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Geographic and ontogenic variability in the venom of the neotropical rattlesnake *Crotalus durissus*: Pathophysiological and therapeutic implications

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Abstract: A comparative study was performed on the venoms of adult specimens of the neotropical rattlesnake, *Crotalus durissus*, from Guatemala, Costa Rica, Venezuela and Brazil, together with the venom of newborn specimens of *C. d. durissus* from Costa Rica. Venoms from Brazil (*C. d. terrificus*) and from newborn specimens of *C. d. durissus* presented an electrophoretic pattern characterized by the predominance of bands with molecular mass of 36 and 15 kDa, whereas those of adult specimens of *C. d. durissus* from Guatemala and Costa Rica, and *C. d. cumanensis* from Venezuela, showed a conspicuous band of 62 kDa, and additional bands of 36, 29 and 15 kDa. Moreover, venoms from *C. d. terrificus* and *C. d. cumanensis* showed a prominent band of < 10 kDa that probably corresponds to crotamine, since a 'crotamine-like' activity was detected in these venoms upon intraperitoneal injection in mice. Venoms of *C. d. terrificus*, *C. d cumanensis* and newborn *C. d. durissus* induced higher lethal and myotoxic effects than those of adult *C. d. durissus*. In contrast, adult *C. d. durissus* and *C. d. cumanensis* venoms induced hemorrhage, whereas venoms of *C. d. terrificus* and newborn *C. d. durissus* lacked this effect. All venoms showed coagulant effect in plasma, the highest activity being observed in the venom of newborn *C. d. durissus*. An anti-crotalic antivenom produced by Instituto Butantan (Brazil), using *C. d. terrificus* venom as antigen, was effective in the neutralization of lethal, myotoxic and coagulant effects of all venoms studied, being ineffective in the neutralization of hemorrhagic activity of the venoms of *C. d. cumanensis* and *C. d. durissus*. On the other hand, a polyvalent antivenom produced by Instituto Clodomiro Picado (Costa Rica), using the venoms of *C. d. durissus*, *Bothrops asper* and *Lachesis stenophrys* as antigens, was able to neutralize lethal, myotoxic, coagulant and hemorrhagic effects of *C. d. durissus* venom, but was ineffective in the neutralization of lethality and myotoxicity of *C. d. terrificus*, *C. d. cumanensis* and newborn *C. d. durissus* venom. The high toxicity of South American and newborn *C. d. durissus* venoms is related to the presence of high concentrations of the neurotoxic phospholipase A2 complex 'crotoxin'. Accordingly, antivenom from Instituto Butantan has a much higher titer of anti-crotoxin antibodies than antivenom from Instituto Clodomiro Picado. *Crotalus durissus* represents an example of intraspecies variation in venom composition and pharmacology that has relevant pathophysiologic and therapeutic implications.

Key words: Snake venom, rattlesnake, *Crotalus durissus*, crotoxin, antivenoms, neutralization.

Snakebite envenomings constitute a relevant public health hazard in Latin America (Fan and Cardoso 1995, Gutiérrez 1995). Most accidents are inflicted by species of the genus *Bothrops*, although envenomings by *Crotalus* sp. occur throughout the region, having particular relevance in South America, where they are usually associated with severe systemic envenomings (Rosenfeld 1971, Azevedo-Marques *et al*. 1987, Fan and Cardoso 1995).

Abundant rattlesnake species have been described in North America, particularly in Mexico (Campbell and Lamar 1989), but *Crotalus durissus* constitutes the most widespread species, ranging in distribution from Mexico to Argentina and presenting 14 subspecies (Campbell and Lamar 1989). Such wide distribution makes this species an interesting case to investigate intraspecies venom variation, as numerous studies have evidenced a significant inter and intraspecies variability in the composition and effects of snake venoms (Chippaux *et al.* 1991), with the consequent clinical and therapeutic implications.

