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Linear B-cell epitopes in BthTX-1, BthTX-II and BthA-1, phospholipase A_2's from *Bothrops jararacussu* snake venom, recognized by therapeutically neutralizing commercial horse antivenom

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ABSTRACT

The benefits from treatment with antivenom sera are indubitable. However, the mechanism for toxin neutralization has not been completely elucidated. A mixture of anti-bothropic and anti-crotalic horse antivenom has been reported to be more effective in neutralizing the effects of *Bothrops jararacussu* snake venom than anti-bothropic antivenom alone. This study determined which regions in the three PLA_2s from *B. jararacussu* snake venom are bound by antibodies in tetravalent anti-bothropic and monovalent anti-crotalic commercial horse antivenom. Mapping experiments of BthTX-I, BthTX-II and BthA-I using two small libraries of 69 peptides each revealed six major IgG-binding epitopes that were recognized by both anti-bothropic and anti-crotalic horse antivenom. Two epitopes in BthTX-I were only recognized by the anti-bothropic horse antivenom, while anti-crotalic horse antivenom recognized four unique epitopes across the three PLA_2s. Our studies suggest that the harmful activities of the PLA_2s present in the venom of *B. jararacussu* are neutralized by the combinatorial treatment with both antivenom sera through their complementary binding sites, which provides a wide coverage on the PLA_2s. This is the first peptide microarray of PLA_2s from *B. jararacussu* snake venom to survey the performance of commercial horse antiophidic antivenom. Regions recognized by the protective antivenom sera are prime candidates for improved venom cocktails or a chimeric protein encoding the multiple epitopes to immunize animals as well as for designing future synthetic vaccines.

1. Introduction

Every year, 2.5 million people are bitten by snakes in South America with approximately 100,000 deaths as a result. Administration of specific antivenoms has been the

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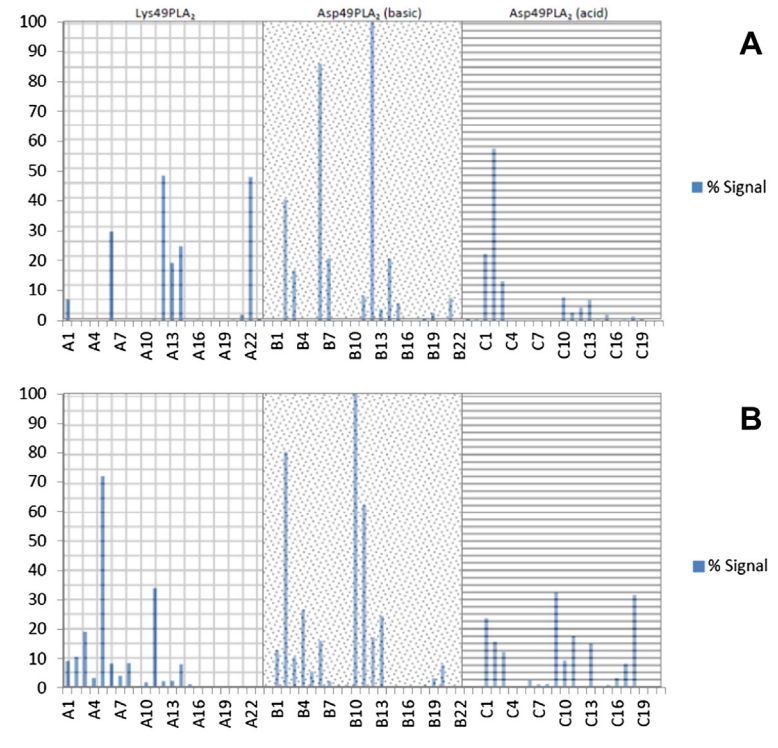

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Lys49PLA$_2$	
A1	S-L-F-E-L-G-K-M-I-L-Q-E-T-G
A2	G-K-M-I-L-Q-E-T-G-K-N-P-A-K
A3	Q-E-T-G-K-N-P-A-K-S-Y-G-A-Y
A4	N-P-A-K-S-Y-G-A-Y-G-C-N-C-G
A5	Y-G-A-Y-G-C-N-C-G-V-L-G-R-G
A6	C-N-C-G-V-L-G-R-G-K-P-K-D-A
A7	L-G-R-G-K-P-K-D-A-T-D-R-C-C
A8	P-K-D-A-T-D-R-C-C-Y-V-H-K-C
A9	D-R-C-C-Y-V-H-K-C-C-Y-K-K-L
A10	V-H-K-C-C-Y-K-K-L-T-G-C-D-P
A11	Y-K-K-L-T-G-C-D-P-K-K-D-R-Y
A12	G-C-D-P-K-K-D-R-Y-S-Y-S-W-K
A13	K-D-R-Y-S-Y-S-W-K-D-K-T-I-V
A14	Y-S-W-K-D-K-T-I-V-C-G-E-N-N
A15	K-T-I-V-C-G-E-N-N-P-C-L-K-E
A16	G-E-N-N-P-C-L-K-E-L-C-E-C-D
A17	C-L-K-E-L-C-E-C-D-K-A-V-A-I
A18	C-E-C-D-K-A-V-A-I-C-L-R-E-N
A19	A-V-A-I-C-L-R-E-N-L-G-T-Y-N
A20	L-R-E-N-L-G-T-Y-N-K-K-Y-R-Y
A21	G-T-Y-N-K-K-Y-R-Y-H-L-K-P-F
A22	K-Y-R-Y-H-L-K-P-F-C-K-K-A-D
A23	R-Y-H-L-K-P-F-C-K-K-A-D-P-C

Asp49PLA$_2$ (basic)	
A24	D-L-W-Q-F-G-Q-M-I-L-K-E-T-G
B1	G-Q-M-I-L-K-E-T-G-K-L-P-F-P
B2	K-E-T-G-K-L-P-F-P-Y-Y-T-T-Y
B3	L-P-F-P-Y-Y-T-T-Y-G-C-Y-C-G
B4	Y-T-T-Y-G-C-Y-C-G-W-G-G-Q-G
B5	C-Y-C-G-W-G-G-Q-G-Q-P-K-D-A
B6	G-G-Q-G-Q-P-K-D-A-T-D-R-C-C
B7	P-K-D-A-T-D-R-C-C-F-V-H-D-C
B8	D-R-C-C-F-V-H-D-C-C-Y-G-K-L
B9	V-H-D-C-C-Y-G-K-L-T-N-C-K-P
B10	Y-G-K-L-T-N-C-K-P-K-T-D-R-Y
B11	N-C-K-P-K-T-D-R-Y-S-Y-S-R-E
B12	T-D-R-Y-S-Y-S-R-E-N-G-V-I-I
B13	Y-S-R-E-N-G-V-I-I-C-G-E-G-T
B14	G-V-I-I-C-G-E-G-T-P-C-E-K-Q
B15	G-E-G-T-P-C-E-K-Q-I-C-E-C-D
B16	C-E-K-Q-I-C-E-C-D-K-A-A-A-V
B17	C-E-C-D-K-A-A-A-V-C-F-R-E-N
B18	A-A-A-V-C-F-R-E-N-L-R-T-Y-K
B19	F-R-E-N-L-R-T-Y-K-K-R-Y-M-A
B20	R-T-Y-K-K-R-Y-M-A-Y-P-D-V-L
B21	R-Y-M-A-Y-P-D-V-L-C-K-K-P-A
B22	A-Y-P-D-V-L-C-K-K-P-A-E-K-C

