Generation and characterization of a recombinant chimeric protein (rCpLi) consisting of B-cell epitopes of a dermonecrotic protein from *Loxosceles intermedia* spider venom

T.M. Mendes [a], D. Oliveira [a], L.F.M. Figueiredo [a], R.A. Machado-de-Avila [a], C.G. Duarte [a], C. Dias-Lopes [a], G. Guimarães [a], L. Felicori [a], J.C. Minozzo [b], C. Chávez-Olortegui [a],*

[a] *Departamento de Bioquímica-Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, CP: 486, CEP: 31270-901, Belo Horizonte, MG, Brazil*
[b] *Centro de Produção e Pesquisa de Imunobiologia (CPPI), Rua Targino da Silva, s/n°, Jardim Primavera, Piraquara, PR, Brazil*

ABSTRACT

A chimeric protein was constructed expressing three epitopes of LiD1, a dermonecrotic toxin from the venom of *Loxosceles intermedia* spider. This species is responsible for a large number of accidents involving spiders in Brazil. We demonstrated that the chimeric protein (rCpLi) generated is atoxic and that antibodies previously developed in rabbits against synthetic epitopes reactive with rCpLi in ELISA and immunoblot assays. The antibody response in rabbits against the rCpLi was evaluated by ELISA and we have detected an antibody response in all immunized animals. Overlapping peptides covering the amino acid sequence of the rCpLi were synthesized on a cellulose membrane, and their recognition by rabbit anti-rCpLi serum assessed. Three different antigenic regions were identified. The percentage of inhibition of the dermonecrotic, hemorrhagic and edematogenic activities caused by the recombinant protein LiD1r in naïve rabbits was assessed by pre-incubation with anti-rCpLi antibodies. Anti-rCpLi induced good dermonecrotic and hemorrhagic protection. The levels of protection were similar to the antiboides anti-LiD1r. In summary, we have developed a polyepitope recombinant chimeric protein capable of inducing multiple responses of neutralizing antibodies in a rabbit model. This engineered protein may be a promising candidate for therapeutic serum development or vaccination.

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1. Introduction

Loxoscelism is the condition resulting from the bite of "brown spiders" from the *Loxosceles* genus. Clinical manifestations of Loxoscelism include dermonecrosis, with gravitational spreading at and near the bite site, and to a lesser extent with systemic involvement, such as acute renal failure, intravascular hemolysis, thrombocytopenia and disseminated intravascular coagulation [1–3]. The spiders of the *Loxosceles* genus have a worldwide distribution and accidents have been described on all continents [4–6]. The spider species *Loxosceles intermedia*, *Loxosceles laeta* and *Loxosceles gaucho* are a group of arachnids with medical importance in the South and South-east of Brazil [7]. The number of human accidents caused by spiders of the *Loxosceles* genus in Brazil amounts to almost 7000 annually [8].

Sphingomyelinases D (SMases D), phospholipase D family, dermonecrotic proteins or Loxtox protein family [9–11] are the main components expressed in *Loxosceles* spp. venom glands [12] and are responsible for local and systemic effects induced by *Loxosceles* venoms. SMase D proteins are also the most antigenic/immunogenic components of the venom [13]. Monoclonal and polyclonal antibodies against *Loxosceles* crude venoms principally recognize dermonecrotic proteins [14,15]. Effective anti-*Loxosceles* antivenoms have been produced by immunization with native or recombinant SMase-D toxins [16–18].

Linear and conformational epitopes mapped from a recombinant dermonecrotic protein (rLiD1) from *L. intermedia* venom induced antibody responses that efficiently neutralize the toxic effects of these venoms [19–22]. These previous results suggest that these synthetic non-toxic immunogens could be used for the production of therapeutic sera or in vaccination [22]. We think that the success of these strategies can be increased by presenting to the immune system of immunized animals a formulation containing recombinant or synthetic multi-epitope proteins consisting of the LiD1 epitopes previously determined. Thus, in this work, we report the design, production, purification and initial immunological characterization of a recombinant chimeric protein (rCpLi), consisting of three binding epitopes (one conformational and two linear) of LiD1. This approach will be a model for the design and development of non-toxic antigens that can be good candidates for the production

of a new generation of therapeutic anti-venoms or vaccines against *Loxosceles* spider venoms.

2. Materials and methods

2.1. Animals, Loxosceles venoms and rLiD1

Adult New Zealand rabbits (2–2.5 kg) were used and obtained from the Escola de Medicina Veterinária of Universidade Federal de Minas Gerais (UFMG), BH, MG, Brazil. All animals received free access to water and food under controlled environmental conditions. Animal manipulations were performed according to the regulations of the UFMG. *Loxosceles* venoms were provided by the Centro de Produção e Pesquisa de Imunobiológicos (CPPI) of the State of Paraná, Brazil. The venoms obtained by electric stimulation (15 V) of adult specimens were pooled, centrifuged, filtered, lyophilized and stored at −20 °C before use. *Tityus serrulatus* scorpions were collected at Belo Horizonte City (Brazil) and maintained at the Laboratory of Immunochemistry of the Biochemistry-Immunology Department, of UFMG. The LiD1 cDNA [16] was subcloned in the pET11a vector, expressed in *E. coli* and purified by reversed-phase HPLC using a C8-Vydac column as described by [19].