Previous studies have documented a conspicuous biochemical and pharmacological venom variation between *C. durissus* from Costa Rica and various subspecies from Brazil (Gutiérrez *et al.* 1991, Dos Santos *et al.* 1993, Santoro *et al.* 1999). The most striking difference has to do with the extremely high toxicity of South American venoms due to the presence of large quantities of the potent neurotoxin crotoxin (Santoro *et al.* 1999), which seems to be present in very low amounts in Central American venoms (Gutiérrez *et al.* 1991). Furthermore, an ontogenic variation was described for the subspecies *C. d. durissus* from Costa Rica, since newborn snake venoms are highly toxic and closely resemble those of South American venoms, in contrast with adult venoms which lack neurotoxicity and induce local inflammatory, hemorrhagic and tissue-damaging effects (Gutiérrez *et al.* 1991).

The present investigation was aimed at further exploring the intraspecies variation in the venom of this rattlesnake, by comparing venoms from specimens classified in various subspecies collected in Guatemala, Costa Rica, Venezuela and Brazil. Our results evidence conspicuous variability in the biochemistry and pharmacology of these venoms, in agreement with the different pathophysiological profiles characteristic of these envenomings. Moreover, the ability of antivenoms produced in Brazil and Costa Rica to neutralize venoms of various subspecies was assessed, demonstrating drastic differences in neutralization, a finding that has relevant practical implications in the selection of appropriate antivenoms to be used in different countries.

MATERIALS AND METHODS

Venoms and antivenoms: *Crotalus d. durissus* venom from Guatemala corresponds to a pool of six adult specimens collected in the Pacific versant of this country. Venoms of this subspecies from Costa Rica correspond to a pool from approximately 40 adult specimens collected in the province of Guanacaste and kept at Instituto Clodomiro Picado. In addition, venom from newborn specimens (20 - 30 days old) of *C. d. durissus* was a pool obtained from three different litters born at the same Institute. Venom of adult specimens of *C. d. terrificus* was obtained from Instituto Butantan, and venom from more than 20 adult specimens of *C. d. cumanensis* was collected in the state of Zulia, Venezuela. All venoms were centrifuged and then lyophilized, and kept at -40°C until used. Crotoxin (provided by Prof. J.R. Giglio, University of Sao Paulo, Brazil) was used in some experiments. Neutralization studies were performed with the following antivenoms: (a) liquid anti-crotalic antivenom, produced at Instituto Butantan, Sao Paulo, Brazil (IB antivenom; batch 8809198) using the venom of *C. d. terrificus* as antigen; and (b) liquid polyvalent antivenom produced at Instituto Clodomiro Picado, Costa Rica (ICP antivenom; batches 2941297 LQ and 2990598 LQ). This antivenom is prepared using a mixture of the venoms of *Bothrops asper*, *C. d. durissus* and *Lachesis stenophrys* as antigen (Bolaños and Cerdas 1980). Antivenoms were used before their expiry dates.

Electrophoresis: Venoms were compared by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), run under reducing conditions in 15% acrylamide gels (Laemmli 1970). Proteins were detected by Coomassie Brilliant Blue R-250. Molecular mass standards were run in parallel.

Pharmacological activities: All *in vivo* experiments were performed in Swiss-Webster mice and in all cases venom was dissolved in phosphate-buffered saline solution, pH 7.2 (PBS). Lethality was determined by the intraperitoneal route in 16 - 18 g mice, using four animals per dose and an injection volume of 0.5 ml. The median lethal dose (LD_{50}) was estimated using the Spearman-Karber method (Anonymous 1981). Lethality experiments were also used to determine the presence of 'crotamine-like' activity in these venoms, evidenced by spastic paralysis of the hind legs (Dos Santos *et al.* 1993). Hemorrhagic activity was assessed by intradermal injection in 18 - 20 g mice; the diameter of hemorrhagic spots was measured 2 hr after envenomation (Gutiérrez *et al.* 1985). Edema-forming activity was determined according to Yamakawa *et al.* (1976) 1 hr after subcutaneous injection of venom into the right foot pad of mice; the left pad was injected with PBS as control. Myotoxic activity was determined by measuring the creatine kinase (CK) activity in the plasma of mice 3 hr after intramuscular injection of venom solutions in the right gastrocnemius muscle, using the Sigma kit 520 (Sigma Chemical Co., Missouri, USA) (Gutiérrez *et al.* 1980). Coagulant activity was evaluated on human citrated plasma (Theaston and Reid 1983, Gené *et al.* 1989). Defibrinating activity was determined after intravenous injection of venom. Blood was collected 1 hr after injection and coagulation observed (Theakston and Reid 1983, Gené *et al.* 1989).