Asp49PLA$_2$ (acid)	
B23	S-L-W-Q-F-G-K-M-I-N-Y-V-M-G
B24	G-K-M-I-N-Y-V-M-G-E-S-G-V-L
C1	Y-V-M-G-E-S-G-V-L-Q-Y-L-S-Y
C2	S-G-V-L-Q-Y-L-S-Y-G-C-Y-C-G
C3	Y-L-S-Y-G-C-Y-C-G-L-G-G-Q-G
C4	C-Y-C-G-L-G-G-Q-G-Q-P-T-D-A
C5	G-G-Q-G-Q-P-T-D-A-T-D-R-C-C
C6	P-T-D-A-T-D-R-C-C-F-V-H-D-C
C7	D-R-C-C-F-V-H-D-C-C-Y-G-K-V
C8	V-H-D-C-C-Y-G-K-V-T-G-C-D-P
C9	Y-G-K-V-T-G-C-D-P-K-I-D-S-Y
C10	G-C-D-P-K-I-D-S-Y-T-Y-S-K-K
C11	I-D-S-Y-T-Y-S-K-K-N-G-D-V-V
C12	Y-S-K-K-N-G-D-V-V-C-G-G-D-D
C13	G-D-V-V-C-G-G-D-D-P-C-K-K-Q
C14	G-G-D-D-P-C-K-K-Q-I-C-E-C-D
C15	C-K-K-Q-I-C-E-C-D-R-V-A-T-T
C16	C-E-C-D-R-V-A-T-T-C-F-R-D-N
C17	V-A-T-T-C-F-R-D-N-K-D-T-Y-D
C18	F-R-D-N-K-D-T-Y-D-I-K-Y-W-F
C19	D-T-Y-D-I-K-Y-W-F-Y-G-A-K-N
C20	K-Y-W-F-Y-G-A-K-N-C-Q-E-K-S
C21	F-Y-G-A-K-N-C-Q-E-K-S-E-P-C

Fig. 1. Array interaction of PLA$_2$s peptide library from *Bothrops jararacussu* snake venom with antibodies in antivenom sera. Anti-bothropic (A) and anti-crotalic (B) horse antivenom were presented to peptides synthesized on membranes by Spot-synthesis. The peptides, listed in (C) represented the coding regions of BthTX-I (A1-A23), BthTX-II (A24-B22) and BthA-I (B23-C21) PLA$_2$s. Each peptide was identified by the Spot-synthesis membrane position numbering. Peptide C22 (positive control, not shown) had the highest signal intensity, which was arbitrarily assigned as 100%. C20 and C21 were negative controls. Spot intensities below 20% were considered as background. The 69 overlapping peptides, synthesized here, encompassed the entire amino acid sequence of the PLA$_2$s, and consisted of 14 residues each with an overlap of 9 residues between peptides.

most efficient treatment for snake envenoming. The effectiveness of anti-bothropic horse antivenom for the neutralization of the toxic and pharmacological effects of *Bothrops jararacussu* venom has been investigated by many groups (dos Santos et al., 1992; de Roodt et al., 1998, 1999; Oshima-Franco et al., 2001; Zamunér et al., 2004; Beghini et al., 2007), yet an understanding of the mechanism has not been elucidated.

Among the bioactive proteins from *Bothrops* sp snake venoms, the phospholipases A$_2$ (PLA$_2$s, E.C. 3.1.1.4) are regarded as one of the most important protein classes. PLA$_2$s are enzymes that catalyze the hydrolysis of 2-acyl ester bonds of 3-sn-phospholipids producing fatty acids and lysophospholipids (Gutiérrez and Lomonte, 1997). In addition to their catalytic role, they show a wide variety of pharmacological activities, such as neurotoxicity,

myotoxicity, anticoagulant and cardiotoxicity (de Paula et al., 2009). An analysis of the *B. jararacussu* venom gland transcriptome showed that 35% of transcripts are PLA_2s with 83% encoding BthTX-I (Bothropstoxin-I, a basic Lys49-PLA_2), 8% BthTX-II (Bothropstoxin-II, a basic Asp49-PLA_2) and 9% BthA-I (Acidic Asp49-PLA_2) (Kashima et al., 2004).

A large number of PLA_2s have been purified, characterized and several three dimensional structures have been solved for PLA_2s from the *Bothrops* genus (revised by Stábeli et al., 2012). Correlative studies have been performed with the predicted structures to understand the sites of pharmacological activity (Soares et al., 2001; Lomonte et al., 2003; Soares and Giglio, 2003; Murakami et al., 2007; Montecucco et al., 2008; Teixeira et al., 2011). The PLA_2s as a family are intriguing because despite the significant sequence and structural similarities between members, they present a diverse spectrum of activities, which may or may not be related to their primary catalytic activity (Higuchi et al., 2007; Tsai et al., 2007). This diverse pharmacological profile is suggested to have been acquired through evolution by a positive darwinian selection in the protein-coding exons by an accelerated evolutionary process that has resulted in many variants with diverse pharmacological effects (Ohno et al., 1998).

The superfamily of PLA_2 can be divided into five principal groups of enzymes: the secreted PLA_2s ($sPLA_2$s), the cytosolic PLA_2s ($cPLA_2$s), the Ca^{2+}-independent PLA_2s ($iPLA_2$s), the platelet-activating factor acetylhydrolases (PAF-AH) and the lysosomal PLA_2s (Schaloske and Dennis, 2006). The $sPLA_2$s are further subdivided into seventeen classifications according to their molecular mass and the number of disulfide bonds (Schaloske and Dennis, 2006). The snake (Viperidae) venom PLA_2s ($svPLA_2$s) are classified as GIIA, which contain seven disulfide bonds and have a molecular mass around 13–15 kDa (Schaloske and Dennis, 2006). The GIIA $svPLA_2$s can be further subdivided into two main types according to the amino acid residue in the 49th position: Asp49-PLA_2 and Lys49-PLA_2 (Arni and Ward, 1996). The Asp49-PLA_2s are the enzymes responsible for cellular membrane disruption through Ca^{2+}-dependent hydrolysis of phospholipids with myotoxicity activity or not. The Lys49-PLA_2s do not display catalytic activity, but can exert a pronounced and localized myotoxic effect that is not neutralized by antivenom therapy (Howard and Gundersen, 1980; Chang, 1985).

In 1911, Brazil demonstrated that the action of the *B. jararacussu* venom could not be efficiently neutralized with a specific antivenom or anti-bothropic horse antivenom alone. For the best treatment of the bite of this snake, it was suggested that therapeutic should be associated with anti-crotalic horse antivenom. Later, experiments were conducted to confirm that the administration of both the anti-bothropic and anti-crotalic horse antivenom provided a more effective neutralization for the myotoxic, coagulant and/or lethal activities than one antivenom used alone (de Roodt et al., 1999; Queiroz et al., 2008). This was not restricted only with bothropic-crotalic antivenom since it was recently observed for venom from Australian snake species (O'Leary and Isbister, 2009).

Other immunochemical studies using rabbit antibodies against a synthetic peptide (residues 1–15) of BthTX-I (Angulo et al., 2001) and an anti-NN-XI_a-PLA_2 from *Naja naja* venom (Basavarajappa et al., 1993) showed that the enzymatic activity of these PLA_2s was inhibited in a dose-dependent manner by antibodies. However, the lethal and neurotoxic symptoms were not neutralized in experimental animals (Basavarajappa et al., 1993). Further studies have demonstrated cross-reactivity between BthTX-I and the crotoxin of *Crotalus durissus cascavella*, but the common and specific antigenic determinants were not identified (Oshima-Franco et al., 2001; Beghini et al., 2007).

