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Innovative immunization protocols using chimeric recombinant protein for the production of polyspecific loxoscelic antivenom in horses

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ABSTRACT

A chimeric protein (rCpLi) was constructed expressing three epitopes of rLiD1, a dermonecrotic toxin from the venom of *Loxosceles intermedia* spider. We have analyzed the neutralization potential of sera obtained by immunization of horses with rCpLi and rCpLi combined with initial doses of venoms and compared these with antivenom traditionally produced in horses using crude *Loxosceles gaucho*, *Loxosceles laeta* and *L. intermedia* venoms as antigens. We have demonstrated by ELISA that horses immunized with three initial doses of crude venom containing mixtures of *L. intermedia*, *L. gaucho* and *L. laeta* followed by nine doses of rCpLi generate antibodies with the same reactivity as those produced following immunization with traditional antivenom, towards the venoms of the three *Loxosceles* sp. species. Results from *in vivo* and *in vitro* neutralization assays showed that the new horse sera are able to neutralize the dermonecrotic activity of *Loxosceles* venoms, which are of medical importance in Brazil and some of these sera are capable of meeting the necessary potency requirements that could allow for their therapeutic use in humans. This immunization strategy combining both antigens used approximately 67% less crude *Loxosceles* venoms compared to traditional immunization protocol and can mean the development of *Loxosceles* antivenoms with the consequent reduction of devastation of arachnid fauna.

1. Introduction

Loxoscelism, the condition resulting from the bite of "brown spiders" from the *Loxosceles* genus, is the most important form of arachneism in some countries and constitutes the third cause of accidents by venomous animals in Brazil (Pauli et al., 2006). In the clinic, antivenom therapy is used to neutralize the circulating venom and reduces the risk of fatal complications following accidents in humans (Ministério da Saúde. Fundação Nacional de Saúde, 2001). The *Loxosceles* antivenom has been produced in Brazil since

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the early 1960s (Furlanetto, 1961) and two antivenoms are available from the Brazilian Ministry of Health: anti-arachnidic serum and antiloxoscelic serum (Pauli et al., 2006). The main loxoscelic antivenom is the polyspecific serum containing antibodies against venoms of the three *Loxosceles* species that are most medically important in the country: *Loxosceles gaucho*, *Loxosceles laeta* and *Loxosceles intermedia* and is produced at Centro de Producão e Pesquisa de Imunobiológicos (CPPI) of the State of Paraná, Brazil.

Sphingomyelinases D (SMases D), phospholipase D family, dermonecrotic proteins or Loxtox family proteins (Tambourgi et al., 1995; Lee and Lynch, 2005; Kalapothakis et al., 2007) are the main components expressed in *Loxosceles* spp. venom glands (Fernandes-Pedrosa et al., 2008) and are mainly responsible for local and systemic effects induced by *Loxosceles* venoms. SMase D proteins are also the most antigenic/immunogenic components of the venom (Barbaro et al., 1994). Monoclonal and polyclonal antibodies against *Loxosceles* crude venoms principally recognize these dermonecrotic proteins (Barbaro et al., 1994; Guilherme et al., 2001; Alvarenga et al., 2003; Araujo et al., 2003; Barbaro et al., 2005). The use of large amounts of crude venoms for the production of *Loxosceles* antivenoms (immunizations and preclinical potency tests) represent a limitation for the production of antibodies for therapeutic purposes, as well as the fact that immunogens are highly toxic for animals (Felicori et al., 2009). Therefore, there is an increasing interest in evaluating the possibility of replacing or reducing the amount of crude venoms used in the production of antivenoms and researches have been undertaken using mice, rabbits or horses as models for immunization with dermonecrotic recombinant proteins (Fernandes Pedrosa et al., 2002; Araujo et al., 2003; Tambourgi et al., 2004; Olvera et al., 2006; de Almeida et al., 2008). In this way, we have recently produced in *E. coli* a chimeric non-toxic protein (rCpLi) containing three epitopes of rLiD1, a dermonecrotic toxin from the venom of *L. intermedia* spider (Mendes et al., 2013). The immunogenicity of rCpLi was initially tested in rabbits and antibodies against this recombinant protein effectively neutralized the toxic effects of rLiD1.

