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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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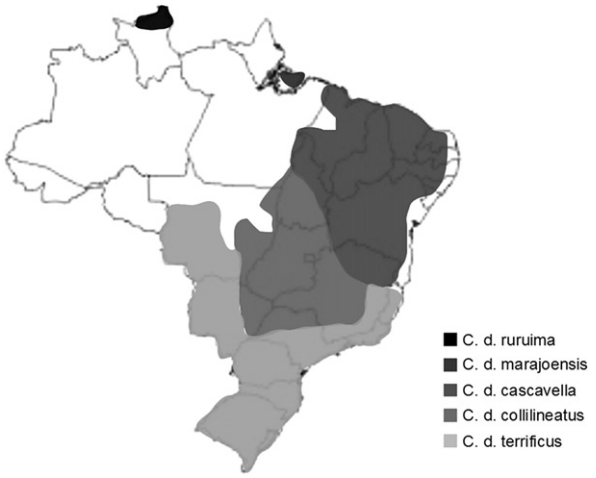

Fig. 1. Distribution of *Crotalus* subspecies in Brazil (modified from Melgarejo, 2003).

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,

100 mM; sodium chloride, 100 mM; magnesium chloride, 5 mM; pH 9.5), NBT solution (NBT, 50 mg; dimethylphormamide, 700 μL; H_2O, 300 μL), BCIP solution (BCIP, 50 mg; dimethylphormamide, 1 mL), developing solution for Western/dot blotting (AP buffer, 5 mL; NBT solution, 33 μL; BCIP solution, 16.5 μL), citrate buffer (citric acid, 0.1 M; monobasic sodium phosphate, 0.2 M; pH 5.0), OPD solution (OPD, 20 mg; citric acid, 1 mL), and substrate buffer for ELISA (citrate buffer, 5 mL; OPD solution, 100 μL; H_2O_2 30 volumes, 5 μL). All the reagents used were obtained from Sigma–Aldrich (USA), except from NBT/BCIP, obtained from Molecular Probes (USA).

2.2. Quantification of proteins

The protein concentration of the venoms and sera was assessed by the bicinchoninic acid method (Smith et al., 1985) with the Pierce BCA Protein Assay Kit (Rockford, IL).

2.3. Venoms

C. d. terrificus, *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms were supplied by "*Laboratório de Venenos, Instituto Butantan*". Each venom batch was a mixture of samples collected from several snake specimens and lyophilized. The lethality (LD_{50}) of crude *Crotalus* spp. Venoms was determined by intraperitoneally injecting male Swiss mice, 18–20 g, with 500 μL of PBS containing 1.0, 2.0, 4.0 or 8.0 μg of the venoms. Four mice were used for each venom dose. The deaths were recorded after 48 h, and the death/survival ratio was determined by probit analysis (Finney, 1992; World Health Organization, 1981).

2.4. Isolation of crotoxin and phospholipase A_2 from C. d. terrificus venom

Samples of *C. d. terrificus* venom (20.0 mg) were applied to a column packed with Mono Q HR 5/5 resin (Amershan Pharmacia Biotech AB/USA), which was previously equilibrated at room temperature with 25 mM Tris, pH 7.4 buffer. After washing the column with the same buffer, a linear gradient of NaCl starting from 0 to 0.1 M was applied under a 30 ml/h flow, and fractions corresponding to each protein peak were collected. Protein concentration and PLA_2 activity in each protein peak were determined using the method described by Price (2007). The absorbance at 280 nm was determined on UPC-900 (ÄKTA FPLC) and by specific hydrolysis of the PLA_2 substrate L-Phosphatidylcholine, Type X-E, minimum 60% TLC (Sigma–Aldrich, Inc., 3050 Spruce Street, St. Louis, MO 63103 USA). Fractions exhibiting PLA_2 activity were pooled together, lyophilized and stored until immunization of the horses.

2.5. Animals

Groups of 20 adult horses (400–450 kg) are regularly used to produce anti-*Crotalus* antivenom. Six horses (two per group) were selected to be immunized with *C. d. terrificus* venom, purified crotoxin or PLA_2 venom components. The animals, not previously immunized, were maintained in a special animal house at the São Joaquim Farm, Instituto Butantan, São Paulo, Brazil. Before immunization, the animals were vaccinated against the most common equine infectious diseases. Male Swiss out bred mice, 18–20 g, (four per group) were used in protocols to determine the lethality (LD_{50}) of the venoms and the neutralizing potency (ED_{50}) of the antivenoms. Mice were kept in standard conditions, with light between 7:00 a.m. and 6:00 p.m., a temperature of 22 ± 2 °C and laboratory food and water *ad libitum*. All animals used in this study were maintained and treated under strict ethical conditions in accordance with the "International Animal Welfare recommendations" (Remfry, 1987) and the "Committee Members, International Society on Toxinology" (1992). This project was approved by the Ethics Committee of Animal Usage in Research (Protocol No: 790/11) of the Instituto Butantan.

2.6. Antigen preparation and immunization schedule

Horses selected to produce regular antivenom received four inoculations of a mixture containing equal amounts of *C. d. terrificus* and *C. d. collilineatus* venoms. Horses were immunized as described in Guidolin et al. (2010). Horses were s.c. injected in the back with 2.0 mL of incomplete MMT80 or complete MMT80 adjuvant mixtures, or with 0.15 M NaCl containing 2.5–5.0 mg of venom. Two vaccinations were performed, with two weeks between the vaccinations. Two weeks after the last immunization, the antisera antibody titers were evaluated: the horses were bled, and a volume of blood corresponding to one-twelfth of each animal's body weight was collected in a sterile plastic bag containing anticoagulant. Plasma and cells were separated by gravity sedimentation, and the cells were reinfused into the corresponding horse through the jugular vein. Plasma from the same horse was pooled and stored at 4 °C. Before immunization, blood samples were drawn by jugular vein puncture, and sera was stored at −20 °C for use as negative controls in the antisera antibody determinations. Three months after bleeding, boosters with similar doses of venom in PBS were given, and blood was collected and processed as described previously (Guidolin et al., 2010). This final procedure was generally repeated for the following two years.

Horses included in the Experimental Groups received four inoculations of the following mixtures. Experimental Group 1 ($n = 2$): 500 μg/animal of crude *C. d. terrificus* venom; Experimental Group 2 ($n = 2$): 200 μg/animal of partially purified crotoxin; and Experimental Group 3 ($n = 2$): 100 μg/animal of partially purified PLA_2. The first inoculation of every group was prepared in Complete MMT80 and the respective antigens. The second inoculation was prepared in Incomplete MMT80 and the respective antigens. The final two inoculations were prepared in 8.0 mL of 0.15 M NaCl containing the respective antigens. The inoculations were performed 15 days apart by subcutaneous injection at four different points of the dorsal region of each animal. Fifteen days after the last inoculation, blood was collected in sterile plastic bags containing anticoagulant solution (citric acid, 1.47 g; sodium citrate, 4.80 g; dextrose, 1.47 g; dissolved in a sufficient amount of distilled water to a final volume of 100 mL) by venipuncture

of the jugular vein. The bags were allowed to stand overnight in a refrigerating chamber (4–8 °C). Plasma samples from each horse were pooled and stored at −20 °C. Blood cells resulting from the bleeding were re-infused in the original horse.