2.2. Recombinant chimeric protein (rCpLi) construction

The rCpLi was constructed containing epitopes from rLiD1 [16]. The continuous epitopes NLGANSIETDVSFDDNANPEYTYHGIP (peptide 27) and CKKYENFNDFLKGLR (peptide 15) [20,21] and one conformational epitope NCNKNDHLFACW (peptide 12) [22] were used for the chimera recombinant production. The DNA was synthesized with the nucleotide sequence of the three epitopes using two glycine codons as spacers between each epitope sequence (ITD – Integrated DNA Technologies). At the 5′ end and 3′ end of the synthetic DNA were added sites for restriction enzymes NcoI and XhoI respectively. The double stranded DNA was subcloned into the easy cloning vector pGEM (Promega) and subsequently treated with restriction enzymes NcoI and XhoI. The DNA fragment was purified and cloned into the expression vector pET 26b (Stratagene), previously treated with NcoI and XhoI and alkaline phosphatase.

2.3. Expression and purification of rCpLi

The vector pET 26b containing the DNA fragment of interest was chemically transformed into bacteria *E. coli* BL21 for expression. Bacterial cultures were grown in 10 mL 2xYT medium containing kanamicin (50 mg/mL) overnight at 37 °C and used to inoculate 100 mL fresh 2xYT medium. When the culture reached a density of Ab600 = 0.6, the recombinant vector was induced by addition of IPTG (0.6 mM final). The cells were grown for an additional 4 h at 37 °C, harvested by centrifugation, and suspended in lysis buffer (50 mM phosphate buffer with 500 mM NaCl) containing 4 mg/mL lysozyme; and incubated for 15 min at 37 °C. The cells were subjected to heat shock (dry ice/water bath at 37 °C by 3×), then were sonicated in short pulses (3 pulses of 10 s with 10 s pause between them, amplitude 40%) and subjected to centrifugation at 8000 × g. The bacterial supernatant and pellet were separated on polyacrylamide gels by [23]. After electrophoresis, gels were stained with Coomassie Blue G-250. The supernatant containing soluble protein was used for purification of rCpLi on a column of nickel (GE) in FPLC apparatus according to manufacturer's specifications. The rCpLi was submitted to MALDI-TOF-TOF analyses to determine the molecular mass.

2.4. Sphingomyelinase activity of rCpLi

Sphingomyelinase activity of rCpLi was determined using the Amplex® Red sphingomyelinase assay kit (Molecular Probes, Invitrogen, USA). Hydrolysis of sphingomyelin was detected indirectly by measuring Fluorescence with the fluorescence micro plate reader (Varian, Agilent Technologies, CA, USA) using excitation at 530 nm and detection at 590 nm.

2.5. Antigenic analysis of rCpLi by ELISA and Western blotting

The antigenic cross-reactivity of rCpLi toward the antibodies anti-*L. intermedia* venom or anti-individual epitopes was determined by ELISA and immunoblotting techniques. Falcon microplates (Becton Dickinson, France) were coated overnight at 4 °C with 100 μL of 5 μg/mL solution of rCpLi or *L. intermedia* venom in 20 mM sodium bicarbonate buffer, pH 9.6. The assays were performed as described by [24]. Absorbance values were determined at 492 nm with a Titertek Multiscan spectrophotometer. All measurements were made in duplicate. For the Western blot experiments samples of rCpLi were solubilized in reducing sample buffer and run on 15% SDS-PAGE [23]. After electrophoresis, the proteins were transferred to a 0.45 μm nitrocellulose membrane [25]. The assays were performed as described by [15].

2.6. Immunization of rabbits with rCpLi incorporated in liposomes

For the preparation of liposomes containing rCpLi, 100 mg of asolectina (Sigma) was dissolved in distilled water (1 mL). rCpLi was added at the ratio 1:1 (mg of asolectina/μg of rCpLi). The solution was lyophilized and stored at −20 °C until use. After collection of pre-immune serum, each animal received an initial subcutaneous injection of 50 μg liposome-entrapped rCpLi in aluminum hydroxide (day 1). Control rabbits received 50 μg of unrelated protein in the same condition. Two similar booster injections (days 15, and 30) and three injections of 100 μg (days 37 and 44) were made. Animals were bled and blood samples were withdrawn one week after the last injection.

2.7. Indirect ELISA for the determination of anti-rCpLi antibodies

Falcon plates purchased from Becton Dickinson France S.A. were coated overnight at 4 °C with 100 μL of a 5 μg/mL solution of the rCpLi, rLiD1 or *L. intermedia*, *L. gaucho*, *L. laeta* spider and *T. serrulatus* scorpion venoms in 0.02 M sodium bicarbonate buffer, pH 9.6. The assays were performed as described before (Section 2.5).

2.8. Immunoassays with cellulose-bound peptides

Sets of 39 overlapping pentadecapeptides frame shifted by two residues corresponding to the amino acid sequence of rCpLi were prepared on cellulose membranes by Spot synthesis [26]. The membranes were obtained from Intavis (Koln, Germany) and the F-moc amino acids from Novabiochem. A Multipep robot was used for peptide synthesis. The immunoassay was performed as described in [21]. The anti-rCpLi rabbit serum was diluted 1:1000 and alkaline–phosphatase conjugated anti-rabbit antibody (Sigma) was diluted 1:3000.

