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Phylogenetic conservation of a snake venom metalloproteinase epitope recognized by a monoclonal antibody that neutralizes hemorrhagic activity

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Abstract

Snake venom metalloproteinases (SVMPs) are present in large quantities in venoms of viper snakes and also in some elapids. Jararhagin is a representative of a P-III multidomain hemorrhagic SVMP present in *Bothrops jararaca* venom. It is comprised of a catalytic, a disintegrin-like and a cysteine-rich domain. Seven anti-jararhagin monoclonal antibodies (MAJar 1–7) were produced, of which six reacted with the disintegrin domain. MAJar 3 recognized an epitope present at the C-terminal part of the disintegrin-like domain, and neutralized jararhagin-induced hemorrhage. In this study, we evaluated the reactivity of these monoclonal antibodies with venoms from 27 species of snakes belonging to different families. MAJar 3 recognized most of the hemorrhagic venoms. By ELISA, MAJar 3 reacted strongly with venoms from Viperidae family and weakly with Colubridae and Elapidae venoms. This recognition pattern was due to bands between 50 and 80 kDa, corresponding to P-III SVMPs. This antibody preferentially neutralized the hemorrhage induced by venoms of *Bothrops* snakes. This fact suggests that the epitope recognized by MAJar 3 is present in other metalloproteinases throughout snake phylogeny. However, slight structural differences in the epitope may result in insufficient affinity for neutralization of biological activities.

Keywords: Snake venom; Metalloproteinase; Hemorrhage; Phylogeny; Monoclonal antibody

1. Introduction

Snake venom metalloproteinases (SVMPs) comprise a series of zinc-dependent enzymes of varying molecular mass which are present in large quantities in venoms of viper snakes and also, although in minor proportions, in Elapidae venoms (Matsui et al., 2000). They are responsible for the conspicuous hemorrhage often associated with snakebites, representing therefore an important antigen to target in antivenom therapy for snakebites. Jararhagin, an archetypical P-III SVMP from *B. jararaca*, is one of the main venom components involved in the local and systemic symptoms observed in envenomed humans (Paine et al., 1992). As all P-III SVMPs, jararhagin is comprised of a catalytic domain plus a disintegrin-like/cysteine-rich domain (Paine et al., 1992). The catalytic activity is involved in hydrolysis of extracellular matrix components and also some plasma proteins important for hemostasis (Kamiguti et al., 1996). These mechanisms would be the main factors contributing to hemorrhagic activity. However, the participation of the disintegrin domain for expression of hemorrhagic activity is also supported in the literature (Harrisson et al., 2000; Bjarnason and Fox, 1994). In a recent

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contribution (Tanjoni et al., 2003), we studied this aspect using monoclonal antibodies directed to the jararhagin disintegrin-like domain. A neutralizing antibody uncovered the presence of an epitope in this domain that is essential for the hemorrhagic activity of the enzyme and also inhibits its binding to collagen. In this study we evaluate the conservation of the epitope recognized by this antibody in venoms from different species throughout venomous snakes' phylogeny. Our data support the presence of this epitope in a large number of venoms from snakes of 27 different species belonging to Viperidae, Colubridae and Elapidae families. However, the neutralizing ability of the monoclonal antibody was variable depending on the genus and species of snake, which represents an important aspect to considerate in order to achieve an efficient broad spectrum serum therapy for snake bites.

