Brazilian J Med Biol Res (1990) 23: 427-435

SOUTH AMERICAN SNAKE VENOM PROTEINS ANTIGENICALLY RELATED TO *BOTHROPS ASPER* MYOTOXINS

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1. The presence of proteins antigenically related to *Bothrops asper* myotoxins in various snake venoms, mainly from South America, was investigated by using polyclonal and monoclonal antibodies.

2. Myotoxin-like components were detected in ten *Bothrops* venoms from South America, and in the venoms of *Crotalus atrox* (North America), *Trimeresurus flavoviridis* (Japan), and *Micrurus alleni* (Costa Rica).

3. Cross-reactive components detected in several *Bothrops* venoms show a common subunit of 15-16 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, although significant charge variations are evident by immunoelectrophoresis.

4. It is concluded that proteins antigenically related to *B. asper* myotoxins are relatively common in the genus *Bothrops* and, in the light of findings discussed, are likely to possess myotoxic activity.

Key words: myotoxin, snake venom, *Bothrops*, cross-reactions.

Introduction

The study of antigenic cross-reactivities between components of snake venoms from different genera and species, and of venoms from the same species but from different geographic regions, is relevant for several reasons. From a basic point of view, it provides information about the evolutionary relationships between the groups studied and, from an applied point of view, the knowledge obtained is essential for the design of immunogen mixtures for antivenom production at the industrial level. These studies also permit the prediction of the cross-neutralizing ability of antivenoms towards heterologous venoms.

The genus *Bothrops* comprises many species of crotaline snakes distributed in the Americas from Mexico to Argentina (Hoge and Romano-Hoge, 1978). There are only a few studies on the antigenic relationships of bothropic venoms at the individual component level (Lomonte et al., 1985, 1987b; Mandelbaum and Assakura, 1988). *Bothrops asper*, a medically

Research supported by Vicerrectoría de Investigación, Universidad de Costa Rica (No. 741-88-009), International Foundation for Science (No. F/1388-1) and UNESCO (No. 1042-R). B.L., G.R. and J.M.G. were the recipients of research career awards from CONICIT (Costa Rican National Scientific and Technological Research Council). Publication supported by FAPESP.

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relevant snake species in Central America, causes prominent local myonecrosis in envenomated patients, among other effects (Bolaños, 1982). This effect is induced mainly by the direct action of myotoxins on skeletal muscle (Gutiérrez and Cerdas, 1984). Other bothropic venoms have also been found to induce myonecrosis (Gutiérrez and Chaves, 1980; Mebs et al., 1983; Queiroz and Petta, 1984; Queiroz et al., 1984, 1985; Homsi-Brandeburgo et al., 1988). Two myotoxins of *B. asper* venom have been isolated and characterized. *B. asper* myotoxin I (Gutiérrez et al., 1984, 1986a) is a basic phospholipase A_2 whereas myotoxin II (Lomonte and Gutiérrez, 1989) is a closely related isoform devoid of phospholipase A_2 activity. These myotoxin isoforms cross-react extensively by immunochemical and neutralization techniques (Lomonte and Kahan, 1988; Lomonte and Gutiérrez, 1989). Antiserum to *B. asper* myotoxin I has been utilized to detect antigenically related components in the venoms of several Central American snakes (Lomonte et al., 1987b). In the present study, the presence of components antigenically related to *B. asper* myotoxins I and II in the venoms of several South American snake species, and some species from other regions, has been investigated by immunochemical techniques, using polyclonal and monoclonal antibodies.

Material and Methods

Venoms

Venoms of South American species were obtained by the serpentarium of the Instituto Clodomiro Picado through exchange with collaborators in Peru, Colombia, and Ecuador. Venoms from Brazilian specimens were obtained from the collection of the Seção de Venenos, Instituto Butantan. Venoms from other continents were obtained from the W.H.O. All venoms were pools obtained from many specimens, lyophilized and stored at -20°C.

