole venom being extremely low when injected by the i.c.v. route.

An important distinction was made here between the neutralizing and protective capacities. Neutralizing usually refers to the ability of antibodies pre-incubated with the toxin to block its binding to the target, and possibly prevent subsequent toxic effects. In neutralizing assays, the toxin is allowed to bind to antibodies *in vitro* before it reaches its receptor. Most authors have limited their investigations to the neutralizing capacity [2, 16, 19]. However, although their findings are of interest, they do not allow us to predict the protective capacity under real envenoming conditions.

In this report, 'protective capacity' refers to the ability to inhibit lethal toxicity when the venom and the antidote are injected independently to animals via two distinct routes. This is a much more stringent test that mimics the latency period required for the distribution of free toxins and antibodies in the body. It allows the venom components to distribute into the tissues according to their individual pharmacokinetic properties, which are still not well understood despite extensive investigations [18, 50]. For instance, even though *Aah*I and *Aah*II share many structural features, they have only 42% sequence identity, and *Aah*II is more hydrophobic than *Aah*I. As a consequence, these toxins display very different immunological and biological properties. Since *Aah*II is much more hydrophobic than *Aah*I, it is more rapidly cleared

from the plasma not due to a rapid transfer into the urine, but probably as a result of non-specific binding to biological surfaces [18].

Our *in vivo* assays under conditions that mimic natural envenomation clearly indicate that T94H6 can protect animals challenged with 1 LD_{100} of freshly calibrated venom, and the need to target *Aah*II toxin as well as *Aah*I. This protection cannot be achieved using a monospecific bivalent antibody, as was demonstrated when diabody 9C2 was used instead of T94H6, even though a large excess was injected. Some clinical signs of envenomation persisted during the first few hours after treatment with T94H6, probably due to the effects of other minor components of the venom, but most if not all the animals survived for 24 h post challenge. The capacity of T94H6 to protect against *A. australis* venom can be estimated to be greater than 200 LD_{50}/mg, indicating a high specific activity, at least four hundred times higher than that of conventional antivenoms. Conventional antivenoms, including those in which anti-toxin antibody fragments have been immunopurified, have at best a neutralizing capacity in the range of 0.5 LD_{50}/mg in the standard mouse assay which implies much less stringent conditions since the antivenom and venom are premixed before being injected into the mice [50, 51].

In conclusion, the T94H6 molecule paves the way for a new generation of immunotherapeutic agents for scorpion envenoming with greater homogeneity, specific activity and possibly safety than conventional polyclonal immune sera. Such a preparation would no longer require the annual collection of venoms for the repeated immunization of horses, extraction of the γ-globulin fraction from hyperimmune serum, fragmentation into Fabs or $F(ab')_2$ or the immuno-purification of the active substances, all of which is subject to increasingly stringent regulations to ensure reproducibility and to avoid various risks, including the transmission of pathogens. The later point is particularly well illustrated currently by the problem posed by the use of polyclonal ovine Fabs (DigiFab™) to treat digoxin overdose.

The murine origin of T94H6 sequence should not prevent its human use. Indeed, all but two therapeutic antibodies approved by the FDA contain mouse protein sequences, and most are indicated for chronic diseases that require the repeated injection of several milligrams of antibody. A bispecific antibody, such as T94H6, would be used solely to treat patients after envenomation, and would not be intended for prophylactic use. This means that it would be injected or infused only once. Although it is theoretically possible, it is unlikely that a given patient would be envenomed and treated twice, which reduces the risk of hypersensitivity reactions. Nevertheless, a number of technologies do exist that can be used to eliminate any residual immunogenicity and improve tolerability. These include CDR grafting within human antibody frameworks and cross-linking to polyethylene glycol [52, 53]. We therefore believe that a new generation of antivenoms containing just one or a few highly specific, recombinant antibody fragments able to neutralize the most abundant and toxic components of a venom can be obtained by means of the procedure reported here. However, before these findings lead to any significant improvement in antivenom serum therapy, it will be necessary to overcome the geopolitical and socio-economic considerations that make scorpion antivenoms orphan drugs.

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