Exploiting Cross-reactivity to Neutralize Two Different Scorpion Venoms with One Single Chain Antibody Fragment

Lidia Riaño-Umbarila, Gabriel Contreras-Ferrat, Timoteo Olamendi-Portugal, Citlalli Morelos-Juárez, Gerardo Corzo, Lourival D. Possani, and Baltazar Becerril

From the Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, Cuernavaca, Morelos 62250, México

We report the optimization of a family of human single chain antibody fragments (scFvs) for neutralizing two scorpion venoms. The parental scFv 3F recognizes the main toxins of Centruroides noxius Hoffmann (Cn2) and Centruroides sulfus sulfus Hoffmann (Css2), albeit with low affinity. This scFv was subjected to independent processes of directed evolution to improve its recognition toward Cn2 (Riaño-Umbarila, L., Juárez-González, V. R., Olamendi-Portugal, T., Ortiz-León, M., Possani, L. D., and Becerril, B. (2005) FEBS J. 272, 2591–2601) and Css2 (this work). Each evolved variant showed strong cross-reactivity against several toxins, and was capable of neutralizing Cn2 and Css2. Furthermore, each variant neutralized the whole venoms of the above species. As far as we know, this is the first report of antibodies with such characteristics. Maturation processes revealed key residue changes to attain expression, stability, and affinity improvements as compared with the parental scFv. Combination of these changes resulted in the scFv LR, which is capable of rescuing mice from severe envenomation by 3 LD50 of freshly prepared whole venom of C. noxius (7.5 µg/20 g of mouse) and C. sulfus (26.25 µg/20 g of mouse), with surviving rates between 90 and 100%. Our research is leading to the formulation of an antivenom consisting of a discrete number of human scFvs endowed with strong cross-reactivity and low immunogenicity.

Scorpion venoms are essentially neurotoxic and contain diverse constituents, including peptide toxins that cause direct impairment of Na+ channel activity (3, 4). This effect results in excessive firing of neuronal axons. Neuronal overstimulation is reflected by both autonomic and neuromuscular symptoms, which may range from local pain, paresthesia, and seizures, to encephalopathy, pulmonary edema, and fatal cardiotoxicity (5). Peptide toxins are related by common ancestry (4). Indeed, the most relevant toxins of the aforementioned species share the same size (66 amino acids) and significant sequence identities (Fig. 1). On the other hand, toxins exhibit significant variation of target specificity. For instance, toxins Css2 and Cn2 bind to the hNav1.6 channel with high specificity (6). In contrast, Cll1 and Cll2 interact with various Na+ channels.2

Current treatment against scorpionism includes the use of antivenoms, which consist of polyclonal antibodies obtained from immunized horses. Such polyclonal antibodies are effective neutralizing agents against diverse scorpion venoms (7). However, neutralization depends on non-human molecules that may become immunogenic (8). Thus, an ongoing research goal is the improvement of antivenom therapy by generating human recombinant antibodies against specific toxins. The combined use of protein engineering and directed evolution further allows the generation of antibody fragments endowed with special features (9–13). Simplified antibodies including Fabs (500 amino acids) and scFvs (250 amino acids) can be generated and evolved experimentally. The resulting antibody variants bind to target antigens with high efficiency and, due to their low sizes, achieve fast diffusion and clearance rates (14), two desirable features in any antivenom against scorpionism.

In a previous work, our group reported the generation of the antibody variant scFv 6009F by directed evolution and phage display for neutralizing the in vivo effects of toxin Cn2 (1). This antibody variant showed increased affinity for the target antigen as compared with its progenitor, the parental scFv 3F. The therapeutic potential of scFv 6009F was further confirmed by neutralizing whole venom of C. noxius (1). Recent tests showed that scFv 6009F has cross-reactivity against toxins Cn3, Cn4, and Cn5 (evidence

2 L. D. Possani and E. Wanke, personal communication.

3 The abbreviations used are: scFv, single chain antibody fragment; CDR, complementarity determining region; C. noxius, Centruroides noxius Hoffmann; C. sulfus, Centruroides sulfus sulfus; C. limpidus, Centruroides limpidus limpidus.
The amino acid sequences of toxins Cll1, Cll2, Cn2, Css2, Css4, and Cn3 are shown. Symbols indicate: identity and physicochemical properties; noxius against whole venom of both activity was also increased against Cn2 and Css2, as well as levels of expression and stability. Furthermore, neutralizing variants. The resulting scFv, named LR, exhibited outstanding properties were identified by comparing the maturation processes and by combining relevant mutations from different sequence contexts.

The above observations suggested that the parental scFv 3F holds structural properties useful to generate antibody variants with neutralizing activity against closely related scorpion toxins. To test this assumption, the parental scFv 3F was matured by directed evolution to attain neutralizing activity against toxin Cn2. The generated antibody (scFv 9004G) neutralized toxin Cn2 and also whole venom of C. suffusus. The maturation process that led to scFv 9004G also resulted in neutralizing activity against toxin Cn2 and whole venom of C. noxius. Thus, scFv 9004G retained the recognition potential of its progenitor, and exhibited a behavior analogous to that of scFv 6009F. The properties of these antibody variants are similar, but not identical. That is, scFv 6009F is better as neutralizing agent, whereas scFv 9004G shows higher expression yields.

