A New Anti-loxoscelic Serum Produced Against Recombinant Sphingomyelinase D: Results of Preclinical Trials

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Abstract. Envenomation by Loxosceles species (brown spider) can lead to local dermonecrosis and to serious systemic effects. The main toxic component in the venom of these spiders is sphingomyelinase D (SMase D) and various isoforms of this toxin are present in Loxosceles venoms. We have produced a new anti-loxoscelic serum by immunizing horses with recombinant SMase D. In the present study, we compared the neutralization efficacy of the new anti-Loxosceles serum and anti-arachnidic serum (the latter serum is used for therapy for loxoscelism in Brazil) against the toxic effects of venoms from spiders of the genus Loxosceles. Neutralization tests showed that anti-SMase D serum has a higher activity against toxic effects of L. intermedia and L. laeta venoms and similar or slightly weaker activity against toxic effects of L. gaucho than that of Arachnidic serum. These results demonstrate that recombinant SMase D can replace venom for anti-venom production and therapy.

INTRODUCTION

Envenomation by Loxosceles spp. spiders is a public health problem in Brazil, and loxoscelism is considered the most dangerous form of araneism in this country. Systemic reactions, including shock, hemolysis, renal insufficiency, and disseminated intravascular coagulation, are rare. In contrast, bite reactions ranging from small areas of erythema to large areas of ulceration and necrosis are frequently observed. The bite is relatively painless and patients often are unaware that they have been bitten. Mild to severe pain, beginning 2–8 hours after envenomation, is probably caused by ischemia. Erythema with itching, swelling, and mild to severe tenderness is also observed. At this point, the patient might consult a physician.

At least three Loxosceles species of medical importance are known in Brazil (L. intermedia, L. gaucho, and L. laeta) and more than 3,000 cases of envenomation by L. intermedia are reported each year. In North America, several Loxosceles species, including L. reclusa (brown recluse), L. apachae, L. arizonica, L. unicolor, L. deserta, and L. bonetti, are the principal cause of numerous incidents of envenomation. In South Africa, L. parrami and L. spinulosa are responsible for cutaneous loxoscelism and in Australia, a cosmopolitan species, L. rufescens, is capable of causing ulceration in humans.

A variety of treatments such as systemic steroids, anti-venom, phentolamine, heparin, chloroprophénylamine maleate, dapsone, hyperbaric oxygen, and other substances have been used for therapy, with little or no benefit and, in some cases, with undesirable collateral effects. Most treatments have attempted to reduce infiltration of polymorphonuclear (PMN) leukocytes, the hallmark of cutaneous loxoscelism. PMN leukocyte infiltration, in part recruited by indirect activation of the complement system, is a major contributor to tissue damage. Treatments to reduce PMN leukocyte infiltration are fraught with side effects and, in some cases, can increase tissue injury.

In Brazil, serum therapy combined with corticosteroids constitute the most common intervention for loxoscelism, and the Brazilian Ministry of Health recommends its use in moderate and severe cases with systemic illness and to reduce severity of the reaction and healing time. The antiserum most commonly used for treatment of loxoscelism in Brazil is anti-arachnidic serum. This serum is produced by the Instituto Butantan (São Paulo, Brazil) by hyperimmunization of horses with venoms of the spiders L. gaucho and Phoneutria nigriventer and the scorpion Tityus serrulatus. Several studies have indicated that sphingomyelinase D (SMase D) in venom of Loxosceles spp. spiders is the main component responsible for local and systemic effects observed in loxoscelism.

The difficulty in obtaining large amounts of venom and purified venom components is one of the limiting factors in studying the mechanisms involved in loxoscelism and raising an effective therapeutic antiserum. We have recently cloned and expressed one of the sphingomyelinases from L. laeta venom (SMase I), which displayed all biological activities endowed by whole venom. An antiserum raised in rabbits against this recombinant protein effectively neutralized L. laeta venom toxicity. We have also cloned and expressed two functional isoforms of SMase (P1 and P2) from L. intermedia and showed that the recombinant proteins display all functional characteristics of whole venom, e.g., dermonecrotic and complement-dependent hemolytic activities and ability to hydrolyze sphingomyelin (SM). We have also compared the cross-reactivities of antisera raised in rabbits against SMase D from different Loxosceles species and showed that the cross-reactivity is high when toxins are from the same species but lower when the toxins are from different species (L. intermedia versus L. laeta).