2. Material and methods

2.1. Venoms

Bothrops jararaca, *B. erythromelas*, *Lachesis muta muta*, *B. jararacussu*, *B. atrox*, *Crotalus durissus terrificus*, *Philodryas olfersii* and *P. patagoniensis* venoms were provided by Dr. M. Fátima D. Furtado from the Herpetology Laboratory of the Instituto Butantan. Venoms of *Echis piramidum leakeyi*, *Daboia russelli*, *Trimeresurus albolabris*, *Agkistrodon contortrix contortrix*, *C. atrox*, *C. adamanteus adamanteus*, *Bitis arietans* and *Calloselasma rhodostoma* were provided by Dr Gavin D. Laing from Liverpool School of Tropical Medicine, England. Venoms of *Naja mossambica*, *N. melanoleuca*, *Pseudechis porphyriacus*, *Hoplocephalus stephensii*, *Notechis scutatus*, *Oxyuranus microlepidotus* were provided by Dr Peter Mirtschin (Venom supplies, Australia) while *Bitis caudalis*, *Vipera ammodytes ammodytes*, *C. vergrandis* and *Micrurus lemniscatus* venoms were provided by Dr Patrick Spencer from IPEN, Brazil and *B. asper* venom by Dr José Maria Gutiérrez from Instituto Clodomiro Picado, Costa Rica. The venoms were lyophilized and stored at −20 °C.

2.2. Monoclonal antibodies

Seven anti-jararhagin monoclonal antibodies, named MAJar 1–7, were produced and characterized as described previously (Tanjoni et al., 2003). These antibodies were produced from hybridomas cultured in RPMI 1640 medium supplemented with 10% FCS (GibcoBRL) at 37 °C and 5% CO_2. They were purified from supernatants by affinity chromatography on protein-A Sepharose (Pharmacia) equilibrated in Borate buffered saline (BBS), pH 8.5. IgG1 was eluted in citrate buffer 0.15 M, pH 5.0 since MAJars belonged to this isotype, showing Kds between 10^{-7} and 10^{-10} (Tanjoni et al., 2003).

2.3. Immunochemical methods

Antigenic cross-reactivity was evaluated by Western blotting, dotblot and ELISA. For ELISA, plates were coated with whole venoms (10 μg/ml) and blocked with 3% bovine serum albumin. Monoclonal antibodies were then added at increasing dilutions (2 times factor) starting with 10 μg/ml. Bound antibodies were detected using anti-mouse IgG-peroxidase conjugate and *ortho*-phenylenediamine (1 mg/ml, Sigma) and H_2O_2 as enzyme substrate. For Western blotting, venoms were first fractionated by SDS-PAGE (15% acrylamide resolution gels, under non-reducing conditions) according to Laemmli (1970). The gels were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes according to Towbin et al. (1979). For dotblots, the venom samples (2 μg/2 μl) were dotted on nitrocellulose membranes, diluted in PBS, without previous fractionation. After blocking, membranes were incubated with the solutions containing antibodies followed by incubation with sheep IgG anti-mouse IgG labeled with horseradish peroxidase (1:2000). The dots were developed by addition of 0.05% 4-chloro-1-naphthol in 15% methanol (v/v), in presence of 0.03% H_2O_2 (v/v).

2.4. Hemorrhagic activity

Venoms (10 μg) were injected i.d. into the dorsum of Swiss mice. Mice were euthanized 3 h after injection, the dorsal skin was removed and the area of the hemorrhagic spots was determined by multiplying the largest diameter by its perpendicular. For neutralization of hemorrhage, 10 μg of venoms were incubated with 400 μg of MAJar 3 or with isotype control (monoclonal antibody anti-*Taenia crassiceps;* Espindola et al., 2002), for 1 h at 37 °C. Mixtures contained in 100 μl were centrifuged and the supernatants injected i.d. into the dorsum of Swiss mice. Hemorrhage was calculated as above and results are shown in $cm^2 \pm$ SD and percentage neutralization, taking as positive value samples pre-incubated with isotype control.

2.5. Statistics

All analyses were carried out in triplicates with results obtained from a minimum of two independent experiments. The data were analyzed by the Student's *t*-test.

