Brazilian J Med Biol Res (1991) 24: 701-710 ISSN0100-879X

NEUTRALIZATION OF CORAL SNAKE *MICRURUS NIGROCINCTUS* VENOM BY A MONOVALENT ANTIVENOM

J.M. GUTIÉRREZ, G. ROJAS, A. PÉREZ, I. ARGUELLO and B. LOMONTE

Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

1. The neutralizing ability of a monovalent anti-*Micrurus nigrocinctus* (coral snake) antivenom produced in Costa Rica was tested against the lethal, myotoxic and phospholipase A_2 activities of homologous venom. In addition, immunodiffusion and Western blot analyses were performed.

2. In experiments where venom and antivenom were incubated prior to the test, antivenom was effective in neutralizing lethal, myotoxic and phospholipase A_2 activities, with Effective Doses 50% of 2700 µl antivenom/mg venom, 1840 µl antivenom/mg venom, and 3630 µl antivenom/mg venom, respectively.

3. When coral snake antivenom was administered at different times after coral snake venom injection, neutralization of lethality was achieved when antivenom was injected *iv* immediately and 15 min after venom. In contrast, lethality was not reduced when antivenom was administered by the *im* route. Only partial neutralization of myotoxicity was observed even when antivenom was injected *iv* immediately after envenomation.

4. Immunodiffusion and immunoblot analyses demonstrated the presence of antibodies in antivenom against several, but not all, venom components.

Key words: venom, *Micrurus*, coral snake, antivenom, neutralization.

Introduction

Envenomations by coral snakes (genera *Micrurus, Leptomicrurus* and *Micruroides*) are not very frequent in America (Parrish, 1967; Rosenfeld, 1971; Bolaños, 1982; Amaral et al., 1987; Kitchens and Van Mierop, 1987). However, they can be severe and cause neurotoxic symptoms (Rosenfeld, 1971). Anti-coral snake antivenoms are produced in several countries for the treatment of these envenomations (Chippaux and Goyffon, 1983).

There is evidence of little cross-reactivity between monovalent anti-coral snake antivenoms (Bolaños et al., 1978). Therefore, specific antivenoms have to be prepared for different species in different countries. In Central America, the most abundant coral snake is *Micrurus nigrocinctus* (Bolaños, 1982). A monovalent antivenom against the venom of

---

Research supported by Vicerrectoría de Investigación, Universidad de Costa Rica (Nos. 741-87-121 and 741-89-057) and the International Foundation for Science (No. F/0883-3). J.M.G., G.R. and B.L. are research fellows of the Costa Rican National Council for Science and Technology (CONICIT).

Correspondence: Dr J.M. Gutiérrez, Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica.

this species is produced at the Instituto Clodomiro Picado, Costa Rica. In the present investigation we studied the neutralizing ability of this antivenom against the lethal, myotoxic and phospholipase A_2 activities of *M. nigrocinctus* venom. In addition, the presence of antibodies against venom components was assessed by immunochemical procedures.

Material and Methods

Venom and antivenom

Venom was obtained from more than 60 specimens collected in both Atlantic and Pacific regions of Costa Rica, corresponding to the subspecies *M. n. nigrocinctus* and *M. n. mosquitensis* (Roze, 1970). Venom was lyophilized and stored at -40°C. Venom weight reported here includes salts. Monovalent antivenom (batch 158) was produced in horses at the Instituto Clodomiro Picado, according to the immunization schedule and the fractionation procedure described by Bolaños and Cerdas (1980).

