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Research Article

Design and evaluation of a diabody to improve protection against a potent scorpion neurotoxin

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Abstract. Diabodies are recombinant, dimeric, antibody-based molecules composed of two non-covalently associated single-chain antibody fragments that bind to an antigen in a divalent manner. In an attempt to develop more effective therapeutic molecules against scorpion venoms, we designed a diabody derived from monoclonal antibody 9C2, which neutralizes the toxicity of scorpion neurotoxin AahI in mammals. The recombinant diabody produced in the periplasm of *Escherichia coli* was purified to homogeneity in a single step by protein L-agarose affinity chromatography. It was functional, and possessed a high binding affinity to AahI (8×10^{-11} M). The bivalence of the diabody was confirmed by size-exclusion chromatography, isoelectrofocussing and electron microscopic observations. Finally, the diabody showed high thermal stability in serum and demonstrated protective activity when injected intraperitonally in mice experimentally envenomed with toxin AahI. In conclusion, the diabody format gives the 9C2 molecule advantageous properties that are particularly important for potential clinical applications in the treatment of envenomations.

Key words. Diabody; scFv; immunotherapy; scorpion; toxin; antibody engineering.

During the past decade, molecular engineering has made possible the development of antibody-based molecules with great biological and therapeutic potential. These advances are reflected in the increased number of engineered antibodies undergoing clinical trials and in the appearance on the market of products used to treat a wide range of disorders, including chronic immune and inflammatory diseases, cardiovascular diseases, infectious diseases and solid and hematological tumors [1]. However, in the field of envenomations, which are of serious concern in many tropical and subtropical regions, serum therapy is the only specific treatment available, and is still based on the administration of heterologous polyclonal antibody fragments. Improvements are required in antivenom production, safety and efficacy [2–5]. Antivenoms are usually polyclonal Fab or F(ab)$'_2$ prepared from the serum of immunized animals. These molecules are not produced in a reproducible manner. Their neutralizing efficacy depends on the immune response of the immunized animal and expensive precautions are required to ensure that they are free of pathogenic agents, including viruses and non-conventional pathogens, which makes antivenoms unaffordable in many developing countries [6, 7]. Finally, the risks of serum sickness and anaphylaxis because of the heterologous origin of these antibodies have sometimes been considered to be serious grounds for not using them [8, 9]. Required is a standardized source of high-titer antibodies targeted against the limited number of the venom compo-

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nents responsible for its lethality. The toxic effects of the venom of the most dangerous Tunisian scorpion (*Androctonus australis hector*) are essentially due to the toxicity of three small proteins with 63/64 residues, which interact with the sodium channels of excitable cells but which correspond to less than 5% of the total protein fraction of the venom [10, 11]. These molecules are very similar; they belong to two distinct immunological groups, and neutralizing murine monoclonal antibodies are now available for each of these groups [12, 13].

Replacing therapeutic polyclonal antibody fragments with monoclonal mouse or human antibodies is probably not possible for several reasons, including the risk of a human anti-mouse antibody (HAMA) reaction, the instability of immortalized human cell lines and the difficulty preparing high-affinity, toxin-neutralizing antibody fragments by phage-display technology [14–16]. However, advances in molecular engineering have provided several alternative methods for constructing and producing novel antibody-based molecules with great potential for combating infectious and toxic agents [17, 18]. Recently, the activity of the lethal toxins of the scorpion venom has been successfully blocked using mouse IgG-derived recombinant scFvs [19, 20]. scFvs are the smallest structures likely to retain the ability to bind antigens. They consist of the VH domain of an antibody joined to the VL domain in a single polypeptide chain via an artificial peptide linker, which ensures the equal expression of both domains and their stability. A 15-amino-acid residue linker, such as the $(Gly_4Ser)_3$ peptide, is usually appropriate to provide resistance to proteases and ensure correct docking of the VH and VL domains in the natural Fv orientation with a functional antigen-combining site. scFvs molecules have better tissue penetration than full-size IgGs, with rapid blood clearance, together with lower immunogenicity as a result of the elimination of the constant domains of the antibody [21, 22]. scFvs are mainly produced as monomeric structures, and usually display monovalent antigen-binding activity, but they tend to have short in vivo half-lives, making them unsuitable for clinical use which requires the antibody to be present in the bloodstream for an extended period of time. Further engineering is required to circumvent the drawbacks of scFvs and to obtain better clearance properties. scFvs can serve as building blocks to generate novel, multivalent antibody fragments of intermediate size with therapeutic potential. Shortening the linker length between the VH and VL domains creates steric restrictions and progressively prevents natural Fv formation, while still allowing the formation of higher-order oligomers with antigen-binding activity [23]. With a five-residue linker, (Gly_4Ser), complementary VH/VL pairs can combine from two adjacent scFv molecules to form predominantly a stable dimer, which is also known as a diabody [24].

In this study, we engineered a diabody from antibody 9C2 raised against toxin AahI from the scorpion *A. australis hector*. The structural and functional properties of the recombinant protein produced in bacteria were investigated, as was as its stability and its ability to protect mice challenged with toxin AahI under conditions that mimic those of natural envenomation.

Material and methods

Animals, antibodies and toxins

C57BL/6 mice were raised and housed in the conventional animal facilities of our laboratory. Animal care complied with European Guidelines on animal welfare (86/609/CEE).

Anti-AahI IgGs secreted by hybridomas 9C2 and 2G3 were prepared as reported elsewhere [13].

Scorpion toxin AahI was isolated from the venom of the scorpion *A. australis hector*. It was carefully purified and characterized in the laboratory [25].

