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Synthetic peptides for *in vitro* evaluation of the neutralizing potency of *Loxosceles* antivenoms

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ABSTRACT

An important step in the development of therapeutic antivenoms is the pre-clinical testing using *in vivo* methods to assess their neutralizing potency. For spider antivenoms (*Loxosceles* species), horse serum potency against the necrotizing activities of *Loxosceles intermedia* crude venom is currently tested in rabbits. These procedures are time consuming and involve a large number of animals. The aim of this study was to develop an *in vitro* method to assess the neutralizing potency of anti-*Loxosceles* sera. We first demonstrated that it was not possible to establish a correlation between the ELISA antibody reactivity of horse anti-*Loxosceles* serum and their neutralizing potency. We then showed that the antivenoms recognized several peptide epitopes from different regions of SMase-D proteins, which are toxic antigens from *Loxosceles* venoms. The recognition of some peptides was observed only when high neutralizing potency sera was used. Based on these results, three peptides (peptide 1, DNRRPIWNLAHMVNA and peptide 3, DFSGPYLPSLPTLDA corresponding to residues 2–16 and 164–178, respectively, of SMase-1 protein from *Loxosceles laeta*, and peptide 2, EFVNLGANSIETDVS corresponding to residues 22–36 of A1H – LoxGa protein from *Loxosceles gaucho* and LiD1 protein from *L. intermedia*) were selected. The peptides were synthesized, coupled to bovine serum albumin (BSA), and used as antigens in indirect ELISA to test their reactivity with horse anti-*Loxosceles* serum of varying neutralizing potencies. We found certain assay conditions that discriminated between the high and low neutralizing potency sera. This study introduced an *in vitro* and peptide-based neutralization assay for anti-*Loxosceles* antivenoms.

1. Introduction

Spiders of the genus *Loxosceles*, commonly known as brown spiders, have a worldwide distribution with more than 100 species present in Europe, Africa, Oceania, Asia, North America, Central America, and South America (Vetter, 2008). In Brazil, especially in the southern and southeastern regions, the predominant species are *Loxosceles intermedia*, *Loxosceles gaucho*, and *Loxosceles laeta* (Pauli et al., 2006). Over the last decade, research studies, motivated by the growing number of envenomation cases have reported that the spider distribution has become

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heterogeneous and includes urban areas (da Silva et al., 2004; Hogan et al., 2004; Chatzaki et al., 2005; Tambourgi et al., 2010).

Envenomation cases in humans are characterized by two clinical manifestations: cutaneous and systemic loxoscelism. The former is characterized by the formation of a dermonecrotic lesion. The second, which is also known as cutaneous-visceral loxoscelism, presents clinical manifestations that may cause, in some situations, disseminated intravascular coagulation, acute renal failure and in rare cases, generalized rash and death (Futrell, 1992; Swanson and Vetter, 2005).

Several protocols for the treatment of *Loxosceles* envenomation have been proposed and tested. However, the use of antivenom is the only treatment available that can effectively neutralize the action of the venom. The ability of specific antibodies to neutralize the dermonecrotic activity has been reported by several authors (Pauli et al., 2006; Furlanetto, 1961; Theakston et al., 2003; de Almeida et al., 2008).

Prior to their use, it is important to have a thorough assessment of the neutralizing potency of therapeutic antivenoms. This assessment of the neutralizing potency is currently achieved by *in vivo* tests that evaluate the neutralization of the dermonecrotic activity of *Loxoceles* antigens by horse serum in rabbits (Pauli et al., 2006; Furlanetto, 1961). The procedure is laborious, expensive, and results in the scarification of many animals. Due to animal cruelty laws, which prohibit the induction of pain and suffering in animals, this procedure is not allowed in many countries (Meier and Stocker, 1989). Therefore, the development of alternative methods for the evaluation of the antivenom neutralizing potency is of outmost importance.