Neutralization of lethal effect

For the determination of lethal dose 50% (LD_{50}), groups of four Webster white mice (16-18 g) were injected either intraperitoneally or subcutaneously with different doses of venom dissolved in 0.5 ml sodium phosphate-buffered saline, pH 7.2. Death was scored at 72 h and the LD_{50} was estimated by the Spearman-Karber method (WHO, 1981). In the study of neutralization of lethal effect, two types of experiments were performed: (A) experiments with preincubation of venom and antivenom and (B) experiments with independent injection of venom and antivenom. In the former, a dose of 52 µg venom, corresponding to four times the *ip* LD_{50}, was mixed with various volumes of antivenom in a constant final volume, in order to provide several antivenom/venom ratios. Incubations were carried out at 37°C for 30 min, after which 0.5 ml of the mixture (containing four LD_{50} of venom) was injected *ip* into groups of four white mice (16-18 g body weight). Death was scored at 72 h and neutralizing ability is reported as Effective Dose 50% (ED_{50}), defined as the µl antivenom/mg venom ratio that protects half of the population of injected mice. ED_{50} was calculated by the Spearman-Karber method (WHO, 1981) using a computer program (Gené and Robles, 1987).

In the second type of experiment, 50 µg of venom was injected subcutaneously into the back of white mice (16-18 g body weight). This dose corresponds to 1.4 subcutaneous LD_{50} and was selected because it induced acute envenomation, with animals dying 50-80 min after venom injection. Then, at three times after envenomation (0 min, 15 min and 30 min), different volumes of antivenom (100 µl, 200 µl and 400 µl) were injected either *iv* into the tail vein or *im* into the left thigh. Death was recorded at 72 h.

Neutralization of myotoxic activity

A dose-response curve was performed for the neutralization of myotoxic activity by injecting various amounts of venom *im* into the right gastrocnemius and plasma

creatine kinase levels were determined 3 h later using the Sigma kit 520 (Sigma Chemical Co., St. Louis, MO, USA). Activity is reported as units/ml, one unit corresponding to the phosphorylation of 1 nmol creatine/min at 25°C. The dose-response curve showed that a dose of 15 µg venom caused a submaximal response and this dose was selected for neutralization studies. Histologically, previous studies have shown that this dose induces extensive myonecrosis (Gutiérrez et al., 1983).

For the study of the neutralizing ability of antivenom, experiments with preincubation and experiments with independent injection of venom and antivenom were carried out. In the former, a constant amount of venom was incubated with various volumes of antivenom at 37°C for 30 min. Then, 100 µl of the mixtures (containing 15 µg of venom) was injected *im* into the right gastrocnemius of four white mice (18-20 g body weight). Mice were bled from the tail 3 h later and plasma creatine kinase levels were measured as described. Mice were killed by cervical dislocation 24 h after venom injection, and a sample of injected gastrocnemius was obtained, fixed in 10% formaldehyde and processed for embedding in paraffin. Sections were stained with hematoxylin-eosin for histological observation. A group of four mice was injected with 15 µg venom with no antivenom and another group was injected with saline. Two additional control groups were included, i.e., mice injected with normal horse serum and mice injected with a mixture of normal horse serum and venom. The neutralizing ability is reported as ED_{50}, defined as the µl antivenom/mg venom ratio in which the myotoxic activity of venom was reduced by 50%.

Experiments with independent injection of venom and antivenom were performed by injecting 15 µg of venom *im* as described. Then, 400 µl of antivenom were injected *iv* into the tail vein at four different times (0, 30, 60 and 120 min). Control mice were injected with venom and did not receive antivenom. Blood samples were obtained 3 h after envenomation by cutting the tip of the tail and plasma creatine kinase levels were quantitated as described. At 24 h, tissue samples were collected, fixed and processed for histological observation.

Neutralization of phospholipase A_2 activity

A dose-response study of the phospholipase A_2 activity of venom was carried out by adding 100 µl of various amounts of venom dissolved in sodium phosphate-buffered saline, pH 7.2, to 1.0 ml of an egg yolk suspension. This was prepared by diluting egg yolk 1:5 with 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM $CaCl_2$ and 1% Triton X-100. After 20 min of incubation at 37°C, free fatty acids were extracted and titrated by the method of Dole (1956). From the dose-response curve, it was observed that 15 µg venom gave a submaximal response and this dose was selected for the neutralization studies. These studies were carried out by preparing mixtures containing a fixed amount of venom and different volumes of antivenom in a constant final volume. Mixtures were incubated for 30 min at 37°C and 100 µl (containing 15 µg of venom) were added to an egg yolk