Plasmid construction

Plasmid pSW1-9C2 coding for scFv 9C2 fused to the MRC-OX74 epitope tag was used to assemble the diabody and scFv genes [20]. This plasmid was used as a template in a PCR reaction to modify the flanking regions of the gene encoding the 9C2 VL domain with the primers VLFor, 5′-CTG TAG CTC GAG TTA TTT GAT CTC CAG CTT GGT G, to replace the MRC-OX74 sequence by a stop codon and either Link5Rev, 5′-AGT GTC GGA TCC GAC GTC CAG ATG ACT CAG, to generate subsequently a short rigid (Gly_4Ser) intramolecular linker (PCR fragment 1), or Link15Rev, 5′-GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC GTC CAG ATG ACT CAG, to generate a long $(Gly_4Ser)_3$ linker (PCR fragment 2). These primers carry unique restriction sites for *Bam*HI and *Xho*I (underlined) suitable for cloning in pSW1-9C2. The PCR reaction was carried out under standard conditions, using Taq polymerase (Promega, Charbonnières, France). The expression plasmids pSW1-scFv$_5$9C2 and pSW1-scFv$_{15}$9C2 were constructed by ligation of the *Bam*HI/*Xho*I restriction fragments from pSW1-9C2 comprising the vector backbone and the *Bam*HI/*Xho*I-digested PCR fragments 1 and 2, respectively.

Escherichia coli strain TG1 was used for all the cloning steps. The sequences of all newly constructed genes and plasmids were confirmed by restriction digests and DNA sequencing (Genome Express, Montreuil/Bois, France). All basic molecular biology procedures were carried out as in Sambrook et al. [26]. Restriction enzymes were from New England Biolabs (Beverly, Mass) and T4DNA ligase from Life Technologies (Cergy Pontoise, France). All chemicals were of standard

grade from Sigma (St Quentin Fallavier, France) or equivalent.

Protein expression and purification

For expression of functional recombinant antibody fragments in the bacterial periplasm, the plasmids pSW1-scFv$_5$9C2 and pSW1-scFv$_{15}$9C2 were cloned into *E. coli* strain HB2151 [K12, *ara*, Δ(*lac-pro*), *thi*/F′ *pro*A$^+$B$^+$, lacI^q lacZΔM15]. Transformed bacteria were grown to $A_{600\,nm}$ = 1.2 in 2×YT medium containing 0.05 g/l ampicillin at 37 °C while shaking at 200 rpm. IPTG was then added to yield a final concentration of 0.84 mM, and growth was continued at 16 °C for 20 h. The bacterial cells were then harvested by centrifuging at 4 °C (3500 g, 30 min) and periplasmic extracts were isolated by EDTA treatment combined with osmotic shock. The cell pellet was resuspended in 0.2 M Tris-HCl buffer, pH 8.0 containing 0.5 mM EDTA and 0.5 M sucrose (20 ml per liter of original culture). The cells were then subjected to a mild osmotic shock by adding the same buffer diluted 1:4 with H_2O (30 ml per liter of original culture). After incubating at 4 °C for 30 min, the suspension was clarified by centrifuging (15,000 g, 20 min), and the supernatant containing all the soluble periplasmic proteins was extensively dialyzed against borate-buffered saline (BBS), pH 7.9, centrifuged (15,000 g, 4 °C, 30 min) and filtered through a 0.2-µM pore size membrane. Aliquots containing the recombinant molecules were prepared and stored at −80 °C until use.

The recombinant scFvs were purified by loading periplasmic preparations extracted from 500 ml bacterial culture on a column of protein L-agarose (0.2 ml; Actigen, Cambridge, U.K.). After washing the gel with BBS, the adsorbed protein was eluted in 0.5 ml fractions with 0.1 M glycine pH 3 and immediately neutralized with 1 M Tris pH 8.9 (10 µl). The elution fractions containing the functional recombinant protein were identified by direct ELISA as previously reported [20]. Positive ELISA fractions were pooled and subjected to dialysis against BBS, pH 7.9, for buffer exchange, before being centrifuged and filtered across a 0.2-µm pore size membrane. The purification process was checked by SDS/PAGE on a homogeneous 15% gel. 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 Mr and pI of the recombinant proteins and their extinction coefficients [27]. Thus, the protein content of the solution was measured by UV spectrophotometry at $A_{278\,nm}$ = 2.088 l g^{-1} cm^{-1} for the scFv$_5$ preparation and $A_{278\,nm}$ = 2.037 l g^{-1} cm^{-1} for scFv$_{15}$.

The purified scFv preparations were resolved by size-exclusion FPLC on a Superdex 75 HR 10/30 column (molecular mass range 3000–70,000) (Amersham Biosciences, Les Ulis, France) calibrated with standards from Boehringer Mannheim (Meylan, France). The column was loaded with 200 µl of the sample to be analysed. Proteins were eluted with BBS at a rate of 0.5 ml/min, and detected with a UV detector at 226 nm. Eluted fractions (0.5 ml) were collected and either used immediately for further analysis or stored at 4 °C.

Antigen-binding analysis

1) Radioimmunoassays. RIA was carried out according to the previous report [20]. AahI was radiolabeled with iodine-125. Successive dilutions of scFv preparations (10^{-7}–10^{-14} M) were incubated with [^{125}I]-AahI (5.7×10^{-11} M) in phosphate-buffered saline pH 7.5 containing 0.1% bovine serum albumin. The mixtures were incubated for 90 min at 37 °C and then overnight at 4 °C. Bound and free antigens were separated by adsorption of the free toxin onto activated charcoal [19]. All assays were conducted in triplicate.

For competitive RIA, successive dilutions of unlabeled toxins (10^{-7}–10^{-12} M) were mixed with [^{125}I]-AahI (5.7×10^{-11} M) and incubated with the scFv preparation (1.7×10^{-11} M for scFv$_{15}$, 3.1×10^{-11} M for scFv$_5$, 1.1×10^{-10} M for purified fractions). Results are expressed as B/Bo, where B and Bo are the radioactivity bound to the recombinant scFv protein in the presence (B) and absence (Bo) of unlabeled ligand, respectively.

2) Isoelectrofocusing and gel filtration analysis of immunocomplexes. To form imunocomplexes, 4.14 µg (83 pmol) of the dimeric (scFv$_5$9C2)$_2$ resolved by Superdex 75 gel filtration was incubated with 0.41–1.22 µg (55–170 pmol) of toxin AahI in 200 µl of BBS for 1 h at 37 °C. Samples were analyzed either by isoelectrofocusing (1 µl) using PhastGel IEF 3–9 (Amersham Biosciences) and silver staining or by FPLC gel filtration (200 µl) using the Superdex 75 HR 10/30 column (Amersham Biosciences).