2.7. Antivenoms

Four equine plasma samples (Batches No: #143, #158, #223 and #356) and six $F(ab')_2$ anti-*Crotalus* commercial antivenom preparations (Batch #1006140; Batch #100107119; Batch #1007187; Batch #1009230; Batch #1010282; Batch #1010283) were provided by "*Divisão de Desenvolvimento Tecnológico e Produção – Seção de Processamento de Plasmas Hiperimunes, Instituto Butantan*". Experimental plasma was obtained by separating plasma from the blood collected from the experimental animals, as described in Section 2.7.

2.8. Processing the horse plasma

The procedure presently used to manufacture horse commercial serum from plasma is completely enclosed and automated (Raw et al., 1996). The procedure used in this study, improved with the introduction of additional filtration and chromatography, included ten steps (Guidolin et al., 2010). Before the antivenom was released to treat envenomed victims, the purified $F(ab')_2$ were submitted to a quality control evaluation in order to verify the absence of bacterial contamination, bacterial lipopolysaccharide and toxic substances. The final products were adjusted to contain the desired neutralizing antibody titer in less than 10 mg of protein/ml and were labeled as "*Crotalic Antiserum*". One milliliter of the preparation neutralized 1.5 mg of *Crotalus* venom. Each ampoule contained 10 ml of antivenom. This antivenom, as well as the other antivenoms produced by the "*Divisão de Desenvolvimento Tecnológico e Produção – Instituto Butantan*", was prepared according to the recommendations of the World Health Organization (1981). Serum rich in $F(ab')_2$ fragments was produced as described by Towbin et al. (1979).

2.9. SDS-PAGE and Western blot analyses

Western blot analysis was carried out according to the method previously described by Towbin et al. (1979). Crude *C. d. terrificus*, *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms (10 μg) and partially purified crotoxin and PLA_2 (2 μg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved in a 12.5% polyacrylamide gel. Some preparations were stained with silver sulfate, while others were electroblotted onto nitrocellulose membranes, according the method described by Laemmli (1970). These membranes were blocked with PBS buffer containing 5% BSA at 37 °C for 2 h, washed with PBS, and treated with equine anti-*Crotalus* antivenoms diluted to 1:5000 in PBS plus 0.1% BSA for 1 h at room temperature on a horizontal shaker. After being washed three times with PBS plus 0.05% Tween-20, the membranes were incubated with rabbit anti-horse IgG conjugated to alkaline phosphatase (whole molecule) diluted 1:7500 in PBS plus 0.1% BSA and 0.05% Tween-20. Then, the membranes were incubated for 1 h at room temperature on a horizontal shaker. The membranes were washed three times with PBS plus 0.05% Tween-20 and placed in developing solution for Western blotting. The reaction was terminated by washing with distilled water.

2.10. Quantification of the antivenom antibodies

Polystyrene, high-affinity ELISA plates (96 wells) were coated with 1.0 μg of crude *C. d. terrificus*, *C. d. collilineatus*, *C. d. cascavella* or *C. d. marajoensis* venom in 100 μL of PBS buffer and kept overnight at 4 °C. In some assays, crotoxin or PLA_2 purified from *C. d. terrificus* was used as the antigen. The wells were blocked for 2 h at 37 °C with 200 μL of PBS plus 5% BSA. The wells were washed with 200 uL of PBS. Serial dilutions of horse IgG or $F(ab')_2$ preparations (1:4000 to 2,048,000) in PBS plus 0.1% BSA were prepared, and 100 μL of each dilution was added to individual wells. The plates were then incubated at 37 °C for 1 h, and then, the wells were washed three times with the wash buffer. Rabbit peroxidase-conjugated anti-horse IgG (whole molecule) (Sigma Aldrich, St. Louis, MO) diluted (1:20,000) in PBS plus 0.1% BSA and 0.05% Tween-20 (100 μL/well) was added to the plates. The plates were incubated for 1 h at 37 °C. After three washes with the wash buffer, 50 μL of substrate buffer were added to each well, and plates were incubated at room temperature for 15 min. The reaction was terminated with 50 μL of 4 N sulfuric acid per well. Absorbance was recorded at 492 nm using an ELISA plate reader (Labsystems Multiskan Ex, Thermo Fisher Scientific Inc., Walthan, MA). IgG from horses collected before immunization was always used as a negative control. The IgG dilution giving an optical density of 0.2 was used to calculate the U-ELISA per milliliter of the undiluted IgG solution. One U-ELISA was defined as the smallest dilution of antibody that presented an O.D. of 0.2 under conditions of the ELISA assay, as described previously (Almeida et al., 2008). The value was then multiplied by 10 to correspond to milliliters.

2.11. Antivenom affinity measurement

The affinity of anti-*Crotalus* antibody was measured by ELISA, as described above, with the inclusion of a potassium thiocyanate (KSCN) elution step (Pullen et al., 1986; Romero-Steiner et al., 2005). After the serum incubation step, dilutions of KSCN (0.0–5.0 M, in intervals of 0.50 M) in PBS were added to the wells and incubated for 30 min at room temperature. The remaining bound antibodies were detected with rabbit peroxidase-conjugated anti-horse IgG (whole molecule) (Sigma Aldrich, St. Louis, MO) diluted (1:20,000) in PBS plus 0.1% BSA (100 μL/well). After three washes with the wash buffer, 50 μL/well of substrate buffer was added, and the plates were incubated at room temperature for 15 min. The reaction was terminated with 50 μL/well of 4 N sulfuric acid. Absorbance was recorded at 492 nm using an ELISA plate reader (Labsystems Multiskan Ex, Thermo Fisher Scientific Inc., Walthan, MA). The results are expressed as follows: affinity index (AI) = M KSCN needed to displace 50% of the bound antibodies.

2.12. Neutralization of the Crotalus spp. lethality by antivenoms

A fixed amount of 5 LD_{50} of *C. d. terrificus* venom and various dilutions of antivenoms were incubated for 30 min at 37 °C. Venom samples incubated only with PBS buffer were used as controls. After incubation, 500 μL aliquots of the mixtures were intraperitoneally injected in the mice. Five mice were used per mixture. The death/survival ratio was recorded 48 h after the injection. ED_{50} was estimated by probit analysis (Finney, 1992).