3. Results

The phylogenetic distribution of epitopes recognized by monoclonal antibodies anti-jararhagin (MAJars) was evaluated by dotblot analysis using venoms of snakes from Elapidae, Viperidae (Crotalinae and Viperinae subfamilies) and Colubridae families (Fig. 1). Recognition by MAJars was related to snake phylogeny and

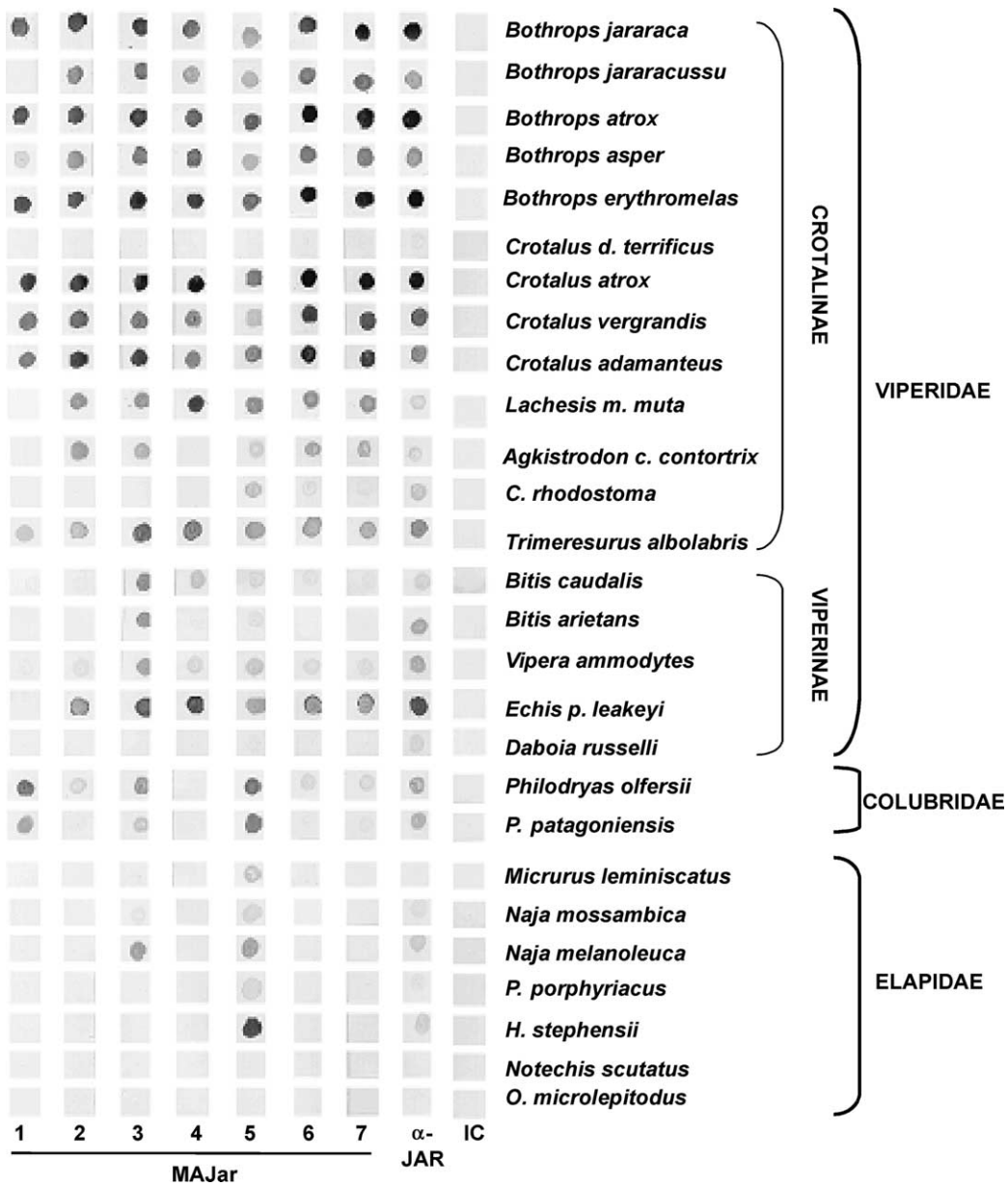

Fig. 1. *Conservation of MAJar epitopes in different snake venoms*. Samples containing 2 μg venoms of different snakes belonging to Viperidae, Colubridae and Elapidae families were dotted on nitrocellulose membranes. After blocking, membranes were incubated with anti-jararhagin polyclonal antibodies (a-JAR), MAJars 1–7 or isotype control (IC). Antigen-antibody reaction was detected by addition of anti-mouse IgG antibodies conjugated with peroxidase followed by the enzyme substrate.

