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Discovery of human scFvs that cross-neutralize the toxic effects of *B. jararacussu* and *C. d. terrificus* venoms

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ABSTRACT

Accidents involving venomous snakes are a public health problem worldwide, causing a large number of deaths per year. In Brazil, the majority of accidents are caused by the *Bothrops* and *Crotalus* genera, which are responsible for approximately 80% of severe envenoming cases. The cross-neutralization of snake venoms by antibodies is an important issue for development of more effective treatments. Our group has previously reported the construction of human monoclonal antibody fragments towards *Bothrops jararacussu* and *Crotalus durissus terrificus*' venoms. This study aimed to select human single-chain variable fragments (scFvs) that recognize both bothropic and crotalic crude venoms following venoms neutralizing capacity *in vitro* and *in vivo*. The cross-reactivity of Cro-Bothrumabs were demonstrated by ELISA and *in vitro* and *in vivo* experiments showed that a combination of scFvs neutralizes *in vitro* toxic activities (*e.g.* indirect hemolysis and plasma-clotting) of crotalic and bothropic venoms as well as prolonged survival time of envenomed animals. Our results may contribute to the development of the first human polyvalent antivenom against *Bothrops jararacussu* and *Crotalus durissus terrificus* venoms, overcoming some undesirable effects caused by conventional serotherapy.

1. Introduction

Around five million people are bitten by snakes every year worldwide and more than 125,000 die from severe envenomings (Chippaux, 1998). In Brazil, there are more than 20,000 incidents involving venomous snakes per year, from which the genera *Bothrops* and *Crotalus* are responsible for over 80% of severe envenoming cases (Chippaux, 2015).

In general, snake venoms, particularly those belonging to the Viperidae family, may induce diverse biological activities such as proteolytic, procoagulant and neurotoxic effects. Venoms from *Bothrops jararacussu* and *Crotalus durissus terrificus* species are included in this snake family and their venoms exhibit indirect hemolytic and coagulant effects (Oliveira et al., 2009; Roncolato et al., 2013; Tamarozzi et al., 2006). Moreover, these two species present similarities among their venom composition and clinical manifestation during envenomings (Boldrini-Franca et al., 2010; Georgieva et al., 2010; Kashima et al., 2004; Kini et al., 2001). This similarity was first described by Vital Brazil in 1909, who verified that the lethal action of *Bothrops jararacussu* venom was better neutralized by a mixture of crotalic and botropic antivenoms than by botropic antivenom, suggesting the use of an antivenom mixture to treat envenoming caused by *B. jararacussu* (Brazil, 1903). Further, many publications have shown experimentally the efficacy in using mixture of these antivenoms (Antunes et al., 1989; De Roodt et al., 1998; dos-Santos et al., 1992).

So far, heterologous serotherapy is the specific treatment for snake envenoming, consisting of antibodies from venom immunized horses. Although serotherapy has proven to be effective in reducing mortality and morbidity from snakebites, it also presents several disadvantages: pyrogenic and anaphylactic reactions; ranges between 60–90% of antivenom antibodies are not directed towards venom toxins; the overproduction of immune complexes (ICs) can cause serum sickness; there is a loss of efficacy if a second administration is needed (Cunningham et al., 1987; Laustsen et al., 2016b; Leon et al., 2013; Segura et al., 2013). In order to avoid these undesired effects, human antibodies should be considered for immunotherapeutic applications.

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Phage display technology allows *in vitro* selection of specific antibodies from large libraries (Hoogenboom, 2005; Winter et al., 1994). Consequently, it can be used to improve the traditional serum therapy (Laustsen, 2016; Marks et al., 1991; Smith, 1985; Winter et al., 1994).

Our laboratory has produced an extensive bibliography on this subject, considering our expertise on phage display and antivenom therapy (Oliveira et al., 2009; Pessenda et al., 2016; Pucca et al., 2012; Roncolato et al., 2015; Roncolato et al., 2013; Tamarozzi et al., 2006). Based on the previously documented cross-reactivity among different antivenoms as well as similarities between crotalic and bothropic toxins (Beghini et al., 2005; Choumet et al., 1991; Claus and Mebs, 1989; Gutierrez and Lomonte, 1995; Isbister et al., 2010; O'Leary et al., 2007; Oshima-Franco et al., 2001; Riano-Umbarila et al., 2011; Rodríguez et al., 2012), this study aimed to select human single-chain variable fragments (scFvs) able to recognize both *Bothrops jararacussu* and *Crotalus durissus terrificus* venoms and to evaluate their neutralizing capacity *in vitro* and *in vivo*.

2. Material and methods

2.1. Venoms, reagents and animals

The venoms of *B. jararacussu* and *C. d. terrificus* were obtained from the Serpentarium of Ribeirão Preto Medical School – FMRP/USP. The snakes collected from different regions of the São Paulo state. Venom milking was carried out by compressing venom glands, applying moderate pressure at the back of the head. Then, venoms were diluted in ultrapure water, lyophilized and stored at −20 °C.

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Horseradish peroxidase-conjugated rabbit antibodies against the VCS-M13 helper phage were produced as previously described (Tamarozzi et al., 2006).

Swiss mice were obtained from the Central Laboratory Animal Facility of Ribeirão Preto Medical School (FMRP), University of São Paulo (USP). Mouse experiments were conducted in accordance with the Ethical Principles in Animal Experimentation (license number – CONCEA n. 117/2014).