These data suggest that to obtain a suitable neutralizing antiserum using recombinant SMase D as immunogen, a mixture of the recombinant toxins from the different species is a requirement. On the basis of these data, we have developed a new anti-loxoscelic serum by horse immunization with a mixture of recombinant sphingomyelinas (P1, P2, and SMase...
I), by a patented process (patent no. 0404765-6–02004006198; 3/11/2004, Brazilian National Institute of Industrial Property [INPI], 2005). In the present study, we have analyzed the neutralization potential of the new serum with that of anti-arachnidic serum in preclinical tests using the venoms of *L. intermedia*, *L. gaucho*, and *L. laeta*.

**MATERIALS AND METHODS**

**Chemicals, reagents, and buffers.** Tween 20, bovine serum albumin (BSA), paraformaldehyde, SM, choline oxidase, horseradish peroxidase (HRP), and 3-(4-hydroxyphenyl)propionic acid were obtained from Sigma (St. Louis, MO). 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) were obtained from Promega (Madison, WI). Buffers used were veronal-buffered saline (VBS**−**), pH 7.4 (10 mM sodium barbital, 0.15 mM CaCl₂, and 0.5 mM MgCl₂; phosphate-buffered saline (PBS), pH 7.2 (10 mM sodium phosphate, 150 mM NaCl); HEPE-BSA-buffered saline (HBS), pH 7.4, 10 mM HEPES 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂; fluorescence-activated cell sorting (FACS) buffer (PBS, 1% BSA, 0.01% sodium azide); and Alsever’s old solution 114 mM sodium citrate, 27 mM glucose, 72 mM NaCl, pH 6.1).

**Antibodies.** Monoclonal antibody against glycoprotein C (GPC) (Bric4) was obtained from the International Blood Group Reference Laboratory (Bristol, United Kingdom). Rabbit anti-mouse IgG labeled with fluorescein isothiocyanate (FITC) was obtained from Amersham Pharmacia Biotech GE (Little Chalfont, Buckinghamshire, United Kingdom). Goat anti-horse IgG labeled with alkaline phosphatase (AP), HRP, or FITC was obtained from Sigma. Goat anti-horse IgGₜ was obtained from Bethyl Laboratories Inc. (Montgomery, TX).

**Animals.** Adult New Zealand white rabbits weighing approximately 3 kg were obtained from Bioindustrial Division, Instituto Butantan. Venoms were obtained from Sigma (St. Louis, MO). Buffers used were veronal-buffered saline (VBS**−**), pH 7.4 (10 mM sodium barbital, 0.15 mM CaCl₂, and 0.5 mM MgCl₂; phosphate-buffered saline (PBS), pH 7.2 (10 mM sodium phosphate, 150 mM NaCl); HEPE-BSA-buffered saline (HBS), pH 7.4, 10 mM HEPES 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂; fluorescence-activated cell sorting (FACS) buffer (PBS, 1% BSA, 0.01% sodium azide); and Alsever’s old solution 114 mM sodium citrate, 27 mM glucose, 72 mM NaCl, pH 6.1).

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**Expression of recombinant protein.** Production of recombinant mature SMases I from *L. laeta* (accession no. AY093599) and P1 and P2 from *L. intermedia* (accession nos. AY304471 and AY304472, respectively) was performed as described.

**Antiseras.** Commercial anti-arachnidic serum (batch no. 0506118), produced by immunizing horses with a mixture of venoms from *L. gaucho* (21.5%), *P. nigritener* (21.5%), and *T. serrulatus* (57%) and experimental anti-SMase D serum, produced by immunizing horses with a mixture of recombinant SMases D from *Loxosceles* spp. spiders (P1 and P2 from *L. intermedia* and SMase I from *L. laeta*), were obtained from the Bioindustrial Division, Instituto Butantan. Production and purification of anti-SMase D serum are patented processes (patent no. 0404765-6, INPI). Normal horse serum was obtained from non-immunized animals.

**Normal human serum and erythrocytes.** Human blood was obtained from healthy donors. Blood samples used for serum were collected without anticoagulant and allowed to clot for two hours at room temperature; normal human serum was stored at –80°C. Blood samples used for obtaining erythrocytes for subsequent use as target cells were collected in Alsever’s old solution.

