

A SENSITIVE AND SPECIFIC IMMUNOASSAY FOR THE MEASUREMENT OF THE ANTIBODIES PRESENT IN HORSE ANTIVENOMS ENDOWED WITH THE CAPACITY TO BLOCK THE PHOSPHOLIPASE A2-DEPENDENT HEMOLYSIS INDUCED BY SNAKE VENOMS

Services on Demand

Article

- Article in xml format
- Article references
- How to cite this article
- Curriculum ScienTI
- Automatic translation

Indicators

- Cited by SciELO
- Access statistics

Related links

Share



More

Permalink

A. C. M. ROCHA CAMPOS¹ ✉, M. C. W. DE FREITAS¹, I. K. YAMAGUCHI², J. R. MARCELINO², W. DIAS DA SILVA¹.

¹ Immunochemistry Laboratory, Butantan Institute, State of São Paulo, Brazil, ² Hyperimmune Plasma Processing Service, Butantan Institute, State of São Paulo, Brazil.

ABSTRACT: Phospholipase A2 (PLA2), a component of most snake venom toxins, cleaves 3-sn-phosphoglycerides releasing lysophosphatidyl-choline. The indirect quantitative assay method for PLA2 was standardized for specific antivenom titration in a fast and sensitive assay by the similarity with the hemolysis induced by PLA2 and by complement system in sheep erythrocytes. The curves obtained by plotting the degree of hemolysis against the doses of snake venom are concave to the abscissa axis following an equation similar to that previously described for the hemolysis induced by the C system. We observed that venoms of some *Bothrops*, *Crotalus* and *Micrurus* species contained around 1×10^3 to 10^4 Z/mg of venom, while the venom of *Naja* contained over one million Z/mg. Antibodies against PLA2 were titrated by incubating amounts of venom predetermined to give 1 to 5 Z with various dilutions of the antivenoms, and the remaining active PLA2 was determined in the hemolytic assay. We observed the following: a) the antivenoms contained specific antibodies against the PLA2 present in the corresponding venoms; b) cross-reactivity was not detected among PLA2 epitopes from venoms and nonspecific antivenoms; and c) the assay quantitatively performed determined the specific antibodies directed to epitopes on the molecule of PLA2. The method described in this paper is highly specific, sensitive and reproducible, besides being fast and inexpensive.

✉ **KEY WORDS:** PLA2, snake venoms, immunoassay, antivenoms, indirect hemolysis.

INTRODUCTION

Phospholipases A2 (PLA2) cleave the 3-sn-phosphoglycerides at the (B)H-C-O-C=O-R

releasing lysophosphatidyl-choline, ethanolamine, inositol or serine. PLA2 are the most commonly ones present in most snake venoms(**1,7,10**). They may exist either as single-chain polypeptides of 110-125 amino acids or as complexes of two or more subunits, but usually as the most toxic components of snake venoms such as taipoxin (LD50=2.0µg/Kg), notexin (LD50=17.0µg/Kg), crotoxin (LD50=25.0µg/Kg), beta-bungarotoxin (LD50=19-130 µg/Kg), and notechin II-5 (LD50=49, µg/kg) (**2,18**). Crotoxin, the main toxic component of *Crotalus durissus terrificus* venom, is a protein composed of two tightly associated nonidentical subunits: a basic and weakly toxic component (B) carrying the phospholipase activity (PLA2), and an acidic, non-toxic component (A) (crotapectin) devoid of enzymatic activity (**1,8,15,16**). Component B is a single polypeptide chain of 123 amino acids presenting a great similarity with the other PLA2, while component A is composed of three polypeptides linked by several disulfide bridges (**5**). The PLA2 present in *Bothrops jararacussu* venoms has been recently isolated and characterized(**12**), while that present in *Micrurus* venoms has been recently cloned(**9**). Some PLA2 act presynaptically inhibiting release, synthesis, storage, or turnover of neurotransmitters, while others damage skeletal muscles or induce indirect hemolysis through the lysolecithin formation from extra erythrocyte lecithin sources(**6**). Some of these phospholipases are efficient immunogens capable of inducing potent neutralizing antibodies with which they combine and react, for instance, the PLA2 subunit of the crotoxin(**3,11**). Thus, the PLA2 is one venom component endowed with quite well defined biochemical, biological and immunogenic properties. The present paper reports experiments showing that: a) the PLA2-dependent hemolysis *in vitro* induced by the venoms of Crotalidae and Elapidae snakes, can be used to quantify hemolytically the PLA2 present in these venoms; b) the curves obtained by plotting the degree of hemolysis (ordinate) versus the doses of venom (abscissa) are concave to the abscissa axis. We suggest that the hemolysis induced by PLA2 could be similar to that induced by the C system, therefore, obeying similar empirical equation; and c) antibodies present in the corresponding horse antivenoms specifically inhibit the PLA2-induced hemolysis.

