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ANTIGENIC CROSS-REACTIVITY OF VENOMS OBTAINED FROM SNAKES OF GENUS *BOTHROPS*

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A. M. MOURA DA SILVA, M. R. D'IMPÈRIO LIMA, A. K. NISHIKAWA, C. I. BRODSKYN, M. C. DOS SANTOS, M. F. D. FURTADO, W. DIAS DA SILVA and I. MOTA. Antigenic cross-reactivity of venoms obtained from snakes of genus *Bothrops*. *Toxicon* **28**, 181–188, 1990.—Antigenic cross-reactivity was studied among the components of venoms from nine species of the genus *Bothrops* using species-specific antivenoms. Sera titration by DOT-ELISA detected similar levels of antibody when either homologous or heterologous antigens were used. Transblotted antigens, after SDS-PAGE fractionation, were also revealed by homologous and heterologous antivenoms. Antigens with mol. wt greater than 30,000 seemed to be the most cross-reactive. Antigens of about 24,000 mol. wt were poorly immunogenic. Antigens between 14–18,000 mol. wt cross-reacted only with *B. moojeni, B. jararacussu, B. neuwiedi* and *B. pradoi* venoms. Neutralization of the lethality of *B. jararaca* venom was observed by homologous and heterologous antivenoms.

INTRODUCTION

SNAKE venoms consist of mixtures of numerous proteins with enzymatic and/or toxic activities. Venoms from snakes belonging to the same family contain components of very similar activities in spite of their heterogeneous electrophoretic patterns (GONÇALVES and VIEIRA, 1950; GONÇALVES and DUETSCH, 1956). In Brazil, most envenomations are due to bites by snakes of the genus *Bothrops*. This genus includes several different species (HOGE and ROMANO-HOGE, 1978/1979). At the Institut Butantan, (São Paulo) bothropic antivenom is produced by immunization of horses with venom obtained from seven species (ROLIM-ROSA *et al.*, 1980/1981). This raises some questions about the efficacy of such antisera in cases of envenomation by snake species whose venom is not included in the pool of venoms used for immunization.

Several groups of researchers have already shown that the venom of each species can be partially or even totally neutralized by heterologous antisera (HOUSSAY and NEGRETE,

1923; MINTON, 1967; TU and SALAFRANCA, 1974; BOLANOS *et al.*, 1975). Double immunodiffusion tests have shown that these venoms share many common components (SILES-VILLARROEL *et al.*, 1974) including haemorrhagic (MANDELBAUM and ASSAKURA, 1988) and coagulant fractions (ROSENFELD and KELEN, 1966), which contribute to this cross-reactivity. However, some venom components are species-specific since they do not show antigenic cross-reactivity (SILES-VILLARROEL *et al.*, 1974).

The present work reports the results of a comparative study of the antigenic composition of the venoms of nine different species of the genus *Bothrops* analyzed by DOT-ELISA and Western Blotting.

MATERIAL AND METHODS

One month old, female A/Sn mice weighing 22–25 g (Instituto Butantan animal house) were used. *B. alternatus, B. atrox, B. cotiara, B erythromelas, B. jararaca, B jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms were extracted from 30–40 adult snakes using standard procedures. Venoms of each species were pooled, dried and stored at 4°C before use.

For immunization 100 μg of each venom was added to 1 ml of a suspension containing 1 mg/ml $Al(OH)_3$ as adjuvant. The mixture was stirred for 15 min to allow adsorption of the antigen onto the adjuvant particles. Nine groups of 18 mice were injected i.p. with four doses of 200 μl of each venom/adjuvant mixture at 7 day intervals. A control group received only $Al(OH)_3$ on the same days as the experimental groups. Blood was collected 28 days after the last immunization dose from the brachial plexus of the animals under light ether anaesthesia. Sera were separated and stored at −20°C until used.

