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Protection against the toxic effects of *Loxosceles intermedia* spider venom elicited by mimotope peptides

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ABSTRACT

The venom of *Loxosceles intermedia* (Li) spiders is responsible for cutaneous lesions and other clinical manifestations. We previously reported that the monoclonal antibody LimAb7 can neutralize the dermonecrotic activity of crude Li venom. In this study, we observed that this antibody recognizes several proteins from the venom dermonecrotic fraction (DNF), including LiD1.

Identifying the epitope of such a neutralizing antibody could help designing immunogens for producing therapeutic sera or vaccination approaches. To this aim, two sets of 25- and 15-mer overlapping peptides that cover the complete amino acid sequence of LiD1 were synthesized using the SPOT technique. None of them was recognized by LimAb7, suggesting that the epitope is discontinuous. Then, the screening of four peptide phage-display libraries yielded four possible epitope mimics that, however, did not show any obvious similarity with the LiD1 sequence. These mimotopes, together with a 3D model of LiD1, were used to predict with the MIMOP bioinformatic tool the putative epitope region (residues C197, Y224, W225, T226, D228, K229, R230, T232 and Y248 of LiD1) recognized by LimAb7. This analysis and the results of alanine-scanning experiments highlighted a few residues (such as W225 and D228) that are found in the active site of different SMases D and that may be important for LiD1 enzymatic activity. Finally, the only mimotope NCNKNDHLFACW that interacts with LimAb7 by SPOT and its analog NSNKNDHLFASW were used as immunogens in rabbits. The resulting antibodies could neutralize some of the biological effects induced by crude Li venom, demonstrating a mimotope-induced protection against *L. intermedia* venom.

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1. Introduction

The spiders *Loxosceles intermedia* (Li), *Loxosceles laeta* and *Loxosceles gaucho* belong to a group of arachnids (known as "brown spiders"), which are found in the South and South-East of Brazil and are responsible for most of the accidents by this genus [1]. Loxoscelism and dermonecrotic arachnidism are two terms used to describe the cutaneous lesions and various clinical manifestations following bites by members of the *Loxosceles* genus. Systemic effects, such as thrombocytopenia, disseminated intravascular coagulation and renal failure, have also been reported [2]. Indeed, the crude venom from *Loxosceles* spiders can degrade the extracellular matrix [3] and induce endogenous responses, such as platelet aggregation [4], hemolysis [5], nephrotoxicity [6], hepatotoxicity [7] and cardiotoxicity [8].

A group of toxins from the Sphingomyelinases D (SMases D) or dermonecrotic factor (DNF) family have been characterized as responsible for most of the toxic effects of *Loxosceles* spider venoms [4]. However, the exact mechanism of action of the venom is not yet fully understood, thus hindering the development of effective medical treatments for loxoscelism. Among the current treatments, serotherapy using the specific anti-venom is considered as having the greatest potential when administered during the first 8 h after envenomation [2,9].

Some monoclonal and polyclonal neutralizing antibodies against a group of 30–35 kDa molecules, which correspond to the DNF family and are considered the most immunogenic

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components of *Loxosceles* venom [10], have been produced [11,12]. We previously reported that the specific monoclonal antibody LimAb7 recognizes these 30–35 kDa proteins and can neutralize the dermonecrotic activity of crude Li venom [12].

Identifying the epitope of such a neutralizing antibody could help in the preparation of immunogens for producing therapeutic sera or vaccination approaches. In the present investigation, the peptide phage display method [13,14], the SPOT synthesis technique [15–17] and a bioinformatic tool [18] were used to delineate the epitope recognized by LimAb7. The immunization of rabbits with synthetic mimotopes encapsulated in liposomes induced an antibody response capable of neutralizing efficiently several of the toxic effects of crude Li venom, suggesting a potential utility of these peptides for producing therapeutic sera or vaccination approaches.

2. Materials and methods

2.1. Animals, L. intermedia spider venom and antibodies

Eight to nine week/old New Zealand rabbits were used. Animals were maintained at the Veterinary School of the Federal University of Minas Gerais (UFMG) (Belo Horizonte, Brazil) and received water and food under controlled environmental conditions. Treatment and handling of all animals was followed by the UFMG Ethics Committee for Animal Experimentation.

Li venom was collected at the Laboratório Interdisciplinar de Pesquisa em Animais Peçonhentos (LIPAPE), UFPR, Curitiba, PR, Brazil by electro-stimulation (15 V) and then frozen until use. For the production of the recombinant dermonecrotic protein rLiD1 [19], LiD1 cDNA was subcloned in the pET11a vector and transformed in *Escherichia coli* BL21 DE3 cells for expressing the recombinant protein [20].

The dermonecrotic fraction of Li venom was purified and used for immunization of horses as described by Chavez-Olortegui et al. [21]. Briefly, one adult horse was immunized with an initial subcutaneous injection of 150 μg of DNF from Li venom diluted in Freund's complete adjuvant. The remaining three injections were performed in Freund's incomplete adjuvant at 15-day intervals. The horse was bled one week after the last injection.

The neutralizing monoclonal antibody LimAb7 was produced as described by Alvarenga et al. [12].