MATERIAL AND METHODS

VENOMS: Venoms of *Crotalus durissus terrificus* (LD50=1,60 µg), *Bothrops jararaca* (LD50=38.38µg), *Naja naja*, *Naja nigricollis*, *Naja melanoleuca*, *Micrurus ibiboboca*, *Micrurus frontalis* (LD50=8.82 µg) and of *Micrurus spixii* were dissolved in 0.15M NaCl at the concentration of 2.0mg/ml and stored in aliquots at -20°C. Starting solutions at 12g/ml also in 0.15M NaCl were prepared before use.

ANTISERA: We used five anti-*Crotalus durissus terrificus* (L.-9210236, ED50=2.35; L.-9208187, ED50=2.22; L.-9209200, ED50=2.40; L.-9211267, ED50=1.99; L.-9211268, ED50=2.54), eight anti-*Bothrops* (L.-9212282, ED50=7.37; L.-9204072, ED50=6.71; L.-9209210, ED50=8.46; L.-9207171, ED50=7.12; L.-9212287, ED50=5.93; L.-9212291, ED50=6.56; L.-9210238, ED50=7.47; L.-9211246, ED50=7.10), two anti-*Micrurus* (L.-63, ED50=24,12, L.-64, ED50=44.08), and seven anti-tetanic sera, (L.-9111259\T.314\, Titer=1200UI/ml; L.-9211261\T.323\, Titer=>1400UI/ml; L.-9103063,\T.308\, Titer=110UI/ml; L.-911256\T.313\, Titer=1400UI/ml; L.-9303038, \T.325\Titer=140UI/ml;L. 9205104\T.318\ Titer= 1400Ui/ml; L.-9203057\ T.317\Titer=> 1400UI/ml). These antisera were prepared using hyperimmunized horses with the respective venoms, as described by Dias da Silva(**2**). All antisera were diluted 1:40 in 0.15M NaCl before use. The ED50 of the antivenoms were determined by the method of Finney(**4**). A lecithin (Merck Sharp Dome, USA) solution at 42µg/ml of saline containing 5µM CaCl₂ was prepared.

SHEEP RED BLOOD CELLS (SRBC): The SRBC were washed five times with Triethanolamine-saline buffer pH 7.4 (TBS) and standardized to 1.5×10^8 cell/ml.