Important mutations for toxin recognition and other properties were identified by comparing the maturation processes of scFv 9004G and scFv 6009F. A key mutation appeared in both processes and resulted in improved recognition toward toxins Css2 and Cn2. Another key mutation appeared during the maturation of scFv 9004G (1). This change was introduced into scFv 9004G to merge the best properties of both antibody variants. The resulting scFv, named LR, exhibited outstanding levels of expression and stability. Furthermore, neutralizing activity was also increased against Cn2 and Css2, as well as against whole venom of both C. noxius and C. suffusus. Thus, we are generating a family of antibody variants against different scorpion venoms. Cross-reactivity is a feature that allows each antibody variant to neutralize different toxins without adverse secondary effects. This model system entails an open ended potential for generating new therapeutic agents against scorpionism. Such potential can be harnessed by directed evolution and by combining relevant mutations from different sequence contexts.

FIGURE 2. Cross-reactivity of scFv 6009F and scFv 9004G against closely related scorpion toxins. An ELISA was used to test recognition toward Cn2, Cn3, Css2, Css4, Cll1, and Cll2. Concentrations used were: toxin, 3 μg ml⁻¹; scFv, 5 μg ml⁻¹. Bars: white, scFv 6009F; black, scFv 9004G. Error bars of four experiments are shown.

EXPERIMENTAL PROCEDURES

Venoms of C. noxius and C. suffusus—Fresh whole venom was obtained from scorpions by electrical stimulation. Samples were dissolved in water and centrifuged at 12,000 × g at 4 °C for 10 min. Insoluble material was discarded, whereas the toxin-containing supernatant was recovered and spectrophotometrically quantified at A = 280 nm, assuming that 1 unit of absorbance is equivalent of 1 mg ml⁻¹ of protein.

Enzymes—Taq polymerase, T4 DNA ligase, and restriction endonucleases SfiI and NotI were purchased from Fermentas (Glen Burnie, MD).

Toxin Purification—Toxins Css2 and Css4 were isolated from whole venom of C. suffusus as described elsewhere (6, 22). Toxins Cn2 and Cn3 were obtained from whole venom of C. noxius Hoffmann as described in Ref. 23. Other venom constituents used in this work, including toxins Cll1 and Cll2, were isolated from C. limpidus limpidus as described elsewhere (24, 25).

Directed Evolution of the Parental scFv 3F—The maturation process consisted of three cycles of directed evolution to obtain antibody variants against toxin Css2 (Fig. 2). Each cycle included the construction of a mutant library by random mutagenesis and four bio-panning rounds by means of phage display. Error-prone PCRs were performed under conditions to obtain different mutation rates (26, 27). Coding sequences used as templates corresponded to the parental scFv 3F in cycle 1; the antibody variants 9D and 5C in cycle 2; and the antibody variant 910F in cycle 3. Amplification products were combined to obtain mutant libraries.
Neutralizing Antibody Fragments against Scorpion Venoms

as described previously (1). In cycle 1 of directed evolution, bio-panning rounds were performed as described by Marks et al. (28); the toxin concentration was 3 μg ml⁻¹ in the first and second bio-panning rounds, 1.5 μg ml⁻¹ in the third round, and 300 ng ml⁻¹ in the fourth round. In cycles 2 and 3 of directed evolution, bio-panning was carried out under stringent conditions as described previously (1). A toxin concentration of 300 ng ml⁻¹ was used in all bio-panning rounds of cycle 2; in the fourth round, phage antibodies were preincubated with 2 M guanidinium chloride at 25 °C for 30 min. In cycle 3, toxin concentration was decreased from 350 to 170 ng ml⁻¹; phage antibodies were preincubated at 40 and 50 °C for 30 min. Antibody variants were evaluated as phage antibodies and soluble protein by ELISA as described previously (1). The sequence of each variant was determined with the primers: forward, 5′-ATACCTATTGCTACGGGC-3′, and reverse, 5′-TTTCAAGCTTACTG-3′. The product sequences were subcloned in vector pSyn1 to transform Escherichia coli TG1 cells by electroporation. Protein expression and purification were carried out as described previously (1). Protein purity and identity were confirmed by mass spectrometry. Protein concentration was determined spectrophotometrically at λ = 280 nm, assuming 1 absorbance unit as equivalent to 0.7 mg ml⁻¹ of protein.

Construction of the Antibody Variant scFv LR by Site-directed Mutagenesis—The coding sequence of scFv 9004G was modified to generate a V101F change. A megaprimer was generated by PCR with the oligonucleotides V101F 5′-CAAAAC-TTCCGAACCCCCCTC-3′ and forward, 5′-TTTCAAGCTTACTG-3′. The product was purified from agarose gel. The scFv 9004G sequence was re-amplified using the megaprimer and the oligonucleotide Myc 5′-TCGATCTTCTTCTGAGATG-3′. The product was gel purified, digested with restriction endonucleases Sfil and NotI, gel purified again, and ligated to appropriately digested vector pSyn1. The coding sequence of the new antibody variant scFv LR was verified by sequencing.