2.9. Neutralization assays

The dermonecrotic, edema and hemorrhagic activities of rLiD1 were determined as described by [27]. The neutralization of these activities was determined in non-immunized rabbits by pre-incubation, 1 h at 37 °C, of 10 μg of the rLiD1 (the MND-minimum necrotizing dose of the rLiD1 used throughout this study was 10 μg)
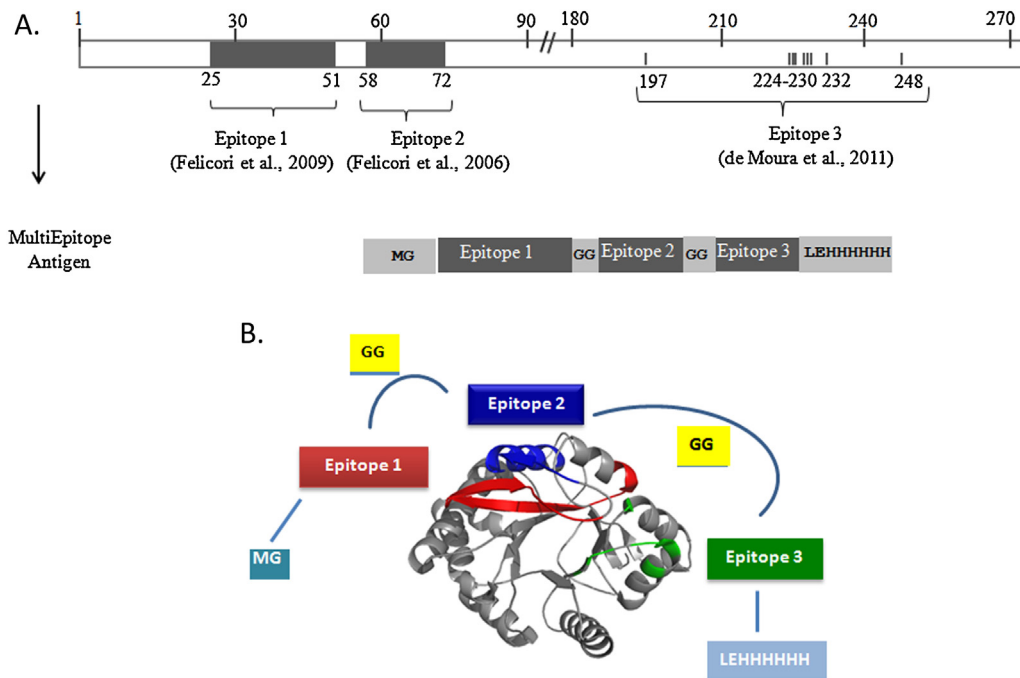
Fig. 1. Recombinant chimeric protein of *Loxosceles intermedia*. (A) LiD1 sequence schema showing the position of the three previously mapped epitopes in LiD1 and the multi epitope antigen that present the three epitopes. Epitope 1, with the sequence NLGANSIETDVSFDDNANPEYTYHGIP, is present in LiD1 in the position 25–51 [20]. Epitope 2, with the sequence CYGSKKYENFNDFLKGLR, is in the position 58–72 [19] and finally the discontinuous epitope 3 (NCNKNDHLFACW) is located close to C-termini of LiD1 [22]. These three segments are linked by the short spacer GG. This gene, corresponding to the 3 epitopes was cloned in the plasmid vector pET-26b (+) and expressed in *Escherichia coli* as a fusion product with a His-tag. For cloning, two amino acid residues were added at the N-terminal portion (MG) and C-terminal portion (LE). (B) Localization of epitopes used for the construction of chimeric protein in the 3D structure of LiD1.

with 200 µg of IgG anti-rCpLi in a final volume of 100 µL. Incubation of the rLiD1 with pre-immune IgG was performed as a negative control. Dermonecrosis, edema and hemorrhage activities were measured 72 h after injection. The diameters of hemorrhagic and edematogenic lesions were measured with a scale meter and tachymeters, respectively.

2.10. Molecular modeling

The three epitopes used for the chimera recombinant production were localized in the three-dimensional model of LiD1 and visualized using swiss-pdb viewer package [28]. The LiD1 model was achieved by utilizing the SMase I dermonecrotic protein (PDB accession code: 1XX1) [29] as template and the molecular modeling package Modeller [30] for models generation.

3. Results

3.1. Design, expression, purification and characterization of chimer protein (rCpLi)

We have previously identified and characterized three neutralizing epitopes of a dermonecrotic protein (rLiD1) from *L. intermedia* venom [19–22]. The nucleotide sequence of two linear epitopes (NLGANSIETDVSFDDNANPEYTYHGIP and CKKYENFND-FLKGLR) was obtained from gene bank [16]. For the conformational epitope (NCNKNDHLFACW) we used the codon preference of *E. coli* to give its nucleotide sequence. We also used a spacer with two glycine codons between the epitope sequences (Fig. 1). Sites for the restriction enzymes Nco I and Xho I were inserted at the 5′ and 3′ ends, respectively, of the synthetic gene for further cloning. The final encoding gene has chimeric 189 nucleotides and was synthesized single stranded by the company IDT. To obtain the double stranded gene PCR was performed using primers covering the full