suspension as described above. For controls, the egg yolk suspension was incubated with 15 µg of venom with no antivenom (100% activity) and with saline (negative control). Enzymatic reactions were carried out for 20 min at 37°C and free fatty acids were extracted and titrated as described. Phospholipase A_2 activity was expressed as µEq fatty acids released mg protein^{-1} min^{-1} and the neutralizing ability of antivenom was expressed as Effective Dose 50%, defined as the µl antivenom/mg venom ratio at which the phospholipase activity of venom was neutralized by 50%.

Immunochemical studies

Immunodiffusion was performed in 0.5% agarose gels by the method of Ouchterlony and Nilsson (1978). Several dilutions of *M. nigrocinctus* venom were confronted with undiluted anti-*M. nigrocinctus* antivenom. After 48 h of incubation at 22-25°C, gels were extensively washed with saline and stained with Amido Black. For Western blot analysis, *M. nigrocinctus* venom (130 µg) was reduced with 2-mercaptoethanol for 4 min at 95°C and then separated by SDS-polyacrylamide gel electrophoresis on a 15% gel by the method of Laemmli (1970). A set of molecular weight markers (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as reference. Proteins were transferred electrophoretically to a nitrocellulose membrane (0.45 µm; Bio-Rad Laboratories, Richmond, CA) as described by Towbin et al. (1979). After transfer, proteins were visualized on the nitrocellulose by mild Amido Black staining (Syu and Kahan, 1977) and the positions of the main bands were marked by punching pinholes. Nitrocellulose was blocked for 2 h with 2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) in sodium phosphate-buffered saline (PBS), pH 7.2. Then, anti-*M. nigrocinctus* antivenom was added, either at 1:25 or 1:50 dilution in 0.2% BSA-PBS for 4 h. After four washings with 0.2% BSA-PBS, bound antibodies were detected by the addition of an anti-horse immunoglobulin-horseradish peroxidase conjugate (Sigma) diluted 1:1000 for 2 h. After washing, color was developed with 4-Cl-1-naphthol (Bio-Rad) and hydrogen peroxide as substrate.

Statistical analysis

When appropriate, the significance of the differences observed between means was determined by the Student *t*-test.

Results

Neutralization of lethality

The estimated LD_{50} was 13 µg (95% confidence limits: 9.1 to 16.9 µg) when the venom was injected *ip* and 35 µg (95% confidence limits: 29.7 to 43.1 µg) when the venom was injected *sc*. In experiments in which venom and antivenom were incubated prior to

injection, anti-coral snake antivenom was effective in neutralizing lethality, with an ED_{50} of 2700 µl antivenom/mg venom.

The results of experiments in which antivenom was injected at different times after *sc* venom injection are shown in Table 1. With the dose of venom used, mice receiving venom and no antivenom died between 50 and 80 min after envenomation. Intramuscular administration of antivenom prolonged survival time, but was ineffective in preventing death, even when 400 µl of antivenom was given immediately after envenomation. In contrast, intravenous administration of antivenom was effective in neutralizing lethality, particularly when antivenom was given immediately or 15 min after envenomation (Table 1).

Table 1- *Effect of the route of antivenom administration on the lethality of* M. nigrocinctus *venom when antivenom was administered after envenomation.*

Venom (50 µg) was injected subcutaneously and antivenom was administered either intravenously *(iv)* or intramuscularly *(im)* at different times after envenomation. Control mice received only venom and died 50-80 min after venom injection. Death was scored 72 h after venom administration.