2.13. Statistical analysis

The obtained data were subject to a one-way ANOVA, followed by the Dunn's multiple comparison test. Differences were considered to be significant for $P < 0.05$.

3. Results

3.1. General characteristics of Crotalus venom

The protein concentrations (μg/mL) and lethality (LD_{50}) of the *C. d. terrificus*, *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms used in this work were determined by using the bicinchoninic acid method and the LD_{50} in mice (Table 1). The electrophoretic profiles of the venoms were determined by the polyacrylamide electrophoresis (Fig. 3a).

Previous studies have shown that the major venom in the *Crotalus* species is crotoxin (Santoro et al., 1999). Although some differences were noted, mainly in terms of the electrophoretic mobility of the protein bands and their intensity, the venoms were similar overall in the four *Crotalus* subspecies. The differences noted, usually in the concentrations of particular components, correlated with the ages of the snake donors at the time of venom collection as well to the particular ecological regions from which the specimens were collected.

3.2. Chromatographic isolation of PLA_2 from the C. durissus terrificus venom

C. d. terrificus crude venom (20.0 mg) was applied to a Mono Q HR 5\5 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), which had been previously equilibrated with pH 7.4 Tris buffer and eluted with a linear gradient of NaCl (0.0–1.0 M) in pH 7.4 Tris buffer. The chromatography resulted in 11 peaks (Fig. 2a). Peak 2 was represented by only one 15 kDa protein band, whereas peaks 5 contained a majority band of 15 kDa and the other of 30 kDa (Fig. 2b). The activity of PLA_2, as assayed on synthetic substrate L-α-phosphatidylcholine, was detected only in peak 2 (data not shown). Upon "dot blotting" using specific mouse anti-crotoxin as the primary antibody, peak 5 reacted positively, indicating the presence of crotoxin (data not shown).

Table 1
Specific activity of antivenoms against *C. d. terrificus* venom.

Antivenom	Protein concentration[a]	Neutralizing potency[b]	Specific activity[c]
IBU serum	93.10 ± 22.96	131.60	0.082
IBU plasma	103.24 ± 15.98	145.30	0.067
Exp. Group 1	105.80 ± 3.10	416.70	0.023
Exp. Group 2	110.22 ± 3.51	320.00	0.029
Exp. Group 3	116.19 ± 6.05	403.00	0.022

[a] Protein concentration is expressed as mg/mL.
[b] Neutralization is expressed as Median Effective Dose (ED_{50}), defined as the volume of antivenom (μL) required to reduce by 50% the mortality of mice injected with 5 LD_{50}.
[c] Specific activity value for each antivenom was calculated by using the formula: SA = (1000/μL corresponding to 1 ED_{50})/(mg/mL of protein concentration).

3.3. Electrophorectic profiles of the different C. d. terrificus venoms

Equal samples of the *C. d. terrificus*, *C. d. collineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms were treated with SDS under reducing conditions and separated by polyacrylamide gel electrophoresis (upper gel, 5%; lower gel, 12.5%). Samples of purified crotoxin and PLA_2 were run in parallel. Fig. 3a shows strong similarities among the protein profiles of all venoms. The presence of crotapotin, PLA_2 and conjugated crotoxin was indicated by the similar mobility of the 10 kDa, 15 kDa and 30 kDa protein bands in the samples with the isolated crotoxin and PLA_2 controls that were run in parallel. A band of 35 kDa, equivalent to gyroxin, could be found in all the venom samples, although not in the purified fractions.

3.4. Antivenom recognition of the antigenic components present in the venom of four Crotalus species

Samples from the antivenom produced by the Instituto Butantan and samples of the *Crotalus* venoms were electrophoretically separated under reducing conditions on polyacrylamide gel electrophoresis (upper gel, 5%; lower gel, 12,5%). The protein bands were transferred to nitrocellulose membranes, treated with samples from the antisera (diluted 1:5000) and exposed to rabbit IgG anti-horse immunoglobulins as the second antibody. The recognition patterns of the plasma and antivenom from the Instituto Butantan were very similar, with the presence of bands near 15 kDa and 30 kDa, corresponding to PLA_2 and crotoxin, respectively (Fig. 3b and c). These proteins were detected in all the venoms with great intensity. Bands at 50 kDa and 60 kDa were also found in the *C. d. terrificus*, *C. d. collilineatus* and *C. d. cascavella* venoms, and a 10 kDa band, corresponding to crotapotin, was detected in the *C. d. collilineatus* venom. In the plasma from Experimental Group 1, bands at 15 kDa and 30 kDa were observed for all the venoms, a 10 kDa band was observed for the *C. d. terrificus* and *C. d. collilineatus* venoms, and a 60 kDa band was observed for the *C. d. terrificus* venom (Fig. 3d). In the plasma from Experimental Group 2, bands at 15 kDa and 30 kDa were observed in all the venoms, a band at 10 kDa was observed for *C. d. collilineatus* venom, and bands at 50 kDa and 60 kDa were observed for the *C. d. terrificus* venom (Fig. 3e). In the plasma from Experimental Group 3, only the 15 kDa band was observed for all the venoms (Fig. 3f).

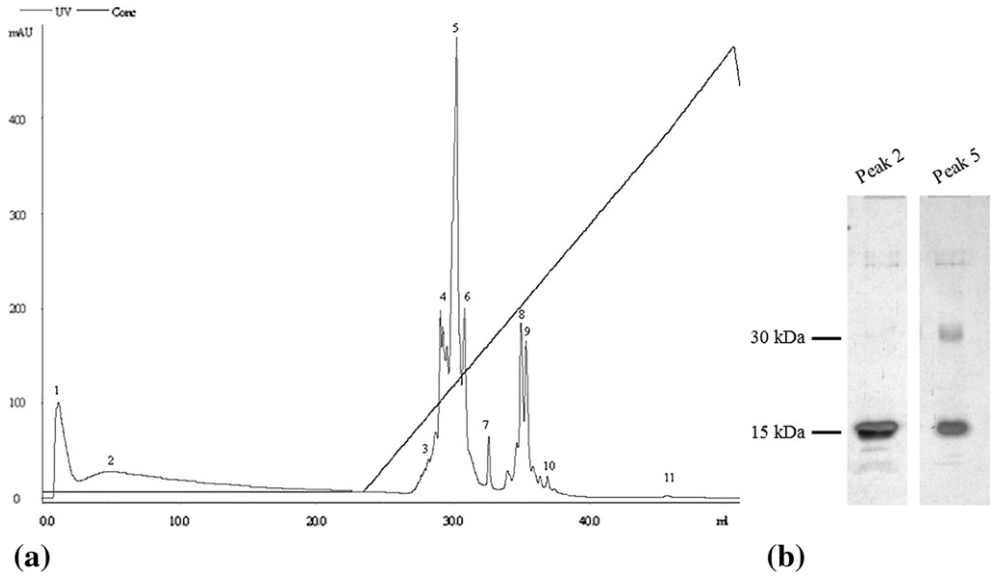

Fig. 2. Partial isolation of phospholipase 2 (PLA_2) and crotoxin from *C. d. terrificus*. a) Electrophoretic profile of the crude *C. d. terrificus* venom. Samples of the venom (20.0 mg) were applied to a chromatography column packed with Mono Q HR 5/5 (Amershan Pharmacia Biotech AB, USA) after equilibration with Tris 25 mM, pH 7.4 buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M. The samples were collected, the protein contents were determined, and the presence of PLA_2 quantified using a synthetic specific substrate. b) SDS-Page, upper gel 5%, lower gel 12.5%, stained with silver, confirming the majority presence of PLA_2 in peak 2 and crotoxin in peak 5. In all, 10 μg of each sample was applied under reducing conditions.