Surface Plasmon Resonance Measurements—Kinetic constants of antibody binding to immobilized toxin were determined in a Biacore biosensor system (BIACORE X, Uppsala, Sweden). 90 ng of toxin dissolved in 10 mM MES (pH 6) were bound to a CM5 sensor chip using a solution of 0.05 μM N-hydroxysuccinimide and 0.2 μM N-ethyl-N-(dimethylaminopropyl)carbodiimide. Approximately 70 to 100 resonance units were coupled. scFv proteins were serially diluted in HBS-EP buffer (Biacore); 100-μl samples were injected over immobilized toxin at a flow rate of 50 μl min⁻¹. Delay between injections of the parental scFv 3F was 400 s. Delay for scFv 6009F, scFv 9004G, and scFv LR was 1000 s. Biosensor measurements were performed at 25 °C. Protein concentrations ranged from 0.5 to 50 nM for scFv 6009F, scFv 9004G, and scFv LR. For the parental scFv 3F, protein concentrations ranged between 1 and 100 nM. Constants were calculated with the Langmuir (1:1) model of the BIAEVALUATION software version 3.1.

Functional Stability Measurements—To test recognition of toxin Cn2 in the presence of strong denaturing conditions, samples of each antibody variant were prepared at 1.5 μg ml⁻¹ with different concentrations of guanidinium chloride (0, 0.9, 1.1, 1.3, and 1.5 M). Samples were incubated overnight at 37 °C and then evaluated by ELISA in a plate with immobilized toxin Cn2 at 1.5 μg ml⁻¹. The ELISA test was followed as described previously (1). Average values (n = 4) were calculated from readings at λ = 492 nm, allowing observation of stability differences among antibody variants.

In Vivo Neutralization Tests—LD₅₀ values used in this work were ~0.25 μg/20 g of mouse and ~0.7 μg/20 g of mouse for toxins Cn2 and Css2, respectively (22, 29). Unless otherwise noted, 2 LD₅₀ of toxin was used. Groups of 8 to 10 female CD4 mice weighing ~20 g were injected with toxin by the intraperitoneal passage, thus providing a control population. Intoxication symptoms were followed until death or symptom remission was observed. In the experimental population, the neutralizing activity of antibody variants was tested by a common procedure. Toxins were mixed with each antibody variant to different toxin-to-antibody molar ratios (1:10, 1:2, or 1:1). Mixtures were preincubated for 30 min at room temperature (~25 °C) prior to their injection into mice by the intraperitoneal passage. To test the neutralization activity against freshly prepared whole venom, two different tests were performed. In test 1, an amount of venom equivalent to 2 or 3 LD₅₀ was mixed with antibody to a final 1:3 toxin-to-antibody molar ratio. (Ratios were calculated relative to the main toxin of the venom.) A LD₅₀ of ~8.75 μg/20 g of mouse was determined for the venom of C. suffusus suffusus, because ~2.8% of the total venom corresponds to toxin Css2. The LD₅₀ of the venom of C. noxius was calculated at ~2.5 μg/20 g of mouse, because toxin Cn2 represents ~6.8% of the total content (29). Mixtures were preincubated at room temperature for 30 min prior to their injection into mice. Test 2 was aimed at determining the ability of the antibody to rescue mice from developing envenomation. An amount of freshly prepared whole venom equivalent to 3 LD₅₀ was injected into mice. A time lapse of 5–10 min was allowed to pass before injecting antibody in a 1:3 toxin-to-antibody molar ratio relative to the main toxin of venom.

RESULTS

The parental scFv 3F was previously isolated against toxin Cn2 from a human non-immune antibody library. However, this scFv was unable to neutralize the in vivo effects of the toxin. The scFv 3F was subjected to a maturation process to increase its affinity to Cn2. The process culminated in variant scFv 6009F, which showed neutralizing activity against Cn2 and whole venom of C. noxius (1). scFv 6009F did not show recognition toward toxins Cll1 and Cll2 (1). More recently, ELISA tests revealed that scFv 6009F cross-reacts with other toxins, including Cn3, Css2, and Css4 (Fig. 2). Cross-reactivity against Css2 was relevant, because this toxin is the major con-
Neutralizing Antibody Fragments against Scorpion Venoms

Maturation of scFv Antibody Variants

The parental scFv 3F was used again as starting point of directed evolution, but this time to achieve neutralizing activity against toxin Cn2. This new maturation process was expected to reveal mutations fundamental to cross-reactivity (30). Conceivably, however, it could have also resulted in modified specificity and even loss of recognition toward the toxin for which the parental antibody was first isolated, as it has occurred in other cases (18). The maturation process consisted of three cycles of directed evolution and multiple rounds of bio-panning. Fig. 3a compares the maturation processes from the parental scFv 3F to improve its recognition toward toxins Cn2 and Css2.