Overall, the mechanisms associated with the capacity to neutralize myotoxic and anticoagulant activities of snake venoms remain unknown along with the observed protective synergic effects of combining therapeutic antivenom. In this study, we report the identification and structural characterization of the linear B-cell epitopes of the three PLA_2s from *B. jararacussu* snake venom recognized by neutralizing anti-bothropic and anti-crotalic commercial horse antivenom. The results suggest that the best performance of the monovalent anti-crotalic antivenom to neutralize *B. jararacussu* PLA_2s may be due to the recognition of different epitopes rather than cross-reactivity or other factors such as the affinity of the antibodies. Our observations reinforce the importance of defining the mechanisms leading to the neutralization of the highly toxic proteins in venom by commercial antivenom to drive production of more protective treatments.

Table 1
Summary of the epitopes identified using commercial anti-bothropic and anti-crotalic horse antivenom. The PLA_2s sequences from *Bothrops jararacussu* snake venom were obtained from the Swiss-Prot database (http://www.uniprot.org/).

Sequences accession number	Protein identification	Anti-crotalic horse serum	Anti-bothropic horse serum
Q90249	BthTX-I Lys49-PLA_2	Gln11–Lys20	–
		Cys27–Gly30	Cys27–Gly30
		Gly59–Tyr73	Gly59–Tyr73
		–	Cys84–Asn89
		–	Lys116–Asp130
P45881	BthTX-II Asp49-PLA_2 basic	Leu17–Tyr25	Leu17–Tyr25
		Pro37–Cys45	Pro37–Cys45
		Thr69–Glu77	–
		Gly79–Thr89	Gly79–Thr89
Q8AXY1	BthA-I Asp49-PLA_2 acidic	Ser17–TyrY25	Ser17–TyrY25
		Tyr52–Tyr73	–
		Phe106–Phe119	–

2. Material and methods

2.1. Material

Amino acids for peptide synthesis were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Super SignalR West Pico chemiluminescent substrate was from Pierce Biotechnology (Rockford, IL, USA). Amino-PEG_{500}-UC540 cellulose membranes were obtained from Intavis Bioanalytical Instruments (Koeln, Germany). Pyperidine, acetonitrile and trifluoracetic acid were from Fluke. A peroxidase-labeled rabbit anti-horse immunoglobulin serum was from KPL (Gaithersburg, MD, USA). Bovine serum albumin, 3,3,5,5′ tetramethylbenzidine

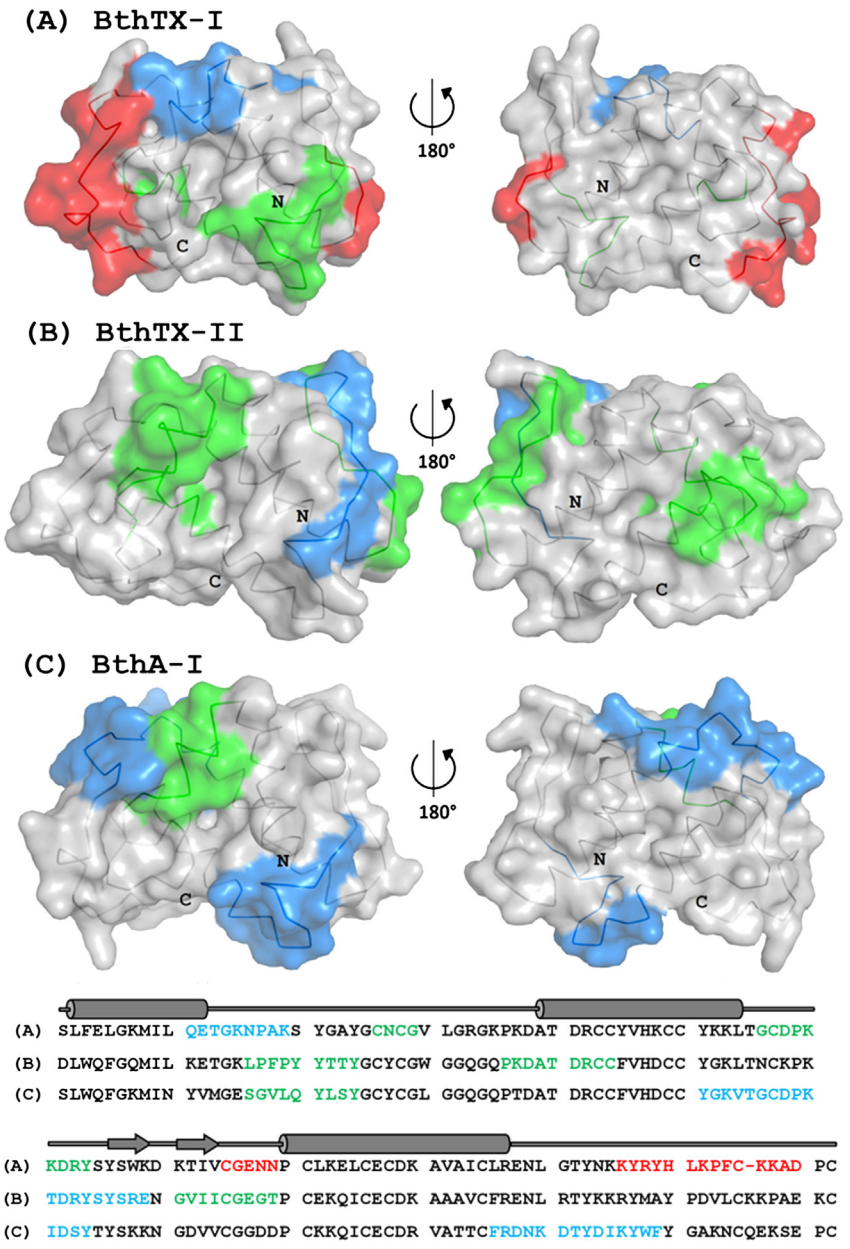

Fig. 2. Spatial localization of the specific reactive epitopes in three dimensional structures of BthTX-I, BthTX-II and BthA-I from *Bothrops jararacussu* snake venom. The residues in PLA_2s bound by antibodies are highlighted in red (anti-bothropic horse serum), blue (anti-crotalic horse serum) and green (both antivenom). The structures of the BthTX-I (PDB ID: 3I3I), BthTX-II (PDB ID: 2OQD) and BthA-I (PDB ID: 1U73) were obtained from protein data bank (http://www.pdb.org) and drawn using PyMol (DeLano, 2002).

(TMB) and Tween 20 were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). Amicon centricon 10 filters were from Millipore (Billerica, MD, USA). CDP-Star® Substrate was from Applied Biosystems (Foster City, CA, USA). Sequence reagents and all other reagents and chemicals were from Calbiochem-Merck (Darmstadt, Germany).

2.2. Anti-bothropic and anti-crotalic horse antivenom

Tetravalent anti-bothropic (*B. jararacussu*, *Bothrops jararaca*, *Bothrops neuwiedi* and *Bothrops alternatus*) and monovalent anti-crotalic (*C. d. terrificus*) horse antivenom were produced and kindly provided by the Vital Brazil Institute, Niteroi, RJ, Brazil.