Antibodies to myotoxins

Antisera to *B. asper* myotoxins I and II were prepared as previously described (Lomonte et al., 1987a), by immunization of rabbits with purified myotoxins. Monoclonal antibodies MAb-3 and MAb-4 were obtained from murine hybridomas as described by Lomonte and Kahan (1988). MAb-3 recognizes both *B. asper* myotoxins I and II, whereas MAb-4 reacts with myotoxin I, but not with myotoxin II, in several immunochemical assays (Lomonte and Kahan, 1988; Lomonte and Gutiérrez, 1989). In some cases, a polyclonal antiserum to myotoxin I produced in mice was utilized as a control for assays involving anti-mouse immunoglobulin conjugates.

Dot-blotting

Binding of polyclonal and monoclonal antibodies to components of snake venoms was tested by dot-blotting (Towbin and Gordon, 1984). Drops of 2 µl of each venom (2.5 mg/ml) were applied onto a nitrocellulose membrane (Bio-Rad Laboratories, California,

USA) and allowed to dry for 5 min. The membrane was immersed in blocking solution (1%, 10 mg/ml, w/v, bovine serum albumin and 1%, 10 mg/ml, v/w, casein in sodium phosphate containing 0.04 NaCl, pH 7.2, PBS) for 2 h at room temperature, with slow agitation. The membrane was then immersed in a solution containing the antibodies (rabbit anti-myotoxin I, rabbit anti-myotoxin II, MAb-3, MAb-4, mouse anti-myotoxin I) diluted 1:100. Control membranes were immersed in normal rabbit serum and normal mouse serum solutions (1:100). All dilutions were prepared in washing solution (0.1% blocking solution in PBS). After 3 h of incubation, the nitrocellulose membranes were washed four times (10 min each time). Antibody binding was detected by the addition of a protein A-horseradish peroxidase conjugate (1:100; Lomonte et al., 1987b) in the case of rabbit antibodies, or an anti-mouse IgG-alkaline phosphatase conjugate (1:500; Sigma Chemical Co., Missouri, USA) in the case of mouse antibodies. Peroxidase was detected by the addition of H_2O_2 and 4-chloro-1-naphtol (Bio-Rad), whereas alkaline phosphatase was detected by the addition of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitrobluetetrazolium (Sigma). *B. asper* venom was included as a control. Experiments were performed twice.

Gel immunodiffusion

Rabbit polyclonal antibodies to myotoxin II were tested against venoms by gel immunodiffusion (Ouchterlony and Nillson, 1978). Thirty μl of venom solutions (0.5, 1 and 2 mg/ml) or antisera were applied to wells made in 1% agarose gels dissolved in PBS. Plates were incubated 24-30 h at room temperature, washed with PBS and stained with Coomassie blue R-250. *B. asper* venom (0.5 mg/ml) was included as a control. Experiments were performed twice.

Immunoelectrophoresis and immunoblotting

A group of venoms from Brazilian species that gave a positive result by dot-blotting and gel immunodiffusion against anti-myotoxin sera was further characterized by immunoelectrophoresis (Ouchterlony and Nilsson, 1978) and by immunoblotting (Towbin and Gordon, 1984). Immunoelectrophoresis was carried out in 1% agarose gels with 0.1 M Tris-HCl and 0.3 M glycine buffer, pH 8.6. Thirty μl of venoms (10 mg/ml) were separated at 80 volts for 50 min, troughs were filled with 300 μl of rabbit anti-myotoxin II serum, and diffusion was allowed to occur for 24-30 h at room temperature. Plates were washed and stained as described above. For immunoblotting, venom samples (200 μg) were electrophoresed on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the procedure of Laemmli (1970). All samples were reduced with 2-mercaptoethanol. Molecular weight standards (Pharmacia Fine Chemicals, Uppsala, Sweden) were included in the gels. After separation, proteins were transferred to nitrocellulose as described by Towbin et al. (1979). Then, positions of molecular weight standards and relevant venom bands were visualized on the nitrocellulose by reversible non-denaturing amidoblack staining (Syu and Kahan, 1987) and marked by punching pinholes. Immunodetection of specific bands was carried out as described above for dot-blotting, using rabbit polyclonal antibodies. *B. asper* venom was included as a control. Experiments were performed twice.