the presence of SVMPs in the venoms: MAJars 1–7 preferentially recognized venoms of Crotalinae snakes followed by Viperinae and Colubridae snake venoms. Elapidae venoms were recognized only by MAJar 5 and MAJar 3. Reactivity was also related to SVMP venom content: no reaction was observed with *C. durissus terrificus* venom, which presents only minor amounts of SVMPs (Rosenfeld, 1971). However, minor reaction was detected with *D. russelli* and *Calloselasma rhodostoma* venoms, despite their content of SVMPs. Interestingly, the antibodies that recognized venoms of a greater number of species of snakes were MAJar 3 and 5, which reacted with conformational epitopes (Tanjoni et al., 2003).

Due to the importance of MAJar 3 in neutralization of hemorrhagic activity, the next experiments comprised

Table 1
Reactivity of MAJar 3 with different venoms

Venom	Antibody reactivity (M)[a]	Neutralization of hemorrhagic activity		
		Venom + isotype control[b]	Venom + MAJar 3[b]	%
B. jararaca	4.0×10^{-13}	12.8 ± 0.9	0*	100
B. erythromelas	2.0×10^{-13}	13.3 ± 2.9	0.4 ± 0.3*	97
B. asper	4.0×10^{-13}	4.7 ± 2.2	0.2 ± 0.3*	96
C. atrox	2.0×10^{-13}	11.6 ± 4.4	9.6 ± 3.2	NSD
L. muta muta	6.7×10^{-12}	2.0 ± 1.7	0.3 ± 0.6	NSD
T. albolabris	4.0×10^{-13}	9.5 ± 0.9	15.2 ± 1.9	NSD
B. arietans	6.7×10^{-13}	15.6 ± 3.8	18.7 ± 4.6	NSD
V. ammodytes ammodytes	8.3×10^{-13}	11.9 ± 2.1	5.0 ± 4.9	NSD
E. piramidum leakeyi	8.3×10^{-13}	6.2 ± 3.0	9.7 ± 1.3	NSD
P. olfersii	2.1×10^{-9}	8.5 ± 3.2	16.8 ± 1.5	NSD
N. melanoleuca	6.7×10^{-9}	0.1 ± 0.1	1.3 ± 1.4	NSD

*Statistically significant differences in comparison to isotype control (paired *t*-test, $p < 0.05$); NSD: differences are not statistically significant.
[a] Minimal molar concentration of antibody that reacts with venoms in a typical ELISA assay
[b] Area of hemorrhagic spots of three mice (mean ± SD).

the level of reactivity by ELISA and neutralizing ability of this monoclonal antibody using the reactive venoms in dotblot tests. An ELISA assay was carried out in order to quantitatively characterize the reactivity of MAJar 3 to different venoms. Using antigen excess, MAJar 3 antibody titers were similarly high with venoms of all Viperidae snakes and low for Elapidae and Colubridae venoms (Table 1). In Western blots (Fig. 2), MAJar 3 preferentially reacted with bands presenting the molecular mass of P-III SVMPs. The multiple bands recognized by this antibody included *Bothrops* and *Crotalus* venoms 50–80 kDa antigens, and two major bands above 60 kDa in *B. arietans*, *V. ammodytes*, *B. caudalis* and *E. p. leakeyi* venoms. In Colubridae venoms, MAJar 3 revealed three bands around 54, 69 and 85 kDa of *P. olfersii* venom. A band of around 69 kDa in *P. patagoniensis* venom was also detected. In Elapidae venoms, MAJar 3 recognized bands of about 59 and 69 kDa of *N. mossambica* and around 59, 69 and 74 kDa of *N. melanoleuca*. Bands around 20–30 kDa were weakly recognized, suggesting a reactivity of MAJar 3 with disintegrin-like/cysteine-rich proteins, as jararhagin-C, which is originated by proteolysis of jararhagin (Moura da Silva et al., 2003).