Neutralization studies: For each effect to be evaluated, a 'challenge dose' of venom (Gutiérrez *et al.* 1990) was selected. The 'challenge doses' for each effect were: (a) lethal, 4 LD_{50}; (b) hemorrhagic: five minimum hemorrhagic doses (MHD); (c) coagulant, two minimum coagulant doses (MCD); and (d) myotoxic, three minimum myotoxic doses (MMD). Definitions of these minimum doses are shown in the footnotes of Table 1. Mixtures, containing a constant amount of venom and various dilutions of antivenom, were prepared to attain various antivenom: venom ratios (expressed as µl antivenom per mg venom). Mixtures were incubated for 30 min at 37°C. Then, aliquots of the mixtures, containing a 'challenge dose' of venom, were tested in the corresponding experimental systems referred to above. Controls included venom incubated with no antivenom, antivenom incubated without venom and PBS alone. Neutralization was expressed as Effective Dose 50% (ED_{50}), defined as the ratio µl antivenom per mg venom at which the effect induced by venom was reduced 50% (Gutiérrez *et al.* 1990). In the case of coagulant and defibrinating activities, neutralization was expressed as Effective Dose (ED), as defined by Gené *et al.* (1989).

Quantification of anti-crotoxin titer in antivenoms: Anti-crotoxin titers of Costa Rican polyvalent and Brazilian anti-crotalic antivenoms were assessed by enzyme immunoassay. Briefly, microplates were coated with 0.4 µg crotoxin per well, and various dilutions of each antivenom were assayed. Bound antibodies were detected by an anti-equine immunoglobulin-peroxidase conjugate. Absorbances at 490 nm were recorded in a Dynatech MR 5000 microplate reader.

Statistical analysis: Results are presented as mean ± S.D. The significance of the differences between the mean values of two experimental groups was determined by the Student's *t* test.

RESULTS

Electrophoresis: Venoms of adult *C. d. durissus* from Guatemala and Costa Rica and *C. d. cumanensis* had a conspicuous yellow color, whereas those of newborn *C. d. durissus* and adult *C. d. terrificus* were colorless. Evident variations were observed in the electrophoretic patterns of the various venoms analyzed. Venoms of adult *C. d. durissus* specimens from Guatemala and Costa Rica showed predominant bands corresponding to proteins of 62, 42, 36, 29 and 15 kDa, and a faint band of 100 kDa. In addition, Guatemalan venom

showed a faint band of < 10 kDa (Fig. 1). In contrast, *C. d. terrificus* and newborn *C. d. durissus* venoms presented two conspicuous bands of 15 and 36 kDa, together with faint bands of higher molecular mass. Venom of newborn *C. d. durissus* had a 40 kDa band, and *C. d. terrificus* venom had a prominent low molecular mass band (< 10 kDa) (Fig. 1). The electrophoretic pattern of *C. d. cumanensis* venom included predominant bands of 62, 42, 36, 29 and 15 kDa, together with a band of < 10 kDa that migrated identically to a band in the venom of *C. d. terrificus* (results not shown). Crotoxin migrated as a band of 15 kDa (result not shown), corresponding to the molecular mass of crotoxin subunit B (Breithaupt *et al.* 1974).