2.2. One- and two-dimensional separation of crude L. intermedia venom

High-resolution 2D electrophoresis was performed according to the manufacturer's specifications (Amersham Biotech) with some modifications. Briefly, crude Li venom was lysed in lysis buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM dithioerythritol, 40 mM Tris, 0.002% bromophenol blue, protease inhibitors and 1% of IPG buffer. Precast, non-linear immobilized pH 3–10 gradient (IPG) strips (18 cm) were rehydrated with 100 μg or 200 μg of venom proteins for 4 h (no electric field) and then for 12 h at 30 V. Isoelectric focusing was carried out using a gradient mode to a total of 50 kVh. Strip equilibration was done with 65 mM DTT and then 135 mM iodoacetamide. In the second dimension, proteins and molecular standards (20–200 kDa) were electrophoresed on 12% SDS-PAGE gel. One of the gels was stained with silver nitrate and the other was blotted as follows: proteins were electro-transferred to a nitrocellulose membrane that was blocked for 1 h in 2% non-fat milk in 0.05% Tween 20 in PBS and incubated with the LimAb7 antibody (10 μg/ml) at room temperature for 1 h. Antibody binding was detected with a horseradish peroxidase (HRPO)-conjugated rabbit anti-mouse secondary antibody and visualized with a chemiluminescent substrate.

One-dimensional PAGE was conducted as follows: molecular weight markers or rLiD1 (5 μg) were mixed with sample buffer under non-reducing and reducing conditions and electrophoresed on a 12% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane that was blocked with 0.3% Tween 20 in phosphate-buffered saline (PBS) for 1 h and incubated with 10 μg/ml LimAb7 antibody at room temperature for 1 h and then with a HRPO-conjugated rabbit anti-mouse secondary antibody. Blots were developed with 0.025% 4-choro-1-naphtol in 5% methanol (v/v), 0.05% diaminobenzidine and 0.04% H_2O_2 (v/v).

2.3. SPOT synthesis

Overlapping synthetic peptides corresponding to the amino acid sequence of LiD1 (GenBank accession number AAQ16123) were prepared using the SPOT technique [15]. Two series of membrane-bound peptides (overlapping 25-mer peptides with a frameshift of two residues and 15-mer peptides with a frameshift of three residues) were synthesized according to the procedure described by Laune et al. [17]. After synthesis, non-specific binding to the membranes was blocked by incubation with blocking buffer (Roche, Germany) at 4 °C overnight, and then membranes were probed with a horse serum against DNF of Li crude venom (diluted 1:3000 for the membrane with the 25-mer peptides and 1:1000 for the membrane with the 15-mer peptides) or with the LimAb7 antibody at a concentration of 1 or 10 μg/ml in blocking buffer at room temperature for 90 min. Antibody binding was revealed by incubation (at room temperature for 90 min) with alkaline phosphatase-conjugated rabbit anti-horse, or goat anti-mouse secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) plus 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as substrate. For further use, membranes were sequentially treated with dimethylformamide, 1% SDS, 0.1% 2-mercaptoethanol in 8 M urea, ethanol/water/acetic acid (50:40:10, vol/vol/vol) and, finally, methanol to remove the precipitated dye and molecules bound to the peptides.

2.4. Isolation of phage clones binding to the LimAb7

Four different M13 phage libraries expressing 15-mer (X_{15}), 30-mer (X_{30}), 17-mer with a fixed cysteine residue (X_8CX_8) and 12-mer peptides with two fixed cysteine residues (XCX_8CX) were employed [22]. Three rounds of biopanning were performed as described previously [13] with some modifications. Briefly, 96-well microtiter plates (Falcon 353912, Becton Dickinson, Oxnard, CA) were coated at 4 °C with 100 μl of 5 μg/ml (for the first two rounds of biopanning) and 0.5 μg/ml (for the last one) LimAb7 antibody in 0.1 M NaHCO3, pH 8.6 overnight. Plates were then washed with 0.05% Tween 20 in 50 mM Tris-buffered saline, pH 7.5 (TBS-T) and blocked with 3% BSA in TBS-T at 37 °C for 2 h. For the first biopanning, 1.5×10^{11} transducing units (TU) of phages that expressed linear 15-mer (X_{15}), 17-mer (X_8CX_8) and 30-mer (X_{30}) peptides and 2.5×10^{10} TU of phages that expressed constrained 12-mer (XCX_8CX) peptides were incubated in TBS-T with the immobilized LimAb7 at 4 °C overnight. Unbound phages were removed by washing with TBS-T. Bound phages were eluted with 0.1 M glycine, 1 mg/ml bovine serum albumin (pH 2.2) and neutralized with 2 M Tris–HCl, pH 9.0. *E. coli* K91 cells were infected with the eluted phages and grown overnight at 37 °C. Phages particles were precipitated with 20% PEG 8000, 2.5 M NaCl on ice overnight, resuspended in 0.05 M Tris–HCl/0.15 M NaCl, pH 7.5 and used for the next round of biopanning (2×10^{11} TU). After three rounds, phage clones were isolated and screened by ELISA.