sequence of the chimera and using chimeric DNA as a template. The DNA obtained was purified and inserted into the expression vector pET 26b containing a His$_6$-tag in its C-terminus (Stratagene). The expression of the chimer gene in the pET 26b vector was performed in *E. coli* strain BL21 (DE) and produced after 4 h of induction with IPTG. After cytoplasmic lysis the recombinant protein was found both in the supernatant and in the pellet, but was present at a higher concentration in the pellet, indicating that the protein was expressed largely in an insoluble form (Supplementary Fig. 1). rCpLi was purified from the supernatent using a nickel column on an FPLC system. A product of the correct size for rCpLi (7.7 kDa) was identified using mass spectrometry. The rCpLi did not show sphingomyelinase activity in the concentrations tested using the Amplex® Red sphingomyelinase assay kit (Fig. 2).
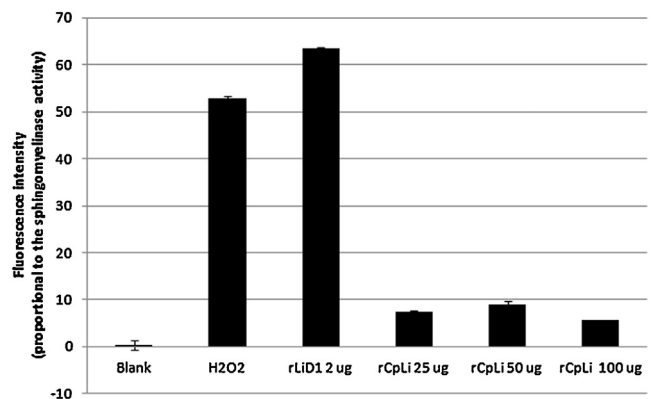

Fig. 2. Sphingomyelinase activity of the recombinant protein rCpLi. The SMase activities using different amounts (25, 50, 100 µg) of rCpLi were assessed by Amplex Red Sphingomyelinase Assay Kit (Invitrogen). The rLiD1 was used as control.
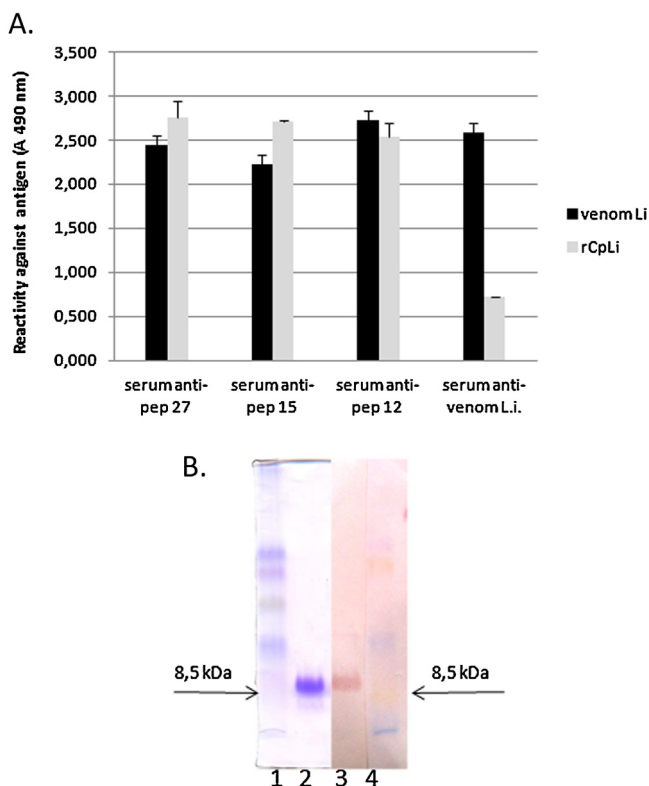
A.

B.

Fig. 3. Immunoreactivity of the rCpLi by ELISA and Western blotting. (A) ELISA showing the reaction of rCpLi and *Loxosceles intermedia* whole venom against anti-peptide antibodies and anti-venom *L. intermedia*. ELISA plates were coated with 5 μg/mL solution of each antigen. Sera were diluted at 1:100 and values given are the means of duplicates. The absorbance of the samples was determined at 492 nm. (B) Samples were applied to a 15% polyacrylamide SDS gel and recombinant protein was transferred to a nitrocellulose membrane. The rCpLi was probed with mouse antibody anti-rLiD1 (diluted 1:5000). The molecular weight standard was applied to lanes 1 and 4.

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3.2. Antigenicity and immunogenicity of the rCpLi

Antigenic functionality of rCpLi and its cross-reactivity toward antibodies against *L. intermedia* venom, rLiD1 or their epitopes was studied by ELISA and immunoblot analysis. The interaction of rCpLi with four antibodies: rabbit anti-peptide 27, anti-peptide15, anti-peptide 12 and anti-*L. intermedia* crude venom was detected by ELISA. As shown in Fig. 3A, the reactivity of rCpLi and *L. intermedia* whole venom against anti-peptide antibodies have similar values (absorbance at 490 nm); however anti-*L. intermedia* serum antibodies recognized strongly the whole venom antigens and moderately the chimeric protein. Western blot analysis (Fig. 3B, lane 3) showed that specific mouse polyclonal antibody anti-rLiD1 reacted with rCpLi. From these results, we conclude that the rCpLi exhibits good antigenic properties.

To assess the capacity of rCpLi to stimulate a protective immune response, rCpLi was entrapped into liposomes which were used as immunogens in rabbits. One week after the fourth injection, the reactivity of serum samples toward the rCpLi, rLiD1 protein and Li whole venom was tested by indirect ELISA (Fig. 4). Serum samples from immunized rabbits showed reactivity against rCpLi (Fig. 4A). rCpLi was able to elicit the production of antibodies which were cross-reactive with the cognate antigen, rLiD1. Similar reactivities were obtained with the pool of the two immune sera (Fig. 4B). The rabbit anti-rCpLi antibodies cross-reacted with venom from the *L.*

Table 1
Neutralization in vitro activities. Rabbits were injected i.d. with 1 MND of rLiD1 which was pre-incubated for 1 h, at 37 °C with pre-immune rabbit IgG or immune rabbit IgG anti-rCpLi (200 μg). The neutralization of the dermonecrotic activity, hemorrhagic area and edema were measured 72 h after the injection. N = 4.