Route of administration and antivenom dose (µl)	Delay in antivenom administration (min)	Lethality (dead mice/ total mice)
Intravenous		
100	0	4/4
200	0	0/4
400	0	0/4
100	15	2/4
200	15	2/4
400	15	0/4
100	30	3/4
200	30	4/4
400	30	4/4
Intramuscular		
100	0	4/4
200	0	4/4
400	0	4/4
100	15	4/4
200	15	4/4
400	15	4/4
100	30	4/4
200	30	4/4
400	30	4/4

Neutralization of myotoxicity

Intramuscular injection of 15 µg *M. nigrocinctus* venom induced an increase in plasma creatine kinase levels of 739 Units/ml (N = 4) 3 h after envenomation. Normal horse serum did not reduce the myotoxic activity of venom, whereas antivenom was effective in neutralizing this activity in experiments with preincubation of venom and antivenom. The ED_{50} was 1840 µl anti-venom/mg venom (Figure 1). There was good correlation between plasma creatine kinase levels and muscle tissue histology. Muscle from mice injected with venom and receiving no antivenom showed prominent myonecrosis by histological analysis, with 90-95% of the fibers being necrotic 24 h after envenomation. When mice were injected with a mixture of venom and antivenom at a ratio of 2000 µl antivenom/mg venom, myotoxic activity was reduced significantly, with 40-45% of all fibers showing necrosis. Almost complete neutralization was obtained at an antivenom/venom ratio of 4000 µl/mg, with only 4-8% of muscle fibers showing necrosis. No myonecrosis was observed in muscle injected with saline solution or with normal horse serum.

In contrast, when antivenom was administered *iv* at various times after envenomation, neutralization of myotoxicity was only partial (Figure 2). Again, there was a good correlation between plasma creatine kinase levels and histological extent of myonecrosis. Despite the difficulty in neutralizing myotoxicity under these conditions, there was partial neutralization even when antivenom administration was delayed 120 min (Figure 2).

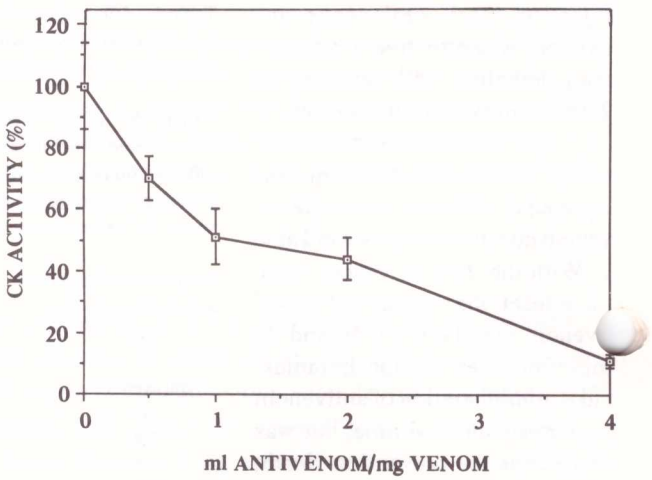

Figure 1- Neutralization of the myotoxic effect of *M. nigrocinctus* venom (determined by quantitation of plasma creatine kinase activity) by antivenom in preincubation experiments. Mixtures containing various ratios of ml antivenom/mg venom were prepared and 0.1 ml of each mixture, containing 15 µg venom was injected *im* into mice. Creatine kinase (CK) levels in serum were quantitated 3 h later. Myotoxic activity is expressed as percent activity, using as reference the plasma creatine kinase levels in mice injected with venom alone (100%). The plasma creatine kinase activity of venom-treated mice was 739 units/ml 3 h after venom administration. Each value is the mean ± SEM for 4 animals.

Neutralization of phospholipase A_2 activity

M. nigrocinctus venom had a phospholipase A_2 activity of 52 ± 3 µEq NaOH mg^{-1} min^{-1}. Antivenom neutralized this activity, with an ED_{50} of 3630 µl antivenom/mg venom (Figure 3).

Immunochemical studies

When *M. nigro-cinctus* venom and anti-venom were submitted to immunodiffusion, five precipitin arcs were observed (Figure 4). When venom was analyzed by SDS polyacrylamide gel electrophoresis under reducing conditions, several protein bands migrating mainly in the molecular mass region below 26 kDa were observed. Western blot analysis demonstrated the presence of antibodies in antivenom against the following venom components: a) one band located in the migration front whose molecular mass was less than 10 kDa; b) one band of molecular mass 14 kDa; c) two bands of molecular mass between 16 and 18 kDa. All of these bands showed moderate staining intensity. In addition, there were two bands of molecular mass 23 and 70 kDa which stained for protein but not with antibodies present in the antivenom.