For the antibody assay 2 μg of each venom were dotted onto nitrocellulose strips (Trans-Blot, BIO-RAD, Laboratories, Richmond, CA, U.S.A.). After drying at room temperature, the nitrocellulose was blocked with 5% dehydrated skimmed milk (Molico-Nestlé, São Paulo, Brazil) in Tris-buffered saline and then placed into incubation chambers containing titrated sera anti-specific and anti-heterologous *Bothrops* venom. After 1 hr at room temperature, strips were incubated for 1 hr with horseradish peroxidase-conjugate anti-mouse IgG (Cooper Biomedical Inc., Malvern, PA 19355, U.S.A.) diluted 1:1000 in Tris-buffered saline. The affinity staining for the horseradish peroxidase was performed with DAB (3, 3′ diaminobenzidine, Sigma Chemical Company, St Louis, MO, U.S.A.) at a concentration of 250 μl of the stored solution (20 mg/ml) in 30 ml of Tris buffered saline in the presence of 30% H_2O_2 (20 μl). Titres were visually determined (Table 1).

Venoms were analyzed by SDS polyacrylamide gel electrophoresis and Western blot using 8–18% gradient polyacrylamide slab gels under non-reducing conditions. Samples (80 μl) were boiled in SDS (5 min) at a concentration of 1000 μg/ml. The gels were stained with Coomassie Brilliant Blue R-250 (BIO-RAD). Western blots were obtained by transblotting from the polyacrylamide gels onto nitrocellulose paper. The blots were blocked with 5% dehydrated milk in Tris buffered saline. Serum samples (1:100 dilutions) were added and were incubated for 1 hr at room temperature. After washing with Tris buffered saline, the strips were incubated with peroxidase conjugate anti-mouse IgG (1:1000 dilution). The affinity staining was performed as described above.

TABLE 1. ANTIBODY TITERS ($\times 10^{-3}$) OF THE ANTIVENOM TESTED AGAINST HOMOLOGOUS AND HETEROLOGOUS VENOMS AS ANTIGENS BY DOT-ELISA

Venoms	Antivenoms against								
	ALT	ATR	COT	ERY	JAR	JUS	MOO	NEU	PRA
ALT	512	256	128	128	128	64	128	256	128
ATR	512	256	128	128	128	128	128	256	128
COT	256	256	256	128	128	128	128	256	128
ERY	512	256	128	256	128	128	128	256	128
JAR	16	32	128	128	128	128	128	256	128
JUS	64	128	64	32	32	128	64	128	64
MOO	64	128	64	128	128	128	256	128	64
NEU	64	64	64	128	128	128	128	128	64
PRA	64	64	64	128	128	128	128	128	64

ALT = *B. alternatus*; ATR = *B. atrox*; COT = *B. cotiara*; ERY = *B. erythromelas*; JAR = *B. jararaca*; JUS = *B. jararacussu*; MOO = *B. moojeni*; NEU = *B. neuwiedi*; PRA = *B. pradoi*.

Lactalbumin (14,000), trypsinogen (24,000), egg albumin (43,000), bovine serum albumin (68,000) and murine IgG (150,000) were used as molecular weight markers.

For the neutralization test of lethality *B. jararaca* venom was mixed with antiserum to the venom of either *B. jararaca, B. erythromelas, B. moojeni* or serum of mice which received only adjuvant or saline. The mixtures were incubated for 1 hr at 37°C and then injected i.p. into groups of eight mice. The individual dose was 70 μg of venom (2 LD_{50}) and 200 μl of antiserum. The number of dead animals was assessed after 1, 2, 3, 4, 24 and 48 hr. Animals were examined for peritoneal haemorrhagic lesions immediately after death or, in the case of surviving mice, 48 hr after injection when they were killed.

RESULTS

Cross titration of the species-specific antivenoms

Antibodies present in species-specific antivenoms prepared in mice were estimated by the DOT-ELISA technique using homologous and heterologous venoms as antigens. Table 1 shows the antibody titres obtained against either homologous or heterologous antigens. Levels ranged from 1:64,000–1:512,000 and did not differ significantly. However, there were a few exceptions represented by the anti-*B. alternatus* and anti-*B. atrox* sera against *B. jararaca* venom and anti-*B. atrox* serum against *B. jararacussu* venom. In these cases, the antibody titres were respectively 32 and 16 fold lower than the titres of the homologous antisera.