Immunogen	Activity inhibition (%)			Reference
	Dermonecrotic	Hemorrhagic	Edema	
rLiD1 (in vitro)	100	100	70	Felicori et al. [20]
Pep 15 (in vitro)	80	40	40	Felicori et al. [20]
rCpLi (in vitro)	95	75	10	This paper

intermedia and *L. gaucho* sub-species, but not with venoms from *L. laeta* or *T. serrulatus* (Fig. 4C).

In order to map the epitope recognized by the anti-rCpLi antibodies, we used Spot synthesis to prepare a set of 39 overlapping peptides (15 residues, frameshifted by 2 residues) corresponding to the amino acid sequence of the rCpLi. At the end of the synthesis, the peptides remain covalently bound to the membrane and can be collectively assayed for antibody reactivity. Fig. 5A shows the binding pattern of a rabbit anti-rCpLi serum with the overlapping peptides. Three series of peptides were strongly recognized by antibodies in the N-terminal part (spots 18–22), the central region (spots 25–30) and in the C-terminal part of the protein (spots 37–39), indicating that the three continuous epitopes are displayed on a large part of the protein (Fig. 5B). The binding was deemed to be specific since neither the pre-immune rabbit serum nor the alkaline–phosphatase conjugated anti-rabbit antibody showed reactivity with the membrane (results not shown).

3.3. Protective immune response induced by antibodies after pre-incubation with rLiD1

The ability of anti-*rCpLi* rabbit IgGs to block the toxic activity of rLiD1 was assessed by measuring the reduction of three different biological effects induced by rLiD1 in naïve rabbits. The dermonecrotic, hemorrhagic and edema forming activities were assessed in rabbits after injection of rLiD1 previously incubated with serum IgGs from immunized rabbits. As can be seen in Table 1, 72 h after the injections IgGs from immunized rabbits were able to effectively neutralize the dermonecrotic and hemorrhagic effects. However, the edema effects were not neutralized by the IgGs from anti-rCpLi. Previous results derived from rabbits immunized with the rLiD1 of *L. intermedia* were used as a neutralization reference [20]. These rabbits demonstrated 100% protection against necrotic and hemorrhagic activities and 70% against oedematogenic activities. Similarly, groups that received the chimeric protein as an immunogen displayed about 95% protection against necrotic and 75% against hemorrhagic activities. Notably, for reasons that are not clear to us, inhibition of edema was particularly low (10%). For comparative purposes IgGs from rabbits immunized with the epitope peptide [20], displayed about 50% protection against dermonecrotic activity, whilst the hemorrhagic and oedematogenic activities were 40% neutralized. Therefore, antibodies prepared from rabbits immunized with the chiemeric multiepitopic protein were protective against the toxic effects of rLiD1 and were more efficient in their neutralizing properties than antibodies against individual epitopes.

4. Discussion

The production of anti-venoms used for therapeutic purposes in humans is problematic due to the fact that the animals required for their production suffer from the effects of the venom [31]. In an attempt to improve these conditions we have identified alternative
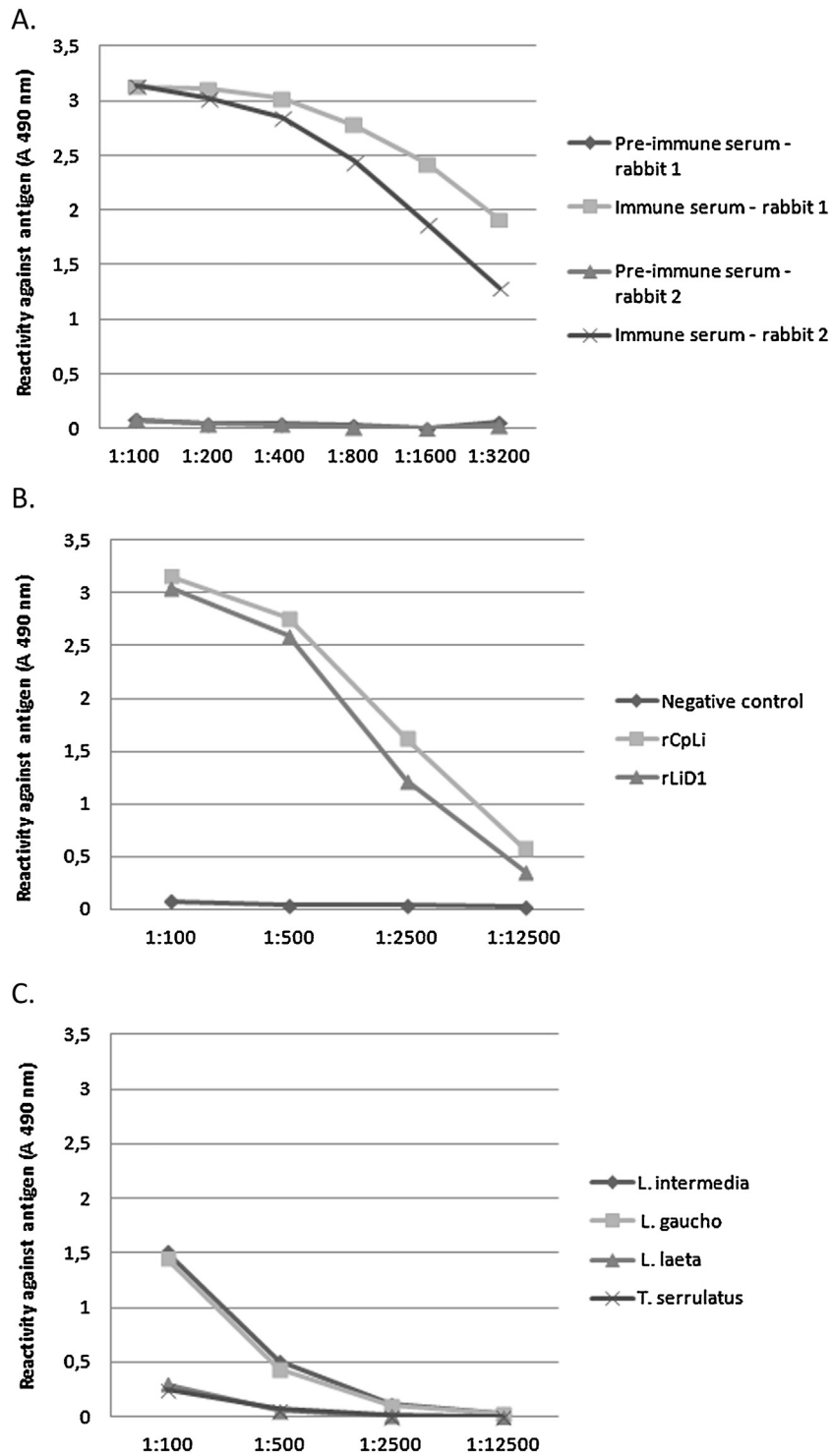
Fig. 4. Titration of sera from rabbits immunized with the recombinant protein rCpLi. (A) An ELISA plate was pre-coated with rCpLi (5 μg/mL) and sera were diluted between 1:100 and 1:3200. (B) ELISA showing the reaction of pool rabbit antibody anti-rCpLi against rLiD1 and rCpLi. Plates were coated with 5 μg/mL solution of each antigen. Anti-rCpLi serum was tested at 4 different dilutions (1:100; 1:500; 1:2500; 1:12,500). (C) Recognition of the arachnidic venoms (*L. intermedia*, *L. gaucho*, *L. laeta* and *Tityus serrulatus*) by rabbit antibody anti-rCpLi (dilutions – 1:100; 1:500; 1:2500; 1:12,500). The values given are the means of duplicates and absorbance of the samples was determined at 492 nm. $N = 2$.