2.3. Synthesis of the cellulose-membrane-bound peptide array

Two libraries of sixty-nine, 14-mer peptides were designed to represent a consecutive overlapping coverage that was offset by nine amino acids across the entire coding region (121–122 amino acids) of the three PLA_2s present in

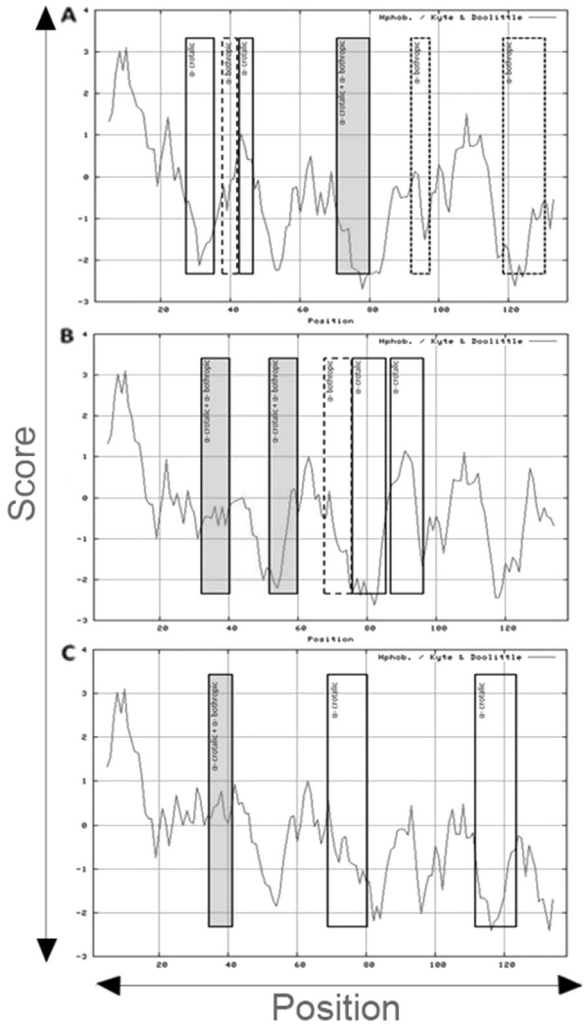

Fig. 3. Hydrophobicity plots of the three PLA_2 proteins. Panels A (BthTX-I), B (BthTX-II) and C (BthA-I) were determined with window sizes between 7 and 11 residues according to Kyte and Doolittle (1982) to maximize the information content for the plots. Gray rectangles mark the epitopes recognized by both antivenom sera. Solid rectangles encompass epitopes recognized exclusively by the anti-crotalic horse serum and the dashed rectangles enclose the epitopes recognized solely by the anti-bothropic horse serum.

the venom of *B. jararacussu*. Sequences were obtained from the UniProtKB – Protein knowledgebase (http://www.uniprot.org/): BthTX-I (Swiss-Prot ID.: Q90249), BthTX-II (Swiss-Prot ID.: P45881) and BthA-I (Swiss-Prot ID.: Q8AXY1). The peptides were automatically prepared onto Amino-PEG_{500}-UC540 cellulose membranes according to standard SPOT synthesis protocols (Frank, 2002) using an Auto-Spot Robot ASP-222 (Intavis Bioanalytical Instruments AG, Köln, Germany). In brief, coupling reactions were followed by acetylation with acetic anhydride (4%, v/v) in *N*, *N*-dimethylformamide to render peptides unreactive during the subsequent steps. After acetylation, Fmoc protective groups were removed by the addition of piperidine to render nascent peptides reactive. The remaining amino acids were added by this same process of coupling, blocking and deprotection until the expected desired peptide was generated. After the addition of the last amino acid in the peptide, the amino acid side chains were deprotected using a solution of dichloromethane–trifluoracetic acid–triisobutylsilane (1:1:0.05, v/v/v) and washed with methanol. Membranes containing the synthetic peptides were either probed immediately or stored at −20 °C until needed. Negative controls [without peptide; IHLVNNESSEVIVHK (*Clostridium tetani*) precursor peptide] and positive controls were included in each assay.

2.4. Screening of SPOT membranes

SPOT membranes were washed with TBS (50 mM Tris-buffer saline, pH 7.0) and blocked with TBS-CT (50 mM Tris-buffer saline, 3% casein, 0.1% Tween 20, pH 7.0) at room temperature under agitation or overnight at 4 °C. After extensive washing with TBS-T (50 mM Tris-buffer saline, 0.1% Tween 20, pH 7.0), two membranes presenting the same peptide library were incubated separately for two hours with either horse anti-crotalic or anti-bothropic antivenom (1:250) in TBS-CT and them washed again with TBS-T. Afterward, the membranes were incubated with alkaline phosphatase-labeled sheep anti-horse IgG (1:5000 in TBS-CT) for one hour, and then washed with TBS-T and CBS (50 mM citrate-buffer saline, pH 7.0). Chemiluminenscente CDP-Star® Substrate (0.25 mM) with Nitro-Block-II™ Enhancer (Applied Biosystems, USA) was added to complete the reaction.

2.5. Scanning and measurement of spot signal intensities

Chemiluminescent signals were detected on MF-ChemiBis 3.2 (DNR Bio-Imaging Systems, Israel) at a resolution of 5 MP. The digital image file was analyzed with TotalLab Software (Nonlinear Dynamics, USA) to quantify signal intensities and to define the empirical probability that the intensity from a spot was distinct from that of the background signal using algorithms that compared the intensities between background, spot area and negative controls.

2.6. Sequence analysis

Fifty PLA_2s isolated from the venom of snakes from *Bothrops*, *Crotalus* and *Lachesis* genera were selected and their amino acid sequences were aligned, compared and analyzed. The sequences were obtained from the UniProt Knowledgebase (http://www.uniprot.org/). The sequences were clustered using two criteria: physical–chemical property (acidic or basic) and the amino acid residues at position 49 (lysine or aspartic acid). The theoretical isoelectric point (p*I*) of all the selected sequences was calculated according to the amino acids sequence with the ProtParam tool (Gasteiger et al., 2005) from the ExPASy Proteomics Server (http://www.expasy.ch/tools/protparam.html). The multiple sequence alignment (MSA) of the selected sequences was generated within the web server T-Coffee (Notredame et al., 2000), using the program default parameters. Manual improvements were made to adjust the alignment performed by T-Coffee with the numbering system proposed by Renetseder et al. (1985).