3.5. Antivenom antibody quantification

Equal samples from the antivenoms were diluted (1:4.0 × 10^3 to 1:2.048 × 10^6) and assayed by ELISA. The obtained O.D. values at 492 nm were plotted against the corresponding serum dilutions, and dilutions giving O.D. values of 0.2 were used to calculate the number of U-ELISA/mL (Fig. 4). Antivenoms produced by the Instituto Butantan obtained the highest titers against the *C. d. terrificus*, *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms. Although no significant difference could be observed, there was a gap between the titers obtained against the crude venoms and those obtained against the purified components, suggesting that the high titers observed were related to the recognition of components other than crotoxin and PLA_2. The titers obtained with plasma from Experimental Group 1 were the lowest against all the antigens tested. Plasma from Experimental Groups 2 and 3 showed high titers against the antigens tested. Of interest, no difference could be found between the titers against crude venoms and purified components, indicating that crotoxin and PLA_2 were the components being recognized. Antibody activity against the *C. d. collilineatus*, *C. d. cascavella* and *C. d. marajoensis* venoms were found with all the antivenoms, although those venoms were not used in the immunization schedules (with the exception of *C. d. collilineatus*, which was used in the Instituto Butantan's immunization schedule).

3.6. Antibody affinity evaluation

The antibody affinity for *C. d. terrificus* crude venom, crotoxin and PLA_2 was evaluated by ELISA with the addition of KSCN in increasing concentrations as a chaotropic agent (Fig. 5). The antivenoms provided by the Instituto Butantan showed the highest affinity for the antigens used. The affinity scores from the three Experimental Groups were lower, and there was no difference between them.

3.7. Neutralization of venom lethality

The lethal dose 50% (LD_{50}) of *C. d. terrificus* venoms was calculated to be 1.2 μg per animal. Neutralizing activity was assessed by injecting Swiss mice (18–20 g) with serial dilutions of antivenoms and 5 LD_{50} of venom, and neutralization was calculated by probit analysis. Results are expressed as the volume of antivenom (mL) required to neutralize 1 mg of venom (Fig. 6). Antivenom and plasma provided by the Instituto Butantan showed a great neutralizing capacity, with 2.18 mL and 2.42 mL required to neutralize 1 mg of venom, respectively. Plasma from Experimental Group 1 displayed a low neutralizing action, with 6.15 mL required to neutralize the venom. Plasma from Experimental Group 2 showed the highest neutralization capacity among the three Experimental Groups, although it was still lower than the commercial antivenoms, requiring 3.80 mL to neutralize 1 mg of venom. Plasma from Experimental Group 3 showed the lowest neutralizing capacity, with 6.68 mL needed to neutralize the venom.

Using the *in vivo* neutralization data and the protein concentration of the antivenoms, we were able to calculate the specific activity against *C. d. terrificus* venom (Table 1).

4. Discussion

The production of anti-snake venom antibodies to treat victims bitten by venomous snakes was originally