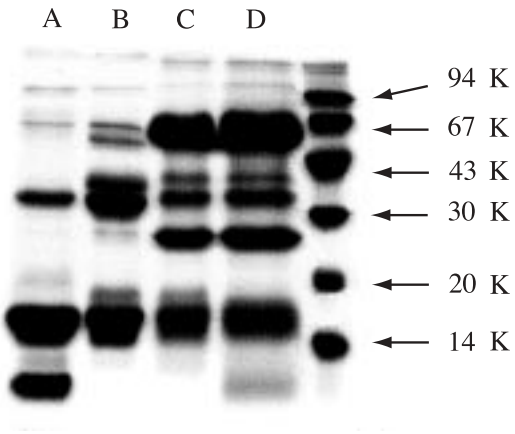

Fig. 1. SDS-polyacrylamide gel electrophoresis of the venoms of: (A) adult *C. d. terrificus* (Brazil); (B) newborn *C. d. durissus* (Costa Rica); (C) adult *C. d. durissus* (Costa Rica); (D) adult *C. d. durissus* (Guatemala). Electrophoresis was run under reducing conditions in 15% acrylamide gels and proteins were stained with Coomassie Brilliant Blue R-250. The right lane corresponds to molecular mass markers (in kDa).

Pharmacological activities: Table 1 depicts the conspicuous variations in the pharmacological activities of *C. durissus* venoms. Those of *C. d. terrificus* and newborn *C. d. durissus* present a similar pattern, characterized by their potent neurotoxicity, *i.e.* lethality, and myotoxicity, as well as by their lack of hemorrhagic activity. In contrast, venoms of adult *C. d. durissus* from Guatemala and Costa Rica displayed a similar profile of lower lethal and myotoxic effects, but with hemorrhagic activity. Venom of *C. d. cumanensis* presented a somehow mixed pattern, since it showed high lethality and myotoxicity but also induced hemorrhagic effect, although being less potent in this regard than the venom of *C. d. durissus*. All venoms assayed presented coagulant activity,

TABLE 1
Pharmacological activities of the venoms of various subspecies of C. durissus

Venom	Lethality[1]	Hemorrhagic[2]	Coagulant[3]	Myotoxic[4]
C. d. durissus (newborn)	2.0 (1.7 - 2.3)	(-)[5]	5.9 ± 0.8	338 ± 25
C. d. durissus (adult,Costa Rica)	16.0 (13.9 - 18.4)	5.0 ± 0.3	27.0 ± 0.2	65 ± 7
C. d. durissus (adult, Guatemala)	18.0 (14.6 - 23.0)	5.2 ± 0.7	19.0 ± 0.7	82 ± 9
C. d. cumanensis	3.0 (2.1 - 4.3)	24.0 ± 1.8	31.4 ± 2.3	521 ± 48
C. d. terrificus	1.3 (1.1 - 1.4)	(-)	26.4 ± 1.0	595 ± 68

1 Lethality: Median Lethal Dose (LD50), determined by the intraperitoneal route and expressed in µg venom per 16-18 g mouse; 95% confidence limits are included.

2 Hemorrhagic effect: Minimum Hemorrhagic Dose (MHD), defined as the amount of venom (in µg) which induces a hemorrhagic lesion of 10 mm diameter after 2 hr.

3 Coagulant: Minimum Coagulant Dose (MCD), defined as the amount of venom (in µg) which induces clotting of citrated human plasma in 60 sec.

4 Myotoxic: Plasma creatine kinase (CK) activity (units/ml) in mice 3 hr after injection of 10 µg venom in the right gastrocnemius muscle. One CK unit results in the phosphorylation of 1 nmol of creatine per min at 25ºC. CK activity of control mice injected with PBS was 22±4 units/ml.

5 (-): These venoms did not induce hemorrhage in the skin at the highest dose tested (50 µg).

with newborn *C. d. durissus* showing the highest effect when tested on human plasma. Only the venoms of *C. d. terrificus* and *C. d. cumanensis* displayed crotamine-like activity, as evidenced by the spastic paralysis developing in the hindlimbs of mice after intraperitoneal venom injection. No such activity was observed with the venoms of newborn and adult *C. d. durissus*. These observations correlate with the presence of a conspicuous low molecular mass band, probably corresponding to crotamine, when the venoms of *C. d. terrificus* and *C. d. cumanensis* were analyzed by SDS-PAGE.

