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Characterization of anti-*crotalic* antibodies

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ABSTRACT

Crotalus durissus terrificus, *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* are responsible minor but severe snake bites in Brazil. The venoms of these snakes share the presence of crotoxin, a neurotoxin comprising of two associated components, crotapotin and phospholipase A2 (PLA2). Treatment of the victims with specific antiserum is the unique effective therapeutic measure. The ability of anti-*Crotalus antisera* produced by the routine using crude venom to immunize horses or purified crotoxin and PLA2 as individual immunogens was compared. Antisera obtained from horses immunized with *C. durissus terrificus* crude venom were able to recognize and neutralize not only the toxins presents in *C. durissus terrificus*, but also the ones present in the venoms from *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis*. Antisera from horses immunized with individual crotoxin or PLA2, although in lesser titers, were also able of recognizing the toxins in all four Crotalus species and neutralize the lethality of the *C. d. terrificus* venom.

1. Introduction

Envenomation caused by *Crotalus* snake bites represents 6.2% of reported cases of envenomation in Brazil, with an estimated mortality rate of 1.8% per year (Ministério da Saúde, Brasil, 2001). As is shown in Fig. 1, five geographic subspecies of *Crotalus* are found in Brazil. *Crotalus durissus terrificus*, although common in the southern states of São Paulo, Minas Gerais, Paraná and Rio Grande do Sul, is also present in the areas of Mato Grosso, Rondônia, Amazonas and Pará to the west, including Paraguai, Uruguai and Argentina. *Crotalus durissus cascavella* is found in the dry "*caatinga*" region, ranging from southern Maranhão, Piauí, Ceará and Rio Grande do Norte. *Crotalus durissus collilineatus* is present in central and northern Brazil, including parts of Rondônia, Mato Grosso, Goiás, southWestern Bahia, Western Minas Gerais, and São Paulo (where it intermingles with *C. durissus terrificus*), and its presence may extend southward into Western Paraná (Fig. 1). *Crotalus durissus marajoensis* is restricted to the "*cerrado*" of Ilha de Marajó in the state of Pará. *Crotalus durissus ruruima* is also present in Roraima (Melgarejo, 2003). The general pharmacological and composition of the venom from the various *Crotalus* species in Brazil is very similar (Santoro et al., 1999; Boldrini-Franca, 2010). The toxins in *Crotalus* venoms are crotoxin, crotamin (Gonçalves, 1956) and gyroxin (Barrio, 1961; Barrabin et al., 1978). Crotoxin is responsible for both the neurotoxic and systemic myotoxic effects characteristic of this venom. Crotoxin was first isolated from the venom of *C. d. terrificus* (Slotta and Fraenkel-Conrat, 1938). Crotoxin comprises two sub-units that are non-covalently linked: the non-catalytic crotoxin A (CA), or crotapotin, and the catalytic unit, crotoxin B (CB), which is also known as PLA_2. Crotapotin is an acidic polypeptide with no detectable enzymatic activity (Harris, 1991). Crotapotin, working as a chaperon, potentiates the toxicity of PLA_2 by about 35-fold. PLA_2 is a basic single-chain polypeptide formed by 123 amino acid residues. PLA_2 binds pre-

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[1] Dr. Rosalvo Guidolin contributed to improving the quality of antisera and vaccines throughout his fruitful, professional life (*in memoriam*).

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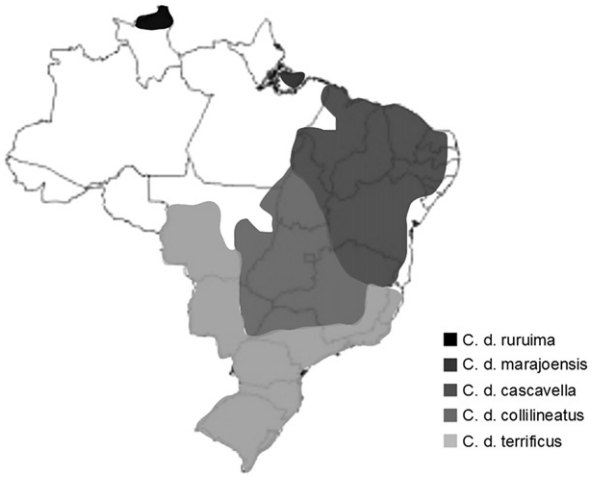

Fig. 1. Distribution of *Crotalus* subspecies in Brazil (modified from Melgarejo, 2003).

synaptic receptors, inhibiting acetylcholine release (Marlas and Bon, 1982). Mice and horses immunized with purified PLA_2 are protected from the lethal effects of the *C. d. terrificus* crude venom (Dos Santos et al., 1988, 1989). While antibodies specific to crotapotin are unable to neutralize crotoxin activity, antibodies specific to PLA_2 neutralize crotoxin but do not cross-react with crotapotin (Choumet et al., 1998).