Discussion

Very few studies have been carried out on the neutralizing ability of coral snake antivenom. Most of them have dealt with neutralization of lethal effect in experiments in

which venom and antivenom were incubated prior to injection (Cohen et al., 1968; Bolaños et al., 1978; Siles-Villarroel et al., 1980/81). In the present study we have demonstrated the ability of the monovalent anti-*M. nigrocinctus* anti-venom produced in Costa Rica to neutralize the lethality, myotoxicity and phospholipase A_2 activity induced by homologous venom.

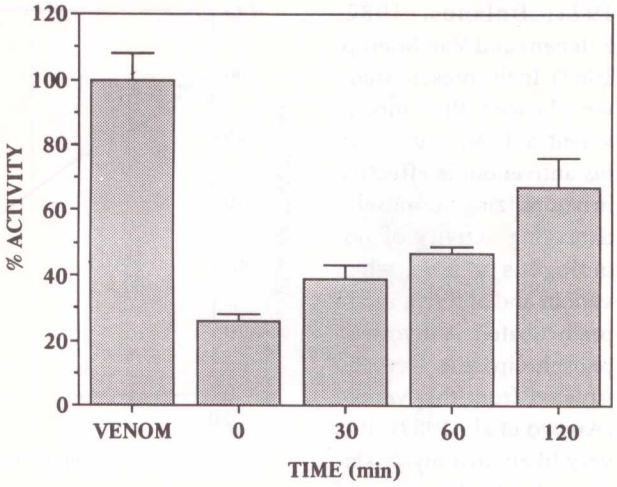

Figure 2 - Neutralization of myotoxic activity (plasma creatine kinase levels) of *M. nigrocinctus* venom by antivenom. Venom (15 µg) was administered *im* into the right gastrocnemius, followed by *iv* administration of 400 µl antivenom at various times (0, 30, 60 and 120 min) after envenomation. Three h after venom injection, blood samples were collected and plasma creatine kinase levels were determined. Myotoxic activity is reported as percent plasma creatine kinase activity increase in response to the venom (see legend to Figure 1). Each value is the mean ± SEM for 5 animals. Myotoxic activity was significantly reduced ($P<0.05$) in all groups receiving antivenom (Student *t*-test).

Our data also show that coral snake antivenom is effective in experiments with independent injection, provided it is administered *iv* early in the course of envenomation. These findings agree with data reported by Cohen (1966) for *M. fulvius* venom. In contrast, under the present experimental conditions, which resulted in an acute envenomation that killed mice 50-80 min after venom injection, *im* administration of antivenom did not prevent lethality, even when this was done immediately after envenomation. This experimental finding confirms observations of the ineffectiveness of the intramuscular route for antivenom administration (Gutiérrez et al., 1981; Amaral et al., 1987) and suggests that, in cases of coral snake envenomation, anti-venom should be given *iv*.

Lethality induced by *M. nigrocinctus* venom is probably due mainly to the action of alpha-neurotoxins which act postsynaptically at the neuromuscular junction, by blocking cholinergic receptors at the motor end-plate (Moussatché and Meléndez, 1979; J.P. Rosso, unpublished results). In addition, there is evidence that this venom also exerts a presynaptic action (Rodrigues-Simioni et al., 1990), as has also been demonstrated for other coral snake venoms (Vital Brasil and Dias-Fontana, 1983/84).