**Electrophoresis and Western blotting.** Samples of venoms and recombinant proteins were solubilized in reducing sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and silver staining. Alternatively, gels were blotted onto nitrocellulose membranes. After transfer, membranes were blocked with PBS containing 5% BSA and incubated with anti-arachnidic or anti-SMase D horse sera (diluted 1:2,000) for one hour at room temperature. Membranes were washed three times (10 minutes/wash) with PBS/0.05% Tween 20 and incubated with goat anti-horse IgG labeled with AP (diluted 1:3,000) in PBS/1% BSA for one hour at room temperature. The membranes were then washed three times (10 minutes/wash) with PBS/0.05% Tween 20 and blots were developed using NBT/BCIP according to the manufacturer’s instructions (Promega).

**Enzyme-linked immunosorbent assay (ELISA).** Microtiter plates were coated with 100 μL of *Loxosceles* spp. venom or recombinant proteins (10 μg/mL) overnight at 4°C. Plates were blocked with 5% BSA in PBS and dilutions of anti-arachnidic or anti-SMase D horse sera were added. After incubation for one hour at room temperature, plates were washed with PBS/0.1% Tween 20 and incubated with goat anti-human IgG labeled with HRP (diluted 1:1,000) for one hour at room temperature. Plates were washed and developed with o-phenylenediamine dihydrochloride (OPD) substrate according to the manufacturer’s (Sigma) recommendations. Alternatively, titers of IgGₜ from anti-arachnidic and anti-SMase D horse sera were determined by coating microtiter plates with 100 μL of goat anti-horse IgG against horse IgGₜ (250 μg/mL) overnight at 4°C. Plates were blocked with 5% BSA in PBS and dilutions of anti-arachnidic and anti-SMase D sera made in PBS were added. After incubation for one hour at room temperature, plates were washed with PBS/0.1% Tween 20 and incubated with goat anti-horse IgG labeled with HRP RP (diluted 1:3,000) for one hour at room temperature. Plates were washed and developed with OPD substrate according to the manufacturer’s (Sigma) recommendations. Titers were established as the highest antiserum dilution in which an absorbance was five times greater than that for normal serum.

**Neutralization of dermonecrotic activity by sera.** The ability of the anti-arachnidic and anti-SMase D sera to neutralize dermonecrotic activity was measured by an in vivo method. Two hundred microliters of *L. gaucho*, *L. intermedia*, or *L. laeta* venoms in PBS (15 μg/mL) were injected intradermally into shaved backs of adult rabbits. Simultaneously, 1 mL of normal or test sera was injected intravenously. The size of the
lesions was measured over a period of 72 hours after injection. Neutralization was expressed as percentage reduction in size of the dermonecrotic lesion and calculated using as reference the extent of lesions developed by animals injected with the venoms and treated with normal serum.

Neutralization of hemolytic activity by sera. The ability of the anti-arachnidic and anti-SMase D sera to block hemolytic activity induced by *Loxosceles* spp. venoms was assessed by analyzing inhibition of GPC cleavage on the erythrocyte cell membrane as described. Human erythrocytes were washed and resuspended at a concentration of 2% in VBS++ and incubated with *Loxosceles* spp. venom for 30 minutes at 37°C. Control samples were incubated with VBS++. Cells were washed three times, resuspended in their original volume in VBS++, and analyzed in a hemolysis assay.

For this assay, 100 μL of 2% erythrocytes pre-treated with *Loxosceles* spp. spider venom or VBS++ were mixed with 100 μL of normal human serum (1:2 in VBS++). Background or total cell lysis was evaluated by incubation of erythrocytes with VBS++ or water, respectively. After incubation for one hour at 37°C, unlysed cells were centrifuged and the absorbance of the supernatant was measured at 414 nm and expressed as percentage of lysis. Because high concentrations of serum were used in the assays, the absorbance of serum without erythrocytes was subtracted from all samples. Percentage lysis was calculated as: (absorbance<sub>water</sub> − absorbance<sub>VBS++</sub>) × 100. Mean and standard deviations were determined from duplicate samples. Erythrocytes and normal human serum were always obtained from the same donor.