Neutralization by antivenoms: Anti-crotalic antivenom produced at Instituto Butantan showed a very high effectiveness in the neutralization of the lethal effect of venoms from all the subspecies studied, including that of newborn *C. d. durissus* (Table 2). In contrast, polyvalent antivenom produced by Instituto Clodomiro Picado was only effective in the neutralization of lethality of venoms of *C. d. durissus* from Guatemala and Costa Rica, being ineffective against venoms of *C. d. terrificus* and *C. d. cumanensis*. Moreover, this antivenom showed a very low neutralizing activity against the venom of newborn *C. d. durissus* from Costa Rica. Butantan antivenom was effective in the neutralization of myotoxic activities of all venoms assayed, whereas Instituto Clodomiro Picado antivenom was effective only against the venoms of adult *C. d. durissus* from Costa Rica and Guatemala (Table 2). Since neurotoxicity, *i.e.* lethality, and myotoxicity induced by *C. d. terrificus* are due to the action of crotoxin, a phospholipase A_2 complex (Gopalakrishnakone *et al.* 1984, Bon 1997), the anti-crotoxin antibody titers of the two antivenoms were investigated by ELISA. As evidenced in Fig. 2, Butantan antivenom has a high antibody titer against crotoxin, whereas Instituto Clodomiro Picado antivenom has a very low titer.

Both antivenoms were effective in the neutralization of the coagulant activity of all venoms tested, Butantan antivenom showing again a higher neutralizing potency (Table 2). In contrast, Instituto Clodomiro Picado antivenom effectively neutralized hemorrhagic

TABLE 2

Neutralization of pharmacological activities of venoms of C. durissus *by antivenoms produced at Instituto Butantan (IB) and Instituto Clodomiro Picado (ICP)*[1]

Venom	Antivenom	Lethal	Hemorrhagic	Coagulant	Myotoxic
C. d. durissus (newborn)	ICP	No neutral.[2]	(-)[3]	267 ± 5	No neutral.
	IB	< 150[4]	(-)	58 ± 1	Neutralization
C. d. durissus (Costa Rica)	ICP	454 (408-514)	122 ± 9	221 ± 5	Neutralization
	IB	< 150	No neutral	21 ± 1	Neutralization
C. d. durissus (Guatemala)	ICP	380 (276-492)	181 ± 19	160 ± 2	Neutralization
	IB	< 150	No neutral	9 ± 0.5	Neutralization
C.d. cumanensis	ICP	No neutral.	558 ± 23	508 ± 15	No neutral.
	IB	< 150	No neutral	4 ± 0.1	Neutralization
C. d. terrificus	ICP	No neutral.	(-)	208 ± 11	No neutral.
	IB	500 (412-560)	(-)	33 ± 1	Neutralization

1 In the case of lethal and hemorrhagic effects, neutralization is expressed as Effective Dose 50% (ED_{50}), defined as the ratio µl antivenom/mg venom at which the effect is neutralized by 50%. In the case of coagulant activity, neutralization is expressed as Effective Dose (Gené et al. 1989). In the case of myotoxic activity, only one antivenom/venom ratio was tested (2000 µl antivenom/mg venom). Neutralizing ability is therefore expressed as either 'neutralization' or 'no neutralization' at this ratio. 'Neutralization' means that myotoxic activity was reduced more than 50%.

2 'No neutral.' means that antivenom did not neutralize the effect at the highest antivenom/venom ratio tested (2000 µl antivenom/mg venom).

3 (-) means that the venom did not exert the activity being tested.