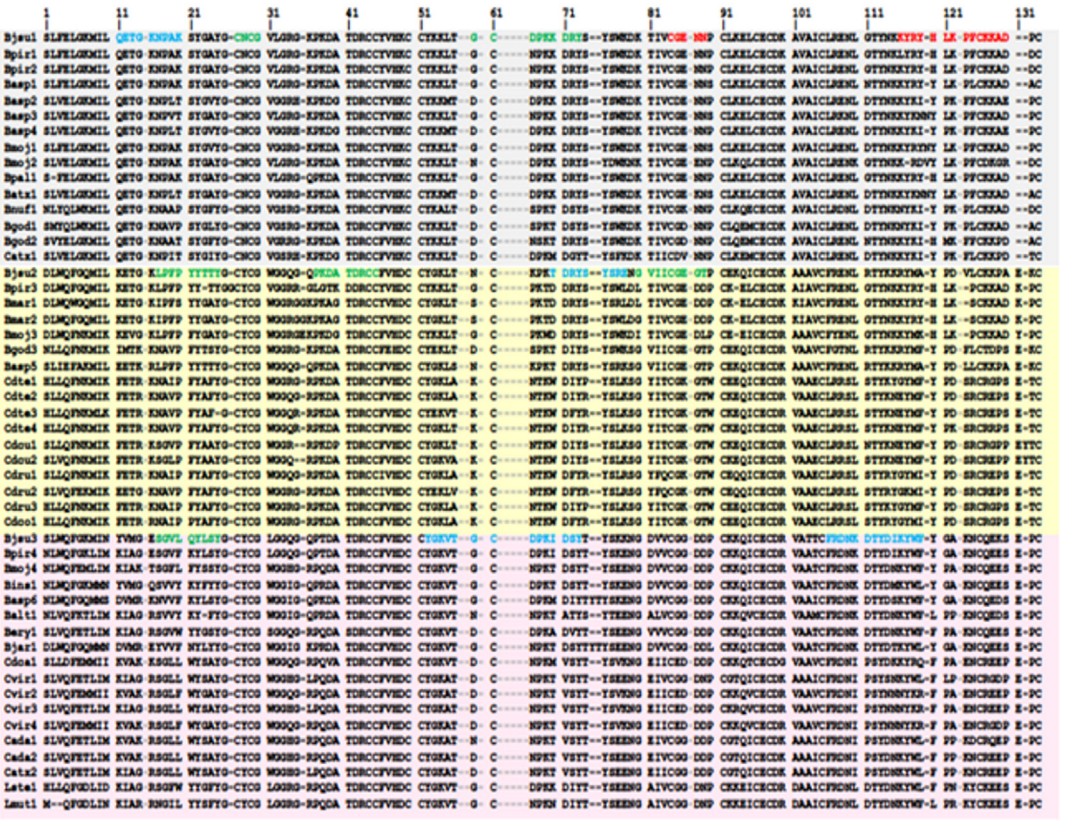

Fig. 4. Multiple sequence alignment of PLA_2s amino acid sequences of the snake venoms from the *Bothrops*, *Crotalus* and *Lachesis* genera. The sequences were clustered into three groups according to their physical-chemical property (acidic or basic) and the type of amino acid (lysine or aspartic acid) at position 49. Basic Lys49-PLA_2s are gray, basic Asp49-PLA_2s basic are yellow and acidic Asp49-PLA_2s are shown in pink. The gray gaps were included to adjust the sequences for the numbering system proposed by Renetseder et al. (1985) and the black gaps were used to adjust the alignment for all sequences. The amino acid residues from BhtTX-I, BthTX-II and BthA-I that bound antibodies are highlighted in red (anti-bothropic horse serum), blue (anti-crotalic horse serum) and green (both antivenom sera).

2.7. Hydropathy

A hydropathy plot with a window size of nine was used to span the epitopes throughout the hydrophobicity of the PLA_2s over the length of the peptide sequence (Kyte and Doolittle, 1982).

3. Results

3.1. Identification and mapping of linear epitopes using synthetic peptides

The epitopes recognized by therapeutic horse antivenom sera in the three major PLA_2s present in the venom of *B. jararacussu*, BthTX-I, BthTX-II and BthA-I, were mapped using the parallel Spot-synthesis strategy. Two peptide libraries were designed to more precisely define the epitopes recognized by anti-bothropic and/or anti-crotalic horse antivenom. Each consisted of 69 peptide sequences of fourteen amino acids each that overlapped by nine amino acids and covered the entire protein sequences of the three PLA_2s. A representative experiment, which shows results identical to three independent assays, is presented in Fig. 1.

The analysis of spot signal intensity for the synthesized peptides from the three PLA_2s sequences in cross-reactivity with the anti-crotalic and anti-bothropic horse antivenom showed a total of 12 epitopes. Two of the epitopes were specifically recognized by the anti-bothropic horse antivenom, while four epitopes were restricted to the activity of the anti-crotalic horse antivenom. The other six epitopes interacted with antibodies in both antivenom sera, however there were differences in the signal intensities.

The two immunodominant antigenic determinants present in the BthTX-I (Cys84–Asn89 and Lys116–Asp130) were recognized exclusively by the anti-bothropic horse antivenom, while one (Gln11–Lys20) was bound by the anti-crotalic horse antivenom and two others (Cys27–Gly30 and Gly59–Tyr73) by both horse antivenom sera. On the other hand, four major IgG-binding epitopes were recognized in BthTX-II: three (Leu17–Tyr25, Pro37–Cys45 and Gly80–Thr89) by both antivenom sera and one (Thr70–Glu78) solely by the anti-crotalic horse antivenom. In the BthA-I, two epitopes (Tyr52–Tyr73 and Phe106–Phe119) were recognized specifically by the anti-crotalic horse antivenom and one (Ser17–Tyr25) by both of antivenom (Table 1).

Overall, each of the epitopes displayed a relatively strong reactivity (containing 4–14 amino acids extension).

Table 2
List of the individual snake venom PLA_2s from *Bothrops sp*, *Crotalus sp* and *Lachesis sp* used for comparative analysis with the species name, individual identifiers, their accession numbers for Swiss-Prot, TrEMBL or PDB and theoretical isoelectric points. Enzymes are divided in three groups; Lys49-PLA_2s, basic Asp49-PLA_2s and acidic Asp49-PLA_2s.

Species	Individual identifier	Accession number	Isoelectric point
Lys49-PLA_2s			
Bothrops jararacussu	Bjsu1	sp|Q90249|	9.03
Bothrops pirajai	Bpir1	sp|P58399|	8.97
Bothrops pirajai	Bpir2	sp|P82287	9.03
Bothrops asper	Basp1	sp|P24605|	9.10
Bothrops asper	Basp2	sp|P0C616|	8.70
Bothrops asper	Basp3	sp|Q9PVE3|	9.10
Bothrops asper	Basp4	tr|B0FM89|	8.70
Bothrops moojeni	Bmoj1	sp|Q9I834|	8.95
Bothrops moojeni	Bmoj2	sp|P82114|	8.97
Bothropoides pauloensis	Bpal1	sp|Q9IAT9|	8.97
Bothrops atrox	Batx1	sp|Q6JK69|	8.87
Atropoides nummifer	Bnuf1	sp|P82950|	8.71
Cerrophidion godmani	Bgod1	sp|P81165|	8.61
Cerrophidion godmani	Bgod2	sp|Q8UVU7|	8.90
Crotalus atrox	Catx1	sp|Q8UVZ7|	8.60
Basic Asp49-PLA_2s			
Bothrops jararacussu	Bjsu2	sp|P45881|	8.50
Bothrops pirajai	Bpir3	pdb|1GMZ|	8.35
Bothrops marajoensis	Bmar1	sp|P86804|	8.61
Bothrops marajoensis	Bmar2	sp|P86803|	8.49
Bothrops moojeni	Bmoj3	sp|P0C8M1|	8.17
C. godmani	Bgod3	tr|Q6EER5|	8.36
Bothrops asper	Basp5	sp|P20474|	8.72
C. durissus terrificus	Cdte1	sp|P62022|	8.74
C. durissus terrificus	Cdte2	sp|P24027|	8.74
C. durissus terrificus	Cdte3	sp|P0CAS7|	8.74
C. durissus terrificus	Cdte4	sp|P0CAS5|	9.16
C. durissus cumanensis	Cdcu1	sp|P86806|	8.83
C. durissus cumanensis	Cdcu2	sp|P86805|	8.62
C. durissus ruruima	Cdru1	sp|P0CAS3|	8.75
C. durissus ruruima	Cdru2	sp|P0CAS4|	8.51
C. durissus ruruima	Cdru3	sp|P86169|	8.74
C. durissus collilineatus	Cdco1	sp|P0CAS2|	8.85
Acidic Asp49-PLA_2s			
Bothrops jararacussu	Bjsu3	sp|Q8AXY1|	5.20
Bothrops pirajai	Bpir4	sp|C9DPL5|	4.90
Bothrops moojeni	Bmoj4	tr|G3DT18|	4.91
Bothrops insularis	Bins1	sp|Q8QG87|	5.05
Bothrops asper	Basp6	sp|P86389|	5.05
Bothrops alternatus	Balt1	sp|P86456|	6.69
Bothrops erythromelas	Bery1	tr|Q2HZ28|	4.67
Bothrops jararaca	Bjar1	sp|P81243|	4.58
C. durissus cascavella	Cdca1	tr|C9E7C4|	5.14
Crotalus viridis viridis	Cvir1	tr|Q800C4|	4.99
Crotalus viridis viridis	Cvir2	tr|Q800C3|	5.39
Crotalus viridis viridis	Cvir3	tr|Q800C1|	5.10
Crotalus viridis viridis	Cvir4	tr|Q800C2|	5.78
Crotalus adamanteus	Cada1	sp|P00623|	5.06
Crotalus adamanteus	Cada2	tr|F8S100|	4.91
Crotalus atrox	Catx2	sp|P00624|	4.64
Lachesis stenophrys	Lste1	sp|P84651|	4.95
Lachesis muta muta	Lmut1	tr|D3IVZ4|	5.38