DETERMINATION OF INDIRECT HEMOLYTIC ACTIVITY: PLA2-dependent hemolysis was assayed according to Tambourgi *et al.*(**17**). Briefly, increasing amounts of venoms starting from 120ng and increasing to 3000ng in a volume of 100 µl were added to a series of test tubes. To 100 µl of venom solution, 100 µl of lecithin solution in saline plus 0,01 M CaCl₂, and 200 µl SRBC at 1.5×10^8 in TBS were added to each tube. Three control tubes each containing 200µl of TBS (blank), TBS plus lecithin (lecithin control) or 200µl of distilled water (100% lysis) were always included. After incubation for 1 h at 37°C, the reaction was stopped by adding 2.0 ml of cold saline to all tubes, except the 100% lysis tube to which 2.0 ml of water was added. The tubes were centrifuged for 10 min, the hemoglobin released in the supernatants was determined spectrophotometrically at 412 nm and the percentage of hemolysis was calculated. As the hemolytic curve obtained by plotting the percentage of hemolysis versus the doses of venom was concave to the abscissa axis, and following the von Krogh equation derived from the hemolysis induced by the C system, this equation was used to titrate the active PLA2. The presumptive of hemolytically active sites (Z) of phosphatidyl-choline molecules released from the lecithin by PLA2 can be represented by $Z = -\ln(1-Y)$, the negative natural logarithm of the number of non-lysed erythrocytes, since Y is equal to the degree of lysis. For 62.3% hemolysis, Z=1, which corresponds to one hemolytically active site per erythrocyte (**14**). For the neutralization

experiments with antivenoms, each venom solution previously titrated for its PLA2 content, was adjusted to contain 50 Z per milliliter.

NEUTRALIZATION OF THE PLA2-DEPENDENT HEMOLYSIS ACTIVITIES PRESENT IN SNAKE VENOMS: One hundred microliter samples of the venom solutions containing the amount of venom able to produce hemolysis correspondent to 1 to 5 Z were mixed with equal volumes of different dilutions of the antivenoms. The mixtures were allowed to stand at room temperature for 1h. Then, the samples were centrifuged for 30 min at 3,000rpm to remove the immunoprecipitates. The active PLA2 that remained in each experimental sample was assayed for its capacity to promote indirect SRBC hemolysis, and the residual number of Z was calculated as described above. Control samples containing venom (1 to 5Z) and the serum sample (lowest dilution) alone were always included. The necessary antibody dose to inhibit the hemolysis in each assay by the amount of venom used was chosen arbitrarily as the neutralization unit, which was referred one ED-100%.

DETERMINATION OF EFFECTIVE DOSE - 100% (ED-100): Using predetermined LD50 (*B. jararaca*, 38.38µg; *C. durissus terrificus*, 1.60µg; *M. frontalis*, 8.8µg), a range of doses of each venom was analyzed. For neutralization studies, venom and antivenoms were mixed together in saline and incubated for 1 h at room temperature before injection into groups of 4 or 5 mice. Control mice received an equivalent venom dose and control antivenom in saline, also incubated for 1 hour at room temperature. The mice were injected intraperitoneally with 100 to 1000 µl of solution. The animals were observed for 24h following the injections, and the results analyzed by the probit test.

RESULTS

PLA2-DEPENDENT LYSIS ACTIVITY PRESENT IN SNAKE VENOMS: In [Figure 1](#) we represent one curve obtained after preliminary trials to determine the adequate dose range of each venom for optimal hemolysis under the standard assay conditions. The curves representing each venom are concave to the abscissa axis as in C-mediated lysis. [Table 1](#) shows the number of Z per mg of the venom used. It is clear from these results that the analyzed venoms can be divided into two groups in regard to the PLA2-dependent hemolysis activity: the venoms of the genera *Bothrops*, *Crotalus* and *Micrurus* were the less active containing around 1×10^3 to 10^4 Z/mg of venom, while the venoms of *Naja* contained over a million Z/mg of venom.