4 In these cases, all mice survived at the lowest antivenom/venom ratio tested (150 µl antivenom/mg venom).

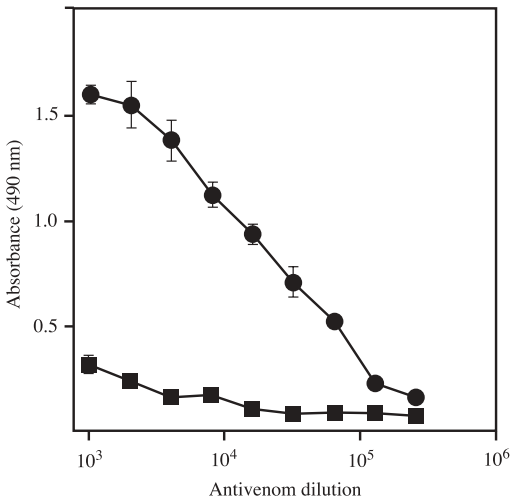

Fig. 2. Titration of antibodies to crotoxin in Instituto Clodomiro Picado (■) and Instituto Butantan (●) antivenoms by enzyme immunoassay. Microplates were coated with 0.4 µg crotoxin per well, and various dilutions of each antivenom were assayed. Bound antibodies were detected by an anti-equine immunoglobulin-peroxidase conjugate. Absorbances at 490 nm were recorded in a Dynatech MR 5000 microplate reader. Each point represents mean ± SD (n = 4).

activity of the venoms of adult *C. d. durissus* and of *C. d. cumanensis*, whereas Butantan antivenom failed to neutralize this effect. As described above, venoms from *C. d. terrificus* and newborn *C. d. durissus* were devoid of hemorrhagic activity at the doses tested.

DISCUSSION

The present study evidenced a conspicuous biochemical and pharmacological geographic and ontogenetic variability in the venom of *C. durissus*, further extending previous observations on this subject (Lomonte *et al.* 1983, Gutiérrez *et al.* 1991, Dos Santos *et al.* 1993, Santoro *et al.* 1999). The case of *C. durissus* constitutes one of the most conspicuous examples on how biochemical differences in venom composition may bring prominent pathophysiological and therapeutic implications in snakebite envenomings. The venom of *C. d. terrificus* contains high amounts of crotoxin (Hendon and Bieber 1982) and induces neurotoxicity and systemic myotoxicity, in contrast with the venom of adult *C. d. durissus* from Central America which contains low amounts of this phospholipase A_2 complex (Gutiérrez *et al.* 1991). In addition, *C. d. terrificus* venom contains crotamine, a low molecular mass myotoxic protein that induces spastic paralysis in mice (Jiménez-Porras 1970). These observations agree with clinical reports, since signs and symptoms of *C. durissus* envenomings in Central and South America drastically differ (Rosenfeld 1971, Bolaños *et al.* 1981, Azevedo-Marques *et al.* 1985, 1987, Fan and Cardoso 1995, Gutiérrez 1995). Rattlesnake envenomings in Brazil and other parts of South America are characterized by their high severity, usually associated with conspicuous neurotoxicity, systemic myotoxicity and acute renal failure (Azevedo-Marques *et al.* 1985, 1987, Fan and Cardoso 1995), together with defibrination (Sano-Martins *et al.* 2001). In contrast, accidents by this species in Central America are less severe, being mainly associated with local manifestations, *i.e.* edema, hemorrhage and necrosis, and moderate systemic alterations such as coagulopathies and bleeding (Bolaños *et al.* 1981). Thus, our findings using a rodent laboratory model agree in qualitative terms with the clinical observations.

Two previous studies have addressed the issue of venom variability between the various South American subspecies of *C. durissus* (Dos Santos *et al.* 1993, Santoro *et al.* 1999). It was shown that the venoms of *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* were very similar (Santoro *et al.* 1999), and that a 'white' venom of *C. d. ruruima* also showed strong similarities with that of *C. d. terrificus* (Dos Santos *et al.* 1993). Interestingly, the 'yellow' venom of *C. d. ruruima*, a subspecies distributed in northern Brazil and Venezuela (Campbell and Lamar 1989), exerts hemorrhagic and dermonecrotic activities, in addition to neurotoxic, coagulant and myotoxic effects (Dos Santos *et al.* 1993). Our observations on *C. d. cumanensis* venom indicate that it has similarities with the 'yellow' venom of *C. d. ruruima*, since it also induces

hemorrhage and presents a yellow coloration. Moreover, venom of *C. durissus* from Colombia was also reported to induce hemorrhagic activity in mice (Otero *et al.* 1992), and a hemorrhagic proteinase was isolated from the venom of *C. vegrandis*, a South American species closely related to *C. durissus* (Aguilar *et al.* 2001). Thus, at least some *C. durissus* venoms from northern South America may induce local tissue damage associated with hemorrhage and inflammation, besides provoking the neurotoxic, myotoxic and defibrinating activities typical of other subspecies in the region. These observations urge caution when diagnosing rattlesnake bites in northern South America, since local tissue damage might be expected in these cases, besides the characteristic neurotoxic manifestations.