SDS-PAGE and immuno blotting

Figure 1 shows the distinct electrophoretic pattern of the nine venoms used for immunization. Figures 2, 3 and 4 show immuno blots of *B. erythromelas, B. jararaca* and *B. moojeni* venoms respectively, where the antigens were revealed with the nine species-specific antivenoms.

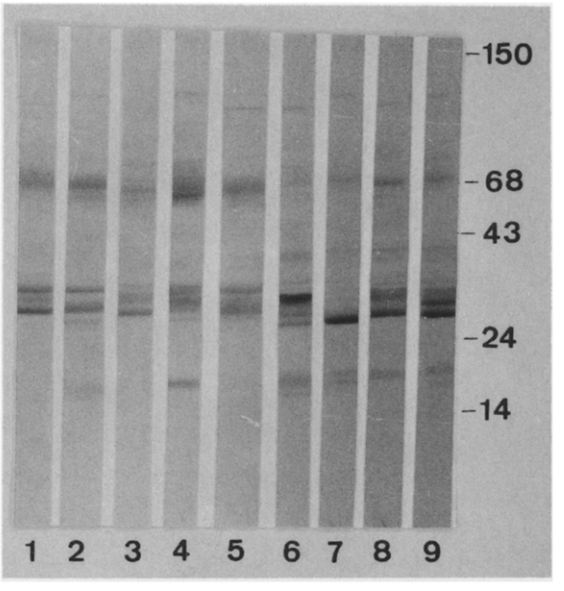

FIG. 1. VENOMS OF *B. alternatus* (1), *B. atrox* (2), *B. cotiara* (3), *B. erythromelas* (4), *B. jararaca* (5), *B. jararacussu* (6), *B. moojeni* (7), *B. neuwiedi* (8) AND *B. pradoi* (9) FRACTIONATED BY SDS-PAGE. Proteins were stained by Coomassie Blue. Mol. wt values are shown on the right.

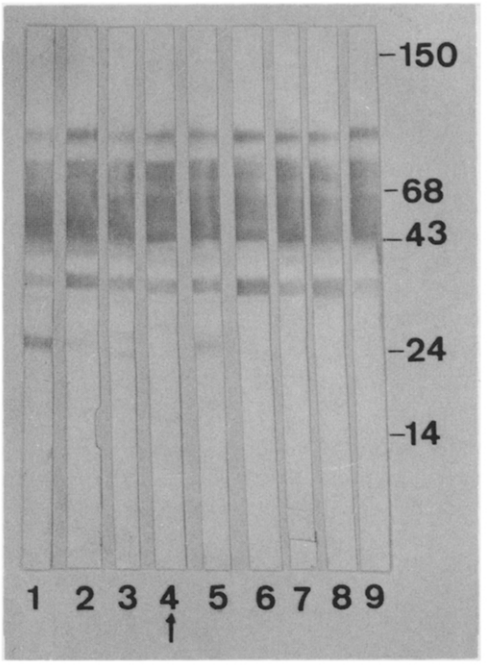

FIG. 2. ANTIGENS OF *B. erythromelas* VENOM WERE FRACTIONATED BY SDS-PAGE AND REVEALED BY WESTERN BLOTTING BY ANTI-*B. alternatus* (1), ANTI-*B. atrox* (2), ANTI-*B. cotiara* (3), ANTI-*B. erythromelas* (4), ANTI-*B. jararaca* (5), ANTI-*B. jararacussu* (6), ANTI-*B. moojeni* (7), ANTI-*B. neuwiedi* (8) AND ANTI-*B. pradoi* (9) SERA.

Fractionated components of *B. erythromelas* venom (Fig. 2) were recognized almost identically by all nine antisera. No difference could be detected in the antigens of mol. wt greater than 30,000, except for the 42,000 band which was not detectable by the anti-*B. jararacussu* sera. However, each sera recognized different bands between 20 and 30,000 mol. wt. Thus, anti-*B. alternatus* intensely stained a band of 23,000; anti-*B. atrox* stained the same band weakly; anti-*B. cotiara* stained two bands with 29 and 21,000; anti-*B. erythromelas* stained only a 29,000 band while anti-*B. jararaca* stained 29 and 23,000 mol. wt bands. No bands were stained in this region by the anti-*B. jararacussu*, anti-*B. moojeni*, anti-*B. neuwiedi* and anti-*B. pradoi* sera.