However, the strongest intensity was observed with the antigenic determinant Thr70–Glu78, from the basic Asp49-PLA_2 (BthTX-II) either with the anti-bothropic and anti-crotalic horse antivenom (Fig. 1A and B, spots B12 and B11, respectively). Fig. 1C shows the list of synthesized peptides. Fig. 1A and B present the immunological assay and the signal intensity of reactivity for each peptide with anti-bothropic and anti-crotalic horse antivenom, respectively.

3.2. Spatial location of the most reactive heavy chain hIg-epitopes

The oligomeric structure of BthTX-I, BthTX-II and BthA-I proteins were solved by X-ray crystallography and are available in the protein data bank (http://www.pdb.org) under the PDB accession numbers: 3I3I (Fernandes et al., 2010), 2OQD (Correa et al., 2008) and 1U73 (Magro et al., 2004), respectively. Fig. 2 displays the spatial localization of the epitopes identified by the SPOT-synthesis array experiments. Two of the BthTX-II epitopes (Thy70–Glu78 and Gly80–Thr89) were localized in a β-wing region, while all of other linear epitopes were located in coil/loop structures in the PLA_2s protein structures. The hydropathy plots of the three proteins, shown in the Fig. 3, also suggested that all of the epitopes were present on the surface of the proteins.

3.3. Sequence analysis

The sequences of fifty PLA_2s were selected and grouped into three sub-groups: *a*. Lys49-PLA_2 (fourteen from the *Bothrops* genus and one from the *Crotalus* genus); *b*. basic Asp49-PLA_2 (seven from the *Bothrops* genus and ten from the *Crotalus* genus); *c*. acidic Asp49-PLA_2 (eight from the *Bothrops* genus, eight from the *Crotalus* genus and two from the *Lachesis* genus) (Fig. 4). Individual identifiers, accession numbers and theoretical isoelectric points (p*I*) of the PLA_2s sequences are presented in Table 2.

Shared amino acids sequence from the 12 epitopes recognized by the reaction between the *B. jararacussu* PLA_2s and anti-crotalic/anti-bothropic horse antivenom were analyzed by a multiple sequence alignment between the fifty PLA_2s selected sequences.

Two antigenic determinants present in the Lys49-PLA_2s, which reacted positive only for the anti-bothropic horse antivenom, were identified as Cys84–Asn89 and Lys116–Asp130. The ^{84}CGENN^{89} epitope of BthTX-I was identified in the three-dimensional structure within a β-wing region (Fernandes et al., 2010), which was considered to have an acidic characteristic (theoretical pI = 4.0). The Glu86, Asn88 and Asn89 residues could be important for the specific interaction between BthTX-I and the anti-bothropic horse antivenom, since the replacement of Asn88 → Gly and Asn89 → Thr was observed as an epitope in BthTX-II that was recognized by both antivenom antivenom. The 116KYRYHLKPFCKKAD130 epitope was situated in the C-terminal region (Fernandes et al., 2010) which, in *Bothrops* genus proteins, is considered responsible for the myotoxic activity observed in Lys49-PLA_2s (Chioato et al., 2007). The Lys116–Asp130 epitope has a basic characteristic (theoretical pI = 9.75) that was rich in positively charged amino acids and differed from most of the acidic Asp49-PLA_2s, which presented theoretical pI's of approximately 4.0. This positively charged region could exert a strong influence on the binding of antibodies in the anti-bothropic horse antivenom with BthTX-I.

Four epitopes were specifically recognized by the anti-crotalic horse antivenom: Gln11–Lys20 (BthTX-I), Thy70–Glu78 (BthTX-II), Tyr52–Tyr73, and Phe106–Phe119 (BthA-I) (Fig. 4). For BthTX-I, the sequence ^{11}QETGKNPAK^{20} was located in a transition region within the three dimensional

model that corresponded with the end of an alpha helix I, which was followed by the Ca^{2+}-binding loop (Fernandes et al., 2010). This epitope showed a basic characteristic (theoretical p*I* equal to 8.59). The comparative analysis of snake venom PLA_2s amino acid sequences showed that the glutamine in position 11 was conserved in all of the Lys49-PLA_2s from the *Bothrops* genus. Therefore, this residue may be responsible for the interaction between this epitope and the anti-crotalic horse antivenom, since this is the only amino acid with an observed change when compared with the same region in BthTX-II, which is not recognized by this antivenom. The proline in position 18 was present in almost all Lys49-PLA_2s or was replaced by alanine, serine or glycine in basic PLA_2s.

In BthTX-II, the acidic ^{70}TDRYSYSRE^{78} (theoretical pI = 5.73) epitope was three-dimensionally located in the β-wing region (Correa et al., 2008). The comparative analysis showed that the ^{77}RE^{78} → ^{77}WK^{78} replacement observed in Lys49-PLA_2s was not recognized as an epitope from the absence of observed interactions between this same region and the anti-crotalic horse antivenom. In BthA-I, the ^{52}YGK**V**TGCDPKIDSY^{73} epitope (theoretical pI = 8.14) was located in three dimensional model between the final alpha helix II and the beginning of the β-wing (Magro et al., 2004). The Val55 was conserved in acidic PLA_2s from the *Bothrops* genus. When it was replaced by leucine or methionine in the sequence of the basic PLA_2s, no interactions were measured for this region with the anti-crotalic horse antivenom.

The other BthA-I epitope, Phe106–Phe119, had an acidic characteristic (theoretical pI = 6.04). In the three dimensional model, it was located in the C-terminal loop of this protein (Magro et al., 2004). The comparative sequence analysis for the residues 106–119 from BthA-I and others PLA_2s highlighted the following amino acids: Phe106 (absent in basic PLA_2s from *Crotalus* genus), Lys110 (present only in acidic Asp49-PLA_2s from *Bothrops* genus and Bmoj2), Asp114 (present only in acidic Asp49-PLA_2s from *Bothrops* genus) and Trp118 (exclusive to the acidic Asp49-PLA_2s from *Bothrops* and *Lachesis* genera and present in most of acidic PLA_2s from *Crotalus* genus). All of these amino acids may be essential for the recognition of this region exclusively by anti-crotalic horse antivenom.

