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CROSS-REACTIVITY OF HORSE MONOVALENT ANTIVENOMS TO VENOMS OF TEN *BOTHROPS* SPECIES

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ABSTRACT: Horses were immunized with *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms. Antibodies recognizing the venom antigenic components were either immunochemically detected by the enzyme-linked immunosorbent, double immunodiffusion and quantitative precipitation methods or biologically by the assays measuring the venoms indirect hemolytic and lethal toxic activities. Specific and cross-reacting antibodies against the *Bothrops* venoms were found in all ten monovalent antivenoms. Modifications in the serum electrophoretic patterns characterized by a reduction of the albumin peak and by a correspondent increase of the γ-globulins with a patent or no modification of the α or β globulins were found in these sera.
KEY WORDS: Antivenoms, *Bothrops* venoms, monovalent *Bothrops* antivenoms, cross-reactivity reactions.

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The "Comissão de Soros, Ministério da Saúde, Brasil", has recommended studies to determine the satisfactory *Bothrops* venoms mixture to be used as antigen to produce the corresponding antivenom. Therefore, a study group at the "Instituto Butantan, São Paulo, Brasil" was convened and charged with these studies and this paper contains the result obtained.
Dedicated to Dr. Saul Schenberg's 70th birthday.
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INTRODUCTION

Horse antivenoms against *Bothrops* venoms have been produced using as antigen a mixture composed of venoms from seven of this snake species. At least ten well characterized snake species, belonging to the genus *Bothrops*, have been identified in Brazil and it is well known that the venoms from these snakes as complex mixtures, contain various enzymes in addition to toxic elements. There are evidences that some of these components are largely distributed among the majority whereas others are restricted to some species. This raises questions concerning the ability of antisera routinely produced for therapeutic purposes to neutralize the relevant components responsible for the pathophysiologic action of the venom as a whole. In order to clarify these practical posed questions two experimental protocols were delineated: a) production of monovalent antisera against each of the ten *Bothrops* venoms and analysis for the presence of antibodies against the components found both in the venom used for immunization and in the venoms from the other *Bothrops* species; and, b) to test in parallel assays, the polyvalent *Bothrops* antivenoms routinely prepared in the Instituto Butantan for therapeutic purposes for their capacity to combine with the components present in each individual *Bothrops* venom.

ABBREVIATIONS

LD_{50}.....lethal dose 50%
BVM.....*Bothrops* venoms mixture
FCA.....Freund's complete adjuvant
FIA.....Freund incomplete adjuvant
PBS.....phosphate saline buffer
BSA.....bovine serum albumin
NPGB.....p-nitrophenyl-p' guanidino benzoate
PMSF.....phenyl-methyl sulphonyl-phluoride
EDTA.....ethylene-diamine tetraacetic acid

MATERIAL AND METHODS

Animals. Adult horses with 400-450kg of body weight maintained at the São Joaquim farm, Instituto Butantan were to produce both, the monovalent and the *Bothrops* polyvalent antivenoms. Outbred albino mice weighing 18-22 g from Biotério Central, Instituto Butantan were used for the neutralization test.

Venoms. Bothrops venoms from the *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* were pooled venoms from several adult snakes maintained in the "Seção de Venenos" of the Instituto Butantan. Venoms were extracted, desiccated and stored at 4°C before use. Stock solutions of venoms from each *Bothrops* species were, as otherwise indicated, prepared by diluting 100mg of dried venom in 10 ml of 0.15M phosphate buffer at pH 7.2. The *Bothrops* venom mixture used to produce the polyvalent antivenoms was prepared by adding one volume 10mg/ml solution of *B. jararaca* venom to an equal volume of a 10 mg/ml solution containing equal parts

of venoms from *B. alternatus, B. neuwiedi, B. jararacussu, B. cotiara, B. moojeni* and *B. pradoi*. The immunizing mixtures were prepared just before use, by diluting stock solutions to a final concentration of 5 mg/ml in: a) Freund's complete adjuvant (FCA); b) Freund's incomplete adjuvant (FIA); c) alginate; d) 0.15 M NaCl. The LD_{50} values for each *Bothrops* species were previously determined by the ''Seção de Controle'' of the Instituto Butantan by the probit analysis Finney[4]. The values for each *Bothrops* species are indicated in parenthesis: *B. alternatus* (66.0 μg); *B. atrox* (145.0 μg); *B. cotiara* (46.79 μg); *B. erythromelas* (70.0 μg); *B. insularis* (50.0 μg); *B. jararaca* (33,0 μg); *B. jararacussu* (76.0 μg); *B. moojeni* (115.2 μg); *B. neuwiedi* (35.5 μg) and *B. pradoi* (61.56 μg).

Antivenom production. Monovalent antivenoms were obtained from horses subcutaneously injected with one *Bothrops* venom each: *B. alternatus* (horse nº 120), *B. atrox* (horse nº 144), *B. cotiara* (horse nº 134), *B. erythromelas* (horse nº 25 and 37), *B. insularis* (horse nº 54 and 154), *B. jararaca* (horse nº 141), *B. jararacussu* (horse nº 162), *B. moojeni* (horse nº 290), *B. neuwiedi* (horse nº 980) and *B. pradoi* (horse nº 131). The animals were previously sensitized by a primary stimulus with 5.0 mg of the venoms in FCA. Six months later horses were reinjected subcutaneously with 3 mg of the corresponding venom in 10 ml of alginate. After three months a third reinjection was performed with 5 mg venom in FIA followed by three injections of 2,5mg venom in 0.15 M NaCl, at 7 days intervals. Seven liters of blood were collected one week later and the plasma separated and stored at 4°C. Polyvalent plasma with antibodies, against the mixture of venoms from *B. alternatus, B. cotiara, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* were obtained by immunizing horses with BVM essentially by the same immunization schedule.

Purification of horse IgG and its F(ab')$_2$ fragments. All purifications were carried out using the same pool horse plasma.