Once the venom concentration to be used in the neutralization assays was determined (3 μg/mL), 25 μL of 2% human erythrocytes were incubated with *Loxosceles* spp. venoms in the presence or absence of normal, anti-arachnidic, or anti-SMase D sera for 30 minutes at 37°C. Control samples were incubated with VBS++. Cells were washed three times, resuspended in their original volume in VBS++, and prepared for flow cytometry analysis. Cells were incubated for 30 minutes with 25 μL of the primary monoclonal antibody (MAb Bric4: anti-GPC; 1 μg/mL) in FACS buffer. After washing, cells were incubated with FITC-labeled secondary antibodies for 30 minutes. Cells were washed and fixed in FACS buffer containing 1% paraformaldehyde and analyzed by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA).

Neutralization of SMase activity by sera. The SMase D activity in *Loxosceles* spp. venoms was measured as described. Briefly, samples containing increasing concentrations of the *Loxosceles* spp. venoms were incubated with 50 μg of SM diluted in 1 mL of HBS for 20 minutes at 37°C. One unit/mL of choline oxidase, 0.06 units/mL of HRP, and 50 μM of 3-(4-hydroxy-phenyl)propionic acid in HBS was added and incubated for 10 minutes. The choline liberated was oxidized to betaine and H<sub>2</sub>O<sub>2</sub>. The oxidation products were measured by fluorometry at λ<sub>ex</sub> = 405 nm and λ<sub>em</sub> = 320 nm by using 96-well microtiter plates in a spectrofluorimeter (Victor 3M; Perkin-Elmer, Waltham, MA). Once the venom concentration used in neutralization assays (1 μg) was determined, venom samples were incubated for 30 minutes at 37°C with increasing dilutions of normal, anti-arachnidic, or anti-SMase D sera. Samples were then centrifuged for 20 minutes at 10,000 × g and supernatants were analyzed for SMase D activity in the samples as described above.

**Statistical analysis.** Data were analyzed by using the Student’s t-test. A P value < 0.05 was considered significant.

**RESULTS**

Immunochemical cross-reactivity and serum titers. Analysis of venoms from *L. gaucho*, *L. laeta*, and *L. intermedia* by SDS-PAGE and silver staining showed differences in composition, number and intensity of bands. However, all venoms contained components with molecular masses of 32–35 kD, which includes the main toxic components of *Loxosceles* spp. venoms, the SMases D (Figure 1). Western blot analysis showed that anti-arachnidic serum recognized most of the components in venoms and that antibodies to SMase D identified only components with molecular masses of 32–35 kD in *L. laeta* and *L. gaucho* venom (Figure 1). In *L. intermedia* venom, antibodies to SMase D reacted with 32–35-kD SMase D proteins and with proteins with molecular masses twice that of the 32–35-kD proteins, which may indicate the presence of SMase D dimers.

Cross-reactivities of the two sera were tested by ELISA using *L. gaucho*, *L. intermedia*, and *L. laeta* venoms as antigens. As shown in Figure 2A, both sera recognized all tested antigens. Although anti-arachnidic serum contained higher levels of antibodies against venom of *L. gaucho* than against *L. intermedia* and *L. laeta* venom, antibodies to SMase D contained the highest level of antibodies against *L. intermedia* venom. The antibody titer against *L. gaucho* was much lower in the anti-SMase D serum than that in the anti-arachnidic serum. Both antisera had the same antibody titer against *L. laeta* venom. Because horse IgG<sub>2</sub> is considered the most important antibody isotype in the neutralization of toxins, the anti-arachnidic and anti-SMase D sera were compared by ELISA for their contents of IgG<sub>2</sub>. Figure 2B shows that the anti-SMase D serum had much higher titers of IgG<sub>2</sub> than the anti-arachnidic serum.

Analysis of neutralization potential of anti-arachnidic and anti-SMase D sera. To determine whether toxins responsible for the main toxic effects of *Loxosceles* spp. venoms could be
neutralized by the anti-arachnidic and anti-SMase D sera, in vivo and in vitro-in vivo neutralization experiments were performed.