3) Immunoelectron microscopic analysis. Freshly purified dimeric diabody (scFv$_5$9C2)$_2$ (70 pmol) was incubated with toxin AahI (140 pmol) in 200 µl BBS, pH 7.9 for 1 h at 37 °C and then incubated for another hour with purified IgG 2G3 (70 pmol). Immunocomplexes formed before or after adding the IgG preparation were isolated by FPLC gel filtration (200 µl) using a Superose 12CL 10/30 column (Amersham Biosciences). Samples were negatively stained with 2% aqueous uranyl acetate, pH 4.5. They were observed under an electron microscope (Jeol 1010, Croissy, France) at an accelerating voltage of 80 kV and a magnification of about 50,000 [28]. Free IgGs were observed under the same conditions.

Analysis of the stability of scFv in vitro

The protein L-agarose affinity-purified scFvs were diluted in rabbit serum to concentrations of 0.24 and 2.40 µg/ml. Aliquots of 100 µl were prepared at once under sterile

conditions and kept frozen at –20 °C until use. At given time points, triplicate samples were thawed and incubated at 37 °C over a period of 0–8 days. The activities of the samples after incubation at 37 °C were determined by direct RIA, using 25 µl of serum as a source of scFv.

Analysis of the stability of scFvs in vivo

Three female C57BL/6 mice each weighing approximately 20 g were injected intraperitonally with 7 µg of protein L-agarose-purified diabody (200 µl). The animals were bled at given time points (from 5 to 2880 min) after antibody fragment injection. Blood collected on heparin was centrifuged at 2800 g for 10 min at 4 °C and supernatants were kept at –20 °C. Antigen-binding activity of the plasma was determined after storage by direct RIA at a dilution of 1:50. The concentration of the active antibody fragment in the plasma was deduced from these data using a standard curve plotted with calibrated diabody.

Protection of mice against toxin challenge

AahI was injected into mice by the subcutaneous route at varying quantities higher than 665 ng per animal (1 LD_{50} = 320 ng for 20 g C57BL/6 mice). Ten minutes later, antibody fragments (19.5–39 µg) (or BSA 0.1 p. 100) were intraperitoneally injected in a volume of 200 µl. Six animals were used for each test condition, and the survival was recorded after 24 h.

Results

Design of genes encoding the single-chain antibody molecules scFv and $(scFv)_2$

A recombinant plasmid for the diabody $(scFv_5 9C2)_2$ was constructed (fig. 1). The genetic construction consisted of a PCR amplification of a cassette encoding part of the (Gly_4Ser) linker and the entire light-chain variable region of antibody 9C2 with primers VLFor and Link5Rev. Following restriction with *Bam*HI and *Xho*I, this gene was inserted in the pSW1-9C2 vector restricted in the same manner. The constructed vector was named pSW1-$scFv_5$9C2. Recombinant clones carrying the plasmid containing the insert fused in frame with the *Pel*B sequence were selected by PCR screening, and DNA sequencing was carried out to check that no mutation had occurred as a result of PCR amplification. pSW1-$scFv_5$9C2 encodes diabody 9C2, in which the VH C-terminus Ser^{H113} is joined to the VL N-terminal residue Asp^{L1} by the short rigid (Gly_4Ser) intramolecular linker. The cDNA sequence encoding $scFv_5$9C2 is now registered in the EMBL data bank (Accession number AJ534441).

For direct comparison of diabody properties with the scFv that was originally produced as a fusion protein with the MRC-OX74 epitope tag, a novel vector encoding the $scFv_{15}$9C2 free of tag was constructed as described in Materials and methods (fig. 1B).

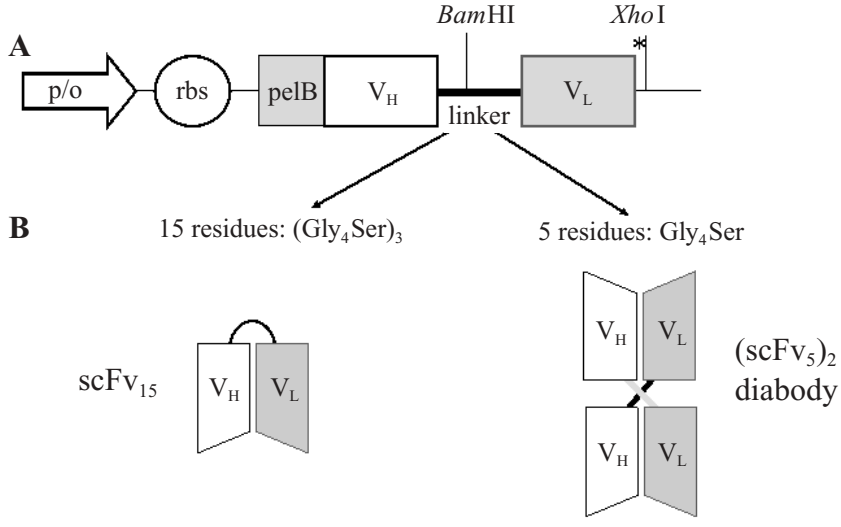

Figure 1. Engineering of recombinant antibody fragments. (*A*) Diagram of plasmid vectors pSW1-$scFv_5$9C2 and pSW1-$scFv_{15}$9C2 used for expression of anti-AahI scFv constructs: coding regions are shown as boxes. A cDNA encoding a peptide linker of 15 (in pSW1-$scFv_{15}$9C2) or 5 (in pSW1-$scFv_5$9C2) residues was genetically introduced between the VH and VL cassettes. The locations of the LacZ promoter, operon (p/o), ribosome binding-site (rbs), stop codon (*) and important restriction sites are indicated. (*B*) Diagram of recombinant scFvs 9C2 produced essentially as a monomeric structure with a 15-residue linker ($scFv_{15}$) or as a homodimeric diabody with a 5-residue linker $[(scFv_5)_2]$.

