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A novel human recombinant antibody fragment capable of neutralizing Mexican scorpion toxins

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ABSTRACT

Using phage display and directed evolution, our group has progressed in the construction of a second family of human single chain variable fragments (scFv) which bind to scorpion toxins dangerous to mammals. It was observed that scFv C1 only bound initially to toxin Cn2, which constitutes 6.8% of whole venom from the scorpion *Centruroides noxius* Hoffman. Only a few amino acid changes were necessary to extend its recognition to other similar toxins and without affecting the recognition for its primary antigen (Cn2 toxin). One variant of scFv C1 (scFv 202F) was selected after two cycles of directed evolution against Cll1 toxin, the second major toxic component from the venom of the Mexican scorpion *Centruroides limpidus limpidus* Karsh (0.5% of the whole venom). scFv 202F is also capable of recognizing Cn2 toxin. Despite not having the highest affinity for toxins Cll1 ($K_D = 25.1 \times 10^{-9}$ M) or Cn2 ($K_D = 8.1 \times 10^{-9}$ M), this antibody fragment neutralized one LD_{50} of each one of these toxins. Additionally, scFv 202F moderately recognized Cll2 toxin which constitutes 1.5% of the venom from *C. limpidus*. Based on our previous experience, we consider that these results are promising; consequently, we continue working on generating new optimized variants from scFv C1 that could be part of a recombinant scorpion anti-venom from human origin, that might reach the market in the near future.

1. Introduction

In order to improve the quality of anti-venoms used in therapy, several procedures have been developed to ensure the effectiveness and safety of anti-venoms from non-human origin. Some examples are: the optimization of the immune response of animals challenged with venoms; the purification of the horse immunoglobulins (IgGs) and their enzymatic digestion with pepsin to remove the crystallizable fragment (Fc) which is immunogenic for humans (reviewed in Espino-Solis et al. (2009)). Due to significant advances in antibody engineering in recent decades, it has been possible the generation of human antibody fragments capable of toxin neutralization, with the advantage of being less immunogenic than whole immunoglobulins (Holliger and Hudson, 2005).

A scFv is constituted by the variable domains of a light chain and a heavy chain linked by a peptide linker, usually 15 amino acids (aa) long (Bird et al., 1988; Huston et al., 1991). This format has a similar functional activity as compared to that of a whole antibody. Due to its

Abbreviations: aa, amino acid(s); CDR, complementarity determining region (s); *C. limpidus*, *Centruroides limpidus limpidus* Karsh; Cll1 and Cll2, toxins 1 and 2 from *Centruroides limpidus limpidus* venom; *C. noxius*, *Centruroides noxius* Hoffman; Cn2 and Cn3, toxins 2 and 3 from *Centruroides noxius* venom; *C. suffusus*, *Centruroides suffusus suffusus*; Css2, toxin 2 of *Centruroides suffusus suffusus*; FW, framework(s); scFv, single chain antibody fragment; VH, variable domain of heavy chain; VL, variable domain of light chain.

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comparatively small size (30 kDa), a scFv would have a greater spreading in any organism, compared with an IgG or a F $(ab')_2$, which would be an important advantage in acute cases of poisoning by scorpions. It is worth noting that the genetic information of scFvs is relatively easy to manipulate. By means of phage display, directed evolution and rational panning procedures, scFvs with optimized properties and level of expression can be isolated against specific targets.

Scorpion venom toxicity for mammals is due to the presence of toxins that affect the activity of the sodium channel (reviewed in Rodriguez de la Vega and Possani (2005)). Each venom contains one or several main toxic components, in terms of its toxicity and abundance, meaning that neutralization of them is sufficient to achieve the effect of inhibiting whole venom. Three out of the 8 most dangerous Mexican scorpions are: *Centruroides suffusus suffusus* which is distributed in the Durango state and its major toxic component is Css2 toxin (2.8% from whole venom); *Centruroides noxius* is distributed in Nayarit state and its major toxic component is Cn2 (6.8% from de whole venom); and *Centruroides limpidus limpidus* distributed mainly in the states of Morelos and Guerrero. This last venom contains two main components Cll1 and Cll2 with abundances of 0.5% and 1.5% respectively.