Inhibition of dermonecrotic activity of Loxosceles spp. venoms by sera. The ability of Loxosceles spp. venoms to induce dermonecrotic lesions was investigated by injecting rabbits with different venoms. Typical loxoscelic lesions developed in the skin area injected with all venoms within a few hours of injection, as shown by the presence of edema, erythema, and mild tenderness. Approximately 24-hours post-injection, necrosis at the inoculation site was observed. A more potent dermonecrotic activity was observed with L. laeta and L. gaucho venoms than with L. intermedia venom (Figure 3A) (P < 0.05).

Neutralizing ability of the two antisera on Loxosceles spp. venom dermonecrotic activity was determined by an in vivo test. Loxosceles gaucho, L. intermedia, or L. laeta venoms were injected intradermally into the backs of adult rabbits and, simultaneously, antisera were injected intravenously. Size of the lesions was measured during a 72-hour after injection. Groups of animals were injected intradermally with venoms and intravenously with normal horse serum were used as controls. Figure 3B–D shows that anti-SMase D serum was more active than anti-arachnidic serum in neutralizing the dermonecrotic activity of L. intermedia and L. laeta (P < 0.05) venoms, and the neutralization activity of the two antisera for the L. gaucho venom was the same.

Inhibition of hemolytic activity of Loxosceles spp. venoms by sera. We previously showed that venoms/sphingomyelinases from L. intermedia, L. gaucho, and L. laeta spiders...
transform human erythrocytes into activators of the complement system. We subsequently elucidated the mechanism of complement susceptibility and showed that the toxins facilitate activation of the alternative pathway of complement on human erythrocytes by removal of GLPs as a consequence of activation of an endogenous metalloproteinase and activation of the classic pathway of complement possibly by alteration of the membrane asymmetry with exposure of phosphatidylserine.

**Figure 4A** shows that all *Loxosceles* spp. venoms tested induced autologous complement lysis of human erythrocytes in a dose-dependent manner. To determine if toxins responsible for the removal of GPC and autologous complement hemolysis could be neutralized by the antisera, the venoms from *Loxosceles* spp. were mixed with human erythrocytes in the presence or absence of control serum and experimental antisera. **Figure 4B** shows that the anti-arachnidic serum was more efficient at neutralizing sphingomyelinase activity of *L. gaucho* venom than anti-SMase D at dilutions of 1:1 to 1:75 (*P* < 0.05). At the highest dilutions, both sera showed similar neutralization values. For *L. intermedia* venom, the anti-arachnidic serum was more efficient in neutralization than anti-SMase D at dilutions of 1:1 to 1:15 (*P* < 0.05). The two sera showed similar neutralization at dilutions of 1:30 to 1:75. At dilutions of 1:90 and 1:120 (*P* < 0.05) the anti-SMase D serum was more effective. For *L. laeta*, anti-SMase D serum was more efficient in neutralization than anti-arachnidic serum, mainly at the highest serum dilutions (*H*11350; *P* < 0.05). Under the same experimental conditions, the control serum was not able to inhibit the sphingomyelinase activity of the venoms.

**DISCUSSION**

Envenomation by *Loxosceles* spp. spiders is a well-documented cause of necrotic skin lesions in humans. Although systemic loxoscelism is less common than the cutaneo-
ous form, it is the main cause of death associated with _Loxosceles_ spp. envenomation. Most deaths occur in children and are related to the South American species _L. laeta_.¹ _Loxosceles_ spp. are the most poisonous spider in Brazil and more than 3,000 cases of envenomation by _L. intermedia_ are reported each year. Sphingomyelinase D is the primary enzyme in _Loxosceles_ spp. venom responsible for its major clinical effects in humans.

It is difficult to obtain large quantities of _Loxosceles_ spp. venom. In addition, only venom from _L. gaucho_ is used for the anti-arachnid serum and success of toxin neutralization by an antivenom depends on abundance of specific antibodies against the principal lethal toxin of the venom and its binding affinity for the toxin.⁴⁵,⁴⁶ Because we have recently cloned and expressed the main toxic components of _L. intermedia_ and _L. laeta_ (both considered to cause more severe pathologic effects than _L. gaucho_), we have produced a new anti-loxoscelic serum by immunizing horses with a mixture of recombinant SMase D from venoms of _L. intermedia_ and _L. laeta_, which are the main agents of loxoscelism in Brazil and South America, respectively. In the present study, we have analyzed the neutralization potential of this new anti-loxoscelic serum against the toxic effects of venoms from spiders of the genus _Loxosceles_ of medical importance in Brazil and compared it with the anti-arachnidic serum used for human therapy in this country.