Bacterial expression and purification

Recombinant HB2151 clones expressing the active proteins were identified by a direct ELISA using crude periplasmic extract of induced bacteria as a source of scFv. This enabled demonstration that the V domain was correctly folded and disulfide bonds formed whether the bacterial clones were transformed with pSW1-scFv$_5$9C2 or with pSW1-scFv$_{15}$9C2. Large-scale production of the pSW1-scFv$_5$9C2 and scFv$_{15}$9C2 constructs was then carried out as reported in Materials and methods. These experimental conditions were selected empirically after testing several induction temperatures and times. We and others have previously described the difficulty finding standard conditions suitable for the periplasmic production of all sorts of scFvs in bacteria. Both recombinant proteins were produced as soluble proteins in the periplasm of recombinant bacteria, even though insoluble protein aggregates remained sequestred in the cytoplasmic compartment (data not shown). There were no apparent differences in the periplasmic expression levels of these two molecules. Purification was easily achieved by affinity chromatography on a Protein L-agarose gel column. Yields of 0.5–0.8 mg of soluble affinity-purified protein per liter of culture were typically obtained for each recombinant molecule. Preparations were shown by SDS-PAGE under reducing conditions to present essentially homogeneous scFv, with a single protein band at about 31 kDa for scFv$_{15}$ and slightly lower for scFv$_5$ (fig. 2). This difference in protein mobility on SDS-PAGE was due to the presence of ten additional amino acid residues in the sequence of the linker used to generate scFv$_{15}$. No minor bands of higher electrophoretic mobility were detected, indicating the high purity of the preparations.

Structural properties

The purity of the scFv preparations isolated by Protein L-agarose affinity chromatography was confirmed by mass spectrometry, which indicated an experimental relative molecular mass $(M+H)^+$ of 24,960 for scFv$_5$9C2 and 25,577 for scFv$_{15}$9C2. These results closely matched the theoretical Mr of 24,929 and 25,560, respectively, calculated from the amino acid sequences of these molecules. They also confirmed that the N-terminal signal peptide had been correctly processed, and that no degradation products were co-purified. The formation of multimeric structures was further characterized by submitting samples (0.33 mg/ml) of affinity-purified scFv proteins to size-exclusion chromatography on a calibrated Superdex 75 column (fig. 2B). Gel filtration of the diabody preparation revealed the presence of a main peak (69%) eluted at 10 ml with an apparent molecular mass of about 40 kDa, which was probably the scFv$_5$ dimer, preceded by two minor peaks, one eluting at 9 ml (18%) and the other at 8.5 ml (13%). These minor peaks could correspond to

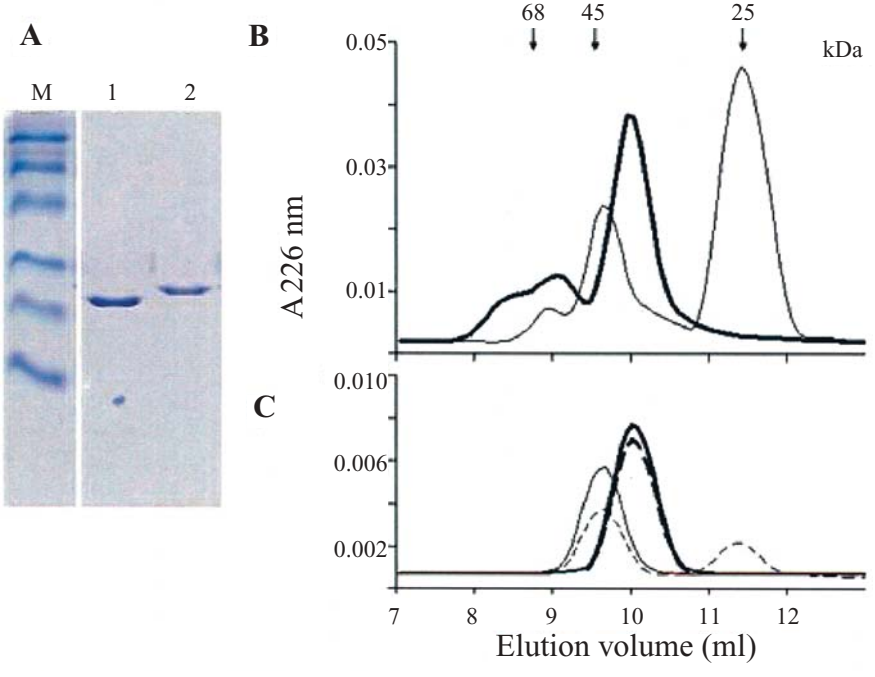

Figure 2. SDS-PAGE and size-exclusion chromatography on a calibrated Superdex 75 HR 10/30 column of the Protein L-agarose-purified scFvs 9C2. (*A*) SDS-PAGE of the scFv$_5$ (lane 1) and scFv$_{15}$ (lane 2) preparations. Molecular markers (M) are 112, 81, 49.9, 36.2, 29.9 and 21.3 kDa. (*B*) Chromatography of affinity-purified scFv$_5$ (bold) and scFv$_{15}$ (plain). (*C*) Rechromatography of collected fractions corresponding to $(scFv_5)_2$ (bold) and $(scFv_{15})_2$ (plain) after storage at 4 °C for 1 h (continuous line) or 8 days (dashed line). The column was calibrated with chymotrypsinogen A (25 kDa), ovalbumin (45 kDa) and bovine serum albumin (68 kDa).

trimeric and multimeric structures. Direct RIA demonstrated that all the fractions contained functional antigen-binding protein (not shown). These results indicated a minimal tendency to form higher-order oligomers either in vivo during production in *E. coli*, or in vitro, during the extraction process. No fraction corresponding to the monomeric $scFv_5$ was eluted from the column. The $scFv_5$ dimeric fraction was resubmitted to chromatography after prolonged storage for up to 8 days at 4 °C in diluted solution (20 µg/ml) (fig. 2C). Once again, the retention volume was 10 ml, and it was eluted free of other molecular mass species, demonstrating the high stability of the dimer $(scFv_5)_2$. Conversely, analysis of the fractions corresponding to the multimeric forms of $scFv_5$ showed that they were relatively unstable, as they partially reverted back to the trimer and dimer when stored at 4 °C for 8 days (not shown).

