Toxicon 117 (2016) 59–68

Contents lists available at ScienceDirect

Toxicon

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

Micrurus snake species: Venom immunogenicity, antiserum cross-reactivity and neutralization potential

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ARTICLE INFO

Article history:
Received 26 February 2016
Received in revised form
24 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
Micrurus spp.
Snake venoms
Immunogenecity
Antivenom
Cross-reactivity
Neutralization potential

ABSTRACT

Micrurus snakebites can cause death by muscle paralysis and respiratory arrest a few hours after envenomation. The specific treatment for these snake envenomations is the intravenous application of heterologous antivenom. In Brazil, this antivenom is produced from horses that are immunized with a mixture of *Micrurus corallinus* and *Micrurus frontalis* venoms, which are snakes that inhabit the south and southeastern regions of the country. Previously, we demonstrated that the coral antivenom, which is used in human therapy, was not able to neutralize several of the toxic venom effects from some *Micrurus* species that inhabit the country, as measured by *in vitro* and *in vivo* assays. The present study aimed to investigate the immunogenic properties of *Micrurus* spp. venoms, as well as the cross-reactivity and neutralization potential of experimental monovalent and polyvalent sera that were produced in different animal species. The present data showed that *Micrurus* venoms exhibited the same immunogenicity pattern in the three utilized animal species and that the specific antisera presented a large cross-reactivity when analyzed with ELISA and Western blot assays. Nonetheless, these positive results were not well correlated with the neutralizing potential of the antisera. Thus, the establishment of a new antigenic mixture to produce novel more efficient therapeutic *Micrurus* antivenom is not a simple task. Further studies, particularly with the *Micrurus lemniscatus*, *Micrurus altirostris and Micrurus surinamensis* venoms, are necessary to establish new strategies for the production of antivenoms with broad neutralizing activity for the treatment of accidents involving coral snakes throughout the country.

1. Introduction

The Elapidae snake family has approximately 250 cataloged species. In the Americas, there is a group of more than 120 species and subspecies that are distributed into three genera, including *Micruroides*, *Leptomicrurus* and *Micrurus* (Roze, 1967, 1983; Roze and Bernal-Carlo, 1987; Scrocchi, 1990; Campbell and Lamar, 1989, 2004). The genus *Micrurus* can be found in terrestrial habitats from the southern United States to southern South America.

The medically important *Micrurus* species are *Micrurus fulvius* in the United States; *Micrurus nigrocinctus* and *Micrurus alleni* in Central America; and *Micrurus mipartitus*, *Micrurus corallinus, Micrurus frontalis, Micrurus spixii, Micrurus dumerilii carinicauda, Micrurus surinamensis,* and *Micrurus isozonus* in South America (Russel, 1983; Bolaños, 1984; Kitchens and Ven Mierop, 1987). In Brazil, *M. corallinus* and *M. frontalis* are responsible for the majority of coral snake envenomations (Bucaretchi et al., 2016), and both species inhabit highly populated areas in the central, south and southeast regions of the country.

In the Americas, there are 200,000 cases of snakebite envenomations reported *per* year, being 3000 of which are fatal (Kasturiratne et al., 2008). Most of these cases are caused by species from Crotalinae subfamily (Chippaux, 1998), while coral snakebites cause 1–2% of the events (de Roodt et al., 2004, 2013). In Brazil, 191 coral accidents were notified in 2014 (Brasil, 2001).

The high toxicity of the coral venoms combined with their neurotoxic symptomatology can lead to respiratory paralysis and death (de Roodt et al., 2004; da Silva and Aird, 2001; Vital Brazil, 1987). The *Micrurus* spp. venoms are basic, composed of enzymes, non-enzymatic proteins and peptides (Aird, 2002). The most

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http://dx.doi.org/10.1016/j.toxicon.2016.03.020
0041-0101/

abundant groups reported in these venoms are phospholipases A_2 (PLA_2) and non-enzymatic three-finger toxins (3FTx) (Alape-Girón et al., 1996; Correa-Netto et al., 2011). These groups act on the neuromuscular endplate, either by injuring the nerve terminal, by means of hydrolysis of phospholipids, causing release and depletion of the synaptic vesicles, as in the case of some phospholipases (β-neurotoxins), or blocking its binding to the postsynaptic receptor (α-neurotoxins), thus causing acid paralysis of skeletal muscle (Kini, 2003; Nirthanan and Gwee, 2004; Rigoni et al., 2008). Both protein families have members with additional pharmacological activities, including myotoxicity in the case of PLA_2 (Kini, 2003; Montecucco et al., 2008) and cardiotoxicity, in the case of three-finger toxins (Kini and Doley, 2010). Venom proteomes of *Micrurus altirostris* and *M. corallinus* were established by combining snake venomics and venom gland transcriptomic surveys. In both coral snake species, 3FTx and PLA_2 were the most abundant and diversified toxin families (Correa-Netto et al., 2011). Recently, Fernandez and collaborators (Fernández et al., 2015) have demonstrated that the venoms of *M. alleni* and *Micrurus mosquitensis*, coral snakes inhabiting the Caribbean region of Costa Rica, exhibit divergent compositional patterns. The first presents mainly 3FTx and the second PLA_2s, as their most abundant venom proteins. Analyses of proteomic data from other *Micrurus* spp. suggest that these two venom patterns are recurrent, and may represent a general divergent trend for the venom compositions of New World elapids (Fernández et al., 2015).