2.2. Recovery and amplification of antibody-displaying phages

In this study we used the previous selection performed by Tamarozzi et al. (Tamarozzi, Soares et al., 2006) and Oliveira et al. (Oliveira, Soares et al., 2009). Both used the genomic library of bacteriophages “Griffin. 1” (H. Griffin, MRC, Cambridge, UK, unpublished data) with the courtesy of Dr. Greg Winter (Medical Research Council, Laboratory of Protein Engineering, Cambridge, UK) to select specific antibody-displaying phages for *B. jararacussu* and *C. d. terrificus* venoms, respectively. The adopted protocol was the designated by the MRC. Briefly, individual monoclonal phages were amplified from wells containing *Escherichia coli* TG1 suppressor strain [k12,D9lac-pro), supE, thi, hsdD/F'traD36, proAþBþ, laclq, lacZDMIS]) (Carter et al., 1985) from each round (96-well plates per selection round, total of four rounds for each venom). An inoculum of approximately 2 ml of these wells was transferred to a second 96-well platecontaining 200 µl of LB medium, 100 µg/ml ampicillin and 1% glucose per well. 96-well plates were left shaking for 1 h at 37 °C. Subsequently, 25 µl of LB medium containing 100 µg ampicillin, 1% glucose and 10^9 pfu of the helper phage VCSM-13 was added to each well. Plates were allowed to stand for 30 min at 37 °C and then shaken for 1 h at the same temperature. They were then centrifuged for 10 min at 1800 *x* g (IEC, OM2438, Needham Heights, MA, USA), and the supernatant was aspirated and discarded. The remaining pellet was suspended in 200 µl of LB medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. 96-well plates were incubated under shaking at 30 °C for one night and centrifuged at 1800 *x* g for 10 min. The supernatant was used for Enzyme-Linked Immunosorbent Assay (ELISA) of monoclonal antibody-displaying phages and screening of the clones with venom cross-reactivity.

2.3. Selection of monoclonal antibody-displaying phages presenting cross-reactivity with both crude venoms

Briefly, 96-well plates (flat bottom, Costar®, Corning Incorporated, Oneonta, NY, USA) were sensitized with 10 µg per well of each venom diluted in carbonate/bicarbonate buffer 0.05 M pH 9.6 (total volume 100 µl) and incubated at 4 °C overnight. As positive control, wells were sensitized with the helper phage VCSM13 in a concentration of 10^9 pfu/well, and as a negative control, wells were filled with 100 µl of carbonate/bicarbonate buffer. The content of each well was discarded and plates were washed three times with PBS pH 7.2. After blocking using 200 µl of 2% MPBS (2% solution of skimmed milk in PBS) for two hours at 37 °C, plates were washed three times with PBS pH 7.2. Sensitized 96-well plates with the venom of *B. jararacussu* were incubated with 50 µl of monoclonal antibody-displaying phages of selection rounds against *C. d. terrificus*, and 96-well plates sensitized with the venom of *C. d. terrificus* were incubated with 50 µl of monoclonal phage antibodies from selection rounds of *B. jararacussu*. The plates were incubated for 90 min at room temperature and then washed three times with 0.05% PBS Tween pH 7.2, and three times with PBS pH 7.2. After washing, rabbit polyclonal antibodies anti-VCSM13 labeled with peroxidase and diluted 1:1000 in 1% MPBS, pH 7.2, were added. After incubation (60 min at room temperature), plates were washed again with PBST and with PBS pH 7.2. To each well, 100 µl of OPD–H_2O_2 substrate (2 mg of OPD, Acros Organics, Fisher Scentific International, Inc., Hampton, NH, USA, in 10 µl of PBS and 30 µl of H_2O_2 30%) were added. After 20 min, the reaction was interrupted by the addition of 50 µl of 1 M sulfuric acid and the plates were analyzed in an ELISA reader at 490 nm (Molecular Devices, Sunnyvale, CA, USA).

2.4. DNA sequencing

The positive antibody-displaying phages clones (2.3. item) were used for plasmid DNA sequencing. Plasmid samples were extracted employing a DNA extraction kit from Qiagen, QIAprep Spin Miniprep (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The primers used were pHEN2 F: 5′ ATG ATT ACC ACG CCA AG 3′ and pHEN2 A: 5′ GCC TTC CCA AGA TCT TCC TC 3′. The labeling reaction was performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and sequencing was conducted with the ABI 3500xL Genetic Analyzer (Applied Biosystems) by the Nucleus of Services in Biotechnology (NSB) from Hemocenter Foundation (*Núcleo de Serviços em Biotecnologia (NSB) from Fundação Hemocentro*) from Ribeirão Preto, SP, Brazil.

2.5. Production and purification of soluble scFvs presenting cross-reactivity with both venoms

The antibody-displaying phages clones displaying cross-reactivity were chosen for the production of scFvs through infection of the non-suppressor *E. coli* HB2151 (Carter et al., 1985), [k12, ara, D(lac–-pro),thi/F'proA + B, laclqZDM15. An inoculum of *E. coli* HB2151 was added to 5 ml of LB medium, and left at 37 °C and 300 rpm overnight. On the next day, 5 ml of the saturated culture were transferred to 5 ml of LB. The culture was incubated at 37 °C under constant agitation 300 rpm up to reach O.D. of 0.5 at 600 nm. After reaching the O.D required, 200 µl were transferred to wells of a 96-well plate. Subsequently, 10 µl of the supernatant from each previously selected antibody-displaying phages were added to each well and the plate was incubated at 37 °C for 30 min. In sequence, 5 µl of each infected culture were transferred to 5 ml of LB supplemented with 100 µg/ml ampicillin and 1% glucose, which were maintained at 37 °C, 300 rpm overnight. On the following day, 1 ml of each culture was added to 1 ml of cryo