Crotamin was isolated as a basic protein, i.p. 10.3, from *C. d. terrificus* (Gonçalves, 1956). The biological and biochemical molecular features of crotamin suggest that crotamin is related to myotoxins (Bieber and Nedelkov, 1997). Crotamin was purified (Seki et al., 1980), and its nucleotide sequence was determined (Rádis-Baptista et al., 1999). *In vitro* and *in vivo* studies indicate that crotamin is a cell membrane-penetrating protein with nuclear localization. Although the nature of the interaction between crotamin and cells has not been investigated at the molecular level, the suggested mechanisms differ from those of DAPI or 5-BrdU. Cumulatively, the data indicate that crotamin could be a marker for actively proliferating cells (Kerkis et al., 2004).

Gyroxin was described by Barrio (1961), and it was subsequently isolated from the venom of *C. d. terrificus* (Barrabin et al., 1978). This toxin was first identified by its ability to induce a loss of equilibrium and the subsequent complete revolutions of the body around the longitudinal axis upon experimental injection into mice (barrel roll). These manifestations are preceded by systemic skeletal muscle contractions (Kosako et al., 2000). In addition to these typical neurological toxic effects, gyroxin exhibits a thrombin-like activity fibrinogen A cleavage at its N-terminal peptide region (Raw et al., 1986).

Victims of *C. d. terrificus* exposure exhibit almost no local symptoms but do present grave neurotoxic and myotoxic symptoms (Azevedo-Marques et al., 2003). The neurotoxic effects include eyelid heaviness; facial muscle paralysis, specifically around the mouth; blurred vision; ptosis; external ophthalmoplegia; and progressive respiratory muscle paralysis. The myotoxic effects include diffuse muscular pain, red or brown urine, decreased blood coagulation, and increased serum levels of creatine kinase (CK), lactic dehydrogenase (LDH), aminotransferase aspartase (AST) and aldolase. Acute renal failure (ARF) is the most important systemic symptom. Histopathological analyses of muscle fragments collected distal from the bite location show myonecrosis with lysis of the myofilaments. The induction of myonecrosis by *C. d. terrificus* venom has been experimentally confirmed, and this effect was demonstrated to be caused by the sub-units of crotoxin (Kouyoumdjian et al., 1986). Neurotoxicity (Vital Brazil, 1966), nephrotoxicity (Hadler and Vital Brazil, 1966), myotoxicity (Breithaupt, 1976) and cardiotoxicity (Santos et al., 1990) have been also ascribed to crotoxin. The variety of local and systemic effects resulting from *Crotalus* venom injection is likely the result of the combined action of the toxic components of the venom.

Current antiserum production still relies on the use of whole snake venom as an immunogen. This strategy results in the production of antibodies against both the toxic and non-toxic components of the venom, resulting in an antiserum that contains both relevant and non-relevant therapeutic antibodies. The injection of irrelevant antibodies into victims of snake bites can increase the risk adverse reactions (Cardoso et al., 1993). Thus, using purified toxic venom components instead of whole venom during antiserum production is the first step to obtaining more specific antivenoms. To promote the selection and expansion of high-affinity naïve and memory lymphocyte subsets, the immunization period and the amount of injected immunogen should be reduced. Steiner and Eisen (1966) demonstrated that smaller quantities of antigen result in antibodies with high titers and higher affinity. Highly specific antivenom antibodies exhibiting high avidity and high-affinity will likely result in more efficient and reliable therapeutic tools.

This work aims to compare the quality between sera produced by injecting crude *Crotalus* venom into horses and antivenoms produced using purified crotoxin and phospholipase A_2 as immunogens. Evaluated parameters include the antibody titers against whole venom and the toxic components of venom, the levels of neutralizing antibodies, the antibody affinity and the ability of these antibodies to cross-react with venoms from related *Crotalus* subspecies found in Brazil.

2. Materials and methods

2.1. Reagents

Tris buffer (Tris HCl, 25 mM; pH 7.4), complete MMT80 (Marcol Montanide ISA 50, 2 mL; sodium chloride 0.15 M, 5 mL; Tween 80, 1 mL; lyophilized BCG, 1 mg), incomplete MMT80 (Marcol Montanide ISA 50, 2 mL; sodium chloride 0.15 M, 5 mL; Tween 80, 1 mL), solution A for SDS buffer (Tris, 6.25 mM; SDS, 6.94 mM; pH 6.8); SDS buffer for reduction conditions (solution A, 8.5 mL; glycerol, 1 mL; β-mercaptoethanol, 0.5 mL; bromophenol blue 1%, 2 mL), PBS buffer (potassium chloride, 2.6 mM; monobasic potassium phosphate, 1.5 mM; sodium chloride, 76 mM; disodium phosphate, 8.2 mM; pH 7.2–7.4), AP buffer (Tris HCl,