Cycle 1 of Directed Evolution—A mutant library was constructed using the parental scFv 3F as template for error-prone PCR. The resulting library exhibited 1.3 × 10⁷ sequences and a mutagenic rate of 1.6%. Four bio-panning rounds against Cn2 were performed. Two antibody variants, 9D and 5C, were isolated and further characterized: 5C exhibited one amino acid change (N74D), whereas 9D showed two changes (A23T and N74D) (Fig. 3b). Interestingly, the N74D change was present in both variants and emerged independently in the maturation process of scFv 6009F (1). This mutation was sufficient to increase recognition of both antibody variants to Cn2, Cn3, and Cn3, as demonstrated by phage-antibody ELISA tests (data not shown). The sufficiency of a single amino acid change to achieve significant binding activities has been observed before with a mutant Fab fragment. Such mutant recognizes digoxin and three of its analogs (30).

Cycle 2 of Directed Evolution—Antibody variants 5C and 9D were used as templates to construct a second mutant library (2.5 × 10⁷ variants; mutation rate, 0.4%). Four bio-panning rounds against Cn2 were performed under stringent conditions. The antibody variant 910F showed the best recognition to Cn2 and Cn3, as demonstrated by phage-antibody ELISA tests (data not shown). The sufficiency of a single amino acid change to achieve significant binding activities has been observed before with a mutant Fab fragment. Such mutant recognizes digoxin and three of its analogs (30).

Cycle 3 of Directed Evolution—A third mutant library was constructed using antibody variant 910F as template (1.8 × 10⁷ variants; mutation rate, 0.4%). Bio-panning rounds against Cn2 were performed under stringent conditions. Twelve antibody variants showed good recognition to Cn2 (A₄₉₂ nm ~2). Sequence analyses revealed that an antibody variant, named scFv 9004G, was isolated in three independent occasions. The coding sequence of scFv 9004G was cloned into plasmid pSyn1 to obtain soluble protein. Cross-reactivity was evaluated in an ELISA test that included scFv 6009F as control (Fig. 2). Both antibody variants showed similar recognition to Cn2 and Css2, as well as to Cn3 and Css4. Neither scFv showed recognition to toxins ClI1 and ClI2 of C. limpidus.

Characterization of scFv 9004G

The neutralizing activity of scFv 9004G was evaluated with CDI female mice. Table 1 indicates that 1 LD₅₀ of Cn2 killed 60% of individuals in the control population. By mixing Cn2 and scFv 9004G in a 1:10 toxin-to-antibody molar ratio, the fraction of surviving individuals in the experimental population was 100%. No symptoms of intoxication were observed throughout the experiment. The neutralizing ability of scFv 9004G was further tested with 2 LD₅₀ of freshly prepared whole venom of C. suffusus. A LD₅₀ of ~8.75 μg/20 g of scFv 9004G was used as templates to construct a second mutant library (2.5 × 10⁷ variants; mutation rate, 0.4%). Four bio-panning rounds against Cn2 were performed under stringent conditions. The antibody variant 910F showed the best recognition to Cn2 and Cn3, as demonstrated by phage-antibody ELISA tests (data not shown). The sufficiency of a single amino acid change to achieve significant binding activities has been observed before with a mutant Fab fragment. Such mutant recognizes digoxin and three of its analogs (30).
Detailed Characterization of scFv 9004G and scFv 6009F

Expression Yield and Binding Kinetics—At the level of expression, scFv 9004G yielded 2 times more protein than scFv 6009F. Kinetic constants of binding to Cn2 and Cn2 were also determined for both antibody variants and the parental scFv 3F (Table 2 and supplemental Fig. S1). scFv 3F showed slightly higher affinity for Cn2 than for Cn2: $K_d$ values were similar in both cases, whereas the $K_d$ for Cn2 was 1.5 times lower than for Cn2. That is to say, the parental scFv 3F binds both toxins, but has higher affinity toward the antigen for which it was isolated. With Cn2, binding kinetics measurements were strikingly similar for scFv 6009F and scFv 9004G. The situation was to some extent different with Cn2. scFv 6009F showed a slightly lower dissociation constant, and consequently an affinity increase relative to the values observed for scFv 9004G. Interestingly, $K_d$ values for scFv 9004G and scFv 6009F were increased by 2 orders of magnitude relative to the $K_d$ value determined for the parental scFv 3F with both toxins. This indicates that independent maturation processes can result in antibody variants with similar binding properties.

Neutralization—The neutralization activity was challenged against 2 LD$_{50}$ of Cn2 or Cn2 in different toxin-to-antibody molar ratios (Table 3). scFv 6009F and scFv 9004G provided full protection in a 1:10 molar ratio. However, scFv 6009F showed superior neutralizing activity when antibody concentration was decreased to a 1:2 molar ratio. The surviving rate was 100%, although mice manifested mild intoxication symptoms. In contrast, scFv 9004G failed to protect half of the experimental population, and survivors suffered severe symptoms. Apparently, the maturation process of scFv 9004G resulted in an antibody variant with minor neutralizing activity as compared with that of scFv 6009F. Nevertheless, scFv 9004G exhibited a comparatively superior expression yield (Table 3), an aspect that would be important for its production as a therapeutic agent.