Our previous studies have demonstrated that *M. nigrocinctus* venom induces prominent myotoxicity when injected in mice, as indicated by histological analysis, increase in plasma creatine kinase levels and myoglobinuria (Gutiérrez et al., 1980, 1983, 1986). Although this effect has not been described clinically, some reports suggest that myotoxicity might occur in human victims of coral snake bites (Machado and Rosenfeld,

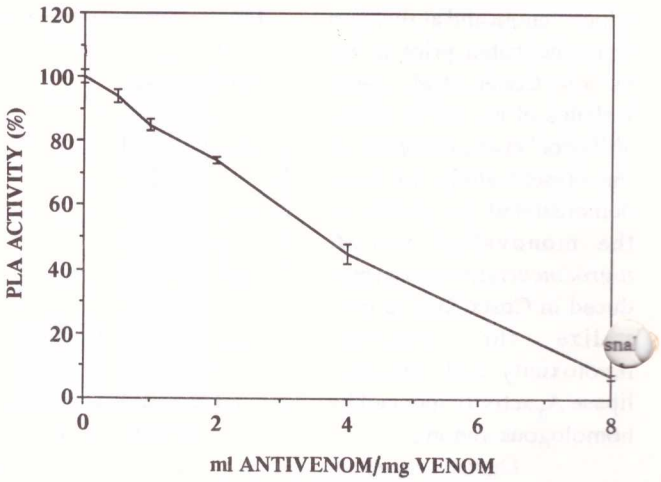

Figure 3 - Neutralization of *M. nigrocinctus* venom phospholipase A_2 activity by antivenom in experiments in which both were preincubated prior to the test. Phospholipase A_2 (PLA) activity is reported as percent activity relative to its specific activity in the absence of antivenom (52 μEq min^{-1} mg^{-1}). Data are reported as mean ± SEM (N = 3).

1971; Bolaños, 1982; Kitchens and Van Mierop, 1987). In the present study we showed that monovalent anti-*M. nigrocinctus* antivenom is effective in neutralizing the muscle-damaging activity of homologous venom when venom and antivenom are preincubated. A myotoxic phospholipase A_2 has been isolated from this venom (Arroyo et al., 1987). It is very likely that myotoxicity is due to the action of phospholipase A_2, as is the case for other elapid venoms (Harris et al., 1980; Mebs, 1986). Thus, antivenom contains antibodies against these myotoxins. In support of this conclusion, our results showed that antivenom neutralizes phospholipase A_2 activity of this venom when tested using an egg yolk suspension as source of phospholipids. In addition, Western blot analysis clearly showed the presence of antibodies in antivenom which react against components of molecular mass similar to that of phospholipase A_2, i.e. between 14 and 18 kDa.

When antivenom was injected *iv* after envenomation, neutralization of myotoxicity was only partial. This agrees with previous studies carried out on other venoms, myotoxins and antivenoms (Gutiérrez et al., 1981; Ownby et al., 1986), and is probably due to the extremely rapid development of muscle cell damage after injection of coral snake venom (Gutiérrez et al., 1980, 1986). Under these conditions, neutralization is achieved only partially, despite the rapid administration of antivenom. However, even after a 120-min delay in administering antivenom, there was a significant reduction of the myotoxic effect. The role of myotoxicity in human envenomation following coral snake bites has not been studied. Thus, the routine evaluation of plasma CK levels and myoglobinuria in these patients should be encouraged.

No information is available about other components of coral snake venoms in addition to neurotoxins, myotoxins and phospholipases. However, the observation of five precipitin arcs in immunodiffusion plates, together with the detection of several bands by Western blot, indicates the presence of additional antigenic components in these venoms. The nature and pharmacological activity of these components remain to be investigated. Neutralization studies with anti-coral snake antivenoms are urgently required in order to

define the neutralizing profile of commercially available antivenoms.

Acknowledgments

The authors thank Jorge Sanabria, Javier Núñez (Instituto Clodomiro Picado, Universidad de Costa Rica) and Carlos Cambronero (Facultad de Medicina, Universidad de Costa Rica) for technical support. Thanks are due to J.P. Rosso (Faculty of Medicine, Marseille) for permitting the authors to use his unpublished data.