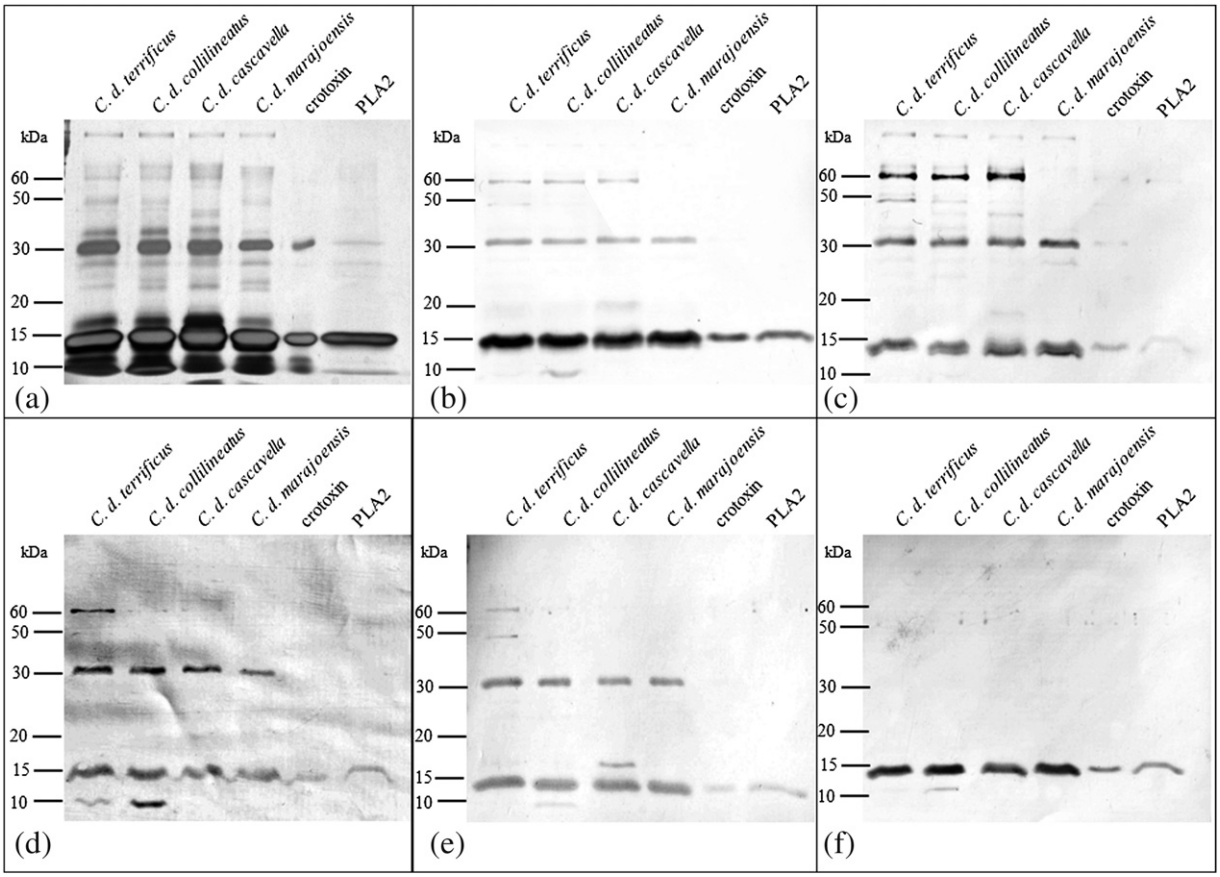

Fig. 3. Electrophoretic profile of different crotalic venoms and recognition by antivenoms as assessed by Western blotting (WB). a) SDS-Page, upper gel 5%, lower gel: 12.5%, stained with silver. b) WB using detection by the Instituto Butantan serum. c) WB using detection by the Instituto Butantan plasma. d) WB using detection by Experimental Group 1. e) WB using detection by Experimental Group 2. f) WB using detection by Experimental Group 3. In all, 10 μg of crude venom or 2 μg of partially purified fractions was applied, under reducing conditions. Serum and plasma samples were diluted to 1:5000.

developed in France at the Institute Pasteur (Calmette, 1894) and later developed and greatly expanded by Vital Brazil (Brazil, 1901, 1903). Crude venoms and horses were the immunogens and animals producing the antibodies, respectively. Once the antivenom effectiveness was demonstrated, the original procedure, although preserved in essence, evolved as dictated by progress in fields such as carbohydrate, lipid, and protein chemistry and basic immunology. For example, the serum protein cleavage by pepsin (Pope, 1936), with the clear objective of reducing the amount of heterologous protein injection into the victims. In addition to cleaving several non-antibody proteins, pepsin cleaves the Fc region of the IgG molecule generating a single, active, bivalent antigen-binding fragment, $F(ab')_2$ (Nisonoff et al., 1960). This fragment retains its original antigen-binding capacity and loses its ability to activate the complement system by the classical pathway, two properties that are absolutely required to neutralize venom components in a victim's circulation (WHO, 1981). Periodically, therefore, methods must be re-evaluated to take into account the advances of relevant basic science disciplines.

In this work, *Crotalus* antivenom was selected for improvement for several reasons. First, *Crotalus* venoms contain a limited number of relevant toxic components; second, these components can be isolated as reasonably homogeneous forms, and they can be titrated using trusted techniques; third, animals can be immunized with separated components; fourth, the envenoming symptoms are quiet clear, allowing precise evaluation of both venom lethality and antivenom neutralizing potency.

To improve the usual methodology, we first characterized six anti-*Crotalus* antivenom batches with respect to specificity, potency, affinity and specific activity. Although the analyzed antivenoms exhibited the required overall neutralization capabilities recommended by WHO (1981), the affinity and specific activity needed to be improved. Antivenoms lacking these qualities are prone to induce unavoidable adverse reactions by the presence of unnecessary contaminating protein and non-specific antibody.

Groups of horses were immunized according current protocols using as antigen crude venoms or isolated crotoxin or PLA_2. Blood samples were collected at strategic times throughout the immunization, as dictated by the antibody evolution during the immune response.

The antibody titer, neutralizing potency and affinity were evaluated by immunochemical and *in vitro* and *in vivo*

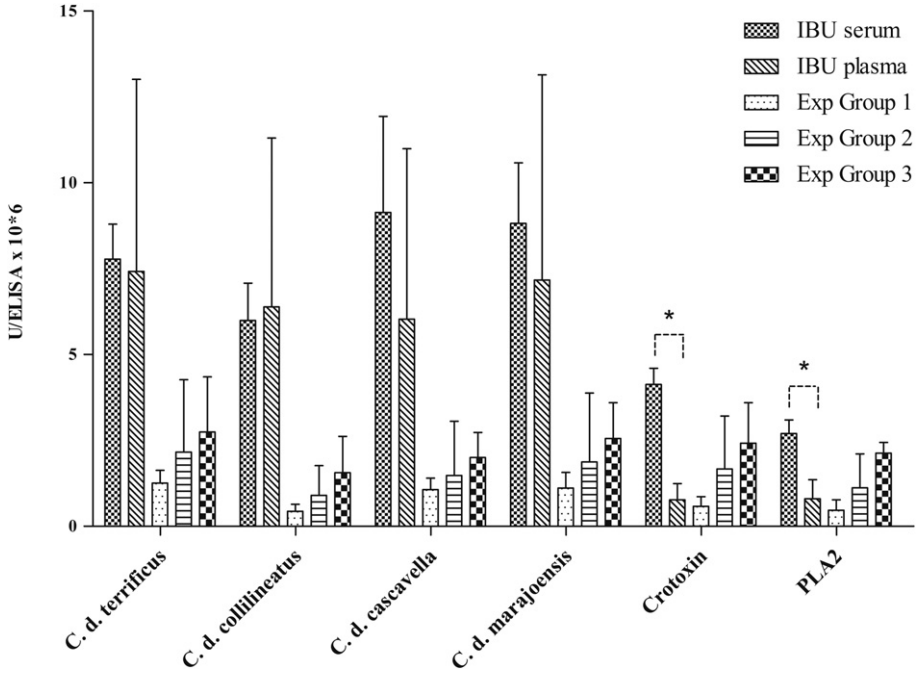

Fig. 4. Titration of plasma and sera from the Instituto Butantan and experimental plasma against venom from *C. d. terrificus*, *C. d. collilineatus*, *C. d. cascavella*, *C. d. marajoensis*, purified crotoxin and purified phospholipase A_2. Plasma and sera were pooled within their respective groups and dilutions ranged from 1:4000 to 1:2,048,000. Titers are expressed as Units-ELISA/mL $\times$ 10^6. Medians were compared by a one-way ANOVA followed by Dunn's test, * $P > 0.05$. Data are representative of two independent experiments.