Optimization of a scFv by Site-directed Mutagenesis

scFv 6009F and scFv 9004G have similar affinity constants and cross-reactivity, we attempted to generate an improved scFv by combining their mutations. The V101F change was inserted by site-directed mutagenesis in the sequence context of scFv 9004G. This change appeared previously in the maturation process of scFv 6009F, and it has been shown to be a determinant for binding to Cn2 (1). The resulting antibody variant, scFv LR, confirmed that the properties of antibodies can be dramatically improved by simply combining key mutations.
Neutralizing Antibody Fragments against Scorpion Venoms

FIGURE 4. scFv recognition to Cn2 upon treatment under strong denaturing conditions. Antibody variants were incubated with increasing concentrations of guanidinium chloride before testing their recognition to toxin by ELISA. Bars: white, scFv 9004G; black, scFv 6009F; hatched, scFv LR. Error bars of four experiments are shown.

Neutralization Challenge with 3 LD$_{50}$ of Whole Venom

The best antibody variants were challenged against 3 LD$_{50}$ of whole venom in two ways. First, we followed the common protocol by incubating toxin/antibody mixtures prior to their injection into mice (Table 4A). The second way was injecting venom alone, waiting 5 to 10 min to allow the onset of severe envenomation symptoms, and finally attempting rescue by antibody injection (Table 4B).

Neutralization Test 1—After preincubating venom with either scFv 6009F or scFv LR, the mixture was injected by the intraperitoneal passage. In the case of the C. suffusus venom, a survival rate of 100% was obtained, even at a molecular ratio as low as 1:3 with respect to the main toxin of this venom (Table 4A). No intoxication symptoms were observed with both antibody variants. In the case of C. noxius venom, differences between scFv 6009F and scFv LR were observed. In a 1:3 molecular ratio, scFv LR protected all individuals. scFv 6009F failed to protect half of the experimental population, but nevertheless, delayed death for more than 3 h as compared with the control population.

Neutralization Test 2—In this challenge, mice were rescued from actual envenomation by injection of 3 LD$_{50}$ of whole venom. Antibody was injected after the onset of severe symptoms. With the venom of C. suffusus, rescue was carried out with antibody in a 1:10 molar ratio. scFv 6009F did not allow a significant survival rate, because 7 of 8 individuals died. By contrast, a significant reduction of symptom intensity was observed, together with a survival rate of 100%, when scFv LR was injected in a 1:10 molar ratio. After injecting C. noxius venom into mice, survival rates of 50 and 60% were observed with scFv 6009F and scFv LR, respectively (Table 4B). These results suggested that increased amounts of antibody could be necessary to rescue more animals from this demanding situation. We tested a 1:18 ratio and found that scFv 6009F was able to rescue 80% of envenomed mice, whereas scFv LR rescued 90% of mice. This difference was slight, but it was found repeatedly in independent experiments.

### TABLE 4

Neutralization tests: (A) Test 1: neutralization challenge with 3 LD$_{50}$ of whole soluble venom of C. suffusus and C. noxius. An amount of freshly prepared whole venom equivalent to 3 LD$_{50}$ was injected into mice or as a preincubated mixture with antibody at the indicated toxin-to-antibody molecular ratio. (B) Test 2: rescue from a challenge with 3 LD$_{50}$ of whole soluble venom of C. suffusus and C. noxius. A time lapse of 5–10 min was allowed to pass before injecting antibody in a several toxin-to-antibody molecular ratios, relative to major component from venoms.

| Fresh whole soluble venom 3 LD$_{50}$ | scFv | Molar ratio toxin:scFv | Survivors/total |
|--------------------------------------|------|------------------------|-----------------|
| **A**                                |      |                        |                 |
| C. suffusus                          | 0/10 |                        |                 |
| C. suffusus                          | 1/8  |                        |                 |
| C. suffusus                          | 1/10 |                        |                 |
| C. noxius                            | 0/10 |                        |                 |
| C. noxius                            | 5/10 |                        |                 |
| C. noxius                            | 8/10 |                        |                 |
| C. noxius                            | 9/10 |                        |                 |
| **B**                                |      |                        |                 |
| C. suffusus                          | 0/8  |                        |                 |
| C. suffusus                          | 1/8  |                        |                 |
| C. suffusus                          | 8/10 |                        |                 |
| C. noxius                            | 5/10 |                        |                 |
| C. noxius                            | 6/10 |                        |                 |
| C. noxius                            | 9/10 |                        |                 |
| C. noxius                            | 9/10 |                        |                 |
Neutralizing Antibody Fragments against Scorpion Venoms

DISCUSSION

The optimization of antibodies for therapeutic purposes can be obtained by diverse methodologies. Interesting proposals have been developed to neutralize the effect of scorpion venoms. Such proposals share two common aspects. First, they were based on the rational approach of neutralizing the major toxic and abundant constituents of the whole venom of a particular species. Second, animals previously immunized with specific toxins were employed as source of antibodies. Several monoclonal antibodies have been generated by immunization of mice (23, 32, 33). By means of recombinant DNA technology, different simplified antibody formats (scFv, Fab, dimer, and tandem repeats) have been constructed (34–38). Another antibody source has been the dromedary immune system. Recent papers showed the ability of antibody fragments, known as nanobodies, to neutralize the most abundant toxins of the scorpion Androctonus australis Hector (39, 40). The whole venom has been neutralized by a bi-specific antibody fragment (41). Exceptional work has reported the possibility of neutralizing whole venom with a single antibody. However, in the majority of instances, whole venom neutralization has depended on at least two antibodies, or on equivalent bi-specific molecular constructions. We propose the in vitro maturation of human scFv antibodies, by directed evolution and phage display, for generating variants capable of neutralizing the main toxins and venoms of different species.