IgG. IgG was prepared as described by Steinbuch and Audran[14]. Horse hyperimmune plasma at a concentration of 70 mg/ml, was heated at 56°C for 15 min and subsequently centrifuged at 900 x g for 10 min. The pH of the supernatants was adjusted to 5.0 with 0.1 N acetic acid and caprylic acid (Merck, Darmstadt) was then added, to a final concentration of 8.7%, under vigorous stirring for 30 min at room temperature. After centrifugation (15 min, 1000 x g) the supernatant was filtered through a 0,45 μm Millipore membrane (Millipore Corporation, Bedford, MA 01730), and dialysed overnight against 0.85% NaCl at 4°C. The IgG preparation was stored at -20°C. *F(ab')$_2$. The method of Pape[12] Slightly modified was used to obtain F(ab')$_2$ fragments from both monovalent or polyvalent horse hyperimmune plasma according to the procedures established by the Instituto Butantan (unpublished data): Briefly, Bothrops* antivenom plasma diluted to 40 mg/ml with distilled water was heated at 30°C. The pH was adjusted to 3.1 with 2.5 M HCl, and pepsin (INLAB, São Paulo, Brazil) was then added under slow stirring (5.0 gr per liter of plasma). The mixture was incubated for 40 min at 30°C, followed by the addition of tetrasodium pyrophosphate (Labsynth, São Paulo, Brazil) (0.1% final concentration) and toluene (INLAB, São Paulo, Brazil) (0.1% final concentration). Temperature was then elevated to 55°C and the pH was adjusted to 5.2 with 20% NaOH. After adding ammonium sulphate (Quimis, São Paulo, Brazil) to a final concentration of

17.5%, the solution was stirred during 90 min at 55°C and then centrifuged for 10 min at 900 x g. The supernatant was removed and the pH adjusted to 6.9-7.1 with NaOH. Ammonium sulphate was again added to the mixture to a final concentration of 28% which was stirred at room temperature for 60 min. After centrifugation, the precipitate was resuspended in 0.85% NaCl and the resulting solution dialysed for 48h against 0.85% NaCl at 4°C. The $F(ab')_2$ rich preparation was stored at -20°C. The protein content was measured by the method of Lowry *et al.*[9] using bovine serum albumin as a standard. The preparations obtained were termed monovalent or polyvalent *Bothrops* antivenoms.

The ELISA method Theakston et al.[15] One hundred ul of *Bothrops* venoms (1 μg/ml) were absorbed to the wells of Nuclon plates (Delta, Denmark), at 4°C, overnight, blocked with 3% BSA in phosphate buffered saline containing 0.05% Tween 20 for 3h at room temperature. The plates were washed with 0.05% of BSA in phosphate buffered saline containing 0.05% of Tween 20. One hundred μl of several dilutions of each serum sample (1/100-1/12,000) were added to the wells and allowed to incubate for 45 min at room temperature. The plates were washed again as mentioned and 100 μl of peroxidase conjugated anti-mouse immunoglobulin diluted 1/1,000 (Cappel, Cochanville, Pa) were added to each well. Plates were incubated for 45 min at room temperature. After washing the wells as previously described, 100 μl of ortho-phenylenediamine (Sigma Co., U.S.A) (1 mg/ml) and 4 μl of H_2O_2 were added to wells and plates were allowed to stand at room temperature for 15 — 20 min before spectrophotometric determination of colour change.

Double Immunodiffusion Ouchterlony[11]. Agarose was dissolved in 0.056 M sodium veronal buffer, pH 8.6, at 0.8% concentration and overlaid on glass plates. The plates contained one center well and eight outer wells (3mm in diameter) at 4mm distance. Twenty microlliters of *Bothrops* crude venoms were added to central wells and tested against eight dilutions of the corresponding horse antisera pipetted in the outer wells. The plates were allowed to develop for 24h at room temperature and stained with Coomassie blue. Antivenoms were arbitrarily classified as weak reactors (WR) and strong reactors (SR) according to the presence of precipitin bands at dilutions up to 1:64 and 1:128, respectively.

Cellulose acetate electrophoresis. Electrophoresis of horse sera obtained immediately before and after the immunization with each *Bothrops* venom was performed in 0.04M sodium veronal buffer pH 8.6 during 25 minutes at 200 Volts. Strips were stained for 8 minutes in 0.5% amidoblack 10B dissolved in a mixture of 47.5% methanol and 5% acetic acid, and destained in the same mixture. They were dried in absolute methanol, and treated with a solution containing 85% methanol, 14% acetic acid, 1% glycerol. Absorbance was determined in a densitometer. In control samples the typical plasma protein bands were clearly seen: albumin and the globulins $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$ and $\gamma 2$.

Ability of Bothrops antivenoms to neutralize the indirect hemolytic activity of Bothrops venoms. 0.3 ml of packed sheep erythrocytes were washed four times with 0.15 M NaCl and pelleted. To these pellets were added 0.3 ml of egg yolk diluted 1:4 in 0.1M NaCl containing 0.25 ml of 0.01 M $CaCl_2$, resuspended and mixed with 25 ml of 0.8% agarose dissolved in phosphate-

buffered saline solution, pH 8.1. 18 ml of this solution were overleyed on 40 x 180 mm glass plates and allowed to solidify. 4mm diameter wells were punched in the middle of the plates 40mm apart and filled either with 15 μl of a standard solution of venoms previously standardized to give hemolytic haloes of 20 mm or a mixture containing these amounts of venom plus the antivenoms at different dilutions. The plates were incubated at 37°C for 24h, the hemolytic haloes measured and the percentage of inhibition was calculated.