Total neutralization of the venom by means of specific antibodies depends on several factors such as complexity of the venom, cross reactivity and affinity. There are cases in which a single antibody is able of neutralizing up to two different scorpion venoms (Riano-Umbarila et al., 2011). In other reported cases two antibodies are required to neutralize one scorpion venom (Clot-Faybesse et al., 1999; Hmila et al., 2010). The goal of our group is to generate a set of human antibody fragments like those already developed against the venoms of *C. suffusus* and *C. noxius* (Riano-Umbarila et al., 2011, 2005) against other Mexican scorpions. The whole set of scFvs should be equivalent to the commercial polyclonal and polyvalent anti-venom. The effectivity of each scFv is based on their cross reactivity level, which is favored by the high aa sequence identity of these toxins, and the conservation of the disulfide bridges (Fig. 1).

In this contribution, the parental scFv C1 (which recognizes Cn2 toxin) was subjected to two cycles of directed evolution against Cll1 and Cll2 toxins. We obtained several scFv C1 variants which showed better recognition to Cll1 and Cll2. From this process, scFv 202F was isolated. This variant was able of neutralizing toxins Cll1, and surprisingly, Cn2 toxin as well. The maturation process (through mutagenic PCR and affinity selection) once again was successfully enough to increase the recognition capacity and the improvement of the affinity in a second scFv family.

2. Materials and methods

2.1. Toxins

Toxins were purified from scorpion venoms using previously described methodologies: Cll1 and Cll2 were obtained from venom of *C. limpidus* (Dehesa-Davila et al., 1996; Ramirez et al., 1994); Cn2 and Cn3 toxins from *C. noxius* Hoffmann (Zamudio et al., 1992) and Css2 from *C. suffusus* (Hernandez-Salgado et al., 2009; Schiavon et al., 2006).

2.2. Directed evolution

2.2.1. Mutant libraries construction

Each maturation process included the construction of a mutant library by random mutagenesis and three or four rounds of bio-panning. Error-prone PCR reactions were performed under conditions that permit different mutation rates along the coding sequence of scFv C1 (Cadwell and Joyce, 1992; Leung et al., 1989). The primers used were the following: Forward (5′-ATACCTATTGCCTACGGC-3′) and Myc (5′-TCAGATCCTCTTCTGAGATG-3′). The PCR products were gel-purified, digested with restriction enzymes *Sfi*I and *Not*I and ligated into pSyn2 phagemid predigested with the same enzymes. The ligation product was inserted into *Escherichia coli* strain TG1 electro-competent cells. The library was characterized and evaluated by bio-panning against Cll1 and Cll2 toxins as described previously (Marks et al., 1991). The DNA segments of four clones isolated from the first cycle of directed evolution were used as templates for a new step of random mutagenesis (Meyerhans et al., 1990). The construction of the second library was performed using the conditions already described.

2.2.2. Bio-panning against toxins

For the first cycle of directed evolution three bio-panning rounds were performed. For the first bio-panning step, 2 $\mu g\ mL^{-1}$ of Cll1 or Cll2 toxins were used to coat each immuno-tube. BSA (1%) was used as blocking agent and $1 \times 10^{13}\ mL^{-1}$ phage-antibodies were poured into immune-tubes. The unspecific or weak binding phages were eliminated by several washes with phosphate-buffered saline (PBS)-Tween 20 (Marks et al., 1991). Recovering of specific phages was made by adding TG1 cells to the washed immuno-tubes (Riano-Umbarila et al., 2005). Each phage sample was subjected to independent rounds of bio-panning. Toxin concentration was kept in the following rounds, changing the blocking agents which were gelatin (second round) and milk (third round). Several clones from

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      1.......10........20........30........40........50........60......
Cll1  KEGYIVNLSTGCKYECYKLGDNDYCLRECKQQYGKGAGGYCYAFGCWCTHLYEQAVVWPLPKKTCT
Cll2  ....L..H........F.............................N.................N
Cn3   ....L.E.G.......F.............AR.................Q..........KN...R
Cn2   ....L.DKN.......L.............................A.........I.....N.R.S
Css2  ....L.SK........L...................SS.......A................N...N
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Fig. 1. Amino acid sequence alignment of homologous toxins. Cll1 and Cll2: toxins 1 and 2 from *C. limpidus* venom; Cn2 and Cn3: toxins 2 and 3 from *C. noxius* venom. Css2: toxin 2 from *C. suffusus*. Dots indicate identical residues. All sequences contain 66 aa residues and the same array of cysteine residues.

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