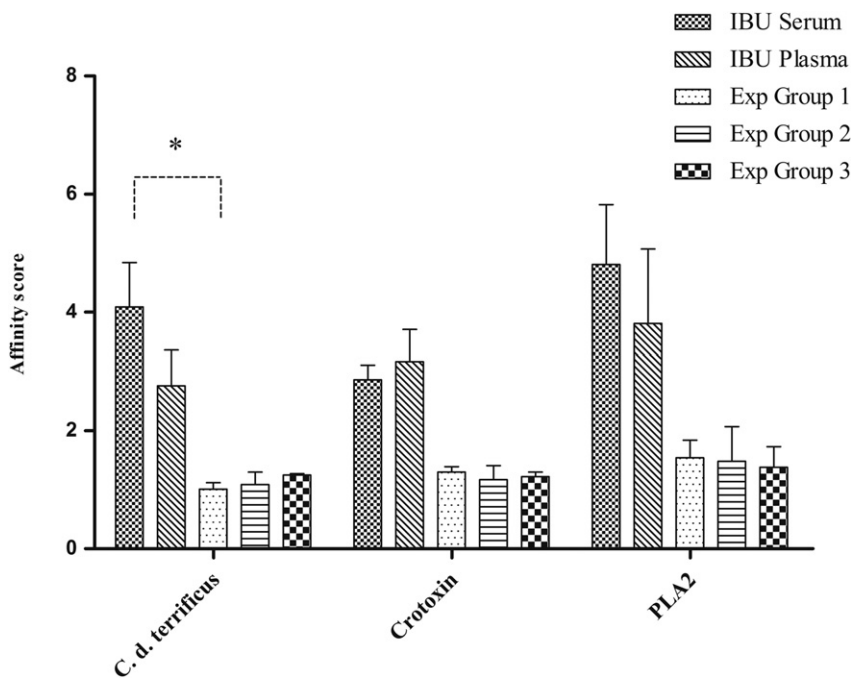

Fig. 5. Affinity determination of antivenoms for *C. d. terrificus* venom, crotoxin and PLA_2. Plasma and sera were pooled within their respective groups, and the dilution was fixed at 1:50,000. KSCN dilutions ranged from 0 M to 5 M in intervals of 0.5 M. The affinity score is expressed as the concentration of KSCN necessary to reduce to 50% the O.D. obtained with the controls (KSCN 0 M). Medians were compared by a one-way ANOVA followed by Dunn's test, * $P > 0.05$. Data are representative of two independent experiments.

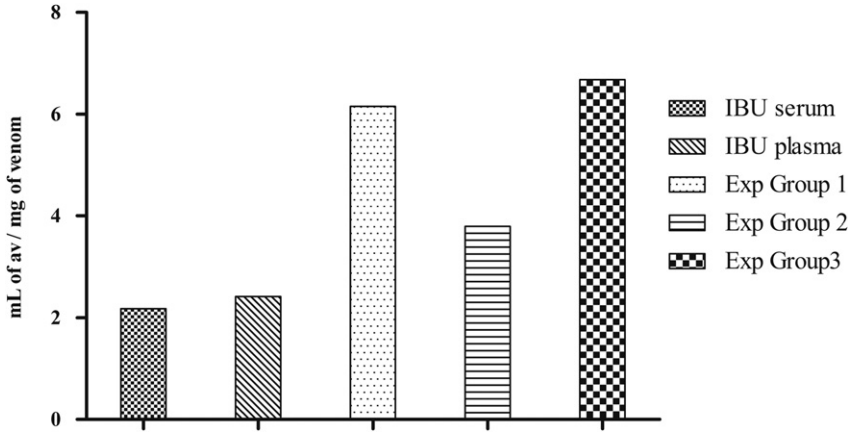

Fig. 6. Serum neutralization of *C. d. terrificus* lethality *in vivo*. Swiss mice, 18–20 g, were injected intraperitoneally with mixtures of 5 DL_{50} of *C. d. terrificus* venom with antivenom dilutions (1:5, 1:10, 1:20 or 1:40). Plasma and sera were pooled within their respective groups. Deaths were recorded after 48 h, and ED_{50} was calculated by probit analysis. Neutralization is expressed as volume of antivenom (mL) required to neutralize 1 mg of venom.

assays. The ability of the antibodies to recognize purified crotoxin and PLA_2 was an additional and important data readout, and it was clearly successful.

The antivenoms provided by the Instituto Butantan were able to recognize proteins present in the venoms of the main Brazilian *Crotalus* snakes, and they showed high titers against those venoms. Cross-reaction was expected, as the venoms from those animals have a very similar composition and biological activity (Santoro et al., 1999; Rangel-Santos et al., 2004). This antivenom also provided the highest neutralization of lethality *in vivo*, even though titers against the most toxic components were relatively low. The high-affinity found for those components, however, might have acted to counterbalance the low titers and therefore increase the neutralizing capacity. The antivenoms also recognized components in the other venoms, as evidenced by both Western blotting and ELISA, which corroborated our presupposition that the antivenoms currently produced have antibodies that bind to non-toxic proteins and that are therefore irrelevant in the treatment.

In plasma from Experimental Group 1, obtained from animals immunized with crude *C. d. terrificus* venom, 500 μg/animal, we observed components in the different crotalic venoms tested in a profile similar to that of Experimental Group 2. The use of a small quantity of antigen might have directed the response toward the most prevalent component in the venom, crotoxin. The plasma yielded very low titers and neutralizing capacity. The venom from *C. d. terrificus* is known to possess immunosuppressive components (Cardoso and Mota, 1997) and, as our results and the literature suggest, is not an effective immunogen. The use of other venoms or proteins in addition to or as a substitute for *C. d. terrificus* venom could result in an antivenom of higher quality.

In plasma from Experimental Group 2, obtained from animals immunized with crotoxin at 200 μg/animal, we observed components present in the different crotalic venoms with a specificity for conjugated crotoxin (30 kDa) and PLA_2 (15 kDa). There was no difference between titers against those components and titers against the crude venoms, strongly indicating a great specificity in the binding of the most toxic proteins. The plasma also showed a high protective capacity *in vivo*, which was more effective than that of the other Experimental Groups, and neutralization of crotalic venom by anti-crotoxin antibodies has also been shown by other groups (Freitas et al., 1990; Oshima-Franco et al., 1999; Beghini et al., 2004). The use of crotoxin as an immunogen has to be carefully planned, as crotoxin is the most toxic component present in the venom. The use of low dosages, as shown here, and/or adjuvants such as liposomes (Freitas and Frézard, 1997) could prevent injuries and adverse reactions in the serum-producing animals.

Plasma from Experimental Group 3, obtained from animals immunized with phospholipase A_2 (100 μg/animal), showed a great specificity for that component, and we observed almost exclusively that enzyme in the venom of the different *Crotalus* snakes tested. The plasma also yielded the highest titers when compared to the other Experimental Groups. However, the neutralizing capacity of this plasma *in vivo* was the lowest. The dissociation of the crotapotin/PLA_2 complex plays a major role in the neutralization of the crotalic venom (Choumet et al., 1996). Free phospholipase, like that used for the immunization, might present epitopes that differ from those presented by the complexed phospholipase, resulting in antibodies that were incapable of binding to the complexed enzyme and therefore unable to promote the dissociation of the complex. Antibodies against epitopes generated on the PLA_2-crotapotin complex interface, which were absent when the animals were immunized with PLA_2 alone, could be the most effective neutralizing antibodies.

The immunization protocols tested were able to produce antibodies with high titers and cross-reactive capacity, yet there was no increase in affinity. Changes in the schedules, with an increase in time between injections and use of alternate adjuvants, are still open for testing and could result in even better antivenoms.