In the present study, we have analyzed the neutralization potential of sera obtained by immunization of horses only with a chimeric protein containing epitopes, another one using two antigens (loxoscelic venoms for initial doses followed by the chimeric protein) and compared this with antivenom traditionally produced at CPPI, using crude *L. gaucho*, *L. laeta* and *L. intermedia* venoms as antigens (loxoscelic antigen). The immunization schedule combining both antigens used 67% less crude *Loxosceles* venoms compared to traditional immunization protocol.

2. Materials and methods

2.1. Venoms and recombinant proteins

The venoms were obtained from adult specimens of *L. intermedia, L. laeta* and *L. gaucho* spiders. Spiders were maintained in the climatized aracnidarium of CPPI. The venoms obtained by electric stimulation (15 V) were pooled, centrifuged, filtered, lyophilized and stored at −20 °C before use. The rLiD1 cDNA was sub cloned in the pET11a vector, expressed in *E. coli* and rLiD1 purified by reversed-phase HPLC using a C8-Vydac column as described by Felicori et al., (2006). The rCpLi-containing epitopes of rLiD1 were constructed, expressed and purified using a slightly modified procedure described by Mendes et al., (2013). Inclusion bodies, containing significant portions of rCpLi, were solubilized in phosphate 20 mM, 500 mM NaCl, 8 M urea, pH 7.5 (denaturing buffer) and purified in a nickel column utilizing the refolding procedure with phosphate 20 mM, 500 mM NaCl, 30 mM imidazole, pH 7.5 (equilibration buffer) to remove gradually the urea. The recombinant protein was eluted with phosphate 20 mM, 500 mM NaCl, 500 mM imidazole, pH 7.5 (elution buffer). Purified rCpLi was desalted using the column HiPrep™ 26/10 Desalting (GE Healthcare) using phosphate buffer 20 mM, 0.9% (w/v) NaCl. Finally, rCpLi was submitted to reversed-phase chromatography on a C18 Shimadzu column (250 × 20 mm, 15 μm particle diameter). The fractions were eluted with a linear gradient of 0–100% acetonitrile in 0.1% trifluoracetic acid at flow rate of 5 mL/min, lyophilized and dissolved in the desalting buffer.

2.2. Immunization protocols

Nine naïve horses were used for the production of antivenoms at the Immunobiological Production Unit of CPPI. Three horses were used for each group of immunization protocol, which has started with the hyperimmunization protocol. After collection of pre-immune sera, six animals (groups 1 and 2) received an initial subcutaneous injection of 5 mg of a mixture of *Loxosceles intemedia, L. laeta* and *L. gaucho* venoms (loxoscelic antigen) (1.66 mg of each venom), whilst the third group received 5 mg of rCpLi in complete Freund's adjuvant (as shown in Table 1A). Two subcutaneous booster injections of loxoscelic antigen

Table 1
Immunization schedule.

Time (days)	Adjuvant	Dose (mg)	Group 1	Group 2	Group 3
A					
0	AFC	5	Lox. ant.	Lox. ant.	rCpLi
30	AFI	5	Lox. ant.	Lox. ant.	rCpLi
45	AFI	5	Lox. ant.	Lox. ant.	rCpLi
60	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
67	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
74	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
81	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
88	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
95	$Al(OH)_3$	5	Lox. ant.	rCpLi	rCpLi
B					
270	AFI	5 (4 of protein)	Lox. ant.	Lox. ant.	rCpLi
285	$Al(OH)_3$	5 (4 of protein)	Lox. ant.	rCpLi	rCpLi
292	$Al(OH)_3$	5 (4 of protein)	Lox. ant.	rCpLi	rCpLi

A: hyperimmunization schedule. B: Re-immunization schedule. For both tables, time is shown continuously. AFC – Complete Freund's adjuvant; AFI – Incomplete Freund's adjuvant; $Al(OH)_3$ – Aluminum hydroxide; Lox. ant. – loxoscelic antigen (mixture de *L. intermedia*, *L. gaucho* and *L. laeta* crude venoms). rCpi – recombinant chimeric protein from *Loxosceles intermedia* venom.