Quantitative precipitin reaction Heidelberger[6]. The IgG immunoglobulins used were obtained from the different *Bothrops* monovalent antivenoms purified with aid of caprylic acid and the corresponding *Bothrops* venoms were used as the source of antibody and antigen, respectively. The IgG preparations containing 1 mg protein/ml were dissolved in phosphate-saline buffer (PBS) pH 7,4 and diluted 1:4 before use. In order to minimize the possible IgG cleavage by the proteolytic enzymes present in the *Bothrops* venoms this buffer contained 5 μM p-nitrophenyl — p'-guanidino benzoate (NPGB), 5 μM phenyl-methyl sulphonyl-phluoride (PMSF) and 10mM ethylenediamine tetracetic acid (EDTA). To a series of tubes (13 x 70 mm) were added 0.5 ml of IgG solution and 0.5 ml of the corresponding *Bothrops* venom solution containing, 20, 40, 80, 160, 320, 640, 1,000 and 1,280 μg, mixed and allowed to stand overnight at 4°C. Controls containing IgG or the highest amount of venom alone were rumning in parallel. The precipitates were washed three times with cold PBS, ressuspended in 0.1 N NaOH and the total protein contents determined by the Lowry's method (1951)[9].Knowing the amounts of antigen (Ag) added in each tube, the corresponding values for antibody (Ab) and the ratios Ab:Ag were calculated. Graphics were constructed by plotting the Ag values on ordinates (Y) and the ratios Ab:Ag (X) on abscissae. A straight line was obtained following the equation $\frac{Ab}{X} = a - bx$, or $Ab = a - b(x)^2$ in whic "x" is the amount of Ag added and the "a" and "b" constants representing the intercept of the line on the "Y" axis and "slope", respectively. Using these values graphicaly obtained and the values for the known maximal Ag precipitated as substitutes for "a", "b" and "X", respectively, in the above derived equation, the maximal values for specific Ab in each monovalent IgG preparation were obtained. As the venoms are complex mixtures of several immunogenic components and the correspondent antisera shall have antibodies specific for distinct epitopes and antigens, the precipitin curves obtained in each venom-antivenom assay was logistically transformed in order to determine their parallelism and coincidence. The values for Ab on the precipitin curves were applied to the equation $y = 1n.\frac{Ab/2,000}{1-Ab/2,000}$, Bussab[2].

The linear function y=A (μg of venom) + B was obtained where A is the angle coeficient and B the linear coeficient, these values being calculated by the "SAS square minimal program Ray[13]. The parallelism and the coincidence among the retified curves were determined by the F test Choi[3], according to the criteria used by Murata[10] in their analyses of horse anti-*Crotalus durissus terrificus* venom when assayed against gamma radiated venom.

Mouse protection test. This assay was performed strictly obeying the following conditions and methodology:

a) Outbred albino mice with 18-22g body weight were used.

b) The venom solutions containing 5 LD_{50} were incubated with an equal volume of different antivenom dilutions (dilution factor of 1.2) at 37°C for 30 min. Control mixtures containing 0.15M NaCl instead of antivenom were running in parallel. Eight mice per dilution point or for the control were intraperitoneally injected with 0.5 ml of each solution, the animals being observed for 48h and the number of alive animals recorded. The probits were calculated with the aid of the percentage of survival values obtained for each serum dilution, plotted on a semi-log paper (in abscissae) against the volume of antivenom (in ordenates) and the 50% mortality determined. With the aid of the formule TV-1 in which TV = 5 LD_{50} in μg, the amount of venom in mg was calculated.

RESULTS

Antibodies detected by the ELISA assay.

According to their reactivity the *Bothrops* monovalent antivenoms were classified for descriptive purposes as weak reactors (WR), intermediate reactors (IR) and strong reactors (SR), with antibody titers below 64 x 10^3, up to 128 x 10^3 and over this latter values, respectively. From the data presented in Table 1 three observations can be drawn: a) *B. cotiara* venom produces WR antisera against all venoms; b) *B. alternatus* and *B. insularis* venoms produce SR antisera either when tested against the specific venom or in cross-testing one against each other; and c) the remaining venoms produce IR or WR antisera both in specific and in cross-testing assays.

Antibodies detected by the double diffusion method.

The *Bothrops* monovalent antivenoms were also classified as WR (titers up to 64) and SR (titers over 128) according to their maximal dilution giving visible precipitin bands. Table 2 shows: a) *B. cotiara* produced SR antiserum against all venoms; b) *B. alternatus, B. insularis, B. jararaca, B. moojeni, B. neuwiedi* and *B. pradoi* produced SR antisera at least when tested against one of the *Bothrops* venoms; and, c) *B. alternatus, B. atrox, B. erythromelas* and *B. jararacussu* venoms produced WR antisera.

Electrophoretic profiles of horse **Bothrops** *antivenoms.*

In comparison with normal horse serum the relative quantities of the serum proteins in *Bothrops* antivenoms as determined by electrophoresis on cellulose acetate strips were the following:

a) Albumin was unequivocally reduced in *B. atrox, B. moojeni* and in BVM antivenoms but only slightly reduced in *B. cotiara, B. insularis, B. jararaca, B. jararacussu* and *B. pradoi* or unchanged in *B. alternatus* and *B. neuwiedi* antivenoms; b) excepting for *B. cotiara, B. insularis* and *B. moojeni*, α -globulins were decreased in the (ileg.) *Bothrops* antivenoms; c) the β --globulins, excepting for *B. atrox, B. cotiara, B. jararaca, B. pradoi* and BVM antivenoms, β-globulins were reduced; and, d) the γ-globulins, excepting for the *B. erythromelas* antivenom, were increased in the other *Bothrops* antivenoms. Table 3 and Fig. 1 show these results.