When *B. jararaca* venom was used as antigen (Fig. 3), the immuno blot pattern was slightly different for each antiserum. High mol. wt bands (120,000) were stained only by *B. jararaca*, *B. jararacussu*, *B. moojeni* and *B. neuwiedi* antivenoms. *B. erythromelas* antivenom did not recognize the 66,000 antigen and *B. jararacussu* antivenom failed to recognize the 47,000 band. *B. alternatus* and *B. pradoi* antivenoms revealed the blots weakly. Most cross reactive antigens seemed to be located in the 48–60,000 mol. wt region. Antigens of mol. wt in the region of 22,000 again showed a different pattern of recognition by each sera: anti-*B. alternatus*, anti-*B. atrox* and anti-*B. jararaca* stained a 21,000 band, anti-*B. cotiara* stained 25 and 21,000 bands. No band was stained in this region by the anti-*B. erythromelas*, anti-*B. jararacussu*, anti-*B. moojeni*, anti-*B. neuwiedi* and anti-*B. pradoi*.

B. moojeni venom showed a different electrophoretic pattern than *B. jararaca* and *B. erythromelas* venoms (Fig. 1). Blots stained with homologous sera (Fig. 4) showed that

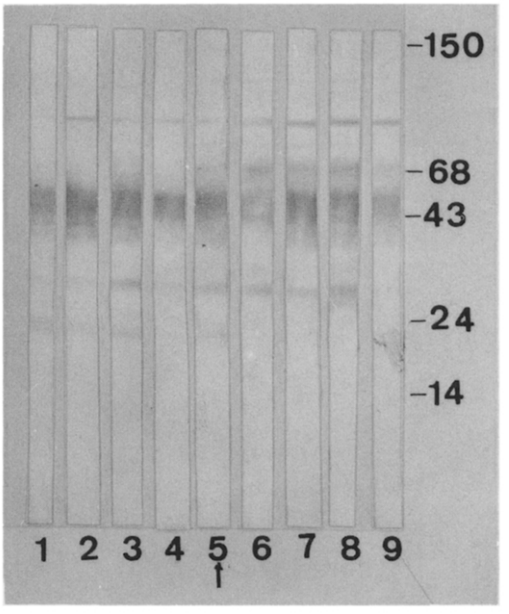

FIG. 3. ANTIGENS OF *B. jararaca* VENOM WERE FRACTIONATED BY SDS-PAGE AND REVEALED BY WESTERN BLOTTING BY ANTI-*B. alternatus* (1), ANTI-*B. atrox* (2), ANTI-*B. cotiara* (3), ANTI-*B. erythromelas* (4), ANTI-*B. jararaca* (5), ANTI-*B. jararacussu* (6), ANTI-*B. moojeni* (7), ANTI-*B. neuwiedi* (8) AND ANTI-*B. pradoi* (9) SERA.