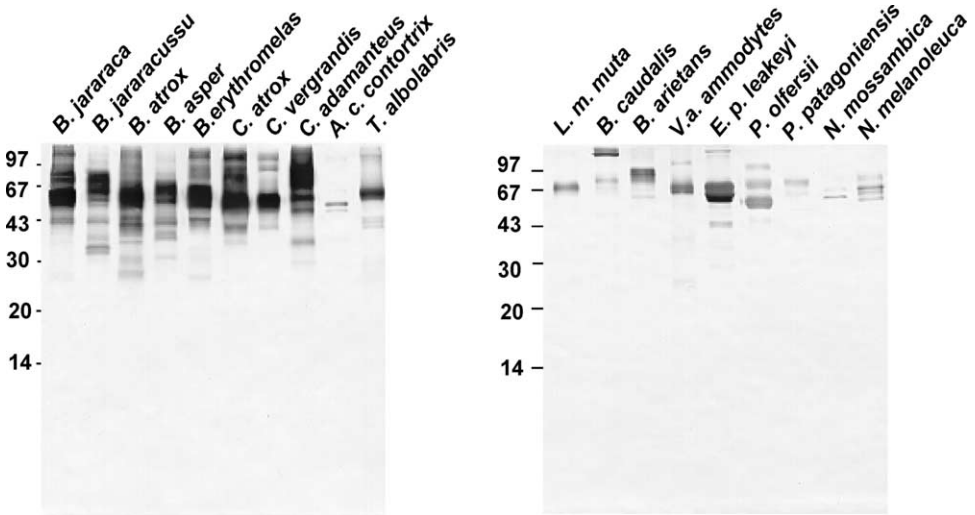

Fig. 2. *MAJar 3 recognizes preferentially P-III SVMPs by Western Blotting*. Samples containing 7 μg of venoms of different species of snakes were fractionated by SDS-PAGE (15%) and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with MAJar 3. Antigen-antibody reaction was detected by addition of anti-mouse IgG antibodies conjugated with peroxidase followed by the enzyme substrate.

Table 2
Sequence alignment of jararhagin fragment containing MAJar 3 epitope (JD49) with homologous region in SVMPS from different snake species

Protein	Gene Bank reference (gi)	Snake species	Alignment	% Identity
Jararhagin	231997	*B. jararaca*	GSQCGHGDCCEQCKFSKSGTECRASMSECDPAEHCTGQSSECPADVFHK	100
Bothropasin	4895110	*B. jararaca*	.....	100
B. insularis SVMP	20069135	*B. insularis*	.....	100
Catrocollastatin	7512197	*C. atrox*	.....	100
Cobrin	6006966	*N. naja*	EA..DSEE...KC..KGA.A....AKDD..LP.L.....A...T...QR	56
Mocarhagin 1	21435683	*N. m. mossambica*	EA..DSGE...KC..KGA.A....AKND..FP.L...R.AK.PK.S.QR	52
EcH-I	1086019	*E. p. leakeyi*	.S..AD....N.CR.RPART...RKIDD..VP.Y.....G...L...QR	57
Ecarin	2134246	*E. carinatus*	.AE..N....DKC.IRKA.T...PARDD..V..H.....A...RNE.QR	57
EcH-II	1364104	*E. p. leakeyi*	WVE.EF.H..D.CR.KPA.T...GIR....LP.YC....A..LR-M.STR	60
VAP	14325767	*T. flavoridis*	.A..AE.L..D.CR.KAA......ATD...M.DLC.GR.A..T-.R.QR	59
VAP 1	25331213	*C. atrox*	.A..AE.L..D.CR.KGA......AKD...M.DVC.GR.A..T-.R.QR	59
A. c. laticintus SVMP	2231613	*A. c. laticintus*	....AD.V..D.CR.TRA.....QAKDD..M.DLC....A...T.R.QR	57
Berytractivase	17865171	*B. erythromelas*	....VE.L..D.CR.RKT....RAAKHD..LP.SC....AD..M.D.QR	59
HR1A	20530119	*T. flavoridis*	WVE.ES......CR.RTA....RARR....I..SC..H.AD..T.R..R	61
HF3	18379369	*B. jararaca*	WVE.ES....D.CR.KGA......AK....I..SC....AD..T.D.KR	59
HR1B	462301	*T. flavoridis*	WVK.ES....D.CR.RTA......AE....IP.SC....AD..T.R..R	59
Atrolysin A	542663	*C. atrox*		57