TABLE 1

Antibody production in horses immunized with different *Bothrops* venoms, measured by the enzyme linked immuno-absorbent assay (ELISA) and classified as strong (SR), intermediate (IR) and weak reactors (WR)[a]

Bothrops antivenoms		*Bothrops* Venoms									
		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	SR	WR	WR	WR	SR	WR	IR	IR	WR	IR
B. atrox	(atr)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. cotiara	(cot)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. erythromelas	(ery)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. insularis	(ins)	SR	WR	WR	WR	SR	WR	WR	WR	WR	WR
B. jararaca	(jar)	WR	WR	WR	WR	WR	IR	IR	IR	IR	IR
B. jararacussu	(jsu)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. moojeni	(moo)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. neuwiedi	(neu)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR
B. pradoi	(pra)	WR	WR	WR	IR	WR	IR	IR	IR	IR	IR

[a]WR, titers of 64 x 10^3; IR, titers of 64 x 10^3 to 128 x 10^3; SR, titers over 128 x 10^3

TABLE 2

Cross-reactivity of *Bothrops* antivenoms with different *Bothrops* venoms as determined by double immuno-diffusion and classified accordingly as strong reactor (SR) and weak reactors (WR)[a]

Bothrops antivenoms		Bothrops Venoms									
		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	WR	WR	SR	SR	WR	WR	WR	SR	WR	WR
B. atrox	(atr)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. cotiara	(cot)	SR	SR	SR	SR	SR	SR	SR	SR	SR	SR
B. erythromelas	(ery)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. insularis	(ins)	SR	WR	WR	WR	WR	SR	SR	WR	WR	WR
B. jararaca	(jar)	SR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. jararacussu	(jsu)	WR	WR	WR	WR	WR	WR	WR	WR	WR	WR
B. moojeni	(moo)	SR	SR	WR	WR	SR	WR	SR	WR	WR	SR
B. neuwiedi	(neu)	SR	WR	WR	WR	WR	WR	SR	WR	WR	WR
B. pradoi	(pra)	WR	SR	WR	WR	WR	WR	WR	WR	WR	WR

[a]Strong reactors (SR): titers of 128 and over; weak reactors (WR): titers up to 64.

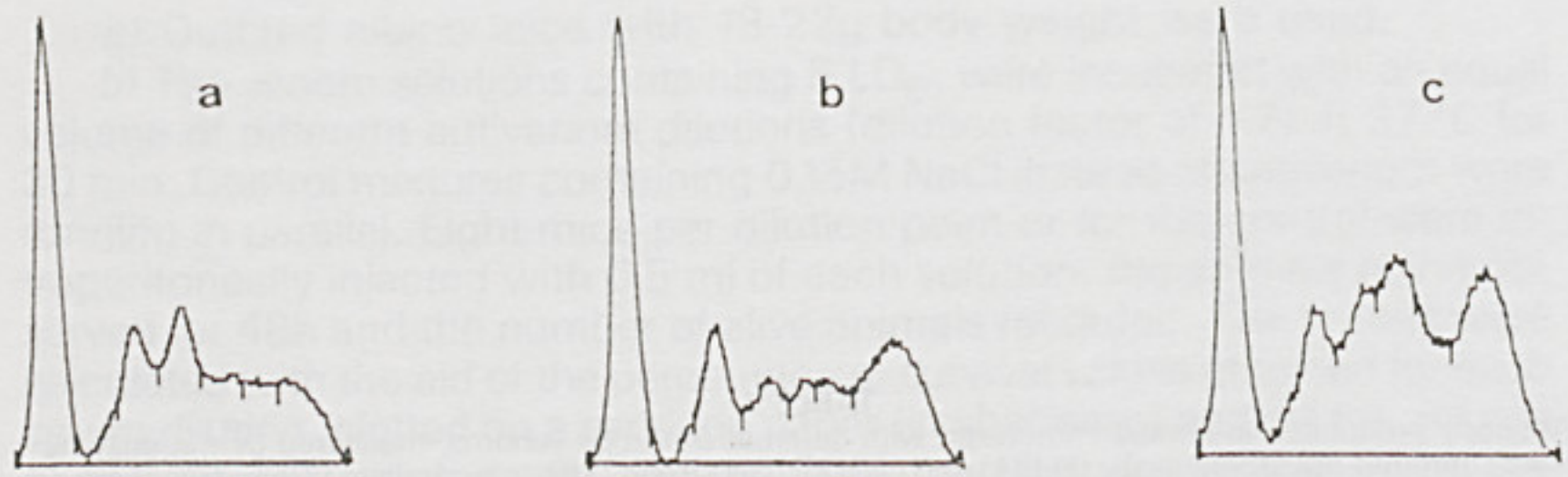

Fig. 1: Cellulose acetate electrophoresis of sera obtained from normal (panel a) or from hyperimmunized horses with venoms of *B. alternatus* (panel b), *B. atrox* (panel c), *B. cotiara* (panel d), *B. erythromelas* (panel d), *B. insularis* (panel e), *B. jararaca* (panel f), *B. jararacussu* (panel g), *B. moojeni* (panel i), *B. neuwiedi* (panel j), *B. pradoi* (panel l) and the mixture of *Bothrops* venoms composed of 50% of a solution containing *B. jararaca* venom and 50% of a solution containing equal parts of venoms from *B. alternatus, B. cotiara, B. jararaca, B. moojeni, B. neuwiedi* and *B. pradoi*.
Samples of horse sera were applied on cellulose acetate strips and electrophoresed in 0.04M sodium veronal buffer, pH 8.6, for 25 min at 200V. The numbers at right at each panel indicate, from the top to the **bottom**, the concentration (%) values for albumin and for the globulins α 1 and α 2, β 1 and β 2, γ 1 and γ 2.

TABLE 3

Electrophoretic distribution of proteins from horses hyperimmunized with *Bothrops* venoms. Comparison with sera from non immunized horses.

