Toxicon 119 (2016) 52–63

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Broadening the neutralizing capacity of a family of antibody fragments against different toxins from Mexican scorpions

Everardo Remi Rodríguez-Rodríguez [a], Timoteo Olamendi-Portugal [a], Hugo Serrano-Posada [b], Jonathan Noé Arredondo-López [a], Ilse Gómez-Ramírez [a], Guillermo Fernández-Taboada [a], Lourival D. Possani [a], Gerardo Alfonso Anguiano-Vega [c], Lidia Riaño-Umbarila [d, **], Baltazar Becerril [a, *]

[a] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, Cuernavaca, Morelos, 62250, Mexico
[b] CONACYT-Research Fellow, Laboratorio de Bioingeniería, Universidad de Colima, Km. 9 Carretera Coquimatlán-Colima, C.P. 28400, Coquimatlán, Colima, Mexico
[c] Laboratorio de Biomedicina Molecular, Facultad de Ciencias Químicas, Durango, Universidad Juárez del Estado de Durango, Av Veterinaria S/N, Circuito Universitario, CP 34120, Durango, Dgo, Mexico
[d] CONACYT-Research Fellow, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Mexico

ARTICLE INFO

Article history:
Received 10 March 2016
Received in revised form
12 May 2016
Accepted 18 May 2016
Available online 20 May 2016

Keywords:
Cross reactivity
Human scFvs
Phage display
Scorpion toxin neutralization

ABSTRACT

New approaches aimed at neutralizing the primary toxic components present in scorpion venoms, represent a promising alternative to the use of antivenoms of equine origin in humans. New potential therapeutics developed by these approaches correspond to neutralizing antibody fragments obtained by selection and maturation processes from libraries of human origin. The high sequence identity shared among scorpion toxins is associated with an important level of cross reactivity exhibited by these antibody fragments. We have exploited the cross reactivity showed by single chain variable antibody fragments (scFvs) of human origin to re-direct the neutralizing capacity toward various other scorpion toxins. As expected, during these evolving processes several variants derived from a parental scFv exhibited the capacity to simultaneously recognize and neutralize different toxins from *Centruroides* scorpion venoms. A sequence analyses of the cross reacting scFvs revealed that specific mutations are responsible for broadening their neutralizing capacity. In this work, we generated a set of new scFvs that resulted from the combinatorial insertion of these point mutations. These scFvs are potential candidates to be part of a novel recombinant antivenom of human origin that could confer protection against scorpion stings. A remarkable property of one of these new scFvs (ER-5) is its capacity to neutralize at least three different toxins and its complementary capacity to neutralize the whole venom from *Centruroides suffusus* in combination with a second scFv (LR), which binds to a different epitope shared by *Centruroides* scorpion toxins.

1. Introduction

The severity of envenomations caused by scorpion stings reported in Mexico and around the world (Chippaux, 2012) is associated with the presence of abundant and potent neurotoxins in the venom. Therefore, medical importance of these beta-neurotoxins relies on two key elements, abundance and toxicity reflected as their affectation of voltage-dependent sodium (Na_v) channels (Cestèle et al., 1998; Possani et al., 1999) that are present in the central and peripheral nervous systems and are responsible for the transmission of nerve impulses. The fatal effect of the toxins relates

Abbreviations: scFv, single chain antibody fragment; CDR, complementarity determining region; FW, framework region; V_L, light chain variable domain; V_H, heavy chain variable domain; *C. noxius*, *Centruroides noxius* Hoffmann; Cn, toxin from *C. noxius*; *C. suffusus*, *Centruroides suffusus*; Css, toxin from *C. suffusus*; *C. limpidus*, *Centruroides limpidus*; Cll, toxin from *Centruroides limpidus*; *C. tecomanus*, *Centruroides tecomanus*; Ct1a, toxin from *C. tecomanus*; LD_{50}, Median lethal dose.

* Corresponding author. Av. Universidad No. 2001, Colonia Chamilpa, Cuernavaca, 62210, Mexico.
** Corresponding author. Av. Universidad No. 2001, Colonia Chamilpa, Cuernavaca, 62210, Mexico.
E-mail addresses: umbarila@ibt.unam.mx (L. Riaño-Umbarila), baltazar@ibt.unam.mx (B. Becerril).

http://dx.doi.org/10.1016/j.toxicon.2016.05.011
0041-0101/

to the impairment of this transmission affecting some vital functions, which can lead to death.

The current antivenom applied in Mexico is produced by hyperimmunizing horses with the venoms from four venomous scorpion species. Serum antibodies are enzymatically processed to obtain $F(ab')_2$ type fragments (Dehesa-Dávila and Possani, 1994). The high homology shared by scorpion toxins (de la Vega et al., 2013; Rodríguez De La Vega and Possani, 2005), would favor the generation of cross reacting antibodies. This antivenom consisting of the whole content of cross reacting antibodies, is expected to be polyclonal and polyvalent. Its polyvalent character is revealed by the capacity to neutralize the whole venoms of at least 7 venomous scorpion species from Mexico: *Centruroides noxius, Centruroides limpidus, Centruroides suffusus, Centruroides tecomanus, Centruroides infamatus, Centruroides elegans*, and *Centruroides sculptures*.