non-toxic immunogens [19–22] for the production of anti-sera against *Loxosceles* spider venoms. Peptide epitopes were identified by epitope mapping of LiD1, a dermonecrotic protein found in the venom of the Brazilian brown spider *L. intermedia* [16]. LiD1 protein showed biological toxic activities which were comparable with the activities evoked by the crude *L. intermedia* venom [19]. Many

reasons justify the use of peptides instead of entire proteins for the production of anti-venoms. Firstly, unlike toxic antigens, peptide formulations are not harmful to the immunized animal. Secondly, the presentation of formulations containing chimeric proteins consisting of LiD1epitopes to the immune system of immunized animals can increase the success of immunization protocols.
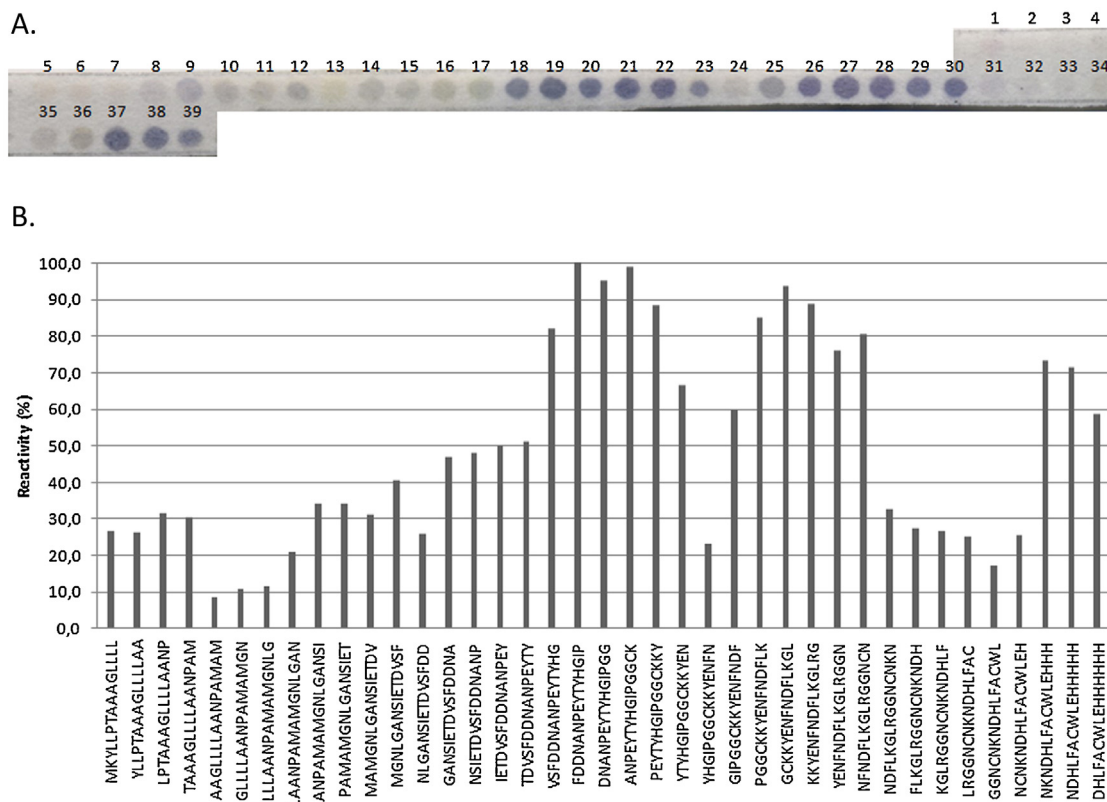
Fig. 5. Binding pattern of a rabbit anti-rCpLi serum with the overlapping peptides. (A) Reactivity of 15-mer overlapping peptides derived from the amino acid sequence of rCpLi. Peptides were prepared by the Spot method on cellulose membranes and rabbit anti-rCpLi binding (diluted 1:1000) to cellulose-bound peptides was detected by an alkaline phosphatase-coupled anti-rabbit antibody (diluted 1:3000). (B) Percentage of reactivity of each peptide recognized by antiserum rCpLi. The first 12 peptides are corresponding to the signal peptide vector pET 26b.