Bothrops antivenoms	Horse plasma proteins (%)			
		Globulines		
	albumin	α	β	γ
(1) *B. alternatus*	33.1	16.1	16.7	34.1
(2) *B. atrox*	23.9	13.9	28.6	33.6
(3) *B. cotiara*	27.6	17.9	24.4	30.1
(4) *B. erythromelas*	45.5	15.1	15.6	23.8
(5) *B. insularis*	27.9	17.9	22.1	32.1
(6) *B. jararaca*	25.5	15.4	25.7	33.4
(7) *B. jararacussu*	27.4	14.5	15.0	43.1
(8) *B. moojeni*	21.8	16.6	16.4	45.2
(9) *B. neuwiedi*	32.1	15.3	12.3	40.3
(10) *B. pradoi*	25.1	12.9	24.9	37.1
Mixture of (2) + (5) + (7) + (8) + (9) (BUM)	20.5	14.9	30.2	34.4
Normal horse serum	34.3	18.1	27.4	20.2

ANTI-INDIRECT HEMOLYTIC ACTIVITY

The *in vitro* ability of different *Bothrops* antivenoms to neutralize the indirect hemolytic activity of *Bothrops* venoms was assayed. The following results were obtained: a) *B. cotiara* venom, at least under the conditions used in these experiments, was free of indirect hemolytic activity even when high concentrations up to 60 µg were used; b) the anti-*B. alternatus* serum at 1:100 dilutions was able to block 50% of the hemolytic activity present in the venom from all nine *Bothrops* species tested; c) the antisera against the venoms from the other *Bothrops* species have very weak or no anti-hemolytic activity; d) the anti-hemolytic activity present in the *Bothrops* polyvalent antivenom was manifested against the *B. alternatus* venom but not against the venom from the other *Bothrops* species.

Antibodies detected by the quantitative precipitin assay

Fig. 2 shows that in all *Bothrops* venom-antivenom systems analyzed with the addition of increasing quantities of venoms the amount of the corresponding antigen-antibody precipitate increases until it reaches a maximum and then declines. Inspection of the shapes of these curves shows that both the maximum quantity of precipitated antibodies and the antigen-antibody ratio vary for those representing the antivenom analyses against the individual *Bothrops* venoms or against the BVM mixture of *Bothrops* venoms used to produce antivenoms for therapeutic purposes, shows that both the maximum quantity of precipitated antibodies and the ratio antigen-antibody vary. In order to ascertain if each antivenom has comparable precipitation activity against the specific venom and against the *Bothrops* venom

mixture the corresponding curves were transformed in straight lines and the ''F test'' applied on their respective intercepts and slopes. The values were calculated for the intercepts and slopes and for the parameters used to determine ''Fa'' and ''Fb'' used to verify the parallelism and coincidence among the straight lines for each individual antivenom assayed against the specific venom and against the *Bothrops* venoms Table 4. The values for ''Fa'' and ''Fb'' being below the calculated criticals $F_{1.4} = 7.71$ and $F_{1.5} = 6.61$ respectively, indicate parallelism and coincidence of the corresponding precipitin curves experimentally obtained for each venom-antivenom assayed in parallel against the specific-venom and the BVM mixture. Accordingly, all curves are parallel with these parameters while all but the *B. cotiara* venom-antivenom system are coincidents. Table 5 shows the Ag_{mx}, the Ab_{mx} precipitated, and the ratio Ab_{mx}: Ag_{mx} for all monovalent and polyvalent IgG antibodies against *Bothrops* venoms. The data suggest that: a) the precipitation curves obtained with IgG are similar to those obtained with whole horse antisera and described many years ago Heidelberger[6], against *Clostridium tetani* or *Corynebacterium diphtheriae* toxins, since insoluble precipitates were not detected in large Ab excess; b) as expected those values vary accordingly to the venom used for immunization; c) the Ab:Ag molecular ratios can not be calculated since the venoms are complex mixtures and the molecular weight for most of their components has not yet been determined; d) the IgG antibodies produced by a mixture of *Bothrops* venoms form good amounts of Ag-Ab complexes with a high Ab:Ag ratio.

Venom neutralization. Table 6 shows these results. Three observations can be drawn from these assays: a) There was a good correlation among the results obtained when the specific and the *B. jararaca* venom where used for the monovalent antivenoms produced with *B. insularis*, *B. alternatus* and *B. atrox* venoms; b) *B. pradoi*, *B. atrox* and *B. jararacussu* seen not to be good immunogens in terms of their capacity to produce neutralizing antibodies, as compared with the other venoms; c) The horse sera anti-*B. pradoi* and anti-*B. neuwiedi* venoms were more active against *B. jararaca* venom than against the corresponding venoms used for their production.