Results

Dot-blotting analysis

A summary of the dot-blotting tests of all venoms and antibodies studied is presented in Table I. An example of the reactions of a group of venoms, using rabbit

Table 1 - *Dot-blot detection of proteins antigenically related to* Bothrops asper *myotoxins in snake venoms.*

ra-I, Rabbit anti-myotoxin I serum; ra-II, rabbit anti-myotoxin II serum; MAb-3, MAb-4, monoclonal antibodies 3 and 4; ma-I, mouse anti-myotoxin I serum; -, no reaction; +, moderate reaction; ++, intense reaction; NT, not tested.

Venom	Origin	Reaction with antibodies				
		ra-I	ra-II	MAb-3	MAb-4	ma-I
Bothrops asper	Costa Rica	++	++	++	++	++
B. atrox	Ecuador	++	++	-	-	++
B. atrox	Peru	++	++	-	-	++
B. atrox	Brazil	+	+	-	-	NT
B. atrox	Colombia	++	++	++	+	NT
B. jararacussu	Brazil	++	+	+	-	NT
B. erythromelas	Brazil	-	-	-	-	NT
B. neuwiedi	Brazil	++	+	-	-	NT
B. cotiara	Brazil	-	-	-	-	NT
B. marajoensis	Brazil	+	+	-	-	NT
B. jararaca	Brazil	+	+	+	-	NT
B. alternatus	Brazil	-	-	-	-	NT
B. moojeni	Brazil	+	+	+	-	NT
B. castelnaudi	Peru	-	-	-	-	-
B. colombiensis	Venezuela	++	++	++	-	++
B. barnetti	Peru	-	-	-	-	-
B. pictus	Peru	+	+	-	-	+
B. bilineatus	Peru	++	++	-	-	+
B. xanthograma	Ecuador	++	++	++	-	++
B. brazili	Peru	++	++	++	-	++
Lachesis muta	Peru	-	-	-	-	-
Crotalus atrox	USA	+	+	-	-	+
C. d. terrificus	Brazil	-	-	-	-	-
Trimeresurus flav.	Japan	++	+	-	-	+
Micrurus alleni	Costa Rica	+	+	+	-	+
M. mipartitus	Costa Rica	-	-	-	-	-
M. surinamensis	Colombia	-	-	-	-	-
M. frontalis	Argentina	-	-	-	-	-
M. carinicauda	Colombia	-	-	-	-	-
Naja n. kaouthia	Thailand	-	-	-	-	-
Echis carinatus	Iran	-	-	-	-	-
Notechis scutatus	Australia	-	-	-	-	-
Vipera russelli	India	-	-	-	-	-

anti-myotoxin I as a probe, is shown in Figure 1. Positive reactions of variable intensities were obtained with ten *Bothrops* species, using polyclonal sera against myotoxins I and II (Table 1; Figure 1). Venoms from *Crotalus atrox, Trimeresurus flavoviridis,* and *Micrurus alleni* also gave positive reactions (Table 1; Figure 1). Rabbit polyclonal antibodies to myotoxins I and II, as well as mouse anti-myotoxin I polyclonal antibodies, all gave identical reactivity patterns, with only slight differences in the intensity of the reactions. Monoclonal antibodies reacted with fewer venoms than polyclonal antibodies (Table 1).

Gel immunodiffusion

All venoms giving a positive reaction in the dot-blotting test also precipitated by gel immunodiffusion against rabbit anti-myotoxin II serum. Precipitin reactions varied in intensity and sharpness, making it difficult to establish identity or partial identity relationships with the homologous antigen.