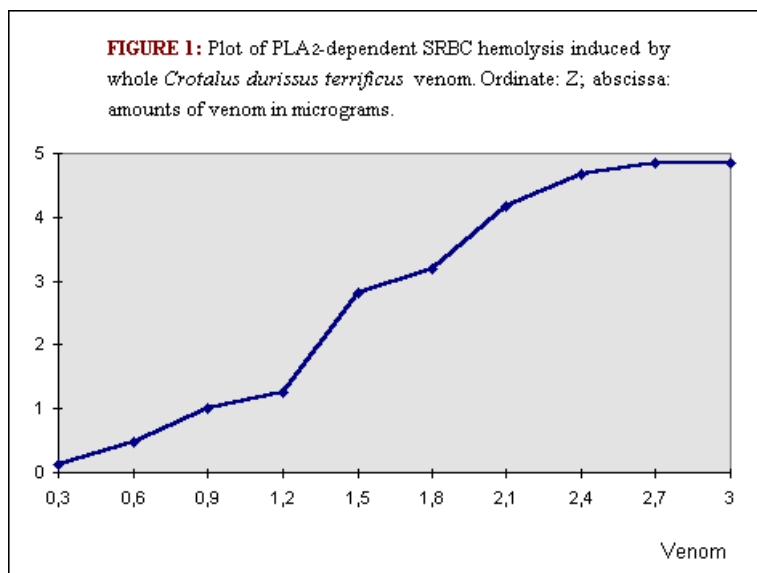


TABLE 1. Determination of the number of effective molecules (Z) of lysophosphatidyl-choline generated from lecithin by some snake venoms.

VENOMS	NUMBER OF Z/mg OF VENOM
<i>B. jararaca</i>	1.0×10^3
<i>C. d. terrificus</i>	1.0×10^3
<i>M. ibiboboca</i>	2.0×10^4
<i>M. spixii</i>	21.3×10^4
<i>N. naja</i>	3.546×10^6
<i>N. nigricolis</i>	14.981×10^6
<i>N. melanoleuca</i>	39.9×10^9

NEUTRALIZATION OF THE PLA₂-DEPENDENT HEMOLYSIS BY HYPERIMMUNE HORSE

ANTIVENOMS: The ability of the horse hyperimmune antivenom to block the PLA₂-dependent hemolysis was measured by preincubating constant amounts of the venoms at room temperature with a volume of the serum serially diluted and by measuring the remaining active PLA₂ activity as described above. Typical results obtained for the PLA₂-dependent hemolysis neutralization by horse hyperimmune antivenoms are shown in [Figure 2](#). Antivenoms against *Bothrops*, *Crotalus* and *Micrurus* snake venoms specifically inhibited the PLA₂-induced hemolysis by the corresponding venoms ([Figure 2](#), [Figure 3](#) and [Figure 4](#)). Antivenoms against *Micrurus* snake venoms were also able to inhibit *Naja naja* venom ([Figure 3](#)). Unspecific inhibitions were not observed either by the unspecific or by irrelevant antisera ([Figure 5](#)). [Table 2](#) and [Table 3](#) show the calculated numbers of ED-100 units per each antivenom analyzed.

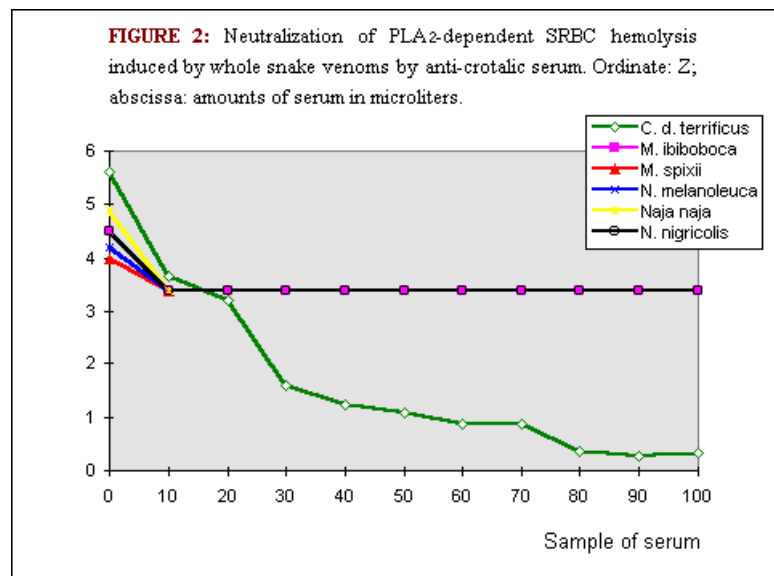


FIGURE 3: Neutralization of PLA₂-dependent SRBC hemolysis induced by whole snake venoms by anti-elapidic serum. Ordinate: Z; abscissa: amounts of serum in microliters.