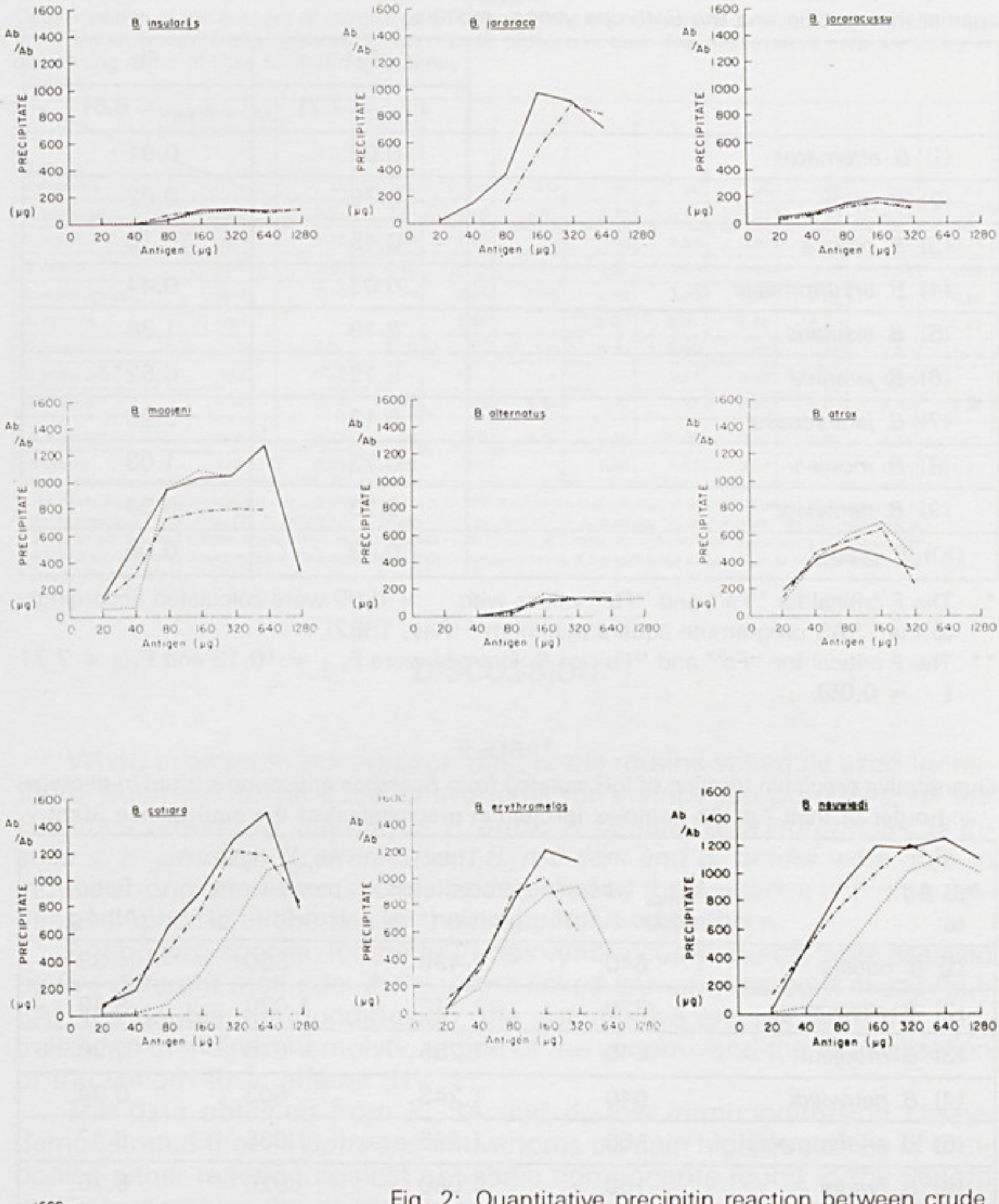

Fig. 2: Quantitative precipitin reaction between crude *Bothrops* venoms and horse antibodies to the venom componentes. 0.5ml of horse IgG dissolved in phosphate saline buffer (PBS) (1.0mg/ml) pH 7.4, was added to 0.5ml of PBS containing different amounts of the specific venom and incubated overnight at 4°C. The precipitates were washed in cold PBS, ressuspended in 0,5ml of 0.1N NaOH and the total protein determined. Control samples containing only venom or IgG were included. The ratio of antibody (Ab) to venom (Ag) found in each precipitate was plotted (axis y) against the amount of venom (axis x).
Each horse antivenom serum was assayed in parallel against the specific venom (————), against the *B. jararaca* venom (• • •), or against the mixture of *Bothrops* venoms (••••••) .

TABLE 4

Values for F critical of "Fa" and "Fb" used to verify the parallelism and the coincidence of the precipitin curves obtained for each *Bothrops* venom-antivenom system assayed both against the specific and the *Bothrops* venom mixture.

	Fa*	Fb
	$F_{1.4} = 7.71$	$F_{1.5} = 6.61$
(1) *B. alternatus*	0.07	0.91
(2) *B. atrox*	0.79	0.02
(3) *B. cotiara*	0.45	16.10
(4) *B. erythromelas*	0.03	0.44
(5) *B. insularis*	2.19	1.38
(6) *B. jararaca*	5.19**	0.52**
(7) *B. jararacussu*	0.12	0.26
(8) *B. moojeni*	0.73	1.03
(9) *B. neuwiedi*	0.23	3.34
(10) *B. pradoi*	0.03	0.06

* The F critical for "Fa" and "Fb" values with = 0.05 were calculated accordingly to the "SAS programme square minimum" (Ray, 1982)

** The F critical for "Fa" and "Fb" of *B. jararaca* were $F_{1.3} = 10.13$ and $F_{1.4} = 7.71$ (= 0.05)

TABLE 5

Quantitative precipitin titration of IgG isolated from *Bothrops* antivenoms listed in decreasing order of their Ag: Ab complex (protein in precipitates) at the equivalence 30ml.

IgG Ab to:	Ag_{max} (venom) added (g)[a]	Total precipitated protein (g)	Ab_{max} (IgG) precipitated protein (g)	Ratio Ab:Ag
(1) *B. cotiara*	640	1.490	850	1.33
(2) *B. alternatus*	320	1.340	1.020	3.19
(3) *B. moojeni*	640	1.255	615	0.96
(4) *B. neuwiedi*	640	1.243	603	0.94
(5) *B. erythromelas*	160	1.195	1.035	6.47
(6) *B. pradoi*	160	1.080	920	5.75
(7) *B. insularis*	320	1.030	710	2.22
(8) *B. jararacussu*	160	885	725	4.53
(9) *B. jararaca*	320	790	470	1.47
(10) *B. atrox*	80	505	425	5.31
(11) *Mixture of (2) + (5) + (7) + (8) + (9)* [b]	160	1.075	915	5.72

[a] Values correspond to Ag_{max} and Ab_{max} precipitated in g of total protein as determined by the Lowry's method.

[b] Solution of 50% *B. jararaca* and 50% of equal parts of the other *Bothrops* venoms designated by the numbers in parenthesis.

TABLE 6

Determination of the amount of specific and *Bothrops jararaca* venoms neutralized by 1.0ml different *Bothrops* antivenoms as estimated by the mouse protection test. The monovalent sera are listed in decreasing order of their neutralizing potency.