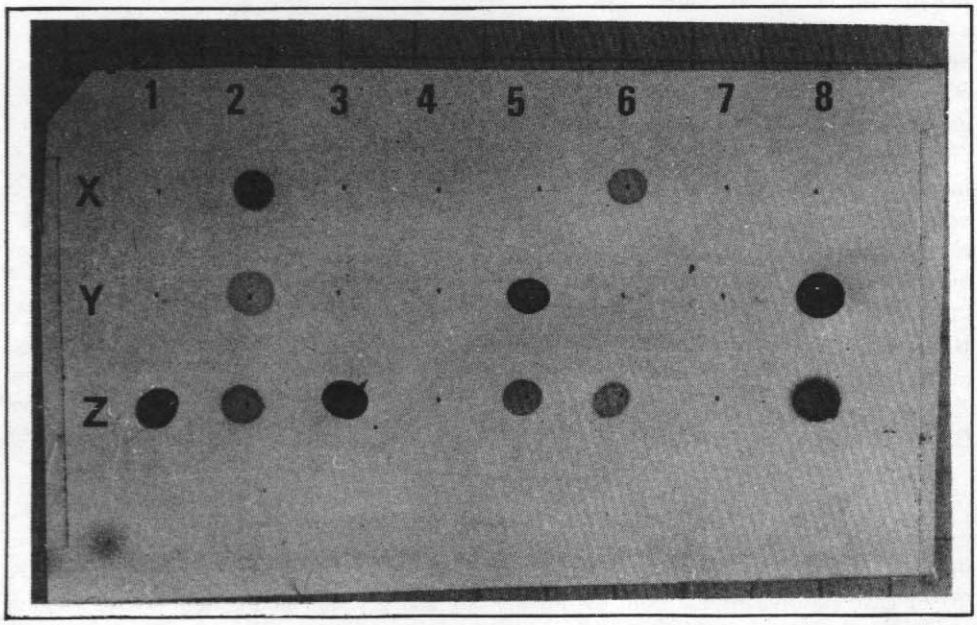

Figure 1 - Dot-blot detection of proteins antigenically related to *Bothrops asper* myotoxins in different venoms, by dot-blotting. Samples were applied onto nitrocellulose membrane and processed as described in Material and Methods. X1, *Bothrops castelnaudi;* X2, *B. colombiensis;* X3, *B. barnetti;* X4, *Micrurus mipartitus;* X5, *Naja naja atra;* X6, *B. pictus;* X7, *Naja n. kaouthia;* X8, *Lachesis muta;* Y1, *Micrurus surinamensis;* Y2, *M. alleni;* Y3, *Echis carinatus;* Y4, *Notechis scutatus;* Y5, *B. bilineatus;* Y6, *Loxosceles laeta* (spider); Y7, *Vipera russelli;* Y8, *B. xanthograma;* Z1, *B. atrox* (Ecuador); Z2, *B. atrox* (Peru); Z3, *B. brazili;* Z4, *Micrurus frontalis;* Z5, *Trimeresurus flavoviridis;* Z6, *Crotalus atrox;* Z7, *Micrurus carinicauda;* Z8, *B. asper.*

Immunoelectrophoresis and immunoblotting

Immunoelectrophoresis showed two types of reaction patterns. *B. neuwiedi, B. jararacussu* (Figure 2), *B. moojeni,* and *B. atrox* (Colombia) venoms, all formed a conspicuous precipitation arc towards the cathode, at the same position as *B. asper* venom. On the other hand, *B. jararaca, B. marajoensis,* and *B. atrox* (Brasil) venoms (Figure 2) formed a precipitation arc near the origin, slightly towards the anode. By immunoblotting, *B. moojeni, B. neuwiedi, B. jararacussu,* and *B. atrox* (Colombia) venoms gave positive reactions, with a cross-reactive band migrating to the same position as *B. asper* myotoxins I and II, corresponding to an estimated molecular weight of 15-16 kDa. *B. atrox* venom from Brazil did not react by immunoblotting.