When the control affinity-purified $scFv_{15}$ protein (0.35 mg/ml) was analyzed on the same column, it was eluted mainly as the monomer at 11.4 ml (58%), with a small amount of dimer at 9.6 ml (36%) and higher-order oligomers (less than 8.8 ml) (6%), in agreement with previous observations made with the same $scFv_{15}$ carrying an epitope flag at the C terminus (fig. 2B) [20]. After storage at 4 °C, the isolated dimeric fraction was resubmitted to gel filtration (fig. 2C). This resulted in a two-peak elution profile: one peak corresponding to the dimer and the other to the monomer. The proportion of monomer increased when the dimer fraction was left for several days before being reapplied, demonstrating the instability of $scFv_{15}$ dimers.

Antigen-binding activity

Diabody $scFv_5$9C2 recognized [^{125}I]-AahI in a direct RIA (fig. 3). The affinity and specificity of the recognition were assessed using the affinity-purified preparation or the isolated dimeric form $(scFv_5)_2$ eluted from the Superdex 75 column in a competitive RIA. Affinity-purified $scFv_{15}$9C2, monomeric $scFv_{15}$9C2 and dimeric $scFv_{15}$9C2 were used as controls. All forms exhibited high affinity for AahI, with a K_D of the order of 10^{-11} M ($scFv_5$ dimer: $K_D = 8.08\ 10^{-11}$ M; protein L-agarose-purified $scFv_5$: $K_D = 8.79\ 10^{-11}$ M; $scFv_{15}$ dimer: $K_D = 9.82\ 10^{-11}$ M; monomeric $scFv_{15}$: $K_D = 12.72 \times 10^{-11}$ M; protein L-agarose purified $scFv_{15}$: $K_D = 13.51 \times 10^{-11}$ M).

To determine whether the diabody was bivalent, the FPLC-eluted fraction corresponding to the $scFv_5$ dimer was incubated with increasing amounts of AahI before being submitted to isoelectrophoresis (IEF) (fig. 4A). This resulted in an increasing fraction of shifted diabody due to antibody-antigen complex formation. Under the conditions used for IEF, two additional bands with higher mobility were observed after adding AahI to the diabody. One band appeared after incubating the diabody with moderate amounts of AahI, and probably corresponded to binary complexes of one toxin molecule bound to the diabody [AahI-$(scFv_5)_2$]. The other band, at the very top of the gel, was observed after incubating the diabody with an equimolar amount of toxin AahI, and obviously corresponded to ternary immunocomplexes of the type AahI-$(scFv_5)_2$-AahI. Finally, free toxin AahI, which is a very basic protein (pI > 10) was not detectable on the gel under these experimental conditions. The addition of toxin AahI to the monomeric $scFv_{15}$ fraction, when analyzed in the same manner, led to a single additional band, corresponding to binary AahI-$scFv_{15}$ immunocomplexes, regardless of the amount of toxin added (fig. 4A). The electrophoretic mobility of this band was the same as that of the ternary complexes AahI-$(scFv_5)_2$-AahI, and indeed both types of complex are likely to have the same pI. From this observation, we concluded that most if not all the diabody molecules were fully functional and bivalent.

The bivalency of the diabody was also demonstrated by size-exclusion chromatography of the immunocomplexes

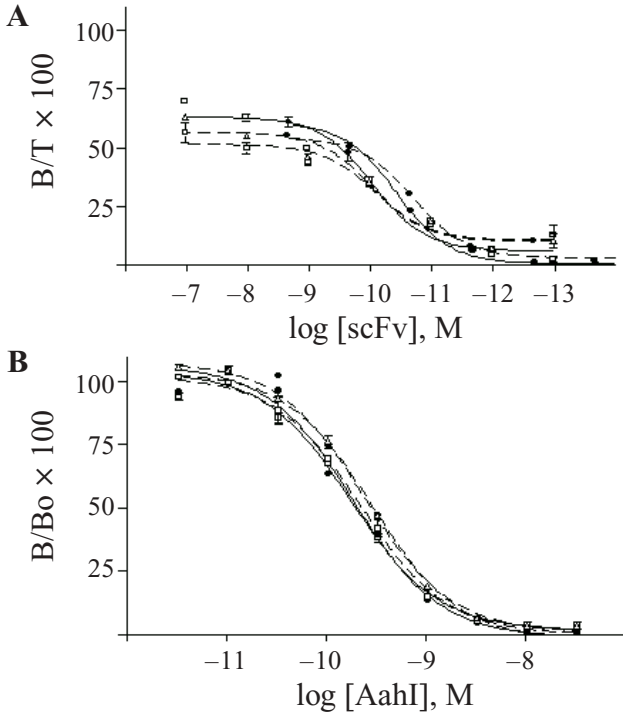

Figure 3. Reactivity of scFv 9C2 preparations with AahI toxin tested by RIA. Continuous lines represent $scFv_5$ preparations: protein L-agarose-purified $scFv_5$ (circle), diabody $(scFv_5)_2$ (square). Dashed lines represent $scFv_{15}$ preparations: protein L-agarose-purified $scFv_{15}$ (circle), dimeric $scFv_{15}$ (square) and monomeric $scFv_{15}$ (triangle). (*A*) Serial dilutions of scFv were incubated with [^{125}I]-AahI. Results are expressed as B/T, where B is the radioactivity bound to scFv and T the total radioactivity. (*B*) Inhibition of [^{125}I]-AahI binding to scFv was performed with unlabeled AahI. Results are expressed as B/B_0, where B and B_0 are the radioactivity bound to scFv in the presence (B) and absence (B_0) of unlabeled ligand respectively.