Six other epitopes were recognized by both antivenom sera: Cys27–Gly30 and Gly59–Tyr73 from BthTX-I; Leu17–Tyr25, Pro37–Cys45 and Gly80–Thr89 from BthTX-II; and Ser17–Tyr25 from BthA-I. The 27CNCG30 region corresponded to the Ca^{2+}-binding loop within the three dimensional structure of BthTX-I (Fernandes et al., 2010). The acidic Cys27–Gly30 epitope (theoretical pI = 5.51) was a conserved region in Lys49-PLA_2s that was recognized by both antivenom sera and presented a single change that differentiated it from Asp49-PLA_2s. The Asn28 was conserved in Lys49-PLA_2s, but this position in the Asp49-PLA_2s was occupied exclusively by tyrosine and this amino acid residue could be responsible for its interaction with both of antivenom sera. The replacement of Asn28 → Tyr Asp49-PLA_2s did not demonstrate an interaction with either antivenom sera. The other epitope from BthTX-I that was recognized by both of the antivenom sera was ^{59}GCDPKKDRY^{73} (theoretical pI = 8.18), which was located near to a β-wing (Fernandes et al., 2010). The preceding region of the β-wing (70KDRY73) in BthTX-I interacted with both of antivenom sera. This same region in BthTX-II (70TDRY73) and BthA-I (70IDSY73) interacted only with the anti-crotalic horse antivenom. In BthTX-I, the lysine at position 70 could be crucial due to its positive charge for the interaction of this sequence with both of the antivenom sera. Furthermore, this amino acid was present in the Lys49-PLA_2s from *Bothrops* genus with the exception of the sequences Bnuf1, Bgod1 and Bgod2.

Moreover, the comparative analysis with the selected PLA_2s showed that the Gly59 and Asp67 could be important amino acids residues for interactions with the antivenom sera based on the replacements of Gly59 → Asn and Asp67 → Lys that are present in BthTX-I. These changes eliminated measurable interactions. The epitopes Leu17–Tyr25 (BthTX-II – theoretical pI = 5.52) and Ser17–Tyr25 (BthA-I – theoretical pI = 5.24) represented the same regions in both of the Asp49-PLA_2s and were located near the Ca^{2+}-binding loop, an important catalytic region in PLA_2s. Two other epitopes from BthTX-II were located at the end of the Ca^{2+}-binding loop (37PKDATDRCC45) and in the β-wing (80GVIICGEGT89). Each was determined to have acidic characteristic with theoretical p*I*'s of 5.95 and 4.0, respectively.

4. Discussion

The therapeutic action of antivenom serum is based on neutralizing the normal, detrimental activity of enzymes present in venom. Neutralization most likely occurs by the formation of complexes between antibodies in the antivenom and their corresponding target antigens in the venom. This complex formation can lead to structural changes that prevent the normal function of the native antigen either by (a) steric hindrance that interferes with the interaction of the antigen with cellular targets, (b) binding of antibodies to the toxic activity site precluding the interaction of toxin's active site with their targets or (c) causing allosteric changes of the toxin. In either case, identification of the epitopes bound by antivenom serum antibodies will improve the quality of antivenoms.

In the case of *B. jararacussu* snake venom, the most effective treatment involves the administration of a combination of anti-bothropic and anti-crotalic antivenom to neutralize the myotoxic, coagulant and lethal activities of the venom than when one of these antivenom sera is used alone (dos Santos et al., 1992; de Roodt et al., 1998, 1999). It is evident that each of the individual antivenoms delivers antibodies that are necessary for neutralizing the effect of the venom. Considering the proteins present in venom, the PLA_2s are the main enzymes responsible for the harmful effects. Since the performances of the individual antivenom sera are not well understood, we focused on determining the antigenic determinants present in the PLA_2s proteins from *B. jararacussu* venom that are bound by antibodies present in the individual anti-bothropic and anti-crotalic horse antivenom.

The mapping experiments presented in Fig. 1 showed the immunogenicity of the array of peptides that was

synthesized to represent the three PLA_2s from *B. jararacussu* snake venom. Two antigenic determinants were recognized by the anti-bothropic horse antivenom, four antigenic determinants by the anti-crotalic horse antivenom and six peptides were recognized by both antivenom sera (Table 1). While cross reactivity has been described for distinct proteins from snake venoms (de Roodt et al., 1998; Oshima-Franco et al., 2001; Beghini et al., 2007), which may reflect genetic relationship within proteins of the same family in various species and/or repetitive segments in distinct toxins, the use of spot synthesis peptide array employed here provided more detail of the common and unique epitopes bound by the two commercial horse antivenom sera. The advantages of this micro-immunoassay employing cellulose immobilized peptides over other different assays as classical ELISA for screening of antigenic peptide-arrays has been extensively discussed (Copeland et al., 2004; Henderson and Bradley, 2007). In our assays it was employed a cellulose membrane derivatized with amino-PEG_{500} to attach the amino acids. The advantage of this link over that using beta-alanine is the neglected background generated.

The Lys49-PLA_2s are proteins that exhibit various toxic effects including oedema, membrane depolarization (Kihara et al., 1992) and myonecrotic activity (Montecucco et al., 2008). The chemical cleavage of the N-terminal octapeptide of myotoxic PLA_2s from *Bothrops* genus by cyanogen bromide caused a drastic reduction of the myotoxicity, cytotoxicity, edema formation, bactericidal effect and the ability to disrupt liposomes (Soares and Giglio, 2003). The results from the peptide array demonstrate that the amino acid sequence for one of the epitopes recognized by anti-crotalic horse serum was from the sequence ^{11}QETGKNPAK^{20}, which encompasses this N-terminal region (Table 1). A comparative analysis of this epitope with the selected snake venoms sequences indicated that these residues are conserved in Lys49-PLA_2s and may exert strong influence on the toxic and pharmacologic actions exhibited by this family of proteins (Selistre-de-Araujo et al., 1996; Soares and Giglio, 2003). Angulo et al. (2001) showed that rabbit antibodies obtained against the N-terminal peptide ^{1}SLFELGKMILQETGK^{15} of myotoxin-II from *Bothrops asper* snake venom was able to block the myotoxic activity of the toxin. This suggests that the neutralization of the myonecrotic action caused by Lys49-PLA_2s could occur by the interaction with the anti-crotalic horse serum with this specific region, which is present only in BhTX-I. Furthermore, the three dimensional molecular model (Fig. 2) placed this epitope between the alpha-helix I and the beginning of the Ca^{2+}-binding loop suggesting a possible molecular mechanism for the action of binding of an antibody.

The myotoxic activity is an important and severe behavior displayed by Lys49-PLA_2s, which was associated with the significant number of positively charged residues located in the C-terminal region (Arni and Ward, 1996). Experiments that included site-directed mutagenesis (Ward et al., 2002; Chioato et al., 2007) and synthetic peptide immunogenicity (Lomonte et al., 2010) suggested that the C-terminal region of Lys49-PLA_2s acts as a heparin-binding site (Lomonte et al., 1994) and as a domain for myotoxic activity (Calderón and Lomonte, 1998). Our results showed that the C-terminal of BthTX-I contains the epitope ^{116}KYRYHLKPFCKKAD^{130}, which was specifically recognized by anti-bothropic horse serum. The myotoxic activity have been attributed to this segment however it contributes several positively charged residues, a critical fact that may determine the specific neutralization of this important region by the anti-bothropic horse serum.

Kini and Iwanaga (1986) suggested that residues between the positions 83–95 were involved in the myotoxic pre-synaptic action and neurotoxicity of PLA_2s and in our studies, the epitope ^{84}CGENN^{89} were neutralized specifically by the anti-bothropic horse antivenom. This specificity may be related with the physical chemical characteristics of the amino acid residues that constitute this sequence, especially the conserved Glu86. The Glu86 is conserved in basic PLA_2s from *Bothrops* genus along with the asparagine dyad (Asn88/89) can be observed only in Lys49-PLA_2s. However, in acidic Asp49-PLA_2s, the Glu86 was substituted by the amino acid residues glycine or aspartic acid. In basic Asp49-PLA_2s from *Crotalus* genus, this residue was replaced by an oppositely charged amino acid (Lys86) (Fig. 4). The replacement of charged residues by a glycine at position 86 in the acidic Asp49-PLA_2s from *Bothrops* genus is probably responsible for the absence of interaction between these regions in BthA-I with either antivenom sera studied.