This work resulted in the production of antibodies with high titers and specificities against the most toxic components of *C. d. terrificus* venom. The obtained antibodies were capable of cross-reacting with components present in the venom of other Brazilian *Crotalus*. Our next goal is the identification of CDRs present in the hypervariable regions of these antibodies with specificity to crotoxin, crotapotin and PLA_2. Variability of CDR1 and CDR2 is encoded by the germline and further diversified by somatic mutation while each one of CDR L3 and CDR H3 is somatically diversified by rearrangement of the V segment with the (J) L or diversity (D) H and JH segments, respectively (Wu and Kabat, 1970; Alazari et al., 1988; Padlan, 1994). Recognition of individual antigen (Ag) is mainly mediated by CDR H3 (Kabat and Wu, 1972). The amino acid sequences of these regions will be used to construct homologous peptides potentially capable of recognizing toxin domains.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

References

Alazari, P.M., Lascombe, M.B., Poljak, R.J., 1988. Three-dimensional structure of antibodies. Annu. Rev. Immunol. 6, 555–580.

Almeida, C.M.C., Silva, C.L., Pena-Couto, H., Escocard, R.C.M., Rocha, D.G., Sentinelli, L.P., Kipnis, T.L., Dias da Silva, W.D., 2008. Development of process to produce polyvalent IgY antibodies anti-African snake venom. Toxicon 52, 293–301.

Azevedo-Marques, M.M., Hering, S., Cupo, P., 2003. Acidente Crotálico. In: Costa Cardoso, J.L., Siqueira França, F.O., Wen, F.H., Málaque, C.M.S., Haddad Jr., V. (Eds.), Animais Peçonhentos no Brasil: Biologia, Clínica e Terapêutica dos Acidentes. Sarvier-Fapesp, São Paulo, pp. 91–98.

Barrabin, H., Martiarena, J.L., Vidal, J.C., Barrio, A., 1978. Isolation and characterization of gyroxin from *Crotalus durissus terrificus* venom. In: Rosemberg, P. (Ed.), Toxins: Animals, Plant and Microbial. Pergamon Press, New York, pp. 113–133.

Barrio, A., 1961. Gyroxin, a new neurotoxin of *Crotalus durissus terrificus* venom. Acta Physiol. Latinoamer. 11, 224.

Beghini, D.G., Hernandez-Oliveira, S., Rodrigues-Simioni, L., Novello, J.C., Hyslop, S., Marangoni, S., 2004. Anti-sera raised in rabbits against crotoxin and phospholipase A_2 from *Crotalus durissus cascavella* venom neutralize the neurotoxicity of the venom and crotoxin. Toxicon 44, 141–148.

Bieber, A.L., Nedelkov, D., 1997. Structural, biological and biochemical studies of myotoxin A and homologous myotoxins. J. Toxicol. Toxin. Rev. 16, 33–52.

Boldrini-França, J., Corrêa-Netto, C., Silva, M.M.S., Rodrigues, R.S., De la Torre, P., Pérez, A., Soares, A.M., Zingali, R.B., Nogueira, R.A., Rodrigues, V.M., Sanz, L., Calvete, J.J., 2010. Snake venomics and antivenomics of Crotalus durissus subspecies from Brazil: assessment of geographic variation and its implication on snakebite management. J. Proteomics 73, 1758–1776.

Brazil, V., 1901. Contribuição ao estudo do veneno ophidico. III. Tratamento das mordeduras das cobras. Rev. Med. São Paulo 4, 375–380.

Brazil, V., 1903. Contribuição ao estudo do veneno ophidico. III. Tratamento das mordeduras das cobras. Rev. Med. São Paulo 6, 265–278.

Breithaupt, H., 1976. Neurotoxic and myotoxic effects of Crotalus phospholipase A_2 and its complex with crotapotin. Naunyn Schmiedberg's Arch. Pharmacol. 292, 271–278.

Calmette, A., 1894. Límmunisation artificelle des animaux contre le venin des serpents et la therapeutic experimentale de morsures venimeuses. CR Soc. Biol. 46, 120–124.

Cardoso, D.F., Mota, I., 1997. Effects of Crotalus venom on humoral and cellular imune response. Toxicon 391, 197–202.

Cardoso, J.L.C., Fan, H.W., França, F.O.S., Jorge, M.T., Leite, R.P., Nishioka, S.A., Avila, A., Sano-Martins, I.S., Tomy, S.C., Santoro, M.L., Chudzinski, A.M., Castro, S.C.B., Kamuguti, A.S., Kelen, E.M.A., Hirata, M.H., Mirandola, R.M.S., Theakston, R.D.G., Warrell, D.A., 1993. Randomized comparative trial of three antivenoms in the treatment of envenoming by lance-headed vipers (*Bothrops jararaca*) in São Paulo, Brazil. Q. J. Med. 86, 315–325.

Choumet, V., Bouchier, C., Delot, E., Faure, G., Saliou, B., Bon, C., 1996. Structure and function relationship of crotoxin, a heterodimeric neurotoxic phospholipase A_2 from the venom of a South American rattlesnake. Adv. Exp. Med. Biol. 391, 197–202.

Choumet, V., Lafaye, P., Mazié, J.C., Bon, C., 1998. A monoclonal antibody directed against the non-toxic subunit of a dimeric phospholipase A_2 neurotoxin, crotoxin, neutralizes its toxicity. Biol. Chem. 379, 899–906.

Dos Santos, M.C., Diniz, C.R., Whitaker-Pacheco, M.A., Dias da Silva, W., 1988. Phospholipase A_2 injection in mice induces immunity against the lethal effets of *Crotalus durissus terrificus* venom. Toxicon 26, 201–213.

Dos Santos, M.C., Yamaguchi, I.K., Caricatti, C.P., Higashi, H.G., Dias da Silva, W., 1989. Immunization of equines with phospholipase A_2 protects against the lethal effects of Crotalus durissus terrificus venom. Braz. J. Med. Biol. Res. 22, 509–512.

Finney, D.J., 1992. Probit Analysis, third ed. Cambridge University Press, Cambridge.

Freitas, T.V., Frézard, F., 1997. Encapsulation of native crotoxin in liposomes: a safe approach for the production of antivenom and vaccination against *Crotalus durissus terrificus* venom. Toxicon 35, 91–100.

Freitas, T.V., Fortes-Dias, C.L., Diniz, C.R., 1990. Protection against the lethal effects of Crotalus durissus terrificus (South American rattlesnake) venom in animals immunized with crotoxin. Toxicon 28, 1491–1496.

Gonçalves, J.M., 1956. Purification and properties of crotaminc. In: Buckley, E.E., Porges, N. (Eds.), Venoms. A. A. A. A. S., Washington DC, p. 261.