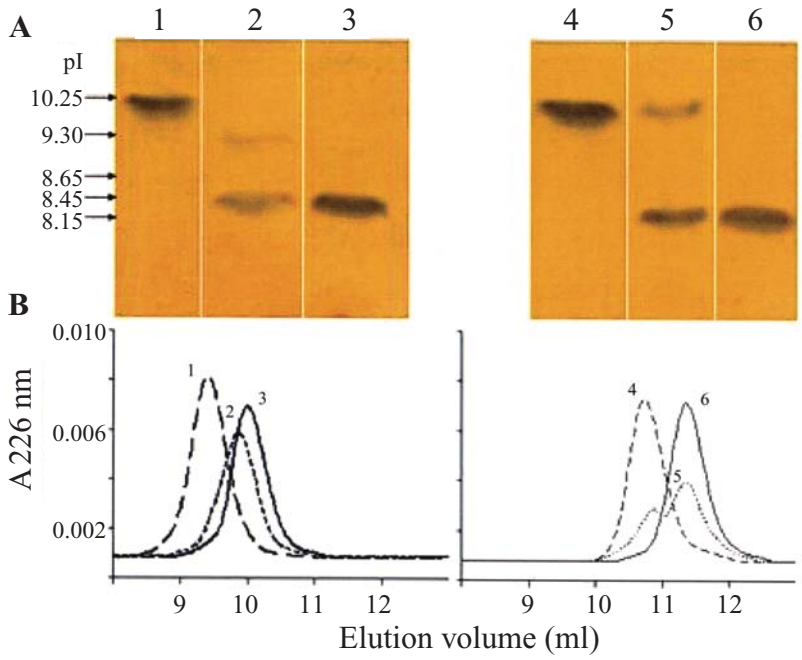

Figure 4. Analysis of the antigen-binding status of scFvs 9C2 by isoelectrofocussing (*A*) and size-exclusion chromatography Superdex 75 HR 10/30 (*B*). Lanes/columns 1–3: FPLC-purified diabody $(scFv_5)_2$ incubated with AahI in a 1 : 2 molar ratio (1), 3 : 2 molar ratio (2) or alone (3). Lanes/columns 4–6: FPLC-purified monomeric $scFv_{15}$ incubated with AahI in a 1 : 1 molar ratio (4), 3 : 1 molar ratio (5) or alone (6).

resulting from incubating purified FPLC $(scFv_5)_2$ with increasing amounts of toxin AahI (fig. 4B). When an excess of toxin AahI was added, the elution peak from the Superdex column was completely shifted to the left, whereas a single peak with intermediate mobility (not detected with $scFv_{15}$) was observed when samples resulting from the incubation of diabody with moderate amounts of AahI were loaded onto the column. We concluded that both binding sites of the diabody were active, and that the preparation did not contain any detectable amounts of inactive or partially active protein.

Direct evidence of the bivalency of diabody 9C2 could not be provided by a simple electron microscopic observation of immunocomplexes formed with AahI, mainly because of the small size of the AahI toxin (fig. 5A). However, the existence of ternary immunocomplexes was confirmed as follows. Immunocomplexes resulting from the incubation of $(scFv_5)_2$ and AahI were mixed with IgG 2G3, a monoclonal antibody previously shown to be specific for a single epitope of AahI which does not overlap with epitope 9C2 [22]. Unbound material was removed by gel filtration and immunocomplexes were adsorbed to thin carbon films, negatively stained and examined under the electron microscope. Figure 5B, C show selected views of free IgG 2G3 and of complexes resulting from incubating AahI with $(scFv_5)_2$ and then with IgG 2G3. The size of the immunocomplexes observed clearly demonstrated that they did not consist simply of one IgG with a binary complex AahI-$(scFv_5)_2$ bound to each Fab arm. The most frequent structure was a characteristic flattened annular complex, which probably consisted of two IgG molecules bridged via a pair of ternary immunocomplexes [AahI-$(scFv_5)_2$-AahI]. Each ternary immunocomplex maintained the link between the Fab arms of two distinct IgG molecules, as suggested in figure 5D. These annular complexes were

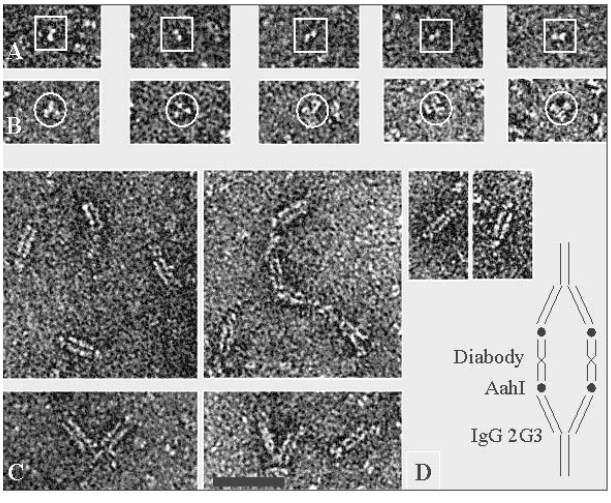

Figure 5. Analysis of the antigen-binding status of $(scFv_5\,9C2)_2$ by electron microscopy. Bar, 25 nm. (*A*) Immunocomplexes resulting from incubating diabody $(scFv_5)_2$ with AahI. (*B*) Gallery of free IgG 2G3 molecules. (*C*) Fields of diabody 9C2/AahI complexes after incubation with IgG 2G3. (*D*) Selection of flattened ring complexes and schematic interpretation.

flattened, demonstrating that the angle between the two arms of the IgG molecules was considerably reduced, and this may be related to constraints imposed by the negative stain, but also by the close vicinity of epitopes 9C2 and 2G3 on the surface of AahI. These observations provided direct and unambiguous evidence for the bivalence of the recombinant diabody.

Stability in vitro and in vivo

To test the long-term stability of $scFv_5$9C2, protein L-agarose-purified samples were diluted in rabbit serum and incubated at body temperature (37 °C) for 1–8 days. RIA determination of the antigen-binding properties of samples taken at several time points showed no change over a period of 6 days. The antigen-binding activity then decreased slightly, but the samples still retained 90% of their activity after 8 days (data not shown).

The in vivo stability and diffusibility of the diabody were investigated after intraperitonal injection of mice (fig. 6). Blood samples were taken at various time points and analyzed by RIA. Significant AahI-binding activity was very soon detected in the plasma (only 5 min after the intraperitoneal injection) demonstrating a high rate of diffusion of the diabody to the vascular compartment. The concentration of functional diabody in the plasma reached a maximum value (C_{max}) after a short time (T_{max}) of 30–60 min, and then declined slowly over 24–32 h.