Several reports have shown that an antibody may recognize structurally related antigens, but with significant affinity variations. It has also been demonstrated that affinity and cross-reactivity are readily modulated by mutations in the variable domains (16, 18, 30, 42–45). scFv 6009F is an interesting case, because its maturation was aimed at increasing recognition toward toxin Cn2. However, scFv 6009F showed cross-reactivity against 3 scorpion toxins (Cn3, Css2, and Css4) and the ability to neutralize two of them (Cn2 and Css2). Such behavior with diverse toxins was an interesting byproduct of the maturation process, because the increase of affinity would normally be expected to trade-off with cross-reactivity. Interestingly, directed evolution resulted in an antibody variant with increased potential of recognition toward toxins of different scorpion species.

Cross-reactivity was pre-existent in the progenitor of scFv 6009F. The parental scFv 3F showed recognition against Cn2 and Css2 in ELISA tests (data not shown). Consequently, this antibody family recognizes a key epitope for neutralizing Cn2, Css2, and probably other related toxins. A second relevant aspect of this family is that its structural scaffold derives from the germ-line of domains VH3 and Vκ3, which has been shown to be the domain combination with the best thermodynamic stability (46). This suggested that scFv 3F could be used as a plastic template to generate new neutralizing variants and for identifying residues associated with important properties, as affinity and stability.

Optimization of the antibody family started with the decision of exploring again the sequence space of the parental scFv 3F. Mutant libraries were generated by random mutagenesis, and multiple bio-panning rounds were performed to isolate antibody variants with increased binding to Css2. The antibody variant scFv 9004G was isolated. This variant did not lose the binding activity of its progenitor, and thus recognized Cn2 (Fig. 2). Furthermore, scFv 9004G showed the ability to neutralize Css2 (the antigen targeted by directed evolution) and Cn2 (the antigen for which the parental antibody was isolated) (Table 1). The result of this new maturation process confirmed that the recognition potential toward Cn2 can be preserved, despite 10 mutations that separate the sequences of scFv 9004G and scFv 6009F (Fig. 3b). It is likely that important contact surfaces were not significantly altered and that conserved structural geometry account for the high level of cross-reactivity of scFv 6009F and scFv 9004G.

Noteworthy is the observation that all amino acid changes of scFv 9004G were located in the framework regions (Fig. 3b, maturation against toxin Css2). Of particular importance is the N74D mutation, which appeared recurrently during the first cycle of directed evolution. This change appeared independently in the evolutionary line of scFv 6009F (Fig. 3a, maturation against toxin Cn2). The N74D change affected the recognition for both toxins in a significant manner. Position 74 was subjected to saturation mutagenesis to reveal amino acid residues that could be important to increase recognition to Css2 (data not shown). Invariably, the best residue was aspartic acid, so it can be concluded that this residue plays an important role for a good interaction with toxins Cn2 and, in both scFv 6009F and scFv 9004G.

Mutations located near the CDRs may be relevant for its conformation and interaction with amino acid residues of the antigen (47). This is the case of a mutation that was isolated in the second cycle of directed evolution. The antibody variant 910F exhibited a G59D change that increases recognition to Css2 in a significant manner. On the other hand, mutations located in frameworks may contribute to increase affinity and specificity (48), and therefore may lead to scFv variants that recognize related toxins without loosing binding to Cn2. This was observed after using scFv 9004G in recognition tests that included toxic fractions from venoms of Centruroides sculpturatus and Centruroides elegans (supplemental Fig. S2). These species have medical importance, and the tested fractions include the most abundant and potent toxins of their venoms.

Further characterization of scFv 6009F and scFv 9004G showed that recognition and affinity toward Css2 and Cn2 were similar, and that significant cross-reactivity emerged repeatedly (Fig. 2 and Table 2). For both antibody variants, \( K_D \) values were in the range of \( 10^{-10} \) m. Indeed, sensorgrams of scFv 6009F and scFv 9004G can be hardly distinguished from each other when superimposed (supplemental Fig. S1). For this reason, protein identity was confirmed by mass spectrometry (supplemental Fig. S3). The antibody variants did not show different neutralizing activities when tested against 2 LD_{50} of either toxin in a 1:10 toxin-to-antibody molar ratio (Table 3). However, conspicuous differences were found in more challenging tests with 1:2 molar ratios. On the one hand, scFv 6009F provided full protection, reflected by a survival rate of 100% and the prevention of intoxication symptoms. On the other hand, scFv 9004G provided partial protec-
Neutralizing Antibody Fragments against Scorpion Venoms