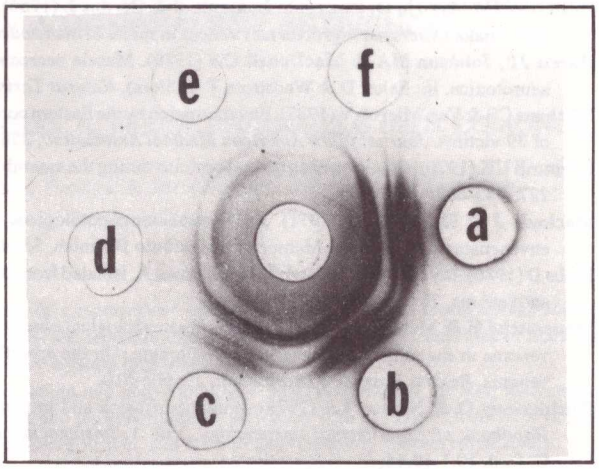

Figure 4 - Double immunodiffusion of anti-*M. nigrocinctus* anti-venom against *M. nigrocinctus* venom. Central well: Undiluted anti-venom. Peripheral wells: *M. nigrocinctus* venom: *a*, 20 mg/ml; *b*, 10 mg/ml; *c*, 5 mg/ml; *d*, 2.5 mg/ml; *e*, 1.25 mg/ml; *f*, 0.62 mg/ml.

References

Amaral CF, Dourado HV, Kouyoumdjian JA, Cardoso JL, Campos JA, Azevedo-Marques M & Lopes PF (1987). Manual de Diagnóstico e Tratamento de Acidentes Ofídicos. Ministério da Saúde, Brasil.

Arroyo O, Rosso JP, Vargas O, Gutiérrez JM & Cerdas L (1987). Skeletal muscle necrosis induced by a phospholipase A_2 isolated from the venom of the coral snake *Micrurus nigrocinctus nigrocinctus*. *Comparative Biochemistry and Physiology*, 87B: 949-952.

Bolaños R (1982). Las serpientes venenosas de Centroamérica y el problema del ofidismo. Primera parte. Aspectos zoológicos, epidemiológicos y biomédicos. *Revista Costarricense de Ciencias Médicas*, 3: 165-184.

Bolaños R & Cerdas L (1980). Producción y control de sueros antiofídicos en Costa Rica. Boletín de la Oficina Sanitaria Panamericana, 88: 189-196.

Bolaños R, Cerdas L & Abalos JW (1978). Venenos de las serpientes coral (*Micrurus* spp): Informe sobre un antiveneno polivalente para las Américas. Boletín de la Oficina Sanitaria Panamericana, 84: 128-133.

Chippaux JP & Goyffon M (1983). Producers of antivenomous sera. *Toxicon*, 21: 739-752.

Cohen P (1966). *In vivo* neutralization of coral snake venom. *Memórias do Instituto Butantan*, 33: 881-886.

Cohen P, Dawson JM & Seligman EB (1968). Cross neutralization of *Micrurus fulvius fulvius* (coral snake) venom by anti-*Micrurus carinicauda dumerilii* serum. *American Journal of Tropical Medicine and Hygiene*, 17: 308-312.

Dole VP (1956). A relation between non-sterified fatty acids in plasma and the metabolism of glucose. *Journal of Clinical Investigation*, 35: 150-154.

Gené JA & Robles A (1987). Determinación de la dosis letal 50% por el método de Spearman-Karber. *Revista Médica del Hospital Nacional de Niños* (Costa Rica), 22: 35-40.

Gutiérrez JM, Chaves F, Rojas E & Bolaños R (1980). Efectos locales inducidos por el veneno de la serpiente coral *Micrurus nigrocinctus* en ratón blanco. *Toxicon*, 18: 633-639.

Gutiérrez JM, Chaves F, Bolaños R, Cerdas L, Rojas E, Arroyo O & Portilla E (1981). Neutralización de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon*, 19: 493-500.

Gutiérrez JM, Lomonte B, Portilla E, Cerdas L & Rojas E (1983). Local effects induced by coral snake venoms: Evidence of myonecrosis after experimental inoculations of venoms from five species. *Toxicon*, 21: 777-783.