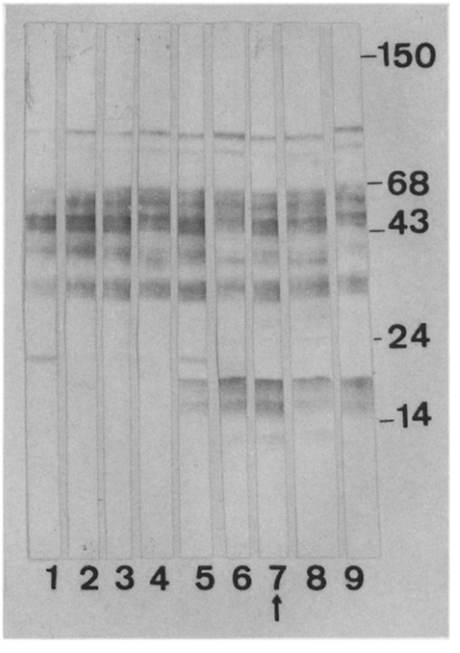

FIG. 4. ANTIGENS OF *B. moojeni* VENOM WERE FRACTIONATED BY SDS-PAGE AND REVEALED BY WESTERN BLOTTING BY ANTI-*B. alternatus* (1), ANTI-*B. atrox* (2), ANTI-*B. cotiara* (3), ANTI-*B. erythromelas* (4), ANTI-*B. jararaca* (5), ANTI-*B. jararacussu* (6), ANTI-*B. moojeni* (7), ANTI-*B. neuwiedi* (8) AND ANTI-*B. pradoi* (9) SERA.

the major antigens were located in the 17,000 region and between 28 and 60,000 mol. wt. Many other bands could also be detected. Antigens with mol. wt greater than 31,000 were stained similarly by all nine sera. However, *B. alternatus* antivenom did not detect the 80 and 37,000 bands and the latter was also not stained by the *B. atrox* and *B. cotiara* antivenoms. The 22,000 band was stained strongly by *B. alternatus* and *B. jararaca* antivenoms and weakly by *B. atrox* and *B. cotiara* antivenoms. The most significant differences when *B. moojeni* venom was used was observed with antigens of mol. wt in the region of 14,000. Bands of 14 and 18,000 mol. wt were strongly stained only by *B. jararaca*, *B. jararacussu*, *B. moojeni*, *B. neuwiedi* and *B. pradoi* antivenoms and the 12,000 mol. wt band only by *B. jararacussu*, *B. moojeni* and *B. neuwiedi* antivenoms.

When *B. alternatus*, *B. atrox* and *B. cotiara* venoms were used as antigens they showed similar results to those obtained with the *B. erythromelas* blots. On the other hand, *B. jararacussu*, *B. neuwiedi* and *B. pradoi* venoms behaved similarly to *B. moojeni* (data not shown). Under our experimental conditions antigens recognized only by homologous serum were never detected.

Neutralization experiments

The large extent of antigenic cross reactivity observed in Western blotting suggests that each individual *Bothrops* venom could induce the production of antivenoms capable of protecting mice against the lethal effects of the other cross-reacting venoms. To test this, homologous and heterologous sera were reacted *in vitro* with the reference venom of *B. jararaca* for 1 hr at 37°C. The mixtures were subsequently injected in groups of mice. Figure 5 shows that groups of mice receiving 2 LD_{50} of the venom mixed with saline or