On the other hand, little variation was observed when comparing the venoms of adult *C. d. durissus* from Guatemala and Costa Rica, further strenghtening previous observations on the lack of prominent intraspecies variability in the venoms of various Central American crotaline snakes (Saravia *et al.* 2000, Rojas *et al.* 2001). Adult *C. durissus* venoms induce a pathophysiological profile similar to that of *Bothrops* venoms, characterized by local tissue damage, *i.e.* necrosis, hemorrhage, edema and pain, together with systemic disturbances such as defibrination and bleeding (Bolaños *et al.* 1981), being noticeable the lack of neurotoxic manifestations. However, the venom of newborn specimens of *C. d. durissus* induces an experimental pathophysiology similar to that of South American venoms, due to the relatively high crotoxin concentration in this venom (Lomonte *et al.* 1983, Gutiérrez *et al.* 1991). Thus, a complex developmentally-regulated pattern of protein venom expression occurs in *C. d. durissus*. Although there are no published reports on envenomings by newborn and juvenile specimens of *C. durissus* in Central America, our observations strongly suggest that these cases might be associated with lack of local effects and with neurotoxic manifestations, therefore resembling coral snake (*Micrurus*) envenomings (Gutiérrez 1995). Physicians must be aware of this possibility and laboratory blood clotting tests are recommended for differential diagnosis, since newborn *C. d. durissus* venom probably induces defibrination whereas *Micrurus* venoms do not.

Owing to the prominent intraspecies variability in venom composition and action, it is highly important to assess the efficacy of various *Crotalus* antivenoms produced in the Americas. Antivenoms produced using Central American rattlesnake venom failed to neutralize lethal, *i.e.* neurotoxic, and myotoxic activities of the venoms of South American subspecies of *C. durissus*. This is associated with an extremely low anti-crotoxin antibody titer in this antivenom, since the venom from adult *C. d. durissus* has very little content of this neurotoxic and myotoxic phospholipase A_2 (Gutiérrez *et al.* 1991). In contrast, Butantan antivenom is highly effective in the neutralization of lethal and myotoxic effects induced not only by Brazilian rattlesnake venoms, but also by those of *C. d. cumanensis* and *C. d. durissus*. Moreover, it effectively neutralizes lethality of venom of newborn *C. d. durissus*, as this venom contains high amounts of crotoxin (Gutiérrez *et al.* 1991). However, Butantan antivenom failed to neutralize hemorrhagic activity of the venoms of *C. d. durissus* and *C. d. cumanensis*, probably due to the lack of hemorrhagic components in the venom of *C. d. terrificus* used as immunogen in the preparation of anti-crotalic antivenom in Brazil. The lack of neutralization of hemorrhagic effect would preclude the use of this antivenom for rattlesnake envenomations in Central America and in some regions in northern South America. It was previously observed by Dos Santos *et al.* (1993) that Brazilian anti-crotalic antivenom was ineffective in the neutralization of hemorrhagic activity of 'yellow' *C. d. ruruima* venom whereas a bothropic-crotalic antivenom was effective in neutralizing not only lethality but also hemorrhagic effect of this venom. On the basis of our observations and those of Dos Santos *et al.* (1993) it is suggested that a bothropic-crotalic antivenom, using *Bothrops* sp. and *C. d. terrificus* venoms

in the immunizing mixture, would be effective in the neutralization of the most relevant toxic effects of all subspecies of *C. durissus* in South, Central and North America. Alternatively, antivenoms prepared using the venoms of *C. d. durissus* and *C. d. terrificus* as antigens for immunization should be effective in the neutralization of venoms of the various subspecies of *C. durissus*.