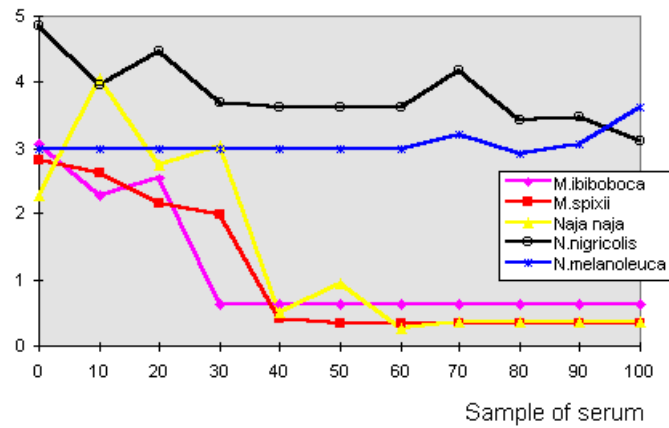


FIGURE 4: Neutralization of PLA₂-dependent SRBC hemolysis by whole snake venoms by anti-bothropic serum. Ordinate: Z; abscissa: amounts of serum in microliters.

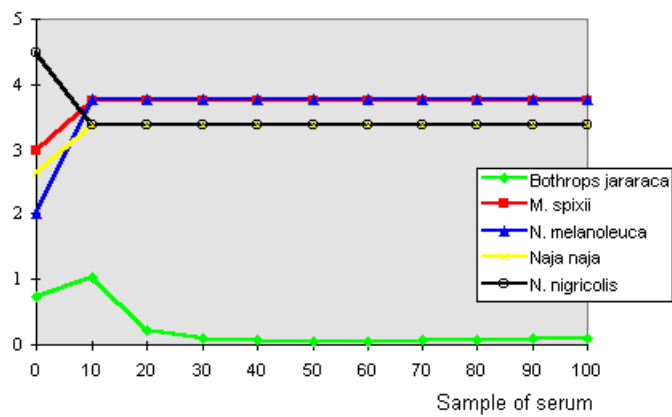


FIGURE 5: Negative control of neutralization of PLA₂-dependent SRBC hemolysis induced by snake venoms. Ordinate: Z; abscissa: amounts of anti-tetanic serum in microliters.

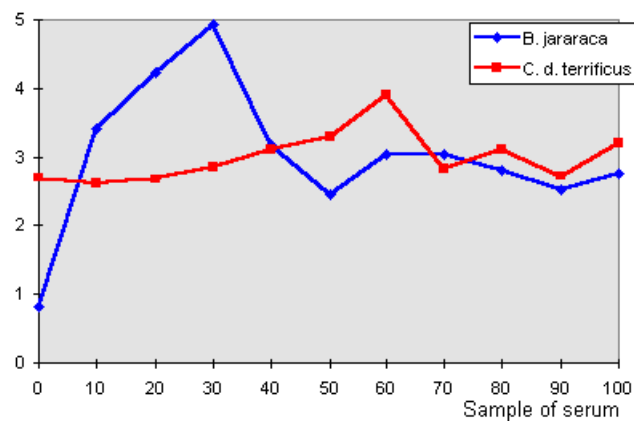


TABLE 2. Comparison between the *in vivo* neutralization of lethality and *in vitro* inhibition of hemolysis producing activities of snake venoms by anti-bothropic and anti-crotalic sera. Heterologous venoms: **a:** *N. melanoleuca*; **b:** *N. nigricolis*; **c:** *N. naja*; **d:** *M. spixii*; **e:** *M. ibiboboca*.