Guidolin, R.G., Marcelino, R.M., Gondo, H.H., Morais, J.F., Ferreira, R.A., Silva, C.L., Kipnis, T.L., Silva, J.A., Fafetine, J., Dias da Silva, W., 2010. Polyvalent horse F(Ab′)2 snake antivenom: development of process to produce polyvalent horse $F(Ab')_2$ antibodies anti-african snake venom. Afr. J. Biotechnol. 9, 2446–2455.

Hadler, W.A., Vital Brazil, O., 1966. Pharmacology of crotoxin. IV. Nephrotoxicity. Mem. Inst. Butantan 33, 1001–1008.

Harris, J.B., 1991. Phospholipases in snake venoms and their effects on nerve and muscle. In: Harvey, A. (Ed.), Snake Toxins. Pergamon Press, New York, pp. 91–129.

Kabat, E.A., Wu, T.T., 1972. Construction of a three-dimensional model of the polypeptide backbone of the variable region of kappa immunoglobulin light chains. Proc. Natl. Acad. Sci. U.S.A. 69, 960–964.

Kerkis, A., Kerkis, I., Rádis-Baptista, G., Oliveira, G.E.B., Vianna-Morgante, A.M., Pereira, L.V., Yamane, T., 2004. Crotamine is a novel cell-penetrating protein from the venom of rattlesnake *Crotalus durissus terrificus*. FASEB J. 18, 1407–1409.

Kosako, T., Kawachi, A., Cheng, S.B., Kuchiiwa, S., Motoya, T., Nakagawa, S., Yamada, K., 2000. Role of the vestibular nuclei in endothelin-1-induced barrel rotation in rats. Eur. J. Pharm. 454, 199–220.

Kouyoumdjian, J., Harris, J., Johnson, M., 1986. Muscle necrosis caused by the sub-units of crotoxin. Toxicon 24, 575–583.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680–685.

Marlas, G., Bon, C., 1982. Relationship between the pharmacological action of crotoxin and its phospholipase activity. Eur. J. Biochem. 125, 157–165.

Melgarejo, A.R., 2003. Serpentes Peçonhentas do Brasil. In: Costa Cardoso, J.L., Siqueira França, F.O., Wen, F.H., Málaque, C.M.S., Haddad Jr., V. (Eds.), Animais Peçonhentos no Brasil: Biologia, Clínica e Terapêutica dos Acidentes. Sarvier-Fapesp, São Paulo, pp. 33–61.

Ministério da Saúde, 2001. Manual de Diagnóstico e Tratamento de Acidentes por Animais Peçonhentos, 1ª ed. Fundação Nacional da Saúde, Brasília.

Nisonoff, A., Wissler, F.C., Lipman, L.N., Woernley, D.L., 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule

by reduction of disulfide bonds. Arch. Biochem. Biophys. 89 (2), 230–244.
Oshima-Franco, Y., Hyslop, S., Prado-Francheschi, J., Cruz-Höfling, M.A., Rodrigues-Simioni, L., 1999. Neutralizing capacity of antisera raised in horses and rabbits against *Crotalus durissus terrificus* (South American rattlesnake) venom and its main toxin, crotoxin. Toxicon 37, 1341–1357.
Padlan, E.A., 1994. Anatomy of the antybody molecule. Mol. Immunol. 31, 169–172.
Pope, C.G., 1936. The action of proteolytic enzymes on antitoxins and proteins in immune sera. I. True digestion of the proteins. Br. J. Exp. Pathol. 20, 132–149.
Price III, J.A., 2007. A colorimetric assay for measuring phospholipase A_2 degradation of phosphatidylcholine at physiological pH. J. Biochem. Biophys. Methods 70, 441–444.
Pullen, G.R., Fitzgerald, M.G., Hosking, C.S., 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods 86, 83–87.
Rádis-Baptista, G., Oquiura, N., Hayashi, M.A., Camargo, M.E., Grego, K.F., Oliveira, E.B., Yamane, T., 1999. Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37, 973–984.
Rangel-Santos, A., Lima, A., Lopes-Ferreira, M., Cardoso, D.F., 2004. Immunosupresive role of principal toxin (crotoxin) of *Crotalus durissus terrificus* venom. Toxicon 44, 609–616.
Raw, I., Rocha, M.C., Esteves, M.I., Kamiguti, A.S., 1986. Isolation and characterization of a thrombin-enzyme from the venom of *Crotalus durissus terrificus*. Braz. J. Med. Biol. Res. 19 (3), 333–338.
Raw, I., Higashi, H.G., Kelen, E.M., 1996. Antivenom production and organization of the public health system for the treatment of envenoming in Brazil. In: Bon, C., Goyffon, M. (Eds.), Envenomings and Their Treatments. Foundation Marcel Mérieux, Lyon, pp. 155–160.
Remfry, J., 1987. Ethical aspects of animal experimentation. In: Tuffery, A.A. (Ed.), Laboratory Animals: An Introduction for New Experimenters. Interscience, New York, pp. 5–9.
Romero-Steiner, S., Holder, P.F., de Leon, P.G., Spear, W., Hennessy, T.W., Carlone, G.M., 2005. Avidity determinations for Haemophilus influenzae Type b anti polyribosylribitol phosphate antibodies. Clin. Diagn. Lab. Immunol. 12, 1029–1035.
Santoro, M.L., Sousa-e-Silva, M.C., Gonçalves, L.R., Almeida-Santos, S.M., Cardoso, D.F., Laporta-Ferreira, I.L., Saiki, M., Peres, C.A., Sano-Martins, I.S., 1999. Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (*Crotalus durissus terrificus*, *C. durissus cascavella* and *C. durissus collilineatus*). Comp. Biochem. Physiol. 22, 61–73.
Santos, P.E., Souza, S.D., Freire-Maia, L., Almeida, A.P., 1990. Effects of crotoxin on the isolated guinea pig heart. Toxicon 28, 215–224.
Seki, C., Vidal, J.C., Barrio, A., 1980. Purification of gyroxin from a South American rattlesnake *Crotalus durissus terrificus* venom. Toxicon 18 (3), 235–247.
Slotta, K.H., Fraenkel-Conrat, H.L., 1938. Schlangengifte. III Mitteiling: Reinnigung und Krystallisationdes Klapperschlangen-Giftes. Ber. Disch. Chem. Ges. 71, 1076–1081.
Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150 (1), 76–85.
Steiner, L.A., Eisen, H.N., 1966. Variations in the immune response to a simple determinant. Bacteriol. Rev. 30 (2), 383–396.
Towbin, H., Staechelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
Vital Brazil, O., 1966. Pharmacology of crystalline crotoxin-II. Neuromuscular blocking action. Mem. Ins. Butantan 33, 981–992.
World Health Organization, 1981. Progress in the Characterization of Venoms and Standardization of Antivenoms. WHO Offset Publication, Geneva. No 58.
Wu, T.T., Kabat, E.A., 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. J. Exp. Med. 132 (2), 211–250.