Analysis of venoms from _Loxosceles_ spp. by SDS-PAGE showed differences in composition, number, and intensity of bands. Western blotting demonstrated that although anti-arachnidic serum was developed against venom from _L. gaucho_, this serum recognized most components in the three venoms. As expected, antibodies to SMase D recognized only components with molecular masses of 32–35 kD, which corresponded to SMase D in these venoms, although with less intensity than with these components in _L. gaucho_ venom. In _L. intermedia_ venom, antibodies to SMase D also reacted with some proteins with double the molecular of SMase D, which suggested the presence of SMase D dimers.

Results of ELISA also showed cross-reactivity of the anti-arachnidic serum, but reactivity was highest within the same species. For antibodies to anti-SMase D, the highest titer was obtained against _L. intermedia_ venom because in this horse serum preparation two SMases D, P1 and P2, were used in the immunization pool. Antibody titers against venoms in anti-arachnidic serum and anti-SMase D serum indicate that anti-arachnidic serum was raised against whole venom. Thus, antibody titers against active toxins (SMase D in venom) may be much lower; this result was confirmed by Western blotting (Figure 1), which showed that anti-arachnidic serum recognizes not only SMase D but other proteins in venom.

We have shown that the amount of total IgG, which is found in all antibodies to toxin,⁴⁷–⁵⁰ was higher in antibodies to SMase D. This result indicated a higher neutralization potential for this serum than for anti-arachnidic serum. To confirm this hypothesis, _in vivo_ and _in vitro_ neutralization assays were performed. Results showed that, as previously suggested,²²,²⁵ the new anti-loxoscelic serum, which was raised against only the toxic component (SMase D), is able to control the toxic action of _Loxosceles_ spp. venoms. Comparative analysis indicated that the anti-arachnidic serum, although able to neutralize heterologous venoms, such as those from _L. intermedia_ and _L. laeta_, is less efficient than anti-

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**FIGURE 5.** Neutralization of sphingomyelinase activity of _Loxosceles_ spp. venoms. A, Sphingomyelinase (SMase) activity: SMase (50 µg) was incubated with buffer or with increasing amounts of _Loxosceles_ spp. venoms. After incubation at 37°C for 20 minutes, the choline formed was oxidized to betaine and measured fluorimetrically. B–D, Serum neutralization: SMase was incubated with 1 µg of _Loxosceles_ spp. venom or veronal-buffered saline in the presence or absence the anti-arachnidic (□) or anti-SMase D (●) sera for 30 minutes at 37°C. Samples were centrifuged and supernatants were analyzed for SMase D activity as described above. Results are representative for three experiments and expressed as percentage reduction ± SD of fluorescence of duplicate samples.
SMases D. Conversely, for *L. gaucho*, anti-arachnidic serum, as expected, showed a better neutralization potential because its formulation venom was from *L. gaucho* and was used in the immunization pool.

In conclusion, using recombinant *Loxosceles* spp. toxins of the SMase D family, we have generated an effective anti-*Loxosceles* serum with higher in vivo neutralizing capacity against *L. intermedia* and *L. laeta* venoms than the most widely used anti-arachnidic antiserum. This development represents an important proof of the concept that recombinant SMases D can replace whole venom for anti-venom production and therapy. The fact that antibodies against a single toxin type are able to neutralize the major deleterious activities of a venom containing many other toxic components is not surprising because of the amount of SMase D in the venom glands, representing 16% of all mRNAs and orders of magnitude higher than most other toxins. The remaining components may be important for other biological needs of the spider and may not play a role in the pathophysiologic effects of venom in humans.

If one considers that *L. intermedia* and *L. laeta* cause most cases of envenomation in Brazil and South America, respectively, antiserum used in therapy is effective against these venoms. Therefore, we recommend use of anti-SMase D serum. Local knowledge of the distribution of different *Loxosceles* spp. may also help in the choice of antiserum used. Inclusion of SMase D isoforms from *L. gaucho* venom in the immunization formulation to obtain a fully neutralizing horse antiserum against the three predominant *Loxosceles* spp. spiders that cause envenomation in Brazil would be beneficial.

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