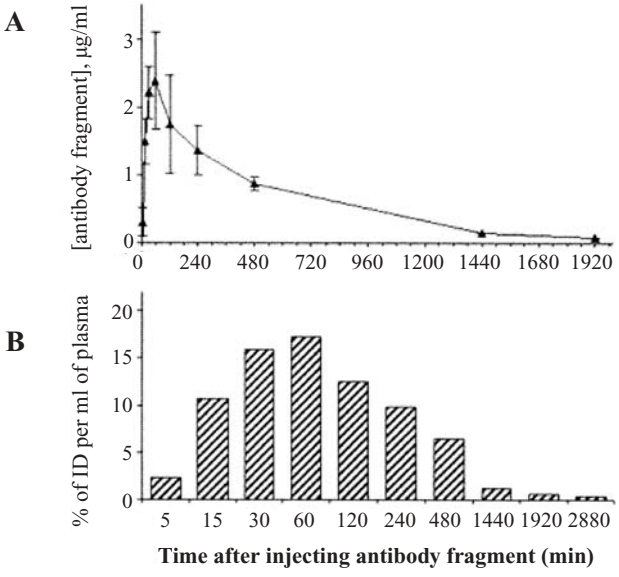

Figure 6. In vivo plasma distribution of functional diabody after intraperitoneal injection in mice. (*A*) Concentration of active antibody fragment as deduced from direct RIA data. (*B*) Percentage of injected dose (ID) of diabody (14 µg/20 g mouse) recovered per milliliter of plasma.

Toxin neutralization in vivo

The in vivo interaction of diabody with toxin AahI was investigated in the murine model under conditions that mimic natural envenomations (table 1). Purified toxin AahI was injected into mice subcutaneously at doses equal to or higher than the LD_{50}. Then, 10 min after the experimental envenomation, which is when the first clinical symptoms usually appear, the mice were treated with an intraperitoneal injection of diabody. All mice treated with the diabody preparation survived the injection of 665 ng (2 LD_{50}) when the toxin/$scFv_5$ molar ratio injected was 1/8. The protective capacity of the $scFv_5$ remained significant when injected in the same quantity (19.5 µg per mouse) after experimental envenomation with 4 LD_{50} (table 1).

The protective capacity of the diabody was therefore between 102 and 205 LD_{50} per milligram of preparation (between 0.125–0.250 nM of AahI/nM of $scFv_5$) under these assay conditions. No protection was observed in the control experiments, where the diabody preparation was replaced by 0.1% bovine serum albumin.

Discussion

Toxin-binding antibodies have been known to be potent therapeutics since the landmark studies of Behring and Kitasato [30]. Passive immunotherapy is still the only specific treatment available for neutralizing biological toxins in vivo, and polyclonal immunoglobulins are still used as antitoxins in the treatment of venomous bites, some infectious diseases such as diphtheria, tetanus and botulism, and to reverse colchicine poisoning [31–33]. However, the administration of heterologous polyclonal antibody fragments [Fab and $F(ab)'_2$] to humans is associated with major drawbacks concerning their production and use, including (i) lot-to-lot heterogeneity, (ii) risk of contamina-

Table 1. Protection of mice against toxin challenge by intraperitoneal injection of protein L-agarose purified diabody 9C2.

AahI injected (s.c.)		$scFv_5$ injected (i.p.) 10 min later (µg per couse)	Molar ratio AahI : $scFv_5$	Protected/ injected mice	Protective capacity
ng per mouse	LD_{50}				
665	2	0		0/6	
665	2	19.5	1 : 8	6/6	>102 LD_{50}/mg
1330	4	19.5	1 : 4	4/6	<205 LD_{50}/mg
2660	8	19.5	1 : 2	2/6	

tion with infectious agents, (iii) low specific antibody content, making it necessary to inject a high, and potentially toxic dose and (iv) life-threatening early anaphylactic shock and late serum sickness. Recombinant antibody fragments are emerging as promising therapeutics, which outperform polyclonal serum in homogeneity, specific activity and, possibly, safety. They can be tailored to design novel therapeutics with various formats and multiple valencies which are potentially effective in detoxification [18]. They includes animal (scorpion, snake), bacterial (pertussis, botulinum, anthrax) and vegetal (digoxin, colchicin) toxins, and may be extended to other drugs such as tricyclic antidepressants and narcotics [20, 34–39].

We are engaged in designing molecules that are more appropriate than polyclonal antibodies for the treatment of human envenomations. Recently, we engineered recombinant scFvs from existing scorpion toxin-neutralizing IgG molecules and demonstrated their efficacy in vitro and in an animal model [19, 20]. However, the conditions of the assay were purely experimental, very different from natural envenomations, and the very rapid clearance of scFvs is usually viewed as a major drawback for therapeutic application [40]. Here, the diabody (≈ 50 kDa) we engineered is significantly larger than scFv monomers. It is similar in size to Fabs, which are currently considered to be one of the most suitable formats of antibody fragments for antivenom therapy in terms of neutralizing efficacy and bioavailability [3, 5].

The fully functional neutralizing diabody 9C2 was easily purified from the periplasm of recombinant bacteria in a one-step affinity chromatography using protein L-agarose. This procedure did not require the fusion of the diabody to an epitope flag, which is undesirable for therapeutic applications. Polyacrylamide gel electrophoresis and mass spectrometry analysis confirmed the purity and integrity of the recombinant protein. The bacterial production yield of the purified diabody was sufficient to allow us to investigate its features in more detail even if further modifications of the production process might be required for clinical applications. One could consider the use of fermenters for large-scale production as is the case for pharmaceutical insulins produced from recombinant bacteria. Recent studies have also demonstrated that transgenic tobacco plants might be a competent production system for clinically relevant antibody fragments [41].