Both Costa Rican and Brazilian antivenoms were effective in the neutralization of coagulant activity on plasma of all venoms tested, evidencing immunological similarities between clotting enzymes in the venoms of these subspecies. The Brazilian antivenom was particularly effective in this regard. Coagulant activity of *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* is due to the action of thrombin-like enzymes (Santoro *et al.* 1999), and such an enzyme was isolated from the venom of *C. d. terrificus* (Raw *et al.* 1986). It is likely that similar components are present in *C. d. cumanensis* and *C. d. durissus*.

In conclusion, there is a prominent geographic and ontogenic variability in the biochemistry and pharmacology of the venom of *C. durissus*. As a consequence, drastically different pathophysiological profiles are associated with envenomations by this species in Central and South America. Moreover, antivenoms produced in Central and South America show different neutralizing efficacy, depending on the nature of the venom being neutralized. This is an example of conspicuous intraspecies venom variability with evident pathophysiological and therapeutic implications that clinicians and antivenom producers need to be aware of.

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RESUMEN

Se efectuó un estudio comparativo de los venenos de especímenes adultos de la serpiente cascabel neotropical, *Crotalus durissus*, de Guatemala, Costa Rica, Venezuela y Brasil, junto con el veneno de ejemplares recién nacidos de *C. d. durissus* de Costa Rica. Se observaron drásticas diferencias intraespecíficas, dado que los venenos de Brasil (*C. d. terrificus*) y los recién nacidos de *C. d. durissus* presentaron un patrón electroforético caracterizado por bandas predominantes de 36 y 15 kDa, en tanto los venenos de especímenes adultos de *C. d. durissus* de Costa Rica y Guatemala, así como el de *C. d. cumanensis* de Venezuela, presentaron bandas predominantes de 62, 36, 29 y 15 kDa. Además, los venenos de *C. d. terrificus* y *C. d. cumanensis* presentaron una banda prominente de < 10 kDa, la cual probablemente corresponde a 'crotamina', dado que se observó actividad 'tipo-crotamina' cuando estos venenos se inyectaron en ratones por la vía intraperitoneal. Los venenos de *C. d. terrificus*, *C. d. cumanensis* y recién nacidos de *C. d. durissus* presentaron actividades letal y miotóxica más fuertes que los de ejemplares adultos de *C. d. durissus*. Por otra parte, los de adultos de *C. d. durissus* y *C. d. cumanensis* indujeron hemorragia, en tanto que este efecto no se observó con los de *C. d. terrificus* y recién nacidos de *C. d. durissus*. Todos los venenos mostraron actividad coagulante sobre plasma. El antiveneno anti-crotálico producido en el Instituto Butantan (Brasil), usando veneno de *C. d. terrificus* como antígeno, fue eficaz en la neutralización de los efectos letal, miotóxico y coagulante de todos los venenos estudiados, aunque fue ineficaz en la neutralización de la actividad hemorrágica de los venenos de *C. d. cumanensis* y *C. d. durissus*. Por otra parte, el antiveneno polivalente producido por el Instituto Clodomiro Picado (Costa Rica), utilizando los venenos de *Bothrops asper*, *C. d. durissus* y *L. stenophrys* como antígeno, fue eficaz en la neutralización de los efectos letal, miotóxico, hemorrágico y coagulante del veneno de *C. d. durissus*, pero fue ineficaz en la neutralización de la letalidad y la miotoxicidad de los venenos de *C. d. terrificus*, *C. d. cumanensis* y recién nacidos de *C. d. durissus*. La alta toxicidad de los venenos de cascabeles sudamericanas y de ejemplares recién nacidos de *C. d. durissus* se debe al alto contenido de 'crotoxina', un complejo bimolecular con actividad fosfolipasa A_2 con potentes acciones neurotóxica y miotóxica. En

concordancia, el antiveneno brasileño tiene un título de anticuerpos anti-crotoxina mucho mayor que el antiveneno de Costa Rica.

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