C-terminal region of Jararhagin disintegrin domain (JD 49 fragment) was aligned with the homologous regions of P-III SVMPs from snake species available at Gene Bank using the ClustalW program. Conserved residues are denoted with a (·).

Neutralization of hemorrhagic activity of venom components recognized by MAJar 3 was then carried out. As shown in Table 1, hemorrhage induced by venoms of *Bothrops* snakes was completely neutralized by MAJar 3, while neutralization of hemorrhage induced by venoms from snakes of other genera of Viperidae family was partial and the differences observed were not statistically significant in relation to control samples. No significant neutralization was observed for Colubridae and Elapidae venoms (Table 1).

The data of antigenic cross-reactivity and neutralizing ability of MAJar 3 were then correlated with SVMPs primary structure by the alignment of their fragments corresponding to JD49 jararhagin sequence that comprises the C-terminal segment of the disintegrin domain and includes the MAJar 3 epitope (Tanjoni et al., 2003). In Table 2, we can see that some SVMPs from *Bothrops* snakes (as bothropasin and a metalloproteinase from *B. insularis* venom) share all residues within the disintegrin-like C-terminal region (100% identity) while others like HF3 and beritractivase present only 59% identity. For SVMPs from venoms of snakes belonging to other genera, the identity at this region varied between 52 and 61%, however 100% of identity was observed for catrocollastatin-C, from *C. atrox* venom (Table 2). The similarity of these proteins in the epitope-containing fragment could then explain the high reactivity in solid-phase assays. However, several amino-acid substitutions were detected, what could result in lower affinity of epitope interaction with the antibody, that would not be enough for neutralization of hemorrhagic activity.

4. Discussion

Monoclonal antibodies are important tools for identification of shared epitopes in a protein family. They react with a specific region of the protein, and discriminate very subtle immunological differences among proteins from the same group, as the SVMPs. In this paper, the antigenic cross-reactivity of PIII SVMPs was easily detected in venoms of snakes from different genera, subfamilies or families by reactivity of seven monoclonal antibodies that react with the jararhagin disintegrin domain (Tanjoni et al., 2003). This observation confirmed the presence of PIII SVMPs in most of the venoms studied, showing the importance of this protein family for the toxicity of snake venoms. However, the intensity of the reaction of the monoclonal antibodies was variable, thus suggesting a structural variability in this group of toxins, which may be related to phylogeny, variable degrees in expression of genes coding for SVMPs in different species of snakes or even amino acid substitutions in residues involved in the epitopes.

One interesting aspect was that conformational epitopes seemed to be more conserved along snake phylogeny than sequential ones. Supporting this, MAJar 2, 6 and 7 recognized SVMPs present mostly in venoms of viper snakes while MAJar 1, 3 and 5 detected antigens also in Elapidae and Colubridae snake families. This is expected since the scaffold of the molecules is strongly conserved during the divergent evolution of a protein family. Considering MAJar 3 epitope, its conservation may be related not only to molecule scaffold but also with conservation of its function: collagen binding and hemorrhagic activity.