The specific treatment for snakebites from the genus *Micrurus* is the intravenous administration of coral antivenom. This antivenom is produced, in Brazil, by hyperimmunization of horses with a mixture of *M. corallinus* and *M. frontalis* venoms (Raw et al., 1991). The production of this antivenom is hampered by the small amount of venom that is obtained during the extraction and the difficulty of capturing coral snakes and the maintaining in captivity (Jorge and Ribeiro, 1990; Brasil, 2001).

The envenomation severity that is caused by snakes of this genus has sparked the development of studies to assess the neutralizing potential of commercially available antivenoms. Cohen and collaborators (Cohen and Seligmann, 1966; Cohen et al., 1968, 1971) noted the existence of cross-reactivity among venoms from the genus *Micrurus* because of the presence of antigenically related components. However, monovalent and polyvalent antivenoms do not always neutralize venoms from heterologous species (Bolaños et al., 1978; Moraes et al., 2003), indicating that, although there are immunologic similarities, there are also significant differences amongst venoms species. In a previous study, we demonstrated the existence of a large range of variations in the toxic composition of the Brazilian *Micrurus* venoms, which probably reflect the adaptation of the snakes from this genus to different habitats, preys and diets (Tanaka et al., 2010). We also showed that the antivenom that is used for human therapy was not fully able to neutralize the main toxic activities that are present in the venoms from various *Micrurus* species, suggesting that modifications in the immunization scheme, with the inclusion of other venoms in the antigenic mixture, are needed to generate an effective broad spectrum therapeutic coral snake antivenom.

In the present study, we quantitatively and qualitatively analyzed the immunogenic properties of various *Micrurus* venoms, the antigenic cross-reactivity of monovalent and polyvalent experimental antisera and their neutralizing potential.

2. Material and methods

2.1. Chemicals and reagents

Tween 20, bovine serum albumin (BSA), ortho-phenylenediamine (OPD), and goat antibodies anti-mouse IgG (GAM), labeled with horseradish peroxidase (IgG-HRP), were purchased from Invitrogen (Carlsbad, California, USA). Goat antibodies anti-rabbit IgG (GAR), labeled with horseradish peroxidase (IgG-HRP), were purchased from Pierce (Rockford, Illinois, USA). Rabbit antibodies anti-horse IgG (RAH), labeled with horseradish peroxidase (IgG-HRP), were purchased from Sigma (St. Louis, Missouri, USA). Goat antibodies against mouse IgG (GAM), labeled with alkaline phosphatase (IgG-AP), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT), were purchased from Promega Corp. (Madison, Wisconsin, USA). Phosphate buffered saline (PBS) was utilized and contained the following: 145 mM NaCl, 3 mM Na_2HPO_4 and 2.5 mM Na_2HPO_4, pH 7.4.

2.2. Venoms

M. spixii, *Micrurus ibiboboca*, *M. frontalis*, *M. corallinus*, *M. altirostris*, *Micrurus lemniscatus* and *M. surinamensis* venoms were supplied by the Herpetology Laboratory from Butantan Institute, SP, Brazil. The authorization to access the *Micrurus* venoms was provided by the Brazilian Institute of Environment and Renewable Natural Resources - IBAMA - a Brazilian Ministry of the Environment's enforcement agency (permission no. 01/2009). The determination of the protein content present in the coral snake venoms was performed with the BCA method (Protein Assay Kit, Pierce Biotechnology Inc., USA), according to the manufacturer's recommendations, and the concentration was adjusted to 1 mg/mL with PBS. The samples were aliquoted and stored at −80 °C.

2.3. Animals

All of the procedures involving animals were in accordance with the animal research ethical principles that were adopted by the Brazilian Society of Animal Science and the National Brazilian Legislation no.11.794/08. The protocol was approved by the Animal Care and Use Committee from Butantan Institute (permission no. 207/05). Experimental monovalent sera were produced in high responder male mice (H_{III} line) (Sant'Anna et al., 1982), weighting between 18 and 22 g, provided by the Immunogenetics Laboratory from Butantan Institute. New Zealand male rabbits, weighing between 2.5 and 3 kg, were supplied by the Butantan Institute animal facilities, and used for the production of the experimental monovalent and polyvalent sera. Male horses from the Butantan Institute São Joaquim farm, weighing approximately 500 kg, were used for the production of experimental monovalent and polyvalent sera. The serum neutralization assays were performed, *in vivo,* using Swiss male mice, weighing between 18 and 22 g, supplied by the Butantan Institute animal facilities.

2.4. Coral snake antisera

2.4.1. Brazilian therapeutic coral horse antivenom

The coral antivenom, which was produced from horses following *M. corallinus* (50%) and *M. frontalis* (50%) venom immunizations, was provided by the Plasma Processing Section of the Butantan Institute, SP, Brazil (Lot 0312104).

2.4.2. Experimental sera produced in mice

For the analysis of the immunogenicity of the *Micrurus* spp. venoms, H_{III} mouse groups (three *per* group) were inoculated subcutaneously with 20 μg of the venom, which was mixed with aluminum hydroxide (diluted 1:25 in PBS) to a final volume of 100 μL. Both groups received two inoculations at an interval of 90 days, and the bleeding was performed at various times after the first and second immunizations. The sera were stored at −20 °C until use.