By analyzing the maturation process of scFv 6009F, it was inferred that the V101F change has a positive effect on affinity toward Cn2 (Cycle 2, variant 610A), without being sufficient to provide neutralizing activity (1). This mutation was introduced in the sequence context of scFv 9004G, which shows a comparatively higher expression yield. The resulting scFv LR was notorious in four aspects. First, affinity toward Css2 and Cn2 was significantly increased. This was reflected by $K_D$ values in the picomolar range, and by low dissociation constants in the range of $10^{-5} \text{s}^{-1}$ for both toxins (Table 2). In our experience, a $K_D$ value does not reflect the real potency of an antibody in the absence of an analysis of both $k_d$ and $k_i$ rate constants. Good neutralizing antibodies are those with off rates in the range $10^{-5} \text{s}^{-1}$ to $10^{-5} \text{s}^{-1}$. This recognition level is superior to that observed in antibodies obtained by immunization processes (49). Second, scFv LR showed the highest protein yield (2.4 mg liter$^{-1}$). Conceivably, expression and purification protocols can be improved still further. Third, scFv LR prevented intoxication of 50% of individuals within a population injected with $2 \text{LD}_{50}$ of either toxin in 1:1 toxin-to-antibody molar ratio. Fourth, recognition of Cn2 was tested after treatment with the denaturing agent guanidinium chloride, revealing significant stability differences between antibody variants. scFv 9004G was less stable than scFv 6009F. scFv LR showed a higher stability as compared with scFv 6009F (Fig. 4). This observation further confirmed the importance of the V101F change in the sequence context of scFv 9004G.

The neutralization activities of scFv 9004G and scFv 6009F were challenged (Table 1) (1) against 2 $\text{LD}_{50}$ of freshly prepared whole venom of either C. noxius or C. suffusus. Although mild envenomation symptoms were observed, both antibody variants conferred full protection. Symptoms could be associated to the presence of toxins that were not fully neutralized, but that are nevertheless, recognized to a significant extent as a result of cross-reactivity. This effect was more evident in neutralization tests with C. noxius venom, which is more potent. For this reason, more drastic conditions were imposed to the best antibody variants, scFv 6009F and scFv LR. Neutralization was attempted against 3 $\text{LD}_{50}$ of freshly prepared whole venom. Whole venoms were collected between 1 and 2 h prior to starting the neutralization test. This requirement is explained by the heterogeneous toxicities that have been observed with stored venoms. Thus, we wanted to guarantee that the venom was fully active when injected. In neutralization test 1, scFv 6009F allowed a survival rate of 100% against C. suffusus venom. The same antibody allowed a survival rate of only 50% against C. noxius venom. The observed differences may be attributed to the potent nature of the venom of C. noxius. In stark contrast, neutralization with scFv LR was absolutely successful. Full protection was observed in tests with both venoms at 1:10 and 1:3 molar ratios.

Additional experiments were performed by injecting mice with 3 $\text{LD}_{50}$ of whole venom, allowing the onset of symptoms, and then rescuing mice by injecting antibody (neutralization test 2). This gave valuable information, because venom was injected by the intraperitoneal passage. In this way, toxins are expected to reach all tissues faster than they could possibly do when venom is subcutaneously injected by a scorpion sting. Rescue was attempted after toxins were already bound to target channels and symptoms were developing. Antibody was administered by the intraperitoneal passage, and not by the ideal route to treat envenomation, which is by endovenous injection. Put in other words, venom diffusion was faster than it could have possibly happened after a scorpion sting, whereas antibody diffusion was slower than it would be wanted to counteract envenomation.

Differences were clearly observed between neutralizing activities of scFv 6009F and scFv LR after mice were injected with whole venom of C. suffusus. Only one individual survived when rescue was attempted with scFv 6009F. In stark contrast, a survival rate of 100% was observed with scFv LR. This antibody variant caused a decrease of symptoms as soon as it was injected; full recovery was observed after 2 to 3 h. With the venom of C. noxius, scFv 6009F and scFv LR rescued between 50 and 60% of the experimental population when used in 1:10 molar ratio. Because this venom is more potent, the molar ratio was increased to 1:18. In this condition, scFv LR allowed a survival rate of 90%; full recovery was observed after 6 to 7 h. It is worth pointing out that the calculated $\text{LD}_{50}$ for the venom of C. noxius is 3.5 times higher than that for the venom of C. suffusus. Accordingly, the $\text{LD}_{50}$ for toxin Cn2 is twice as high as that for Css2. Envenomation associated with C. noxius is definitely more potent, and this situation is explained at least in part by the activity of the main toxin Cn2. Therefore, rescue tests were carried out with different toxin-to-antibody molar ratios to find the amount of circulating antibody that could inhibit the ongoing interaction of toxins with target channels. In contrast, preincubating venom (or toxin) with antibody allows the capture of toxic constituents even when low molar ratios are used.