Finally, the production of engineered peptides is generally easier than procuring and preparing crude venom from spiders.

In the present study we present the development of a chimeric protein immunogen of LiD1, a dermonecrotic toxin from *L. intermedia* spider venom, the most prevalent species with medical importance in Brazil. In our first attempt a chimeric protein construct expressing the epitopes NLGANSIETDVSFDDDANPEYTY-HGIP comprising residues 25–51, SKKYENFNDFLKGLR comprising residues 58–72 and the conformational epitope NCNKNDHLFACW were generated by cloning the respective synthetic genes in pET 26b vector. We demonstrated that a non-toxic miniprotein expressing epitopes of LiD1from *Loxosceles* spider venoms can be generated. This is not in fact the first time that a synthetic chimeric, multi-epitope immunogen has been constructed for the stimulating venom toxin-specific IgGs. A synthetic immunogen consisting of a string of epitopes identified in viper venom SVMPs induced IgGs capable of neutralizing the hemorrhagic effects of the venom [32]. ELISA and Immunoblot assays revealed that the mini-protein displayed antigenic activity against antibodies anti-individual epitopes. Anti-*L. intermedia* crude venom also reacted with rCpLi. The antibody response of each immunized rabbit against the chimeric protein rCpLi and recombinant protein rLiD1 were evaluated by ELISA. Through these assays, we were able to detect a good antibody response in both the immunized rabbits. Surprisingly, similar antibody reactivity was observed for rCpLi and rLiD1. Rabbits immunized with the chimeric construct reacted significantly to *L. intermedia* and *L. gaucho* venom antigens; however no reactivity was observed with *L. laeta*, other *Loxosceles* species or with *T. serrulatus* scorpion venom. This finding is not surprising as proteins similar to LiD1 from *L. intermedia* are found in *L. gaucho* venom [33].

An experimental approach to map rCpLi epitopes was undertaken. Overlapping peptides covering the sequence of the protein were synthesized, and their recognition by rabbit anti-rCpLi serum assessed. Three different antigenic regions were identified, as expected. The percentage of inhibition of the dermonecrotic, hemorrhagic and oedematogenic activities caused by the recombinant protein rLiD1 in naïve rabbits was assessed by pre-incubation with anti-rLiD1 antibodies and anti-rCpLi chimeric protein antibodies. IgG purified from the serum of each group were pre-incubated with rLiD1. The anti-rLiD1 as well as by the anti-rCpLi antibodies provided good dermonecrotic protection (100% and 95% respectively). The protection against hemorrhagic and oedematogenic activities was smaller for the rCpLi antibodies compared to the anti-rLiDi antibodies. Hemorrhagic protection given by the anti-rLiD1 antibodies was 100%, whereas anti- rCpLi antibodies gave 75% protection. The oedematogenic activity seems to be more difficult to neutralize than the other two studied activities as already observed in previous studies [20].

In summary, we have developed a recombinant chimeric protein immunogen against LiD1, a dermonecrotic toxin of *L. intermedia* spider venom consisting of B-cell epitopes. This polyepitope miniprotein is capable of inducing multiple responses of neutralizing antibodies in a rabbit model. Therefore, this engineered protein may be a promising candidate for therapeutic serum development or vaccination in the future.

References

[1] Futrell JM. Loxoscelism. Am J Med Sci 1992;304:261–7.

[2] Schenone H. Cutaneous loxoscelism with edematous predominance. Bol Chil Parasitol 1998;53:78–83.

[3] Tambourgi DV, Paixao-Cavalcante D, Goncalves de Andrade RM, Fernandes-Pedrosa Mde F, Magnoli FC, Paul-Morgan B, et al. Loxosceles sphingomyelinase induces complement-dependent dermonecrosis, neutrophil infiltration, and endogenous gelatinase expression. J Invest Dermatol 2005;124:725–31.

[4] da Silva PH, da Silveira RB, Appel MH, Mangili OC, Gremski W, Veiga SS. Brown spiders and loxoscelism. Toxicon 2004;44(7):693–709.

[5] Hogan CJ, Barbaro KC, Winkel K. Loxoscelism: old obstacles, new directions. Ann Emerg Med 2004;44(6):608–24.

[6] Swanson DL, Vetter RS. Loxoscelism. Clin Dermatol 2006;24(3):213–21.

[7] Eickstedt VRD. Aranhas de importância médica no Brasil. In: Barravieira B, editor. Venenos animais: uma visão integrada. Rio de Janeiro: EPUC; 1994. p. 151–72.

[8] Ministério da Saúde Sistema Nacional de Notificação de Agravos. Ministério da saúde, Brasil; 2007.