Neutralized venoms / antivenoms mg/ml — *Bothrops* antivenoms		alt	atr	cot	ery	ins	jar	jsu	moo	neu	pra
B. alternatus	(alt)	9,5	1,44	4,48	4,8	4,72	9,1	0,91	0,82	5,14	0,77
B. atrox	(atr)	1,15	1,09	1,87	–	1,42	0,94	0,97	–	1,54	0,74
B. cotiara	(cot)	2,20	0,83	8,1	–	3,37	0,53	0,97	0,92	2,96	0,65
B. erythromelas	(ery)	2,50	1,73	2,65	5,80	2,86	2,70	0,86	0,82	–	0,88
B. insularis	(ins)	–	–	2,65	–	8,0	8,0	0,86	0,82	–	0,88
B. jararaca	(jar)	4,0	1,20	2,55	–	6,30	3,40	0,91	0,82	2,06	1,03
B. jararacussu	(jsu)	0,84	–	1,0	–	–	0,53	0,97	–	3,28	–
B. moojeni	(moo)	0,77	1,41	2,19	1,62	1,45	2,17	0,91	4,8	3,70	2,96
B. neuwiedi	(neu)	1,78	1,20	2,65	5,6	4,86	5,05	0,91	0,82	2,81	1,02
B. pradoi	(pra)	0,85	0,83	2,66	–	1,38	3,43	0,97	1,18	4,30	1,31

* The mixture containing monospecific anti-*Bothrops* venom or 0.15M NaCl plus equal volumes of different dilutions of the specific or the *B. alternatus* (1), *B. atrox* (9), *B. cotiara* (2), *B. pradoi* (8), *B. insularis* (5), *B. jararacussu* (7) or *B. moojeni* (8) was incubated at 37°C for 30 min. Half milliliter of each mixture was injected i.p. in groups of 8 mice, the animals maintained under observation for 48h, the mortality ratio determined and the amount of neutralized venom in terms of *mg* calculated as a function of their correspondent probits (Finney, 1971).

DISCUSSION

When injected in horses according to the routine schedule used in Instituto Butantan to produce antivenoms for therapeutic purposes, all ten *Bothrops* venoms, *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni and B. pradoi* were able to induce strong immune response as detected by the presence of high quantities of specific antibodies in their respective antivenom.

Antibodies, specific for the *Bothrops* venom components, were assayed by five different methods: the enzyme-linked immunobsorbent assay (ELISA), the double immunodiffusion, the quantitative precipitation, the neutralization of indirect hemolytic action of the venoms and the neutralization of the venom toxic effects (NVTE).

The data obtained from ELISA and double immunodiffusion assays demonstrated that all *Bothrops* antivenoms contain high quantities of antibodies either reacting against antigenic components found in the venom used to immunize the animal or cross-reacting with components present in the other venoms. The discrepancies observed among the titers obtained by the ELISA and by the double immunodiffusion assays can be explained by the sensitivity limit inhibited by these two methods: for example, the ELISA assay is capable of detecting antibodies in the range of μg to ng N Ab/ml whereas in the double immunodiffusion assay this limit goes up to 5 to 10 μg N Ab/ml Bier *et al.*[1].

The high quantities of antibody in the *Bothrops* antivenoms was confirmed by the quantitative measurement of precipitins. Each antivenom was assayed by the precise analytical method introduced by Heidelberger[6], either against the specific venom, or against the *B. jararaca* venom or against the BVM. Fig. 2 shows that the precipitation curve comprises three distinct segments: an initial ascending portion, a plateau corresponding to the precipi-

tation maximum, and a descending terminal segment. The shape of these curves (floculation type) is very similar to that described for horse antisera to diphteria and tetanus toxins since they do not appear to go through the origin (Heidelberger, 1949). The presence of a soluble antigen and antibody complex at the antibody excess region as well as in the antigen excess region explaing this kind of curve. The mechanism acounting for the floculation type of curve may be related with the floculation antibody heterogeneity and with the relative binding affinities of these antibodies as compared to the precipitin antibodies. Inspection of the three floculation curves obtained for each *Bothrops* antivenom indicates that they follow similar patterns. There are, therefore, indications suggesting that the antibody population found in each antivenom recognizes similar antigens found either in the specific venom or in the *B. jararaca* one as well as in the BVM mixture. To assert this suspicion the floculation curves obtained for each horse monovalent *Bothrops* antivenom assayed both against the corresponding venom and the BVM were transformed in straight lines and their intercepts and slopes were submitted to F test. With the aid of the parameters used to calculate the values for ''Fa'' and for ''Fb'' and their corresponding ''critical F'' (Table 4), it was possible to conclude that all paired curves were parallel and coincident. The *B. cotiara* antivenom pair was an exception for although being parallel it was not coincident.

With the exception of *B. atrox* antivenom the amount of antigen antibody complexes found at the zone of equivalence with the other nine monovalent antivenoms was over 1,0 mg/ml of the total protein content. These values were comparable to those obtained with the polyvalent antivenom (Table 5).

The immunochemical assays indicate therefore that each *Bothrops* venom per se was able to induce the production of antibodies capable of mutually cross-react against one each other. The presence of such complex antibody populations was reflected on their ability to neutralize the biological properties of the venoms. For instance, the monovalent antivenoms with higher number of precipitating properties detected by quantitative measurement of antigen-antibody interactions (anti-*B. alternatus*, anti-*B. cotiara*, anti-*B. insularis*, anti-*B. erythromelas* and anti-*B. moojeni*) possess also strong neutralizing activity against the venom lethal effects. On the other hand, the *B. jararacussu* antivenom contains both small amounts of precipitating antibodies and low neutralizing activity (Table 5 and 6). However, such correlation was not found when the anti-indirect hemolytic activity was assayed Lachmann *et al.*, Gutierrez *et al.*,[8,5]; such activity was almost exclusive of the *B. alternatus* antivenom.