Antivenom	ED ₅₀ (in vivo)	ED ₁₀₀ (in vitro)	ED ₁₀₀ (in vitro)				
Anti-bothropic			Homologous venoms		Heterologous venoms		
					a	b	c d e
9212291	6.56	769.53	0	0	0	0	0
9204072	6.71	747.59	0	0	0	0	0
9211246	7.10	740.85	0	0	0	0	0
9210238	7.47	716.18	0	0	0	0	0
9212287	6.93	746.63	0	0	0	0	0
9212282	7.37	731.31	0	0	0	0	0
9207171	7.12	762.98	0	0	0	0	0
9209210	8.46	747.12	0	0	0	0	0
Anti-crotalic					a	b	c d e
9211267	1.99	622.91	0	0	0	0	0
9211268	2.54	663.09	0	0	0	0	0
9210236	2.35	610.26	0	0	0	0	0
9208187	2.22	473.84	0	0	0	0	0

TABLE 3. *In vivo* neutralization of lethality and *in vitro* inhibition of hemolysis producing activities of elapidic venoms (**b:** *N. nigricolis*; **c:** *N. naja*; **d:** *M. spixii*; **e:** *M. ibiboboca*) by anti-elapidic sera. The anti-tetanic sera were assayed with *B. jararaca*(**f**) and *C. durissus terrificus*(**g**) venoms as a negative control.

Antivenom	ED ₅₀ (in vivo)	ED ₁₀₀ (in vitro)	ED ₁₀₀ (in vitro)
Anti-elapidic		Homologous venom	
			Heterologous venom
63	24.12	0.00 (b)	
63		607.86 (c)	
63		742.88 (d)	
63		822.22 (e)	
64	44.08	0.00 (b)	
64		652.21 (c)	
64		489.68 (d)	
64		822.22 (e)	
Anti-tetanic		f, g	
9111259		0	
9211261		0	
9103063		0	
911256		0	
9303038		0	
9203057		0	
9205104		0	

DISCUSSION

The PLA2-mediated hemolysis of SRBC can mimic *in vitro* the intravascular hemolysis observed in envenomation by animal venoms. In this paper, we showed that the lysis of SRBC induced by snake venoms in the presence of an excess of lecithin and Ca⁺⁺ ions followed a curve similar in shape to that induced by the C system, thereby, following the von Krogh equation. Thus, this equation could be used to calculate the presumptive number of lysophosphatidyl-choline active molecules generated from the extracellular lecithin source and, indirectly, the effective number of lysophosphatidyl-choline molecules present in the venoms. Upon using this method, the number of lysolecithin molecules generated by the PLA2 present in seven snake venoms was shown to vary enormously, *B. jararaca* (1.0 x 10³ molecules) and *N. melanoleuca* (39.9 x 10⁹) standing on the lowest and highest extremes, respectively.

Upon knowing the presumptive number of lysophosphatidyl-choline active molecules generated by standard preparations of some snake venoms, a simple protocol to titrate antibodies against PLA2 present in these venoms was delineated. After preincubation of several dilutions of the antivenoms with a fixed amount of the corresponding venom, previously determined to generate 1 to 5 effective molecules of lysophosphatidyl-choline, the residual unblocked PLA2 molecules were determined by SRBC assay. The antibody titer necessary to neutralize the PLA2 responsible for the release of lysophosphatidyl-choline molecules was used to determine the neutralization unit and referred empirically as

effective dose 100% units (ED-100).