This study describes the development of an *in vitro* method to evaluate equine hyperimmune sera (anti-*Loxosceles* sera). Peptide epitopes of representative toxins from venoms of three species of *Loxosceles* (*L. intermedia*, *L. gaucho*, and *L. laeta*) were identified by assessing the reactivity of overlapping peptides (Spot method) with anti-*Loxosceles* sera with different neutralizing potencies. Three synthetic epitopes were selected to establish a synthetic peptide-based ELISA, which allows the discrimination between high and low neutralizing potency sera.

2. Materials and methods

2.1. Venoms

Venoms were obtained from the *L. laeta*, *L. gaucho*, and *L. intermedia* spiders. The spiders, which were taxonomically identified and captured in various areas of Curitiba city, were provided by the Center for Production and Research of Immunobiological Products (CPPI; Piraquara, PR, Brazil). The venoms were obtained by electrical stimulation applied to the cephalothorax of the spiders. Subsequently, the venoms were vacuum dried, filtered, and stored at −20 °C. The total protein determination was performed according to the Lowry's method (Lowry et al., 1951).

2.2. Antivenoms

Nine anti-*Loxosceles* horse sera and a pre-immunized horse serum were provided by CPPI. They were obtained from the plasma of hyperimmunized horses that received a mixture of *L. intermedia*, *L. laeta*, and *L. gaucho* venoms, following the conventional immunization procedures carried out at CPPI. Briefly, after the collection of the pre-immunized horse sera, each horse received an initial subcutaneous injection (5 mg) of a mixture of the venoms in a complete Freund's adjuvant. After 30 days, two additional injections in incomplete Freund's adjuvant were administered with a 15-day interval in between the injections. Additionally, six subsequent doses were administered in $Al(OH)_3$ with a 7-day interval in between the doses. After 60 days, the animals underwent a new round of immunizations; 3 doses were administered (5 mg of venom/dose) at 15- and 7-day intervals. The horses were subjected to at least two cycles of re-immunizations. All doses were diluted in sterile saline buffer. The horses were bled one week after the last injection. Approximately 50 ml of blood was collected and subjected to hemosedimentation at 37 °C for 1 h and the supernatant was centrifuged. The fraction obtained (anti-*Loxosceles* serum) was stored at −20 °C.

2.3. In vivo *neutralization assays*

Forty-eight New Zealand rabbits were used to assess the neutralizing potency of the ten horse sera used in this study. The neutralizing capacity of the anti-*Loxosceles* sera was assessed using the methodology described by Furlanetto (1961). The quantity of the venom that was inoculated into the rabbits was based on the minimum necrotic dosage (MND) as reported by Theakston et al. (2003). For this test, only the venom from *L. intermedia* was inoculated (intradermally) on the inner ear of a rabbit (3 animals/dilution of sera tested) with 1 ml of intravenous injection (marginal vein of the opposite ear) of serum (1:8 and/or 1:6 dilutions) in saline buffer. Initially, the serum dilution was 1:8 and the animals were observed for 72 h for the appearance of necrosis. During this time period, the appearance of necrosis indicated that the diluted horse serum was not sufficient to neutralize the venom. In that case, a 1:6 serum dilution was then used. Sera, which were not able to neutralize 6 MND of the venom, were considered to be of low neutralizing potency.

2.4. ELISA procedures

ELISA was performed by coating plates (Falcon, Becton Dickinson) overnight at 4 °C with 100 μl of a 2.5 μg/ml solution of crude venom from the three species of *Loxosceles* (*L. intermedia*, *L. gaucho*, and *L. laeta*) in carbonate buffer (0.05 mM) at pH 9.6. After washing and blocking (with 2% casein for 1 h at 37 °C) the plates, they were incubated in diluted sera (1:1000; 1:5000; 1:20 000; and 1:40 000) under the same conditions. Peroxidase-conjugated anti-horse IgG antibody (Sigma, 1:30 000) was added and the plates were incubated for 1 h at 37 °C. After rinsing the plates, a substrate (citrate buffer pH 5.0, hydrogen peroxide, and ortho-phenyldiamine) was added. The reaction was stopped by adding 20 μl of 5% H_2SO_4; the antibody reactivity was determined by the intensity of the staining. Absorbance values were determined at 492 nm using an ELISA Bio-Rad 550. All measurements were done

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