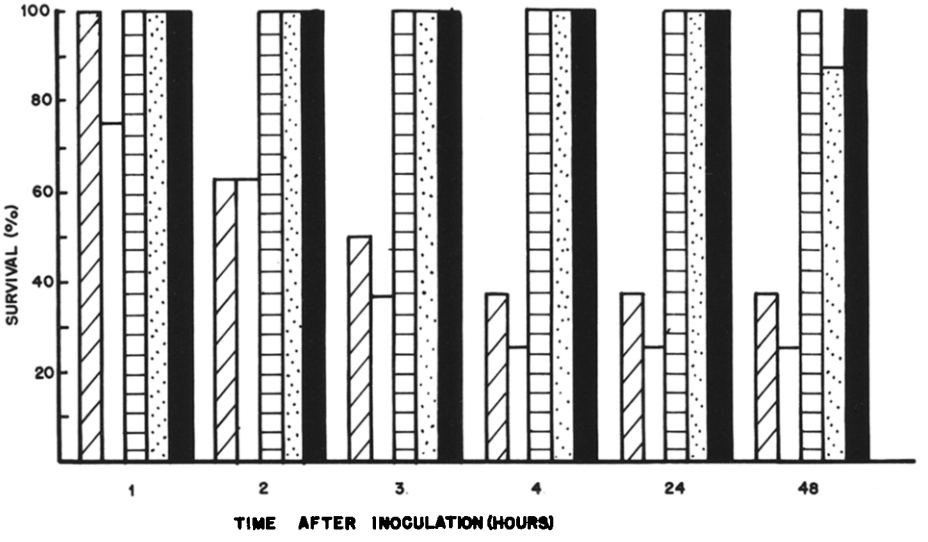

FIG. 5. DECREASE IN THE LETHALITY INDUCED BY 2 LD_{50} DOSES OF *B. jararaca* VENOM BY 200 µl OF EITHER ANTI-*B. jararaca* SERUM (▤), ANTI-*B. moojeni* SERUM (▨), ANTI-*B. erythromelas* SERUM (■), SERUM OF ANIMALS THAT RECEIVED ONLY ADJUVANT INJECTION (▨) OR SALINE (□). Percentage of survived animals was determined 1, 2, 3, 4, 24 and 48 hr after inoculation.

serum from animals injected only with adjuvant had survival rates of 38% and 25% respectively 4 hr after injection. The survival rate did not decrease over 48 hr. However, mice that received the mixture containing *B. jararaca* venom plus its homologous or heterologous sera were strongly protected against the venom lethal effect as all the mice belonging to these groups survived for 48 hr, except one that received *B. moojeni* antivenom and died 48 hr after injection. After 48 hr, all surviving animals were sacrificed and the peritoneal cavity was visually examined for haemorrhage. Mice which received *B. moojeni* and *B. erythromelas* antivenoms did not show signs of haemorrhage in the peritoneal cavity, while mice which received *B. jararaca* antivenom showed variable degrees of haemorrhage, especially at the site of injection. Dead mice all showed intense haemorrhage in the peritoneal cavity.

DISCUSSION

These results indicate a high degree of antigenic cross reactivity among the nine bothropic venoms studied. Seven of the venoms are from species occurring in the southern, central and southeastern regions of Brazil and are included in the pool of venoms used for immunization of horses at the Instituto Butantan for *Bothrops* antivenom production. *B. atrox* is the species of *Bothrops* most involved in ophidic accidents in the northern region of Brazil and *B. erythromelas* in the northeast. Thus, these venoms were included in order to study species from all the Brazilian regions.

In general, antigens with apparent mol. wt above 30,000 appeared to be the most cross-reactive. This region of the gels includes the most immunogenic antigens as detected by titration of antivenoms specific for antigens separated by SDS-PAGE and transferred to nitrocellulose membranes (unpublished results). It is likely that these are the antigens mainly responsible for the antigenic cross reactivity detected earlier by double immunodiffusion (SILES-VILLARROEL *et al.*, 1974). This region also includes the haemorrhagic factor (MANDELBAUM *et al.*, 1984), coagulant proteinase (GABRIJELCIC *et al.*, 1982), thrombin-like enzymes (KOSUGI *et al.*, 1986) and other toxins. Proteins of 24,000 mol. wt were strongly stained in all venoms by Coomassie Blue. In spite of this, they are weak immunogens in *B. alternatus, B. atrox, B. cotiara, B. erythromelas* and *B. jararaca* venoms, and non immunogenic in *B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms.

Interesting results were observed for proteins in the region of 14–18,000 mol. wt. The gel stained by Coomassie Blue, stressed bands in this region for *B. atrox, B. erythromelas, B. moojeni, B. neuwiedi, B. jararacussu* and *B. pradoi* venoms. However, they appeared only to be immunogenic in *B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms. Bands belonging to the last four venoms may not represent the same antigens as the ones with the same mol. wt present in *B. atrox* and *B. erythromelas* venoms since in *B. erythromelas* and *B. cotiara* blots they were not stained by any antivenom. These bands are not present in *B. jararaca* venom; however anti-*B. jararaca* serum is able to recognize such antigens in *B. moojeni* (Fig. 3) and also in *B. neuwiedi, B. jararacussu* and *B. pradoi* blots (not shown). This strongly suggests the existence of epitopes shared by 14–18,000 mol. wt antigens of *B. moojeni* venom and other proteins of *B. jararaca* venom. The participation of these antigens in the toxic action of the venoms needs to be established. The presence of 14–18,000 mol. wt antigens only in *B. jararacussu, B. moojeni, B. neuwiedi* and *B. pradoi* venoms cannot be explained by similarity in geographic distribution since the four related species, *B. alternatus, B. cotiara* and *B. jararaca* occur also in the central-southern region of Brazil. On the other hand, phylogenetic differences among

these species are poorly understood and the available data (BRATTSTROM, 1964) does not provide enough information to elucidate such data.