[9] Tambourgi DV, Magnoli FC, Von Eickstedt VR, Benedetti ZC, Petricevich VL, da Silva WD. Incorporation of a 35-kilodalton purified protein from Loxosceles intermedia spider venom transforms human erythrocytes into activators of autologous complement alternative pathway. J Immunol 1995;155(9):4459–66.

[10] Chaim OM, Sade YB, da Silveira RB, Toma L, Kalapothakis E, Chávez-Olortegui C, et al. Brown spider dermonecrotic toxin directly induces nephrotoxicity. Toxicol Appl Pharmacol 2006;211:64–77.

[11] Kalapothakis E, Chatzaki M, Goncalves-Dornelas H, de Castro CS, Silvestre FG, Laborne FV, et al. The Loxtox protein family in Loxosceles intermedia (Mello-Leitao) venom. Toxicon 2007;50:938–46.

[12] Fernandes-Pedrosa Mde F, Junqueira-de-Azevedo Ide L, Gonçalves-de-Andrade RM, Kobashi LS, Almeida DD, Ho PL, et al. Transcriptome analysis of Loxosceles laeta (Araneae, Sicariidae) spider venomous gland using expressed sequence tags. BMC Genomics 2008;9:279.

[13] Barbaro KC, Eickstedt VR, Mota I. Antigenic cross-reactivity of venoms from medically important Loxosceles (Araneae) species in Brazil. Toxicon 1994;32:113–20.

[14] Guilherme P, Fernandes I, Barbaro KC. Neutralization of dermonecrotic and lethal activities and differences among 32-35 kDa toxins of medically important Loxosceles spider venoms in Brazil revealed by monoclonal antibodies. Toxicon 2001;39:1333–42.

[15] Alvarenga LM, Martins MS, Moura JF, Kalapothakis E, Oliveira JC, Mangili OC, et al. Production of monoclonal antibodies capable of neutralizing dermonecrotic activity of Loxosceles intermedia spider venom and their use in a specific immunometric assay. Toxicon 2003;42:725–31.

[16] Kalapothakis E, Araujo SC, de Castro CS, Mendes TM, Gomez MV, Mangili OC, et al. Molecular cloning, expression and immunological properties of LiD1, a protein from the dermonecrotic family of Loxosceles intermedia spider venom. Toxicon 2002;40:1691–9.

[17] Olvera A, Ramos-Cerrillo B, Estevez J, Clement H, de Roodt A, Paniagua-Solís J, et al. North and South American Loxosceles spiders: development of a polyvalent antivenom with recombinant sphingomyelinases D as antigens. Toxicon 2006;48:64–74.

[18] de Almeida DM, Fernandes-Pedrosa Mde F, de Andrade RM, Marcelino JR, Gondo-Higashi H, de Azevedo Ide L, et al. A new anti-loxoscelic serum produced against recombinant sphingomyelinase D: results of preclinical trials. Am J Trop Med Hyg 2008;79:463–70.

[19] Felicori L, Araujo SC, de Avila RA, Sanchez EF, Granier C, Kalapothakis E, et al. Functional characterization and epitope analysis of a recombinant dermonecrotic protein from Loxosceles intermedia spider. Toxicon 2006;48:509–19.

[20] Felicori L, Fernandes PB, Giusta MS, Duarte CG, Kalapothakis E, Nquyen C, et al. An in vivo protective response against toxic effects of the dermonecrotic protein from Loxosceles intermedia spider venom elicited by synthetic epitopes. Vaccine 2009;27:4201–8.

[21] Dias-Lopes C, Guimaraes G, Felicori L, Fernandes P, Emery L, Kalapothakis E, et al. A protective immune response against lethal, dermonecrotic and hemorrhagic effects of Loxosceles intermedia venom elicited by a 27-residue peptide. Toxicon 2010;55:481–7.

[22] de Moura J, Felicori L, Moreau V, Guimaraes G, Dias-Lopes C, Molina L, et al. Protection against the toxic effects of Loxosceles intermedia spider venom elicited by mimotope peptides. Vaccine 2011;29:7992–8001.

[23] Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[24] Chavez-Olortegui C, Amaral DA, Rochat H, Diniz C, Granier C. In vivo protection against scorpion toxins by liposomal immunization. Vaccine 1991;9(12):907–10.

[25] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76(9):4350–4.

[26] Molina F, Laune D, Gougat C, Pau B, Granier C. Improved performances of Spot multiple peptide synthesis. Peptide Res 1996;9:151–5.

[27] Furlanetto WBRS, Rosa RR, Saliba F, Navas J. Observação dos efeitos do veneno loxoscélico e avaliação da sua atividade. Ciênc Cult 1962;14:242.

[28] Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 1997;18(15):2714–23.

[29] Murakami MT, Fernandes-Pedrosa MF, Tambourgi DV, Arni RK. Structural basis for metal ion coordination and the catalytic mechanism of sphingomyelinases D. J Biol Chem 2005;280(14):13658–64.

[30] Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct 2000;29:291–325.

[31] Theakston RDG, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 2003;41:541–57.

[32] Wagstaff SC, Laing GD, Theakston RDG, Papaspuridis C, Harrison RA. Bioinformatics and multiepitope DNA immunization to design rational snake antivenom. PLoS Med 2006;3(6):832–9.

[33] Binford GJ, Bodner MR, Cordes MH, Baldwin KL, Rynerson MR, Burns SN, et al. Molecular evolution, functional variation, and proposed nomenclature of the gene family that includes sphingomyelinase D in sicariid spider venoms. Mol Biol Evol 2009;26:547–66.