Independent maturation processes were directed to isolate antibody variants with neutralizing activity against toxins Cn2 or Css2. Each isolated antibody variant exhibited neutralizing activity against both toxins and two different venoms. This increased potential is associated with few sequence changes, each one conceivably exerting a structural effect from the frameworks or the CDRs. This discrete number of mutations allows dissection of individual effects. Furthermore, by segregating relevant mutations in different sequence contexts we can obtain new antibody variants with improved properties, as has been shown for scFv LR. Thus, the rationale of our research is to exploit the strong cross-reactivity of antibody variants for neutralizing different venoms. The case in point is not unique, because the generation of a second antibody family is on the way. This second family exhibits a different pattern of cross-reactivity. Its members recognize a different epitope of Cn2 (supplemental Fig. S4), and neutralize toxins CII1 and CII2 (the main toxins of C. limpidus limpidus). Our ultimate goal is the generation of a new antivenom, equivalent or superior to the commercially available. We are sure to do so by optimizing human antibody families that recognize key epitopes of toxins from the venoms of different Centruroides spp.
Acknowledgments—We thank Dr. Humberto Flores for critical reading of the manuscript. Dr. Georgina Gurrola and Dr. Fernando Zamudio for toxin purification and mass determinations. We are indebted to DVM Elizabeth Mata, IBL, and Marcela Ramírez Yarza and Sergio González for invaluable help in animal provision and indebted to DVM Elizabeth Mata, IBI, and Marcela Ramírez Yarza and Cipriano Balderas and Fredy Coronas for technical assistance on surface plasmon resonance measurements. We also thank Dr. Paul Gaytán, Eugenio López, and Santiago Becerra for oligonucleotide synthesis and purification and Cipriano Balderas and Fredy Coronas for technical assistance.

REFERENCES

1. Riaño-Umbarila, L., Juárez-González, V. R., Olamendi-Portugal, T., Ortiz-León, M., Possani, L. D., and Becerril, B. (2005) FEBS J. 272, 2591–2601
2. Dehesa-Dávila, M., and Possani, L. D. (1994) Toxicol 32, 1015–1018
3. Possani, L. D., Becerril, B., Delepiere, M., and Tytgat, J. (1999) Eur. J. Biochem. 268, 284–290
4. Rodríguez de la Vega, R. C., and Possani, L. D. (2005) Toxicol 46, 831–844
5. Chippaux, J. P., and Goyffon, M. (2008) Acta Trop. 107, 71–79
6. Schiavon, E., Sacco, T., Cassulini, R. R., Gurrola, G., Tempia, F., Possani, L. D., and Wanke, E. (2006) J. Biol. Chem. 281, 20326–20337
7. Espino-Solis, G. P., Riaño-Umbarila, L., Becerril, B., and Possani, L. D. (2009) J. Proteomics 72, 183–199
8. Ishister, G. K., Graudins, A., White, J., and Warrell, D. (2003) J. Toxicol. Clin. Toxicol. 41, 291–300
9. Bodet, E. T., Midedfort, K. S., and Wittrup, K. D. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 10701–10705
10. Graff, C. P., Chester, K., Begent, R., and Wittrup, K. D. (2004) Protein Eng. Des. Sel. 17, 293–304
11. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A. M., Marks, J. D., and Weiner, L. M. (1998) Cancer Res. 58, 485–490
12. Chao, G., Lau, W. L., Hackel, B. I., Szajinsky, S. L., Lippow, S. M., and Wittrup, K. D. (2006) Nature Protocols 1, 755–768
13. Seo, M. J., Jeong, K. J., Leysath, C. E., Ellington, A. D., Iverson, B. L., and Georgiou, G. (2004) Protein Sci. 13, 259–267
14. Batra, S. K., Jain, M., Wittel, U. A., Chauhan, S. C., and Colicher, D. (2002) Curr. Opin. Biotechnol. 13, 603–608
15. Oppezzo, P., Dumas, G., Bouvet, J. P., Robello, C., Cayota, A., Pizarro, J. C., Dighiero, G., and Pritsch, O. (2004) Eur. J. Immunol. 34, 1423–1432
16. Dubreuil, O., Bossus, M., Graillle, M., Bilous, M., Savatier, A., Jolivet, M., Ménez, A., Stura, E., and Ducancel, F. (2005) J. Biol. Chem. 280, 24880–24887
17. Harvey, B. R., Shanafelt, A. B., Baburina, I., Hui, R., Vitone, S., Iverson, B. L., and Georgiou, G. (2006) J. Immunol. Methods 308, 43–52
18. Miyazaki, C., Iba, Y., Yamada, Y., Takahashi, H., Sawada, J., and Kurosawa, Y. (1999) Protein Eng. 12, 407–415
19. Weaver-Feldhaus, J. M., Miller, K. D., Feldhaus, M. J., and Siegel, R. W. (2005) Protein Eng. Des. Sel. 18, 527–536
20. Mian, I. S., Bradwell, A. R., and Olson, A. J. (1991) J. Mol. Biol. 217, 133–151
21. Braden, B. C., and Poljak, R. J. (1995) FASEB J. 9, 9–16
22. Hernández-Salgado, K., Estrada, G., Olvera, A., Coronas, F. I., Possani, L. D., and Corzo, G. (2009) Immunol. Lett. 125, 93–99