The antibodies present in the horse antivenoms were capable of specifically neutralizing the PLA2 present in the corresponding venoms in a dose-effect-related fashion. No cross-reactivity was detected among antivenoms and non-corresponding venoms. A horse antiserum against tetanus toxin digested by pepsin and processed as the antivenoms was also unable to interfere with the venom PLA2 activity. The elapidic antivenom, however, besides being effective in the neutralization of the venoms of *M. ibiboboca* and *M. spixi*, cross-reacted with the venom of *N. naja*, but not with the venom of *N. nigricollis*.

We hypothesize that antibodies against PLA2 present in the antivenoms can inhibit the enzyme by combining with epitopes surrounding the active site, thereby, preventing the enzyme appropriate interaction with the substrate. In this study, antibodies against the enzyme active site are not apparently involved in the antibody-PLA2 interaction, since lack of cross-reactivity was the rule. It has already been described that PLA2 of different snake venoms are recognized by heterologous anti-sera by ELISA and Western Blotting assays (13). This fact suggests that besides the molecular homology among these phospholipases, there is a heterogeneity among the enzymatic sites of PLA2 molecules or adjacent epitopes. Thus, antibodies produced against one species of venom can bind to heterologous venoms, but the binding does not affect the heterogenic region next to the enzymatic site, therefore, does not affect the enzymatic activity. In contrast, inhibition of the PLA2 present in *N. naja* venom by antivenom against *Micrurus* venoms can not be explained by zoological proximity among snakes of the two genera, since the PLA2 present in the other species tested such as *N. melanoleuca* were not inhibited. The higher PLA2 activity of *N. melanoleuca* venom can not account for the indirect hemolysis assay of the venoms because the latter were equalized to give 1 to 5 Z in the mixture.

The number of ED100 units per each antivenom reflects the anti-PLA2 antibody contents in the antivenoms. The high specificity and correlation degree with the neutralization of lethality indicate the use of the *in vitro* inhibition of the PLA2-mediated hemolysis as a substitute for the *in vivo* neutralization method. The *in vitro* methods are usually more sensitive, accurate and feasible besides being cheaper than the methods using animals. Furthermore, the use of *in vitro* methods in place of experimental animals meets the recommendations of the ethical principles on the use of animals in basic and in applied research. Therefore, the inhibition of PLA2-mediated hemolysis can be used to titrate antivenoms during the hyperimmunization of animals and the preparation of antisera.

REFERENCES

- 01 BREITHAUPT H., RUBSAMEN K., HABERMANN E. Biochemistry and pharmacology of the crotoxin complex. Biochemical analysis of crotapotin and basic *crotalus* phospholipase A. **Eur. J. Biochem.**, **1974**, **49**, 333-45. [[Links](#)]
- 02 DIAS DA SILVA W., GUIDOLIN R., RAW I., HIGASHI HG., CARICATI CP., MORAIS JF., LIMA MLRS., YAMAGUSHI IK., STEPHANO MA., MARCELINO JR., PINTO JR., SANTOS MJ. Cross-reactivity of horse monovalent antivenoms to venoms of ten *Bothrops* species. **Mem. Inst. Butantan**, **1989**, **51**, 153-68. [[Links](#)]
- 03 DOS SANTOS MC., DINIZ CR., WHITAKER PACHECO MA., DIAS DA SILVA W. Phospholipase A2 injection in mice induces immunity against the lethal effects of *Crotalus durissus terrificus* venom. **Toxicon**, **1988**, **26**, 207-13. [[Links](#)]
- 04 FINNEY DJ. **Probit analysis**. 3 ed. Cambridge: Cambridge University Press, **1971**. [[Links](#)]
- 05 FRAENKEL-CONRAT HL., JENG TH., HSIANG M. Biological activities of crotoxin and amino acid sequence of crotoxin B. In: EAKER D., WADSTRÖM T. Eds. **Natural toxins**. Oxford: Pergamon, **1980**: 561-8. [[Links](#)]
- 06 GRASSMANN W., HANNING K. Elektrophoretische untersuchungen an schlinger und insektentoxin **Hoppe Seyler's Z. Phys. Chem.**, **1955**, **296**, 30-44. [[Links](#)]
- 07 HARRIS JB. Phospholipases in snake venoms and their effects on nerve and muscle. In: HARVEY, A.L. Ed. **Snake toxins**. Oxford: Pergamon Press, **1991**: 91-129. [[Links](#)]
- 08 HENDON RA., FRAENKEL-CONRAT H. Biological roles of the two components of crotoxin. **Proc. Natl. Acad. Sci.**, **1971**, **68**, 1560-3. [[Links](#)]
- 09 LEE HO P., SOARES MB., YAMANE T., RAW I. Reverse biology applied to *Micrurus corallinus*, a south american coral snake. **J. Toxicol. Toxin Rev.**, **1995**, **14**, 327-37. [[Links](#)]
- 10 MEBS D. **List of biologically active components from snake venoms**. Frankfurt: University of Frankfurt, **1985**. [[Links](#)]
- 11 MIDDLEBROOK JL. Immunological cross-reactions and cross-neutralizations of phospholipase A2 neurotoxins. **J. Toxicol. Toxin Rev.**, **1990**, **9**, 7. [[Links](#)]
- 12 MOURA DA SILVA AM., PAINE MJ., DINIZ MRU., THEAKSTON RDG., CRAMPTON JM. The molecular cloning of a phospholipase A2 from *Bothrops jararacussu* snake venom: Evolution of venom group II phospholipase A2's may imply gene duplications. **J. Mol. Evol.**, **1995**, **41**, 174-9. [[Links](#)]