Several aspects of scFv dimerization are important for effective antivenom therapy. First, the higher molecular weight should contribute to extending the persistence of molecules in the circulation by slowing their renal clearance, thus increasing the chance that these molecules will bind and redistribute the target toxin. Conversely, this increase in size could reduce the diffusion of the antibody fragment to the tissues and therefore its ability to trap the toxin before it binds to its receptor. To bypass this drawback, a design compromise must be found with respect to the molecular weight of therapeutic molecules, and the optimal molecular design can only be determined experimentally. Another point to consider in dimerization is the greater valency compared to scFv or Fab fragments and thus the potentially higher avidity. This is of particular interest for scFvs with moderate affinity [42, 43]. We compared the structural stability of the dimeric diabody 9C2 to that of the dimeric form of $scFv_{15}$. The results we report clearly confirm that a 5-amino-acid residue linker between the variable domains of an scFv promotes the formation of highly stable bivalent dimers. Size-exclusion, fast-performance liquid chromatography after prolonged storage at 4 °C showed that dimers were the predominant stable form of diabody $scFv_5$. Monomeric, non-functional $scFv_5$ was never observed, and higher-order oligomers (trimers and oligomers) reverted back to the dimer after storage in dilute solution. Such high stability of the diabody (dimeric form of $scFv_5$) was not observed with the conventional $scFv_{15}$ construct, which results from a back-to-back dimerization and spontaneously dissociates into monomeric structures, which are the thermodynamically stable form [44, 45]. Thus, the dimeric $scFv_{15}$ structures are not appropriate for clinical use, since they rapidly dissociate into monomers which are still functional but have inappropriate bioavailability and pharmacokinetics [46]. Another advantage of the diabody format of antibody 9C2 is its high thermal and serum stability, which contrasts with most scFvs that rapidly lose their activity in biological fluids at 37 °C [22]. We found that 90% of AahI-binding activity was retained by diabody 9C2 after incubating for 8 days at 37 °C in serum.

Crystal structure analysis of a diabody clearly showed that the VH domain of one chain is paired with the VL domain of the other chain and vice versa [24]. The antigen-binding sites point in opposite directions and are separated by approximately 65 Å, which is less than half the distance in IgG. Such molecules may lack the flexibility to bind simultaneously to two epitopes with fixed spatial orientations relative to each other, such as multivalent antigenic molecules or multiple cell surface antigens. However, we clearly demonstrated here by complementary approaches that both binding sites displayed by the diabody molecule are fully functional when incubated with the free toxin in a 1 : 2 diabody to toxin molar ratio. This is undoubtedly an advantage over the Fab format that has the same size, but is only capable of detoxifying in an equimolar ratio. Moreover, binding of the toxin to the diabody molecule is likely to reinforce the stability of the dimer, since the antigen-binding site is formed by the pairing of one VH domain and one VL domain from the two different subunits. We observed here that the diabody ($K_D = 8 \cdot 10^{-11}$ M) and protein L-agarose affinity-purified $scFv_5$ preparations had extremely high functional affinity. This high affinity is es-

sential for detoxification and to reverse the normal distribution of the toxin in the body [47].

The ultimate goal of the present study was to demonstrate the therapeutic efficacy in vivo of the scFv 9C2 in the diabody format. First, we demonstrated that the diabody molecule entered the systemic circulation very rapidly after intraperitoneal injection, with a high level of activity (> 50% of C_{max}) between 10 min and 6 h. The persistence of significant high toxin-binding activity in the plasma is important since (i) any toxin remaining in the vascular compartment can be sequestered, forming stable ternary immunocomplexes in the same way that $F(ab)'_2$ does, but here the complexes are small enough (64 kDa) to be filtered by the kidneys, and (ii) it certainly helps to redistribute toxins from deep compartments into the vascular space. This study also provides direct evidence that the diabody can be used as an antitoxin in the mouse model under conditions that perfectly mimic natural envenomation. Here, complete protection was observed when mice envenomed with AahI (2 LD_{50}) by the subcutaneous route were intraperitonally injected 10 min later with antibody fragments in a 1 : 8 molar ratio of AahI to $scFv_5$ monomer. The protective capacity of the diabody was therefore estimated to be between 102 and 205 LD_{50} per milligram of diabody. This must be compared to the protective capacity of scorpion antivenoms, which are at their best in the range of 0.5 LD_{50} per milligram of antivenom in the standard mouse assay [48, 49]. In addition, we must stress that in vivo toxin neutralization is usually performed by injecting a premix of toxin and antibody [36, 49]. This allows immunocomplexes to be formed before the mouse is injected, and means that the result reflects the neutralizing titer of the antivenom, but not its protective capacity in natural envenomations, where the antidote is injected to the patient after the toxins have already diffused throughout the whole body. Thus, we concluded that neutralization with conventional pharmaceutical products requires the administration of much larger quantities of therapeutic proteins, even though the manufacturing process of some antivenoms now includes the selective extraction of horse scorpion-venom-specific IgGs from other immunoglobulins, as in the case of the polyspecific scorpion antivenom LABS 50 (Aventis, Marcy l'Etoile, France). Such antivenoms continue to be made from antibody fragments with varying affinities for the different components of the venom. Moreover, relatively few of the specific antibodies that recognize the different epitopes of these toxins possess significant neutralizing activity [50]. The results reported here clearly indicate that the high activity of specific and homogeneous recombinant fragments should make the use of a smaller amount of antigen-binding protein possible and thus avoid the toxic events occasionally reported with high-dose antivenom serum therapy.

In conclusion, this study provides the first experimental evidence of a diabody that is a potentially effective agent for treating envenomations and diseases caused by pathogens and biological threat agents. The binding (affinity and specificity) and pharmacokinetic properties of diabody 9C2 could be further enhanced by molecular 'tailoring' [22, 51, 52]. For clinical applications in human medicine, the humanization of the variable domains of the diabody by grafting the 9C2 CDRs onto variable chain frameworks of homologous human antibodies may further improve tolerance and reduce the drawbacks of residual immunogenicity [53, 54]. Finally, the construction of a heterodimeric diabody using humanized murine scFv 4C1 (directed against toxin AahII) and 9C2 (directed against AahI, but also able to cross-react with AahIII) as building blocks, may enable the use of a single molecule to neutralize the overall toxicity of the venom.

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