Other important point was the discrepancy between MAJar 3 neutralizing ability and reactivity in solid phase assays: Although MAJar 3 recognized most venoms with high ELISA antibody titers, the neutralization was achieved mostly in venoms of *Bothrops* snakes. Similar results were obtained with a neutralizing monoclonal antibody raised against mutalysin-II, a *Lachesis muta muta* hemorrhagic SVMP, which showed variable potencies in neutralizing the hemorrhagic activity of *Bothrops* snakes venoms (Estêvão-Costa et al., 2000). These data could be explained by the defined reactivity of monoclonal antibodies for a given epitope. However, restricted heterologous neutralization was also observed using polyclonal antibodies raised against SVMPs from *Bothrops* snakes (Mandelbaum et al., 1989; Rucavado et al., 1995). This could be explained by the fact that during evolution, genes coding for P-III SVMPs underwent duplication and divergence through accelerated evolution, generating a multiple number of similar but not identical toxins in each venom (Moura da Silva et al., 1996), thus increasing the spectrum of substrates for these enzymes. The primary structure of these proteins is slightly different, interacting with matrix components or neutralizing antibodies with different affinities/specificities. It has also to be considered that the proportion of expression of each SVMP gene copy may vary in each venom. In the case of *C. atrox*, we detected a high antibody titer and a low neutralization of hemorrhage by MAJar 3. The high antibody titer should correspond to catrocollastatin reactivity and the preservation of hemorrhage should be due to lack of neutralization of atrolysin A, which shows lower identity to jararhagin at the JD49 region (57%). In the case of *L. m. muta* and *V. a. ammodytes,* MAJar 3 partially neutralizes the hemorrhagic activity probably due to the presence of SVMPs with dissimilarities in the epitope recognized by the monoclonal antibody. Unfortunately, SVMPs isolated from these species have not been sequenced yet to confirm this hypothesis (Leonardi et al., 2002; Bjarnason and Fox, 1995). In *Trimeresurus*, *Echis*, and *Naja* venoms, SVMPs have low similarity with jararhagin ranging from 52 to 61%, thus limiting the efficiency of the neutralizing antibody. Therefore, the fact that venoms recognized by MAJar 3 are not neutralized by this antibody could be explained by the affinity of antibody interaction. Since MAJar 3 recognizes a conformational

epitope, a single substitution might alter the epitope conformation, and consequently the affinity for the antibody. This may not interfere in a solid assay, but when these molecules are tested in a hemorrhagic test, MAJar 3 might be displaced by the high affinity of the natural target. Confirming this hypothesis, in our previous study, MAJar 1 reacted with a proximal epitope of MAJar 3 and blocked jararhagin binding to collagen. However, affinity of MAJar 1 was 10 fold lower than MAJar 3 affinity for jararhagin and this antibody was not able to neutralize hemorrhagic activity (Tanjoni et al., 2003).

Another possibility that should be addressed is the location of MAJar 3 epitope in the disintegrin domain of PIII SVMPs (Tanjoni et al., 2003). Neutralization of hemorrhagic activity by antibodies directed to disintegrin domain of jararhagin has been previously reported (Harrison et al., 2000). PIII SVMPs are the most potent hemorrhagic factors in viper venoms (Bjarnason and Fox, 1994). These evidences could explain the complete neutralization of hemorrhage induced by whole venoms from *Bothrops* snakes by MAJar 3. However, hemorrhagic PI SVMPs could be highly expressed in venoms of snakes from other genera and since they are devoid of the disintegrin domain, they would not be neutralized by MAJar 3 antibody.

Concluding, sequence similarities between the toxins are not enough to predict their mechanisms of action and neutralizing ability of antibodies. Fine structural and conformational constraints and expression of different gene products may vary according to the snake species and must also be regarded. The conformational epitope, recognized by MAJar 3, is present in most hemorrhagic venoms, but in only a few it can interact with heterologous antibodies with high affinity, leading to neutralization of the biological activity of the toxin. This has a particular implication in order to produce efficient antivenoms. Despite the high conservation of toxin structures in different snake species, efficiency of antivenoms should be tested against venoms of all the different species they will be directed for.

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