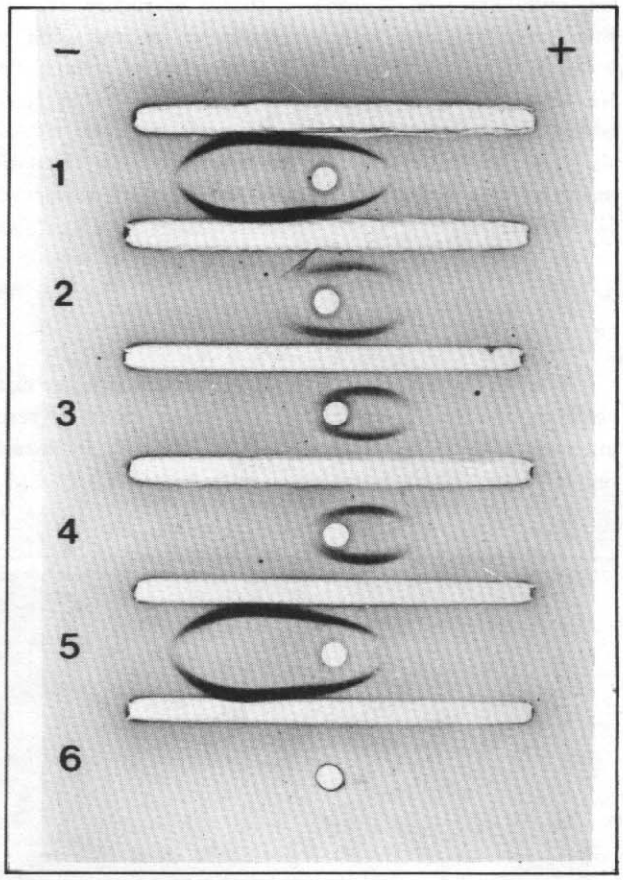

Figure 2 - Immunoelectrophoretic analysis of components antigenically related to *B. asper* myotoxin in different snake venoms. Venoms were analyzed by immunoelectrophoresis against rabbit antiserum to *B. asper* myotoxin II, as described in Material and Methods. 1, *B. neuwiedi;* 2, *B. atrox* (Brazil); 3, *B. jararaca;* 4, *B. marajoensis;* 5, *B. jararacussu;* 6, *B. alternatus.*

Discussion

The present results demonstrate the presence of proteins antigenically related to *B. asper* myotoxins (I and II) in the venoms of several South American snake species. Of 16 *Bothrops* species tested, 10 (62%) gave positive results both by dot-blotting and gel immunodiffusion. These findings expand the list of bothropic venoms possessing myotoxin-like components (Lomonte et al., 1985, 1987b) and support the conclusion that this type of toxin is relatively frequent and conserved among *Bothrops* venoms. Most bothropic venoms have been found to induce myonecrosis (Gutiérrez and Chaves, 1980; Mebs et al., 1983; Queiroz and Petta, 1984; Queiroz et al., 1984, 1985; Homsi-Brandeburgo et al., 1988). Since in the case of *Bothrops asper* venom, myotoxins are the most important factors in skeletal muscle damage (Lomonte et al., 1987a), it would be interesting to investigate the role of cross-reacting components

from other venoms in the pathogenesis of this local effect. Previous studies identified proteins antigenically related to *B. asper* myotoxin I in the venoms of several Costa Rican *Bothrops* species (Lomonte et al., 1987b). Of these, a cross-reactive protein from *B. nummifer* has been isolated and characterized as a myotoxin devoid of phospholipase A_2 activity (Gutiérrez et al., 1986b) and resembling *B. asper* myotoxin II. In addition, a cross-reactive protein from the venom of *B. godmani* also has myotoxic activity (C. Díaz, personal communication). Thus, searching for myotoxic factors in bothropic venoms by an immunochemical approach, using antibodies to *B. asper* myotoxins as a probe, has proved to be an effective method. On the basis of the present results, myotoxic factors from the venoms of *B. moojeni* from Brasil and *B. atrox* from Colombia are currently being purified in our laboratory.