Moreover, the 80GVIICGEGT89 region from BthTX-II interacted with both antivenom sera suggesting that the hydrophilic dyad composed by Asn88 and Asn89, present in BthTX-I, mediated the interactions only with antibodies present within anti-bothropic horse antivenom. However, the amino acid sequence analysis suggested that the residues Glu86, Asn88 and Asn89 are critical for the neutralizing of the myotoxic activity carried on Lys49-PLA_2s by interaction with the anti-bothropic horse antivenom.

The 27CYCG30 region is conserved within the Asp49-PLA_2s and in the three dimensional model corresponded to a Ca^{2+}-binding loop that coordinates the Ca^{2+} ion, an essential cofactor to the catalytic action of PLA_2s (Selistre-de-Araujo et al., 1996). The Ca^{2+}-binding domain was not present in Lys49-PLA_2s due to a substitution of the tyrosine residue at position 28 by asparagine. This specific adjustment caused a conformational change in the Ca^{2+}-binding loop and, consequently, a loss of the catalytic activity of PLA_2s (Kaiser et al., 1990). As indicated by the results of the spot synthesis experiments, both of the antivenom sera interacted with the epitope 27CNCG30 from BthTX-I. It can be suggested that the presence of an aromatic amino acid at position 28 prevented the interaction of the Asp49-PLA_2s with the antivenom sera analyzed.

The BthA-I presents a highly catalytic, platelet aggregation inhibition, oedema induction, hemolytic and hypotensive activities (Fully et al., 2004). However, it is not myotoxic, cytotoxic or lethal (Magro et al., 2004). It was proposed that the lysine at position 69 and the glycine or glutamic acidic at position 53 are essential for the anticoagulant effect displayed by this acidic Asp49-PLA_2 (Carredano et al., 1998). In addition, it appears that the key

regions related to the pharmacological effects of this acidic Asp49-PLA_2 is in the C-terminal loop, the region 17SGVLQYL23 (between alpha helix I and Ca^{2+}-binding loop) and the lysine at position 69 (Magro et al., 2005). Our results showed that two regions of BthA-I was specifically bound by anti-crotalic horse antivenom (52YGKVTGCDPKIDSY73 and 106FRNDKDTYDIKYWF119) and only one region (17SGVLQYALSY25) reacted with both antivenom sera. Thus our results indicated that the major pharmacological activities of BthA-I are most likely neutralized by the anti-crotalic horse antivenom more than by the anti-bothropic horse antivenom, but that the association of both antivenom could better inhibit the pharmacological activity of this toxin.

The comparative analysis of PLA_2s sequences allowed a survey of the glycine residue at position 53. It was highly conserved in Asp49-PLA_2s except for Cdte3 and Bjmo3, which have a glutamic acid at this position. The lysine residues at positions 54 and 69 were conserved in PLA2s from snake venoms. In addition, we observed that the amino acid residues Phe106, Lys110, Asp114 and Trp118 were conserved in the acidic Asp49-PLA2s from the *Bothrops* genus. However, the epitopes Tyr52–Tyr73 and Phe106–Phe119 were specifically recognized by anti-crotalic horse antivenom and not by anti-bothropic horse antivenom, which suggests that the anticoagulant activity of BthA-I was best neutralized by the anti-crotalic horse antivenom.

Toxins with similar biological actions usually present structural similarities, which are reflected in their antigenic cross-reactivity and consequent neutralization by heterologous antivenom sera. Only a few reports have shown antigenic cross-reactivity between *B. jararacussu* and *C. durissus ssp* venoms that specifically focused on the PLA_2s from both venoms (de Roodt et al., 1998, 1999; Oshima-Franco et al., 2001; Beghini et al., 2007; Correa-Netto et al., 2010). One report identified linear B-epitopes in myotoxin II, a Lys49-PLA_2 from *B. asper* snake venom, by PepSets™-ELISA assays using a specifically generated rabbit antitoxin serum and a therapeutic polyvalent Crotalinae horse antivenom (Lomonte, 2012). Their therapeutic antivenom was generated against a mixture of *B. asper*, *Crotalus simus* and *Lachesis stenophys* snakes venoms, which precluded an analysis of cross-reactivity of antibodies against one venom recognizing epitopes in a different venom, a major aim of this study. Our use of two therapeutic antivenom generated independently against *bothropic* and *crotalic* venoms permitted our analysis of cross reactivity. While it was difficult to directly compare results, the differences highlight the need for careful attention to the sources of venoms and antivenom.

The results of our antigenic map also reinforce the need for the application of multiple antivenom sera; only two epitopes were detected specifically by the anti-bothropic horse antivenom in relation to four epitopes to the anti-crotalic horse antivenom. Together, it is proposed that; (1) the improved performance observed with the application of both antivenom sera compared to a single antivenom is a result of synergism from expanded specificity rather than shared antigenic determinants, (2) the therapeutic contributions of the anti-crotalic horse antivenom can be linked to the interaction of its antibodies to important regions of BthTX-II and BthA-I and (3) the anti-bothropic horse antivenom appears to neutralize the sites of BthTX-I that are proposed to be myotoxic.

The commercial anti-bothropic horse antivenom produced in Brazil by the Vital Brazil Institute and other institutes is prepared by hyperimmunization of horses with a pool of venoms from *B. jararacussu*, *B. jararaca*, *Bothrops moojeni*, *B. alternatus* and *B. neuwiedi* while the anti-crotalic antivenom is produced using only *C. durissus terrificus* venom. A major question that remains unresolved is why the immunization of horses with distinct antigenic proteins (*Crotalus* sp proteins x *Bothrops* sp proteins) results in a product that, individually, is deficient to overcome the detrimental effects of a snake bite, but when applied jointly gives a neutralizing response. It is possible that intraspecies variations exist in the composition of specific snake venoms such that there are major implications in the preparation of uniform pools of venom used for the generation of antivenoms, as suggested recently (Gutiérrez et al., 2010). Furthermore, some epitopes could give a more dominant immune response than others and when mixing different *Bothrops* sp snake venoms to create pools used for immunization effectively creates a dilution effect. Additional experiments are needed to determine the mechanisms that drive the need for generating multiple and separate antivenom preparations. The identification of the individual epitopes presented here that are involved in the neutralization of the PLA_2s observed with the commercial antivenom sera provides a new direction for the design of immunization protocols to generate more effective treatments.

5. Conclusions

In conclusion, the peptide arrays formed directly onto cellulose membranes allowed the identification of the major antigenic determinants in the three most important PLA_2s (BthTX-I, BthTX-II and BthA-I) isolated from *B. jararacussu* snake venom recognized by commercial anti-bothropic and anti-crotalic horse antivenom. The cross-reactive epitopes located in the Lys49-PLA_2, the major protein of this venom, recognized two specific epitopes located in a region of the enzyme responsible for the myotoxic action, which contributes to the deleterious effects of snake venom. In addition, the ability of the anti-crotalic horse antivenom to neutralize the anticoagulant activity was most likely associated with the acidic Asp49-PLA_2. This study provides proof that the mixture of anti-crotalic and anti-bothropic horse antivenom is qualitatively more effective in neutralizing the effects unleashed of *B. jararacussu* snakebite.

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Conflict of interest

The authors declare no conflict of interest.

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