Intensive research on scorpion venom components, has revealed that usually there are one to three major neurotoxins in each venom (Bahraoui et al., 1988; Clot-Faybesse et al., 1999; Martin et al., 1987; Possani et al., 1981; Ramírez et al., 1994; Zamudio et al., 1992). As already mention, medical importance of these beta-neurotoxins is based on their abundance and toxicity (lethality) (Possani et al., 1999; Rodríguez De La Vega and Possani, 2005). It has been shown that polyclonal antibodies are not essential for neutralizing the toxic effect of the lethal components of scorpion venoms. For example, in *C. noxius* venom the main toxin (Cn2) represents 6.8% of the whole venom and shows an LD_{50} of 0.25 μg/20 g mouse. A monoclonal antibody (BCF2) was able of neutralizing Cn2 toxin and the whole venom (Zamudio et al., 1992). Another example, corresponds to *Androctonus australis* scorpion venom which contains two main neurotoxins (AaHI and AaHII). This venom is neutralized by two fragments of antibodies directed against each of these toxins (Hmila et al., 2010). These examples and others documented in the literature (reviewed by Alvarenga et al., 2014; Rodríguez-Rodríguez et al., 2015) indicated that neutralization of the main toxins would be sufficient to neutralize the whole venom, meaning that it is possible to generate novel specific antivenoms with a few antibody fragments. Using a single antibody would be sufficient to neutralize each main beta-neurotoxin. However, the presence of two or more different neutralizing antibodies against each toxin would increase the therapeutic efficiency of the new antivenoms. In support of these observations, we have demonstrated that the neutralization of the venom of *Centruroides noxius* with two antibody fragments (LR and RU1), that recognize different epitopes on the Cn2 toxin is more efficient (Riaño-Umbarila et al., 2016). Smaller amounts of these scFvs mixed compared when injected separately, were sufficient to protect envenomed mice. Innovative antivenoms would have several advantages: a homogenous composition, limited batch-to-batch variation, better safety and efficacy. Furthermore, these products do not depend on the immune response of animals and the probability of occurrence of secondary effects associated with the heterologous origin of antivenoms might be minimized (Chippaux, 2012). Results reported by our group, indicate that single-chain variable fragments (scFv) of human origin capable of neutralizing the major beta-neurotoxins present in the venoms, represent a promising alternative to be used as a new antivenom against *Centruroides* scorpions stings (Riaño-Umbarila et al., 2016, 2013, 2011, 2005).

Initially, we isolated two non-neutralizing scFvs, 3F and C1 against toxin Cn2 from a human scFv library (Riaño-Umbarila et al., 2005). These parental antibody fragments were affinity matured by directed evolution and phage display. As a result of these processes, variants capable of neutralizing Cn2 toxin were generated. scFvs LR and RU1 were the best variants obtained from parental scFvs 3F (Riaño-Umbarila et al., 2011) and C1 (Riaño-Umbarila et al., 2016), respectively. It was shown that these fragments were capable of neutralizing a second toxin. scFv LR was able to neutralize Css2 toxin from the venom of *Centruroides suffusus*, while scFv RU1 neutralized Cll1 toxin from *Centruroides limpidus*. It is worth mentioning that these two scFvs recognize different epitopes. Both non-overlapping epitopes are supposed to be involved in the envenoming effects of the toxins (Riaño-Umbarila et al., 2016). The main aim of our group is to optimize the different antibody fragments derived from both families of scFvs (3F and C1) to develop variants being able of neutralizing the main toxins present in Mexican scorpion venoms. We suggest that a set of scFvs that bind to both epitopes will guarantee protection from envenoming. At the same time the polyclonal and polyspecific nature of the current antivenom would be imitated.

As mentioned above, scFv LR neutralizes the Css2 toxin. To further optimize the neutralization of this toxin, we sought to generate a second scFv derived from scFv C1 which would be able to neutralize Css2 toxin through a second epitope. At the same time, based on the high homology among *Centruroides* scorpion toxins, we would be broadening the neutralization capacity of the derived scFvs against other toxins. To achieve these goals, two mutagenesis processes were implemented in parallel; random and site-directed mutagenesis, using some of the variants derived from scFv RU1 taken as substrate. It is worth noting that scFv RU1 recognizes but does not neutralize Css2 toxin (data not shown). Two variants derived from scFv RU1 (scFvs RJI-1 and RJI-2) were subjected to *in vitro* maturation by means of directed evolution and semi-rational approaches. Generated libraries were subjected to several rounds of selection against Css2 toxin using phage display. Selected variants showed several changes that were combined, resulting in a new set of scFvs that were evaluated for their ability to bind to several toxins. The results of these experiments revealed that the incorporation of a few key mutations allowed to expand the binding capacity of the new scFvs toward various toxins; including Css2 (*C. suffusus*), Cn2 (*C. noxius*), Cll1, Cll2 (*C. limpidus*) and Ct1a (*C. tecomanus*). scFv ER-5 which was evolved toward Css2 toxin, was capable of neutralizing three different toxins. Structural analyses of the complexes of toxins with scFvs helped us to understand how this set of selected mutations significantly increased binding capacity, cross reactivity and affinities of the new variants against several toxins.

2. Experimental procedures

2.1. Venom preparation

The scorpion venoms used in this work were obtained from *Centruroides suffusus*, *Centruroides noxius*, *Centruroides limpidus* and *Centruroides tecomanus*. Fresh venom was obtained from individuals of each species by electrical stimulation. The samples were diluted in bidistilled water and centrifuged at 14,000 rpm for 10 min at 4 °C. The insoluble material was discarded, and the toxin-containing supernatant was recovered and spectrophotometrically quantified ($\lambda = 280$ nm).

2.2. Purification of the toxins

The beta-neurotoxins were purified from the scorpion venoms using previously described methodologies. Css2 and Css4 were obtained from *C. suffusus* (Espino-Solis et al., 2011), Cll1 and Cll2 were obtained from *C. limpidus* (Alagón et al., 1988; Ramírez et al., 1994), Cn2 was obtained from *C. noxius* (Zamudio et al., 1992) and Ct1a was obtained from *C. tecomanus* (Olamendi-Portugal et al., 2005).