Gutiérrez JM, Arroyo O, Chaves F, Lomonte B & Cerdas L (1986). Pathogenesis of myonecrosis induced by coral snake (*Micrurus nigrocinctus*) venom in mice. *British Journal of Experimental Pathology*, 67: 1-12.

Harris JB, Johnson MA & MacDonell CA (1980). Muscle necrosis induced by some presynaptically active neurotoxins. In: Eaker D & Wadstrom T (Editors), *Natural Toxins*. Pergamon Press, Oxford, 569-578.

Kitchens CS & Van Mierop V (1987). Envenomation by the Eastern coral snake (*Micrurus fulvius fulvius*). A study of 39 victims. *Journal of the American Medical Association*, 258: 1615-1618.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Machado J & Rosenfeld G (1971). Achados anátomo-patológicos em necroscopia de paciente falecido por envenenamento elapídico. Memórias do Instituto Butantan, 35: 41-53.

Mebs D (1986). Myotoxic activity of phospholipases A_2 isolated from cobra venoms: Neutralization by polyvalent antivenoms. *Toxicon*, 24: 1001-1008.

Moussatché H & Meléndez T (1979). Some pharmacological observations with Elapidae and Crotalidae snake venoms in the guinea pig denervated diaphragm. On the specificity of the cholinergic blockade by their venoms. *Revista Brasileira de Biologia*, 39: 605-610.

Ouchterlony O & Nilsson LA (1978). Immunodiffusion and immunoelectrophoresis. In: Weir DM (Editor), *Handbook of Experimental Immunology*, Vol. 1, *Immunochemistry*. Blackwell Scientific Publications, Oxford, 19.1-19.44.

Ownby CL, Colberg TR & Odell GV (1986). *In vivo* ability of antimyotoxin *a* serum plus polyvalent (*Crotalidae*) antivenom to neutralize prairie rattlesnake *(Crotalus viridis viridis)* venom. *Toxicon*, 24: 197-200.

Parrish HM (1967). Bites by coral snakes: Reports of 11 representative cases. *American Journal of Medical Science*, 253: 561-566.

Rodrigues-Simioni L, Cogo JC, Gutiérrez JM & Prado-Franceschi J (1990). *Micrurus nigrocinctus* venom induced effects on isolated mouse phrenic-nerve diaphragm and chick biventer cervicis preparations. In: *I Simpósio da Sociedade Brasileira de Toxinologia: Peçonhas e Envenenamento no Brasil* (Abstracts). Instituto Butantan, São Paulo, 7.

Rosenfeld G (1971). Symptomatology, pathology and treatment of snake bites in South America. In: Bucherl W & Buckley E (Editors), *Venomous Animals and Their Venoms*. Vol. II. *Venomous Vertebrates*. Academic Press, New York, 345-384.

Roze J (1970). *Micrurus*. In: Peters JA & Orejas-Miranda B (Editors), *Catalogue of the Neotropical Squamata*. Part 1. *Snakes*. Smithsonian Institution Press, Washington DC, 196-220.

Siles-Villarroel M, Rolim-Rosa R, Zelante F & Jardim Vieira EG (1980/81). Perspectivas de padronização das titulações de venenos e antivenenos elapídicos, em camundongos. *Memórias do Instituto Butantan*, 44/45: 289-297.

Syu WJ & Kahan L (1987). Use of protein-stained immunoblots for unequivocal identification of antibody specificities. *Journal of Immunological Methods*, 103: 247-252.

Towbin H, Staehelin T & Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, USA*, 76: 4350-4354.

Vital-Brasil O & Dias-Fontana M (1983/84). Ações pré-juncionais e pós-juncionais da peçonha da cobra coral *Micrurus corallinus* na junção neuromuscular. *Memórias do Instituto Butantan*, 47/48: 13-26.

World Health Organization (1981). *Progress in the Characterization of Venoms and Standardization of Antivenoms*. World Health Organization, Geneva.

Received September 11, 1990
Accepted July 9, 1991