The increase of the γ -globulins at the end of the immunization procedure is compatible with the high quantities of antibodies detected in the *Bothrops* antivenoms.

In conclusion: a) all ten *Bothrops* venoms were able to induce antibody formation wich mutually cross-react as assayed by three different immunochemical methods; b) the venoms from *B. alternatus, B. jararaca, B. moojeni* and *B. neuwiedi*, four of the most spread species of *Bothrops* snakes in Brazil Hoge *et al,*[7], behaved as good immunogens for horses and the antivenoms elicited, although in different degree, are capable to neutralize the lethal activity present in the other venoms; c) in contrast, *B. jararacussu* venom behaved as poor immunogen and its lethal activity was not efficiently

blocked neither by the specific antivenom nor the antivenoms produced by the other *Bothrops* venoms. These results agree well with previous work showing that venoms from six *Bothrops* species contain common antigenic components (Villarroel et. al[16]; Villarroel et al[17]). Taken together the observations described in this work indicate that the *Bothrops* venom mixture to be used to immunize horses to produce antivenom for therapeutic purposes should contain venoms from *B. alternatus, B. jararaca, B. jararacussu, B. moojeni* and *B.neuwiedi. Table 6 shows that the antivenoms elicited by these five Bothrops* venoms contain antibodies capable to neutralize the lethal activities for mice present in all ten *Bothrops* venoms used in this work.

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RESUMO: Cavalos foram imunizados com veneno de *B. alternatus, B. atrox, B. cotiara, B. erythromelas, B. insularis, B. jararaca, B. jararacussu, B. moojeni, B. newiedi,* e *B. pradoi*. Anticorpos específicos para componentes antigênicos dos venenos foram detectados pelos métodos imunoenzimático, dupla-difusão e precipitação quantitativa enquanto que os anticorpos neutralizantes foram analisados pelos métodos da hemólise indireta em placas e pela neutralização de seus efeitos letais. Anticorpos, tanto específicos como dando reações cruzadas com venenos botrópicos foram encontrados em todos os dez soros monovalentes. Modificações nos padrões eletroforéticos, caracterizadas por uma redução no pico da albumina e por um correspondente aumento das γ -globulinas com modificações ora acentuadas ora pouco perceptíveis nas frações das α e β globulinas, foram detectadas em todos esses soros.
UNITERMOS: Antivenenos; antissoros botrópicos monovalentes; venenos botrópicos.

REFERENCES

1. BIER, O.G.; DIAS DA SILVA, W.; GOTZE, D.; MOTA, I. Antigen antibody interaction. In: *Fundamentals of immunology*. 2. ed. Berlin, Springer-Verlag, 1986, p. 179
2. BUSSAB, W.O. *Análise de variância e de regressão. São Paulo, Atual, 1986. p. 92-100.*
3. *CHOI, S.C. Introductory applied statistics in science*. Englewood Cliffs, N.J., Prentice-Hall, 1978. p. 123-159.
4. FINNEY, D.J. Probit analysis. 3. ed. Cambridges, Cambridge University, 1971.
5. GUTIÉRREZ, J.M.; ÁVILA, C.; ROJAS, E.; CERDAS, L. An alternative in vitro method for testing the potency of the polyvalent anti-venom produced in Costa Rica. *Toxicon, 26*: 411-413, 1988.
6. HEIDELBERGER, M. Quantitative absolute method in the study of antigen-antibody reactions. *Bact. Rev., 3*: 49-95, 1949.
7. HOGE, A.R.; ROMANO-HOGE; S.A.R.W.L. Sinopse das serpentes peçonhentas do Brasil. 2. ed. *Mem. Inst. Butantan, 42/43:* 373-496, 1978/79.
8. LACHMANN, P.J.; HALBWACHS, L.; GEWURZ, A.; GEWURZ, H. Purification of cobra venom factor from phospholipase A_2 contaminant. *Immunology, 31:* 961-968, 1976.
9. LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L.; RANDALL, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem., 193:* 265-275, 1951.
10. MURATA, Y. Efeitos da radiação gama no veneno do *Crotalus durissus terrificus*. São

Paulo, 1988 (Dissertação de Mestrado — Instituto de Pesquisas Energéticas e Nucleares)

11. OUCHTERLONY, O. Antigen-antibody reactions in gels. *Acta path. microbiol. scand., 26:* 507, 1949.
12. POPE, C.G. The action of proteolytic enzymes on the antitoxins and proteins in immune sera. I. True digestion of the protein. *Brit., J. exp. Pathol., 20:* 132, 1939.
13. RAY, A.A., ed. *SAS user's guide; statistics. 1982 edition.* cary, North Caroline, SAS Institutte, 1982.
14. STEINBUCH, M. & AUDRAN, R. The isolation of IgG from mammalian sera with the aid of Caprylic acid. *Arch. Bioch. Biophy., 134:* 279 - 284, 1969.
15. THEAKSTON, R.D.G.; LLOYD-JONES, M.J.; REID, H.A. Micro-ELISA for detecting and assaying venom and venom-antibody. *Lancet, 2:* 639 — 641, 1977.
16. VILLARROEL, M.S.; ZELANTE, F.; FURLANETTO, R.S.; ROLIM-ROSA, R. Contribuição ao estudo imunoquímico de venenos botrópicos: I. Análise comparativa dos componentes antigênicos de seis espécies de venenos frente a seus respectivos antivenenos, através das técnicas de dupla difusão e imunoeletroforese em gel de ágar. *Mem.Inst. Butantan, 38:* 13-30, 1974.
17. VILLARROEL, M.S.; FURLANETTO, R.S.; ROLIM-ROSA, R.; ZELANTE, F.; NAVAS, J. Contribuição ao estudo imunoquímico de venenos botrópicos. II. Análise comparativa dos componentes antigênicos comuns de seis espécies de venenos botrópicos. *Mem.Inst.Butantan, 38:* 31-40, 1974.