The most important point was that the high degree of antigenic cross reactivity observed *in vitro* could be confirmed *in vivo* since *B. jararaca* venom was neutralized similarly by homologous and heterologous sera. However, studies concerning cross neutralization of each toxic activity of the venoms must be carried out in order to clarify completely the antigenic cross reactivity of *Bothrops* venoms.

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REFERENCES

BOLAÑOS, R., CERDAS, L. and TAYLOR, R. (1975). The production and characteristics of a coral snake antivenom. *Toxicon* **13**, 139–142.

BRATTSTROM, B. H. (1964) Evolution of the pit vipers. *Trans. San Diego Soc. nat. Hist.* **13**, 185–268.

GABRIELCIC, D., DRUJAN, B. and GUBENSEK, F. (1982) Coagulation proteinase from *Bothrops colombiensis* venom. *Toxicon* **20**, 275–278.

GONÇALVES, J. M. and DEUTSCH, H. F. (1956) Ultracentrifugal and zone electrophoresis studies of some Crotalidae venoms. *Archs Biochem. Biophys.* **60**, 402–411.

GONÇALVES, J. M. and VIERA, L. G. (1950) Estudos sobre venenos de serpentes brasileiras. I-Análise electroforètica. *Anais Acad. bras. Ciênc.* **32**, 141–150.

HOGE, A. R. and ROMANO-HOGE, S. A. R. W. L. (1978/79) Sinopse das serpentes peçonhentas do Brasil. *Mem. Inst. Butantan* **42/43**, 373–496.

HOUSSAY, B. A. and NEGRETE, J. (1921) Proporciones en que el suero antiofidico neutraliza la ponzona de las serpientes. *Revta. Asoc. méd. argent.* **34**, 845–861.

KOSUGI, T., ARIGA, Y., NAKAMURA, M. and KINJO, K. (1986) Purification and some chemical properties of Thrombin-like enzyme from *Trimeresurus flavoviridis* venom. *Thromb. Haemostas.* **55**, 24–32.

MANDELBAUM, F. R. and ASSAKURA, M. T. (1988) Antigenic relationship of hemorrhagic factors and proteases isolated from the venoms of three species of *Bothrops* snakes. *Toxicon* **26**, 379–385.

MANDELBAUM, F. R., ASSAKURA, M. T. and REICHL, A. P. (1984) Characterization of two haemorrhagic factors isolated from the venom of *Bothrops neuwiedi*. *Toxicon* **22**, 193–206.

MINTON, S. A. (1967) Observations on toxicity and antigenic makeup of venoms from juvenile snakes. In: *Animal Toxins*, pp. 211–222 (RUSSELL, F. E. and SAUNDERS, P. R., Eds). Oxford: Pergamon Press.

ROLIM-ROSA, R., VIEIRA, E. G. J., SILES-VILLARROEL, M., SIRACUSA, Y. Q. and IIZUKA, H. (1980/81) Anàlise comparativa entre os diferentes esquemas de hiperimunização empregada na produção de soros antiofidicos pelo Instituto Butantan (1957–1979). *Mem. Inst. Butantan* **44/45**, 259–270.

ROSENFELD, G. and KELEN, E. M. A. (1966) Cross neutralization of the coagulant activity of some snake venoms by antivenins. *Toxicon* **4**, 7–15.

SILES-VILLARROEL, M., FURLANETTO, R. S., ROLIM-ROSA, R., ZELANTI, F. and NAVAS, J. (1974) Contribuição ao estudo immunoquímico de venenos botrópicos. *Mem. Inst. Butantan* **38**, 31–40.

TU, A. T. and SALAFRANCA, E. S. (1974) Immunological properties and neutralization of the sea snakes venoms (II). *Am. J. trop. med. Hyg.* **23**, 135–138.