Of the venoms studied by immunoblotting, those of *B. moojeni, B. neuwiedi, B. jararacussu,* and *B. atrox* (Colombia) showed positive bands of about 15-16 kDa molecular weight. This finding was expected since, with the single exception of *B. picadoi,* all cross-reactive components described so far for *B. schlegelii, B. nummifer, B. godmani,* and *Agkistrodon bilineatus* venoms show a molecular weight of 15-16 kDa by SDS-polyacrylamide gel electrophoresis (Lomonte et al., 1987b). Immunoelectrophoretic analysis of venoms showed heterogeneity in the net charge of the cross-reactive components. One group of venoms (see Results) had myotoxin-like components with a basic isoelectric point similar to that of *B. asper* myotoxin, whereas another group had myotoxin-like components with a more neutral isoelectric point, as judged by their migration in immunoelectrophoresis. This finding agrees with results of previous studies which demonstrated that, although molecular weight of components antigenically related to *B. asper* myotoxins from other venoms is conserved, significant variations in charge occur (Lomonte et al., 1987b). Homsi-Brandeburgo et al. (1988) described the isolation of a myotoxin (bothropstoxin) from the venom of *B. jararacussu* which has a basic isoelectric point and a molecular weight and amino acid composition similar to those of *B. asper* myotoxins. It is likely that the cross-reaction observed between this venom and anti-*B. asper* myotoxin antibodies is due to bothropstoxin.

In addition to the reactivity of anti-*B. asper* myotoxin antibodies towards several bothropic venoms, positive reactions were obtained in three non-bothropic venoms. *Trimeresurus flavoviridis* is closely related to *Bothrops* species (Cadle, 1987), and immunochemical studies using crude venoms and antivenoms have demonstrated antigenic similarities between these two genera (Tu et al., 1980). *T. flavoviridis* venom is known to induce severe myonecrosis (Okonogi et al., 1964), and contains a myotoxin with physicochemical and enzymatic properties similar to those of *B. asper* myotoxin I (Mebs and Samejima, 1986). Thus, cross-reactivity with *T. flavoviridis* venom is not surprising and suggests that myotoxic phospholipases A_2 originated early in the evolutive history of the *Bothrops* and *Trimeresurus* group. More unexpected was cross-reactivity with the venom of *Crotalus atrox,* especially the strong positive reaction obtained with the venom of *Micrurus alleni,* an elapid snake. These two venoms are known to induce myonecrosis (Ownby and Colberg, 1988; Gutiérrez et al., 1983).

Monoclonal antibodies were more selective in their reactivity towards venoms that gave positive cross-reactions using polyclonal antibodies. MAb-3 reacted with the venom of *B. atrox* from Colombia, but not with the venom of *B. atrox* from Brazil. Interestingly, these

two venoms showed a clear difference in the charge of their cross-reactive components. MAb-3 also reacted with several other bothropic venoms. On the other hand, MAb-4 reacted only with the venom of *B. atrox* from Colombia. These results suggest that MAb-3 is directed at a relatively conserved epitope of myotoxins, whereas MAb-4 is directed at a different, non-conserved epitope. In this regard, it is interesting to point out that MAb-3 reacted with the venom of *M. alleni*.

In conclusion, the present results demonstrate that *B. asper* myotoxin-like components are relatively common and conserved in the genus *Bothrops*, constituting a group of proteins with a subunit molecular weight of about 15-16 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis, but with significant charge heterogeneity. The physicochemical and immunochemical similarities observed between *B. asper* myotoxins and the myotoxin-like proteins of other bothropic venoms suggest the possibility of a common mechanism of action on skeletal muscle. The isolation and characterization of new bothropic myotoxins will allow this hypothesis to be tested.

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Received August 3, 1989
Accepted March 23, 1990