- 13 NASCIMENTO N., SPENCER PJ., DE PAULA R., CARDI BA., ROGERO JR. Cross-reactivity and phospholipase A2, neutralization ability of anti-irradiated *Bothrops jararaca* venom antibodies. In: **REUNIÃO DA SOCIEDADE BRASILEIRA DE TOXINOLOGIA**, **3**, Belo Horizonte, 1994. Belo Horizonte: Segrac, **1994**: 69. [[Links](#)]
- 14 RAPP HJ., BORSOS T. **Molecular basis of complement action**. New York: Appleton-Century-Crofts, **1970**. [[Links](#)]
- 15 SLOTTA KH., FRAENKEL-CONRAT HL. Schlangengifte III. Mitteilung: Reinigung und Krystallisation des Klapperschlangengiftes. **Ber. Dt. Chem.**, **1938**, **71**, 1076-81. [[Links](#)]
- 16 SLOTTA KH., FRAENKEL-CONRAT HL. Crotoxin. **Nature**, **1939**, **144**, 290-1. [[Links](#)]
- 17 TAMBOURGI DV., SANTOS MC., FURTADO MFD., FREITAS MCW., DIAS DA SILVA W., KIPNIS TL. Pro-inflammatory activities in elapid snake venoms. **Br. J. Pharmacol.**, **1994**, **112**, 723-7. [[Links](#)]
- 18 TAMIYA N. A comparison of amino acid sequences of neurotoxins and phospholipases of some Australian elapid snakes with those of other proteroglyphous snakes. In: GRIGG G., SHINE R., EHMAN H. Eds. **Biology of australian frogs and reptiles**. Chipping Norton: Surrey Beatty, **1985**: 209-19. [[Links](#)]

✉ **CORRESPONDENCE TO:**

A.C.M. ROCHA CAMPOS - Laboratorio de Imunoquímica, Instituto Butantan, Avenida Vital Brasil, 1500, CEP: 05503-900, São Paulo, SP, Brasil.



All the contents of this journal, except where otherwise noted, is licensed under a [Creative Commons Attribution License](#)

Caixa Postal 577
18618-000 Botucatu SP Brazil
Tel. / Fax: +55 14 3814-5555 | 3814-5446 | 3811-7241



jvat@cevap.org.br